Original Article Pharmacology

To Assess the In vitro Genotoxicity of Metformin and Aspartame alone & In Combination

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ABSTRACT

Objective: Metformin is a known oral antidiabetic agent belonging to the class of biguanides, widely prescribed for the treatment of type 2 Diabetes Mellitus (DM). In this study the genotoxic potential of metformin was studied alone and in combination with an artificial sweetener aspartame as most of the diabetic patients utilized this low calorie sweetener to reduce their sugar consumption per day. Many complaints regarding its potential to cause DNA damage have been submitted to FDA

Study Design: Experimental study

Place and Duration of Study: This study was carried out at the Department of Pharmacology and Toxicology, University of Veterinary and Animal Sciences Lahore from 1st Jan 2011 to Dec 2011.

Materials and Methods: Peripheral Blood Lymphocytes were exposed to various concentrations of metformin, aspartame and their combination. DNA damage was checked by comet assay. The data was analyzed by using Analysis of Variance (ANOVA) by Statistical Package of Social Sciences SPSS

Results: Exhibited dose dependant rise in comet tail lengths. Moreover the data advocates that tail lengths of lymphocytes after exposure to aspartame were high as compared to metformin. When lymphocytes were exposed to combination of aspartame and metformin and DNA damage was checked by comet assay, the results were significantly different ($p<0.05$) as compared to metformin and aspartame alone.

Conclusion: It can be concluded from the present study that aspartame is posing great genotoxic threat to the cells as compared to metformin and the combination is even more toxic to DNA, so the drug regime of diabetic patient must be closely monitored. There is further need of more studies in this regard.

Key Words: Metformin, Aspartame, Diabetes Mellitus, Comet Assay. DNA damage.

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INTRODUCTION

Among the antidiabetic agents, metformin (1-(diaminomethylidene)-3,3-dimethyl-guanidine) is reported to have several properties, such as it reduces formation of Advance glycation endproducts(AGE), helps to regain the reduced levels of glutathione in DM and increases the antioxidant defense[1]. A recent study demonstrates that metformin increases AMPK activation in MIN6 cells responsible for insulin production and primary rat beta cells in dose dependant manner. Prolonged exposure of metformin (>24h) resulted in a gradual rise in apoptotic beta-cells similar to the effect which was documented for AMPK-activator AICAR[2].

AMPK is an enzyme that plays a role in cellular energy homeostasis and its persistent activation has recently been concerned with apoptosis of beta cells of pancreas and in cell death of islets of langerhans[3].

Activation of AMPK by metformin might be mediated by the mitochondria-derived RNS in vivo in C57BL6 mice[4]. There are certain studies supporting that metformin stimulate cell death in p53-deficient HCT116 cells in condition of limited glucose availability. So metformin can be beneficial to be used in patients with p53 deficient tumors which have become resistant to chemotherapy[5].

The antidiabetic drug has also been reported to cause a decline in the occurrence of mammary carcinomas in hamsters when administered in therapeutic doses. Most probably it would have damaged the genetic material of the carcinoma cells to reduce chances of cancer prevalence in mammary cells of hamster[6]. Certain reports support combination therapy of metformin and doxorubicin which kills both cancer stem cells and non–stem cancer cells in culture, and cause reduction in tumor mass and delays relapse more efficiently than metformin of doxorubicin can do alone[7].

Onaran et al. (2006) suggested that therapeutic concentrations of metformin could not check DNA damage caused by oxidative stimulus in cultured human lymphocytes regardless of its anti oxidant characteristic[8].

The use of artificial sweetener aspartame is increasing day by day by both diabetic as well as non diabetic population as a sugar substitute. Aspartame is mainly composed of phenylalanine (50%), aspartic acid (40%) and methanol (10%). In this study different doses of aspartame was administered to check the cellular effects of aspartame on brain and it was revealed that

consistent use of aspartame might cause various mental disorders due to damage to the brain cells [9].

The effect of an artificial sweetener Aspartame was studied to investigate oxidative stress and brain monoamines in normal conditions and also after administration of lipopolysaccharides intraperitoneally in mice. The results revealed that aspartame alone and in combination with any mild systemic inflammatory response enhance inflammation and oxidative stress in brain cells[10].

MATERIALS AND METHODS

Chemicals: Tested chemicals i.e., Aspartame and Metformin were provided by Popular Laboratories, Lahore Pakistan. Dimethyl Sulfoxide (DMSO) was purchased from Merck Pakistan. All other chemicals and media used were of analytical grade.

Comet Assay: Cavity slides were layered with 1% Normal melting Agarose and placed in refrigerator for 2-12 hours. Peripheral blood lymphocytes were separated by using Lymphocyte separating medium. 3ml of 1:1 ratio of peripheral blood with PBS was layered onto 4ml of Lymphocyte separating medium and after centrifuging lymphocyte suspension was made in RPMI-1640[11]. Now the lymphocytes were exposed to various concentrations of test chemicals i.e. metformin, aspartame and combination doses of aspartame and metformin. The exposed cells were mixed 1% low melting point agarose and layered onto the precoated slides. After solidification 3rd layer of Agarose 1% was layered onto the same slide and allowed to solidify. Subsequently the slides were dipped in the lysing solution (pH 10) consisting of high salt concentration and detergent for 2 hours. After that slides were exposed to alkaline buffer (pH > 13) consisting of 1mM EDTA and 300mM NAOH for DNA unwinding for 20 minutes. The cells were subjected to electrophoresis for 20 minutes at 25 volts and 300mA. Slides were washed with 0.4M TRIS buffer/ Neutralizing buffer (pH 7.5) 3 times and then stained with Ethidium bromide[12].

PBS was used as negative control and DMSO 20% was used as positive control[13].

Microscopic Analysis: The slides were visualized by using Fluorescent Microscope at 40X objective. The DNA damage was quantified by measuring tail lengths of 25 consecutive cells and quantification was done in triplicate manner. Four damage categories were established according to the method described by Valencia-Quintana and coworkers (Valencia-Quintana et al. 2012).

Class 0= Undamaged cells

Class 1= Tail length ≤Head Diameter

Class 2= Tail Length > Head Diameter BUT Tail Length ≤Double of Head Diameter

Class 3= Tail Length > Double of Head Diameter

The damage to the DNA was expressed as %age fragmentation and ultimately finding the DNA damage index of each concentration according to the following formula.

Damage Index= No. of cells in Class.1+ (2×No. of cells in Class.2) + (3×No. of cells in Class.3)

Fragmentation %= No. of cells in Class.1 + No. of cells in Class.2 + No. of cells in Class.3 ×100
Total Number of cells under observation

Statistical Analysis: The data was analyzed by using Analysis of Variance (ANOVA) by Statistical Package of Social Sciences SPSS for windows (version 16; SPSS Inc; Chicago IL; USA) and Tukey's test was applied. The value of $p<0.05$ was considered significant.

RESULTS

The results of comet assay on lymphocyte sampled from peripheral blood treated with different concentrations of metformin are presented in Table 1. Treatment with different doses of metformin induced a significant increase in DNA damage at 200, 250, 300, 350 and 400μg/ml as these values were significantly higher ($p < 0.01$) than that found in the negative control. It is noted that percentage of fragmentation of DNA and damage index increases as the concentration of metformin increases reaching a maximum value at 400μg/ml. The effect observed with 10, 20, 80, 100 and 150μg/ml do not show significant differences ($p < 0.05$) when compared with the negative control.

Lymphocytes were exposed to 10 different concentrations of aspartame presented in the Table 2. The treatment with various doses of aspartame showed same order of genotoxicity as that of metformin. Aspartame caused significant rise ($p < 0.01$) in DNA damage at 500, 1000, 2000, 4000, 8000 μg/ml as compared to the DNA damage caused by negative control. The percentage fragmentation of DNA and Damage Index becomes high when the exposed concentration of aspartame increases and the maximum DNA damage index is found at 8000 μg/ml. However the results with lower doses i.e. 12.5, 25, 50, 100 and 250μg/ml did not show any significant rise ($p < 0.05$) in the DNA damaging potential as compared to negative control.

When the cells were exposed to the combination of aspartame and metformin, the results were significantly high ($p < 0.05$) as compared to metformin and aspartame individually. Here again dose dependant rise in genotoxicity is observed in manner similar to the individual drugs. We can see a significant rise ($p < 0.01$) in DNA damage index at 500:200, 1000:250, 2000:300, 4000:350, 8000:400 μg/ml as compared to the negative control (Table 3). Percentage fragmentation and DNA damage index increases as the concentration of aspartame and metformin increases with maximum DNA damage index obtained at 8000:400 μg/ml which has highly significant difference ($p < 0.001$) with negative control.

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Table No.1: DNA Damage induced by different concentration of Metformin in lymphocytes evidenced from Comet assay.

Sr. No.	Conc.	Class 0	Class 1	Class 2	Class 3	Fragmentation %	Damage Index
A	DMSO 20%	2	2	6	15	92	59
B	-ve Control	24	1	0	0	4	1
1	10µg/ml	24	1	0	0	4	1
2	20 µg/ml	23	2	0	0	8	2
3	80 µg/ml	22	3	0	0	12	3
4	100 µg/ml	22	3	0	0	12	3
5	150 µg/ml	22	2	1	0	12	4
6	200 µg/ml	20	2	3	0	28	8*
7	250 µg/ml	16	5	4	0	36	13*
8	300µg/ml	14	4	6	1	44	19*
9	350 µg/ml	13	3	7	2	48	23*
10	400 µg/ml	12	3	8	2	52	25*

***=significant difference (p<0.01) as compared to negative control analyzed by SPSS Windows Version 16 Tukey's Test.**

Table No.2: DNA Damage induced by different concentration of Aspartame in lymphocytes evidenced from Comet assay.

Sr. No.	Conc.	Class 0	Class 1	Class 2	Class 3	Fragmentation %	Damage Index
A	DMSO 20%	2	2	6	15	92	59
B	-ve Control	24	1	0	0	4	1
1	12.5 µg/ml	24	1	0	0	4	1
2	25 µg/ml	23	2	0	0	8	2
3	50 µg/ml	23	2	0	0	8	2
4	100 µg/ml	22	3	0	0	12	3
5	250 µg/ml	22	2	1	0	12	4
6	500 µg/ml	19	3	3	0	24	9*
7	1000 µg/ml	16	2	7	1	40	19*
8	2000 µg/ml	14	2	7	2	44	22*
9	4000 µg/ml	12	2	8	3	52	27*
10	8000 µg/ml	9	1	11	4	64	35*

***=significant difference (p<0.01) as compared to negative control analyzed by SPSS Windows Version 16 Tukey's Test.**

Table No.3: DNA Damage induced by different concentration of Combination of ASP: MET in lymphocytes evidenced from Comet assay.

Sr. No.	Conc.	Class 0	Class 1	Class 2	Class 3	Fragmentation %	Damage Index
A	DMSO 20%	2	2	6	15	92	59
B	-ve Control	24	1	0	0	4	1
1	12.5:10µg/ml	24	1	0	0	4	1
2	25:20 µg/ml	23	2	0	0	8	2
3	50:80 µg/ml	23	1	1	0	8	3
4	100:100 µg/ml	23	1	1	0	8	3
5	250:150 µg/ml	22	2	1	0	12	4
6	500:200 µg/ml	15	4	3	3	56	19*
7	1000:250 µg/ml	10	4	9	2	60	28*
8	2000:300µg/ml	8	3	10	4	68	35*
9	4000:350 µg/ml	6	2	12	5	76	41*
10	8000:400 µg/ml	4	2	14	5	84	45*

***=significant difference (p<0.01) as compared to negative control analyzed by SPSS Windows Version 16 Tukey's Test.**

When the all the three results were analyzed statistically for multiple comparison by applying Analysis of Variance (ANOVA) Post Hoc Test LSD by Statistical Package of Social Sciences SPSS for windows (version 16; SPSS Inc; Chicago IL; USA) it was revealed that combination doses were significantly ($p < 0.05$) highly

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genotoxic as compared to metformin. However the results of combination doses were not significantly ($p < 0.05$) different as compared to aspartame individually.

DISCUSSION

The prime objective of therapy for type 2 diabetes mellitus is to obtain and sustain good glycemic control, and to reduce the risk of secondary complications associated with DM. Metformin is considered a gold standard treatment of DM along with certain interventions in lifestyle of patients. Metformin also have efficacy against polycystic ovary syndrome and various neoplasms[14].

The antidiabetic drug has been reported to cause a decline in the occurrence of mammary carcinomas in hamsters when administered in therapeutic doses. Most probably it would have damaged the genetic material of the carcinoma cells to reduce chances of cancer prevalence in mammary cells of hamster[6]. The results of comet assay on various concentrations of metformin exhibited dose dependent rise in its potential to damage DNA. Percentage fragmentation of DNA and damage index increases as the concentration of metformin increases reaching a maximum value at 400µg/ml. The results of this study are in accordance with the fact that prolonged exposure of metformin may become a potential threat of genotoxicity. A study was conducted to check the genotoxic potential of metformin in Chinese Hamster Ovary cells and DNA damage was checked by Chromosomal Aberration Assay and single cell gel alkaline electrophoresis/comet assay. Results showed significant damage to the DNA by metformin[15]

However data is available which supports that metformin is neither cytotoxic nor genotoxic when various concentrations were administered in vivo condition to diabetic and non diabetic rats[16]. Another study was conducted to evaluate the prevention of DNA damage in human lymphocytes by metformin. Cells were exposed to various concentrations of metformin and subsequently they were incubated with cumene hydroperoxide a known DNA damaging substance. The results were evaluated by comet assay revealing that metformin is unable to prevent DNA damage by any chemical causing oxidative stress to the cells[8]. Metformin kills cancer stem cells in genetically different type of breast cancer so combination therapy of metformin and doxorubicin is used to reduce tumor mass and delays relapse more efficiently that metformin and doxorubicin can do alone[7].

The underlying mechanism responsible for DNA damage by metformin may lie in the fact that metformin may inhibit mitochondrial respiration cells and ultimately there is rapid rise in the superoxide level because of inhibition of cellular respiration due to insult to the mitochondria [17]. According to Warburg theory of cancer, the key reason for development of tumor is an inadequate cellular respiration due to insult to mitochondria[18].

The use of sweetening agent by diabetic individuals is common. A survey of our diabetic clinic population showed that 65% regularly use these products[19]. It is widely used by the diabetic population for the sweet taste avoiding high calories through sugar consumption[20]. The results of comet assay revealed that aspartame exhibited significant potential of genotoxicity when different concentrations of the sweetener were exposed to the lymphocytes. Damage Index of DNA revealed that aspartame was causing more damage to the DNA of peripheral blood lymphocytes as compared to metformin when compared with the negative control. The results of comet assay are in agreement with a study exhibiting significantly high ($p < 0.01$) carcinogenic potential on prolonged use, demonstrating that these artificial sweeteners are not entirely safe although they are FDA approved[21].

Results also state that there is dose dependant rise in the DNA damaging potential of aspartame endorsing the statement that aspartame can cause chromosomal aberrations at all concentrations (500, 1000 and 2000 µg/ml) and treatment periods, in human lymphocytes, in a dose dependant manner[22]. The underlying mechanism of DNA damage caused by aspartame may lie in the fact that prolong exposure of aspartame may result in detectable amount of methanol in blood[23]. As aspartame consists of 3 components aspartic acid, phenylalanine and methanol which is most dangerous of all[24]. Various studies revealed the data that aspartame is responsible for increased frequency of lymphomas and leukemias and is also responsible in significant rise in prevalence of transitional cell carcinomas of renal pelvis at doses approximated with the ADI of aspartame for humans. These results indicate that aspartame proves to be the multipotential carcinogen greatly affecting the quality of life[25].

As far as results of the combination doses are concerned they cause severe damage to DNA when exposed to the lymphocytes and cells were quantified using comet assay. %age fragmentation and DNA damage index were significantly high ($p < 0.05$) as compared to individual results of metformin and aspartame. The data advocates the same threshold level of genotoxicity but the intensity of the genotoxic effect was relatively high in case of combination as compared to the individual drugs. The additive effect of both aspartame and metformin on DNA damage may be accredited to the fact that aspartame is responsible for increased oxidative stress to the cells[10]. Along with that metformin is associated with production of reactive nitrogen species from mitochondria[4] which ultimately hinder the cellular respiration leading to cell damage.

CONCLUSION

It can be concluded from the present study that aspartame is posing great genotoxic threat to the cells as compared to metformin. When combination of aspartame and metformin were exposed to the peripheral blood lymphocytes the results showed significantly high ($p < 0.05$) genotoxicity as compared to metformin and aspartame individually. Most of the diabetic patients utilize aspartame as an artificial sweetener along with their daily regimen of antidiabetic drug metformin. So caution must be taken while using

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both these products together as it may cause significant damage to the cells of body.

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