Abstract

Introduction
In the setting of inflammatory bowel disease, curcumin supplementation to prevent inflammatory flares has shown promise. However, the outcome of curcumin exposure, when there is existing inflammation, is not clear. The aim of this study was to compare the anti-inflammatory properties of curcumin when added at differing times to an inflammatory stimulus, in an established in vitro intestinal epithelial cell model of intestinal inflammation.

Materials and Methods
HT-29 and INT407 cells were incubated with a range of concentrations of curcumin prior to, or at the same time as, the addition of tumour necrosis factor-α. Following incubation, cell viability, interleukin-8 levels and cytoplasmic inhibitor of kappa B (IkB) were assessed.

Results
High concentrations of curcumin reduced epithelial cell viability. At lower concentrations, curcumin had no effect on cell viability; however, curcumin (with or without pre-incubation) reduced interleukin-8 levels and inhibited the phosphorylation and degradation of IkB.

Conclusion
Curcumin can reduce interleukin-8 and IkB response to tumour necrosis factor-α in an in vitro model of intestinal inflammation. This response is not dependent on curcumin pre-incubation, but does depend on curcumin concentration. These findings support the role of curcumin as an anti-inflammatory therapy and suggest it may be effective in both reducing existing inflammation and preventing inflammatory relapse. Further research is needed to assess optimal in vivo curcumin dosing.

Introduction
Turmeric (the common name for Curcuma longa) is an Indian spice that belongs to the ginger family. In ancient times, turmeric powder was utilised as a traditional and natural remedy for various health conditions, such as joint pain, ulcers, liver disease, wounds and skin diseases. The active ingredient of turmeric is curcumin with chemical name diferuloylmethane. Curcumin exhibits anti-microbial, anti-inflammatory, anti-oxidant and anti-neoplastic properties, and has been extensively investigated for its proposed benefits in managing chronic inflammatory conditions. In inflammatory bowel disease (IBD), curcumin is proposed as an attractive alternative therapy to the conventional medicines. Curcumin is considered to be safe and inexpensive and emerging evidence from preclinical studies indicates the potential benefits of curcumin supplementation in IBD. Clinical trials of curcumin have included a pilot study involving 5 patients with chronic ulcerative proctitis and 5 patients with Crohn’s disease (CD). Curcumin was administered for 2 months to patients with proctitis and for 3 months to the patients with CD. All patients with proctitis experienced symptom relief, which was consistent with a significant reduction in inflammatory indices; in addition, four out of five patients were able to reduce concomitant medications. Four of the CD patients who completed the study showed a significant drop in disease activity and erythrocyte sedimentation rate.

Discussion
In these studies, curcumin-treated mice exhibited symptomatic relief, improved survival rates and decreased inflammatory indices. Addition of curcumin to mice diets resulted in a significant amelioration in the histological intestinal injury seen in the inflamed mucosa and lower mucosal cytokine levels.

However, the role of curcumin in the management of active inflammation is less clear. The aim of this study was to investigate whether timing of exposure to curcumin and/or curcumin concentration influenced the inflammatory response in an in vitro model of intestinal inflammation.
Materials and Methods
The protocol of this study has been approved by the relevant ethical committee related to our institution in which it was performed.

Cell culture and induction of inflammation
HT-29 cells (ATCC HTB-38) were seeded in 24-well plates (Becton Dickinson, NSW, Australia) at a concentration of 5x10^3 cells per well and maintained in McCoy's 5A medium (Gibco®, Invitrogen, Victoria, Australia) containing 10% foetal bovine serum (FBS: Gibco®, Invitrogen) and 100 U/mL penicillin/streptomycin (Gibco®, Invitrogen). INT407 (HeLa derivative) cells were also grown in 24-well plates and maintained in Basal Medium Eagle (Gibco® Invitrogen) containing 10% FBS and 100 U/mL penicillin/streptomycin. Cells were incubated at 37°C with 5% CO₂, with media changed every alternative day. Experiments were conducted after 5 days of incubation or if 90% confluence was reached. Inflammation was induced by exposing cells to 50 ng/ml tumour necrosis factor-alpha (TNF-α; Gibco®, Invitrogen) and incubating HT-29 cells for 6 hours or INT407 cells for 24 hours.

Curcumin treatment protocol
Curcumin was first dissolved in dimethyl sulfoxide (DMSO: Sigma-Aldrich, NSW, Australia), then mixed with a culture medium to give a final concentration of 10, 25, 50, 60, 75 or 100 µM curcumin with a final DMSO concentration of 0.1% v/v in culture media.

Assessment of cell viability by trypan blue exclusion
Following experimentation, cells were washed twice with warm phosphate-buffered saline (Gibco®, Invitrogen), then incubated with trypsin- Ethylenediaminetetraacetic acid (EDTA) (Gibco®, Invitrogen) 0.5 mL per well for 15 minutes. Warm PBS (0.5 mL per well) was then added to inhibit trypsin. A total volume of 1 mL of cells in suspension was transferred to a separate tube and centrifuged at 1200g for 5 minutes. Supernatant was discarded and the pellet containing the cells was resuspended in warm media gently. Equal volumes of cells (25 µL) and trypan blue (Sigma-Aldrich) (25 µL) were mixed and cell viability counts were conducted using a haemocytometer and a light microscope. The viability was expressed as the percentage of unstained cells (viable) among the total cells.

Assessment of cell viability by MTT colourimetric assay kits
The assay was conducted according to the manufacturer's instructions (Sigma-Aldrich). In brief, after 24 hours of incubation with curcumin, culture media was replaced with 1 ml per well phenol red-free McCoy’s 5A medium (Banksia Scientific Company, QLD, Australia). One hundred microlitres of MTT (3-[4,5-Dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide; Thiazolyl blue) solution (5 mg/ml) was added to each well of cells and incubated further for 4 hours at 37°C with 5% CO₂. In metabolically active cells, the MTT was converted to an insoluble purple formazan by cleavage of the tetrazolium ring by mitochondrial dehydrogenase enzymes. Following the incubation, the dye was solubilised in DMSO and 200 µl of the mixture was transferred to a well of a 96-well plate. Well absorbance was read at a wavelength of 570 nM by a Micro-plate reader (Bio-Rad, NSW, Australia). The amount of converted dye, representing metabolic activity, was determined by comparing to a standard curve.

Enzyme-linked immunosorbent assay to measure interleukin-8
The concentration of interleukin (IL)-8 in culture supernatant was measured using an enzyme-linked immunosorbent assay (ELISA) kit, according to manufacturer’s protocol (Novex-Invitrogen, Victoria, Australia). Briefly, following experiments, cell culture supernatants were collected and tested in duplicate. Samples were added to 96-well microtitre plates (MaxisorpNunc, Victoria, Australia) coated with monoclonal IL-8 antibody and detected with biotinylated secondary antibody and streptavidin-horseradish peroxidase (HRP) conjugate. After development of the colourimetric reaction using tetramethylbenzidine substrate (Thermo-Fischer Scientific, Victoria, Australia), the reaction was stopped with the addition of 1.8 N H₂SO₄. Absorbance was measured at 450 nm by a Micro-plate reader (Bio-Rad). Absorbance readings were then converted to picograms per millilitre based on standard curves obtained with the recombinant cytokine (lower detection limit of assay was 31.5 pg/ml).

Western blot analysis of 1xB
HT-29 cells were seeded in a 6-well plate at a concentration of 10⁴ cells per well and grown for 5 days before experiments. For some experiments, cells were treated with 50 µM curcumin for 1 hour before or at the same time during the administration of 100 ng/ml TNF-α. Cells were then incubated with TNF-α for 5, 15 and 30 minutes. Proteins were extracted from cells using radioimmuno precipitation assay buffer (150 mM sodium chloride, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 50 mM Tris, pH 8.0) containing 10 mM NaF and a mixture of protease inhibitors (2 µg/ml Aprotinin, 10 µg/ml Leupeptin and 1 µg/ml Pepstatin A). Cell lysates were centrifuged at 12,000 g for 10 minutes, and supernatants were collected. The total protein concentration of the cell lysates was measured by the bicinchoninic acid method (Micro BCA protein assay reagent kit; Pierce Biotechnology-Thermo-Fischer Scientific). Equal amount of protein (40 µg) for all samples was loaded and run onto Tris-Glycine NB...
10% gel (NuSep Ltd, NSW, Australia) for 1 hour. Proteins were then transferred onto PVDF nitrocellulose membranes using the Trans-Blot Turbo Transfer System (Bio-Rad Co), and then blocked with 5% bovine serum albumin in Tris-buffered saline solution with 0.1% polysorbate 20 (Tweeen 20; Sigma-Aldrich). Separate membranes were probed with the primary antibodies rabbit anti-IκB and anti-phosphorylated IκB (1:1000 dilution) for overnight incubation at 4°C (Abcam, Cambridge, UK); and total protein levels were assessed with anti-β-actin antibodies (Abcam, 1:1000 dilution). Subsequently, membranes were incubated with the secondary antibody goat anti-rabbit IgG (Bio-Rad, 1:25000 dilution) conjugated with horseradish peroxidase (HRP) (Bio-Rad) for 1 hour at room temperature before bands were detected by chemiluminescent detection method using the Immun-Star HRP Chemiluminescent Substrate Kit (Bio-Rad) and visualised by GelDoc (Bio-Rad).

Statistical analysis
GraphPad Prism software (version 6.0 for windows; GraphPad Software, San Diego, CA, USA) was used for statistical analysis. Results are presented as mean standard errors of mean (SEM). One-way ANOVA test with a Ficsher’s least significance post-test was used for analysis. A p-value of $< 0.05$ was considered as statistically significant.

Results
Curcumin reduces epithelial cell viability at high concentrations
HT-29 and INT407 cells were treated with increasing concentrations of curcumin (0, 10, 25, 50, 75 and 100 µM) in DMSO for 24 hours with cell viability measured. Additional DMSO only controls (cells exposed to 50 ng/ml TNF-α and DMSO without curcumin) were included. The final concentration of DMSO for all experiments remained constant at 0.1% v/v, which did not have an effect on cell viability (Figure 1). Increasing concentrations of curcumin, (up to 50 µM) had no significant effect on cell viability, with viability remaining above 90% for both the cell lines ($p > 0.05$) (Figure 1). However, curcumin concentrations of 75 µM and above significantly decreased cell viability ($p < 0.05$ for both 75 and 100 µM curcumin) (Figure 1).

Cell viability results were further supported by the MTT assay experiments that measured the mitochondrial dehydrogenase enzyme activity of the viable cells. HT-29 cells were treated with increasing concentrations of curcumin dissolved in DMSO (0, 10, 25, 50, 60, 75 and 100 µM) for 24 hours with cell activity measured. The final concentration of DMSO for all experiments including 0 µM curcumin treatment group (DMSO-control group) remained constant at 0.1% v/v. Curcumin, up to 50 µM concentrations, showed an increase in the mitochondrial dehydrogenase enzyme activity, while those treated with 60 µM or higher concentration of curcumin exhibited a significant drop in the activity as compared to the control group ($p < 0.05$ for 60, 75 and 100 µM treated groups) (Figure 2). Therefore, for further experimentation, curcumin concentrations of up to 50 µM were used.

Curcumin inhibits IL-8 production from TNF-α-exposed HT-29 and INT407 cells
Curcumin exhibited a strong anti-inflammatory effect in response to TNF-α exposure of intestinal epithelial cells. In HT-29 cells, curcumin at low concentration (10 µM) in all the indicated pre-incubation time points (24, 6, 1 and 0 h) had no significant effect ameliorating IL-8 production from the cultured cells ($p > 0.05$ in all treated groups vs positive control group) (Figure 3A). However, at higher concentrations (25 and 50 µM), curcumin did significantly attenuated IL-8 levels in a dose-dependent fashion. Cell exposed to 25 µM curcumin showed significant reductions in IL-8 levels compared to the positive control ($p < 0.01$) (Figure 3B). Interestingly, IL-8 levels were not significantly different between cells with varying curcumin pre-incubation time ($p > 0.05$ for all comparisons at 24, 6, 1, and 0 hours) (Figure 3B), but by increasing curcumin concentration to 50 µM, the strongest anti-inflammatory response was observed with maximal reduction in IL-8 levels ($p < 0.001$ for all comparisons to positive controls;
Cellular activity assay of curcumin-treated cells. HT29 cells were exposed to curcumin at 0, 10, 25, 50, 60, 75 and 100 µM dissolved in DMSO (0.1% v/v in culture media) and incubated for 24 hours. Media was replaced with phenol red-free media and MTT substrate, incubated further and absorbance was read at 570nM (*p < 0.01; **p < 0.001 vs. 0 µM treated group).

Discussion

These in vitro investigations have demonstrated that curcumin can prevent TNF-α-mediated production of IL-8 in human intestinal epithelial cells. Further, inhibition of IL-8 was comparable whether cells were pre-incubation with curcumin or if cells were exposed to curcumin at the same time as TNF-α administration. These results also indicated that curcumin impaired the degradation of IκB and thereby modulated the nuclear factor (NF)-κB signal transduction pathway.

In numerous experimental murine models of colitis, there is evidence that curcumin has potential therapeutic and preventive benefits that may be applicable in the treatment of IBD. Previously, using the human intestinal cell line (HCT116, HT29 and CaCO₂), it was shown that curcumin pre-treatment inhibited and modulated gene expression of IL-8 in response to inflammatory cytokine exposure. However, it was not investigated whether curcumin had a similar effect if given simultaneously with inflammatory inducers. The results presented in this paper indicate that curcumin pre-incubation had no measurable additional benefit compared to when curcumin was given with the inflammatory stimulus. However, curcumin concentrations did significantly affect IL-8 suppression. This finding indicates that curcumin concentration, but not the timing of supplementation, likely determines the efficacy of curcumin in reducing inflammation.

Curcumin inhibits the activity of cyclooxygenase, lipoxygenase and inducible nitric oxide synthetase enzymes; thereby reducing pro-inflammatory cytokine production and each time point following TNF-α exposure, which was consistent with less phosphorylated IκB detected (Figure 5). There was no difference in the IκB response whether or not cells were pre-incubated with curcumin prior to TNF-α exposure.
down-regulating mitogen activated and Janus kinases21,25–27. These effects are primarily a result of influencing the NF-κB signal transduction pathway21,25–27. The NF-κB/Rel family of transcription factors plays a role in regulating the expression of a large number of genes, particularly those determining immune functions and acute-phase reactions28. Inhibition of NF-κB is of particular importance and is considered a putative target for intervention in IBD29,30. In unstimulated cells, most of the Rel/NF-κB factors are sequestered in the cytoplasm as inactive complexes by association with a group of inhibitory proteins (IκB)23. Once phosphorylated, IκB dissociates from the NF-κB complex and permits the subsequent translocation of NF-κB to the nucleus32.

Thus, activity of the NF-κB pathway is determined by the state of the IκB protein. In this paper we report that a phosphorylated IκB band was evident shortly after the addition of TNF-α, which was consistent with the initial drop in IκB. Curcumin, with or without pre-incubation, appeared to prevent the rapid and initial loss of IκB. The lack of IκB degradation in curcumin-treated cells indicates inhibition of NF-κB signal transduction. A previous report involving an intestinal epithelial cell line indicated that curcumin treatment blocks IL-8 expression through inhibition of NF-κB signal transduction33. Of interest, in that study, curcumin was pre-incubated with cells prior to exposure to inflammatory mediators. However, in the present study results have indicated that curcumin inhibits IκB degradation whether given prior to or at the same time as an inflammation inducer. This suggests that curcumin gains rapid entry into the cells and become active and does not need to be metabolised to be activated.

It is important to note that, according to these experiments curcumin may have a narrow safe window. Cells treated with curcumin concentration greater than 50 µM had a significant drop in cell viability that was consistent with the reduction in the mitochondrial dehydrogenase enzyme activity. In contrast clinical trial has reported that patients were able to tolerate up to 8g of curcumin daily for 3 months with no reported toxicity34. This apparent discrepancy is likely due to poor pharmacokinetic properties of curcumin, including very low bioavailability, poor absorption and rapid metabolic elimination35 when curcumin is delivered in the absence of a vehicle. Another study has found that the highest achieved peak serum concentration of curcumin in the peripheral blood was only 139 nM measured 4 hours after a single oral dose of 12 g curcumin35. Further, limited transepithelial flux and rapid metabolism in the gut epithelium are likely to impair curcumin exerting its beneficial effect locally on gut epithelium. Thus, this may explain why curcumin has shown better outcomes in both in vitro and ex-vivo settings than in clinical trials and may also explain the lack of toxicity despite high doses. Therefore, to improve clinical outcomes and to fully utilize the properties of this agent, future clinical application of curcumin may benefit from further investigation of curcumin solubility and targeted delivery of curcumin to the gut. However, improving delivery in vivo must also be done cautiously and with concurrent investigation of cell toxicity.

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Figure 4: Effect of curcumin on IL-8 production from INT407 cell line in response to TNF-α stimulation. INT407 cells were grown to confluence and pre-treated with varying concentrations of curcumin (10 [A], 25 [B] and 50 [C] µM) for varying times (24, 6, 1 and 0 [D] h). TNF-α (50 ng/ml) was added at time 0 and incubated for a further 6 h. Supernatants were collected and assayed for IL-8 by ELISA. [Analysis of data was conducted using one-way ANOVA test followed by Fischer’s least significance (*p < 0.001; **p < 0.01, ns > 0.05 vs. positive control group)].

Figure 5: IκB response in HT29 cells. Confluent cells were treated with DMSO-dissolved curcumin either for a 1-hour pre-incubation or at the same time as TNF-α exposure. Following TNF-α (100 ng/ml) exposure, cells were incubated for 5, 15 and 30 minutes, then cell lysates were collected. IκB levels in lysates were analysed by Western blot technique using anti-IκB (37 KDa) and anti-phosphorylated IκB (40 KDa), and total protein levels were assessed with anti-β-actin antibodies (40 KDa). Curcumin treatments with or without pre-incubation block IκB phosphorylation and thereby its degradation in TNF-α-stimulated HT29 cells.

Limitations of this study are related to the use of HT-29 and INT407 epithelial moolayers. HT-29 cells are tumour-derived colonic epithelial cells and INT407 cells are human embryo small intestinal epithelial cells. These cell lines provide an approximation of the human gut epithelial surface, but may not precisely reflect normal human intestinal physiology in vivo. However, due to their critical role in the gut mucosal immune response, epithelial cell lines are very often used as an in vitro model to reflect in vivo events. Intestinal epithelial cells act as a protective barrier against different invasive infectious agents, toxins and antigenic factors within the gut lumen, and are also in contact with cytokines secreted by other cells in the intestinal mucosa. In addition, intestinal epithelial cells secrete numerous cytokines involved in intestinal injury and damage. Thus, the in vitro results presented here can be of value when contemplating in vivo curcumin supplementation.

Conclusion

In summary, curcumin prevented and ameliorated TNF-α-induced inflammation in two intestinal cell lines. This action was mediated through inhibition of IκB degradation, and therefore the NF-κB signal transduction pathway is also blocked in a similar way as with or without pre-incubation. This study further adds support that curcumin has the potential to improve intestinal diseases associated with inflammation such as IBD. This study suggests that curcumin does not likely need to be metabolised and is active in its natural state. Therefore, curcumin supplementation may be useful to both prevent inflammatory relapse episodes and treat current inflammatory flare. However, curcumin concentration and delivery method are likely to be essential for the successful therapeutic utilisation of curcumin, and definitive clinical studies

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are now required to establish the optimal dose and delivery regimen to ensure efficacy and safety.

**Abbreviations list**

- CD, Crohn's disease; DMSO, dimethyl sulfoxide; EDTA, Ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; FBS, foetal bovine serum; HRP, horseradish peroxidase; IBD, inflammatory bowel disease; iKB, inhibitor of kappa B; NF-κB, nuclear factor-κB; SEM, standard errors of mean; TNF-α, tumour necrosis factor-alpha

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**References**

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Research study

Therapeutic and preventative anti-inflammatory benefits of curcumin in vitro.


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