Research Article

Black Tea Consumption Enhance Antioxidant Status, Reduce Inflammatory Stress vis-a-vis Insulin resistance: Hint from a Small Clinical Cohort Study on Pre-diabetic Subjects

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ABSTRACT

Progression of type-2 diabetes may include various inflammatory mechanisms which can induce insulin resistance and beta cells dysfunction. In diabetic pathophysiology, the pancreatic beta cells can no longer produce enough insulin to overcome insulin resistance, causing blood glucose level rise above the normal range. Epidemiological evidences suggest that daily consumption of black tea may be associated with the reduced pro-inflammatory stress by altering the level of specific cytokines. In this context, our present study aims at evaluation of the effects of black tea consumption on surrogate markers of insulin resistance. This is a prospective cohort study including 14 pre-diabetic subjects, selected from OPD of KPCMCH on the basis of waist circumference: male>90cm, female>80cm, BP>130/85 mm Hg, IFG (impaired fasting glucose) 100 mg/dl-125 mg/dl, HB1AC (5.7%-6.4%). 9 pre-diabetic subjects out of 14, consumed 5 cups of black tea (2gm tea brewed in 100 ml of hot water) for 4 weeks in a randomized manner after 7 days deprivation of tea consumption. Results showed that the black tea consumption down regulated the serum lipid peroxides level, pro-inflammatory cytokines (TNF-α, IL-1β) level and up regulated the anti-inflammatory cytokine (IL-10) significantly. Though black tea consumption for one month had no significant change in fasting glucose level, but the value of the two indexes, HOMA-IR and QUICKI changed significantly ($p<0.5$). Therefore, black tea may play an important role in reducing surrogate markers of insulin resistance and for long period of tea consumption may modulate the fasting glucose / insulin level to slower the progression of diabetes.

Keywords: Black Tea; HOMA-IR; IL1-β; IL-10; Prediabetes; QUCKI; TNFα

INTRODUCTION

Diabetes is a chronic disorder of carbohydrate, fat and protein metabolism characterized by increased fasting and post prandial blood sugar levels. The global prevalence of diabetes is estimated to increase, from 4% in 1995 to 5.4% by the year 2025. World Health Organization has predicted that the major burden will occur in developing countries.[1]

In the last few years, numerous studies have shown that low-grade inflammation is associated with the risk of developing type-2 diabetes.[2,3] Furthermore, nowadays it is accepted that chronic subclinical inflammation is a part of the insulin resistance syndrome.[4,5] and is strongly related to features of the metabolic syndrome. In diabetic pathophysiology, the pancreatic beta cells can no longer produce enough insulin to overcome insulin resistance, causing blood glucose level rise above the normal range.[6,7] It is observed that approximately 5-10% of the pre-diabetic subjects become diabetic every year.[8] Prolonged low-grade inflammation during pre-diabetic state facilitate onset of diabetes.[2,9] Epidemiological evidences also support the facts that inflammatory markers predict the development of diabetes and glucose disorders.[10] Two major pro-inflammatory cytokines TNF-α and IL-1β have been implicated for obesity associated insulin resistance and pathogenesis of Type 2 Diabetes[11-13] where as plasma IL-10 concentration is directly proportional to insulin sensitivity.[14]

Use of medicinal plants in disease remediation is an ancient practice. However, herbal medicine is gaining popularity both in developing and developed countries in recent era because of their natural origin and less side effects.[15,16] There are a lot of medicinal plants with antidiabetic and related beneficial effects such as, Babhul (*Acacia arabica*), Bengal Quince, GBel or Bilva (*Aegle marmelos*), Onion (*Allium cepa*), Garlic (*Allium sativum*), Neem (*Azadirachta indica*), Indian gooseberry, jamun (*Eugenia jambolana*), Bhuiawala (*Phyllanthus amarus*), Tea (*Camellia sinensis*)[17] etc., have been identified. Among them Tea (Camellia sinensis) is one of the world's most popular beverages, especially in Asian countries including Korea, China, Japan and India. Because of the high rate of tea consumption in these populations, even small effects on an individual basis could have a large public health impact.[18,19] There are some evidences suggest that tea is a hypoglycaemic agent.[20,21] Tea has immunomodulatory effect on animal model and human PMBM cell.[22] L-theanine, an unique amino acid of tea was found to modulate Th1/Th2 cytokine level and reduce pro-inflammatory stress in gastric ulcer disease.[23]

Therefore, in this backdrop it is important to know whether black tea consumption has any beneficial effect

on inflammatory stress mediated insulin resistance. Hence, this population based cohort study will focus on the effect of black tea on pre-diabetic subjects.

MATERIALS AND METHODS

Chemicals: L-Theanine spectrofluorometric assay kit, Total Antioxidant Status-Assay kit, from Cayman Chemicals, Ann Arbor. TNF-alpha; IL-1β; and IL-10 ELISA assay kit from Ray Biotech and Thermo Scientific (USA). Other chemicals used were: ethanol and methanol; 35% hydrogen peroxide (H_2O_2) (E. Merck, Mumbai, India); disodium hydrogen phosphate and sodium dihydrogen phosphate (BDH, Poole Dorset, UK); All other chemicals (analytical grade) were procured from reputed manufactures.

Preparation of the tea infusion

5 black tea samples were purchased from the local market of Kolkata and biochemical analysis were carried out before prescribed to pre-diabetic subjects. Leaf teas (2 g) were stirred with hot water (100 ml) at 90°C for 5 min. After filtration, the tea solution was allowed to cool to room temperature. The extraction method was performed in duplicate.[23]

Determination of polyphenolics content

Total water soluble polyphenolics content per cup was determined according to the standard procedure.[24] The method was downscaled to 1 ml final volume was followed to determine the amounts of total phenolics in the test samples. The test samples (each 100 µl) were mixed with 500 µl of 1:10 Folin-Ciocalteau's reagent followed by addition of Na_2CO_3 (400 µl, 7.5%). After incubating the reaction mixture at 24 °C for 2h, the absorbance at 765 nm was recorded. Gallic acid monohydrate was used as the standard. The total phenolic contents of the test samples are expressed as milligram gallic acid equivalents (mg GAE) /cup black tea decoction.

Determination of total water soluble flavonoids contents: Total water soluble flavonoids content per cup black tea decoction was determined according to Jia et al.[25] but the known method downscaled to 1 ml. In brief, the sample (100 µl) was added to 0.4 ml distilled water followed by $NaNO_2$ (0.03 ml, 5%). After 5 min at 25 °C, $AlCl_3.6H_2O$ (0.03 ml, 10%) was added followed by aqueous NaOH (0.2 ml, 1 M) after 6 min. The mixture was diluted with water to 1 ml and the absorbance at 510 nm was read. Quercetin was used as the standard and the total flavonoids content of the test samples was expressed as milligram Quercetin equivalents (mg QE) /cup black tea decoction.

Determination of Theaflavins content

Theaflavins content was determined using standard procedure.[26] In brief, 30 ml of the tea infusion was mixed with equal volume of ethyl acetate in a separating funnel and shaken for 5 min. 15 ml of the ethyl acetate layer was withdrawn and mixed with 2.5% (w/v) $NaHCO_3$ (15 ml) and shaken for 30s. The aqueous layer was discarded and part of the ethyl acetate layer (4 ml) was diluted to 25 ml with 95% (v/v) ethanol. The absorption of this ethanol solution was measured with a spectrophotometer at 380 nm and was recorded as A_T. 95% (v/v) of ethanol was used as a blank.

$$Calculation: TF\% = \frac{(2.25 \times A_T)}{(1-M)}$$

M is the moisture content of the tea sample that was determined using standard procedure. From the % value, TF content per cup black tea decoction (2g/100 ml) was determined.

Determination of L-Theanine contents

L-Theanine content in the test samples were estimated using commercially available spectrofluorometric assay kit. The 50 µl of each tea sample along with the standards were taken separately to 1.5 ml microfuge tube at an appropriate dilution. 50µl L-theanine initiator and 50µl L-theanine detector were added, and the mixture was incubated for 30 mins at 25°C. Analyzed at an excitation wavelength of 430-440nm and an emission wavelength of 485-495nm. From L-Theanine standard curve, L-Theanine content in cup black tea decoction was determined.[23]

Clinical Study

Inclusion criteria of patients

A total of 14 pre-diabetic subjects were selected from OPD of KPC medical college and Hospital on the basis of the following inclusion criteria; these are waist circumference: male>90cm, female>80cm, BP>130/85 mm Hg, IFG (impaired fasting glucose) 100 mg/dl-125 mg/dl, HB1AC (5.7%-6.4%). Blood was collected from selected patients and their routine blood tests were done on the pathology lab at KPC medical college and Hospital.

Study design

14 subjects were divided into two groups randomly; the case (9 subjects) and the control (5 subjects). After one week of depriving tea drinking to both the groups, cases were advised to drink 5 cups of tea (2 gm tea leaf brewed in 100 ml hot water/cup without milk and sugar) for 4 weeks. However, control subjects were advised to continue without tea for the same period. To both the groups the low or nil flavonoids diet was advised by the dietician.

Sample collection & processing: After 4 weeks, blood samples were collected from the control and case subjects, into vacutainer tubes. Once collected, blood samples were kept at 4 °C and prior to work centrifuged at 2000 rpm at 4°C but within 2 hrs of collection.

Estimation of Fasting and PP Blood Sugar

Fasting and PP blood sugar were done on the pathology lab at KPC medical college and Hospital using Kit based

method (GOD POD method). Fasting insulin was measured using Human Insulin ELISA assay kit.

Determination of Insulin resistance and Insulin sensitivity

Insulin resistance and Insulin sensitivity were determined by Homeostasis Model of Assessment (HOMA-IR) and Quantitative Insulin sensitivity check index (QUICKI) methods respectively.

HOMA-IR was calculated by standard formula (Fasting Insulin X Fasting Glucose)/405. (Fasting insulin expressed in µU/ml; fasting glucose expressed in mg/dL) High HOMA-IR scores symbolize lower insulin resistance. All statistical analysis and graph work were done on Microsoft Excel 2007 and Graph Pad software.

QUICKI was calculated by standard formula as stated below;

$$\text{QUICKI} = \frac{1}{\log \text{fasting insulinemia (µU/ml)} + \log \text{fasting glycemia (mg/dl)}}$$

Total antioxidant status (TAS) of serum

The total antioxidant status levels (TAS) was measured[27] using the Cayman chemical antioxidant assay kit (USA); the assay relies on the ability of the antioxidants in the sample to inhibit the oxidation of ABTS (2,2 1-azinodi- [3-ethylbenzthiazoline suiphonate]) to ABTS® by metmyoglobin. The amount of ABTS+ produced can be monitored by reading the absorbance at 750 nm, under the reaction condition used, the antioxidants in the sample cause suppression of the absorbance at 750 nm to a degree which was proportional to their concentration. The capacity of the antioxidants in the sample to prevent ABTS® oxidation was compared with that of Trolox, water soluble tocopherol analogue and was quantified as millimolar Trolox equivalents. The plate reader used was Bio-Tek instrument plate reader ELX800.20 µl sample and 1 ml of chromogen were required for the assay. TAS was obtained using the formulae:

$$TAS = factor \times (absorbance\ of\ blank - absorbance\ of\ sample)\ \frac{mmol}{l}$$

$$factor = \frac{concentration\ of\ standard}{(absorbance\ of\ blank\ absorbance\ of\ standard)}$$

Assay of Lipid peroxides

The extent of lipid peroxidation in tissue samples during in vitro incubation was measured by quantitating the amount of malondialdehyde (MDA) formed by 2-TBA reaction[28]; in brief 1ml serum sample was treated with 1ml of 24% TCA. It was mixed gently and incubated at room temperature and centrifuged. 2 ml of protein free supernatant and 0.671% freshly TBA was added. Samples were incubated for 1 h at 95 °C. The supernatant was then cooled at room temperature. Pink colour supernatant was extracted in 2 ml n-butanol, mixed by vortexing and reading was taken at 535nm by spectrophotometer. The amount of malondialdehyde produced was calculated using the molar extinction coefficient of MDA-TBA adduct as 1.56 $10^5 cm^2 mmol^{-1}$.

Serum Insulin and Cytokine Analysis

Serum insulin, pro / anti inflammatory cytokines (TNF-alpha, IL-1β and IL-10 respectively) by using ELISA assay kit (Ray Biotech and Thermo Scientific). The plate reader used was Bio-Tek instrument plate reader ELX800.

Statistical Analyses

Data were expressed as mean ± S.D. unless mentioned. Comparisons were made between case and control groups by paired t Test using the software GraphPad InStat (GraphPad Software Inc., San Diego, CA) (http://www.graphpad.com/quickcalcs/ttest1.cfm).

RESULTS AND DISCUSSION

Phytochemical Analysis of Black Tea

Nutritional support is an emerging advancement in the domain of diet-based therapies. Black tea and its constituents like polyphenols, flavonoids, theaflavins and L-theanine are the significant components of these strategies to maintain the health.[29,30] In the present study we have taken an initiative to check whether black tea supplementation can help pre-diabetic subject to control their insulin resistance and inflammatory stress. Phytochemical analysis of 5 black tea decoction were carried out before prescribed to pre-diabetic subjects under study. Total soluble polyphenolics, flavonoids and two main ingredients of black tea, viz: Theaflavins and L-theanine were analysed. However, among the five black tea samples, one of the black tea was prescribed to case subjects. Table 1 shows the important phytochemicals concentration of the prescribed black tea.

Table 1: Composition of per cup black tea (2g extracted 100 ml hot water at 90 °C for 5 mins):

Black Tea Biochemicals	Biochemical composition per cup of black tea decoction
Polyphenolics	198.6 ±10.2 mg GAE
Flavonoids	57.3 ± 4.8 mg QE
Theaflavins	2.86 ± 0.38 mg
L-Theanine	8.36 ± 0.76 mg

The **Table 1** highlights that from a cup of tea a person can intake a good proportion of all the important health promoting biochemicals such as, polyphenols, flavonoids, theaflavins and L-theanine.

Several research investigations, epidemiological studies, and meta-analyses suggest that tea and its bioactive polyphenolics constituents have numerous beneficial effects on health, including the prevention of many diseases such as cancer, diabetes, arthritis, cardiovascular disease, stroke, genital warts and obesity.[29] However, the results from epidemiological and clinical studies of the

relationship between tea and health are mixed.[3]

Clinical Study

Selection of Pre-Diabetic Subjects

A group of individuals whose glucose levels did not meet the criteria for diabetes, but were too high to be considered normal, were recognised as pre-diabetic. They generally diagnosed by impaired fasting glucose (IFG) (FPG levels 100–125 mg/dL, or impaired glucose tolerance (IGT) (2-h PG OGTT values of 140–199 mg/dL).

However, in our present course of study 14 pre-diabetic subjects were included based on the BMI, WHR, HB1AC% and IFG. The mean ± SD value for BMI, WHR (Hip-Waist ratio), HB1AC% and IFG were 26.74±2.63, 0.98±0.87, 6.2±8.02 and 119.35±13.36 mg/dL. These values have suggested that patients are in range of Pre-diabetic.[32] 14 pre-diabetic patients were divided in two groups at random. 5 patients were in control group (-tea) and rest of patients have on the test (+tea) group.

Biochemical Parameters before and after tea drinking

Emergence of Type2 Diabetes Mellitus from Pre Diabetes is the greatest proof of insulin resistance.[33] It consists of impaired fasting glucose and/or impaired glucose tolerance and is a significant risk factor for the development of type 2 diabetes, micro-vascular and macro-vascular disease.[34] Predicting development of type2 diabetes from pre-diabetes state remains a challenging task. Insulin resistance can be estimated in course of dynamic tests like clamp studies or by way of static mathematical expressions.[35] Current approaches for assessing insulin sensitivity and resistance in vivo relies on several studies and calculations viz. G/I ratio (glucose/insulin ratio); HOMA-IR (homeostasis model assessment of insulin resistance); QUICKI (quantitative insulin sensitivity check index); OGTT (oral glucose tolerance test); G_{mean} (mean plasma glucose concentration during OGTT); I_{mean} (mean insulin concentration during OGTT); G_o (plasma glucose concentration during fasting); G_{120} (plasma glucose concentration at 120 min); BW (body weight); I_o (plasma insulin concentration during fasting); and I_{120} (plasma insulin concentration at 120 min) etc.

In the present study, using fasting glucose and fasting insulin values we have calculated HOMA-IR and QUICKI for analyzing our data.

Table 2 have shown that black tea consumption for one month has no effect on fasting glucose or insulin level. However, the value of two indexes viz. HOMA-IR and QUICKI before and after tea consumption changed significantly ($p<0.05$). However, change in the parameters for control group is nonsignificant ($p>0.05$). Hence it can be concluded that black tea consumption for long period may modulate the FG and FI level to slower the progression of diabetics.

Table 2: Effect of black tea drinking on fasting glucose, fasting insulin and two indexes viz. HOMA-IR and QUICKI of case and control group were measured. P>0.05 was treated as nonsignificant.

Exp.Gr.	PARAMETERS	BEFORE (1 MONTH)	AFTER (1 MONTH)	Statistical significance
Control-tea	Fasting Glucose (mg/dL)	116.8±11.81	116.2±13.00	NS
	Fasting Insulin(mIU/ml)	7.582±1.523	7.648±1.637	NS
	HOMA-IR	2.184±0.52	2.192±0.60	NS
	QUICKI	0.341 ±0.014	0.340 ± 0.01	NS
Case+ tea	Fasting Glucose	120.78±14.62	117.11±12.9	NS
	Fasting Insulin	8.02±0.74	8.25±1.43	NS
	HOMA-IR	2.38±0.24	2.29±0.32	P<0.05
	QUICKI	0.33±0.0047	0.34 ±0.006	P<0.05

Table 3: Effect of black tea drinking on serum total antioxidant status (TAS), lipid peroxidation (LP) and inflammatory markers viz., TNF-α, IL1-β and IL-10

Exp.Gr.	Parameters	Before (1 month)	After (1 month)	Statistical significance
Control-tea	TAS (mmol/L)	0. 91±0.2	0.90±0.15	NS
	LP (nmol/ml)	1.92±0.052	2.01±0.29	NS
	TNF-α (pg/ml)	5.158±2.85	5.188±2.93	NS
	IL-1β (pg/ml)	3.508±1.20	3.504±1.207	NS
	IL-10 (pg/ml)	7.22±0.39	7.12±0.457	NS
Case + tea	TAS (mmol/L)	0.98±0.14	1.21±0.30	P<0.05
	LP (nmol/ml)	2.21±0.19	1.94±0.08	P<0.05
	TNF-α (pg/ml)	5.8±3.21	4.2±2.99	p<0.0001
	IL-1β (pg/ml)	3.09±0.42	1.99±0.33	p<0.0001
	IL-10 (pg/ml)	8.737±0.86	10.65±1.29	P<0.01

Effect of Black Tea consumption on Surrogate Markers of Insulin Resistance

Progression from pre-diabetic state to Type2 Diabetes Mellitus is a slow process and therefore we choose to evaluate the effect of tea on surrogate markers of insulin resistance such as serum total antioxidant status, lipid peroxidation, pro and anti inflammatory cytokine concentration. In this study we observed that tea plays a significant role in reducing surrogate markers of insulin resistance thereby potentially indicating the role of tea in preventing Type2 Diabetes Mellitus. However not all markers showed significant change which could be because of smaller number of subjects and short duration.

Table 3 have shown that black tea consumption for one month has significant effect on serum total antioxidant status level as well as surrogate markers of diabetes. Serum total antioxidant status ($p<0.05$) and anti-

inflammatory cytokine IL-10 level ($p<0.01$) up-regulated significantly whereas lipid peroxides level ($p<0.05$) as well as expression of inflammatory cytokines viz. TNF-α, IL-1β ($p<0.0001$) were down regulated significantly after 4 weeks consumption of black tea extract.

CONCLUSION

Black Tea may play a role in reducing surrogate markers of insulin resistance with particular reference to QUICKI and HOMA-IR independent of the values of glycemic status. It could perhaps achieve by the way of reducing inflammation in the body exemplified by significantly reduced the value of pro-inflammatory cytokines like TNF-∞, IL-1β and increased the value of anti-inflammatory cytokine IL-10.

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