



In vitro assessment of the mutagenic effect of Metformin

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ABSTRACT

Metformin drug has a widespread usage in diabetes mellitus , it prevents hepatic glucose release and increases tissue insulin sensitivity. Metformin toxicity is established in metabolic failure of liver and kidney disorders, Lactic acidosis toxicity induced by metformin, may lead to central nervous system dysfunction, cardiovascular collapse, renal failure, and death. The studies investigated the genotoxicity of metformin are few either *in vivo* and *in vitro* and are controversial. The possible cytotoxic and mutagenic effects that may be induced by metformin had been investigated *in vitro* models and compared it with the standard cytotoxic drug , cyclo phosphamide. Metformin did not cause an obvious genotoxic effect or increased oxidative stress and this was indicated by absence of changes in malondialdehyde(MDA), 8-hydroxy-2-deoxy Guanosine (8-OH-dG) levels and reduced glutathione (GSH) as compared to control normal group. So ,metformin did not cause any cytotoxic effect on comparing with the standard cytotoxic cyclophosphamide drug .Metformin does not express *in vitro* toxic effects .

Key words: anti diabetic drug, DNA, Blood lymphocytes

INTRODUCTION

Metformin is an anti diabetic biguanide drug . It produces an antihyperglycemic effect through prevention of hepatic glucose synthesis, associated with stimulation of tissue insulin sensitivity [1]. The mode of action of metformin is incompletely explained however, it appears to have anti-inflammatory and antioxidant actions included in its medical uses in insulin resistance and polycystic ovary syndrome [2,3].

Metformin toxicity is an established metabolic failure due to liver and kidney diseases, it has been associated with lactic acidosis, in both acute and chronic exposures. This lactic acidosis, may lead to the central nervous system changes, cardiovascular collapse, and renal impairment, Results investigated the genotoxicity of metformin are few either *in vivo* and *in vitro* and are controversial [4] . DNA damage or genotoxicity can initiate different biological effects[4]. Micronucleus test has been used to determine the genotoxic effects of sulfamethoxazole and carbamazepine. This simple *in vitro* test used in many cell types as it is a good method to evaluate cytochromosomal alteration [5]. Micronuclei (MN) test is also measuring chromosomal aberration [6].

Therefore this work aimed to examine the association between the use of metformin and chromosomal alteration.

EXPERIMENTAL SECTION**MATERIALS***Human blood samples*

Ten ml fresh venous blood samples were taken from 18 adult donors after consent. All donors were of both sexes (age 20-45 years). Apparently healthy, non-smoking, non-alcoholic and did not take any medications recently. The donors were obtained from the blood-banking center of Mansoura University Hospital. All blood samples were taken on heparinized sterile tubes to prevent clotting.

The study was done according to The Medical Research Ethics Committee of Mansoura University, Egypt.

Chemicals

Metformin was obtained as metformin hydrochloride solution 1mg/ ml/ ampoule (Sigma- Aldrich company, USA). Cyclophosphamide 1gm vial was obtained from (Multipharma company, Egypt).

The chemicals used in this work were purchased from Sigma-Aldrich company, USA.

METHOD*Isolation and culture of human lymphocytes:*

Lymphocytes were isolated from blood samples and cultured as described by Durante et al [6] and Chung et al. [7] with minor modification. Ten ml of whole blood was collected in an isolation tube for blood cells. The sample was centrifuged (20 min).

The mononuclear cells layer and platelets was gathered in a pipette and transfused to centrifuge tube (10 ml) . RPMI 1640 (Roswell Park Memorial Institute 1640) medium was put till 8 ml and the sample was centrifuged (10 min) . After the supernatant removal , the cell pellet was re-suspended in 8 ml RPMI 1640 medium at a density of 1.0×10^6 with cells/ml supplemented with 20% fetal calf serum, 0.1 ml phytohaemagglutinin-M, phytohaemagglutinin-M, 2 mM L-glutamine, 100 U/ml penicillin, 100 ug/ml streptomycin (antibiotics for sterilization) and 5 IU/ml sodium heparin (complete medium). Cells were kept in a culture flask in an incubation at 37°C in a 5% CO₂ humidified atmosphere.

The study plan:

Isolated lymphocytes were cultured in 10 ml RPMI 1640 culture medium for 72 hours at 37°C in the dark [8]. They were separated randomly into 3 groups: (6 samples each). The potential genotoxic drugs were added twice, at 24 hour and 48 hours ,at the beginning of cultivation period and after induction of mitotic division with phytohaemagglutinin that was added at the start of the period ,for mitosis within 24 hours, the maximum time for drugs addition , which if are added before 24 hours, the response to phytohaemagglutinin is affected, leading to delay in mitosis[7,9,10].

Grouping of isolated lymphocytes and Drug doses:

The groups consisted of lymphocyte cells treated with high concentration of metformin (114.4 µg/ml) [4] , control group consisted of untreated cell cultures and cyclo phosphamide treated lymphocyte cells group (cyclophosphamide , 6 µg/ml) [4].

*Evaluation of the drug effects:**1 -Micronucleus (MN) assay:*

At 72 h of incubation, the cultures were collected by centrifugation (10 min) . Then the cell pellet was treated with a hypotonic solution (5 min, 0.075 M KCl at 37 C), in order to remove red cell and to keep the cytoplasm . Cells were centrifuged, then Carnoy's fixative (methanol: acetic acid, 3:1, v/v) solution was added. This was done five times. then, the pellets of cells were resuspended in a small volume of fixative solution and dropped on to cold slides stained with 10% Giemsa dye solution.

Micronucleus cytokinesis-block assay scoring criteria:

To determine binucleate cells with micronuclei (BNMN) frequency and the total number of MN in lymphocytes (MNL), a two thousands of binucleate cells with preserved cytoplasm were scored for each subject on coding slides. MN were accepted, if

(i) they were separated from the main nuclei, but still in their cytoplasm, (ii) they had a chromatin substance as the main nucleus, (iii) they were coplanar to the main nuclei. Toxicity was assayed by

classifying cells depending on the nuclei numbers.

The cytotoxicity index was applied for measuring the cell proliferation, (the cytokinesis-block proliferation index) [11].

The cytokinesis-block proliferation index = $MI + 2MII + 3(MIII, MIV) / 100$

MI–MIV = the numbers of cells with one to four nuclei, respectively, (MIII and MIV are considered to be in their third cell cycle). The cytokinesis-block proliferation index is a relevant index in evaluating cell toxicity or cell-cycle delay [11,12,13].

2- Comet assay was done under alkaline conditions [14,15], the cell suspension (30 μ l) was added to 90 μ l low-melting point agarose (37°C) and evenly layered on to the pre-coated (with 300 μ l high-melting point agarose) microscope slides. Slides were put on ice to solidify and to stop cell metabolism. Then, they were immersed in chilled lysis buffer (2.5 M NaCl, 100 mM sodium-EDTA, and 10 mM Tris, pH 10) at 4°C. They were kept overnight. Then, they were put in a horizontal electrophoresis tank having freshly formed and chilled electrophoresis buffer (300 mM NaOH and 1 mM EDTA, pH 13). The slides were kept for 30 min to allow the unwinding of DNA and then electrophoresis was done at 30 V and 300 mA at 4°C for 40 min. The slides were rinsed with double distilled water (ddH₂O) and then put in a neutralizing buffer (0.4 M Tris-HCl, pH 7.5) for 15 min at 4°C. The slides were stained with ethidium bromide.

- The slides were examined using fluorescence microscope (with an excitation filter of 565 nm and a barrier filter of 590 nm). Images were taken by a digital camera (saved as TIFF/JPEG files). These steps were done in the presence of reduced light to decrease DNA damage.
- Cells images were performed by a computer-assisted image-analysis system (Comet Assay IV) for the comet parameters. Results were expressed as tail length (TL; the migrated distance of DNA, in μ m), DNA percentage in tail (% Tail; the intensity of migrated DNA) and tail moment (TM) (tail length x DNA percentage in tail / 100).

3-Assay against chromosomal breakage The chromosomal culture method described by Rooney and Czepulkowski [16]. 2 milliliter of sodium heparinized whole blood was collected from normal individuals. 0.5 cc of individual's blood sample was added to 5 cc of a complete media containing RPMI 1640, fetal calf serum PHA (10 μ g/ml), L- glutamate (2 mM), penicillin (10%) (unit/ml), and gentamycin (50 μ g/ml). Each sample was cultured three times, the first was free, metformin solution (114.4 μ g/ml) was added to the second culture and the cytophosphamide solution (6 μ g/ml) was added to the third culture. After 72 hours of incubation in 37°C, colcemide was added (0.2 μ g/ml) After 90 min, the cells were harvested by centrifugation (150g for 10 min). Then, 5 ml of 0.075 M KCl solution was added and mixed and incubated at 37°C for 15 min. After centrifugation (150 \times g for 10 min), hypotonic supernatant was removed. Then, 5 cc cold, fresh fixative solution (3:1 methanol acetic acid) was added drop wise to the cell pellet. Centrifugation was done afterwards and the supernatant removed. These two latter steps were repeated until a clear pellet was obtained. Finally, cells obtained were dropped on distinct slides. Staining with Giemsa was performed for some of the slides prepared from each individual. Fifty metaphases were analyzed for chromosomal breakage. The number of breaks per cell for each culture was calculated.

4- Assay of intracellular reduced glutathione (GSH):

Reduced glutathione was done by the colorimetric method according to the method described by Beutler et al [17]. This method utilized metaphosphoric acid for protein precipitation and the water soluble 5,5'-dithiobis 2-nitrobenzoic acid, (DTNB) for color development.

5. Assay of malondialdehyde level (MDA):

Lipid peroxidative products (MDA) were released from isolated lymphocytes by sonication, then lipid peroxidation products (MDA) were measured by thiobarbituric acid (TBA) test according to the method of Draper and Hadley [18,19], employing the colorimetric method using a spectrophotometer (JENWAY 6405). The sample under test is treated with TBA at low pH, one molecule of MDA reacts with two molecules of TBA to produce a pink chromogen pigment that was absorbed by spectrophotometer.

6- Assay of 8-hydroxy-2-deoxy Guanosine (8-OH-dG):

The 8-OH-dG was assayed using Cayman 8-hydroxy-2-Dixie, Guanosine enzyme-linked immunosorbent assay (Elisa = EIA) Kit (Cayman Chemical's ACE™, USA).

This assay is based on the competition between 8-OH-dG and an 8-OH-DG-acetylcholinesterase (AChE) conjugate (8-OH-dG Tracer) for 8-OH-dG monoclonal antibody [20].

Statistical Analysis

The SPSS for Windows 11 package program was used for statistical analysis. Data (means + standard deviation SD) was compared by one-way analysis of variance (ANOVA). Multiple comparisons were performed by (Tukey's post-hoc) test. $P < 0.05$ was the level of significance between the tested groups. While comet assaying results were expressed as median (minimum-maximum) (non parametric), statistical difference involving multiple group comparisons were determined by Kruskal Wallis test, differences between individual groups were determined with MannWhitney test. $P < 0.05$ was the level of significance between the tested groups.

RESULTS***Effect of Metformin on MN assay in cultured lymphocytes:***

Table 1, demonstrates the data of the in vitro evaluation of metformin by using the micronucleus test. The results indicated that metformin has no cytogenetic activity with statistically significant differences from cyclophosphamide group. A cell-cycle delay, and a decrease in cell proliferation was observed in cyclophosphamide group and not in metformin group ($p < 0.05$).

Effect of Metformin against chromosomal breakage

There was chromosomal breakages induced by cyclophosphamide (6 $\mu\text{g/ml}$), while metformin did not produce any breakages in its highest dose (table 4, figures 3,4)

Effect of Metformin on GSH level in cultured lymphocytes:

In table 2, metformin significantly increased the level of reduced glutathione as compared to cyclo-phosphamide group (12.72 \pm 1.48 vs 2.57 \pm 0.97), ($p < 0.05$) and this was insignificant as compared with the control group (12.72 \pm 1.48 vs 12.93 \pm 1.21, $p > 0.05$),.

Effect of Metformin on MDA level in cultured lymphocytes:

Metformin significantly reduced the level of MDA as compared to cyclophosphamide group (3.85 \pm 0.79 vs 9.94 \pm 1.08), ($p < 0.05$), table 2.

Effect of Metformin on 8-hydroxy-2-deoxy Guanosine (8-OH-dG) in cultured lymphocytes:

In comparison to cyclophosphamide treated group, metformin significantly decreased (8-OH-dG) level, (51.02 \pm 11.03 vs 170.27 \pm 4.53, respectively), ($p < 0.05$), table 2.

Effect of Metformin on comet assaying :

Statistical analysis of the mean values of the tail length, tail intensity and moment showed a significant rise in cyclophosphamide and not metformin group as compared with the non treated group ($p < 0.05$, table 3, figure 1,2).

Table 1, MN and CBPI frequencies values in lymphocyte cultures treated with metformin
(6 samples each, mean \pm SD)

Groups	MN assay %	CBPI%
Control group	2.62 \pm 0.31	2.03 \pm 0.03
Cyclophosphamide group	8.62 \pm 0.51*	1.68 \pm 0.07*
Metformin group	2.94 \pm 0.12 [#]	1.96 \pm 0.08 [#]

*statistically significance versus untreated control group, ($p < 0.05$)

[#]statistically significance versus cyclophosphamide group, ($p < 0.05$)

Figure 1, normal comet assay for control lymphocytes with normal DNA spot

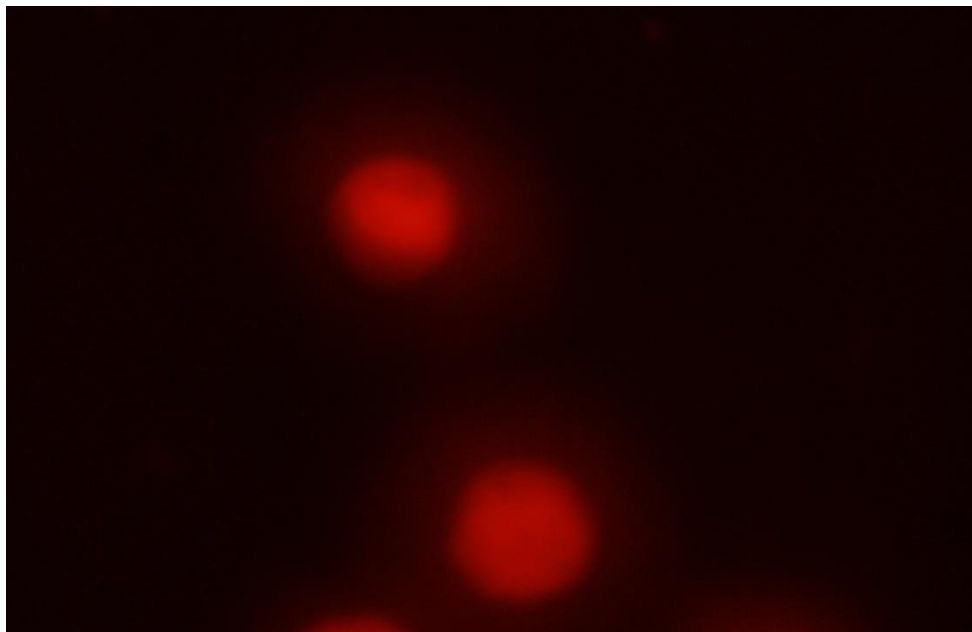


Figure 2, the comet tail of lymphocytes with damaged DNA spot : migration towards the anode

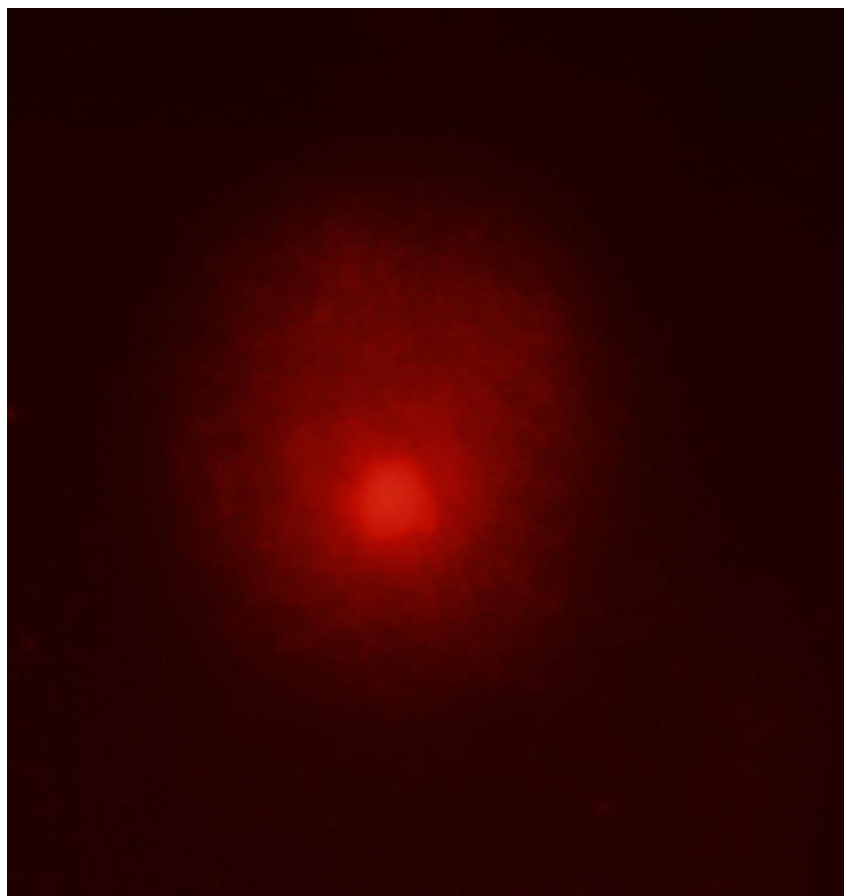


Table 2, Metformin effect on GSH(nmol/10⁶cell),MDA(nmol/10⁶cell) and 8-OH-dG(pg/10⁶cell) in lymphocytes cultures (6 samples each,Mean±SD)

Groups	GSH (nmol/10 ⁶ cell)	MDA(nmol/10 ⁶ cell)	8-OH-dG(pg/10 ⁶ cell)
Control	12.93±1.21	1.96±0.29	42.63±8.23
Cyclophosphamide group	2.57±0.97*	9.94±1.08*	170.27±4.53 [#]
Metformin group	12.72±1.48 [#]	2.35±0.79 [#]	51.02±11.03 [#]

*statistically significance versus untreated control group , (p <0.05)

[#]statistically significance versus cyclophosphamide group , (p <0.05)**Table 3: Comet assay percentage of the different studied groups**

		Control group	Cyclophosphamide group	Metformin group
Tail length μm (median range)	Median	45.05	100.45*	57.95 [#]
	Range	20.20-70.10	77.90-170.50	30.20-71.30
Tail intensity (% Tail)	Median	34.30	72.30*	23.63 [#]
	Range	9.80-50.30	46.70-94.00	10.90-40.70
Tail moment	Median	11.52	75.27*	11.34 [#]
	Range	6.87-22.28	45.88-152.26	5.00-24.54

*statistically significance versus untreated control group

[#]statistically significance versus cyclophosphamide group**Table 4.a. No chromosomal breakage with 50, 100, 200 μl of metformin (114.4μg/ml) (not shown)
b.Chromosomal breakage which was induced by cyclophosphamide (6 μg/ml)**

no	50μl	100μl	200μl
1	-	0.9	2.1
2	-	1.3	1.9
3	-	0.8	2.0
4	-	1.1	2.3
5	-	1.0	1.8
6	-	1.4	2.2
Mean± SD	- ±-	1.08±0.23	2.05±0.18

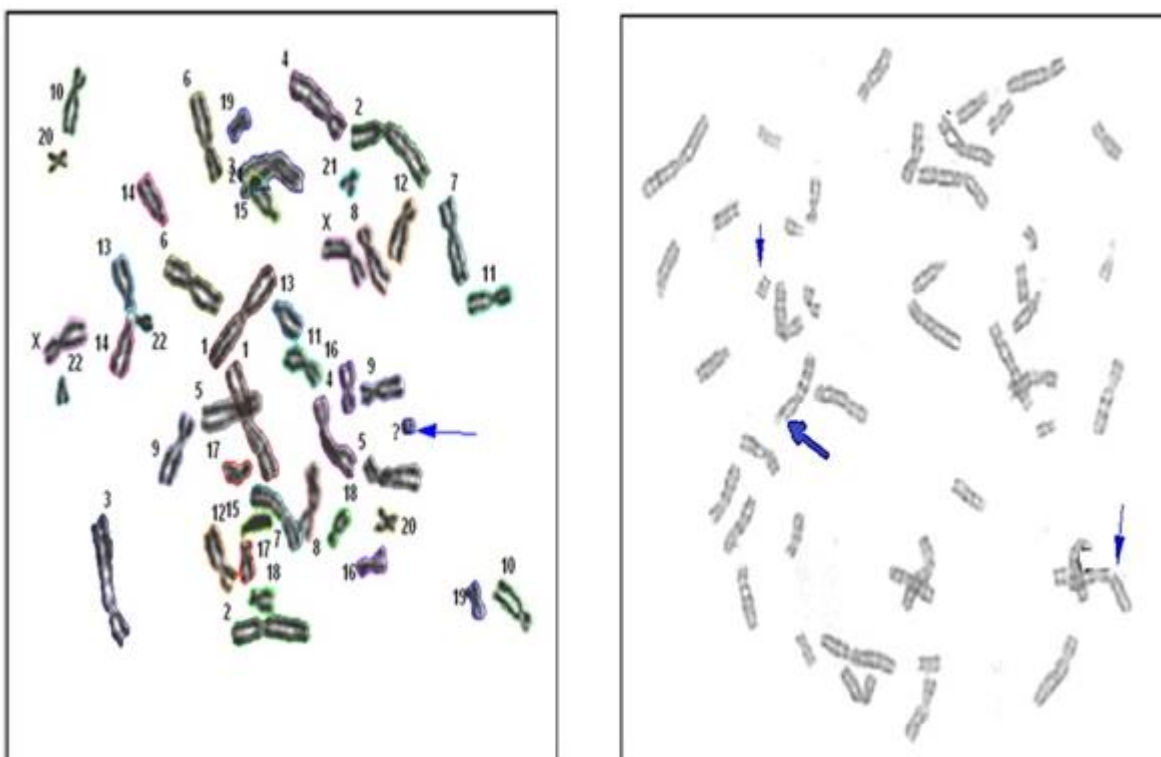
**Figure 3, (a) Metaphase spread from a healthy individual and chromosomal breakages induced by 100 μl of cyclophosphamide (6 μg/ml)
(b) Metaphase spread from a healthy individual showing chromosomal breakages induced by 200 μl of cyclophosphamide (6 μg/ml)**

Figure 4, Metaphase spread from a healthy individual and no chromosomal breakages induced by metformin (50, 100, 200 μ l of metformin 114.4 μ g/ml)



DISCUSSION

Metformin is an oral antidiabetic drug that has been used for human conditions suffering from an insulin-resistance, diabetic states, impaired glucose tolerance, and weight gains. It is also used in treating anovulation, and induce conception in polycystic-ovary syndrome. However, there is a strong debate around the metformin-induced genotoxicity [4].

Among different cytogenetic techniques, micronucleus is an index for genotoxicity, and the reduction in the cytokinesis-block proliferation, is an index for the cytotoxic effect. The technique is relevant because it detects the clastogenic effect via induction of cytokinesis block, and its applicability have made its usage very promising in chromosomal alteration studies [21,22]. Micronucleus assay is only working when DNA strands are broken [21].

The dose used by Amador *et al* [4] was small, and was re-evaluated in the present work and other different doses are needed to be investigated in further works. In the present study, metformin did not show significant oxidative effects as indicated by non significant changes in the levels of MDA, GSH, and 8-OH-dG.

This coincides with different *in vivo* studies showed that metformin at therapeutic concentrations could destroy hydroxyl (\cdot OH) free radicals.

Metformin is able to scavenge ROS directly or by modulating the intracellular production of superoxide anion, that NADPH oxidase constitutes the major source. Metformin decreases the oxidative process in the cardiovascular and renal systems, delays diabetes mellitus onset and prevents early manifestations of vascular disorders [23,24].

The data obtained from metformin treated cultures proved that this drug does not increase significantly, the frequency of binucleate cells with MN (BNMN) in lymphocytes, at the concentration used of metformin. More over metformin treated lymphocyte cells did not have any significant changes in their percentages or in tail length, as compared with cyclophosphamide that induced a significant increase in lymphocytes percentage and tail length having the studied comet assay. Metformin in this work did not show any effect on DNA oxidative destruction through the free radicals formation.

Hirsh et al [25] showed that metformin in low doses inhibit cellular transformation and kills cancer-stem cells in four genetically different types of the cancer of the breast tissue . They proposed that metformin combination with chemotherapeutic drugs may be a novel treatment for cancer .

In the study of Onaran et al metformin administration in pharmacological dose was unable to prevent DNA destruction under the effect of pro-oxidant stimulus in cultured lymphocytes, in spite of its antioxidant properties [26] .

While , Kefas et al proved the genotoxicity effect of metformin in mammalian cells using comet assay through the activation of AMPK ,which increases nitric oxide synthase and mitochondria-derived reactive-nitrogen species. Furthermore, blood lymphocyte cultures challenged with cumene hydroperoxide (CumOOH) showed that in vitro high dose of metformin produces DNA damage, despite its antioxidant effect [26,27].

On the other hand, Kanigür-Sultuybek et al [28] in vitro results, indicated the protective effect of metformin against prooxidant stimulus-induced DNA damage in lymphocytes from elderly subjects.

CONCLUSION

This study concluded that metformin does not cause any oxidative DNA desrtuctive effects in vitro.

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