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EVALUATION OF THE PROTECTIVE EFFECT OF CHITOSAN IN MICE TREATED WITH CYCLOPHOSPHAMIDE USING GENOTOXIC ASSAYS AND BIOCHEMICAL MARKERS.

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ABSTRACT

The protective effect of Chitosan in mice treated with Cyclophosphamide has been evaluated in this study using cytogenetic analysis, DNA fragmentation, biochemical markers and molecular genetic assay. The experimental animals divided into two groups as male and female, each group divided into four subgroups. Two subgroups received saline of 0.5ml/ animal as control. Two subgroups received a single i. p. injection of 50 mg/kg Cyclophosphamide in saline for 24 hours as positive control. Two subgroups received Chitosan low dose (15 mg/ kg) for 3 weeks followed by a single i. p. injection of 50 mg/kg Cyclophosphamide in saline for 24 hours. Two subgroups received Chitosan high dose (30 mg/ kg) for 3 weeks followed by a single i. p. injection of 50 mg/kg Cyclophosphamide in saline for 24 hours. The results of different parameters used confirmed that the treated subgroups ($G♂^2$ and $G♀^2$) with Cyclophosphamide induced significant different in the chromosomal aberration of bone marrow, the frequency of micronucleus, DNA damage% and all tested biochemical markers compared to the control and the other treated subgroups. The changes in RAPD markers between the control subgroups and treated Cyclophosphamide subgroups were obvious, including the changes in the polymorphic pattern and molecular size. The results of chromosomal aberration, the frequency of micronucleus, DNA fragmentation, biochemical markers analysis, and polymorphic pattern of RAPD markers in the treated male and female subgroups with Cyclophosphamide and Chitosan (low and high doses) showed a significant improvement compared to the treated Cyclophosphamide subgroups. The improvement increased toward the levels of the control subgroups in pretreated Chitosan at high dose. In conclusion, the results of this study suggested that the pretreatment with Chitosan at dose 30

mg/ kg could act as anti-mutagenic and chemo-protective agent against Cyclophosphamide mutagenic effect.

KEYWORDS: Cytogenetic analysis, DNA fragmentation, Biochemical marker, Molecular genetic assay, Cyclophosphamide, Chitosan.

INTRODUCTION

Chemotherapeutic agent is a chemical agent used for treating various forms of cancer generally by directly killing the cancer cells that divide rapidly. Alkylating agents are the oldest group of chemotherapeutics in use today.[1] Cyclophosphamide (INN, trade names Endoxan, Cytoxan, Neosar, Procytox, Revimmune) is a nitrogen mustard alkylating agent from the oxazaphosphorine group.[2] It is used in the treatment of lymphomas, some forms of brain cancer, leukemia,[3] and some solid tumors.[4] Although that Cyclophosphamide itself is carcinogenic and may increase the risk of developing lymphomas, leukemia, skin cancer, transitional cell carcinoma of the bladder and other malignancies especially at higher doses.[5] Chitosan is obtained from the hard outer skeleton of shellfish, including crab, lobster, and shrimp. It has various biological activities including antimicrobial,[6, 7, 8] antibacterial,[9] and antioxidant activity.[10, 11] Also, the biodegradability, biocompatibility and non-toxicity of Chitosan and its derivatives allow for widespread applications in wound healing,[12, 13] immuno-enhancing effects[14, 15] and antitumor activity.[12, 16] The study aimed to evaluate the protective role of the Chitosan against the harmful effect induced by Cyclophosphamide. To achieve this task a cytogenetic analysis, DNA fragmentation %, biochemical markers and molecular genetic assay were investigated.

MATERIALS AND METHODS

Drug

Cyclophosphamide (trade name Endoxan) was supplied by Baxter oncology (Germany) as solution for injection. Each ampule contains 200 mg/ml. The Cyclophosphamide treatment for experimental animals was a single dose of 50mg/kg intraperitoneally (i.p) 24 hours before the investigated work according to Tripathi et al.[17] and Rudrama et al.[18] Chitosan (trade name Merti) was supplied by, Pharaonia Pharmaceuticals, Egypt. Chitosan doses for experimental animals (low and high) were calculated according to Paget and Barnes.[19]

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. Animals were divided into two groups as male and female, each group divided into four subgroups. Each subgroup was six animals. All animals were received humane care in compliance with the guidelines of the Animal Care and Use Committee of the National Research Centre, Dokki, Giza, Egypt.

Experimental design

Male group

- Subgroup 1($G^{♂1}$) received saline of 0.5ml / animal as control.
- Subgroup 2 ($G^{♂2}$) received a single i. p. injection of 50 mg/kg Cyclophosphamide in saline for 24 hours as positive control.
- Subgroup 3 ($G^{♂3}$) received Chitosan low dose (15 mg / kg) for 3 weeks followed by a single i. p. injection of 50 mg/kg Cyclophosphamide in saline for 24 hours.
- Subgroup 4 ($G^{♂4}$) received Chitosan high dose (30 mg / kg) for 3 weeks followed by a single i. p. injection of 50 mg/kg Cyclophosphamide in saline for 24 hours.

Female group

- subgroup 1 ($G^{♀1}$) received saline of 0.5ml / animal as control.
- subgroup 2 ($G^{♀2}$) received a single i. p. injection of 50 mg/kg Cyclophosphamide in saline for 24 hours as positive control.
- subgroup 3 ($G^{♀3}$) received Chitosan low dose (15 mg / kg) for 3 weeks followed by a single i. p. injection of 50 mg/kg Cyclophosphamide in saline for 24 hours.
- subgroup 4 ($G^{♀4}$) received Chitosan high dose (30 mg / kg) for 3 weeks followed by a single i. p. injection of 50 mg/kg Cyclophosphamide in saline for 24 hours.

Cytogenetic analysis

Chromosome Preparations

For chromosome analysis, both treated and control animals were sacrificed by cervical dislocation at the end of the experiment. Two hours before sacrifice, mice were injected i. p with 0.05 gm colchicine/ kg Femurs were removed and the bone marrow cells were aspirated using saline solution. Chromosome preparation metaphase spreads and slides stain were prepared according Preston et al.[20]

Micronucleus Assay

The bone marrow of the femur was flushed with fetal bovine serum into tubes, smeared on clean slides, and fixed with methanol. The slides were air-dried and stained with 5% Giemsa for 5 minutes.[21]

Quantitation of DNA Fragmentation

DNA fragmentation was quantified by diphenylamine (DPA) as described by Paradones et al.[22] 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).

Molecular genetic assay

DNA extraction

DNA was extracted from the liver of tested animals according to the Sharma et al.[23] The concentration of DNA and its relative purity were determined using a spectrophotometer based on absorbance at 260 and 280 nm, respectively. The integrity of extracted genomic DNA was checked by electrophoresis in 0.8% agarose gel.

RAPD-PCR electrophoresis

The RAPD primers codes and sequences were listed in table 1. The polymerase chain reaction (PCR) solution in total volume 25 µl. A PCR cocktail consists of 0.2 µM primer and 2xpower Taq PCR master mix (Bioteke corporation) were placed into tubes with 50 ng of DNA. The reaction ran in a Coy Temp Cycler II (Coy Corporation, Grass Lake, USA) and was cycled for 1 cycle at 94°C for five minutes and 45 cycles at 94°C for one minute, annealing temperature for each primer for two minutes (table 1), and 72°C for one minute. Final extinction was at 72°C for ten minutes.

PCR products were subjected to electrophoresis on 1.5% agarose gels containing 0.05% ethidium bromide. The polymorphic bands of RAPD were scored on the basis of the band mobility, clear bands were scored using Gel-Doc (Bio-Rad) Gel analysis program as (1) for presence and (0) for absence in a binary data form, the unclear unidentified bands were excluded.

Table 1: RAPD primer codes and sequences used for RAPD-PCR and their annealing temperature.

Primer code	Primer Sequence (5'- 3')	annealing temperature
OPA-01	CAGGCCCTTC	34.0
OPA-03	AGTCAGCCAC	32.0
OPA-04	AATCGGGCTG	32.0
OPA-07	GAAACGGGTG	32.0
OPB 13	TTCCCCCGCT	34.0

Biochemical analysis

Enzyme activities of GOT and GPT in liver tissue were determined according to Reitman and Frankel.[24] The sera were separated from blood samples of the experimental animal groups using cooling centrifugation and stored at -20°C until used. Superoxide dismutase (SOD) activity was assayed according to Nishikimi and Yagi.[25]

Biochemical estimation of cholesterol was developed according to Richmond,[26] Glucose was determined by a differential pH technique according to Ripamonti et al.[27] and Triglyceride was measured according to Fossati and Prencipe.[28]

Scoring and statistical analysis

For each animal, 100 well-spread metaphases were examined for structural chromosomal aberrations. Chromosomal aberrations were scored by following the guideline of Brusick [29] and Preston et al.[20] In each animal, 2000 cells were examined for measuring micronucleus (MN) formation. The results of chromosomal aberrations, micronucleus formation, DNA fragmentation and biochemical analysis were analyzed using the arithmetic mean, standard error. Statistical analysis was performed with SPSS software. Data were analyzed using one-way analysis of variance (ANOVA) followed by Duncan ´s post hoc multiple comparisons tests .[30] Moreover Chi-Square test was used for comparison between male and female mice for inducing micronuclei and chromosome aberrations .The differences were considered statistically significant when p-value ≤ 0.05.

RESULTS

Cytogenetic results

The results of chromosomal aberrations involved breaks, gaps, deletions and fragments, ring, and end to end association. The frequency of different types of structural chromosomal

aberrations in bone marrow cells in all experimental animals of male and female groups illustrates in table 2 and 3, respectively.

In male group (table 2), the animals treated with Cyclophosphamide ($G^{♂2}$) showed high significant difference in deletion, breaks and fragments aberrations compared to the other experimental subgroups. The total structural aberrations in animals treated with Cyclophosphamide/Chitosan, low dose ($G^{♂3}$) was high significant different (22.33 ± 0.88) compared with the control subgroup (2.33 ± 0.33). In $G^{♂4}$, however, the total structural aberrations was decreased compared to the other treated subgroups, a significant difference was observed between this subgroup and the control subgroup ($G^{♂1}$). The ring aberration was observed in all treated animals with a significant difference compared to the control subgroup.

Table 2: The frequency of chromosomal aberrations in control and treated male group.

treatment	Structural chromosomal aberrations						Total structural aberrations
	Gap	Break	Fragment	Deletion	End to end association	ring	
$G^{♂1}$	0.67 ±0.33 a	0.33± 0.33 a	1.00 ±0.57 a	0.33 ±0.33 a	00 a	00 a	2.33 ± 0.33 a
$G^{♂2}$	2.33±0.33 b	5.67 ±0.33 c	9.00 ±1.15 b	4.67 ±0.66 c	4.67 ±0.88 b	2.67 ± .33 c	29.00 ±0.57 d
$G^{♂3}$	1.67 ± 0.33 ab	3.33±0.33 b	8.33±0.0.88b	3.67 ± 0.33 bc	3.33 ±0.33 b	2.00 ±0.00 bc	22.33 ± 0.88 c
$G^{♂4}$	1.33±0.33 ab	3.00±1.00 b	7.33 ±1.20 b	2.33 ±0.67 b	1.33 ±0.33 a	1.67± 0.33 b	17.00 ± 1.53 b

Data were expressed as mean ± S.E.

Means with different superscript letters (a, b, c,d) are significantly different ($P<0.05$)

In female group (table 3), the animals treated with Cyclophosphamide ($G^{♀2}$) showed high significant difference in deletion, breaks and fragments aberrations compared to the control subgroup. The animals treated with Cyclophosphamide/Chitosan, low or high doses showed a significant difference in gaps and breaks aberrations compared to the control subgroup. Deletion aberration in differently treated subgroups observed with a high frequency compared to the frequency of other chromosomal aberrations screened. The total structural aberrations between differently treated subgroups was a significant different, $G^{♀4}$ recorded the lowest frequency (15.67 ± 0.88) between the treated subgroups but with a significant different compared to the control subgroup (3.00± 0.58).

Table 3: The frequency of chromosomal aberration in control and treated female group.

treatment	Structural chromosomal aberrations						Total structural aberrations
	Gap	Break	Fragment	Deletion	End to end association	ring	
$G^{♀1}$	1.33 ±0.33 a	0.33 ±0.33 a	1.00 ±0.58 a	0.33 ±0.33 a	00 a	00 a	3.00± 0.58 a
$G^{♀2}$	3.000±0.57 a	3.67 ±0.66 b	9.33 ±0.67 c	7.33 ±0.67 c	5.00 ±0.57 c	3.00 ± 0.57 c	31.33 ±1.33d
$G^{♀3}$	2.67 ± 0.88 a	2.00±0.57 a	7.33±1.20b c	6.67 ± 0.88 c	2.33 ±0.33 b	2.00 ± 0.57bc	23.00 ±1.15 c
$G^{♀4}$	2.00±1.15 a	1.33±0.33 a	5.67 ±0.88 b	4.00 ±0.57 b	2.00±0.57 b	0.67 ± 0.33ab	15.67 ±0.88 b

Data were expressed as mean ± S.E.

Means with different superscript letters (a, b, c,d) are significantly different (P<0.05)

Comparative study of the frequency of chromosomal aberrations between male and female subgroups

The results of table 4 are shown that there was a non-significant difference between female and male subgroups except in deletion aberration, the significant was decreased in pretreated female subgroup with high dose of Chitosan compared to pretreated female with Chitosan low dose.

Table 4: Comparison the chromosomal aberrations in male and female mice in control and treated subgroups.

Treatment	Structural chromosomal aberrations						Total structural
	Gap	Break	Fragment	Deletion	End.	Ring	
$G^{♂1}$	0.67 ±0.33	0.33± 0.33	1.00 ±0.57	0.33 ±0.33	00 ± 00	00 ± 00	2.33 ± 0.33
$G^{♀1}$	1.33 ±0.33	0.33 ±0.33	1.00 ±0.58	0.33 ±0.33	00 ± 00	00 ± 00	3.00± 0.58
X2 values	1.74	00	00	00	00	00	0.67
$G^{♂2}$	2.33±0.33	5.67 ±0.33	9.00 ±1.15	4.67 ±0.66	4.67 ±0.88	2.67 ± 0.33	29.00 ±0.57
$G^{♀2}$	3.000±0.57	3.67 ±0.66	9.33 ±0.67	7.33 ±0.67	5.00 ±0.57	3.00 ± 0.57	31.33 ±1.33
X2 values	0.674	3.4	0.05	4.72*	2.83	0.15	0.7
$G^{♂3}$	1.67 ± 0.33	3.33±0.33	8.33±0.0.88	3.67 ± 0.33	3.33 ± 0.33	2.00 ± 0.00	22.33 ±0.88
$G^{♀3}$	2.67 ± 0.88	2.00±0.57	7.33±1.20	6.67 ± 0.88	2.33 ±0.33	2.00 ± 0.57	23.00 ±1.15
X2 values	1.84	2.66	0.51	6.96**	0.20	00	0.079
$G^{♂4}$	1.33 ± 0.33	3.00±1.00	7.33 ±1.20	2.33 ±0.67	1.33 ±0.33	1.67± 0.33	17.00 ± 1.53
$G^{♀4}$	2.00 ± 0.15	1.33±0.33	5.67 ±0.88	4.00 ±0.57	2.00±0.57	0.67± 0.33	15.67 ± 0.88
X2 values	1.08	5.15	1.7	5.52*	0.24	3.42	0.433

*Significant at P<0.05. **Significant at P<0.01.

Micronucleus assay results

The results of micronucleus assay of male and female groups present in table 5 and 6, respectively. In male group, the frequency of micronucleus in animals treated with Cyclophosphamide ($G^{♂2}$) and Cyclophosphamide/Chitosan low dose ($G^{♂3}$) showed a significant difference compared to $G^{♂1}$ and $G^{♂4}$. In the subgroup treated with

Cyclophosphamide/Chitosan high dose ($G^{♂4}$) the result observed with a significant difference than that observed in $G^{♂2}$ and $G^{♂3}$, however, the difference between $G^{♂4}$ and the control ($G^{♂1}$) was a significant difference (table 5).

Table 5: The frequencies of micronucleated polychromatic erythrocytes in control and treated male group

Treatment	Number of examined cells	Mean values of MNPCE
$G^{♂1}$	2000	3.67 ±0.67 a
$G^{♂2}$	2000	20.67 ± 1.20 c
$G^{♂3}$	2000	18.33 ± 0.88 c
$G^{♂4}$	2000	12.33 ± 0.67 b

Data were expressed as mean ± S.E.

Means with different superscript letters (a, b, c) are significantly different (P<0.05)

In female group, the frequency of micronuclei in animals treated with Cyclophosphamide ($G^{♀2}$) showed high significant difference compared to the other experimental subgroups. The frequency of micronucleus in $G^{♀3}$ was a significant difference compared to the $G^{♀1}$ and $G^{♀4}$. In the subgroup treated with Cyclophosphamide/Chitosan high dose ($G^{♀4}$) the result observed with a significant difference than that observed in $G^{♀2}$ and $G^{♀3}$, however, the difference between $G^{♂4}$ and the control ($G^{♀1}$) was a significant difference (table 6).

Table 6: The frequencies of micronucleated polychromatic erythrocytes in control and treated female group

Treatment	Number of examined cells	Mean values of MNPCE
$G^{♀1}$	2000	4.67 ± 0.33 a
$G^{♀2}$	2000	25.33 ± 1.76 c
$G^{♀3}$	2000	21.33 ± 0.33 d
$G^{♀4}$	2000	15.33 ± 1.20 b

Data were expressed as mean ± S.E. Means with different superscript letters (a, b, c, d) are significantly different (P<0.05)

Comparative study of the frequency of micronuclei between male and female groups

Table 7 is shown that there was a significant different in Cyclophosphamide treated subgroups male and female, while a non-significant difference in the frequency of micronuclei was observed between the other male and female subgroups.

Table 7: Comparison the frequency of micronuclei between male and female mice in control and treated subgroups

Treatment	MNPE
$G^{♂1}$	3.67 ±0.67
$G^{♀1}$	4.67 ± 0.33
X2 values	0.96
$G^{♂2}$	20.67 ± 1.20
$G^{♀2}$	25.33 ± 1.76
X2 values	3.77*
$G^{♂3}$	18.33 ± 0.88
$G^{♀3}$	21.33 ± 0.33
X2 values	1.82
$G^{♂4}$	12.33 ± 0.67
$G^{♀4}$	15.33 ± 1.20
X2 values	2.6

*Significant at P<0.05

DNA fragmentation results

DNA fragmentation in all experimental animals of male and female groups is shown in table 8. In male group, the DNA fragmentation in animals treated with Cyclophosphamide ($G^{♂2}$) and Cyclophosphamide/Chitosan low dose ($G^{♂3}$) was high significantly different compared to the control subgroup. DNA fragmentation of $G^{♂4}$ was decreased compared to the other treated groups but with a significant different compared with the control subgroup.

In female group, the DNA fragmentation in animals treated with Cyclophosphamide ($G^{♀2}$) and Cyclophosphamide/Chitosan low dose was high significant different compared to the control subgroup. The same observation as in male, the DNA fragmentation in $G^{♀4}$ was decreased compared to the other treated subgroups but with significantly different compared with the control subgroup.

Table 8: DNA fragmentation in mice's livers of control and treated groups

Sex	Treatment	Rate of DNA fragmentation	Change %
Males	$G^{♂1}$	6.0 ± 0.31^{a}	–
	$G^{♂2}$	24.32 ± 0.49^{e}	18.32
	$G^{♂3}$	21.04 ± 0.72^{d}	15.04
	$G^{♂4}$	16.92 ± 0.47^{b}	10.92
Females	$G^{♀1}$	7.1 ± 0.48^{a}	–
	$G^{♀2}$	26.27 ± 0.34^{f}	19.17
	$G^{♀3}$	23.28 ± 0.73^{e}	16.18
	$G^{♀4}$	18.75 ± 0.76^{c}	11.65

Data were expressed as mean ± S.E. Means with different superscript letters (a, b, c,d,e,f) are significantly different (P<0.05)

Molecular assay results

The results of five RAPD primers illustrated that the primer OPA-1 molecular size of amplified bands ranged from 400 to 900-bp. The polymorphic pattern in $G^{♂1}$ and $G^{♀1}$ subgroups was five bands with three bands in different sizes between the two subgroups (figure 1). In treated subgroups $G^{♂2}$ and $G^{♀2}$, the polymorphic pattern was four bands for each subgroup and at molecular size different compared to the control subgroups. The polymorphic pattern in $G^{♂3}$ and $G^{♀3}$ was five bands but at molecular size different compared to the control subgroups. In treated subgroups $G^{♂4}$ and $G^{♀4}$, the polymorphic pattern was five bands and almost at the same molecular size of the similar bands in control subgroups.

Primer OPA-3 of RAPD analysis was polymorphic with molecular size bands ranged from 475- 900-bp (fgure 1). The polymorphic pattern in $G^{♂1}$ and $G^{♀1}$ was four bands at the same molecular sizes in the two subgroups. The treated subgroups with Cyclophosphamide and Cyclophosphamide/Chitosan low dose were observed with three bands for each subgroup at the same molecular size of the similar bands in control subgroups. In treated subgroups $G^{♂4}$ and $G^{♀4}$, the polymorphic pattern was four bands at the same molecular size of the similar bands in control subgroups.

Primer OPA-4 of RAPD analysis was polymorphic with molecular size bands ranged from 375- 900-bp (Fig. 1). The polymorphic pattern in $G^{♂1}$ and $G^{♀1}$ subgroups was five bands at the same molecular size in the two subgroups. In treated subgroups $G^{♂2}$ and $G^{♀2}$, the polymorphic pattern was four bands in $G^{♂2}$ and decreased to two bands in $G^{♀2}$. In treated subgroups $G^{♂3}$ and $G^{♀3}$ the polymorphic pattern was five and two, respectively. In treated subgroups $G^{♂4}$ and $G^{♀4}$, the polymorphic pattern was five bands at the same molecular size of the similar bands in the control subgroups.

Primer OPA-7 of RAPD analysis was polymorphic with molecular size bands ranged from 200- 800-bp (figure 1). The polymorphic pattern in $G^{♂1}$ and $G^{♀1}$ subgroups was eight and five bands, respectively. In treated subgroups $G^{♂2}$ and $G^{♀2}$, the polymorphic pattern decreased to four bands in the two subgroups at different sizes compared to the control subgroups. The treated subgroups with Cyclophosphamide/Chitosan low dose and

Cyclophosphamide/Chitosan high dose were polymorphic with five bands compared to the control subgroups.

Primer OPB-13 of RAPD analysis was polymorphic with molecular size bands ranged from 300-1200-bp (figure 1). The polymorphic pattern in control subgroups was five bands. In treated subgroups $G^{\male 2}$ and $G^{\female 2}$ the polymorphic pattern decreased to four bands compared to the control subgroups. The treated subgroups with Cyclophosphamide/Chitosan low dose and Cyclophosphamide/Chitosan high dose were polymorphic with five bands for male subgroups and four bands for female subgroups compared to the control subgroups.

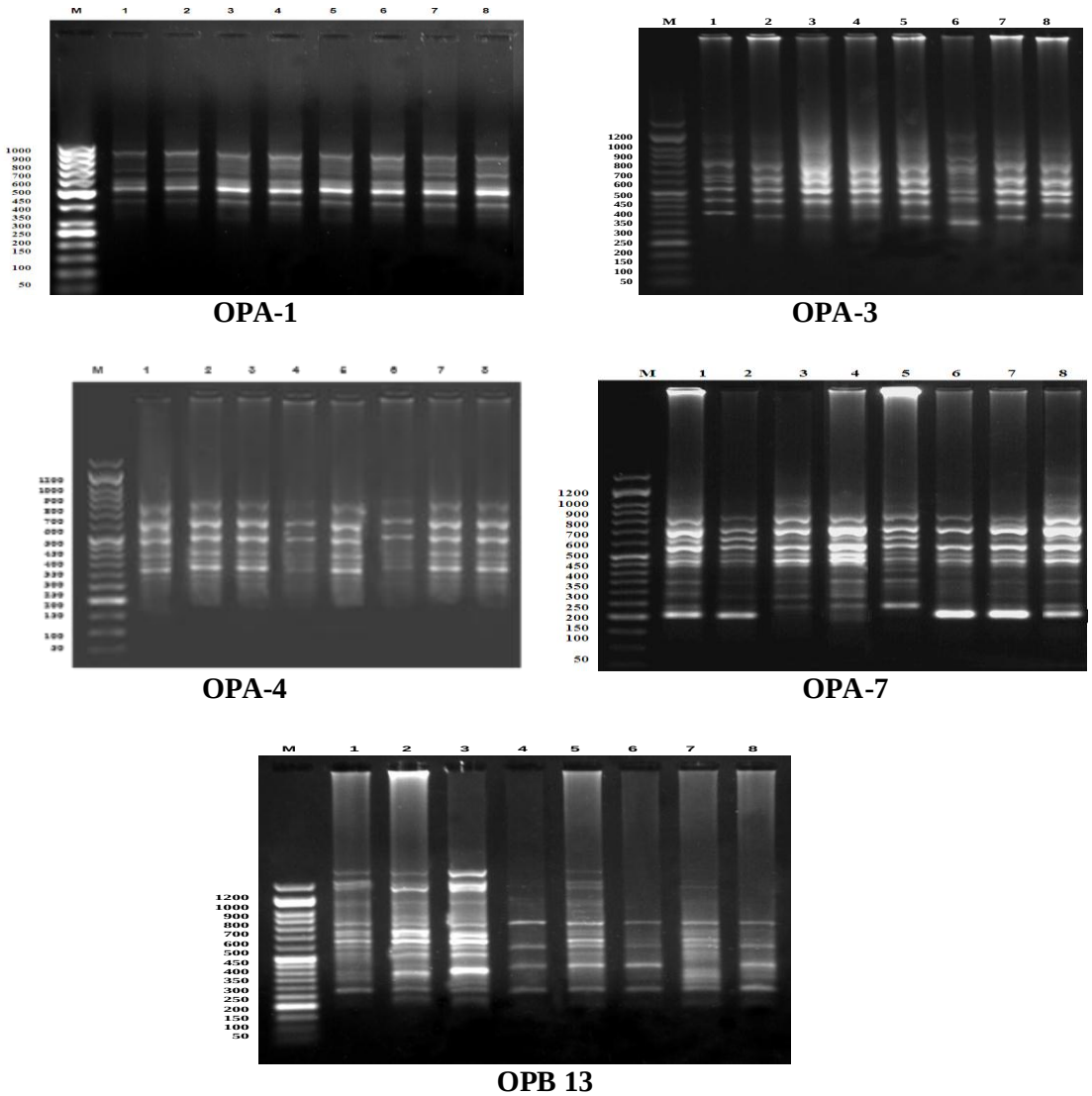

Figure 1: RAPD – PCR pattern of primers OPA-01, OPA-03, OPA-04, OPA-07, OPB 13, in all experimental groups. M: 50-bp Ladder, Lanes 1to 8: $G^{\male 1}$, $G^{\female 1}$, $G^{\male 2}$, $G^{\female 2}$, $G^{\male 3}$, $G^{\female 3}$, $G^{\male 4}$, $G^{\female 4}$, respectively.

Biochemical analysis results

The chemo-protective effects of chitosan were evaluated on male and female mice treated with Cyclophosphamide as strong cytotoxic agent by measuring standard biochemical markers. Table 9, 10 illustrated the levels of SOD, GOT, GPT, Glucose, Cholesterol and Triglycerides of experimental male and female subgroups. The results showed that the estimated biomarkers activities in male and female different subgroups had almost the similar significant different between the treated and control subgroups.

Cyclophosphamide treated subgroups ($G^{♂2}$ and $G^{♀2}$) induced strong damage to liver cells as indicated by highly increase of liver enzymes (GOT and GPT) compared to the control subgroups. These subgroups reported with significant different in the levels of Glucose, Cholesterol and Triglycerides compared to the control and the treated subgroups with Cyclophosphamide/Chitosan at low and high doses.

The treated male and female subgroups with Cyclophosphamide/Chitosan at low dose showed improved in the levels of biochemical markers (SOD, GOT, GPT, Glucose, Cholesterol and Triglycerides) compared to Cyclophosphamide treated subgroups ($G^{♂2}$ and $G^{♀2}$). The improvement increased towards the levels of the control subgroups in treated subgroups $G^{♂4}$ and $G^{♀4}$ with Cyclophosphamide/Chitosan at high dose.

Table 9: Biomarkers detection of control and treated male subgroups.

Parameters / Treatment	SOD U/ml	GOT U/mg	GPT U/mg	Glucose mg/dL	Cholesterol mg/dL	Triglycerides mg/dL
$G^{♂1}$	91.98±1.15 a	50.33±1.20 a	69.00±0.57 a	60.41±0.43 a	149.73±0.67 a	80.04± 0.20 a
$G^{♂2}$	68.10±1.02 c	70.00±1.15 d	87.66±1.45 c	76.63±0.69 d	167.66±0.55 d	91.65±0.33 d
$G^{♂3}$	69.48±0.99 c	65.33±1.45 c	76.66±0.88 b	70.25±0.48 c	162.40±0.70 c	86.25± 0.34 c
$G^{♂4}$	7 6.19±1.42b	56.33±1.20 b	71.33±0.88 a	64.52±0.82 b	157.19±0.56 b	81.67± 0.44b

Data were expressed as mean ± S.E.

Means with different superscript letters (a, b, c,d) are significantly different (P<0.05)

Table 10: Biomarkers detection of control and treated female subgroups.

Parameters / Treatment	SOD U/ml	GOT U/mg	GPT U/mg	Glucose mg/dL	Cholesterol mg/dL	Triglycerides mg/dL
$G^{♀1}$	98.05±0.75 a	52.00±1.52 a	72.33±0.88 a	61.42±0.40 a	152.80±0.80a	81.26±0.45 a
$G^{♀2}$	72.94±0.94 d	75.66±1.20 d	95.33±2.02 c	79.50±0.68 d	169.73±0.30 d	96.23±1.70 c
$G^{♀3}$	77.48±0.78 c	70.00±0.57c	80.00± 0.57 b	72.64±0.39 c	162.89±0.96 c	87.92±0.21 b
$G^{♀4}$	81.81±0.75 b	60.66±1.20 b	74.00 ±0.57 a	67.65±1.01 b	159.25±0.28 b	84.15±0.19 a

Data were expressed as mean ± S.E.

Means with different superscript letters (a, b, c,d) are significantly different (P<0.05)

DISCUSSION

The protective effect of Chitosan in mice treated with Cyclophosphamide has been evaluated in this study using cytogenetic analysis, DNA fragmentation, biochemical markers and molecular genetic assay. The results of different parameters used confirmed that the treated subgroups ($G^{♂2}$ and $G^{♀2}$) with Cyclophosphamide induced significant different in the chromosomal aberration of bone marrow, the frequency of micronucleus, DNA damage% and all tested biochemical markers compared to the control and the other treated subgroups (tables 2- 10). The highly significant observed in chromosomal deletion compared with other aberrations illustrate the mutagenic effect of Cyclophosphamide. The results also revealed that the mutagenic effect on female group was significant difference compared with male group (table 4). The results of chromosomal aberration and the frequency of micronucleus are in agreement with previous studies reported by Raja et al.,[31] Sharma et al.,[23] and Rudrama et.al.[32] Hales[33] suggested that Cyclophosphamide gets metabolized to phosphoramide mustard and acrolein before it can act as a mutagenic agent. The chromosomal aberrations induced due to the lesions in DNA caused by phosphoramide mustard which lead to discontinuities of the DNA helix. The results of DNA fragmentation and biochemical analysis confirmed the cytotoxic effect of Cyclophosphamide on liver cells (tables 8-10). The biomarkers results of SOD, glucose, Cholesterol and Triglycerides in treated Cyclophosphamide subgroups clarified the adverse effect of Cyclophosphamide on body function performance. These results suggested that the Free radicals due to Cyclophosphamide lead to high chemical reactivity which induce cellular damage in a number of ways, causing a number of pathological conditions.[34, 35] At the molecular level the genotoxic effect of Cyclophosphamide was estimated by Randomly Amplified Polymorphic DNA (RAPD) which is a polymerase chain reaction (PCR)-based technique used to amplify randomly DNA fragments with several short-chain primers of arbitrary nucleotide sequence.[36] Unlike conventional PCR, RAPD does not need to obtain sequence data for primer design, which makes experimental manipulation much easier. Compared with other techniques of genetic toxicology, such as comet, micronucleus and chromosome aberration assays, RAPD measures genotoxicity directly on DNA with the advantages related to the sensitivity and short response time.[37, 38] The molecular genetic results of cluster and genetic diversity analysis was performed based on the changes of polymorphic RAPD pattern. The changes in RAPD polymorphic pattern between control subgroups and treated Cyclophosphamide subgroups were obvious, including the changes in the polymorphic pattern and molecular size (figure 1). The results of RAPD showed that the polymorphic

pattern of primers OPA-1, OPA-3, OPA-4, OPA-7 and OPB-13b showed decreases in the number of bands in Cyclophosphamide treated subgroups compared to the control subgroups. These bands were at different molecular sizes compared to the molecular size bands in control subgroups. The results suggested that the appearance of new fragments may be attributed to some sites becoming accessible to the primer after point mutations and/or large rearrangements occur in genomic DNA[36] while the disappearance of bands possibly resulted from the presence of DNA photoproducts (pyrimidine dimmers), which can act to block or reduce DNA polymerization in the PCR reactions.[39] Previous studies proved that changes in DNA fingerprint could reflect DNA alteration in genome from single base changes (point mutations) to complex chromosomal rearrangements.[38] The results of the molecular assay confirmed the results obtained by cytogenetic and biochemical analysis and proved that the Cyclophosphamide had mutagenic effect at the molecular level. According to the mutagenic effect of Cyclophosphamide, Chitosan was selected in our study as a protective agent against Cyclophosphamide mutagenic effect. The animals were pretreated with Chitosan at low does 15 mg/ kg for subgroups $G^{♂3}$ and $G^{♀3}$, and at high dose 30 mg/ kg for subgroups $G^{♂4}$ and $G^{♀4}$. The results of chromosomal aberration, the frequency of micronucleus, DNA fragmentation, biochemical markers analysis, and polymorphic pattern of RAPD markers in the treated male and female subgroups with Cyclophosphamide and Chitosan (low and high doses) showed a significant improvement compared to the treated Cyclophosphamide subgroups. The improvement increased toward the levels of the control subgroups in pretreated Chitosan at high dose (table 2- 10 and figure 1). These results suggested that Chitosan can protect against Cyclophosphamide mutagenic effect in dose-dependent manners in both female and male mice. The results are in agreement with previous studies reported that Chitosan significantly inhibited mercury induced MN and chromosomal aberrations in mice.[40] In another study Chitosan treatment prevents CCl4-induced genotoxicity and apoptosis in the bone marrow and liver of mice.[41] Eshak and Osman[42] recorded that the Chitosan treatment counteracted the effects of Bisphenol and decreased the liver inflammation and necrosis. Antitoxic activity mediated by the derivatives of Chitin and Chitosan was also reported.[43, 44] Also, Chitosan have shown potential as scavenging agents, due to their ability to abstract hydrogen atoms free radicals.[45] This ability has been reported as directly correlated with their structural properties (amino and hydroxyl group) that can react with unstable free radicals to form stable macromolecules radicals.[46, 47]

CONCLUSION

The results of this study suggested that the pretreatment with Chitosan at dose 30 mg/ kg could act as anti-mutagenic and chemo-protective agent against Cyclophosphamide mutagenic effect.

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