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Full Length Research Paper

BISPHENOL A DEPLETES THE CELLULAR GLUTATHIONE SYSTEM IN FEMALE WISTAR RATS

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ABSTRACT
BisphenolA (BPA) is an environmental contaminant with two (2) phenolic groups. It is used as plasticizer in plastic and rubber industries. Free BPA is released and it migrates into the food, beverages, as it is used as lacquer in the linning of beverage cans, its migration is enhanced by repeated washing, rubbing and sterilization. Continous exposure to BPA exerts oxidative stress effects on cells. This study investigated the possibility of total glutathione, oxidized glutathione and reduced glutathione perturbations at prevailing low exposure rates in female albino Wistar rats, following exposure for the period of three (3) months. To eleven experimental groups each containing ten (10) non-pregnant female rats were administered 0.05, 0.1, 00.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 1 mg of BPA/kgbw/day. To the twelveth (12^{th}) control group was given water. Blood was collected from animals at the end of every week of the study and serum sample specimens analyzed by routine diagnostic procedures for total glutathione, oxidized glutathione and reduced glutathione using Autochemical Analyzer. Significantly increased concentrations of serum total glutathione, oxidized glutathione and decreased reduced glutathione were observed at all concentrations of BPA exposure suggesting that bisphenol A upsets total glutathione, oxidized glutathione and reduced glutathione balance. Hence, inducing cellular toxicity and then apoptosis.

Key words: BPA, total glutathione, oxidized glutathione (GSSH), reduced glutathione (GSH), exposure.

INTRODUCTION

BPA accumulates into adipose tissue (Rubin, 2011) and is present in body fluids of the normal population. BPA has been detected in the human placenta (Schonfelder *et al*., 2002), cord blood (Wan *et al*., 2010), amniotic fluid (Yamada *et al*., 2002; Nishikawa *et al*., 2010), fetal liver (Cao *et al*., 2012) and breast milk (Sun *et al*., 2004; Carwile and Michels 2011), and in fetus (Nishikawa *et al*., 2010). BPA is well distributed in a wide range of organs, in the

following order, predominantly detected in the lung, followed by kidneys, thyroid, stomach, heart, spleen, testes, liver, and brain. Ratios of the organ to serum BPA concentrations for the organs ranged from 2.0–5.8, except for brain ratio, 0.75, (Yoo *et al*., 2000). BisphenolA (BPA) is a long and well-known endocrine disruptor (ED),that has increasingly been receiving a considerable amount of attention from the scientific community as well as the general public, mainly because of its ubiquity in our environment and uncertainties about its effects on humans. Bisphenol A is well absorbed by the oral route. The ester bond connecting the BPA molecules in polycarbonates or epoxy resins is hydrolyzed during heating or in acidic or alkaline medium. As a result, free BPA is released and it migrates into the food, beverages and into the environment. In addition, migration is enhanced by repeated washing with detergents, rubbing and sterilization (EFSA, 2010). Glutathione provides a first line of defence against (ROS), as it can scavenge free radicals and reduce H_2O_2 (Burgoyne *et al*., 2012). GSH acts directly as an antioxidant and also participates in catalytic cycles of several antioxidant enzymes such as glutathione peroxidase and glutathione reductase. GSH is consumed during the conversion of hydrogen peroxide into hydrogen oxide. exposure to BPA causes oxidative stress by disturbing the balance between ROS and antioxidant defenses system in liver. GST protects cells or tissues against oxidative stress and damage by detoxifying various toxic substrates derived from cellular oxidative processes (Sharma *et al*., 2004). The aim of this study is to unveil/establish the possible effects of Bisphenol A on glutathione in female wistar albino rats, which will lead to the understanding of how BPA might potentiate apoptosis and facilitates/potentiates the response to other cellular death stimulus.

MATERIALS AND METHODS

One hundred and ten (110) non-pregnant female rats of age 5 weeks were acclimatized in the laboratory for seven days. Animals were housed in aluminum wire-mesh cages in a well-ventilated animal house with a 12 h dark/light cycle and at room temperature and were provided commercial rat pellets (Vital feed from Vital group of Company, Nigeria) and water *ad libitum*.

The animals were randomly divided into twelve (12) groups of 10 rats each and Eleven (11) groups were respectively administered 0.05, 0.1, 00.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 1 mg of BPA/kgbw/day. The first group which served as control did not receive any treatment but water instead. The BPA was dissolved in distilled water and administered by oral gavage using intubation canular. Blood was obtained from the tail of the animal by capillary action weekly, for thirteen (13) weeks. Blood samples were processed for clinical assay.

At the end of each blood sample collection, essential blood total glutathione, oxidized glutathione and reduced glutathione were assayed using Auotanalyser. All reagents used, were commercially obtained as already prepared kits. The kits for total glutathione, oxidized glutathione and reduced glutathione were purchased from Enzo Life Sciences, Inc., USA. Individual tests were carried out according to the kit specifications

Materials Supplied with the kit

1. **Clear Microtiter Plate Pack, 2 each:** Two packs of Four Plates of 96 Wells, Clear uncoated solid plates.
2. **25X Assay Buffer:** 12 mL,
3. **Reaction Mix:** 8 bottles, One vial is sufficient for 53 wells of a 96 well plate.
4. **GSSG (4 µM):** 2.5 mL,
5. **Glutathione Reductase:** 80 µL,

6. **Total Glutathione Assay Layout**

Reagent Preparation (as described by Enzo Life Sciences, Inc., US, 2007)

1. **1X Assay Buffer:** the assay buffer was allowed to come to room temperature for aminimum of 1hr:30 minutes. Then the 25X Assay Buffer was diluted to 1X (1:25) with distilled water. The 1X Assay Buffer was then used toprepare dilutions of Total Glutathione Standard Curve and todilute each experimental sample.

2. **Reaction Mix:** Two bottles of Reaction Mix was reconctituted with 8 mLof distilled water. The bottle was periodically swirl over a 30 minute period to dissolve contents. Immediately before use in the assay, the vial ofGlutathione Reductase was vortex and 10 µL of the Glutathione Reductase was added to the bottle of ReactionMix**.** Each bottle of Reaction Mix is sufficient for 53 wells ina 96-well plate, or little more than half a plate. Thereconstituted Reaction Mix was pooled together into one tube because morethan one bottle is used.

3. **5% (w/v) Metaphosphoric acid:** 5% (w/v) Metaphosphoric acid was prepared in distilled water.

4. **2M 4-Vinylpyridine:**2M 4-vinylpyridine solution was prepared by mixing 108 µL 4-vinylpyridinewith 392 µL ethanol. This preparation andsubsequently used of the reagent was done in a chemical fume hood, immediately after preparation and unused portion was discarded.

Sample Handling (as described by Enzo Life Sciences, Inc. US, 2007)

All samples was treated with 5% (w/v) Metaphosphoric acid to remove proteins which might interfere with the assay.

Plasma Preparation

Four volumes of ice-cold 5% (w/v) Metaphosphoric acid was added to the Collected plasma and mixed thoroughly and store on ice for 15 minutes. It was centrifuged at 14,000xg for 15 minutes at 4°C. The clarified supernatant was collected and stored on ice for immediate use in the assay for Glutathione.

Experimental Samples

After preparing the samples as outlined in the SampleHandling section above, the sample obtained was further dilutedto 1:10 with1X Assay Buffer.

Assay Procedure (as described by Enzo Life Sciences, Inc. US, 2007)

Total Glutahione Assay

The Glutathione standard curve was first set up. fifty 50 µL of 1X Assay Buffer was added to all the wells in rows Athrough E, columns 1, 2, and 3 of the microtiter plate(see Figure 1). Then 50 µL of the 4 µM GSSG was added to wells A1, A2, and A3with a multichannel pipetor. The wells were mixed by pipeting thesolution up and down for15 times. 50 µL of the resulting mixture was transferred from wells A1, A2, and A3 to wells B1,B2, and B3, respectively. The wells were mixedfor 15 timesand 50 µL from row B was transferred to row C. This continued to row D. after mixing and the last 50 µLfrom row D discarded.

Wells E1, E2, and E3 were set aside asBlank wells. The GSSG content in rows A, B, C, and D, was 100 pmoles/well, 50 pmoles/well, 25 pmoles/well,and 12.5 pmoles/well, respectively.

Then 50 µL of the diluted experimental samples was added to thewells in columns 4 to 12.The parameters to be measured, the required wells to be read, and 10 seconds orbital shake was then set up on

the plate reader section to measure absorbance at 414nm.Using a multichannel pipetor, 150 µL of freshlypreparedReaction Mix was added to each well and immediately,the record the absorbance in the wells at 414 nm inthe plate reader section was carried out at 2 minute intervalsover a 10 minute period.

Oxidized Glutathione Assay

One (1) µL of 2M 4-vinylpyridine was added per 50 µL of sample and 4µM GSSG. The mixture was incubated for one hour at room temperature. The4-vinylpyridine-treated GSSG standard was serially diluted asdescribed above in the total glutathione assay protocol.

The 4-vinylpyridine-treated experimental sample was serially diluted as described above in the total glutathione assayprotocol.

Then 50 µL of the diluted experimental samples was added to the wells in columns 4 to 12. The parameters to be measured, the required wells to be read, and 10 seconds orbital shake was then set up on the plate reader section to measure absorbance at 414 nm. Using a multichannel pipetor, 150 µL of freshly prepared Reaction Mix was added to each well and immediately, the record the absorbance in the wells at 414 nm in the plate reader section was carried out at 2 minute intervals over a 10 minute period.

	1	2	3	4	5	6	7	8	9	10	11	12
A	GSSG 100 pmole	GSSG 100 pmole	GSSG 100 pmole	Sample 2 1:10	Sample 2 1:10	Sample 2 1:10	Sample 4 1:40	Sample 4 1:40	Sample 4 1:40	Sample 7 1:20	Sample 7 1:20	Sample 7 1:2 0
B	GSSG 50 pmole	GSSG 50 pmole	GSSG 50 pmole	Sample 2 1:20	Sample 2 1:20	Sample 2 1:2 0	Sample 5 1:10	Sample 5 1:10	Sample 5 1:10	Sample 7 1:40	Sample 7 1:40	Sample 7 1:40
C	GSSG 25 pmole	GSSG 25 pmole	GSSG 25 pmole	Sample 2 1:40	Sample 2 1:40	Sample 2 1:40	Sample 5 1:20	Sample 5 1:20	Sample 5 1:2 0	Sample 8 1:10	Sample 8 1:10	Sample 8 1:10
D	GSSG 12.5 pmole	GSSG 12.5 pmole	GSSG 12.5 pmole	Sample 3 1:10	Sample 3 1:10	Sample 3 1:10	Sample 5 1:40	Sample 5 1:40	Sample 5 1:40	Sample 8 1:20	Sample 8 1:20	Sample 5 1:2 0
E	Back - ground	Back - ground	Back - ground	Sample 3 1:20	Sample 3 1:20	Sample 3 1:2 0	Sample 6 1:10	Sample 6 1:10	Sample 6 1:10	Sample 8 1:40	Sample 8 1:40	Sample 8 1:40
F	Sample 1 1:10	Sample 1 1:10	Sample 1 1:10	Sample 3 1:40	Sample 3 1:40	Sample 3 1:40	Sample 6 1:20	Sample 6 1:20	Sample 6 1:2 0	Sample 9 1:10	Sample 9 1:10	Sample 9 1:10
G	Sample 1 1:20	Sample 1 1:20	Sample 1 1:2 0	Sample 4 1:10	Sample 4 1:10	Sample 4 1:10	Sample 6 1:40	Sample 6 1:40	Sample 6 1:40	Sample 9 1:20	Sample 9 1:20	Sample 9 1:2 0
H	Sample 1 1:40	Sample 1 1:40	Sample 1 1:40	Sample 4 1:20	Sample 4 1:20	Sample 4 1:2 0	Sample 7 1:10	Sample 7 1:10	Sample 7 1:10	Sample 9 1:40	Sample 9 1:40	Sample 9 1:40

Figure 1. Suggested 96-well plate format for GSSG Standardsand samples.

Calculation of Results.

Determination of Total Glutathione Concentration

The average of the triplicate absorbance readingsfor each standard, sample, and Blank at each time point was taken. Then the average of each standard, sample, and backgroundabsorbance (A414 nm) was plotted against incubation timeand the slope from the linear portion of eachcurve was determined. The background slope was subtracted from the slopes of thestandards and the experimental samples. The net slopes of the GSSG standards versus pmoles of Glutathione was plotted.The net slopes of the experimental sampleswith those of the standard curve were compared to determinethe pmoles of GSSG (equivalent to total glutathione)for each experimental sample.

Determination of Oxidized Glutathione Concentration

Follow the procedure described above for generating theStandard GSSG curves for the 4-vinylpyridine treated standards. The net slopes of the 4-vinylpyridine-treatedexperimental samples with those of the 4-vinylpyridinetreatedstandard curve were compared to determine thepmoles of oxidized Glutathione for each experimentalsample. The pmole of oxidized glutathione in the sample was subtracted from the pmole of total glutathione to obtain thepmole of reduced glutathione in the sample.

Reduced GSH = Total glutathione - Oxidized GSSG

Statistical analysis of obtained values was carried out by one-way analysis of variance (ANOVA) using SPSS software version 17.0 followed by the Tukey-Kramer multiple comparison test. A $p \leq 0.05$ was taken as a criterion for a statistically significant difference.

RESULTS AND DISCUSSION

There is a significant increase in the total glutathione level in all the groups when compared with the control at $p \leq 0.05$, except for group 6(0.5mg/kg) (Fig. 1a). Total glutathione result showed that the groups that were administered 0.5mg/kg to 1mg/kg of BPA revealed a dose dependent increase in total glutathione except at week-10 to 13, where the group exposed to 0.8mg/kg showed a decrease in total glutathione level relative to those exposed to 0.7mg/kg of BPA (Fig. 1a). The groups exposed to 0.05mg/kg to 0.2mg/kg also showed dose-dependent increase in total glutathione, which was consistent over time (Fig. 1b). The test group 0.3mg/kg, 0.9mg/kg and 1mg/kg BPA showed no sensitivity to time (Fig. 1b).

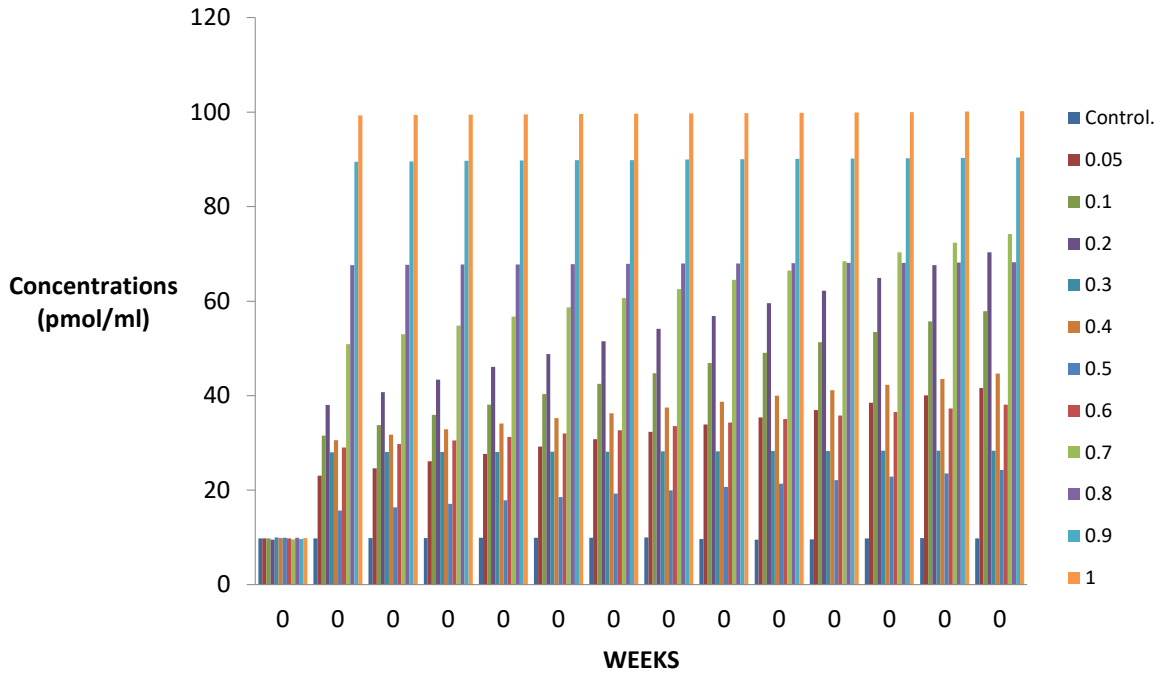

Fig. 1a; Chart of concentration against weeks (durations) for total glutathione level. The legend represents the control group and the respective dose (µg/kg body weight) of BPA administered to the eleven (11) test groups (that is Group 2 to group 12).

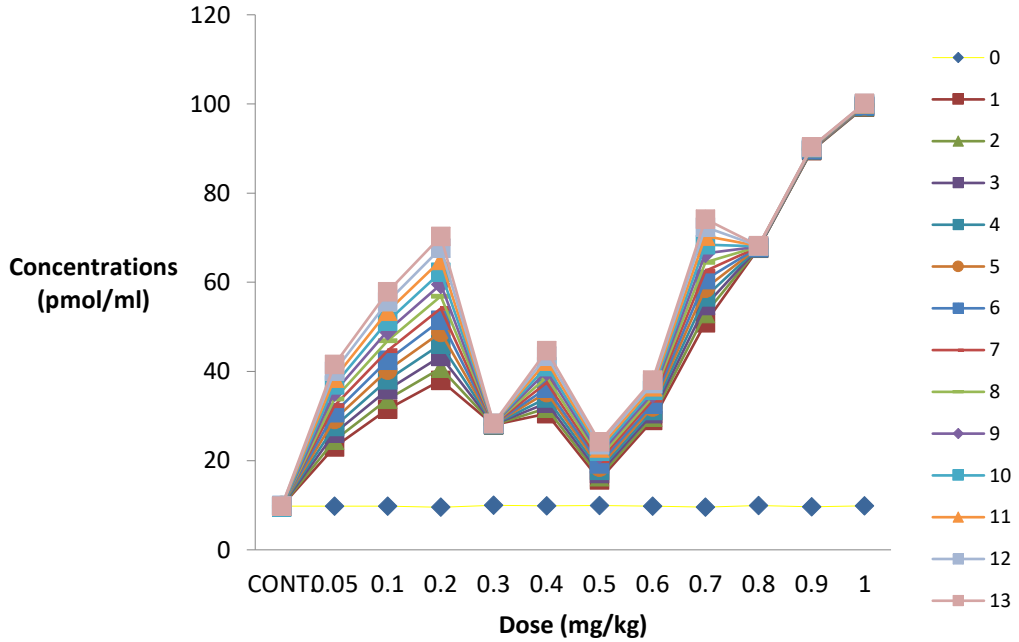

Fig. 1b;Graph of concentration against dose for total glutathione level. The legend represents the duration (in weeks) of the experiment, with the baseline/control week as zero (0) and experiment week as 1 to 13.

There is a significant increase in the oxidised glutathione level in all the groups when compared with the control at $p \leq 0.05$ (Fig. 2a). The result of oxidised glutathione level revealed that the test group that was administered 0.05mg/kg of BPA showed lower oxidised glutathione relative to that exposed to 0.1mg/kg of BPA, except at week-9 where the reversed was observed (Fig. 2a). At the first five weeks (1-5) of exposure, the test group exposed to 0.2mg/kg to 0.9mg/kg showed a dose dependent in increases in the serum level of oxidised glutathione, with their 1mg/kg exposure group showing a decline in oxidised glutathione level relative to that of 0.9mg/kg (Fig. 2a), also at week-5, the 0.5mg/kg exposure group showed a rise in the oxidised glutathione level relative to that of 0.6mg/kg exposure group (Fig. 2a). At week-6 to 13 the test group that received 0.2mg/kg to 0.5mg/kg and 0.6mg/kg to 1mg/kg of BPA revealed a dose dependent increase in the serum concentration of oxidised glutathione (Fig.2a). The group exposed to 0.9mg/kg of BPA showed the level of oxidised glutathione at the first five weeks (1-5) of exposure, while the exposure group of 1mg/kg showed the maximumlevel of oxidised glutathione at week - 6 to 13 (Fig. 2a). The test group exposed to 0.2mg/kg to 0.4mg/kg and 0.6mg/kg to 0.9mg/kg showed no sensitivity to time of exposure (Fig. 2b). The test group exposed to 0.05mg/kg, 0.1mg/kg, 0.5mg/kg and 1mg/kg showed time sensitivity and time dependent effect except that the 0.05mg/kg exposure group showed peak at week-9 of the study (Fig.2b).

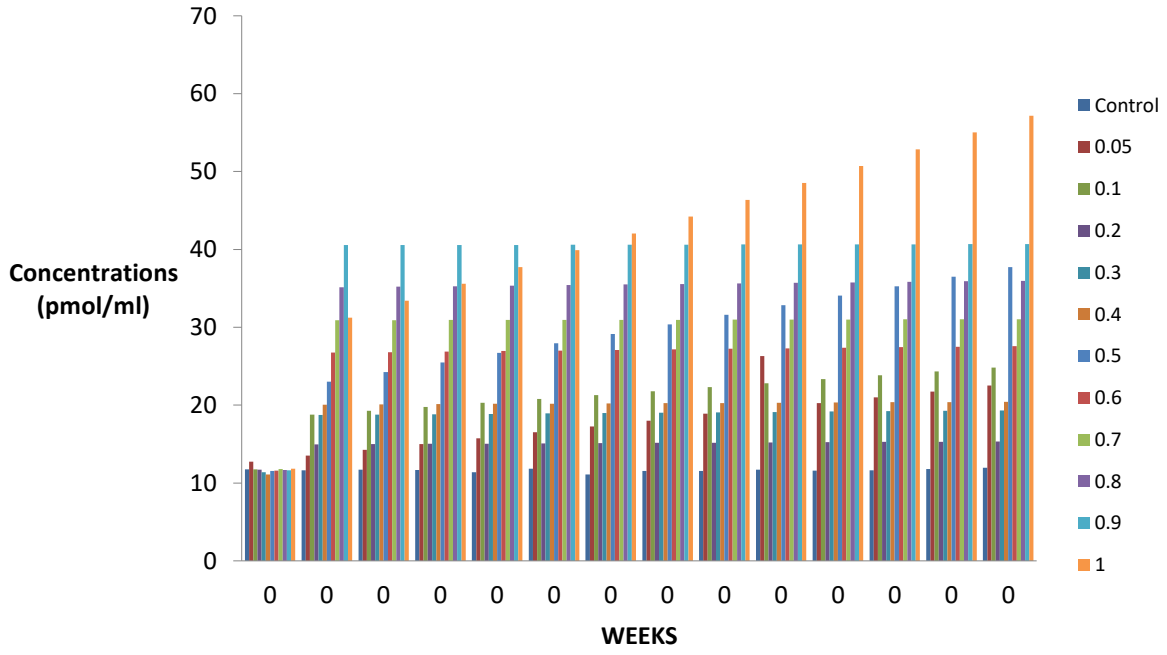

Fig. 2a; Chart of concentration against weeks (durations) for oxidised glutathione level. The legend represents the control group and the respective dose (mg/kg body weight) of BPA administered to the eleven (11) test groups (that is Group 2 to group 12).

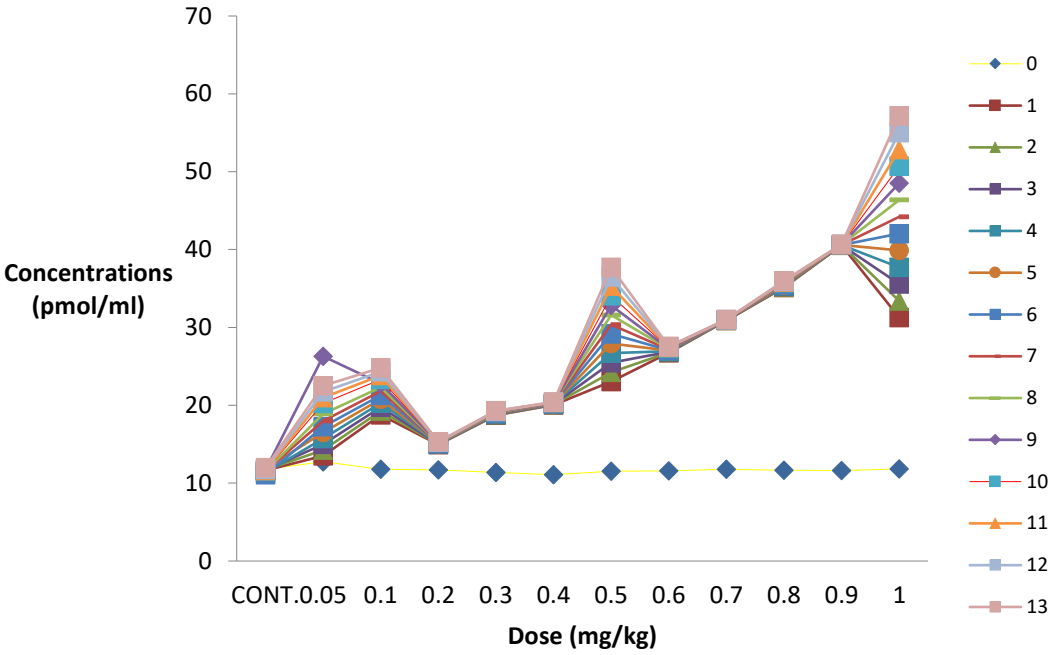

Fig. 2b;Graph of concentration against dose for oxidised glutathione level. The legend represents the duration (in weeks) of the experiment, with the baseline/control week as zero (0) and experiment week as 1 to 13.

There is a significant decrease in the reduced glutathione level when compared with the control at $p \leq 0.05$ (Fig. 3a). The result of reduced glutathione revealed that the test group exposed to 0.05mg/kg of BPA showed lower serum level of reduced glutathione relative to those of 0.1mg/kg (Fig. 3a). The group that received 0.4mg/kg to 0.6mg/kg of BPA showed a dose dependent decrease while the group that received 0.7mg/kg to 1mg/kg of BPA showed a dose dependent increase except at week-10 to 13, where those of 1mg/kg dropped relative to of 0.9mg/kg (Fig. 3a). The test group that were exposed to 0.3mg/kg, 0.8mg/kg and 0.9mg/kg of BPA showed no sensitivity to time of exposure (Fig. 3b). While the test groups exposed to 0.05mg/kg to 0.2mg/kg and 0.4mg/kg to 0.7mg/kg showed increased reduced glutathione over time, those of 1mg/kg showed a decline in reduced glutathione over time (Fig. 3b).

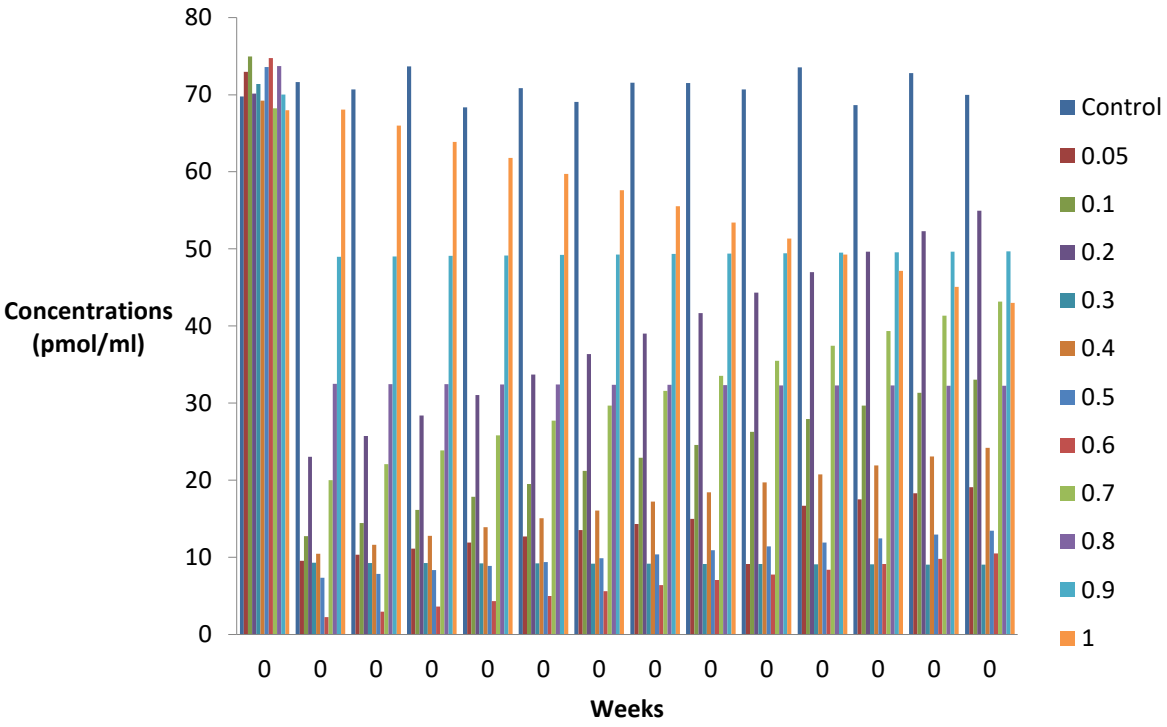

Fig. 3a; Chart of concentration against weeks (durations) for reduced glutathione level. The legend represents the control group and the respective dose (mg/kg body weight) of BPA administered to the eleven (11) test groups (that is Group 2 to group 12).

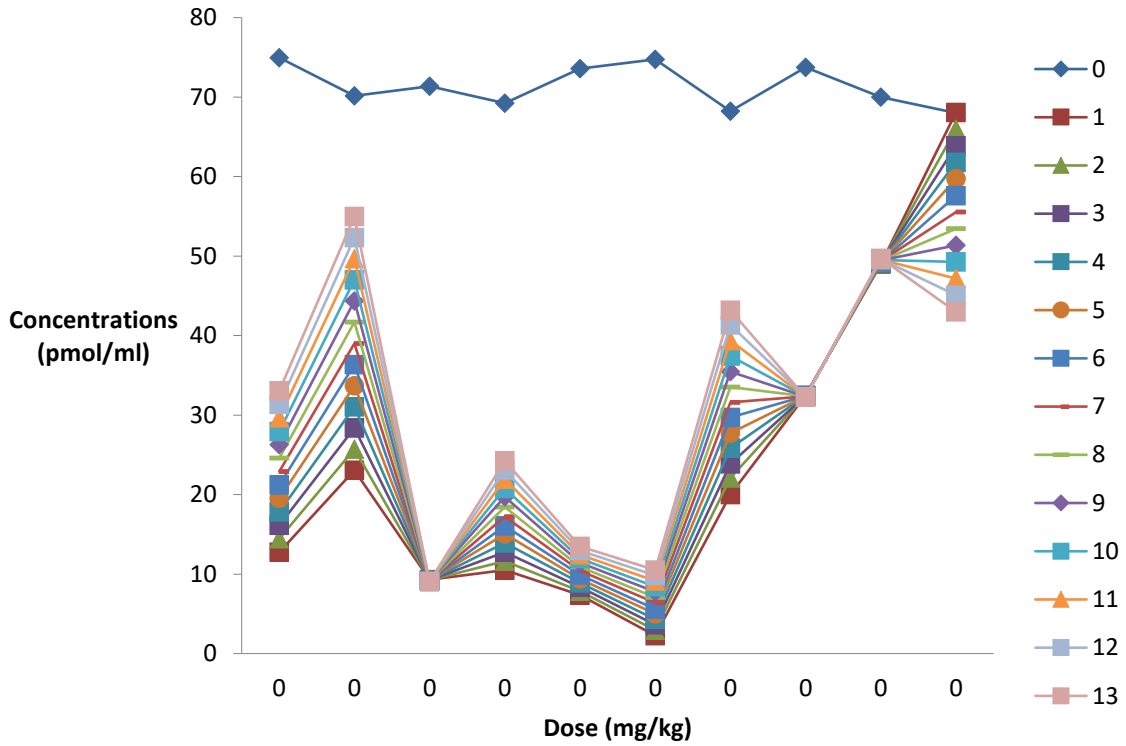

Fig. 3b;Graph of concentration against dose for reduced glutathione level. The legend represents the duration (in weeks) of the experiment, with the baseline/control week as zero (0) and experiment week as 1 to 13.

The findings of these experiment showed an increase in total glutathione and oxidized glutathione (GSSH), but reduced glutathione (GSH) were decreased after BPA exposure. In concordance with these results, Hassan *et al*., (2012) showed a significant decreases in reduced glutathione (GSH), Wu *et al*., (2011), showed significant decrease in the levels of GSH in BPA group. Korkmaz *et al*., (2010) reported the decrease in GSH. Aboul-Ezz*et al*., (2015) revealed that BPA administration induced a state of oxidative stress by decreasing GSH levels. The depletion of GSH and GSH/GSSG ratio in blood has been reported (Vaziri *et al*., 2000; Husain and Hazelringg 2002). Hassan *et al*., (2012) also showed a significant down regulation in the GST gene expression levels and a number of compounds that includes BPA, lead to induce activity and expressions of GST isoenzymes (Fenichel *et al*., 2013).The reduction of GSH shows the failure of primary antioxidant system to act against free radicals. Decreased GSH concentration indicates an increased generation of ROS (Nandi *et al*., 2005).

Glutathione system is essential for cell survival and provides the major antioxidant defence mechanism in cells. Glutathione system directly scavenges diverse free radicals such as superoxide anions, hydroxyl radicals, nitric oxide and carbon radicals. Glutathione system catalytically detoxifies hydroperoxides, and lipid peroxides. Its depletion increases the cellular susceptility to apoptosis. This study revealed depletion of reduced glutathione (GSH), and accumulations of oxidized glutathione (GSSH), and total glutathione.

Depletion of glutathione (GSH) is capable of complete alteration of the T-cell activation and enhancement of hypersentivity response. The accumulation of GSSH due to oxidative stress resulting from exposure to BPA, is directly toxic to cells (Nandi *et al*., 2005). The accumulation of GSSH toxicity to cells is by the activation of the SAPK/MAPK pathway, thereby inducing cellular toxicity and then apoptosis. A change in GSSH- to -GSH ratio is highly indicative of high levels of oxidative stress radicals. A shift in the ratio towards the oxidized pool tend to induce glutathioylation of the cysteine located in the DNA binding site of C-JUN as well as produce a disulfide bond between cysteines proximal to the leucine zipper motif. The impact of these changes is the capacity to induce a direct and reversible control of transcriptional regulation through these stress kinase pathways as earlier stated. The GSH thiol group of cysteine quenches free radicals and ROS generated in cell by donating a reducing equivalent. Its depletion could be a cause of exposure to environmental toxin (BPA), chronic liver condition/damage and inflammation as a result of induced oxidative stress by BPA exposure (Hassan *et al*., (2012). GSH depletion either induce or potentiate apoptosis and facilitates/potentiates the response to other cellular death stimulus (Sharma *et al*., 2004).

This finding has demonstrated that there is an imbalance between oxidized and reduced glutathione in the sytem, Showing therefore, that the exposure to BPA causes oxidative stress by disturbing the balance between ROS and antioxidant defenses system.

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