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Genotoxic Impacts of Gluten on Chromosomes and DNA of Mice

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ABSTRACT
Background: Cereal grains contain a group of proteins known as gluten, which is a significant ingredient of the endosperm of mature cereal grains, making up around 80% of the protein content.
Aim: This study investigated the genotoxic effects of gluten on male albino mice's chromosomes and DNA.
Materials and methods: The study included four groups of mice: a control group, a negative control group given glacial acetic acid, and two groups given gluten doses of 1.5 and 3 g/kg body weight. These treated groups were given oral injections 3 times/week over 4 weeks. To assess the chromosomal abnormalities of the bone marrow cells, the research utilized C-banding and G-banding techniques.
Results: The study found that giving mice gluten at both low and high doses caused abnormal changes in bone marrow chromosomes. The effects were more severe with the higher dose of gluten. These changes involved chromatid abnormalities such as deletion and fragments, and chromosome abnormalities such as centromeric attenuation, centric fusion, ring formation, end-to-end association, and chromosomal gap, as well as numerical abnormality like polyploidy. These changes suggested that gluten treatment may have genotoxic effects. Additionally, the study found that the treated mice experienced DNA damage, which indicated that gluten can negatively impact DNA integrity.
Conclusion: Limiting gluten intake is important to avoid damage to chromosomes and DNA and prevent potentially harmful effects on human health. Further research is necessary to understand the genotoxicity mechanisms caused by gluten.
Keywords: C-banding, Chromosomes, DNA, G-banding, Gluten, Mice.

INTRODUCTION

The technological quality of common wheat is significantly determined by the gluten protein composition of cereal grains [(1)]. When making bread and pasta, gluten proteins play a crucial role in providing the necessary cohesion in the dough. Wheat contains gliadins (monomers) and glutenins (polymers), while barley contains hordeins and rye contains secalins. These proteins, also known as prolamins, are rich in glutamine and proline amino acids [(2)]. The properties of dough's viscoelasticity are determined by gluten, a type of storage protein found in grains. The gluten network in dough provides the necessary cohesion for making bread and pasta [(3)]. When wheat dough is rinsed, the water-soluble components like starch are removed, leaving behind the viscoelastic gluten [(4)].

Wheat allergy, nonceliac gluten sensitivity, and celiac disease (CD) are the three primary disorders associated with gluten intolerance [(5)]. In individuals with a genetic predisposition, consuming gluten can lead to celiac disease. The small intestine mucosa is affected by CD, which is a chronic autoimmune disorder that leads to villi atrophy [(6)]. Gluten intake in individuals with CD results in an immune response that causes damage to the small intestine epithelia. The recommended treatment involves adopting a diet that is free of gluten [(7)].

Consuming gluten can lead to an increased occurrence of chromosomal abnormalities that are detected through karyotyping of peripheral blood lymphocytes (PBL). The identification of this event is acknowledged as a cancer risk biomarker in humans, as it can indicate either the initial biological consequences of genotoxic substances or an individual's predisposition to cancer [(8)].

Patients with CD who followed a free diet of gluten showed a significant decrease in the frequency of chromosomal abnormalities in their PBL [(9)]. The two main proteins in gluten, gliadins, and glutenins, are known to be detrimental to individuals with CD. Glutenins form a mesh of fibers that trap globular gliadins [(10)]. Individuals with CD experience DNA damage, pro-apoptotic stimulation, and cellular oxidative stress in their cells and the mucosa of their duodenum as a result of gliadins [(11)].

MATERIALS AND METHODS

Chemical used:

For the experiment, gluten powder obtained from Sigma-Aldrich Corporation in Cairo was used. The gluten powder had a protein basis assay of ≥75% and a CAS number of 8002-80-0. The gluten powder was made more soluble by dissolving it in glacial acetic acid (0.02 mM) to produce a concentrated solution. This concentrated solution was then given to the animals through an oral feeding tube at doses of 1.5 and 3 g/kg body weight (b.wt.). Mice were orally given 3 non-consecutive days/week for 4 weeks to perform the gluten challenge. The control group consisted of mice that were not treated with gluten or acetic acid, while a negative control group, which received only 0.02 mM acetic acid [(12, 13)].

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The molecular formula of gluten is C30H38N6O7 (Figure **(1)** [14].

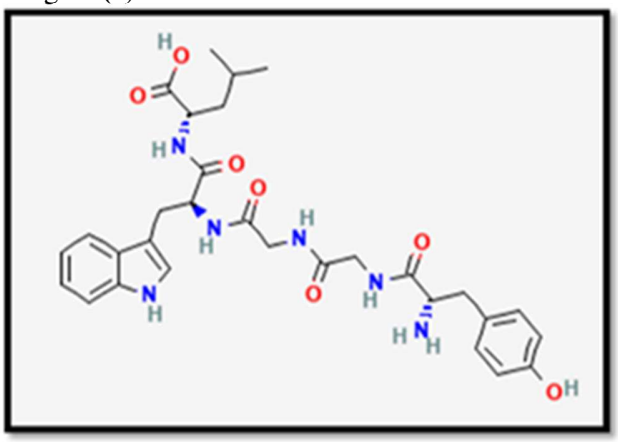

Figure (1): Chemical structure of gluten [14] (NCBI 2023).

Experimental animals: The study involved 40 male albino mice aged between 16-18 weeks that weighed between 25-30 g. The mice used in the experiment were purchased from Theodor Bilharz Research Institute's animal house in El-Giza-Egypt. The mice were cadged in clean plastic containers filled with wood shavings and were given a standard rodent pellet diet, with unrestricted access to room temperature water (25 ± 2 °C) a 12 hour light and dark cycle, and relative humidity of 55 ± 5%. Before the experiment began, the mice were given a week to acclimatize to their new environment. The study was conducted by international animal laboratory treatment standards.

Experimental design: The animals were divided into four groups, each group consisting of ten mice. 1st group, the control group did not receive any treatment. The 2nd group, the negative control group, was given oral doses of glacial acetic acid. 3rd group received oral doses of gluten (1.5 g/kg b.wt.) dissolved in 0.02 M glacial acetic acid. 4th group received oral doses of gluten (3 g/kg b.wt.) in 0.02 M glacial acetic acid. The doses were given three times a week, but not consecutively for four weeks.

Chromosome C-banding and G-banding techniques: The bone marrow chromosomes were prepared for analysis using a well-established protocol [15, 16, 17, 18].

The experimental procedure proposed by **Preston *et al.*** [15] and **Barch *et al.*** [19] was followed to prepare the chromosomal C-banding and G-banding techniques. Chromosomes were prepared from the collected bone marrow and observed under a bright field microscope. Most of the photos were taken with oil immersion at 100x magnification. The best photograph of a well-spread metaphase stage was used to create a karyotype. Cells from all groups, including the control, were examined for structural and numerical chromosome abnormalities to observe the impacts of gluten on the chromosomes of bone marrow cells after four weeks of treatment.

Mitotic index

During the study, we examined at least 1000 metaphase spreads for each group, where each group consisted of five animals. The % cells undergoing division among the full number of cells that were examined was determined to calculate the mitotic index. This measurement provides crucial information about the cell division and proliferation rate in the studied samples.

Comet assay:

A comet assay was conducted following a previously established protocol [20]. The length of the tail comet was measured in micrometers and then compared to the diameter of the nuclei of undamaged cells in the same field to determine the severity of the DNA damage. To assess the influence of gluten on the degree of DNA damage, the mean ± standard deviation of the percentage of DNA in the tail length of the total DNA migration was calculated after analyzing a hundred cells per animal.

Ethics approval and consent to participate: Animal care and use protocols were conducted according to animal care guidelines approved by the Authorities of Ain Shams University. The committee's reference number was Sci1312306009.

Statistical analysis

Data collected from chromosomal abnormality assay, mitotic index, and comet assay were analyzed using statistical techniques. Results for each group were expressed using mean and standard deviation. To assess group differences, a one-way ANOVA was used with IBM SPSS Statistics for Windows, version 16, followed by the SCHEFFE test. $p \leq 0.05$ was considered statistically significant.

RESULTS

C and G-banding chromosomes:

The bone marrow chromosomal G-banding analysis revealed that all chromosome pairs had prominent G-bands that differed in number, thickness, and staining intensity. Two types of chromosomal abnormalities were detected namely structural and numerical abnormalities. The structural abnormalities included chromosomal and chromatid-type abnormalities, while the numerical abnormality was a change in the normal diploid number of chromosomes. Figure (3) showed that no significant differences ($p \geq 0.05$) were found in the total number of abnormalities between the control mice and the mice treated with glacial acetic acid. On the other hand, both low-dose and high-dose gluten-treated mice showed significantly higher numbers ($p \leq 0.05$) of total abnormalities, with 44.7% and 58.2%, respectively, compared to the control group.

The treatment with gluten resulted in several structural abnormalities (**Figure (2** A, B, C, and D), including deletions (D), which were seen as a lack of a segment of chromosome resulting in one chromatid being longer than its sister chromatid in the same chromosome. The chromosomes appeared to consist of a continuous series of light and dark bands along their entire length, with their centromere regions appearing as darkly stained regions. The presence of fragments (F), centromeric attenuations (Ca), and centric fusions (Cf) was also observed, where the centromeres of abnormal chromosomes stretched and darkly stained (C-bands). Sister chromatids were faintly stained and appeared to be joined by very thin chromatin threads. Additionally, a unique pattern of dark and light G-bands was observed consecutively along each chromosome. The centromeric region appeared wide and C-bands were darkly stained, while the chromosomes showed a continuous series of light and dark G-bands. Ring formations (R), end-to-end associations (Ee), and beaded chromosomes (Bch) were also observed. Furthermore, gaps were observed in one chromatid called chromatid gaps (Cg) or in both sister chromatids called chromosomal gaps (Chg), where the gap was an achromatic non-staining region. Numerical abnormalities such as polyploidy (Po) were also evident.

The results of the study, as shown in Figure (3) indicate that the administration of glacial acetic acid alone did not have a significant impact on chromosomal abnormalities ($p \geq 0.05$) compared to the control group. However, mice treated with low and high doses of gluten showed significant increases ($p \leq 0.05$) in chromosomal abnormalities, specifically centromeric attenuations (95% and 99% respectively), deletions (40% and 40.86% respectively), and ring formations (29.8% and 35.6% respectively). Moreover, the number of fragments and centric fusions in mice treated with the high gluten dose was significantly different ($p \leq 0.01$) from those of the control group (360% and 75.8%, respectively).

The study also found that the consumption of gluten affected the number of chromosomes in the bone marrow cells of mice, increasing polyploidy cells. Mice treated with low and high doses of gluten showed significant increases ($p \leq 0.05$) in the number of polyploidy cells (3% and 12% respectively) compared to the control group.

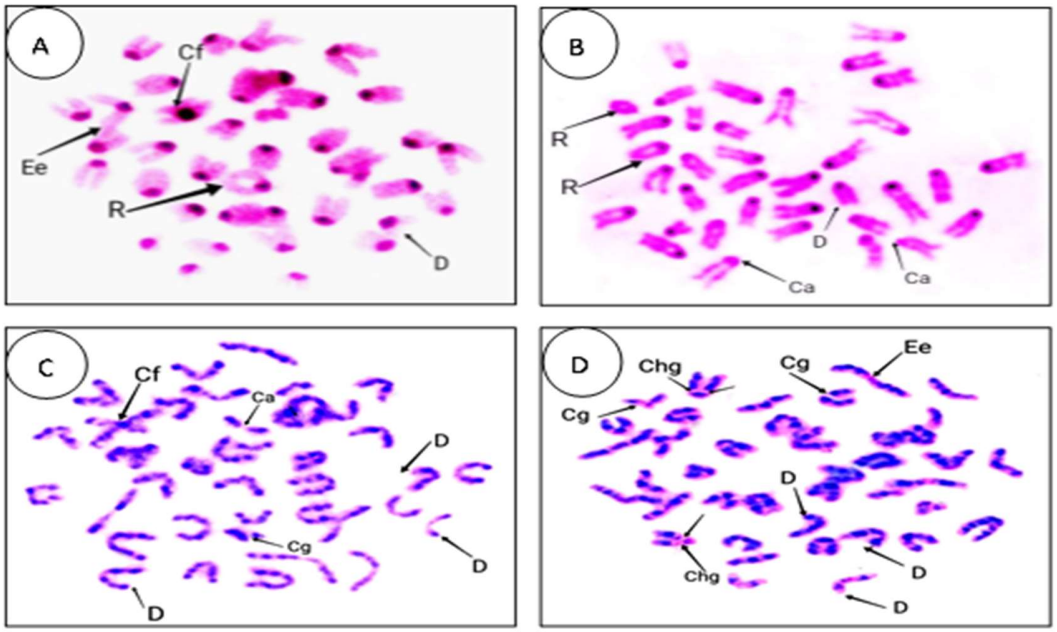

Figure (2): Photomicrographs of C & G-banding metaphase chromosomes of mice: Groups treated with an oral doses of gluten of 1.5 or 3 g/kg b.wt. for three days a week for four weeks: A- deletions (D), end-to-end associations (Ee), ring shape chromosomes (R), and centric fusion (Cf). B - deletions (D), ring-shaped chromosomes (R), and centromeric attenuations (Ca). C - Centric fusions (Cf), deletions (D), chromatid gaps (Cg), and centromeric attenuations (Ca), and chromosomal gaps (Chg), D - deletions (D), chromatid gaps (Cg), chromosomal gaps (Chg), and end-to-end associations (Ee). [X: 2400].

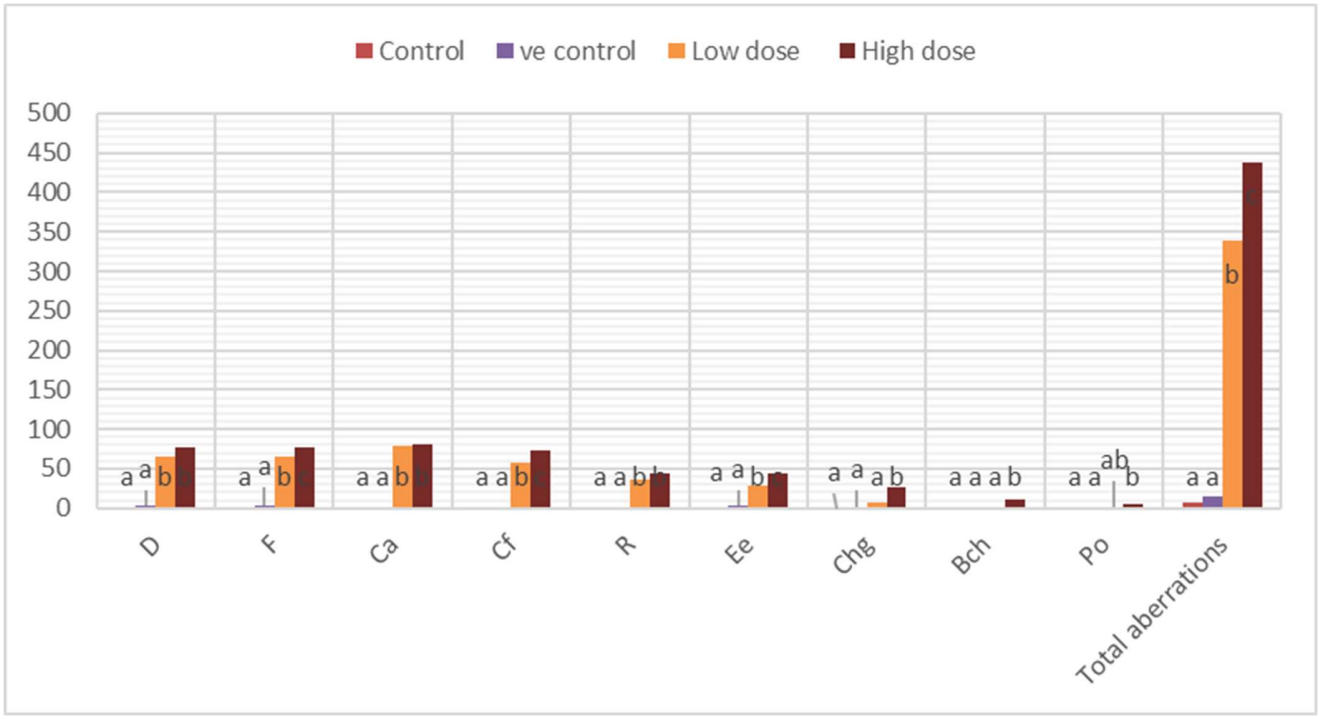

Figure (3): Histogram showing the mean numbers of chromosomal abnormalities.
Deletions (D), fragments (F), centromeric attenuations (Ca), centric fusions (Cf), ring forms (R), end-to-end associations (Ee), chromosome gaps (Chg), beaded chromosomes (Bch), polyploidy (Po) and total abnormalities in metaphase cells of mice treated with gluten 1.5 g/kg body weight (Group3), or gluten 3 g/kg body weight (Group 4) for three non-consecutive days a week for four weeks. The negative control group was treated with 0.02 M glacial acetic acid only (Group 2) and the control group (Group 1). The data are expressed as means ±Std. deviations. Significant at $p < 0.05$ when compared using a one-way ANOVA.

Mitotic index: The potential toxicity of gluten on cell proliferation was evaluated using the mitotic index. The results, presented in Table (1) and Figure (4) showed a significant decrease ($p < 0.001$) in the percentage of the mitotic index. Results were compared to the corresponding control group after administering gluten orally at a dose of 3 g/kg for four weeks.

Table (1): Mean and standard deviation of divided cells and percentage of mitotic index (MI%) of male albino mice (Mus musculus) treated with gluten 1.5 g/kg b.wt. (Group 3), gluten 3.0 g/kg b.wt. (Group 4), and glacial acetic acid 0.02 M (Group 2), and untreated mice (Control group).

	No. of examined mice	**No. of examined cells/mice**	**Score of divided cells**		**Percentage of mitotic index (MI%)**	
			Mean	±Std. D.	Mean	±Std. D.
Control	5	1000	183.6^{a}	±14.3	91.8^{a}	±7.15
ve Control	5	1000	149.2^{b}	±10.6	74.6^{b}	±5.33
Low dose	5	1000	133.2^{b}	±4.08	66.6^{b}	±2.04
High dose	5	1000	108.0^{c}	±11.8	54.0^{c}	±5.94
ANOVA Sig.			0.000		0.000	

$p < 0.05$ was considered significant.

• Statistically significant means ($p < 0.05$) are given different letters, a, b, and c. The groups that showed a non-significant change from each other took the same letter, but the group that showed a significant change compared to the other groups took a different letter.

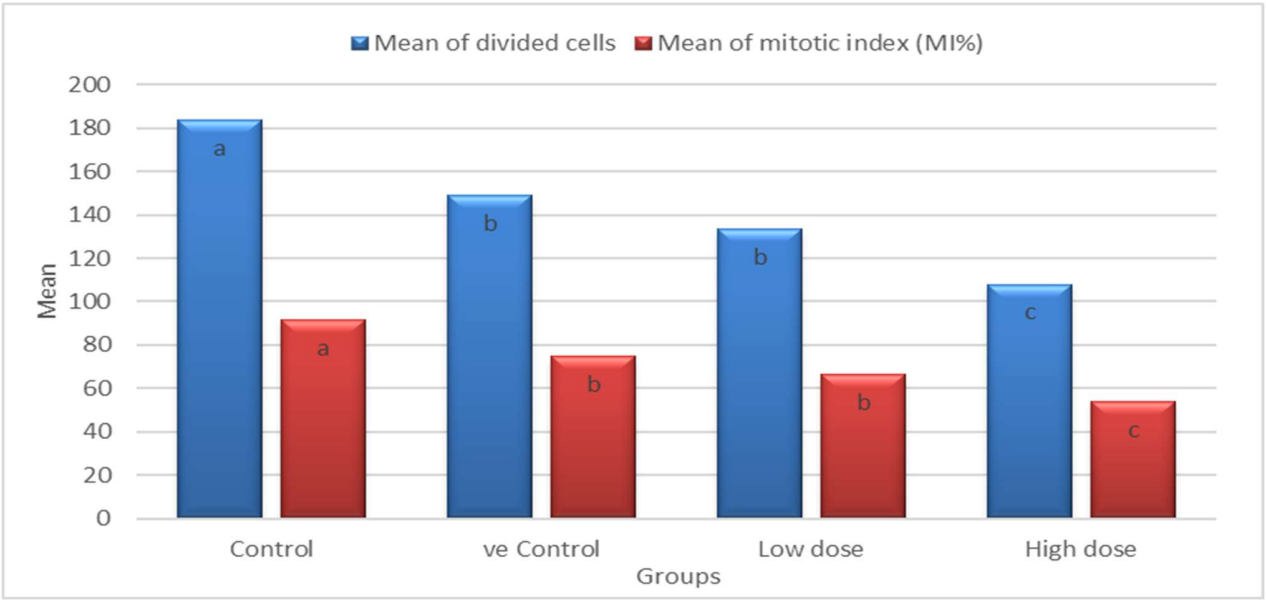

Figure (4): Histogram showing the mean of divided cells and mitotic index (MI%) of bone marrow cells of mice in control and all treated groups.

Comet assay: The use of single-cell gel electrophoresis images revealed that cells from mice that were fed with gluten exhibited a high degree of DNA damage. This damage was indicated as a "comet-like" appearance resulting from fragmented DNA migration through electrophoresis. The DNA damage extent was indicated by the tail formation observed in Figure (5). This study analyzed the comet classes by fluorescent microscope with a 515-560 nm filter. Four comet shapes were observed and are described in Table (2) and Figure (5) as follows: Class 0 - no tail, Class 1 - slightly damaged, Class 2 - moderately damaged, and Class 3 - extensively damaged. Cells that were damaged beyond recognition, represented by hepatocytes without heads, were not included in the analysis.

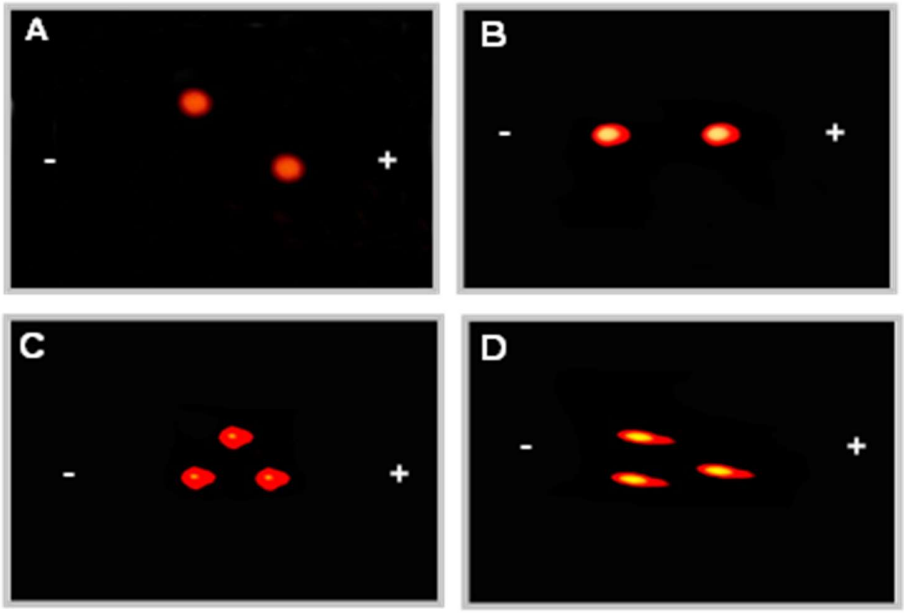

Figure (5): Photomicrographs of hepatocytes smear of mice.

A - Control group, showing comet Class 0, with no damage, a head diameter of 37.44 µm, and no tail. B - negative control group, which received acetic acid showing Class 1 with slight damage, a head diameter of 17.28 µm, and a tail length of 1.44 µm. C - group which received gluten 1.5 g/kg b.wt showed Class 2 moderate damage with a head diameter of 37.44 µm and a tail length of 19.44 µm. D - group which received an oral dose of gluten 3 g/kg b.wt. showing comet Class 3 in which heavy damage occurred with a head diameter of 16.56 µm and a tail length of 19.08 µm. The symbols – and + represent the cathode and anode during the electrophoresis. [X: 1000].

The objective of this study was to evaluate the distribution of DNA damage in liver cells using the comet assay. After four weeks of gluten treatment with three treatments per week, the damage was assessed by counting the number of damaged cells from 100 randomly selected and not overlapping cells on slides for each animal. The DNA damage severities for the control group, negative control group, and treated groups (1.5 g/kg and 3 g/kg b.wt. of gluten) are displayed in Table (2). The means ± standard deviations are provided for better representation. According to Table (2) there was a significant increase ($p < 0.01$) in the average percentage of DNA in the tail for animals treated with high-dose gluten (1.088%) compared with control animals. Furthermore, compared to negative (0.32%) and control animals, there was a significant increase ($p < 0.05$) in the average tail length of 1.033%.

Table (2): Mean and standard deviation of % DNA in tail, tail length, and classes of mice treated with gluten 1.5 g/kg b.wt. (Group 3), gluten 3 g/kg b.wt. (Group 4), and glacial acetic acid 0.02 M, and untreated mice (control group).

	%DNA TAIL		IN CLASSES	TAIL LENGTH	
	Mean	±Std. D		Mean	±Std. D.
CONTROL	9.103a	±0.86	0	1.8a	±0.496
VE CONTROL	9.87a	±0.45	1	2.38ał	±0.896
GLUTEN (1.5 g/Kg)	12.03a	±1.51	2	2.74ał	±0.238
GLUTEN (3 g/Kg)	19.01b	±3.71	3	3.66b	±1.009
SIG.	0.001			0.001	

DISCUSSION

This study aimed to investigate the potential genotoxic impacts of gluten on mice bone marrow cells. C-banding and G-banding techniques, mitotic index, and comet assay were used in the investigation. The study found a diploid number of chromosomes in a male albino mouse that were 40 chromosomes, all of which are telocentric. This result is consistent with previous findings [16, 21, 22, 23, 24]. Our findings on chromosomal abnormalities are consistent with those reported by **Kolacek *et al.*** [25], revealing that the abnormalities are structural, such as Ca, D, F, Cf, Chg, Cg, R, and numerical chromosomal abnormalities in the form of Po. Consuming gluten can increase the frequency of chromosomal abnormalities in PBL, which are biomarkers for cancer risk in humans. This may indicate the early biological impacts of genotoxic agents or an individual's susceptibility to cancer [8]. Centromeric attenuation is a common biomarker of cancer risk in humans. It reflects either the early impacts of genotoxic carcinogens or individual cancer susceptibility [8, 26].

Children who have been newly diagnosed with CD showed a significantly high number of chromosomal abnormalities. These abnormalities included breaks, fragments, and gaps. Gluten has been found to cause seven types of structural abnormalities and one type of numerical abnormalities. The most significant structural abnormalities include Ca, C break, Ch break, and F. Additionally, polyploidy, which is a numerical abnormality, can also be detected [27]. The relationship between chromosomal instability and malignancy has been proven through genetic disorders such as ataxia telangiectasia and Fanconi syndrome anemia, which exhibited increased chromosomal instability and a higher risk of cancer. Moreover, a recent study discovered that lymphocytes from patients diagnosed with cancer had more chromosomal damage than those from healthy individuals. People with CD are reported to have an excessive risk of developing cancer than the general population [8,28].

The study indicated that gluten, the protein present in wheat, rye, and other grains, has harmful effects on the bone marrow cells and DNA of male mice, causing damage to genetic material. The decrease in the mitotic index suggests that gluten had a negative impact on cell division and growth. Gliadin, a component of gluten, has been appearing to induce oxidative stress and inflammation [29, 30]. In addition, gliadin peptides can accumulate in cells and induce high levels of ROS in the cells [31, 32], and continuous exposure can cause additional harm to the DNA [33, 34]. Oxidative stress occurs when oxidizing substances surpass the body's antioxidant defenses due to increased ROS or decreased antioxidant levels [35,36,37]. Consuming gluten has been demonstrated to increase cellular oxidative stress, proapoptotic signals, and DNA damage in CD patients and duodenal mucosa [9]. The study discovered that gluten treatment caused cytotoxicity and genotoxicity in mice, resulting in chromosomal abnormalities and DNA damage. These findings are reliable with previous research on CD patients [38].

CONCLUSION

The study found that mice treated with gluten had significantly increased structural and numerical abnormalities in the chromosomes of their bone marrow cells. Additionally, they had a decreased mitotic index and increased DNA damage in their liver cells. These findings suggest that gluten damages the genetic material of mice and may have harmful effects on their health. Therefore, it is recommended to limit the usage of gluten to a narrow dosage and time range to ensure it remains within a safe and acceptable range.

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- **Conflicts of interest:** No conflicts of interest.

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