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Assessment of Some Oxidative Stress Biomarkers in Automobile Mechanics in Selected Local Government Areas in Ibadan, Oyo State, Nigeria

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Abstract: **The adverse health effects associated with occupational exposure to toxic heavy metals is a serious global concern and has been associated with oxidative stress. This study assessed total antioxidant status (TAS) and some oxidative stress markers among automobile mechanics in Ibadan North and Oluyole Local Government areas of Ibadan. Thirty male participants (25-60 years) with at least five years working experience as automechanic were recruited as exposed participants. 30 apparently healthy volunteers were also recruited as controls. The ethical approval was obtained from the University College Hospital (UCH) ethical Committee. Ten millimeters of venous blood was collected from each participant into heparinized and plain bottles. The concentrations of vitamins A, C, E, TAS, hydrogen peroxide (H_2O_2) and Thiobarbituric acid reactive substances (TBARS) were determined in the plasma and serum obtained. Vitamins A, C and E were analysed using High Performance Liquid Chromatography (HPLC) while TAS, H_2O_2 and TBARS were analysed using standard procedures. Statistical analysis was done using Independent T-test and Pearson's correlation at $p<0.05$. Significantly higher concentration of H_2O_2 was observed in the exposed. The concentration of H_2O_2 was significantly higher in auto mechanics that have worked for more than 10 years relative to those who have worked for less years. TAS concentration was significantly lower in the exposed when compared with the controls. Vitamins A, C and E levels were lower among the exposed participants. This study shows that exposure of mechanics to toxicants resulted in oxidative stress which increased with duration of exposure.**

Keywords: **Automechanics, Free radicals, Occupational exposure, Oxidative stress, Total antioxidant status, Vitamins.**

I. INTRODUCTION

More than ever before the pivotal role of oxidative stress in the etiology and progression of several pathologies is becoming evident. Oxidative stress is a cellular condition or phenomenon which occurs as a result of imbalance between free radicals (reactive species) and the antioxidant defense system of the body (Poljsak *et al*., 2013). Under normal physiological condition, there is a homeostasis between the level of the body antioxidant molecules (enzymatic or non-enzymatic) and the generation of free radicals. A disturbance in the equilibrium status of free radicals/ antioxidants is caused by either an overproduction of reactive species or low levels of antioxidants in living organisms (Ighodaro and Akinloye, 2017; Tiwari *et al*., 2010; Krajeir *et al*., 2008). The levels of free radicals are minimized by the action of the

body's antioxidant defense mechanisms which delays, prevents or removes oxidative damage to a target molecule (Bolajoko *et al*., 2017). Sources of antioxidants include minerals and vitamins which are required by the body in small quantities while reactive species comprise of free radicals and other non-free radical molecules that are made up of oxygen such as hydrogen peroxide (H_2O_2), singlet oxygen (1/2 O_2·), hydroxyl radical (·OH) and superoxide ($O2^{-}$) (Granados-Silvestre *et al*., 2014).

Increased generation of free radicals is promoted by environmental and occupational exposure to certain toxic chemicals containing carbon monoxide, benzene and heavy metals such as cadmium (Cd), Lead (Pb), Arsenic (As) and mercury (Hg) (Arinola and Akinbiinu, 2006). Workers around the world are daily facing global health challenges due to occupational exposure to these hazardous chemicals (Oche *et al*., 2020). This exposure is frequent among professions such as automechanics which are trained personnels or technicians involved in the repair, maintenance and diagnostic testing of automobiles such as cars and light trucks (Saliu *et al*., 2015; Anetor *et al*., 2009). They are often found in groups of painters, panel beaters, vehicle electrical repairer (termed "rewire"), welders and mechanical parts repairer in open or enclosed spaces in Nigeria. Exposure among automobile mechanics to hazardous toxicants in their workplaces occurs through dermal contact, inhalation and accidental ingestion (Prüss-Ustün *et al*., 2011).

Many chemicals used in the manufacture of automobile batteries and paints, brake fluids, lubricants, detergents, radiator coolants, degreasers, paints removers, metal cleaners, antiknock agents and gasoline have been demonstrated to contain hazardous substances that are capable of eliciting a number of biochemical responses (Saliu *et al*., 2015). They cause damage to cellular components such as DNA, lipids, enzymes and other structural protein molecules through lipid peroxidation thereby inhibiting their normal physiologic functioning (Kamal *et al*., 2011). The oxidative degradation of lipids, termed lipid peroxidation, which results from excessive reactions of free radicals with polyunsaturated fatty acids in cell membranes and have been implicated in several diseases such as diabetes (Okuonghae *et al*., 2015), chronic kidney disease (Reyes *et al*., 2013), cardiovascular disease (Rysz *et al*., 2020), cancer (Valavanidis *et al*., 2009), and neurodegenerative diseases (Mendez-Armenta *et al*., 2014). The importance of Total Antioxidant Status (TAS) in protecting man against free radical-induced pathologies cannot be over emphasized. A compromise or decline in the status (TAS) undoubtedly makes human highly susceptible to disease conditions initiated by reactive species or free radicals. Hence, it is important to assess the free radical generating capacity of chemicals or substances that individuals are occupationally exposed to. Exposure to chemicals in occupational environments and associated disorders has not received significant attention (Anetor *et al*., 2008). This study therefore sought to assess antioxidant status and certain biomarkers of oxidative stress among auto-mechanics and compare the parameters with the length of work exposure.

II. MATERIALS AND METHODS

A. Subjects

This is a case-control study carried out on 30 apparently healthy male automobile mechanics from different mechanic communities within Oluyole and Ibadan North Local Government areas of Ibadan, Oyo state Nigeria. They were between the ages of 25 and 60 years with at least 5 years working experience. The controls (30) were apparently healthy volunteers selected from among the staff and students of University College Hospital (UCH), Ibadan. Participants were recruited only after obtaining their consent. Ethical approval was obtained from the joint University of Ibadan/University College Hospital Institutional Review Committee. Questionnaire was used to obtain all necessary information for the study. Participants with known chronic illness such as diabetes mellitus, hypertension, kidney failure as well as cigarette smokers were excluded from the study. Those on regular use of analgesic, alcohol, herbal concoction, multivitamin supplements were also excluded. Each participant's height in meters and weight to the nearest kilogram were measured. Body mass index (BMI) was calculated as weight (kg)/height^2 (m^2).

B. Collection of Blood and Storage of Sample

Ten (10) milliliters of venous blood was aseptically collected from each participant into heparinized and plain sample bottles and centrifuged at 3000 revolution per minute (rpm) for 10 minutes at room temperature to obtain the plasma and serum respectively. The plasma and serum obtained were stored at about (-20ºC) prior to analysis. Hydrogen peroxide (H_2O_2), Thiobarbituric acids reactive substances (TBARS), were measured in serum samples, Total antioxidant status (TAS), Vitamin A, C and E were determined in plasma samples. All chemical reagents for analysis were purchased from Sigma Aldrich (Germany).

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C. Analytical Methods

1. Hydrogen peroxide (H_2O_2) determination

Hydrogen peroxide was estimated based on the coupled oxidation method described by Wolff, (1994). Peroxidase combines with hydrogen peroxide as it is liberated; the complex thus formed then brings about an oxidation of substances such as nitrite, ethanol, cytochrome C or manganese ions in the presence of P-cresol. The extent of the oxidation is measured spectrophotometrically at a wavelength of 560nm.

TABLE 1: THE STANDARD PROCEDURE FOR HYDROGEN PEROXIDE ASSAY

Reagents	**Volume**
Buffer	2.5ml
Ammonium ferrous sulphate	250µl
Sorbitol	100µl
Xylenol orange	100µl
Sulphuric acid (H_2SO_4)	25µl
Sample	50µl

The mixture was incubated at room temperature for 30minutes and the absorbance read in a spectrophotometer at 560nm. Hydrogen concentration (µmole) was extrapolated from standard curve of Absorbance (560nm) against concentration (µmole).

2. Determination of Lipid Peroxidation using Thiobarbituric Acid (TBARS) as an Index

Level of Thiobarbituric acid reactive substances was determined according to the method described by Ohkawa *et al*, 1979 and modified by Carregosa *et al*, 2014. It is based on the principle of auto-oxidation of unsaturated fatty acids which involves formation of semi-stable peroxides which then undergo a series of reactions to form malondialdehyde (MDA). Malondialdehyde reacts with thiobarbituric acid (TBA) to form a pink-coloured chromogen, the absorbance of which is read at 535nm using a spectrophotometer.

TBA was pipetted into test tube followed by 500µL of 20% trichloroacetic acid (TCA), 100µL of plasma was then added. The mixture was incubated at 100^0C for 20 minutes and centrifuged at 12,000rpm for 5minutes. The absorbance of the supernatant was read at 535nm. TBARS was determined by using a molar extinction coefficient of 1.56 X $10^5 m^{-1}cm^{-1}$ and the values expressed as nanometer (nm).

3. Determination of Total Antioxidant Status (TAS)

Plasma total antioxidant status (TAS) was determined by a method described by Koracevic *et al*., 2001. A standardized solution of Fe-EDTA complex reacts with hydrogen peroxide by a Fenton-type reaction leading to the formation of hydroxyl radicals (·OH). These reactive oxygen species degrade benzoate, resulting in the release of TBARS. Antioxidants from the added human fluid cause a suppression of the production of TBARS. This reaction is measured spectrophotometrically at 532nm and the rate of inhibition of color is proportional to the concentration of antioxidant status.

TABLE 2: THE STANDARD PROCEDURE FOR TOTAL ANTIOXIDANT STATUS ASSAY

	A_i	**A_0**	**K_i**	**K_0**	**UA_i**	**UA_0**
Sample	0.01	0.01	-	-	-	-
Uric acid	-	-	-	-	0.01	0.01
Buffer	0.49	0.49	0.50	0.50	0.49	0.49
Na-benzoate	0.50	0.50	0.50	0.50	0.50	0.50
Acetic acid	-	1.00	-	1.00	-	1.00
Fe-EDTA	0.20	0.20	0.20	0.20	0.20	0.20
H_20_2	0.20	0.20	0.20	0.20	0.20	0.20

The mixture was mixed properly and incubated for 60 minutes at 37^0C and the procedure continued as follows:

Acetic acid	1.00	-	1.00	-	1.00	-
TBA	1.00	1.00	1.00	1.00	1.00	1.00

The mixture was again incubated for 10 minutes in a boiling water bath then cooled in an ice bath. Absorbance was then read at 532nm using deionized water as blank.

Calculation:

Total antioxidant status (nmol/litre) = $\frac{(C_{UA})\ (K-A)}{(K-U_A)}$

Where: K = Absorbance of control (K_i-K_0)

A= Absorbance of sample (A_i-A_0)

UA= Absorbance of uric acid solution (UA_i-UA_0)

C_{UA}= Concentration of Uric acid (mmol/L)

4. Determination of Vitamin A, C and E

Vitamin A, C and E were determined using Waters 616/626c system of High-Performance Liquid Chromatography (HPLC) as described by Bates, (1997). The working standards for each vitamin was prepared using the formulae

$C_1V_1 = C_2V_2$

i. Vitamin A analysis

0.125µL of each of the plasma sample was pipetted into clean test tubes and made up to 500µL with distilled water. 10g/L of ascorbic acid was then added and shaken for 15 minutes followed by sonication for 5 minutes. O.5g/L of triton x 100 was added as detergent, after which 400µL of acetonitrite was added and mixed thoroughly. 400µL of n-hexane which contains butylated hydroxyltoluene (BHT) was also added and the mixture vigorously shaken for 4 minutes and centrifuged for 2 minutes at 8000rpm. The supernatant was collected and injected into the High-performance Liquid Chromatography (HPLC) with a flow rate fixed at 1.5ml/minute and measured at a wavelength of 325nm

ii. Vitamin C analysis

25ml of plasma sample was pipetted into 250ml volumetric flasks and 50ml distilled water was added and whirled gently. 20ml of 20% metaphosphoric acid was added and the mixture shaken gently. 2.5ml of 0.5% oxalic acid was then added and carefully shaken. 25ml acetone was also pipetted into the mixture and made up to 250ml with distilled water. The mixture was shaken on a mechanical shaker for 30 minutes and then centrifuged for 20 minutes at 5000rpm. The supernatants were transferred into vials for analysis on a HPLC machine. 100ppm stock of ascorbic acid was prepared from1000ppm reference standard using

$C_1V_2 = C_2V_2$

Giving 25ml of 1000ppm reference standard pipetted into 250ml volumetric flask and made to volume with a mixture of distilled water + oxalic acid + acetone in a ratio of 2:1:1. Working standards of 0.0, 0.25, 0.50, 0.75, 1.00ppm were prepared from the 1000ppm stock standard.

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iii. Vitamin E analysis

0.25ml of each sample was pipetted into a 100ml volumetric flask and 20ml of distilled water was added followed by 25ml of benzene solution and shaken properly. A solution of dehydrated ethanol: diethyl ether: HCl in the ratio 4:3:1 was prepared. 25ml of the extraction mixture was added into the sample solution and mixed properly. Each of the whole set up was vigorously shaken for 20 minutes and centrifuged for 20 minutes at 5000rpm. The supernatant was transferred to a set of vials and analysed using HPLC equipment. Vitamin E stock standard of 0.0, 1.0, 2.0, 3.0, 4.0 ppm were prepared from 100ppm stock standard.

III. STATISTICAL ANALYSIS

Results were analysed using the Independent T test and Chi square. Comparison was determined from the mean±standard deviation. Correlation was done using the Pearson's product moment correlation coefficient. Differences between values in exposed groups and controls were accepted as significant at 5% confidence level ($p<0.05$).

IV. RESULTS

Table 4 shows H_2O_2 (µmoles) was significantly higher in the exposed participants (26.12 ± 14.15) than in the controls (14.45± 5.06). There was no significant difference in the concentration of TBARS between the exposed and controls. A significant difference was observed in the TAS level among automechanics that have worked between 6 - 9years (13.445± 3.647) and those who have worked for more than 10 years (28.064± 14.183) $p<0.05$.

Vitamins A (µg/dl), C (mg/dl), and E (ng/dl) were all significantly lower among the automechanics (78.58±13.02; 0.81±0.13; 0.59±0.19) when compared with the controls (101.80±10.72; 0.96±0.19; 0.74±0.19) respectively.

TABLE 3: DEMOGRAPHIC CHARACTERISTICS, SOCIAL HABITS AND DIETARY HISTORY OF STUDY RESPONDENTS.

Variable	Response	Exposed n (%)	Controls n (%)	X^2	p
Demography					
Marital status	Single	2(6.7)	17(56.7)	17.041	0.000*
	Married	26(93.3)	13(43.3)		
Ethnic group	Yoruba	29(96.7)	25(83.3)	2.762	0.097
	Igbo	0(0)	3(10.0)		
	Others	1(3.3)	2(6.7)		
Educational status	Primary	11(36.7)	0(0)	36.999	0.000*
	Junior Sec.	3(10.0)	0(0)		
	Senior Sec	16(53.3)	7(23.3)		
	Graduate	0(0)	16(53.3)		
	Postgraduate	0(0)	7(23.3)		
Dietary History					
Vegetables and fruits	Daily	9(30.0)	5(16.7)	0.086	0.770
	Weekly	8(26.7)	17(56.7)		
	Occasionally	13(43.3)	8(26.7)		
Analgesic	Occasionally	21(70.0)	15(55.6)	1.252	0.263
	No				

***significant at $p<0.05$, X^2 = Chi square, p = probability value**

TABLE 4: COMPARISON OF BIOCHEMICAL PARAMETERS IN EXPOSED PARTICIPANTS AND CONTROLS

Variables	Exposed (n=30)	Controls (n=30)	t	p
Oxidative stress Parameters				
H_2O_2 (µmoles)	26.12±14.15	14.45±5.06	4.252	0.000*
TBARS (nM)	0.23±0.20	0.16±0.14	1.574	0.121
Antioxidants parameters				
TAS (mmol/L)	0.81±0.26	0.41±0.26	7.089	0.000*
Vitamin A(µg/dl)	78.58±13.02	101.80±10.72	-7.089	0.000*
Vitamin C(mg/dl)	0.81±0.13	0.96±0.19	-5.412	0.000*
Vitamin E(ng/dl)	0.59±0.19	0.74±0.19	-3.173	0.002*

H_2O_2 - Hydrogen peroxide; TBARS – Thiobarbituric acid reactive substances; TAS – Total antioxidant status; * - significant at $p<0.05$

TABLE 5: COMPARISON OF THE MEAN VALUES OF BIOCHEMICAL MEASUREMENTS IN THE EXPOSED PARTICIPANTS IN TERMS OF DURATION OF EXPOSURE

Variables	6 – 9 years (n=4)	≥ 10years (n=26)	t	p
H_2O_2 (µmoles)	13.45±3.65	28.06±14.18	-2.121	0.049*
TBARS (nM)	0.37±0.33	0.21±0.18	1.50	0.145
TAS (mmol/L)	1.13±0.48	0.80±0.19	2.92	0.007*
Vitamin A (µg/dl)	87.01±16.57	77.29±12.27	1.42	0.168
Vitamin C (mg/dl)	0.86±0.16	0.80±0.13	0.81	0.425
Vitamin E (ng/dl)	0.66±0.23	0.58±0.18	0.821	0.419

***significant at $p < 0.05$.

V. DISCUSSION

Exposure to heavy metals at work places is becoming a major health concern due to their implication in a number of diseases. Similarly, oxidative stress is increasingly being recognized as a possible mechanism in the pathogenesis of various heavy metal-associated diseases (Bhattacharya *et al*, 2014; Anetor *et al*., 2009). Increased oxidative stress is a major paradigm in the pathophysiology of different disease states. The utility of oxidative stress biomarkers in prognostication and guidance of individualized treatment is driven by their potential to act as an indicator, reflecting the total health status of the individual (Ho *et al*., 2013). The effect of oxidative stress on the total antioxidant status is important in determining the health status of an individual and it is considered also as an indicator of risk from diseases associated with ROS generation.

In this study, anthropometric and demographic characteristics of the participants were evaluated and compared with controls. 53.3% of automobile mechanics recruited had secondary school leaving certificate, 10.0% had junior secondary education while 36.7% had only primary education. This is significantly different from the controls where 53.3% had first degree education, 23.3% had postgraduate certification and 23.3% had senior secondary education. This showed that majority of workers in the automobile repair profession has limited educational exposure. Limited educational exposure and reduced access to information could be responsible for poor personal hygiene and working conditions among these workers. The habit of eating with bare hands and washing of hands with gasoline is prominent among them as well as sucking of petrol through rubber hoses from gallons and car fuel tanks. Meals are carelessly placed, served and eaten in their workshops in the midst of heavy automobile pollution during working hours. These habits may increase their exposures to toxicants through inhalation and dermal contact. In a study by Saliu *et al*., 2015, they also observed that automechanics have poor personal hygiene and contaminated work environments. They also reported that gasoline, which auto mechanics are daily exposed to, is rich in tetraethyl lead which can escape through the mucosa and thus increase

blood levels of lead and other heavy metals. Bioaccumulation of heavy metals can generate the excessive production of free radicals which subsequently overwhelm the body's antioxidant status.

Hydrogen peroxide is an important intermediate of endogenous free radical activity which is toxic to cells when produced in excess and could lead to the generation of more potent free radicals that cause macromolecular damage (Lobo *et al*., 2010). In this study, hydrogen peroxide concentration was observed to be significantly higher among the automobile mechanics when compared with the controls. Bio accumulation of H_2O_2 in the body may results from different processes, including dismutation of superoxide radical by superoxide dismutase (SOD). It may also be directly generated by a number of oxidase enzymes such as glycollate and monoamine oxidases as well as through peroxisomal pathway for L-oxidation of fatty acid (Halliwell *et al*., 2000). H_2O_2 is a cytotoxic agent whose levels must be minimized by the action of antioxidant defense enzymes, catalase in particular. Through the process of Fenton reaction, H_2O_2 oxidizes the reduced metal ion (Fe^{2+}) to produce hydroxide (OH^-) and the highly reactive hydroxyl radical ($H^{\cdot}$). This must be removed from the body as it is toxic to tissues in high concentration (Lemire *et al*., 2013). Increased level of hydrogen peroxide is therefore indicative of suppressed activity of catalase, an integral component of the body's antioxidant defense grid.

Thiobarbituric acid reactive substances (TBARS) are formed as a degradation products of lipid peroxidation and their measurement is an established method for screening and monitoring lipid peroxidation (Meagher and Fitzgerald, 2000). The non-significant difference in the level of TBARS determined in the exposed and controls despite the high concentration of H_2O_2 could be because only certain lipid peroxidation products generate Malondialdehyde (MDA) (Marnett, 1999).

The body uses endogenous non enzymatic antioxidants to combat excessive generation of free radicals. The higher the free radicals sequestered by the body, the greater the demand on the antioxidants. Vitamins are micronutrients which play important roles in the general metabolic activities and defence system of the body (Bolajoko *et al*., 2017). They are established as endogenous antioxidants which protects against free radicals. The significant decrease in vitamins A, C and E in the exposed participants compared to the controls is suggestive of excess free radical production subsequent upon heavy metal exposure. This agrees with a study by Anetor et al., 2009 which found low level of vitamins in workers exposed to mixed chemicals when compared with unexposed controls.

Moreover, total antioxidant status (TAS) is the reflection of the body's enzymatic (primary) and the vitamins (secondary) antioxidants. It is believed that it takes into account both known and unknown antioxidants in the body and also the relationship between them. Hence, the estimation of TAS is preferable to the analysis of the known antioxidants separately as it is a useful and better indicator of health status, particularly in respect to the activities of free radicals (Akinosun and Bolajoko, 2007). It reflects the ability of the body to neutralize the generation of excess ROS and thus prevent lipid peroxidation and other free radical-associated damages. The relatively low concentration of TAS in the exposed participants could be adduced to heavy metal-induced free radical generation. This was in disagreement with the observation of Arinola and Akinbiinu, 2006 which found high level of total antioxidants in exposed participants and explained that it might be a compensation for low vitamin E or response to raised toxic metals

VI. CONCLUSION

The auto-mechanics assessed in this study showed that occupational exposure to heavy metals results in increased oxidative stress and lowered total antioxidant status which positively correlates with the duration of exposure. Automobile mechanics can be protected from exposures to hazardous substances by the use of appropriate personal protective equipment such as hand gloves and nose masks. Personal hygiene should be encouraged as well as the development of appropriate ecofriendly equipment by automobile and chemical companies.

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