

Cytotoxic Effect of Luteolin on Human Colorectal Cancer Cell Line (HCT-15): Crucial Involvement of Reactive Oxygen Species

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Abstract

Background: Colorectal cancer, a major health concern worldwide, is the third most common form of cancer and second leading cause of cancer-related deaths. The flavonoids are naturally occurring diphenylpropanoids ubiquitous in plant foods and important components of the human diet. Luteolin, a bioflavonoid, possesses many beneficial effects including antioxidant, anti-inflammatory, anti-allergic activities.

Methods: We used the HCT-15 colon adenocarcinoma cell line in this study. Cells were treated with luteolin (100 μ M).

Results: Membrane damage markers such as alkaline phosphatase and lactate dehydrogenase were analyzed in a time-dependent manner. Luteolin increased reactive oxygen species in a time-dependent manner. DNA damage, a hallmark of apoptosis, was induced by luteolin as analyzed by agarose gel electrophoresis.

Conclusion: Luteolin acts as a potential cytotoxic agent that can be used to treat colorectal cancer.

Keywords: Colon cancer, Luteolin, ROS, HCT-15

Introduction

Colorectal cancer (CRC), a major health problem worldwide, is the third most common form of cancer and second leading cause of cancer-related deaths.¹ While continuing efforts have been made for discovering new molecular target-based molecules, there is an emerging interest in chemotherapeutic applications of natural substances such as tea polyphenols and

resveratrol for cancer treatment.² Many signaling pathways are altered during the pathogenesis of CRC.³

The flavonoids are naturally occurring diphenylpropanoids ubiquitous in plant foods and important components of the human diet.⁴ Flavonoids appear in animal and human cells following consumption of vegetables, fruits and beverages such as tea and wine.^{4,5} Although generally considered to be

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non-nutritive agents, interest in flavonoids has arisen because of their potential role in prevention of human cancer.⁴ Luteolin (LUT) is bioactive flavonoid with demonstrated effect on various tumors such as a human leukemia cell line and pancreatic tumor cells.^{6,7} It exhibits a wide spectrum of pharmacological properties, however little is known about its biochemical targets. Luteolin has antioxidant⁸ and anti-proliferative/antitumor⁹ activities, controls glycoprotein levels,¹⁰ modulates the status of thiols¹¹ and induces apoptosis¹² during chemically-induced carcinogenesis.

Reactive oxygen species (ROS) such as the superoxide anion and hydroxyl radicals, hydrogen peroxide and single oxygen, are formed as a result of normal metabolic activity¹³ or as by products of oxidative reactions that involve xenobiotics¹⁴. *In vivo*, such species are securely coupled at their site of generation or are detoxified by endogenous antioxidative defenses so as to preserve optimal cellular function. In pathological conditions, however, the detoxifying mechanisms are often inadequate as excessive quantities of ROS can be generated. This resulting pro-oxidant shift, a

process known as oxidative stress, can result in the degradation of cellular components such as DNA, carbohydrates, polyunsaturated lipids and proteins, or precipitate enzyme inactivation, irreversible cellular dysfunction and ultimately cell death if the pro-oxidant-antioxidant balance is not restored.¹⁵

Earlier study in our laboratory have shown that diallylsulfide induces ROS, thereby it acts as a cytotoxic agent to the Colo DM320 colon adenocarcinoma cell line.¹⁶ In the present study, we intend to analyze the cytotoxic effect of LUT on an HCT-15 colon adenocarcinoma cell line. This study has confirmed the cytotoxic effects by production of ROS as well as DNA fragmentation.

Materials and Methods

Cell line and culture conditions

HCT-15 colon cancer cell line was purchased from the National Center for Cell Sciences (NCCS), Pune, India and maintained on RPMI medium (Himedia, Mumbai) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Himedia, Mumbai) in an atmosphere of 95% air and 5% CO₂.

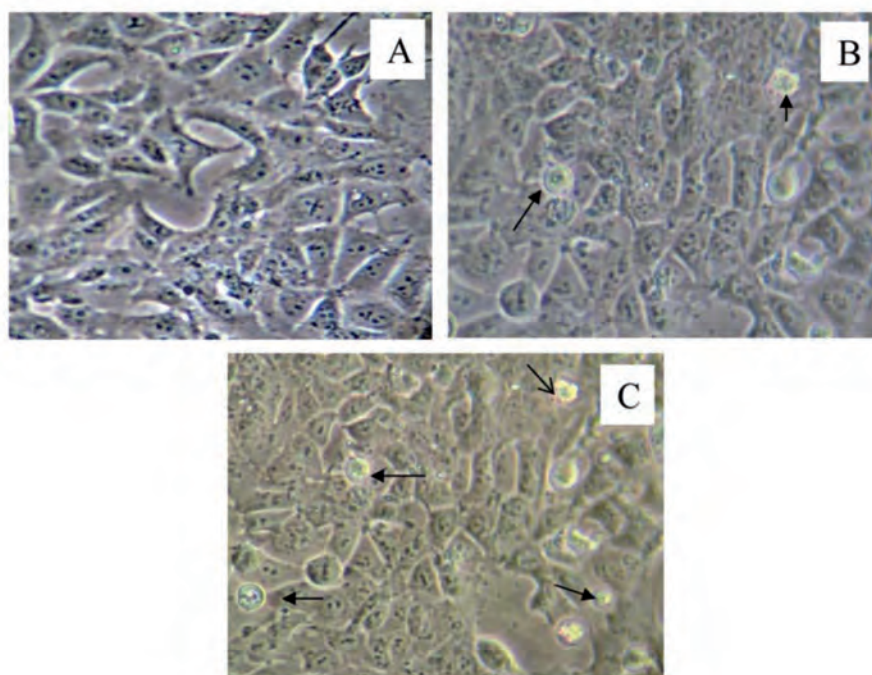


Figure 1. Influence of luteolin on cell morphology; Microscopic analysis of HCT-15 cell morphology after treatment with luteolin(LUT) for 12 and 24 h. (a) Control cells, (b)LUT(100μM at 12 h), (c)LUT(100μM at 24h) induced morphological changes in HCT-15 cells. Arrows indicate floating cells (Magnification: 20x; Scale: 100μM).

Alkaline phosphatase (ALP) assay

Alkaline phosphatase (ALP) activity was measured in control and LUT-treated cells and used as a marker of cell differentiation.¹⁷ The assay was performed according to the method described by Sriram et al.¹⁶ Briefly, the cells were centrifuged at 4000g, resuspended in phosphate buffered saline, and sonicated with an ultrasonic cell disrupter. Cellular debris was pelleted by centrifugation, after which the supernatants were transferred to new tubes and stored at -70°C until assayed. Enzyme activity was measured at 37°C using p-nitrophenyl phosphate as the substrate. The amount of p-nitrophenol liberated was determined spectrophotometrically at 405 nm using a Shimadzu spectrophotometer.

Lactate dehydrogenase (LDH) assay

Lactate dehydrogenase (LDH) activity was measured in both cell lysate and in the conditioned medium. After 12 and 24 h incubation the cultured medium was removed and attached cells were lysed by the addition of 0.1% Triton X-100 and subjected to two freeze thaw cycles. LDH activity was measured according to the method of King. Briefly, the substrate reaction buffer (0.5mM lactic acid +0.1 N NaOH +0.1 M glycine buffer) was added to the cell lysate and medium. Dinitrophenylhydrazine (0.02%, DNPH) was added as the chromogenic agent and absorbance values at 460

nm were read in a Shimadzu UV-Visible spectrophotometer.

Determination of production of reactive oxygen intermediates

Intracellular ROS generation was assessed by the oxidation-sensitive fluorescent probe, DCFH-DA. Briefly, after treatment, HCT-15 cells were harvested and suspended in 0.5 ml PBS that contained 10 mM DCFH-DA for 15 min at 37°C in the dark. DCFH-DA was taken up by the cells and deacetylated by cellular esterase to form a non-fluorescent product, DCFH, which was converted to a green fluorescent product (DCF) by intracellular ROS produced by treated HCT-15 cells. The intensity of DCF fluorescence was measured by fluorimetry with excitation settings of 488 nm and emission settings of 530 nm.

DNA fragmentation analysis

After treatment with LUT, HCT-15 cells were lysed in a buffer that contained 10 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA and 0.5% Triton X-100 for 30 min on ice. Lysates were vortexed and cleared by centrifugation at 12000 g for 20 min. Fragmented DNA in the supernatant was extracted with an equal volume of a neutral phenol: chloroform: isoamyl alcohol mixture (25:24:1) and analyzed by agarose gel electrophoresis.

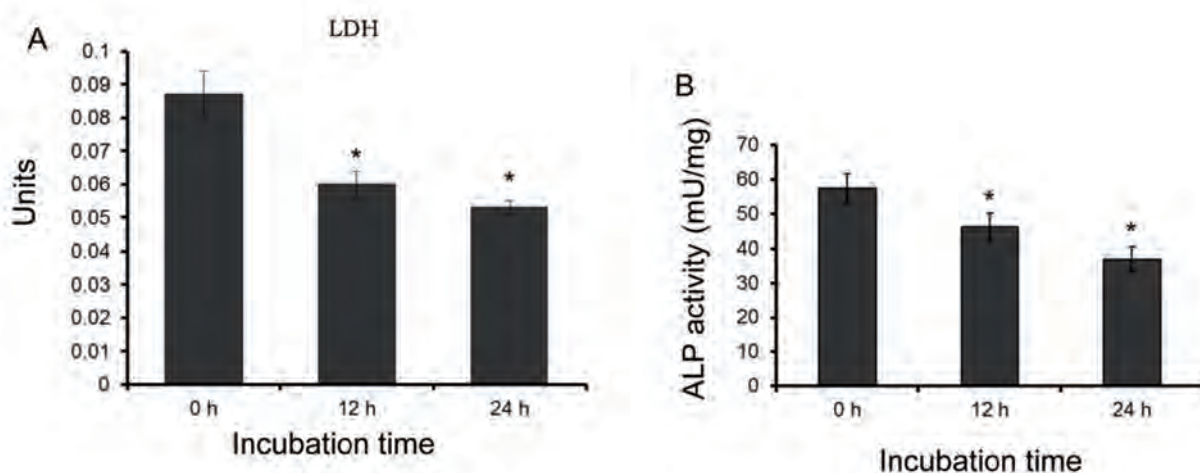


Figure 2. Assay of lactate dehydrogenase (LDH) and alkaline phosphatase (ALP) activity in control and luteolin (LUT)-treated HCT-15 cells; (A) LDH-activity was expressed as μ M of pyruvate liberated/min/mg protein. (B) ALP levels were expressed as mU/mg protein. Values represent the mean of three independent experiments. Values statistically significant at * P <0.05 compared with control.

Statistical analysis

Data were expressed as mean \pm SD. Comparisons between control and treated cells were made using SPSS/20.0, $P < 0.05$ values were considered statistically significant.

Results

Morphological analysis of HCT-15 cells is shown in Figure 1. HCT-15 cells were treated with 100 μ M of LUT for 12 and 24 h. At the time periods of 12 and 24 h, treated cells floated in the culture medium. The maximum numbers of floating cells were observed at 24 h of treatment.

Luteolin (LUT) modulates alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) activities

ALP and LDH levels of untreated and LUT-treated HCT-15 colon adenocarcinoma cells are shown in Figure 2. Control cells showed increased ALP and LDH activities. Colon cells treated with LUT showed significant reductions in the activities of ALP and LDH at 12 and 24 h. There was membrane damage in the colon cells due to the administration of LUT.

Luteolin enhances the production of reactive oxygen species (ROS)

The effect of LUT on intracellular hydrogen peroxide production was represented in Figure 3. We incubated the HCT-15 cells with 100 μ M of LUT at a different time interval and measured

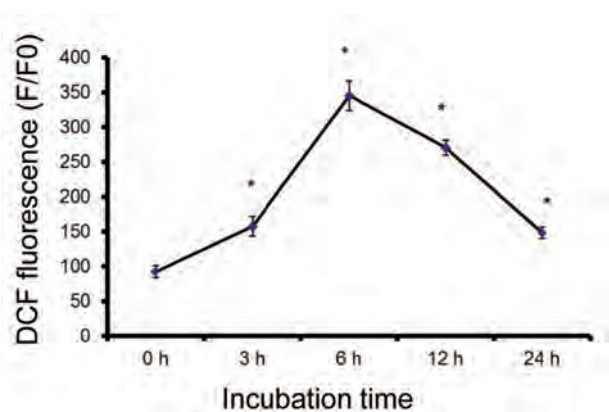


Figure 3. Luteolin (LUT) increases intracellular reactive oxygen species (ROS) production. HCT-15 cells were treated with LUT at various timepoints. ROS production was quantified using DCFH in fluorimetry. At 6 and 12 h, ROS levels increased as shown in the graph.

ROS production. At 6 h there was increased ROS production, however at 12 and 24 h the levels of ROS gradually decreased. Thus, from this observation it is clear that LUT produces ROS in HCT-15 cells.

Luteolin induces DNA fragmentation

The typical DNA fragmentation was observed as evidenced by characteristic ladder formation in cells treated with 100 μ M of LUT after 6 and 12 h (Figure 4, lanes 2 and 3). This result demonstrated that LUT induces apoptosis in a time-dependent manner.

Discussion

Numerous researchers have studied cancer chemoprevention derived from natural compounds. By using these compounds bioactive flavonoid compounds which are widely distributed in numerous beverages and food products have been implicated in the prevention of human carcinogenesis, possibly through their inhibitory activities of cell proliferation or survival.¹⁸ Cancer cells generally exhibit highly proliferative, migrative and matrix-invasive potentials often by modulating signaling molecules.^{19,20}

Promising anti-cancer agents with strong inhibitory properties of survival-related proteins or activating capacities of apoptotic proteins are scrutinized for cancer treatments. Those derived from natural compounds are considered safe as

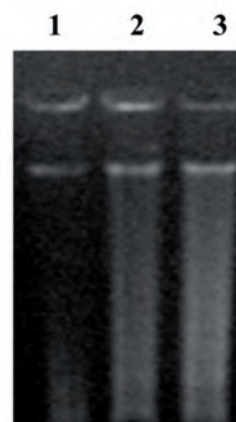


Figure 4. Luteolin (LUT) induces DNA damage in HCT-15 cells. Induction of apoptosis at 6 and 12 h in HCT-15 cells by LUT. Cells were treated with 100 μ g for 6 and 12 h. DNA fragmentation LUT-treated cells was analyzed by agarose gel electrophoresis.

they are generated from commonly consumed food stuff. Luteolin is one such compound considered to be safe and nontoxic to cells. This compound also possesses many beneficial effects that include anti-oxidant, anti-inflammatory, anti-proliferative and anti-allergic properties. This study has analyzed the cytotoxic effect of LUT against the HCT-15 colon adenocarcinoma cell line.

Colon specific ALP expression is a marker of maturation and differentiation of the colon's epithelial cells.²¹ ALP has been extensively characterized as an *in vivo* marker of absorptive cell differentiation. In the present study, addition of LUT to cells resulted in a decline in the colonic ALP and LDH activities. Administration of LUT reduced the activity of LDH in HCT-15 cells. This might be due to increased permeability of the plasma membrane coupled with excessive leakage of LDH from the cells to the medium.

To show the importance of ROS for HCT cell apoptosis induced by LUT, we quantified ROS production using dichlorodihydro fluorescein (H₂DCF-DA). Normal intracellular esterases convert this compound to H₂DCF. In the presence of ROS, H₂DCF is converted to highly fluorescent dichlorofluorescein (DCF). The fluorescence intensity is directly proportional to the quantity of ROS. The present study has shown increased fluorescence intensity and an increase in the level of ROS upon incubation with LUT, which showed the capability of LUT to entail ROS production in colon cancer cells and might lead to apoptosis. Reports from previous studies indicated that ROS could potentially induce apoptosis in various cell lines.^{22,23,16} NSAIDs such as indomethacin and salicylic acid induced ROS in HT-29 cells lead to apoptosis.²⁴

We performed DNA fragmentation analysis to further confirm apoptosis induced by LUT. The results indicated that the HCT-15 cell line treated with LUT induced DNA shear, which was characteristic of apoptotic cell death. DNA fragmentation is considered the hallmark of apoptosis.^{25,26} A previous report has suggested that LUT induces DNA fragmentation in HepG2

cells.²⁷ LUT has an ability to intercalate DNA and inhibit the topoisomerase I.²⁸

Conclusion

In conjunction, the results of this study have shown that LUT is cytotoxic to HCT-15 colon adenocarcinoma cells. Luteolin increases ROS production, which subsequently induces DNA fragmentation. LUT-induced cell damage has been confirmed by ALP analysis- a marker of membrane damage. We conclude that LUT may act as a potential drug for the treatment of colorectal cancer.

Conflict of interest

The authors declare they have no conflicts of interest.

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