ANTIMUTAGENIC EFFECTS OF CHITOSAN AGAINST THE CYTOLOGICAL AND BIOCHEMICAL CHANGES INDUCED BY AFLATOXINS IN RATS

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Aflatoxins (AFs) are extremely toxic mycotoxins to which human are exposed. It is produced by *Aspergillus flavus* and *A. parasiticus* which occur as natural contaminants of foods and feeds. Experimental studies have shown that aflatoxins is a potent mutagen and hepatocarcinogen (Abdel-Wahhab and Aly, 2003 and 2005; Abdel-Wahhab *et al.*, 2007). A number of epidemiological investigations have also implicated its role in the etiology of human liver cancer (Van Rensburg *et al*., 1985; Yeh *et al*., 1989). A study with cultured human lymphocytes suggested that aflatoxin B1 was able to induce chromosomal damage through the release of Reactive Oxygen Species (ROS) (Amstad *et al*., 1984). The toxicity of AFB varies significantly between animal species. Whereas rats are sensitive to AFB hepatocarcinogenesis, many mice strains can tolerate exposure to the mycotoxin (Eaton and Gallagher, 1994; Hengstler *et al.*, 1999). The most applied method for protecting animals against mycotoxicosis is the utilization of adsorbents mixed with the feed which are supposed to bind the mycotoxins efficiently in the gastro-intestinal tract (Huwig *et al.*, 2001).

Chitosan is a natural polymer that is a versatile biopolymer exhibiting various unique biological properties; hence it has wide applications in food, biomedical and chemical industries (Shahidi *et al.*, 1999). The precursor to chitosan; chitin, was first discovered in mushrooms. It was also isolated from insects. The most abundant source of chitin is the shells of shellfish such as crab and shrimp. Chitosan is a deacetylated product of chitin with one of the polysaccharides having a free amino group at the C2 position of the glucose residue of cellulose (Kim *et al.*, 1997 and 1999). The degree of deacetylation provides significant effect on the antimicrobial activity of chitosan (Hongpattarakere and Riyaphan, 2008). Shon *et al*. (2001) reported that chitosan oligosaccharide showed an inhibitory effect on the mutagenic activity of the cooked food mutagen. Yoon *et al.* (2005) investigated the effects of chitosan oligosaccharide on mercury induced genotoxicity in mice using the micronuclei and chromosomal aberrations.

There was no difference between the untreated and experimental groups.

The present study was conducted to determine the ability of chitosan to reduce the genotoxic effects due to consumption of aflatoxins contaminated diet in male albino rats through cytological and biochemical studies.

MATERIALS AND METHODS

This work was carried out in Biological and Ecological Science Lab., Fac. of Home Economics, El-Azhar Univ. and Dept. of Genetics, Fac. of Agric., Kafer El-Sheikh Univ., Egypt.

Experimental Animals

Sixty male albino rats (Sprague-Dawley) weighting 60-70 g were purchased from the Biological Products & Vaccines Holding Company, Helwan Farm, Cairo, Egypt. Rats were kept under the laboratory conditions for one week as an acclimatization period. They were housed in stainless steel cages and kept through the experimental period under good ventilation and hygienic conditions. Rats were maintained on *ad libitum* diet and water. All animals received humane care in accordance with the protocol of National's Animal Care and Use Committee and Guidelines for the Care and Use of Experimental Animals and in accordance to Helsinki Declaration.

Aflatoxins

Aflatoxins were extracted from *Aspergillus flavus* which grow on some food and feed samples. Fourteen food and feed samples were collected from different sources from Tanta (Gharbia Governorate, Egypt) during winter season, 2008. The dilution plate method of Czapek (1902) was used for isolation and counting the total count of fungi. Fungal genera and species were identified according to Gilman (1957), Barnett (1972) and Booth (1977). Isolates of *A. flavus* recovered from the studied samples were screened for their ability to produce aflatoxin(s) on yeast extract sucrose agar plates, using the fluorescent agar technique of El-Bazza *et al*. (1982). The best isolates; that produce the highest amount of aflatoxin, was selected to produce aflatoxin as described by Stubblefield *et al.* (1967).

Chitosan

Chitosan was extracted from Shrimp which were collected from the branches of Nile River (Gharbia Governorate, Egypt) during winter fall 2009. The shell material were separated, dried at 50°C in oven for 24 h. and homogenized. Chitosan were extracted by the method of Khanafari *et al.* (2008).

Experimental Design

Rats used in this study were divided randomly into four groups (15 rats/group) which received the feeding administration for 45 and 90 successive days as follows:

a- The first experiment: (up to 45 days)

Group 1: Control fed on the basal diet.

Group 2: Rats were treated orally by stomach tube with chitosan (0.5 g/kg b.wt.).

Group 3: Rats were fed on aflatoxin-contaminated diet (1.650 µg/kg diets) and treated orally by stomach tube with chitosan (0.5 g/kg b.wt.).

Group 4: Rats were fed only on aflatoxin contaminated diet (1.650 µg/kg diets).

Feeding administration was lasted for 45 successive days. At the end of the experiment, eight rats were scarified for the later studies. The rest were kept for the next experiment.

b- The second experiment: (up to 90 days)

Seven animals from the first experiment were employed. The feeding period lasted for 90 days. Rats were fed on the same treatments as the previous experiment except for the dose of aflatoxin which was 3.300 µg/kg diets in the 3rd and 4th groups. At the end of the experimental period, all animals were fasted for 12 h. and blood samples were collected for isozymes analysis. After that, animals were killed and bone marrows were removed for cytological studies, while liver was maintained for total protein analysis.

Cytological studies

Animals were scarified after 2 h. from injection with 4 mg/Kg b.wt. colchicine according to Anwar *et al*. (1994). Metaphase chromosomes from bone marrow were prepared according to Rabellow-Gay and Ahmed (1980) and were stained with Giemsa (10%). Fifty metaphase spreads/treatment were checked and chromosomal aberrations were scored using a standard light microscope.

Biochemical studies

a. Liver total protein

Liver samples were mixed from five rats/treatment, then 250 mg of the mix were homogenized according to El-Fadly *et al*. (1990) for total protein profiles. Total protein banding patterns were separated electrophoretically using SDS-discontinuous gel as slabs (4% and 7.5% for stacking and separating gels, respectively) according to Laemmli (1970). The gel was placed in staining solution containing Commassie Brilliant blue-R250 as a protein chromocouple reagent, then it was transferred to the destaining solution twice until background became clear.

b. Blood serum isozymes

Blood samples were collected and the sera were separated according to Abdel-Wahhab *et al*. **(**2007) for isozymes analysis. Continuous standard slabs of 7.5% polyacrylamide gel were used for isozyme determination of esterases, peroxidase, acid phosphatase and alkaline phosphatase enzymes. The gel was stained for esterases and peroxidase activities according to Scandalios (1964), while, acid phosphatase and alkaline phosphatase were detected as described by Ahmed (1994).

Statistical analysis

The data were presented as means ± standard deviation (SD). Analysis of variance was conducted using a one-way ANOVA test followed by Student's *t*-test using SPSS computer program (version 9.0).

RESULTS AND DISSECTION

Chromosomal abnormalities

Table (1) presents the numbers and types of chromosomal aberrations induced in rat bone marrow cells after feeding rats for 45 days on the treatments of aflatoxin, chitosan and combination between them. The results revealed that all of the applied treatments induced both structural and numerical chromosomeal aberration. Ten types of structural chromosomal aberrations were observed. They were chromatid deletions (D), fragments (F), rings (R), centric fusions (CF), centric separations (CS), endomitosis (EM), end to end associations (E), chromatid breaks (B), chromatid gaps (G) and stickiness (S).

The results indicated that the highest average number of chromosomal aberrations (8.64) was detected as a result of aflatoxin treatment. The induced aberrations by this treatment were highly significantly different from the control group (1.45). These results agree well with the earlier findings of Anwar *et al.* (1994), who reported that chromosomal aberrations and micronuclei were significantly increased in rats treated with AFB1 at doses above 0.1 µg/g compared to the control group. These results were also supported by Ezz El-Arab *et al.* (2006) who found that oral administration for 2 months with 1 µg/kg b.wt/day of aflatoxins induced structural and numerical chromosomal aberrations in bone marrow and germ cells of male mice which differ significantly from the control group ($p \leq 0.05$). Shen *et al.* (1994) suggested that oxidative damages caused by AFB1 may be one of the underlining mechanisms for AFB1-induced cell injury and DNA damage, which eventually lead to tumorigenesis.

The average number of aberrations induced by chitosan treatment (3.45) did not differ significantly as compared to the control. On the other hand, treatment with combination of aflatoxin and chitosan (average number of 2.64) showed clearly positive effect for reducing the injury effect of either chitosan or aflatoxin when applied separately. Thus, the presence of chitosan with aflatoxin significantly reduces the side effects of aflatoxin.

Cytological examination of the chromosomal aberrations induced in rat bone marrow cells after feeding rats for 90 days on the treatments of aflatoxin, chitosan and combination between them are shown in Table (2). The same types of structural chromosomal aberrations seen in the first experiment were observed except for the ring (R) type of aberrations.

Figure (1) showed the different types of chromosomal abnormalities induced as results of 45 and 90 days treat-

ments which were manifested in Tables (1 and 2).

The results indicated that aflatoxin has side effects on chromosomes and these effects were highly significant than control. The average number of aberrations induced by aflatoxin was 8.27. There were also significant differences between control (1.81) and chitosan treated rats (3.09). These significant differences were not observed after 45 days of treatment. This can be attributed to the long exposure to chitosan. It can be noticed that the presence of chitosan with aflatoxin (average number of 3.36) has positive effect on repairing the injury of the chromosomes and decrease the side effects of aflatoxin, which means that chromosomal aberrations induced by aflatoxin were suppressed by the presence of chitosan. These results suggested that chitosan itself caused a mutagenic effect, while in the presence of aflatoxin, it promoted DNA repair activity and resulted in an antimutagenic effect. The results of the effect of chitosan and its side effects did not agree with Koo *et al.* (2002) and Chen *et al.* (2006), who reported that chitosan was able to protect normal cells against apoptosis challengs. Similar results were reported by Anraku *et al.* (2011), who reported that chitosan was found to protect cells from the damaging effects of oxygen radicals.

The reduction of the aflatoxin side effects may due to chitosan which has adsorption properties to adsorb the toxin or has antioxidant properties to protect oxidative damages of aflatoxin. This is consistent with the conclusions of Lee *et al.* (2001) and Coulombe *et al.* (2005), who reported that since lipid peroxidation plays a major role in the toxicity of AF, a protective effect of antioxidants is possible. This also agreed well with the results of Sugiyama *et al.* (2002), who reported that chlorophyllin fixed on chitosan (chl-chitosan), which is insoluble in water, can efficiently and tightly trap polycyclic compounds; e.g., aflatoxin B1. These results were also supported by Khodagholi *et al.* (2010) who decided that chitosan, an antioxidant oligosaccharide, is a protective agent against $H_2O_2/FeSO_4$-induced cell death in the NT2 neural cell line. Abdel-Aziem *et al.* (2011) evaluated the chemopreventive effects of carboxymethyl chitosan (CMC) against carbon tetrachloride (CCl4)-induced genotoxicity in male Balb/c mice. They concluded that both the low and high doses of CMC significantly ($p<0.01$) reduced the percentages of aberrant cells compared to the control group in a dose dependent manner.

Biochemical parameters

a. SDS-PAGE of liver protein

In order to study the effects of different treatments on total protein of liver, the protein banding patterns was evaluated following treatment with chitosan, aflatoxin and combination between them for 45 and 90 days (Fig. 2). It could be noticed that there were few differences between the different treatments and the control.

Corresponding to the banding patterns of liver protein of rat fed for 45 days on aflatoxin (1.650 µg/kg diet), chitosan (0.5 g/kg b.wt.) and combination between them, eight different bands were exhibited in control group (2, 3, 4, 5, 6, 8, 9 and 11). Meanwhile, band No. 4 was absent as a result of chitosan treatment. In general the intensity of the separated bands in the control experiment was higher than those detected following chitosan application. On the other hand, four bands (2, 5, 6 and 11) out of the eight detected bands following the combined treatment exhibited the same intensity as in the control experiment. It was noticeable that bands No. 4, 5, 6 and 11 following aflatoxin treatment did not differ from the control, while the remaining bands increased in their intensity.

When the rats were administrated for 90 days with the different external supplies of chitosan (0.5 g/kg b.wt.), aflatoxin (3.300 µg/kg diet) and combination between them, it was noticed that there were no differences between the control and all treatments in number of bands. However, bands of the chitosan and combination treatments exhibited higher intensity than in the control experiment for bands No. 1, 2, 3, 4, 5, 6, 8, 9 and 10. On the contrary, following aflatoxin treatment, the majority of the detected bands showed low intensity compared to the control experiment.

It could be concluded that the differences between the control and other treatments were increased after 90 days of exposure compared to 45 days. It could be observed that the new bands No. 1, 7 and 10 were presented, which were not present in the period of 45 days. On the other hand, some bands were very faint in some treatments in the 45 days while became very dark in the period of 90 days (No. 2, 4, 8, 9 and 11). It could be concluded that the total protein increased after 90 days as shown by bands intensity.

The present results agreed well with Verma and Nair (2004) who reported that oral administration of 25 and 50 mg of aflatoxin/mice/day for 45 days caused reduction in protein contents which could be due to formation of aflatoxin adduct. Abdel-Wahhab *et al.* (2007) reported that aflatoxin reaction in rat liver showed a significant decrease in total protein content. Denli *et al.* (2009) concluded that AFB1 decreased the concentration of albumin in broilers when treated with 1 mg of AFB1/kg diet for 42 days. The decline in protein concentration in the testis of aflatoxin-treated mice could be due to a decline in the protein biosynthesis by forming adducts with DNA, RNA and proteins, an inhibition of RNA synthesis or DNA-dependent RNA polymerase activity, as well as degranulation of endoplasmic reticulum (Cullen and Newberne, 1994; Groopman *et al.*, 1996).

b. Blood serum isozymes

Activities of esterases, peroxidase, acid phosphatase and alkline phosphatase in serum were performed to study the genetic effect of aflatoxin and chitosan on

gene expression in rats after 45 days and 90 days of treatments.

b.1. Esterases isozymes (Est)

Figure (3-Est) illustrates the esterases electrophoretic banding patterns in serum from rats treated with chitosan (0.5 g/kg b.wt.), aflatoxin with chitosan, aflatoxin alone (1.650 µg/kg diets and 3.300 µg/kg diets for 45 and 90 days, respectively) and control treatments. Two bands of esterases isozymes were observed when rats were fed on different tretments for 45 and 90 days. The results indicated that aflatoxin treatment (lane 4 and 8) increased the intensity of band No. 2 than the control (1 and 5, respectively) for both experiments of 45 and 90 days. However, band intensity was decreased in chitosan treatment (lane 2 and 6) compared to the control. After the combined treatment for 45 days (lane 3), the band intensity was decreased than the two separate treatments, while after 90 days the intensity was increased than chitosan treatment but was less than that treated with aflatoxin.

b.2. Peroxidase isozymes (Prx)

The electrophorotic patterns of peroxidase enzyme revealed marked differences in treated rats with chitosan (0.5 g/kg b.wt.), aflatoxin with chitosan and aflatoxin (1.650 µg/kg diets and 3.300 µg/kg diets after 45 and 90 days, respectively) compared to the control treatment as shown in Fig. (3-Prx). It can be clearly observed that the intensity of bands was greatly decreased compared to the control as a result of the three different treatments for either 45 or 90 days.

b.3. Acid phosphatase isozymes (Acph)

Figure (3-Acph) shows the changes in the activity of acid phosphatase isozymes after 45 and 90 days of treating rats with different treatments. Four bands were observed in both the periods; 45 and 90 days. For experiment of 45 days, bands No. 1 and 2 did not differ than control in its intensity, while band No. 3 was very faint in the combination and aflatoxin treatments. On the other hand, the intensity of band No. 4 was the same in the control and chitosan treatment, while was increased in aflatoxin treatment. The intensity of this band was decreased in the combined treatment than the control and the single treatments. Corresponding to the experiment of 90 days, three bands (No. 1, 2 and 3) with the same activities were seen in all treatments as well as the control. Band No. 4 was increased in intensity in aflatoxin treatment than the control and other treatments, while chitosan treatment has the lowest intensity after 90 days of administration. It is clear that these results were similar to the activity of esterases isozymes.

These results were in agreement with those of Rastogi *et al.* (2001) who reported that the administration of aflatoxin B1 (2 mg/kg intraperitoneally) for six weeks to rats caused significant elevations in the levels of acid phosphatases. Verma and Nair (2001) found that the activity of acid phosphatase was significantly increased in the testis of adult

male albino mice orally administered with 25 or 50 g of aflatoxin/animal/day (750 or 1500 g/kg b.wt.) for 45 days. This rise in acid phosphatase activity in liver was dose-dependent (Verma and Nair, 2004).

b.4. Alkline phosphatase isozymes (Aph)

The alkaline phosphatase isozymes in serum of rats treated with chitosan, aflatoxin and combined treatments were also investigated after 45 and 90 days (Fig. 3-Aph). The results revealed six common bands for the control and other treated samples after either 45 or 90 days. With respect the experiment at 45 days, it could be noted that the intensity of band No. 6 in the aflatoxin treatment was decreased followed by treatment of chitosan in comparison to the control. However, the combined treatment exhibits the highest decline in the intensity of this band of alkaline phosphatase. These results were similar to esterases and acid phosphatase isozymes. After 90 days, an increase in the intensity of band No. 6 was noticed for aflatoxin treatment followed by the combined treatment then chitosan treatment compared to the control.

Mehta *et al.* (1993) reported that the treated male Fischer 344 rats with 250-600 µg/kg AFB1 exhibited an increase in serum levels of alkaline phosphatase. However, at 100 µg/kg AFB1, these levels remained at the control levels. The studies of Rastogi *et al.* (2001) and Preetha *et al.* (2006) indicated that administration of aflatoxin B1 to rats (2 mg/kg and 1 mg/kg body mass, respectively) was found to significantly increase the levels of alkaline phosphatases in the rats serum. Moreover, Denli *et al.* (2009) found that the treatment with 1 mg of AFB1/kg feed; for 42 days, increased the serum activity of alkaline phosphatase in broiler chicks. On the other hand, Lee *et al.* (2003) reported that alkaline phosphatase activity was significantly increased in primary rat calvarial cells *in vitro* as a result of chitosan treatments.

Based on all the previous results, it could be concluded that chitosan has an antimutagenic effects against cytological and biochemical changes induced by aflatoxin.

SUMMARY

The purpose of this study was to investigate the possible antimutagenic effects of chitosan against genotoxic effects induced by aflatoxin in different mammalian cell systems. The chromosomal abnormalities in bone marrow cells as well as liver total proteins and blood serum isozymes in male rats were assayed after 45 and 90 days of treatments. The results indicated that aflatoxin significantly ($p<0.01$) increased chromosomal abnormalities in bone marrow cells after both of 45 and 90 days of treatments, while the effect of chitosan was significant ($p<0.05$) only after 90 days of treatment. Treating rats with aflatoxins contaminated diet in addition to chitosan resulted in a significant decrease in chromosomal aberrations compared to the single treatments for both experimental periods. The results of electrophoretic separation of total protein and isozymes revealed the

presence of some bands with higher intensity in aflatoxin group, while it was less for the combined treatments compared to the corresponding bands of the control group. The results suggested that chitosan has an antimutagenic effect against aflatoxin.

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Table (1): Chromosomal aberrations induced in bone marrow cells after treating rats for 45 days with chitosan, aflatoxin and combined treatments.

Treatments	No. of examined metaphases	No. of aberrant metaphases	Deletion (D)	Fragment (F)	Ring (R)	Centric fusion (CF)	Centric seperation (CS)	Endomitosis (EM)	End to end (E)	Break (B)	Gap (G)	Stickiness (S)	Numerical aberration	Total number of abnormalities	Average No. of aberrations ± S.D
Control	50	14	2	5	0	0	0	0	0	4	1	4	-	16	1.45 ± 1.97
Chitosan	50	21	11	1	0	0	1	0	1	8	7	1	8	38	3.45 ± 4.13
Aflatoxin	50	45	19	24	3	2	12	1	3	8	4	10	9	92	8.64 ± 7.38**
Chit+AF	50	24	6	0	0	1	1	1	0	9	4	7	0	29	2.64 ± 3.29

(**) indicate highly significant differences, compared to control proup (*t*-test).

Table (2): Chromosomal aberrations induced in bone marrow cells after treating rats for 90 days with chitosan, aflatoxin and combined treatments.

Treatments	No. of examined metaphases	No. of aberrant metaphases	Deletion (D)	Fragment (F)	Centric fusion (CF)	Centric seperation (CS)	Endomitosis (EM)	End to end (E)	Break (B)	Gap (G)	Stickiness (S)	Numerical aberration	Total number of abnormalities	Average No. of aberrations ± S.D
Control	50	18	8	0	0	0	0	1	3	0	6	2	20	1.81 ± 2.79
Chitosan	50	26	10	4	1	4	2	1	4	1	6	1	34	3.09 ± 2.95*
Aflatoxin	50	44	23	9	3	7	4	3	15	6	10	11	91	8.27 ± 6.50**
Chit+AF	50	30	10	6	0	4	0	2	3	1	4	7	37	3.36 ± 3.26

(*) and (**) indicate significant and highly significant differences; respectively, compared to control proup (*t*-test).

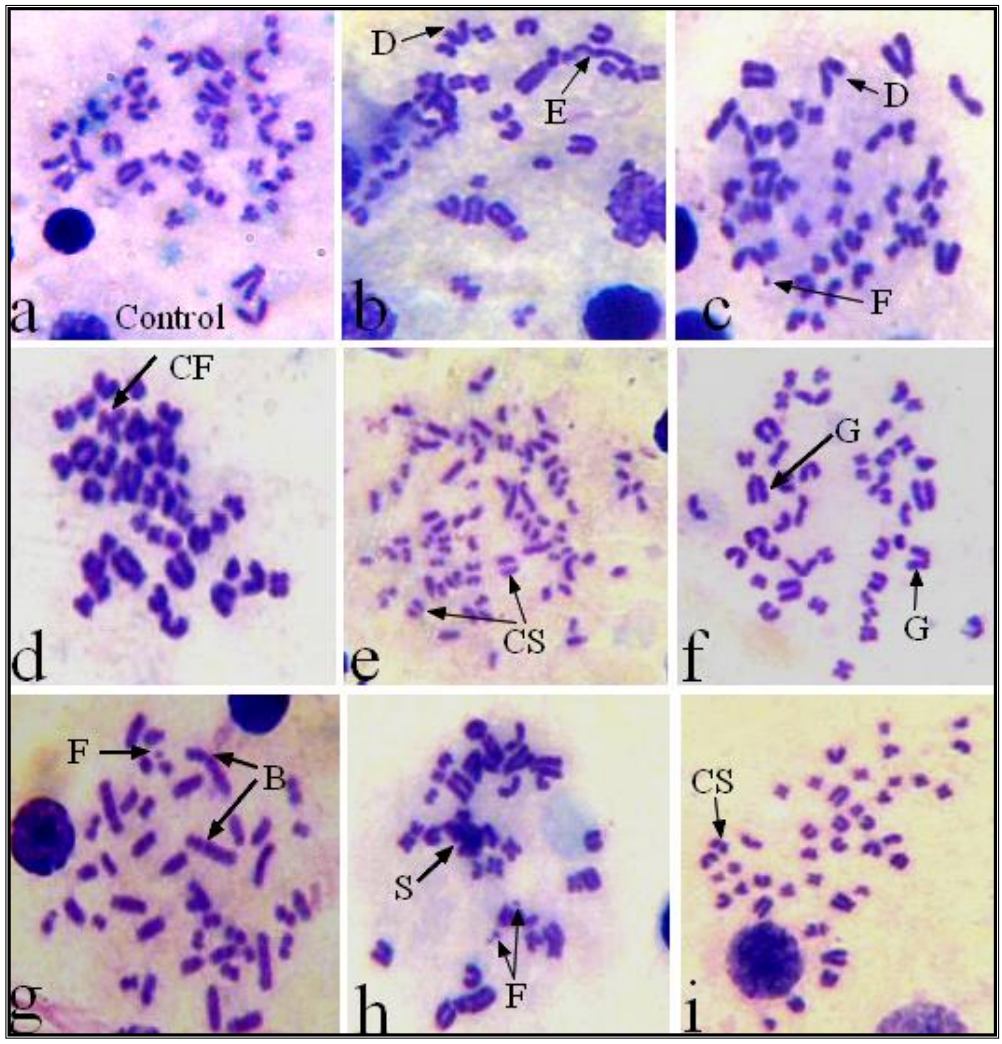

Fig. (1): Micrograph of different types of chromosomal aberrations induced in rat bone marrow cells as a result of different treatments with chitosan, aflatoxin and combination treatments. (a) Metaphase spread from boon marrow cells of control grpup, (b) Deletion and end-to-end associations, (c) Deletion and fragment, (d) Centric fusion, (e) Centric separation, (f) Gap, (g) Endomitosis with fragment and break, (h) Fragment and stickiness, (i) 41 chromosome with centromeric separation.

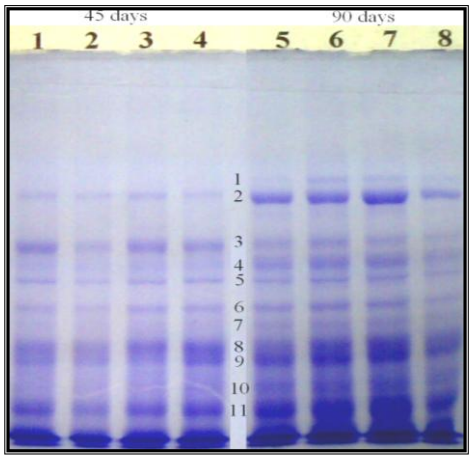

Fig. (2): Electrophoretic banding patterns of total proteins from rat liver treated for 45 and 90 days with chitosan, combination and aflatoxin treatments (lane 2,3,4 & lane 6,7,8 respectively) as well as control (lane 1 & 5).

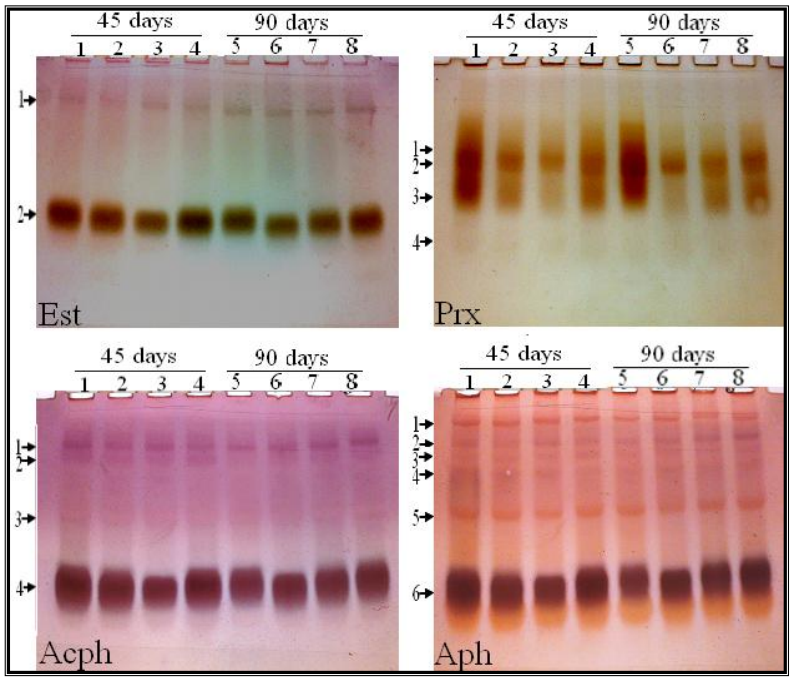

Fig. (3): Electrophoretic banding patterns of esterase (Est), peroxidase (Prx), acid phosphatase (Acph) and alkline phosphatase (Aph) in blood serum of rats treated for 45 and 90 days with chitosan, combination and aflatoxin treatments (lane 2, 3, 4 & lane 6, 7, 8, respectively) as well as control (lane 1 & 5, respectively).