

Luteolin, a bioflavonoid inhibits azoxymethane-induced colon carcinogenesis: Involvement of iNOS and COX-2

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ABSTRACT

Colon cancer (CRC) is a serious health problem through worldwide. Development of novel drug without side effect for this cancer was crucial. Luteolin (LUT), a bioflavonoid has many beneficial effects such as antioxidant, anti-inflammatory, anti-proliferative properties. Azoxymethane (AOM), a derivative of 1, 2-Dimethyl hydrazine (DMH) was used for the induction of CRC in Balb/C mice. CRC was induced by intraperitoneal injection of AOM to mice at the dose of 15 mg/body kg weight for 3 weeks. Mouse was treated with LUT at the dose of 1.2 mg/body kg weight orally until end of the experiment. The expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 were analyzed by RT-PCR and immunohistochemistry. The expressions of iNOS and COX-2 were increased in the case of AOM induction. Administration of LUT effectively reduced the expressions of iNOS and COX-2. The present study revealed that, LUT suppresses both iNOS and COX-2 expressions and act as an anti-inflammatory role against CRC.

Key words: Azoxymethane, colon cancer, COX-2, iNOS, Luteolin

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INTRODUCTION

Colon cancer (CRC), a complex multi-step process involving progressive disruption of homeostatic mechanisms^[1] controlling intestinal epithelial proliferation/inflammation, differentiation and programmed cell death, is the third most common malignant neoplasm worldwide.^[2] Several epidemiological studies have indicated the influence of lifestyle factors, particularly nutritional factors, on the development of certain forms of cancers, including the colon carcinomas.^[3,4] AOM, a metabolite of DMH, has been used extensively to induce CRC in rodents.^[5] AOM-induces cancer to both rat and mouse models were the main animal models used to study the effect of dietary agents.^[6,7] It is necessary to measure the ability of certain natural product or pharmaceutical agent to hinder the carcinogenesis process.^[8]

Luteolin, a 3',4',5,7-tetrahydroxyflavone, is usually found in a glycosylated form in celery, green pepper, perilla leaf and seed, chamomile tea and *Lonicera japonica*. Luteolin possesses anti-inflammatory, anti-oxidant^[9] and phytoestrogen like

activities.^[10] Several researchers had found that LUT possess anti-neoplastic activities against several human cancers.^[11-13]

There are two isoforms of cyclooxygenase (COX), namely COX-1 and COX-2. COX-1 is constitutively expressed in a number of cell types and tissues and plays a major role in homeostasis. COXs are the rate-limiting enzymes in the conversion of arachidonic acid into prostaglandins (PGs). COX-2 catalyzes the conversion of arachidonic acid into PGH₂ and it is further converted into Prostaglandins and thromboxane A₂ by prostaglandin synthase and thromboxane synthase respectively. The formation of PGH₂ occurs in two steps; initially, two oxygen molecules are incorporated into arachidonate, thus generating PGG₂. The synthesis of PGH₂ is the second step and it involves reduction of PGG₂ catalyzed by the peroxidase activity of COX-2. COX-2 is not detected in most normal tissues but is rapidly induced in response to mitogens, cytokines and tumor promoters, leading to increased accumulation of prostanoids in neoplastic and inflamed tissues.^[14] Accumulated evidence suggests that COX-2 selective inhibitors such as non-steroidal anti-inflammatory drugs (NSAIDs) induce apoptosis by suppressing the COX-2.^[15]

Variety of natural compounds have the potential to control the neoplasms. Our own observations revealed that Luteolin inhibit CRC *in vivo*.^[16-18] Hence, our present

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investigation focused to study inhibitory effect of LUT against the crucial inflammatory molecules involved in CRC such as iNOS and COX-2.

MATERIALS AND METHODS

Animals

Male Balb/c mice weighing approximately 25 to 30gm were obtained from the Laboratory Animal Maintenance Unit, Tamilnadu Animal Science and Veterinary University (TANUVAS), Madavaram, India. The animals were acclimatized to the laboratory conditions for a period of two weeks. They were maintained at an ambient temperature of $25 \pm 2^\circ\text{C}$ and 12/12h of light-dark cycle and given a standard rat feed (Hindustan Lever Ltd., Bangalore) and water *ad libitum*. The experiments involved with animals were conducted according to the ethical norms approved by Ministry of Social Justices and Empowerment, Government of India and Institutional Animal Ethics Committee Guidelines.

Experimental procedure

The animals were divided into four groups ($n = 6$ per group). Group 1 - Control animals received intra peritoneal injections (*i.p.*) of physiological saline. Group 2 animals were administered with AOM (15mg/kg body weight) intraperitoneally (*i.p.*) once in week for three weeks. Group 3 animals (AOM + LUT) were treated with a single dose with 1.2mg/kg body weight of LUT orally until end of the experiment, after AOM administration as mentioned in group 2.^[9] Group 4 received the same dose of LUT alone as mentioned in group 3.

Assay of Alkaline Phosphatase and Lactate Dehydrogenase

Lactate dehydrogenase was assayed according to the method described by King (1965a).^[9] Alkaline phosphatase was assayed by the method described by King (1965b).^[20]

Immunohistochemical analysis of iNOS and COX-2

Immunohistochemical analysis of iNOS and COX-2 were analyzed by the method Ashokkumar and Sudhandiran, (2011).^[21] The stained slides were visualized under a light microscope (Nikon XDS-1B). To measure the relative intensity, scoring was done as arbitrary units 4 as intensely stained, 3 as moderately stained, 2 as mild staining, 1 as poorly stained in control and experimental groups.

RNA Isolation and PCR analysis

Isolation of RNA and RT-PCR analysis of iNOS and COX-2 were followed by the method Ashokkumar and Sudhandiran, (2011).^[21] Total cellular RNA was extracted from colon tissues using the TRIzol reagent (Bangalore Genei, Bangalore, India). cDNA was synthesized from 2 μg of total RNA using M-MuLV reverse transcriptase (Bangalore

Genei, India). The cDNA for iNOS, COX-2 and actin were amplified by PCR with gene specific primers [Table 1]. PCR products were analyzed in an agarose gel electrophoresis and visualized by ethidium bromide staining.

Statistical analysis

All the data were statistically evaluated with SPSS/10.0 software. Hypothesis testing methods included one-way analysis of variance followed by least significant difference (LSD) test $*P < 0.05$ was considered to indicate statistical significance. All the results were expressed as mean \pm S.D.

RESULTS

Luteolin reduces the levels of Alkaline phosphatase and Lactate Dehydrogenase

ALP is a non-specific phosphomonoester hydrolase that catalyses the hydrolysis of wide variety of organic monophosphatases and it is a useful marker for the determination of differentiation in colon cancer cells. LDH is recognized as a potential tumor marker enzyme in assessing the proliferation of malignant cells. The possible reason for elevated levels of LDH may be due to higher glycolysis in cancerous conditions, which is the only energy producing pathway for the uncontrolled proliferating malignant cells. Figure 1 shows the levels of ALP and LDH in control and experimental groups of mice. Administration of AOM (Group 2) facilitates the increase in the levels of ALP and LDH. LUT (group 3) treatment substantially reduced the levels of ALP and LDH. In control (Group 1) and LUT (Group 4) state no such alteration in the levels of ALP and LDH enzyme were observed.

Luteolin decreased the expression of iNOS

Figure 2a shows expression of iNOS in colon tissue. Colon tissue section from control (a) and drug control (d) mouse showed a small level of immunostaining for iNOS. AOM produced an increased expression of iNOS in colon tissues, as shown in (b). The LUT treatment reduced the AOM-induced expression of this iNOS (c). The immunohistochemical staining of iNOS was quantified and the result of the same is represented in Figure 2b.

Table 1: Primer sequence of iNOS and COX-2

| Gene | Primer sequence | Base pair |
|-------|-------------------------------------|-----------|
| iNOS | Forward-5'-agaaggaatggctgcagaa-3' | 194 |
| | Reverse-5'-gctcggctccagattgag-3' | |
| COX-2 | Forward-5'-cctgtgtccaccaggagat-3' | 247 |
| | Reverse-5'-ccctggctagtgtctcagac-3' | |
| Actin | Forward-5'-tggtaccaactgggacgaca-3' | 842 |
| | Reverse-5'-acatctgctggaagggtggac-3' | |

iNOS: Inducible nitric oxide synthase; COX-2: Cyclooxygenase

Similar results were observed in RT-PCR analysis of iNOS [Figure 2c]. The image] analysis of PCR bands were quantified and illustrated in Figure 2d.

Luteolin decreased the expression of COX-2

An elevated level of COX-2 has been reported in CRC and selective inhibitors of COX-2 could greatly reduce the tumor burden. Control mouse colon tissue section showed a small degree of immunostaining for COX-2 [Figure 3a]. AOM induction increased the levels of COX-2 protein in the colon tissues, as evident from intense and wide spread brown staining in colon tissues (b). However treatment with LUT significantly reduced the expression of COX-2 (c), when compared to AOM induced group. The immunohistochemical staining of COX-2 was quantified and the result of the same is represented in Figure 3b. Similar results were observed in RT-PCR analysis of COX-2 [Figure 3c]. The Image] software analysis of PCR bands were quantified and illustrated in Figure 3d. The results of this study exhibited that LUT could reduce the expressions of these inflammatory markers during CRC.

DISCUSSION

Flavonoids constitute the largest and most important group of polyphenolic compounds in plants. They are widely distributed in many frequently consumed beverages and food products of plant origin such as fruit, vegetables, wine, tea and cocoa.^[22,23] Intake of beverages or food products containing flavonoids has been frequently associated with a reduced risk for developing various cancers.^[22,24]

Several studies showed increased expression and activity of iNOS in human colon adenomas.^[25-27] In the present investigation, the expression of iNOS was increased in AOM-induced mice. The expression of iNOS and nitrotyrosine accumulation (marker of peroxynitrite, the product of NO and superoxide) in inflamed mucosa of patients with ulcerative colitis and gastritis demonstrate the production of NO and its potential involvement in the pathogenesis of these diseases.^[28] Studies in experimental models of CRC indicate that AOM-induced colon tumors have higher expression and/or activity of iNOS compared to levels found in adjacent colonic tissue.^[29,30] But, LUT have a strong ability to suppress iNOS and indirectly prevent the production of NO in disease conditions,^[31-33] which was concordant with our present study.

The role of COX-2 as an enhancer of carcinogenesis in many organs including the colon is receiving increasing

attention. Hence, assays of COX-2 expression may be used to monitor the process of carcinogenesis, and the suppression of COX-2 expression has become an important target for treatment and prevention of CRC.^[34-36] COX-2 is induced by inflammatory cells, by various stimuli including cytokines, growth factors and tumor inducing factors. Overexpression of COX-2 results in dedifferentiation, adhesion to extra-cellular matrices and inhibition of programmed cell death in untransformed rat intestinal epithelial cells.^[37] Inducibility of COX-2 in response to mitogenic stimuli, oncogenes and tumor

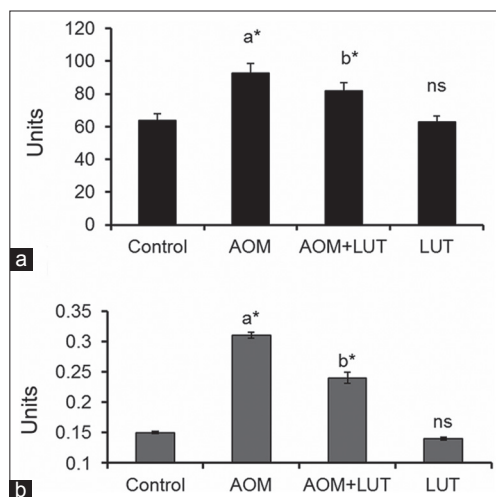


Figure 1: Luteolin reduced the levels of ALP and LDH Units: (a): Activity of ALP (mU/mg protein), (b): activity of LDH (µM of pyruvate liberated/min/mg protein). Values are expressed as mean ± S.D. for 6 mice in each group. ^aControl Vs AOM, ^bAOM Vs AOM+LUT, ns-non significant *P* < 0.05

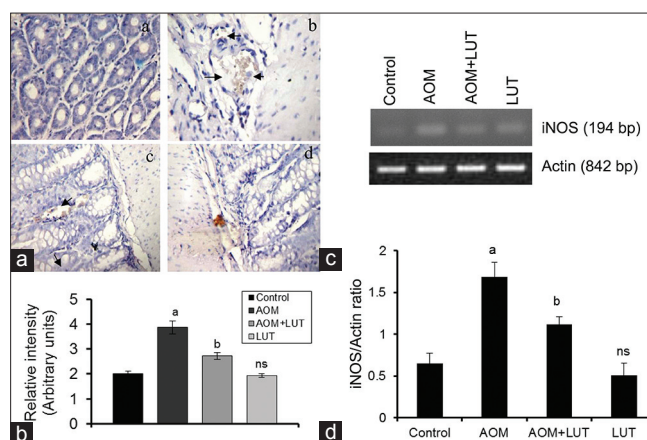


Figure 2: Luteolin decreases the expression of iNOS (a): (Control) Normal expression of iNOS, (b): (AOM) high expression of iNOS (brown color) was observed, (c): (AOM+LUT) lesser degree of expression of iNOS was noticed, (d): (LUT) Expression was similar to that of control. → shows the expression of iNOS. (2B), Quantification of the expressions of iNOS. Values are expressed as mean ± S.D. Comparisons: ^aControl Vs AOM, ^bAOM Vs AOM+LUT, ns-non significant **P* < 0.05. (2C): RT-PCR analysis of iNOS. The band was detected at the 194 bp and Actin was at 842 bp. The quantification of iNOS was normalized with Actin shown in 2D

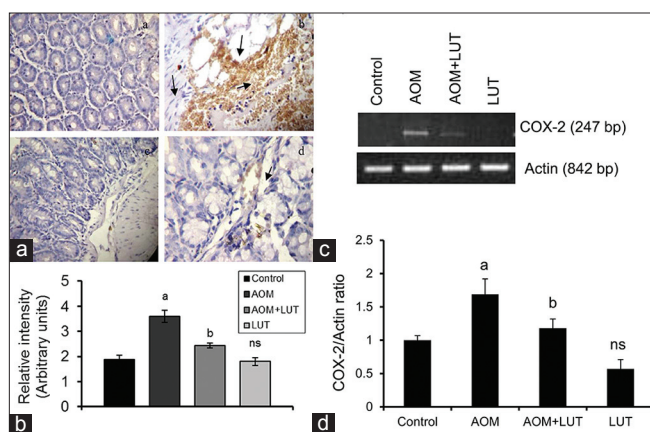


Figure 3: Luteolin decreases the expression of COX-2 (a): (Control) Normal expression of COX-2, (b): (AOM) high expression of COX-2 (brown color) was observed, (c): (AOM+LUT) lesser degree of expression of COX-2 was noticed, (d): (LUT) Expression was similar to that of control. → indicates the expression of COX-2 protein. (3B): Quantification of the expression of COX-2. Values are expressed as mean ± S.D. Comparisons: ^aControl Vs AOM, ^bAOM Vs AOM+LUT, ns-non significant **P* < 0.05. (2C): RT-PCR analysis of COX-2. The band was detected at the 247 bp and Actin was at 842 bp. The quantification of COX-2 was normalized with Actin shown in 3D

promoters link it to cell proliferation.^[38] Increased COX-2 expression leads to the elevated levels of PGE₂, that is correlated with increased MDA, which forms an adduct with DNA in human colon leads to carcinogenesis^[39] or inhibit apoptosis in epithelial tumor cells.^[40] In the present study, the expression of COX-2 protein in colon tumors was higher than that of normal colonic mucosa. Abundant evidences supports, the role of COX-2 is involved in CRC^[37,41,42] and provides the basis for the development of COX-2 selective inhibitors for CRC prevention and treatment.^[43] Many reports stating that, LUT selectively inhibits COX-2 in various disease conditions.^[33,44] Administration of LUT directly suppresses the expression of COX-2 and thereby reduced the inflammation in colon. Recently we reported that LUT modulates the status of ATPases in AOM-induced CRC^[45] and inhibits wnt/ β -catenin signaling *in vitro*.^[46]

Our own observations indicated that the LUT, potentially inhibit CRC in rodents. The results obtained from the above findings clearly indicated that, Luteolin inhibits the expression both iNOS and COX-2 in AOM-induced colon carcinogenesis. These two proteins are important mediator of inflammation associated cancer. Hence, LUT act as a novel chemotherapeutic agent to treat CRC.

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