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Genoprotective Effect of Indian Gentian in Type 2 Diabetes Mellitus (T2DM): Comet Assay, Sister Chromatid Exchange and Protein Oxidation Studies

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ABSTRACT The present study was undertaken to study the effect of Indian Gentian (*Enicostemma littorale Blume*), a herb, as a genoprotective agent in type 2 diabetes mellitus (T2DM) patients. For this, a total of 52 T2DM patients were investigated, of which 38 received 500 mg - 1 g Indian Gentian thrice daily escalated over three months (Group 1). The remaining 14 patients were not given the herb (Group 2). Fifteen age and sex matched non diabetic healthy volunteers served as controls (Group 3). All three groups were studied for DNA damage by comet assay and Sister Chromatid Exchanges (SCEs); Group 1 was also investigated for protein oxidation. Paired and unpaired *t* tests were performed at 95% confidence interval. Results of comet assay and SCE studies revealed that in Group 1, post Indian Gentian treatment, normal cell population increased, whereas moderately damaged, highly damaged and apoptotic cell population and SCE decreased as compared to Group 1 (pre-treatment patients) and Group 2 (without treatment patients). In comet assay, statistically significant difference between Group 1 (post-treatment patients) and Group 3 (controls) suggested that the herb was able to decrease the DNA damage but not as low as non-diabetic healthy controls. On the other hand, SCE analysis showed that the herb can reduce such exchanges to as low as the controls. In protein oxidation assay, no significant difference was found between the pre- and post-treatment T2DM patients of Group 1. The present study therefore indicated that overall Indian Gentian may have a significant effect on reducing DNA damage and attenuating SCEs in T2DM patients.

INTRODUCTION

Diabetes mellitus, a chronic metabolic disorder, is known to have several micro- and macrovascular complications that contribute to an increase in the morbidity and mortality (Giugliano et al. 1996). Increased generation of Reactive Oxygen Species (ROS) due to hyperglycemia causes oxidative stress. This results in endothelial damage that leads to vascular complications (Giugliano et al. 1996; Son 2007). The ROS induced Advanced Glycation End products (AGEs) damage several macromolecules, including lipids, proteins, and nucleic acids (Son 2007). In addition, the release of pro-inflammatory cytokines by ROS leads to chronic inflammation. The latter mechanism is emerging as an important causative consequence of oxidative stress leading to DNA damage that predisposes to age related diseases, including diabetes, atherosclerosis, osteoporosis and cancer (Khansari et al. 2009; Hamada et al. 2009). The damage to DNA in the peripheral blood lymphocytes can be revealed by the comet assay (single cell gel electrophoresis) (Sheth et al. 2006). Beside double-strand and single-strand breaks, this technique measures DNA damage in somatic cells after a variety of genotoxic insults, including *in vivo* and *in vitro* radiation.

The Sister Chromatid Exchange (SCE) refers to the exchange of certain homologous stretches of DNA sequence between two pairing chromatids and higher frequency of such exchanges is associated with certain pathological conditions. A study conducted by Sheth et al. (2006) revealed an increased frequency of SCEs in diabetes as compared to the healthy controls. Carbonyl groups result from protein oxidation and their level in tissues and plasma is indicative of AGEs due to oxidative damage. The quan-

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tification of these proteins in the peripheral blood is widely used to measure the extent of AGEs (Trombetta et al. 2006).

Enicostemma littorale Blume (Family: Gentianaceae) is an herbal medicinal plant, commonly known as Indian Gentian and is widely used in West India for the treatment of diabetes. The herb has been reported to have blood glucose lowering potential in alloxan induced diabetic rats and humans (Maroo et al. 2002; Upadhyay and Goyal 2004). Treatment with Indian Gentian has been shown to decrease the elevated cholesterol, triglyceride and creatinine levels in non insulin dependent diabetes mellitus (NIDDM) rats (Murali et al. 2003). In the present study, genoprotective effect of Indian Gentian in T2DM patients has been studied by comet assay and SCEs tests, in addition to assessment of protein oxidation in the treated patients.

MATERIAL AND METHODS

Selection of Study Subjects

An independent Ethics committee approved the study of Indian Gentian trial in diabetes mellitus prior to the patient recruitment. After written informed consent, recruitment of the subjects was carried out by organizing camps in Gujarat state and patients were selected as per the inclusion and exclusion criteria listed in Table 1.

Table 1: Inclusion and exclusion criteria for Groups 1 and 2 T2DM patients

Inclusion Criteria	
Age	25 to 65 yr
Fasting Blood Sugar (FBS)	> 126 mg %
Post Prandial Blood Sugar (PPBS)	> 162 mg %
Glycosylated Hemoglobin (HbA1c)	7 to 9.5 %
Body Mass Index (BMI)	19 to 35 kg/m^2
Exclusion Criteria	
Type 1 diabetic patient	
Pregnant woman	
Lactating mother	
Patient with recent stroke or unstable angina or coronary artery disease in previous 6 months	
Presence of ketone bodies in patient's urine	
Patient receiving any type of thiazolinedione group drug(s)	
Patient suffering from major systemic illness(es)	

The criteria of selection of patients with T2DM were that the upper limit of fasting plasma glucose was 234 mg % and post prandial it was 360 mg % to avoid the hyperosmolar problem or associated complications. It was also felt that higher exclusion levels would not be advisable for early study of a standardized natural product such as Indian Gentian. The highest dose of oral hypoglycemic agents (OHAs) taken by the patient was 20 mg sulphonyl urea and 1.5 to 2 g of metformin.

A total of 52 clinically diagnosed T2DM patients were selected for the study. Thirty eight of these patients were considered for Indian Gentian treatment (Group 1) and the remaining 14 patients (Group 2) were not given any such treatment. Fifteen, age, sex and Body Mass Index (BMI) matched non diabetic volunteers were selected as healthy controls (Group 3).

Herbal drug Indian Gentian was selected as an insulin sensitizer for T2DM patients, under a CSIR funded research project. The herb is commercially available and is manufactured by Shree Dootpapeshwar Ltd (Panvel), Mumbai. All the three groups were studied for genotoxicity by comet assay and SCEs, and Group 1 also for protein oxidation by protein carbonyl estimation. In Group 1, post-treatment follow up was done after 12 weeks. A battery of biochemical parameters including plasma glucose, lipid profile, serum insulin and glycosylated haemoglobin (HbA1c) were measured in both pre- and post-treatment patients in Group 1.

Comet Assay

The comet assay was performed under alkaline conditions following the protocols of Klaude et al. (1996) and Tice et al. (2000) with minor modifications. A freshly prepared cell suspension from the buffy coat of the centrifuged blood sample was mixed in 0.5% low melting agarose and casted on microscope slide pre-coated with 1% normal melting agarose. The cells were then lysed for 1 hour at 4ºC in a buffer composed of 1.25 M NaCl, 100 mM Tris, 50 mM EDTA, 1% Triton X-100 and 10% Dimethyl sulfoxide (DMSO) pH 10. After lysis, DNA was allowed to unwind for 20 minutes in electrophoresis buffer consisting of 10 N NaOH, 200 mM EDTA and 10% DMSO pH >13. Electrophoresis was carried out at 0.7 to 1.0 V/cm for 20 minutes in a refrigerator in dark. The slides were neutralized with 0.4 M Tris pH 7.0 and stained with 10 µg/ml ethidium bromide and covered with cover slips and scanned under a fluorescence micro-

scope (Olympus BX-51) attached with a CCD camera. Using appropriate filters 10 images were captured per slide and comet tail DNA was measured using the Adobe Photoshop. According to tail length, the cells were differentiated as normal or as mildly, moderately and highly damaged, and apoptic cells.

Sister Chromatid Exchanges (SCEs)

Peripheral blood was subjected to the standard cytogenetic culture technique. Differential chromatid staining method of Schneider et al. (1978) was employed and 10 µl / ml Bromodeoxy-uridine (BrdU) was added after 24 hours of the initiation of the culture and harvested after 96 hours. The slides were prepared and mounted with Bisbenzimide solution (150 µg / ml) (Hoechst), followed by exposure to sunlight for 7-8 hours. These were dipped in 2 X saline sodium citrate solution for 30 minutes at 50ºC in a water bath, followed by staining with Giemsa. SCEs were observed under a microscope and a minimum of 25 metaphases were scored in second cell cycle. Results were recorded as SCEs per metaphase and SCEs per chromosome.

Protein Carbonyl Estimation

Protein carbonyl estimation was carried out as per the method of Reznick and Packer (1994). The assay involves derivitization of the carbonyl group with dinitrophenylhydrazine (DNPH), followed by an anti-DNP antibody detection. DNPH reacts with protein carbonyls and the amount of protein-hydrazone produced was measured spectrophotometrically at 375 nm using Shimandzu UV-1700.

Statistical Analysis

Data on Group 1 pre- and post- treatment T2DM patients using Indian Gentian for the comet assay, SCEs, protein carbonyl, plasma glucose, lipid profile, serum insulin and glycosylated haemoglobin (HbA1c) were analysed using paired *t* test at 95% confidence interval. Such data on Group 1 post-treatment T2DM patients were compared with Groups 2 and 3 using unpaired *t* test at 95% confidence interval. All *t* tests were performed using GraphPad QuickCalcs Web sites http://www.graphpad.com/quickcalcs/ttest1.cfm?Format=C and http://www.graphpad.com/quickcalcs/ttest1.cfm?Format=SD (accessed June 2011).

RESULTS

Paired *t* test results (Table 2) of Group 1 pre- and post- treatment T2DM patients with Indian Gentian using comet assay, SCE and protein carbonyl tests showed variable results. Statistically the herb was found to significantly increase normal cells ($p = 0.0020$), and decrease moderately damaged ($p = 0.0279$), highly damaged ($p = 0.0165$) and apoptic ($p = 0.0014$) (Table 2, Fig. 1) cells. It also decreased mildly damaged cells but the result was not appreciable ($p = 0.9309$). Significant decrease was observed in SCEs ($p = 0.0002$) (Table 2, Fig. 2), while no significant difference was observed in protein carbonyl levels ($p = 0.9038$) (Table 2). In addition, paired *t* test results showed that there is no significant effect of Indian Gentian in Group 1 post-treatment T2DM patients for fasting plasma glucose and lipid levels ($p > 0.05$). However, fasting and post prandial insulin ($p = 0.0308$ and 0.0187, respectively) and HbA1c levels ($p = 0.0070$) did decrease significantly after 12 weeks of the treatment (Table 2).

Table 3 shows comparison of the comet assay and SCE results of Group 1 post-treatment with Groups 2 and 3. The unpaired *t* test between Group 1 post-treatment and Group 2 showed highly significant difference in normal ($p < 0.0001$), moderately damaged ($p = 0.0141$), highly damaged ($p = 0.0001$) and apoptic ($p < 0.0001$) cells, and SCEs ($p < 0.0001$), while mildly damaged cells showed no such difference ($p = 0.6424$). Similarly, unpaired *t* test between Group 1 post-treatment and Group 3 showed highly significant difference in normal ($p < 0.0001$), mildly damaged ($p = 0.0001$), moderately damaged ($p = 0.0003$), highly damaged ($p < 0.0001$) and apoptic ($p < 0.0001$) cells, while SCEs showed no significant difference ($p = 0.6070$).

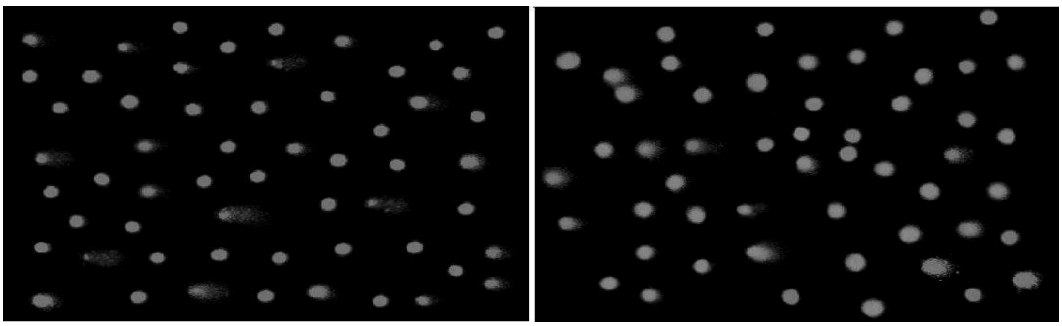
DISCUSSION

DNA damage and impaired DNA repair have been shown in T2DM by Blasiak et al. (2004). In an *in vitro* study, OHA gliclazide showed DNA repair (Sliwinska et al. 2008). The significant antiglycemic effect of Indian Gentian has been well documented in T2DM patients. The present

Table 2: Paired *t* test results for Group 1 pre- and post-treatment T2DM patients

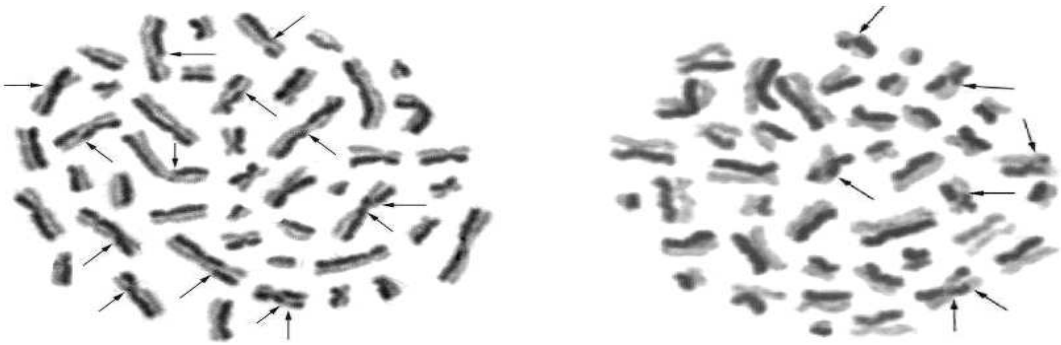
Test	*Parameter*	*Pre-treatment*		*Post-treatment*		*t*	*d.f.*	*p*
		n	*Mean ± S.D.*	*n*	*Mean ± S.D.*			
Comet assay	Normal cells (%)	38	57.18 ± 13.95	29	68.90 ± 8.36	3.3991	28	0.0020*
	Mildly damaged cells (%)	38	16.33 ± 7.83	29	13.57 ± 5.17	0.0875	28	0.9309
	Moderately damaged cells (%)	38	13.01 ± 6.16	29	9.01 ± 4.14	2.3198	28	0.0279*
	Highly damaged cells (%)	38	9.61 ± 4.70	29	6.28 ± 2.50	2.5519	28	0.0165*
	Apoptic cells (%)	38	3.85 ± 2.26	29	2.09 ± 1.51	3.5501	28	0.0014*
SCE	SCEs per metaphase	38	8.82 ± 1.24	29	7.32 ± 1.10	4.3075	28	0.0002*
Protein oxidation	Protein carbonyl (nmol/mg)	38	1.20 ± 0.56	27	1.25 ± 1.41	0.1221	26	0.9038
Plasma glucose (mg/dl)	Fasting Blood Sugar (FBS)	29	134.03 ± 27.60	27	131.78 ± 35.06	0.3490	26	0.7299
	Post Prandial Blood Sugar (PPBS) (mg/dl)	29	208.24 ± 37.34	27	191.22 ± 41.29	1.6290	26	0.1154
Lipid profile	Cholesterol (mg/dl)	29	201.90 ± 23.46	27	198.93 ± 28.12	0.8715	26	0.3914
	Triglycerides (TG) (mg/dl)	29	145.10 ± 40.93	27	145.44 ± 54.83	0.1161	26	0.9085
	High Density Lipoprotein (HDL) (mg/dl)	29	43.97 ± 5.14	27	43.56 ± 4.93	0.8534	26	0.4013
	Low Density Lipoprotein (LDL) (mg/dl)	29	135.93 ± 24.55	27	127.89 ± 20.30	1.9064	26	0.0677
Serum insulin	Fasting Insulin (ìIU/ml)	29	17.63 ± 11.77	27	11.87 ± 8.46	2.2836	26	0.0308*
	Post Prandial Insulin (ìIU/ml)	29	61.90 ± 35.29	26	44.02 ± 31.95	2.5159	25	0.0187*
Glycosylated haemoglobin	Glycosylated Haemoglobin (HbA1c) (%)	29	7.68 ± 00.72	27	7.19 ± 0.70	2.9311	26	0.0070*

*Statistically significant ($p \leq 0.05$)



(a) (b)

Fig. 1. Comet assay results of a Group 1 subject: (a) pre-treatment showing large number of damaged cells (b) post-treatment showing reduced number of damaged cells



(a) (b)

Fig. 2. SCEs results of a Group 1 subject: (a) pre-treatment showing excessive chromatid exchanges (b) post-treatment showing less chromatid exchanges

Table 3: Unpaired *t* test results for Group 1 (post-treatment T2DM patients) with Group 2 (T2DM patients without Indian Gentian treatment) and Group 3 (healthy controls) using comet assay and SCE tests

Test	*Parameter*	*Group 1*		*Group 2*		*t*	*d.f.*	*p*
		n	*Mean ± S.D.*	*n*	*Mean ± S.D.*			
Comet assay	Normal cells (%)	29	68.90 ± 8.35	14	48.61 ± 21.89	4.4117	41	< 0.0001*
	Mildly damaged cells (%)	29	13.57 ± 5.16	14	14.38 ± 5.58	0.4678	41	0.6424
	Moderately damaged cells (%)	29	9.01 ± 4.14	14	13.31 ± 6.83	2.5653	41	0.0141*
	Highly damaged cells (%)	29	6.28 ± 2.50	14	13.33 ± 8.34	4.2224	41	0.0001*
	Apoptic cells (%)	29	2.09 ± 1.51	14	10.85 ± 10.23	4.5673	41	< 0.0001*
SCEs	SCEs per metaphase	29	7.32 ± 1.10	4	13.00 ± 5.05	5.6436	31	< 0.0001*
Test	*Parameter*	*Group 1*		*Group 3*		*t*	*d.f.*	*p*
		n	*Mean ± S.D.*	*n*	*Mean ± S.D.*			
Comet assay	Normal cells (%)	29	68.90 ± 8.36	15	87.79 ± 10.60	6.4801	42	< 0.0001*
	Mildly damaged cells (%)	29	13.57 ± 5.16	15	6.39 ± 5.75	4.2092	42	0.0001*
	Moderately damaged cells (%)	29	9.01 ± 4.14	15	3.83 ± 4.28	3.8915	42	0.0003*
	Highly damaged cells (%)	29	6.28 ± 2.50	15	1.75 ± 2.84	5.4373	42	< 0.0001*
	Apoptic cells (%)	29	2.09 ± 1.51	15	0.22 ± 0.58	4.6099	42	< 0.0001*
SCEs	SCEs per metaphase	29	7.32 ± 1.10	8	7.10 ± 0.85	0.5191	35	0.6070

*Statistically significant ($p \leq 0.05$)

study showed decline in mean glycation of haemoglobin, which is likely to be due to glucose induced insulin release through K(+) - ATP channel dependant pathway as observed in diabetic rats (Maroo et al. 2002). However, the cellular effect of Indian Gentian was not known which has been demonstrated by comet assay, SCE and protein oxidation tests.

In the present study, in comet assay, a significant increase in normal cell population and decrease in damaged cell population was observed in T2DM patients after treatment with Indian Gentian as compared to that observed in pretreatment, and Group 2 patients receiving only OHAs. Among DNA repair pathways, nucleotide excision repair (NER) is able to recognize and process a wide variety of DNA lesions. Effect of Indian Gentian could be comprehended as an increased DNA repair due to significant role of the herb in NER activity. There was a significant difference between Group 1 (post-treatment) and Group 3 (controls), which suggested that the herb was able to decrease the DNA damage but not as low as in non diabetic healthy subjects.

Significant decrease in SCEs in T2DM patients receiving Indian Gentian treatment can be explained by two different mechanisms of SCEs, one operating at replicating points probably utilizing the machinery of DNA replication, and the other acting only in the post replication DNA stage. Cells can achieve error-free repair of repair DNA double-strand breaks (DSBs) by homologous recombination through gene conversion with or without crossover (Kato 1977). Eukaryotes have developed several mechanisms to repair DSBs, including non-homologous DNA end-joining (NHEJ) and homologous recombination (HR) (Paques and Haber 1999). It can be postulated that Indian Gentian may have efficiency to play significant role in DNA repair activity by inducing homologous recombination, with or without cross over, that reduces SCEs *per se*. No significant difference between Group 1 (post-treatment T2DM patients) and Group 3 (controls) was observed in this study suggesting that the herb reduced SCEs per metaphase as low as the latter group.

In conclusion, the present results indicated that Indian Gentian may have potential beneficial effect in correcting DNA damage produced by oxidative stress in T2DM patients. It would be desirable to confirm these findings in a larger sample elucidating the possible molecular/cellular mechanism(s) involved in genoprotective effect of the herb.

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