Arch. Bas. App. Med. 9 (2021): 59– 68
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Research Article

Influence of Atrazine and Diclofenac Co-exposure on Hypothalamic-Pituitary-Testicular Axis Function in Rats.

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Abstract
Humans and animals are commonly exposed to numerous chemicals through diverse sources causing unpredictable real-life health effects. This study evaluated the influence of joint exposure to the herbicide atrazine (ATZ) and the NSAID diclofenac (DCF) on the hypothalamic-pituitary-testicular axis function in pubertal rats. The animals were jointly exposed to ATZ (20 and 40 mg/kg body weight) and DCF (10 and 20 mg/kg body weight) for 42 days. In comparison with individual exposures, the current data illustrated that combined exposure to ATZ and DCF exacerbated the reductions in follicle stimulating hormone (FSH), luteinizing hormone (LH), serum and intra-testicular testosterone levels with testosterone/LH ratio. Additionally, co-exposure to ATZ and DCF worsened the sperm quality and quantity with marked disruption in the testicular function marker enzymes activities. The diminution in the epididymal, testicular and hypothalamic antioxidant defense mechanisms was intensified in animals co-exposed to ATZ and DCF. Moreover, the induction of reactive oxygen and nitrogen species, lipid peroxidation, inflammatory stress and histopathological lesions in the epididymal, testicular and hypothalamic tissues was intensified in co-exposed animals. These data accentuate the possible male reproductive dysfunction related to ATZ and DCF co-exposure in mammals and, by extension, provide useful insights into the public health threats associated with combined exposure to pesticides and pharmaceuticals.

Key Words: Atrazine; Diclofenac; Joint exposure; Oxido-inflammatory stress; Reproductive axis

INTRODUCTION

The increasing concern about the male reproductive dysfunction is related to the numerous reports that human semen quality has deteriorated globally in recent decades (Mann *et al*., 2020; Pizzol *et al*., 2021). Various factors that have been implicated in the decline of male reproductive health include environmental pollutants, stress, dietary pattern, pharmaceuticals, and lifestyles (Ilacqua *et al*., 2018; Salas-Huetos *et al*., 2019). Atrazine (ATZ) is an herbicide widely used to control weeds in agriculture and urban golf courses (Sherchan and Bachoon, 2011). The annual consumption of ATZ is reported to be greater than 3,000 and 33,000 tons in Australia (Radcliffe 2002; Warne *et al*., 2020) and the United States (Farruggia *et al*., 2016) respectively. The mobility, ubiquity, and persistence of ATZ in the environment are of emerging concern globally (Cheng *et al*., 2020). Exposure of animals and humans to ATZ occurs mostly through ingestion of contaminated food or drinking water. The potential negative impact of ATZ on human reproductive health has been associated with its detection in human bodily fluids including semen (Swan *et al*., 2003). Several animal studies have demonstrated that ATZ elicits endocrine disrupting effect in model organisms (Hayes *et al*., 2011; Vandenberg *et al*., 2020). Chronic oral exposure of rats to different doses (50-200 mg/kg) of ATZ reportedly induced reproductive dysfunction via decrease in the sperm motility and antioxidant enzymes activities with simultaneous upsurge in oxidative stress indices (Jin *et al*., 2013; Martins-Santos *et al*., 2018; Abarikwu *et al*., 2020).

Diclofenac (DCF), a non-steroidal anti-inflammatory drug, is widely prescribed for the treatment of gout, trauma, pain, fever, dysmenorrhea, migraine headache and osteoarthritis in both humans and animals (Khan and McLean, 2012; Ahmed *et al*., 2019). Owing to its therapeutic efficacy, there is a remarkable increase in the global consumption of DCF to about 1443 ± 58 tons per year (Acuna *et al*., 2015). In general, the global health concern about pharmaceuticals is associated with their high consumption quantities, environmental pollution and noxious effects (Wen *et al*., 2014, Adedara *et al*., 2020). Previously reported data have indicated some toxicological responses in humans (Simon and Evan Prince 2017; Ramachandran *et al*., 2018; Moore and Scheiman 2018). Several previous studies have also shown that varying doses (10-100 mg/kg) of DCF induced toxicological effects in laboratory rodent models. Administration of DCF has been associated with induction of hepatotoxicity, nephrotoxicity, ulceration, and cardiotoxicity via oxidative stress mechanism (Aycan *et al*., 2018; Mostafa *et al*., 2020; Motawi *et al*., 2020;Dolanbay *et al*., 2020; Xu *et al*., 2021). Moreover, the

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placental transfer of DCF previously documented in rodents and humans evidenced its potential reproductive toxicity (Siu *et al*., 2000, Shintaku *et al*., 2009, Arslan *et al*., 2016). Adult Wistar rats exposed to DCF exhibited significant diminution in sperm parameters and hormonal status with marked testicular and epididymal degeneration (Adegbegi *et al*., 2014; Owumi *et al*., 2020).

Indeed, exposure of organisms to xenobiotics frequently happens in combinations. Exposure of individuals on medication to environmental pollutants is possible. The non-prescription use of DCF by agriculturalists is mostly unsupervised and usually not the same in all countries. The co-existence of both ATZ and DCF in the aquatic and terrestrial environments due to the intensive anthropogenic actions and poor removal by wastewater treatment plants represents another source of exposure to these contaminants of emerging concern. Hitherto, there is no study in literature on the cellular responses to ATZ and DCF co-exposure. The systemic responses of organisms to chemical mixtures can be assuaged (in case of inhibition or antagonism) or aggravated (leading to additive or synergistic effects) compared with individual chemical in the mixtures (Adedara *et al*., 2017, Hernandez *et al*., 2019). The aim of the present study was to characterize, for the first time, the impact of ATZ and DCF co-exposure on the hypothalamic-pituitary-testicular axis in pubertal rats with arrays of parameters including sperm characteristics, endocrine status, antioxidant defense system, oxido-inflammatory indices and tissue microscopic assessment.

MATERIALS AND METHODS

Chemicals: Diclofenac (DCF) and 2′,7′-dichlorofluorescein diacetate (DCFH-DA) were obtained from Sigma Aldrich (St. Louis, MO, USA). Technical grade atrazine (ATZ) was obtained from Shandong Vicome Greenland Chemicals Company Limited (Shandong, China). Enzyme-Linked Immunosorbent Assay (ELISA) kit (Elabscience Biotechnology Company, Beijing, China) was used for interleukin-1β (IL-1β) assay.

Animal maintenance: A total of sixty male Wistar rats (10 weeks old, 163 ± 6 g) gotten from the Faculty of Veterinary Medicine, University of Ibadan were kept in plastic cages with adequate quantity of wood shavings (beddings) within a well-ventilated vivarium in the Department of Biochemistry, University of Ibadan. They were maintained under a 12 h light and 12 h dark photocycle with free access to water and rat foods. Moreover, the rats were acclimatized for one week prior to treatment. The experimentation was in consonance with the approved guidelines of the University of Ibadan Ethical Committee as well as the U.S. National Institute of Health.

Research design: Six groups of ten animals each were exposed to the test compounds for 42 consecutive days as specified:
Control: Animals orally administered vehicle alone.
ATZ40: Animals orally exposed to ATZ alone at 40 mg/kg body weight.
DCF20: Animals orally exposed to DCF alone at 20 mg/kg.
ATZ20 + DCF10: Animals orally co-treated with ATZ at 20 mg/kg and DCF at 10 mg/kg.
ATZ20 + DCF20: Animals orally co-treated with ATZ at 20 mg/kg and DCF at 20 mg/kg.
ATZ40 + DCF20: Animals orally co-treated with ATZ at 40 mg/kg and DCF at 20 mg/kg.

Taking into consideration the previously reported doses of ATZ and DCF, this investigation reported the impacts of DCF (10 and 20 mg/kg) and ATZ (20 and 40 mg/kg) doses selected from the pilot studies in our laboratory. Twenty-four hours succeeding the final administration of test compounds, the animals were weighed. Blood collected from the retro-orbital venous plexus was allowed to clot before it was processed to obtain the serum. Serum reproductive hormone concentrations were thereafter assessed. The excision of the epididymis, testes and hypothalamus were carefully done and processed for biochemical and histopathological evaluations following euthanization of the rats under light ether anesthesia. Moreover, testosterone concentration was assessed in the interstitial fluid obtained from the testes as previously reported (Adedara *et al*., 2019).

Assessment of sperm quality and quantity: Epididymal sperm concentration and the progressive motility of the sperm cells were evaluated according to established methodologies of WHO (1999) and Zemjanis (1970), respectively. Sperm morphological abnormalities and viability were evaluated according to standard procedures (Wells and Awa, 1970; Adedara *et al*., 2019). Testicular sperm count and daily sperm production were analyzed in the frozen left testes in line with established protocols (Blazak *et al*., 1993).

Reproductive hormones concentration assay: Concentrations of serum LH and FSH as well as intra-testicular and serum testosterone were analyzed using rats specific ELISA kits: LH (E-EL-R0026), FSH (E-EL-R0391) and testosterone (E-EL-R0033) as per the directions from Elabscience Biotechnology Company (Beijing, China). The sensitivities of testosterone, LH and FSH were 0.21, 0.35 and 0.28 ng, respectively. The intra-assay coefficients of variations were 3.7%, 2.9%, and 3.5 % for testosterone, LH and FSH, respectively.

Sample preparation for biochemical endpoints: Epididymal, testicular and hypothalamic tissues excised from the animals were separately homogenized in 0.05 M Tris-HCl buffer, pH 7.4. The homogenates were then centrifuged for 15 minutes at 12,000 g. The supernatant was obtained and used to assay biochemical endpoints. Protein concentration of the samples was analyzed using established procedure (Bradford 1976).

Testicular function indices: Testicular function was assessed by monitoring specific enzyme activities in the testes supernatant. Alkaline phosphatase (ALP) and acid phosphatase (ACP) activities were analyzed using established methodologies (Malymy and Horecker 1966, Vanha-Perttula and Nikkanen 1973). Lactate dehydrogenase-X (LDH-X) and glucose-6-phosphate dehydrogenase (G6PD) activities were analyzed using standard protocols (Vassault 1983, Salihu *et al*., 2017).

Oxidative and inflammatory stress indices assay: Testicular, epididymal and hypothalamic levels of reactive oxygen and nitrogen species (RONS) was analyzed at 488 nm

(excitation) and 525 nm (emission) according to standard procedure (Adedara *et al*., 2016). Estimation of lipid peroxidation (LPO) level using malondialdehyde (MDA) was done at 532 nm according to Farombi *et al*. (2000). Moreover, activities of antioxidant enzymes catalase (CAT) was analyzed at 240 nm as previously reported by (Aebi 1984), superoxide dismutase (SOD) at 480 nm by Misra and Fridovich (1972), glutathione-S-transferase (GST) at 340 nm by Habig *et al*. (1974) and glutathione peroxidase (GPx) at 412 nm by Rotruck *et al*. (1973). Level of glutathione (GSH) was evaluated at 412 nm according to standard procedure (Jollow *et al*., 1974). In addition, markers of inflammatory response namely nitric oxide (NO) level and myeloperoxidase (MPO) activity were analyzed in line with standard protocols (Green *et al*., 1982; Granell *et al*., 2003). The assessment of IL-1β concentration in the investigated tissues was done using ELISA Kits as stated by the manufacturer (Elabscience Biotechnology Company, Beijing, China). Analysis of CAT and SOD activities were done using 752S UV-VIS Spectrophotometer (Ningbo, China) whereas the remaining biochemical endpoints were analyzed with a SpectraMax microplate reader (Molecular Devices, CA, USA).

Tissue microscopic evaluation: Microscopic evaluation of the epididymal, testicular, and hypothalamic tissues excised from the animals were done in consonance with Bancroft and Gamble (2008). In brief, the excised tissues were fixed in Bouin's solution prior to dehydration, paraffin embedment and sectioning to 5 µm pieces with a microtome. Hematoxylin and Eosin staining of the sections was done on clean slides which were subsequently coded before histological evaluation by pathologists with a light microscope (Leica DM 500, Germany) and capturing of images with a digital camera (Leica ICC50 E, Germany).

Statistical analyses: The verification of the normal distribution and homogeneity of data was done according to Kolmogorov-Smirnov and Bartlett's tests, respectively. Data analysis by one-way analysis of variance (ANOVA) and subsequently, Bonferroni's post-hoc test was done with GRAPHPAD PRISM 5 software (Version 4; GraphPad Software, La Jolla, California, USA). Statistically significant values were set at $P < 0.05$.

RESULTS

Reproductive hormonal concentrations in ATZ and DCF co-exposed animals: Figure 1 portrays the influence of joint exposure to ATZ and DCF on the levels of serum testosterone, LH and FSH as well as intra-testicular testosterone concentration in the exposed animals. In comparison with the control, the serum concentrations of testosterone, LH and FSH with intra-testicular testosterone concentration were significantly ($p < 0.05$) decreased in animals exposed to ATZ or DCF whereas these effects were largely exacerbated in animals exposed to ATZ20 + DCF20 or ATZ40 + DCF20. Moreover, the influence of ATZ and DCF joint exposure on Leydig cell function was investigated by calculating the serum testosterone/LH ratio. The marked diminution in serum testosterone/LH ratio by individual exposure to ATZ or DCF was predominantly exacerbated in animals exposed to ATZ20 + DCF20 or ATZ40 + DCF20.

Activities of testicular function marker enzymes in ATZ and DCF co-exposed animals: Figure 2 portrays the effect of co-exposure to ATZ and DCF on the activities of testicular function marker enzymes in the experimental animals. When compared with the control, exposure to ATZ and DCF elicited similar effects on the testicular ALP, ACP and G6PD activities but differentially affected LDH activity. The significant ($p < 0.05$) increases in testicular ALP and ACP activities due to ATZ alone and DCF alone were intensified in animals co-exposed to ATZ20 + DCF10, ATZ20 + DCF20 or ATZ40 + DCF20. Moreover, ATZ alone significantly increased LDH activity whereas it was markedly reduced in DCF-exposed animals. The individual effects of ATZ and DCF were markedly abated in the co-exposed animals.

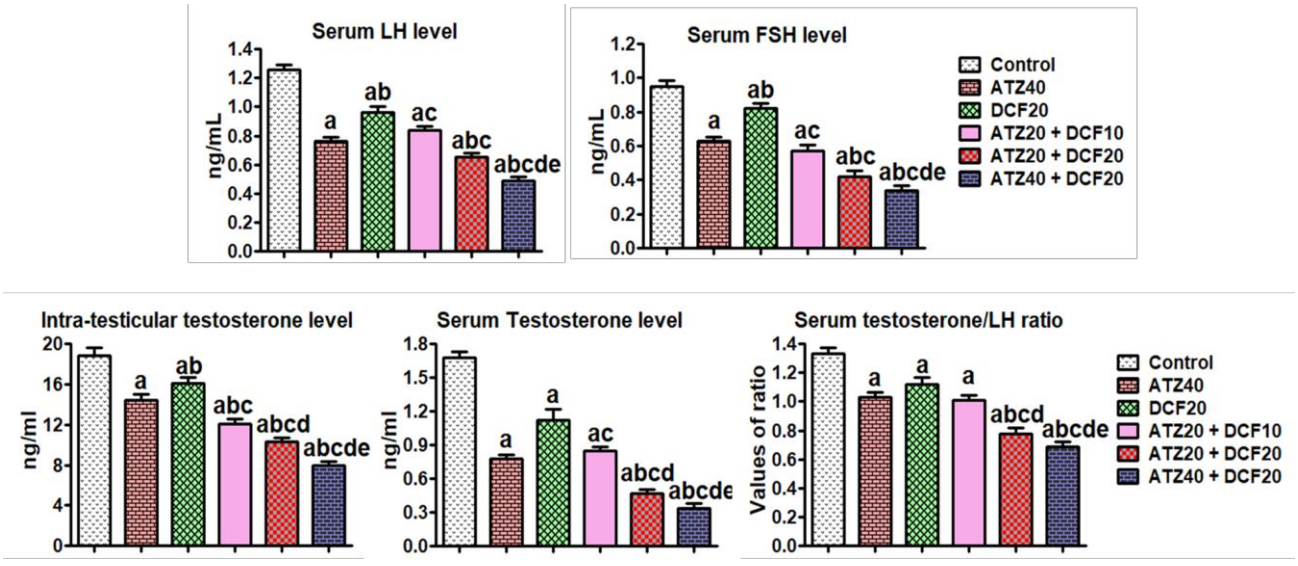

Figure 1: Influence of ATZ and DCF co-exposure on gonadotropins, serum and intra-testicular testosterone and index of Leydig cell function in rats. ATZ denotes Atrazine. DCF denotes Diclofenac. a: Significantly vary from control. b: Significantly vary from ATZ40. c: Significantly vary from DCF20. d: Significantly vary from ATZ20 + DCF10. e: Significantly vary from ATZ20 + DCF20.

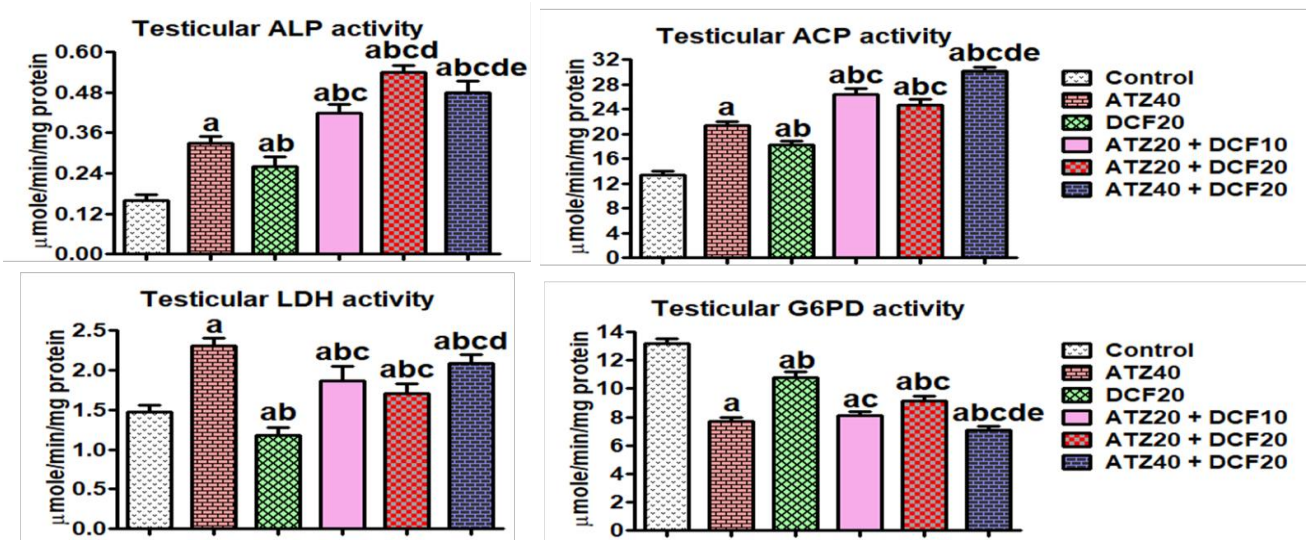

Figure 2:
Effect of ATZ and DCF joint exposure on testicular function marker enzymes activities in rats. ATZ denotes Atrazine. DCF denotes Diclofenac.. a: Significantly vary from control. b: Significantly vary from ATZ40. c: Significantly vary from DCF20. d: Significantly vary from ATZ20 + DCF10. e: Significantly vary from ATZ20 + DCF20

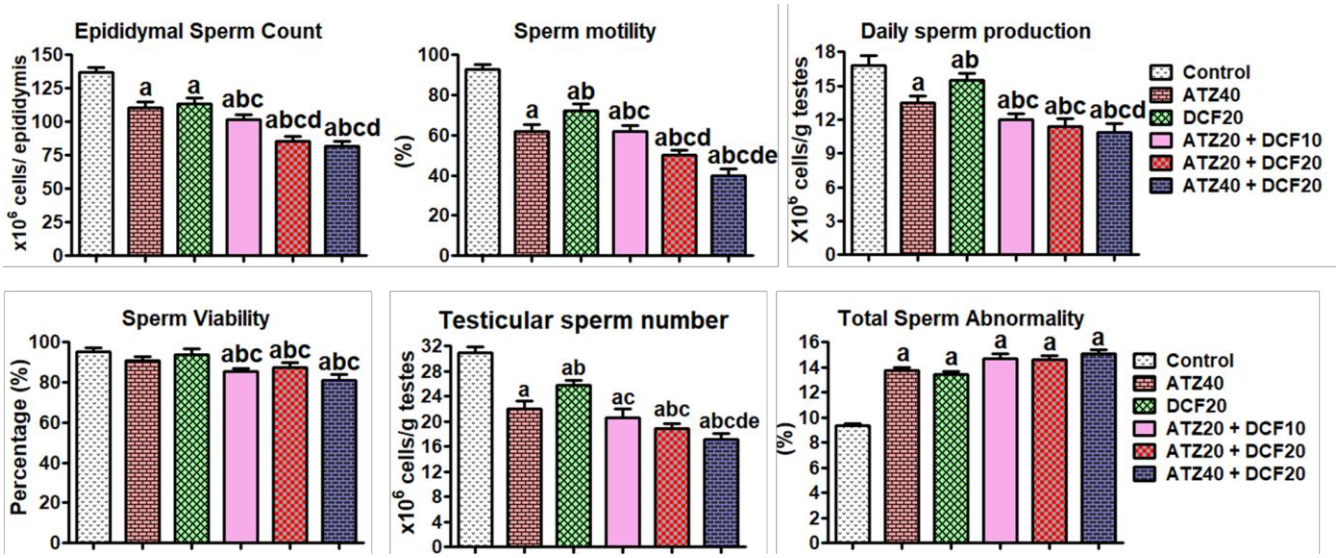

Figure 3: Influence of ATZ and DCF co-exposure on indices of sperm quantity and quality in rats. ATZ denotes Atrazine. DCF denotes Diclofenac.. a: Significantly vary from control. b: Significantly vary from ATZ40. c: Significantly vary from DCF20. d: Significantly vary from ATZ20 + DCF10. e: Significantly vary from ATZ20 + DCF2

Indices of sperm quality and spermatogenesis in ATZ and DCF co-exposed animals: Figure 3 portrays the harmful effects of co-exposure to ATZ and DCF on the indices of spermatogenesis and sperm function in the exposed animals. Animals singly exposed to ATZ and DCF exhibited significant reductions in epididymal sperm count and motility compared with the control. The reductions in the sperm count and motility were exacerbated in co-exposed animals. Further, the sperm viability which not affected following individual exposure to ATZ and DCF was markedly diminished in the co-exposed animals. The marked reductions in the testicular sperm count and daily sperm production following separate exposure to ATZ and DCF were exacerbated in the co-exposed animals. Separate and co-exposure to ATZ and DCF similarly elevated the sperm defects which were mainly bent tails and curved mid-piece in the exposed animals.

Oxido-inflammatory stress markers in ATZ and DCF co-exposed animals: Figures 4-8 portray the influence of co-exposure to ATZ and DCF on the biomarkers of oxidative and inflammatory stress in the exposed animals. In comparison with the control, the epididymal, testicular and hypothalamic activities of antioxidant enzymes specifically SOD, CAT, GPx and GST with GSH level were significantly ($p < 0.05$) decreased in animals singly exposed to ATZ or DCF. These effects were mainly worsened in animals exposed to ATZ20 + DCF20 or ATZ40 + DCF20. Conversely, the elevation in the biomarkers of oxidative stress namely RONS and LPO in epididymal, testicular and hypothalamic tissues of animals singly exposed to ATZ and DCF were intensified in the co-exposed animals. Also, the elevation in inflammatory stress endpoints (i.e., MPO activity and levels of NO and IL-1β) were significantly augmented in epididymal, testicular and hypothalamic tissues of co-exposed animals.

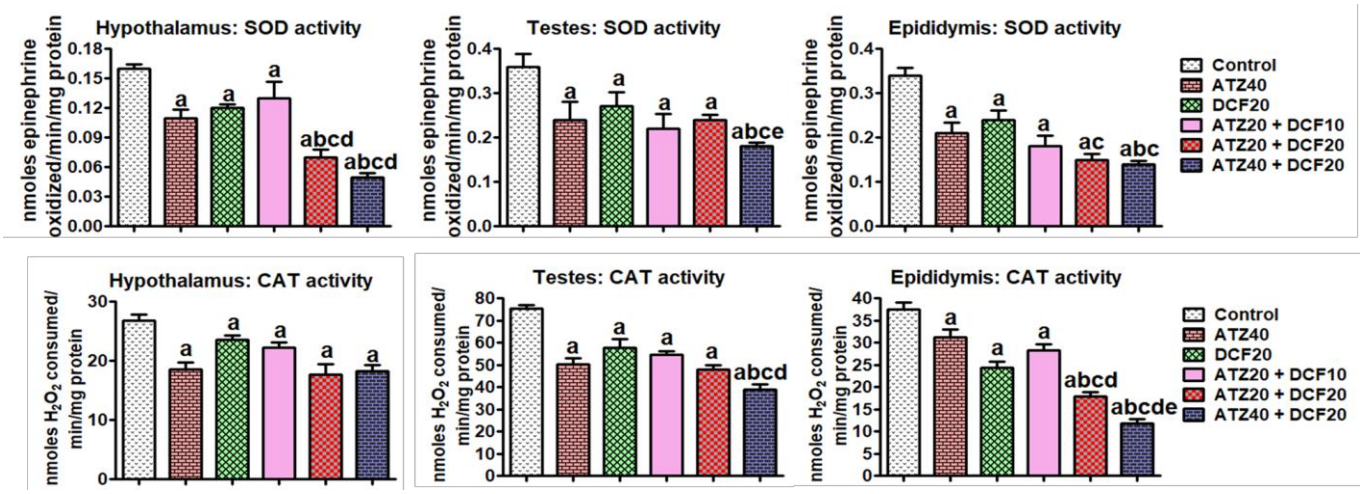

Figure 4: Influence of ATZ and DCF co-exposure on SOD and CAT activities in epididymis, testes and hypothalamus of rats. ATZ denotes Atrazine. DCF denotes Diclofenac.. a: Significantly vary from control. b: Significantly vary from ATZ40. c: Significantly vary from DCF20. d: Significantly vary from ATZ20 + DCF10. e: Significantly vary from ATZ20 + DCF20.

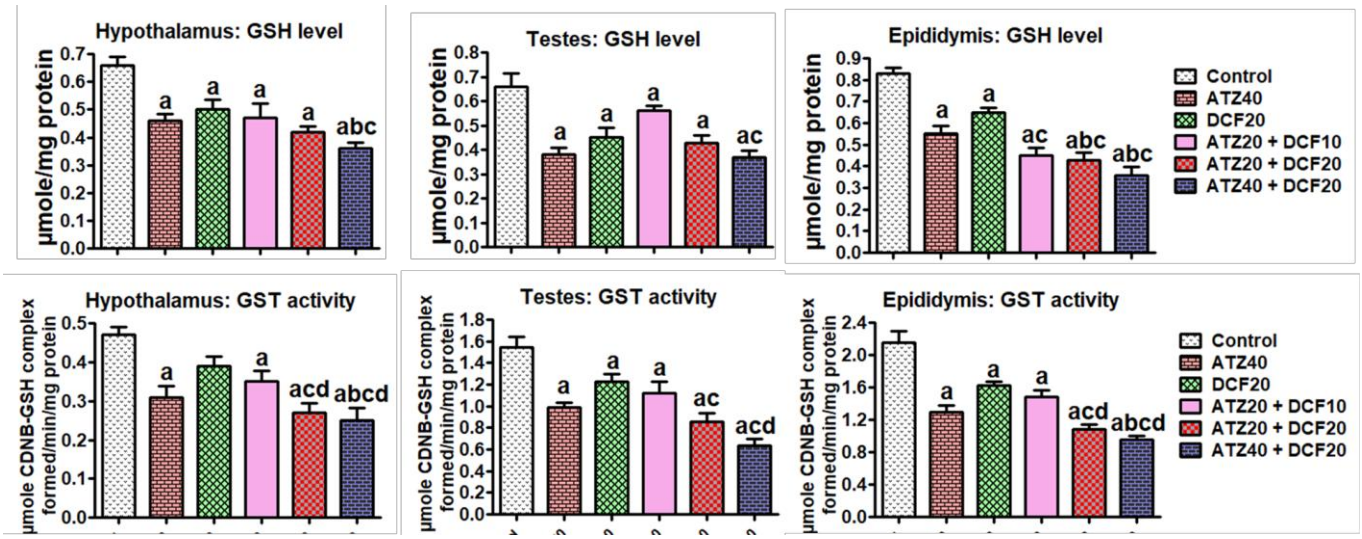

Figure 5: Influence of ATZ and DCF co-exposure on GST activity and GSH level in epididymis, testes and hypothalamus of rats. ATZ denotes Atrazine. DCF denotes Diclofenac.. a: Significantly vary from control. b: Significantly vary from ATZ40. c: Significantly vary from DCF20. d: Significantly vary from ATZ20 + DCF10. e: Significantly vary from ATZ20 + DCF20.

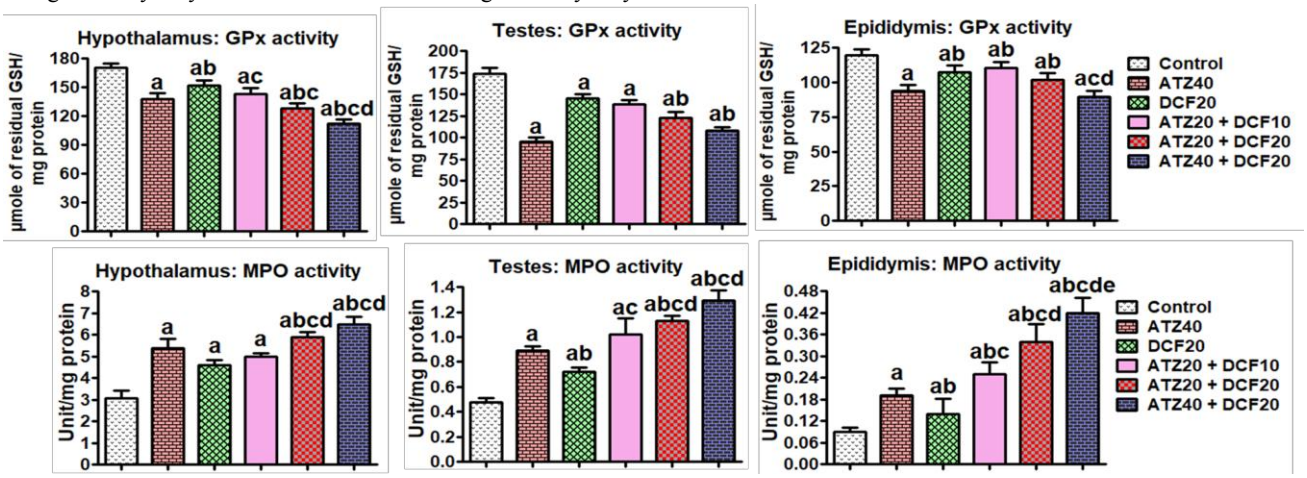

Figure 6: Influence of ATZ and DCF co-exposure on GPx and MPO activities in epididymis, testes and hypothalamus of rats. ATZ denotes Atrazine. DCF denotes Diclofenac.. a: Significantly vary from control. b: Significantly vary from ATZ40. c: Significantly vary from DCF20. d: Significantly vary from ATZ20 + DCF10. e: Significantly vary from ATZ20 + DCF20.

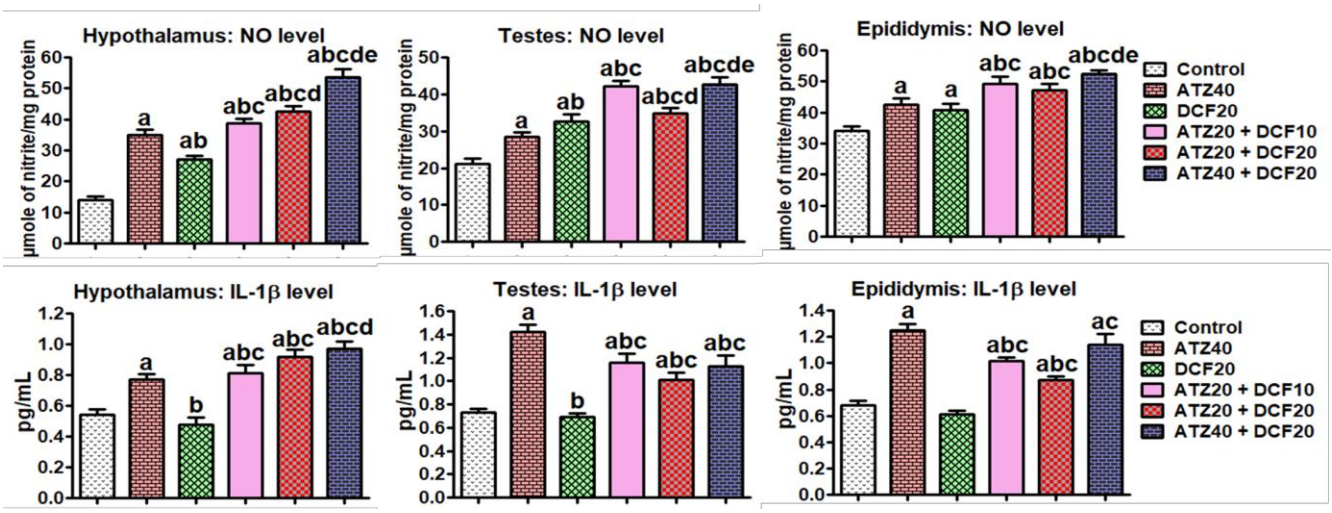

Figure 7: Influence of ATZ and DCF co-exposure on NO and IL-1β levels in epididymis, testes and hypothalamus of rats. ATZ denotes Atrazine. DCF denotes Diclofenac.. a: Significantly vary from control. b: Significantly vary from ATZ40. c: Significantly vary from DCF20. d: Significantly vary from ATZ20 + DCF10. e: Significantly vary from ATZ20 + DCF20.

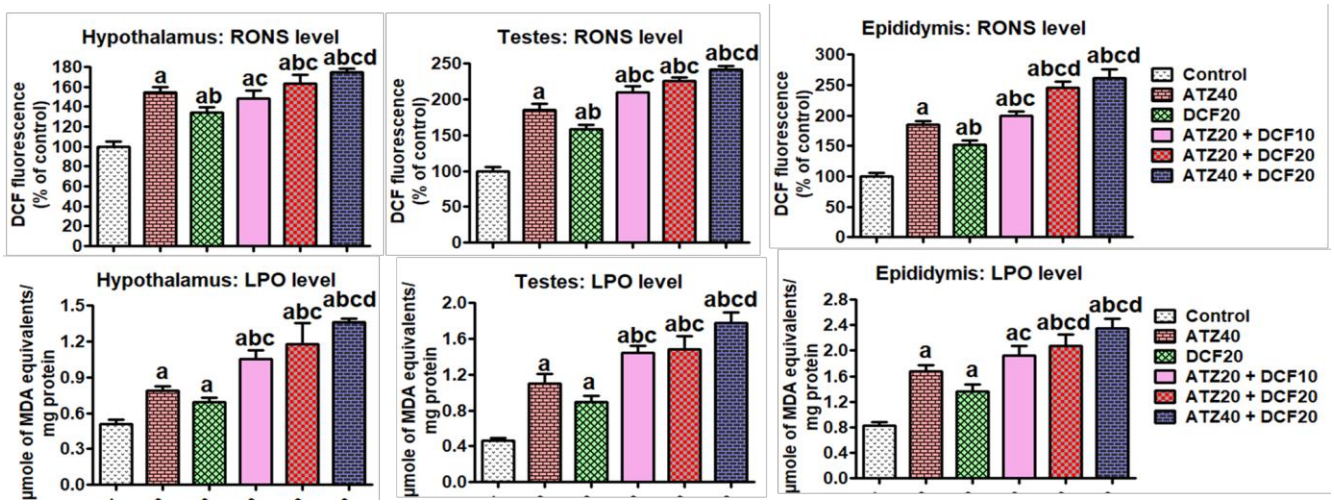

Figure 8: Influence of ATZ and DCF co-exposure on RONS and LPO levels in epididymis, testes and hypothalamus of rats. ATZ denotes Atrazine. DCF denotes Diclofenac.. a: Significantly vary from control. b: Significantly vary from ATZ40. c: Significantly vary from DCF20. d: Significantly vary from ATZ20 + DCF10. e: Significantly vary from ATZ20 + DCF20.

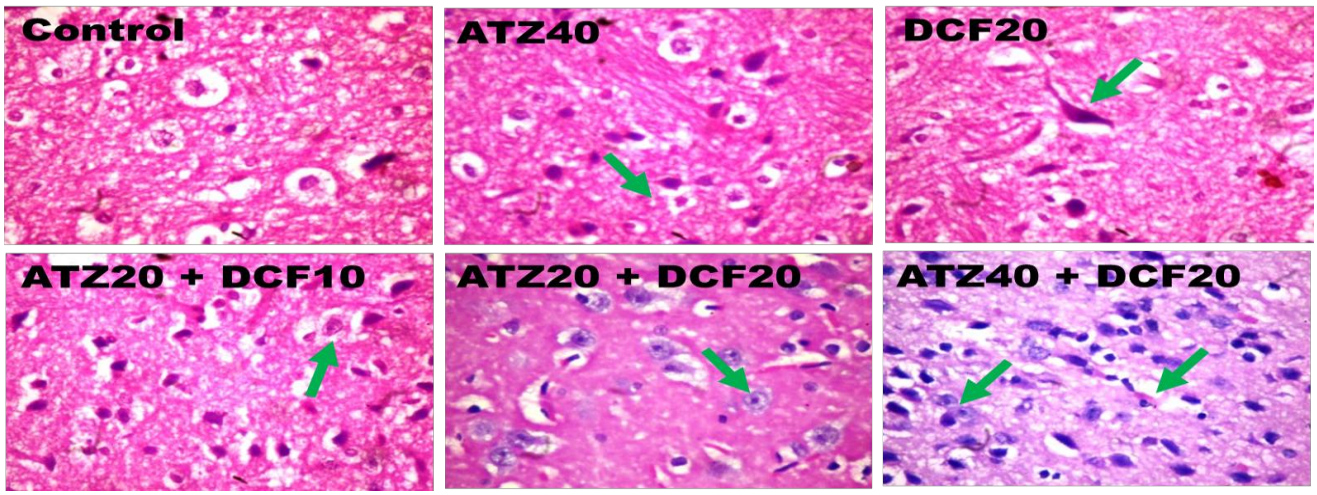

Figure 9: Representative histological lesions identified in hypothalamus of animals co-exposed to ATZ or DCF. The microscopic appearances of hypothalamus of control animals were normal. However, the severity of hypothalamic neuronal degeneration (green arrows) in animals singly exposed to ATZ and DCF was worsened in the co-exposure groups. Mag: x400.

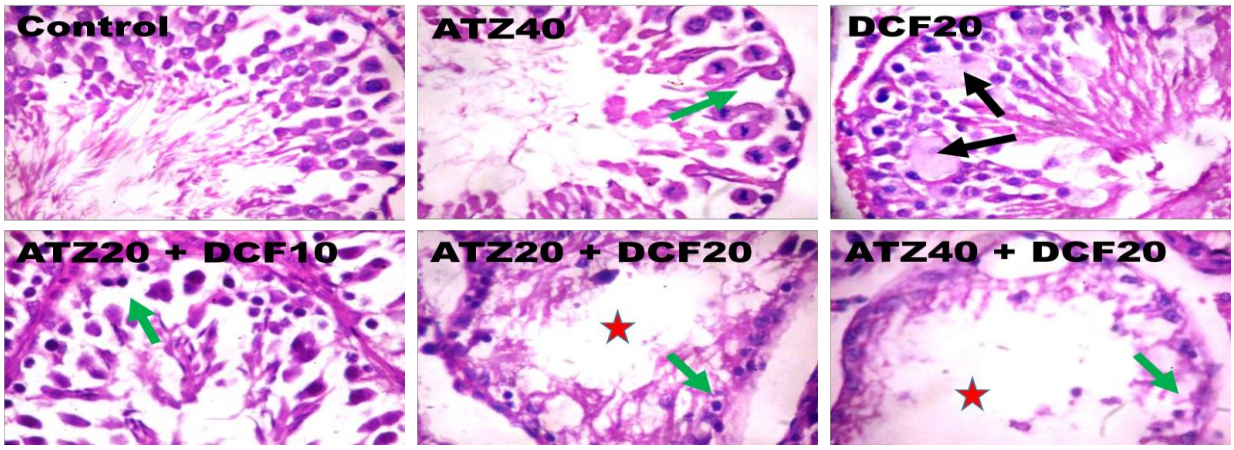

Figure 10: Representative histological lesions identified in testes of animals co-exposed to ATZ and DCF. The microscopic appearances of seminiferous tubules of control animals were normal. Testicular toxicity elicited by individual exposure to ATZ or DCF were worsened as characterized by severe seminiferous tubules degeneration (black arrows), reduced sperm cells (green arrows) with a few multifocal tubules and vacuolization (red stars) in the co-exposure groups. Mag: x400.

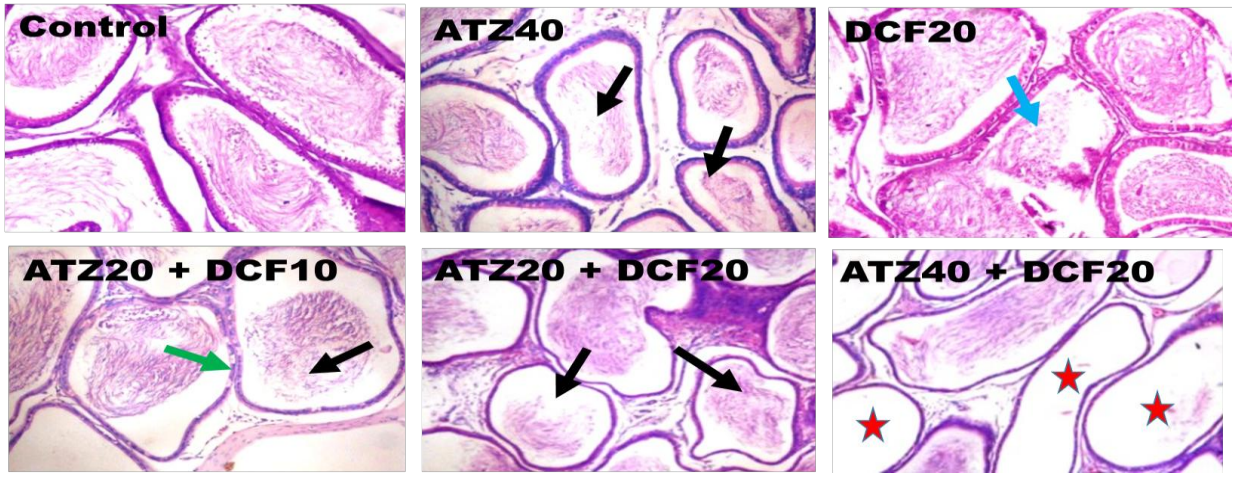

Figure 11: Representative histological lesions identified in epididymis of animals co-exposed to ATZ and DCF. Epididymal morphology of control animals was normal. The lesions associated with separate exposure to ATZ or DCF were intensified in the co-exposure groups and characterized by reduced epididymal sperm cells (black arrow) tubular degeneration (blue arrow), reduced epididymal lining (green arrows) with a few vacuolization (red stars). Mag: x400.

Histological lesions in epididymal, hypothalamic and testicular tissues of ATZ and DCF co-exposed animals: Figures 9-11 portray typical histological appearances of the epididymis, testes and hypothalamus of the exposed animals. The histological appearances of epididymis, testes and hypothalamus of control animals appeared normal. However, the severity of hypothalamic neuronal degeneration in animals singly exposed to ATZ or DCF was worsened in the co-exposure groups. Similarly, the testicular injuries induced by separate exposure to ATZ or DCF were exacerbated as characterized by severe seminiferous tubules degeneration, reduced sperm cells with a few multifocal tubules and vacuolization in the co-exposed groups. The epididymal lesions resulting from separate exposure to ATZ or DCF were intensified in the co-exposure groups and characterized by reduced epididymal sperm cells tubular degeneration, reduced epididymal lining with a few vacuolization

DISCUSSION

The noxious effects of separate exposure to pesticides including ATZ and pharmaceuticals like DCF have been previously reported; however, scientific information on the possible outcome of co-exposure to these contaminants of emerging concern due to their high consumption and discharge to the environment is scarce. This scientific information is important for strategic risk assessment of exposure to pesticides and pharmaceuticals.

The pituitary gland releases LH which is responsible for the initiation of testosterone secretion by Leydig cells whereas FSH triggers testicular growth and Sertoli cells maturation. Moreover, FSH stimulates the Sertoli cells to synthesize androgen-binding protein which is essential for spermatogenesis (Sharpe, 1994; Walker and Cheng, 2005; Adedara *et al*., 2014; Shiraishi and Matsuyama 2017). The current research evidenced that co-exposure to ATZ and DCF mediated greater depletion in the serum LH and FSH levels more than separate treatments with ATZ or DCF. These findings indicate that co-exposure to these compounds elicited greater interference and detrimental impact on the pituitary function than separate exposure. The diminished serum pituitary hormone levels may modify Sertoli cell maturation and consequently result in the impairment of Leydig cell function. The marked decrease in both serum and intra-testicular testosterone levels which was intensified in co-exposure group is related to the LH diminution and impaired

Leydig cell function as substantiated by reduced serum testosterone/LH ratio, an acknowledged index of Leydig cell function (Holm *et al*., 2003, Muerköster *et al*., 2020).

The impact of ATZ and DCF co-exposure on spermatogenesis was further investigated by assessing the testicular LDH, G6PD, ALP and ACP which are acknowledged metabolic enzymes central to the maintenance of germ cell growth and spermatogenesis (Salihu *et al*., 2017). Testicular ACP is predominantly located in the Sertoli cells, the nurse cells which give nutritional and structural support during spermatogenesis whereas ALP converts 6-phosphoglucose to free glucose which is utilize during proliferation and differentiation of spermatogenic cells. The findings from the present research indicate that co-exposure to ATZ and DCF elicited greater detrimental impact via induction of testicular phosphatase activities than their separate exposures. The marked increases in these enzymes may thus indicate an adaptive response to recover spermatogenic cell maturation and spermatogenesis from testicular degeneration following exposure to these compounds.

In the current investigation, co-exposure to ATZ and DCF markedly alleviated the increase in testicular LDH activity compared to ATZ alone but significantly increased LDH activity in comparison to control and DCF alone. These findings denote unfavorable modification of this important metabolic enzyme as well as an additive noxious effect of the co-exposure on lactate metabolism and maturation of spermatogenic cells in the animals. The wane effects of ATZ alone, DCF alone and their combined exposure on the testicular G6PD activity in the current research indicates an interference of these compounds with its role in the pentose phosphate pathway to synthesize NADPH and regenerate GSH, a potent endogenous antioxidant, during oxidative stress in the testes of the exposed animals.

Moreover, the impact of co-exposure to ATZ and DCF on semen was investigated by evaluating sperm quantity and quality in the animals. The current research demonstrated that the marked reductions in sperm count, and motility was accompanied by significant elevation in sperm abnormalities in animals exposed to ATZ alone or DCF alone compared with control. These observations were further worsened with concomitant significant reduction in sperm viability in animals co-exposed to ATZ and DCF, thus indicating that ATZ and DCF elicited greater detrimental impact on sperm functional indicators in the animals. Similarly, co-exposure to ATZ and DCF exacerbated the reductions in the daily sperm production and testicular sperm count induced by ATZ alone and DCF alone. These findings indicate that co-exposure to ATZ and DCF mediated greater testicular toxicity and disruption of spermatogenesis than separate exposure to ATZ or DCF.

Endogenous antioxidant enzymes and non-enzymatic antioxidant are key defense mechanisms which maintain normal cellular redox status by minimizing ROS concentrations (Chen *et al*., 2013; Kruk *et al*., 2019). The present research demonstrated that co-exposure to ATZ and DCF elicited greater induction of oxidative damage with concomitant decrease in the activities of CAT, SOD, GST and GPx as well as GSH level in the epididymis, testes and hypothalamus of the animals. These observations indicate that the marked increase in RONS production overwhelmed the antioxidant capability of the epididymal, testicular and hypothalamic locale and consequently, elicited oxidative injury related to histological alterations detected in these tissues.

Additionally, the current investigation showed that animals co-exposed to ATZ and DCF exhibited greater inflammatory stress response than separate exposure to ATZ or DCF as evidenced by the enhanced NO level, MPO activity and IL-1β concentration in the epididymis, testes, and hypothalamus of the animals. Undue cellular production of NO during exposure to toxicants is associated with protein nitration and impaired signal transduction pathways (Kapil *et al*., 2020), whereas MPO which possesses cytokine-related activity excites neutrophils and consequently induces ROS production and inflammation (Lau *et al*., 2005). The injury induced by co-exposure to ATZ and DCF in the animals specifically excites secretion of pro-inflammatory cytokine IL-1β which is well-known to suppress the testicular immune microenvironment. Thus, the significant elevation in the inflammatory biomarkers indicates the contribution of inflammation to the hypothalamic-pituitary-gonadal axis dysfunction and consequently, the suppression of spermatogenesis in the exposed animals.

In conclusion, ATZ and DCF co-exposure aggravated their separate reprotoxic effects by communal mechanisms involving endocrine disruption, depletion of antioxidant status, elevated RONS production and inflammatory stress. The findings from the current research accentuate the possible public health threats related to concurrent exposure to pesticides and pharmaceuticals.

Acknowledgment

This research was done without specific grant from any funding agency in the public, commercial, or not-for-profit sectors

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Acknowledgement

The publication of this issue of Archives of Basic and Applied Medicine was funded by the University of Ibadan Medical Education Partnership Initiative Junior Faculty Research Training Program (UI-MEPI-J), Award Number D43TW010140 from the Fogarty International Centre. The content is solely the responsibility of the authors and does not necessarily represent the official views of Fogarty International Centre or the National Institute of Health.