Original Article

Neuromorphological and Biochemical Effects of Co-exposure to Bisphenol A and Cadmium in Insulin-resistant Rats

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Abstract

Background: Cadmium (Cd) and bisphenol A (BPA) are known industrial additives and environmental toxicants that have been extensively reported for their various deleterious effects on biological systems, particularly endocrine disruption and neurotoxicity. In high-fat diet-induced insulin-resistant model rats, we studied the neurotoxicity and oxidative stress effects of co-exposure to Cd and BPA. **Aims:** This study aims to look at prefrontal microarchitecture and antioxidant profiles in insulin-resistant rats. **Materials and Methods:** Twenty-five adult Wistar rats were randomly assigned into five groups (A– E; $n = 5$). With A receiving normal saline; B: 40 mg/kg. bw $CdCl_2$ + high-fat diet (HFD) + Suc; C: 40 mg/kg. bw BPA + HFD + Suc; D: 40 mg/kg. bw BPA + 40 mg/kg. bw $CdCl_2$ + HFD + Suc; and E: HFD + Suc orally for 56 days. Finally, brains were excised from each group and the medial prefrontal cortex was dissected from both hemispheres with right hemisphere samples processed for hematoxylin and eosin histology and left hemisphere samples homogenized for biochemical evaluation of oxidative stress markers. One-way analysis of variance and Tukey's *post hoc* test were used for data analysis with $P < 0.05$ considered statistically significant. **Results:** From our findings, prefrontal glutathione levels were significantly lower ($P < 0.05$) in the insulin-resistant rats (Cd + BPA + HFD + Suc: 120.9 ± 21.89, HFD + Suc: 93.27 ± 17.29) compared with control rats (244.0 ± 11.57), while prefrontal glutathione reductase activity was significantly elevated (Cd + BPA + HFD + Suc: 41.02 ± 5.5, HFD + Suc: 41.09 ± 1.68, $P < 0.05$) compared to the control rats (20.17 ± 3.27). Prefrontal neurons showed nuclear condensation, cytoplasmic vacuolations, and clumping of cells. **Conclusion:** Morphological and biochemical evidence from the present study suggests that environmental and metabolic factors do combine to induce profound adverse effects on prefrontal microanatomy and antioxidant system.

Keywords: *Bisphenol A, cadmium, insulin resistance, oxidative stress, prefrontal cortex*

Introduction

An extensive body of research has identified bisphenol A (BPA) as a well-known endocrine-disrupting chemical.[1] Environmental pollutant to which humans and living organisms get exposed in varying concentrations and durations can reach toxic levels and has been confirmed to lead to oxidative stress due to the build-up of reactive oxygen species, increased lipid peroxidation, and alteration of DNA structure through nucleotide base modifications that disrupts DNA synthesis and repair pathways.[2-5] Exposure and contamination with BPA has been reported to be through leaching from various consumer goods and wearable products that find their way into the bloodstreams and membranes through nasal, oral, and dermal routes and has been detected in hazardous levels in urines of individuals in a wide range of population studies.[1,6]

Another candidate of interest due to its occurrence in the environment, industrial production and usage in manufacturing, and other commercial purposes is cadmium which is a member of a class of naturally occurring elements known as heavy metals.[7] Heavy metals have been described as metallic elements having higher densities relative to water which thus confers their toxicities that can be induced following exposure even to low concentrations.[8] The commercialization of cadmium which increases its concentrations

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in the environment further increases its potential for human exposure.[9] The public health concern to heavy metals generally, is with respect to biological systems that include plants and animals, is due to their reported disruption of cellular components and morphology as well as critical biochemical and metabolic processes through the alteration of normal enzyme-mediated pathways and cellular processes.[10] Thereby, leading to tissue damage, uncontrolled cell division, and increasing the potential for tumourigenesis, unprogrammed cell death (apoptosis) affecting normal tissue development.[11] Likewise, cerebellar ataxia induced by environmental toxins that include heavy metals have been reported to be owed to the perturbation to cerebellar cortical and Purkinje neurons.[12] Meanwhile, affluent and busy lifestyles have continued to exhibit far-reaching impacts on our day-to-day quality of life. Moreover, this is because it has led to an increasing palate for fast foods (also known as western diets [WDs]) which are essentially high fat based and the prolonged consumption of which has been widely associated with insulin resistance (IR) and accompanying diabetes leading to precocious cognitive and memory impairments which are functions of the hippocampus and parts of the prefrontal cortex (PFC).[13] Arnold *et al*. reported that, in addition to inducing brain IR in both cerebral cortical tissue and hippocampus, high-fat diets (HFDs) produced impairment in spatial working memory due to observed decreased T-maze alternation.[14] Although there are other underlying genetic and epigenetic permutations such as gene polymorphism that increase the risk of Alzheimer's disease (AD), however, prolonged HFD consumption has been established as a major influence in the pathophysiology. A study by Del Olmo and Ruiz-Gayo highlighted the relationship between hippocampal learning/memory deficits and nutritional/endocrine inputs derived from HFDs on juvenile hippocampal morphology and neurotransmission.[15] Since the earliest description of HFD, extensive studies have established it as a promoter of hyperglycemia and whole-body IR and thus accepted as a valid rodent model for simulating the metabolic syndrome and its associated IR and compromised functions of the pancreatic beta cells.[16] Diets high in fats and calories are known as HFDs or WDs and as the rate of their consumption continues to rise globally, especially in the more industrialized nations, it has become a major source of public health concern due to its attending obesity which is associated with a myriad of metabolic diseases that includes type II diabetes, cardiovascular diseases, stroke, gastrointestinal and respiratory diseases as well as several kinds of cancers.[17,18] Meanwhile, Akinola *et al*. linked poorly treated diabetes mellitus with neural complications and varying degrees of neurobehavioral manifestations.[19] Moreover, the mechanism of such neural complication could not be far from the fact that fatty acids being components of phospholipids cell membranes and thus their involvement in the interaction between proteins and lipid and their influences on membrane properties, cellular processes, and susceptibility to cell death as a result of the length of their carbon atom chains.[20] In other words, diet-induced obesity (and IR) has been linked with cognitive deficits and neurodegenerative diseases such as AD through mechanistic processes that involve the exacerbation of brain inflammation and acceleration of brain aging.[17]

Insulin-dependent diabetes mellitus, also known as type I diabetes, and insulin-independent diabetes mellitus (type 2 diabetes) are metabolic diseases that manifest in chronic hyperglycemia in the body. Type I diabetes is a condition that is characterized by low or complete lack of pancreatic insulin production, while type 2, on the other hand, is propagated by insulin production which is insufficient enough to effectively keep up with the glucose metabolic demands of the body. An imbalance in this systemic glucose–insulin dynamics precipitates abnormal glucose metabolism secondary to the development of IR. This is due to problems with the transport of glucose into the cells and its normal metabolism which, therefore, keeps glucose levels in the bloodstream elevated, which is characteristic of both types I and II diabetes. The resulting metabolic complications, such as neuropathy, renal failure, retinopathy, cardiovascular diseases, and peripheral vascular diseases, make it of significant concern.[21,22]

We, therefore, aimed to study the effects of simultaneous oral exposure to BPA and Cd on prefrontal cortical function and oxidative stress in a model of insulin-resistant rats.

Materials and Methods

Ethics committee approval was granted for this study the Postgraduate Ethical Review Committee of the University of Ilorin on 12.09.2019 with the number (UERC/ASN/2019/1854).

All experimental protocols and animal handling were in accordance with the guidelines of the University Ethical Review Committee and the Institutional Animal Care and Use Committee.

Chemicals and high-fat diet

Cadmium chloride (Kermel Chemical Reagent Co., Ltd., Tianjin, China) was obtained from Labtrade (Nig) Co, and BPA (Loba Chemie Pvt Ltd, India) was procured from Mich-Mikedenson Nig. Ltd. Other chemicals used are of analytical grade. High-fat feed was compounded at Ogo-Oluwa Livestock and Aqua Feeds Enterprises, Ilorin.

Animals and experimental design

Twenty-five adult male Wistar rats (*Rattus norvegicus*) (95–120 g) (Ogo-Oluwa Livestock and Feed Mills, Ilorin) were used for this study. The animals were kept in cages at the animal holding facility of the university with a 12-h light/dark cycle under standard room temperature/humidity with liberal access to rat pellets (Ogo-Oluwa Livestock and Feed Mills, Ilorin) and distilled water.

Treatment plan

The rats were randomly assigned into five groups of five animals each as follows: Group A (control: free access to distilled water), Group B (daily oral $CdCl_2$ at 40 mg/kg) + HFD + Suc.), Group C (daily oral BPA at 40 mg/kg) + HFD + Suc, Group D (daily oral $CdCl_2$ at 40 mg/kg + BPA at 40 mg/kg + HFD + Suc), and Group E (daily oral HFD + Suc daily). The administration lasted for 56 consecutive days.

Evaluation of brain and body weight

Body weights of rats were recorded on arrival at the animal house and every week of the 8-week (56 days) duration of the study. Weekly weights were taken to study the changes in weights across the groups through the study duration. Body weight changes were calculated as follows:

Change in body weight (%) = (Body weight at day 56– Body weight at day 0)/Body weight at day 56 × 100%

Prefrontal oxidative stress

On the last day of exposure, animals fasted overnight, final body weights were taken, tails were pricked to collect arterial blood, and fasting blood glucose was estimated by the glucose oxidase method using Accu chek (Roche, Belgium) and animals were anesthetized with intraperitoneal injection of ketamine (20 mg/kg). Blood samples from the left ventricle of the heart were collected through cardiac puncture into appropriate plain bottles and left to clot at room temperature (23°C) for 30 min. Blood samples were subsequently centrifuged at 3000 × g for 15 min. The serum was frozen at −20°C pending hormonal and glucose analysis. The animals were decapitated and brain tissues were harvested and separated into the right and left hemispheres. Hemispheres were dissected on cold plate to excise the PFC. Right hemisphere tissues for histological protocol were immediately fixed in 4% paraformaldehyde (Central Research Laboratories, Tanke, Ilorin). The left hemispheres were used for enzymatic analysis and preserved in 0.25 M sucrose solution at 4 ºC and were homogenized using Omni Prep Homogenizer (Omni International, GA, USA). The subsequent homogenates were centrifuged at 1957 × g for 10 min using a hematocrit centrifuge to obtain supernatants and pellets. Finally, the supernatants were aspirated into fresh tubes and both were stored at −20°C until further processing.[22] Protocol for hematoxylin and eosin (H and E) was carried out for histological studies.

Assay for fasting serum insulin and glucose

Serum glucose was assayed using the glucose oxidase method diagnostic enzyme kit (Span Diagnostic Chemicals, India), and insulin levels were assayed using the AccuBind enzyme-linked immunosorbent assay Microwells Insulin Test System (Monobind Inc. CA, USA) per manufacturer's instruction.

Estimation of insulin resistance

IR was estimated using the homeostasis model assessment of IR (HOMA-IR) method as previously reported[23] as follows:

(Fasting serum insulin [μU/L] × fasting serum glucose [mg/dL]/405)

Prefrontal photomicrography

The harvested brain tissues were fixed with 10% phosphate-buffered formaldehyde and subjected to the routine method for paraffin wax embedding to produce the required paraffin wax-embedded tissue blocks. To obtain the tissue blocks for staining, the fixed tissues were taken through the routine tissue processing protocol for H and E. Photomicrographs were taken with an Axiocam ERc 5s camera attached to a Carl Zeiss AX10 microscope and analyzed using the ZEN Core 3.5 software.

Data analysis

To analyze body weight and prefrontal oxidative stress parameters, GraphPad Prism software version 9 (GraphPad Software, Inc., San Diego, CA, USA) was used. One-way analysis of variance was used to compare differences in means, followed by Tukey's multiple comparison tests where necessary. All data are presented as mean ± scanning electron microscopy with a significance value set at $P < 0.05$.

Results

Body weight

The result from this study [Table 1] shows weight increase and percentage change in body weight was significantly higher in the HFD + Suc (116.0 ± 1.8, 29.96 ± 1.13) and BPA + HFD + Suc (109.4 ± 1.6, 26.12 ± 1.12) groups. Meanwhile, Cd seems to prevent much weight gain as the groups exposed to the chemical recorded the lowest mean weight and percentage increase in weight, Cd + BPA + HFD + Suc (80.2 ± 0.7, 3.18 ± 0.83) and Cd + HFD + Suc (82.2 ± 1.9, 22.22 ± 2.11), respectively.

Fasting blood glucose

HFD + Suc exposed group recorded the highest significant ($P < 0.05$) fasting blood glucose as well as the highest significant increase throughout the period of administration among all the groups. The fasting blood glucose level of the negative control (Ngtv Ctrl) group remained relatively stable throughout the duration of the study [Figure 1].

Fasting serum insulin

Cd + HFD + Suc recorded the highest fasting serum insulin levels. There was a significant increase ($P < 0.05$) in the levels of serum insulin in both Groups B and C relative to Group A (Ngtv Ctrl). There was, however, no significant difference in the levels of serum insulin in Groups D and E relative to the Ngtv Ctrl Group (A) at the end of 56 days of exposure [Figure 2].

Fasting serum glucose

There were significant differences ($P < 0.05$) in fasting serum glucose levels of Group D (Cd + BPA + HFD + Suc) and E (HFD + Suc) relative to the Ngtv Ctrl group. Group D (47.4 ± 0.6) and E (51.5 ± 1.2) both have higher fasting serum glucose levels than group control group (37.3 ± 0.6). However, there was no significant difference in fasting serum glucose levels between the control group and other insulin-resistant groups [Figure 3].

Insulin resistance

All the rat groups (B, C, D, and E) exposed to the agents HFD and Suc recorded significantly higher ($P < 0.05$) HOMA-IR scores compared to the Ngtv Ctrl group [Figure 4].

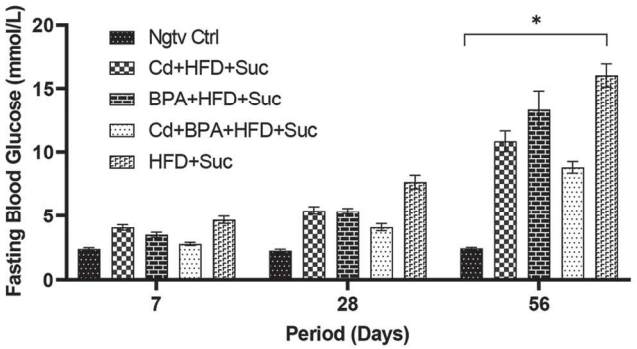

Figure 1: Fasting blood glucose levels across the groups following exposure for 7 days, 28 days, and 56 days. Ngtv Ctrl: Negative control, Cd: Cadmium, HFD: High-fat diet, BPA: Bisphenol A, Suc: Sucrose

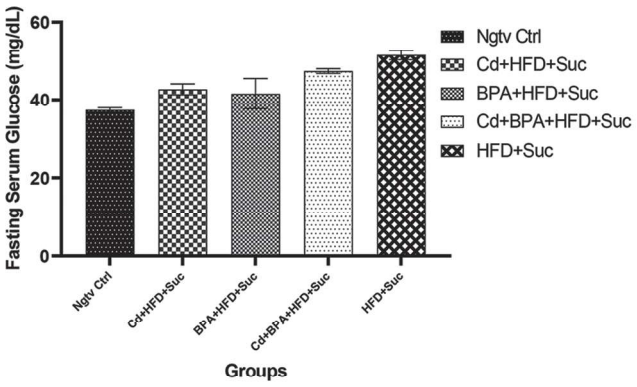

Figure 3: Fasting serum glucose levels across the groups following exposure for 56 days. Ngtv Ctrl: Negative control, Cd: Cadmium, HFD: High-fat diet, BPA: Bisphenol A, Suc: Sucrose

Prefrontal superoxide dismutase

There were significant differences ($P < 0.05$) in the mean levels of prefrontal superoxide dismutase (SOD) across the groups. The group exposed to BPA + HFD + Suc (298.1 ± 45.8) had the highest prefrontal SOD levels relative to every other group with the group exposed to HFD + Suc (196.3 ± 41.1) recording the lowest SOD level [Figure 5].

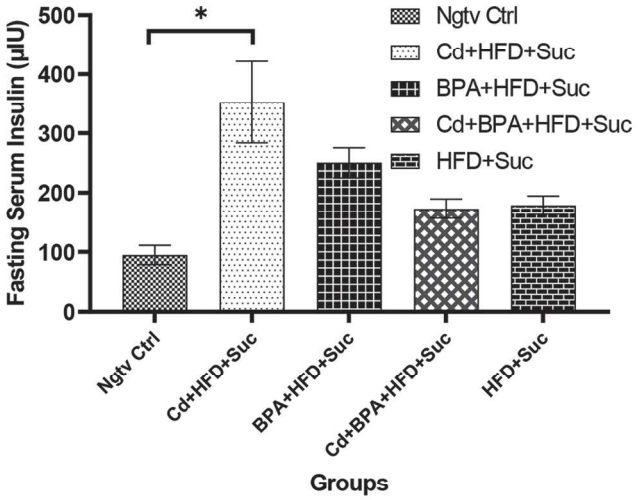

Figure 2: Fasting serum insulin levels across the groups following exposure for 56 days. Ngtv Ctrl: Negative control, Cd: Cadmium, HFD: High-fat diet, BPA: Bisphenol A, Suc: Sucrose

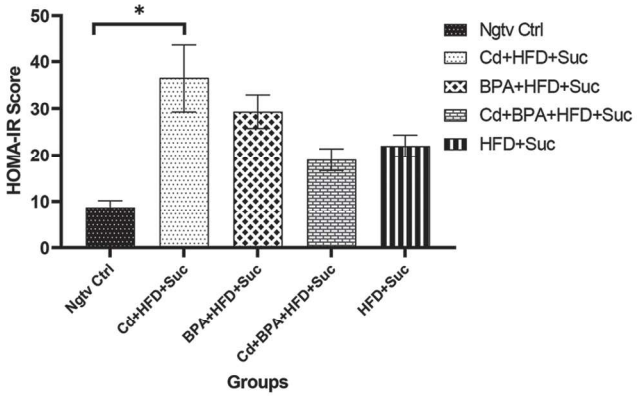

Figure 4: HOMA IR score across all groups following 56 days of exposure. Ngtv Ctrl: Negative control, Cd: Cadmium, HFD: High-fat diet, BPA: Bisphenol A, Suc: Sucrose

Table 1: Comparison of weight (g) and percentage change in weight across groups (%)

Groups	Initial weight (g)	Final weight (g)	Weight difference (%)
Ngtv Ctrl	73.4±1.8	98.4±2.4	25.14±3.17
Cd + HFD + Suc	63.3±1.4	82.2±1.9	22.22±2.11
BPA + HFD + Suc	80.8±1.3	109.4±1.6*	26.12±1.12
Cd + BPA + HFD + Suc	78.2±1.3	80.2±0.7	3.18±0.83
HFD + Suc	81.8±1.6	116.0±1.8*	29.96±1.13

Values are expressed as mean±SEM with the level of significance shown as *(P<0.05). SEM: Standard error of mean, Ngtv Ctrl: Negative control, Cd: Cadmium, HFD: High-fat diet, BPA: Bisphenol A, Suc: Sucrose

Prefrontal glutathione levels

The result in Figure 6 shows glutathione level was significantly higher ($P < 0.05$) in the control group (244.0 ± 11.57) compared to every other group exposed to the various toxicants. The exposed groups recorded lower glutathione levels suggesting that they are undergoing various degrees of oxidative stress.

Prefrontal glutathione reductase

Figure 7 shows glutathione reductase (GR) activity was higher in the HFD + Suc exposed group, followed by the group administered Cd + BPA + HFD + Suc. The GR activity level was lowest in the Ngtv Ctrl group. The higher GR activity in the exposed groups suggests an increased antioxidant response to the oxidative stress from the toxicants.

Histomorphology of the prefrontal cortex

All groups exposed to metabolic and environmental toxicants showed various forms and degrees of perturbations from the normal histoarchitecture of the PFC. These perturbations range from apparent nuclear condensation of granule cells of layer II, which is one of the early signs of apoptosis, clumping together of neurons, loss of granule cell density in some groups as well as necrotic blood vessels [Figure 8a].

External granular layer shows evenly stained and well-differentiated nuclei in the Ngtv Ctrl group. Cd + BPA + HFD + Suc treated group presents with aggregations of larger and deeply pigmented granule cells. There, however, appears to be fewer granule cells across the external granular in the BPA + HFD + Suc exposed group. Furthermore, sections from the treatment groups, especially the Cd + BPA + HFD + Suc and HFD + Suc groups, present with apparent histoarchitectural alterations that can be described as isolated aggregations of darkly stained nuclei present across the layers which is consistent with cellular pyknosis [Figure 8b].

Discussion

Industrialization has contributed to the increased exposure of humans to heavy metals such as cadmium, aluminum, lead, arsenic, and others.[24] Through primarily oxidative stress and mitochondrial dysfunction, these metals induce various levels of toxicities with manifestations ranging from motor to cognitive impairments.[25-27]

Results from this study indicate that exposure to HFD and sucrose drink precipitates IR in animals which leads to consequently higher serum insulin and serum glucose levels in the exposed groups compared to the control. Patience Ojo *et al*. (2022) reported increased glucose and diminished insulin sensitivity in a study of high fructose (sugar) diet exposure.[28] The metabolic imbalance that results from insulin insensitivity (IR) is exhibited by the observed significant

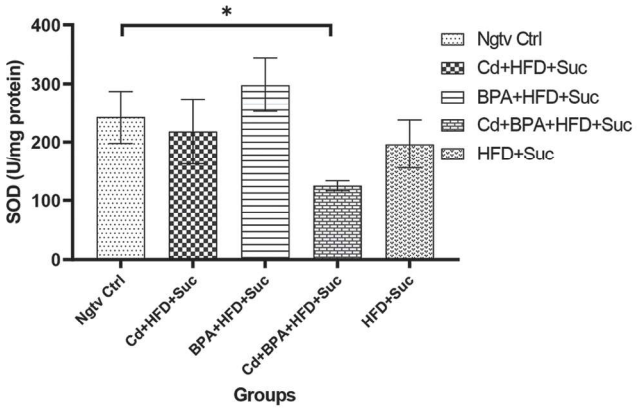

Figure 5: SOD readings of all groups at the end of the 56 days of simultaneous exposure to metabolic and environmental toxicants. Ngtv Ctrl: Negative control, Cd: Cadmium, HFD: High-fat diet, BPA: Bisphenol A, Suc: Sucrose

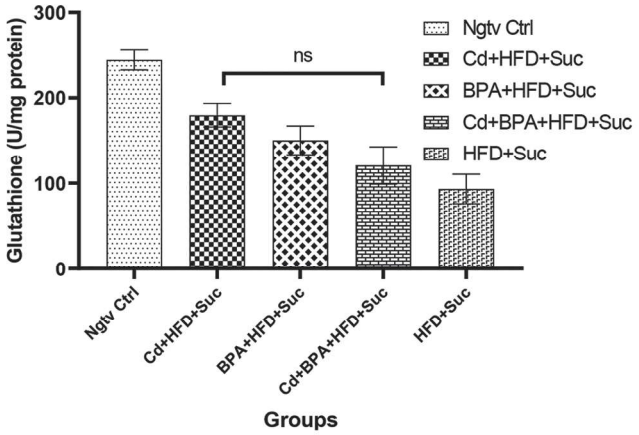

Figure 6: Glutathione levels across the groups at the end of the period of administration of Cadmium, bisphenol A, high fat, and sucrose diet. Ngtv Ctrl: Negative control, Cd: Cadmium, HFD: High-fat diet, BPA: Bisphenol A, Suc: Sucrose

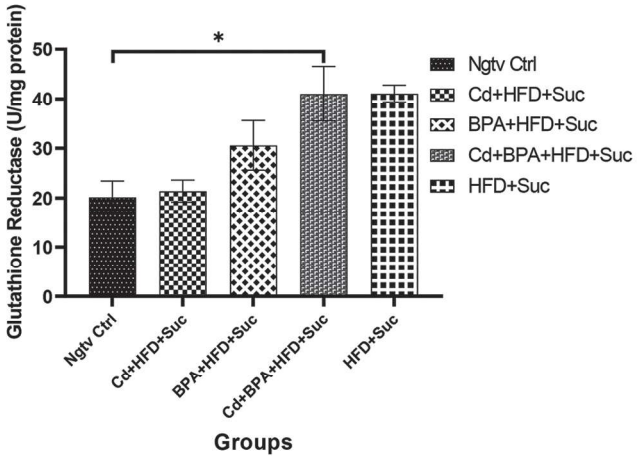

Figure 7: Glutathione reductase levels across the rat groups at the end of administration. Ngtv Ctrl: Negative control, Cd: Cadmium, HFD: High-fat diet, BPA: Bisphenol A, Suc: Sucrose

increase and higher percentage gain in body weight in the insulin-resistant groups. Moreover, this finding is consistent

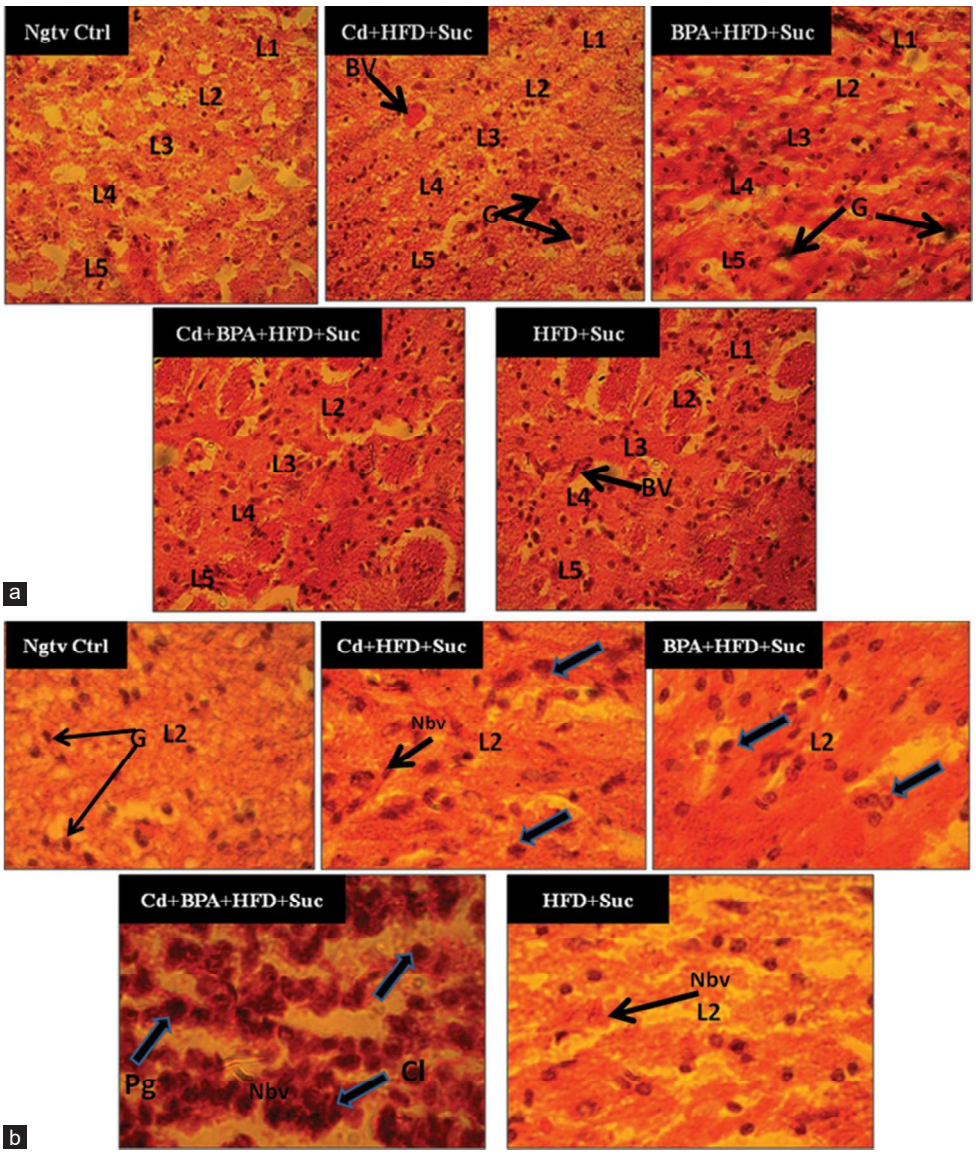

Figure 8: (a) Representative light micrograph of the prefrontal cortex of all groups exposed for 56 days. Stain: H and E, ×100. (b) Representative light micrograph of the prefrontal cortex of all groups exposed for 56 days. Stain: H and E, ×400. L1: Layer 1, L2: Layer 2, L3: Layer 3, L4: Layer 4, L5: Layer 5, BV: Blood vessel, G: Granule cell, V: vacuolation, Nbv: Necrotic blood vessel, Cl: Clumping, Tg: Tangling, Pg: Deeply pigmented cells. Ngtv Ctrl: Negative control, Cd: Cadmium, HFD: High-fat diet, BPA: Bisphenol A, Suc: Sucrose

with reports from studies conducted by Mohammed *et al*. and Akbari *et al*.[13,29] Likewise, exposure to the heavy metal, cadmium, and the endocrine-disrupting chemical, BPA resulted in notable oxidative stress on the treated animals. For instance, GR activity was significantly elevated in BPA-exposed groups bar the positive control. The expression of the antioxidant and SOD was also most highly elevated in the group exposed to BPA. Kobayashi *et al*. also reported on oxidative effects of BPA in a study where it contributed to a significant reduction in free radical scavenging capacity in plasma after 2- and 4-week exposure to BPA as well as significantly increased levels of SOD1 after 8 weeks of BPA treatment and alteration of ROS-induced signaling pathways in the brain.[30] Serum glucose and serum insulin were the insulin–glucose homeostasis markers analyzed and the reported result suggests that combined exposure to HFD and sucrose results in high serum glucose and insulin levels. Moreover, this observation is further buttressed by the reported higher score for assessment of IR (HOMA-IR) in the HFD and sucrose-treated groups. On histopathological examination, neuronal loss, nuclear condensation, and cytoplasmic vacuolations of granule cells characterized the PFC. Imam *et al*. also reported global cerebellar neurodegenerative changes characterized by numerous perineural spaces and reductions in neural density indicative of cellular shrinkage following aluminum chloride exposure.[25]

Conclusion

Reports from this work lends further credence to previous studies on the oxidative stress-inducing effects of heavy

metals and neuroendocrine-disrupting effects of HFD, sucrose (high sugar) drink, and endocrine-disrupting chemical, BPA. Overall, we demonstrated that isolated or co-exposure to cadmium chloride and BPA in high fat and sucrose diet-induced insulin-resistant rats precipitated prefrontal cortical lesions that could progress to neurodegenerative changes with the associated perturbation of cognitive and affective functions.

Patient informed consent

There is no need for patient informed consent.

Ethics committee approval

Ethics committee approval was granted for this study the Postgraduate Ethical Review Committee of the University of Ilorin on 12.09.2019 with the number (UERC/ASN/2019/1854).

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Conflict of Interest

There is no conflict of interest to declare.

Author Contributions subject and rate

- Abdulwasiu Taiwo Lawal (35%): Concept and design of the study, definition of intellectual content, experimental studies, literature search, collection of data, analysis and interpretation of data, manuscript preparation, editing and submission of manuscript.
- Ahmed O Sharafadeen (30%): Concept and design of the study, experimental studies, literature search, collection of data and analysis.
- Oluwole Busayo Akinola (35%): Concept and design of the study, provision of laboratory, definition of intellectual content, analysis and interpretation of data, manuscript editing and review and final approval of the version to be published.

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