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Green Tea Reduces Indomethacin-induced Damage in AGS Cells

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Abstract

Non-steroidal anti-inflammatory drugs (NSAIDs) disrupts mucosal defense through inhibition of cyclooxygenase (COX) and depletion of endogenous prostaglandins, thus exerts its adverse effects. Tea flavonoids; catechins and its derivatives, in particular, are known to prevent various systemic diseases like ulcer, cancer, atherosclerosis, hypertension, and diabetes that occur due to oxidative stress. Green tea is the most abundant source of catechins and its derivatives. In the present study, we aimed to determine the dose dependent toxicity of conventional NSAIDs, indomethacin on cultured AGS cells using MTT assay and morphological deformities by DAPI and acridine orange/ethidium bromide (AO/EtBr) fluorescence staining where aqueous extract of green tea (aGTE) provided significant protection. In the future, aGTE will be useful in designing and development of a novel protective supplement against NSAIDs that have reduced gastrointestinal toxicity while maintaining their essential therapeutic efficacy to inhibit pain and inflammation.

Keywords: AGS cells; Green tea extract; Indomethacin; MTT assay; ROS

Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are the important family of therapeutics and commonly prescribed medications globally which inhibit COX activity, and prostaglandin synthesis thus reduces pain and inflammation [1,2]. However, long-term use of NSAIDs exert deleterious effects to gastrointestinal system ranging from gastric and duodenal perforated ulcers, abdominal pain, bleeding apoptosis of mucosal cells, antioxidant level, ROS, mitochondrial oxidative stress and modulates autophagy [3]. It resulted in the reduction of mucus and/or bicarbonate release and decreased mucosal blood flow, which often hampers metabolic homeostasis and tissue integrity. NSAIDs specifically target the epithelial cells of the gastrointestinal tract. Researchers reported that NSAIDs cause lysosomal dysfunction in cultured cells. It is well established that excess generation of ROS due to the development of oxidative stress is the primary cause of NSAID-induced gastropathy[4,5]. NSAID-induced ulcers account for approximately 22% of gastric ulcers [6].

White tea, green tea, oolong tea, and black tea are produced from the leaves of Camellia sinensis, but for maintaining different oxidation state, their names are different. Their chemical components, percentage, and properties vary due to the time of year the tea leaves are harvested, and the fermentation methods used [7]. The efficacy of aGTE has been extensively studied over the past years for its various beneficial activities in systemic diseases. Tea is mainly composed of several catechin compounds, whereas green tea contain contains morecatechins than that of others [8]. Green tea is now widely consumed worldwide as part of a regular diet. Emerging literature suggests that green tea is associated with a vast array of biological activities, including antioxidation, anti-obesity, anti-inflammatory, antihyperlipidemic, anticancer, and antimicrobial effects [9]. However, its potential for limiting NSAID-induced gastric mucosal injury has not been experimentally elucidated.

Upon exposure to indomethacin, gastric cells show a series of changes leading to gastric injury. In this study, we used indomethacin as a representative NSAID for induction of cell damage, and aGTE as the potent rescuer in cultured AGS cells. Generation of reactive oxygen species (ROS) was investigated by fluorescence spectroscopy with and without treatment with aGTE.

Materials and Methods

Cell culture

AGS cell line (human gastric adenocarcinoma epithelial cell line) was obtained from NCCS, Pune. The cells were routinely cultured in RPMI medium supplemented with 10% FBS, 1% penicillin-streptomycin, and 0.2% sodium bicarbonate at 37 °C with 5% CO2 in a humidified atmosphere. The cells were cultured in 75 cm2 culture flask. When the culture flasks became nearly 70–80% confluent,

the cells were harvested by 0.25% trypsin-EDTA and reseeded into fresh flasks at one-third density for continued passage. Trypan blue dye exclusion principle was used for determination of Cell viability before performing each experiment. For treatment purposes, cells were incubated in serum-free culture medium [10].

Preparation of aGTE

Twinings tea leaves were obtained from the local market of Kolkata, India. Ten grams of tea leaves were added in 100 ml of boiling water, allowed to stand for 3-4 min and filtered by Whatmann filter paper.

Cell viability by MTT assay

Cytotoxicity of different concentrations of indomethacin at different time points was determined via colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay, which is based on the conversion of MTT to formazan crystals by mitochondrial dehydrogenases [11]. AGS cells (1 × 104 cells/well) were cultured in a 96 well plate at ambient temperature and conditions described before. On confluency, sets of wells were treated with different concentrations of indomethacin media for different time points. In other sets, aGTE was added 12 h prior addition of indomethacin media. After the treatment, MTT was added to each well, and the cells were incubated for 3-4 h in the dark. The media were replaced with 150 ml of DMSO of each well to dissolve the formazan crystals. The absorbance of the 96-well plate was analyzed at 490 nm on a microplate reader (Robonik, India). Percentage of cell viability was determined by the following formula:

% Cell Viability = (Mean absorbance in Test cells)/(Mean absorbance in Control Cells) × 100

mean DATA of three experiments (run in duplicate) is presented.

DAPI staining

The damaging effect of indomethacin and preventive effects of aGTE were assessed by analyzing the morphology of cells using DAPI staining [12]. Cells were grown to ~50% confluence on sterile glass coverslips, and they were incubated with 400 µM of indomethacin for 4 hrs, or with aGTE prior addition of indomethacin, cells were stained with DAPI (4',6-diamidino-2-phenylindole) for 5 minutes at room temperature; washed with PBS and observed under Andor confocal microscope at 60X magnification, for nuclear fragmentation, shrinkage or condensation. The extent of cellular damage (or protection) is reflected by nuclear state.

Ethidium Bromide (EtBr)-Acridine orange (AO) fluorescent staining

To confirm the result (rate of apoptosis) obtained from flow cytometric data, dual staining with EtBr-AO was carried out and visualized under a fluorescent microscope [13]. EtBr-AO staining combines the differential uptake of fluorescent DNA binding dyes AO and EtBr, and the morphologic aspect of chromatin condensation in the stained nucleus, which allows distinguishing between viable, apoptotic and necrotic cells [14]. Viable cells possess uniform bright green nuclei. Early apoptotic cells show bright green areas of condensed or fragmented chromatin in the nucleus and necrotic cells show uniform bright orange nuclei. AGS cells were treated with aGTE, followed by indomethacin on the selected dose. Cells cultured in indomethacin-free media and aGTE treatment were used as control. After the exposure time, 100µg/ml of EtBr-AO was added to the cell monolayer in each separate well, and the plates were incubated for 30°C mins in the dark at room temperature. The stained cells were then observed under an Olympus fluorescence microscope at 20X magnification.

Cell Extraction

Cultured and treated cells were directly lysed in buffer containing EDTA free protease inhibitor cocktail and centrifuged at 12,000 g for 15 min.

Detection of mitochondrial ROS generation

Mitochondria were isolated from cultured cells by differential centrifugation method, and ROS level was measured. Fluorescence was measured from tissue mitochondria through spectrofluorometer (LS 3B, Perkin Elmer, USA) by using 499 nm as excitation and 520 nm as emission wavelengths. The data were normalized, and the normal was expressed as a value of 100% (**Figure 1**).

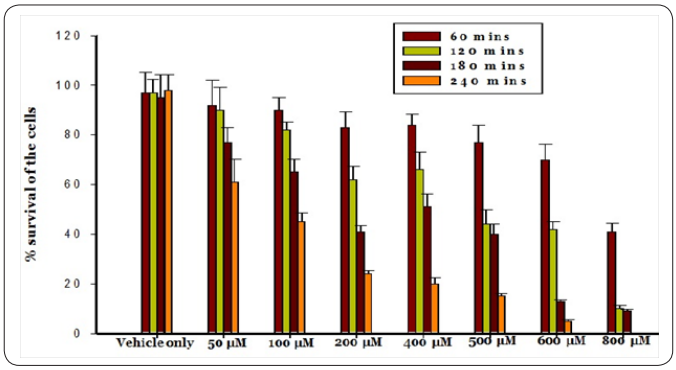

Figure 1. AGS Cells were maintained in RPMI media as described in 'Materials and Methods' section. MTT assay was used to assess cell viability with different concentrations of indomethacin from 50 µM to 800 µM for varied time points. It was seen that the viability of cells did not get affected when the cells were treated with only vehicle Na2CO3. When the exposure time was increased upto 240 min., the viability decreased by 5% in case of 800 µM indomethacin whereas viability decreased to 52% in 400 µM indomethacin.

Results

Indomethacin-induced damage in AGS cells

Figure 2. On treatment with 400 µM indomethacin on AGS cells, viability decreases almost 42%, but on pre-treatment with aGTE, viability increased by approximately 70%. To standardize the damaging dose and duration of indomethacin, AGS cells were exposed to 50 µM, 100 µM, 200 µM, 400 µM, 500 µM, 600 µM and 800 µM of indomethacin for a varied time span, i.e. 1, 2, 3 and 4 h. As assessed by MTT assay, exposure to 400 µM of indomethacin for 3 h showed 52% cell survival and was chosen as the damaging dose for further assays. Then, the cells were pretreated with aGTE 1h prior to

indomethacin treatment to assess its preventive potential.

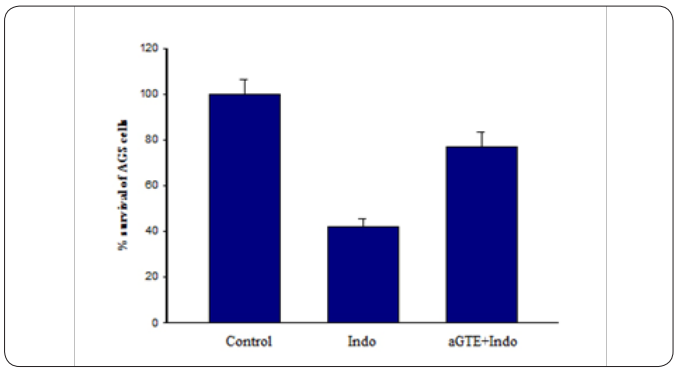

Figure 2. On treatment with 400 µM indomethacin on AGS cells, viability decreases almost 42%, but on pre-treatment with aGTE, viability increased by approximately 70%.

Pretreatment with 1µL of aGTE for 1 h showed almost 77% cell viability. This result showed that aGTE has the efficacy to prevent indomethacin-induced gastric cell damage.aGTE maintained cellular morphology and nuclear integrity against indomethacin-induced damage(Figure 2).

AGS cells were exposed to 1 µL of aGTE followed by 400 µM indomethacin for 3h. DAPI (Panel A) and EtBr-AO (Panel B) staining of those cells were depicted in Figure 2. The cells revealed a shrunken nuclear membrane along with chromatin aberration and fragmentation when treated with indomethacin alone. Pretreatment of aGTE provided significant protection to the cells, which were evident from DAPI staining. aGTE treated cells were quite compa¬rable to the control counterpart. Panel B represented the various apoptotic states of cells stained with EtBr-AO cocktail. The treatment period was the same with DAPI pictures. More than 50% of cells became yellowand approxi¬mately 30% cells became orange to red on EtBr-AO staining, which signified that the cells became pro-apoptotic to necrotic. aGTE protected the cells significantly by reducing the extent of apoptosis (**Figure 3**).

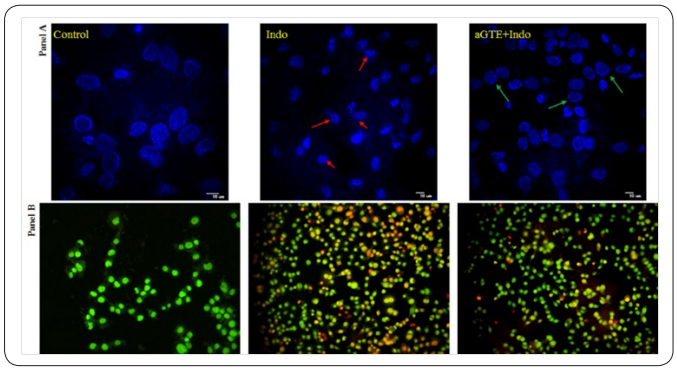

Figure 3. AGS cells were stained with DAPI and EtBr-AO cocktail staining solution separately which revealed the nuclear morphology as well as the apoptotic states of the cells on exposure with 1µL of aGTE pretreatment fol¬lowed by 400 µM indomethacin.

Prevention of ROS generation

Overproduction of mitochondrial ROS level is an indication of increased oxidation in the cells, which ultimately leads to inflammatory response and cellular damage. Increased ROS generation is the characteristic feature of indomethacin exposure in the cells with respect to the control group. Fluorescence intensity produced by H2DCFDA on oxidation to H2DCF was proportional to the amount of ROS produced in the mitochondria. The ROS values for control cells (121 ± 6.3) were taken as the reference value. The relative fluorescence intensity was increased in indomethacin-treated cells (278 ± 6.25). aGTE pretreatment reduced the values near the basal value significantly (185 ± 8.77) (**Table 1**).

Treatment Groups	ROS (DCF fluorescence intensity)
Control	121 ± 6.3
Indomethacin treated	278 ± 6.25
aGTE+ Indomethacin treated	185 ± 8.77

Table 1: AGTE pretreatment reduced the values near the basal value significantly

Discussion

The major findings in this study are aGTE shows significant potential in preventing epithelial cell damage induced by indomethacin. aGTE prevented indomethacin-induced oxidative damage, promoted survival of AGS cells, and maintained the nuclear integrity of the cells. It also prevented cellular apoptosis, which is evident from AO/EtBr staining. In addition, aGTE showed ROS scavenging potential in vitro during the prevention of gastric cell damage. The green tea concoction is a complex mixture of many bioactive polymers and flavonoids, including catechins and derivatives. Literature suggested that both green and black tea extracts have pronounced disease preventive potential caused by several ulcerogens, but the mechanism is not fully understood[15,16]. It is also reported that purified catechins have intense free-radical scavenging activity, both in vitro and in vivo. As green tea is a real source of catechin, we wanted to investigate its anti-inflammatory potential on AGS cells. Fluorimetric data from cell extracts showed lesser ROS generation when the aGTE was administered prior to indomethacin on AGS cells. MTT assay further confirmed that aGTE had pronounced potential on cell survival.

In summary, these results evidenced that aGTE significantly reduced ROS generation in indomethacin-treated cells, thus inflammatory response. In this context, further research and clinical trials are needed to evaluate the impact of these findings in patients suffering from indomethacin-induced gastropathy.

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