Testicular Toxicity of the Concurrent Administration of Cadmium And Arsenic Through The Food Chain

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ABSTRACT

BACKGROUND
This study examined the effects of Cadmium (Cd) and Arsenic (As) through a controlled food chain on testes antioxidant status, protein concentration and expression of mRNA of Bax and Bcl - 2 genes.

METHODS
Catfish exposed to both metals at a concentration of 0.4 mg/ metal / 100 mL for 1 month served as source of protein for the experimental diet which rats were exposed to for 1 and 3 months. The metal burden on the feed and testes, activities of antioxidant enzymes, mRNA expression of Bax and Bcl - 2 in the testes were then carried out using standard procedures.

RESULT
The activities of the antioxidants enzymes and reduced glutathione (GSH) were significantly ($p < 0.05$) decreased after 3 months. Testes total protein and MDA levels were significantly ($p < 0.05$) increased after both periods of exposure. Increase in the level of mRNA expression of Bax gene and a decrease in Bcl - 2 gene in the test groups compared to the control after 3 months of exposure were recorded.

CONCLUSION
These results showed exposure to these metals through the food chain increased oxidative damage leading to alteration in the expression of Bax / Bcl - 2 ratios. These have significant consequences in the induction of apoptosis and other associated biochemical cascade.

KEYWORDS
Bcl, Bax, Testes, Cadmium, Arsenic

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How to Cite This Article:

Theresa Ezedom, Samuel Ogheneovo Asagba. Testicular toxicity of the concurrent administration of cadmium and arsenic through the food chain. J Evid Based Med Healthc 2022;9(7):40.

Received date: 08-March-2022;
Manuscript No: JEBMH-22-50711;
Editor assigned date: 11-March-2022;
PreQC No. JEBMH-22-50711(PQ);
Reviewed date: 25-March-2022;
QC No. JEBMH-22-5071;
Revised date: 30-March-2022;
Manuscript No. JEBMH-22-50711(R);
Published date: 05-April-2022;
DOI: 10.18410/jebmh/2022/09.07.40

INTRODUCTION

Several studies on the decline in male reproductive ability as a result of environmental toxicants have been reported. Of the different contaminants present in the environment, Cadmium (Cd) and Arsenic (As) are among the top list. Cd is an environmental contaminant gotten primarily from mining, plastics manufacturing, paint pigments, electroplating, alloy preparation, and batteries. For the non-smoking and non-occupationally exposed population, food is the most important source of exposure to Cd. It can exert both carcinogenic and non-carcinogenic effects on various tissues such as the lung, bone, liver, kidney etc. Cd has also been found to have adverse effects on the endocrine and reproductive systems and exerts its toxicity through several biochemical mechanisms associated with oxidative stress and cell death. Arsenic is a well - known environmental pollutant whose exposure could occur through drinking As-contaminated water. Other important sources of exposure to this chemical is through food and inhalation.[1-7] Arsenic is a carcinogen which is capable of causing tumors in a variety of tissues including the skin, liver, kidney, lung, prostate etc. It has also been shown to be capable of inducing oxidative stress and cell death. Cell death could occur through apoptosis, which is characterized by loss of plasma membrane phospholipids asymmetry, enzymatic cleavage of the DNA, condensation of nuclear chromatin and cell segmentation into apoptotic bodies. The main regulators of this process are the Bcl - 2 family proteins. These help in the suppression of cell death. Bax - like subgroup such as Bax, Bak, Bok and Bik has been reported to promote cell death. Although the exact mechanism through which Bcl - 2 family proteins regulate the apoptotic pathway has not been fully elucidated, it has been shown to be dependent on protein-protein interactions. This protein-protein interaction could be induced by mixtures of chemicals in the environment. According to a mixture is defined as the combination of two or more environmental agents. The toxicological actions of components in a mixture could be described using the concept of additively and interaction (which could be synergism or antagonism). In order to describe the combined action of the components in the mixture of metals, the most common approach utilized is to carry out experimental studies to compare the effects of the individual components to the effects of the mixture. However, a study on the possible effects of metals and mixture of metals when provided through the food-chain is scarce in literature. The importance of the food-chain as one of the major sources of human exposure to metals and the increasing rate of male infertility underscore the need for the present study. The aims of the study were to evaluate total protein concentration, antioxidant status as well as expression of Bax and Bcl - 2 genes of the testes of rats exposed to Cd and As (singly and in mixture) through a controlled food-chain and to evaluate the comparative effect of both metals when administered singly and in combination through a controlled food-chain.[8-15]

MATERIALS AND METHODS

Catfish (first trophic level) were obtained from a local fish pond located in Imoje-Orogun, Delta State, Nigeria. Exposure of the fishes to the metals was done using plastic troughs for a period of 1 month. The fishes were divided into four groups. Group A which served as the control had fish kept in fresh water. In Groups B, C and D, fishes were kept in water contaminated with a nominal concentration of 0.4 mg of metal / 100 mL of water using Cd, as and Cd + As, respectively. The concentration of metal to which the fishes were exposed to is in accordance with previous studies. The water, in which the fishes were kept, was changed and re-contaminated every 24 hours. During the 1 month exposure, fishes were provided with normal commercial feed before being sacrificed, dried and used as a source of protein in compounding the experimental diet (Table 1). The Cd and as contents of these diets were determined by atomic absorption spectrophotometry.[16-21]

	Percentage (%) composition			
Ingredients	Control	Cd-contaminated feed	As-contaminated feed	(Cd + As) contaminated feed
Control Fish	20	-	-	-
Cd treated fish	-	20	-	-
As teated fish	-	-	20	-
Cd + As treated fish	-	-	-	20
Carbohydrate	55	55	55	55
Fats and oil	10	10	10	10
Fibre (Cellulose)	10	10	10	10
Multi vitamin/ mineral mix	5	5	5	5

Table 1. Composition of Experimental Diet.

Treatment of animals

The sixty-four (64) adult male albino rats with average weight of 126.25 ± 3.59 g which were used for the study were gotten from the Animal house of College of Health Sciences, Delta State University, Abraka, Nigeria. The rats were acclimatized for 1 week, given a 12 h light and dark cycle with free access to normal rat chow pellet and water. Thereafter, the animals were categorized into 4 experimental groups with 16 rats per group. Groups A served as the control. Rats in Group B were exposed to the Cd contaminated diet while rats in Groups C and D were exposed to the As and Cd + As compounded diets, respectively. After exposure to the experimental diet for 1 month, half the number of rats in each group were sacrificed and the other half after 3 months of exposure. The animals were sacrificed under chloroform anesthesia and their testes were excised and used immediately for biochemical analysis. The use of rat models in the present study was subject to approval by the ethical committee of the College of Health Sciences, Delta State University, Abraka, Nigeria. All sections of this report adhere to the arrive Guidelines for reporting animal research.[22-25]

Metal Analysis

Weighed samples of the feed and testes of experimental rats of each group were digested separately in beakers with 20 ml of concentrated acid mixture (98 % w/ v HNO_3 / $HClO_4$, 4:1 v / v) at 100 ˚C. After digestion of samples, the Cd and as concentrations in the tissues and feeds were measured using a Varian AA 1475 spectrophotometer. An International Atomic Energy Agency (IAEA) reference biological sample was used for the evaluation of the accuracy and precision of the analysis.[26]

Biochemical assays

The testes of the rats were weighed, homogenized, centrifuged and the supernatants obtained were used for the determination of the activities of superoxide dismutase (SOD), Catalase (CAT), Glutathione Transferase (GST) based on the methods the levels of reduced glutathione (GSH), total

protein and lipid peroxidation were determined using the methods respectively.[27]

Preparation of guanidine isothiocyanate (GITC) lysate

Weighed samples of testes from rats in each group and for both durations of study were pounded aseptically using mortar and pestle under cold conditions and then transferred into 2 mL eppendorf tube. GITC solution was activated and the GITC lysate prepared based on the method. The lystate were stored at – 16 °C prior to use.[28]

Isolation and purification of RNA from GITC lysate

Total RNA of the GITC lysate of testes were extracted using Reliaprep RNA Kit, a product of Promega Corporation Madison, Wisconsin, USA. The integrity and purity of RNA obtained was electrophoretically verified by formaldehyde agarose gel stained with ethidium bromide based on the method of Lehrach (1977).[29]

cDNA Synthesis Protocol

In a reverse transcription reaction mixture containing 1 x PCR buffer, 0.5 mMdeoxy-nucleoside triphosphates (dNTPs), 1 unit of RNase inhibitor, 2.5 µM of olio d (T) 16, and 2.5 units of MuLV reverse transcriptase (Perkin-Elmer, Roche Molecular Systems, Branchburg, New Jersey, USA). One microgram 1 µg of RNA was reverse transcribed into cDNA. This was incubated for 10 mins at room temperature to allow primer annealing, then, the reaction mixture was incubated at 42 °C for 15 min, heated to 95 °C for 5 min, and chilled at 4 °C for 5 min in a GeneAmp thermal cycler (Applied Biosystems, Foster City, CA, USA). Two microliters 2 µL of the resultant cDNA products was used for PCR amplification.[30]

Real time quantitative RT-PCR

A Light cycler 2.0 system (Roche Applied Systems, USA) was used for the Real time quantitative RT-PCR to analysis the expression levels of Bax and Bcl-2 gene relative to glyceraldehyde - 3- phosphate dehydrogenase (GAPDH). The real-time quantitative PCR probe design software (Roche Applied Systems, Branchburg, New Jersey, USA) were used to design primer sets for GAPDH, Bax and Bcl-2. PCR reactions for these primers were first optimised using conventional PCR.[31]

Gene	Primer
GAPDH	F: GGCTCTCTGCTCCTCCCTGTTCTA
	R: TGCCGTTGAACTTGCCGTGG
Bc^{l2}	F: CTG GTG GAC AAC ATC GCT CTG
	R: GGT CTG CTG ACC TCA CTT GTG
Bax	F: TTCATC CAGGAT CGA GCA GA
	R: GCA AAG TAG AAG GCA ACG

Table 2. Primers Used For The Amplification of Bax And Bcl-2 Mrna In The Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction (qrt - pcr)

For the quantitative Real-Time PCR, 20 µL amplification mixtures (Light Cycler Fast start DNA MasterPLUS SYBR Green Reaction Mix; Roche Applied Science) were prepared as per manufacturer's instructions, containing cDNA (equivalent to 100 mg reverse transcribed RNA) and 0.5 µM of each primer. The cycling conditions were: 10 min polymerase activation at 95 °C and 40 cycles at 95 °C for 15 s, 58 °C for 15 s, and 72 °C for 15 s. After thermal cycling, the gel was run with 3 % agarose gel (that is, 3 g of agarose powder in 100 mL of TBE buffer). On cooling, 20 µL EtBr was added. Electrophoresis was run at 100 V for 30 min, watching the controls.[32]

Statistical Analysis

All the data are expressed as mean ± standard deviation (SD). Statistical comparisons were performed by one way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) comparison when ANOVA results indicates statistically significant difference between groups. The SPSS software (version 20) was used in the statistical analysis using multiple comparison tests. A p - value of less than 0.05 ($p < 0.05$) was considered significant.[33]

RESULTS

The results of metal analyses carried out on the feed and testes of rats are shown in (Table 3). There were trace amount of the metals in the control most especially for as the level of Cd was below detection limit. The test groups also had slight contamination of these metals as evident by the mole ratio obtained. The results obtained further showed that the metals accumulated in the testes of rats after both period of exposure.

	Metal Concentration (mg / g)				
Groups	Cadmium	Arsenic	Mole ratio (Cadmium : Arsenic) x 10 - 7	Metal Concentration in testes (mg/g tissue)	
A	Not Detected	0.02 ± 0.56a	-	0.02 ± 0.09a	
B	3.68 ± 0.62b	0.03 ± 0.07a	81.75:1	3.01 ± 1.01b	
C	0.01 ±0.32a	1.82 ± 0.18b	05:33.0	0.38 ± 0.01 c	
D	3.50 ±0.14b	1.52 ±0.26b	1.5:1	Cd in group D diet	As in group D diet
				2.21 ± 0.14d	0.16 ± 0.01c

Table 3. Concentration of Metals in Feed and Testes if Rats.

Results are expressed as Mean ± SD. Values not sharing same superscript in same column differs significantly at ($P < 0.05$). A, Control; B, Cd - contaminated diet; C, as contaminated diet, Cd + As contaminated diet (Table 4). Presents the changes in the body weight and testes / body weight ratio of rats used in the present study for the experimental period of 1 and 3 months. There was a significant ($p < 0.05$) reduction in the body weights of rats in all test groups relative to controls after both periods of study. The weight loss in the 3 months exposure was higher than that of the 1 month exposure and this effect was more severe in rats fed a combination of both metals in diet. There was a significant ($p < 0.05$) decrease in the testes/body weight ratio of rats fed with Cd-contaminated diet and Cd + As - contaminated diet relative to control after 1 month exposure. Similarly, the testes/

body weight ratio of rats exposed to Cd and as singly or in combined form in diet was significantly decreased relative to control. Again, in both durations of exposure, the effect of the metals on testes / body weight ratio was more severe when combined in diet. The effects of the treatment on total protein and antioxidant status of the testes are shown in (Table 5).

The levels of total protein and malondialdehyde (MDA) (an index of lipid peroxidation) were significantly ($p < 0.05$) increased when compared to the control for both periods of exposure. After 1 Month exposure, there was a significant ($p < 0.05$) increase in the level of GSH and activities of SOD, CAT and GST but after 3 months exposure, these were significantly ($p < 0.05$) decreased.

Group	Body Weight Gain (g)	Testes/body weight ratio
1 month exposure		
Control	(+)3.19 ± 0.96a	1.46 ± 0.13a
Cd - contaminated diet	(-)2.22 ± 0.38b	1.15 ± 0.18b
As - contaminated diet	(-)2.91 ± 0.67b	1.34 ± 0.11a
Cd + As - contaminated diet	(-)3.48 ± 0.64c	0.87 ± 0.06b
3 months exposure		
Control	(+)9.24 ± 1.84a	1.63 ± 0.28a
Cd - contaminated diet	(-)6.52 ± 1.34b	0.70 ± 0.07b
As - contaminated diet	(-)7.81 ± 0.26c	1.03 ± 0.06c
Cd + As - contaminated diet	(-)13.08 ± 0.46d	0.61 ± 0.03b

Table 4. Effect of Food Chain Mediated Exposure To Cd And As On Body Weight Gain And Testes/ Body Weight Ratio Of Rats.

Results are expressed as Mean ± SD. n = 16. Values not sharing same superscript in same column differ at ($P < 0.05$).

	Parameter					
Group	MDA (units/g testis)	Total Protein (mg/ml)	SOD (units/g testis)	CAT (µmole H_2O_2/ min/mg protein)	GST (µmol CDNB – GSH complex formed/ min/mg protein)	GSH (mg/ g testis)
1 Month Exposure						
A	56.86±1.56a	6.33 ±0.70a	34.47± 0.20a	67.45 ± 1.48a	7.75 ± 1.14 a	31.00± 1.41a
B	72.53± 1.25b	8.75 ±1.09b	44.27± 0.24b	88.30 ± 1.83a	10.48 ± 1.58b	39.33± 2.31b
	-27.56%	-38.23%	-28.43%	-30.91%	-35.23%	-26.87%
C	69.38± 0.70c	7.50 ±0.21b	40.29± 0.28b	71.85 ± 4.77b	8.10 ± 0.10 a	33.04± 2.82a
	-22.02%	-18.48%	(16.88%)	-6.52 %	- 4.52 %	-6.58%
D	89.51± 5.30d	8.25 ±0.26b	57.49± 1.25c	88.00 ± 5.65c	13.76 ± 3.83c	49.00± 4.24d
	-57.42%	-30.33%	-66.78%	-30.46 %	-77.54 %	-58.06%
3 Months Exposure						
A	59.10± 1.75a	8.00 ±0.08 a	30.73± 0.24a	145.40 ± 1.30a	8.15 ± 0.20 a	32.34± 3.82a
B	86.44±1.30b	17.00 ± 0.17b	18.74± 0.91b	92.30 ± 1.97b	3.23 ± 0.13b	22.02± 2.82b
	-46.26%	-112.50%	(-39.02%)	(- 36.52 %)	(- 60.37 %)	(-31.91%)
C	81.04± 0.62b	15.00 ±0.14c	24.13± 0.66c	102.89 ± 4.14c	7.17 ± 0.06 a	29.21± 4.24c
	-37.12%	-87.50%	-21.48%	(-29.24 %)	(-12.02 %)	(-9.68%)
D	97.02± 3.70c	19.33 ± 0.11d	14.46± 0.59b	78.15 ± 3.18d	2.38 ± 0.12b	20.03± 4.24b
	-64.16%	-141.62%	(-52.45%)	(- 46.25 %)	(-70.80 %)	(-38.06%)

Table 5. Effect of Treatment On Total Protein And Antioxidant Status of Testes.

Results are expressed as Mean ± SD. Values not sharing same superscript in same column differs significantly at ($P < 0.05$). A, Control; B, Cd-contaminated diet; C, As-contaminated diet; D, Cd + As contaminated diet. The changes in the expressions of Bax And Bcl - 2 genes mRNA in the testes of rats exposed to Cd and As contaminated diet for both periods of exposure are shown in (Figure 1). There was a significant ($p < 0.05$) increase in the level of Bax gene mRNA expression for the Cd and Cd + As

contaminated group when compared to the control after 1 month of exposure. However, there was a significant ($p < 0.05$) increase in the mRNA expression of Bax gene in the testes for all test groups when compared to the control after 3 months exposure. The expression of Bcl - 2 genes mRNA was found to be down regulated when compared to the control for all groups. In the group exposed to As contaminated diet, this reduction was not significant ($p < 0.05$) after 1 month exposure when compared to the control. However, after 3 months exposure, there was a significant ($p < 0.05$) decrease in the level of Bcl - 2 mRNA for all test group.

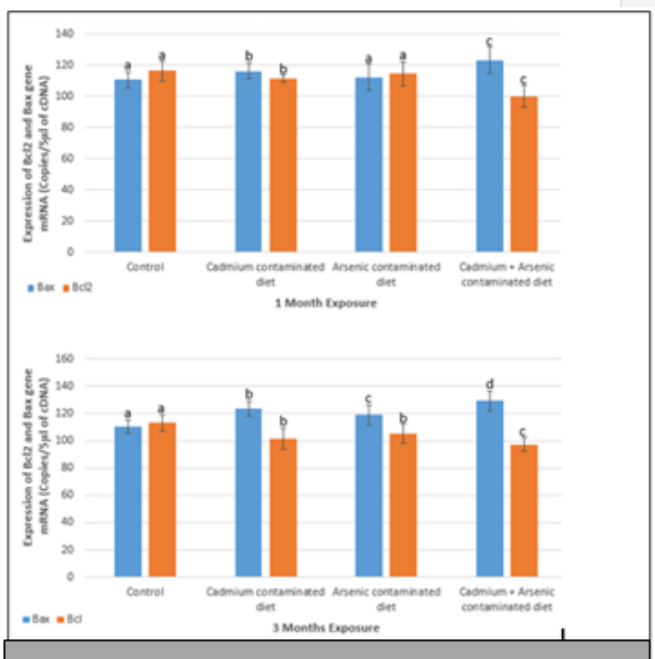

Figure 1. Changes in the Expression of $BCL_{2\,and}$ BAX Gene in the Tissues of the Rat After 1 and 3 Month Exposure to Experimental Diet. Values not Sharing Sane Superscript with in a Tissue Differs Significently at ($p < 0.05$)

DISCUSSION

Humans are exposed to Cadmium (Cd) and Arsenic (As) at low concentrations either voluntarily through supplementation or involuntarily through intake of contaminated food and water or contact with contaminated soil, dust, or air. These metals could have adverse impacts on male reproductive health. Metal analysis on the compounded experimental feed showed trace contamination of the control and the test groups. The mole ratio clearly indicated the trace presence of as in the Cd-contaminated diet and the trace presence of Cd in the As-contaminated diet. It was observed in the present study, that the control testes also had some very minimal level of contamination of the metals. This could be attributed to the pervasiveness of Cd and as in the general environment. The present study revealed a reduction in the body weight and testes/body weight ratio of rats exposed to the experimental diets for both periods of exposure. The severe effect on body weight gain and testes / body weight ratio of rats observed after 3 months exposure could be attributed to toxicity of the metals due to their increased accumulation relative to 1 month exposure. Again, the findings also indicated that combination of the metals in Group D had a more pronounced effect on the weight gain of rats. This is in accordance with the report of who showed that both As and Cd decreased body weight gain and food utilization with a more pronounced effects for the mixture. It has been shown that testicular germ cells are susceptible to oxidative damage by free radicals due to the presence of polyunsaturated fatty acids in the plasma membrane. This leads to the excessive generation of ROS which could outweigh the cell's antioxidant defense system. Thus, markers of oxidative stress and antioxidant enzymes were analyzed in the testes of experimental rats. After 1 month exposure, the activities of the antioxidant enzymes (SOD and CAT), and GST, a phase 1 drug metabolizing enzyme were all increased in the testes by the Cd and as (singly and in mixture) contaminated die. This is apparently traceable to the low level of oral dose of the metals in the diet as well as the short duration of exposure. This is in consonance with the study of where an induction of SOD and CAT activities were recorded following exposure to Cd and Cd and As, respectively at low dose. Increase in the activities of the antioxidant enzymes could also be attributed to the ability of the testes to tolerate the metal stress have also reported increase in oxidative enzymes in testes of rats after 1 month exposure of rats to Cd and As contaminated diets. The reduction in the activities of these enzymes after 3 months exposure may imply enzyme inactivation caused by excess reactive oxygen species (ROS) formation, displacement of essential cofactor such as Zn or Cu or binding to thiol groups of the enzyme. The current study also showed that exposure to Cd and As caused a significant ($p < 0.05$) increase in membrane lipid peroxidation (LPO) in the testes of exposed rats for both durations of study which is an indication of oxidative stress and could lead to intra and inter-molecular cross-links in proteins and nucleic acids occasioned by LPO products such as MDA. In the present study, significant ($p < 0.05$) increase in GSH levels in the testes was observed after 1 month exposure however, at the end of 3 months exposure, there was a significant ($p < 0.05$) decrease in the level of GSH. The increase in tissue GSH content after 1 month exposure might be attributed to the system trying to mop up free radicals generated by the metals in these organs. High level of GSH as observed after 1 month exposure could protect against cell death. The decreased GSH level observed after 3 months exposure could be due to the oxidation of GSH by free radicals or as a result of depletion of the sulfhydryl group of cysteine moiety in GSH due to its high affinity for Cd and As forming Cd / As-GSH complex or its electron donor ability. A reduction in the level of GSH, might initiate the onset of cytotoxicity. After 1 month exposure through the food-chain, non-significant ($p < 0.05$) increase in the level of Bax gene mRNA in the testes of rats was recorded but down regulated for Bcl - 2. After 3 months exposure, Bcl - 2 was significantly ($p < 0.05$) down regulated in all test groups while Bax was significantly ($p < 0.05$) up-regulated in all test groups. Interactions between Bcl - 2 and Bax regulate cytochrome c release from mitochondria and establish baseline sensitivity to apoptotic stimuli. Induction of apoptosis has been utilized for the treatment of cancer, but cancer cells have developed different strategies to resist death by apoptosis. One of such strategy is an increase in the expression of anti-apoptotic Bcl - 2 family proteins. Upon binding to Bax, Bcl - 2 can prevent pore formation and cytochrome c release. On the other hand, increase in the expression of Bax can induce cell death and is utilized in eliminating tumor cells. Based on different reports showing a reduction in the expression of Bax and a corresponding increase in the expression of Bcl - 2 in different drug-resistant tumor cells, it could be hypothesized that induction of mitochondrial apoptosis pathway by Cd and As in the testes of rats through the food chain might be mediated through the Bcl - 2 and Bax proteins. The disruption of the blood-testis

barrier (BTB) could be responsible for the sensitivity of the testes to Cd and as toxicity. Rodent testes under normal conditions have higher levels of metallothionein (MT) than other organs (liver and kidney excluded), therefore, the high susceptibility of the testes to Cd toxicity could possibly be related to genetic background. MT is a metal binding protein that protects cells from toxicity of metals by binding to them. Environmentally, humans and animals are exposed to the combinations of various risk elements. The present study thus took account of the effects of long-term exposure to low levels of these elements through the food chain. Very few information is available on the interactive effects of as and Cd upon ingestion have shown using sub-chronic dietary studies in rats that neither metal significantly affected accumulation of the other in kidney, liver or brain tissue found that both As and Cd increased RBC count and decreased haematocrit; responses to the mixture were less than additive. One of the most striking findings from this study was the pronounced effect of the diet containing a combination of Cd and As compared to diets containing the individual metals on most of the parameters studied which is evident in the percentage changes from the controls. These findings may have arisen from the additive effect and/or synergism of the two metals on the parameters studied. Thus, the result of the present study is in consonance with the observation of that showed that As (as arsenite) and Cd were more lethal to rats when administered as a mixture than when injected alone. In the studies of each metal lowered the LD50 for the other, and the two metals at single fixed doses caused greater than additive lethality as compared to either alone.[35]

CONCLUSION

In conclusion, this study has provided evidence that consumption of food contaminated with Cd and as through the food-chain could be a contributory factor to reproductive disorders. The results of the present study showed that Cd and As accumulated through the food chain can potentially affect negatively the weight gain and testes / body weight ratioof rats most especially as the duration of exposure increases with the mixture of metals having greater effect than either of the metals alone. Exposure to the metalsalso altered the activities of antioxidant enzymes leading to increased level of LPO by ROS, induced histological changes and altered the normal ratio of the mRNA expression of Bax and Bcl – 2 genes in the testes of exposed rats and these effects could lead to loss of reproductive functions. The up regulation of the mRNA expression of Bax and down regulation of Bcl – 2 gene expressions after both periods of exposure could be hypothesized as mediating induction of apoptotic pathway leading to cell death and consequently, loss of cell function. The findings of the study also revealed that the combined metals had a more pronounced biochemical and histological effects on the testes of the exposed rats than the individual metals especially in the three months exposed rats.

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