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Flaxseed extract exhibits mucosal protective effect in acetic acid induced colitis in mice by modulating cytokines, antioxidant and antiinflammatory mechanisms

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ABSTRACT

New treatments for inflammatory bowel disease are of interest due to high rate of remission failure. Natural products have been effective in IBD therapeutics as they have multiple constituents. The aim of the present study was to evaluate the effect of Flaxseed extract (Fs.Cr) on ulcerative colitis and identify the possible mechanisms involved. Colitis was induced by intrarectal administration of 6% AA in BALB/c mice. Colonic mucosal damage was assessed after 24 h by calculating disease activity index (DAI), macroscopic and histological damage scores, biochemical measurement of myeloperoxidase (MPO), malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), and total glutathione activities. Since cytokines are involved in exacerbating inflammatory cascade with emerging role of innate immune cytokines in IBD therapeutics, we hence assessed the effect on the levels of TNF-α, IFN-γ and IL-17, at 6, 12 and 24 h by ELISA.
Fs.Cr ameliorated the severity of AA colitis as evident by improved DAI, macroscopic damage and the histopathological scores along with restoration of goblet cells. Fs.Cr decreased MDA and MPO activities and enhanced antioxidant activity compared to the AA group. Finally, Fs.Cr in doses (300 and 500 mg/kg) decreased TNF-α and IFN-γ levels at all time points with simultaneous increase in IL-17 levels at 24 h as compared to the AA group.
These results suggest that Fs.Cr ameliorates the severity of AA colitis by reducing goblet cell depletion, scavenging oxygen-derived free radicals, reduce neutrophil infiltration that may be attributed due to decreasing IFN-γ and TNF-α and increasing IL-17 levels.

1. Introduction

Inflammatory bowel disease (IBD) comprises of Ulcerative colitis (UC) and Crohn's disease (CD) that manifests in a genetically susceptible host when exposed to either microbes or other environmental factors. CD and UC were considered prototypically as T helper (Th)1 or Th2-associated disease respectively, although recently Th17 cells have been identified as the common player in both disease phenotypes [1]. Traditionally it has been believed that the adaptive immune system is the key contributor to the pathogenesis of IBD [2]. However, the recent discovery that cells mediating innate immunity are altered in IBD patients [3] along with proven efficacy of anti-TNF-α drugs in UC [4]; a crucial role for defective innate immunity has also been established for therapeutic interventions.

Innate immune pathogenesis causes activation of the inflammatory cytokine cascade that leads to chemotaxis and reactive oxygen metabolite (ROM) cascade. This further causes oxidative stress, resulting in further aggravation of the inflammation initiated tissue damage [5]. The cytokine most commonly involved in innate immune pathogenesis of IBD are tumour necrosis factor (TNF) -α, interferon (IFN) -γ, interleukin (IL) -8, IL1-β and IL-12. Additionally, IL-17A released from innate lymphoid cells (ILCs) [3] has a controversial role as an effector or protector. Some studies have shown its pathogenic role [6], however treatment with their antibodies in CD patients was not very effective [7]. On the contrary, IL-17A has been reported to have a protective role in different models of IBD [8–10]. IFN-γ, another important cytokine has an established pro-inflammatory role in the pathogenesis of IBD. Surprisingly, the clinical trials with IFN-γ antibody were also not promising,

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hence suggestive that combating a single cytokine may not be able to control diseases effectively as compared to animal studies where single anti-cytokine therapies have proven effective. This led to testing the combination therapy of TNF-α and IFN-γ antibodies or other combination of cytokines as an alternative approach to target the disease [11].

However a limitation of cytokine therapy is that it possesses serious side effects that require extensive monitoring, especially in developing countries that have burden of tuberculosis and hepatitis, which can be reactivated as a result of immune suppression. [12]. That's the reason why cytokine therapy are usually kept as a third line of action when the escalating response is required, despite being the most effective in the available treatment options [13].

Recently, there has been a lot of interest generated in alternative and complementary medicine for IBD [14]. Natural products hold a lot of promise as they are mostly in common use and are perceived as relatively safe when taken along regular medications. Natural products have been tried in clinical trials in IBD patients based on their successful effect in different experimental settings. The use of natural products have proven effective in IBD therapeutics, for example, curcumin capsules improved patient symptoms as well as decreased the duration of corticosteroid therapy [15] whereas in active mild to moderate UC patients along with amino salicylic acid, it induced remission in 4 weeks as compared to none of the patients receiving placebo only [16].

In the traditional Iranian medicine, Flaxseed is documented to have a beneficial role in IBD [17]. We have several evidences that prompted us to hypothesize that Flaxseed is effective in IBD. Those evidences include antispasmodic activity mediated via phosphodiesterase 4 (PDE-4) enzyme inhibition, antibacterial activity against wide range of Gram negative gut pathogens especially enteropathogenic *Escherichia coli*, entero toxigenic *E. coli* and enteroaggregative *E. coli* (implicated in IBD) [unpublished] antidiarrheal antispasmodic activities [18]. Hence we hypothesized that methanolic aqueous (70:30) extract of Flaxseed (Fs.Cr) is effective in mice model of colitis. The primary objective therefore was to assess the effectiveness in acetic acid (AA) induced colitis model in mice. In order to evaluate effectiveness we tested the experimental animals for key manifestation of IBD that is antiinflammatory and antioxidant effects with focus on modulation of multiple cytokines- as this is the current advancement in the field of IBD. AA colitis model was used because it is an easy and reproducible method in laboratory for induction of colitis and resembles human features of UC [19] with alteration of cytokines causing inflammation and oxidative stress.

2. Materials

2.1. Reagents

Acetic acid, prednisolone, periodic acid, Schiff reagent and DPX were purchased from Sigma. DMSO and Tween-80 were purchased from Scharlau chemicals. MPO, Lipid peroxidation (MDA); glutathione peroxidase and catalase activity assay kits were purchased from Biovision whereas SOD and glutathione assay kits were purchased from Sigma. Elisa kits and tissue extraction reagent for cytokines estimation were purchased from Invitrogen, Life sciences. Cenogenics single strip kit for occult blood test. Sigma Fast Protease inhibitor tablets were purchased from Sigma.

2.2. Preparation of the crude extract of flaxseed (Fs.Cr)

Linum usitatissimum L. (Flaxseed) was purchased from an authentic herb supplier in the local market of Karachi, Pakistan. The plant name and physical characteristics matched the description when checked with http://www.theplantlist.org. It appeared as deep brown flat and oval seed with a pointed tip, and measured about 4 mm. A sample of the plant material was deposited to the herbarium of the Natural Products Research Unit at the Department of Biological and Biomedical Sciences of The Aga Khan University, with a voucher number LU-SE-0812-106. The seeds were made free of dirt and other adulterants and were ground to coarse powder by an electrically driven mill. Approximately 1 kg ground seeds were soaked in the aqueous-methanol (30:70 v/v) at room temperature for three days with occasional shaking [20]. It was filtered through a double layered muslin cloth and subsequently through a filter paper. The residue was re-soaked in the fresh solvent and the process was repeated twice to get maximum yield of crude extract from the plant material. The combined filtrate was concentrated in a rotary evaporator at 40 °C under reduced pressure (−760 mm Hg) to a thick, semisolid, dark brown colored mass, labeled as the crude extract of Flaxseed (Fs.Cr), with approximated yield of 8% and was stored at −20 °C until used. The extract was tested for bacterial contamination in vitro and for pyrogen activity in vivo. No bacterial growth were observed when extract was plated on nutrient agar plate and grown aerobically at 37 °C in an incubator for 16 h. Animals were then administered the extract i.p. for 10 days to observe for pyrogen activity. No toxicity signs were observed in animals indicating that the extract was pyrogen free.

2.3. Working stocks

For the in vivo and ex vivo experiments, the extract was solubilized in 10% DMSO–5% Tween-80 and subsequent dilutions were made in distilled water on the day of administration. For the sham control animals, 10% DMSO-5% Tween-80 in distilled water was prepared. Prednisolone was dissolved in peanut oil. Acetic acid was diluted to 6% in normal saline.

2.4. Ethical statement

The study protocol (005-Ani-BBS-13) was approved by ECACU (Ethics Committee for Animal Care and Use) of The Aga Khan University, and experiments performed complied with the rulings of the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council [21]. Our study design is in compliance with ARRIVE guidelines and a checklist has been followed while designing the study [22].

2.5. Experimental animals and diet

6–8 weeks female BALB/c mice (22–30 g) were obtained and maintained in animal house of Aga Khan University (Karachi), in the rectangular cages ($47 \times 34 \times 18$ cm^3) with sawdust (changed at every 48 h). Animals were given standard diet consisting of flour (5 kg), bran (5 kg) molasses (150 g), salt (75 g), nutrivet L (33 g), potassium meta bisulphate (15 g), oil (500 g), fish meal (2.25 kg) and powdered milk (2 kg) for a total of 13 kg of the food material and maintained under standard conditions (temperature 22–25 °C, humidity 70–75%, lighting regimen of 12 hour light and dark cycle). Animals also had access to food and water ad libitum throughout the study except that food was withdrawn 24 h prior to experiments. Mice were killed after 24 h by cervical dislocation and colonic biopsies were taken for different purposes after assessing for disease activity index (diarrhea, weight loss and rectal bleed), macroscopic and microscopic damage.

2.6. Study design

There were total of 6 groups named as sham control, diseased control (AA group), treatment groups (Fs.Cr 150 mg/kg, 300 mg/kg and 500 mg/kg) and the positive control group (Prednisolone). Because of high mortality rate (20%–30%), 12 animals in each group (except the negative control) were selected. Studies with power calculation of 80% show that it will require at least 10 mice per group in order to guarantee that this effect is detected in 80% of cases with a p value of 0.05. The