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ANTI-INFLAMMATORY EFFECTS OF ACACETIN IN IL-1β INDUCED COLON EPITHELIAL (CACO-2) CELLS

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ABSTRACT

Aim: *To evaluate anti-inflammatory effect of a bioflavanoid Acacetin on IL-1ß-induced inflammation in colon cancer (CaCo-2) cells.* ***Methods:*** *CaCo-2 cells, an in-vitro IBD model, were stimulated with IL-1ß (25 ng/ml) to assess anti-inflammatory effects of the phytochemical acacetin. Trypan blue dye exclusion assay was performed to examine cytotoxicity, Western blotting to assess protein expression of transcription factor NF-κB-p50 and inflammatory mediator COX-2, and secreted PGE_2 levels were determined using PGE_2 colorimetric assay.*

Results: *The results demonstrate that when treated with acacetin CaCo-2 cells did not show cytotoxicity and their tight junction morphology did not alter as they maintained the colony morphology intact. Further, acacetin exhibited anti-inflammatory effects in IL-1ß-induced CaCo-2 cells by decreasing the activation of NF-κB (p50) and expression of inflammatory enzyme COX-2. Acacetin treatment of CaCo-2 cells also reduced IL-1 ß-induced PGE-2 levels which is known to cause inflammation and initiation of colon cancer in IBD model.*

Conclusion: *In conclusion, Acacetin significantly decreased IL-1ß-induced cytotoxicity and expression of inflammatory markers in Caco-2 cells, and thus can be an effective anti-inflammatory agent in the treatment of IBD.*

KEY WORDS

Acacetin, IL-1ß, anti-inflammatory, CaCo-2 cells, colon cancer, COX-2, PGE_2, NF-κB (p50).

INTRODUCTION:

Inflammatory bowel disease (IBD) is a chronic inflammatory condition of the intestine. Normally intestinal immune system remains in homeostasis and a tight balance exists between pro-inflammatory and anti-inflammatory cells, which prevents intestinal tissue damage [1]. However, due to various factors including genetic, environment and immunoregulatory factors, this balance is disturbed and intestinal immune mechanism gets stimulated resulting in overproduction and release of inflammatory cytokines and chemokines [2]. The release of inflammatory mediators results in establishment of IBD, which if not cured and persists for longer duration, may increase the risk of developing colorectal cancer [3]. Hence, resolving the inflammation can mitigate the IBD and could also provide protection against the development of colorectal cancer.

Interleukin-1β (IL-1β) is an important pro-inflammatory cytokine, which has been elevated in the patients suffering from IBD [4]. IL-1β plays important role in initiation and amplification of inflammatory cascade in intestinal epithelial cells. Various *in vitro* [5] and *in vivo* [6] studies have shown that IL-1β disrupts the epithelial cell's tight junction and increases the permeability of epithelial cells resulting into abrupt mucosal immune response. Further, IL-1β also activates the redox-sensitive transcription factor NF-κB which results in transcriptional activation of inflammatory enzyme

cyclooxygenase-2 (COX-2) [7]. COX-2 is an inducible enzyme whose expression increases during chronic inflammation and it catalyzes the conversion of arachidonic acid into lipid mediator prostaglandin-2 (PGE_2). PGE_2 is known for increasing disease severity by causing cancer initiation and immunomodulation [8]. Hence, inhibition of IL-1β-induced NF-κB-mediated inflammatory pathway can be effective therapeutic strategy to control chronic inflammation in IBD and prevention of inflammation-induced colorectal cancer initiation, termed as primary chemoprevention.

Although several types of anti-inflammatory drugs including corticosteroids and immunosuppressive molecules are available for the management of IBD, however these unnatural and synthetic drugs are associated with severe side-effects [9, 10]. Hence, there is an urgent need of therapeutic strategy that is less toxic and shows least side effects. Recently phytochemicals have been recognized as anti-inflammatory and chemopreventive agents in colon-related diseases. Dietary phytochemicals such as curcumin, epigallocatechin gallate and resveratrol have demonstrated effective anti-inflammatory effects with no side effects on normal cells [10]. Acacetin, a bioflavonoid extracted from *Turnera diffusa* (damiana), *Betula pendula* (silver birch), *Asplenium normale* fern and several other plants, has been shown to possess anti-inflammatory activities in various inflammatory models. In a study, acacetin decreased airway inflammation by modulating eosinophil infiltration and expression of eotaxin-1 and Th2 cytokines in a mouse model of asthma [11]. Further, acacetin has also been shown to possess protection against LPS-induced neuroinflammation in *in vitro* and *in vivo* models [12]. In the present study, we have investigated the anti-inflammatory effects of acacetin against IL-1β -induced inflammation in colon epithelial (CaCo-2) cells, a well-known *in-vitro* model for IBD.

MATERIAL AND METHODS:

Chemicals and Reagents

Acacetin, trypan blue dye and Dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich (St. Louis, MO, USA). COX-2 and NF-κB (p105/50) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). MEM media, penicillin/streptomycin solution and fetal bovine serum (FBS) were purchased from Gibco Life Technologies, Invitrogen (Grand Island, NY, USA). PVDF membrane and ECL were purchased from Merck Millipore (Billerica, MA, USA). PGE_2 kit was purchased from Cayman chemicals (Michigan, USA)

Cell culture and Treatments

The human CaCo-2 cell line was procured from National Centre from Cell Science (Pune, India). The cells were grown in MEM medium supplemented with sodium bicarbonate (2g/L), penicillin and streptomycin solution (100 units/ml) and 10% FBS. Cells were incubated for growth and maintenance in humidified CO_2 (5 %) incubator at 37^0 C.

Stock solution of acacetin was prepared by dissolving it in molecular grade DMSO. While treatment with different doses of acacetin final concentration of DMSO in cell culture was maintained at 0.01% in all experimental groups, which was non-toxic to cells.

Trypan blue cell viability assay

Approximately $1x10^5$ cells/well were seeded in 60 mm plates and after 24 h cells were treated with different doses of acacetin and incubated in starving medium containing 0.5 % FBS for 24 h. After indicated time of incubation, the cells were trypsinized and collected by centrifugation at 1200 rpm. Cell pellet was re-suspended in PBS and trypan blue dye was added. Live and dead cells were counted using hemocytometer under light microscope [13] .

Western Blotting

Approximately $5x10^5$ cells were seeded in 100 mm plates and allowed to adhere for 24 h in complete media. After 24 h, media was replaced by 0.5% serum starving media and incubated for 18 h. Where needed acacetin (10 µM) pre-treatment was given for 6 h in serum free media. The cells were treated with vehicle (DMSO), IL-1β (25 ng/ml), Acacetin (10 µM) and IL-1β (25 ng/ml) + Acacetin (10 µM) and incubated for 24 h. After 24 h, cells were washed and lysed on ice with RIPA lysis solution (50 mM Tris, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 150 mM NaCl) containing protease and phosphatase inhibitors. Cell lysates were collected and centrifuged at 14,000 rpm for 30 minutes at 4°C. The supernatant containing whole cell proteins was collected and equal amount of proteins (40 µg) were electrophoresed onto 10% SDS-PAGE gels. The separated proteins were transferred to PVDF membrane followed by blocking in 5% nonfat milk containing blocking solution for 1 h at room temperature or for overnight at 4°C. Membranes were probed using specific primary antibodies against COX-2,

NF-κB (p105/p50) or GAPDH, followed by peroxidase-conjugated secondary antibody. The specific bands were visualized by the ECL detection system using X-ray film. Alpha-imager software was used for densitometry of the western blot images.

PGE-2 assay

The Caco-2 cells ($1x10^5$) were seeded in 6-well plate and after 24 h cells were treated as described above. After 24 h incubation, cell culture media was collected and centrifuged at 1500 rpm to remove debris and processed for PGE-2 assay according to manufacturer's protocol (Cayman chemicals, Michigan, USA).

Statistical analysis

Statistical analysis was performed with Graph Pad Prism 5 software version 5. Quantitative data are presented as mean ± SEM. Statistical significance between control and treated group was determined by Student's t-test and $p<0.05$ was considered significant.

RESULTS:

Acacetin induced no toxicity in CaCo-2 cells and maintains the tight junction morphology

The CaCo-2 cells resemble the intestinal enterocyte cell and are used as cellular model to study IBD. These cells form uniform monolayer colony by forming tight junctions. First of all, we assessed the toxicity of acacetin on CaCo-2 cells using trypan blue cell viability assay. Acacetin (0-10 µM) did not show any significant adverse effect on cell viability (Fig 1A), percent cell death (Fig 1B) and total cell number (Fig 1C) in CaCo-2 cells. This result confirmed that acacetin is non-toxic to intestinal epithelial cells. Further, microscopic images (100x) of CaCo-2 cells treated with 0-10 µM dose of acacetin showed that the cells maintained the normal colony morphology and did not disrupt epithelial tight junction formation (Fig 2). Thus, in the subsequent studies, we used 10 µM dose of acacetin to study its anti-inflammatory effects.

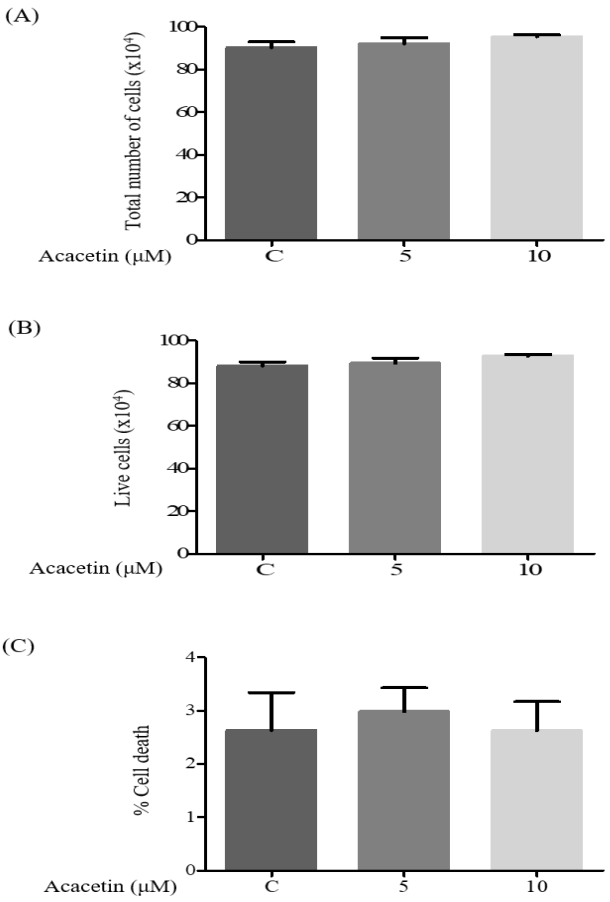

Figure 1: Effect of acacetin on viability of CaCo-2 intestinal epithelial cells: Viable cells and dead cells were counted using Trypan blue dye exclusion method using haemocytometer. Representative values of total cell number (A), live cells (B) and percent cell death (C) are shown as bar diagramme at 24 h. The bars show mean ± SEM (n=6) vs. respective controls.

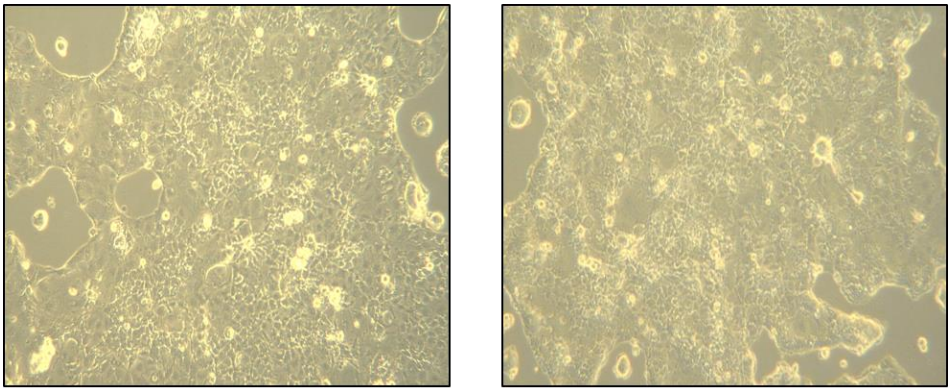

Figure 2*:* Effect of acacetin on tight junction morphology of CaCo-2 cells*:* Cells were grown and treated with 10 µM dose of acacetin and after 24 h microscopic images were acquired, and representative images are shown *(*n=3*)*, magnification 100X.

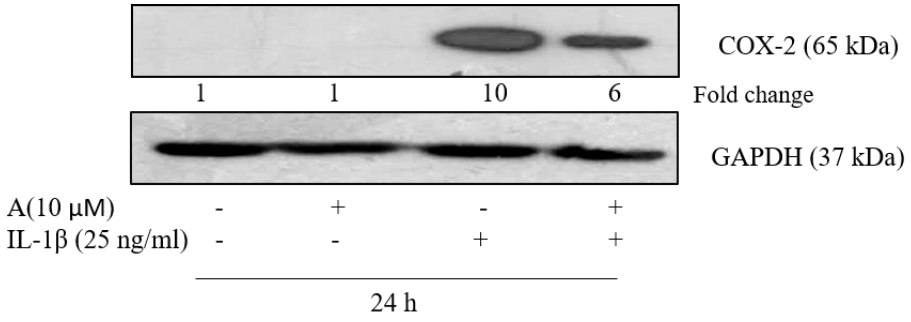

Figure 3*:* Effect of acacetin on expression of COX-2 enzyme in IL-1β *(*25 ng*/*ml*)* induced CaCo-2 cells*:* Respective Western blot image of COX-2 protein is shown *(*n=3*)*. Numbers below the bands represent fold changes corrected for GAPDH loading control.

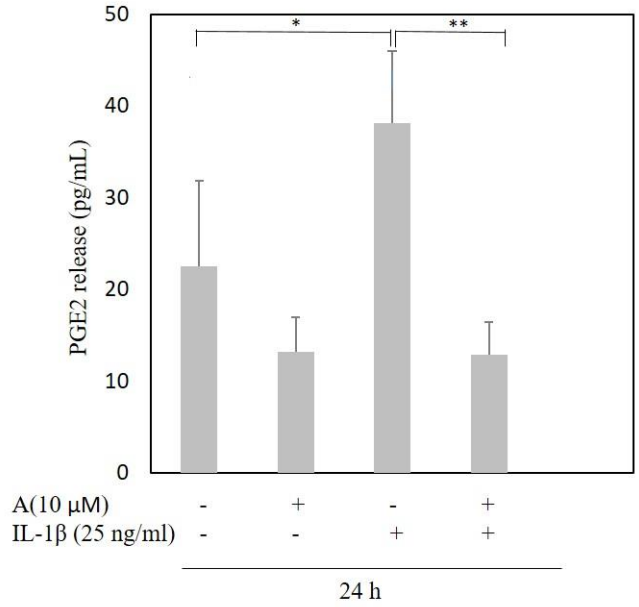

Figure 4*:* Effect of acacetin on PGE_2 levels in IL-1β *(*25 ng*/*ml*)* induced CaCo-2 cells*:* Cells were pre-treated with or without acacetin *(*10 µM*)* and induced with IL-1β *(*25ng*/*ml*)* and secreted PGE_2 in cell culture media were estimated using PGE-2 kit. The bars show PGE_2 level as mean ± SEM *(*n=6*)*, **p*<0.05 vs control; ***p*<0.01 vs IL-1β.

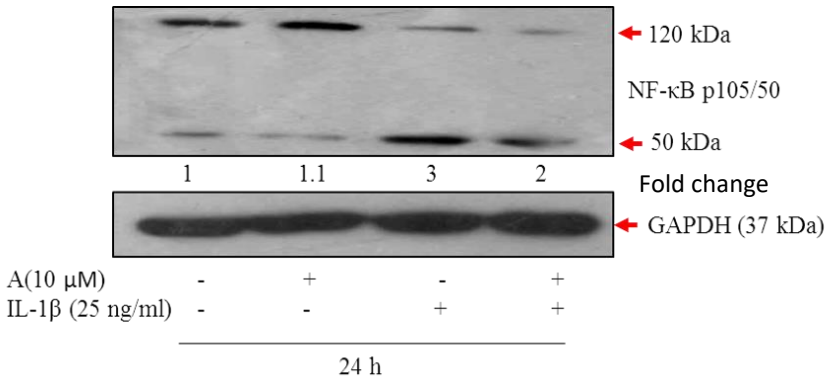

Figure 5: Effect of acacetin on expression of NF-κB-p50 in IL-1β (25 ng/ml) induced CaCo-2 cells: Representative Western blot image of NF-κB-p50 is shown (n=3). Numbers below the bands represent fold changes in protein levels corrected for GAPDH loading control

Acacetin decreased the expression of COX-2 protein in IL-1β induced CaCo-2 cells

We next assessed the anti-inflammatory effects of acacetin on IL-1β-induced CaCo-2 cells inflammation by examining the expression of COX-2, an inducible inflammatory mediator. We measured the expression of COX-2 in Caco-2 cells after IL-1β induction by Western blotting and observed that IL-1β treatment caused 10-fold increase in COX-2 expression which was significantly decreased to 6-fold when cells were pre-treated with acacetin (Fig 3). This result indicated that acacetin had an anti-inflammatory role in IL-1β-induced CaCo-2 cells.

Acacetin decreased the secreted PGE_2 level in IL-1β induced CaCo-2 cells

COX-2 enzyme is known to catalyze the synthesis of PGE-2, which plays an important role in inflammation and cancer initiation. Therefore, we examined the level of released PGE-2 in IL-1β-induced CaCo-2 cells culture media. Treatment of Caco-2 cells with IL-1β resulted in 38 pg/ml (*p<0.05) secretion of PGE-2 as compared to nearly 20 pg/ml in control cells. Further, pretreatment of CaCo-2 cells prior to induction with IL-1β significantly (**p<0.01) decreased PGE-2 level to 12 pg/ml (Fig 4). This result indicated that acacetin not only regulates the expression of COX-2, but also regulates its enzyme activity leading to decreased PGE-2 synthesis in CaCo-2 cells.

Acacetin decreased expression of IL-1β induced NF-κb (p50) activation

NF-κB (p105/p50) in normal condition exists as 120 kDa protein in the cytosol, however upon reception of inflammatory signals it gets cleaved into an active form of 50 kDa protein. This activation is necessary for its translocation into nucleus where it begins the transcription of its target inflammatory genes such as COX-2. IL-1β (25 ng/ml) induction of CaCo-2 cells resulted in increased level of active p50 by 3-fold, which was decreased to nearly 2-fold when pre-treated with acacetin (Fig 5). Thus, our results suggest that acacetin exhibits its anti-inflammatory effects through the regulation of NF-κB pathway.

DISCUSSION:

The precise etiology and molecular mechanism responsible for IBD is unknown, however the key features of disease pathology include upregulated inflammatory process due to secretion of chemokines and cytokines by intestinal epithelial and resident immune cells. It suggests that IBD is chronic inflammation of gastrointestinal tract. The CaCo-2 cells are intestinal epithelial cells that grow by forming tight junction and resemble the enterocyte cells of colon. Accordingly, they are widely used in *in-vitro* IBD studies [14]. During IBD, disruption of epithelial tight junction increases the epithelial cell permeability which results in movement of luminal bacterial product into lamina propia layer of intestine where immune cells resides. This lead to immune cell activation and excessive release of cytokines and chemokines leading to chronic inflammation [15]. Hence, maintenance of tight junction and reducing inflammation is crucial to prevent the progression of IBD. Several synthetic drugs including anti-inflammatory drugs such as corticosteroids and amino salicylates, immunosuppressant drugs such as azathioprine and methotrexate are available to decrease disease severity, but they are associated with several side-effects. Alternatively, acacetin has recently

been observed to have potent anti-oxidant, anti-cancer and anti-inflammatory properties [16]. Therefore, we have evaluated the anti-inflammatory effects of acacetin in an *in-vitro* IBD model.

First, we examined whether acacetin is toxic for CaCo-2 cells. Our results showed that acacetin (0-10 µM) did not cause cell death and there was no change in total cell number after 24 h. Further, microscopic image revealed that tight junction between cells remained intact and thus acacetin was non-toxic to CaCo-2 cells in culture. Hence, acacetin (10 µM) could be safe to use for further IBD study.

IL-1β is an important pro-inflammatory cytokine which is elevated in IBD patients [4]. Increased production of COX-2 enzyme is observed in intestine of IBD patient and inflamed tissue [8], hence, it is considered as an important molecular target for IBD and colitis associated cancer. COX-2 enzyme converts the arachidonic acid present in cell membrane into prostaglandins (PG) and thromboxanes. Several studies has shown that among various prostaglandins, PGE_2 plays important role in inflammation and tumor initiation [18]. Our results showed that acacetin significantly decreased the level of COX-2 expression in IL-1β induced CaCo-2 cells. Further, acacetin also inhibited the activity of COX-2 enzyme as observed in terms of decreased PGE-2 levels. IL-1β treatment lead to significant increase in secreted PGE-2 level which was significantly reduced when treated with acacetin. Hence, acacetin decreased both expression as well as activity of COX-2 enzyme indicting its potent anti-inflammatory property.

Studies has shown that IL-1β increases the severity of IBD either by compromising intestinal tight junction or by activating redox sensitive NF-κB pathway [17]. We thus next assessed transcriptional regulator of COX-2 i.e. NF-κB, which remains sequestrated in cytoplasm in normal conditions. When activated, the active subunits translocate to nucleus and start the transcription of pro-inflammatory genes including cytokines, chemokines and inflammatory mediator such as COX-2. In our study IL-1β induction resulted in increased level of NF-κB p50 subunit which in normal condition remains as inactive 120 kDa protein. Acacetin treatment resulted in significant decrease in activation of IL-1β-induced NF-κB p50 subunit. Although, p50 subunit alone does not have trans-activation domain but it forms heterodimer with p65 and Rel A subunit and causes transcription of cytokines and chemokines resulting in inflammation. Our results, thus confirmed the anti-inflammatory potential of acacetin in IL-1β-induced CaCo-2 cells through regulation of NF-kB pathway that leads to transcription of pro-inflammatory enzyme COX-2.

CONCLUSION:

Overall, our results showed that acacetin could be a potent anti-inflammatory agent and may be useful in the treatment of IBD as well as in the primary chemoprevention of colitis-associated colorectal cancer by exerting its inhibitory effect on NF-κB pathway and its target inflammatory genes COX-2.

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