Study the Effect of Indian Gentian, Genoprotective Agent in Type 2 Diabetes Mellitus Patients

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Abstract: **The present study was undertaken to study the effect of Indian Gentian, 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.**

Keywords: **Indian Gentian, T2DM, Comet Assay, SCEs, Protein Oxidation, DNA etc.**

Introduction

The Diabetes mellitus, a chronic metabolic disorder, is known to have several micro- and macro- vascular complications that contribute to an increase in the morbidity and mortality (Giugliano et al. 2006). 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. 2006; Son 2007).

The ROS induced Advanced Glycation End products (AGEs) damage several macromolecules, including lip- ids, proteins, and nucleic acids. 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 doublestrand 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 DNA sequences between two paired chromatids, and the higher frequency of such exchange is associated with certain pathological conditions. A study by Sheth et al. (2006) showed an increase in the frequency of SCE in diabetes compared to healthy controls. Carbonyl groups are produced by oxidation of proteins, and their levels in tissues and plasma indicate AGEs due to oxidative damage. Quantification of these proteins in peripheral blood is widely used to measure the extent of AGEs (Trombetta et al., 2006).

Material and Methods

The 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 Haryana state and patients were selected as per the inclusion and exclusion criteria listed in Table 1.

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/m2

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)

Table 1: Showing Inclusion & exclusion criteria for Groups 1 & 2 T2DM Patients

The criteria for selecting patients with T2DM were a fasting blood glucose upper limit of 234 mg% and a postprandial upper limit of 360 mg% to avoid hyperosmolar problems or related complications. Others believe that higher levels of exclusion are not advisable for early studies of standardized natural products such as Indian gentian. The highest dose of oral hypoglycemic agents (OHAs) administered by the patient are 20 mg sulfonylurea and 1.5-2 g metformin.

A total of 52 clinically diagnosed T2DM patients were selected for the study. Of these, 38 patients were considered for Gentian treatment in India (Group 1) and the remaining 14 patients (Group 2) did not receive any such treatment. Fifteen non-diabetic volunteers matched for age, gender and body mass index (BMI) were selected as healthy controls (Group 3). Under the CSIR-funded research project, the herbal Indian gentian was selected as an insulin sensitizer for patients with T2DM. The herb is commercially available from Shree Doot papeshwar Ltd (Panvel) in Mumbai.

All three groups were tested for genotoxicity by comet assay and SCE, and group 1 was also studied for protein oxidation by protein carbonyl estimation. In group 1, follow-up after treatment was performed after 12 weeks. A set of biochemical parameters including plasma glucose, blood lipids, serum insulin, and glycosylated hemoglobin (HbA1c) were measured in pre-treatment and post-treatment patients in Group 1.

Comet Assay

The comet assay was performed under alkaline conditions following the protocols with minor modifications. A freshly prepared cell suspension from the buffycoat 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 4°C 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.

The Electrophoresis was carried out in the dark at a temperature of 0.7 to 1.0 V / cm for 20 minutes in the refrigerator. The slides were neutralized with 0.4 M Tris pH 7.0 and stained with 10 ml of ethidium bromide and covered with a coverslip and scanned under a fluorescence microscope (Olympus BX-51) with a CCD camera. Ten images were captured per slide using an appropriate filter and the comet tail DNA was measured using Adobe Photoshop. According to the tail length, the cells differentiate into normal or mild, moderate and highly damaged, as well as apoptotic cells.

Sister Chromatid Exchanges (SCEs)

Standard cytogenetic culture techniques are applied to peripheral blood. Differential chromatid staining method 10 µl/ml bromodeoxyuridine (BrdU) was added 24 hours after the start of the culture, and harvested 96 hours later. Slides were prepared and fixed with bisbenzoquinone solution (150 µg/ml) (Hoechst) and then exposed to sunlight for 7-8 hours. They were immersed in a 2 x sodium citrate solution in a water bath at 50 ° C for 30 minutes and then stained with Giemsa. SCE was observed under a microscope and at least 25 metaphase cells were scored in the second cell cycle. Record the results of each mid-term SCE and SCE for each chromosome.

Protein Carbonyl Estimation

Protein carbonyl estimation was carried out as per the method of Reznick and Packer (2004). 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

The 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 glycosyla- ted 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 www.graphpad.com/quickcalcs/ttest1.cfm?Format=SD..

Results

The 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, Figure 1) cells. It also decreased mildly damaged cells but the result was not appreciable ($p = 0.9309$).

The 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 as-say 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

The 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.

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	Fasting Blood Sugar (FBS)	29	134.03 ± 27.60	27	131.78 ±	35.06	0.3490	26	0.7299
(mg/dl)									
	Post Prandial Blood Sugar	29	208.24 ± 37.34	27	191.22 ±	41.29	1.6290	26	0.1154
	(PPBS) (mg/dl)								
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	29	43.97 ± 5.14	27	43.56 ±	4.93	0.8534	26	0.4013
	(HDL) (mg/dl)								
	Low Density Lipoprotein (LDL) 29		135.93 ± 24.55	27	127.89 ±	20.30	1.9064	26	0.0677
	(mg/dl)								
Serum insulin	Fasting Insulin (iIU/ml)	29	17.63 ± 11.77	27	11.87 ±	8.46	2.2836	26	0.0308*
	Post Prandial Insulin (iIU/ml)	29	61.90 ± 35.29	26	44.02 ±	31.95	2.5159	25	0.0187*
Glycosylated hemoglobin	Glycosylated Haemoglobin (HbA1c) (%)	29	7.68 ± 00.72	27	7.19 ±	0.70	2.9311	26	0.0070*

***Statistically significant ($p < 0.05$)**

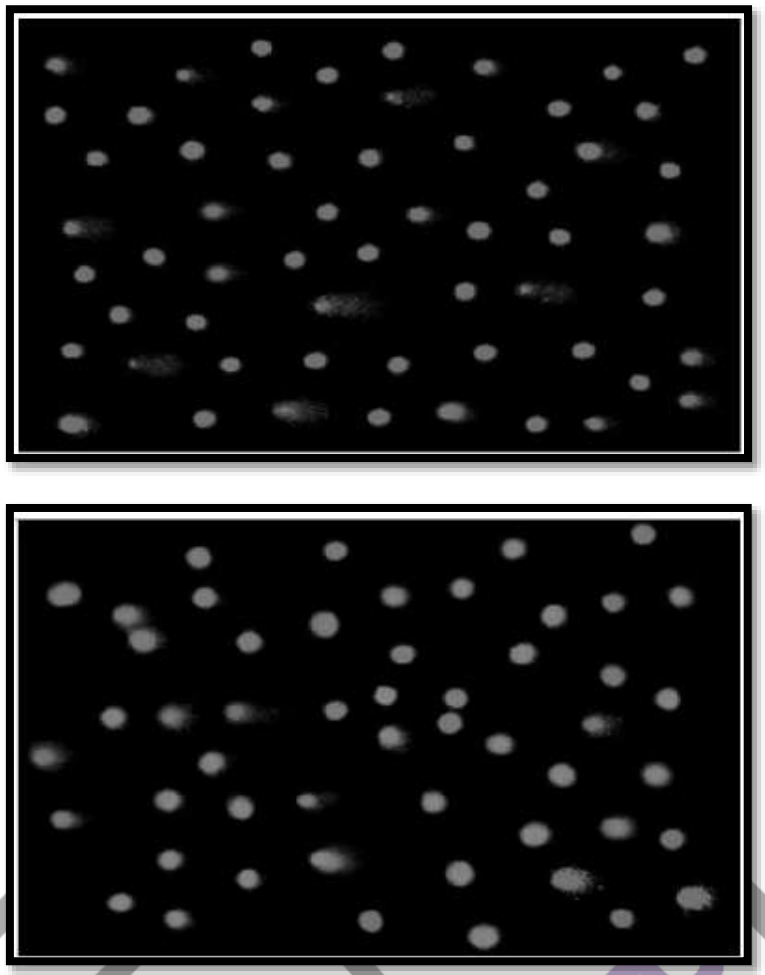
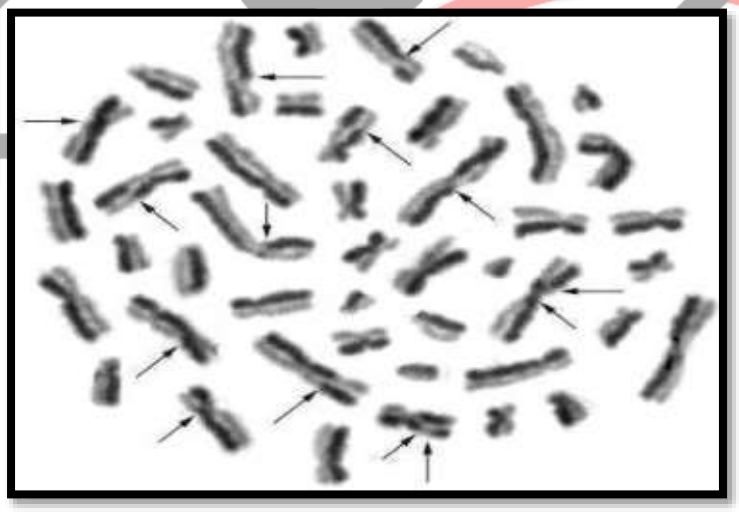


Figure 1: Showing Comet assay results of a Group 1 (a) pre-treatment showing large number of damaged cells (b) post-treatment showing reduced number of damaged cells



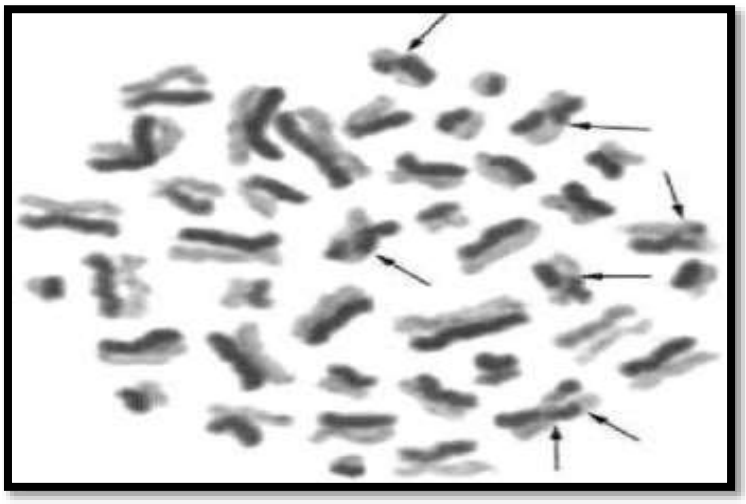


Figure 2: Showing SCEs results of a Group 1 (a) pre-treatment showing excessive chromatid exchanges (b) post treatment showing less chromatid exchanges

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	df	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 ($p < 0.05$)

Table 3: Showing Unpaired *t* test results for Group 1 (post-treatment T2DM patients) with Group 2 (T2DM) and Group 3 (healthy controls) using comet assay and SCE tests

The 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 .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, a significant increase in normal cell population was observed in T2DM patients treated with Gentian in India compared to T2DM patients used for pre-treatment observations and Group 2 patients receiving OHAs only in the comet assay and a reduction in damaged cell populations. In the DNA repair pathway, nuclear resection repair (NER) can identify and treat a variety of DNA damage. Due to the important role of herbs in NER activity, the effects of Indian gentian can be understood as increased DNA repair. There was a significant difference between Group 1 (post-treatment) and Group 3 (control), indicating that the herb can reduce DNA damage but not lower than non-diabetic healthy subjects.

The Significant reduction in SCE in patients with type 2 diabetes treated with Indian gentian can be explained by two different SCE mechanisms, one at the point of replication, possibly using a DNA replication mechanism, and the other only at the DNA stage after replication. effect. Cells can be genetically modified by homologous recombination to achieve error-free repair and repair of DNA double-strand breaks (DSB), with or without exchange.

Eukaryotes have developed several mechanisms for repairing DSB, including non-homologous DNA end joining (NHEJ) and homologous recombination (HR). It can be speculated that Indian gentian may play an important role in DNA repair activity by inducing homologous recombination. With or without crossover, this reduces the SCE itself. No significant differences between Group 1 (post-treatment T2DM patients) and Group 3 (control) were observed in this study, indicating that the drug reduced each intermediate SCE to as low as the latter.

Conclusion

The current research results indicate that Indian gentian may have a potential beneficial effect on correcting DNA damage caused by oxidative stress in T2DM patients. It is desirable to confirm these findings in larger samples, illustrating possible molecular/cellular mechanisms involved in the genetic protection of herbs.

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