Journal of Biological Sciences and Bioconservation
Volume 9, Number 2, 2017
ISSN: 2277-0143

PERCENTAGE CARBOMONOXIDE ESTIMATION AND MEASUREMENT OF MALONDIALDEHYDE, CATALASE VITAMIN E AND VITAMIN C IN RELATION TO OXIDATIVE STRESS IN EXHAUST FUME IN HUMANS.

Idoko Alexander[1*], Muhammad Gwarzo Yalwa[2], Ibrahim Usman Muhammad[3], Rita Ogochukwu Ngwu[1] and Nwali Onubuiwe Nelson[1]
[1]Department of Biochemistry, Caritas University Amorji – Nike, Enugu, Nigeria.
[2]Department of Medical Laboratory Science, Bayero University, Kano, Nigeria.
[3]Department of Biochemistry, Bayero University, Kano, Nigeria.
E-mail: idokoalexander1@gmail.com

ABSTRACT

Health threatening effects of exhaust fumes have been voluminously reported. However, the need to specifically determine the percentage estimation of Carbon monoxide (as Caroxyhemoglobin, (COHB), along with Malondialdehyde (MDA), Catalase (CAT), Vitamin E and Vitamin C becomes imperative. A total of 120 apparently healthy non smokers commercial tricyclists and non-drivers in Kano Metropolis, were used to achieve this investigation. This research was designed in phases I and II, of sixty subjects each for the investigation of possible oxidative stress. Each phase was grouped into two of thirty five and twenty five each, group I served as test control (commercial tricyclists, N = 35) and group II served as normal control (non-drivers, N = 25). A significant increase ($p<0.05$) in Malondialdehyde (MDA), Vitamin E and Vitamin C was observed in test control group compared to normal control in both phases, and exceptionally, Catalase (CAT) in phase II. In both phases, there was no statistical significant difference between the test group compared to control group in Carboxyhemoglobin (COHB), and Catalase (CAT) in phase I. Higher plasma Malondialdehyde in test group was suggestive of higher oxidative stress in the subjects. The observed increase levels of the

biomarkers of oxidative stress are strongly related to the free radicals generating potential of CO in exhaust fumes inhaled by these subjects, which also indicate the danger of exposure of exhaust fumes at any minute quantity.

Keywords: Exhaust Fumes; Carbon monoxide; Free Radicals; Oxidative Stress; Antioxidants.

INTRODUCTION

Exhaust emissions from mobile sources, stationary area sources (oil & gas production and industrial), and stationary point sources including industrial, electric utilities (electric generators), commercial and institutional sources [1], emit 60 percent of nitrogen oxides (NO), 17 percent of hydrocarbons, and close to 90 percent of total particulate emissions [1]. Exhaust fume is a complex mixture of gases and particulate matter (PM). Components include; carbon monoxide, carbon dioxide, sulphur dioxide, lead, nitrogen oxides, aldehydes including benzene and formaldehyde, hydrocarbons, polycyclic aromatic hydrocarbons (PAHs), and soot (carbon). The exhaust contains 38 components that are hazardous pollutants and is listed as a probable carcinogen by the NIOSH (National Institute for Occupational Safety & Health) [2], and IARC (International Agency for Research on Cancer) [3]. Diesel engine exhaust fumes has been classified as probably carcinogenic to humans, evidence as high risk of death from lung cancer in exposed underground mine workers [3]. In addition 80% of carbon monoxide and 40% of the nitrogen oxides are produced when gasoline and diesel fuels are burnt and Petrol creates 28 times more CO than diesel [4].The air we breathe should normally contain 21% oxygen, 78% nitrogen and 1% trace amounts of rare gases [4]. Air pollution introduces
abnormal gases like nitrogen oxides (NO_X), sulphur oxides (SO_X), carbon monoxide (CO), particulate matter (PM), and Lead (Pb) into the atmosphere at a much higher concentration than normal. Most of

these pollutants are emitted from incomplete combustion of hydrocarbons in internal combustion engines like motor cycle [5]. Carbon monoxide is one of the most common and widely distributed air pollutants in the world. It is a colourless, odourless, and tasteless gas with poor solubility in water and slightly low density than air [6]. This can aggravate Pre-existing diseases, such as emphysema, asthma, or heart disease. The two main sources of CO are the natural sources examples are; volcanic eruption, forest fire, wood burning, natural organic and inorganic decays or vegetation decay and the man-made (anthropogenic) sources examples are; exhaust of internal combustion engines, especially motor vehicles and gasoline-powered generators, various industrial processes, power plants using coal, and waste incinerators [7,8].

Bernard used the gas to poison dogs, noticing the scarlet appearance of their blood. Later, Haldane demonstrated that a high partial pressure of O_2 can counteract the interaction between Hb and CO, despite the high affinity [9]. There were a lot of cases of CO poisoning during World War II, many of which were due to the rampant use of wood as fuel [9]. In Nigeria, the most important sources are exhaust from motor vehicles, motor cycles and gasoline-powered generators; kerosene stoves, wood burning and cigarette smoke [10]. In Nigeria, CO was first identified as CAC after the formation of Federal Environmental Protection Agency (FEPA) in 1992 and since then, Nigerian Ambient Air Quality standards was set, Carbon monoxide became a major air pollutant [10]. Lipid peroxidation and oxidative damage to brain have been implicated to CO, free oxygen and nitric oxide radical production [11].Evidences for the implication of reactive oxygen species (ROS) to oxidative stress, resulting in many inflammatory disorders have been published [12,13,14].

Free radicals are chemical substances (atoms or groups of atoms) which contain one or more unpaired electrons. They are highly

reactive, capable of initiating a chain reaction in the cellular damage [15]. The participation of free radicals generation by CO in the involvement of toxicity happens to be associated with the decreased oxygen delivery sensed at the central nervous system, resulting in ventilator stimulation and further increased uptake of CO, results in elevation in Carboxyhemoglobin, and eventually respiratory alkalosis. Thus, CO is toxic because of its capacity to reduce both the oxygen-carrying capacity and the oxygen-unloading function of the hemoglobin molecule [16].

Oxidative stress occurs when generation of free radicals (i.e. substances with one or more unpaired electrons) exceed the capacity of antioxidant defense mechanisms (i.e. Pathways that provide protection against harmful effect of free radicals) [17]. Some pathophysiological conditions of oxidative stress in the body include; inflammatory diseases, heart failure, heart attack, gene mutations, chronic fatigue syndrome, fragile X syndrome, heart and blood vessels disorders, atherosclerosis etc. [18]. Chemically, oxidative stress is associated with increased production of oxidizing species or a significant decrease in the effectiveness of antioxidant defenses, such as catalase, Malondialdehyde. The effects of oxidative stress depend upon the size of these changes, with a cell being able to overcome small perturbations and regain its original state. Oxidative stress plays an important role in progression of CO-induced tissue damage during ischemic and reperfusion phases of CO-induced damage. Carbon monoxide may cause oxidative damage and may affect leukocytes, platelets and endothelium [19;20].

Antioxidants are substances that inhibit or cause delay in oxidation of a substrate while present in a little amounts. Endogenous antioxidant defences include; Non-enzymatic (uric acid, glutathione, bilirubin, thiols, albumin, and nutritional factors, including vitamins and phenols) and Enzymatic, (such as the superoxide dismutases, the

glutathione peroxidases GSHPx, and catalase). However, under normal condition, the endogenous antioxidant defenses balance the reactive oxygen species production, but for the above-mentioned 1% daily leak. The most important source of antioxidants is provided by nutrition, many belonging to the phenol family [21]. The body antioxidant defense can be approximated by measuring antioxidant plasma levels (micronutrients, enzymes, and other antioxidant), keeping in mind that the circulating compartment only reflects the flow between organs and tissues. The tissue levels of the various antioxidants remains limited to research protocols as tissue biopsies are required [21]. Vitamin C is the major water-soluble antioxidant and acts as first defence against free radicals in whole blood and plasma. It is a powerful inhibitor of lipid peroxidation and regenerates vitamin E in lipoproteins and membranes. A strong inverse association has been shown between plasma ascorbic acid and isoprostanes [22]. Vitamin E is a lipid-soluble vitamin found in cell membranes and circulating lipoproteins. It protects against oxidative damage by acting directly with a variety of oxygen radicals. Its antioxidant function is strongly supported by regeneration promoted by vitamin C [23].

STATEMENT OF THE PROBLEM

As the world population is increasing so are the activities of humans increasing. The measurement of ambient air concentrations in urban areas, have been seen to have high concentrations, and depend greatly on the density of vehicles, topography and weather conditions. In the streets, CO concentration varies greatly according to the vehicle density on the road and distance from the traffic [24]. In Nigeria, people rely on coal and biomass in the form of wood, dung and crop residues for domestic energy supply. These materials are burnt indoors in an open fires or poorly functioning stoves [25]. There is general over reliance on biomass as source of energy in the developing countries [26].In Nigeria, air pollution is greatly attributed the use of automobile, mostly motor cycle and electric

generator, as an alternative quick and easier sources, and industrial activities, have all unpredictably been increased [25].

Limitation of the Research

The scope of this research is focused on the biochemical evaluation of the effects of exhaust fumes on human, associated with oxidative stress caused by per oxidants inorganic substances, specifically CO, present in the exhaust fume, as a result of free radical generated. This research is limited only to assessing some specific bio-indicators of oxidative stress.

Approaches to the Research Problem

This research was aimed at investigating the effects of exhaust fume on apparently healthy non smoker commercial motorcyclists in Kano municipal. This was achieved by the determination of the following;

- Plasma malondialdehyde (MDA) of commercial motorcyclists
- Catalase activity in serum of commercial motorcyclists
- Vitamin C (ascorbic acid) level in serum and
- Determination of carboxyhemoglobin (COHb) in whole blood as a biomarker of carbon monoxide (CO) fraction in whole blood.

Thus, the percentage determination of Carbomonoxide as carboxyhemoobin and evaluation of its toxic effects as exhaust fumes alongside Plasma Malondialdehyde level, Catalase activity and Vitamin C levels becomes an imperative approach to measure the effects of oxidative stress.

MATERIALS and METHODS

Ethical Approval and Informed Consent

This study was conducted according to the guidelines laid down in the Medical Ethics Manual, 2009 [27].All procedures involving human subjects were approved by the Hospital management board of Kano

State ethical committee. Similarly, both written and oral informed consent from the subjects recruited for this research was sought for before blood sample was collected.

Chemicals
All chemicals unless otherwise stated were purchased from Sigma Chemical Company (St Louis, MO) USA.

Subjects
One hundred and twenty (120) apparently healthy male non smokers, aged 18-50 years, among which seventy (70) persons who are occupationally exposed to exhaust fumes were recruited and the remaining fifty (50), Non motorcyclists non smokers were used as control group in Kano metropolis

Blood Collection
Six milliliters (6ml) of blood sample was collected from each subjects by vein puncture. Two milliliters (2ml) was put in EDTA vacutainer tube, for carboxyhemoglobin determination. And the remaining 4ml was centrifuged at 300rpm for 5 minutes to obtain serum. The samples in bottles were kept in refrigerator until required.

Determination of Carboxyhaemoglobin
Carboxyhaemoglobin (COHb) was determined as an indicator of carbon monoxide (CO) in the blood. This was assayed by spectrophotometric analysis as described by Canfield [28], Doglas [29], Ernest and Carol [30].

Measurement of Plasma Malondialdehyde
Plasma malondialdehyde was measured by the method of Ohakawa [31], with absorbance at 532nm caused Lipid peroxidation, which generates peroxide intermediates which upon cleavage releases MDA, a product which reacts with thiobarbitutic acid (TBA).

Measurement of Catalase

The method described by Goth [32], was used by measuring the UV absorbance change of H_2O_2 at 405nm against a blank.

Vitamin C measurement

Serum vitamin C was assayed by the method of Roe and Kuether [33], in absorbance at 540nm. It generally takes advantage of its reducing ability (ease of oxidation), to form keto product.

Statistical Analysis of Data

Standard error of mean (SEM) from the mean standard deviation were obtained for all data collected during analysis and experiment. Differences between the groups were analyzed statistically, using one – way analysis of variance (ANOVA) and t-test statistical analysis. Instat statistical software was used for the analysis. A confidence level of 95% ($p < 0.05$) was considered significant. Results are mean± standard deviation.

RESULTS

Table 1 present the percentage estimations of Carboxyhemoglobin (COHb) and measurement of Catalase (CAT), Malondialdehyde (MDA) and Vitamin C in phase I. A significant ($P < 0.05$) increase in MDA and Vitamin C was observed in test group compared to control, but no significant ($P < 0.05$) increase was observed in test group and control of COHb and CAT.

Table 1: percentage Estimation of COHb, and Measurement of CAT, MDA and Vitamin C in Phase I.

Phase1	COHb (%)	Catalase (Ku/L)	MDA (µM)	Vitamin C (mg/dL)
Test n=35	0.46±0.36	9.40±9.25	12.98±5.16[a]	2.18±1.20[b]
Control n=25	0.55±0.36	9.48±7.81	6.25±4.59[a]	1.52±0.85[b]

Results are mean ± standard deviation, (n= 35, test group; n= 25 control group). Values bearing superscripts in the same column are significantly different ($p<0.05$). Key: COHb: Carboxyhemoglobin, MDA: Malondialdehyde.

Table 2 shows the values of COHb, CAT, MDA and Vitamin E in phase II. There was a significant ($P <0.05$) increase in MDA and Vitamin E in test group compared to control, but no significant ($P <0.05$) increase was observed in test group and control of COHb.

Table 2: Percentage measurement of COHb, and determination of CAT, MDA and Vitamin E in phase 2.

Phase 2	COHb (%)	Catalase (Ku/L)	MDA (µM)	Vitamin E (µg/L)
Test n =35	0.58±0.42	10.13±8.11[a]	12.90±4.69[b]	31.93±8.82[c]
Control n =25	0.48±0.41	6.95±6.55[a]	5.25±4.16[b]	24.11±7.97[c]

Results are mean ± standard deviation, (n= 35, test group; n= 25 control group). Values bearing superscripts in the same column are significantly different ($p<0.05$). Key: COHb: Carboxyhemoglobin, MDA: Malondialdehyde CAT: Catalase.

Table 3 presents values from combination of phase I and II COHb, CAT, MDA, Vitamin C and Vitamin E. A significant (P <0.05) increase in MDA, Vitamin E and Vitamin C was observed in test group compared to control, but no significant (P <0.05) increase was observed in test group and control of COHb and CAT.

Table 3: Values from combination of phase I and II COHb, CAT, MDA, Vitamin C and Vitamin E

PI & PII Combined	COHb (%)	Catalase (Ku/L)	MDA (μM)	Vitamin C (mg/dL)	Vitamin E (μg/L)
Test n = 70	0.52±0.39	9.97±8.97	12.92±4.89[a]	2.02±0.99[b]	32.05±10.66[c]
Control n = 50	0.49±0.35	8.22±7.23	5.88±4.91[a]	1.35±0.84[b]	21.42±8.55[c]

Results are mean ± standard deviation, (n= 70, test group; n= 50, control group). Values bearing superscripts in the same column are significantly different (p<0.05). Key: COHb: Carboxyhemoglobin, MDA: Malondialdehyde, CAT: Catalase, PI: Phase I, PII: Phase II.

DISCUSSION

Tables 1, 2 and 3, shows the non statistical significance difference of the COHb experimental and control group, with the value of experimental slightly high, shows that the subjects in test group were exposed to fume from the aforementioned sources, whose number is high [34] and coupled with the activity (occupation) of the subjects, as they inhale fumes (out door source) from all road users, especially in a traffic jam [35]. On the other hand, the control group, who are in rural area (villagers), whose occupation is farming, inhale carbon monoxide from the occupational activities of bush burning. Burning of wood, corn cobs and stems of dried maize (indoor source) as sources of energy to cook their food, in the process may as well have been the sources of increase exposure to CO [36]. The

indoor sources mentioned here tend to release more carbon monoxide, due to the proximity of diffusion of the gas to subjects, hence the non-significant difference between the experimental and control [37]. It was reported that, it is probably sufficient to check the COHb content of blood from one or two nonsmokers. If values between 0.5 and 2% are obtained, then the instrument to be used is adequate for clinical purposes. This is true since values obtained in this study, are within this range [30]. The half life of COHb is only 4 to 6 hours, and COHb normal level measured for weeks and months, following a single acute CO exposure is 2% for non – smokers and 10% for smokers [38]. It has been pointed out that living cells can tolerate CO in the concentration range of 0.01% (100 ppm) for several hours [39].

However, while a high COHb level confirms significant exposure to one or more sources of CO, a normal value cannot rule out chronic low-level exposure. Because chronic low-level CO poisoning impairs oxygenation of tissue, any organ may be affected, with the brain, heart and lungs being most sensitive to the effects of CO [40]. Hemoglobin ($Hbfe^{2+}$) is oxidized to methemoglobin ($metHbfe^{3+}$) in the living organism by the action of CO free radical, H_2O_2 and drug, (Fig.1). The presence of fe^{3+} makes metHb unstable when it binds with O_2 and it can be converted back to hemoglobin ($Hbfe^{2+}$) by the enzyme catalyzed activity of methemoglobin reductase [41].

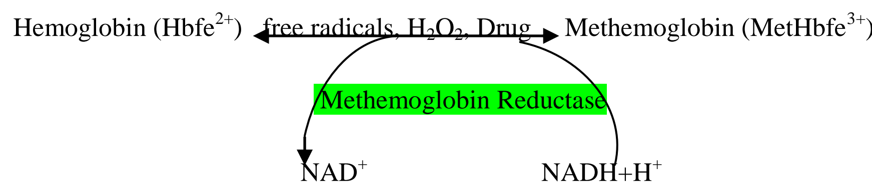

Figure 1: Reversible conversion of hemoglobin to methemoglobin

In equation 1, the free $Hbfe^{2+}$ combines with O_2 from the inhaled air to form oxyhemoglobin ($oxyHbfe^{2+}$) in the erythrocytes. This makes oxygen available and transported to tissues [41].

$$Hb_{(aq)} + 4O_{2(g)} \longrightarrow Hb(O_2)_{4(aq)} \quad \ldots\ldots\ldots\ldots\ldots (I)$$

The high affinity (200 times more) of CO than O_2 for binding with Hb, makes CO to displace O_2 to form carboxyhemoglobin HbCO (Eqn. 2) in a similar reaction as above, thus toxicity of CO is impacted. However, the therapeutic use of pure O_2 to a person with CO poison reverses the effects of HbCO in the blood by converting it to oxygenated blood and the CO (Eqn. 3) is dissipated via exhaled air, where it is converted to carbon dioxide CO_2 (Eqn.4) in the lungs [41].

$$Hb_{(aq)} + 4CO_{(g)} \longrightarrow Hb(CO)_{4(aq)} \quad \ldots\ldots\ldots\ldots\ldots (II)$$

$$Hb(CO)_{4(aq)} + 4O_{2(g)} \longrightarrow Hb(O_2)_{4(aq)} + 4CO_{(g)} \ldots\ldots\ldots (III)$$

$$4CO_{(g)} + 2O_{2(g)} \longrightarrow 4CO_{2(g)} \quad \ldots\ldots\ldots\ldots\ldots\ldots (IV)$$

In the lungs, when the pH is low (increasing H^+ concentration), the affinity of oxygen to hemoglobin decreases, or an increase partial pressure of CO_2 (pCO_2). This effect is known as Bohr effect, and causes the oxygen dissociation curve to shift to the right. This therefore, releases O_2 from the oxyhemoglobin complex to the tissue (Fig. 2). Although, most common symptoms of chronic CO poisoning are actually the same as those of acute poisoning, except that they may vary considerably over time as they wax and wane in response to not just exogenous CO exposures but also in response to any chronically stressful stimuli, since all such stimuli induce hemeoxygenase (HO-1) to breakdown heme proteins into CO [42]. Typical levels of COHb in healthy nonsmokers have been shown to be <2%. The results of this study suggest that these highly reactive entities (free radicals), might not have been generated in increased amounts in the subjects studied since carboxyhemoglobin, a biomarker of carbon monoxide was not increased in test subjects [6]. And the various duration of exposure to exhaust fumes,

irrespective of age does not raise the level of carboxyhemoglobin as it could be attributed to life style such as eating habit, inhalation of oxygen which reverses the effects [43,44], observation of break, which may allow inhaled fumes to wax and wane, due to oxygen circulation [44,45,46]. The measured % of COHb indicated low level of CO and as such an associated decrease in vitamin C and catalase activity [47], and that the duration of exposure to the fume irrespective of the age of the subjects, might not have been enough for any significant effects [43].

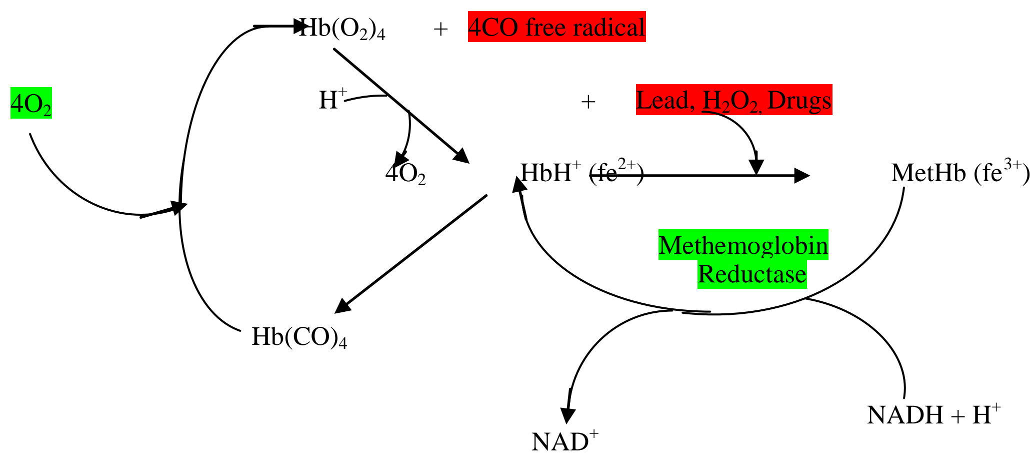

Figure 2: Conversion of HbCO to HbO_2 through the mediation of O_2 (MetHb – Methemoglobin; Hb – Hemoglobin; HbO_2 – Oxyhemoglobin)

The end product of lipid peroxidation has been extensively attributed to Malondialdehyde (MDA), as a good marker of free radical mediated damage and oxidative stress [48]. There was a significant difference in malondialdehyde test and control group, as a result of the exhaust fumes inhaled. As a result of exposure to exhaust fumes (Table1, 2 and 3), the subjects in the two phases showed raised level of plasma malondialdehyde, as this may be implicated by free radical agents of toxicity [47]. This probably reflects the increase in lipid oxidation due to either increased production of free oxidative radicals or decreased antioxidant defense mechanisms, or both, as a result of residence free radical generating agents in the body [47, 49].

As a result of inhaled exhaust fume, MDA content increases even with values of COHb as small as 0.5%, thus, indicating a concentration-dependent free radical generation [38]. The level of MDA in the tissue is considered a measure of lipid peroxidation status (Table 1). The exhaust fumes level, though low as compared to acute exposure (smokers), was able to raise the level of malondialdehyde (MDA), as seen by a corresponding slight increased level of Catalase (Table 2), which showed that lipid peroxidation took place, as a result of the free radicals generated. The Level of the MDA would have been much higher among the test group if the two groups have the same level of vitamin intake [50].
The slightly raised levels of Catalase between experimental and control group (Table 2), seems to confirm the result of a research on catalase of its instability and slowest of antioxidants enzyme to respond to an increased level of free radical in blood, a research conducted between smokers (test group) and non smokers (control group) that values (catalase activity) of smokers returned as those of control subjects [51].

Values of vitamin E in this study are significantly high and (Tables 1, 2 and 3), as antioxidants in the test compared with control group. This may be due to the fact that the test subjects (Tricyclists), by virtue of their occupation and earnings, are able to afford food rich in antioxidants (Vitamin E). The per capita income of Nigeria is $2,722 [52]. Nakamura et al., [53], mention some Food sources rich in vitamin E to include; nuts and seeds, spinach, pumpkin seeds, bell pepper, pine nuts, asparagus, avocados, tomatoes, vegetable oils, papaya, butter, cereals and peanuts. The high values of vitamin E could also be connected to the quality of food and manner of eating [54]. Antioxidants activities have been reported to be enhanced by the effects of high values of vitamin E, and thus, reducing the level of free radicals and their damages to tissues. [54]. Increased values

of Vitamin E (Phase II, Table 2) could explain why symptoms of lipid peroxidation may not be seen obviously in these subjects, despite their occupational exposure to exhaust fume, thus, exerting its protective effects [55,56].

This study reveals low levels of vitamin C, however, slightly significantly high in Tricyclists compared with control (Table 3). It has been reported that the major role of Vitamin C, however little, is to neutralize free radicals, since it is in a unique position to scavenge aqueous peroxyl radicals before these destructive substances have a chance to damage the lipids [57], meanwhile, reduced levels of vitamin C in this study is an indication of level of oxidative stress [58]. It can function with vitamin E, a fat-soluble antioxidant, and the enzyme glutathione peroxidase in order to stop free radical chain reactions [59]. Thus, though malondialdehyde (MDA) values are high, which shows lipid peroxidation, the corresponding increase in values of Vitamin E in the test (occupational workers) in this study may suggest higher intake of food rich in antioxidants (vitamin E) among the subjects due to a better income generated by the occupational activity than the control [53,60].

ACKNOWLEDGEMENT
All acknowledgements go to God almighty, the one who made of all things. Appreciations to everyone who made this work a possibility.

CONCLUSION
This study reveals the harmful health effects of occupational exposure to exhaust fumes on human, after the evaluation of COHb as harmful parameter of CO, and biomarkers of oxidative stress, such as plasma Malondialdehyde, Catalase activity, Vitamin E and C. The percentage of carbon monoxide was assayed as carboxyhemoglobin (COHb) in the blood. From the results obtained, the significant differences observed in the serum mean of the

various parameters evaluated between the test group and control group, revealed a significant level of free radical and peroxidation, as indicated by the rise in the level of Plasma Malondialdehyde (MDA) and decrease in Catalase activity couple with increase in the levels of non- enzymatic antioxidants (Vitamin E) in both phases.

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Reference to this paper should be made as follows: Idoko Alexander, et al. (2017), Percentage Carbomonoxide Estimation and Measurement of Malondialdehyde, Catalase Vitamin E and Vitamin C in Relation to Oxidative Stress in Exhaust Fume in Humans. *J. of Biological Science and Bioconservation,* Vol. 9, No. 2, Pp. 1-24