Original Article

Combination Therapy of DM

In vitro Evaluation of Mutagenicity of Metformin and Aspartame alone and in Combination

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ABSTRACT

Objective: To assess the Mutagenicity of Metformin and Aspartame in vitro.
Study Design: Observational / Analytical study
Place and Duration of Study; This study was carried out at Department of Pharmacology and Toxicology, University of Veterinary and Animal Sciences Lahore from 1st Jan 2011 to Dec 2011.
Materials and Methods: Ames Salmonella/ Microsome Mutagenicity Assay was used to check the mutagenic potential of test chemicals & control. The data was analyzed by using Statistical Package of Social Sciences.
Results: Metformin was found to be highly mutagenic against TA100 and TA98 both in the presence and absence of metabolic activation system. The results were significant because there was 2 fold rise in number of revertants as compared to the negative control. Overall metformin exhibited more mutagenicity against TA 100 as compared to TA 98 strain of Salmonella Typhimurium. Aspartame showed significant rise in mutagenicity at 100µg/plate and 250µg/plate in dose dependant manner against TA 100 in presence of metabolic activation system. When combination doses of aspartame and metformin were studied, even those doses became mutagenic which were not mutagenic alone. The data advocates that combination doses showed significant additive effect ($p < 0.05$) in the intensity of mutagenic index as compared to the mutagenic index of metformin and aspartame alone.
Conclusion: Both of these products alone & together may cause significant damage to the cells of body as well. Combination therapy of these products should be monitored closely.
Key Words: Diabetes Mellitus, Metformin, Aspartame, Ames Assay, Mutagenicity, S-9.

INTRODUCTION

Diabetes Mellitus (DM) is a chronic disease characterized by high levels of blood sugar. Different oral antidiabetic drugs are being utilized as single as well as combination therapy. Among them Metformin is the most common one. Moreover diabetic patients utilize various low calorie sweeteners to decrease their sugar consumption per day. Aspartame is most widely used artificial sweetener used by diabetic as well as non- diabetic population.

Diabetes Mellitus is a progressive disease and about 6% population in Westernized countries is currently being affected with Diabetes. The ratio is expected to be doubled by 2020[1]. Pakistan stands amongst the highly predominant region, presently having 6.9 million affected people, probably expected to be increased twofold by 2025 and will have an effect on 11.5 million people[2]. Recently Pakistan is ranked at 7th position in the list of countries with problem of DM and it may exceed to 4th position if same circumstances prevail throughout[3]. Mostly the patients of Diabetes are obese at the time of diagnosis and it will be difficult for them to achieve normal glucose level without using oral antidiabetic agents. Metformin prevents obesity and also has advantageous effects on various cardiovascular risk factors. Therefore, Metformin is extensively prescribed as the drug of choice for type 2 Diabetes Mellitus[4]. Metformin is also known to be used to induce and maintain pregnancy in Polycystic Ovary Syndrome[5]. According to a study it is assumed that metformin is associated with the production of oxidative stress in cells leading to DNA fragmentation[6]. In another study it is concluded that metformin is responsible for oxidative stress in white adipocytes, by increasing the levels of reactive oxygen species[7].

Epidemic rates of obesity and type II DM are taking place in the United States and even other areas of the world. Basically this outbreak of obesity have come into view by modification in our dietary habits and decreased physical activities. An important yet not well-supported dietary change is the gradual rise in the quantity of various sweeteners which are used in the food industry[8].

Among these low calorie sweeteners Aspartame is the most widely used sweetener almost capturing 50% of the consumer market in the world. It was discovered in 1965 and now it is easily available in more than 5000 commercial products in not less than 90 countries. Since 1988 almost 80% complaints about various food additives submitted to FDA belong to aspartame[9].

After approval from FDA Aspartame consumption as low calorie sweetener and sugar substitute is being controversial because of its ill effects on health such as carcinomas of brain[10].

MATERIALS AND METHODS

Chemicals: Test chemicals i.e., Aspartame and Metformin were provided by Popular Laboratories, Lahore Pakistan. Metabolic Activation System (S9) was obtained from Environmental Biodetection Products Inc. (EBPI, Canada) in lyophilized form along with its co factors. All other chemicals and media used were of analytical grade.

Mutagenicity Assay: Ames Salmonella/ Microsome Mutagenicity Assay was used to check the mutagenic potential of test chemicals. Standard pour plate incorporation assay and Pre incubation Assay were performed according to the method illustrated by Mortelmans and Zeiger[11]. Standard pour plate incorporation assay was performed to check the mutagenic potential of test drugs without addition of metabolic activation system S9 mix, while Pre incubation assay was used to evaluate the mutagenicity in presence of S9 mix. Two mutant strains of Salmonella Typhimurium TA 100 and TA 98 were used to check reversion caused by test drugs. TA 100 is sensitive to the Base pair substitution mutation whereas TA 98 is used to evaluate the test chemicals which are responsible for Frame shift mutation. Both the strains were cryopreserved at -80°C in nitrogen cylinder according to the protocol described in Mortelmans and Zeiger[11].

Evaluation of mutagenicity was performed by using glucose minimal (GM) agar plate containing glucose minimal medium, Vogel Bonner Salts, Top agar supplemented with 0.5mM Histidine/biotin 2ml, test chemical dilution, 0.05-0.1ml bacterial culture and 0.5ml S9 mix was poured onto the GM agar plate and allowed to solidify for 3 -5 minutes. Now the plates were inverted and incubated at 37°C for 48 hours. In each experiment negative control plate containing top agar supplemented with 0.5mM Histidine/biotin and bacterial culture was poured on GM agar plate without addition of any test chemical. Positive control used to check mutagenicity against TA 100 was sodium azide (5µg/plate) whereas against TA 98 the positive control was 2 nitroflourene (1µg/plate). After the given period of incubation the number of revertant colonies per plate were counted and compared with the number of revertants in negative control plate.

Mutagenic Index of Test Chemicals: Mutagenic Index was measured by applying following formula:

$$\text{Mutagenic Index (M.I.)} = \frac{\text{Number of Revertant Colonies per Plate with Test Chemical Dose}}{\text{Number of Natural Revertant Colonies of Negative Control Plate}}$$

If Mutagenic Index is greater than 2, it means the test concentration will be mutagenic.

Mutagenic response was considered positive when number of colonies in test chemical plate were more than or equal to two fold than the natural revertants of –ve control or background colonies[12].

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

RESULTS

10 different concentrations of Metformin in range of 10µg/plate to 400µg/plate were checked for mutagenic response. Similarly 10 different concentrations of Aspartame ranging from 12.5µg/plate to 8000µg/plate were checked for mutagenicity. 10 combination doses of Aspartame: Metformin in range of 12.5:10 µg/plate to 8000:400 µg/plate were checked for their mutagenic potential. In case of Metformin, mutagenicity was observed only when the test concentration ranging from 80µg/plate, 100µg/plate and 150µg/plate were evaluated in Pre Incubation Assay using metabolic activation system S9 mix using TA 100 strain. Whereas the concentrations of 100µg/plate and 150 µg/plate were also found mutagenic while assaying with the same TA 100 strain but without metabolic activation system. Results were considered significant because the revertant colonies showed two fold rise in number of revertant colonies as compared to the negative control plates.

Mutagenicity was observed only when the test concentrations 80µg/plate and 100µg/ plate were evaluated in Pre Incubation Assay using metabolic activation system S9 mix against TA 98 strain. Whereas the concentration of 100µg/plate was also found mutagenic while assaying with the same TA 98 strain but without metabolic activation system (Table 1).

In case of Aspartame Mutagenicity was observed only when the test concentrations of 100µg/plate and 250µg/plate were evaluated in Pre Incubation Assay using metabolic activation system S9 mix with TA 100. Whereas same doses were proved to be non-mutagenic when evaluated in Standard Plate Assay without S9 mix with TA 100. All the 10 doses of Aspartame were found to be non mutagenic in both Pre Incubation Assay and Standard Plate Assay with TA 98 as number of revertants were insignificant because no two fold rise in number of revertant colonies were found as compared to the negative control plates (Table 2).

The concentrations of combination doses of 25:20, 50:80, 100:100 and 250:150µg/plate were found mutagenic with S-9 Mix while assaying with TA 100. Whereas the concentrations of the combination 50:80, 100:100 and 250:150µg/plate were found to be mutagenic while checking the combination without S9

Table No.1: Mutagenic Potential of Metformin

Metformin									
Sr. No	Conc.µg per plate	Revertant Colonies Per Plate							
		TA 100				TA 98			
		+ S-9	M.I.	-S-9	M.I.	+S-9	M.I.	-S-9	M.I.
1	10	122	0.67	61	0.64	65	0.81	36	0.72
2	20	157	0.94	75	0.79	80	1.001	43	0.86
3	80	392	2.35*	99	1.03	184	2.30*	49	0.97
4	100	479	2.85*	265	2.79*	224	2.80**	105	2.09*
5	150	618	3.69**	341	3.54**	148	1.85	77	1.54
6	200	330	1.81	171	1.78	112	1.40	67	1.33
7	250	278	1.53	142	1.48	85	1.06	52	1.01
8	300	174	1.043	96	1	75	0.93	42	0.84
9	350	141	0.85	74	0.78	53	0.66	28	0.58
10	400	83	0.49	44	0.46	39	0.49	16	0.33
-Control	0	182		96		80		51	
+Control	5	1928	10.57	936	9.75	410	5.13	200	3.94

-control × 2*, -control × 3**, -control × 4 and above***

Table No.2: Mutagenic Potential of Aspartame

Aspartame									
Sr. No.	Conc. µg/plate	Revertant Colonies per Plate							
		TA 100				TA 98			
		+S-9	M.I.	-S-9	M.I.	+S-9	M.I.	-S-9	M.I.
1-	12.5	185	0.71	66	0.68	55	0.69	31	0.62
2-	25	212	1.16	87	0.90	66	0.82	36	0.71
3-	50	248	1.36	110	1.17	68	0.85	39	0.76
4-	100	389	2.14*	115	1.21	82	1.025	41	0.83
5-	250	546	3**	140	1.48	95	1.19	44	0.88
6-	500	289	1.59	149	1.55	107	1.32	48	0.93
7-	1000	244	1.34	122	1.28	87	1.09	38	0.73
8-	2000	207	1.14	105	1.10	77	0.97	29	0.55
9-	4000	179	0.98	87	0.93	52	0.65	26	0.50
10-	8000	108	0.595	55	0.58	48	0.60	19	0.37
-ve	0	182		96		80		51	
+ve control	5	1928	10.57	936	9.75	410	5.13	200	3.94

-control × 2*, -control × 3**, -control × 4 and above***

Table No.3: Mutagenic Potential of Combination of Aspartame and Metformin

Aspartame : Metformin									
Sr. No.	Conc. µg per plate	Revertant Colonies Per Plate							
		TA 100				TA 98			
		+ S-9	M.I.	-S-9	M.I.	+S-9	M.I.	-S-9	M.I.
1	12.5:10	189	1.04	85	0.89	66	0.82	35	0.74
2	25:20	395	2.17*	102	1.07	164	2.05*	45	0.89
3	50:80	587	3.22**	190	2.04*	250	3.13**	119	2.24*
4	100:100	814	4.47***	288	3.03**	329	4.12***	185	3.72**
5	250:150	915	5.03***	340	3.58**	154	1.93	90	1.78
6	500:200	346	1.90	165	1.72	139	1.74	69	1.35
7	1000:250	315	1.73	133	1.39	118	1.47	58	1.14
8	2000:300	279	1.53	108	1.14	95	1.13	53	1.05
9	4000:350	185	1.02	82	0.86	64	0.79	37	0.74
10	8000:400	150	0.83	67	0.71	43	0.54	25	0.49
-Control	0	182		96		80		51	
+Control	5	1928	10.57	936	9.75	410	5.13	200	3.94

-control × 2*, -control × 3**, -control × 4 and above***

mix using TA 100 strain as significant difference ($p < 0.05$) was found in no. of colonies of revertants as compared to the negative control. The concentrations of combination doses of 25:20, 50:80 and 100:100µg/plate were mutagenic with TA 98 using metabolic activation system whereas 50:80 and 100:100µg/plate were also mutagenic using TA 98 strain without metabolic activation system i.e. S-9 mix (Table 3).

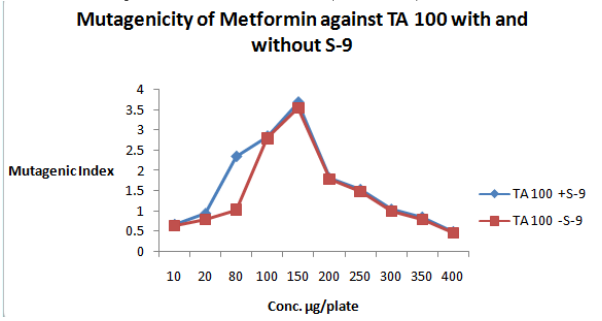

Figure No.1: Mutagenic Potential of Metformin against TA 100 with and without S-9

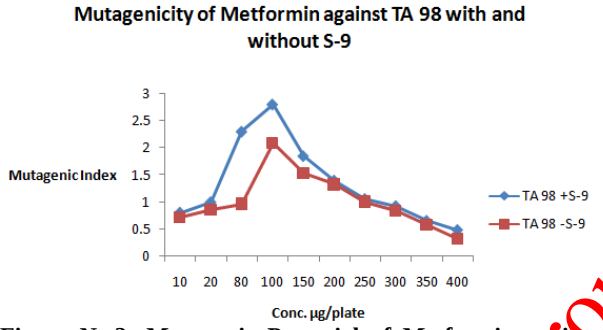

Figure No.2: Mutagenic Potential of Metformin against TA 98 with and without S-9

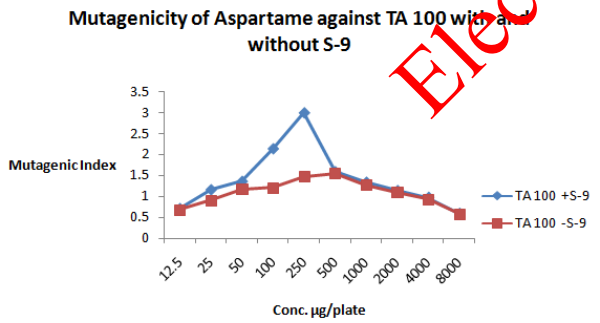

Figure No.3: Mutagenic potential of Aspartame against TA 100 with and without S-9

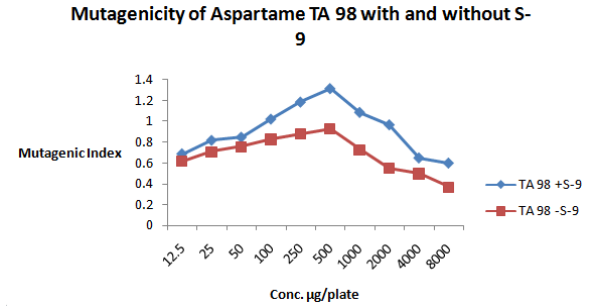

Figure No.4: Mutagenic Potential of Aspartame against TA 98 with and without S-9

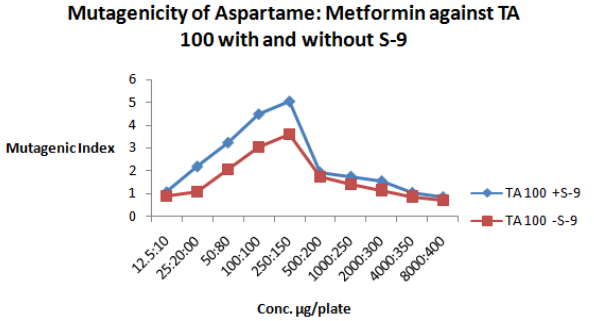

Figure No.5: Mutagenic Potential of ASP: MET against TA 100 with and without S-9

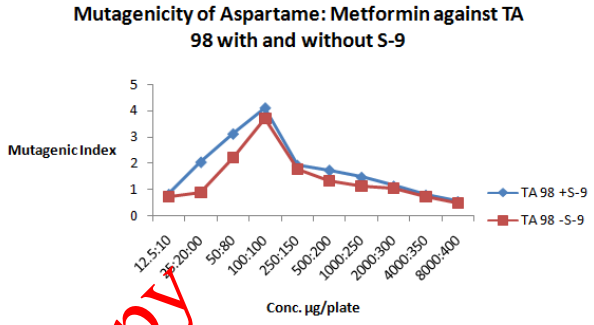

Figure No.6: Mutagenic Potential of ASP: MET against TA 98 with and without S-9

DISCUSSION

Diabetes mellitus Type 2 is a progressive disorder and incidence of this disease is rising rapidly. In the US alone, 41 million individuals of the total population have symptoms of prediabetes, posing them at elevated risk for the established disease of diabetes. The pathological symptoms of type 2 diabetes include insufficient insulin discharge and resistance to the action of insulin[13]. High rates of type 2 Diabetes Mellitus in Pakistan are affecting the quality of life socially as well as financially. This may be due to the poor monitoring criteria as well as high rates of complications associated with the patients of Diabetes Mellitus[14]. A survey conducted in Pakistan concluded that newly diagnosed DM patients were 5.0% in men and 4.8% in women in rural areas 5.1% in men and 6.8% in women in urban areas[15].

This frightening situation may lead to severe consequences. So to treat this progressive disease effective oral antidiabetic agents must be prescribed to deal with the challenge of Diabetes Mellitus. Metformin is the most commonly prescribed drug against DM as it can help to decrease various secondary disorders associated with Diabetes Mellitus such as obesity. Metformin reduces blood glucose level and also cause reduction in obesity. Diabetic population routinely utilizes some low calorie sweetener to reduce their daily intake of sugar. Aspartame is the most common artificial sweetener utilized by diabetic population in

their daily diet schemes. So it is important to determine the toxicological data associated with Metformin and Aspartame. Current study was an attempt to provide the toxicological Index of metformin alone and in combination with aspartame by performing Ames mutagenicity assay to evaluate their mutagenic potential.

Results of Ames test revealed that both metformin and aspartame were mutagenic at different concentrations and the combination doses exhibited significantly high ($p<0.05$) mutagenic index as compared to the metformin and aspartame alone. Among metformin and aspartame, metformin proved to be more mutagenic. A significant ($p<0.05$) dose dependent rise in the mutagenicity against TA 100 and TA 98 was exhibited at 100 and 150µg/plate as compared to the negative control plate. While the dose of 80µg/plate was mutagenic only in presence of metabolic activation system S-9 mix. Maximum mutagenicity was observed at 150 µg/plate against TA 100 both in the presence and absence of S9 mix. Whereas in case of TA 98 maximum mutagenicity was observed at 100 µg/plate both in presence and absence of S9 mix. The results were in accordance with the study results revealed that metformin may have a significant mutagenic potential in pregnant females and their embryos[16]. Moreover metformin may cause DNA damage to Chinese Hamster Ovary cells which supports the hypothesis that metformin may cause DNA damage both directly and indirectly via various unforeseen mechanisms[17]. These studies are in line with the fact that metformin may inhibit the phenomenon of mitochondrial respiration[18]. This fact supports the Warburg theory of cancer that the key factor for tumorogenesis is an inadequate cellular respiration caused by insult to mitochondria[19].

Aspartame caused significant mutagenic response against TA 100 with metabolic activation system having maximum mutagenicity at 250µg/plate. Over all mutagenic potential of aspartame was relatively high against TA 100 as compared to TA 98 strain of Salmonella typhimurium. These results are in accordance with the study in which aspartame and saccharin were checked for their mutagenic potential and aspartame was proved to be more mutagenic as compared to saccharin. Moreover the mutagenic potential was more profound when drug was assayed with TA 100 in presence of metabolic activation system[20]. Similarly aspartame treatment resulted in dose dependant chromosomal aberrations at all concentrations whereas it did not cause Sister Chromatid Exchange[21]. However the data is also available which contradicts the result of this research work such as Ames assay was conducted on aspartame, acesulfame-K and sucralose with and without metabolic activation system and the results revealed that these sweeteners were non mutagenic[22]. The reason for the mutagenic response of aspartame may be credited to the fact that aspartame is mainly composed of 3 components 50% aspartic acid, 40% phenylalanine and 10% methanol. The last component, methanol, is the most dangerous as it is converted to formaldehyde and formic acid[23]. Formaldehyde is a known carcinogen causing severe damage to DNA affecting the process of DNA replication. If the linkages between formaldehyde and DNA become permanent, it may hinder DNA replication resulting in gene mutations[24].

When the combination doses were subjected to Mutagenicity Assay, the results revealed significantly high mutagenic index as compared to metformin and aspartame alone. The order of mutagenicity was same in all 3 assays as large number of revertants were found in case of TA 100 as compared to TA 98. Moreover the mutagenic potential was high in the presence of metabolic activation system S9 mix during Pre incubation Assay as compared to Standard Pour Plate Incorporation Assay.

When combination doses of Aspartame and Metformin were administered, mutagenicity was revealed at 50:80 and 100:100 µg/plate against both mutant strains of Salmonella typhimurium. Relatively large number of revertant colonies was found when assay was performed in presence of S9 mix. The dose ratio of 25:20 µg/plate was only found mutagenic in Pre incubation assay against TA 100 as well TA 98. The results of this study reveal that the threshold level of mutagenicity caused by metformin, aspartame and their combination was same. But the intensity of mutagenic index with combination doses was relatively high as compared to the individual drugs. The reason for this significantly high mutagenicity as compared to aspartame and metformin might lay in the fact that metformin is responsible for increased production eNOS and NO in endothelial cells[25]. On the other hand it was reported that when aspartame was nitrosated for 30 minutes, tremendous rise in mutagenicity in both TA 100 and TA 98 strains of Salmonella typhimurium was observed. So care must be taken by taking into account the risk posed by endogenous nitrosation of foods in human stomach[26].

CONCLUSION

It can be concluded from the present study that metformin is causing more mutagenicity to both strains of Salmonella Typhimurium as compared to aspartame. When combination of aspartame and metformin were exposed to check their mutagenic potential, the results showed significantly high ($p < 0.05$) mutagenicity 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 care must be taken while using both these products together as it may cause significant damage to the cells of body.

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