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Molecular Genetic, Cytogenetic and Sperm Studies on the Protective and Therapeutic Role of Vinasse against Toxicity of Lead Acetate in Mice.

Abeer H. Abd El-Rahim[1], Heba A.M. Abd El-Kader[1], Omaima M. Abd-El-Moneim[1], Hasnaa A. Radwan[1], M. Fadel[2], and Ibrahim M. Farag[1].

[1]Department of Cell Biology, National Research Centre, Dokki, Cairo, Egypt.
[2]Department of Microbial Chemistry, National Research Centre, Dokki, Cairo, Egypt.

ABSTRACT

The present study was carried out to evaluate the role of vinasse as a protective or therapeutic agent against lead acetate toxicity. Six groups of mice (8 mice each) were used. The mice groups involved normal control fed basal diet, positive control fed basal diet polluted with lead acetate (0.87 g/kg diet) for three weeks, the third and fourth groups fed basal diet containing vinasse at low (7.5 %) or high (9.5 %) levels (used as a protective agent) along treatment with lead acetate, the fifth and sixth groups fed basal diet containing vinasse at the same dose for two weeks (as a therapeutic agent) after cessation of lead acetate treatment. Molecular genetic, cytogenetic and sperm analysis were investigated. The results revealed in mice fed diet containing lead acetate high significant elevation of proportions of DNA fragmentation, genetic polymorphism in DNA of RAPD-PCR profiles, MNPCE, chromosome aberrations and sperm shape abnormalities as well as significant reduction of sperm count as compared to control group. Vinasse treatment as a protective or therapeutic agent significantly decreased the genetic alterations and sperm-shape abnormalities and significantly increased the sperm count in comparison with treatment of lead acetate alone. Better results were obtained by using basal diets supplemented with vinasse along treatment (as a protective agent) with lead acetate especially the vinasse treatment with high dose (9.5 %). In conclusion: the present study proves that the supplementation of vinasse to animal diets is a potent protective agent against genotoxicity induced by pollution with lead acetate. Where the use of this by-product had capability for reducing DNA damage, polymorphism of RAPD-PCR, cytogenetic changes and sperm-shape abnormalities and ameliorating the sperm count.

Keywords: vinasse, lead acetate, DNA damage, RAPD-PCR, cytogenetics, sperm analysis, mice.

Corresponding author

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INTRODUCTION

Lead is considered to be a wide-spread potent environmental pollutant. It is used for the preparation of a number of industry and household-based products. Due to extensive use of this metal in different industries, its contents have increased folds during recent years in the air, food and tap water. So, the general populations may be chronically exposed to lead by contaminated food or water intake and by inhalation [1]. Lead is represent as a significant ecological and a major public health concern due to its toxicity and it has a very strong ability to accumulate in several living organisms [2] such liver, kidney, lung , heart, brain and bone marrow. Moreover, its toxic effects extend to other organs as the reproductive systems [3], by passing through the blood–testis barrier and consequently accumulate in the epididymis and testis causing anomalies in the germinal diploid (spermatogonia) and haploid (primary spermatocytes, spermatids, spermatozoa or sperms) cells [4,5,3].

The toxicity of lead compounds was found to be a like with that of all other heavy metals, have been implicated in the etiology of different disorders in man and other mammals [6-9]. It causes decreasing in the levels of endogenous antioxidants [10-12,3] and through it metabolites in liver, the generation of reactive oxygen species (ROS) have been arisen causing severe damage to proteins, lipids and nucleic acid and inducing genotoxicity [13]. ROS or lead ions can attack the genomic DNA in somatic and germinal cells inducing DNA fragmentation, micronuclei formation, chromosomal aberrations and sperm abnormalities [14-18].

The harmful effects or the generation of oxidative processes due to lead toxicity may be avoided by using artificial and natural subjects that have strong antioxidant activity against several oxidative systems. The important of artificial antioxidants include butaleted hydroxyanisole (BHA), butaleted hydroxytoluene (BHT) and n-propylgallate**.** However, these artificial antioxidants were found to have many risks in vivo and their use has been restricted in a lot of countries [19]. In contrast several natural antioxidants were revealed to be safe and capable for minimizing or eliminating most traces of toxic materials and disorder metabolites without dangerous side effect or having harmful residues [20,21]. The use of vinasse on the basis of its biological protection [22-24] for detoxification of pollution with lead metal is a one approach of many to this problem.

Vinasse is considered to be a by-product of industrial production of alcohol, or other substances through the fermentation of molasses [25,26]. Generally the vinasse has a high organic matter and potassium content [27]. The composition of vinasse was found to possess yeasts, polysaccharides and minerals as well as vitamins and glutamic and aspartic acids [24,26]. These compounds have been revealed to be potent antioxidants and strong free radical-scavenging activities, as well as they have antimutagenic effects [28,29].

Oliveira et al. [30] demonstrated the ability of vinasse to stimulate cell growth and mitigate the toxic effect of aluminum toxicity to Saccharomyces cerevisiae. Also Eshak et al. [31] reported that vinasse supplementation in quail rations, in case of contamination with phenol minimized the toxic effects of such toxicant by reducing DNA damage and ameliorating IGFBPs gene expression, and consequently increased the productive performances of the birds.

Moreover, the use of vinasse as additive in poultry and farm animals were reported to improve the productive and reproductive performances as well as decrease the mortality cases [32-34,24].

So, the present study was designed to assess the protective and therapeutic effects of vinasse to modulate the frequency of lead acetate-induced genotoxicity in male mice.The study was investigated through determination the percentages of DNA fragmentation and polymorphic bands in RAPD-PCR. The assaying of frequencies of micronuclei and chromosome aberrations were also evaluated in bone marrow cells. Moreover, the sperm abnormalities were analyzed.

MATERIALS AND METHODS

Chemicals

Lead acetate, also known as lead diacetate, plumbous acetate, sugar of lead, salt of Saturn, and Goulard's powder, is a water soluble white crystalline chemical compound with a sweetish taste and was purchased from Sigma–Aldrich. Lead acetate was added and mixed well to the basal diet at level 0.87 g / Kg of

diet. This dose of lead acetate has been considered as a toxic experimental dose that affects mice organs [35,36].

Vinasse Diets Preparation

Distillery vinasse was obtained from the Sugar and Integrated Industries Company, Hawamdyia City, Egypt. The Microbial Chemistry Department, National Research Center, Egypt supplied by-product at two levels, 7.5% (600ml/8kg of diet) and 9.5% (760ml/8kg of diet). These rates of vinasse were added and mixed well to the feed [24]. Lead acetate was incorporated into the mixed feed before vinasse was added.

Experimental animals

Albino mice of Swiss strain weighing 25-30 g were obtained from the Animal House, National Research Centre, Egypt. Animals were housed in an ambient temperature of 25 ± 3.2°C on light/dark cycle of 12/12 hours. All mice were kept in clean polypropylene cages and administered food and water *ad libitum*.

Experimental design

The mice were divided into 6 equal groups, 8 mice each. Group 1, fed normal basal diet (control group). Group 2, fed basal diet containing lead acetate at level 0.87g/Kg of diet for three weeks. The third and fourth groups fed basal diet containing vinasse at low (7.5 %) or high (9.5 %) levels along treatment with lead acetate. The fifth and sixth groups fed basal diet containing vinasse at the same levels for two weeks after cessation of lead acetate treatment. The groups (3, 4) used to evaluate the protective role of vinasse against the potential mutagenic effects of lead acetate. The groups (5, 6) were used to evaluate the therapeutic effect of vinasse against the potential mutagenic effects of lead acetate. Mice had free access to food and drinking water during the study. At the end of the experiment, mice were sacrificed by cervical dislocation for studying of molecular genetics (DNA fragmentation and RAPD-PCR), cytogenetics (micronuclei and chromosome aberrations) and sperm examination (sperm-shape and count).

Molecular genetic studies

Assaying of DNA fragmentation using spectrophotometer

DNA fragmentation was quantified by diphenylamine (DPA) as described by Paradones et al. [37] in the liver cells of all investigated groups. The percentage of DNA fragmentation was taken as the ratio of DNA absorbance reading in the supernatant to the total amount of DNA in pellet and supernatant. Absorbance was measured at 600 nm using a UV double beam spectrophotometer (Shimadzu, 160A).

$$\text{DNA fragmentation} = \frac{OD\ of\ fragmented\ DNA\ (S)}{OD\ of\ fragmented\ DNA\ (S) + OD\ of\ intact\ DNA\ (P)} \times 100$$

DNA extraction and Random Amplification of Polymorphic DNA (RAPD-PCR) analysis

DNA extraction using salting out method

Cell (liver tissue) were pelleted and then diluted in 600 µl extraction buffer: 10 mM Tris-HCL pH 8.0, 2 mM Na_2EDTA (Sigma-Aldrich), pH 8.2 [38], 500 mM NaCL with 0.5% SDS, and incubated at 65°C in a water path for 20 min. 180 µl 5 M potassium acetate (Sigma-Aldrich) was added and the mixture centrifuged at 13000 rpm for 15 min to purify DNA; the supernatant was transferred to a 1.5 ml graduated microcentrifuge tube and incubated with proteinase K at 37°C for 2 h then DNA (aqueous phase) was precipitated with isopropanol and then with 70 % ethanol [39].

DNA amplification

Three commercial primers with sizes 10 bases, with variable nucleotide proportion were used for the amplification process. The primers (supplied by Operon, Almeda, CA, USA) including: OPC05

(5′GATGACCGCC3′), OPC02 (5′GTGAGGCGTC3′), OPA06 (5′GGTCCCTGAC3′). The PCR protocol for RAPD analysis was followed as described by Williams et al. [40], amplification was performed in 15 µl reaction volumes containing 1.5 µl (50 ng genomic DNA), 1.5 µl of 10X PCR reaction buffer, 1.5µl DNTPs (200 µM), 1.5 µl primer (1pmo1) (Operon, CA, USA), IU Taq DNA polymerase. The final reaction mixture was placed in a DNA thermal cycler (Eppendorf). The PCR program included an initial denaturation step at 94°C for 4 min followed by 45 cycles with 94°C for 1 min for DNA denaturation, 1 min at 36°C, at 72°C for 2 min and final extension at 72°C for 5 min were carried out. Approximately 3 µl of the amplified DNA product plus 2 µl of 1 X loading dye were loaded on 2 % agarose gel and then subjected to electrophoresis in 1 X TBE buffer and stained with ethidium bromide (0.5 µl/ml) for verification. A BIO-RAD XR+Molecular imager apparatus was used to visualize the PCR products. The ladder DNA (100 pb) (Fermentas) and labImage were used for determination of molecular size of bands.

RAPD profiles and data analysis

In randomly amplified polymorphic DNA (RAPD) analysis, the concentration of DNA and its relative purity were determined using a spectrophotometer based on absorbance at 260 and 280 mm, respectively. The integrity of extracted genomic DNA was checked by electrophoresis in 0.8 % agarose gel using DNA molecular weight marker (Fermentas). All mutagen that presented a RAPD profile was defined by loss or addition of bands compared with the control. Only reproducible and clear amplification bands were scored. The marked changes observed in RAPD profiles as disappearance and/or appearance of bands in comparison with untreated control treatments (b/a bands) were evaluated [41].

Cytogenetic Analysis

Micronucleus Test

Bone marrow slides were prepared according to the method described by Krishna and Hayashi [42]. The bone marrow was washed with 1 ml of fetal calf serum and then smeared on clean slides. The slides were left to air dry and then fixed in methanol for 5 minutes, followed by staining in May-Grunwald- Giemsa for 5 minutes then washed in distilled water and mounted. For each animal, 2000 polychromatic erythrocytes (PCEs) were examined for the presence of micronuclei.

Chromosome Preparation

For chromosome analysis both treated and control animals were sacrificed by cervical dislocation at the end of experiment. One hour and half or two hours before sacrifice, mice were injected i. p with 0.5 gm colchicine / kg. b.w. Femurs were removed and the bone marrow cells were aspirated using saline solution. Metaphase spreads were prepared using the method of Preston et al. [43]. Fifty metaphase spreads per animal were analyzed, for scoring the different types of chromosome aberrations.

Sperm Analysis

For sperm-shape analysis, the epididymis excised and minced in about 8 ml of physiological saline, dispersed and filtered to exclude large tissue fragments. Smears were prepared after staining the sperms with Eosin Y (aqueous), according to the methods of Wyrobeck and Bruce [44] and Farag et al. [45]. At least 3000 sperms per group were assessed for morphological abnormalities. The sperm abnormalities were evaluated according to standard method of Narayana [46]. Epididymal sperm count was also determined by hemocytometer as described by Pant and Srivastava [47].

Statistical analysis

Statistical analysis was performed with SPSS software. Data were analyzed using one-way analysis of variance (ANOVA) followed by Duncan′s post hoc test for comparison between different treatments. Results were reported as mean ±S.E.

The polymorphism values were performed according to method of Taspinar et al. [48] as follows:

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$$\text{Polymorphism value}=\frac{Total\ No.of\ polymorphic\ bands\ of\ treated\ group}{Total\ No.of\ observed\ bands\ of\ control} \times 100$$

Polymorphic bands=appearance of new bands (based on control bands) +disappearance of bands (based on control bands).

RESULTS

Results of DNA fragmentation

The present findings (table 1) observed that the rate of DNA fragmentation significantly increased in male mice fed diet containing lead acetate than that found in normal control. In the protective cases, this aberration significantly lowered in mice fed diet containing lead plus vinasse at levels 7.5% or 9.5% as compared with that revealed in a group of lead alone. The high level of vinasse greatly ameliorated the genetic material and gave the lowest value of DNA fragmentation than low level of vinasse. On the other hand, in the therapeutic cases, the rate of DNA fragmentation was significantly decreased in mice fed diet containing lead then fed diet containing vinasse at level 7.5% or 9.5% in comparison with a group of lead alone. The effect of high dose of vinasse for decreasing the DNA aberration was more than the effect of low dose of vinasse. In general, best results for decreasing the DNA damage were observed by using vinasse as a protective agent than its using as a therapeutic subject.

Table 1: DNA fragmentation in mice fed diets containing vinasse as a protective or therapeutic agent against lead toxicity

Treatment	% of DNA fragmentation
Control	11.3±0.3 [c]
Lead	32.2±1.0 [a]
Protective effect: Lead+V1	26.9±0.9 [b]
Lead+V2	25.4±0.5 [b]
Therapeutic effect: Lead then V1	27.7±0.5 [b]
Lead then V2	26.5±0.5 [b]

All data are expressed as mean±S.E.; a, b, c means with different letters are significantly different (P<0.05)
Lead treatment: The feeding on basal diet polluted with lead acetate.
Lead +V1: The feeding on basal diet polluted with lead acetate plus low level (7.5%) of vinasse.
Lead +V2: The feeding on basal diet polluted with lead acetate plus high level (9.5 %) of vinasse.
Lead then V1: The feeding on basal diet polluted with lead acetate then the feeding on basal diet containing only low level (7.5 %) of vinasse.
Lead then V2: The feeding on basal diet polluted with lead acetate then the feeding on basal diet containing only high level (9.5 %)of vinasse.

Results of RAPD-PCR

The results of RAPD-PCR fingerprint are shown in figure 1 and listed in table 2. These results were generated by three primers, OPC05, OPC02 and OPA06. The primers have been observed to display a strong amplification with distinct bands profile.

Protection treatment

In mice groups that fed on each of basal diet (control), basal diet plus lead, basal diet containing lead plus low level of vinasse (lead+V1) and basal diet containing lead plus high level of vinasse (lead+V2), the three primers amplified a total of 88 different bands. The molecular size of obtained bands has been ranged from 1565 to 194 (for OPC05 primer), 1475 to 268 (for OPC02 primer) and 1754 to 160 (for OPA06 primer). Of the 88 different bands, 45 (51.14%) were polymorphic in lead, lead+V1 and lead+V2 groups. These polymorphisms in RAPD profiles might be due to the gain (new appearance) and / or loss (disappearance) of the amplified bands as compared to control RAPD profile. The primer OPC02 gave a highest rate of genetic polymorphism

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(42 amplifies bands) in genomic DNA. In mice group that fed basal diet containing lead had the high rate (77.2 %) of genetic polymorphism. In contrast, this degree of genetic polymorphism was reduced to 68.18 % or 59 % in mice groups that fed diet lead +V1 or lead +V2, respectively. The treatment of lead plus high level of vinasse (lead+ V2)has been shown to be more effective for improve the genetic material by reducing the genetic polymorphism value than the treatment of lead plus low level of vinasse (lead + V1).

Therapeutic treatment

On the other hand, in mice groups that fed on each of basal diet (control), basal diet containing lead and basal diet containing lead then basal diet containing low (V1 group) or high (V2group) levels of vinasse, the used three primers had amplified a total of 102 different bands. The molecular size of generated bands ranged from 1565 to 170 (for OPC05 primer), 1475 to 288 (for OPC02 primer) and 1480 to 160 (for OPA06 primer). Of the 102 different bands, 51 (50%) were polymorphic in lead, lead then V1 and lead then V2 groups. The primer C2 generated a highest degree of genetic polymorphism (45 amplified bands) in genomic DNA. The degree of genetic polymorphism in treatment with lead or treatment with lead then low (V1 group) or high (V2 group) levels of vinasse was similar, this degree was 77.2 %.

From the present results, it can be shown that there are best findings by using the diet containing vinasse (especially at high level) as a protective subject than using the diet containing vinasse as a therapeutic agent, where the percentages of polymorphic bands were decreased more in protection groups (lead+V1 and lead+V2) than in therapeutic groups (V1 and V2).

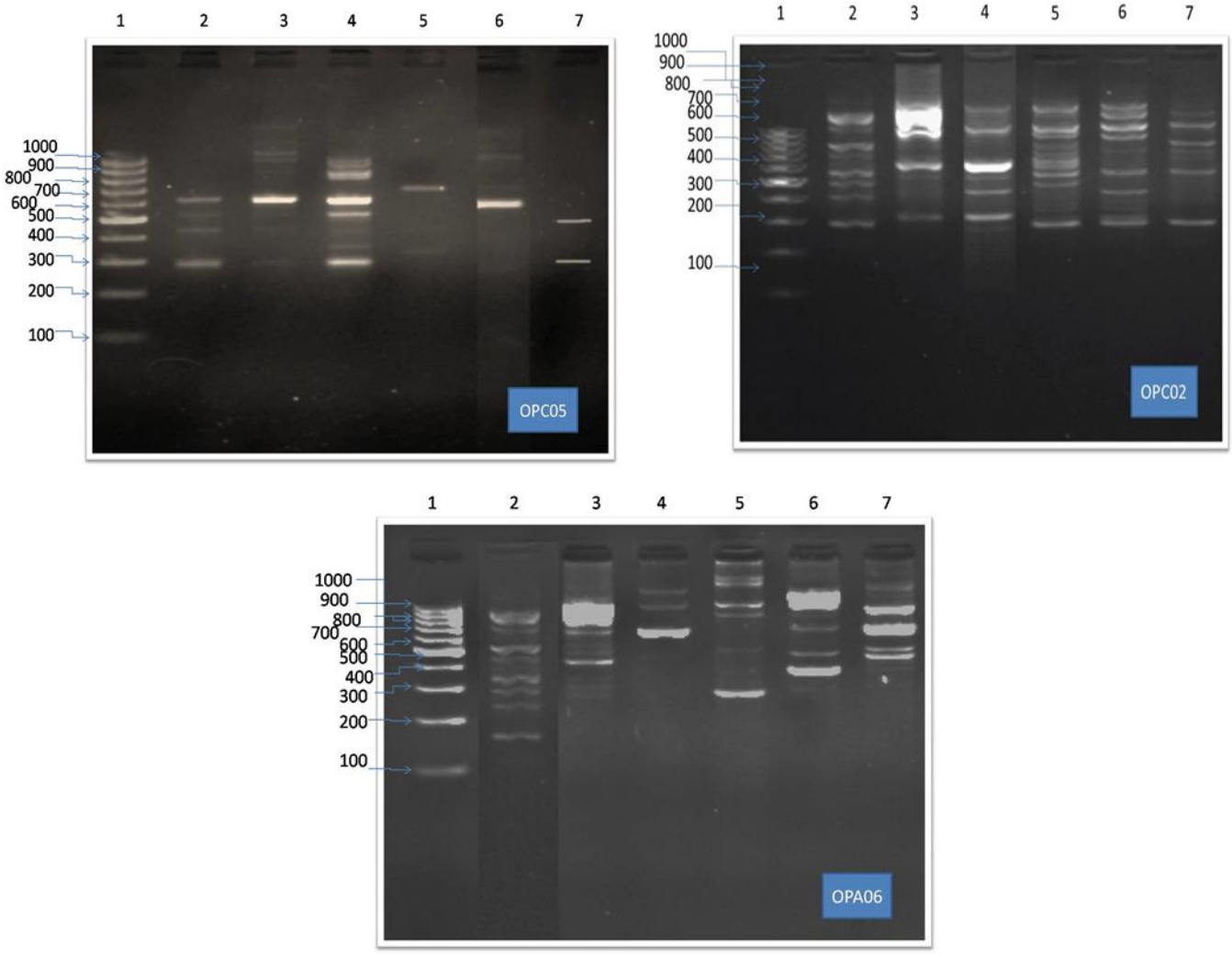

Figure 1: Control bands and polymorphic bands inRAPD-PCR fingerprints generated by OPC05, OPC02 and OPA06 of genomic DNA isolated from male mice. Lane 1(ladder DNA (100pb). Lane 2 (control), Lane 3 (Lead), Lane 4 (Lead+V1), Lane 5 (Lead +V2), Lane 6 (V1), Lane 7 (V2).

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Table(2):Control bands and polymorphic bands generated by RAPD-PCR of genomic DNA isolated from mice fed diets containing vinasse as protective or therapeutic agent against lead toxicity

Primers	Control	Lead		Lead+v1		Lead+v2		Total no.of bands	Band size	Polymorphic bands
		a	B	a	B	a	B			
OPC05	4	5	-	3	-	1	2	15	1565-194	11
OPC02	9	-	4	2	3	3	1	42	1475-268	13
OPA06	9	5	3	1	6	2	4	31	1754-160	21
Total	22	10	7	6	9	6	7	88		45(51.14%)
a+b		17		15		13				
Polymorphism		77.2%		68.18%		59%				
Primers	**Control**	**Lead**		**V1**		**V2**		**Total no. of bands**	**Band size**	**Polymorphic bands**
		a	B	A	b	a	B			
OPC05	4	5	-	3	2	-	2	20	1565-170	12
OPC02	9	-	4	3	1	4	2	45	1475-288	14
OPA06	9	5	3	4	4	4	5	37	1480-160	25
Total	22	10	7	10	7	8	9	102		51(50%)
a+b		17		17		17				
Polymorphism		77.2%		77.2%		77.2%				

(a) means appearance of new bands.
(b) means disappearance of bands.

Results of MNPCE

The results (table 3) showed that the percentage of micronucleated polychromatic erythrocytes (MNPCE) in mice fed diet containing lead was significantly elevated than those found in normal control. Using the diet containing vinasse as a protective or therapeutic agent significantly decreased the rate of MNPCE as compared to effect of diet containing lead alone. In all cases, the effect of high level of vinasse resulted in more decreasing of rate of MNPCE than the low level of vinasse. Using diet containing vinasse along (as a protective agent) with lead gave much better results. It decreased the percentage of MNPCE (especially in high level of vinasse) more than the using diets containing vinasse as therapeutic agent.

Table 3: The rate of micronucleated polychromatic erythrocytes (MNPCE) in mice fed diets containing vinasse as a protective or therapeutic agent against lead toxicity

Treatment	MNPCE
Control	3.8±0.5 ^{d}
Lead	14.8±0.3 ^{a}
Protective effect: Lead+V1	9.3±0.5 ^{b}
Lead+V2	6.3±0.5 ^{c}
Therapeutic effect: Lead then V1	10.3±0.9 ^{b}
Lead then V2	9.3±0.9 ^{b}

All data are expressed as mean±SE; a, b, c, d means with different letters are significantly different (P<0.05)

Results of chromosomal aberrations

The present results (table 4) revealed that the feeding on diet containing lead induced in male mice the high significant (P<0.001) increase and significant elevation (P<0.05) of total structural aberrations and aneuploidy, respectively as compared to feeding on basal control diet. This damaging effect especially total

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structural aberrations significantly reduced by feeding on diet containing vinasse (at level 7.5% or 9.5% level) as protective or therapeutic agent against toxicity of lead in respect to feeding on diet containing lead alone. Also, the percentages of aneuoploidy were decreased in mice fed diet containing vinasse as a protective or a therapeutic agent than those observed in lead diet alone; however, these decreases were not significant. In all cases, better results were observed in mice fed vinasse diet as a protective agent, where the percentages of chromosome aberrations were decreased more than using the vinasse as a therapeutic agent. The group of mice that fed diet containing high level of vinasse as a protective agent had the lowest percentage of chromosome aberrations.

Table 4: The frequencies of chromosome aberrations in mice fed diets containing vinasse as protective or therapeutic agent against lead toxicity

Treatment	Structural aberrations								Numerical aberrations
	Gaps	Breaks	Deletions	Fragments	Centric fusions	Centromeric attenuations	Endomitosis	Total structural aberrations	Aneuoploidy
Control	0.5±0.3 ^{b}	0.5±0.3 ^{a}	0.5±0.3 ^{b}	0.5±0.3 ^{b}	0 ^{c}	1.8±0.5 ^{a}	0 ^{d}	3.8±0.3 ^{d}	2.0±0.6 ^{b}
Lead	2.0±0.4 ^{a}	1.8±0.3 ^{a}	3.0±1.0 ^{a}	2.3±0.3 ^{a}	3.3±0.6 ^{a}	3.3±0.5 ^{a}	3.0±0.4 ^{a}	18.5±1.8 ^{a}	4.5±0.6 ^{a}
Protective effect: Lead+V1	1.3±0.3 ^{ab}	1.3±0.5 ^{a}	2.0±0.7 ^{ab}	1.5±0.3 ^{ab}	0 ^{c}	2.3±0.5 ^{a}	1.5±0.3 ^{bc}	9.8±1.1 ^{bc}	2.8±1.0 ^{ab}
Lead+V2	1.0±0.4 ^{ab}	1.0±0.6 ^{a}	1.0±0.4 ^{b}	1.3±0.8 ^{ab}	0 ^{c}	1.8±0.3 ^{a}	0.5±0.3 ^{cd}	6.5±0.9 ^{cd}	2.8±0.3 ^{ab}
Therapeutic effect: Lead then V1	1.3±0.5 ^{ab}	1.3±0.5 ^{a}	1.8±0.3 ^{ab}	1.8±0.3 ^{ab}	1.5±0.5 ^{b}	2.8±0.8 ^{a}	2.0±0.6 ^{ab}	12.3±1.7 ^{b}	3.0±0.4 ^{ab}
Lead then V2	1.0±0.4 ^{ab}	1.0±0.4 ^{a}	1.8±0.5 ^{ab}	1.5±0.3 ^{ab}	1.5±0.6 ^{b}	2.3±0.8 ^{a}	1.5±0.3 ^{bc}	10.5±1.3 ^{bc}	3.0±0.4 ^{ab}

All data are expressed as mean±SE; a, b, c, d means with different letters are significantly different (P<0.05)

Results of sperm examination

The present results (table 5) showed that feeding on diet containing lead caused high significant increases of sperm abnormalities in head or in tail, as well as significant decrease in sperm count in comparison with feeding on normal base diet. In contrast, the addition of vinasse (at low level 7.5% or high level 9.5%) to lead diet as a protective or a therapeutic agent significantly reduced the sperm-shape abnormalities and significantly increased the sperm count as compared to using the diet containing lead alone. The best results were obtained by adding vinasse to lead diet as a protective agent, where the feeding on these diets greatly decreased the sperm-shape abnormalities and increased the sperm count in respect to feeding on vinasse diet as a therapeutic agent. The mice fed diet with high level of vinasse (as protective agent) had the lowest percentages of sperm-shape abnormalities and the highest rate of sperm count.

Table 5: Sperm abnormalities in mice fed diets containing vinasse as a protective or therapeutic agent against lead toxicity

Treatment	Head abnormalities						Tail abnormalities	Total abnormalities (head & tail)	Sperm count
	Amorphous	Without	Small	Big	Banana	Total head abnormalities	Coiled		
Control	10.0±0.0 ^{d}	4.5±0.5 ^{d}	0.0 ^{c}	0.5±0.5 ^{a}	0.0 ^{a}	15.0±0.0 ^{e}	1.5±0.5 ^{d}	16.5±0.5 ^{e}	76.83±1.47 a
Lead	31.5±0.5 ^{a}	10.5±0.5 ^{a}	4.5±0.5 ^{a}	2.0±1.0 ^{a}	1.0±0.0 ^{a}	49.5±1.5 ^{a}	5.5±0.5 ^{a}	55.0±1.0 ^{a}	42.35±1.65 c
Protective effect: Lead+V1	24.5±0.5 ^{c}	7.5±0.5 ^{c}	1.0±0.0 ^{c}	0.5±0.5 ^{a}	0 ^{a}	33.5±0.5 ^{d}	3.5±0.5 ^{bc}	37.0±1.0 ^{d}	64.40±0.4 b
Lead+V2	23.5±0.5 ^{c}	7.5±1.5 ^{c}	0.5±0.5 ^{c}	0.5±0.5 ^{a}	0 ^{a}	32.5±1.5 ^{d}	3.0±0.0 ^{c}	35.5±1.5 ^{d}	66.75±0.8 b
Therapeutic effect: Lead thenV1	29.5±0.5 ^{b}	9.5±0.5 ^{ab}	2.5±0.5 ^{b}	1.0±0.0 ^{a}	0.5±0.5 ^{a}	43.0±1.0 ^{b}	4.5±0.5 ^{ab}	47.5±1.5 ^{b}	63.90±0.4 b
Lead thenV2	28.5±0.5 ^{b}	8.5±0.5 ^{bc}	1.0±0.0 ^{c}	0.5±0.5 ^{a}	0 ^{a}	38.5±0.5 ^{c}	4.0±0.0 ^{bc}	42.5±0.5 ^{c}	64.75±0.8 b

All data are expressed as mean±SE; a, b, c, d, e means with different letters are significantly different (P<0.05)

DISCUSSION

The present study found that mice fed diet polluted with lead had significant elevation of rate of DNA fragmentation and proportions of genetic polymorphism in genomic DNA of RAPD profiles as compared to mice fed basal normal diet. Also, several studies by Xie et al. [49], Shaike et al. [50] and Azoz and Raafat [51] reported that exposure to lead induced DNA damage in different mammalian cells. Al Makawy et al. [18] reported by using comet assay that mice which exposed to lead had significant increase of DNA damage of comet tail length and tail DNA% as well as olive tail moment (OTM). On the other hand, the changes of RAPD profile in the present work were similar with that reported in another study by Abd El-Rahim et al. [52] who found that mice fed diet containing aflatoxin had high proportions of genetic polymorphism of amplified bands of DNA in comparison with mice fed normal basal diet. Moreover, in plants, Liu et al. [53], [54] and Taspinar et al. [48] revealed that the exposure to cadmium led to significant increases of changes in RAPD profiles. These changes were observed by inducing high degrees of genetic polymorphisms of amplified bands of DNA in respect to control plants. The mutagenicity of lead might be arisen during its metabolites in liver by inducing the generation of reactive oxygen species (ROS) that include hydrogen peroxide (H_2O_2), superoxide radicals (O_2^-) and lipid peroxidation (LPO) [55,56]. The ROS components can interact with DNA causing strand breaks, significant damage in base pairs, unscheduled synthesis or adducts formation resulting in high levels of genetic instability [56,3]. Moreover, some studies by Salma and Kshama [57] and Marchlewicz et al. [58] reported that lead treatment depleted the antioxidant enzymes causing inhibition of DNA mismatch repair and consequently resulting in DNA damage like DNA fragmentation or RAPD profile changes. Therefore, The DNA fragmentation and genetic polymorphism in DNA of changes in RAPD profile that were observed in the present study might be due to strand breaks of DNA or base pair alterations in genomic DNA that induced by toxic effect of lead.

The present results also revealed that the feeding on diet containing lead acetate induced high significant elevation in percentages of MNPCE and chromosome aberrations as compared to feeding on control basal diet. Concerning the induction of MNPCE, our results are in agreement with that reported in other previous studies. In these studies the exposure to lead acetate has been observed to be a strong effective agent for inducing high percentages of MNPCE in different mammalian cells, including kidney cells, leukocytes and erythrocytes in rats [59-61,29,51], bone marrow cells in mice [62,3,18] and melanoma cells in human [63].

Considering the induction of chromosome aberrations, the present results were supported by several previous studies: Hanada and Muramatsu [36] revealed that the structural chromosome aberrations in leukocyte cells significantly increased in goats fed diet containing lead acetate in respect to that observed in goats fed normal basal diet. Also, Lorencz et al. [64] and Nehez et al. [65] found in rats that exposed to lead acetate significant elevation of numerical chromosome anomalies in bone marrow cells as compared to those found in the control. Moreover, high significant elevation of structural aberrations in chromosomes of bone marrow and spermatocytes were observed by Aboul-Ela [14] in mice that administrated with lead acetate. Al-Khalid et al. [66] found that the mice that treated with lead acetate had significant increase ($P<0.05$) of structural chromosome aberrations and significant decrease ($P<0.05$) in mitotic index in comparison with control group.

The inducing of micronuclei and chromosome aberrations might be due to the mutagenic effect of lead ions that can interact with genomic DNA causing small to large changes. These changes are DNA strand breaks forming micronuclei [60,61,3] or chromosome aberrations [60,61,65,66,3,14]. Micronucleus assaying is considered to be a cytogenetic form. This form used for measuring the chromosome aberrations specially when DNA breaks are induced in both strands [67,68].On the other hand, several studies by Durgut et al. [69], Nabil et al. [70], Azoz and Raafat [51] and Al-Khalid et al. [66] revealed that during the metabolic processes of lead in liver, ROS are generated. ROS attack different cellular components such as proteins, lipids and nucleic acids causing cytotoxicity and genotoxicity and consequently resulting in micronuclei, DNA fragmentation and chromosome aberrations.

Also, ROS interact especially with guanine base forming DNA adducts. This adduct is responsible for inducing structural chromosomal aberrations that might be occurred by DNA breakage, replication in damage region of DNA template, and suppression of synthesis or repair of DNA by inhibiting the DNA polymerase [71-73,60].

Furthermore, lead ions could interfere in processes of calcium regulation that affect replication and repair of DNA causing DNA damage and consequently induce chromosome aberrations [60]. On the other hand, lead ions could also interfere in forming processes of spindle fibers causing disturbance of mitotic division and inducing chromosome numerical aberrations [66]. Moreover, the lead treatment cause high rate of inducing of LPO and reduction of antioxidant enzymes that scavenge the free radicals in bone marrow cells leading to genotoxicity [18]. Therefore, the genotoxic effect of lead acetate in the present study might be due to interacting the lead ions with DNA or inducing ROS during the metabolic processes of lead in liver causing DNA damage and resulting in micronuclei and chromosome aberrations.

The present results showed that feeding on diet containing lead acetate caused high significant increases of sperm abnormalities in head or in tail as well as significant decrease in sperm count in comparison with feeding on normal basal diet. Our findings are coincidence with that reported in previous several studies on different mammals such humans [74,75,3] and rats [76]. These studies revealed that the exposure to lead caused adverse effects on sperm parameters that included significant increases of sperm-shape abnormalities and significant decreases in sperm count. Apostoli et al. [4] reported that lead ions can across and pass through the barrier of blood testis and consequently accumulate in testis cells and epididymis affecting on diploid cells (spermatogonial cells) and haploid cells including (spermatocytes, spermatids and spermatozoa) causing sperm shape abnormalities and decrease of sperm count. Also, Garu et al. [75] and Aitken and Roman [77] stated that the induction of oxidative stress due to toxic effect of lead cause damage in spermatogenesis and leydig cells as well as in lastly sertoli cells in which sperm maturation is occurred resulting in sperm-shape abnormalities. Moreover, Acharya et al. [73] observed that the generation of reactive oxygen species (ROS) due to oxidative stress of lead acetate had been found to induce mutation of certain segment of the genes causing chromosome aberrations in germ cells and consequently inducing sperm-shape abnormalities and decreasing in sperm count. On the other hand, Sanchez et al. [78] reported that the plasma membrane of sperm has a high amount of polyunsaturated fatty acids that very sensitive to oxidative stress. So, the generation of ROS due to lead toxicity might attack such fatty acids inducing alterations in the plasma membrane and occurring LPO. LPO can affect DNA of sperm causing sperm anomalies in shape and number. Therefore, the induction of sperm abnormalities in the present study might be due to interacting the lead ions to germinal cells or induction the generation of ROS by oxidative stress of lead acetate, these ROS attack the diploid and haploid of germinal cells causing increases of sperm-shape abnormalities and decreases of sperm count.

The present results revealed that the use of the diet containing vinasse as a protective or a therapeutic agent significantly decreased the rates of inducing of DNA fragmentation, genetic polymorphism of RAPD profiles, MNPCE and chromosome aberrations. Also, the vinasse diets significantly reduced the sperm-shape abnormalities and significantly increased the sperm count. The use of diets containing vinasse along (as a protective agent) treatment with lead treatment gave much better results. These results proved that vinasse is considered to be a strong protective agent on genetic and reproductive parameters against the toxic effect of lead acetate. Our findings are supported by Eshak et al. [31] who revealed that the addition of vinasse to quail diet that polluted with phenol caused reduction of rate of DNA fragmentation and enhancement of gene expression level for IGFBPs gene as compared to diet polluted with phenol alone.

The ameliorations that observed on genetic and reproductive parameters in the present study by using vinasse might be due to its constituents of polysaccharids, yeasts, vitamins, minerals and glutamic and aspartic acids [22-24]. These constituents of vinasse have been found to be potent antioxidants and possess strong activities of free radical-scavenging [79-81,29] and their capability for generation of biological enzymes that can interact with different toxicants and minimizing the dangerous effects [82,83].

The supplementation of Saccharomyces cerevisiae (Sc) (a main constituent of vinasse) to bird diets contaminated with AFB1 significantly decreased the adverse effect of such toxicant and resulting in improvement of the growth performance in broiler chickens [84]. Also, the Sc treatment could inhibit the aflatoxicosis by decreasing the generation of ROS and improving the level of GSH and activity of SOD in mice exposed to aflatoxin B1 [85]. These ameliorations of yeast (Sc) might be due to its content of Tsal (which a major constituent of peroxiredoxins). This constituent was found to be possessing the strongest activity to scavenge H_2O_2 [86]. Furthermore, the Tsal constituent was revealed to be a strong protective agent to genomic DNA and make it more stability against toxic or mutagenic effects of different mutagenes [87].

Eshak et al. [88] revealed that addition of yeast Sc to quail diet polluted with AFB1 significantly ameliorated the gene expression of neural and gonadal genes as compared to basal diet contaminated with AFB1 alone. On the other hand, Janisch et al. [89] reported that the vitamins (that are considered to be the main constituents of vinasse) such vitamins C and E were found to be strong antioxidants by scavenging reactive oxygen species that generated by toxicants. In previous study, Hsu et al. [90] observed that the treatment of vitamins C and E to rats minimized the lead toxicity and resulted in amelioration of sperm shape and count. Moreover, Hamadouche et al. [76] concluded that treatment with vitamin E along treatment with lead acetate significantly decreased the sperm-shape abnormalities as compared to the treatment of lead alone. Furthermore, the treatment of vitamin E to rabbits [91] and rams of sheep [92] led to increase of sperm count and concentration.

CONCLUSION

The present study proves that the supplementation of vinasse to animal diets is a potent protective agent against genotoxicity induced by pollution with lead acetate. Where this by-product had capability for reducing DNA damage, polymorphism of RAPD-PCR, cytogenetic changes and sperm-shape abnormalities and ameliorating the sperm count.

REFERENCES

[1] Glalipour MJ, Roshandel DRG, Ghafari S, Ghafari S, Kalavi S, Kalavi M Kalavi K. . In J Morphol. 2007; 25 (4): 717-722

[2] Vaglenov A, Creus A, Laltchev S, Petkova V, Pavlova S, Marcos R. . Environ Health Perspect. 2001;09: 205-298.

[3] Abd El-Monem DD. The Journal of Basic & Applied Zoology. 2012.;65: 223–231.

[4] Apostoli P, Porru S, Bisanti L. Scand J Environ Health.1999; 25: 40–43.

[5] Telisman S, Colak B, Pizent A, Jurasovi J, Cvitkovi P. Environ Res. 2007; 105: 256-266.

[6] Perlstein MA, Attala R. Clin Pediatr. 1966;5: 292-298.

[7] Saxena DK, Hussain T, Lal B, Chandra SV. Ind Health.1986 ; 22: 105-109.

[8] Kumar BD, Krishnaswamy K. Bull Environ Contam Toxicol.1995 ; 54: 863-869.

[9] Correa M, Roig-Navarro AF, Aragon CM. Life Sci.2004 ; 74: 2009–2021.

[10] Reichlmayr-lais AM, Kirchgessner M. O'dell Bl. Sunde, R. A. Marcel Dekker, Inc., New York.1997 ; 479–792.

[11] Zhao ZY, Li R, Sun L, Li ZY, Yang RL. J Zhejiang Univ Sci.2004 ; 5: 1001–1004.

[12] Wala'a J, AL-Khalid Majdy F, Majeed Adel J, Hussein . Bas J Vet Res.2012 ; 11 (2): 173-185.

[13] Mittler R .Trends in Plant Science.2002 ; 7:405-410.

[14] Aboul-Ela EI . Mutat Res. 2002; 516: 1-9.

[15] Wozniak K, Blasiak J . Mut Res. 2003;535: 127–139.

[16] Shaik AP, Jamil K. J Hazard Mater.2009 ; 168: 918-924

[17] Chen Z, Lou J, Chen S, Zheng W, Wu W, Jin L. Toxicology.2006 ; 223: 219–226.

[18] El Makawy AI, Ashoush IS, Abd-Elmoneim OM. Pol J Food Nutr Sci.2015 ; 65 (1): 39–47.

[19] Tepe G, Dietrich T, Grafen F, Brehme U, Muschick P, Dinkelborg LM, Greschniok A, Claussen CD, Duda SH. Cardio vasc Inter vent Radiol. 2005; 28: 632-637.

[20] Leeson S, Dizy G, Summers JD. Aflatoxins. In: poultry metabolic disorders and mycotoxins. S. Leeson; G. Diaz and J.D. Summers (eds.). Guelph, Ontario, University Books, 1995.pp: 248-279.

[21] Kubena LF, Harvery RB, Balitey RH, Buckley SA, Rottinghaus GE. Poultry Science.1998 ; 77: 1502-1509.

[22] Lewicki W. Introduction to vinasses (cms) from sugarbeet and sugar cane molasses fermentation. International Sugar Journal. 2001;103: 126.

[23] Stemme K, Gerdes B, Hams A, Kamphues J. Journal of Animal Physiology and Animal Nutrition.2005 ; 89: 179.

[24] Hidalgo K, Rodriguez B, Lopez M, Iben C, Albelo A, Cardenas M. In XXII Latin American Poultry Congress, 2011;pp: 1-7.

[25] Haanon K, Trenkle A. Journal of Animal Science.1990 ; 68: 2634-2641.

[26] Iranmehr M, Khadem AA, Rezaeian M, fzalzadeh A, Pourabedin M. Krmiva.2011 ; 53 (1): 3-8.

[27] Ferreira ES, Monteiro AO. Boletim Técnico Copersucar. 1987; 36: 3-7.

[28] Hussein AS, Cantor AH, Peascatore AJ, Johnson TH . Department of animal Sciences, University of Kentucky, Lexington, Kentucky,1996 ; pp: 40215-40546.

[29] Celik S, Erdogan Z, Erdogan S, Bal R. Turk J Vet Anim Sci. 2005; 29: 909-916
[30] Oliveria SA, Okoshi K, Lima-Leopoldo AP, Leopoldo AS, Campos DH, Martinez PF, Okoshi MP, Padovani CR, Pai-Silva MD, Cicogna AC. ArquivosBrasileiros de Cardiologia.2009 ; 93: 526-533.
[31] Eshak MG, Farag IM, Fadel M, Stino FKR.Global Veterinaria. 2013;11 (5): 657-673.
[32] Sarria P, Preston TR. Livestock Research for Rural Development.1992 ; 4: 80.
[33] Nakano T, Shimuzu M, Fukushima I. Biotechnology and Biochemistry.1999 ; 63: 1569-1575.
[34] Javierre J. Acidificantes Sinergicos en Avicultura: aplicacion 64.76.120.161/ acidificantes_sinergicos_aviculturaespecilicapara el manejo del //: estres de calor. Disponibleen: http. 2006.
[35] Sitting M. Handbook of toxic and hazardous chemicals Park Ridge, NJ: Noyes Data Corporation pp: 185.1981 .
[36] Hanada H, Muramatasu S. Food and agriculture organization of the United Nations. 1981;52 (8): 619-621.
[37] Paradones CE, Illera VA, Peckham D, Stunz LL, Ashman RF. J Immunol.1993 ; 151(7): 3521- 32529.
[38] Miller SA, Dykes DD, Polesky HF. Nucleic acid res. 1988; 16 (3):1215.
[39] Sambrook L, Fritsch EF, Manitatis T. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Press, Cold Spring Harbor, N.Y.1989 .
[40] Williams JKG, Kubelik AR, Livak KJ, Rafalsky JA,Tyngey SV. Nucl Acids Res.1990; 18: 6531–6535.
[41] El-Alfy NZ, Mahmoud MF, Abdullah AM. Global Journal of Pharmacology.2014 ; 8 (4): 532-541.
[42] Krishna G. and Hayashi M. Mutation Research.2000 ; 455-(1-2): 155-166.
[43] Preston RJ, Dean BJ, Galloway AF, Mcfee S. Mutant Research.1987 ; 189: 157-165.
[44] Wyrobek AJ, Bruce WR. Chem Mutagens.1978 ; 5: 237-285.
[45] Farag IM, Abdou HSA, Ayesh AM, Osfr MMH. Al-Azhar Bull Sci. 2002; 13: 105-120.
[46] Narayana K. J Toxicol Sci. 2008; 33: 85-96
[47] Pant N, Srivastava SP. J Appl Toxicol. 2003; 23 (4):271-274.
[48] Taspinar MS, Agar G, Yildirim N, Sunar S, Aksakal O, Bozari S. Journal of Food Agriculture and Environment.2009 ; 7 (3&4): 857-680.
[49] Xie H, Wise SS, Holmes AL, Xu B, Wakeman TP, Pelsue SC. Mut Res.2005 ; 586: 160– 172.
[50] Shaik AP, Sankar S, Reddy SC, Das PG, Jamil K. Drug Chem Toxicol.2006 ; 29, 111–124.
[51] Azoz HA, Raafat RM. Aust J Basic Appl Sci.2012 ; 6: 497–509.
[52] Abd El-Rahim A H, Abd El-Kader HA, Abd El-Moneim O M, El- Desouky TA, Farag IM. Int J Pharm Sci Res. 2014; 28 (2): 69-82.
[53] Liu W, Lip J, Qi XM, Zhoug OX, Zheng L, Sun TH, Yang Ys. Chemosphere.2005 ; 61 (2): 158-67
[54] Liu W, Yang Ys, Lipm J, Zhoug OX, Xie LJ, Han YP. J Hazard Mater.2009 ; 3, 161 (2-3): 878-83.
[55] Wozniak K, Blasiak J. Mut Res.2003 ; 535, 127–139.
[56] Nisha K, Deshwal RK. Int J Pharm Pharm Sci.2011 ; 3, 28–32.
[57] Salma K, Kshama D. Journal of food Agriculture and Environment.2005 ; 3 (1): 31-33.
[58] Marchlewicz M, Wiszniewska B, Gonet B, Baranowska-Bosiacka I, Safranow K, Kolasa A, Glabowski W, Kurzawa R, Jakubowska K, Rac ME. Biometals.2007 ; 20 (1): 13-9.
[59] Robbiano L, Carrozzino R, Puglia CP, Corbu C, Brambilla G. Toxicol Appl Pharmacol.1999 ; 161, 153–159.
[60] Kasuba V, Rozgaj R, Fucˇic A, Varnai VM, Piasek M. Biologia Bratislava.2004 ; 59, 779–785.
[61] Piao F, Cheng F, Chen H, Li G, Sun X, Liu S, Yamauchi T, Yokoyama K. Ind Health.2007 ; 45 (4): 546-51.
[62] Tapisso JT, Marques CC, Mathias ML, Ramalhinho MG. Mutat Res.2009 ; 678, 59–64.
[63] Poma A, Pittaluga E, Tucci A. Melanoma Res. 2003;13, 563–566.
[64] Lorencz R, Nehez M, Desi I. Abstracts of the XXVII th Meeting of the Hungarian Society of Hygiene, Blatonfoldvar, Hungary, September 25-27, P. 116.1996 .
[65] Nehez M, Lorencz R, Desi I. Ecotoxicol Environ Saf. 2000;45, 55–60.
[66] Al-Khalid J, Majdy F, Adel M, Hussein J. Bas J vet Res.2012 ; 11 (2): 173-185.
[67] Slamenova D, Gabelova A, Ruzekova L, Chalupa I, Horvathova E, Farkasova T, Bozsakyova E, Stetina R. Mutat Res.1997 ; 383: 243-252.
[68] Zein O, Scarfi MR. Method Mol Biol.2010 ; 625: 109-19.
[69] Durgut R, Koc A, Gonenci R, Bal R, Celik S, Guzaf M, Altug ME, Atesoglu O. J Appl Biol Sci.2008 ; 2, 11–18.
[70] Nabil M, Ibrahim, Esam A, Eweis, Hossam S, El-Beltagi, Yasmin E, Abdel-Mobdy. Asian Pacific Journal of Tropical Biomedicine.2012 ; 2 (1): 41-46.

[71] Sorsa M, Wilbourn J, Vainio H. *Mechanisms of Carcinogenesis in Risk Identification,* Vainio H., Magee P.H., McGregor D.B., McMichael A.J. (Eds.) pp. 543-554, IARC Scientific Publication no. 116 (WHO), ISBN 978-9283221166, Lyons and International Agency for Research on Cancer, New York. 1992.
[72] Albertini RJ, Anderson D, Douglas GR, Hagmar L, Hemmimki K, Merlo F, Natarajan AT, Norppa H, Shuker DE, Tice R, Waters MD, Aitio A. International Programme on Chemical Safety. *Mutation Res.* 2000;111-172.
[73] Acharya UR, Acharya S, Mishra M. Ind Health. 2003; 41 (3): 291–294.
[74] Bonde JP, Joffe M, Apostoli P, Dale A, Kiss P, Spano M, Caruso F, Giwercman A, Bisanti L, Parru S, Vanhoorne M, Comhaire F, Zschiesche W. Occup Environ Med. 2000;59: 234-242.
[75] Garu U, Sharma R, Barber I. IJPSR. 2011; 2 (9): 2403–2407.
[76] Hamadouche NA, Sadi N, Kharoubi O, Slimani M, Aoues A. Arch Biol Sci Belgrade. 2013; 65 (4): 1435-1445.
[77] Aitken RJ, Roman SD. Oxid Med Cell Longev. 2008; 1 (1): 15–24.
[78] Sanchez EET, Marquette M, Brown DB, Ansari NH. Fertile. Steril.2006 ; 86 (sup 1). S 444. In: Am Soe Reprod Med 62 nd Annual meeting.
[79] Parlat SS, M Ozcan, Oguz H. Research in Veterinary Science. 2001; 71: 207-211.
[80] Denli M, Celik K, Okan F. Inter J Poultry Science. 2003;2 (2): 174-177.
[81] Huang ME and Kolodner RD. Molecular cell. 2005;17: 709-720.
[82] Stanley VG, Ojo R, Moldesenbet S, Hutchinson DH, Kubena LP. Poultry Science.1993 ; 12: 1867-1872.
[83] Raju MV, Devegowda G. British Poultry Science. 2000; 41: 640-650.
[84] Abousadi AM, Rowghami E, Honarmand ME. Iranian Journal of Veterinary Research. 2007; 8: 144-150.
[85] Darwish HR, Omara EA, Abdel-Aziz KB, Farag IM, Nada SA, Tawfek NS. Report and Opinion. 2011 ; 3(12): 32-43.
[86] Park SG, Cha MK, Jeong W, Kim IH. J Biol Chem. 2000; 275: 5723-5732.
[87] Huang D, Koshiand D. Genes Dev. 2003;17: 1741-1754.
[88] Eshak MG, Khalil WKB, Hegazy EM, Farag IM, Fadel M, Stino FKR. J American Science.2010 ; 6 (12): 824-838.
[89] Janisch KM, Milde J, Schempp H, Elstner EF. De Dphthalmol. 2005; 38: 59-69.
[90] Hsu PC, Liu MY, Hsu CC, Chen LY, Guo YL. Toxicology.1998 ; 128 (3): 169-79.
[91] Youssef MI. Food and Chemical Toxicology. 2010; 48 (5): 1152–1159.
[92] Yue DB, Yan LY, Luo HL, Jin XX, Xu X. Anim Reprod Sci. 2010; 118: 217-222.