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Studies on the effects of 4-hydroxy isoleucine in experimentally induced inflammatory bowel disease

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Abstract

Objective: The present study was designed to evaluate the effects of 4-Hydroxyisoleucine in experimentally induced Inflammatory Bowel Disease (IBD) in rats.
Method: Wistar Albino rats weighing about 150-250 gm were employed in the study for induction of acetic acid and indomethacin induced IBD and assessed for various parameters like body weight, colon and intestinal damage, oxidative stress, total proteins and inflammation.
Results: In this study 4-hydroxyisoleucine at two different doses was found to be successful in managing the serum and tissue homogenate changes in acetic acid induced UC and indomethacin induced CD possibly through its antioxidant and anti-inflammatory profile.
Conclusions: 4-hydroxyisoleucine proved its antioxidant and anti-inflammatory profile by reducing the oxidative stress and inflammation, further it also improved the integrity of intestine thus amino acid, 4-hydroxyisoleucine was successful in mediating the changes induced by acetic acid and indomethacin induced experimental IBD in rats.
Keywords: IBD, 4-hydroxyisoleucine, acetic acid, indomethacin.

1. Introduction

Inflammatory bowel disease (IBD) is referred as inflammatory and ulcerative disease of small and large intestines and comprises of two different but closely related diseases, Ulcerative colitis (UC) and Crohn's disease (CD) [1]. These are most common inflammatory disorders only after rheumatoid arthritis, with millions of patients all over the world [2]. The two forms have high rate of claiming lives as these disease increases the risk of adenocarcinoma of the colon/ileum in the affected areas. Indian population shows an incidence rate of 6.02 per 100,000 annually [3].

Initiation and progression of IBD is influenced by multiple immune, genetic and environmental factors [4-5]. Ulcerative colitis is a chronic disease of the large intestine/colon with slightly higher incidence than CD. Inflammation begins in the rectum and extends proximally in an uninterrupted fashion to the proximal colon and could eventually involve the entire length of the large intestine [6-7]. The inflammation of colon, mucous or pus formation, bloody stools abdominal pain and weight loss are some effects seen in UC [8]. There is involvement of reactive oxygen metabolites and inflammatory mediators such as cytokines, eicosanoids in the development and persistence of this disease [9-10].

Crohn's Disease or ileitis may affect any part of the gastrointestinal tract, from mouth to anus but usually involves either the colon or the ileum of the small intestine [11]. It spreads deep into the effected tissues [7]. Symptoms and signs arise from a robust, cytokine-driven inflammation of the gut [12]. The intestinal microbiota strongly plays a role in initiating and triggering the immune system, leading to characteristic inflammation [13]. The progression of inflammation to stricturing or fistuliziation seen in crohn's disease is also due to the influence of smoking [14]. Genetic component involved in CD is mutated gene that encodes NOD2 (nucleotide-binding oligomerization domain 2) protein [15-16]. Although CD and UC share many clinical and pathological characteristics, yet two diseases are distinct due to distinct immunologic phenotypes[17].CD is usually described as a prototypical T-helper (Th1) disease because the primary mediators of inflammation are the Th1, cytokines like interleukin 12 (IL-12), interferon-gamma (IFN-γ), and tumor necrosis factor- α (TNF-α). However, UC is often viewed as a Th2-type condition because of the reports of increased mucosal expression of the Th2 cytokine like interleukin -5 (IL-5)[18-19].

Several antibiotics and vaccines can cause damage to the intestinal mucosa of the stomach, small bowel and colon [4]. Induction of IBD in animal models are carried by nutritional depletion, application of toxins, vasoconstriction induced by adrenaline, several chemicals like trinitrobenzene, sulfonic acid, alcohol. Non-steroidal anti-inflammatory drugs (NSAIDs), too cause ulceration [20]. NSAIDs such as indomethacin and aspirin can cause gastrointestinal injury regardless of route of administration [21]. Indomethacin, a non-selective COX inhibitor produces enterocolitis [22]. Indomethacin

develops acute intestinal inflammation, manifested by a thickening of the bowel wall, mesenteric haemorrhage, mesentery adhesion and multiple mucosal ulcers of small intestine and colon [23]. Several studies demonstrated different mechanisms followed by indomethacin for generating colitis, i.e, inhibition of the protective prostaglandins PGE1, PGE2 and prostacyclin [24], contribution of luminal bacteria and their products [25], indomethacin induced apoptosis of the intestinal epithelial cells accompanied by an increased production of reactive oxygen species (ROS) [26-27]. Recent studies showed that indomethacin treatment induced oxidative stress in rat gastric mucosa by the irreversible inactivation of gastric peroxidase [28], high iNOS mRNA expression and excessive interleukin-18 (IL-18) formation [29]. TNF-α also participate in indomethacin-induced small intestinal damage [30]. CRP is an inflammatory marker, observed to be high in indomethacin treated animals [31].

Acetic acid can cause non-trans mural inflammation, which is characterized by increase in neutrophil infiltration into the intestinal tissue [32-33]. Neutrophils release proteases and lipid mediators that contribute to intestinal injury [34]. Inflammation following acetic acid instillation was characterized by edema, diffuse inflammatory cell infiltration and necrosis. The colonic inflammation induced by acetic acid was result of significant increase in TNF-α levels and NOS activity [35]. As UC and CD are due to inflammation and oxidation therefore, the herbal plants, plants extracts and their phytoconstituents, those which exhibit anti-inflammatory and antioxidant property are now a day evaluated against IBD.

Fenugreek (*Trigonella foenum graecum*) belongs to the family *Leguminosae* [36]. It is well known spice used in world cuisine due to its flavor and nutritional value. The seeds and extracts of *Trigonella foenum* are having well established anti-diabetic and hypocholesterolaemic effects [36-37], used in treatment of arthritis, asthma and bronchitis [38]. Fenugreek seed extract is beneficial in management of neuropathic pain [39]. Polysaccharide gel and flavonoid composition of fenugreek seeds exhibit antisecretory and gasoprotective activity [40]. Beside this Fenugreek constituents are useful in improving digestion, maintain a healthy metabolism, gastric mucosal ulcer in sore throat and cure acid reflux. Fenugreek contains active constituents such as alkaloids, saponins, flavonoids and mucilaginous fiber [41-42]. It contains 4-hydroxyisoleucine, lysine, L-tryptophan, diosgenin, yamogenin, tigogenin and neotigogenin, trigonelline, galactomannans. 4-hydroxyisoleucine constitutes about 80% of total content of free amino acid in *Trigonella foenum graecum* [43]. 4-hydroxyisoleucine is a type of isomer, an atypical action [44]. 4-hydroxyisoleucine has effect on glucose and lipid metabolism and can be used for control of type- II diabetes, obesity and dyslipidemia [45]. 4-hydroxy isoleucine exhibited antidepressant effect by increasing turnover of serotonin in the brain [43]. Although Fenugreek has efficiency to decrease the values of gastric ulcers, volume of gastric juice and total acidity [42], but the effect of 4-hydroxyisoleucine is still unknown on ulcers. Therefore, the present work was designed to study the effects of 4-hydroxyisoleucine in experimentally induced Inflammatory bowel disease in rats.

2. Materials and Methods

The experimental protocols employed in the present study were approved by the 'Institutional Animal Ethics Committee' in accordance with the guidelines given by the 'Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA)' New Delhi, India (RITS/IAEC/2014/04/04). Wistar Albino rats weighing about 150-250 gm were employed in the present study. The rats were acclimatized in the institutional animal house and maintained on rat chow (Ashirwad Industries, Mohali, India) and tap water. Rats were allowed for ad libitum access to food and water. They were exposed to normal day and night cycles. CPCSEA registration no. of College– 888/PO/Re/S/05/CPCSEA.

2.1. Drugs and Chemicals

Sulfasalazine was obtained as a gift sample from Posh Chemicals Pvt. Ltd., Hyderabad, which was given as a suspension in 0.5% carboxy methyl cellulose (0.5% CMC). Indomethacin, as a marketed product of micro labs, Banglore was dissolved in 5% of sodium bicarbonate (NaHCO3). Acetic acid was purchased from Rankem, New Delhi, India and, reduced glutathione (GSH) were purchased from SD Fine-Chem Ltd. (SDFCL), Mumbai. 5, 5'-Dithio-bis (2-nitrobenzoic acid) (DTNB) was purchased from Sigma Aldrich, St Louis, MO, USA. Thiobarbituric acid (TBA) was purchased from Otto Chemika- Biochemica, Mumbai, India.

2.2. Inflammatory Bowel Disease animal models

2.2.1. Induction of Ulcerative Colitis in Rats:

Acetic acid-induced colitis in the rat was established using the modification of method described by Millar *et al*., 1996 [46]. The rats were fasted for 24 hr with access to water *ad libitum*. Each rat was lightly anaesthetized with diethyl ether, and a polyethylene catheter of 2 mm diameter (Paramount Surgimed Ltd., New Delhi, India) was inserted through the rectum into the lumen of the colon and advanced so that its tip was 6-8 cm proximal to the anus. Initially, each rat was lavaged with 2 ml of saline for enema followed by manual palpation of the abdomen to remove the fecal matter, if any. 2 ml of 3% acetic acid in saline was instilled into the colon lumen through the rubber catheter, and the rat was maintained in a head-down position for 30 seconds to limit the expulsion of solution, after which the fluid was withdrawn. 4-Hydroxy

isoleucine and sulfasalazine treatment was given orally 24 hrs. after acetic acid administration and continued for 7 days. On the 8th day all the rats were sacrificed and their distal colon was removed for the evaluation of macroscopic, microscopic and biochemical parameters.

2.2.2. Induction of Crohn's disease in rats:

Indomethacin induced enterocolitis in the rat was established using the modification of method described by Millar *et al.*, 1996. 4-Hydroxy isoleucine and sulfasalazine treatment was given orally for 7 days. On the 7th day, after 1 hour of treatment with sulfasalazine and 4-hydroxyisoleucine, indomethacin was given subcutaneously. Animals were sacrificed on 8th day and their distal duodenum was removed for the evaluation of macroscopic, microscopic and biochemical parameters [46].

2.3. Experimental Protocol:

In the present investigation, the rats were randomly divided into 10 groups. Each groups comprised of 6 rats. Pre and post treatment with 4-hydroxy isoleucine was carried in indomethacin and acetic acid groups respectively.

Group I (Normal group): Rats were maintained on standard food and water and no treatment was given.

Group II (Acetic acid group): Rats were administered single dose of acetic acid (2 ml of 3% v/v in saline, *i.r.*).

Group III (Indomethacin group): Rats were administered single dose of indomethacin (10 mg/kg, *s.c.*).

Group IV (4-Hydroxy isoleucine per se): Rats were treated with 4-Hydroxy isoleucine (50 mg/kg/day, *p.o.*) for 7 consecutive days.

Group V (Sulfasalazine + Acetic acid group): Single acetic acid (2 ml of 3% v/v in saline, *i.r.*) administration in rats on day Ist was followed by sulfasalazine (500 mg/kg/day, *p.o.*) dissolved in 0.5% w/v of CMC for 7 consecutive days.

Group VI (Sulfasalazine + Indomethacin group): Rats were administered with sulfasalazine (500 mg/kg, *p.o.*) dissolved in 0.5% w/v of CMC for 7 consecutive days following single indomethacin (10mg/kg, *s.c.*) administration.

Group VII & Group VIII (4-Hydroxy isoleucine treated acetic acid group): Rats administered acetic acid (2ml of 3%v/v, *i.r.*) were treated with 4-Hydroxy isoleucine (25 and 50 mg/kg/day, *p.o.*) and the treatment was started after 24 hr and continued for 7 days.

Group IX & Group X (4-Hydroxy isoleucine treated indomethacin group): Rats administered indomethacin (10 mg/kg, *s.c.*) were treated with 4-Hydroxy isoleucine (25 and 50 mg/kg, *p.o.*) and the treatment was administered 60 min. prior to indomethacin administration.

2.4. Assessment of IBD

2.4.1. Assessment of physical parameter: Body weight (Before and final)

2.4.2. Assessment of Colonic Damage and Small Intestine damage:

It was done by macroscopic and microscopic assessment of tissue.

2.4.2.1 Macroscopic assessment:

a) Weight of the tissue

b) Weight/length ratio of the tissue

2.4.2.2 Microscopic assessment using histopathological studies:

Distal colon and proximal ileum were excised and immersed in 10% buffered formalin. They were then dehydrated in the graded concentrations of ethanol, immersed in xylene, and then embedded in paraffin. From the paraffin blocks, 4 mm thin sections were cut, and staining was done using with haematoxylin (0.6% w/v) for 15 min followed by counterstaining with eosin (1% w/v) for 2 min. They were then examined using light microscopy to analyze integrity using miotic.

2.4.3. Assessment of Oxidative stress:

Oxidative stress was assessed by estimations of Thiobarbituric acid reactive substances (TBARS) and reduced glutathione reactive substances (GSH) in blood serum and tissue homogenate. Tissue/serum TBARS were estimated as per followed by H. ohkawa, 1979(47) and GSH content in tissue/serum was estimated using method of Jollow *et al.*, 1974(48).

2.4.4. Assessment of total protein in serum:

Total Protein were assessed by Biuret method using the commercially available kit (Agappe diagnostics Ltd. Kerala, India).

2.5. Assessment of Inflammation:

It was done by estimation of C- reactive protein(CRP) in serum. CRP was carried by CRP-turbilatex method; it is a quantitative measurement of CRP in serum or plasma. Latex particles coated with specific anti-mouse CRP are agglutinated, when mixed with sample containing CRP. The agglutination causes an absorbance change, dependent upon the CRP contents of the sample that can be quantified by comparison from a calibrator of known CRP concentration. The reagents and the photometer (cuvette holder) where maintained at 37˚C. Wavelength was adjusted to 540 nm, temperature 37˚C and with path length of 1 cm. Instrument was adjusted to zero with distilled water. 5.0 µL of brain homonenate / Calibrator was mixed with 900 µL diluent (Tris buffer 20 mmol/L pH 8.2) and 100µL of latex (particles coated with IgG

anti mouse CRP, pH 7.3). The absorbance was measured immediately (A1) and after 2 minutes (A2) of the sample addition.

2.6. Statistical analysis:

The results were expressed as mean ± S.E.M. The macroscopic, microscopic, biochemical and anti-inflammatory values were analyzed by one-way analysis of variance (ANOVA) followed by Dunnet' test using statistical Graph Pad Prism software. $p < 0.01$ was set to be statistically significant.

3. Results

3.1. Effect of 4-hydroxyisoleucine on body weight of acetic acid induced Ulcerative Colitis (UC) and indomethacin induced Crohn's disease (CD):

The body weight in the acetic acid and indomethacin treated rats was found to be decreased, when compared to normal rats. Post and pretreatment with 4-hydroxyisoleucine (25mg/kg and 50 mg/kg) for 7 days significantly and dose dependently increased the body weight of rats. (Table 1)

Table 1: Effect of 4-hydroxyisoleucine on body weight of acetic acid and Indomethacin induced IBD Ulcerative Colitis (UC) and Crohn's Disease (CD)

S. No	Groups	Body Weight Before (gram) with S.E.M.	Body Weight After (gram) with S.E.M.
1	Normal	157.5 ± 6.551	160.00 ± 6.583
2	Acetic Acid	175.83 ± 13.255	168.50 ± 12.369
3	Indomethacin	175.00 ± 10.328	166.66 ± 10.220
4	4-Hydroxyisoleucine *Perse*	178.33 ± 4.216	181.16 ± 6.853
5	Sulfasalazine + Acetic Acid	158.00 ± 7.024	168.00 ± 11.619
6	Sulfasalazine + Indomethacin	143.00 ± 2.944	146.83 ±5.326
7	25mg/kg 4-Hydroxyisoleucine + Acetic Acid	150.31 ± 2.901	157.0 ±2.947
8	50mg/kg 4-Hydroxyisoleucine + Acetic Acid	152.50 ± 3.152	153.17 ±3.154
9	25mg/kg 4-Hydroxyisoleucine + Indomethacin	157.50 ± 8.241	162.50 ± 11.162
10	50mg/kg 4-Hydroxyisoleucine + Indomethacin	167.50 ± 8.827	177.50 ± 10.468

3.2. Effect of 4-hydroxyisoleucine on weight of colon in acetic acid induced UC:

Administration of acetic acid was found to be highly significant in increasing colon weight (1.58±0.013; $p<0.001$) as compared to normal group (0.70±0.003). Post treatment with 4-hydroxyisoleucine (25mg/kg and 50mg/kg; *p.o*) for 7 days was highly significant reducing (1.27±0.005, 0.84±0.001; $p<0.001$) the colon weight, when compared to acetic acid group. 50mg/kg of 4-hydroxyisoleucine produce dose dependent effects in reducing colon weight. Significant reduction in colon weight (0.77±0.003; $p<0.001$) was also observed, when post treatment of sulfasalazine, a standard drug (500mg/kg; *p.o.*) was given for 7 days.

3.3. Effect of 4-hydroxyisoleucine on wet weight/length ratio of colon in acetic acid induced UC:

Colon weight/length after the administration of acetic acid was found to be significantly increased (263±1.1; $p<0.01$) as compared to normal group (116±1.6). Post treatment with 4-hydroxyisoleucine (25mg/kg and 50mg/kg; *p.o*) for 7 days significantly reduced (210±0.959, 140.8±2.9; $p<0.01$) the colon weight/length ratio, when compared to acetic acid group. 50mg/kg of 4-hydroxyisoleucine produce dose dependent effects in reducing weight/length ratio. Significant reduction in colon weight/length ratio (129.8±2.7; $p<0.01$) was also observed, when post treatment of sulfasalazine, a standard drug (500mg/kg; *p.o.*) was given for 7 days.

3.4. Effect of 4-hydroxyisoleucine on colon mucosal damage index (CMDI) in acetic acid induced UC

CMDI after the administration of acetic acid was found to be significantly increased (3.9±0.069; $p<0.01$), when compared to normal group (0.00±0.00). Post treatment with 4-hydroxyisoleucine (25mg/kg and 50mg/kg; *p.o*) for 7 days significantly reduced (2.89±.046, 1.62±0.066; $p< 0.01$) CMDI, when compared to acetic acid group. 50mg/kg of 4-hydroxyisoleucine produce dose dependent effects in reducing CMDI. Significant reduction in CMDI (1.41±0.049; $p<0.01$) was also observed, when post treatment of sulfasalazine, a standard drug (500mg/kg; *p.o*) was given for 7 days.

3.5. Effect of 4-hydroxy-isoleucine on macroscopic features in acetic acid and indomethacin group

Macroscopic aspect of longitudinally opened rat intestine showed erythema and hemorrhage with perforation in acetic acid group when compared to normal group. 4-hydroxyisoleucine at the dose of 25mg/kg and 50mg/kg dose reduced hemorrhage. 50mg/kg dose successfully abolish the signs of hemorrhage indicating its dose dependent effect (Figure 4a). Indomethacin group showed severe ulceration and perforation in longitudinally opened proximal ileum, when compared to

normal. 4-hydroxyisoleucine at the dose of 25mg/kg and 50mg/kg dose reduced ulceration. 50mg/kg dose successfully healed ulcers and no perforations were seen indicating its dose dependent effect.

3.6. Effect of 4-hydroxyisoleucine on microscopic features in acetic acid induced UC and indomethacin induced CD

Acetic acid induced UC showed massive necrotic destruction of epithelium, submucosal edema, disappearance of glands, areas of hemorrhages and inflammatory cellular infiltration. 4-hydroxyisoleucine at low dose of 25mg/kg showed severe damage of the mucosa with slight submucosal edema and mild inflammatory cell infiltration. 4-hydroxyisoleucine at 50 mg/kg showed remarkable recovery of colonic mucosa from Acetic acid induced colitis damage. Subcutaneous administrations of indomethacin in normal rats induced severe necrosis with complete disruption of superficial epithelium and diffuse infiltration of inflammatory cells in the mucosa, submucosa and lamina propria. Rats treated with 4-hydroxyisoleucine (25mg/kg and 50mg/kg; *p.o*) significantly reversed the histological changes in the indomethacin administered rats as indicated by the reduced necrosis and mild inflammatory cell infiltration.

3.7. Effect of 4-hydroxyisoleucine on weight of proximal ileum in indomethacin induced CD.

Proximal ileum weight after the administration of indomethacin was found to be significantly increased (1.62±0.002; $p<0.001$) as compared to normal group (0.528±0.002). However, pretreatment with 4-hydroxyisoleucine (25mg/kg and 50mg/kg; *p.o*) for 7 days significantly reduced (1.2875±0.001, 0.62±0.001; $p<0.001$) the proximal ileum weight, when compared to indomethacin group. Dose dependent effects were observed with 50mg/kg of 4-hydroxyisoleucine. Significant reduction in proximal ileum weight (0.6718±0.00612; $p<0.001$) was also observed, when pretreatment of sulfasalazine, a standard drug (500mg/kg; *p.o*) was given for 7 days.

3.8. Effect of 4-hydroxyisoleucineon wet weight/length ratios of proximal ileum in indomethacin induced CD

Proximal ileum weight/length after the administration of indomethacin was found to be significantly increased (162.33±1.054; $p<0.01$) as compared to normal group (52.74±1.00). Pretreatment with 4-hydroxy-isoleucine (25mg/kg and 50mg/kg; *p.o*) for 7 days significantly reduced (128.63±2.00, 62.31±1.076; $p<0.01$) the proximal ileum weight/length ratio, when compared to indomethacin group. Dose dependent effects were observed with 50mg/kg of 4-hydroxyisoleucine. Significant reduction in proximal ileum weight/length ratio (66.97±1.532; $p<0.01$) was also observed, when pretreatment of sulfasalazine, a standard drug (500mg/kg; *p.o*) was given for 7 days.

3.9. Effect of 4-hydroxyisoleucine on mucosal damage index in indomethacin induced CD

Indomethacin caused severe hemorrhagic lesions in the small intestine within 24 h, mostly in the proximal ileum and the lesion score was found to be significantly high (3.47±0.085; $p<0.01$), when compared to normal group (0.00±0.00). Pretreatment with 4-hydroxyisoleucine (25mg/kg and 50mg/kg; *p.o*) for 7 days significantly reduced hemorrhagic lesions (1.8±0.024, 1.39±0.030; $p<0.01$), when compared to indomethacin group. Dose dependent effects were observed with 50mg/kg of 4-hydroxyisoleucine. Significant reduction in hemorrhagic lesions was also observed, when pretreatment of sulfasalazine, a standard drug (500mg/kg; *p.o*) (1.24± 0.056; $p<0.01$) was given for 7 days.

3.10. Effect of 4-hydroxyisoleucine on serum parameters in acetic acid induced UC

3.10.1. Effect of 4-hydroxyisoleucine on serum thiobarbituric acid reactive substances (TBARS) level in acetic acid induced UC

Induction of UC in acetic acid group produced a significant increase in serum TBARS (36.28±1.30;$p<0.01$), when compared with normal group (22.53±0.479). However post treatment with 4-hydroxyisoleucine (25mg/kg and 50 mg/kg) attenuated the level of TBARS (29.45±0.496, 24.67±0.379;$p<0.01$), when compared to acetic acid group. A dose dependent response was obtained with 50mg/kg dose of 4-hydroxyisoleucine. Sulfasalazine as standard drug markedly reduced the level of TBARS (22.64±0.284), when compared with acetic acid group.

3.10.2 Effect of 4-hydroxyisoleucine on serum reduced glutathione (GSH) level in acetic acid induced UC

Induction of UC in acetic acid group produced a significant decrease in GSH level (35.08±0.087; $p<0.01$), when compared with the normal group (90.55±0.995). However, post treatment with 4-hydroxyisoleucine (25mg/kg and 50mg/kg) significantly increased (79.03±1.4, 85.73±0.603; $p<0.01$) GSH content, when compared with acetic acid group. A dose dependent response was obtained with 50mg/kg dose of 4-hydroxyisoleucine. Sulfasalazine as standard drug markedly attenuated the level of GSH (90.0±0.053), when compared with acetic acid group.

3.10.3. Effect of 4-hydroxyisoleucine on serum total protein level in acetic acid induced UC

Induction of UC in acetic acid group produced a significant increase in serum total protein level (3.80±0.379; $p<0.01$), when compared with the normal group (10.94±0.391). However, post treatment with 4-hydroxyisoleucine (25mg/kg and 50mg/kg) significantly reduced (5.53±0.541; $p<0.05$, 9.03±0.432; $p<0.01$) total protein content as compared with acetic acid group. Highly significant results were obtained with 50 mg/kg dose of 4-hydroxy isoleucine. Sulfasalazine as standard drug markedly attenuated the level of total protein (8.57±0.149; $p<0.01$), when compared with acetic acid group.

3.10.4. Effect of 4-hydroxyisoleucine on serum C-reactive protein in acetic acid induced UC

Induction of UC in acetic acid group produced a significant increase in serum CRP (29.72±3.294; p<0.01), when compared with the normal group (4.68±0.591). However, post treatment with 4-hydroxyisoleucine (25mg/kg and 50mg/kg) significantly decreased (18.91±0.510, 18.60±1.721; p<0.01) CRP level, when compared with acetic acid control group. Significant results were obtained with 50mg/kg dose of 4-hydroxyisoleucine. Sulfasalazine as standard drug markedly attenuated the level of CRP (5.89±0.751), when compared with acetic acid group.

3.11. Effect of 4-hydroxyisoleucine on serum parameters in indomethacin induced Crohn's disease (CD)

3.11.1. Effect of 4-hydroxyisoleucine on serum thiobarbituric acid reactive substances (TBARS) level in indomethacin induced CD

Induction of CD in indomethacin group produced a significant increase in serum TBARS (37.13±2.909; p<0.01), when compared with normal group (22.53±0.479). However, pretreatment with 4-hydroxyisoleucine (25mg/kg and 50mg/kg) attenuated the level of TBARS (29.19±0.512, 24.67±0.412; p<0.01), when compared to indomethacin group. A dose dependent response was obtained with 50mg/kg dose of 4-hydroxyisoleucine. Sulfasalazine as standard drug markedly attenuated the level of TBARS (21.90±0.518), when compared with indomethacin group. (Figure 13)

3.11.2. Effect of 4-hydroxyisoleucine on serum reduced glutathione (GSH) level in indomethacin induced CD

Induction of CD in indomethacin group produced a significant decrease in serum GSH level (35.12±0.1168; p<0.01), when compared with the normal group (90.55±0.995). However, pretreatment with 4-hydroxyisoleucine (25mg/kg and 50mg/kg) significantly increased (79.78±1.235, 84.66±1.593; p<0.01) GSH level, when compared with indomethacin group. A dose dependent response was obtained with 50 mg/kg dose of 4-hydroxyisoleucine. Sulfasalazine as standard drug markedly attenuated the level of GSH (88.37±1.508), when compared with indomethacin group.

3.11.3. Effect of 4-hydroxyisoleucine on serum total protein level in indomethacin induced CD

Induction of CD in indomethacin group produced a significant increase in serum total protein level (3.73±0.578; p<0.01), when compared with the normal group (10.94±0.390). However, pretreatment with 4-hydroxy-isoleucine (25mg/kg and 50mg/kg) significantly reduced (5.43±0.362; p<0.05, 8.45±0.446; p<0.01) total protein content as compared with indomethacin control group. Highly significant results were obtained with 50mg/kg dose of 4-hydroxy isoleucine. Sulfasalazine as standard drug markedly attenuated the level of total protein (8.68±0.125; p<0.01), when compared with indomethacin group.

3.11.4. Effect of 4-hydroxyisoleucine on serum C-reactive protein in indomethacin induced CD

Induction of CD in indomethacin group produced a significant increase in serum CRP 24.84±2.168; p<0.01), when compared with the normal group (4.68±0.591). However, pretreatment with 4-hydroxyisoleucine (25mg/kg and 50mg/kg) significantly decreased (17.63±0.601, 11.24±0.682; p<0.01) CRP level, when compared with indomethacin group. Highly significant results were obtained with 50mg/kg dose of 4-hydroxyisoleucine. Sulfasalazine as standard drug markedly attenuated (6.91±0.637) the level of CRP, when compared with indomethacin group.

3.12. Effect of 4-hydroxyisoleucine on tissue homogenate parameters in acetic acid induced UC

3.12.1. Effect of 4-hydroxyisoleucine on colonic TBARS level in acetic acid induced UC

TBARS level were found to be significantly increased in tissue homogenate (51.4±1.18; p<0.01) after the administration of acetic acid, when compared to normal group (20.9±0.67) in tissue homogenate. However, post treatment with 4-hydroxyisoleucine (25mg/kg and 50mg/kg; *p.o*) for 7 days significantly reduced the TBARS level, (33.7±0.873, 23.58±;p<0.01), when compared to acetic acid group. 50mg/kg of 4-hydroxyisoleucine produce dose dependent effects. Significant reduction in TBARS level was also observed, when post treatment of sulfasalazine, a standard drug (500mg/kg; *p.o*) (21.5±0.842; p<0.01) was given for 7 days .

3.12.2. Effect of 4-hydroxyisoleucine on colonic GSH level in acetic acid induced UC

GSH level were found to be significantly decreased (20.7± 0.621; p<0.01) after the administration of acetic acid, when compared to normal group (38.1±0.547; p<0.01) in tissue homogenate. Post treatment with 4-hydroxyisoleucine (25mg/kg and 50 mg/kg; *p.o*) for 7 days significantly increased (29.5±0.595, 37.7±1.40; p<0.01) the GSH level, when compared to acetic acid group. 50 mg/kg of 4-hydroxy isoleucine produced dose dependent effects. Significant increase in GSH level (34.9±0.857; p<0.01) was also observed when post treatment of sulfasalazine, a standard drug (500 mg/kg; *p.o*) was given for 7 days.

3.13. Effect of 4-hydroxyisoleucine on tissue homogenate parameters in indomethacin induced CD

3.13.1. Effect of 4-hydroxyisoleucine on TBARS level in indomethacin induced CD

TBARS level were found to be significantly increased (51.45±1.188; p<0.01) after the administration of indomethacin, when compared to normal group (20.93±0.679). However, pretreatment with 4-hydroxyisoleucine (25mg/kg and 50mg/kg; *p.o*) for 7 days significantly reduced (33.70±0.873, 23.58±0.358; p<0.01) the TBARS level in tissue homogenate, when compared to indomethacin group. Dose dependent effects were observed with 50mg/kg of 4-hydroxy

isoleucine. Significant reduction in TBARS level was also observed (21.5±0.844; p<0.01), when pretreatment of sulfasalazine, a standard drug (500mg/kg; *p.o*) was given for 7 days.

3.13.2. Effect of 4-hydroxyisoleucine on GSH level in indomethacin induced CD

GSH level were found to be significantly decreased (17.08±0.674; p<0.01) after the administration of indomethacin, when compared to normal group (36.06±0.273; p<0.01). However, pretreatment with 4-hydroxyisoleucine (25mg/kg and 50mg/kg; *p.o*) for 7 days significantly increased (25.19±0.4112, 33.362±0.6874; p<0.01) the GSH level in tissue homogenate, when compared to indomethacin group. Dose dependent effects were observed with 50mg/kg of 4-hydroxyisoleucine. Significant increase in GSH level (35.28±0.1717; p<0.01) was also observed, when pretreatment of sulfasalazine, a standard drug (500mg/kg; *p.o*) was given for 7 days.

4. Discussion

Inflammatory bowel disease is an inflammatory disorder of the colon and small intestine. Ulcerative colitis and Crohn's disease represent a group of heterogeneous inflammatory and ulcerative diseases of the large and small intestines associated with many gastrointestinal and systemic complications [1]. In ulcerative colitis, inflammatory lesions usually affect the large intestine, involving the rectum (proctitis), extending proximally to the entire colon [48,49]. Crohn's disease can affect any part of gastrointestinal tract, but the most commonly affected regions include terminal ileum or the perianal region [50].

Occurrence of gastrointestinal toxicity is a major complication, which arises due to intake of NSAIDs [51]. Indomethacin induced enterocolitis in the rat was established using the modification of method described by Millar [46]. Acetic acid induced colitis is commonly employed and easily inducible model [52-53], while screening drugs active against inflammatory bowel disease. In the present study, carried out to evaluate the effects of 4-Hydroxyisoleucine in experimentally induced inflammatory bowel disease in rats, intra-rectal administration of acetic acid induced ulcerative colitis and administered single dose of indomethacin subcutaneously induced crohn's disease.

In IBD, oxidative stress plays a major role in disease initiation and progression [54]. Oxidative stress causes abrupt generation of reactive oxygen species (ROS) like superoxide radicals, hydroxyl radicals, nitrite radicals [46]. Experimentally induced colitis using acetic acid in animals is due to an imbalance between oxidant and antioxidant substances [55, 80]. Similarly indomethacin induced apoptosis of the intestinal epithelial cells was accompanied by an increased production of reactive oxygen species [26-27]. In the present work, acetic acid and indomethacin generated ROS cause lipid peroxidation, as indicated by elevated TBARS and reduced GSH levels in serum as well as in tissue homogenate, which was significantly managed by post treatment and pretreatment of 25mg/kg and 50mg/kg 4-hydroxyisoleucine. Oxidative stress was managed by 4-hydroxyisoleucine by successfully reducing thiobarbituric acid reactive substances and increasing reduced glutathione levels in serum as well as tissue homogenate. Dose dependent results were obtained for 50mg/kg, 4-hydroxyisoleucine.

Acetic acid induces colitis involves the entry of protonated form of acid into the epithelium, where it dissociates to liberate protons causing intracellular acidification that might account for the epithelial injury [57]. Weight of colon is raised due to the inflammation and also because of the increased activity of the fibroblasts leading to the overgrowth of muscularis mucosa in acetic acid treated animals [58]. Significant increases in duodenum, caecum and colon weights were evident in rats administered indomethacin [59]. In present study results obtained for colon and duodenum weight in acetic acid and indomethacin groups were in harmony with previous findings. These weight increase may represent mucosal, submucosal or muscularis hyperemia and oedema, potentially combined with inflammatory cell infiltration. 4-hydroxyisoleucine treatment with 25mg/kg and 50mg/kg dose successfully reduced colon and duodenum weight indicating reduction in odema and inflammation, when compared to acetic acid and indomethacin groups. 50mg/kg dose dependently showed changes in both cases.

From morphological analysis and lesion scoring of tissue in acetic acid and indomethacin treated groups, significant mucosal damage and hemorrhagic lesions were assessed. Both the doses of 4-hydroxyisoleucine were effective in reducing intestinal damage in IBD as clearly shown by reduction in colon damage severity index and lesions, when compared to acetic acid and indomethacin group. CRP is an acute-phase protein that has been identified as an important biomarker for various inflammatory, degenerative, and neoplastic diseases [60]. Elevated levels of CRP have been found in the blood during virtually all diseases associated with active inflammation or tissue destruction, particularly in patients with rheumatoid arthritis [61]. Increase production of CRP was found to be under the influence of interleukin (IL)-6, tumour necrosis factor a (TNF-α). CRP has been shown to be an objective marker of inflammation for predicting disease course and outcome in IBD specifically CD[62-63] and to less extent in UC [64]. Elevated C-reactive protein (CRP) levels observed in indomethacin treated arthritic animals were restored in fenugreek mucilage treated rats [31] Results of present study regarding CRP are in consistence with above reference. Levels of CRP were decreased in 25mg/kg and 50mg/kg 4-

hydroxyisoleucine treatment groups, when compared to acetic acid and indomethacin groups indicating reduction in inflammation. CRP levels were reduced in dose dependent manner by 50mg/kg dose.

Total protein test measures the total amount of two kinds of protein in our body that is Albumin and Globulin [65]. Serum total protein was found to be reduced in ulcerative colitis and crohn's disease as suggested by Pawar and coauthor [66]. Decrease in albumin is associated with decrease in serum total protein [67].

In acetic acid induced model, Plasma total protein level was decreased significantly in colitis control group which was found to be increased significantly after treatment with 4-hydroxy isoleucine, which indicates that 4-hydroxy isoleucine is useful in treatment of IBD. Sulfasalazine as a standard drug used in IBD managed the level of TBARS, GSH and CRP, when compared to acetic acid and indomethacin groups. Sulfasalazine improved intestinal damage severity scorings.

5. Conclusion

4-hydroxyisoleucine proved its antioxidant and anti-inflammatory profile by reducing the oxidative stress and inflammation, further it also improved the integrity of intestine thus amino acid, 4-hydroxyisoleucine was successful in mediating the changes induced by acetic acid and indomethacin induced experimental IBD in rats.

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