*Research Article*

# Evaluation of genotoxic potential and effects on body weight of Glimepiride and Pioglitazone combination after 4 weeks in Swiss albino mice

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**Abstract**

**Objective:** Combination of thiazolidinediones and sulfonylureas as antidiabetic treatment is beneficial in terms of delaying the progression of type 2 diabetes that is the need for exogenous insulin therapy and insulin sensitizing effects. However, antidiabetic treatment is needed to be continued for longer period of time, it may interact with DNA & genetic material to produce genotoxic effects. In the present study, genotoxic potential of pioglitazone and glimepiride was evaluated at chromosomal, cellular and germinal level. **Material and methods:** Genotoxic potential of glimepiride & pioglitazone was evaluated by performing 4 weeks of genotoxicity study with the use of mammalian bone marrow chromosomal aberration assay, mammalian bone marrow erythrocyte assay and sperm abnormality assay. Three different doses of the pioglitazone & glimepiride combination as well as carboxymethylcellulose (0.5 %) as negative control and cyclophosphamide (40 mg/kg) as positive control groups were used in the study. Body weight was also measured to check systemic effects. **Results and conclusion:** The results revealed that pioglitazone and glimepiride combination produce significant structural chromosomal aberrations as well as sperm abnormalities moreover, the combination induce cytotoxicity only at higher dose. However, no significant changes were observed in numerical chromosomal aberrations in the form of micronucleus formation as well as in case of body weight. This controversial reports stress the need to evaluate the genotoxic potential of the combination after chronic treatment.

***Keywords*:** Pioglitazone, Glimepiride, Chromosomal aberrations, Micronucleus formation, Sperm abnormality

## Introduction

Oral hypoglycemic drugs are used globally and there are nine different categories of FDA approved medicines have been used in the treatment of diabetes mellitus (DM) (Batsaki, 2005; Chaudhary, 2017; Agius, 2014; Tripathi, 2013). Among all the approved drugs, metformin, sulfonylureas (SUs) and thiazolidinediones (TZDs) are most studied medicines. They play prominent role in the treatment of type 2 diabetes (T2D) as per the algorithm advised by American diabetes association (ADA) and European diabetes association for the study of diabetes (EASD) (Staphenie et al., 2013). Moreover, combined effects of SUs and TZDs can delay progression of T2D, the need of exogenous insulin therapy, reduce the risk of CVD as well as shows sustained glycemic control (Hanefeld, 2007). Although having much of the benefits in DM, hepatic metabolism of SUs, possibility of initiation of cancer by TZDs as well as longer duration of therapy as oral hypoglycemic drugs, they might produce detrimental effects on genetic material and DNA termed as genotoxicity (Agius, 2014; Carpio, 2014). Glimepiride (GMP) is the newest drug from SUs category having possible insulin sensitizing effect and longer duration of effect however, pioglitazone (PIO) is well tolerated in patients with renal failure and not associated with hypoglycemia (Craig, 2011; Matsuki, 2007; Richardson, 1976).

Combination of GMP and PIO can give promising therapeutic strategy and the combination is effective in addressing pathophysiological defects in T2D (Hanefeld, 2007). Genotoxic potential of the combination can be determined with the use of different testing procedures- as per different regulatory authorities like European Union (EU), Organization of Economic Co-operation and development (OECD) and International Conference of Harmonization (ICH). Moreover, WHO scientific group stressed the need to reevaluate the mutagenicity and

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antimutagenicity of timely tested drugs and report even the negative results is obtained (OECD, 2008; OECD-474, 2016; OECD-475, 2016; FDA et al., 2012; ICH S2 & S3, 2011; WHO, 1961) To get more insight into the genotoxic potential of GMP and PIO combination and as per the guidelines, standard battery of approach was used in the present study as no single test is capable to detect overall genotoxic potential of any drug. Three different *in vivo* tests i.e. mammalian bone marrow chromosomal aberration assay, mammalian bone marrow erythrocyte assay and sperm abnormality assay were used to assess genotoxicity at chromosomal, cellular and germ line level as well as to give precise results regarding pharmacokinetic parameters & DNA repair mechanisms. Moreover, systemic effects of PIO+GMP combination were determined in the form of their effects on body weight.

## Materials and methods

### Drugs and chemicals

Pioglitazone was received as gift sample from Alembic Pharmaceuticals Vadodara and Glimepiride was obtained from Yarrow Chem. Products Mumbai. Cyclophosphamide was received as gift sample from K. R. Mehta & Co Ahmadabad. All other chemicals used for the study were of lab reagent grade and purchased from commercial sources.

### Animals

Swiss albino mice (6-8 weeks old) were procured from institutional animal house of Shri Sarvajanik Pharmacy College Mehsana, Torrent Pharmaceuticals Limited, Research Center, Zydus Research Center Cadila Healthcare Limited. They were accustomed for 7 days under standard husbandry conditions, i.e. room temperature of 25±10°C; relative humidity 45-55% and a 12:12h light/ dark photo period, with *ad libitum* access to food (commercial mouse pellets) and water throughout the experiments. Approval from Institutional Animal Ethical Committee (IAEC) of Shri Sarvajanik Pharmacy College, Mehsana had been taken prior to the experiments for the animal experimentation. All the protocols and the experiments were carried out in strict compliance according to ethical principles and guidelines provided by CPCSEA, OECD and ICH guidelines (ICH, 1997; ICH S2 & S3, 2011; OECD, 2008; OECD-452, 2008; OECD-474; 2016; OECD-475; 2016).

### Experimental protocol

Animals were divided into 5 different groups of positive control, negative control and three different doses of PIO & GMP combination. Dose selection of both the drugs was based on the average human daily doses of both the drugs according to OECD guidelines. The average of the human daily dose of pioglitazone is 30 mg/kg/day, however, for glimepiride it is 4 mg/kg. Interpretation of the dose in to present study gave the top dose for PIO+GMP combination which is PIO: 2 mg/kg + GMP: 0.26 mg/kg. Other dose levels are PIO: 1 mg/kg + GMP: 0.13 mg/kg and PIO: 0.2 mg/kg + GMP: 0.026 mg/kg. Moreover, cyclophosphamide 40 mg/kg was used as positive control where as carboxymethylcellulose (CMC- 0.5%) was given to the animals included in the negative control group. For doses of PIO & GMP combination, suspension of the combination was prepared in the CMC. All the doses were given to the animals for 4 weeks of duration.

### Mammalian bone marrow chromosomal aberration assay

Intraperitoneal injection of 0.4 ml 0.05% colchicine solution before 90 minutes of sacrifice of animals. The animals then sacrificed by cervical dislocation and femurs were extracted out. The material was flushed with hypotonic solution (0.56 % KCL) and cell suspension was vortexed and keep aside for 20 minutes. The cell suspension was centrifuged at 1000 rpm for 8 minutes for five times and fixed with cold fixative (methanol: Glacial acetic acid, 3:1) after each centrifugation. The slides were prepared by heat fix and stained with the use of diluted Giemsa stain. The slides were observed at 100x magnification; oil immersion lens. Hundred well spread metaphase per animal were observed for aberrations in chromosomes like deletions, acentric fragments, chromatid & chromosomal breaks, gaps, polyploidy, exchanged fragments and ring formation. Chromosomal aberrations per cell (CAs/cell) were calculated by including and excluding gaps. Moreover, mitotic index was calculated from the same slides among 1000 cells by taking the percentage ratio of dividing per total number of cells (Williams, 1984; IPCS, 1985; Preston, 1981; Tice RR, 1987; Perez, 2008).

### Mammalian bone marrow micronucleus assay

Animals were sacrificed with cervical dislocation and both femurs were extracted out. The cells were collected by flushing 0.2 ml 5 % bovine serum albumin solution. The cell suspension was centrifuged at 1000 rpm for 5 minutes and with again agitating of the cells, slides were made by preparing smear. Then the slides were prepared by diluted May-Gruenwald and Giemsa stain. The slides were observed at 100x magnification of oil immersion lens and the ratio of polychromatic to normochromatic erythrocytes as well as presence of micronucleus formation was determined by scoring 1000 erythrocytes per animal (Cole, 1979; Schmid, 1975).

### Sperm abnormality assay

Animals were sacrificed by cervical dislocation and both the cauda epididymus was removed and mix with previously prepared 1 ml phosphate buffer saline. The

solution was filtered and mixed with 1 % aqueous solution of eosin Y and allow it to stain. The slides were prepared by making smear and observed at 100x magnification of oil immersion lens. Approximately 1000 perm cells per animals were scored for sperm head abnormalities like amorphous, banana, hookless and folded (Bruce, 1974; Wyrobek, 1975).

### Statistical analysis

For statistical analysis of data on chromosomal aberrations/cell in bone marrow cells, the mitotic index micro nucleated cells and percentage sperm abnormality of each treatment group was compared with the negative control by one-way ANOVA followed by Dunnett's test using Prism software (PRISM 6) as "a posteriori" test was used in all the experiments. The significance of differences were examined at the p<value of 0.05.

## Results

### Effect on body weight

The effects of PIO+GMP on body weight of Swiss albino mice were observed and recorded. No statistical significance was observed between initial and final body weight after 4 weeks of treatment of PIO+GMP in any of tested doses in comparison with NC (Table 1; Figure 1).

### Mammalian bone marrow chromosomal aberration assay

PIO+GMP treatment induced dose dependent and statistically significant changes in chromosomal aberrations per cell (CAs/cell- including gaps and excluding gaps) were observed in the bone marrow cells.

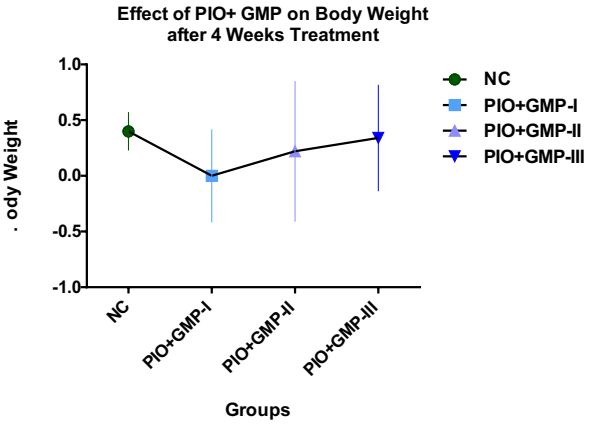



**Figure 1.** Effect of PIO + GMP on body weight after 4 weeks treatment

**Table 1.** Effects of Pioglitazone and Glimepiride combination on body weight (g)

| Groups | Dose (mg/kg) | Body Weight (g) (mean ± S.D.) | | |
|---|---|---|---|---|
| | | Initial | Final | Difference |
| NC | CMC (0.5 %) | 24.66 + 0.88 | 25.06 + 0.60 | 0.4 + 0.17 |
| PIO + GMP- I | 0.2 + 0.026 | 27.06 + 0.60 | 27.06 + 0.73 | 0.0 + 0.41 |
| PIO + GMP- II | 1 + 0.13 | 26.98 + 1.50 | 27.2 + 1.67 | 0.22 + 0.63 |
| PIO + GMP- III | 2 + 0.26 | 30.34 + 0.73 | 30.68 + 0.91 | 0.34 + 0.47 |

Data are expressed as Mean ± SD (n=5); Abbreviation: NC: Negative Control, PIO + GMP: Pioglitazone + Glimepiride

**Table 2.** Chromosomal aberrations after pioglitazone and glimepiride combination treatment

| Groups | Dose (mg/kg) | Total AM | % of total AM | Break | | G | D | A | P | E | R | CA/Cell (mean ± S.D.) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Ch (t) | Ch (b) | | | | | | | Including Gaps | Excluding Gaps |
| NC | CMC(0.5%) | 88 | 22.16 | 8 | 9 | 29 | 29 | 29 | 16 | 0 | 0 | 0.24 ± 0.01 | 0.18 ± 0.01 |
| PC | CP (40) | 397 | 100 | 22 | 17 | 62 | 183 | 192 | 82 | 8 | 0 | 1.13±0.09*** | 1.00±0.07*** |
| P+G-I | 0.2 +0.026 | 137 | 34.50 | 6 | 12 | 22 | 52 | 42 | 15 | 2 | 0 | 0.30 ± 0.05 | 0.25 ± 0.04 |
| P+G-II | 1 + 0.13 | 159 | 40.05 | 11 | 4 | 30 | 56 | 40 | 13 | 5 | 1 | 0.32 ± 0.04 | 0.26 ± 0.05 |
| P+G-III | 2 + 0.26 | 157 | 39.54 | 12 | 5 | 24 | 67 | 54 | 11 | 4 | 0 | 0.35 ± 0.02 | 0.30 ± 0.03* |

Data are expressed as Mean ± SD (n=5) Abbreviations: NC: Negative Control; PC: Positive Control; P+G: Pioglitazone+Glimepiride; AM: Aberrated Metaphase; D: Deletion; A: Acentric Fragment; P: Polyploidy; Ch (t): Chromatid Break; Ch (B): Chromosomal Break; E: exchange; R: Ring; G: Gap; CA: Chromosomal Aberrations. Significance: * $p < 0.05$; *** $p < 0.0001$ significant when compared to NC

The results show that chromosomal aberrations/cell- including gaps in PC was significantly higher ($p< 0.0001$) as compare to NC for both the parameters- CA/cell- including gaps as well as CA/cell- excluding gaps. No statistical differences in any of the three doses were observed for chromosomal aberrations/cell including gaps when compare to NC. In CA/cell- excluding gaps, lower and middle doses were not significant as compare to NC however, higher dose showed statistical significance ($p< 0.05$) in comparison with NC. The observed chromosomal aberrations per cell- excluding gaps were more in higher dose (0.30 ± 0.03) as compared to NC (Table 2; Figure 2).

**Mitotic Index**

To observe effect of PIO+GMP treatment on cytotoxicity as well as cell proliferation rate in bone marrow cells of mice, mitotic index was calculated by taking percentage ratio of number of dividing cells per total number of observed cell. The suppression of percentage ratio of mitotic index indicates decreased cell proliferation rate in bone marrow cells of mice. The summarized data of obtained results are given in the table in dose dependent and in statistical significant manner**.** Percentage mitotic index was significantly lower in PC ($p<$ 0.0001) as compare to NC after all sampling times. No statistical significance was observed in percentage mitotic index in lower and middle doses when compare to NC however, in higher dose significant suppression of mitotic index ($p< 0.05$) was observed in comparison with NC. The value of mitotic index in higher dose was lower (7.68 ± 1.59) as compare to negative control (CMC- 0.5 %) group (11.76 ± 1.33) (Table 4; Figure 3 and 4).

**Mammalian bone marrow micronucleus assay**

Results of polychromatic to normochromatic erythrocytes ratio as well as micronucleus formation are summarized in

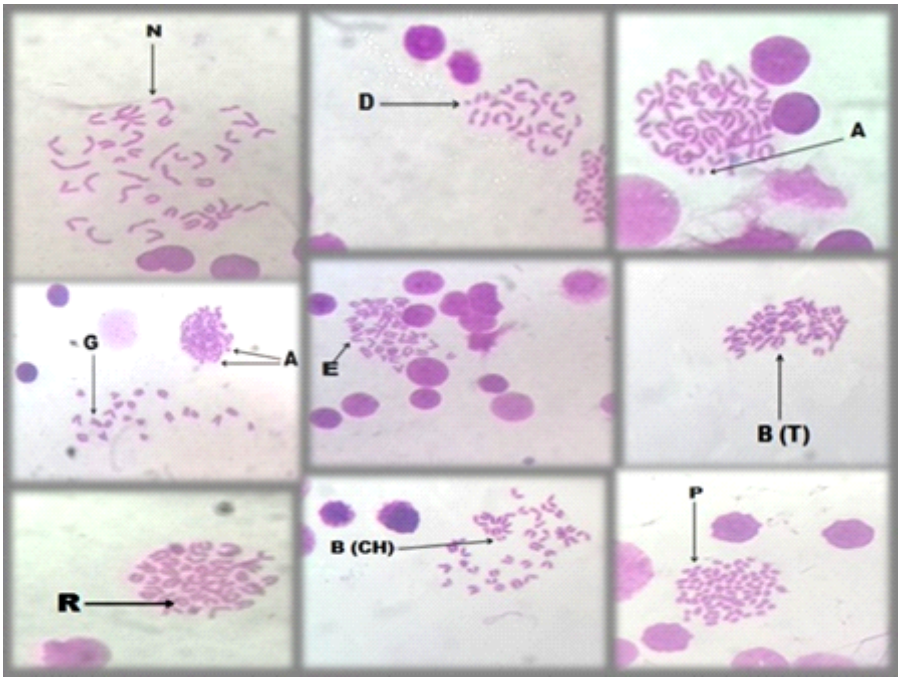



**Figure 2.** Chromosomal Aberrations in which N: normal metaphase; D: deletions; A: acentric fragments; G: gap; E: exchange; B (T): chromatid breaks; R: ring formation; B (CH): chromosomal breaks; P: polyploidy at 100x magnification; oil immersion lens

**Table 3.** The mitotic index after pioglitazone and glimepiride treatment

| Groups | Dose (mg/kg) | No. of Metaphase Analyzed | No. of Dividing Cells | % Mitotic Index (mean ± S.D.) |
|---|---|---|---|---|
| NC | CMC (0.5 %) | 5000 | 588 | 11.76 ± 1.33 |
| PC | CP (40) | 5000 | 240 | 4.8 ± 0.45*** |
| PIO+GMP- I | 0.2 + 0.026 | 5000 | 532 | 10.64 ± 3.09 |
| PIO+GMP- II | 1 + 0.13 | 5000 | 452 | 9.04 ± 1.57 |
| PIO+GMP- III | 2 + 0.26 | 5000 | 384 | 7.68 ± 1.59* |

Data are presented as Mean ± SD (n=5). % Mitotic Index= Number of dividing cell per total number of observed cells x 100. Abbreviations: NC: Negative Control; PC: Positive Control; CP: Cyclophosphamide; PIO+GMP: Pioglitazone + Glimepiride Significance: * $p< 0.05$; *** $p< 0.0001$ significant when compared with the NC

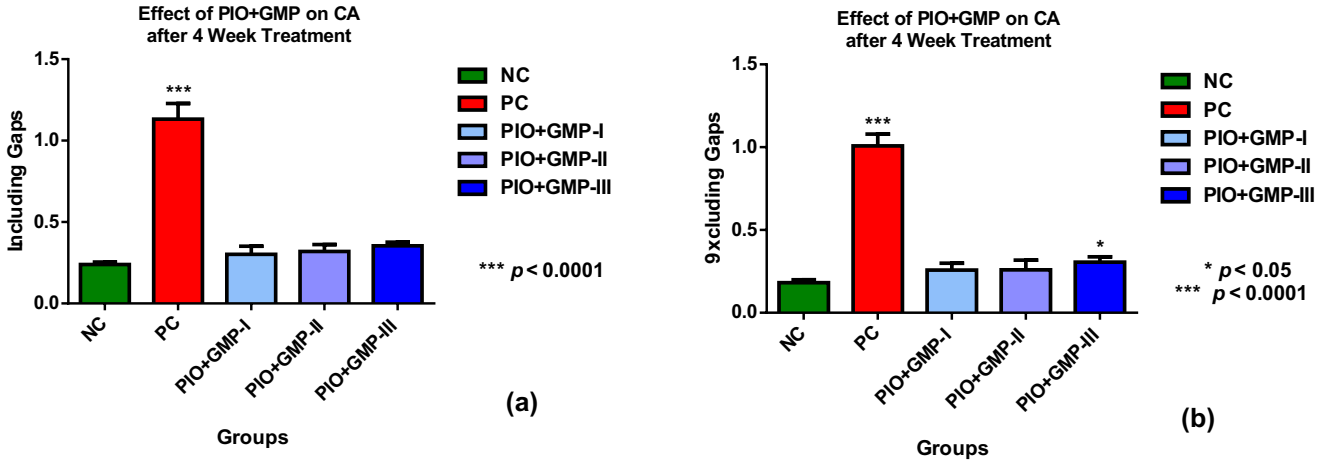



**Figure 3**. (a) Chromosomal aberrations- including gaps after 4 weeks treatment of PIO+GMP (b) Chromosomal aberrations- excluding gaps after 4 weeks treatment of PIO+GMP

**Table 4.** The Micronucleus assay after pioglitazone and glimepiride treatment

| Groups | Dose (mg/kg) | Individual Animal Scores/1000PCE | PCE/NCE (mean ± S.D.) | MNPCE (mean ± S.D.) |
|---|---|---|---|---|
| NC | CMC (0.5 %) | 1, 2, 1, 2, 1 | 1.84± 0.10 | 1.4 ± 0.54 |
| PC | CP (40) | 24, 20, 12, 19, 18 | 0.24 ± 0.01*** | 18.6 ± 4.33*** |
| PIO+GMP- I | 0.2 + 0.026 | 1, 2, 0, 1, 0 | 1.62 ± 0.07** | 0.8 ± 0.83 |
| PIO+GMP- II | 1 + 0.13 | 2, 1, 1, 1, 1 | 1.66 ± 0.08* | 1.2 ± 0.44 |
| PIO+GMP- III | 2 + 0.26 | 1, 3, 2, 1, 4 | 1.58 ± 0.08** | 2.2 ± 0.30 |

Data are presented as Mean ± SD (n=5, % ). **Abbreviations:** NC: Negative Control; PC: Positive Control; CP: Cyclophosphamide; PIO+GMP: Pioglitazone + Glimepiride; PCE: Polychromatic Erythrocytes; NCE: Normochromatic Erythrocytes; MNPCE: Micronucleated Polychromatic Erythrocytes. Significance: * $p$< 0.05; ** $p$< 0.01; *** $p$< 0.0001 significant when compared with the NC

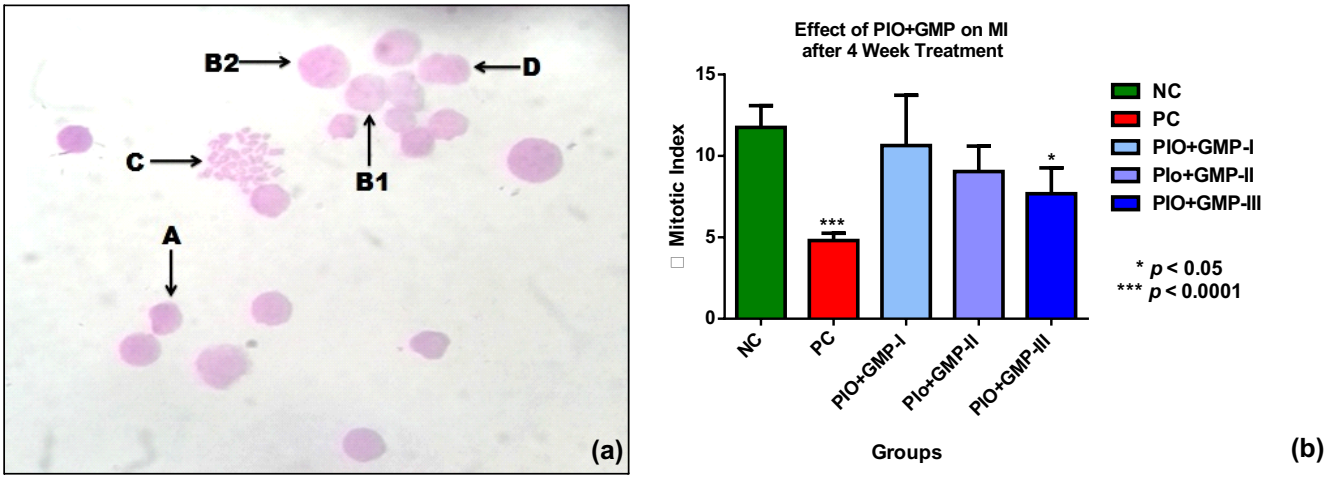
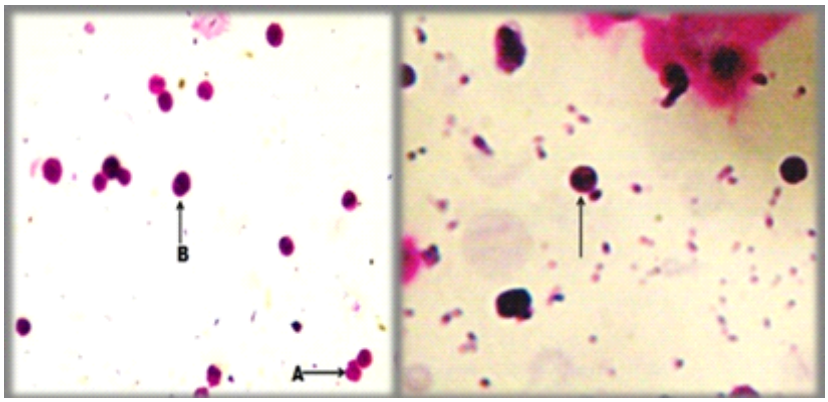
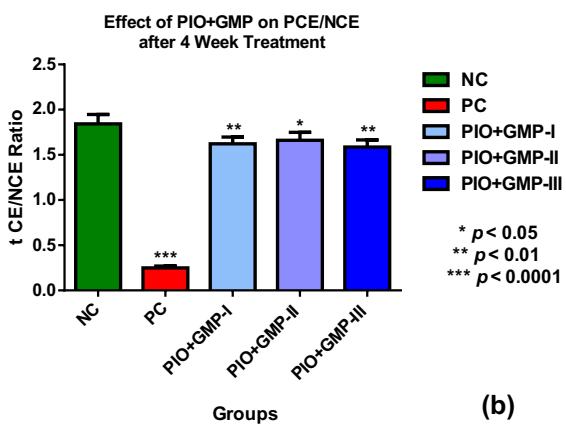



**Figure 4.** (a) Different phases of cells in the cell cycle in which A, represents normal cell, B1, represents early prophase, B2, represents late prophase, C, represents metaphase, D, represents anaphase; at 100x magnification, oil immersion lens (b) % Mitotic index after 4 weeks treatment of (PIO + GMP)

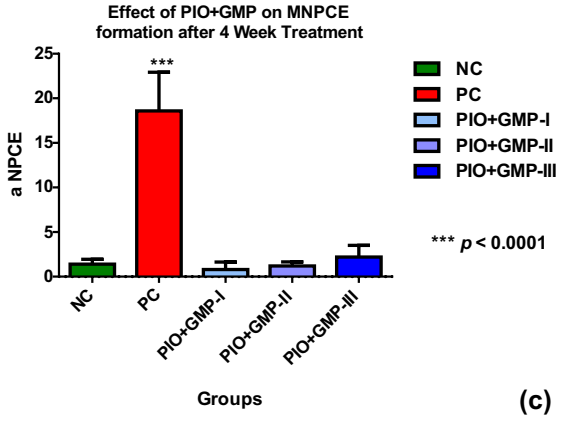
table. Suppression in the PCE/NCE ratio represents induction of cytotoxicity in bone marrow cells of mice. The obtained results show that in PC, ratio of PCE/NCE was significantly decreased ($p$<0.0001) when compared to NC. Moreover that formation of micronucleus was also highly significant ($p$<0.0001) in PC in comparison with NC whereas significant suppression in




(a)




(b)




(c)

**Figure 5. (a)** Normochromatic cells represented as A & polychromatic cells represented as B where as pointed cell represent micronucleus formation at magnification 100x, oil immersion lens **(b)** PCE/NCE after 4 weeks treatment of PIO + GMP **(c)** MNPCE after 4 weeks treatment of PIO + GMP

**Table 5.** Sperm abnormality assay after pioglitazone and glimepiride treatment

| Groups | Dose (mg/kg) | Abnormal Sperms | A | B | H | F | % of Abnormal Sperms (mean ± S.D.) |
|---|---|---|---|---|---|---|---|
| NC | CMC (0.5 %) | 761 | 320 | 178 | 197 | 67 | 15.22 ± 0.40 |
| PC | CP (40) | 1851 | 791 | 417 | 513 | 131 | 37.02 ± 0.30*** |
| P+G- I | 0.2 + 0.026 | 848 | 420 | 166 | 65 | 207 | 16.96 ± 0.67 |
| P+G- II | 1 + 0.13 | 907 | 445 | 239 | 103 | 120 | 18.14 ± 2.36 |
| P+G- III | 2 + 0.26 | 998 | 517 | 261 | 96 | 124 | 19.96 ± 2.43** |

Data are presented as Mean ± SD (n=5, % )

**Abbreviations**: NC: Negative Control; PC: Positive Control; CP: Cyclophosphamide; PIO+GMP: Pioglitazone + Glimepiride; A: Amorphous; B: Banana; H: Hookless; F: Folded; Significance: ** $p < 0.01$; *** $p < 0.0001$ significant when compared with the NC

PCE/NCE ratio was observed in all the three doses. In lower dose, the significant lower PCE/NCE ratio was observed ($p < 0.01$) (1.62 ± 0.07) which then enhanced ($p < 0.05$) (1.66 ± 0.08) in middle dose and further the value was suppressed when higher dose was given ($p < 0.01$) (1.58 ± 0.08). All doses were compared with NC (1.4 ± 0.54). Moreover, no statistical significance was observed in any of the three doses in micronucleus formation when compare to NC (Table 4; Figure 5).

### Sperm abnormality assay

Sperm abnormality assay was conducted to determine potential of PIO+GMP to induce germ cell abnormalities. The obtained results are summarized in table and indicate that statistically higher ($p < 0.0001$) was observed in PC in all durations of treatment as compare to NC. However, among three different doses, lower and middle dose were not statistically significant in comparison with NC where as higher dose was statistically significant ($p < 0.01$) (19.96 ± 2.43) when compared with NC (15.22 ± 0.40) (Table 5; Figure 6).

## Discussion

Controversial reports of genotoxicity study of pioglitazone, glimepiride as well as pioglitazone and glimepiride combination was obtained from reviewed literature

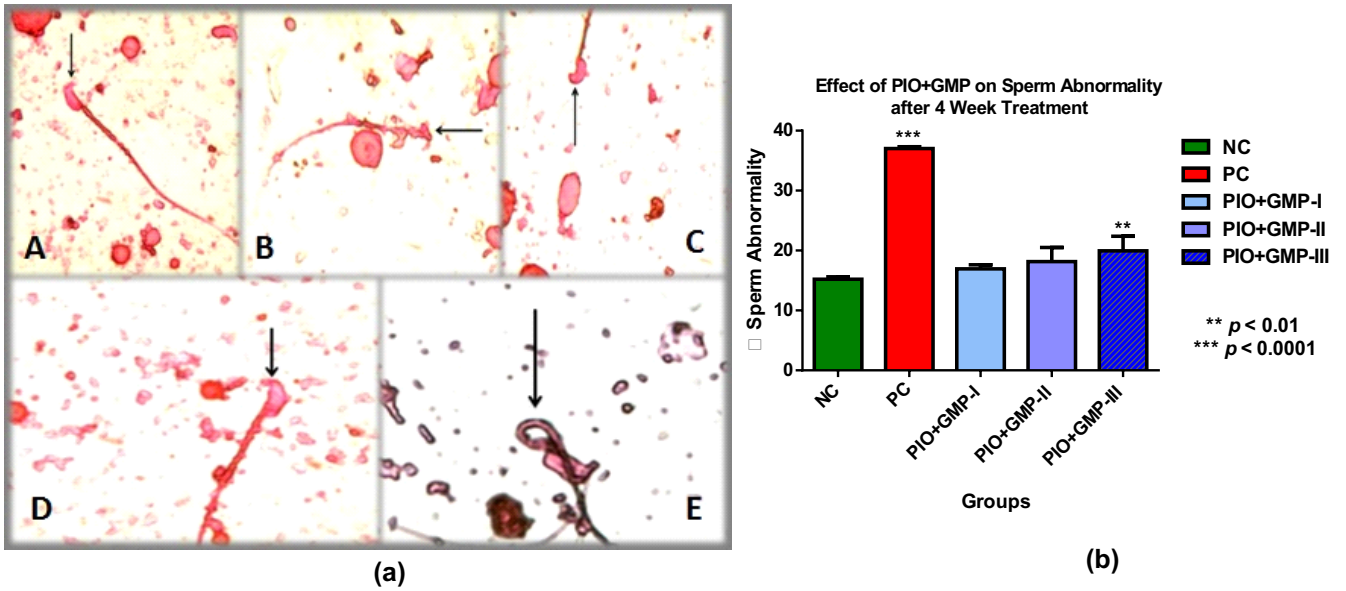



**Figure 6. (a)** Sperm abnormality in which A: normal; B: amorphous, C: banana, D: hookless, E: folded sperm cell at 100x magnification; oil immersion lens **(b)** SA after 4 weeks treatment of PIO + GMP

(Brambilla, 2009; Bedir, 2008; Friedrich, 2011; Gurbuzel, 2014; Oz Gul O, 2013; Rabbani, 2008; Rabbani, 2009; Rabbani, 2010; Shaik, 2010). In the present study evaluation of genotoxic potential of the combination was done in more specific way at chromosomal, cellular and germ line level. For all types of assessment three doses of PIO+GMP as well as NC and PC groups were included for more specific comparison and dose dependent and statistically significant results after four weeks of sampling time.

According to the obtained results, no changes in the body weight were observed which indicates that there is absence of systemic side effect produced by the combination after four weeks of treatment. However, in relevance to the genotoxic potential, cyclophosphamide (CP) produces significant changes in all the parameters that might be attributed to DNA binding ability of CP and hepatic metabolic activation by microsomal cytochrome P450 mixed function oxidase system. In case of chromosomal aberrations (CAs/cell), only significant changes were observed in CAs/cell-excluding gaps in higher dose which clearly indicate clastogenic potential of the combination. Structural changes in the chromosomes are the primary step in the development of cancer. It is complicated process and might be attributed to direct DNA damage or replication of DNA damage or replication of damaged DNA template or inhibition of DNA synthesis or inhibition of topoisomerase II. Breakage of chromosomal fragment in the presence of acentric fragment predispose to micronucleus formation. Although in the present study, analysis of metaphase showed more numbers of deletions & acentric fragments and presence of breaks, gaps, polyploidy, exchanges & ring formation; no significant micronucleus formation was observed. The responsible mechanism might be the presence of cell cycle checkpoints and DNA repair mechanisms that remove damaged DNA by repair or apoptosis. Cytotoxic effects of the combination were determined by calculation of mitotic index as well as polychromatic erythrocytes to normochromatic erythrocytes ratio (PCE/NCE ratio) from chromosomal aberration and micronucleus assay. As per the results, significant suppression in percentage mitotic index in higher dose as well as fluctuations in the significance in PCE/NCE ratio shows the cytotoxic potential of the compound. However, the above results of both the assays give confirmation of presence of DNA repair mechanisms during cell cycle. According to the previous studies and literature review, if any compound has potential to produce somatic cell mutations they might produce germ cell mutations, too. Detection of germ cell mutations was done from sperm abnormality assay due to ease of performing assay and precise results. As per the results of the assay, higher dose gave significant abnormality in sperm cells.

In summary, PIO+GMP combination have capability to produce structural chromosomal damage, cytotoxicity & germ cell mutations at higher dose but it does not alter numerical chromosomal changes as well as body weight as per the obtained results after 4 weeks of treatment.

## Conflicts of interest

The authors declare that there are no conflicts of interest.

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