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Oleic acid, hydroxytyrosol and *n*-3 fatty acids collectively modulate colitis through reduction of oxidative stress and IL-8 synthesis; *in vitro* and *in vivo* studies

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ABSTRACT

Our recent study has demonstrated that medium chain triglycerides (MCT) and monounsaturated fatty acids potentiate the beneficial effects of fish oil on risk factors of cardiovascular disease. In the present study, we have investigated the influence of MCT or olive oil on the protective and mucosal healing ability of fish oil in ulcerative colitis using cell simulation and animal models. Caco-2 cells grown in medium chain fatty acids enriched medium has exaggerated *t*-butyl hydroperoxide induced cell damage, GSH depletion, and IL-1β induced IL-8 synthesis, compared to the cells grown in oleic acid & hydroxytyrosol (OT) enriched medium. Further, combined treatment of cells with eicosapentaenoic acid, docosahexaenoic acid, and OT has remarkably attenuated the cell damage, and IL-8 synthesis, compared to individual treatments. To evaluate the effect of these lipid formulations *in vivo*, adult Wistar rats were fed diet enriched with high amount of medium chain triglycerides (MCT), virgin olive oil, or their combination with fish oil. Colitis was induced in rats using dextran sulfate sodium (DSS) for 7days followed by 10-days of recovery period. Rats of MCT group exhibit severe disease activity, higher levels of inflammatory cytokines in the colon compared to the olive oil group. Furthermore, there was persistent body weight loss, loose stools, higher levels of inflammatory cytokines in the rats of MCT group, even after DSS was withdrawn from drinking water. Conversely, fish oil has remarkably attenuated the DSS induced alterations in both MCT and olive oil diet groups with significantly greater effect in the olive oil group. Thus, MCT increase the susceptibility to colitis through oxidative damage and IL-8 synthesis in intestinal epithelial cells. The beneficial effects of virgin olive oil could be partially attributed to hydroxytyrosol. Combined treatment of hydroxytyrosol, oleic acid and *n*-3 fatty acids exhibit huge therapeutic benefits in colitis.

1. Introduction

Ulcerative colitis (UC) is a chronic inflammatory disease characterized by a diffuse inflammation involving part of, or the entire colon extending proximally from the rectum in a uniform uninterrupted pattern. The pathogenesis of the disease is not completely understood, but, is believed to be multifactorial involving genetic, environmental and immunological factors. It is considered that the mucosal barrier limits the immune response against the commensal flora and food antigens in the luminal surface of the colon. The disruption of mucosal barrier causes activation of underlying immune cells culminates with an excessive production of detrimental inflammatory cytokines including TNF-α, IL-1β, IL-6 and INF-γ. Subsequent infiltration of neutrophils intensifies the damage and results in ulceration of mucosa, blunting and loss of crypts [1]. Current therapies for UC include 5-aminosalicylic acid, sulfasalazine, and glucocorticoids. However, prolonged administration with a higher dosage of these drugs manifests serious adverse effects. Therefore, there is a growing interest for alternative remedies for the management of colitis.

Growing evidence suggests that dietary fats influence intestinal inflammation and regulate mucosal immunity. Epidemiological and animal studies have been emphasized that dietary intake of dietary long-chain *n*-3 PUFA associated with a reduced risk of colitis [2–5]. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), the two major long-chain *n*-3 PUFAs of fish oil, incorporates in plasma and cellular phospholipid pool at the expense of arachidonic acid (ARA) [6]. It has been considered that *n*-3 PUFA reduces the synthesis of ARA-derived proinflammatory eicosanoids such as leukotriene B_4 (LTB_4), thromboxane A_2 (TXA_2), prostaglandin E_2 (PGE_2), and also inflammatory cytokines such as TNF-α, IL-1β and IL-6, whose levels are reported to be elevated during colitis [7–8].

Medium chain triglycerides (MCT) with 8–10 carbon atoms are found mainly in coconut oil and are bestowed with huge therapeutic applications in cystic fibrosis, epilepsy and fat malabsorption diseases [9]. The MCT are absorbed, transported to the liver via the portal venous system and rapidly undergo β-oxidation [10–11]; whereas, PUFA

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remain as triglycerides (TG) in the hepatocyte. Recently, we have demonstrated that combined feeding of FO along with MCT or olive oil remarkably elevates systemic EPA and DHA levels and attenuate serum TNF-α, IL-6, lipid peroxides and *C*-reactive protein levels in rats. This was associated with a significant increase in serum IL-10 levels in rats [12]. Olive oil, a Mediterranean oil rich in oleic acid (18:1 *n*-9), is reported to be atheroprotective and also suppress inflammation in colitis [13–14]. Diet rich in virgin olive oil is reported to mitigate cardiovascular disease through reduction of endothelial dysfunction, inflammation and oxidative stress. These effects are partially attributed to minor components of virgin olive oil. The main phenolic compounds of virgin olive oil are tyrosol, hydroxytyrosol and lignans. They are absorbed by the human intestine and are bioavailable. The biological properties of these minor components overlap with the *n*-3 fatty acids including the antioxidant and chemopreventive activity, inhibition of nitric oxide synthesis, quenching of intracellular free radicals and reduction of the expression of cell adhesion molecules [15]. Therefore, the combined treatment of virgin olive oil rich in phenolics along with *n*-3 fatty acids may improve the anti-inflammatory and mucosal healing ability of *n*-3 fatty acids during colitis.

IL-8 is one of the proinflammatory cytokine secreted mainly by monocytes and macrophages as well as intestinal epithelial cells. Unlike eicosanoids, which are short-lived in the inflammatory microenvironment, IL-8 has a persisted action especially towards neutrophils [16–17]. Many of the cytokines such as TNF-α and IL-1β can stimulate IL-8 secretion [18]. Furthermore, IL-8 is a potent chemotactic agent for neutrophils and augmented expression of IL-8 may correlate to histological grade of active colitis [19].Therefore, the molecules which inhibit IL-8 synthesis are shown to be promising therapeutic agents in UC. Several lines of evidence suggest that dietary intake of MCTs are beneficial during colitis. Caprylic acid (C_8:0), a medium chain fatty acid, is reported to inhibit IL-8 secretion and suppress inflammation in colitis [20]. In contrast, capric acid (C_{10}:0) increase the synthesis of IL-8 in Caco-2 cells [21]. Medium chain fatty acids (MCFAs) as such are not incorporated into the membranes; since, a majority of MCFAs rapidly undergo β-oxidation, and few are converted to higher chain fatty acids ($C_{10,12,14}$) [22]. Increased saturated fatty acid content in the colon mucosa collectively alters the membrane fluidity, mucosal permeability, IL-8 synthesis and increase the susceptibility to colitis.

In this study, we aimed to evaluate the impact of dietary medium chain triglycerides, monounsaturated fatty acids and their combination with fish oil on colon inflammation and mucosal healing ability during colitis in rats.

2. Materials and methods

2.1. Materials

Sodium caprate, Sodium laurate, Sodium EPA, Sodium DHA, t-BHP, hydroxytyrosol, sodium oleate, the antibodies of HRP-conjugated goat anti-rabbit IgG, β-actin, and boron trifluoride (BF_3) in methanol are procured from Sigma Chemical Co, Bangalore, India. Fish oil (Sea cod oil, Sanofi Pharmaceuticals, India), virgin olive oil (Delmonte brand, Spain), coconut oil (cooking grade) were purchased from the commercial vendors in Mysore, India. Bernhart Tommerali salt mixture and heparin were purchased from SRL Laboratories, Bangalore, India. Dextran sulfate sodium (DSS; 36,000–50,000 MW) was obtained from MP Biomedicals, India Ltd. The polyvinylidene fluoride membrane was obtained from Pall Corporation, Pensacola, FL. The antibody of iNOS was procured from Santa Cruz Biotechnology, USA. The antibody of COX-2 was procured from Abcam, USA. Novex Enhanced Chemiluminescence detection kit was procured from Invitrogen, Carlsbad, CA, USA. The reference fatty acid methyl esters (FAME) mix was purchased from Nuchek, Waterville, MN, USA.

2.2. Caco-2 cell culture

Caco-2 cells were obtained from the National Centre for Cell Sciences (NCCS), Pune, India and were grown in Dulbecco's modified Eagle's medium containing 15% fetal bovine serum (FBS; Hi Media Laboratories, Mumbai, India), 2 mM glutamine (Gibco, U.S.A.), 1% non-essential amino acids (Sigma Chemical Co. Bangalore, India) and 1% antibiotic reagent (100 U/ml penicillin and 100 μg/ml of streptomycin, Hi Media Laboratories, Mumbai, India). The cells were allowed to reach 80% confluence and sub-cultured once in a week. The medium was changed for every 48 h. The cells at passage number 28–35 were used in all the experiments. On day-14, the cells were allowed to grow in a serum free medium, and all experiments were carried out in a serum free medium using the cells at day-21.

2.3. Cell viability assay

Cell viability was determined using a 3-(4,5-dimethylthiazol-2-yl)2–5-diphenyl-tetrazolium bromide (MTT) assay [16]. Briefly, 4×10^4 cells were plated in a 96-well plate with 200 μl of medium/well. The cells were pretreated with different fatty acids for 5 h in a 37 °C, 5% CO_2 humidified atmosphere. The cells were exposed to *t*-BHP for 5 h; subsequently, 10 μl of MTT (5 mg/ml) was added to each well and incubated for another 4 h. The medium was discarded and the crystals were dissolved in 100 μl of DMSO. The optical density was measured at 570 nm using a plate reader.

2.4. Lactate dehydrogenase (LDH) assay

The cells were seeded in a 24-well plate containing 1 ml of medium at a density of 5×10^5/ml. The cells were preincubated with different fatty acids for 5 h in a 37 °C, 5% CO_2 humidified atmosphere. The cells were exposed to *t*-BHP for 5 h. The culture medium (0.1 ml) was transferred to a 96-well plate and lactate dehydrogenase in the medium was determined using LDH assay kit (Sigma Chemical Co. Bangalore, India). The absorbance was measured at a wavelength of 450 nm by a microplate reader (Bio-Rad, CA). Lactate dehydrogenase (LDH) activity in the culture medium was expressed as a percentage of total LDH activity, which was obtained by treating cells with 2.0% TritonX-100 in PBS.

2.5. Glutathione (GSH) assay

The cells were seeded in a 24-well plate containing 1 ml of medium at a density of 5×10^5/ml. The cells were preincubated with different fatty acids for 5 h in a 37 °C, 5% CO_2 humidified atmosphere. The cells were exposed to *t*-BHP for 5 h. The medium was discarded and the cells were collected by incubating the plate on ice for 15 m. The cells were lysed by recurrent freeze-thawing. The cell lysate was collected and centrifuged at 2000 ×*g* for 15 min at 4 °C. GSH level in the supernatant was estimated using a colorimetric GSH assay kit (Sigma Chemical Co. Bangalore, India). The absorbance was measured at 450 nm by a microplate reader. Total GSH levels were estimated according to the manufacturer's instructions.

2.6. Assay of IL-8

The cells were plated in a 24-well plate containing 1 ml of medium at a density of 5×10^5/ml. The cells were cultured in the presence or absence of fatty acids for 5 h, followed by stimulation with 10 ng/ml of IL-1β (Sigma Chemical Co. Ltd MO, U.S.A.) for 24 h. IL-8 levels in the culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA; Sigma Chemical Co. Ltd). Sodium derivatives of lauric acid, capric acid, oleic acid, EPA and DHA, and hydroxyl tyrosol are solubilized directly in the medium and added to the cells.