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Editorial

The Changing Faces of a Novel Disease

Corona viruses had been with man for close to sixty years. It was regarded as a simple, non-fatal virus with few mortalities. Thus, when flu-related deaths were reported in the last quarter of 2019 in China, the world thought another season of Severe Acute Respiratory Syndrome (SARS) caused by corona had come. The last time the world experienced high-level corona virus-related deaths was between 2003 and 2004 when mortally of more than 1000 patients was reported. Although the World Health Organisation (W.H.O) Country Office in the People's Republic of China first picked up a media statement by the Wuhan Municipal Health Commission from their website on cases of 'viral pneumonia' in Wuhan, People's Republic of China on 31 December 2019, it wasn't until 3rd January 2020 that Chinese officials provided information to WHO on the cluster of cases of 'viral pneumonia of unknown cause' identified in Wuhan. This was also followed by an announcement on January 15, 2020 by the Japanese Ministry of Health, Labour and Welfare which informed WHO of a confirmed case of a novel coronavirus in a person who travelled to Wuhan, becoming the second confirmed case detected outside of the People's Republic of China. By the end of January 2020, it was clear from laboratory and clinical observations that the deaths were caused by a strain of coronavirus having similar features like the general corona virus family. Since the particular corona virus strain had not been identified in humans, it was referred to as a 'novel' coronavirus. Also, through surveillance and epidemiological data, the initial suspicion of animal-to-person spread was confirmed after studies indicated the initial outbreak among people who had a link to a large seafood and live animal market in Wuhan, China. On February 11, 2020, WHO announced that the disease caused by the novel coronavirus would be named COVID-19.

The world began to observe the spread of the virus and its disease as it was being transmitted. For example, Thanks to WHO and several institutional disease monitoring centres around the globe, the world began to monitor updates on the detection and spread of the virus in different countries. The first five months of the year 2020 saw a world less prepared for a pandemic. With lockdowns in many countries, economy of nations started crumbling and personal businesses began to nosedive. Several myths and facts emerged of a relatively unknown disease and deaths rose from a total of 213 in January 2020 to about 180,000 mid-June. Also, it was initially believed (without much scientific proof) that the higher environmental temperatures in most African countries would confer protection from covid-19. This hope was dashed in Nigeria on 27 February 2020 when the first confirmed case announced via an Italian citizen who tested positive for the virus in Lagos. Since then, the number of cases in Nigeria has grown, with Lagos being the epicentre of the pandemic in the country. By June 1, 2020, the Nigerian total cases had risen to 10,578 with 299 deaths recorded.

That Covid-19 pandemic is characterised by many uncertainties is an understatement. At the early stage, some world leaders even doubted the existence. However, what is now certain is the identity of the pathogen responsible. Also, several questions remained unanswered (or is it unanswerable?) for some time. Why do we have more cases and deaths in some countries? Is it because tests are not being carried out? Are plant-based therapies okay? Will a vaccine against Covid-19 be ready before the end of 2020? The world is waiting for answers.

As expected, several suggestions on therapeutic approaches to Covid-19 were proposed and later debunked. Recently, WHO welcomed initial clinical trial results from the United Kingdom that showed dexamethasone, and hydroxychloroquine could be lifesaving for patients critically ill with COVID-19. Plant-based therapeutic remedies are also being researched into, and it is expected that more insight will be emerging soon. It is therefore not a surprise that three review articles in this issue of the *Nigerian Journal of Physiological Sciences* are focussed on covid-19. The first article looked at the worldwide lockdown and movement restriction, highlighted the consequences of such on the cardiovascular health on a physically inactive African population. The authors recommended culturally related indoor physical activities in Africa such as *ampe* or *tente* that could enhance health. They also suggested dog walking, tending backyard farm and catering for indigenous chicken and small ruminants as means of increasing physical activity. The second review explored the potential of Bromelain, a potent inflammatory and anticoagulatory agent as a potential candidate that may be used to inhibit or prevent the symptoms of Covid-19. The third review paper provides immunological angles to the studies needed by researchers to unravel many unanswered questions on the pathophysiology of the virus.

Three articles are featured on side effects of exposure to potential toxicants, two of which were on mosquito coil, a remedy used to repel mosquitoes in order to prevent malaria in many malaria-endemic countries. With a report that burning mosquito coil for 15 minutes produced up to 312 parts per million (ppm) of CO and raised the blood carboxy-hemoglobin level by 15.8%. In another paper, mosquito coil fumes were shown to be toxic to the stomach and delayed the healing of experimental gastric ulcer. Another article reported the effects of occupational exposure to spray paints.

Apart from the afore-mentioned articles, this issue has many interesting articles in many specialties of physiological sciences that should interest the open-minded researcher.

S. B. Olaleye
Editor-in-Chief

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Tribute

An Editor Bows Out - Tribute to Prof. Anthony Ebeigbe as He Retires

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Summary: This tribute was written to appreciate the longest-serving Editor-in-Chief in the history of this journal as he retires from active teaching and research. His contributions to the Physiological Society of Nigeria is outstanding and his relationship with colleagues highly commendable.

Keywords*: Tribute, Anthony Ebeigbe, Retirement, Nigerian Journal of Physiological Scienecs*

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Prof. Anthony B. Ebeigbe
Editor-in-Chief (1987 – 2001)

Let those who sleep wake up
Let them arise as the listening skies
To appreciate God's love for him who lives and works so hard
Him through whom God's love washes
Lift up your glass to celebrate with us
One who deserves the best of everything

The first time I met Prof. Anthony B. Ebeigbe was at a Scientific meeting of the Physiological Society of Nigeria about 32 years ago. I was at the meeting as a young student of Physiology and President of Physiology Students Association. I had gone to the meeting to seek for the support of alumni of Ibadan Physiology programme for a student association that needed a direction, focus and encouragement of those who passed through the programme. We asked our lecturers at that time to give us names of those who they thought were worthy Physiologists and who would be approachable. Prof Ebeigbe's name was mentioned by all of them. He did not disappoint us when we eventually met him after he gave a very interesting presentation at one of the sessions. His counsel made a lot of difference to my understanding of Physiology and he encouraged me to go all out to pursue a career in Physiology. Generations of students and young faculties in several Nigerian Universities have similarly been impressed with the way Prof Ebeigbe relates and picks interests in their academic and professional pursuits. He is indeed a friend of the young generation. His formal exit from the University as he takes a bow out of the ivory tower on August 22, 2020 is therefore expected to be a celebration of fulfilled years of mentoring, service and administrative excellence.

Born on August 22, 1950 at Igbanke in Edo State, Anthony Ebeigbe attended Nigeria's premier University and graduated with a degree in Physiology in 1974. He joined the services of the newly established University of Benin in 1975 as a Graduate Assistant. He belonged to the elite group of Nigerians who had the opportunity of pursuing a higher degree at the famous Institute of Physiology at the University of Glasgow in Scotland, from where he got a doctoral degree in Physiology in 1980. He returned to his station in Benin and rose through the ranks to become an Associate Professor of Physiology in 1984. Even after his return to Benin, 'Tony, as he is fondly called by his peers, had research exposures in several laboratories around the world. He was at the Physiology Department. University of Michigan Medical School, USA as a visiting Research Scientist and later as a Fulbright Scholar in 1982. In 1985, he was Research fellow at the Preclinical Research laboratories of Sandoz Ltd., Basel, Switzerland. Between 1987 and 2005, he was visiting Professor to Universities and Pharmaceutical companies in Brussels (Belgium), Basel (Switzerland), Glasgow

(U.K), London (U.K) and Newcastle (U.K). 1n 2004 – 2005, he was Richard A. Bernstein distinguished Professor at the Department of Natural Sciences, University of Maryland, Eastern Shore, Maryland, U.S.A. Prof Ebeigbe was a recipient of many research grants and awards both within and outside Nigeria.

After traversing the globe for laboratory research and teaching experiences, he settled down to contribute his quota to the administrative development of the University of Benin, where he held several administrative positions. In 1990, he was appointed Professor of Physiology at the Bendel/Edo State University (now Ambrose Alli University), Ekpoma. At Ekpoma, he was Dean, Faculty of Basic Medical Sciences between 1991 and 1993. He later returned to base at the University of Benin as professor (by appointment) in 1993. He was elected Provost, College of Health Sciences, University of Benin between 1993 and 1994. He also served as the acting Vice Chancellor of PAMO University of Medical Sciences, Port Harcour, on its establishment in 2018.

Prof Ebeigbe has been a great and reliable pillar within the Physiological Society of Nigeria. He is perhaps one of the very few (not more than five) who would dare all consequences to attend the Annual General Meetings of the Society (except when not in Nigeria). He was the Society's National Secretary between 1983 and 1987. He became the second Editor-in-Chief of the Nigerian Journal of Physiological Sciences in 1987, a position he held till 2001, when he became the Vice President. He is thus the longest serving Editor-in-Chief of the Journal till date. During his tenure as editor, he exposed the Nigerian Journal of Physiological Sciences to several University libraries around the globe. He also laid the foundation for the Journal's inclusion in Medline and many other Indexing services. Prof Ebeigbe became the President, Physiological Society of Nigeria in 2005 at the Society's meeting in Port Harcourt and served in that capacity till 2010. He is a member of both the Board of Trustees (B.O.T) and also heads the Body of Fellows (B.O.F), of the Society.

Though Prof Ebeigbe is retiring from active duties, it is sure he will not withdraw from our midst in the Physiological Society of Nigeria. His jokes, historical interjections at meetings, his intelligent suggestions on how to move PSN forwards and his regular lectures to upcoming Physiologists will continue to be focal points at meetings.

We in the Board of the Nigerian Journal of Physiological Sciences will continue to celebrate a man whose impact has been well felt in his career, an honest reviewer of manuscripts, experienced editor, an excellent teacher and researcher who has produced several Professors, someone whose amicable and approachable manner made him a helpful resource for junior colleagues and lastly, a proud family man.

We congratulate Prof Ebeigbe and wish him a long, healthy and happy life in retirement.

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Combating COVID-19 Lockdown Inactivity in the African Population: Use of Cultural Practices and One Health Approach

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Summary: A novel pulmonary illness caused by coronavirus 2019 (COVID-19) of unknown origin was first reported in China. This disease has claimed over a million human lives worldwide. This pandemic respiratory disease spread through droplets on surfaces and community spread. Government of different countries adopted a total lockdown to reduce human to human contact and keep families safe from the disease. This compulsory movement restriction reduces physical activity of individuals which could pose cardiovascular risk to physically inactive African population. This brief states the various cultural and one health approaches that could be adopted to increase physical activity within the home setting. Literature search using PubMed, Scopus and google scholar and views of one health personnel in the promotion of physical activity at home during the compulsory lockdown were sought for to identify some simple approaches and gaps that need to be researched on. The overview identified culturally related indoor physical activities in Africa such as ampe or tente that could enhance health. It encouraged dog walking, tending backyard farm and catering for indigenous chicken and small ruminants as means of increasing physical activity. Counselling was proffered by nurses to increase health promotion activities such as setting reminders for physical activities and routine house chores. This submission bring to bear indigenous, flexible and simple measures to combat boredom, promote cardiovascular health by increasing physical activity during the compulsory lockdown currently being experienced in Africa, a known cardiovascular risk, physically inactive population.

Keywords*: Physical activities, COVID 19, Lockdown, indigenous approach*

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INTRODUCTION

A lower respiratory tract febrile illness (novel coronavirus, pulmonary illness, coronavirus disease 2019 (COVID-19)) of unknown origin was reported in a cluster of patients in Wuhan City, Hubei Province of China in December, 2019. COVID-19 has been declared as a pandemic health condition (Gates, 2020). As at April 11, 2020, records showed that over 1,518,518 individuals were affected and 88,495 human lives claimed worldwide by the deadly disease (Hosseiny *et al*., 2020). Headache, persistent pain or pressure in the chest, dry cough, confusion, fever, diarrhea, myalgia, excessive drowsiness, and dyspnea were presently identified clinical indicators of COVID-19 where multi-organ failure and fatal pulmonary contribution has arisen from most infected people (Zahra et al., 2020). Although, the developed world has the enormous ratio of COVID-19 cases and death rate compared to developing countries, its threatening crisis would be disproportionately hit the former (UNDP, 2020).

Africa reported the first case of COVID-19 in Egypt on 14 February, 2020. Presently, 45 countries (approximately 60.0%), including power house of African countries, have reported COVID-19 cases where confirmed cases has risen above 9,147 and more than 413 deaths (WHO, 2020). The exponential growth of COVID-19 infection suggests that soonest it would reach every country in Africa which might put the continent at indescribable health risk.

Several pragmatic measures/guidelines/ approaches were presented by standard international health regulatory bodies such as WHO and OSHA to curtail its spread globally including frequent hand washing with soap under running water for at least 20

seconds, maintaining more than 2 metres (6 feet) physical (social) distance, avoidance of eyes, nose and mouth touching, respiratory hygiene practices including covering coughs and sneezes, those having fever, cough and difficulty in breathing to seek medical care early, avoiding close contact with people who are sick, staying home if sick, staying informed by recognizing personal risk factors and following advice given by healthcare provider (OSHA, 2020). The envisaged approaches in this submission were considered under lockdown and physical activities, culturally related indoor physical activity in Africa as complement of health, pets as a tool to increase physical activity, backyard farming and home nursing perspectives.

This study employed thematic review method where current literature search on peculiar issues were conducted. Database search platforms of Pubmed, Scopus, AJOL and Google scholar were consulted. Search terms used included "culturally related" and "indoor physical activity" or "indoor exercise" or "games". Information (data) from titles, abstracts and main body of articles were analysed. Opinions of exercise physiologists, experts in veterinary medicine and nurses were gathered through teleconferencing for 30minutes per day for a week, connected and integrated together.

Lockdown and physical activities

Apart from the above guidelines, most threatened countries-China, Spain, USA, Italy, South Africa, Ghana, Nigeria, etc., pronounced partial or total lockdown (an emergency protocol that prevents people from leaving home) on its citizens to curtail the spread of the virus. The lockdown was not only made compulsory but enforced militarily on recalcitrant citizens. Critical analysis of the 'lockdown' protocol suggests that sedentary (inactive) citizens were made to stay healthy in doors safe thereby reducing the exposure to the virus as recommended by World health Organization (OSHA, 2020). Activities that would appeal to most of the inactive citizens in African countries will be sleeping, playing inactive games and watching of video/films significantly. These activities are regarded as insufficient physical activity (not meeting the WHO recommendations on physical activity for health (WHO, 2010) which may have deleterious effects on their health and wellbeing.

Those who are active will at least remain active by dancing to favourite music, engaging in garden works where available and do some work outs within the compound where available which might not be sufficient if not monitored while vast majority will persistently be inactive. Although the sample population of the report considered was 10-19 years, its findings showed that boys in Sub-Saharan Africa region has the second highest prevalence of insufficient activity (83·9% [82·3–85·0]) while girls from the central Asia, Middle East, and north Africa regions has the second highest prevalence (89·9% [88·6–90·9]) (Guthold et al., 2020). The physical activity behaviours of the generation may reflect that of their parents. Studies have shown significant correlations between parental support and child physical activity level (Guthold et al., 2020).

Although sleeping, hypothesized as one of the major activities that families/individuals during the 'lockdown', has positive effects on the mechanisms defense of the body systems and inflammatory response's magnitude and characteristics (Tan *et al*., 2019); improves memory recall, regulates metabolism, and reduces mental fatigue, repairs tissue, synaptic homeostasis, and immune-inflammatory control (Chennaoui *et al.,* 2019), it adversely contributes to weight gain when caloric consumption is greater than energy expenditure. Furthermore, other earlier hypothesized physically inactive activities also play major roles in weight gain.

It has been established that weight gain >2% is an indicative defensive connotation with cardiovascular diseases (CVD) and an expressive opposing suggestion with increased mortality (Strelitz *et al.,* 2019). There is the need to scientifically admit, to be best of authors' knowledge, that African continent has vast citizens who are physically inactive, overweight, obese and at risk of CVD. Another perspective is with those who would engage in sitting activities due to the 'lockdown' that would expose them to low back pain either acute or chronic. Low back pain (LBP) has the potential of signaling CVD. This submission is presented in line with studies where chronic LBP had higher prevalence of myocardial infarction and coronary heart disease even when controlled for genetics and early shared environment (Fernandez *et al.,* 2016). The envisaged inactivity suggest a need to critically look inward for cultural practices and other one health measures that could be put in place to avoid public health problems during and post COVID-19 periods.

Cultural Physical Activity in Africa as complement of Health

There is evidence of many culturally related leisure time physical activities among African countries (Thind *et al.,* 2015). Culturally related indoor games vary in names like Senet (Egypt)/Ayo olopon (Nigeria)/Kigogo (Kenya)/Oware (Ghana)omweso (Uganda), Igisoro (Rwanda), Enkeshui (Kenya), Kudoda (Zimbabwe), Nyama-nyama-nyama (Kenya), and Nngapi / Bao (Tanzania) appear to provide perceptions of physical activity conception patterns in African setting.

The conceptualizations of African culturally related indoor games (not for festivals) practically are passive in nature rather than active which could suggest insight of native and emotional intelligence

(Oyibo *et al.*, 2018). Physical activity is an effective measure for preventing diseases in children and adolescents across all ethnic and socioeconomic subgroups (Ahmadinejad *et al.*, 2014). The playing patterns of the few active indoor games, like Stockings (across Africa), Bojuboju and Talowa ninu ogba naa (Nigeria) and *Tente* (Nigeria)/*Ampe* (Ghana), present health enhancing opportunities.

Generally, the health benefits of being physically active, although depend on intensity and duration among other things, include reduction in the risk of chronic cardiovascular diseases, symptoms such as myocardial infarction (within 6 months), coronary heart disease associated with related chest pain, heart valve disorder, cardiomyopathy, stroke, hypertension, obesity, arthritis, and diabetes (Rhodes *et al*., 2017). Analyzing specific health benefits of each of all the culturally related indoor physically active games may be challenging but attempt is currently on to scientifically assess the usefulness of a*mpe (*Ghana*) or tente* (Nigeria) in increasing physical activity, the scientifically analysed health benefits which are scarce in literature. Given its mechanism of execution, ampe is not difficult to learn, requires readily available natural surface and a presence of at least two individuals. Recent experimental study conducted among school children showed that ampe exercise programme significantly decreased waist circumference, hip circumference, waist-to-hip ratio, percent body fat, blood pressure, and heart rate in school children (Moses *et al*., 2020). *Ampe* can be executed both indoor and outdoor to reduce the risk of cardiovascular diseases, has potentials that could boost psychological stressors and enhance wellbeing of all irrespective socio-economic status during lockdown.

Pets as a tool to increase physical activity for Health

The lockdown in Africa has brought family together and emphasis is on what can be done within the home setting to promote physical activity. One of such is the use of pets in increasing physical activities.

It is a known fact that mutual benefits and robust relationship between people and animals termed human–animal interaction had been the focus of many researchers with very few highlighting the psychological, physiological, and social benefits (Walsh, 2009) of such interaction especially in an African setting. The enhancement of some physiological traits through this interaction such as walking dogs has been identified among children with resultant good outcome on social benefits at old age (Bergroth *et al*., 2012). This clearly showed that a history of dog ownership often benefit such owners in later life than during youth. Reports also showed that dog walkers at baseline were 1.65 times active physically than non–dog owners (Thorpe *et al*., 2006) with resultant better social and psychological benefits. Various reports abound in developed counties of community-dwelling older dog owners having greater motor fitness and walk more, with resultant higher social function than never owners. Dog walking had been identified to increase walking time for older persons and help maintain motor fitness and social activities, regardless of family support or financial resources. Caring for a dog might be an effective health promotion strategy to increase dog walking, physical activity and facilitate social participation among older adults (Taniguchi *et al*., 2018).

Information on dog walking abound in literature particularly in Western countries as a feasible approach for increasing physical activity especially for the household that owned dog which are 23% of UK (Bergroth *et al*., 2012) and 35.1 % of Ghana (Tasiame *et al*., 2019). This is important because large proportions of the population in many developing countries are not sufficiently active for health benefits (Guthold *et al*., 2020). In Sub-saharan Africa, even before the lockdown, 83.9% of boys and 89.9% of girls are not sufficiently active (Sorek *et al*., 2018). To achieve recommended level of physical activity in adults, effort should be tailored towards the use of dogs for briskly walking for at least 30 minutes each day. It has also been established that the dog-owner relationship has the potential to enhance health by reducing stress to a greater degree than if walking alone or with a person and attributed to effects on parasympathetic neural activity (Westgarth *et al*., 2014).

Many reports also showed that the exercise levels of dogs correlate well with their owners' activity levels which are inversely associated with obesity. This evidences in the literature showed clearly that dog walking should be an approach to reduce boredom, physical inactivity during this COVID 19 lockdown especially in an African setting. Though there are various religious and cultural issues that influence dog walking and especially dog ownership as opined by Suluku *et al.*, (2019), but embracing this simple activity within the home will no doubt increase the rate of physical activity

Backyard farming and Health

Apart from dog walking, tending household or backyard farming has been identified as a means of increasing physical activity especially tending vegetable garden and caring for indigenous small ruminants and poultry varieties. Backyard ruminant and poultry farming is gaining prominence because 70% of the poultry and small ruminant production are raised in the rural and some urban households. The care for the animals is often by children and women who sell them for immediate cash need or for religious ceremonies. During this COVID19 lockdown period, catering for these domestic animals will reduce physical inactivity or boredom often associated with this time. The scientific evidence of the level of

reduction of physical activity using this approach in the African population has not been fully elucidated. This approach has been practiced in various communities in Australia with tremendous increase in physical activity and interaction within the community (Kingsley *et al*., 2009). The economic gains associated with backyard poultry production was well marked during this period when demand for their demand and eggs increased as coronavirus shopping frenzy empties supermarkets in US. Though this simple approach can be adopted especially for those that have backyard, while those with no facilities could make do with dog walking.

Role of Nurses in health promotion at Home

Sedentary behaviour which is common during this period is any activity involving sitting, reclining or lying down with resultant low energy expenditure. This unhealthy behaviour if left uncurbed will likely increase the risk of individuals to chronic health conditions such as cardiovascular diseases, obesity, type 2 diabetes, deep vein thrombosis and mental health disorders. Cardiovascular diseases and diabetes mellitus constitute more than half of non-communicable diseases in sub-Saharan Africa (Hamid *et al*., 2019) and this may get worse considering the current rate of inactivity individuals are being subjected to. Prior to this time, Africa had a high prevalence of obesity, hypertension and diabetes which usually go undiagnosed and resulted into preventable death.

The WHO recommends a minimum of 150 minutes period of physical activity or an equivalent of 75 minutes of vigorous activities per week or a combination of both to stay healthy. However restrictions of this nature on movement and social distancing may not make this achievable unless conscious efforts are made by individuals to remain physically fit because remaining physically fit enhances healthy living and longer lives (Wolfson *et al*., 2019).

Physical activity is health promoting and disease preventing. It promotes mental alertness of individuals as it has been linked to improved immune system and weight maintenance. For physical activity to produce the desired results as enumerated, it must be done regularly, consistently and preferably in a family group. Sedentary living and poor dietary habit of individuals during this lockdown can have deleterious effects on cardiovascular health which may lead to early death (Kandola *et al*., 2020). This makes the need for improved physical activity very imperative for adults and children during Covid-19 lockdown.

The role of nurses in the prevention of cardiovascular diseases among the populace during this period is that of health promotion which is aimed at enabling and encouraging people to increase control over and improve their health. Provision of health teaching on COVID 19 to increase individuals' awareness can be done in form of short but educative video clips as well as encouraging people to seek for correct and accurate information from appropriate media. This will serve to give them relief from preventable psychological stress. People who are already on treatment for chronic diseases are encouraged to maintain their health through strict compliance to their medications. Zhai and Baran, (2019) recommended walking round the house at intervals, doing household chores, moving round the house to receive calls, taking a break during television commercials and setting reminders to stand up every thirty minutes in order to remain physically active. These conscious efforts in turn improve concentration and reduce behavioural problems even in children.

Nurses' role in the prevention of sedentary living also include promotion of physical activity such as providing dietary advice on appropriate nutrition, encouraging active play among children and sustaining physical activity in both children and adults to promote physical and mental wellbeing. Enhancement of behavioural change counselling among the populace and persuading them to make appropriate decisions that support a healthy lifestyle in order to cope with the current challenges of COVID19 is equally important.

Conclusion

This overview clearly identified some cultural and one health approaches that could be adopted to increase physical activity within the home setting. They include cultural dances and exercise known in Africa such as ampe, ten-ten, it also encouraged indigenous chicken and small ruminant as source of increasing physical activity while dog walking and tending backyard vegetable garden was identified as some activities which can be intensified to increase physical activities especially for youth and old adults. Some health promotion activities such as reminders to walk couple with routine house chores that demand for some physical activity were also advised. This bring to bear indigenous, flexible and simple measures to combat boredom and increase physical activities during the compulsory lockdown currently being experienced in Africa, a known Cardiovascular Risk Physically inactive population.

Already known facts

- African population is a physical inactive population
- Sedentary life style results in cardiopulmonary risks
- The lockdown is a challenge to adequate physical activity

This review adds

- Ingenious approach such as use of cultural dance and exercise

- Dog walking should be encouraged in the African population to increase physical activity
- Tending backyard farming and simple routines within the home should employed

Authors' contributions; All authors (MOM, AOE, MKM and BOE) contributed to the study's design. MOM, AOE, MKM and BOE drafted of the manuscript. Other authors (MOM and BOE) critically revised the manuscript and provided valuable inputs. All authors (MOM, AOE, MKM and BOE) read and approved the final version of the manuscript.

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Review Article

Bromelain: A Review of its Potential as a Therapy for the Management of Covid-19

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Summary: Coronavirus Disease 2019 is a wide-spreading severe viral disease caused by the Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-COV-2) virus that needs to be urgently eradicated. SARS-COV-2 has infected millions of people worldwide and results in more than three hundred thousand deaths. Several repurposed drugs have failed to successfully eradicate the infection. Multiorgan failure caused by pronounced inflammation and systemic coagulation accounts for severe complications and death associated with diseases. Bromelain appears to be a potential candidate that may be used to inhibit or prevent the symptoms of the disease. Its anti-inflammatory and anticoagulatory properties make it a potential agent that may slow the progression of the disease. In this review, we highlighted the beneficial effects of bromelain based on both experimental and clinical evidence that make bromelain a good candidate for the treatment of symptoms of CoVID-19 infection.

Keywords*: Anticoagulant; Bromelain; Covid-19; Inflammation; SARS-COV-2*

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INTRODUCTION

The sudden emergence of severe cases of pneumonia with an unknown etiological cause that was later identified to be a novel coronavirus was reported in late 2019 at Wuhan province of China. The disease caused by this novel coronavirus was later labeled as Coronavirus 2019 (CoVID-19) by the World Health Organization (WHO) on the 11th of February, 2020. This novel coronavirus is highly contagious and the disease spread like wildfire around the world, currently affecting more than five million people with over one thousand and three death worldwide.

CoVID-19 is caused by Severe Acute Respiratory Syndrome Coronavirus – 2 (SARS-CoV-2) which belongs to the β-coronavirus subtype. It is a single RNA positive strand of size 26 - 32 kbs in length and a diameter of 65 – 125 nm (Adnan *et al*., 2020). It belongs to the same class of deadly viruses, SARS-Cov and the Middle East Acute Respiratory Syndrome Coronavirus (MERS-CoV) which causes severe types of lower respiratory tract infection and acute respiratory distress syndromes (ARDS). They all belong to the family of Coronaviridae order Nidovirales. The emergence of coronavirus was first noticed in 2002 when there was a SARS-CoV outbreak that infected more than 8000 in 37 countries and causing 774 fatalities. Another wave of coronavirus pandemic occurred in 2012 caused by MERS-CoV which infected 2494 individuals and led to 858 deaths (Lu *et al*., 2020; Wit *et al*., 2016).

STRUCTURE OF SARS-COV-2

SARS-CoV-2 is less than 80% nucleotide identical and 89.1% nucleotide similarity to SARS-CoV genes (Tao *et al*., 2020; Zhou *et al*., 2020). It possesses two untranslated regions (5' cap structure and 3'-poly-A-tail) and a single open reading frame (ORF) that encodes some polyprotein (fig. 1a) (Ge *et al*., 2020). The complete genome of SARS-CoV-2 contain replicate complex (ORF-1a and ORF-1b) encoding the non-structural protein (NSPs); spike protein (S) gene, an envelope protein (E) gene, membrane protein (M) gene, and nucleocapsid protein (N) gene all of which encode structural protein; accessory protein gene (ORF 3a, 7 and 8) that were inserted in the gees of structural proteins (Chan *et al*., 2020; Lu *et al*., 2020; Paraskevis *et al*., 2020); and several other unidentified non-structural open reading frames (ul Qamar *et al*., 2020). Spike protein gene is made up of two major subunits (S1 and S2 subunits) which are critical to the survival of the virus in the infected host. S1 subunit comprises of N-terminal domain and receptor-binding domain (RBD) used in forming a strong bond with the receptor of the host cell.

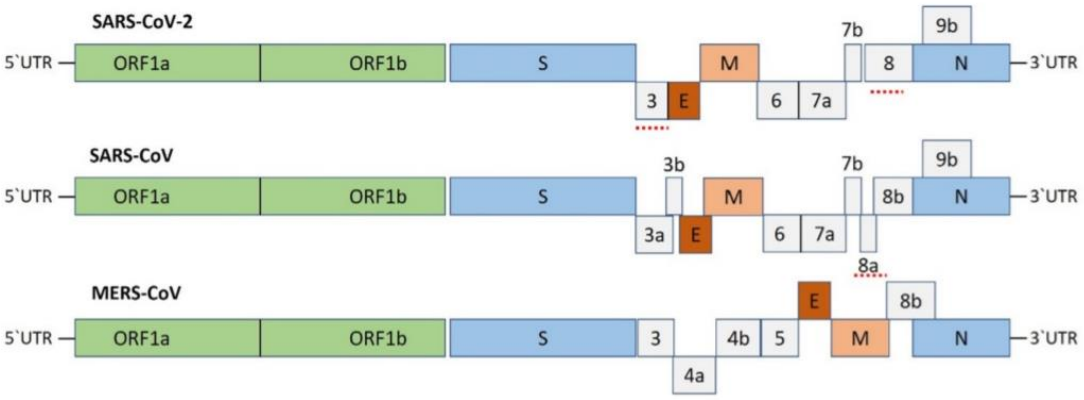

Figure 1:
Complete genomic sequencing of SARS-COV-2. (Adapted from Shereen *et al*., 2020).

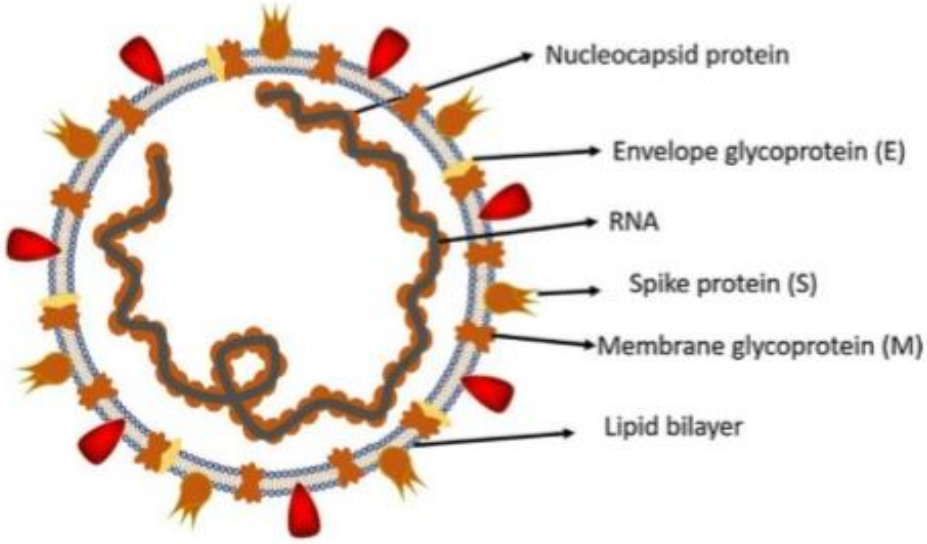

Figure 2:
Structure of SARS-COV-2 (Adapted from Shereen *et al*., 2020).

S2 subunit contains three functional domains (fusion peptide, heptad repeat-1, and heptad repeat-2) which change conformation to aid the fusion of the virus to the host cell membrane (Ge *et al*., 2020).

TRANSMISSION of SARS-COV-2

CoVID-19 is transmitted mainly through droplets, human-human close contact, and aerosol via self-inoculation of the mucus membrane of the eyes, nose, and mouth. SARS-CoV-2 has been detected in the tears, conjunctiva secretion, saliva, urine, and stool of the infected patient (Wang *et al*., 2020; Xia *et al*., 2020; Xiao *et al*., 2020). SARS-CoV-2 can survive on an inanimate material for up to nine days. Temperature ranges from 30 – 40 ^{0}C has been said to reduce the survival of SARS-CoV-2 while the temperature of 4 ^{0}C or below increases its persistence (Kampf *et al*., 2020).

SARS-CoV-2 infects the host cells by binding to the angiotensin-converting enzyme-2 (ACE-2) which is widely expressed in the cell membrane of the heart, kidney, lungs, gastrointestinal tract (GIT), brain, and liver. ACE-2 has also been found to be expressed in the eyes, nostrils membrane lining, and mouth. SARS-CoV-2 uses 394 glutamine residue in the RBD of the S1 motif which is recognized by the lysine-31 residue on the ACE-2 to establish a binding with the host cells while the S2 motif of the virus fuses firmly with the cell membrane (Adnan *et al*., 2020). Some cellular proteases such as furin, human airway trypsin-like protease (HAT) cathepsin, and transmembrane protease serine-2 (TMPRSS2) mediate proteolytic processes that aid penetration of the viral RNA into the host cell through the splitting of the spike protein (Bertram *et al*., 2011; Glowacka *et al*., 2011; Rizzo *et al*., 2020). In the infected cell, 3-chymotrypsin-like protease enzymes regulate the viral replication and it is essential for the life cycle.

ORIGIN AND EPIDEMIOLOGICAL STUDY

SARS-CoV-2 has been presumed to originate from the human seafood in the Wuhan market, China. Genomic

evidence has shown that full genome sequencing of SARS-CoV-2 is 96% march of that of bat SARS-CoV (L. Wang *et al*., 2020). This finding suggested that SARS-CoV-2 may be of a zoonotic source having bat as a potential reservoir with intermediate host yet to be ascertained.

CoVID-19 is the third wave of a pandemic caused by coronavirus named SARS-CoV-2. After its emergence in China in 2019, it quickly spread around the globe from Asia to Europe, America, Australia, and Africa. It has infected over five million individuals with more than three thousand mortality. Elderly people and people with predisposing underlying health conditions such as diabetics, heart diseases, hypertension, etc. are 10 times more susceptible to viral infection. CoVID-19 infects the male gender more than females. This may be due to the large expression of ACE2 in males more than females (Sama *et al*., 2020). As at the time of this review, a total of 63,293 cases of coronavirus have been recorded in Africa with 2,290 fatalities. Nigeria has 4399 cases of CoVID-19 with 143 death across the country; South Africa has 10,015 cases, Egypt have 9400 cases, Morocco with 6063 cases, Algeria with 5723 cases, and Sudan with 1365 cases. Lesotho is the only African country that is yet to record any cases of CoVID-19. A study from China showed that the median age of patients infected by CoVID-19 is 47 years of which 87% of cases were in the age range of 30-79 years.

COVID-19 MANIFESTATION

CoVID-19 incubation period in an infected patient ranges from 0 – 24 days with 14 days median time from the symptom onset to death. Individuals with CoVID-19 are best diagnosed by using reverse transcription-polymerase chain reaction (RT-PCR) techniques that perform genomic sequencing and matching it with the SARS-CoV-2 genome. RT-PCR is highly specific but low sensitivity test range of 60-80%, hence, repeated RT-PCR test is recommended for all negative results with a testing window of 24 - 72 hours. A study from China showed that 81% of patients have mild symptoms, 14% were severe while 5% were said to be in critical condition. Most symptoms of CoVID-19 were fever (87.9%), cough (67.7%), fatigue (38.1%) diarrhea (3.7%), and vomiting (5%) (Wang *et al*., 2020). Studies have shown that CoVID-19 affects various organs such as lungs, heart, liver, kidney, central nervous system (CNS), eyes, and gastrointestinal system either directly or as a comorbidity symptom. Cytokine storm, inflammation, pneumonia, and disseminated intravascular coagulation (DIC) have been highlighted as the major complications which lead to multi-organ failure in CoVID-19 patients.

COVID-19 AND INFLAMMATION

One of the major pathophysiological events in CoVID-19 patients is the development of inflammation. The entrance of SARS-CoV-2 into the host cells leads to the massive activation of both neutrophils, dendritic cells, and macrophages which are mobilized to the site of infection. This first line of defense cells increases the production of chemokines and cytokines that promote the onset of inflammation. The resulting cytokines release syndrome is one of the critical factors that aggravate the disease progression and the major complications that result afterward. Interleukins (IL) – 6, IL-1, IL-2, IL-7, IL-10, and TNF-α are the resulting cytokine storms that are released in CoVID-19 patients. Chemokines such as Granulocyte Colony Stimulating Factor, 10 kD Interferon-gamma-induced-protein-10, Monocyte Chemoattractant Protein-1, and Macrophage Inflammatory Protein 1-α have also been reported to be elevated in the plasma of CoVID-19 patients.

Likewise, an increase in replication of SARS-CoV-2 has been linked with reduced interferon secretion. Low level of CD4+T and CD8 +T in most patients account for the imbalance between the type 1 and type 2 T-helper (Th) cells resulting in cytokines storm (Rizzo *et al*., 2020; Wang *et al*., 2020). A study from Chinese laboratory showed that Th1/Th17 promotes IL-6 in patients with CoVID-19 pneumonia (Rizzo *et al*., 2020). IL-6 has been tagged as the predictor of mortality in CoVID-19 Patients. In patients with mild symptoms, the IL-6 level is moderately increased and it reaches up to 25.2pg/ml in patients with a severe form of the illness (Hughes, 2008). The vital overall effect of cytokine storm is increased risk of vascular hyperpermeability, multiorgan failure, and eventually death.

Increased cytokines level has been linked with the activation of the coagulation pathway resulting in multiorgan failure. Studies have shown that the disseminated intravascular coagulation resulting from increased activation of thrombin is a major cause of mortality in CoVID-19 patients. Increased proinflammatory secretion causes impairment of Thrombin regulator (antithrombin III, tissue factor pathway inhibitor, and protein C system) which were evidence in severe CoVID-19 patient. Prevalent pulmonary embolism and venous thromboembolism aggravating ventilation-perfusion mismatch have been observed in CoVID-19 patients.

COVID-19 AND RESPIRATORY SYSTEM

SARS-CoV-2 causes havoc on the lower respiratory tract. One of the major complications of CoVID-19 is the development of acute respiratory distress syndrome which usually occurs on the 9^{th} day starting from the onset of symptoms in the severe patient (Huang *et al*., 2020). Studies have revealed that SARS-

CoV-2 primarily attacks the pneumocyte type II cell of the alveoli which form 83% of the apical epithelial cells (Li, 2020; Xu *et al*., 2020). Replication of SARS-CoV-2 in the cytoplasm of a pneumocyte induces oxidative stress which results in apoptosis of the apical cells. The cumulative effect of this includes the destruction of the alveoli capillary walls, predominant ground-glass opacity, irregular interlobular septal thickening, air bronchogram, and inflammation of the alveoli (L. Wang *et al*., 2020). These are the typical features of CoVID-19 pneumonia.

COVID-19 AND CARDIOVASCULAR SYSTEM

The devastating effect of SARS-CoV-2 on patients does not exempt the cardiovascular system. Due to the high expression of ACE2 in the heart, various degrees of damaging effects have been recorded on the cardiovascular system. Patients with CoVID-19 have been reported with heart arrhythmia, acute heart injury, lymphopenia, and thrombocytopenia (Wang *et al*., 2020). Few reported cases of leukopenia (33.7%) as well as thromboembolism have been noted. Myocarditis, cardiac arrest, and acute heart failure are common symptoms displayed in about 40% of CoVID-19 patients (Chen *et al*., 2020). It is however not ascertained whether cardiovascular diseases are directly provoked by SARS-CoV-2 or are its comorbid effects due to increased cardiometabolic demand. An insinuation that patients under the treatment of angiotensin II receptor blocker (ARB) may be highly susceptible to CoVID-19 has been soundly disproved by some studies that show a lack of linkage with such treatment (Ferrari *et al*., 2020; Sama *et al*., 2020). Likewise, it has been shown that ARB does not increase ACE2 level and hypertension does not increase CoVID-19 infection. It has been strongly recommended that CoVID-19 patients should be on the watch out for the development of cardiovascular heart disease as baseline comorbidity and as a complication.

COVID-19 AND THE NERVOUS SYSTEM

COVID-19 has generally been studied for common symptoms, hence limited studies have reported its neurological effect on patients. A study from China reported that 36.4% of COVID-19 patients showed neurological manifestation (Mao *et al*., 2020). ACE2 is expressed in the central nervous system as well as the ocular tissues. SARS-CoV-2 certainly, finds its way to the nervous system to produce damages due to its neuroinvasive capabilities. It possibly spreads from the respiratory tract to the CNS. SARS-CoV-2 has been found in the cerebrospinal fluid (CSF), tears, and conjunctiva secretion. It can also gain access to the CNS through the olfactory bulb to cause neuroinflammation and demyelination of central neurons (Bohmwald *et al*., 2018). CoVID-19 patients generally complain of fever, body weakness, fatigue, ocular surface infection, headache, body pain, and some degree of dyspnea (Asadi-Pooya & Simani, 2020). Febrile seizures, convulsion, encephalitis, and changes in mental status have also been reported in some patients (Bohmwald *et al*., 2018; Desforges *et al*., 2019).

HERBAL MEDICINES AND COVID-19

The use of traditional herbal medicine as an antiviral remedy started since the emergence of CoVID-19 (Yang *et al*., 2020). The different continental regions have produced herbal concoctions projected for the treatment of CoVID-19. Chinese traditional medicines have been promising in the treatment of CoVID-19, their antiviral effects have been evaluated on SARS-CoV-2 infection in both humans and mice (Luo *et al*., 2020; Yang *et al*., 2020). In particular, Shuanghuanglia oral liquid has been claimed to inhibit the growth of SARS-CoV-2 (Wang *et al*., 2020). Its effects have been linked with its anti-inflammatory properties that significantly reduce inflammatory responses of the body in the course of progression of the infection (Wang *et al*., 2020). In Africa, Madagascar herbal concoction (CoVID-Organics) has been trending because of the claim of its antiviral effect on SARS-CoV-2. Currently, the CoVID-Organics has been distributed around various African countries for more clinical trials.

BROMELAIN

Bromelain is a major cysteine endopeptidase protease inhibitor found in the family of Bromeliaceae of which pineapple is the most popular. Pineapple is a native of South America but it is now widely grown in various parts of the world including Africa. Bromelain contains numerous cysteine proteinase that are closely related but different amino acid sequencing, specificity as well as sensitivity to inactivation. It contains peroxidase, acid phosphatase, several protease inhibitors, and organically-bound calcium. It is made up of 212 amino acids and the molecular weight is 33 kDa (Gautam *et al*., 2010).

Bromelain is classified into stem and fruit bromelain based on the part of the plant it was extracted from. Stem bromelain (EC 3.4.22.32) is the most abundant proteinase derived from pineapple. It is different in specificity compared with fruit bromelain (EC 3.4.22.33) and cleaves preferentially into the Z-Arg-Arg model substrate whereas fruit bromelain cleaves into Bz-Phe—Val-Arg (Napper *et al*., 1994). Stem bromelain has been reported to contain 285 amino acids of which alanine and glycine are the most abundant, while histidine and methionine are present in minute quantity.

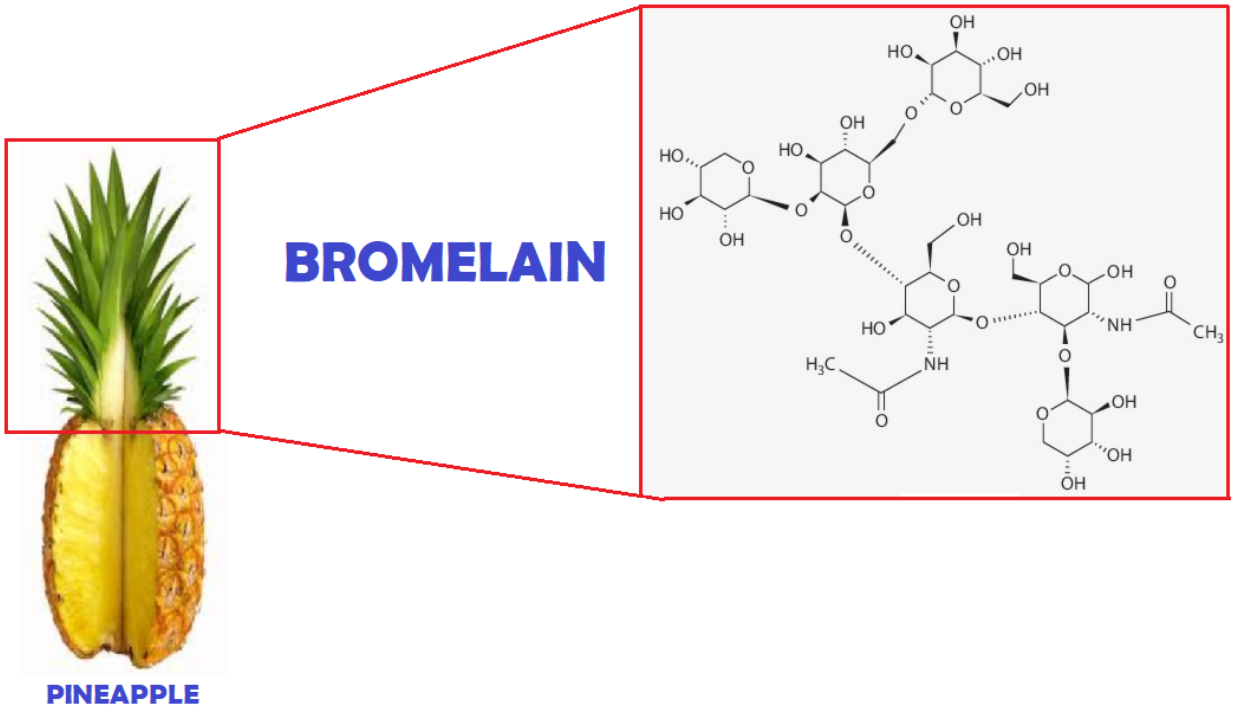

Figure 3:
Molecular structure of bromelain from pineapple stem

Stem bromelain also contains four hexosamines, and 2.1% carbohydrate, meaning it is a glycoprotein. The carbohydrate composition in stem bromelain consists of mannose, fructose, xylose, and glucosamine in the ratio of 3:1:1:4 using gas chromatography analysis (Benucci *et al*., 2011).

The molecular weight of purified stem bromelain is between 23.40 to 35.73 kDa, while fruit bromelain is 31.00 kDa. Bromelain is stable at pH 3.0 - 7.5 and once it has combined with its substrate, the activity is no longer susceptible to the effect of the pH (Grzonka *et al*., 2007). Bromelain is well absorbed from the GIT in a functionally intact form (Babagana & Bala, 2016).

THERAPEUTIC BENEFITS OF BROMELAIN
Bromelain has been widely used for the treatment of numerous pathological conditions which include inflammation, cancer, wounds, analgesic, GIT disorder, microbial infections, and as an anti-oxidant. Both experimental and clinical tests have confirmed the bioactivities of bromelain in *in-vivo* and *in-vitro* studies.

Anti-viral effects: Few studies have reported the antiviral effect of bromelain. Clinical study on acquired immune deficiency syndrome (AIDS) caused by Human immunodeficiency virus (HIV), cervical cancer caused by Human papillomavirus, and hepatitis C patients showed that bromelain substantially increases CD4+ T-cells count (Suthihono *et al*., 2011). A study involving seven HIV infected patients showed that after four months of treatment with pineapple juice, three patients had normal CD4+ T-cells count while two showed low viral count below the detection limit (400 copies/ml) (Maruli *et al*., 2014). An in-vitro study showed that bromelain (10 mg/ml, specific activity of 5.88 U/mg) could kill HIV (Suthihono *et al*., 2011). Bromelain has also been shown to be effective against poliovirus 1 (Comosus & Peel, 2013). More experimental studies are required to ascertain its actual potency and effectiveness.

Anti-inflammatory effects: Clinical and experimental studies have shown that bromelain possesses anti-inflammatory activities (Hale *et al*., 2005; Rathnavelu *et al*., 2016). Bromelain acts as a modulatory agent of cytokines. It can stimulate the release of proinflammatory cytokines in a healthy immune system in response to cellular stress (Barth *et al*., 2005). It increases the release of IL-1β, IL-6, interferon-γ (INT-γ) and TNF-α in mouse macrophages and human peripheral blood mononuclear cells (Rathnavelu *et al*., 2016). Conversely, bromelain inhibits the biosynthesis of pro-inflammatory cytokines and prostaglandins under inflammatory conditions induced by overproduction of cytokines (Onken *et al*., 2008). It reduces the expression of CD44 on the surface of immune cells that regulate lymphocyte homing and migration (Rathnavelu *et al*., 2016; Subramaniam *et al*., 2007). In our yet to be published data, it was found that bromelain inhibited brain and sciatic nerve cytokines and prostaglandin E_2 production in a peripheral model of neuropathic pain. It also showed that bromelain mitigated the activities of nuclear factor-kappa β (NF-κβ) which was significant for the production of proinflammatory cytokines (Bakare & Owoyele,

2019b). Bromelain also inhibited IL-1β, IL-6, and TNF-α in the cerebral cortex and sciatic nerve.

Likewise, Bhui and his colleagues reported that bromelain inhibited COX-2 expression in mouse skin tumorigenesis as well as upregulation of p53 and Bax. (Bhui *et al*., 2009). In their study, bromelain inhibited extracellular signal-regulated protein kinase (ERK ½), p38 mitogen-activated protein kinase (MAPK), Akt, and NF-κβ activities which are key cellular activities that enhance the production of proinflammatory cytokines that mediate inflammation and pain.

It has also been reported that bromelain ameliorated inflammation in the respiratory system (Jr *et al*., 2008). Studies showed that bromelain inhibited the infiltration of the lungs and airway by the eosinophils and leukocytes (Jr *et al*., 2008; Secor *et al*., 2005). In these studies, bromelain also modulated the activities of CD19+ B cells, and CD4+ and CD8+ T lymphocytes. The overall effect of these processes was inhibition of the development of bronchitis and respiratory distress syndrome and aiding of smooth breathing. Bromelain was also found to mitigate the development of allergic airway disease by modulating CD4+ to CD8+ T cells population (Pavan *et al*., 2012).

Antinociceptive effects: Experimental and clinical evidence showed that bromelain possesses analgesic properties. Bromelain has been constantly used as an analgesic agent for the treatment of arthritis pain, muscular pain, episiotomy pain, and perineal pain (Golezar, 2016; Majid & Al-mashhadani, 2014). It has also been reported that bromelain improves the quality of life and subsides pain after mandibular third molar surgery. Walker and his colleagues (Walker *et al*., 2002) reported bromelain to be dose-dependent in its effectiveness for subsiding mild knee pain.

Our study on the antinociceptive effect of bromelain in chronic constriction injury (CCI) model of neuropathic pain in Wistar rats showed that bromelain mitigated hyperalgesia and allodynia after twenty-one days of its administration (Bakare & Owoyele, 2020). In another study using CCI, bromelain was shown to regulate neuronal electrolytes (Ca^{2+}, Na^{+}, K^{+}, and Cl^{-}) imbalance as a mechanism of action (Bakare & Owoyele, 2019a). We also reported that bromelain serves as a good antioxidant that promotes the secretion of antioxidant enzymes (superoxide dismutase, catalase, and reduced glutathione) via increased Nuclear Factor Erythroid-Derived 2-Like 1 and 2 (NrF-1 and NrF-2) Concentration (Bakare & Owoyele, 2020). It mitigated the expression of nitric oxide synthase thereby inhibiting the production of nitric oxide and reactive nitrogen species.

Anti-coagulating effects: The anti-coagulation effect of bromelain in both *in-vivo* and *in-vitro* study have been documented in many studies. Bromelain inhibited platelet aggregation and improved ischemic/reperfusion dysfunction (Hilberg & Gla, 2006; Juhasz *et al*., 2008) and has been shown to prevent angina pectoris and transient ischemic attacks (Zengion *et al*., 2011). It prevented thrombosis formation, thrombophlebitis, and had fibrinolytic activities through increased secretion of plasmin (Errasti *et al*., 2016; Sudjarwo, 2005). It reduced the plasma kininogen level, thereby inhibiting kinins synthesis, swelling, and pains (Zengion *et al*., 2011). The overall effects of bromelain are the improvement in the cardiovascular and circulatory functions of the body system.

COULD BROMELAIN BE USEFUL IN THE MANAGEMENT OF COVID-19?:

Currently, there is no designated drug for the treatment of CoVID-19. Anti-virals, antimicrobials, anti-inflammatory drugs, and some herbal mixtures have been put into trials to curb the progression of the viral infection (Adnan *et al*., 2020). All these drugs had limited success in curing the CoVID-19. Initially, treatment was focused on relieving CoVID-19 associated pneumonia which was one of the major complications of the infection (Jin *et al*., 2020; Shen *et al*., 2020). It was subsequently affirmed that cytokines storm that results in inflammation and disseminated intravascular coagulopathy were mainly responsible for multiorgan failure (Jose & Manuel, 2019). In the bid to combat CoVID-19, novel drug needs to be formulated that will inhibit the viral replications and mitigate the development of SARS-COV-2 pathophysiological reactions in the body system.

The Anti-inflammatory and anti-coagulatory capacity of bromelain has been wildly reported (Suthihono *et al*., 2011). Bromelain inhibits pro-inflammatory cytokines especially IL-6 and TNF-α which have been reported as the hallmark of cytokine storm in CoVID-19 patients (Kakodkar & Kaka, 2020). The various physiological effects of bromelain make it one of the potential candidate drugs that could be deployed for the treatment of symptoms of CoVID-19 infection. Slowing down the advent of inflammation will prevent escalation and the progression of the disease.

Cytokines have also been reported to aid platelet aggregation resulting in thrombosis (Jose & Manuel, 2019). The mitigating effect of bromelain on pro-inflammatory cytokines will inhibit this cascade of reaction that led to platelet aggregation (Errasti *et al*., 2016). The ability of bromelain to inhibit the biosynthesis of kinins that promote the development of inflammation will further prevent the advent of cytokines storms and systemic coagulation (Secor *et al*., 2005) which causes the multiorgan failure that characterizes CoVID-19 infection. Furthermore,

bromelain has been documented to promote fibrinolysis through the plasminogen-plasmin system (Errasti *et al*., 2016). Bromelain inhibits platelet aggregation as well as increases plasmin concentration in the blood that prevents thrombosis. Thrombosis and coagulation reduces erythrocyte transportation and affects ventilation-perfusion rate in CoVID-19 patient which accounts for acute respiratory distress syndrome that the patients develop (Jose & Manuel, 2019). Treatment with bromelain may curb the coagulopathy by promoting the free flow of the blood around the circulating system.

Bromelain is a proven analgesic and neuroprotective agent (Bakare & Owoyele, 2020). CoVID-19 patients have reported experiencing headaches, fever, body weakness, and fatigue (L. Wang *et al*., 2020). Bromelain offers a wide window in abating such neurologic experience via inhibition of prostaglandins (PGE_2 in particular) and bradykinin which is a known mediator of headache and fever. Bromelain reduces oxidative stress in organs via inhibition of cellular peroxidation, nitric oxide synthase, and stimulation of antioxidant enzymes (Bakare & Owoyele, 2020).

Hence bioactivities of bromelain could limit the progression of CoVID-19. However, its combination with one or two antiviral drugs will make it more effective since the limited study has reported its antiviral activities (Suthihono *et al*., 2011). Remdesivir and/or its combination with hydroxychloroquine or other antiviral drugs has yielded the most promising results in infected patients (M. Wang *et al*., 2020). On the other hand, limited study has been conducted on the antiviral effects of bromelain. Its combination with remdesivir or other antiviral drugs may prove effective in curbing CoVID-19 infection in patients.

In conclusion, CoVID-19 is a severe viral disease caused by the SARS-COV-2 virus which results in multiorgan failure and subsequent death. Bromelain shows a lot of potentials that make it a possible candidate for the treatment of symptoms of CoVID-19 infection. Its combination with remdesivir or other anti-viral drugs may provide a golden solution to eradicate the CoVID-19 pandemic. Further research in these directions may yield a lot of dividends.

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Review Article

Immune Responses During Human Coronavirus Infection: Suggestions for Future Studies

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Summary: Severe Acute Respiratory Human Coronavirus 2 (SARS-hCOV 2) infection which began in December 2019 has rapidly disseminated worldwide due to non-availability of anti-viral treatment or vaccine, no knowledge of virus-human interaction, lack of prognostic factors for stages of illness and ability of hCoV 2 to rapidly mutate and infect multiple cell types. Host inflammation and evasion of host immune responses by viruses are believed to play major roles in disease severity of human Corona viruses (hCoVs), thus uses of anti-inflammatory and immune-boosting agents apart from complete multi-disciplinary approach are suggested to combat the ranvaging SAR-hCOV 2 infection. This paper related the structural proteins and life cycle of CoV with host immune responses to CoV. This is to bring out gaps in knowledge for possible future researches.

Keywords*: Antibodies, Coronaviruses, Inflammation, Phagocytes, Vaccine.*

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INTRODUCTION

Coronaviruses are easily spreading, enveloped, nonsegmented, positive-sense single-stranded RNA virus with unelucidated mechanisms of pathogenesis and complex host immune response (Li *et al*., 2020). The causative agent of 2019–2020 ongoing coronavirus disease 2019 (COVID-19) was discovered in January 2020 to be a novel betacoronovirus of the same subgenus as Severe Acute Respiratory Syndrome Coronavirus 2 (WHO, 2020).

Since there are no antiviral treatments or vaccine available, efforts to prevent spread of hCoV includes confinement, screening, restricted body contact and use of nose/mouth mask (UNESCO 2020). Thus, researches on coronaviruses will continue to seek understanding of CoV-host interaction which will significantly improve ability to design vaccines and reduce disease burden. This paper highlights the role of immune responses during coronavirus infection.

Structural Proteins of hCoV

Coronaviruses are enveloped non-segmented, positive-sense and single stranded RNA viruses having four major structural proteins, as follows: Spike (S), Membrane (M), Envelope (E), and Nucleocapsid (N) proteins. The S protein (approximately 150 kDa) makes up the distinctive spike structure on the surface of the virus and mediates attachment to the host receptor. In most, but not all, coronaviruses, S protein is cleaved by a host cell furin-like protease into S1 and S2 (Abraham *et al*., 1990). S1 makes up the large receptor-binding domain of the S protein while S2 forms the stalk of the spike molecule. The M protein is a small (approximately 25–30 kDa) most abundant structural protein in the virion. It has 3 transmembrane domains and is thought to give the virion its shape (Li *et al*., 2020). The E protein is small sized (approximately 8–12 kDa) highly divergent protein with ion channel activity required for pathogenesis. It facilitates assembly and release of the virus (Beniac *et al*., 2006). The N protein is present in the nucleocapsid capable of binding RNA, binds nsp3 a key component of the replicase complex, and the M protein (Chang *et al*., 2006).

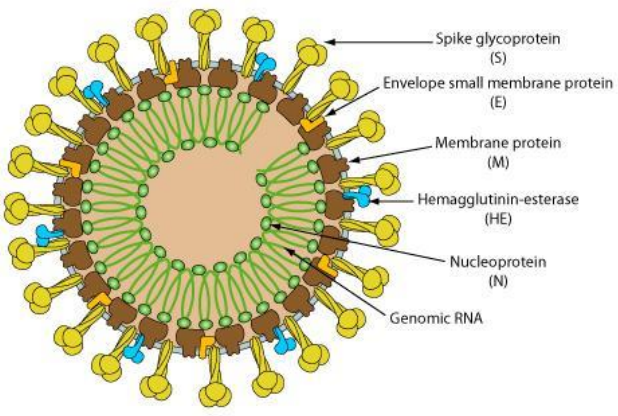

Figure 1:
Diagramatic Structure of a Corona Virus
(Source: Li et al., 2020)

A fifth structural protein, the hemagglutinin-esterase (HE), which binds sialic acids on surface glycoproteins contains acetyl-esterase activity is present in a subset of β-coronaviruses. The HE activities were proposed to enhance S protein-mediated cell entry and virus spread through the mucosa (Molenkamp and Spaan, 1997).

Life Cycle of Coronavirus

It takes 14 days from CoV infection to detection of simplest symptoms as a result of the following stages of CoV life cycle (attachment and entry into cells, replication and transcription of the sub-genomic RNAs, translation and assembly of the viral replicase complexes). Thus, blocking attachment or any of these stages of CoV cycle may be considered in treatment or preventive strategies of COVID-19. SARS-CoV is spread through infectious aerosols containing the virus by breathing, or when someone with the virus sneezes or coughs into their hands which contaminates objects other people touch or when un-infected person(s) breath in air close to an infected patient who sneezes or coughs. CoV from these sources attach to exposed surfaces especially epithelial lining of respiratory system. *A question is "why wont CoV penetrate through intact human skin, eye or mouth" The likely answer is the innate immune factors of these organs which require further investigations. However in the case of eyes, one may think of feeling the taste of an eye-drop in the mouth after dropped in the eye due the intimate link between the eye,nose and throat.*

The initial attachment of the virion to the host epithelial cell is initiated by interactions between the S protein and its receptor (Bosch *et al*., 2003). Many coronaviruses utilize peptidases for instance angiotensin-converting enzyme 2 (ACE2) as their cellular receptor which is commonly found on the surface of cells in the respiratory and digestive systems (Li *et al*., 2003). By binding ACE2, SARS-CoV leads to the downregulation of ACE2 expression (Hamming *et al*, 2004) and might therefore negate the protective effect of ACE2. Following receptor binding, the virus gain access to the host cell cytosol accomplished by acid-dependent proteolytic cleavage of S protein by a cathepsin or another protease. S protein cleavage occurs at two sites. The first cleavage separates the receptor binding domain (RBD) and fusion domains of the S protein (Belouzard *et al*., 2009) and the second cleavage exposes the fusion peptide that inserts into the membrane or within acidified endosomes. This allows for the mixing of viral and cellular membranes, resulting in fusion and ultimately release of the viral genome into the cytoplasm. The next step in the coronavirus lifecycle is the translation of replicase gene from the virion genomic RNA (Snijder *et al*., 2003; Baranov *et al*., 2005).

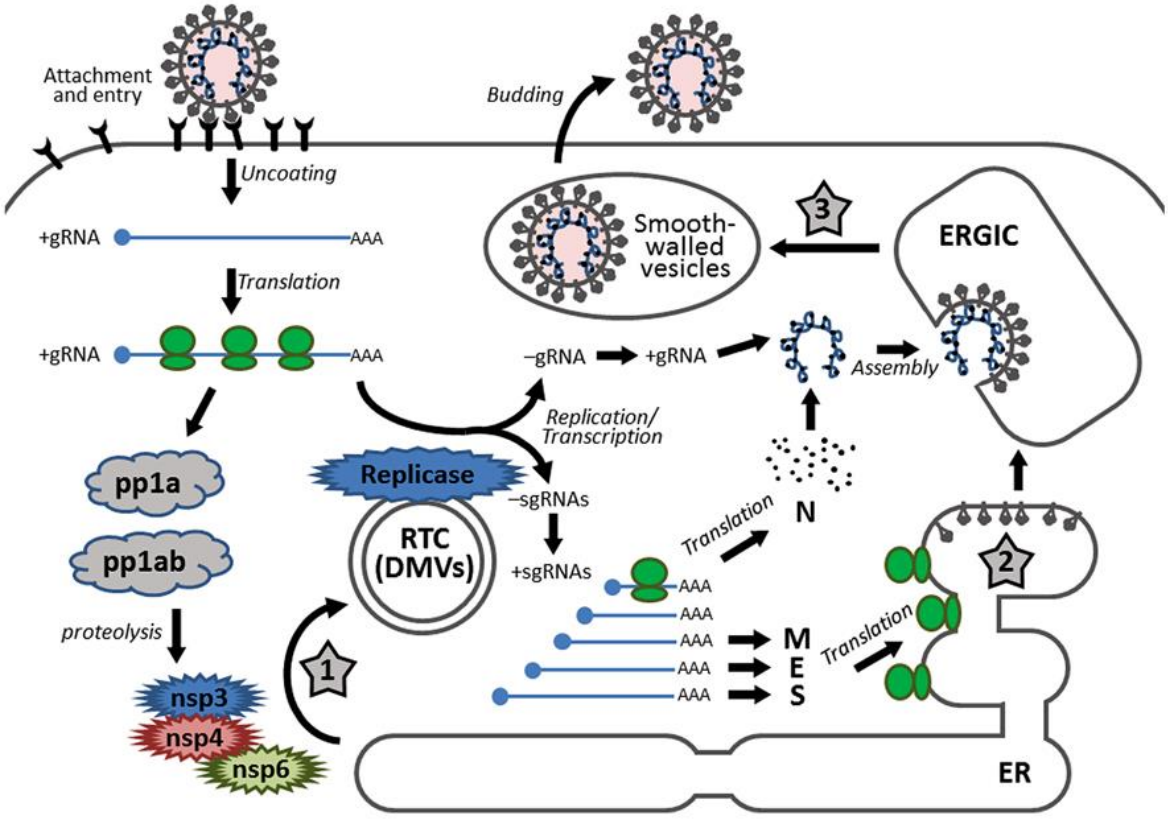

Figure 2:
Schematic Diagramatic of Life Cycle of Coronavirus.
(Source: Fung and Ding 2014).

Viral RNA synthesis produces both genomic and sub-genomic RNAs follow the translation and assembly of the viral replicase complexes.

After replication and subgenomic RNA synthesis, the viral structural proteins (S, E and M) are translated and inserted into the endoplasmic reticulum (ER). These proteins move along the secretory pathway into the endoplasmic reticulum-Golgi intermediate compartment (ERGIC) (Krijnse-Locker *et al*., 1994). There after, viral genomes encapsidated by N protein bud into membranes of the ERGIC containing viral structural proteins, forming mature virions (de Haan and Rottier 2005) After assembly, virions are transported to the cell surface in vesicles and released by exocytosis. *It is not known if the virions use the traditional pathway for transport of large cargo from the Golgi or if the virus has diverted a separate, unique pathway for its own exit.* In several coronaviruses, S protein that does not get assembled into virions transits to the cell surface where it mediates cell-cell fusion between infected cells and adjacent uninfected cells (Fehr and Perlman, 2015). This leads to the formation of giant multinucleated cells, which allows the virus to spread within an infected organism without being detected or neutralized by virus-specific antibodies.

Human Immune responses To CoV

Innate immune factors are chemical, cellular and mechanical/physical barriers that prevent entry and establishment of foreign materials (Edem and Arinola 2015). The nasal cilia filteration, mucus trapping action and muco-ciliary movements are assumed to be relevant during COVID-19, thus the advice to put on nose-mask (example of mechanical/physical barrier). It was also reported that CoV thrives on skin for few hours. *But the relevance of these innate factors requires further investigations.* Prevention of direct entry of CoV into alveoli of the lungs forms part of innate protection against entrance by CoV. However, the ability CoV to attach to the lung epithelial cell through S protein leads to formation of dsRNA of CoV during CoV replication in host cell cytoplasm (Bosch *et al*., 2003), thus S protein may form the basis for the development of a vaccine.

The host innate immune system detects dsRNA (a Pathogen-Associated Molecular Patterns, PAMPs) of CoV using Pattern Recognition Receptors (PRRs). This is followed by NF-kB activation which promotes the synthesis of type I IFNs and other proinflammatory cytokines (Schneider at al., 2014). The most studied members of the Type I family of interferons are the multiple IFNα isotypes and IFNβ. The mammalian types of IFN are designated IFN-α (alpha), IFN-β (beta), IFN-κ (kappa), IFN-δ (delta), IFN-ε (epsilon), IFN-τ (tau), IFN-ω (omega), and IFN-ζ (zeta, also known as limitin) (Cheung CY *et al*., 2005). Type I IFNs promote the release of antiviral proteins for the protection of uninfected cells, limit virus spread, and enhance macrophage phagocytosis, NKC-, T lymphocytes- and B lymphocytes- functions. Other pro-inflammatory cytokines (IL-1, IL-6, IL-8, TNF-β and MCP-1) produced by infected cells in response to CoV infection attract, retain and activate lymphocytes and leukocytes to the site of infection (Edem and Arinola 2015). *An evaluation of these factors during COVID-19 progression is desirable.*

Other PRRs involved in human immune response to CoV are Toll-like receptors TLR 2, TLR 4, mannose receptor, scavenger receptor; mannose-binding lectin and C-reactive protein (Li *et al*., 2020). This stage is associated with high fever, hypoxemia and progression to pneumonia-like symptoms despite progressive decline in virus titers (Peiris*et al*., 2003). However, hCoV evades host immune responses by reaching high titers very early after infection producing multiple proteins that inhibit IFN response and induction of T cells apoptosis (Channappanavar *et al*., 2016).

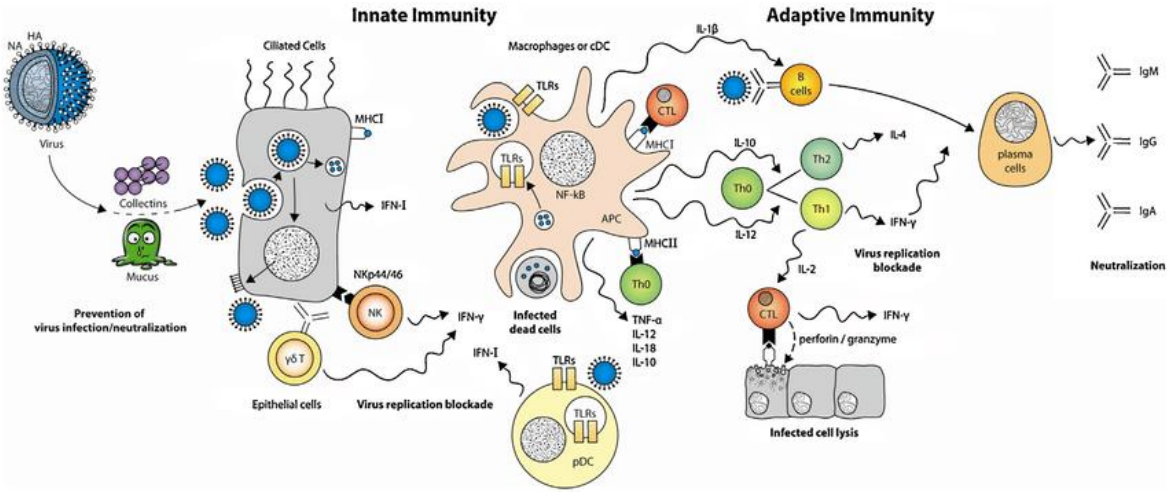

Figure 3:
Schematic Diagram of Human Immune Resposes to hCoV.
(Source: Li et al., 2020)

In the lungs, consequences of rapid virus replication and exuberant pro-inflammatory cytokine/chemokine responses are lung epithelial and endothelial cell apoptosis, compromised lung microvascular and alveolar epithelial cell barrier resulting in vascular leakage and alveolar edema resulting in hypoxia (Rodrigue-Gervais *et al*., 2014). *This may be one of the ways by which CoV leaked to blood circulation.* During this phase, there is progressive decline in virus titers and approximately 20% of patients progressed to ARDS which often resulted in death (van den Brand, 2014) proposed to have resulted from exuberant host production of inflammatory mediators including IL-6, IL-8, IL-1β, and GM-CSF, reactive oxygen species, and chemokines such as CCL2, CCL-5, IP-10, and CCL3 (Jiang Y *et al*., 2005; Zhao J *et al*., 2010; Drosten *et al*., 2013).

It is unknown whether or not people who have recovered, clear CoV infection and mounted strong immune response can get reinfected. However, two immune lung cells [alveolar macrophages (AM) and nerve/airway associated-macrophages (NAM)], four types of blood immune cells (antibody-secreting cells, follicular helper T cells, activated CD4+ T cells and CD8+ T cells) and immunoglobulin M and IgG antibodies that bound the CoV activate immunity to CoV. NAMs are distinct from other lung-resident macrophage subsets and highly express immunoregulatory functions under steady-state and inflammatory conditions (Ural *et al*., 2020). Apart from dissemination of CoV into circulation at the alveoli (Fehr and Perlman, 2015), macrophages present viral peptides to B- and T- lymphocytes leading to adaptive immunity.

A recent study reported increased antibody-secreting cells (ASCs), follicular helper T cells (T_{FH} cells), activated $CD4^+$ T cells and $CD8^+$ T cells and immunoglobulin M (IgM) and IgG antibodies that bound the COVID-19-causing coronavirus SARS-CoV-2 were detected in blood of COVID-19 patients before symptomatic recovery (Thevarajan *et al*., 2020). These immunological changes persisted for at least 7 days following full resolution of symptoms (Wu *et al*., 2007). T cells, CD4+ T cells, and CD8+ T cells particularly play a significant antiviral role by balancing the combat against pathogens (Arinola, 2003) and the risk of developing autoimmunity or overwhelming inflammation (Cecere *et al*., 2018). CD4+ T cells promote the production of virus-specific antibodies by activating T-dependent B cells. However, CD8+ T cells are cytotoxic and can kill viral infected cells. CD8+ T cells account for about 80% of total infiltrative inflammatory cells in the pulmonary interstitium in SARS-CoV-infected patients and play a vital role in clearing CoVs in infected cells and inducing immune injury (Maloir *et al*., 2018). The depletion of CD8+ T cells do not affect and delay viral replication at the time of infection with SARS-CoV (Channappanavar *et al*., 2014; Ng *et al*., 2016). Depletion of CD4+ T cells is associated with reduced pulmonary recruitment of lymphocytes and neutralizing antibody and cytokine production, resulting in a strong immune-mediated interstitial pneumonitis and delayed clearance of SARS-CoV from lungs (Chen *et al*., 2010). Additionally, T helper cells produce proinflammatory cytokines (IL-1, IL-6, IL-8, IL-21, TNF-β, and MCP-1) which recruit monocytes and neutrophils to the site of infection (Arinola *et al*., 2014). SARS-CoV-specific T cells have been screened in SARS convalescent patients. It was found that all detected memory T cell responses were directed at SARS-CoV structural proteins. Further, these reactions are found to last up to 11 years after infection (Li *et al*., 2020). Results of the research showed that the T cell response to S protein and other structural proteins (including the M and N proteins) is long-lasting and persistent. *This provides evidence for the design of the COVID-19 vaccine composed of viral structural proteins with long-term memory cell responses.*

Humoral immunity which is B-lymphocyte mediated controls the spread of infection (Olaniyi and Arinola, 2011). Reports show that humoral immunity is essential to control the persistent phase of CoV infection because antibodies isolated from patients who have survived Middle East Respiratory Syndrome Coronavirus (MERS-CoV) infection have been described (Niu *et al*, 2018a and b). In the peak period of viremia, 75% of the blood samples of patients diagnosed as SARS in the first 1 to 2 weeks before symptoms have virus RNA and circulating IgG. IgG production persists from acute SARS-CoV infection and during recovery (Wu *et al*., 2007). *This is an important subject that needs further study.*

Virus encoded proteins help them evade the detection of the Complement System, suggesting that complements are vital to the antiCoV response. C3a and C5a have potent proinflammatory properties and can recruit or activate neutrophils. Anti-C5a antibody shows protection against SARS-CoV infection activates Complement pathway (Gralinski *et al*., 2018). *These are pointers to the need to assess the levels of these humoral factors in CoV patients. Possiblity of developing monoclocal antibodies against structural or attachment strategy of CoV is worthy since* CoV uses its spike proteins as an adhesion factor to facilitate host entry through a special receptor. Human monoclonal antibody (m336) to receptor-binding region of MES coronavirus spike protein showed high neutralization activity to MES-CoV in vitro (Ying *et al*., 2014) and reduced the MES-CoV RNA titer of lung by 40 000 to 90 000 folds (Houser *et al*., 2016).

Dendritic cells (DCs) play a key role in innate immune and adaptive immune responses by effectively stimulating activation of T-lymphocytes

and B-lymphocytes (Arinola, 2003). DC precursor cells differentiate into DCs in the presence of GM-CSF, IL-4 and TNF-α (Guo *et al*., 2012). In addition, HIV-1 attenuates the major histocompatibility antigen I (MHC I) on the surface of DCs, thereby reducing the ability of DCs to present the viral antigens. HIV-1 infection enhances the expression of DC-specific intercellular adhesion molecule-3-grabbing nonintegrity (DC-SIGN), thus inhibiting CC chemokine receptor 7 (CCR7) and MHC-II, which are important receptors of DC homing (Fairman *et al*., 2012; Cardone *et al*., 2015). *These previous results indicate that CoV infection may interfere with the differentiation and function of DCs*.

Conclusion and suggestions for Further studies

CoVs infection is a periodical unpredictably threat to human life which spread rapidly. Of more concern is lack of approved vaccines or drugs for the treatment of CoV infections. To fulfill the pressing need of effective therapeutic measures, targeted immunotherapy and vaccine development, there is need for further elucidation of host immune responses during hCoV infection. This paper vindicates that inflammation is heightened during hCoV infection, therefore therapeutic interventions to control the inflammatory processes may be useful in the management of hCoV infection. More importantly, startegies directed at reducing the viral load at early stage of hCoV infection is strongly advocated.

Future studies should develop strategies that (a). Develops monoclonal antibodies against structural and non-structural proteins or blocks receptor sites. (b). Improves the understanding of pathways and mediators of inflammation during hCoV-infection in relation to the timing of therapeutic interventions. (c). Determines the roles of antioxidants and inflammatory agents in amelioration of symptoms during hCoV infection. (d). Determines host genetic factors which have been directly implicated in hCoV infection e.g characterise the polymorphisms of host Cathepsin, C3a and C5a or MHC Class 1 gene products (e). Creates forum for interdisciplinary collaborative researches between all professionals. (f). Usefulness or otherwise of surfactants and defensin, salivary and skin proteins during CoV infections.

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Research Article

Mechanisms of Enhanced Vascular Smooth Muscle Contraction Induced by Sickle Erythrocyte Constituents

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Summary: The mechanisms of the increased vascular tone associated with vaso-occlusive crisis of sickle cell disease have not been clearly defined. The goal of the present study was to examine the role of vascular smooth muscle membrane Na+-K+-ATPase enzyme activity as well as nitric oxide synthase inhibition on the contractile responses induced by sickle erythrocyte constituents. 2 mm ring segments of rabbit carotid arterial ring preparations were placed in 20 ml organ baths containing physiological salt solution (PSS) bubbled with 95% O2, 5% CO2, at 37oC and pH 7.4 and isometric contractions recorded, under an initial load of 2g. Arterial rings were exposed to 50 µl of each erythrocyte constituent at an adjusted haematocrit of 0.6. The magnitude of K+-induced relaxation of 10-7 M phenylephrine (PE)-precontracted rings exposed for 30 minutes to K+-free PSS (which inhibits Na+-K+ pump) was estimated in the absence (control) or presence of RBC constituents (ghosts, erythrocytes or haemoglobin solution) from Hb SS subjects. Secondly, the influence of 20-minute exposure of the rings to SS GHOSTS on acetylcholine-induced, endothelium-dependent relaxation of 10-7 M PE phenylephrine-precontraction (in the absence or presence of L-NAME) was evaluated. Our results show that K+-induced relaxation was significantly and differentially attenuated by erythrocyte constituents ($p<0.05$) in the order: SS GHOST > SS HBS > SS RBC. NO synthase inhibition with L-NAME further potentiated the enhanced PE contractions induced by SS GHOSTS and caused a greater attenuation of Ach-induced relaxation (compared with SS GHOSTS alone). The results suggest that SS erythrocyte GHOSTS induce enhancement of vascular smooth muscle tone via impairment of vascular Na+-K+ ATPase enzyme activity as well as attenuate endothelium-dependent relaxation. These functional changes in vascular smooth muscle and endothelial function may contribute to the pathophysiology of vaso-occlusive crisis of sickle cell disease.

Keywords*: Vaso-occlusive crisis, sickle cell disease, Na-K pump, erythrocytes, SS Ghost, nitric oxide, L-NAME.*

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INTRODUCTION

Vascular homeostasis is maintained by the endothelium through the release of endothelium derived relaxing factors (EDRF) including nitric oxide (NO), prostaglandins and endothelium derived hyperpolarizing factor (EDHF) (Reiter and Gladwin, 2003). The released NO could also open K+ channels (Feletou and Vanhoutte, 2006) contributing to the maintenance of adequate vascular function. In many vascular beds, intermediate and small conductance calcium-activated potassium channels play a prominent role in initiating hyperpolarization and modulating electrical conduction along the endothelium (Edwards *et al*., 2010). There is evidence that these channels play a role in the modulation of endothelial calcium signalling and nitric oxide release (Stankevicius *et al*., 2006). K+ channel opening hyperpolarizes smooth muscle, which, leads to vasodilatation by decreasing calcium entry through voltage-dependent Ca2+ channels (Nelson and Quayle, 1995). It was suggested that the relaxation response induced by high concentration of Ca2+ ions in rabbit aortic smooth muscle is endothelium-dependent and possibly mediated by the NO-guanylyl cyclase pathway (Azubuike-Osu and Ebeigbe, 2015). Tonic reduction of smooth muscle tone in vivo is the resultant effect of the vascular release of nitric oxide otherwise called endothelium derived relaxing factor (EDRF) (Furchgott and Zawadski, 1980; Ahmad *et al*., 2018). NO is a labile substance with a half-life of 4-50 seconds (Ebeigbe *et al*., 1990). Nitric oxide (NO) is synthesized from L-arginine by a nitric oxide synthase of the endothelial form in vascular endothelial cells (Tejero *et al*., 2019). Nitric oxide is involved in the regulation of many physiological and pathophysiological functions, including smooth muscle relaxation, platelet inhibition and immune regulation (Nussler and Billiar, 1993; Tykocki *et al*., 2017). NO induces vascular smooth muscle relaxation through the activation of guanylate cyclase leading to the accumulation of guanosine 3',5'-cyclic

monophosphate (cyclic GMP) (Moncada and Higgs, 1993).

Webb and Bohr, (1978) assessed the degree of activity of Na+/K+-ATPase in vascular smooth muscles using potassium-induced relaxations. The endothelium-independent relaxations and hyperpolarizations obtained by re-admitting potassium ions after incubation in potassium-free solution suggest the presence of electrogenic sodium pumping in the smooth muscle of the rat mesenteric artery (Weston *et al*., 2002). There is evidence that Na+-K+ pump activation inhibits Ca2+ mobilization in endothelial cells and endothelium-dependent relaxation (Seol *et al*., 2004) while sodium calcium exchanger contributes to the endothelium-dependent control of vascular contractility (Schneider *et al*., 2002). Stimulation of enzymatic activity of Na+ -K+ ATPase by adenosine 3':5'-cyclic monophosphate (cyclic AMP) may lead to generation of the Na+ gradient necessary to exude Ca2+ via the Na+/Ca2+ exchanger or hyperpolarization of the membrane. An increase in Na+-K+ ATPase activity may induce smooth muscle relaxation by increasing Na+/Ca2+ exchange and reducing the Ca2+ influx through membrane potential-dependent calcium channels (Clausen and Nielsen, 1994). Bondarenko and Sagach, (2006) described interactions between the Na+- K+- pump and relaxations induced by acetylcholine. Ouabain inhibits the Na+-K+-ATPase (Therien and Blostein, 2000) and also induces an intracellular increase in Na+ and Ca2+ concentrations through the inhibition of the Na+/Ca2+-exchanger leading to an increment in vascular tone (Schoner, 2000).

Nitrosylated L-arginine derivatives are mostly used as inhibitors of nitric oxide synthase (Ea-Kim *et al*., 1992). The synthesis of nitric oxide is inhibited by guanidine-substituted L-arginine analogues e.g. L-NNA, L-NAME or L- NMMA. Arginine based nitric oxide synthase (NO) inhibitors constrict isolated, pressurized blood vessels having spontaneous myogenic tone in the absence of intraluminal flow (Undavia *et al*., 2003, Bai *et al*., 2004). The administration of L-arginine analogues in vitro results in a marked inhibition of endothelium dependent relaxations to various agonists including acetylcholine. In vivo, the systemic administration of either L-NMMA or L-NAME causes dose-dependent hypertension and regional vasoconstriction (Rees *et al*., 1990, Gardiner *et al*., 1990) and these effects are attributable to the inhibition of the basal release of nitric oxide from vascular endothelial cells. Feelisch *et al*., (1993) suggested the occurrence of the presynaptic effects of nitric oxide following the release of noradrenaline from sympathetic nerves.

Ghosts are fragmented red blood cell membranes. The red blood cell membrane is composed of: the glycocalyx which is rich in carbohydrates, the lipid bilayer which contains transmembrane proteins and the membrane skeleton which is a structural network of proteins located in the inner surface of the lipid bilayer. Increased wall shear stress, adhesion and the interaction between sickle red blood cells and endothelial cells including an increased viscosity and low oxygen tension are hall marks of endothelial dysfunction in sickle cell disease (Quyyumi *et al*., 1997). According to Stuart *et al*., (1999), the occurrence of vaso-occlusive crisis in sickle cell disease may be result from the increased interaction between sickle erythrocytes and the vascular endothelium. Following intravascular haemolysis is the impairment of nitric oxide bioavailability that results in a diminished blood flow, regional vasoconstriction and a remodelling of the blood vessel (Kato *et al*., 2017). Reduced deformability of red blood cells and accelerated de-oxygenation rates may reduce nitric oxide bioavailability, diminish vasodilatation and oxygen supply (Subashinghe and Spence, 2008). Very importantly, Mosseri *et al*., (1993) reported the attenuation of endothelial nitric oxide-dependent acetylcholine-induced relaxation by sickle erythrocytes. In a previous communication, we have reported a greater red blood cell- induced enhancement of histamine contractions when compared with phenylephrine (in AS and SS haemoglobin genotypes) which suggest a possible role for histamine in the increased vascular tone and vaso-occlusive crisis in sickle cell disease (Azubuike-Osu *et al*., 2017). However, the mechanisms of sodium potassium ATPase and nitric oxide synthase inhibition in sickle cell disease have not been examined. The goal of this study was to establish the mechanisms by which sodium potassium ATPase and nitric oxide synthase inhibition modulate contractile responses following exposure to sickle erythrocyte constituents.

MATERIALS AND METHODS

Blood Samples: Blood samples were obtained from sickle cell subjects attending the University of Benin Teaching Hospital. Erythrocytes (RBCs) were prepared according to the method of Caughley and Watkins, (1985) and as modified by Ajayi and Ebeigbe, (2014) by washing with normal saline, to obtain a clear supernatant. The cells were re-suspended to make up 6-8% packed cell volume. Pre-washed erythrocytes were mixed with distilled water and centrifuged. The supernatant obtained is the haemoglobin solution. To prepare erythrocyte ghosts, 1 ml of blood sample was mixed with 1 ml of distilled water and spun slowly in a centrifuge (1000 rev/min) for 5 minutes. The supernatant was decanted and the resulting sediments washed with normal saline (Ajayi and Ebeigbe, 2014).

Preparation of Arterial Rings: Segments of the carotid arteries were obtained from freshly sacrificed, New Zealand rabbits, cleaned free of adhering connective tissues and cut into 2 mm ring segments. The rings were placed between L-shaped wire loops and suspended in 20 ml organ baths containing physiological salt solution (PSS). The lower loop was attached to the base of the organ bath while the upper end was attached to a Grass model FT03 force transducer connected to a Grass model 7P polygraph (Grass Instrument Co, Quincy, MA, USA). The composition of the normal PSS was (mM): 119 NaCl, 4.7 KCl, 1.6 CaCl2, 1.2 MgSO4, 1.2 KH2PO4, 24.9 NaHCO3 and 11.5 glucose and the composition of K+ free PSS was (mM): 123.7 NaCl, 0 KCl, 1.2 NaH2PO4, 1.2 MgSO4, 1.6 CaCl2, 24.9 NaHCO3 and 11.5 glucose. The PSS was bubbled with 95% O2-5% CO2 gas mixture. The rings were given an initial load of 2g, at 37oC and pH 7.4 and were allowed to equilibrate for 90 minutes.

Experimental protocol:
Two protocols were examined in this study:

K^+-induced relaxation: The rabbit carotid rings were exposed to K+-free PSS in the absence (control) or presence of RBC constituents (ghosts, erythrocytes or haemoglobin solution) from Hb SS subjects for 30 minutes and contracted with 10-7 M PE. Thereafter, re-introduction of K+ (5 mM) to the bath caused relaxation due to increased Na-K pump activity and hyperpolarization of the membrane. The influence of erythrocyte constituents from Hb genotype SS subjects on the sodium-potassium pump activity was examined by estimating the magnitude of such K+-induced relaxation in control rings as well as in rings exposed (separately), to erythrocyte ghosts, red blood cells and haemoglobin solution.

Nitric oxide synthase inhibition: The rabbit carotid rings were exposed to normal PSS in the absence (control) or presence of SS Ghosts for 30 minutes, with or without 10-5 M L-NAME (10-minute exposure). Thereafter, rings were precontracted with EC70 (M) Phenylephrine. Dose-response tests to phenylephrine and acetylcholine were carried out in control rings and following exposure to SS Ghosts. Contractile responses to PE were obtained by cumulative additions of the drug to the organ bath; the next higher concentration was added when response to the previous concentration had stabilized.
Endothelium-dependent acetylcholine-induced relaxation responses were obtained by addition of 10-5M Ach to rings pre-contracted with EC70 (M) Phenylephrine in control rings as well as in rings exposed to SS Ghosts, with or without L-NAME.

Data analysis
Results are presented as means ± SEM. Comparison of the means was done using student's t-test and the MicroCal Origin 5.0 software. A p value < 0.05 was considered statistically significant. EC70 and IC50 (M) values represent the concentrations which produced 70% contraction and 50% inhibition, respectively.

Chemicals: The chemicals used were phenylephrine hydrochloride, acetylcholine, N G-nitro-L-arginine methyl ester (L-NAME), salts, all purchased from Sigma Aldrich.

RESULTS

Dose response to Phenylephrine and Acetylcholine
Cumulative increases in phenylephrine concentrations resulted in concentration-dependent contractions which were significantly enhanced in rings exposed to SS Ghosts (Fig. 1) when compared with control rings that were not exposed to the ghosts. On the other hand, the endothelium-dependent relaxation responses induced by acetylcholine were significantly attenuated following exposure of the rings to SS Ghosts.

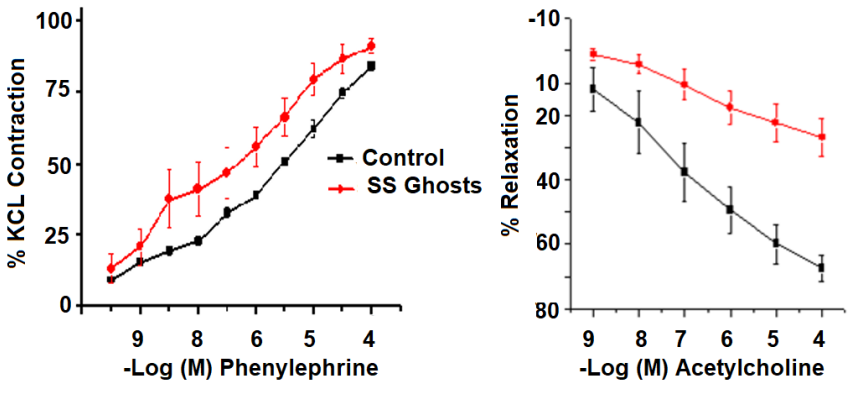

Figure 1:
Concentration-response curves for phenylephrine (PE) contraction (left) and acetylcholine-induced relaxation (right) of rabbit carotid arterial rings in the absence of ghosts (control, n=8; n=7) and following exposure to erythrocyte ghosts from Hb SS subjects (n= 6). Results are expressed as means ± SEM. PE contractions were significantly enhanced by ghosts from Hb SS subjects particularly, at higher doses while SS ghosts attenuated acetylcholine relaxation significantly when compared to the control, $p < 0.05$.

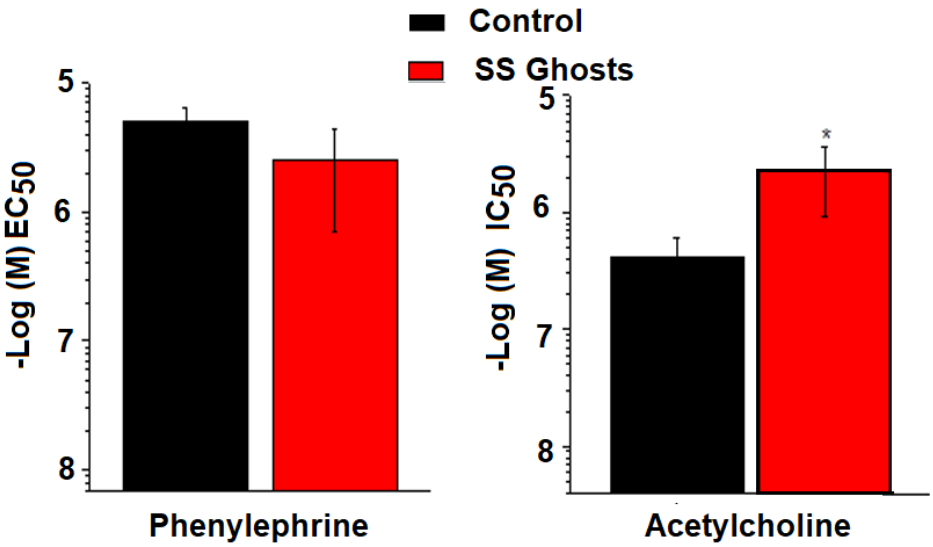

Figure 2:
Comparison of the mean EC_{50} (M) values of phenylephrine contraction and IC_{50} of acetylcholine-induced relaxation in the absence of SS Ghosts (control, n=7; n=8) and following exposure to SS Ghosts (n=6; n=7). While exposure to SS Ghosts enhanced phenylephrine contraction (lower EC_{50}); SS Ghosts significantly attenuated relaxation responses to acetylcholine. Asterisks* denote significant differences, $p < 0.05$.

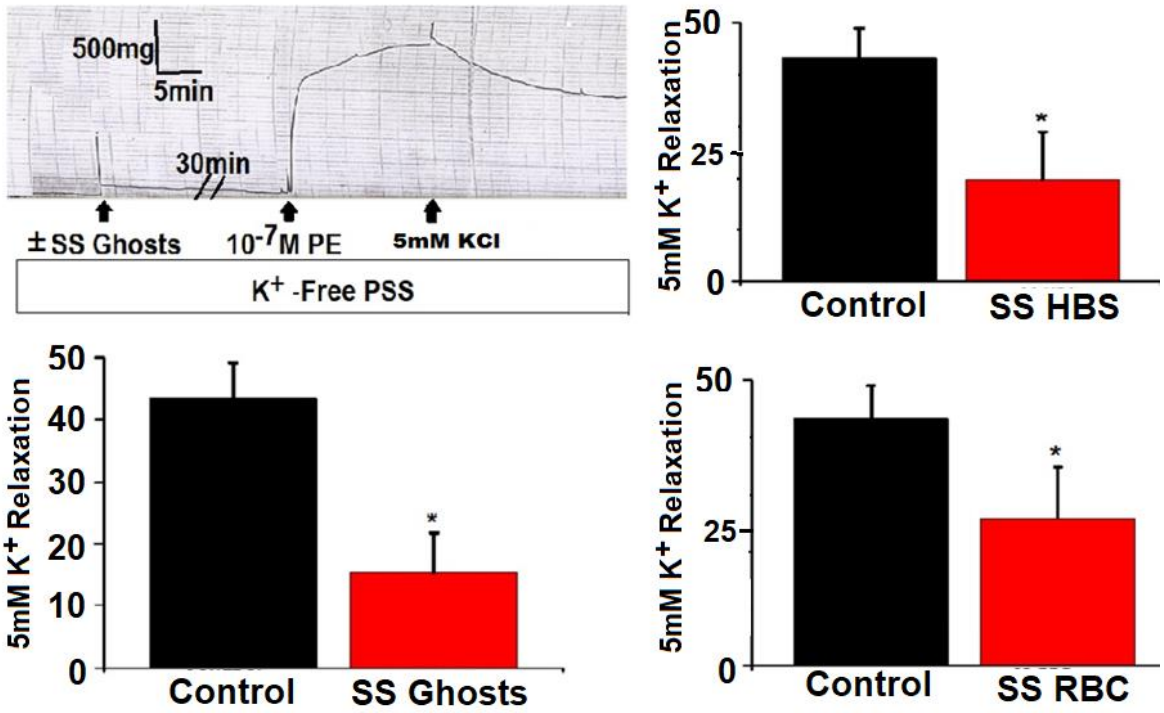

Figure 3:
The tracing (top left) explains the experimental protocol using SS Ghost as an example. The histograms show the magnitudes of 5 mM K^+-induced-relaxation responses of 10^{-7} M PE pre-contracted rabbit carotid arterial rings in the absence of erythrocyte constituents (control, n=7) and following exposure to SS Ghosts, SS HBS and SS RBC from HbSS subjects in K^+ free PSS; n=13, 8, 6 respectively. All data were expressed as means ± SEM. *denotes significant difference, $p < 0.05$.

The respective EC_{50} and IC_{50} values for phenylephrine contraction and acetylcholine relaxation (in the absence or presence of SS Ghosts) are shown in Fig. 2.

Potassium-induced Relaxation: The protocol for studying potassium-induced relaxation is illustrated in the tracing on Figure 3 which shows a typical experimental recording. Re-introduction of 5mM K+ to rings precontracted with PE in K+-free medium results in rapid relaxations. The influence of erythrocyte constituents (SS Hb solution, SS RBC and SS Ghosts) on the magnitude of relaxation responses induced by re-introduction of 5mM K+ (during K+-free exposure) is summarized in the histograms below. K+-induced relaxation responses were attenuated in the order: SS Ghosts > SS HbS > SS RBC.

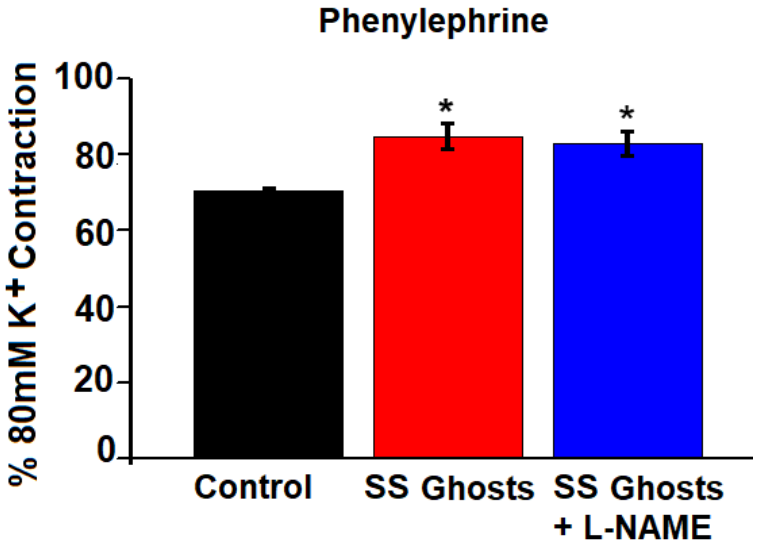

Fig. 4: Contractile responses to 10^{-7} M phenylephrine (PE) in the absence of SS Ghosts (control, n=8), following exposure to ghosts from Hb SS subjects (SS Ghost, n=7) and following exposure to both SS Ghosts and 10^{-5} M L-NAME (SS Ghosts + L-NAME, n=6). *denotes significant difference, $p < 0.05$. Both SS Ghosts and SS Ghosts + L-NAME significantly enhanced PE contractions. Values are means ± SEM.

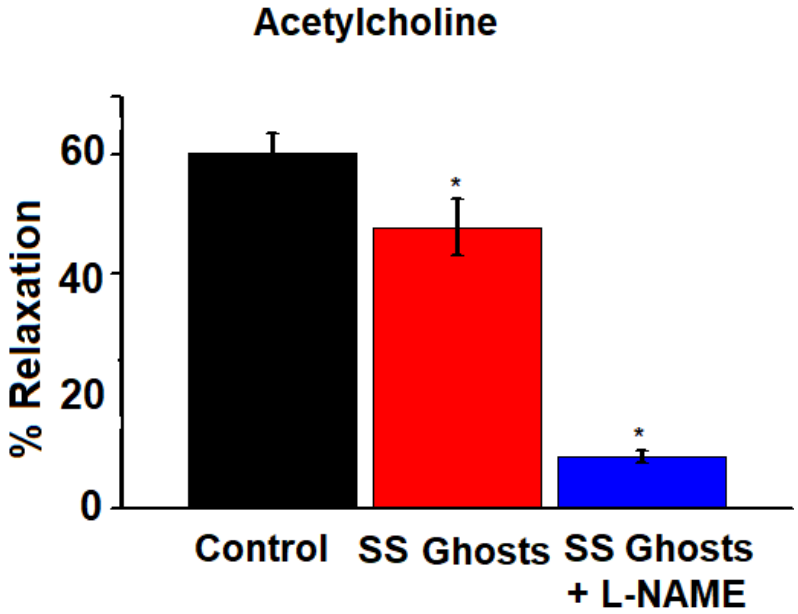

Fig. 5: Relaxation responses to 10^{-5} M acetylcholine in carotid arterial rings precontracted with 10^{-7} M phenylephrine, in the absence of blood constituents (control, n=8), in the presence of SS Ghosts, n=7 and following exposure to both SS Ghosts and L-NAME, n=6. The relaxation responses were significantly attenuated by SS Ghosts + L-NAME. *denotes significant difference, $p < 0.05$. Values are as means ± SEM.

NO Synthase Inhibition: Since SS Ghosts elicited the greatest attenuation of K^+-induced relaxation as compared with SS RBC and SS HbS, we further examined the effects of SS Ghosts and NO synthase inhibition with L-NAME, on phenylephrine contraction as well as endothelium-dependent acetylcholine-induced relaxation responses. L-NAME enhanced the increased contraction induced by SS Ghosts (Fig. 4) but further attenuated the decreased acetylcholine-induced relaxation responses induced by SS Ghosts (Fig. 5).

DISCUSSION

The results of the present study provide support for the notion that enhanced vascular smooth muscle tone as well as impaired endothelium-dependent relaxation following exposure of carotid arterial smooth muscle to erythrocyte constituents, may contribute to the pathophysiology of vaso-occlusive crisis in sickle cell disease.

Vasoactive agents that modulate vascular smooth muscle tone have been well reported to act via the vasoconstrictor or vasorelaxation pathway (Webb, 2003). In this study, we have employed two (2)

protocols: contractile response to phenylephrine as well as relaxation response to acetylcholine and K^+, to examine possible mechanisms by which erythrocyte constituents may alter the reactivity of rabbit carotid arterial smooth muscle.

The enhancement of phenylephrine contractions (Figs. 1 and 4) by exposure of the rings to SS Ghosts and the further potentiation by inhibition of NO synthase with (ω)-nitro-L-arginine methyl ester (L-NAME) suggests that SS Ghosts and reduced NO levels/availability work synergistically to elicit increased vascular tone. L-NAME is known to inhibit the synthesis of nitric oxide by inhibiting nitric oxide synthase (Rees *et al*., 1990). As reported by Ea-Kim *et al*., (1992), inhibition of NO can induce an endothelium-dependent and enantiomerically specific contraction of the vascular smooth muscle, confirming that there is a continuous use for L-arginine for the basal release of NO. Endothelial dysfunction that occurs in sickle cell disease (SCD) may prevent the arteries of patients with SCD from adapting to chronic or acute shear stress elevations (Belhassen *et al*., 2000) hence sickle red blood cell membranes are less deformable. The deformability of red blood cell membranes depends on cellular properties like surface to volume ratio, intracellular calcium concentration, activation of calcium ATPase, sodium-potassium ATPase activation, pH or messenger like prostaglandins and importantly, nitric oxide (Bruckdorfer, 2005).

As shown in Fig. 3 (tracing), K^+-free exposure blocks the Na-K pump, resulting in increased intracellular Na^+ and depolarization, increased calcium influx and contraction. Potassium-induced relaxation following K^+-free exposure results from electrogenic Na^+ pumping and hyperpolarization (Webb and Bohr, 1978) and is an indirect indicator of the Na^+-K^+ ATPase enzyme activity. The Na-K pump is blocked by the cardiac glycoside, ouabain as well as by exposure to K^+-free medium. Studies by various workers have established a relationship between Na^+-K^+ATPase activity, endothelium-dependent relaxation and intracellular calcium concentration. McCaron and Halpern, (1990) reported that Na^+-K^+ pump activation relaxes vascular smooth muscle by hyperpolarizing the membrane. Na^+-K^+ pump inhibition contracts vascular smooth muscle through activating the reverse mode of the Na^+/Ca^{2+} exchanger by Na^+ accumulation in the myoplasm (Fernandez-Alfonso *et al*., 1992). Woolfson and Poston, (1991) reported that Na^+-K^+ pump inhibition affects the synthesis or release of endothelium-derived relaxing factors. Observations from the present study interestingly show that impairment of Na^+-K^+ pump was greatest in rabbit carotid arterial rings exposed to SS Ghosts in comparison with SS HbS and SS RBC. The results also suggest that SS erythrocyte Ghosts mediate the impairment of vascular Na^+-K^+ATPase enzyme activity as well as endothelial dysfunction in sickle cell disease.

The greater attenuation by a combination of SS Ghosts and L-NAME of acetylcholine-induced relaxation is in line with a possible effect of SS Ghosts in mediating endothelial dysfunction in sickle cell disease. It is therefore reasonable to suggest that SS Ghosts inactivate NO function by impairing nitric oxide synthase, thus, providing a role for nitric oxide in modulating vascular reactivity changes induced by exposure to sickle cell ghosts.

In conclusion, our study suggests that sickle erythrocyte ghosts might increase vasoconstriction and vasospasm that characterize vaso-occlusive crisis in sickle cell disease by not only impairing vascular Na^+-K^+ATPase enzyme activity but also by impairment of vascular endothelial function.

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Research Article

Relaxation Responses of Ketamine and Propofol to Vasoactive Agents in Streptozotocin-Induced Diabetic Rats

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Summary: Diabetes mellitus (DM) is a major risk factor for the development of endothelial dysfunction which affects the ability of blood vessels to regulate vascular tone. The study aimed to investigate the mechanisms of vasodilator action of the anaesthetic agents ketamine and propofol in diabetic rat aorta. 30 male Sprague-Dawley rats were randomly divided into two equal groups: (i) non-diabetic control (ii) Streptozotocin-induced diabetic group. DM was induced by a single intra-peritoneal injection of streptozotocin at 50 mg/kg body weight. Blood samples were taken from the tail vein after 24 hours and tested for glucose level using an automated glucose analyser. A blood glucose ≥10 mmol/L confirmed hyperglycaemia and the development of DM. Rats were sacrificed, and the aortae excised. The vascular responses of aortic rings from both groups to ketamine, propofol in the presence of vasoactive agents were studied using standard organ bath procedures. Ketamine and propofol reduced Phe-induced contraction similarly in the diabetic and control groups. Barium chloride, attenuated the relaxation response to propofol in diabetic aorta when compared to ketamine. 4-aminopyridine significantly attenuated the relaxation response to ketamine and propofol in diabetic aorta. Glibenclamide, significantly reduced ketamine-induced relaxation in diabetic aorta when compared to propofol. Activation of K+ channels with nicorandil or NS1619 did not affect the relaxation response to ketamine or propofol in diabetic aorta. The results recommend that propofol can be effective in mitigating the consequences of hemodynamic instability in glibenclamide treated diabetics when compared to ketamine. This response is mediated by propofol-induced inhibition of intracellular calcium influx..

Keywords*: Ketamine, propofol, Vascular reactivity, Potassium Channel Modulators, Diabetes.*

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INTRODUCTION

Diabetes mellitus (DM) and the subsequent development of vascular dysfunction, is one of the leading causes of increased morbidity and mortality among affected humans and animals (Dhanavathy, 2015; Ding, & Tringle, 2005). The prolonged hyperglycaemia results in damage to organs including eyes, heart, kidneys and blood vessels, leading to increase vascular tone, which may also arise due to oxidative stress (Liwa *et al*., 2017; Reid *et al*., 2018). These are high risk factors that must be considered for the diabetic patients undergoing surgery due to hemodynamic instability and the high morbidity and mortality.

The anaesthetic induction agent propofol, produces vasodilation which appears to be caused by blockade of voltage-gated influx of extracellular calcium, independent of the endothelium (Chan *et al*., 2000; Kim *et al*., 2007; Schulingkamp *et al*., 2005), and endothelium-dependent mechanisms (Hao *et al*., 2017; Zhu *et al*., 2001). The vasodilatory effect of propofol is attributed to its ability to cause the production and release of NO from endothelial cells (Schulingkamp *et al*., 2005). On the other hand, ketamine, another anaesthetic agent, is known to cause vasoconstriction through depolarization of the voltage gated (Kv) potassium ion channels (Kim *et al*., 2007). Prior to this vasoconstriction phase, relaxation usually occurs but is short-lived. Its vascular relaxation effect is also independent of the endothelium and is thought to be due partly to alteration in calcium influx (Ibeawuchi *et al*., 2008).

Additionally, other studies have reported that ketamine directly inhibits KATP channel activities in non-diabetic tissues (Kawano *et al*., 2005, 2010). Interestingly, glibenclamide also inhibits KATP channel activities (Jackson, 2000), and is a treatment option for DM (Gribble and Reimann, 2003). Since diabetes affects potassium ion channels and ketamine inhibits KATP channels, it is important to understand if there is exaggeration in the vascular responses to ketamine when glibenclamide is the treatment option in DM.

MATERIALS AND METHODS

Experimental Animals; Male Sprague- Dawley rats (8-10 weeks old) weighing between 170g and 230g were obtained from the Animal House following ethical approval by the FMS/UWI Ethics Committee. Thirty rats were randomly assigned to two groups: (i) Streptozotocin (STZ)-treated, DM rats and (ii) Non-diabetic (control) rats. All animals were fed with standard rat chow and water *ad libitum*

Induction of Diabetes Mellitus; Sprague-Dawley rats assigned to the DM group were given a single intraperitoneal injection (i.p.) of 50 mg/kg body weight STZ (Sigma-Aldrich) dissolved in distilled water, according to previous method used (Dhanavathy, 2015). Basal glucose levels of all experimental animals were determined using an automated glucose analyser (Glucometer Acu-check mini plus, Roche, Germany) and blood glucose levels were assessed at 24-hour intervals following STZ injection. DM was confirmed when blood glucose level was equal to or greater than 10 mmol/L.

Preparation of Aortic Rings; STZ-induced diabetic and non-diabetic (control) rats were sacrificed by cervical dislocation and the thoracic aorta excised and placed in cold (4^0C) physiological Kreb's solution (PSS). Each aorta was cleaned of adhering fat and connective tissues and then cut into rings of 2-3 mm in length. Aortic rings were transferred to an organ bath containing PSS with the following composition (mM): NaCl, 112; KCl 5: $CaCl_2$, 1.8; $MgCl_2$, 1 $NaHCO_3$, 25; KH_2PO_4, 0.5; Glucose 10; and a mixture of 5% carbon dioxide and 95% oxygen at 37ºC passed into the solution to achieve and maintain a pH of 7.4. Aortic ring segments were mounted between two stainless steel wires at optimal length for isometric tension recording, utilizing an isometric force transducer (SS12LA, Biopac Systems Inc., Goleta, CA, USA) connected to a data acquisition unit (Biopac BSL PRO 7 computer software). The tissues were given a passive 1gram tension and allowed to equilibrate for at least 60 minutes. The aortic rings were then equilibrated for another 90 minutes while being rinsed with PSS every 10 minutes (Nwokocha *et al*., 2011, 2012). Following the equilibration period, 10^{-10}-10^{-4} phenylephrine (Phe from Sigma-Aldrich) was added cumulatively to the organ bath until a maximal contracted response was achieved. This plateau response was attained before any further additions of drugs. The ED_{70} concentration (10^{-6} M) obtained for Phe was determined and used throughout the remainder of the experiment.

When contracted rings had attained a steady state plateau, relaxation responses were recorded using cumulative concentrations of one of the following drugs: 10^{-10}-10^{-4} M Acetylcholine (ACh, Sigma-Aldrich), 10^{-9}-10^{-1} M ketamine (Sigma-Aldrich)), 10^{-9}-10^{-1} M propofol (Sigma-Aldrich), 10^{-4} M barium chloride (British Drug House Ltd), 10^{-3} M 4-AP (Sigma-Aldrich), 10^{-5} M glibenclamide (Sigma-Aldrich). Each concentration was added to the organ bath after the previous concentration showed no further change in tissue tension, that is, a steady state had been attained.

Data Analysis; Data were expressed as the mean tension ± SEM. The contractions induced by cumulative concentration of phenylephrine were expressed in terms of percentage of the maximum contraction obtained; while relaxation to the vasoactive agents were expressed as a percentage of the initial tension induced by Phe and the potassium channel modulators. Concentration response curves of each investigated drug were constructed after initial concentration reached a plateau. Statistical analysis of the data was performed using ANOVA, Repeated Measures of ANOVA and Post Hoc test. The Student's T-test was used where appropriate. A p-value <0.05 was considered significant.

RESULTS

Effect of Phenylephrine on Vascular Relaxation Responses to Ketamine and Propofol in Rat Aortic Rings.: Both ketamine (10^{-9} - 10^{-1} M) and propofol (10^{-9} - 10^{-1} M) significantly ($p< 0.5$) reduced contraction induced by Phe (10^{-6} M) at concentrations of 10^{-5} M and higher. However, there were no significant difference ($p> 0.5$) in the relaxation responses to either ketamine and propofol in diabetic group when compared to control group (Figures 1A and 1B). The values of pEC50 for ketamine (control vs. diabetic) were 3.90 ± 0.18 vs. 3.67 ± 0.09. Propofol, though not statistically significant, showed greater attenuation in relaxation in the diabetic group than ketamine in the same group. The pEC50 values were 5.05 ± 0.31 vs. 3.72 ± 0.04.

Effect of Barium Chloride on Vascular Relaxation Response to Ketamine and Propofol in Rat Aortic Rings: Relaxation response to ketamine following barium chloride-induced contraction at concentration (10^{-4} M), showed no significant difference in diabetic rat aorta when compared to the non-diabetic controls. The pEC50 values for control vs. diabetic were 3.55 ± 0.15 vs. 3.33 ± 0.20 as shown in figure 2A. However, the relaxation response to propofol showed significant attenuation in diabetic rat aortic rings compared to control. The pEC50 values control vs. diabetic were 4.77 ± 0.22 vs. 3.60 ± 0.14, p-value ≤ as shown in figure 2B. At concentration of 10-7 M, there was no significant difference with relaxation to propofol in diabetic when compared to control ($p>0.05$).

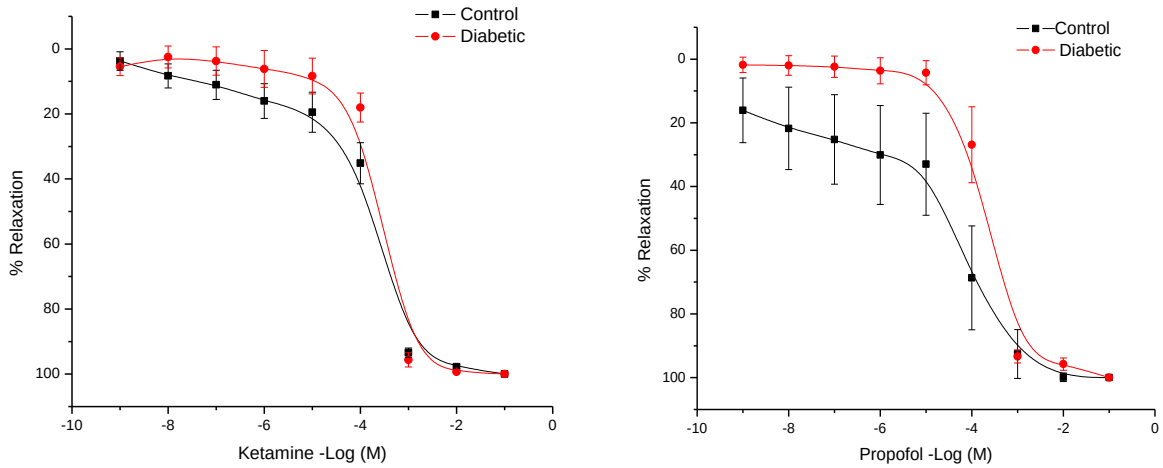

Figure 1
Concentration relaxation response curves for ketamine (1A) and propofol (1B) in diabetic (●) and non-diabetic control (■) rat aortic rings following Phe-induced contraction. Responses are expressed as a percentage of the maximum relaxation evoked by ketamine and propofol (10^{-1} M).

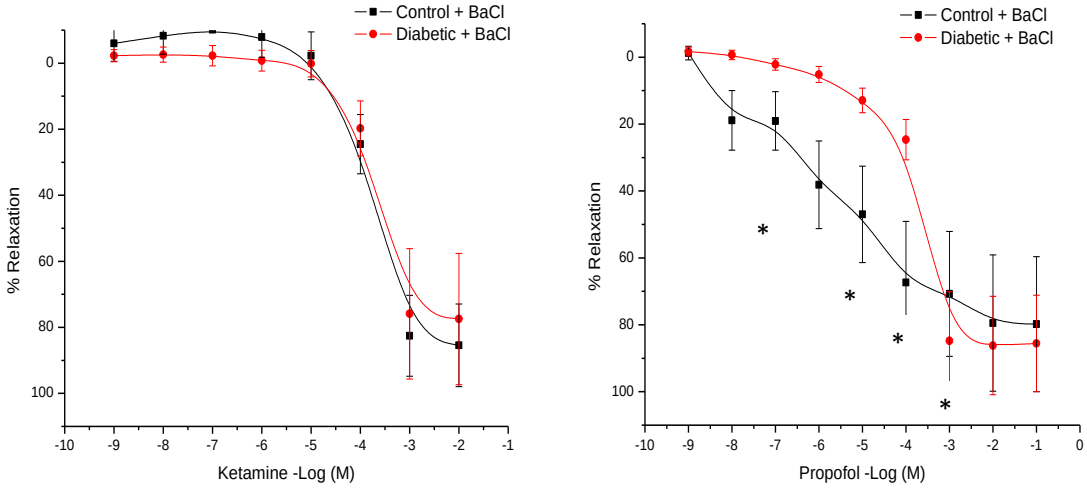

Figure 1
Concentration relaxation response curves for ketamine (A) and propofol (B) in the presence of barium chloride in diabetic (●) and control (■) Sprague-Dawley rat.

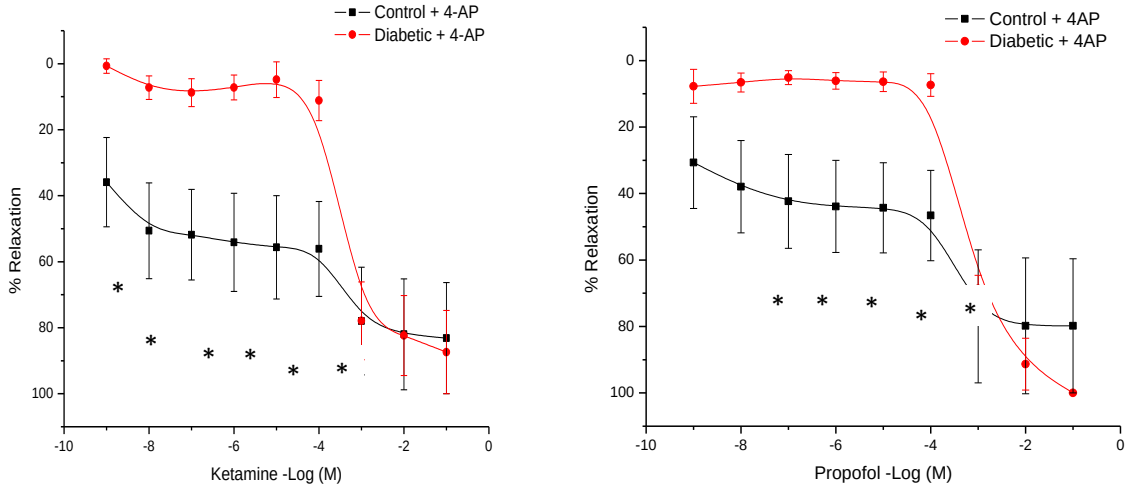

Figure 3
Concentration relaxation response curves for ketamine (3A) and propofol (3B) in the presence of4-AP in diabetic (●) and control (■) Sprague-Dawley rats

Effect of 4-AP on Vascular Relaxation Response to Ketamine and Propofol in Rat Aortic Rings: Relaxation response to ketamine and propofol in the presence of 4-AP (10^{-3} M), showed significant (p.<0,5) reduction in relaxation in the non-diabetic controls when compared to diabetic rat aortic rings . This resulted in a shift of the concentration-response curves to the right (Figures 3A and 3B). The pEC50 values for ketamine were 6.94 ± 0.45 vs.3.37 ± 0.15, p value ≤ 0.05. The values for propofol were 5.62 ± 0.35 vs. 2.78 ± 0.17.

Effect of Glibenclamide on Relaxation Response to Ketamine and Propofol Following Phe-induced Contraction in Rat Aortic Rings: Following pre-treatment of aortic rings with glibenclamide (10^{-4} M) for 15 minutes, then pre-contraction with Phe 10^{-6} M, cumulative concentrations of ketamine (10^{-9}-10^{-1} M) showed a significant (p<0.05) reduction in relaxation in diabetic rings treated with glibenclamide compared to diabetic rings without glibenclamide. The pEC50 values for diabetic rings without glibenclamide vs. diabetic with glibenclamide were 3.67 ± 0.09 vs. 3.11 ± 134.60. However, there was no significant difference between control treated with glibenclamide and diabetic rings treated with glibenclamide (Figure 4A). On the other hand, propofol showed no significant difference between any of the groups except for control without glibenclamide versus control + glibenclamide (Figure 4B).

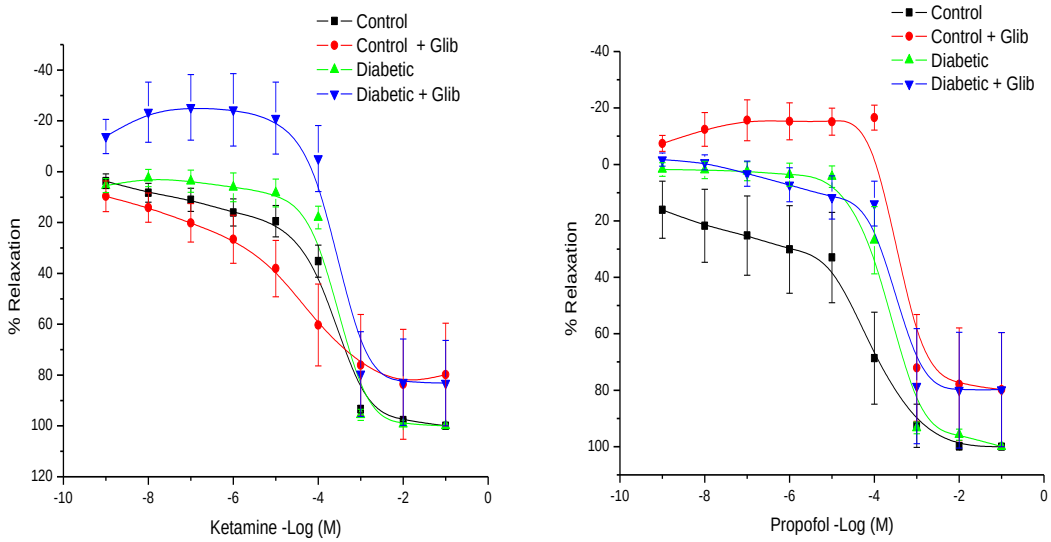

Figure 4:
Concentration relaxation curve of ketamine and propofol following blockade of K_{ATP} channels with glibenclamide in STZ-diabetic and non-diabetic (control) rat aortic rings.

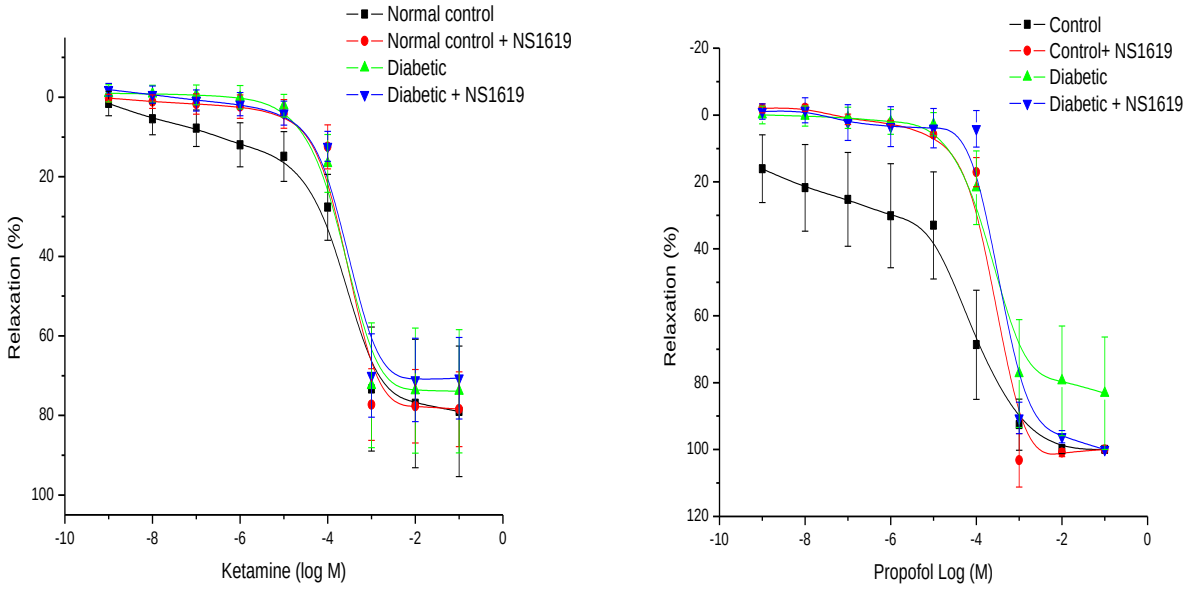

Figure 5:
Relaxation effects of ketamine and propofol in the presence of NS1619 applied to aortic rings obtained from diabetic and non-diabetic (control). Sprague-Dawley rats. N=6 (control) and N=5 (diabetic)

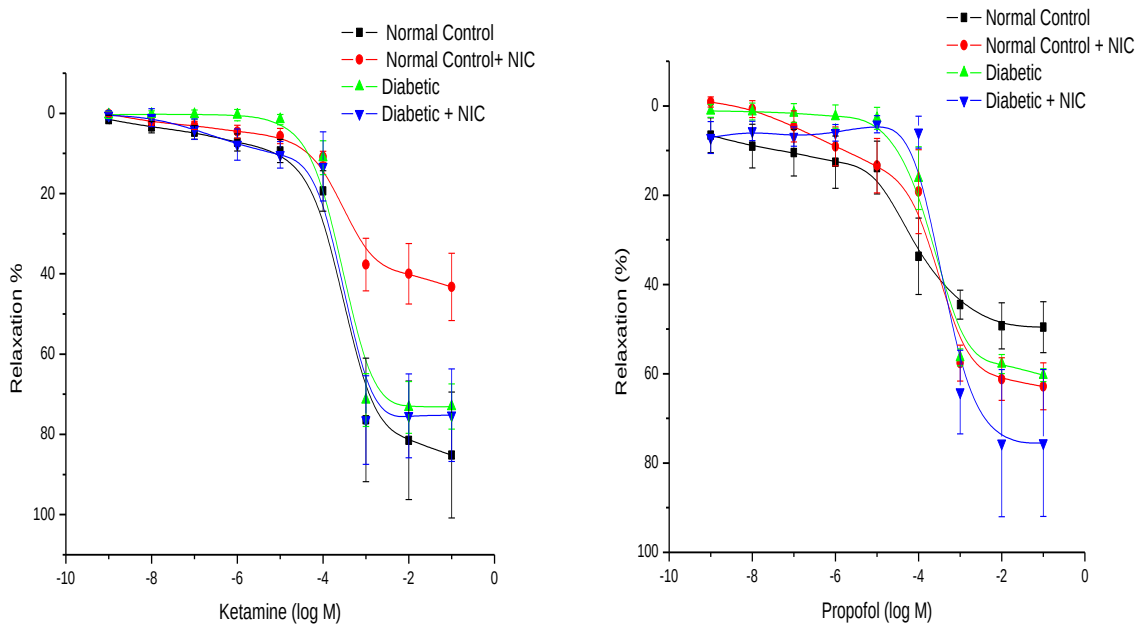

Figure 6:
Concentration response curves for ketamine and propofol in the presence of nicorandil applied to aortic rings obtained from STZ-diabetic (N=6) and non-diabetic (N=6) Sprague-Dawley rats.

Effect of NS1619 on Relaxation Response to Ketamine and Propofol in Rat Aortic Rings: Following pre-treatment with the BK_{Ca} potassium ion channel opener, NS1619 (10^{-7} M) prior to pre-contraction with Phe (10^{-6} M), there was no significant difference in the relaxation induced by ketamine or propofol between diabetic and non-diabetic rat aortic rings at the lower concentrations. However, both anaesthetic agents produced significant ($p< 0.05$) relaxation of the aortic rings at higher concentrations (10^{-4} to 10^{-1} M) Figure 5A and 5B.

Relaxation Response of Ketamine and Propofol in the Presence of Nicorandil: The relaxation effect of ketamine and propofol following activation of K_{ATP} channels with Nicorandil (10^{-6} M) prior to Phe pre-contraction, the relaxation response to ketamine and propofol was similar in both diabetic and non-diabetic rat aortic rings. As observed with NS1619, ketamine and propofol also showed significant ($p< 0.05$) relaxation at the higher concentration (10^{-4} to 10^{-1} M) Figure 6A and 6B respectively

DISCUSSION

The present study compared the vascular responses to ketamine and propofol in the presence of vasoactive agents in STZ-induced diabetic rat aorta. The main findings were that ketamine and propofol similarly reduced Phe-induced contraction in diabetic aorta when compared to control. The contractile response to Phe is increased in DM due to dysfunction in the alpha-adrenergic pathway (Chittari *et al*., 2010; Tsao *et al*., 2012), and an increase in the expression and affinity of adrenoceptors in STZ-induced diabetic rats (Potenza *et al*., 2009). This suggest that ketamine and propofol alter the calcium channels mediated calcium influx involved in the mechanism of Phe-induced contraction. The result further suggests that the reduced NO dependent vasorelaxation associated with diabetes is preserved by propofol, resulting in the similar response to the control group. This may be due to the NO releasing properties of propofol (Sobey, 2001), or that ketamine and propofol act at least in part, directly on VSM cells.

There is supporting evidence that DM progressively leads to impairment of potassium ion channels (Hao *et al*., 2017). These potassium ion channels are major contributors in the maintenance of vascular tone and are inclined towards constriction when there is impairment. Opening of potassium channels leads to efflux of potassium from VSM cells and closure of voltage-activated calcium channels. The closure of these calcium channels prevents calcium entry into cells leading to vasorelaxation, the converse leads to vasoconstriction (Hao *et al*., 2017). Hence, barium chloride blocks Kir channels, causing efflux of potassium from cells and a corresponding influx of calcium into cells, resulting in vasoconstriction. In the presence barium chloride-induced vasoconstriction, the vascular response to propofol in diabetic aorta was reduced, but remains unchanged with ketamine when compared to control. This demonstrates that diabetes impairs Kir channels which alters the vascular response to propofol due to increased calcium influx. Another finding showed significantly reduced relaxation responses to ketamine and propofol in the presence of 4-aminopyridine-induced vasoconstriction

in diabetic aorta. This demonstrates that Kv channels are also impaired in diabetes and supports an earlier study (Chai *et al*., 2005). This showed that the influx of calcium into cells reduced the relaxation response to ketamine and propofol. Propofol-induced relaxation has been attributed to a reduction in intracellular calcium within VSM cells (Sobey, 2001). A study reported a ketamine-induced inhibition of Kv channels resulting in an increase in vascular tone of rat mesenteric arteries (Kim *et al*., 2007). Information on the effects of ketamine on rat aorta is not readily available. However, the result of this study may suggest that ketamine has no significant inhibitory effect on Kv channels in rat aorta. This is evidenced by the similar relaxation responses when Kir channels are blocked.

Pre-incubation of KATP channels with glibenclamide showed a significant attenuation of ketamine relaxation response in diabetic rat aorta. Glibenclamide causes release of NO in cells with endothelium and blockade of calcium influx in cells without endothelium (Chan *et al*., 2000). This may suggest an endothelium dependent attenuated relaxation response to ketamine in the diabetic group due to impairment in glibenclamide-induced NO release. Glibenclamide acts as both a selective inhibitor of KATP channels (Jackson, 2000) in VSM cells and is a VSM relaxant (Ertuna and Yasa, 2005). When compared to ketamine, the vascular response to propofol was not altered in the diabetic rat aorta and indicates that KATP channels are not involved in propofol-induced relaxation. This result also suggests the possible release of propofol-induced NO, compensating for the lack of NO- induced endothelial dysfunction associated with diabetes (Pak *et al*., 2019). The potassium channel blockade with BaCl2 (a non-selective inward rectifier potassium channel blocker in rat aorta (Kir), or glibenclamide (KATP), elevated ketamine and propofol induced contractions in diabetic aortic rings, this is not in keeping with a relaxation or decreased in blood pressure, as may be expected for relaxation inducing agents (Romero *et al*., 2019; Cifuentes *et al*., 2018), and could suggest disruption to calcium channel responses in diabetes. This has previously been reported that both KATP and BKCa activities are attenuated in diabetes mellitus (Owu *et al*., 2013). This may have some clinical benefit as glibenclamide antagonize the protection of cardiac muscles when these channels are open (Tsao *et al*., 2012). However, this protective effect of KATP channel opening may be preserved in the diabetic patient being treated with glibenclamide and to whom propofol is also administered. In this case, propofol may be a better choice of anaesthetic. It has been reported that ketamine reduced the activity of KATP channels and inhibits relaxation induced by KATP channel openers (Kawano *et al*., 2010).

Additionally, the present study showed that opening of KATP and BKCa channels did not alter the vascular response to ketamine or propofol in diabetic aortic rings when compared to control. This suggest that opening of potassium channels preserves the relaxation response to ketamine and propofol in diabetes and stands as a treatment option in maintaining normal vascular tone. Therefore, the benefits derived from nicorandil in preventing angina will not be compromised in the diabetic patient to whom either ketamine or propofol is administered.

In conclusion, the results recommend that propofol can be effective in mitigating the consequences of hemodynamic instability in glibenclamide treated diabetics when compared to ketamine. This may be due to propofol-induced calcium modulation.

Acknowledgments

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Research Article

Micro- and Intermediate Filaments of the Testis of African Catfish (*Clarias gariepinus*) Treated with a Sub Lethal Dose of Carbendazim

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Summary: This study highlighted the effect of Carbendazim on the testicular micro and intermediate filaments adult male African catfish (*Clarias gariepinus*). Previous studies related to carbendazim toxicity in fish have been limited to mortality patterns and degree of sensitivity across species. Literature on actual pathology in fish is scanty. The fish were exposed to a pre-determined sub-lethal concentration (1.4 mg/L) of Carbendazim for seven and fourteen days, 10 fish were sedated by cold shock, and sacrificed on days seven and 14. Another untreated group (control) were sacrificed at the same periods. The testes were harvested and weighed. Testicular actin microfilament, cytokeratin, desmin and vimentin intermediate filaments were determined using standard immunohistochemistry protocols. Variations in the intensity and pattern of immuno-expression of the testicular actin, cytokeratin, desmin and vimentin were significant in a phase dependent manner with day 14 being more pronounced. Immunohistochemical features of degenerated and necrotic germinal and Sertoli cells in the treated group, with loss of wire-mesh network which supported the mature germinal cells in the testicular lumen were also observed. A sub-lethal dose of carbendazim exposure for either seven or 14 days, induced deliterous changes in the testicular micro- and intermediate filaments, of the African catfish. This portends a reduction in the male reproductive potentials of the exposed species and resultant negative impact on production.

Keywords*: Carbendazim, Clarias gariepinus, Histomorphology, Immunohistochemistry, microfilaments, intermediate filaments*

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INTRODUCTION

Intermediate filaments are composed of a family, assembled from a large number of proteins that share related structural and sequence features. In epidermal cells, they feature more frequently than microfilaments or microtubules (Lodish, 2000). Intermediate filaments exist as structures/ layers adjacent to cellular and nuclear membranes, where they form network of associations and interactions with other cytoskeletal structures and receptors on the cell membrane (Kobielak and Fuchs, 2004). In addition, intermediate filaments form extensive, organised structural system that connects the nuclear envelope to the plasma membrane (Djaball, 1999). Some intermediate filaments are found oriented parallel to the cell surface, while others are spread, scattered in the cytosol; together they form an underling framework with respect to the shape and resilience of the cell (Goldman *et al*., 1996).

There is also an existing interconnectivity between the filaments of adjacent cells. Distribution of forces, enhanced by this connectivity provides strength and the entire epithelial structure. The close proximity and connection of filaments with the associated plasma membranes suggest that their principal function is to mechanically support the plasma membrane of cells and the extracellular matrix, structurally reinforce and organize cells into tissues (Lodish, 2000). In spite of the fact that intermediate filaments arose from a vast assemblage of proteins , existing in a dynamic relationship as polymers, they have a more stable structure among the cellular structural proteins. By virtue of these, they are more readily observed intact in cells after tissue processing (Parry and Steinert, 1992; Herrmann and Aebi, 2016; Jones *et al*., 2017).

Actin filaments, also called microfilaments are cytoskeletal proteins with the greatest distribution in cells. The filaments are fairly abundant around the plasma membrane. They provide the foundation of mechanical support, cell shape, and cell kinetics at the surface, thereby enabling acts of migration, phagocytosis, and mitosis (Cooper, 2000). Within the cells they form three-dimensional networks, having properties of semisolid gels. The whole structural array of actin filaments and their relationships within cells are regulated by a variety of actin-binding

proteins, which are critical components of the actin cytoskeleton. Total protein constituents of muscle cells are approximately 20% actin filaments, it is about 5 to 10% of cellular protein in other types of eukaryotic cells (Cooper, 2000).

Cytokeratins are intermediate filaments generally found in the epithelia that line internal body cavities. They are closely related though distinguished from other keratin that forms the "hard" epidermal stratified squamous epithelium hat forms specialised structures of the skin. They can be used as important anatomical markers of normal and abnormal cell differentiation (Oliveira, 2005).

Vimentin, which is the most widely distributed of non-keratin intermediate filaments is typically expressed in, endothelial cells and leukocytes, some epithelial cells, and fibroblasts. The filaments form vital support of cell membranes as well as keep the cellular organelles in situ. Vimentin filaments are frequently seen in parallel network with microtubules. Desmin filaments are, compared to others, much limited in distribution. They are found as sarcomeres stabilizers in contracting muscle cells. Desmin filaments maintain muscle integrity, without them, there would be distruption of the muscle architecture and alignment.

The intermediate filaments are well expressed in the different compartments of the testicular structure and contribute well to the functional output. Their expression is also a useful feature in pathologic conditions (Rogatsch *et al*.,1996) i.e. in heightened expression in tumours and altered arrangement in tissue disruptive events seen in challenges with an environmental toxicant (Cheng, 2014).

Carbendazim (methyl 2-benzimidazole carbamate) is one of the most commonly used systemic pesticides. It is a widely used systemic fungicide with both chemoprotective and chemotherapeutic actions against a wide range of fungi in various vegetables and fruit trees (Mohapatra and Lekha, 2016). The fungicide is implicated as a persistent water pollutant (Fernandez *et al*.,2001; Cuppen *et al*., 2008). Carbendazim dressed fields could lead to contamination of ponds, water ways located near fields where it had been applied (Aina *et al*., 2016). Also, there is the strong possibility carbendazim -contamination of the aquatic environment; earthen ponds, communal rivers related to farms through industrial effluents and farm equipment used in the application of the fungicide. Crops protected with Carbendazim based products end up as part of the raw materials for commercial production of fish feed (Aina *et al*., 2016.

One can infer from all these that degenerative and cellular distruptive conditions likely found in Carbendazim-associated tissue toxicity could negatively impact the architectural milieu of microfilaments and intermediate filament cytoskeleton in cells and tissues. Studies that related environmental toxicity in fish species appears to invest more in mortality pattern, bioaccumulation, respiratory components as well as the vital organs, there are fewer information on the effects on reproductive system.
This present study therefore aims at investigating the presence, expression and the histomorphology of Cytokeratin, Actin, Desmin and Vinmectin filaments in the testicular tissue of the matured African catfish exposed to a sub lethal dose of Carbendazim.

MATERIALS AND METHODS

Testicular tissues obtained from all the exprimental groups of the adult male catfish (C. gariepinus) from control untreated seven day (MW1) , 14 day (MW2) , carbendazim treated seven day (MC1) and 14 day (MC2) groups were fixed in 10% Neutral Buffered Formalin for 48 hours. Fixed tissues were processed routinely for paraffin embedding technique as described by An *et al.,(* 2003).

The immunostaining technique

The immunostaining technique was performed on 5µm-thick testicular sections, using a LSAB-plus kit (Dakocytomation, Denmark) as previously described in our earlier work (Aina *et al*., 2019) . The empirical assessment of the intensities of vimentin, desmin, cytokeratin and actin immunostaining were done. The results were scored as absent (-), weak (+), moderate (++) and strong (+++) , relative to the positive control samples for each intermediate filament and actin microfilament

RESULTS

Actin filament was weakly expressed in the testicular capsule and intertubular connective tissue. The cytoplasm of the maturing germinal cells and the cytoplasm of the Leydig cells reacted with a moderate intensity in all the groups. The carbendazim treated groups did not significantly differ in localization and intensity of the actin filaments, compared with the control (untreated groups). (Tables 1 and 2 ; Figures 1a and 1b)

Desmin was weakly expressed in the testicular capsule and the connecting interstitium, but strongly expressed in the sertoli cell cytoplasmic and its extensions which formed the cysts. There is a weak expression in the lumina mesh work that appears to suspend the mature spermatozoa. (Tables 1 and 2; Figure 3a).

The desmin staining accentuated the pathology of the testis seen at day 14 of carbendazim treatment as clumps of the luminal meshwork and desmin stained cellular debris can be seen clearly (Figure 3b)

Table 1:
Summary of the immunohistochemical localization of the intermediate filaments; cytokeratin, vimentin, desmin and the microfilament-actin in the testis of Carbendazim exposed and Control (unexposed) mature African Catfish at 7th day of Experiment

Cell/Tissue stained	Actin		Desmin		Cytokeratin		Vimentin	
	Carb	Control	Carb	Control	Carb	Control	Carb	Control
Testicular Capsule	+	+	+	+	+	+	--	--
Testicular interstitium	+	+	+	+	-	-	--	--
Basement membrane	--	--	--	--	+++	+++	--	--
Immature Germinal cells	++	++	--	--	--	--	--	--
Mature spermatozoa	--	--	--	--	--	--	--	--
Sertoli Cell Cytoplasm	--	--	+++	+++	+	+	+++	+++
Germinal Cysts	+	+	+++	+++	+++	+++	+	+
Luminal Meshwork	--	--	+	+	--	--	--	--
Leydig cells		++		--		+++		--

a disrupted; b* Free floating cells in the lumina*
Intensities of immunostaining : --, absent; +, weak; ++, moderate;

Table 2:
Summary of the immunohistochemical localization of the intermediate filaments; cytokeratin, vimentin, desmin and the microfilament-actin in the testis of Carbendazim exposed and Control (unexposed) mature African Catfish at 14th day of Experiment

Cell/Tissue stained	Actin		Desmin		Cytokeratin		Vimentin	
	Carb	Control	Carb	Control	Carb	Control	Carb	Control
Testicular Capsule	+	+	+	+	+	+	--	--
Testicular interstitium	+	+	+	+	-	-	--	--
Basement membrane	--	--	--	--	+++	+++	--	--
Immature Germinal cells	++	++	--	--	--	--	--	--
Mature spermatozoa	--	--	--	--	--	--	--	--
Sertoli Cell Cytoplasm	--	--	+++	+++	+	+	+++	+++
Germinal Cysts	+	+	+++	+++	+++	+++	+	+
Luminal Meshwork	--	--	+	+	--	--	--	--
Leydig cells	++	++	--	--	+++	+++	--	--

a Disruptions accentuated by the presence of deeply stained clumps in the lumina*
b Free floating cells in the lumina Intensities of immunostaining : --, absent; +, weak; ++, moderate; +++, s*

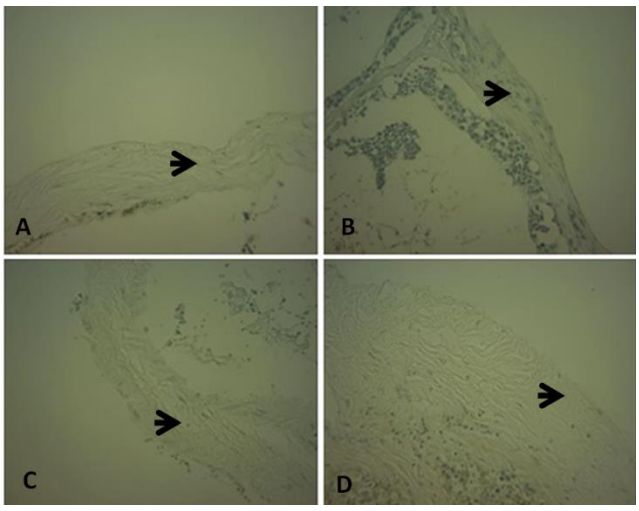

Figure 1a:
Weak expression of Actin filaments in at the capsule (arrows) of groups A : 7th day contol; B: 7th day carbendazim treated; C: 14th day contol; D: 14th day carbendazim treated

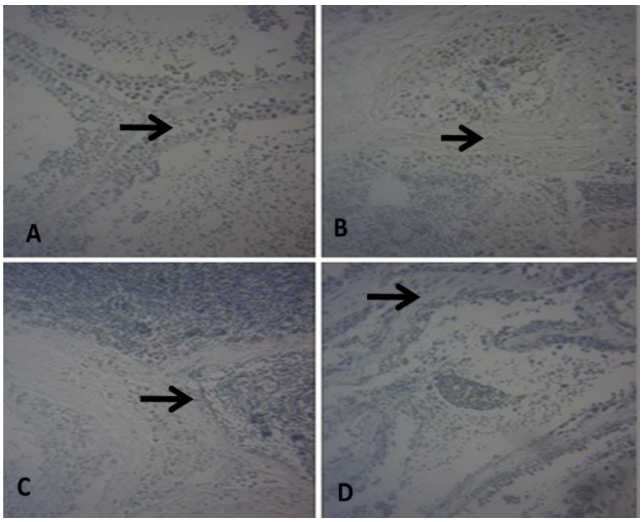

Figure 1b: Localisation of actin filaments in at the intertubular connective tissue (arrows) of groups A : 7th day contol; B: 7th day carbendazim treated; C: 14th day contol; D: 14th day carbendazim treated

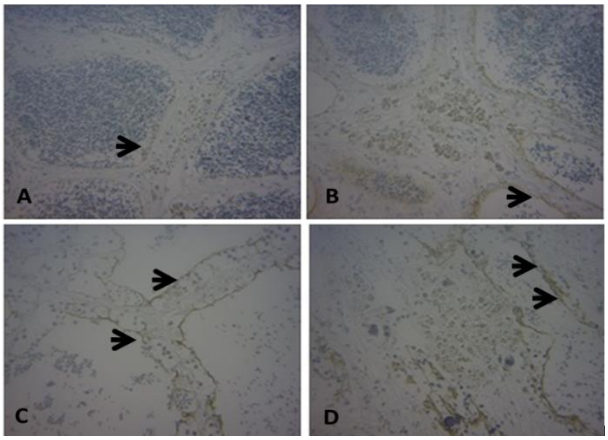

Figure 2a:
Intensity and localisation of cytokeratin filaments in at the basement membrane (arrows) of groups A : 7th day contol; B: 7th day carbendazim treated; C: 14th day contol; D: 14th day carbendazim treated

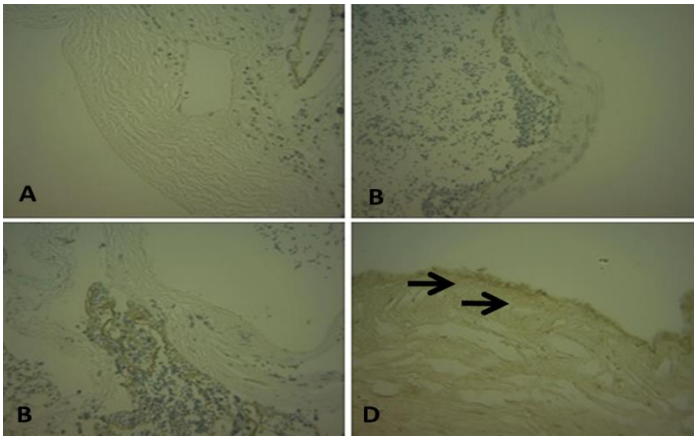

Figure 2b:
Intensity and localisation of cytokeratin filaments in at the testicular capsule (arrows) of group D: 14th day carbendazim treated. Groups A: 7th day contol; B: 7th day carbendazim treated; C: 14th day contol had poor cytokeratin

Cytokeratin filament is strongly the expressed in the basement membrane, germinal cysts and moderately in the leydig cells. In both the 7th and 14th day of carbendazim treatment, cytokeratin stained debris were free floating in the lumina while the cysts are greatly disrupted. Though there was a weak reaction at the capsule for the control groups, there was a relatively strong expression of cytokeratin in the capsule at the 14th day Carbendazim treatment. (Tables 1 and 2; Figures 2a and 2b).

Vimentin expression was seen strongly in the sertoli cell cytoplasm and slightly in its cysts forming cytoplasm. The spread of staining appears to follow the relatively pattern of disruption and scanty presence of sertoli cells in the treatment groups, especially the 14th day carbendazim treated group. (Tables 1 and 2; Figures 4a and 4b).

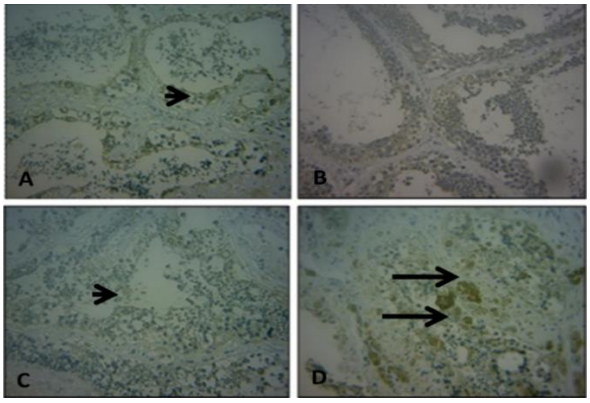

Figure 3b:
Intensity and localisation of cytokeratin filaments in at the testes of Groups A: 7th day contol; B: 7th day carbendazim treated; C: 14th day contol ; Group D: 14th day carbendazim treated. Desmin immunostaining. strongly expressed in the sertoli cell cytoplasmic and its extensions which formed the cysts (short arrows). There is a weak expression in the lumina mesh work that appears to suspend the mature spermatozoa. The desmin staining accentuated the pathology of the testis seen at day 14 of carbendazim treatment as clumps of the luminal meshwork and desmin stained cellular debris can be seen clearly (long arrows)

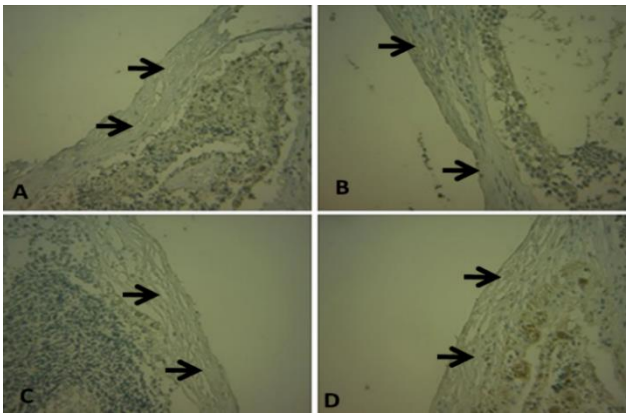

Figure 3a:
Intensity and localisation of desmin filaments showing weak expression at the testicular capsule (arrows) of . Groups A: 7th day contol; B: 7th day carbendazim treated; C: 14th day contol ; group D: 14th day carbendazim treated

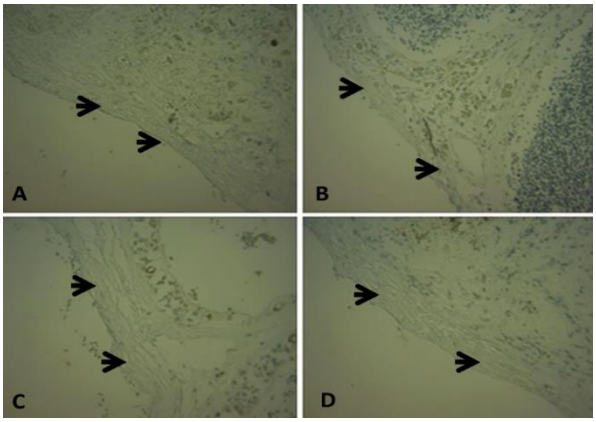

Figure 4a:
Intensity and localisation of Vimentin filaments showing weak expression at the testicular capsule (arrows) of . Groups A: 7th day contol; B: 7th day carbendazim treated; C: 14th day contol ; group D: 14th day carbendazim treated.

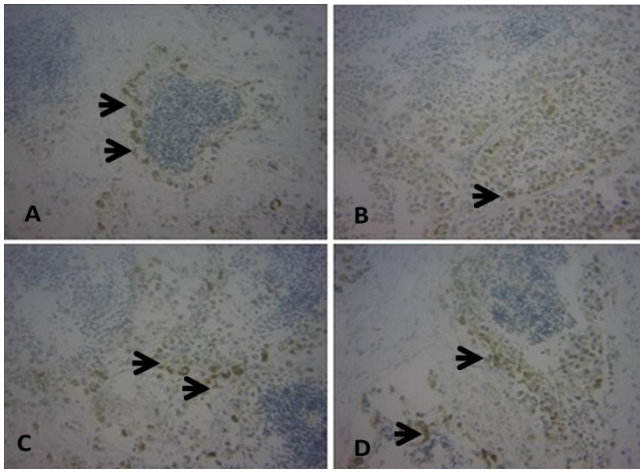

Figure 4b:
Intensity and localisation of Vimentin filaments showing strong expression (arrows) of the sertoli cells . Groups A: 7th day contol; C: 14th day contol , ; Groups B: 7th day carbendazim treated and group D: 14th day carbendazim treated also had strong sertoli expression which appears scattered in the lumen.

DISCUSSION

Significant adverse effects of exposure to potentially toxic chemicals in the environment have been a well-recognised subject of study (Vighi and Villa, 2013). Tools that study the same adverse effects by sub lethal doses of the same chemicals would bring out a greater importance along this line, as drugs that have been confirmed non-poisonous at a given dose may actually carry a plethora of previously unseen effects at the same dose in species of concern. Moreover, in studies that determined lethal concentration of potential environmental toxins, most times, the basis of determination is somewhat limited to mortality pattern (Palawski and Knowles, 1986; Oruc, 2010; Rico *et al*., 2001)

The results present significant phase dependent variations in the intensity of immuno-expression of the testicular actin, cytokeratin, desmin and vimentin with day 14 being more pronounced. In a similar way, the extent of disruption of the filament meshwork is also higher with the later phase (day 14). Immunohistochemical features of degenerated and necrotic germinal and Sertoli cells in the treated group, with loss of wire-mesh network which supported the mature germinal cells in the testicular lumen were also observed. This further corroborates the earlier study that implicates Carbendadim in distruption of microtubule assembly (Winder, 2001)

Immunoexpression of the various cytoskeletal proteins helped to highlight the histomorphological alteration that could be associated with carbendazim exposure. The importance of this immunohistochemical study is not only in the degree of expression of target structures , but also the accentuation of tissue destruction beyond what is generally observed under routine H&E methods. This implies an added advantage of immunohistochemistry, not only as quantification of expression of proteins, but also enhancing tool for the structural features observed in routine H&E methods

The effects seen in the study such as loss in the cytoskeletal framework could be responsible for disruption of microtubule assembly which has been previously reported as a feature of Carbendazim exposure (Lim and Miller,1997; Winder *et al*., 2001).

A sound reproductive system is a cogent factor to the success of the aquatic species in the struggle for survival between pathogens and targets/hosts. The immunohistochemical tool applied for actin filament, desmin, cytokeratin and vimentin microfilaments helped to highlight to a large extent the structure detail of testicular disruption associated with Carbendazim in African Catfish. Simple immunohistochemical techniques therefore could expand and enhance the knowledge base of the pathogenesis of ecotoxicological agents on fish tissues determined through routine H&E methods.

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Research Article

Long-Term Hyperglycaemia Impairs Hormonal Balance and Induces Oxidative Damage in Ovaries of Streptozotocin-Induced Diabetic Wistar Rat

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Summary: Reproductive dysfunction following insulin deficiency in Diabetes Mellitus has been well reported among diabetic patients. However, the mechanism through which Diabetes alters reproductive function remains oblivion. While most studies have focused on diabetes mellitus in male subjects, there have been cases on altered reproductive functions in females. These present study aims to investigate the effect of long term hyperglycemia on diabetic rats' ovary. Female Wistar rats were assigned into control and diabetic group, each consisting of five animals. The later was induced with STZ (50mg/Kg intraperitoneal injection) and the animals were sacrificed after 14 weeks. The blood glucose, body and organ weight, serum hormone level along with oxidative stress parameters of the ovary and uterus were determined. Histology of the ovary and expression levels of CD79 in the ovary was also assessed. The weight of the diabetic rats after the experiment was significantly lower ($p<0.05$) than the control. The level of Follicle Stimulating Hormone, Luteinizing hormone and estrogen was significantly lower in the diabetic group. The antioxidant enzymes catalase, superoxide dismutase (SOD) and glutathione-s-transferase (GST) were significantly lower in the diabetic ovary and uterus while the Malondialdehyde (MDA) concentration significantly increased compared to the control group. Histological observation of the ovary showed signs of chronic inflammation and immunohistochemistry for CD79 showed positive expression in the diabetic ovary. Our research findings suggest that Diabetes mellitus alters ovarian health by altering hormonal balance and stimulating oxidative damage.

Keywords: *Diabetes mellitus, Oxidative stress, Hormonal imbalance, ovarian dysfunction, CD79, Streptozotocin*

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INTRODUCTION

Diabetes mellitus play a critical role in different pathological conditions in humans, including reproductive dysfunction (Chatterjee *et al*., 2013). Females with Diabetes mellitus encounter impaired reproductive functions, although the data are not well established as compared to male (Steger & Rabe, 1997). Diabetic Mellitus is associated with decreased fertility and reproductive losses (Nandi & Poretsky, 2013). Popoola *et al*. reported a biochemical, hormonal and histological change in the prostate of male diabetic rats after three months and six months (Popoola *et al*., 2017).

Several perturbations occur in female diabetic patients ranging from reduced ovulation, low embryo development, abnormal oocyte maturation and altered metabolism (Qiang Wang and Kelle H. Moley, 2016). Of significant note is the increased susceptibility to polycystic ovarian syndrome (PCOS) and menstrual irregularities associated with type 1 diabetes patients (Codner *et al*., 2012). Since the ovaries play a significant role in these functions, an extensive study would provide insight into the pathologies of infertility in female diabetic patients.

Steger and Rabe (1997) reported that diabetes mellitus disrupt endocrine function. According to Tesone *et al*. (1983), decreased hCG receptors may be responsible for ovarian dysfunction in diabetic animals. Deepika and Zaher observed a link between increased advanced glycation end product in diabetic women and increased susceptibility to PCOS (Garg & Merhi, 2015). Zhao *et al*. (2010) reported that Diabetes increased testicular apoptotic cell death via the mitochondria pathway and demonstrated that oxidative stress is the leading cause of testicular detrimental effects. These suggest that reactive oxygen species and hormonal imbalance may play a

significant role in reproductive organ failure in female Wistar rats.

B-lymphocyte has been reported to play a critical role in type 1 diabetes as depletion of B-cells delays diabetes progression in diabetic patients and NOD mice (Hinman *et al*., 2014). While most of these damage has been attributed to CD20 lymphocyte, not much is known about the role of CD79 in Diabetes progression. Hence, this study is aimed at providing insight into the role of oxidative stress and hormonal imbalance and to evaluate the possible role of CD79-B-cell receptor-mediated apoptosis and proliferation in the ovary of diabetic rats.

MATERIALS AND METHODS

Chemicals and Reagents: Streptozotocin was purchased from Santa Cruz, USA. Glutathione, Hydrogen peroxide and 5,5'-dithios-bis-2-nitrobenzoic acid (DNTB) were purchased from Sigma Chemical Company, Saint Louis, USA. Thiobarbituric acid (TBA) and Trichloroacetic acid (TCA) were purchased from British Drug House (BDH), Chemical Limited, Poole, UK. Immunohistochemistry was performed using the Novocastra Kit. Other chemicals and reagents used were of pure quality and analytical grade.

Experimental Animals: Female Wistar rats weighing 130-150g were purchased from the Department of Veterinary Physiology, University of Ibadan. The rats were kept in ventilated cages and housed at room temperature 25±2^{0}C with 12h dark/light cycles. Animals were fed standard rat pellet and were acclimatized for two weeks before the experimental treatment. Animals were handled according to standard ethical procedures stipulated by the Animal Care Unit Research Ethics Committee (ACUREC), University of Ibadan.

Experimental Design: Female Wistar rats were assigned into two groups.

Group1: Control rats fed with standard rat pellet only

Group 2: Diabetic rats that were administered 50mg/Kg body weight intraperitoneal STZ injection.

Rats with blood glucose above 250mg/dl after 72h post-STZ injection were termed diabetic. Animals that remained in this hyperglycemic state for a prolonged duration of 14 weeks were used for the experimental analysis.

The animals in each group were eventually sacrificed, the blood samples were collected in plain bottles, and the uterus and ovary were exercised. The organs weighed, and the percentage relative organ weight was calculated as a ratio of the organ weight to the bodyweight of the animal expressed in percentage. The blood glucose of the animals was determined using a glucometer and standard test strip.

The tissues collected were weighed, and 50% fraction of it was homogenized in four volumes of phosphate buffer and centrifuged at 10,000rpm in a cold centrifuge at 4^{0}C for estimation of oxidative stress parameter. The other tissue section was fixed in 10% formalin for histopathology and immunohistochemistry studies.

Hormonal Assay: FSH (Follicle Stimulating Hormone) and LH (Luteinizing hormone) levels were determined using a solid based enzyme-linked immunosorbent assay as described by Uotila *et al*., (1981), estrogen (EST) and Prolactin (PRL) concentrations were determined by the ELISA method described by Norbert *et al*., (1991).

Oxidative stress Estimation: Protein determination: Protein levels were determined according to the method of Randall and Lewis (1951) using bovine serum albumin as standard.

- The activity of Superoxide dismutase (SOD) was determined according to the method described by Mistra & Fridovich (1972).
- Catalase (CAT) activity was determined using the method of Claiborne Claiborne (1985)
- Glutathione-s-transferase (GST) activity was determined using the method of Habig *et al.* (1974).
- Lipid peroxidation was determined based on Malondialdehyde (MDA) concentration using the method of Buege and Aust (1978).

Histology: Ovarian samples were fixed in Bouin's solution, dehydrated in 95% ethanol and cleared in xylene before embedding in paraffin. 5 µm section were cut, stained with hematoxylin and eosin dye and examined under a light microscope (×400) by a histopathologist who was ignorant of the treatment groups.

Immuno-Histochemical Assay: Fresh ovary sample from control and diabetic rats was fixed in formalin and embedded in paraffin and cut to a thickness of 7µm section. The sections were deparaffinized, rehydrated and immuno-staining for expression of CD79 was performed using mouse primary antibody (Novocastra, New Castle Upon Tyne, England) (Bhargava *et al*., 2007) dilution of 1:100.

Negative and tonsil positive control slides were stained alongside the experimental slides. Results were assigned qualitative values: negative(-), weak (+), moderate (++), strong (+++) based on expression levels of CD79a protein (Fedchenko & Reifenrath, 2014).

Statistical Analysis
Quantitative data were compared using a two-tailed student t-test for data analysis using Graph Pad Prism 5 (Graph Pad Inc. San Diego, USA). The significant difference was set at $p< 0.05$ and results reported as mean ± SD.

RESULTS

Effect of long-term diabetes on Bodyweight and organ weight of reproductive organs: Long term exposure hyperglycemia in female Wistar rats resulted in a statistically significant ($p<0.05$) decrease in body weight compared to the control rat, which showed an increase in body weight. Also observed was a statistically significant decrease in the weight of ovary and uterus compared to the control. The relative percentage weight of the uterus was also statistically different as compared to the control rats ($p<0.05$).

Table 1:
Effect of long-term diabetes on body weight and reproductive organ weight in female Wistar rats

	Measure	Control	Diabetic
Body Weight	Initial	150.0 ±2.59	150.0 ±4.86
	Final	204.14 ±19.65	127.58 ±24.65*
Ovary Weight	Absolute Weight	0.14 ±0.045	0.061 ±0.007*
	% Relative Weight	0.07 ±0.02	0.05 ±0.01
Uterus Weight	Absolute Weight	0.40 ±0.09	0.116 ±0.01*
	% Relative Weight	0.19 ±0.04	0.095 ±0.03*

Values are expressed as mean ± standard deviation (n=5).
** Statistically different compared to control (p<0.05).*

Effect of Long-term diabetes on blood glucose level in female Wistar rats: The final blood glucose of diabetic rats showed a significant increase ($p<0.05$) from the initial blood glucose before STZ induction and from the final blood glucose of the control rats (Figure 1).

Effect of long term diabetes on endocrine levels in female Wistar rats: As shown in Figure 2, the FSH level, LH level and estrogen level of the diabetic rats showed a statistically significant decrease in the plasma of the diabetic rats compared to the control. The level of prolactin was, however, not statistically different compared to control.

Effect of Long term diabetes on Oxidative stress parameters in reproductive organs: The activities of the antioxidant enzyme catalase, superoxide dismutase and Glutathione-s-transferase were significantly lower in the ovary and uterus of the diabetic rats as compared to the control. A resulting marked significant increase in the concentration of Malondialdehyde (MDA) in the ovary and uterus of the diabetic rats as compared to the control was also observed (Table 2).

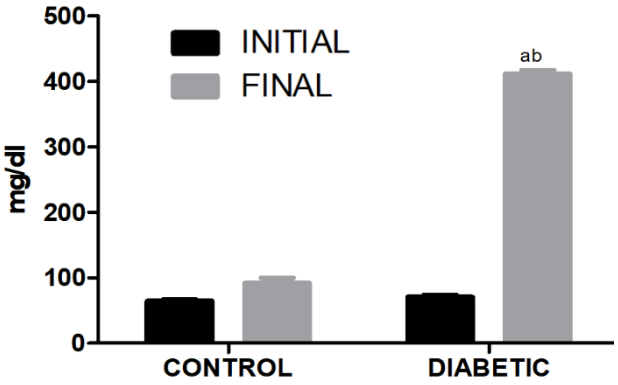

Figure 1:
Effect of long term diabetes on blood glucose level
Values are expressed as mean ± SD.
[a] statistically significant compared to control. [b] Statistically different from initial blood glucose ($P<0.05$).

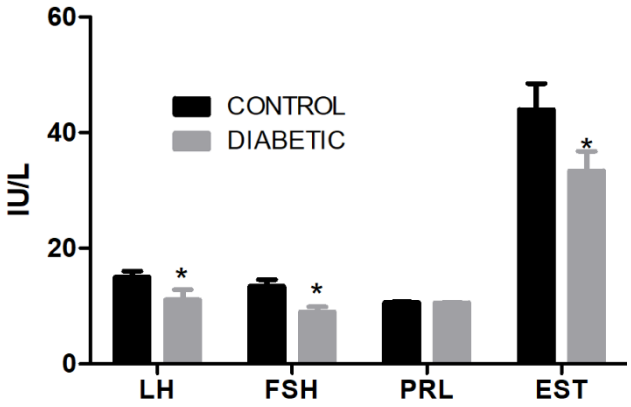

Figure 2:
Effect of long-term diabetes on the level of female reproductive hormones in Wistar rats. Values are expressed as mean ± standard deviation (n=5). * Statistically different compared to control ($p<0.05$).

Table 2:
Effect of long-term diabetes on oxidative stress parameters in ovary and uterus

Measure	Tissue	Control	Diabetic
SOD	Ovary	11±1.95	3.7±0.29*
	Uterus	15.3±0.47	9.46±0.98*
GST	Ovary	8.4±0.58	1.1±0.16*
	Uterus	10.66±0.59	6.54±0.32*
CAT	Ovary	11.62±0.28	3.62±0.40*
	Uterus	5.08±0.24	3.3±0.27*
MDA	Ovary	8.6±0.39	2.2±0.2*
	Uterus	4.36±0.24	0.72±0.13*

Values are expressed as mean ± standard deviation (n=5).
** Statistically different compared to control (p<0.05).*

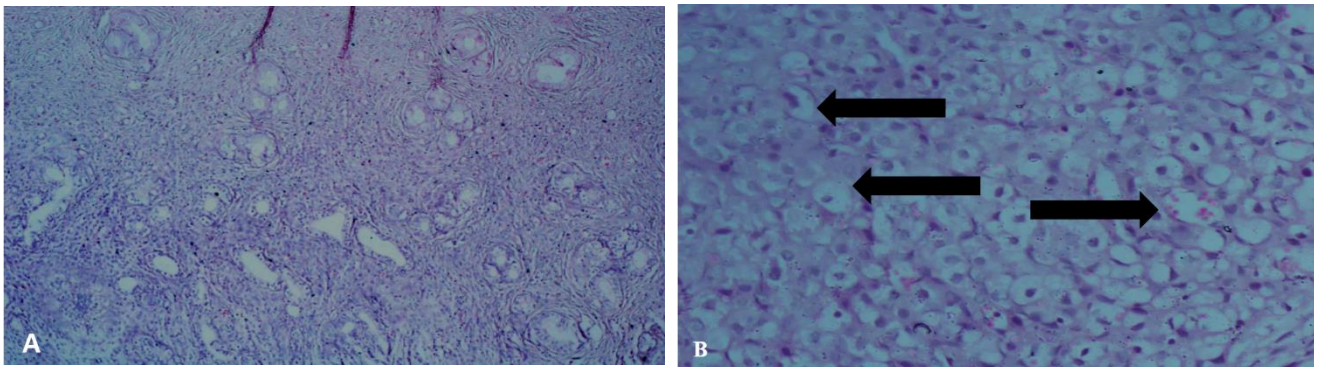

Plate 1:
Histology of the ovary in control and diabetic rats (×400)
A- Control rat ovary showing normal physiology; B-Diabetic rat ovary showing regions of chronic inflammation

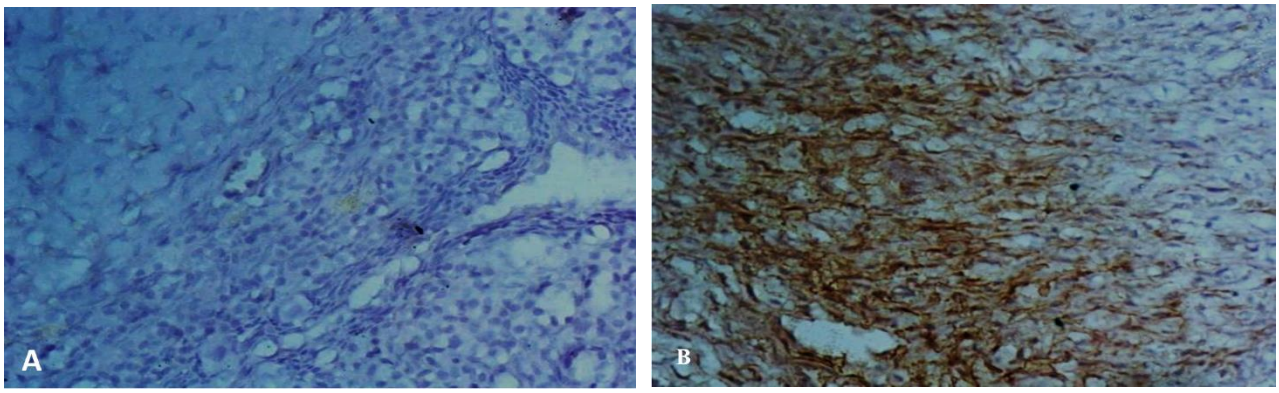

Plate 2:
Expression of CD79a in control and diabetic rats ovary(×400).
A. Control rats showing negative (-) CD79 expression.
B. Diabetic rats were showing moderate positive (++) CD79 expression, as shown by the dark bands. The pattern of staining was cytoplasmic and granular.

Effect of long-term diabetes on histology of the ovary: As shown in Plate 1, the ovaries of the control rat showed normal morphology while the histopathology of the diabetic ovaries showed signs of chronic inflammation of the ovarian cells.

Effect of Long term diabetes on the expression of CD79 in the ovary: The result shows that CD 79a is expressed transiently in the ovary of the diabetic rats and unexpressed in the control rats (Plate 2).

DISCUSSION

Female Patient with Diabetes mellitus has frequently been reported to have various reproductive irregularities resulting in infertility. This dysfunction has been linked to chronic hyperglycemia since the administration of insulin restores the physiological condition of the reproductive organs (Tesone *et al*., 1983). Previous studies have focused mainly on reproductive damage in male diabetic rats (Steger & Rabe, 1997). Owing to the link between prolonged hyperglycemia and reproductive dysfunction, female Wistar rats with prolonged chronic hyperglycemia were studied to evaluate the morphology and physiology of the ovary.

A single-dose administration of 50mg/Kg body weight STZ led to a significant increase in blood glucose of rats in the diabetic group. These state of chronic hyperglycemia remained till the end of 14 weeks before the animals were sacrificed. This is because a single high dose administration of STZ induced diabetes mellitus by destroying pancreatic beta cells (Szkudelski, 2001).

The diabetic rats showed a significant decrease in weight which is similar in human subjects with complications of Diabetes. This is similar to what was observed by Dekel *et al*., who reportedly observed about 40% decrease in weight 14 days post-STZ treatment in mice(MC Deeds, JM Anderson, AS Armstrong, DA Gastineau, HJ Hiddinga, A Jahangir, NL Eberhardt, 2014),(Dekel *et al*., 2009). This is due to massive tissue wasting resulting from hyperglycemia which stems from diabetic ketoacidosis (Umpierrez, 2018).

Decreased ovary and uterus weight can be attributed to a decrease in body mass in diabetic rats. However, the significant decrease observed in the relative weight of uterus indicates an underlying pathological condition. Also, Diabetes has been

reported to decrease the thickness of the myometrium (Tatewaki *et al*., 1989).

Female Diabetes is associated with amenorrhea and disorders with gonadotropin release (Steger & Rabe, 1997). Follicle-stimulating hormone is responsible for ovarian follicle growth, a decreased level in the diabetic rats compared to control rat can affect fertility in diabetic rats. Luteinizing hormone triggers ovulation, and decreased levels in diabetic rats was also observed. Also observed was a significant decrease in estrogen level compared to control. Studies have shown that hypogonadism is associated with chronic hyperglycemia (Komaki *et al*., 2005). In our study, ovarian atrophy (decrease in ovary mass) arises from prolonged hyperglycemia, causing a consequent decline in sex hormones levels.

The level of GST, CAT, SOD, which is antioxidant enzymes were significantly decreased in the diabetic rats and increased MDA concentration in the diabetic rats indicates oxidative stress. This agrees with previous studies conducted in the past, which indicates that oxidative stress contributed to gonadal degeneration in STZ-induced Diabetes in animal models (Shrilatha & Muralidhara, 2007). Prolonged hyperglycemia in the female rat increases free radical concentration resulting in damage in the ovary. The increased reactive oxygen specie concentration may result in accumulation of advanced glycation end-product in granulosa and theca cells resulting in polycystic ovarian syndrome (Diamanti-Kandarakis *et al*., 2007).

The chronic inflammation observed in the cytoplasm of ovarian cells from the histology is a hallmark of cellular necrosis (Allen *et al*., 2005). Hyperglycemia can cause accumulation of advanced glycation end-product resulting in activation of pro-inflammatory marked in the ovarian tissue, which can lead to Polycystic ovarian syndrome (Garg & Merhi, 2015). Female ovarian follicles also undergo apoptosis which is a necessary step for the regulation of follicular pool in the ovary (Vaskivuo & Tapanainen, 2003). However, apoptosis in ovary must be limited to prevent follicular imbalance which can result in infertility. Hyperglycemia has been reported to activate caspases thus increasing the rate of apoptosis (Wu *et al*., 2017), but to the best of our knowledge, the role of CD79 mediated apoptosis in the ovary has not been reported.

Asmita and Vrinda reported an immune-mediated degeneration of ovarian follicular cells via CD45 (Choudhury & Khole, 2015). In our research, we observed a moderate expression of CD79a in diabetic ovary. CD79 is a signalling component B-cell receptor (BCR) commonly used as a marker for leukaemia but can also be expressed in non-b-cell malignancies (Bhargava *et al*., 2007). It is composed of two major component CD79a and CD79b; both of the components have been reported to function synergistically by dimerization stimulating the B-cell receptors (Chu & Arber, 2001). BCR cross-linking has been reported to induce apoptosis or drive cell division in the presence of T-cells (Chu & Arber, 2001). Lower expression of estrogen levels and low tissue mass of ovary of diabetic rats suggest that CD79 expression may play a role of apoptosis in diabetic rat cells rather than cell division. However, the inflammation observed in the ovary gives credence to a possibility of CD79 mediating proliferation in ovary, thereby causing PCOS.

Results from this work showed that prolonged hyperglycemia in STZ induced diabetic in female Wistar rats alters the level of sex hormones via oxidative stress in the ovary which causes damage to ovarian follicular cells which may ultimately lead to infertility. Further studies should be carried out to validate the apoptosis mechanism in the ovary of long term diabetic rats, the expression of CD79 and its role in PCOS and offers new hope for therapeutic targets.

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Research Article

Cardiac and Renal Protective Effect of Vitamin E in Dexamethasone-Induced Oxidative Stressed Wistar Rats

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Summary: Vitamin E is a potent antioxidant used in the management of various ailments arising from oxidative stress. The cardiac and renal protective effect of vitamin E in dexamethasone (Dex)-induced oxidative stress was studied. Twenty four Wistar rats were randomly assigned to four groups of 6 rats each. Group 1 was the control that was administered normal saline placebo. Group 2 was the Dex-induced oxidative stress group (DEX; 30µg/kg b.w i.p). Group 3 was vitamin E group (300 IU/kg administered orally), and group 4 was the Dex (30µg/kg b.w i.p) + vitamin E (300 IU/kg administered orally) group. Administration lasted for 14 days. All animals were fed *ad libitum* with normal rat chow and drinking water. Blood samples were obtained by cardiac puncture and the serum concentrations of nitric oxide, bilirubin, superoxide dismutase, angiotensin converting enzyme and lactate dehydrogenase enzyme activities were analyzed. The heart and kidney were processed for hematoxylin and eosin histological staining. The results show a significant ($p<0.05$) decrease in serum nitric oxide, bilirubin and superoxide dismutase concentration in DEX-only group compared to the control, and were elevated following vitamin E treatment. The angiotensin converting enzyme and lactate dehydrogenase enzyme activities were significantly ($p<0.01$) reduced in DEX+Vit E group. Cardiac and renal histology in DEX-only group showed cardiac hypertrophy and renal injury compared to the control, which were ameliorated following vitamin E treatment. The results of this study suggest that vitamin E may exert ameliorative effects on oxidative stress-induced cardiac and renal impairment in Wistar rats.

Keywords*: Oxidative stress, Vitamin E, Antioxidant, Heart, Kidney.*

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INTRODUCTION

Oxidative stress is an abnormal condition characterized by transient increases in the concentration of reactive oxygen species leading to a disturbance in cellular metabolism, regulation and damage to cellular constituent (Dalle-Donne *et al*., 2016). Reactive oxygen species (ROS) are free radicals produced as a result of cellular metabolism and examples include the hydroxyl radical (OH), superoxide anion (O_2^-) and non-radical molecules such as hydrogen peroxide (H_2O_2) and singlet oxygen ($O_2^{\cdot}$). Proper regulation of ROS has a great impact on many physiological and pathological conditions. ROS level is seen to increase greatly during cell stress and its highly reactive nature can lead to changes in other oxygen species, lipids or proteins, a condition often known as oxidative stress (Bayr, 2005). Increased production or decreased scavenging of reactive oxygen species has been the major cause of diverse diseases such as atherosclerosis, myocardial infarction, hypertension, diabetes and cancer (Wiseman and Halliwell, 1996). Thus, maintaining a normal cellular concentration of ROS is important for proper physiological functions of different cell types in the body (Krötz *et al*., 2004). The various antioxidants present in the body are responsible for this function of reducing ROS in the living system such as vitamins A, C and E.

Evidence abound that abnormal production of ROS results in a cascade of cardiovascular disorders like hypertension, coronary heart disease, neurological disorders, and physiological ageing (Pacher and Szabo, 2008; Vassalle *et al*., 2008). ROS and free radicals also cause damage to biological membranes, modification of proteins, and deactivation of enzymes (Niki, 2010). Endothelial dysfunction is a principal concept of cardiovascular disease pathogenesis mediated by free radicals. It is responsible for the vascular tone regulation, inflammation, thrombosis, platelet activity, and atherosclerosis. The endothelial tone of the vasculature is a responsibility of substances like the endothelins, nitric oxide, endothelium-derived relaxation factor, and prostacyclins (Zorio *et al*., 2008) and is an underlying cause of rise in blood pressure.

Studies have also shown that in renovascular hypertension, essential hypertension, pre-eclampsia and malignant hypertension, there is increased ROS production (Higashi *et al*., 2002; Lip *et al*., 2002). Reactive oxygen species activities result in a direct cardiac injury due to oxidation of cellular constituents, diminishing nitric oxide bioactivity, and disruption of proteins critical for excitation-contraction coupling (Lubos *et al*., 2008).

A precursor of intracellular NOS, L-arginine, improves on the vascular endothelium and causes dilatation of blood vessels in patients with cardiac risk factors (Guoyao and Meininger, 2000). Superoxide dismutase supplementation improves on the endothelium and causes vasodilatation of coronary arteries. Calorie restrictions of superoxide dismutase for 3–12 months enhance cGMP formation and eNOS expression in mice (Nisoli *et al*., 2005).

Vitamin E is the term given to a group of tocotrienols and tocopherols which include alpha (α), delta (δ) and gamma (ϒ) tocopherols that is the most abundant and potent radical-scavenging in vivo antioxidant (Traber and Atkinson, 2007). Vitamin E is the major lipid-soluble component in the cell antioxidant defense system (Comitato *et al*., 2017). It has a role in antioxidant activity and it is derived from dietary component. The antioxidant property of Vitamin E is due to its ability to impede oxidative chain reactions and to scavenge lipid radicals thus, protecting the cardiovascular system from oxidative stress damage (Alshiek *et al*., 2017). Studies have shown that individuals who consume high amounts of vitamin E have decreased rates of chronic diseases like cardiovascular diseases (Rimm *et al*., 1993; Stamfer *et al*., 1993). The antioxidant property of Vitamin E is due to its ability to impede oxidative chain reactions and scavenge lipid radicals thus, protecting the cardiovascular system from oxidative stress damage (Alshiek *et al*., 2017).

Dexamethasone (DEX) is a member of glucocorticoid class of hormones that is strictly controlled for use due to its serious side effects that includes oxidative stress (You *et al*, 2009). Although used to treat inflammatory and autoimmune conditions, study conducted by Hasona *et al*, (2017) revealed that long-term use of dexamethasone reduced the antioxidant capacity of renal tissue and thereby leads to the formation of ROS. DEX suppresses endothelium-dependent vasodilatation of resistance arterioles by inhibiting endothelium nitric oxide synthethase (eNOS), a potent enzyme that helps in vasodilatation (Schafer *et al*, 2005). When eNOS is inhibited, there is vasoconstriction resulting in an increase in blood pressure. This study was designed to investigate cardiac and renal protective effects of vitamin E on dexamethasone-induced oxidative stress using rats as experimental model.

MATERIALS AND METHODS

Drugs and chemicals: Dexamethasone, vitamin E, chloroform and formalin were purchased from Sigma Aldrich (St. Louis, MO, USA). The angiotensin converting enzyme, nitric oxide, lactate dehydrogenase enzyme and bilirubin (ELISA) kits were purchased from Cayman Chemical Company, USA.

Experimental animal: Approval for the animal study was obtained from the Faculty of Basic Medical Sciences Animal Research Ethics Committee, University of Calabar (Approval No: 019PY20317). Twenty-four (24) healthy Wistar rats of both sexes weighing 180-250g were randomly assigned to four (4) groups of six rats each namely; group 1 - Normal Control (NC), group 2 - dexamethasone (DEX) only, group 3 - Vitamin E (Vit E) only and group 4 dexamethasone + Vitamin E (DEX+ Vit E), respectively. The rats were acclimatized for seven (7) days and housed in plastic cages in the animal room of the Faculty of Basic Medical Sciences, University of Calabar. The animals were kept at room temperature of 28±2ºC with a 12-hour light/dark cycle and were fed with normal rat chow and tap water *ad libitum.*

Induction of oxidative stress: Oxidative stress was induced following the method of Safaeian *et al* (2014) using dexamethasone (30µg/kg body weight). The drug was administered intraperitoneally daily for two (2) weeks.

Administration of vitamin E: Vitamin E was dissolved in 1 ml olive oil and administered orally using orogastric tube at a dose of 300 IU/kg body weight for two (2) weeks following the method of Safaeian *et al* (2014). It was administered concurrently with dexamethasone.

Collection of blood sample: All animals were euthanized under chloroform anaesthesia. The rats were then quickly dissected and blood was collected via cardiac puncture into plain sample bottles. The blood samples were allowed to stand for two hours and then centrifuged at 3000 g for ten minutes to obtain the serum. The serum was then collected and stored at - 20ºC for subsequent use for biochemical analysis.

Determination of cardiac and hypertension biomarkers: Biomarkers of hypertension namely nitric oxide (NO), bilirubin, angiotensin converting enzymes (ACE) and lactate dehydrogenase were determined as follows using appropriate methods. Measurement of nitric oxide was carried out by the method of Asl *et al.*, (2008) while serum bilirubin was determined using the method of Powel (1944). Angiotensin converting Enzyme (ACE) was

determined using ELISA kit following the method described by Syed *et al*., (2016) while lactate dehydrogenase was determined following the method of Sobel and Shell (1972).

Histopathological analysis: The hearts and the kidneys were rapidly dissected and fixed in 10% buffered formalin. The tissues were processed following the method of Mohammed and Ismail (2017). Briefly, the tissues were embedded in paraffin, sectioned at 5µm and stained with Haematoxylin and Eosin (H&E). The sections were examined under the light microscope (Zeiss, Germany) and the photomicrographs were captured using digital camera (Sony, Japan) attached to the microscope and connected to a personal computer (iCore 5, 4,00 MB RAM) at x400 magnification.

Determination of body weight

Total body weight of all the rats in each group was measured using a digital weighing balance each day before and after the experimental period with recordings taken as initial and final body weight respectively. The mean body weight was measured for each group from the total body weight and analyzed appropriately.

Statistical Analysis

Results were expressed as mean ± standard error of mean (SEM). Data obtained were analysed using one-way analysis of Variance (ANOVA) followed by Tukey's post hoc test using Graphpad prism software version 5.5 for Windows (Graphpad Software, San Diego, California, USA). For all statistical analysis, results were considered significant at $p<0.05$.

RESULTS

Serum nitric oxide concentration

The result for the normal control group (NC) was 133±1.3 nmol/L. It was 122±0.9 nmol/L in DEX only group and 138±0.4 nmol/L in Vit E group (Table 1). Administration of Vit E significantly ($P<0.01$) increased serum nitric oxide concentration from 122±0.9 nmol/L in dexamethasone-induced oxidative stress group to 134±0.5 nmol/L in DEX+Vit E group. However, serum level of NO in normal rats administered Vit E (138±0.4 nmol/L) was significantly ($p<0.01$) raised when compared to normal control (133±1.3 nmol/L) (Table 1). There was no significant difference between the control compared with DEX+Vit E group.

Serum angiotensin converting enzymes (ACE) activity: The result for serum angiotensin converting enzyme activity in the control group was 17.5±0.5 ng/ml, 24.3±0.1 ng/ml in DEX only group and 18.6±0.6 ng/ml in Vit E only group (Table 1). Administration of Vit E to DEX-induced oxidative stress rats significantly ($P<0.01$) reduced serum ACE activity to 21.0±0.4 ng/ml when compared with DEX only group, reversing the values towards the normal control group. The ACE activity in DEX+Vit E group was significantly ($p<0.05$) compared with control.

Serum total bilirubin level: The serum total bilirubin concentration in the control, Dex only, Vitamin E only, and Dex + Vitamin E groups was 3.20 ± 0.2µmol/L, 2.58 ± 0.1µmol/L,7.35 ± 0.1µmol/L, and 4.40 ± 0.1 µmol/L respectively. The result showed a significant ($p<0.01$) decrease concentration of bilirubin in the Dex only group compared to the control. The concentration was however significantly ($p<0.01$) increased after treatment with vitamin E. Vitamin E only group resulted in a significant ($p<0.01$) increase bilirubin concentration compared to both the control and Dex + vitamin E groups. This is presented in Table 1.

Serum level of lactate dehydrogenase enzyme (LDH): Administration of DEX to rats resulted in a significant ($P<0.01$) elevation (1315 ± 2.9IU/L) of LDH activity compared with control (347 ± 8.2IU/L) and Vitamin E only (397 ± 3.8IU/L) groups. In the Dex + Vitamin E group, LDH activity was significantly ($p<0.01$) decreased (846 ± 1.89IU/L) compared with the Dex only group, but still higher than the control and Vitamin E only groups (Table 1).

Table 1:
Serum levels of biomarkers of cardiac function and oxidative stress in dexamethasone-induced oxidative stressed rats treated with vitamin E

Parameter	Control	DEX only	Vit E only	DEX+Vit E
NO (nmol/L)	133±1.3	122±0.9**	138±0.4^{*a}	134±0.5^{b}
ACE (ng/ml)	17.5±0.5	24.3±0.4**	18.6±0.6	21.0±0.4^{a,c}
Bilirubin (µmol/L)	3.20±0.2	2.58±0.1	7.35±0.1**	4.40±0.1^{a}
LDH (IU/L)	347±8.2	1315±2.9**	397±3.8a	846±1.89^{*b}
SOD (U/ml)	4.7±0.6	0.91±0.7**	8.8±0.8^{a}	5.7±0.7^{ab}
MDA(nmol/L)	0.27±0.01	0.59±0.02**	0.32±0.01	0.42±0.02^{ab}

*** = P< 0.01 compared with control; a = p<0.01 compared with DEX only; b= p<0.01 compared with vitamin E; c=p<0.05 compared with control group; n = 6.*

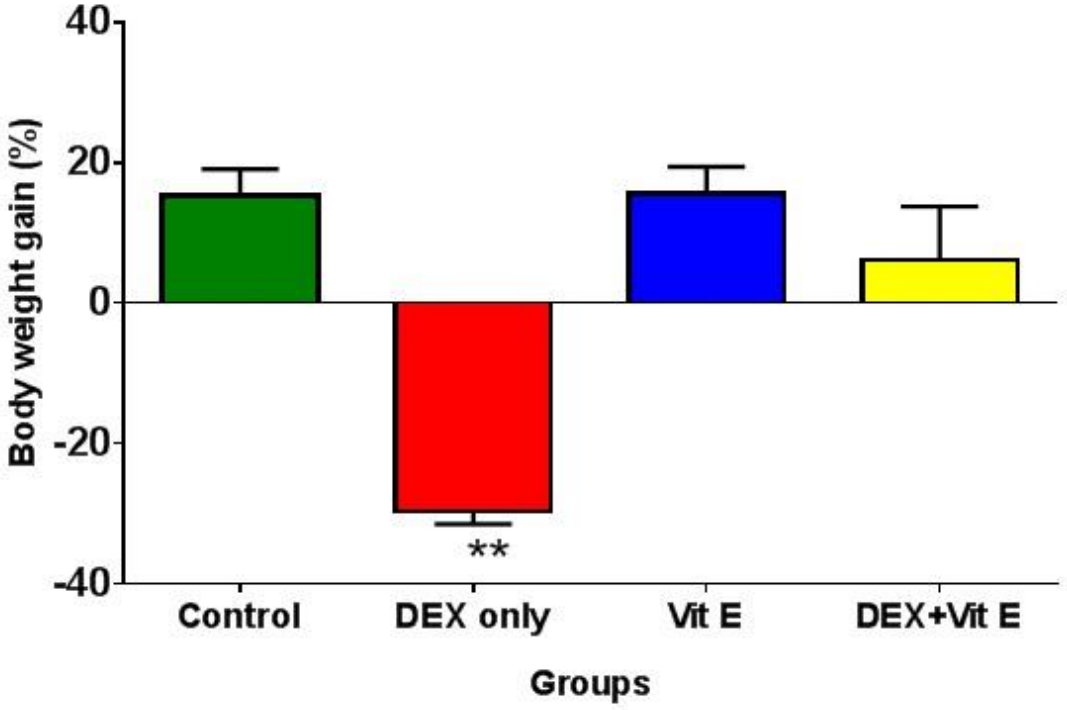

Figure 1:
Body weight gain in dexamethasone-induced oxidative rats treated with vitamin E.
*** = p<0.01 compared with other groups. N=6*

Serum superoxide dismutase (SOD) activity: A significant (p<0.01) reduction of SOD activity in DEX only group was observed compared with the normal control group. Administration of Vit E to DEX-induced oxidative stress rats significantly (P<0.01) increased serum superoxide dismutase activity compared with DEX only group and normal control group. Vitamin E administration also resulted in a significant (p<0.05) increase in SOD activity when compared with DEX only and control groups (Table 1).

Serum malondialdehyde (MDA) level: MDA level is a biomarker of oxidative stress. The result for normal control group as presented in table 1 was 0.27±0.01nmol/L. It was 0.59±0.02nmol/L in DEX only group and 0.32±0.01nmol/L in Vit E only group. Administration of Vit E to DEX-induced oxidative stress rats significantly (P<0.05) reduced serum MDA level to 0.42±0.02nmol/L. No significant difference was observed in Vit E only group (0.32±0.01nmol/L) when compared to the normal control group (0.27±0.01nmol/L). However, serum MDA level increased significantly (P<0.01) in DEX only group (0.59±0.02 nmol/L) when compared with other groups.

Body weight changes: The body weights of the experimental animals were similar at the start of the experiment. At the end of experiment, the body weight in the DEX-induced group (102±6 g) was significantly (P<0.01) reduced when compared with the control group (180±15 g). The body weights in Vit E (180±9 g) group increased significantly (P<0.01) when compared to the DEX-induced group. There was no significant difference in body weight between control and Vit E only groups. Treatment of DEX-induced oxidative groups with vitamin E (163±1 g) led to a significant (p<0.01) increase in body weight when compared with DEX only group.

The result for body weight gain within each group is presented in figure 1. There was a weight gain of 15.3±3.8% in the normal control and 15.5±3.9% in Vit E only group. There was weight loss of 29.7± 1.9% in DEX-induced oxidative group while Vit E treatment in resulted in body weight gain of 6.08±7.7%. The results showed a significant (p<0.01) decrease in body weight gain in the DEX-induced oxidative stressed rats compared to all other groups.

Histological analysis of cardiac muscles: Section of cardiac muscle from the control group showed intersecting bundles of striated muscle fibres running parallel with interdigitation. The individual cardiac myocytes had prominent nuclei with moderate amount of cytoplasm. The separating fibrocollagenous stroma is sparse and contains thin walled blood vessels. The photomicrograph for the normal control group is shown in Fig. 2A.

Section of cardiac muscle in DEX-induced oxidative stress rats showed intersecting bundles of striated muscle fibres running parallel with interdigitation with hypertrophied and widely separated cardiac cells. The cells were plumped with abundant cytoplasm and enlarged nuclei with clumped chromatin pattern. The separating fibrocollagenous stroma showed scanty and thin walled dilated blood vessels (Fig. 2B).

Section of cardiac muscle from Vit E only group showed intersecting bundles of striated muscle fibres running parallel with interdigitation. The individual muscle fibres were stretched out with moderate amounts of cytoplasm and abundant fibrocollagenous stroma within which were congested blood vessels. The cells had enlarged nuclei and clumped chromatin pattern (Fig. 2C).

In DEX +Vit E treated group (figure 2D), section of cardiac muscle showed intersecting bundles of striated muscle fibres running parallel with interdigitation. The fibres showed moderate cardiac hypertrophy with separated thinned fibrocollagenous stroma within which were congested blood vessels. The cells were plumped with abundant cytoplasm and enlarged nuclei having clumped chromatin pattern. The separating fibrocollagenous stroma were scanty and contained thin walled dilated blood vessels

Histological analysis of the kidney: Section of the kidney in control group showed prominent glomeruli and renal tubules. The glomeruli consisted of empty Bowman space surrounding the cellular mesangium comprising of the mesangial cells and arterioles. The renal tubules were lined by tall cuboidal to columnar cells. The intervening interstitium was scanty and contained thin walled blood vessels (figure 3A).

In DEX-induced oxidative stressed group, the section of the kidney showed glomeruli and renal tubules that had empty Bowman spaces surrounding the hypocellular mesangium. The mesangial cells were sparsely populated and atrophic and characterized by arteriolar haemorrhage. The intervening interstitium was scanty and with congested blood vessels suggesting glomerular injury (figure 3B).

In the Vit E only group, the section of the kidney shows glomeruli and renal tubules that were mildly swollen and had empty Bowman spaces surrounding a cellular mesangium. The mesangial cells were also sparsely populated. The renal tubules were preserved but closely packed with empty lumen and their lining epithelium were cuboidal to columnar. The photomicrograph for vitamin E only group is shown in figure 3C.

In the DEX+Vit E group, section of the kidney showed glomeruli and renal tubules. The Glomeruli consisted of an empty Bowman's space surrounding the cellular mesangium. The mesangial cells were prominent. The renal tubules were also preserved but closely packed with empty lumen. The intervening interstitium was scanty and contained thin walled congested blood vessels. The photomicrograph for DEX+ Vit E group is shown in figure 3D.

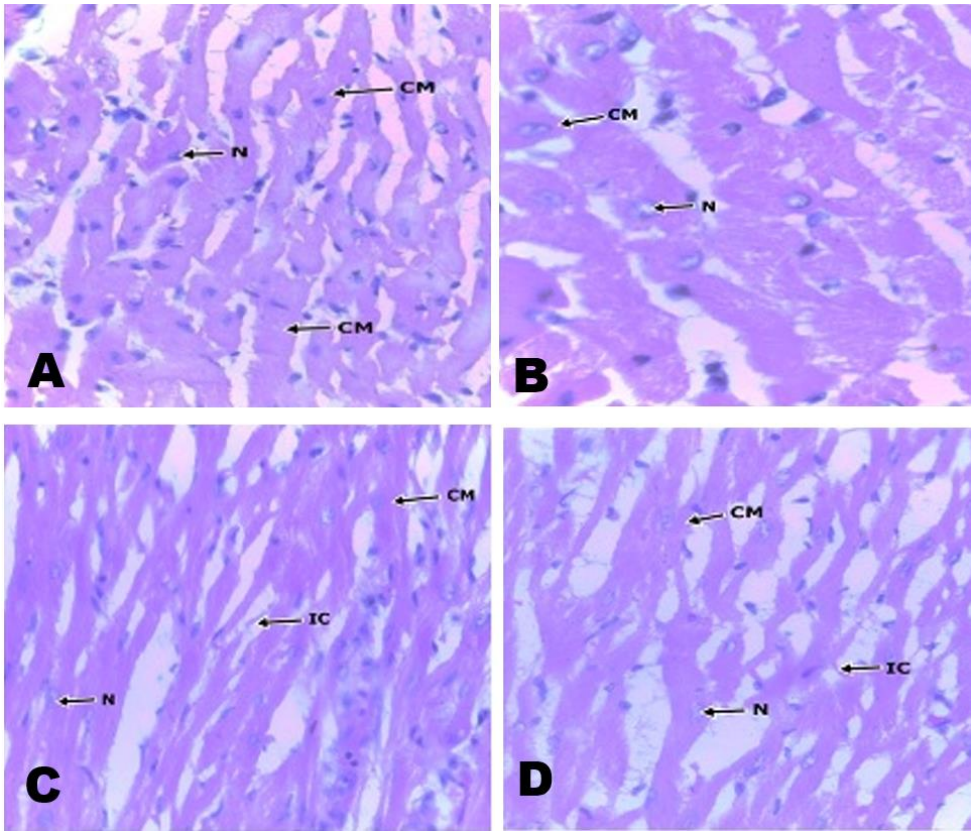

Figure 2: Representative photomicrograph of the cardiac muscles in control and dexamethasone-induced oxidative stress rats in rats treated with vitamin E.
A= Control;
B= DEX only;
C= VIT E group;
D= DEX +Vit E;
CM = cardiac muscle; N = nucleus; IC = intracellular capsule;
H & E stain (X400)

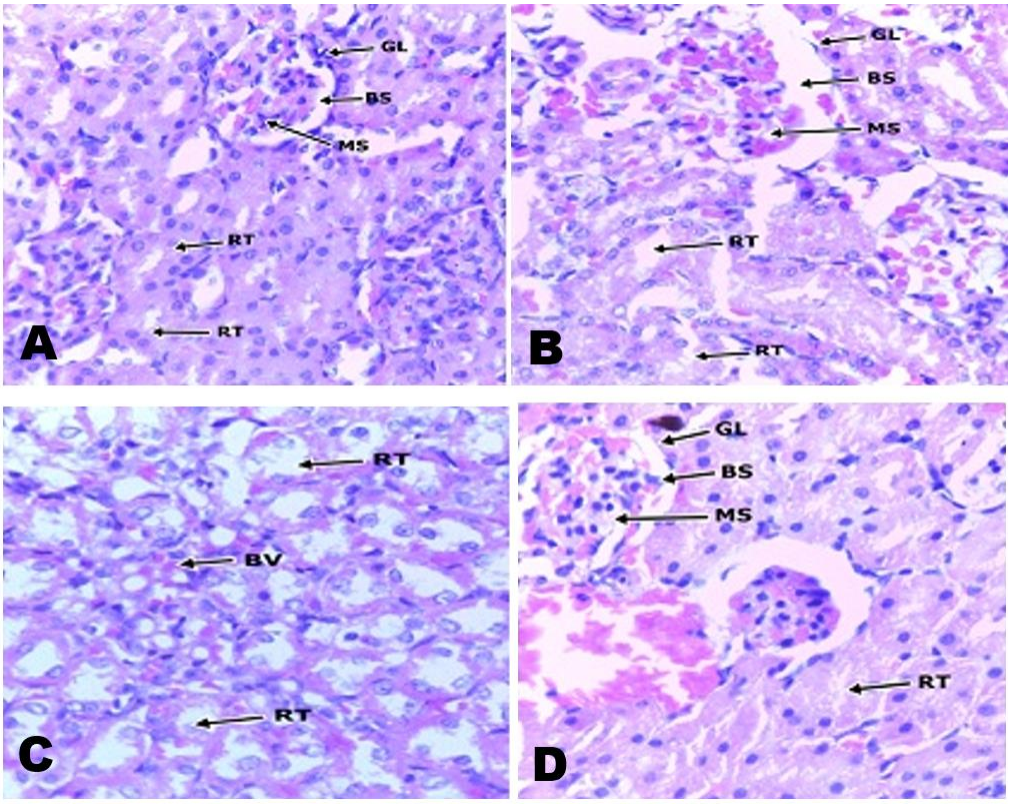

Figure 3: Representative photomicrograph of the kidney in control and dexamethasone-induced oxidative stress rats in rats treated with vitamin E.
A= control;
B= DEX only;
C= VIT E group;
D= DEX +Vit E;
BS= Bowman's space;
RT= Renal tubule;
BV= Blood vessel;
GL= Glomerulus;
MS= Mesengium
H & E stain (X400)

DISCUSSION

This study was aimed at investigating the cardiac and renal protective effects of Vitamin E in dexamethasone-induced oxidative stressed rats. Dexamethasone resulted in oxidative stress and lipid peroxidation of tissues. This is evident in the reduction of the serum concentration of superoxide dismutase (SOD) and elevation in the concentration of malondialdehyde (MDA). The result agrees with previous studies where dexamethasone was used to induce oxidative stress (Bjelavonic *et al*., 2007; Feng and Tang, 2014). The oxidative stress caused by dexamethasone resulted in a reduction in the serum concentrations of nitric oxide and bilirubin, and caused increase in the concentrations of angiotensin converting enzyme and lactate dehydrogenase. However, treatment with vitamin E reversed the effects of dexamethasone tending towards normal.

Increased reactive oxygen species (ROS) production has been reported to reduce the bioavailability of nitric oxide (NO) in addition to the formation of peroxynitrite (Montezano, and Touyz, 2012). Peroxynitrite uncouples the endothelial nitric oxide (NO) synthase to form a harmful superoxide generating enzymes which in turn contributes to vascular damage. Nitric oxide is a vasodilator produced by an enzyme, endothelium nitric oxide synthetase (ENOS) in the endothelium and in the brain by inducible nitric oxide synthatase (INOS) (Alp and Channon, 2004). Inactivation or reduction in NO synthesis is one of the risk factors for cardiovascular diseases including hypertension (Forsterman, 2010). The group treated with vitamin E showed a significant increase in NO concentration when compared to oxidative stress-induced group. This suppression of free radicals by vitamin E could possibly act to reduce the adverse effect of ROS that has vasoconstrictive effects due to a decrease in NO concentration (Tsao, 2010). Vitamin E probably caused a reduction in oxidative stress via its antioxidant activity.

Angiotensin converting enzymes (ACE) is present as a membrane-bound enzyme in endothelial cells other cells of the body. It catalyzes the conversion of Angiotensin I to angiotensin II, a potent vasoconstrictor that raises blood pressure (Skidgel and Erdos, 1993). Activation and increased level of angiotensin converting enzymes (ACE) occurs during stress conditions such as hypertension and myocardial infarction (Barreto-Chaves *et al*., 2000). The results from this study show that there was a significant increase in the ACE activity of rats induced with oxidative stress. Vitamin E administration to oxidative stress group led to a significant decrease in ACE activity. The observed decrease in ACE level suggests the beneficial action of vitamin E in reducing or inhibiting the angiotensin system (RAS) pathway thus reducing the adverse effect of oxidative stress. A study has shown that inhibition of ACE enhances vascular health (Bakris, 2001).

In humans and other mammals, increased serum total bilirubin levels have been reported to decrease the risk of coronary artery disease and atherosclerosis (Montezano and Touyz, 2014). As a risk factor in

arterial hypertension, bilirubin has an inverse relation in hypertension since high level of serum bilirubin decreases blood pressure (Huang *et al*, 2016; Xu *et al* 2017). Bilirubin is also one of the non-enzymatic antioxidant, and function as a chain breaking antioxidant (Stocker *et al*., 1987).

The results showed increased level of serum total bilirubin in rats that were given vitamin E only and in rats treated with vitamin E after inducing oxidative stress. Increased bilirubin level in Vitamin E administration could possibly be attributed to the enhanced activity of haeme oxygenase, a rate limiting enzyme that aids in converting biliverdin to bilirubin. Degradation of heme may mediate beneficial effect such as anti- inflammatory and antioxidant properties. Heme oxygenase mediates antioxidant and anti-inflammatory beneficial effects and has been reported to reduce blood pressure (Biyani *et al*., 2016). Thus, increase bilirubin level reflect an increase in antioxidant activity of vitamin E.

Lactate dehydrogenase is an enzyme found in almost all living cells and it is produced mostly by cardiac and skeletal muscles (Vettor *et al*., 1997). LDH is a marker of myocardial infarction and hypertension (Hu *et al*., 2015). Lipid peroxidation during oxidative stress disrupts cell integrity leading to an increase level of LDH activity (Jovanovic *et al*., 2010). The observed decrease in LDH activity due to vitamin E administration could be due to reduction in oxidative damage. Vitamin E has been shown to reduce LDH activity in oxidative stress induced by various conditions (Ilavazhagan *et al.,* 2001; Pashkow, 2011).

Superoxide dismutase (SOD) is an important endogenous enzyme that exists in several forms and act as a first line of defense system against ROS (Fukai and Ushio-Fukai, 2011). Several studies have revealed a reduction in the activity of SOD in hypertensive patients and in experimental models of oxidative stress (Lassègue and Griendling, 2004; Sousa *et al*., 2008). The significant increase in SOD activity in oxidative stress group treated with vitamin E and vitamin E-only group shows the protective effect of vitamin E against oxidative injury. It has been reported that vitamin E protects cellular membrane from lipid peroxidation and thus reduces oxidative stress caused by ROS (Urso and Clarkson, 2003). Dietary supplementation with antioxidant vitamins such as vitamin C and E has been reported to increase antioxidant enzymes activity (Day and Lal, 2012).

Malondialdehyde (MDA) is one of the end products of oxidative reactions in biological tissues and fluids and is used as a biomarker of oxidative stress (Todorova *et al*, 2005). Increased MDA levels indicates a high rate of lipid peroxidation (Huszar and Vigue, 1994). The significant increase in MDA level in dexamethasone treated rats reflects oxidative stress induced by dexamethasone. Vitamin E administration resulted in a low level of MDA in DEX+VitE suggesting a decrease in lipid peroxidation. A study has shown that vitamin E administration reduces lipid peroxidation by inhibiting various steps in lipid peroxidation (Krajčovičová-Kudláčková *et al*., 2004).

There was a decrease in body weight caused by dexamethasone administration. This finding is in agreement with a previous study that reported body weight loss in rats due to dexamethasone treatment (Amar *et al*, 2013). This body weight loss could be attributed to its inhibitory effect on the appetite centre. It has been reported that dexamethasone down-regulates the hypothalamic appetite centre through the release of neurotransmitters, insulin signaling and neuropeptides leading to a reduction in food intake and ultimate body weight loss (Chruvattil *et al*., 2016). An increased body weight was observed in the groups of animals treated with vitamin E showing the beneficial effect of vitamin E in improving body weight. Studies have reported that vitamin E enhances body weight due to its protective role on the cell membrane and slowing down body metabolism and lipolysis (Azman *et al*., 2001; Shvedova *et al.,* 2007).

Histological analysis on the structural integrity of the cardiac muscle in dexamethasone-induced oxidative stress group showed cardiac injury and hypertrophy. An earlier study has shown that administration of graded doses of dexamethasone causes lesions in the kidney and marked necrosis of the cardiac fibers and cytoplasmic fatty vacuolation (Ahmed and Masoud, 2014). In the vitamin E treated group, there was minimal hypertrophy revealing the cardio-protective property of vitamin E. The hypertrophied cardiac muscle in dexamethasone-induced oxidative stress group may be due to stress, pressure load and damage of the blood vessels caused by increased ROS. Prolonged used of dexamethasone causes cardiac hypertrophy and remodeling (de Vries *et al.,* 2002). Vitamin E reversed the damage of cardiac muscle as evidenced by minimal myocyte loss and mild glomerular injury seen in DEX+Vit E group. This result shows the ability of vitamin E in reducing ROS that may cause oxidative damage to tissues.

In summary, the results of this study have shown that dexamethasone adversely altered biomarkers of hypertension and caused cardiac and renal injury. Vitamin E significantly reduced angiotensin converting enzymes, lactate dehydrogenase and total serum bilirubin level in oxidative stress model, while it increased nitric oxide concentration significantly. Oxidative stress markers such as superoxide dismutase level increased significantly in vitamin E treated groups, while malondialdehyde concentration was reduced significantly in the treated group. In conclusion, vitamin E ameliorates dexamethasone-induced oxidative stress and protects the cardiac and renal tissues against oxidative injury.

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Research Article

Selenium Supplementation Increases Hepatic Glucose-6-Phosphatase and Peroxisome Proliferator Activated Receptor Gamma Coactivator-1α Activity in Male Wistar Rats

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Summary: Increased selenium supplementation has been implicated in diabetes mellitus via peroxisome-proliferator-activated-receptor-gamma-coactivator-1-alpha (PGC-1α) associated pathways. This study was designed to investigate the effect of selenium supplementation on PGC-1α and glucose-6-phosphatase (G6Pase) as well its likely hepato toxicity in male Wistar rats. Animals were randomly divided into 3 groups (n=10/group) and treated orally with water (0.2ml - group 1) or selenium (25µg/day -group 2; 50µg/day - group 3) for 28 and 56days, respectively. Thereafter, blood samples were collected and estimated for glucose, alkaline-phosphate (ALP), gamma-glutamyltransferase (GGT) and aspartate-aminotransferase (AST). Liver homogenates were analyzed for PGC-1α and G6Pase activity. Significant dose-dependent increases in blood glucose, hepatic PGC-1α and G6Pase activities were observed on days 28 and 56 in selenium groups compared to group 1. Serum GGT activity increased in both selenium groups on day 28 however, on day 56 values in group 2 were reduced and increased in group 3, respectively. Compared to control ALP reduced in selenium groups while AST was not significantly different. This study suggests that selenium supplementation increases hepatic peroxisome-proliferator-activated-receptor-gamma-coactivator-1α and glucose-6-phosphatase activity leading to a likely increase in hepatic glucose output. It also shows that though selenium supplementation at the doses used maybe nontoxic to hepatocytes, it may however exert potential toxicity on the biliary tract.

Keywords*: Selenium, Hepatic peroxisome proliferator activated receptor gamma coactivator-1-alpha, Hepatic glucose-6-phosphatase, Liver enzymes*

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INTRODUCTION

Micronutrients are essential inorganic substances needed by the body in small quantities for important processes such as growth, bone and teeth formation, brain development, immune functions and glucose homeostasis (Soetan *et al*, 2010). They are also essential components of enzyme systems and are often required for normal nerve function and development. Examples of these micronutrients include calcium, phosphorus, magnesium, sodium, potassium, chloride, iron, zinc, iodine, selenium and copper (Gernand *et al*, 2016).

Selenium is a micronutrient that is critical for the synthesis of selenoproteins, which play important roles in the antioxidant defense system, reproduction, normal muscle function, tumor prevention, and tumor suppression (Mehdi *et al*, 2012). According to Institute of Medicine, Food and Nutrition Board, Washington DC (2000) and Ogawa-Wong *et al*., (2016) the recommended daily average for selenium is 55 microgram and its supplementary intake has long been publicized, due to its reported cytoprotective properties which arises from its ability to up-regulate antioxidant selenoenzymes (Mehdi *et al*, 2012) and inhibit many inflammatory cell mechanisms (Huang *et al*, 2012). Selenium has also been reported to exert hormesis effects with low doses having beneficial effects and a high dose having toxic effects (Huang *et al*, 2012). Selenium supplementation may have adverse effects in people who are already receiving an adequate intake of selenium (Ogawa-Wong *et al*, 2016). Individuals with high baseline of Selenium have been reported to have an increased risk of type-2-diabetes development (Stranges *et al*, 2007; Vinceti *et al.*, 2018). The actual potential mechanisms underlying this association between selenium status and type 2 diabetes are profuse (McClung *et al*., 2004; Misu *et al*., 2010; Steinbrenner, 2013; Zhou *et al*., 2013; Ishikura *et al*., 2014) however, increased activity of peroxisome proliferator activated receptor gamma coactivator 1 alpha (PGC-1α) has been proposed to be a strong link between high selenium

intake and type-2-diabetes mellitus (Steinbrenner *et.al*, 2011).

PGC-1α is a transcription co-activator which interacts with a broad range of transcription factors to regulate a wide range of biological responses including adaptive thermogenesis, mitochondrial biogenesis, glucose and fatty acid metabolism (Liang and Ward, 2006). It has been reported to control the transcription of rate-limiting gluconeogenic enzymes such as glucose-6-phosphatase. Glucose-6-phosphatase is found mainly in the liver and plays an important role in regulating hepatic glucose output (Wu *et al*, 2016). This study was therefore designed to investigate the effect of sub-chronic and chronic selenium supplementation on hepatic peroxisome proliferator activated receptor gamma co-activator 1 alpha (PGC-1α) and glucose-6-phosphatase activity as these have been observed to regulate hepatic glucose output. The effects of selenium supplementation on the liver toxicity will also be evaluated using the liver function tests

MATERIALS AND METHODS

Animal and grouping: Thirty male (30) Wistar rats were housed in standard well-aerated laboratory cages. They were fed on standard rat chow (Ladokun feeds, Nigeria) and allowed free access to drinking water *ad libitum.* The Applied and Environmental Physiology Unit, Department of Physiology, University of Ibadan approved this experiment. Animals received humane care, and procedures were in accordance with the Guide for the Care and Use of Laboratory Animals (1996, published by National Academy Press, 2101 Constitution Ave. NW, Washington, DC 20055, USA). The animals were randomly divided into 3 groups of 10 rats each consisting of Group 1, that was given distilled water (0.2ml); group 2 which were treated with selenium (as Sodium selenite) at 25µg/day and group 3 that were treated with selenium at -50µg/day (Kang *et al*., 2001), respectively. All treatments were carried out orally once daily for 28 and 56days respectively.

Measurements and Biochemical Assay

Body weight and Assessment of Blood Glucose level: Body weight was monitored throughout the study using a standard laboratory scale while blood glucose, before and after Selenium supplementation, was assessed using the tail tipping method as follows: the tail tip of the animal was punctured with a disposable blood lancet. A drop from the pool of blood on the tip of the tail was collected on an Accu-Check active glucose test strip and thereafter analyzed on an Accu-Check active glucometer (Tack *et al*., 2012) (Roche, Germany), which uses the glucose oxidase method (Barham and Trinder, 1972) as its method of analysis.

Biochemical Assays

Blood samples were collected by cardiac puncture under mild anesthesia (Sodium thiopentone-50mg/kg) into plain sample bottles. The samples were allowed to coagulate and centrifuged at 3500rpm for 15minutes to obtain serum. Aliquots of the clear serum obtained was analyzed for alkaline phosphate (ALP), Aspartate Aminotransferase (AST), and gamma-glutamyltransferase (GGT) respectively.

Assessment of Alanine Aminotransferase (ALT) activity: The ALT activity was determined following the principle described by Reitman and Frankel (1957). Briefly, 0.1ml of diluted serum was mixed with phosphate buffer (100mmol/L, pH 7.4), L-alanine (100mmol/L), and α- oxoglutarate (2mmol/L) and the mixture was incubated for exactly 30mins at 37^0C. 0.5ml of 2,4- dinitrophenylhydrazine (2mmol/L) was then added to the reaction mixture and allowed to stand for exactly 20mins at 25^0C. Thereafter, 0.5ml of NaOH (0.4mol/L) was added and the absorbance was read against reagent blank after 5mins. Reagent blank was prepared as described above replacing sample with 0.1ml of distilled water. The ALT was measured by monitoring the concentration of pyruvate hydrazone formed with 2, 4-dinitrophenylhydrazine at 546nm.

Assessment of Aspartate Aminotransferase (AST) Activity: The AST activity was determined following the principle described by Reitman and Frankel (1957). Briefly, 0.1ml of diluted sample was mixed with phosphate buffer (100mmol/L, pH 7.4), L-aspartame (100mmol/L), and α-oxoglutarate (2mmol/L) and the mixture incubated for exactly 30mins at 37^0C. 0.5ml of 2,4-dinitrophenylhydrazine (2mmol/L) was added to the reaction mixture and allowed to stand for exactly 20min at 25^0C. Then 5.0ml of NaOH (0.4mol/L) was added and the absorbance read against the reagent blank after 5mins at 546nm. The AST activity was measured by monitoring the concentration of oxaloacetate-hydrazone formed with 2, 4- dinitrophenylhydrazine.

Assessment of gamma-glutamyltransferase (GGT) Activity: The GGT activity was determined following the principle described by (Szasz, 1974) in which the rate of increase in absorbance due to release of p-nitroaniline is measured at 405 nm and 37°C. Briefly, 2.90 ml of the substrate solution (0.16M Glycylglycine, 0.016M Magnesium chloride, 0.05M Tris base, 120mg Gamma-glutamyl-p-nitoanilide) was pipetted into a cuvette and incubated in the spectrophotometer at 37°C for 10 minutes to attain temperature equilibration. A blank recording was

taken at 405nm and curve of the change in absorbance per min ($E_{405/min}$) was plotted. 10ml of the sample was thereafter added to the cuvette, mixed and the increase in absorbance at 405nm was taken for 5-8min. The change in absorbance ($\Delta E_{405\ nm/min}$) was calculated from the linear portion of the curve.

Determination of hepatic glucose-6-phosphatase and PGC1-α activity: Liver samples were also excised from each animal and homogenized on ice with ice-cold 0.25M sucrose buffer (for determination of glucose-6-phosphatase activity) and 0.1 M phosphate buffer (1: 4 w/v, pH 7.4) (for determination of PGC1-α activity), respectively. The homogenates obtained was centrifuged at 4000 RPM for 10 minutes at 4^0C and the resulting supernatants was frozen at -4°C until use. Aliquot of the supernatants was assayed for glucose-6-phosphatase activity using the method of Koide and Oda (1959). Briefly, into a test tube, 300µl of 0.1M citrate buffer, 500µl of 150mM glucose-6-phosphate solution and 200µl of sucrose buffer extracted liver homogenate were added. The mixture was thoroughly mixed and incubated at 37^0C for 1 hour. Thereafter 1.0ml of 10% trichloroacetic acid (TCA) was added to stop the reaction and placed on ice. After 10mins on ice, the mixture was centrifuged. 1ml aliquot of the supernatant was pipetted into a test tube to which was added 1.0ml of 1.25% Ammonium molybdate and 9% Ascorbic acid in a stepwise manner. The mixture was incubated at room temperature for 1hour and read at 660nm against a reagent blank. The liberated phosphate was determined by comparing with standards of known concentration.

Aliquots of the supernatant were also evaluated for Hepatic PGC1-α activity using Enzyme-Linked Immunosorbent Assay (ELISA) kits according to the manufacturers (Bioassay Technology Laboratory, China.) directions.

Statistical Analysis

All the data were presented as Mean + Standard Error of Mean (SEM) and subjected to one-way analysis of variance (ANOVA) and Newman-Keil post analysis test using the GraphPad prism version 7.0 (GraphPad software, San Diego, CA USA). Statistical significance was taken at P<0.05.

RESULTS

Effects of selenium supplementation on the body weight (g) and blood glucose level (mg/dL) in control and experimental groups: Body weight in the control group increased consistently with values obtained on day 56 being 7.3% higher compared to day 0 values within same group. Animals in group 2 (Selenium 25µg treated) and 3 (Selenium 50µg treated) also showed body weights that increased consistently with values on day 56 being 19.6% and 20.1% increased (p<0.05) compared with initial values (day 0) within each group respectively (Table 1). Blood glucose values obtained in the control group was relatively stable throughout the study while values in group 2 and 3 increased consistently with values obtained on day 56 being 34.2% and 67.8% increased (p<0.05) respectively compared to their initial day 0 values (Table 2).

Table 1.
Effect of selenium on the body weight (g) in control and experimental groups

	Control	Group 2 (selenium 25µg treated)	Group 3 (selenium 50µg treated)
Day 0	172.0 ± 5.3	171.6 ± 6.5	179.2 ± 7.3
Day 28	180.8 ± 5.2	197.6 ± 2.2	207.0 ± 3.4[a]*
Day 56	184.6 ± 5.7	205.2 ± 1.5[b]*	215.2 ± 1.6[b]*

** Indicates values that are significantly different from day 0 values within same group.[a] indicates values that are significantly different from control on day 28; [b] indicates values that are significantly different from control on day 56.*

Table 2.
Effect of selenium on blood glucose level (mg/dL) in control and experimental groups

	Control	Group 2 (selenium 25µg treated)	Group 3 (selenium 50µg treated)
Day 0	42.0 ±8.1	47.4. ±2.9	49.0 ±2.7
Day 28	50.8 ±2.7	66.6 ±4.5[a]*	71.0 ±4.8[a]*
Day 56	54.6 ±4.5	63.6 ±2.8[b]*	82.2 ±5.7[b]*

** Indicates values that are significantly different from day 0 values within same group. [a] indicates values that are significantly different from control on day 28; [b] indicates values that are significantly different from control on day 56.*

Effects of selenium supplementation on hepatic glucose-6-phosphatase and peroxisome proliferator activated receptor gamma coactivator-1α in control and experimental groups: Glucose-6-phosphatase (G6Pase) activity (µmol Pi liberated/hour/mg protein) was significantly increased (p<0.05) on day 28 and 56 in group 2 (Selenium 25µg treated) and 3 (Selenium 50µg treated) compared to group 1 (control). However, G6Pase values on day 56 in groups 2 (82.8±3.8) and 3 (260.9±12.9) were significantly reduced compared to values obtained in same groups on day 28 (196.4±6.8; 311.5±15.0), respectively (Fig. 1).

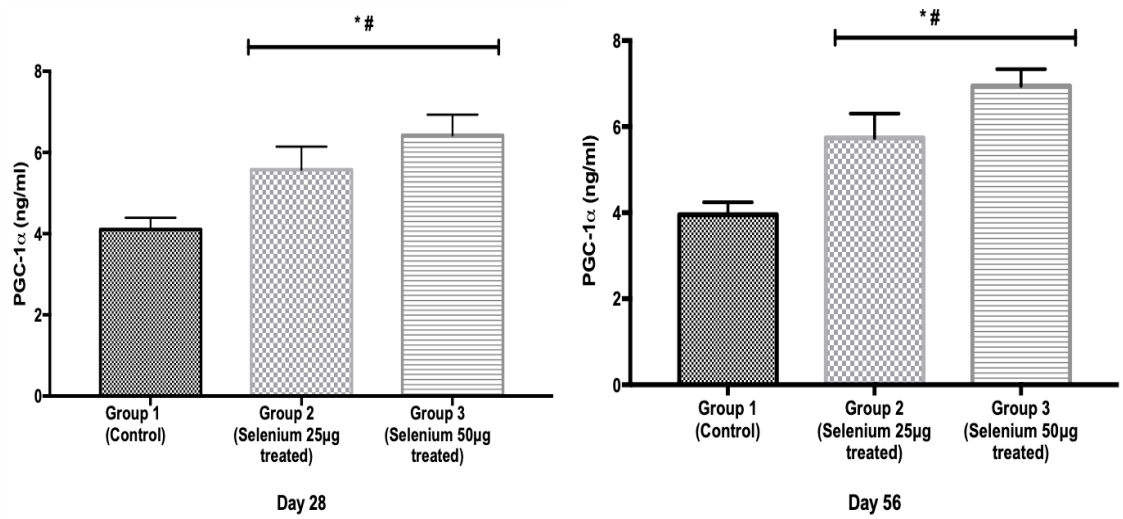

Figure 1.
Effect of Selenium on hepatic glucose-6-phosphatase activity in control and experimental groups.
** Indicates values in group 2 that are significantly different from group 1 . # Indicates values in group 3 that are significantly different from group 1; ª indicates values in groups 2 and 3 on day 56 that are significantly different from values obtained in same group on day 28.*

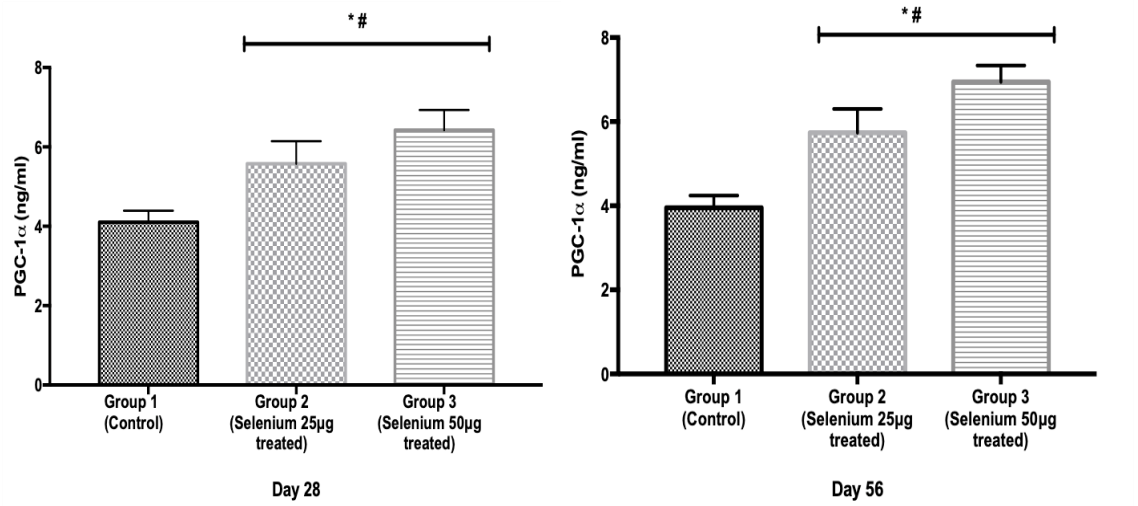

Figure 2.
Effect of Selenium on peroxisome proliferator activated receptor gamma coactivator-1α(PGC1 alpha) activity in control and experimental groups.
** Indicates values in group 2 that are significantly different from group 1. # Indicates values in group 3 that are significantly different from group 1.*

Table 3.
Effect of selenium on Liver function test in control and experimental groups

	Alkaline Phosphate (U/L)		Gamma-glutamyltransferase (U/L)		Aspartate Aminotrasferase (U/L)	
	Day 28	**Day 56**	**Day 28**	**Day 56**	**Day 28**	**Day 56**
Group 1 (Control)	120.0 ±1.8	125.4 ±2.14	1.74 ±0.26	1.55 ±0.35	44.6 ±0.68	47.4. ±2.9
Group 2 (Selenium 25µg treated)	99.0 ±5.50*	111.8 ±2.60*	2.66 ±0.21*	0.96±0.26*	41.0 ±1.76	42.6 ±2.54
Group 3 (Selenium 50µg treated)	112.2 ±2.13#	112.6 ±1.44#	3.00 ±0.23#	2.80 ±0.25#	40.6 ±1.36	43.6 ±1.12

** Indicates values in group 2 that are significantly different from group 1 (control). # Indicates values in group 3 that are significantly different from control.*

Peroxisome proliferator activated receptor gamma coactivator-1α activity (ng/ml) was increased significantly ($p<0.05$) on day 28 and 56 in groups 2 (5.58±0.57; 5.74±0.57) and 3 (6.42±0.51; 6.94±0.39) compared to group 1 (4.10±0.29; 3.95±0.35) on both days respectively (Fig. 2).

Effects of selenium supplementation on liver function tests in control and experimental groups: Alkaline phosphate (U/L) on days 28 and 56 significantly reduced in groups 2 (Selenium 25µg treated) and 3 (Selenium 50µg treated) compared with group 1(control) respectively (Table 3). On days 28 and 56, gamma-glutamyl transferase (U/L) values indicate a significant increase in the experimental groups compared to control (Table 3). However, aspartate aminotransferase (U/L) values on day 28 and 56 in all groups were not significantly different (Table 3).

DISCUSSION

Selenium supplementation in humans has been widely advocated because it is a vital constituent of selenoproteins, which play critical roles in reproduction, thyroid hormone metabolism, DNA synthesis, prevention of oxidative damage and protection against infections (Mehdi *et al*, 2013). However, the likely predisposition of excessive Selenium supplementation to diabetic conditions has been suggested (Steinbrenner *et al*, 2011). This study showed an increase in the body weight of selenium treated animals after sub-chronic (28days) and chronic (56days) supplementation at both low (25µg/day) and high (50µg/day) doses. This is consistent with the previous finding of Hawkes and Keim (2003) who affirmed that high Selenium diet induces subclinical hypothyroidism, which often leads to decreased energy expenditure and increased weight gain. The percentage increase in blood glucose level in the Selenium treated groups compared to control is consistent with the report of Ayaz *et al*, (2005) and suggests a likely hyperglycemic effect of Selenium supplementation which could be dose dependent as treatment with the selenium (50µg/day) treatment group having a higher glucose level than that of the lower dose (selenium, 25µg/day) treatment group.

This study also shows an increase in PGC-1α activity after sub-chronic and chronic selenium supplementation. This is consistent with the reports of Mehta *et al*, (2012) who also reported a significant increase in PGC-1α activity with sodium selenite treatment. An increase in hepatic PGC-1α activity and expression has also been linked to the stimulation of increased hepatic glucose output, and when coupled with its reported inhibitory effect on insulin signaling and secretion (Wu *et al*, 2002; Koo *et al*, 2004; Liang and Ward, 2006) may also account for the increase in blood glucose level observed in the selenium treated groups compared to controls. Furthermore, glucose 6-phosphatase - an enzyme that is found mainly in the liver and kidney – is known to hydrolyze glucose 6-phospate resulting in the creation of a phosphate group and free glucose (Ghosh *et al*., 2002). The activity of this enzyme was increased in the selenium treated groups in this study thus suggesting increased hepatic glucose production and hence the observed differences in blood glucose level between control and selenium treated groups, respectively. Taken together, the increased liver PGC-1α activity, glucose 6-phospatase activity and blood glucose observed in this study suggests a potential diabetogenic activity of selenium supplementation as has been reported in a large number of epidemiologic studies (Vinceti *et al.*, 2018).

This study investigated the likelihood of the existence of a liver disease following acute and chronic selenium supplementation at low (25µg/day) and high doses (50µg/day), respectively. The results obtained in the present study indicates a reduced alkaline phosphate (ALP) level in the selenium treated groups compared to control, which suggests that, the hepatocytes and osteoblasts were not damaged by selenium supplementation (Thapa and Anuj, 2007). However, the increased gamma-glutamyl transferase (GGT) levels observed in the selenium treated animals suggests the likelihood or presence of bile duct problems as it is usually the first liver enzyme to rise in the blood when any of the bile ducts that carry bile from the liver to the intestines become obstructed (Lum and Gambino, 1972;Singh *et al*, 2006). Aspartate aminotransferase (AST) levels were also not significantly different across the groups and this again suggests that selenium supplementation may not be toxic to either liver or muscle cell at the doses used.

In conclusion, this study suggests that sub-chronic and chronic selenium supplementation may increase the activity of both peroxisome proliferator-activated receptor gamma coactivator-1alpha (PGC-1α) and glucose 6-phosphatase leading to an increase in hepatic glucose output. The increased production of gamma-glutamyltransferase (GGT) observed in this study suggests a likely predisposition to biliary track dysfunction following sub-chronic and chronic selenium supplemental intake.

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Research Article

Effects of Mosquito Coil Smoke Inhalation on Spatial Memory in Mice

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Summary: Mosquito coil (MC) is widely used to repel mosquitoes in order to prevent malaria in many malaria-endemic countries. Although we are fully aware and concerned about carbon monoxide (CO) and its toxicity, exposure to CO from common, though occult sources like MC smoke is often overlooked. Equally, the adverse health effects, especially to the brain, are usually underestimated. This work was aimed at assessing the effects of exposure to CO from MC smoke inhalation on spatial memory in mice. Sixteen, adult, male, mice, were randomly assigned to either the experimental or the control group; each having 8 mice. The experimental group was exposed to the MC smoke (Wavetide, China) that was allowed to burn inside the gas chamber (75 cm x 50 cm x 50 cm) for 15 minutes, daily, for 14 days. Digital CO meter (*PCMM05 Pyle*) was used to measure the amount of CO and Barnes maze protocol to assess the spatial memory. Our results indicate that burning MC for 15 minutes produced up to 312 *parts per million* (*ppm*) of CO and raised the blood carboxy-hemoglobin (*COHb)* level by 15.8%. This is higher than the *WHO* recommended limit (<100 mg/m^3 or 87 *ppm* for 15 min.) of CO exposure and the %*COHb* level of <2%. Mosquito coil smoke was also associated with impaired spatial memory. However, the dose and duration of exposure did not significantly affect weight gain in the mice. Although widely used to prevent malaria, MC could serve as a potential source of CO and other neurotoxins that could be harmful to the brain; the use and toxicity of which is mostly overlooked even by the public health professionals.

Keywords*:* mosquito coil; carbon monoxide; carboxyhemoglobin; neurotoxicity; learning and memory

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INTRODUCTION

Although there was a general reduction in the global burden of malaria as shown by the 21% decrease in the incidence and mortality among the populations at risk; there were disproportionately higher malarial cases (90%) and increase in about 92% of malarial death (Organization, 2016). Nigeria alone contributes the highest burden of global malaria morbidity (25% of global malaria cases) and deaths (30% of global malaria deaths) (Arowolo, 2016). The WHO agenda to end malaria in Nigeria identified vector control as one of the key facts to tackle malaria. Among the many control strategies used by the people of Asa local government area of Kwara state in Nigeria, the use of mosquito coil (MC) was the second most popular (15.4%) (Salihu & Sanni, 2013).

Mosquito coil is a coiled substance having one or more insecticides that slowly burns to emit smoke that can drive mosquitoes away (S. H. Garba, Shehu, & Adelaiye, 2007). The most common active ingredients of MC are various pyrethrins (0.3 – 0.4% of the coils mass), organic fillers such as sawdust, binders such as starch gel, and synergist such as piperonyl butoxide (PBO). They are effective against many genera of mosquitoes including Aedes, Anopheles, and Mansonia (Krieger, Dinoff, & Zhang, 2003). Sub-micrometer particles and gaseous irritants such as the aldehydes, sulfates and polycyclic aromatic hydrocarbons released when MC is burning evaporate together with the insecticide (Weili Liu et al., 2003).

Mosquito coils are mostly used by people in the night when they retire to their bedrooms to sleep. Some people burn it in an enclosed room, and later extinguish it and open the windows before they sleep; While some will keep it burning throughout the night when they are asleep. In either case, people (pregnant women, children and the elderly) get exposed to the MC smoke for quite a number of hours in the night. Some rooms may be well or poorly ventilated, especially during the cold season when people use to close most of their windows and doors for warmth. The effectiveness of MC is usually more when used indoors or areas with limited ventilation, however, it is always advised to maintain good ventilation to avoid toxicity.

Generally, CO is produced when burning any carbon-containing substance, depending on the availability of oxygen. Carbon monoxide is considered

to be a highly toxic gas to virtually every organ in the body by either decreasing the oxygen supply to the tissue (hypoxia) (Stewart, 1975), producing reactive oxygen species (ROS) (Hennekens et al., 1996) or via many other unproven mechanisms (Zhang & Piantadosi, 1992). The highly active organs like the brain and heart are mostly affected due to their high energy demand and low energy reserve (Henry et al., 2006). The brain is extremely sensitive to decrease in oxygen supply, as a result, it suffers energy failure only a few minutes after an interruption in oxygen supply (Erecińska & Silver, 2001). This may lead to reversible or permanent tissue damage (Choi, 1983). Acute exposure to mild or moderate amount of CO may result to mild central nervous system (CNS) symptomatology like headache, dizziness, weakness, nausea, confusion, disorientation, and visual disturbances that may often be ignored or considered as part of the daily life chores (Myers, Snyder, & Emhoff, 1985; Raub, Mathieu-Nolf, Hampson, & Thom, 2000). Chronic exposure, however, may cause subclinical symptoms like chronic fatigue, affective conditions, emotional distress, memory deficits, and difficulty in walking (Weaver, 2009). This could be one of the many reasons why CO is considered "the silent killer".

Although a lot of studies were conducted on the toxicities of MC smoke, however, they concentrated more on the effects of major constituents like pyrethrins and its toxicity to organs other than the brain (S. Garba, Adelaiye, & Mshelia, 2007; WK Liu & Wong, 1987). Carbon monoxide is seldom mentioned in the gaseous products of MC. Most CO poisonings are due to acute exposure from fire accidents or exhaust fumes inhalation; however, chronic exposure was fully documented (Townsend & Maynard, 2002; Wilks, Tomashefski, & Clark JR, 1959). While acute poisoning is so glaring and can easily be diagnosed, however, chronic mild exposure is mostly overlooked and may lead to sub-clinical symptoms which can be very difficult to attribute to CO poisoning alone. A substantial amount of CO can be produced by MC especially when used in the poorly ventilated environment.

The aim of the study was therefore to mimic the chronic, mild exposure to CO from MC in humans. Since MC smoke toxicity to other organs is well known, we choose to evaluate its effects on the brain. In the setup, we created a partially ventilated gas chamber similar to poorly ventilated bed rooms used by our people for exposing the animals. Instead of the average of 8 hours of night sleep for humans, we choose a minimal period of exposure that people can get exposed to MC smoke per day, which is about 15 minutes. Our assumption is that if this brief, daily exposure to CO from MC smoke for just 14 days can affect spatial memory in the exposed mice, then an average of 8 hours of daily exposure throughout our lives can have tremendous consequences on our brain health.

MATERIALS AND METHODS

Sixteen, adult, male mice were recruited for the study. They were housed in the animal room for about a week prior to the start of the study in order to acclimatize with the environment. Animals were maintained under natural day and night atmospheric conditions of the savannah region (Kano state, Nigeria). The temperature ranges from 27 - 30^0C throughout the period of the study. They were fed with laboratory animal feed and water *ad libitum*. They were also handled in accordance with the *Ahmadu Bello University* animal use and care guideline. The animals were categorized into either the experimental or control groups (8 mice each) using the simple randomization method. The experimental group was exposed to *MC* smoke (*Wavetide*, *Xiaoshan Yunshi*, China) that was made to burn inside the partially ventilated gas chamber (75 cm x 50 cm x 50 cm) for 15 minutes, daily, for 14 days. The control group was also placed in the gas chamber for 15 minutes, daily, for 14 days, however, they were not exposed to the MC smoke. The exposure was in the mornings (8-9 am). Digital *CO* meter (*PCMM05*, *Pyle*) was used to measure the amount of *CO* produced when the *MC* was burning inside the gas chamber. The peak daily dose of *CO* attained within the period of exposure were recorded. Environmental temperature and that inside the gas chamber were also recorded during each exposure sessions.

Measurement of body weight: on the first and last days of the study, the weights of all the animals were measured and recorded in order to assess the weight gain.

Screening for motor coordination deficits: There is a general requirement for motor strength and coordination before a reliable assessment of cognitive behavior can be made; therefore, all the animals were screened for motor coordination deficits before recruitment into the study (Stanley et al., 2005). The balance beam test (Beam walk), which was used here, is a useful measure of motor coordination and balance deficits that can be used to show gross or subtle motor coordination and balance deficits. It consists of 100 cm long 12 mm and 6 mm flat beams resting on two poles which are 50 cm above the surface of a table. A black escape box is attached to one end of the beam at the finish point. Mouse was forced to move away from the aversive stimulus (60 watt light bulb) at the starting point of the beam towards the escape box; during which the latency and hind-feet slips were recorded as a measure of motor coordination and balance (Carter,

Morton, & Dunnett, 2001; Southwell, Ko, & Patterson, 2009).

Assessment of learning and memory: The Barnes maze was made up of a circular platform (122 cm in diameter) with 40 equally spaced holes (5 cm diameter; 3.5 cm between holes; 2 cm from the edge) along the perimeter and was elevated 90 cm above the floor. There was a small dark recessed "goal box" (28 x 14 x 18 cm) located under one of the holes where mice can escape the aversive stimulus (bright light) and hide. Charts were pasted on the walls to act as visual cues and were kept permanently in their positions throughout the days of the study. A video camera was positioned about 150 cm above the platform to record the activities (Barnes, 1979).

The protocol involved an adaptation and 4 days of training (acquisition/ learning) that was followed by a probe/ retention test on the 5th day of the study. During the acquisition task, the number of primary errors (total number of head deflections into incorrect holes before reaching the target hole), total errors, primary latency (time taken to locate the target hole for the first time), and path length (total length of the path to locate the target hole) were measured by the experimenter. On the 5th day, however, number of pokes/ errors (total number of head deflections into incorrect holes), latency, and path length to reach the virtual target hole were measured.

The search strategies used by mice can be grouped in to 3 different categories. Direct (Spatial) search strategy involves moving directly to the target hole or to an adjacent hole before visiting the target. In a mixed search strategy, the target hole searches were separated by crossing through the center of the maze or an unorganized form of search. In the serial search method, the first visit to the target hole was preceded by visiting at least two adjacent holes in a serial manner; either clockwise or counter-clockwise direction. The strategies were observed by the experimenters during the study and cross-checked with the video recordings at the end of every day's trials.

Assessment of *carbon monoxide* level in blood: About 2.5 *ml* of blood was collected in test tubes containing ethylenediaminetetraacetic acid (EDTA, potassium salt), 1.5 *mg/mL* of blood. Measurement of blood *carboxyhemoglobin* (*COHb)* is a principal biomarker for assessing exposure to *CO* by spectrophotometric method (Ernest & Carol, 1984).

Statistical analyses

Data obtained from the study were expressed as means ± *standard error of the mean* (*SEM*), or as medians and interquartile ranges. Depending on nature and the characteristics of the data, either parametric or non-parametric analysis was employed to analyze the results, followed by an appropriate post hoc test where necessary. For all evaluations, values of $p \leq 0.05$ were considered to imply statistical significance. Microsoft Office Excel version 2013 and statistical package for social scientist (SPSS) version 22.0 software were used in analyzing the data.

RESULTS

The mice in both groups gained weight during the period of the study as shown by the significant increases in the body weight between the initial and final body weights in the MC smoke exposed group (p=0.017) and the control (p=0.012) group (Table 1). There was no significant difference (p=0.681) between the temperature inside the gas chamber and that of the environment (Table 2).

Table I.
Effects of mosquito coil smoke on body weight

Group	*n*	Body weight (g)	Variable	p-value	Z	Percentiles		
						25th	Median	75th
Mosquito coil	8	19.7	Initial Body weight	0.017*	-2.38	15.60	18.55	24.15
		25.6	Final Body weight			21.13	25.45	30.15
Control	8	20.9	Initial Body weight	0.012*	-2.52	15.33	16.85	17.65
		27.5	Final Body weight			19.00	22.00	25.00

Wilcoxon Signed Ranks Test, n=8, p≤0.05, * indicates statistical significance, and its absence indicates insignificance.

Table 2.
Temperature variation between the gas chamber and the environment

Location	*n*	Mean ± SE	Standard Deviation	p-value
Gas chamber	14	27.8^{0}C ± 0.366	1.369	0.681
Environment	14	28.0^{0}C ± 0.363	1.359	

Independent-Samples T-Test, *n*=14, *p*≤0.05, * indicates statistical significance, and its absence indicates insignificance.

Table 3
Screening for motor coordination deficit in the animals

Groups	*n*	Variable	p-value	Z	Percentiles		
					25th	Median	75th
Control and Mosquito coil	8	Left Foot Slip (LFS)	0.52	-.64	0.00	0.00	1.00
		Right Foot Slip (RFS)	0.44	-.77	0.00	0.00	1.00
	8	Latency	0.92	-.11	6.00	6.50	8.00

Mann-Whitney U-test, n=8, p≤0.05, * indicates statistical significance, and its absence indicates insignificance.

In terms of temperature, the two groups were maintained under similar conditions throughout the study period. The mean daily *CO* exposure was 312 *ppm* for the *MC* group and 2 *ppm* for the control group (Figure I). The *COHb* was 15.4% and 1.8% for the *MC* and control groups respectively (Figure 1).

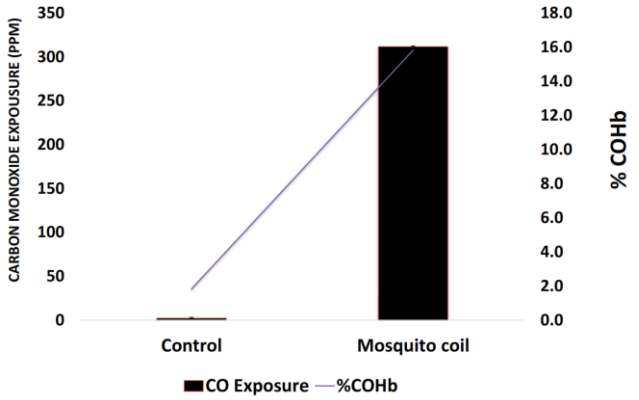

Figure 1:
Carbon monoxide exposure in relation to the percentage carboxy-hemoglobin

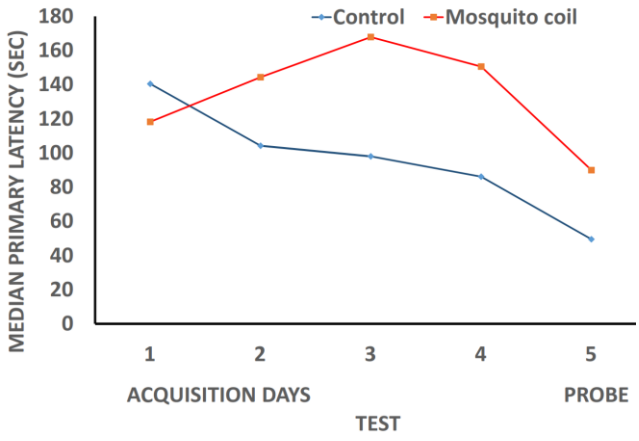

Figure 2:
Changes in the primary latency to reach the target hole**.**
Control: Friedman test indicates significant decrease in the latency [χ^2 (4) = 16.715, p = 0.002] that occurred between D1 and D5 (p=0.01) after post hoc comparison. Mosquito coil: There is a significant change in the latencies [χ^2 (4) = 14.065, p = 0.007], however, no significant difference was observed after post hoc comparison. p≤0.05, n=8.

All the mice were screened for possible motor coordination and balance deficit prior to the start of the study. There were no statistically significant differences in terms of the left foot slip (LFS) (p=0.52), right foot slip (RFS) (p=0.44), and Latencies (p=0.92) between the two groups (*MC* and control) (Table 3). They were all free from any gross motor coordination and balance deficit that might have affected their performance in the Barnes maze tasks.

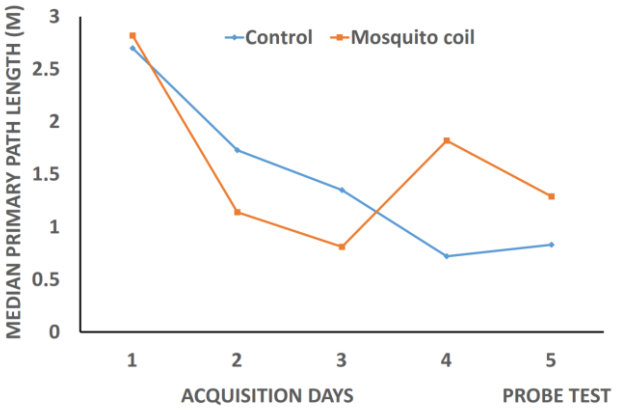

Figure 3:
Changes in the primary path length to locate the target hole.
Control: Friedman test indicates significant [X2(4) =11.190, p=0.025] decrease in the primary path length, however, no significant differences were observed between the daily primary path lengths after post hoc comparison. Mosquito coil: There was no significant [X2(4) =5.800, p=0.215] difference between the daily changes in the primary path lengths. n=8, p≤0.05.

There was significant (p=0.01) decrease in the latency from day 1 (D1) to D5 of the acquisition period in the control group; however, it increases gradually throughout the acquisition period in the MC group (Figure 2). The control animals gradually learn to locate the target hole without wasting time as the training continues (Figure 3). There was a significant (p=0.025) decrease in the primary path-lengths (PPL) over the acquisition days in the control group (Figure 3). Although there was a decrease in the PPL in the MC group, however, it was not significant (p=0.215) and associated with an increase in the PPL after D3 (Figure 3). The number of primary errors made to locate the target hole over the acquisition period decreases significantly in both the control (p=0.000) and MC groups (p=0.001) (Figure 4).

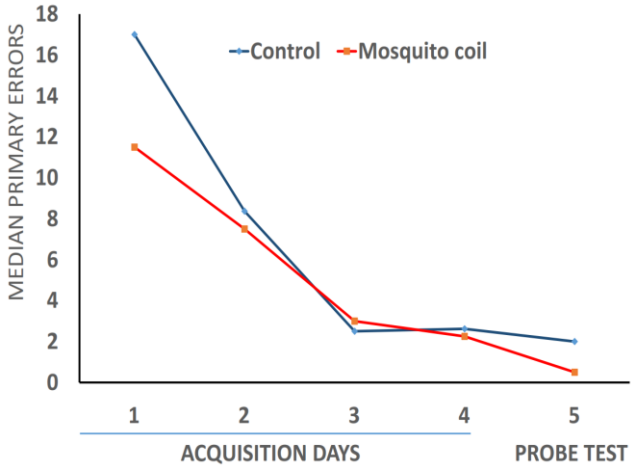

Figure 4:
Changes in the number of errors made to locate the target hole. *Control: Friedman test indicates significant decrease in the errors [X2(4) =22.248, p=0.000] between D1 and D3 (p=0.01) after post hoc comparison. Mosquito coil: Friedman Test indicates significant [X2(4) =18.854, p=0.001] decrease in the errors that occurred between D1 and D5 (p=0.000) after post hoc comparison. n=8, p≤0.05.*

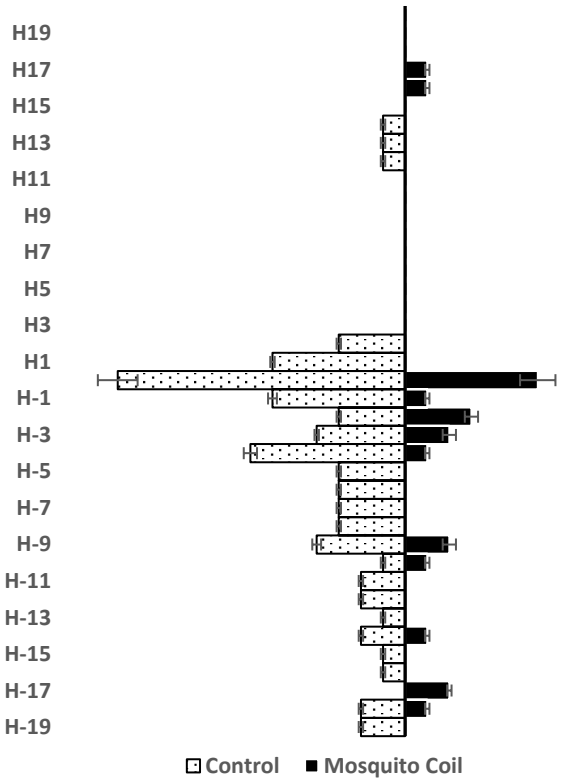

Figure 5: Higher preference for the virtual target hole in the control and mosquito coil groups on the probe test day. *Mann-Whitney U-test,* there was no significant (p=0.102) difference in the preference of the virtual target hole between the control and mosquito coil group. *n=8, p≤0.05*, * indicates statistical significance, and its absence indicates insignificance.

Although mice in both groups prefer the virtual target hole on the probe test (Figure 5), however, the choice of strategy to locate the target hole during the acquisition and probe test days was more of "mixed" in the MC group (Figure 7) as compared to the increase in the "direct" strategy exhibited by the control group (Figures 6).

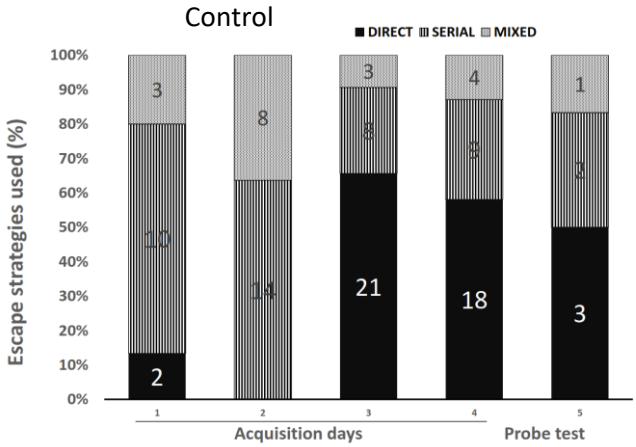

Figure 6:
The preferred escape strategy used by mice to locate the target hole in the control group.

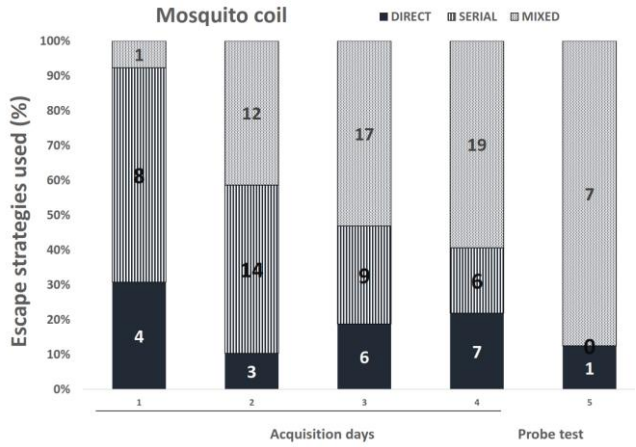

Figure 7:
The preferred escape strategy used by mice to locate the target hole in the mosquito coil group.

DISCUSSION

Body growth is a marker of health, and growth can be objectively assessed through measurement of organ and body weights. During the study period, an initial body weight of all the animals was taken to provide the baseline measure of the weights of the animals. On the final day of the study, another body weight was taken to provide an objective measure of the growth status. Under normal circumstances, and being that the studied animals had no restriction to either water or their feeds, we expect a normal weight gain, with a significant difference between the initial and final body weights. All the animals had significant weight gain over the period of the study. Although there was normal growth in our study, however, many studies of CO poisoning, especially during pregnancy were found to be associated with increased risk of fetal death, developmental disorders, and chronic cerebral

lesions (Raub et al., 2000). Salam and colleagues (2005) also found perinatal exposure to CO as a major risk factor for low birth weight and intrauterine growth retardation (Salam et al., 2005). The normal weight gain observed in our result was not unexpected and could be explained by the short duration of exposure (15 min.), smaller CO dose (312 ppm), and exposure to adult mice. To buttress our point, exposure to a higher dose of CO (up to 1000 ppm) equally lead to 25% mortality and poor growth in those animals that survived (data not shown).

In order to maintain both the control and experimental groups under similar conditions aside from the difference in the treatment; the environmental temperature of the two groups were monitored throughout the period of the study. Our results showed that the temperature inside the gas chamber during the exposure did not differ significantly with the temperature outside the gas chamber, nor was it different from that of the control groups' environment, throughout the study period. We can conclude that both groups were maintained under similar conditions of temperature throughout the period of the study, and any observed behavior couldn't have been caused by the variations in the temperature.

The CO exposure observed in the control group was within the world health organization (WHO) recommended ambient levels (<2.5 ppm); however it was beyond the WHO recommended limit in the MC smoke exposed group (<100 mg/m3 or 87.1 ppm for 15 min) (Carratu et al., 1999). The %COHb was also lower and within the non-smoker, ambient range (<2%) in the control group. However, that of the MC smoke exposed group was much higher than the WHO recommended limit; rather, it falls within the chronic smoker range (>9 ppm). From the literature, patients who were mildly exposed to CO (<1 %COHb) usually had intact memory functions equivalent to that of the control group and were even better in some areas like learning, word recall, and quality of learning by Buschke's verbal memory testing (Deschamps, Geraud, Julien, Baud, & Dally, 2003). Although the mean of the %COHb recorded in our control was up to 1.8%, it was still within the non-smoker range and does not pose much toxicity risk. Such mild exposures were even found to enhance learning by the same author (Kurawa, 2016).

The balance beam test is a useful measure of motor coordination and balance deficits. It can be used to show gross or subtle motor coordination and balance deficits. All the mice were screened for possible motor coordination deficits prior to the start of the study. They were found to be free of any gross motor coordination or balance deficit, and they were fit to undergo the neurobehavioral test. Parameters like the primary latency (PL), primary error (PE), primary path-length (PPL), number of pokes per hole on the probe trial day, and the pattern and kinds of strategies used by mice to locate either real or virtual target holes (VTH) were considered in order to assess learning and memory behavior. The expectations here were significant decreases in the time taken to reach the target hole for the first time (PL), the distance covered before it locates the target hole for the first time (PPL), and the number of errors made before locating the target hole for the first time (PE), as the animal acquire more training over the 4 days of acquisition period; with the least PL, PE, and PPL expected on the probe test day (D5), when all the animals were expected to have mastered the location of the target hole using the visual cues. The target hole, which was closed on D5, should have the highest number of pokes followed by the adjacent holes. This indicates recall of the actual location of the target hole (TH).

The pattern of the strategies exhibited by mice during the acquisition period will indicate whether the animals were learning to locate the target hole as the number of training increases over the days or not. The use of the "serial" form of search strategy initially, followed by a switch to the "direct" form of strategy to locate the target hole is the most appropriate pattern that indicates gradual and progressive learning. The preference for the "mixed" form of search strategy will, however, indicate utter confusion and impaired learning (during the acquisition period) or impaired recall (on the probe test day/ D5). The earlier the animals switch from the use of other strategies to the use of the "direct" strategy indicates faster/ enhanced learning abilities. On day 5, the "direct" strategy should be more preferred/ favored than any other strategy if at all the animals have learned and been able to recall the actual location of the target hole. It appears that neurobehavioral tests can be relied upon in the assessments of subtle neurologic dysfunctions resulting from CO poisoning (Rajiah & Mathew, 2011).

There was significant, progressive decline in the primary latency (Figure II) and also a sharp decline in the primary error (Figure IV) during the acquisition period in the control group. They were able to achieve perfection as early as D3 of the acquisition period; this indicates how fast they were able to learn the location of the maze. In D5, the virtual target hole had the highest number of pokes, followed by the adjacent targets (Figure V). The sharp switch from the use of serial and mixed strategies, to the use of direct strategy on the second day of acquisition period indicate fast learning ability of the animals in the control group (Figure VI). From the literature, patients who were mildly exposed to CO (<1 %COHb) usually had intact memory functions equivalent to that of the control group and were even better in some areas like learning, word recall, and quality of learning by Buschke's verbal memory testing. Attention was also found to be

better in the patients, in whom visual reaction time was shorter than in controls (Deschamps et al., 2003). Although the mean of the % COHb recorded in our control was up to 2% (Figure I), it was still within the non-smoker range and does not pose much toxicity risk. Such mild exposures were even found to enhance learning by the same author (Kurawa, 2016).

Contrary to what was obtained in the control group, the animals in the MC group had impaired learning ability throughout the acquisition period, as shown by the almost horizontal line graph of the primary latency (Figure II). They, however, had a significant gradual decrease in PE over the acquisition days (Figure IV). Although there was more preference to the virtual target hole on D5 (Figure V), the pattern of the choice of strategy during the acquisition period and on the probe test day clearly indicates confusion (Figure VII). In both the acquisition period and probe test, mice in this group nonspecifically tried their luck in locating the target hole, with total disregard to the use of the visual cues. The pattern of the primary path length exhibited by the MC group also clearly shows the gross impairment of memory (Figure III). Patients with CO poisoning usually exhibited impaired memory, attention, and executive functions in many other studies (Penney, 2007).

The impaired learning and memory in the MC group could probably be due to the exposure to CO (up to ≈ 312 ppm) from the MC smoke. Although CO is not the only constituent of MC smoke, and may not be the only toxin responsible for the impaired memory, however, the result is comparable to that of many cases of CO poisoning. It is also worth noting that most CO poisoning doesn't involve CO in isolation; rather, together with many other toxic gases and substances as is always the case in fire accidents. It is important to recall that the duration of exposure rather than the dose plays an important role in determining the level of %COHb in the body and ultimately the toxicity.

Carbon monoxide poisoning impaired memory and attention, and also causes severe white matter damage (WMD) that was associated with Parkinsonism-like features that abate after 13 months later (Sohn, Jeong, Kim, Im, & Kim, 2000). Similarly, CO poisoned subjects were found to have impaired ability to remember new temporal, linguistic, and spatial information while previous knowledge for temporal, linguistic, and spatial information was intact (Hopkins, Weaver, & Kesner, 1993). Significant atrophic changes in the fornix were associated with significant decline in verbal memory; however, visual memory, processing speed and attention, and or concentration did not decline (Kesler et al., 2001). Neuropsychological tests such as memory, new learning ability, attention and concentration, tracking skills, visuomotor skills, abstract thinking, and visuo-spatial, planning, and processing were all impaired after CO poisoning (Amitai, Zlotogorski, Golan-Katzav, Wexler, & Gross, 1998). The recall and recognition memory were significantly impaired in patients who suffered bilateral hippocampal damage and temporal-parietal atrophy after CO poisoning (Bastin et al., 2004).

In most of our communities, cigarette smoking is considered antisocial behavior because it goes against most of the cultures, therefore seriously rejected; however, the use of MC is not stigmatized. Therefore the hazard posed by MC could by far be more dangerous when compared to cigarette smoke. On average, we found that burning either cigarette (Aspen brand) or MC inside the chamber for 15 minutes produces similar concentrations of CO (cigarette ≈347ppm and MC≈312ppm). Therefore; based on the dose of CO produced, families that uses MC in the night will be ≈28 times much more affected by the toxicity of CO (assuming they were exposed for 7 hours equivalent to the average duration of a night sleep), when compared to cigarette smoking which usually last no more than 15 minutes. It is also important to realize that cigarette smokers exhale most of the smoke after inhalation; some even use filters, and mostly smoked in an open space where there is enough ventilation. In contrast, most people use MC indoors to concentrate the smoke; with very poor ventilation, especially during the cold season. Liu et al., (2003) was able to establish that exposure to a single MC was equivalent to burning 75-137 cigarettes in terms of the particulate matter (PM 2.5) and also equivalent to burning 51 cigarettes in terms of formaldehyde exposure (Weili Liu et al., 2003).

The level of community awareness on the hazards of cigarette smoke is quite high; however, that of MC and other commonly ignored indoor air pollutants is quite low even among the high socioeconomic class (Niphadkar et al., 2009). Salvi and colleagues (2016) observed that simply opening the windows and door when MC burns in the night, decreases the CO and PM2.5 levels by 50%. This clearly shows the importance of health education in the fight against MC hazards in our community (Salvi et al., 2016). Nandasena and colleagues (2010) concluded that indoor air quality and pollution may be considered a neglected public health problem in Sri Lanka just like any other developing nations (Nandasena, Wickremasinghe, & Sathiakumar, 2010). Animals that were exposed to MC smoke similar to that of humans for just 60 days, had a lower body weight, histopathological lesions in the respiratory tract, and elevated levels of liver enzyme activities (Ayorinde, Oboh, Otubanjo, Alimba, & Odeigah, 2014). Nephrotoxicity (S. H. Garba et al., 2007), other organs damage (Taiwo, Nwagbara, Suleiman, Angbashim, &

Zarma, 2008) and neurobehavioral changes (Patel, Gupta, & Oswal, 2012) were all documented in MC smoke exposure. Significantly higher frequencies of chromosome aberrations were identified in MC smoke-exposed animals by Vences-Mejía and colleagues in 2012 (Vences-Mejía et al., 2012).

The aim of this study was to mimic the usual human exposure to CO from mosquito coil and the attending neurotoxicity especially in relation to learning and memory process that is often overlooked. Our results indicate that exposure to MC smoke could impair spatial memory. Although there are many other constituents of MC, CO is considered one of the most toxic and the pathology observed here were comparable to that of many other CO poisoning studies. Exposure to CO from common but neglected sources like MC should be considered serious because it can impair learning and memory ability depending of course on the dose, duration of exposure, and other peculiar factors in the subject and the source. Therefore avoiding, or limiting exposure is a key to better health.

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Research Article

Exposure to Mosquito Coil Smoke Delays Healing of Acetic Acid Induced Gastric Ulcer in Male Wistar Rats

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Summary: Mosquito Coil Smoke (MCS is used in several Nigerian homes and some part of the world with reported grave consequences to the respiratory tract majorly. There is paucity of information on its outcome of its exposure on the gastrointestinal tract being a related organ to the respiratory tract. Forty-five male Wistar rats (123.1 ± 7.3g) were used in this study, they were grouped into 3 (n=15/group; 5 each day of experiment). Rats in the first group served as control (no MCS). The second and third groups were exposed to MCS for 8-10 h daily for 2 (2p) and 6 (6p) weeks respectively, in a well-ventilated room of 38.3m3 in size each day. After the period of exposure, chronic gastric ulcer was induced by intraluminal application of 50% acetic acid. The animals were sacrificed on days 0 (no ulcer induction), 3 and 10 post ulceration for complete blood count and ulcer scores. Stomach was excised for histology and biochemical assays, homogenized gastric tissues were analyzed by spectrophotometry for malondialdehyde (MDA), catalase and nitric oxide (NO) estimations. Data were expressed as mean ± SEM. Gross ulcer area (mm3) increased significantly on days 3 and 10 in 6p (167.3±16.03; 65.20±3.93) and 2p (152.7±6.20; 68.70±3.45) compared to control (93.26±2.80; 34.82 ±1.84) respectively. Lymphocytes count (%) decreased significantly on day 3 in 2p (60.60 ±1.97) compared to control (70.60 ± 0.87), Neutrophil count (%) in 6p (36.40 ±1.08: 30.20 ±1.46) increased significantly compared with control (25.60±0.80; 26.00 ±1.58) on day 3 and 10 respectively. MDA concentration in 6p and 2p increased significantly compared to control on day 3. Nitric oxide decreased significantly in 6p and 2p on day 3and 10 compared to control. Mosquito coil fumes proofs toxic to the stomach and more to inflamed rats stomach by delaying healing of gastric ulcer through reduction in NO and raised oxidative stress markers..

Keywords*: Mosquito coil smoke, gastric ulcer, ulcer healing, biochemical assay, oxidative stress*

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INTRODUCTION

The World Health Organization (WHO) predicted about 219 million cases of malaria worldwide in 2017, with expected 435,000 deaths from malaria in 2017 alone (WHO, 2018). The worst hit nations are majorly in Africa and affects mostly under under-5 years' individuals.

Mosquitoes are well known for causing disease in both animal and even human. They have been implicated in ailments such as malaria, West Nile virus, Dengue virus. The knowledge of its hazards has made many adopts multiple methods to reduce its breeding population in the environment. One of the several methods used in achieving this is the use of insecticides products which has increased in hundreds of thousand times (Krieger *et al* 2013).

Man is regularly exposed to environmental toxicants that could damage the biomolecules processes and thereby harm cells and tissues and invariably affects health at various levels. Certain lethal ecological toxicants such as pesticides and insecticides have bounty biological effects, being toxic not only to target organism but also to humans in several ways. The effect of usage of pesticides and insecticides in the ecosystem and has touched every section of life. (Kamsin 1997; Patil *et al*., 2003).

Mosquito coils are used locally because of its relative low cost and convenience (Milla *et al*, 2001). The mosquito coils are burnt indoors and outside the buildings in a manner suggestive of the common practice. Mosquito Coil fume (MCS) is insecticides repellant, and burns with fumes (Krieger *et al* 2003). Pyrethroids are the most abundant dynamic component of mosquito coils. Pyrethroids are active in destroying arthropods (Krieger *et al*., 2003). Studies have described that MCS has potential to alter genes in humans, such genes like tumour suppressor genes which could promote the proliferation of malignant mass within the organ of interest (Keshava *et al*., 1999).

Meanwhile, epidemiologic studies have described substantial exposure to MCS as hazardous and can

promote asthmatic symptoms in children (Aziziz and Henry, 1991, Fagbule and Ekanem 1994: Koo and HO, 1994). Rats expressed focal declination of the tracheal epithelial lining, metaplasia of the cells and mechanical alteration to alveolar macrophages following toxic exposure to MCS, (Liu and Sun, 1988: Liu and wong, 1988).

Despite reports of potential adverse health effects from exposure to MCS, several countries still indulge in the use of mosquito coils regularly to prevent mosquito bites (Liu *et al*., 2003). This is largely so because it is readily available, acceptable and affordable. Previous studies show effects of MCS emissions on irritating and carcinogenic compounds and other pollutants on the respiratory tract, nervous system, metabolic organ such as kidney, liver, immune system, memory and reproductive functions (Liu *et al*., 1987; Azizi *et al*., 1991; Fagbule *et al*., 1994; Cheesebrough 1998; Panda 1999; Abdel-Rahman *et al*., 2001; Okine *et al*., 2004; Garba *et al*., 2007; Wolansky *et al*., 2007;). Erstwhile, study in humans reported the possibility of wave-pattern deposits of particulate matter from the mucocilliary organs of the respiratory tract into the gastrointestinal tract (Moller *et al*., 2004). However, there is dearth of information on the effect of MCS on the gut inflammation and healing processes of gastric ulcer thereby making research in this area pertinent in view of the communications between the respiratory tract and the gut anatomy.

MATERIALS AND METHODS

Drugs and Chemicals

Mosquito coil: Double Rabbit' mosquito coil produced by Gongoni Company limited) were procured and used in this study. It contained pyrethroids (d-trans-allethrin; 0.2 %w/w and insert ingredient 99.8%ww) and was purchased in a retailed store at Bodija in Ibadan. Each mosquito coil has diameter of about 12 cm; 85cm long when straightened and weighs about 15g.

The other chemicals / Reagents: used for this study were gift from the Department of Physiology University of Ibadan. Ketamine was purchased from retail Pharmaceutical Company in Ibadan, Nigeria.

Animal Model and grouping

Animals and Husbandry: This study was carried out in the animal house of Physiology Department College of Medicine, University of Ibadan, Nigeria for a period of 2 -6 weeks. Forty-five (45) adult male Wistar rats (123.1±7.3g) were used. Rats were purchased from the Central Animal House of College of Medicine where they got acclimatized for two weeks. The rats were kept in plastic cages at room temperature of 25 ± 2^0C. They had free access to drinking water and standard laboratory rodent pellet diet (Vital Feeds PLC, Ibadan Nigeria). Ethical approval was sort and obtained from Animal Care and Use Research Ethical Committee (ACUREC), University of Ibadan, Reference number – UI- ACUREC/18/0088, and in accordance with the Guidelines of the National Institute of Health - (NIH, 1985).

Animal Grouping: The experiments were conducted in two rooms (3.8 x 3.6 x 3.0 m), measuring the total surface area of 71.76 m^3 with adequate cross ventilation. Each room was apart in a distance of about 100 meters from another to prevent the fumes from crossing to the control groups. Forty-five rats were randomized into 3 groups (n=15, per group), control, 2p and 6p. The groups 2p and 6p were exposed to MCS for 2 weeks and 6 weeks respectively for 8-12 h daily while control was not exposed to MCS. Five animals each were sacrificed from each group on day 0 (prior to gastric ulcer induction), and another set of 5 animals on days 3 and 10 (post gastric ulcer induction).

At the end of experimental periods, whole blood and gastric tissues were collected for hematological analysis, biochemical variables and histology, accordingly.

Determination of Haematological Variables: Blood samples were collected via retro-orbital route through heparinized hematocrit tube. This was let into an ethylene-diamine-tetra-acetic acid (EDTA) bottle. Consequently, haematological analysis was performed using methods of Dacie and Lewis (1984).

Gastric Ulcer Induction: Gastric ulcer induction was by the method described by Tsukimi and Okabe, (2005) with slight modification. Briefly, the abdomen was opened under ketamine-xylazine cocktail anaesthesia and the stomach was exposed. The anterior and posterior walls of the stomach were clamped together with a pair of 9 mm diameter eye forceps. A 50%acetic acid solution of 0.2 ml was injected slowly into the clamped portion through the anterior end of the stomach via a 23-gauge needle. The acid solution was withdrawn after 60 seconds and the abdomen was closed in two layers with 4-0 nylon suture.

Histological Study: The stomach, kidney, and liver of the sacrificed rats were excised and preserved in 10% formalin solution prior to section of about 5µm of fixed tissue from paraffin wax. Tissues were fixed using Heamatoxylin and Eosin (H&E) stain. The

stained sections were assessed for possible pathologic changes and presence of inflammatory cells.

Determination of Reactive Oxygen Markers and Antioxidant Assays

Protein concentration: Protein concentrations of the samples were determined by the Biuret method as described by Gornal *et al*., (1949).

Lipid peroxidation: Lipid peroxidation was determined from formation of thiobarbituric acid reactive substances (TBARS) by the procedure described by Varshney and Kale (1990). Malondialdehyde was determined as described previously by Adeniyi *et al*., (2014).

Catalase activity: Catalase activity was assessed by the method of Sinha (1972). The method described the role of dichromate in acetic acid when heated in the presence of H_2O_2 leading to the formation of unsteady perchromic acid.

Superoxide Dismutase (SOD) activity: The determination of SOD activity was by the method of Misra and Fridovich (1972).

Reduced Glutathione: The method of Beutler *et al* (1963) was used to assay the quantity of reduced glutathione (GSH) in the homogenized gastric tissues.

Nitric oxide: Nitric oxide was assayed by the method described by Ignarro *et al*., (1987). It relies on a diazotization response reported by Griess, (1879).

Statistical analysis

The results were expressed as mean ± standard error of mean (SEM). Data were analysed using GraphPad Prism software version 7 (Graph Pad Software, San Diego California, USA). One-way analysis of variance (ANOVA) was used to compare means, followed by Newman-Keuls Multiple comparison test. Data was considered significant at $P<0.05$.

RESULTS

Effect of exposure of Mosquito Coil Fumes on percentage mean body Weight (%) changes of Animals.: There was no significant difference in the effect of mosquito coil fumes on percentage change in body weight from the beginning of two weeks exposure to MCS to the sixth week of exposure compared with control.

Effect of Mosquito Coil Fumes on Relative Organ Weight (100g/100) (Liver, Kidney, Lungs): There was no significant change in relative weight of organs of test groups compared to control groups.

Effect of Mosquito Coil Fumes on Relative Stomach weights (100 g/100) of Stomach: The relative stomach weight (100g/100) was significantly increased, $p<0.05$; in the 6p group (1.21±0.13 g/100g) on day 10 post ulceration compared to the 2p (0.85±0.03 g/100g) and control (0.84±0.07 g/100g).

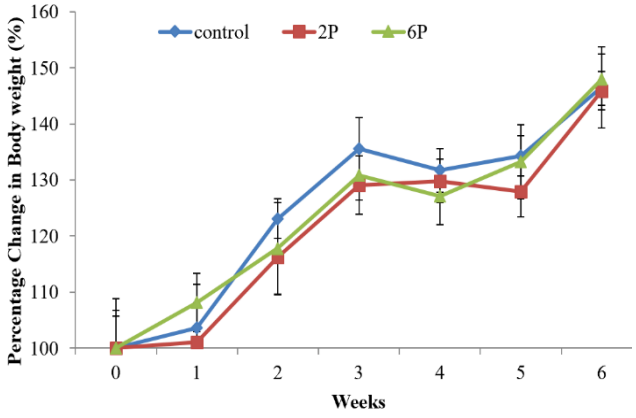


Figure 1:
Effect of exposure of mosquito coil fumes on percentage Mean body weight changes (%) of animals.

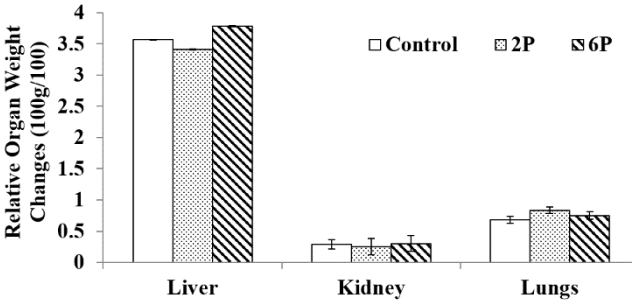


Figure 2:
Effect of mosquito Coil Fumes on relative organ weights.

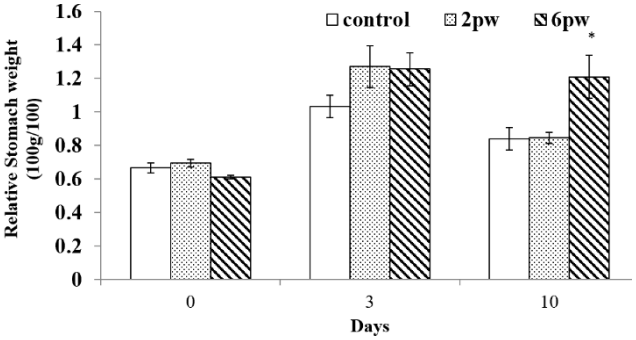


Figure 3:
Effect of mosquito coil fumes on relative stomach weight
***** $p<0.05$ when compared to control and 2pw

Effects of Mosquito Coil Fumes on Ulcer Area, Parietal and Mucous cell count: Different experimental days produced varying ulcer patterns as well as changes in the mucous and parietal cells. On the initial day following ulcer induction (Day 0), 6P group produced significant mucous cell count and parietal count (55.33 ± 4.57; 25.33 ±1.40) compared to

control (48.67 ± 6.94; 18.67 ± 1.39), respectively (Table 1). On day 3 following induction of ulcer, the ulcer area, mucous cell and parietal cell count changed significantly in both exposed groups; 2P (152.7±6.20, 39.33± 7.54, 3.33± 0.30) and 6P (167.3±16.03, 2.44±5.73, 25.33±1.40) compared to control (93.26±2.80, 69.33± 4.66, 26.78 ± 6.73), respectively (Table 2). In a similar pattern, two MCS groups sustained the increased in ulcer area by day 10 of ulcer induction. Groups 2P (68.70 ± 3.45) and 6P (65.20 ± 3.93) ulcer area increased significantly compared to control (34.82 ± 1.84). The parietal cell count decreased statistically in 2P (22.00 ± 5.28) and6P (23.11 ± 2.04) compared to control (12.44 ± 1.47), respectively. The mucous cell count on day 10decreased in 2P (39.33± 7.54) compared to the control (58.89± 7.89), (Table 3)

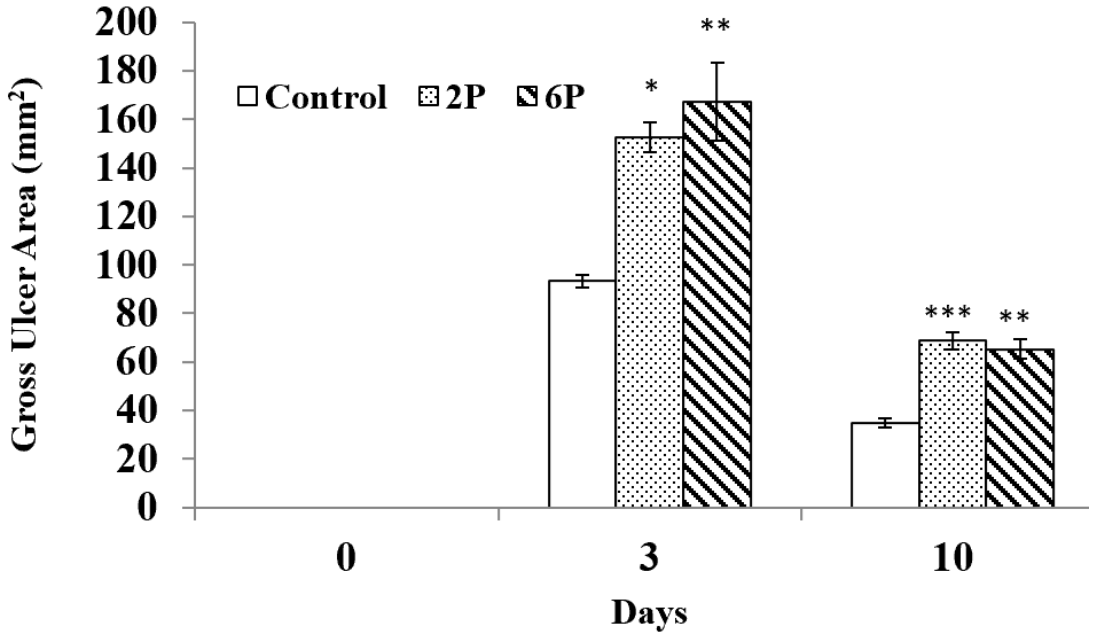
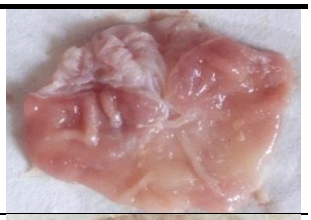
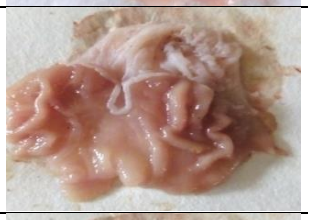
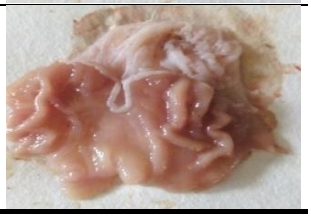


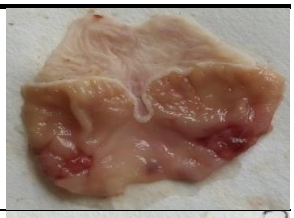
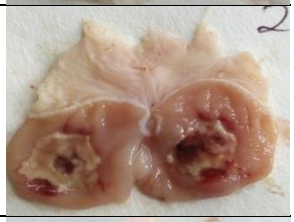
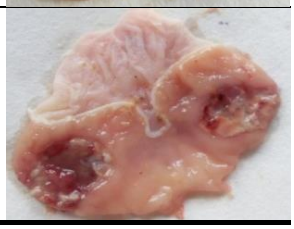
Figure 4:
Effect of mosquito coil fumes on ulcer area
** p<0.05 significant increase in ulcer score in two weeks group on day 3 compared to control.*
*** p<0.01 significant increase in ulcer score in six weeks group on day 3 and 10 compared to control.*
**** p<0.001 significant increase in ulcer score in two weeks group on day10 compared to control.*

Table 1:
Effect of mosquito coil fumes on gastric ulcer area on day 0

Group	Ulcer Area (mm^2)	Mucous cell count	Parietal cell count	Gross stomach Tissue
Control	0.00	48.67± 6.94	18.67 ± 1.39	
2p	0.00	45.44±4.00^{NS}	23.00± 1.84^{NS}	
6p	0.00	55.33±4.57*	25.33±1.40*	

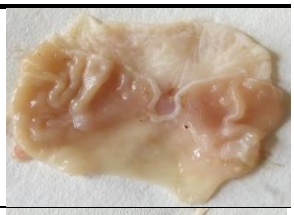
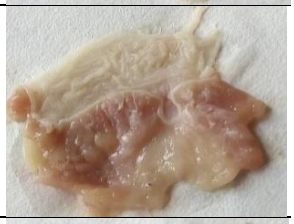
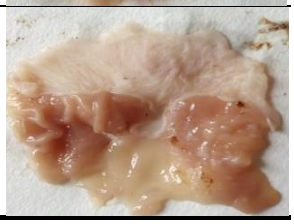
***significant at p<0.05 compared to control, NS – Not significant compared to control*

Table 2:
Effect of mosquito coil fumes on induced gastric Ulcer Area on day 3

Group	Ulcer Area (mm^2)	Mucous cell count	Parietal cell count	Gross stomach Tissue
Control	93.26±2.80	69.33± 4.66	26.78 ± 6.73	
2p	152.7±6.20**	2.21± 0.71***	3.33± 0.30***	
6p	167.3±16.03**	2.44±5.73***	3.06 ± 5.29***	

***significant at p<0.01 compared to control, ***significant at p<0.001 compared to control, NS – Not significant compared to control*

Table 3:
Effect of mosquito coil fumes on induced gastric ulcer area on day 10

Group	Ulcer Area (mm^2)	Mucous cell count	Parietal cell count	Gross stomach Tissue
Control	34.82±1.84	58.89± 7.89	12.44 ± 1.47	
2p	68.70±3.45**	39.33± 7.54*	22.00± 5.28**	
6p	65.20±3.93**	46.89±6.29NS	23.11 ± 2.04**	

**significant at p<0.05 compared to control, **significant at p<0.01 compared to control, NS – Not significant c.f control*

Effect of Mosquito coil fumes on Haematological Parameters: As shown in Table 4, Lymphocytes count was significantly decreased, p<0.05 in the 2p group (60.60±1.97) on day 3 post ulceration compared to control (70.60±0.87). Neutrophils count was significantly increased, p<0.05 in the 2p group (36.60±2.02) on day 3 post ulceration compared to control (25.60±0.81). There was no significant change in the heamoglobin concentration, erythrocyte count and platelets count in all the groups across the different experimental days (Table 5).

Table 4:
Effect of Mosquito coil fumes on Blood Leucocyte Count and Differential WBC

White blood cell and differential	Day 0			Day 3			Day 10		
	Control	**2P**	**6P**	**Control**	**2P**	**6P**	**Control**	**2P**	**6P**
Leucocytes (10^3/µL)	5.06 ±0.83	3.95 ±0.25	5.75 ±0.29	6.67 ±1.15	4.00 ±0.22	6.42 ±0.73	9.61 ±0.63	6.79 ±1.17	5.65 ±0.79
Monocytes (%)	1.50 ±0.50	1.60 ±0.25	2.00 ±0.32	1.80 ±0.37	2.20 ±0.37	1.40 ±0.25	1.20 ±0.20	2.00 ±0.32	1.40 ±0.25
Neutrophils (%)	28.50 ±1.19	25.60 ±1.17	30.60 ±2.48	25.60 ±0.81	36.60 ±2.02*	36.40 ±1.08*	26.00 ±1.58	25.80 ±3.68	30.20 ±1.46*
Lymphocytes (%)	68.00 ±1.47	70.80 ±0.97	66.00 ±2.30	70.60 ±0.87	60.60 ±1.97*	64.40 ±0.81*	72.20 ±1.69	70.40 ±3.70	68.00 ±1.61
Eosinophils (%)	2.00 ±0.41	1.00 ±0.45	1.40 ±0.40	2.20 ±0.37	0.60 ±0.25	1.80 ±0.37	0.60 ±0.25	1.80 ±0.58	0.40 ±0.25

Table 5:
Effect of Mosquito coil fumes on Platelets and Red Blood Cell Variables

	Day 0			Day 3			Day 10		
	Control	**2P**	**6 P**	**Control**	**2P**	**6PW**	**Control**	**2P**	**6P**
Erythrocyes ($x10^6$/µL)	6.70 ±0.23	7.14 ±0.32	6.58 ±0.21	6.90 ±0.21	6.90 ±0.20	6.37 ±0.20	7.08 ±0.69	6.74 ±0.62	6.52 ±0.33
Platelets ($x10^3$/µL)	147.80 ±21.06	129.40 ±14.42	128.60 ±11.06	157.20 ±36.63	123.00 ±6.56	122.60 ±15.07	192.20 ±12.58	163.20 ±7.34	154.80 ±15.16
Heamoglobin (g/dL)	13.15 ±0.32	14.40 ±0.52	13.24 ±0.41	14.00 ±0.14	13.70 ±0.27	12.52 ±0.30	14.48 ±1.15	13.84 ±1.34	13.08 ±0.55
PCV (%)	40.50 ±0.96	43.80 ±1.63	41.20 ±1.66	41.60 ±0.81	41.40 ±1.03	37.20 ±0.66	43.40 ±3.78	40.80 ±3.89	38.80 ±1.53

No significant difference in all comparable

Table 6:
Effect of mosquito coil fumes on activities of tissue endogenous antioxidant enzymes, nitric oxide and reactive oxygen radicals

Biochemical Analysis	Day 0			Day 3			Day 10		
	Control	**2P**	**6 P**	**Control**	**2P**	**6P**	**Control**	**2P**	**6P**
Catalase(µmol/min/mg protein	12.74 ±1.21	14.42 ±1.16	14.91 ±1.54	10.13 ±1.13	10.68 ±2.17	16.00 ±2.51	12.36 ±2.80	15.54 ±1.71	9.30 ±1.80
Glutathione (GSH)	1.54 ±0.07	1.37 ±0.08	1.69 ±0.09	1.58 ±0.136	1.27 ±0.10	1.43 ±0.12	1.39 ±0.128	1.56 ±0.082	1.40 ±0.150
MDA(ng/mg protein)	2.12 ±0.20	2.12 ±0.16	2.49 ±0.27	2.18 ±0.26	2.77 ±0.12*	3.57 ±0.64*	1.04 ±0.10	1.59 ±0.19#	2.34 ±0.33#
NO (µM/mg tissue)	5.78 ±0.24	4.44 ±0.52&	4.81 ±0.08	5.24 ±0.27	2.69 ±0.35ß	2.04 ±0.45ß	5.59 ±0.11	4.25 ±0.19α	3.57 ±0.14α
Protein (mg/g tissue)	5.23 ±0.59	4.20 ±0.19	4.62 ±0.38	4.46 ±0.15	4.12 ±0.17	4.19 ±0.16	4.39 ±0.35	5.09 ±0.43	4.34 ±0.19
SOD(µmol/mg protein)	0.24 ±0.03	0.43 ±0.04**	0.49 ±0.05**	0.70 ±0.04	0.52 ±0.04ß	0.30 ±0.04ß	0.56 ±0.02	0.46 ±0.06 α	0.35 ±0.02 α

** p<0.05 significant increase in 6P group (day 3) and 2P group compare to control,*
α p<0.05 significant decrease in 6P group (day 10) and 2P group compare to control.
 # p<0.05 significant increase in 2P (day 10) and 6P group compared to control.
ß p<0.05 significant decrease in 2P and 6P group (day 3) compared to control.
*** p<0.01 significant increase in superoxide dismutase activity compared to control.*

Effects of mosquito coil fumes on the histology of the liver, kidney and stomach: Changes in the microscopic appearances of the liver, kidney and stomach of control rats and those exposed to mosquito coil fumes are shown in Plates 1 – 7.

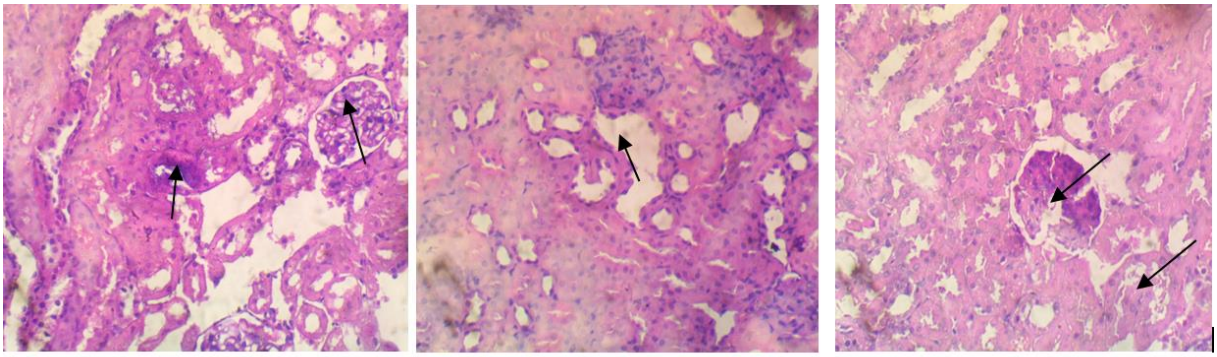


Plate 1:
Representative photomicrograph of the kidney (H&E) X400
Control= the glomeruli appear normal. There are a few foci of mild sloughing off of tubular epithelial cells (arrows). No remarkable vascular changes**.** 2p= the glomeruli appear normal. There are a few foci of tubules lined by flattened epithelial cells (arrow). Vascular changes are mildly congested. 6p= There are multiple foci (arrows) of degeneration of tubular epithelial cells.

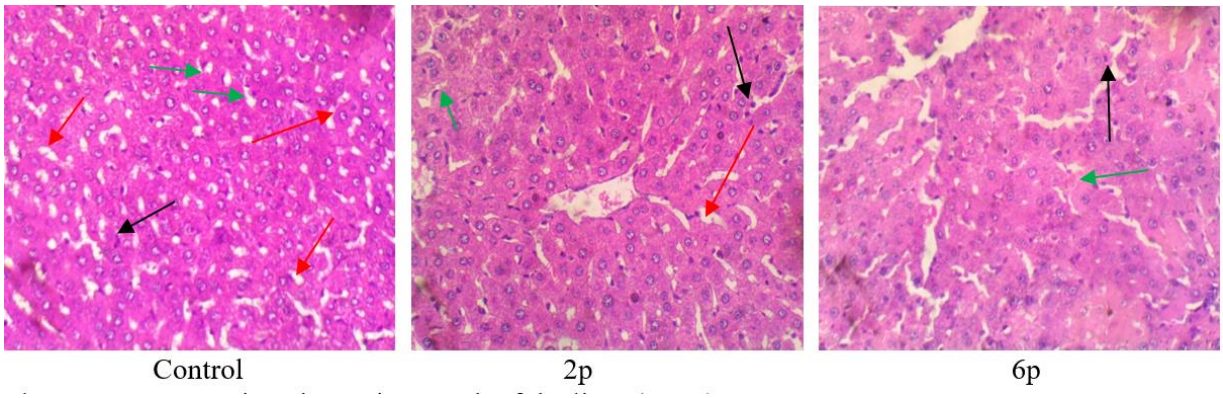


Plate 2:
Representative photomicrograph of the liver (H&E) x400
Control= There are a few foci of mild thinning of hepatic plates (cord atrophy) with resultant dilated sinusoids (red arrows). There are random foci of single-cell hepatocellular necrosis (black arrow). There is moderate Kupffer cell hyperplasia (green arrows). 2p= There are a few foci of mild thinning of hepatic plates (cord atrophy) with resultant dilated sinusoids (red arrow). There are random foci of single-cell hepatocellular necrosis (black arrow). There is moderate Kupffer cell hyperplasia (green arrow). There are no remarkable vascular changes. 6p= Hepatic plates are fairly closely-packed. There are random foci of single-cell hepatocellular necrosis (black arrow). There is moderate Kupffer cell hyperplasia (green arrow). There are no remarkable vascular changes.

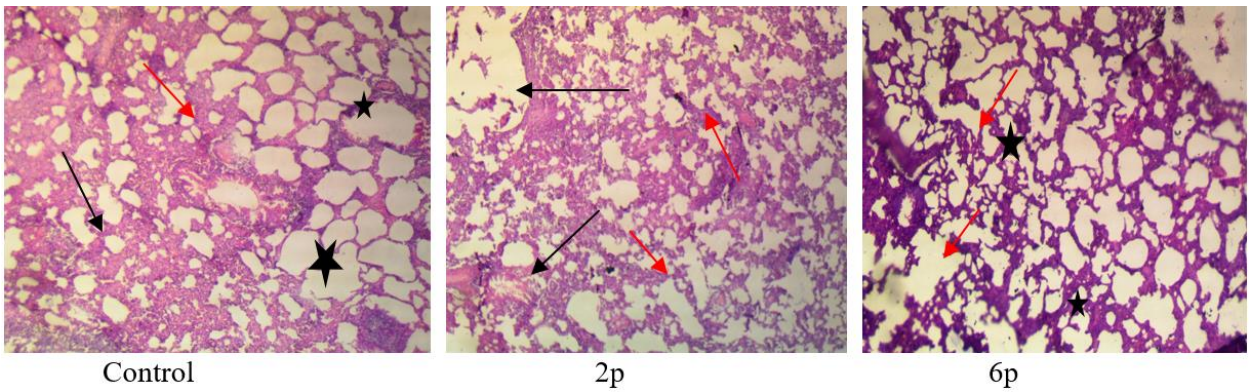


Plate 3:
Representative photomicrograph of the lungs (H&E) x400
Control= There are locally extensive foci of marked thickening of the alveolar interstitium (black arrows). [Compare left of photomicrograph with top-right]. Alveoli and bronchioles are clear (stars). There is moderate congestion of blood vessels (red arrow). 2p= the alveoli and bronchioles are clear and empty. There are a few foci of moderate thickening of the alveolar interstitium (black arrows). Alveolar interstitial capillaries are mildly congested (red arrows).
6p= There are locally extensive foci of marked thickening of the alveolar interstitium (black arrows). [Compare left of photomicrograph with top-right]. Alveoli and bronchioles are clear (stars). There is moderate congestion of blood vessels (red arrow).

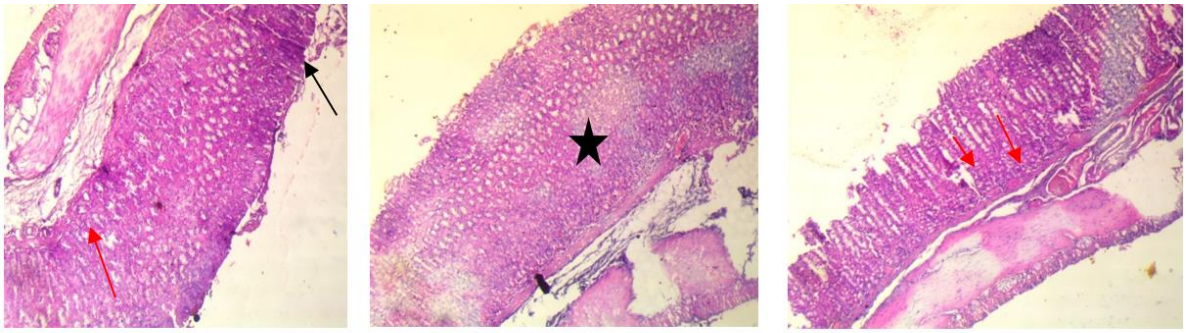


Plate 4: Representative photomicrograph of the stomach on day0 (H&E stain) x10
Control= There is mild loss of covering epithelium (black arrow) at a few foci. There are a few foci of loss of cells of the gastric glands (red arrow) at the base of the mucosa. Vascular changes are unremarkable.2p= There is mild loss of covering epithelium at a few foci. The gastric mucosa (star) appears normal. Vascular changes are unremarkable. 6p= There are a few foci of slight loss of the covering epithelium. The mucosa appears normal. However, there is moderate congestion of blood vessels (red arrows) in the mucosa and submucosa tunics.

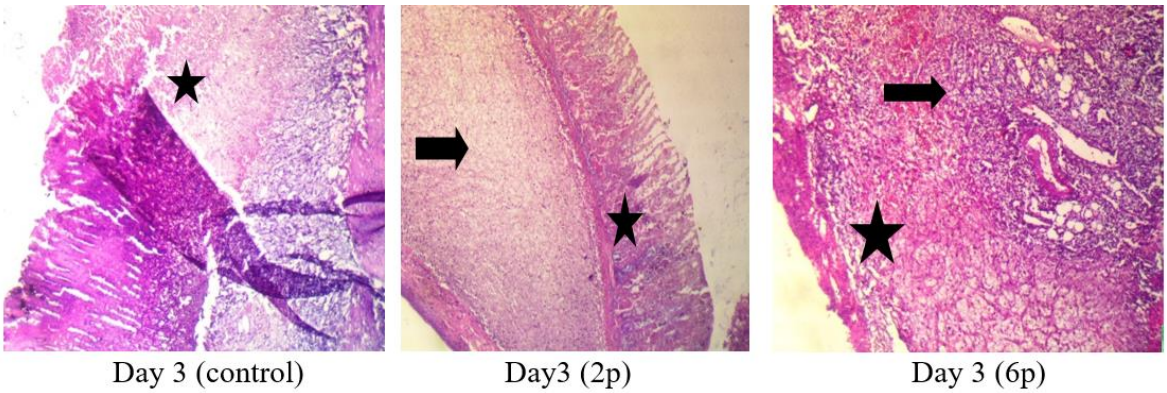


Plate 5: Representative photomicrograph of the stomach on day3 (H&E stain) x100
Control= There is a locally extensive focus of severe ulceration and necrosis of the affected mucosa (black arrow). Adjacent to the ulcerated focus, there are regenerating gastric glands that appear dilated and cystic. The submucosa is markedly expanded by oedema and mild accumulation of inflammatory cells. 2p= There is severe widespread ulceration and coagulative necrosis of the entire mucosa (star). There is expansion of the submucosa (arrow) with inflammatory oedema and mostly polymorphonuclear cells. 6p= There is severe widespread ulceration and necrosis of the entire mucosa with accumulation of inflammatory cells in the submucosa and muscularis (arrow). Inflammatory cells are mostly polymorphonuclear.

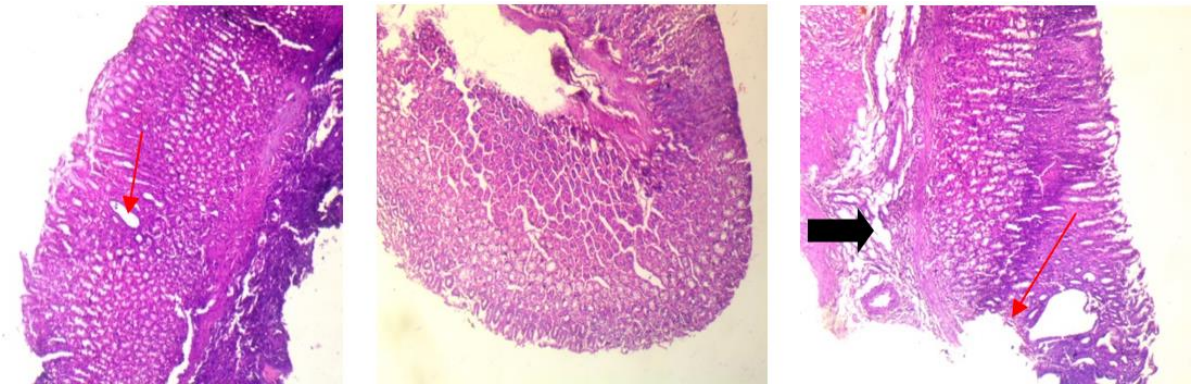


Plate 6:
Representative photomicrograph of the stomach on day 10 (H&E) x100
Control= the mucosa contains a few regenerating dilated glands (red arrow). The covering epithelium is intact. However, the submucosa is mildly expanded by accumulation of mononuclear inflammatory cells trapped in dense fibrous connective tissue. This finding might be suggestive of a healed ulcer. 2p= the covering epithelium is intact. The gastric mucosa, submucosa, muscularis and serosa appear normal. Vascular changes are unremarkable. 6p= There is a locally extensive focus of mild ulceration of the gastric mucosa. Adjacent to the ulcerated focus, there are regenerating gastric glands that appear dilated and cystic (red arrow). There is moderate accumulation of inflammatory cells in the submucosa. There is fibrosis (thick arrow) of the submucosa and muscularis. Inflammatory cells are polymorphonuclear.

DISCUSSION

We designed this study to explore and simulate daily and wanton use of MCS in residential areas using Wistar rat model. The potential health implication of MCS on the stomach of exposed normal gastric mucosa and experimentally induced gastric ulcer was investigated.

The body weight did not change considerably throughout the weeks of exposure to MCS. This is similar to the reports obtained in a previous study where there was no change in body weight compared to unexposed group (Parker *et al*,. 1984; EPA, 1986; Ishmael and Lithchfield, 1988; Schoenig, 1995). The lack of body weight changed observed could be due to the relative short period of exposure which did not reduce appetite for food in the exposed groups when matched with control.

However, there was a relative increased in kidney weight in the 6p group compared to the 2p group which could be attributable to the duration of exposure and suggesting that both forms of exposure could affect the kidney structurally and functionally. More so, with the reported congestion around the glomerular tuft in the histopathological evaluation of the kidneys of animals exposed for six weeks. This agrees with the report of Taiwo *et al*., (2008) which demonstrated glomerular and tubular and vascular damages in the kidneys of rats exposed to mosquito coil and various insecticidal spray fumes. That finding was further buttressed by report of Garba *et al* (2007) which demonstrated severe multifocal congestion, cystic dilation in medulla of kidney tissue exposed to pyrethroid based mosquito coil.

The increase in ulcer area of the 6p and 2P groups suggest a severe ulcer and a delayed healing processes on days 3 and 10 post ulceration respectively. This further reinforced the toxic effects of mosquito coil on stomach mucosa. The increased gross ulcer scores provide backing by the histopathology findings that showed delayed ulcer healing in the exposed groups relative to control. The reported severe ulceration and the subsequent delayed healing in the exposed groups could be associated to the increased lipid peroxidation within the mucosa that was indicated with the raised MDA values. Malondialdehyde results from peroxidation of polyunsaturated fatty acids and related esters within cell membranes and interpreted as oxidative damage to the tissues (Blandizzi *et al*., 2005).

Excessive production of reactive oxygen species (ROS) by the gastric mucosa has been established and it openly results in oxidative damage (Sun and Oberley, 1996; Ali and Harty, 2009). Oxidative stress promotes multiple factors that could activate ROS production or a weakening in antioxidant activities (Hidekazu, 2012).The gut is a major site of generation of ROS. Even with the huge defensive mechanisms from epithelium layers, pathogens can promote formation of inflammatory conditions through interactions of the epithelium, polymorphonuclear neutrophils (PMNs), as well as the macrophages that could promote inflammation and worsened by damaged oxidative cells (Bhattacharyya, 2014). Most popular gut diseases such as gastric ulcers, gut cancers, and inflammatory bowel disease (IBD) promotes oxidative stress (Bhattacharyya, 2014).

The reduced activities of Superoxide Dismutase (SOD) in the exposed group of animals especially on day 3 post-ulceration further highlights the deleterious effect of the fumes on the gastric mucosa and experimental gastric ulcer healing. Reduced SOD activity in the gut worsen gastric ulcer, while an increase in SOD promotes ulcer healing in patients (Naito *et al*., 1992). These responses presented the detrimental effects of ROS on tissue damage and the importance of antioxidant activity in promoting health. Likewise, the responses to nitric oxide from the gastric tissue which was low in the exposure groups compared to control in this study could be playing at least dual roles towards recovery from acetic acid injury. Nitric oxide (NO) is a signaling molecule that plays stimulates inflammation pathways. It provides anti-inflammatory roles normally (Sharma and Al-Omran, 2007). On the other hand, it could be considered a pro-inflammatory facilitator that induces inflammation due to excess production in stressful conditions. NO is synthesized and secreted into the endothelial cells by the help of nitric oxide synthase that convert arginine into citrulline producing NO in the process. Nitric oxide is understood to induce vasodilatation in blood vessels and stimulates immune responses (Sharma and Al-Omran, 2007). The vasodilation property of NO may also be the leading connection towards the faster healing and recovery reported in the control animals compared to the exposed groups.

The increased in leucocyte counts and a counterpart decrease in the lymphocytes in the exposed groups were reported in this study. These changes occurred most significantly on day 3 following ulceration which suggest the peak of ongoing inflammation. This increases neutrophil-lymphocyte ratio which has been reported to be elevated in peptic ulcer patients (Jafarzadeh*et al*., 2013). The quantitative and qualitative differentials in the recruitment of inflammatory cells also play a vital role in accelerating healing and recovery from injury. The histopathology findings in the stomach skewed to the already calculated degree of ulceration and the appropriate healing and recovery that ensued in the study. The persistent elevation of the inflammatory cells observed and the degree of necrotic tissues by day 10 in the exposed groups only added credence to the

toxic effect of the MCS on the stomach. This is not an uncommon finding in acetic acid induced ulcer.

In conclusion, it is apparent that exposure of rats to mosquito coil fumes in normal and experimental gastric ulcer is potentially harmful to the structure and possibly functions of the gastric lining. The delayed healing documented in this study is due to the generation of ROS and a reduced production of NO in the exposed groups. It is anticipated that additional work in this area would be channel to examine the effect of MCS on the human gastrointestinal functions, especially in patients with gastric ulcer.

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Research Article

Effects of Clarithromycin Administration on Gastric Acid Secretion and Cytoprotection in Wistar Rats

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Summary: The incidence of peptic ulcer disease in Nigeria is 28%. Clarithromycin (CLX) is used in the treatment of peptic ulcer owing to its antibacterial effect. Whether CLX has effects on other gastrointestinal parameters that reduce peptic ulcer has not been previously investigated. Therefore, the effects of CLX on gastric acid secretion, mucus secretion and gastric ulcer scores in rats were investigated. A total of 30 albino wistar rats were used for the study. Out of this number, 10 rats each were used for gastric acid secretion, mucus secretion and ulcer scores respectively. In each of these sub groups, 5 rats served as test and were treated with CLX orally and 5 rats served as control. Standard methods were used for the estimation of these parameters of gastric function. The results showed that basal gastric acid, peak acid output following histamine stimulation and mucus secretion were significantly increased ($p<0.001$) in CLX-treated (test) rats than in their control. Furthermore, ulcer scores were significantly reduced ($p<0.001$) in the CLX-treated rats than control. In conclusion, Clarithromycin administration reduced gastric ulcers in rats. This may be attributable to not only its antibiotic property but also its ability to increase gastric mucus which counteracts the aggressive effect of the acid.

Keywords*: clarithromycin, gastric secretion, mucus secretion, ulcer scores, cytoprotection*

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INTRODUCTION

The incidence of peptic ulcer disease in Nigeria is reported to be about 28% (Ndububa and Adeyemi, 2008). Clarithromycin, a macrolide antibiotic discovered in 1980 has been used for treating various infections especially Helicobacter pylori infection of the stomach/duodenum which has been shown to cause peptic ulcerations (Taweesak et al., 2015). The product emerged through efforts to develop a version of the antibiotic erythromycin that did not experience acid instability in the digestive tract, causing side effects, such as nausea and abdominal ache (Greenwood, 2008). CLX is usually used in combination therapy with other drugs in treatment of peptic ulcers (Fashner, 2015). However, H. pylori has developed resistance to these antimicrobial agents (Masaki et al., 2020). CLX may have other anti-ulcerogenic effects such as influence on gastric acidity and gastric mucus secretion. Gastric mucus plays a significant role in physiologic defence against luminal irritants (Toshimitsu et al., 2013). Reduced Gastric mucus secretion is associated with abdominal pains due to non-steroidal drug induced gastropathy (Lijima, 2013). Gastric mucus provides physical protection of the gastric epithelium (Anna et al., 2013). There is paucity of information regarding the effect of administration of CLX on gastric acid secretion and some parameters that may contribute to cytoprotection like mucus secretion.

We therefore engaged in this study to assess effects of CLX on basal gastric acidity, peak acid output, gastric mucus secretion and emergence of gastric ulcers. We believe that if this study shows more benefits of CLX beyond its antimicrobial activities that its use as monotherapy for peptic ulcers may be studied further and encouraged in humans.

MATERIALS AND METHODS

Animals and Drugs

This study used thirty wistar rats obtained from the animal house of the Department of Physiology, University of Calabar, Cross River. The rats were kept in separate cages at 28 ± 2°C, 12 hours light and dark cycles and had free access to water. The rats were fed standard rodent feed. Ranbaxy Pharmaceutical Company, Lagos supplied samples of CLX (Crixan)

used for this study. Dosage of 7.5mg/kg every 12 hours was the clinical dose (Periti, 1999). CLX was dissolved in normal saline.

Drug Administration: Ten Wistar rats each was used for gastric acid, mucus secretion and ulcer score studies respectively. For each experiment, 5 rats were used as control and 5 rats as test. The control group was fed with normal diet while the test group was fed normal diet and CLX solution administered orally in a dose of 7.5mg/kg body weight every 12 hours for 14days. All the animals were allowed water freely. The rats were subsequently sacrificed for the studies. After 14days, Osim modification of Gosh and Schild method was applied for the assessment of gastric acid secretion using histamine as acid secretagogue while method of Tan *et al* was adopted for mucus secretion using acid alcohol and method of Alpin and Wards for ulcer scoring.

Measurement of Gastric Acid Secretion: The method adopted for measurement of gastric acid secretion was a modification of Ghosh and Schild continuous perfusion method (Osim *et al.,* 1991). All animals were fasted for 24 hours prior to the start of the experiment to ensure that their stomachs were empty of food which may contaminate the acid been secreted. The animals were then anaesthetized by intraperitoneal administration of 6 ml/kg of 25 % (v/v) solution of urethane (Sigma, UK). The trachea was exposed and cannulated to allow for adequate air flow into the lungs. Another cannula was passed into the stomach, through the mouth and the esophagus. Both cannulae were tied firmly in place with a ligature. The abdomen was then cut open along the *linea alba* to minimize bleeding. The stomach was exposed and the pyloric end cannulated at its junction with the duodenum. Isotonic (0.9 per cent) saline was introduced gently via the esophageal cannula to wash out the stomach contents. The perfusate was allowed to flow freely after clearing the food particles. The abdominal incision was then covered with a moist cotton wool dipped in normal saline. The stomach was perfused continuously with normal saline at the rate of 1 mL/minute. The pH of the saline was maintained at 7.0 and the body temperature of the rat was maintained at 37 ^{o}C using a heating lamp. The flow was adjusted to give an effluent volume of about 1 mL per minute. The effluent was collected at 10 minutes interval and care was taken not to ligate the blood vessels as this may lead to stained perfusate or interrupt blood flow to the stomach of the rat. Each perfusate obtained after 10 minutes was titrated using two drops of phenolphthalein as indicator against 0.01 N NaOH (May and Baker, UK) to determine its total acidity. The experiments were repeated using histamine as acid secretagogue, administered subcutaneously. The dose of histamine used was 100 mg/kg body weight. Gastric acid output in the effluent sample was measured by titrimetric analysis. The experiments were also repeated using cimetidine as a blocker at a dose of 100 mg/kg body weight to ascertain whether the pathway for acid secretion was via Histamine (H_2) receptors. Administration was via intramuscular route.

Measurement of Gastric Mucus: The adherent gastric mucus was measured by the method of (Tan *et al.*, 2006). The animals were fasted for about 18 hours prior to the experiment, after which they were sacrificed after being anaesthesized with chloroform and their stomachs removed. The stomach was then opened along the greater curvature and pinned on a flat board. Using a spatula, the gastric mucus was scraped off the surface of the mucosa and introduced into pre-weighed sterilized sample bottle containing 3 ml of distilled water. The sample bottle containing distilled water and the collected mucus was then weighed on an electronic balance. Mucus output was calculated as the difference in weights of sample bottle containing water and sample bottle containing water and mucus.

Determination of Ulcer Scores: Gastric ulcer score was assessed using the method of Alphin and Wards, (1967). The animals were anaesthetized by inhalation of 3-4 ml of 5% chloroform poured unto a cotton-wool in a small beaker placed near the nostrils of the animals. The reason for chloroform anaesthesia usage was because of its gastroprotective effects in animals with ethanol-induced gastric ulcers through antioxidant and anti-secretory effects (Zainul *et al.,* 2016). Thereafter, an abdominal incision was made through the *linea alba* and the pylorus exposed. Induction of the ulcers was done using the method by Mizui and Doteuchi, (1983). A pyloric incision was made and a cannula inserted and held in place by tying with a thread. The stomach was infused with 1.5 ml of acid alcohol to induce ulceration. The infusion was made via the pyloric incision. The animals were allowed for an hour. The stomach was then surgically removed, washed, cut open along the greater curvature and rinsed with normal saline. Pins were used to hold the tissue to the dissecting board. A magnifying lens and a Vernier caliper were used to measure the extent of ulceration. Ulcer score was done according to the grading system shown below:

Grade 0.0 - No Lesion (normal stomach)
Grade 0.5 - Pin size ulcer
Grade 1.0 - 2 or more haemorrhagic or small linear ulcers
Grade 2.0 – Ulcer spots greater than 3 mm.

The ulcer score was calculated by multiplying each grade with its frequency of occurrence. The sum of all the values formed the ulcer score for each animal (Koike *et al.*, 2001).

Statistical Analysis

The data were analyzed by unpaired t test using Microsoft excel computer program. The results were presented as mean ± standard error of mean (SEM) and *p* value less 0.05 was considered statistically significant.

RESULTS

This study observed that treatment with CLX resulted in a rise in gastric mucus output in the test group which was significantly higher the control ($P < 0.001$). The mean gastric mucus outputs in the control and test groups were 0.22 ± 0.04g and 0.28 ± 0.02g respectively (Figure 1).

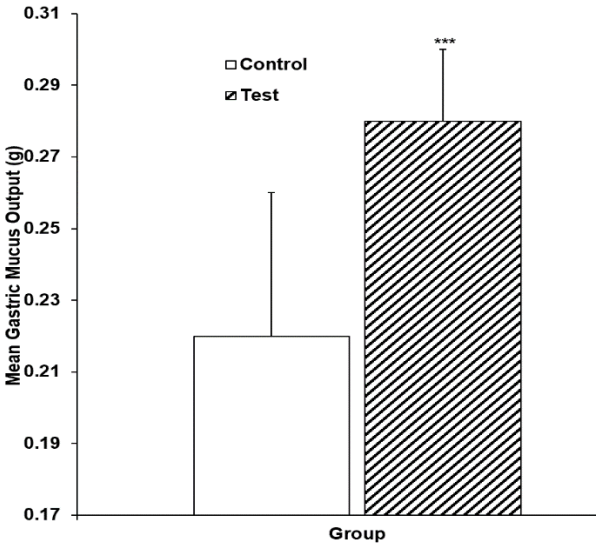

Figure. 1:
Comparison of mean gastric mucus output in the different experimental groups.
Values are mean ± SEM, n = 5. **** = p< 0.001*

With reference to figure 2, there was a rise in basal gastric acid secretion in the test group that was significantly different from control ($p < 0.001$) and with the administration of histamine, the peak acid output (PAO) of the test group was significantly higher than the control group ($p<0.001$). Following the administration of cimetidine, H_2 receptor blocker, gastric acid secretion decreased to levels that were below the basal levels in the control and test groups.

DISCUSSION

Gastric ulcer, one of the most widespread diseases occurs due to an imbalance between protective and aggressive factors (Alkofahi and Atta, 1999). The gastric mucosa is continuously exposed to potentially injurious agents such as gastric acid, pepsin, bile acids, food ingredients, bacterial agents (Helicobacter pylori) and drugs (Peskar and Matricic, 1998).

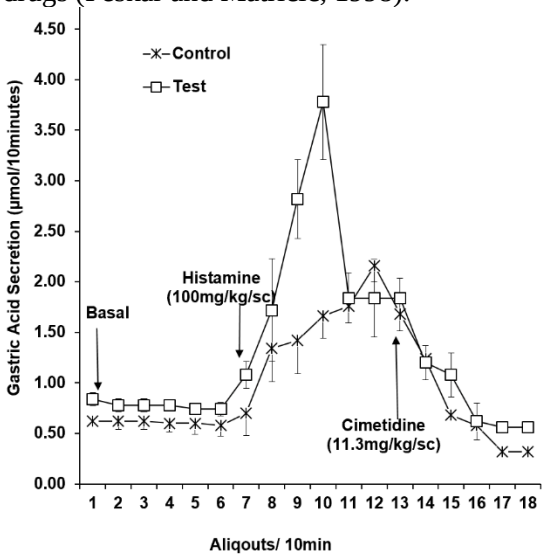

Figure 2:
Gastric acid secretion in control and CLX-treated groups
Values are mean ± SEM, n = 5.

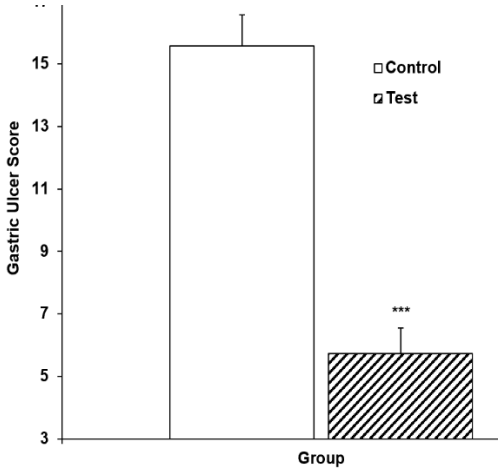

Figure. 3:
Comparison of mean gastric ulcer score in the control and CLX-treated groups.
Values are mean ± SEM, n = 5. ***= p<0.001 vs control.

These agents have been implicated in the pathogenesis of gastric ulcers including enhanced gastric acid and pepsin secretion, inhibition of prostaglandin synthesis, inhibition of cell proliferation, increased generation of free radicals, and diminished gastric blood flow and gastric motility (Toma et al., 2005).

In this study, there was decrease in the gastric ulcer scores in the test group when compared with the control. However, there was a rise in mucus secretion in the test group which was significant when compared with the control. It is likely that this rise in the mucus output contributed to the decrease in ulcer scores in the test groups.

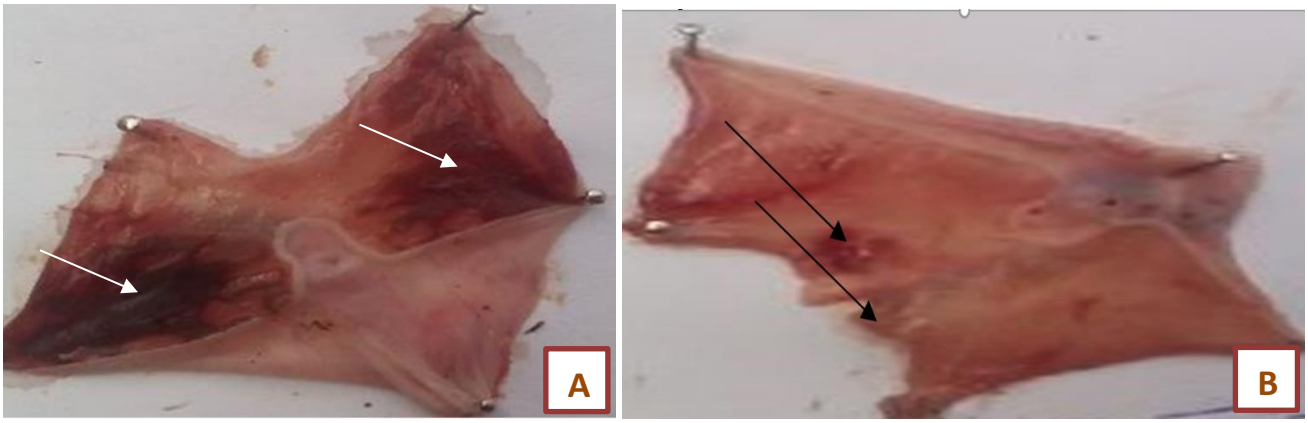

Plate 1:
Gross evaluation of the ulceration on the gastric surface of the Wistar rats
A- is the ulcer-induced group without CLX treatment (control group) showing extensive lesions to the gastric mucosa. They appear as elongated bands of hemorrhage (white arrow).
B – is the ulcer-induced group treated with CLX (test group) showing only mild injuries (black arrow) to the gastric mucosa. (Magnification: 1.8 x) (n=5).

The decrease ulcer scores despite increase acid secretion found in this study may be explained by neutralization of hydrogen ion on the gastric mucosa by the mucus-entrapped bicarbonate ions (cytoprotection), thereby reducing the concentration of the gastric acid (Osim, 2002). In addition, the increased gastric mucus output protects the gastric epithelium from injurious effect of pepsin hence the decrease in ulcer scores.

This therefore ameliorates the ulcerative effects of the acid on the mucosa of the stomach. Similarly, this is supported by a study by Toshimitsu et al., (2013) carried out in humans which reported that increased gastric mucus secretion alleviates non-steroidal anti-inflammatory drug (NSAID)-induced abdominal pain which is likely due to gastric ulceration.

Future studies: To know if CLX alone is adequate to reduce peptic ulceration or if other drugs like Histamine-2 blockers and proton pump inhibitors are necessary.

In conclusion, administration of CLX increased gastric acid and mucus secretions but decreased ulcer scores in the rats. The increase in mucus secretion may be beneficial in the reduction of ulcer scores since presence of mucus is one of the protective factors to the gastric mucosa.

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Research Article

An Assessment of Gaseous Emission from Awotan Dumpsite in Ibadan, South Western Nigeria

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Summary: Gaseous emission, particulate emission, biological molecules and other harmful substances discharge into the atmosphere from dumpsite environment. The carbon dioxide (CO_2) and methane (CH_4) content of the gaseous emission from different platforms and offsets of the Awotan dump site were measured. P Sense CO_2 Meter AZ 7755 was used to measure the level of carbon dioxide gas, temperature and relative humidity. K60 Gas detector was used to measure the level of methane (CH4) gas and Pm 2.5. Thermo-scientific MIE pDR 1500 PM monitor was used to measure the particulate matter on the dumpsite. The CO_2 levels (697±28.84 - 502±2.19) were above the minimum permissible levels of ASHRAE of 400ppm for all platforms at the dumpsite. CH_4 levels range (73.33±3.32 – 18.33±4.27) was above the methane explosive limits (MEL) of 15% for all Platforms, however the level at 25m and 50m offsets (14.83±4.11 – 13.83±2.48) was below the MEL for 75m and 100m offsets. PM2.5 levels were lower in the morning and peaked in the afternoon at Platform 5, 6 and 9 locations with values of 62.76±6.03, 63.9±11.37 and 32.06±3.89 respectively which is not within the WHO minimum permissible limit of 25µg/m3. There was a significant positive correlation between CO2 and CH4 (r=0.7558, p=0.028) but no significant correlation between CO_2 and other meteorological parameters (temperature and humidity) (r=-0.1309, p=0.67and r=0.09644, p=0.754). The carbon dioxide and methane content of the gaseous emission from the Awotan dump site are potential health hazard, hence the need for an engineering design that will reduce the quantum of the emission thereby reducing the hazard.

Keywords*: Dumpsite, Gaseous pollutant, Particulate Matter, Air Quality, Awotan, Ibadan*

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INTRODUCTION

Solid waste and poor management in dumpsite increase the amount of gaseous emission, particulate emission, biological molecules and other harmful substances into the atmosphere (Boardi *et al* 2005, Babs-Shomoye and Kabir 2016). The pollutants so introduced into the atmosphere can have severe effect on humans and the ecosystem in general (Nwafor *et al* 2019). According to United States Environmental Protection Agency USEPA (2012), criteria pollutants are commonly found air pollutants which could have severe health and environmental implications. These pollutants include; particulate matter (PM_{10}), carbon monoxide (CO), nitrogen dioxide (NO_2), sulphur dioxide (SO_2), methane (CH_4), ammonia (NH_3) and hydrogen sulphide (H_2S) (Jha *et al* 2008, Olla *et al* 2015). The gaseous pollutants are colourless and invisible while the particulate matter such as dust and soot are not. Although, pollutants are naturally present in air, human activities such bush burning, industrial processes, decaying of accumulated organisms and domestic wastes increase their atmospheric concentration (Dare 2000, Oladejo and Otene 2018). The quality of the ambient air in any environment is determined by the extent and severity of pollution (Ubuoh and Akhionbare (2011), Agwu and Ozeh (2013) thus air pollution is spatial-temporal. Globally, most municipal wastes are dumped in non-regulated landfills which generate landfill gas (LFG) as a by-product (Johnson 2010).

The impact of these wastes affects health and livelihood of people (Boardi *et al* 2005, Salam 2010, Babs-Shomoye and Kabir 2016). LFG is generated when organic material decomposes anaerobically, consisting of methane (45- 60%), carbon dioxide (40-60 %) and other gases (2-9%) which are mostly emitted to the atmosphere (Metz *et al*. 2007, Jha *et al* 2008). According to the International Panel on Climate Change, methane emission from landfills account for 3-19% of the anthropogenic sources in the world and is considered as a large contributor to global warming

after agricultural activity and losses from fossil fuel distribution (IPCC 1996; Metz *et al*. 2007; Johnsson 2010). The impact of dumpsite solid waste was equally assessed on drinking water sources (Sia Su 2007).

Awotan dumpsite situated in Ido Local Government Area of Oyo State, Nigeria has being in use since 1997 and has adjoining residential and commercial buildings within 300 metre radius. In this study, an assessment of emitted gases from the Awotan dumpsite was carried out with a view to assessing its environmental and health impact on people living in the area.

MATERIALS AND METHODS

Ethical Approval: All applicable international, national, and institutional guidelines for environmental assessment were followed.

Study Area: The study was carried out at Awotan Dumpsite in Ibadan (Latitude 7°27'45.67"N and Longitude 3°50'59.15"E), Nigeria. Awotan dumpsite is located in Ido local Government Area which is a rapidly developing settlement area of more than 2 million populace in Oyo state, Nigeria. The dumpsite has a size of approximately 20.59 hectares and divided into 9 platforms. The fresh waste dumps (yellow areas) were platforms 2,5 and 6 while 1,3,4,7,8 and 9 were old waste dumps (red areas)-Plate 1. The main criterion for the selection of Awotan dump site amongst others was the high carbon and nitrogen content of the waste (Tables 1& 2).

Platforms: Nine (9) Platforms and 4 Offsets on the dumpsite environment served as the sampling locations (SL) of this study. Platform 5, 6 and 9 are of old dumps, new dumps and scavengers gathering respectively and other platforms were abandoned landfills as at the time of this study. 100m offset serves as residential area close to the dumpsite, 75m offset is the access road linking the dumpsite environment and street and other offsets are distances measured at 25m interval as shown in Table 3.

Table 1:
Composition of MSW from the three Dump Sites in Ibadan.

Composition	Percentage contribution		
	Awotan	Ajakanga	Afofunra (Aba Eku)
Paper & Textile	21.99	23.14	19.46
Garden, Park or non food waste	15.64	26.65	25.47
Food waste	36.67	21.80	26.51
Wood/Straw	25.70	28.40	28.57
Total	100	99.99	100

Source: Centre for People and Environment (CPE) for Methane-To-Markets Program USEPA on Landfill Recovery and Use in Nigeria, 2010

Levels of landfill gases comprising carbon dioxide (CO_2) and methane (CH_4) were determined using P-Sense plus CO_2 meter AZ-7755 and K-60 gas detector respectively. The measurements were taken thrice weekly at each location for the entire six week duration of the study. Measurements were carried out at nine platforms (PL1- PL9) and at four offsets from the dumpsite area (25m, 50m, 75m, and100m) and the means of the readings were computed.

The gas monitors were calibrated on the field in a Ziploc-polythene bag which was free of the target gas. The gas monitors were hand held at the sampling locations and stretched at arm's length to determine the ambient levels of the pollutants. The levels of particulate matter at all the study locations were determined by means of $Pm_{2.5}$ and readings recorded morning and evening device. The device was placed on a stable platform and switched on at a height of 1.5m. The measurement was recorded when the readings on the display screen were stable.

Statistics:
Quantitative data were expressed as means ± standard deviation. Two-tailed student t test was used for data comparison and a confidence level of 95% with $P \leq 0.05$ to determine significance. Pearson correlation was used to test the relationship between CO_2 and CH_4 as well as CO_2 and meteorological parameters (temperature and humidity).

Table 2:
Ultimate analysis of the combustible components of Ibadan MSW

Component	Carbon %	Hydrogen %	Oxygen %	Nitrogen %	Sulphur %	Ash %
Food Waste	51.85	3.79	40.23	2.39	1.24	9.75
Paper & Cardboard	56.34	6.13	36.59	0.34	0.21	4.38
Plastic	64.28	6.89	27.44	0.96	0.39	3.04
Textile	53.26	5.76	40.07	0.69	0.18	2.43
Rubber	51.28	5.96	36.22	0.24	0.12	6.13
Wood	46.24	6.08	44.42	0.17	0.03	2.97
Miscellaneous (Dirts,Ashes, etc.)	59.78	2.76	41.79	0.42	0.25	3.79

(Source: Methane Generation Potential of Municipal Solid Waste in Ibadan, 2014)

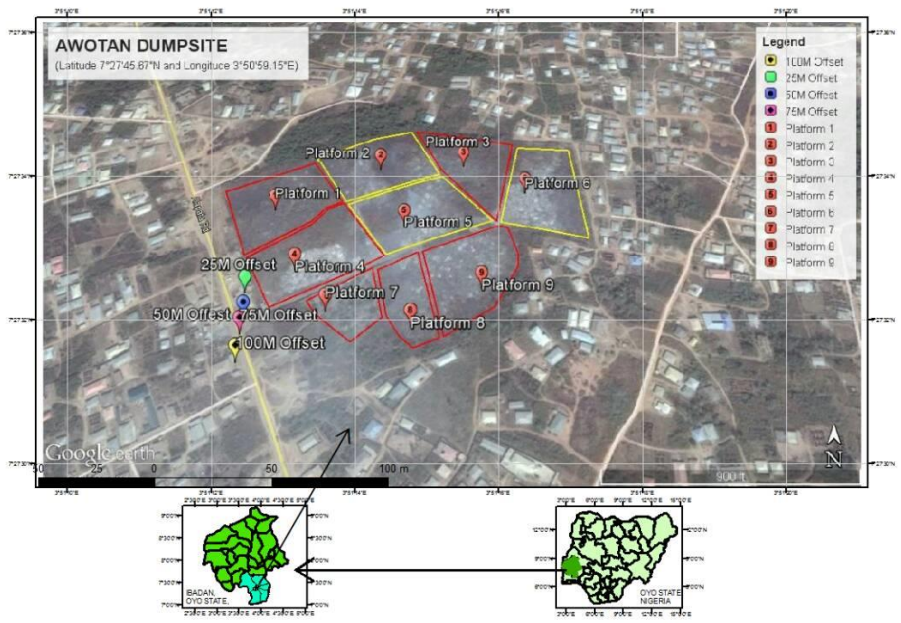

Plate 1:
Location Map of Awotan dumpsite (Source: Arc GIS and Google Map)

Table 3:
Coordinate and Elevation of Sampling Points at Awotan dumpsite

Sampling Point	Longitude	Latitude	Elevation
PL 1	$7^0$27'46.93"N	$3^0$50'53.52"E	730ft
PL 2	$7^0$27'49.07"N	$3^0$50'57.95"E	765ft
PL3	$7^0$27'49.00"N	$3^0$51'02.26"E	769ft
PL 4	$7^0$27'44.17"N	$3^0$50'54.91"E	730ft
PL 5	$7^0$21'46.37"N	$3^0$50'59.59"E	767ft
PL 6	$7^0$21'47.65"N	$3^0$51'05.15"E	752ft
PL 7	$7^0$21'41.79"N	$3^0$50'57.17"E	740ft
PL 8	$7^0$21'42.73"N	$3^0$50'59.37"E	752ft
PL 9	$7^0$27'43.70"N	$3^0$51'01.64"E	756ft
25m offset	$7^0$27'47.77"N	$3^0$50'52.28"E	715ft
50m offset	$7^0$27'42.72"N	$3^0$50'52.85"E	712ft
75m offset	$7^0$27'41.54"N	$3^0$50'52.28"E	708ft
100m offset	$7^0$27'41.30"N	$3^0$50'51.92"E	706ft

RESULTS

The mean carbon dioxide concentrations from all the sampled areas of the dump site significantly exceeded the minimum permitted limit level as prescribed by the American Society of Heating, Refrigerating and Air-conditioning Engineers (ASHRAE) (Table 4).
The mean methane concentrations for the sampled areas apart from offsets 75m and 100 m were significantly higher than methane explosive limit (Table 4).

The moisture content of the organic fraction of the waste being 44.6% could contribute to the high heating value of the municipal waste. The 28.7% moisture content of the MSW might be responsible for the offensive odor being emitted from the dumpsite.

The mean levels of particulate matter were low in the morning but high in the afternoon especially at platform 5,6 and 9 that had regular dumping and frequent burning of wastes. Platform 5, 6 and 9 had respective afternoon levels of 62.76±6.03, 63.9±11.37 and 32.06±3.89 µg/m^3 that were significantly higher than the WHO minimum permissible level of25 µg/m^3 (Figure 4).

Pearson correlation test was carried out between CO_2, CH_4 and the meteorological parameters as shown. The table shows that there was a significant positive correlation between CO_2 and CH_4 (r=0.7558, p=0.0028) and no significant correlation between CO_2 and other meteorological parameter (r=-0.1309, p=0.67) for Co_2 and Temperature likewise (r=0.09644, p=0.754) for CO_2 and Humidity

Table 4:
Air Quality Assessment for CO_2, CH_4, $PM_{2.5}$ and Meteorological parameters

S/N	Platforms /Offsets	Mean	Ashrae Mpl	Mean	Mel	Morning Readings	Afternoon Readings	Who Mpl
		ACR (PPM)	**ACR (PPM)**	**AMR (%)**	**AMR (%)**	**Pm2.5 (µg/m^3)**	**Pm2.5 (µg/m^3)**	**Pm2.5 (µg/m^3)**
1	Platform 1	565.5±24.41*	400	66.333±2.50*	15	0±0	1.36±2.36	25
2	Platform 2	579.333±6.65*	400	62.333±1.50*	15	4.33±1.65	19.63±5.64	25
3	Platform 3	642.166±22.92*	400	67.5±4.88*	15	0±0	9.53±0.40	25
4	Platform 4	563.333±55.05*	400	64.333±2.33*	15	9.0±1.90	19.76±3.65	25
5	Platform 5	587±8.17*	400	67.667±2.80*	15	10.83±1.56	62.76±6.03*	25
6	Platform 6	697±28.84*	400	73.333±3.32*	15	7.43±2.67	63.9±11.37*	25
7	Platform 7	530±22.93*	400	52.667±1.96*	15	5.2±1.47	25.9±4.40	25
8	Platform 8	515.5±3.88*	400	53.667±2.73*	15	0±0	1.96±1.56	25
9	Platform 9	512.666±5.78*	400	52.833±2.99*	15	3.9±2.21	32.06±3.87*	25
10	25m offset	504.5±2.88*	400	20.167±5.07*	15	5.8±2.47	7.63±2.40	25
11	50m offset	504.833±1.47*	400	18.333±4.27*	15	6.33±3.75	8.66±4.25	25
12	75m offset	503.333±3.72*	400	14.833±4.11	15	5.2±1.47	13.83±3.06	25
13	100m offset	502±2.19*	400	13.833±2.48	15	1.9±1.68	2.86±1.22	25

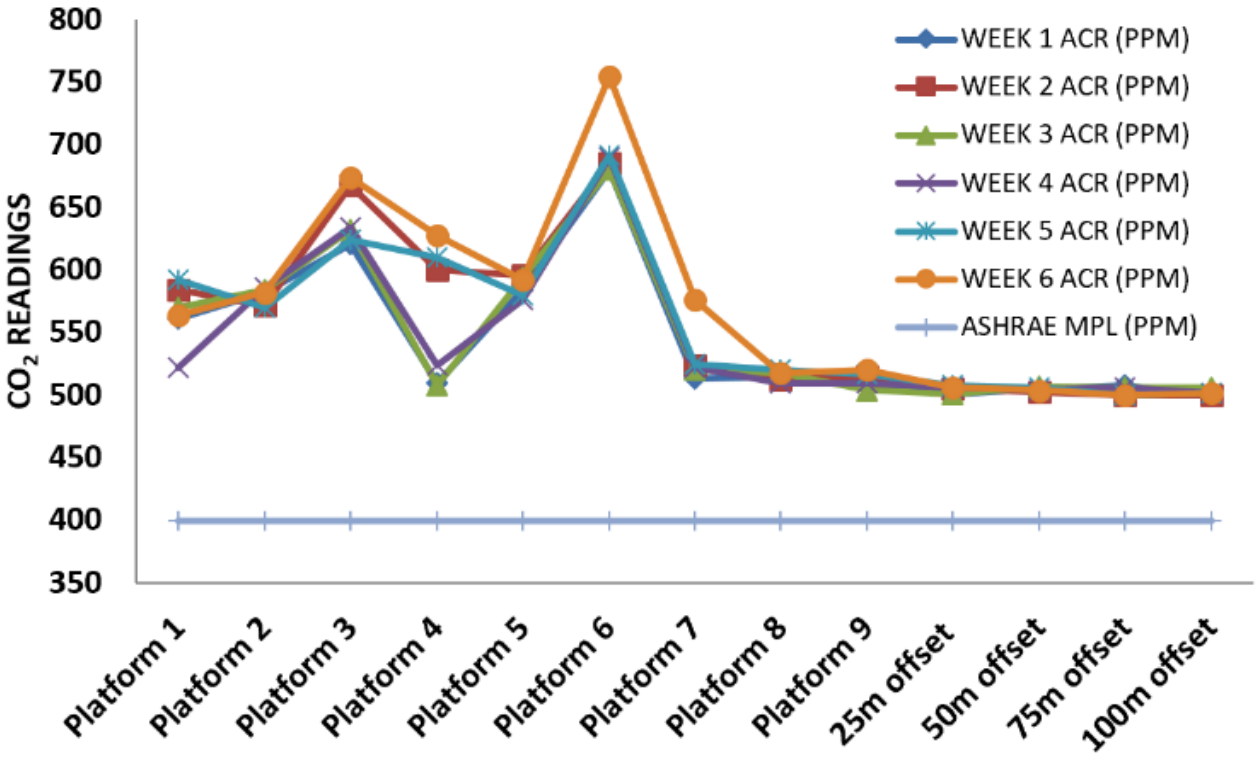

Figure 2:
CO2 Concentrations at sampling locations

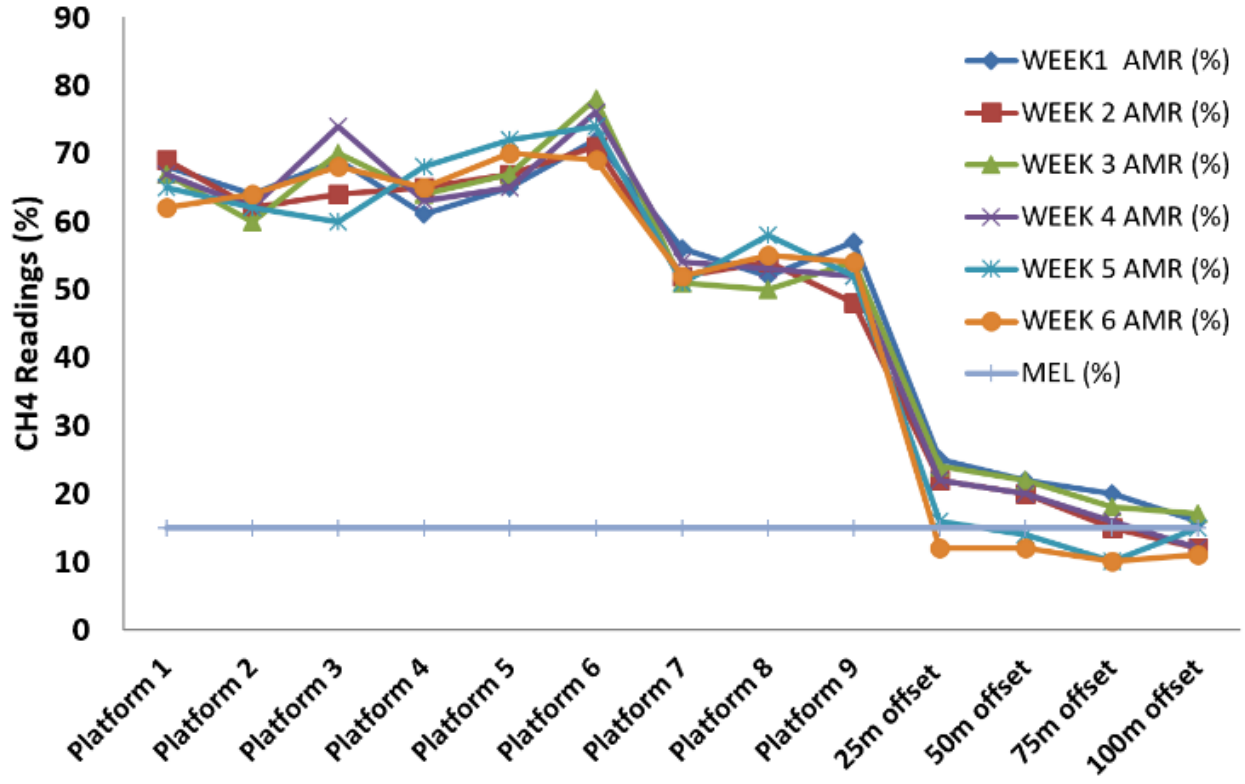

Figure 3:
CH_4 Concentrations at sampling locations

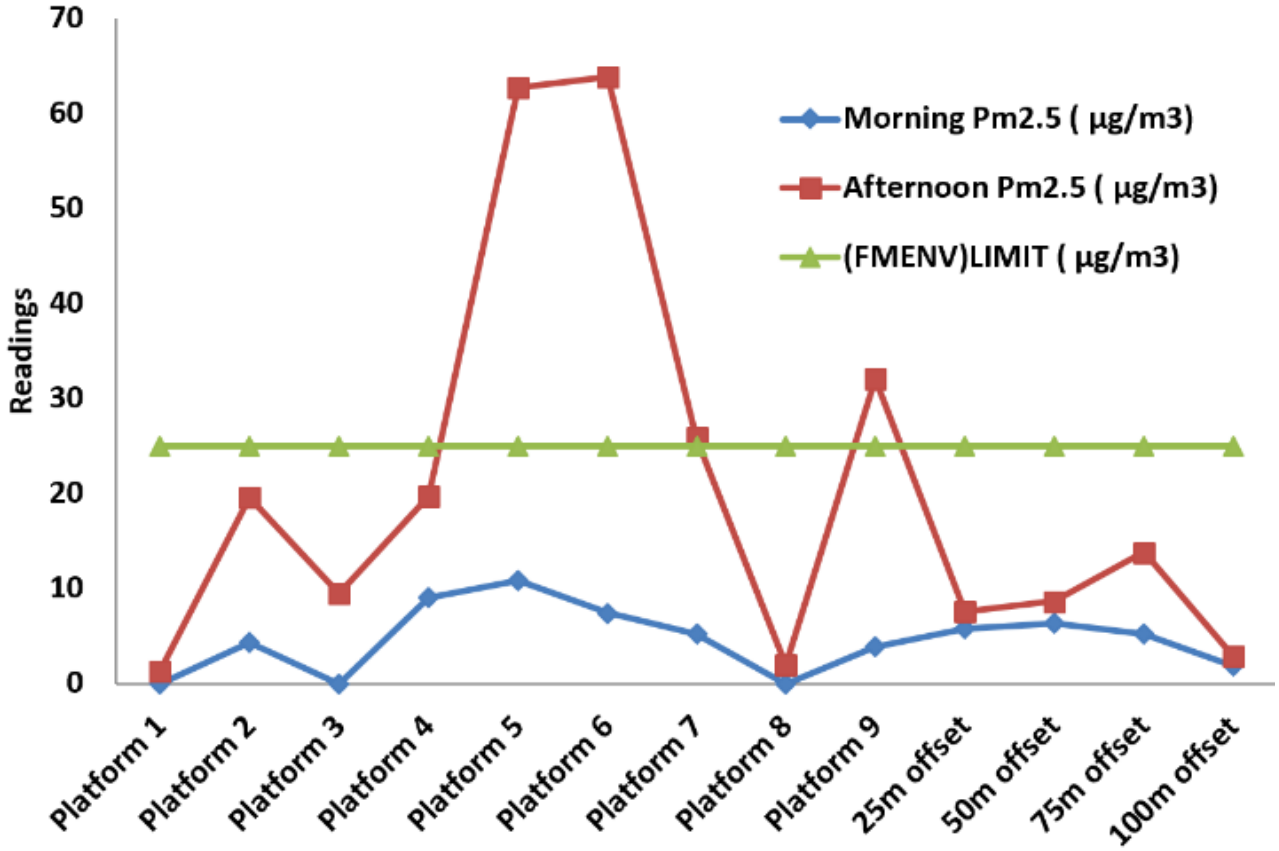

Figure 4:
Concentrations at sampling locations

DISCUSSION

This study investigates the impact of municipal wastes on air quality, recording the variation of pollutants from different platforms and offsets in the dumpsite environment. Both carbon dioxide and methane concentrations were significantly higher than the WHO minimum permissible limits in the Awotan dumpsite. These were likely be consequent upon activities at the dumpsite which included but not limited to anaerobic and aerobic decomposition of organic and biodegradable wastes, burning of combustible wastes such as papers, wooden, plastics and rubbers. Asides from the health hazard which these gases constitute, they also cause increased environmental temperature leading to global warming (Jha *et al*, 2008). This has diverse effects on human population activities such as agriculture, aquaculture and animal husbandry (Salam 2010).

More so, the site has become popular with dump activities. Human settlement and population have really increased in the vicinity which was not hitherto. And the regular visit of humans to the dumpsite to scavenge for renewable waste on the site makes this study paramount for human and animal wellbeing. The moisture content from the waste could be responsible for the high heating value of the municipal solid waste which generates the offensive odor on and around the dumpsite, corroborated by findings of Sia Su (2007) in Payatas dumpsite in Quezon City, Philippines. The diurnal variation of the particulate matter content of the air was a reflection of the offensive odour and amount of humidity on the dumpsite (Boardi *et al* 2005).

These findings corroborated the reports of Akintayo and Olonisakin (2014) which found the typical compositions of the municipal solid waste to comprise over 30% organics, 20% paper and cardboard; tin cans constitute 9%; ashes and other constituents of the waste account for 6%. Similar composition was reported in Payatas dumpsite (Sia Su, 2007) and in Accra metropolitan area (Boardi *et al* 2005), and in Indian cities (Sharma and Jain 2019).

The runoffs and leachates being generated by solid waste have been documented to affect ground water quality (Oyelami *et al* 2013) and to have induced lesions in laboratory animals (Balogun *et al* 2017). Considering the fact that human habitation and activites occur within 300 metres radius of Awotan dumpsite, the ground water might become polluted with disease causing organisms such as viruses, bacteria, fungi and parasites (Adesewa and Morenikeji 2017). This might lead to an outbreak of water borne diseases that might assume epidemic magnitude (Badmus *et al* 2014, Oladejo and Otene 2018). Thus there is a need for re-engineering design of the dumpsite in order to minimize its environmental hazard thereby reducing the human health implication In conclusion, it is obvious that municipal waste management in Awotan dumpsite needs to be improved to international standards of operations, as non-compliance will lead to the generation of smokes, unpleasant odor and general environmental degradation.

New settlement has sprung up in Awotan area very close to the dumpsite and the residents are liable for the negative effects of mismanagement on the dumpsite. To prevent the hazard that may be caused from this mismanagement, pragmatic design and proper management should be done on landfills. Further studies are recommended to investigate the safe offsets from Awotan dumpsite where individuals can reside and they will not be affected by the gaseous emission and particulate matter emission from the dumpsite.

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Research Article

Some Aspects of Neuromorphology, and the Co-localization of Glial Related Markers in the Brains of Striped Owl (*Asio clamator*) from North East Nigeria

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Summary: The striped owl (*Asio clamator*) is unique with its brownish white facial disc and they are found in the north eastern part of Nigeria. Little is known in the literature on the basic neuroanatomy of this species. This study focuses on the histology and glial expression of some brain regions of the striped owl. Five owls were obtained in the wild, and their brains were routinely prepared for Haematoxylin and Eosin, and Cresyl violet staining. Immunostaining was done with anti-Calbindin, anti MBP, anti-GFAP, and anti-Iba-1 antibodies; for the expression of cerebellar Purkinje cells and white matter, cerebral astrocytes and microglia cells respectively. These were qualitatively described. We found that the hippocampal formation of the striped owl, though unique, is very similar to what is seen in mammals. The cerebellar cortex is convoluted, has a single layer of Purkinje cells with profuse dendritic arborization, a distinct external granular cell layer, and a prominent stem of white matter were seen in this study. The astrocytic population in cerebral gray is similar, though lacking in many processes as is typical in protoplasmic astrocytes, while the microglia were not strongly stained. The few stained microglia cells did not, however, show any features of activation. The striped owl's brain reveals some conserved aspects of cellular neuroanatomy in both the avian and mammals that are typical in these species. More work is however needed particularly in age related differences in these structures. This is perhaps the first report of Calbindin immunostaining in the brain of the striped owl.

Keywords*: Striped Owl; Immunostaining; Purkinje cells; Astrocytes; Microglia; Dendritic arborization*

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INTRODUCTION

The Striped Owl is a unique nocturnal bird with frontally placed eyes, facial disk, binocular vision, filoplumes, and a head that can rotate at about 2700 due to a single occipital condyle (Poster and Graphics, 2013). They are a relatively large owl species with a prominent tuft of elongated feathers on the crown resembling ears (Thurber et al., 2009).

Owls are adapted for hunting due in part to the ability to fly almost silently, move very slowly, and have eyes that have the capacity for aiding in capturing of nocturnal prey (Marti, 1974). In addition, they exhibit specialized hearing functions that also aid in hunting (Konig et al., 1999; John, 2013). There are few literature reports on the histology of the brain of the owl. Abd-Alrahman (2012) described the histology of the cerebrum of the barn owl.

The striped owl in particular possesses sophisticated capacity for vocalization used for nocturnal hunting that is developed through a sophisticated learning (Knudson, 2002). There is however, a paucity of information on the neuroanatomy of the striped owl, though it is suggested that her hunting adaptation demands central nervous system co-ordination.

This work is a preliminary study of the neuroanatomy of the striped owl in Nigeria focusing on the histology of some brain regions, and immunohistochemical expression of glial related markers.

MATERIALS AND METHODS

Sample Collection: A total of five owls (3 males and 2 females) were used for this study. They were

obtained by local hunters in the North Eastern of Nigeria. All birds were weighed and rectal temperatures obtained. The birds were subsequently sedated with 50mg/kg of ketamine hydrochloride. All experiments were done based on guidelines of University of Ibadan Ethical Committee.

Thereafter, their intercostal spaces were dissected and they were intracardially perfused using 4% buffered formalin. The brains (Plate 1) were then removed and placed in the same fixative for 24 hrs, replaced in fresh solution for another 24hrs, and thereafter transferred to 0.2% sodium azide solution (preservative against bacterial growth) and kept at 4^0C until processed.

Fixation and Tissue Processing of Samples for Paraffin Embedding, and Staining: The brains were dehydrated in graded concentrations of alcohol for one hour each (70%, 80%, 90%, 100% I, 100% II). The alcohol was in 2 changes of xylene for 2 hours each. Infiltration and impregnation were done in 2 changes of molten paraffin wax for 1 hour each with tissue embedded in the mould. Serial transverse and longitudinal sections of 5-µm thickness were prepared by using a HM330 Micron Microtome. For general histological examination, paraffin representative sections were stained by Hematoxylin and Eosin and Cresyl Violet as depicted by Suvarna *et al* (2013) and Ladagu *et al* (2020).

Immunohistochemistry: The antibodies used, and procedure of Immunohistochemistry (Ladagu *et al*) are as stated in Table 1.

Sections of the paraffin blocks were de-waxed and immediately rehydrated and immersed in distilled water. Retrieval of antigen was done using 10- mM citrate buffer (pH = 6.0) for a duration of 25 min with subsequent peroxidase quenching in 3% H2O2/methanol. After blocking in 3% H2O2/methanol, all the sections were blocked in 2% milk for 1 h after which they were probed with the following antibodies: Anti-GFAP (Glial Fibrillar Acid Protein) Rabbit Polyclonal, 1: 700, Dako Denmark), Purkinje cell aborization (Anti-Calbindin, 1:12,000, Abcam, USA), microglial morphology (Anti-Iba-1 Rabbit Polyclonal antibody, 1:500, Abcam, USA) and myelin expression (Anti-MBP (Myelin basic Protein) 1:500, Abcam, USA) at varying time points after due optimization at 4 °C. The sections were incubated for 2 h at room temperature using the appropriate biotinylated secondary antibodies (diluted 1:500; following Vector Labs' protocols) after washing. Avidin-biotin-peroxidase solution (ABC kit, Vectastain, Vector Labs, USA) were used afterwards for sections' reaction, and 3, 30-diaminobenzidine (DAB) was finally used as chromogen as instructed in manufacturer's protocol.

Experiments were done based on the ethical guidelines of the University of Ibadan for standard care and use of animals in research. All images were obtained using a bright field Leica DM 500 Microscope with in-built camera (Leica Microsystems, Wetzlar, Germany).

Table 1
List, type and dilution factor of antibodies used for immunohistochemistry

Name and Target of Primary Antibody	**Primary antibody**					**Biotinylated secondary antibody**
	Supplier	**Origin**	**Dilution**	**Incubation**	**Antigen retrieval**	
Anti-Calbindin (Purkinje cell aborization)	Abcam, USA	Rabbit	1:12,000	16hrs	Heat-induced epitope retrieval (HIER)	Anti-rabbit
Anti-GFAP (Astrocytic morphology)	Dako Denmark	Rabbit	1: 700	16hrs	Heat-induced epitope retrieval (HIER)	Anti-rabbit
Anti-iba 1 (Microglial morphology)	Abcam, USA	Rabbit	1:500	32hrs	Heat-induced epitope retrieval (HIER)	Anti-rabbit
Anti-MBP (Myelin expression)	Abcam, USA	Rabbit	1:500	16hrs	Heat-induced epitope retrieval (HIER)	Anti-rabbit

RESULTS AND DISCUSSION

The images obtained from this study are presented in Plates 1 - 8.

Animals without a "six-layered" cerebrum, like the owl, display complex intelligence and behaviour, tool utilization and skills comparable to other mammals (Güntürkün and Bugnyar, 2016; Karten, 2015). Recent reports have suggested that the "six-layered" cerebrum has evolved from a common ancestor with a "three-layered" cortex as typified by the hippocampus of varying animal species (Güntürkün et al., 2017; Shepherd and Rowe, 2017).

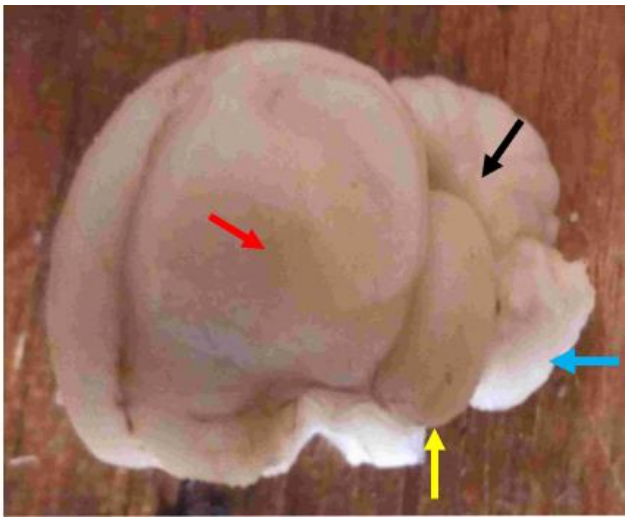

Plate 1:
Photograph of a whole brain of a striped owl, rostrolateral view. Red arrow, Cerebrum; yellow arrow, Rostral Colliculi; black arrow, Cerebellum; blue arrow: Medulla

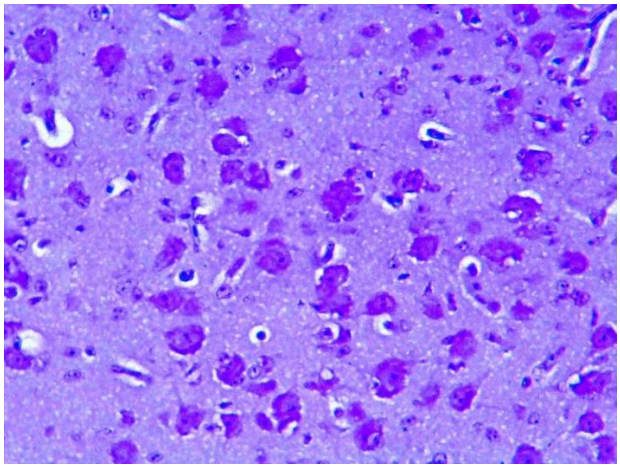

Plate 2:
Photomicrograph showing cortical histology of the brain of the striped owl with polymorphic neurons, Cresyl violet x 400.

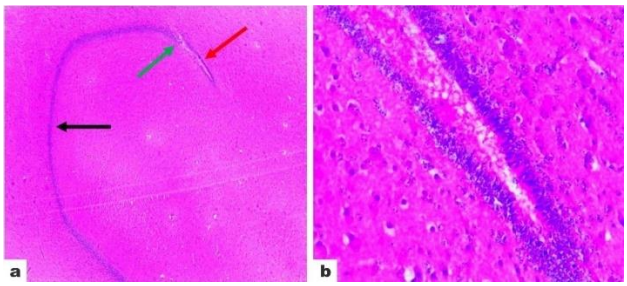

Plate 3:
a): Photomicrograph of the dentate gyrus (red arrow) and the CA1 region (black arrow), CA3 (green arrow) of the striped owl, H&E X 40. B). shows the Dentate Gyrus, H&E X 400.

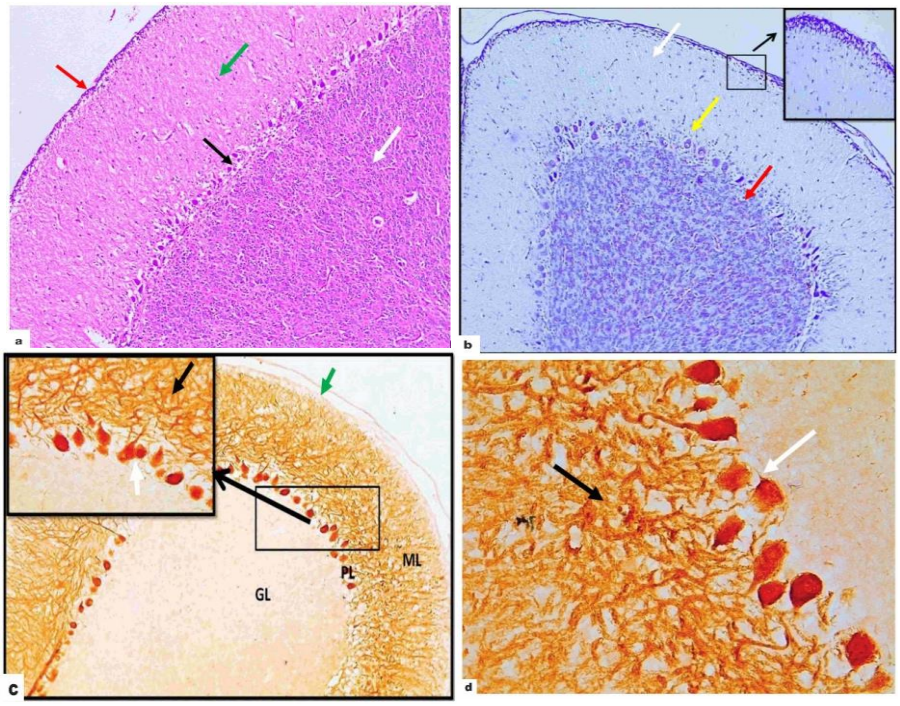

Plate 4:
Photomicrographs of the cerebellum of striped owl. a: Cerebellar cell layers: granular cell layer (white arrow), Purkinje cell layer (black arrow) and molecular cell layer (green arrow). Note the external granular layer (red arrow) H&E x 100. b: Cerebellar cortical layers of molecular (white arrow), Purkinje (yellow arrow) and the granular (red arrow) Cresyl violet x 100, inset x 400 magnifies the external granular layer. c: Purkinje cell layer (white arrow) and dendritic arborisation (black arrow), Anti-Calbindin immunostaining X 100. Note absence of immuno-localization in the external granular layer (green arrow). d: Purkinje cell layer (white arrow) and arborisation (black arrow) into the molecular layer, Anti-Calbindin X 400.

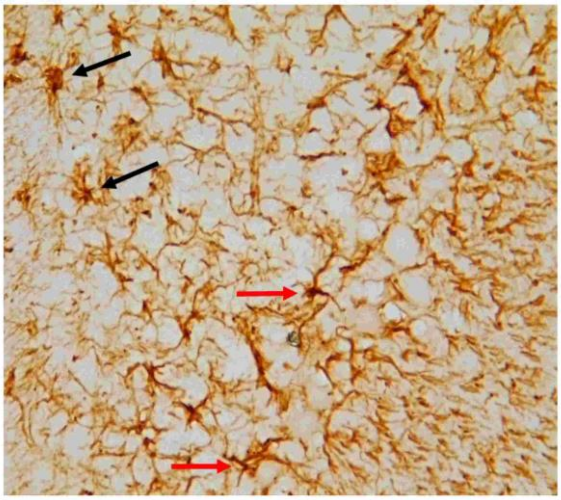

Plate 5:
Photomicrographs of the cerebrum of striped owl showing the fibrous astrocytes (black arrows) and protoplasmic astrocytes (red arrows), Anti-GFAP X 400.

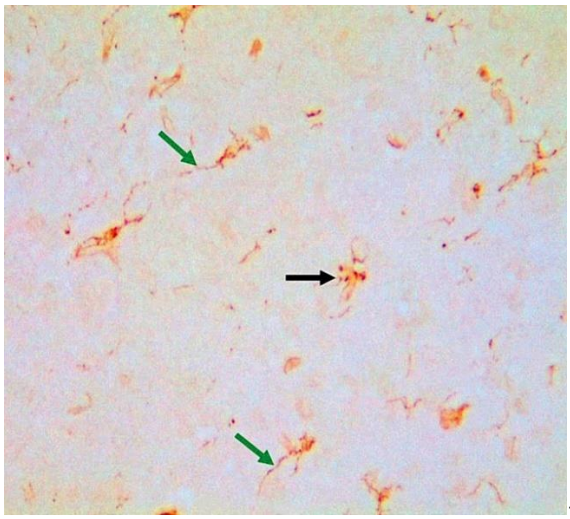

Plate 6:
Photomicrograph of the brain of striped owl showing resting microglia (black arrow) Iba1 X 400. Note the processes of the microglia (green arrows)

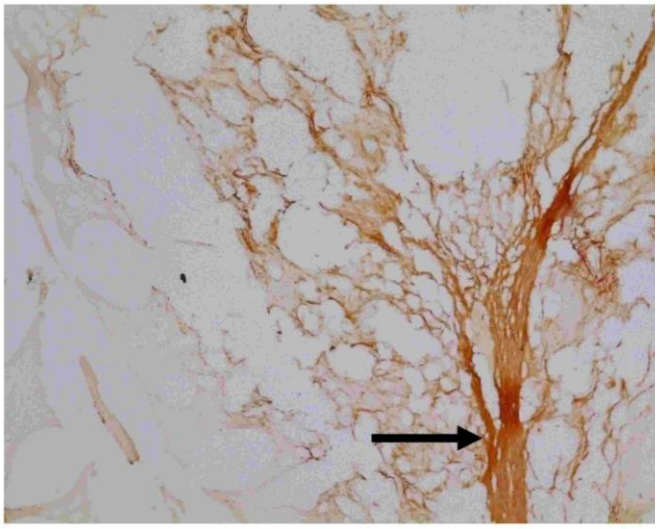

Plate 7:
Photomicrograph of the cerebellum of striped owl showing myelination of white matter (black arrow) MBP X 100.

Though looking unique, the hippocampus of the striped owl in this study (CA1, CA3, and Dentate Gyrus appearance) is similar to what is seen in mammals (Plates 2a and b)

Birds and mammals are the only species with variably convoluted cerebella (Iwaniuk *et al.,* 2006). In this study, the striped owl showed a typical cerebellar histomorphology of outer molecular, middle Purkinje cell and inner granular layers. The presence of the external granular layer (Plates, 4a, and 4b), however, suggests that the owls captured are relatively young. Butt *et al.*, (2014) described that only birds and mammals have granule cells which move in large numbers to form a transient external germinal layer; this cell layer has been shown to consist of stem cells in rodents and is Nestin positive (Bwala *et al.*, 2014). Calbindin localization in the cerebellum is known to clearly show Purkinje cells and dendritic arborization (Ioannidis *et al.*, 2019; Adebiyi *et al.*, 2020). This study shows the owl possess a thick and highly branched dendritic arborisation in the outer molecular layer (Plates 4c and d). Birds have been known to have a significant increase in dendritic arborization in the first 3 days of life (Mori and Matsushima, 2002). It is speculative to affirm that the high dendritic arborization may be a compensation for the reduced cerebellar hemisphere diminished by large rostral colliculi as seen in Plate 1. This is perhaps the first account of Calbindin expression in the brain of the striped owl and shows a lack of localisation of Calbindin to the external granular layer. This suggests that our optimized concentration can be adapted for further investigations on the cerebellum of the striped owl.

The GFAP (astrocytes) localisation in the brain of owls showed fibrous-like and protoplasmic astrocytes (Plate 4) in the cerebrum. Kalman and Pritz (2001) opined that mammals and birds exhibit similar general features in their glial architecture and GFAP distribution. There was a relatively weak expression of Iba-1 (microglia) staining in this study (Plate 5). It is still speculative to determine if they are resting microglia with no inflammatory activation. It is worthy of note, however, that the cells showed processes and devoid of amoebic isotypes as seen during inflammation (Folarin *et al.*, 2017). In addition, the MBP (myelin) expression seen in this study was also typical (Plate 6) as seen in rodents (Usende *et al.*, 2016).

In conclusion, this work has described a histological outlay of some parts of the brain of the striped owl, including the localization of glial related aspects of the brain, showing that some conserved aspects of cellular neuroanatomy in avian and mammals are so typical in these species. In this study, we report to the best of our knowledge, the first attempt at Calbindin localization in the cerebellum of the owl. More work is however

needed particularly in age related differences in all these structures.

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Research Article

Similar Lung Function Impairment in Auto Mechanics Operating in Stand-alone Auto Repair and Auto Repair Shops Shared with Spray Painters

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Summary: Automotive mechanics are reported to have lower lung function indices. Spray painting is associated with impairment of lung function among spray painters. It is a common practice by auto repairers in Calabar to operate in the vicinity of spray-painting shops. Whether such dual exposure to auto repair and spray-painting environments worsens the lung function of auto mechanics is not documented. Lung function was evaluated in 300 males divided into three groups: control (group1), auto mechanics in stand-alone auto repair shops (group 2) and auto mechanics working in the vicinity of spray-painting shops (group 3). Each group consisted of 100 subjects. Forced vital capacity (FVC), forced expiratory volume in the first second (FEV_1), peak expiratory flow rate (PEFR) and forced expiratory volume in the first second expressed as percentage of FVC (FEV_1%) were assessed using a Vitalograph spirometer. Percentage oxygen saturation was evaluated using a pulse oximeter. Results showed no significant differences in age, weight and height among various groups. FVC was significantly reduced in groups 2 and 3 ($p< 0.05$ and $p<0.001$ respectively) compared to control but not between groups 2 and 3. FEV_1 was significantly reduced in groups 2 and 3 ($P <0.001$ each) compared with control but not significantly different between groups 2 and 3. FEV_1% was significantly lower in groups 2 and 3 ($p< 0.001$ each) compared with control but not between groups 2 and 3. The PEFR was significantly reduced in groups 2 and 3 ($p<0.001$ each) compared with control but not significantly different between groups 2 and 3. Percentage oxygen saturation was significantly reduced in groups 2 and 3 ($p<0.05$ and $p<0.001$ respectively) and also significantly lower in group 2 compared with group 1 ($p<0.01$). In conclusion, auto mechanics in auto repair only and stand-alone auto repair shops shared with spray painters have lower lung function compared with control but no significant difference in lung function between auto mechanics in stand-alone auto repair shops compared with those in auto repair shops shared with spray-painters.

Keywords*: Auto mechanics, lung function, spray painting, paints, exhaust fumes, petrol*

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INTRODUCTION

Spirometric evaluation of pulmonary function represents a simple non-invasive method of determining the pulmonary health of an individual and by extension other related systems. There is a strong relationship between pulmonary function and other systems like cardiovascular system, nervous system, urogenital system etc. Reduction in lung function parameters especially obstructive patterns of such pulmonary dysfunction have been associated with increased incidence of atrial fibrillation and other cardiovascular diseases (Fell *et al*, 2014). Chronic obstructive airway diseases arising from occupational exposures have also been linked with increased morbidity and mortality especially in the presence of predisposing factors such as smoking, obesity and infection (Toren *et al* 2007).

Auto mechanics are involved in the repair and maintenance of motor vehicles. These mechanics use garages or open spaces as workshops. By virtue of their occupation, auto mechanics are exposed to organic, inorganic and particulate materials from exhaust fumes, and petrochemical products used in the industry all of which could have negative effects on lung function and quality of life of the individual (Toren *et al* 2007). The quantity of exhaust fumes emitted is dependent on the degree of combustion of the fuel used by the vehicle (Burr and Gregory, 2011). Exhaust fumes are products of fuel combustion in the engines and contain pollutants such as hydrocarbons, nitrogen and nitrogen oxides, carbon monoxide, sulphur oxide, particulate matter and other toxic pollutants (Omidvarbana, 2014). Carbon monoxide (CO) a colorless and odorless gas produced from incomplete combustion of fuels (Ayres and Roberts, 2009), inhalation of which may cause respiratory and non-respiratory symptoms. Carbon monoxide reacts with hemoglobin to form carboxyhemoglobin which impairs normal transport of oxygen and may cause

chronic hypoxia, increased risk of atherosclerosis, neurological diseases, cardiovascular diseases and increased morbidity (Schwela, 2000; Guzman, 2012; Bleecker, 2015).

Sulphur dioxide arises from combustion of sulphur-containing fossil fuel (Bascon, 2008). It is a respiratory tract irritant which may result in the development of cough, wheezing and bronchial asthma (Australian Department of Environment and Energy, 2016). Particulate matter especially Pm10 and Pm2.5 contain reactive oxygen species (ROS) which can penetrate and damage the lungs or the pulmonary tree and may result in lung cancer, asthma and other forms of chronic obstructive airway diseases (Lewtas, 2007, Toren *et al* 2007, Luxham and Nieuwenhuijsen, 2019). Auto mechanics are also exposed to petrol and other petroleum-based byproducts while applying these agents directly on engines or using them to wash engine parts. A recent report revealed that inhalation of petrol is associated with impaired lung function (Meghta *et al*, 2017).

Spray paints are liquid substances made up of solvents, like styrene isocyanates, xylene and alcohol combined with inhibitors and pigments such as acrylates and methylacrylates as well as metal additives (Kopeliovich, 2020). These substances are known to have adverse effect on the respiratory system including, occupational asthma, bronchitis, painters lungs and respiratory depression (Fox, 1984, Pronk *et al* 2007, Kandyala *et al* 2010). Exposure to isocyanates for instance, or paint byproducts remain the commonest cause of occupational asthma (Meredith and McDonald 1994; Bascon, 2008).

Previous reports on lung function evaluation in auto mechanics have demonstrated that the occupation is associated with lower lung function indices with clinical correlates and determinants (Chattopayay, 2007; Krishna and George, 2017; Akintunde *et al*, 2018). Similarly spray painting or exposure to paint products has also been identified as a cause of impaired lung function among spray painters (Randolph *et al* 1997; Pronk, 2007; Aribo and Antai, 2014).

From the fore-going, it could be inferred that auto mechanics and auto body spray painters are prone to developing impairment of lung function from exposure to chemicals in their work environments. There is a possibility that an auto mechanic working in the same vicinity as a spray painter may have more serious effects from exposure to pollutants in the two work environments associated with impairment of lung function. It is a common observation to find auto mechanics operating in the vicinity of or sharing shops with spray painters. The possible synergistic or additive adverse effects of this dual exposure on the lung function of auto mechanics operating in workshops which double as spray painting shops has not been explored and hence this study.

MATERIALS AND METHODS

This was a cross-sectional study carried out among auto mechanics in Calabar metropolis a coastal city which doubles as the capital of Cross River State, Nigeria. It was conducted in September, 2018.

A total of three hundred male participants aged between 18-45 years. Mean ages of groups 1, 2 and 3 were 29.00 ± 0.24, 29.66 ± 0.29 and 29.31 ± 0.45 respectively. They were randomly selected and divided into three groups (groups 1, 2 and 3) of 100 subjects per group. Group 1 was the control and made up of students, business men and civil servants not involved in auto repair, spray painting or similar activities. Group 2 was made up of auto mechanics working in stand-alone auto repair shops while auto mechanics sharing shops with or operating in the vicinity of spray-painting shops constituted group 3. All subjects were resident in Calabar.

Anthropometric parameters (weight and height) were measured. Data about their ages, length of time spent at work, social habits and medical history were taken using interview technique. Subjects with cardiopulmonary symptoms, including bronchial asthma, pulmonary tuberculosis and thoracic deformities were excluded. Also excluded were subjects living in areas heavily polluted with dust or smoke of any nature. Informed consent was obtained from every participant involved in the study.

Assessment of lung function: Lung function indices were evaluated using a Vitalograph spirometer (Alpha Touch Vitalograph, Ireland) with build-in computer program in standard laboratory methods. The spirometer recorded several parameters of lung function. The procedure or spirometric manoeuvres were explained and demonstrated to the subjects. To carry out the procedure, each subject in standing position, was asked to breath in deeply to his full capacity, With his nose clipped, the participant was asked to put the mouth piece into his mouth in such a way as to allow the lips sealed over it and then expel the air from his lungs very fast, forcefully, sustained and completely. Three measurements were recorded for each subject. A participant's best of 3 values was selected as the participant's lung function parameter. The mouth pieces were disinfected with antiseptic solution before another participant was taken through the process. A print-out from the machine was produced which stated among other parameters, forced expiratory volume in the first second (FEV_1), forced vital capacity (FVC), forced expiratory volume in the first second expressed as a percentage of forced vital

capacity (FEV_1%) and peak expiratory flow rate (PEFR) for each subject.

Determination of Percentage Oxygen Saturation: This was done with the aid of a pulse oximeter (Medilab, India) which was clipped on the ring finger of the right hand of each subject. Pulse oximimetry measures oxygen saturation in arterial blood using the differential light absorption at different wave lengths by oxyhaemoglobin (infrared/940nm) and deoxyhaemoglobin (red light/660nm) and the fact that absorbance of light fluctuates with cardiac cycle/arterial pulsation. The relative amount of light that is absorbed at both wave lengths is used to ultimately determine the proportion of haemoglobin bound to oxygen by the oximeter (Chan *et al*, 2013).

Evaluation of Ambient Air Quality at Experimental Sites: This was carried out with the aid of CROWCOM Gasman portable gas monitor. Using this mobile air quality monitoring device, three sites each were randomly selected for control, stand-alone auto repair and auto repair shops located in the vicinity of spray-painting shop sites and analyzed. The control sites were University of Calabar, Marian market and Etim Edem motor park all in Calabar. The air quality assessment was carried out with regards to five criteria of air pollution namely Pm10, Pm2.5, SO_2, NO_2 and radiation. Particulate matter sizes (Pm10, Pm2.5) were determined with a HAZ-DUST particulate monitor (California Analytical Instruments, USA).

Ethical Approval: Ethical approval was obtained from the Cross-River State Health Research Ethics Committee, (CRSMOH/RP/REC/2018/612).

Statistical Analysis: Results were presented as mean ± standard error of the mean (SEM) and analyzed by one-way Analysis of Variance (ANOVA) followed with a post hoc test of least significant difference to assess among groups differences. A P-value of <0.05 was considered to be statistically significant.

RESULTS

Previous studies show that TPO diet has deleterious effects on several body functions with one of such studies associating the diet with male reproductive dysfunction and systemic expression of oxidative stress markers (Aribo *et al*, 2018). The results obtained from our present study are presented in figures and tables as shown below.

Anthropometric parameters: The results did not show any significant differences in the mean ages (yrs.), weights (kg) and heights (m) of the subjects in the different experimental groups as shown in Table 1.

Forced vital capacity (L) in different experimental groups: Forced vital capacity (FVC) of auto mechanics in stand-alone auto repair shops (3.33 ± 0.07) and those in auto repair + spray painting shops (3.33 ± 0.07) were significantly lower ($p< 0.05$ and $P< 0.001$ respectively) compared to control (3.75 ± 0.09). There was no significant difference in the FVC of the two groups of auto mechanics. This is shown in fig 1.

Table 1
Age and anthropometric parameters in various groups of subjects

Parameter	Control	Stand-alone shops	Auto repair+Spray painting
Age (years)	29.00 ± 0.24	29.66 ± 0.29	29.31 ± 0.45
Weight (kg)	66.01 ± 0.85	67.93 ± 0.44	67.52 ± 0.95
Height (m)	1.69 ± 0.01	1.69 ± 0.01	1.68 ± 0.01
Chest circumference (cm)	88.21 ± 0.62	89.77 ± 0.75	88.25 ± 0.63

No significant difference among groups

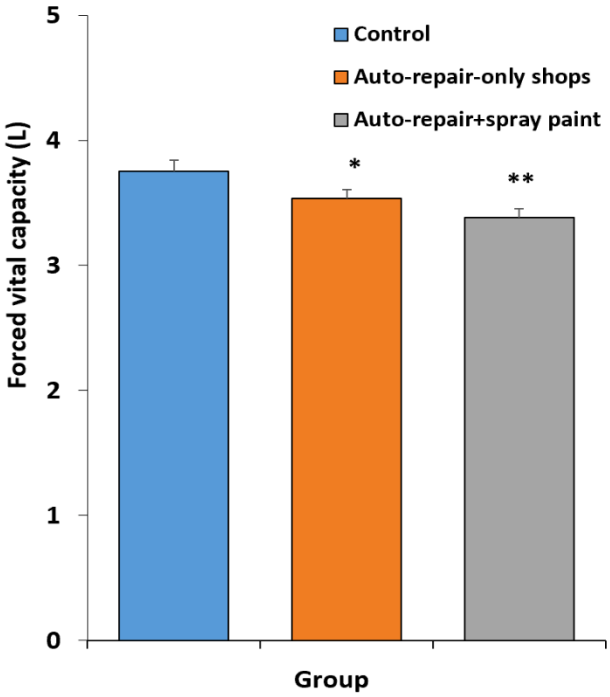

Figure 1:
Forced vital capacity in the different experimental groups.
Values are mean ±SEM, n = 100.
**p<0.05, ***p<0.001 vs control*

Forced expiratory volume in the first second (L) in the different experimental groups: Forced expiratory volume in the first second (FEV_1) was significantly lower in both mechanics in stand-alone

auto repair (1.33 ± 0.03) and mechanics in auto repair + spray painting (1.39 ± 0.06) shops; P< 0.001 and P<0.001 respectively compared with control group (1.90 ± 0.05). There were however no significant differences in this index between the two groups of auto mechanics. This is shown in Fig. 2.

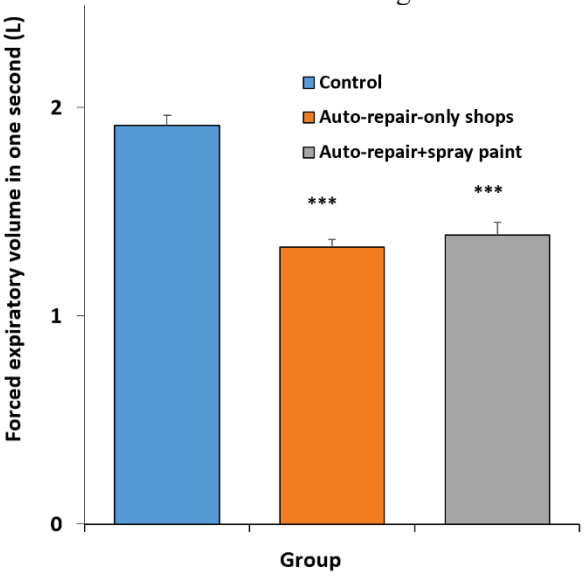

Figure 2
Forced expiratory volume in one second in the different experimental groups. Values are mean ± SEM, n = 100; ***p<0.001 vs control

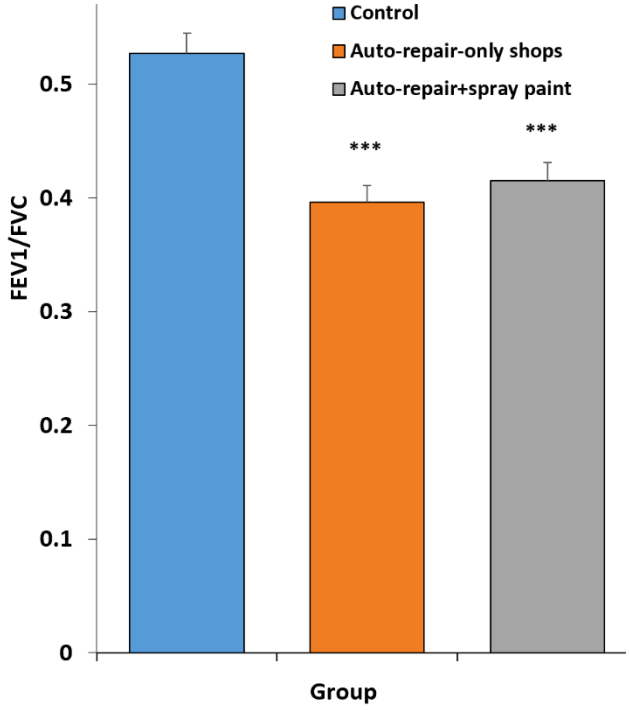

Figure 3
FEV_1/FVC in the different experimental groups. Values are mean ± SEM, n = 100.; ****p<0.001 vs control*

FEV_1/FVC (%) in different experimental groups: The FEV_1% of control, mechanics in stand-alone auto repair and mechanics in auto repair + spray painting shops were 0.53 ± 0.02, 0.30 ± 0.01 and 0.42 ± 0.02 respectively. FEV_1% were significantly lower among mechanics in stand-alone auto repair shops (P<0.001) and those in auto repair shared with spray painting shops (P<0.001) compared with control. No significant difference in FEV_1% between the two groups of auto-mechanics was observed as shown in Fig.3.

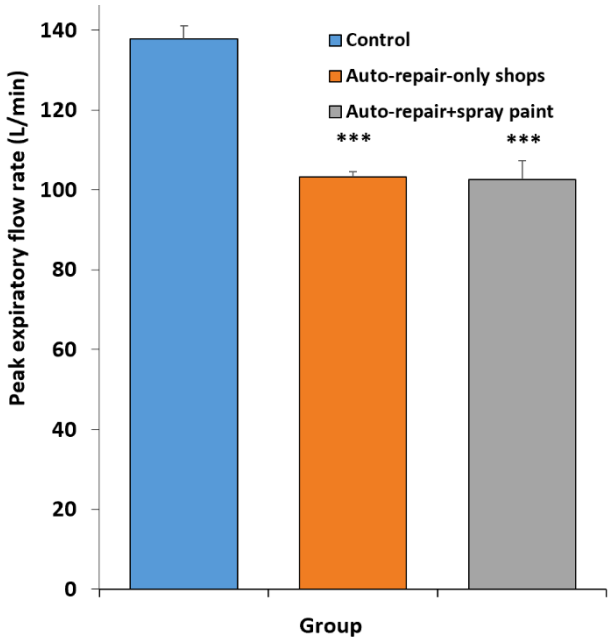

Figure 4
Peak expiratory flow rate in the different experimental groups. Values are mean ± SEM, n = 100.
***p<0.001 vs control

Peak expiratory flow rate (L/Min) in different experimental groups: Peak expiratory flow rate (PEFR) of both mechanics in stand-alone auto repair (103.3 ± 1.28) and auto repair + spray painting (102.56 ± 4.71) shops were significantly decreased (P< 0.001 and P <0.001 respectively) compared with control (137.74 ± 3.39). PEFR were similar in groups 2 and 3 as seen in fig. 4.

Percentage oxygen saturation: Percentage oxygen saturation of both mechanics in stand-alone auto repair (93.1 ± 0.41) and auto repair + spray painting (94.4 ± 0.25) shops were significantly decreased (P<0.001 and P<0.05 respectively) when compared with control (95.39±0.29). It was also significantly reduced among auto mechanics in stand-alone auto repair compared with those in auto repair + spray painting shops P<0.01). This is demonstrated in Fig. 5.

Relationship between length of time in occupation and lung function parameters in both groups of auto mechanics: There was no significant difference between lung function indices and length of time in occupation as shown in Tables 2 and 3.

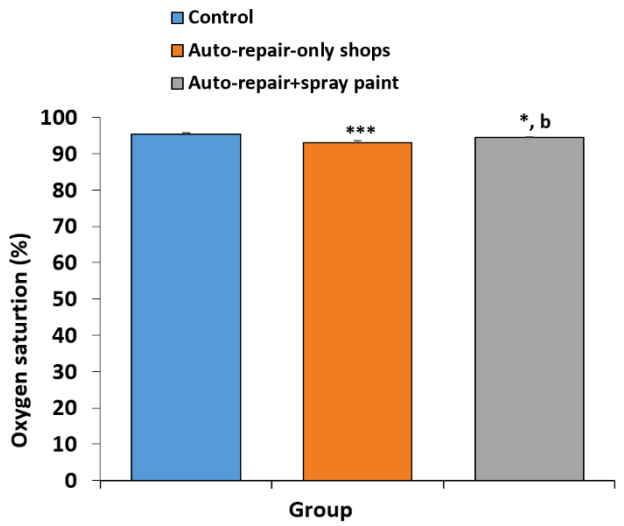

Figure 5:
Oxygen saturation in the different experimental groups. Values are mean ± SEM, n = 100.
*P<0.05, ***p<0.001 vs control; b = p<0.01 vs Auto repair-only

Table 2:
Relationship between length of time in occupation and lung function in mechanics in stand-alone shops

Parameter	r	r^2	P < value	
FVC (L)	-0.084	0.0071	0.406	$p>0.05^-$
FEV_1 (L)	- 0.180	0.0324	0.074	$p>0.05^-$
FEV_1/FVC (%)	0.184	0.0339	0.067	$p>0.05$
PEFR (L/min)	-0.096	0.0093	0.341	$p>0.05^-$
O_2 saturation	-0.062	0.0038	0.341	$p>0.05^-$
Pulse rate (Beat/Minute)	-0.115	0.0132	0.256	$p>0.05^-$

r = correlation coefficient
$p>0.05^-$ *= Non-significant negative correlation*
$p>0.05$ *= Non- significant positive correlation*

Table 3:
Relationship between length of time in occupation and lung function in mechanics in auto repair + spray painting shops

Parameter	R	r^2	P < value	
FVC (L)	-0.120	0.0144	0.234	$p>0.05^-$
FEV_1 (L)	-0.148	0.0220	0.141	$p>0.05^-$
FEV_1/FVC (%)	-0.130	0.0161	0.196	$p>0.05^-$
PEFR (L/min)	-0.116	0.0134	0.251	$p>0.05^-$
Pulse rate (Beats/Minute)	-0.041	0.0017	0.686	$p>0.05^-$

r = correlation coefficient
$p>0.05^-$ *= Non-significant negative correlation*

Air quality assessment in experimental sites: Mean particulate matter, Pm_{10} (µg/M3) in control, (70.68 ± 30.47) stand-alone auto repair (107.33 ± 15.34) and auto repair + spray painting (107.67 ± 13.72) sites did not show any significant difference between the sites (Table 3). Particulate matter, $Pm_{2.5}$ (µg/M3) in control (69 ± 29.54), auto repair- only (84.67± 4.18) and auto repair + spray painting (92.67 ± 1.45) sites were not significantly different (Table 4).

Radiation (Count per minute) in experimental sites
There was no significant differences in radiation in the control (15.1 ± 1.73), stand-alone auto repair (18.33 ± 2.03) and auto repair + spray painting (18 ± 2.08) (Table 4).

Sulphur dioxide (SO_2) in experimental sites
Concentration of SO_2 (m^3) in stand-alone auto repair (0.047 ± 0.003) and auto repair + spray painting (0.037 + 0.009) sites were significantly higher (P < 0.001 and P< 0.001 respectively) compared with control (negligible). It was however insignificantly different between the stand-alone auto repair compared with auto repair + spray painting sites as in table 4.

Nitrogen dioxide (NO_2) in different experimental sites
The concentrations of NO_2 (m^3) in stand-alone auto repair (0.02 ± 0.006) and auto repair + spray painting (0.02 ± 0.006) sites were significantly higher (P < 0.05 and P < 0.05 respectively) compared with control site (0.00) but not significantly different between the two groups of auto repair shop sites as shown in Table 4.

Table 4:
Air quality assessment, radiation and concentrations of NO_2 and SO_2 in the different experimental sites

Parameter		Control	Stand-alone shops	Auto-repair+Spray painting
Particulate matter ($\mu g/m^3$)	$Pm_{2.5}$	69.00 ±28.54	84.67 ±4.18	92.67 ±1.45
	Pm_{10}	70.68 ±30.47	107.33 ±15.34	107.67 ±13.72
NO_2 (m^3)		0.00 ±0.00	0.02 ±0.006*	0.02 ±0.006*
SO_2 (m^3)		0.00 ±0.00	0.047 ±0.003***	0.037 ± 0.009**
Radiation (cpm)		15.00 ±1.73	18.33 ±2.028	18.00 ±2.08

**p<0.0, **p<0.01, ***p<0.001 vs control*
Cpm = count per minute)

DISCUSSION

The environment in auto repair workshops is known to be polluted with a cocktail of gases and particulate matter that can be detrimental to pulmonary health. Auto mechanics are therefore at risk of inhalational injuries (Mirabelli *et al* 2012) and tend to have lower lung function indices (Akintunde *et al* 2018). Spray painting releases toxic substances in aerosols which may also affect pulmonary function and not surprisingly, is associated with a reduction in lung function indices among spray painters. (Aribo *et al*

2018). The combined effects of pollutants in auto shops alone and spray auto shops on mechanics operating in the two shops were examined in this study.

The significantly reduced FVC, FEV_1, FEV_1% and PEFR in both groups of auto mechanics compared with control is in agreement with previous studies that indicate that auto mechanics generally have a lower FVC (Krishna and George, 2017; Akintunde *et al*, 2018) confirming previous findings. Gases and particulate matter present in auto shop environments are toxic ((Toren *et al* 2007; Omidvarbana, 2014) and could have pulmonary and non-pulmonary affectation (Ayres and Roberts, 2009). The impaired lung function indices in this group of workers could be attributed to the effects of inhaled exhaust fumes, particulate matter, gasoline and other petro-chemical products on the respiratory system, which are known to be injurious to the respiratory system (Mirabelli, *et al* 2012, Jewtas, 2007, Mehta *et al* 2017). SO_2 induces inflammatory response (Wigenstam *et al*, 2016) and decreased surfactant production (Wilkins and Fettissoff, 1981) in the lungs. NO_2 causes oxidative stress and associated reduction in surfactant production (Muller *et al*, 2001). Exposure to petroleum hydrocarbon is known to induce lipid peroxidation and impair antioxidant defense system in rats which could lead to alveolitis. interstitial inflammation and bronchial necrosis (Azeez *et al*, 2012).

In spirometry, three forms of lung function defects are recognized which are restrictive, obstructive and mixed patterns of pulmonary dysfunctions (Haynes and Kaminsky, 2015). In obstructive pulmonary dysfunctions FEV_1 and FEV_1% are reduced due to increased pulmonary resistance (Kumar and Clark, 2005; Ranu *et al*, 2011), but FVC and PEFR may be normal or reduced (Ranu *et al*, 2011; Guyton and Hall, 2011). In restrictive dysfunctions there is a low FVC but FEV1% may be normal (Ranu *et al*, 2011; Ponce and Sharma, 2020). Mixed pattern of respiratory dysfunction is characterized by a normal or decreased FVC and a decrease in FEV1 % (Boros *et al*, 2003; Guyton and Hall, 2011). The decreases in FVC, FEV_1, FEV_1% and PEFR in both groups of auto mechanics therefore means that the auto mechanics have a mixed pattern of ventilatory dysfunction. The observed insignificant differences in FVC, FEV_1, FEV_1% and PEFR between mechanics in stand-alone auto repair and auto repair + spray painting shops could be as a result of the design of the auto repair workshops in Calabar which are mainly open space/shops. This allows the gases and particulate matter to be dispersed easily from the work environment reducing their concentration and consequently the inhaled doses of spray paint aerosols and exhaust fumes. Dispersion reduces concentration of gases and particulate matter and as a consequently the dose of pollutants inhaled (Cichowicz *et al*, 2017). Inhalation of exhaust fumes (which contain carbon monoxide) causes elevation of carboxyhaemoglobin (Topacoglu *et al*, 2014). Carbon monoxide reduces oxygen saturation since it binds preferentially with haemoglobin to form carboxyhaemoglobin (Blumenthal, 2001)

Our results did not show any significant differences in the concentrations of particulate matters Pm10 and Pm2.5 in the different groups of sites. Particulate matters are usually contents of exhaust fumes and aerosols from spray paints. The insignificant differences in the particulate matters so observed might be attributed to the open shops/spaces types of shops used by these artisans which allows for fast ventilation and dispersal of the particulates. Dispersal reduces the concentration of pollutants in the air (Samson, 1988; Cichowicz *et al*, 2017).

Petrol, other petroleum-based products as well as combustion of sulphur-containing compounds are major sources of SO_2 (Australian Government Department of the Environment and Energy, 2005). Inhalation of SO2 is known to impair lung function. SO_2 is readily absorbed in the respiratory system because of its high solubility in aqeous media and is converted to sulphuric and sulphureous acids. The decomposition of these acids yield their bi-sulphite and sulphide radicals which are implicated in inflammatory, epithelial damage and hyperplasia in the lungs (Wigenstam *et al*, 2016; World Health Organization Regional Office for Europe, 2006). Nitrogen dioxide is a major air pollutant (United States Environmental Protection Agency, 2016) and is used as inhibitor for acrylates used in the manufacture of spray paints. The difference in concentration of gases observed in both groups of auto repair shops was therefore the result of their emission from fuel combustion and aerosols from spray paints. Inhalation of these gases is known to impact negatively on lung function (United States Environmental Protection Agency, 2016). NO_2 is a toxic free radical gas which on inhalation initiates oxidative damage to the alveolar epithelium and causing denudation followed by proliferation and airway remodeling including fibrosis (Persinger *et al*, 2002). The oxidative damage also results in a decrease in surfactant production (Muller *et al*, 2001) which affects pulmonary function.

Percentage oxygen saturation denotes the percentage of bound oxygen to hemoglobin (Ganong, 2011). At a percentage less than 90%, hypoxia results. Adequate oxygen is supposed to be bound to hemoglobin so that the oxygen can be delivered adequately to tissues for tissue metabolism. Lower percentage oxygen saturation in the two groups of auto mechanics compared with control but which was not significantly different between the two groups of auto mechanics. A decrease in the percentage oxygen saturation can be the result of anemia and chronic

obstructive airway diseases like asthma. It can also be caused by restrictive airway diseases like pulmonary fibrosis from particulate matter or carbon monoxide toxicity (Weaver, 2009) as well as scaring (Guyton and Hall, 2011) and carbon monoxide poisoning. Exposure to exhaust fumes, petrochemical products and spray paint constituents is known to cause varying degrees of obstructive or restrictive lung diseases which may affect the percentage oxygen saturation (Australian Government Department of Environment and Energy, 2005, Bascon 2008, Lewtas, 2007). Another possible explanation for the lower percentage oxygen saturation in the two groups of auto mechanics could be the effects of inhaled carbon monoxide from exhaust fumes. Carbon monoxide has 200 times more affinity with hemoglobin than oxygen (Sembulingam and Sembulingam, 2013). It therefore displaces oxygen from hemoglobin and binds with it to form carboxyhemoglobin, a more stable complex. The percentage oxygen saturation therefore falls (Topakoglu *et al*, 2014).

Contrary to previous reports (Krishna and George, 2017, Mehta *et al*, 2017, Akintunde *et al*.,2018), our findings did not show any significant differences between lung function indices and length of time in occupation among auto mechanics. The probable explanation could be the types of shops (open spaces/ shops) where the repairs take place. Open spaces or shops are well ventilated and as a result, pollutants from the vehicles or spraying guns are quickly dispersed. Dispersion of pollutants in air reduces their concentration (Sampson, 1998; Cichowicz *et al*, 2017)) and so reducing pollutant doses (Manisalidis *et al*, 2020). This might have reduced the pulmonary effect of long term exposure to these pollutants.

In conclusion, auto mechanics in stand-alone auto repair shops and those whose shops are located within the vicinity of spray-painting shops have similar impairment in lung function as demonstrated by reductions in FEV_1, FEV_1%, FVC and PEFR compared with control. These parameters were not significantly different in the two groups of auto mechanics. Auto mechanics in stand-alone auto repair shops have a much-reduced percentage oxygen saturation than those in shops shared with spray painters.

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