Original Article

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The Effects of *Camellia sinensis* Extract on Proliferation, Apoptosis and Oxidative Stress in Insulinoma INS-1 Cells

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ABSTRACT

Objective: Green tea (*Camellia sinensis*) is one of the most consumed beverages in the world. *C. sinensis* consumption may prevent tumor and other diseases. Insulinomas are pancreatic islet cell tumors. In present study, the effects of the extract from *C. sinensis* were investigated in rat insulinoma INS-1 cells.

Methods: *C. sinensis* leaves were collected in Rize, Turkey. The leaves were brewed in tap water for 20 and 40 minutes at 80°C. INS-1 cells were treated with different doses (1-5 mg/mL) of *C. sinensis* extract for 24 h. Cell viability, proliferation, death were examined. The malondialdehyde, glutathione and protein carbonyl levels were measured in INS-1 cells given *C. sinensis* extract.

Results: The cell viability and the percentage of proliferating cell nuclear antigen immunopositive cells decreased, while the percentage of TUNEL positive cells increased in INS-1 cells treated with the extract from *C. sinensis* according to control group. While malondialdehyde and protein carbonyl levels in INS-1 cells treated with *C. sinensis* extract decreased, glutathione levels increased according to control group.

Conclusion: The results indicate that the extracts from *C. sinensis* inhibited proliferation and caused apoptosis in INS-1 cell. *C. sinensis* may be natural agent for supporting the treatment of pancreatic tumors.

Keywords: *Camellia sinensis* extract, proliferation, apoptosis, oxidative stress, INS-1 cells

Introduction

The use of primary beta cells in biochemistry and molecular investigations is limited due to difficulty in obtaining pancreatic endocrine tissue. For this reason, many investigators prefer to study insulin-releasing cell lines, which maintain normal insulin secretion. The most commonly used insulin-releasing cell lines are RIN, HIT, beta TC, MIN6 and INS-1 cells (1). MIN6 and INS-1 cells reflect the physiological situation of beta cells in the best way when stimulated by glucose (2). Although the behavior of these cell lines does not exactly mimic the physiology of the primary beta cells, it is highly valuable for studying the molecular events underlying beta cell function and dysfunction (1). INS-1 exhibits several important characteristics of pancreatic beta cells (2).

There are various types of herbal teas including black and green tea. Green tea (*Camellia sinensis*) is one of the most consumed beverages in the world. People living in different regions have a variety of ways to brew tea (3,4). The previous studies with different brands of tea demonstrated that brewing time is important to increase its antioxidant capacity, total phenolic and catechin content (5). Furthermore, the protective effects of *C. sinensis* were demonstrated in different types of cancers such as breast, ovarian, prostate and pancreatic cancers. The importance of anti-cancer activity of tea was highlighted (6-9)

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In the current study, the effects of the extract from *C. sinensis* on cytotoxicity, proliferation, apoptosis and oxidative stress status were investigated in rat insulinoma INS-1 cells.

Methods

Extraction of *C. sinensis*

C. sinensis leaves were collected in Rize, Turkey as first harvest in May the leaves were dried, and a classic brewing method was applied to the green tea leaves to prepare the extract. The water used as solvent. Two hundred fifty mL water was used for the brew of 5 g of *C. sinensis* leaves. The dried leaves were brewed in tap water for 20, 40 and 60 minutes at 80°C and extracts were obtained. And then, the extracts were cooled at room temperature, and the supernatants were removed after centrifugation at 4000 g for 15 minutes. So, the extract was obtained from *C. sinensis. In vitro* study was not approved.

Cell Culture

Insulinoma INS-1 cell line was a generous gift from Prof. Dr. Claes B. Wollheim (University Medical Center, Geneva). INS-1 cells were cultured in a humidified atmosphere containing 5% CO_2/95% humidified air at 37°C in Roswell Park Memorial Institute (RPMI) 1640 (Invitrogen) containing 10 mM HEPES (Sigma), 50 mM 2-mercaptoethanol (Biorad), 1 μM sodium pyruvate (Sigma), 5% fetal bovine serum (Gibco), 100 IU/mL penicillin and 100 μg/mL streptomycin (Gibco).

The INS-1 cells which were treated with brewed *C. sinensis* leaves in water for 20, 40 and 60 minutes were named as group I, II and III, respectively. Also, the untreated INS-1 cells were named as control group.

Cell Viability Assay

The effects on cell viability of *C. sinensis* extract determined by using 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide (MTT) (0.5 mg/mL) (Applichem). INS-1 cells were seeded, and different doses (1-5 mg/mL) of *C. sinensis* were applied to INS-1 cells for 24 hours. The final concentration of the *C. sinensis* extract was 1-5 mg/mL. After that, MTT was added and incubated for 2 hours. The medium was removed from cells and isopropanol was added to solve formazan (10). Absorbance values were measured at 540 nm. Cell viability % rate was calculated. IC50 value of *C. sinensis* was 4.535 mg/mL for group I, 4,121 mg/mL for group II, 4.041 mg/mL for group III.

Cell Proliferation Assay

Cell proliferation assay was performed by PCNA immune marking. On 24-well plates, $25x10^4$ cells/well were seeded and grown on coverslip. Then it was incubated in 5% CO_2/95% humidified air at 37°C until the cells proliferated. Three and 4 mg/mL final concentration of *C. sinensis* extract were applied to the cells for 24 hours. The cells were washed with phosphate buffered saline (PBS), and fixed with methanol for 5 minimum (min) at -20°C. The cells were incubated with blocking solution for 20 min at room temperature to avoid non-specific immunostaining. PCNA antibody (Neomarkers, USA; dilution 1:300, overnight at 4°C) was used for immunocytochemical labeling by streptavidin-biotin-peroxidase technique in INS-1 cells. After that, the cells were washed with PBS, and biotinylated secondary antibodies and streptavidin, biotinylated horseradish peroxidase (Invitrogen, USA) were applied to INS-1 cells. AEC (Invitrogen, USA) was used to reveal immunopositive cells. The cells were mounted in glycerin gelatin and preserved at 4°C until microscopic examination. The percentage of immunopositive cells were calculated according to following formulas: (The number of immunpositive cells /total cells) x 100.

Cell Death Assay

Cell death assay was determined with TUNEL method by using the *In situ* Cell Death Detection Kit (Millipore, USA). On 24-well plates, $25x10^4$ cells/wells were seeded and grown on coverslip. Then it was incubated in 5% CO_2/95% humidified air at 37°C until the cells proliferated. Three and 4 mg/mL final concentrations of *C. sinensis* extract were applied to the cells for 24 hours. Then the cells were treated with equilibration buffer for 2 min at room temperature. Terminal deoxynucleotidyl transferase (TdT) enzyme and reaction buffer were mixed. It was treated for 1 hour at 37°C. After that, the cells were incubated with the anti-digoxigenin conjugate for 30 min at room temperature. The diaminobenzidine was used to reveal immunopositive cells. The percentage of immunopositive cells was calculated according to the following formulas: (The number of apoptotic cells / total cells) x 100.

Glutathione, Protein Carbonyl and Malondialdehyde Assay

On 6-well plates, 10^6 cells/wells were seeded and incubated in 5% CO_2/95% humidified air at 37°C for determination of biochemical analysis. After incubation, 0.25% tripsin-EDTA was added the cells. It was removed and centrifuged at 1500 rpm for 5 minutes. Lysis buffer (Cell Signaling) was added on pellet. The cells were kept on ice for 5 minutes, and sonicated with ultrasonic water bath. They were centrifuged at 14000 g for 10 minutes. Supernatants were collected and the levels of malondialdehyde (MDA), glutathione (GSH), protein carbonyl (PCO), and protein content were measured by Ledwozyw's (11), Beutler's (12), Reznick and Packer's (13), and Lowry's methods (14), respectively.

Statistical Analysis

The image was captured using an Olympus BX - 50 microscope. Minimum 10 fields were selected randomly and immunopositive cells were counted in each field. Microscopic results were analyzed using One-Way variance (ANOVA), followed by Tukey's multiple comparisons test by using GraphPad Prism version 5.0 computer package for immunohistochemistry. Biochemical results carried out using SPSS software (SPSS, version 21.0). The statistical analysis was performed for statistical significance using ANOVA, followed by Tukey's post-hoc test. A $p<0.05$ was considered significant.

Results

The Cell Viability % of INS-1 Cells Decreased with *Camellia sinensis* Extract

We determined cell viability percentage of INS-1 cells treated with the extract obtained from *C. sinensis* by MTT assay. INS-1 cells were treated with 1-5 mg/mL extract for 3 groups. For group I, the cell viability % significantly decreased in INS-1 cells given *C. sinensis* extract at 3 mg/mL ($p<0.05$), 4 mg/mL ($p<0.001$) and 5 mg/mL ($p<0.0001$) concentrations compared with the control group. The cell viability % significantly decreased in the group II at 3 mg/mL ($p<0.001$), 4 mg/mL ($p<0.0001$) and 5 mg/mL ($p<0.0001$) concentrations of the *C. sinensis* extract compared with the control group. Similarly, for group III, the cell viability % significantly decreased in INS-1 cells given 2 mg/mL ($p<0.001$), 3 mg/mL ($p<0.0001$), 4 mg/mL ($p<0.0001$) and 5 mg/mL ($p<0.0001$) concentrations of *C. sinensis* extract compared with the control group (Figure 1). The results of group II and III were similar for 3 mg/mL, 4 mg/mL and 5 mg/mL doses of extract. Therefore, we studied group I and II brewing *C. sinensis* leaves in tap water for 20 and 40 minutes, respectively in later experiment.

INS-1 Cell Proliferation was Inhibited by Given *C. sinensis* Extract

PCNA antibody was examined as a proliferation marker. The percentage of PCNA immunopositive cells significantly decreased in group I at both 3 mg/mL and 4 mg/mL concentrations of the extract, as well as group II given 4 mg/mL extract from *C. sinensis* compared with the control group ($p<0.001$, $p<0.05$, and $p<0.001$, respectively). Besides, PCNA immunopositive cells significantly decreased in group II given 4 mg/mL extract from *C. sinensis* compared with the group II given 3 mg/mL extract ($p<0.05$) (Figure 2).

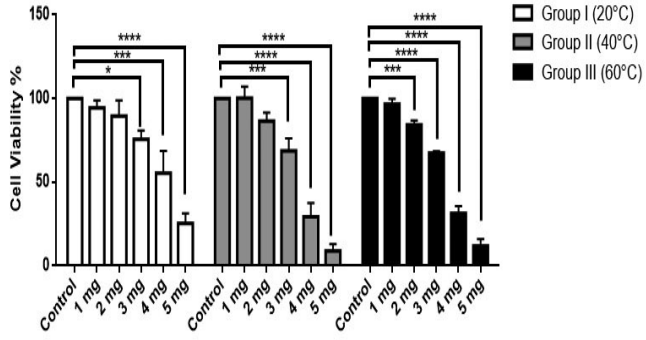

Figure 1. The cell viability % in INS-1 cells with *Camellia sinensis* extract. *p<0.05, ***p<0.001, ****p<0.0001

The Number of Apoptotic Cells Increased after Treating INS-1 Cells with *C. sinensis* Extract

TUNEL method was examined for the cell death detection. The percentage of TUNEL positive cells increased in group II given 4 mg/mL extract from *C. sinensis* compared with the control group ($p<0.001$). Besides, TUNEL positive cells increased in group II given 4 mg/mL extract from C. sinensis compared with group II given 3 mg/mL extract from C. sinensis ($p<0.01$) (Figure 3).

Glutathione, Protein Carbonyl and Malondialdehyde Levels Significantly Changed in INS-1 Cells Given *C. sinensis* Extract

GSH levels showed a significantly increase in group I given 3 and 4 mg/mL extract from *C. sinensis* compared with the control group ($p<0.001$ and $p<0.001$). GSH levels increased in group II given 3 mg/mL extract from *C. sinensis* compared with the control group ($p<0.001$), while GSH levels in group II given 4 mg/mL extract from *C. sinensis* were lower than both control group ($p<0.001$) and group I given 3 mg/mL extract from *C. sinensis* ($p<0.001$) (Table 1).

PCO levels significantly increased in group I given 3 mg/mL extract from *C. sinensis* ($p<0.001$), while PCO levels decreased in group I given 4 mg/mL extract from *C. sinensis* compared with the control group ($p<0.001$). Besides, PCO levels decreased in group II given both 3 and 4 mg/mL doses of *C. sinensis* extract compared with the control group ($p<0.001$ and $p<0.001$) (Table 1).

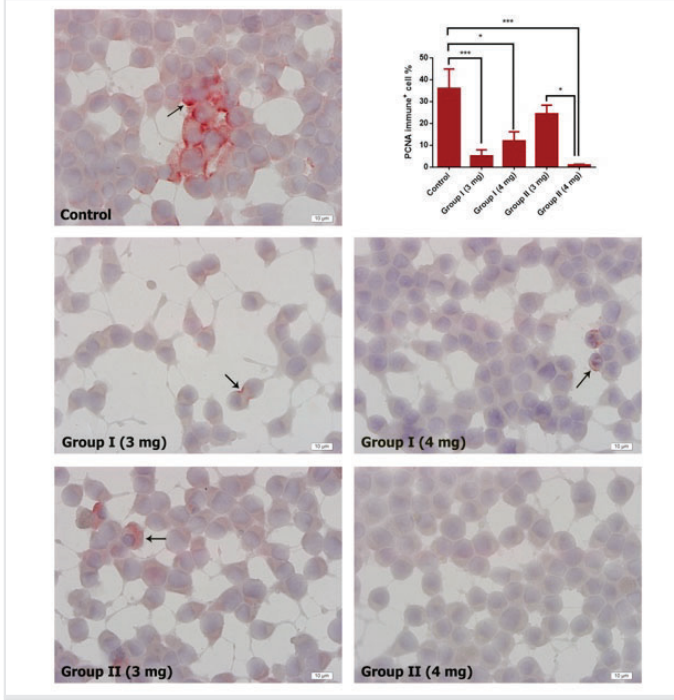

Figure 2. Proliferating cell nuclear antigen immunopositive cell number % in INS-1 cells with *Camellia sinensis* extract. Ò: Proliferating cell nuclear antigen immunopositive cells, *p<0.05, **p<0.001

Table 1. Glutathione, protein carbonyl, and malondialdehyde levels in INS-1 cells with *Camellia sinensis* extract

	GSH		PCO		MDA	
	3 mg/mL	4 mg/mL	3 mg/mL	4 mg/mL	3 mg/mL	4 mg/mL
Control	5.43±0.00	5.43±0.00	1.94±0.00	1.94±0.00	2.33±0.05	2.33±0.05
Group I	6.25±0.00[a]	5.95±0.05[a]	2.86±0.05[a]	1.79±0.00[a]	1.46±0.00[a]	1.01±0.00[a]
Group II	7.89±0.05[a,b]	4.69±0.00[a,b]	0.95±0.00[a,b]	1.72±0.00[a,b]	1.88±0.00[a,b]	1.98±0.00[a,b]
P_{ANOVA}	<0.001	<0.001	<0.001	<0.001	<0.001	< 0.001

* Means ± SEM, [a]p<0.001 versus control group, [b]p<0.001 versus group I, GSH: Glutathione, PCO: Protein carbonyl, MDA: Malondialdehyde

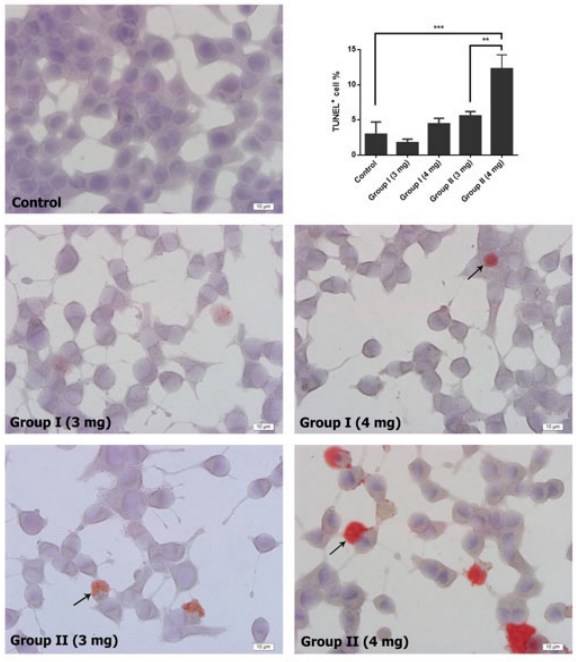

Figure 3. TUNEL positive cell number % in INS-1 cells with *Camellia sinensis* extract. Ò : TUNEL immunopositive cells, **p<0.01, ***p<0.001

MDA levels showed a significant decrease in both group I and II given 3 mg/mL and 4 mg/mL concentration of *C. sinensis* extract compared with the control group (p<0.001 and p<0.001). Furthermore, MDA levels were higher in group II given 4 mg/mL extract from *C. sinensis* compared with group II given 3 mg/mL extract (p<0.001) (Table 1).

Discussion

Green tea (*C. sinensis*) is widely use in the world. There are phytochemicals and functional components in this species of tea. These components have significant benefits on human health. Therefore, consumption of tea at effective levels is important (15). *C. sinensis* contains polyphenols which are related with antioxidant activity. Brewed green tea contains high levels of catechins (16). Catechin levels are important factors indicating the quality of the tea (17). Catechins and caffeine were shown in green tea in several studies (18-20). There are five catechins including catechin, (2)-epicatechin, (2)-epicatechin gallate, (2)-epigallocatechin and (2)-epigallocatechin gallate. Different types of tea may have different caffeine and catechin contents (21). Oxidative damage can be cured with phytochemicals found in tea in diseases such as cancer (15,22). Healing properties of *C. sinensis* have been investigated in many types of cancers such as metastatic breast (6), human ovarian (7), lung (23), and prostate cancers (8). The effects of *C. sinensis* were investigated in pancreatic carcinoma cells (9,24). However, there is no investigation of cell viability in insulinoma INS-1 cells. We applied extract of *C. sinensis* at different doses (1-5 mg/mL) and brewing times (20, 40 and 60 minutes) at 80°C to the cells to investigate killing effect of *C. sinensis* in insulinoma INS-1 cells. The brewing time is important to increase the antioxidant capacity. Therefore, we continued to study with groups I (20 minutes) and II (40 minutes), due to similar results in groups II and III (60 minutes). Furthermore, 3 mg/mL and 4 mg/mL concentration of the extract were considered as effective doses in these groups.

In a previous study, the authors showed that green tea inhibited proliferation and also induced apoptosis in 4TI metastatic breast cancer cells (6) and in A549 lung carcinoma cells (23). These effects have not been determined in insulinoma INS-1 cell line. Our results indicated that extract from *C. sinensis* inhibited proliferation, and caused apoptosis in insulinoma INS-1 cell. It was observed that the increase of brewing time changed cell proliferation and death. The increase in brewing time reduced the cell proliferation, and elevated apoptosis in INS-1 cells.

The authors have shown in a study that green tea can regulate the antioxidant capacity in people exposed to oxidative stress (25). Besides, *C. sinensis* decreased oxidative stress in patients with prostate cancer (26). In a study, it was suggested that green tea showed protective effects through its antioxidant properties against oxidative stress and DNA damages, (27). Furthermore,

antioxidant capacity in *C. sinensis* was increased depending on the duration of brewing. Our data showed a decrease in the levels of MDA and PCO that are known as markers of oxidative stress in insulinoma INS-1 cells treated with extract of *C. sinensis*. Besides, glutathione is an important antioxidant and protects cells against oxidative stress. According to our results, extract of *C. sinensis* increased the glutathione levels. We hypothesized that *C. sinensis* has a positive protective effect on insulinoma cells.

Our aim was to prevent the proliferation of tumor cells and to induce cell death. We observed both inhibition of cell proliferation, and increase in apoptotic cells. We have also shown that the oxidative stress may cause decreased MDA, PCO levels and increased GSH levels in insulinoma cells. So, we can say that *C. sinensis* may be natural agent to support the treatment of various carcinomas.

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Ethics Committee Approval: *In vitro* study was not approved.

Informed Consent: *In vitro* study was not approved.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Concept: A.K.K., M.E., I.S., Z.M.C., Design: M.E., Z.M.C., Data Collection or Processing: M.E., Z.M.C., Analysis or Interpretation: A.K.K., M.E., I.S., Z.M.C., Literature Search: A.K.K., I.S., Writing: A.K.K.

Conflict of Interest: No conflict of interest was declared by the authors.

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