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**Beneficial effect of butyrate, Lactobacillus casei and L-Carnitine combination in preference to each in experimental colitis**

Moeinian M *et al.* A new combination for IBD

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**Abstract**

**AIM:** To investigate the beneficial effect of the combination of butyrate, Lactobacillus casei, and L-Carnitine in murine colitis model.

**METHODS:** The animals were divided into seven groups. Four groups received oral butyrate, L-Carnitine, L. casei and the combination of three agents for 10 consecutive days. The remaining groups included the negative and positive controls and the sham. Macroscopic, histopathological examinations, and biomarkers such as tumor necrosis factor-alpha (TNF-) and interlukin-1β (IL-1β), myeloperoxidase (MPO), thiobarbituric acid reactive substances (TBARS), and ferric reduced ability of plasma (FRAP) were determined in colon.

**RESULTS:** The combination therapy exhibited a significant beneficial role in alleviation of colitis comparing to controls. Overall changes in reduction of TNF- (114.66 ± 18.26 *vs* 171.78 ± 9.48 pg/mg protein, *P* < 0.05), IL-1β (24.9 ± 1.07 *vs* 33.06 ± 2.16 pg/mg protein, *P* < 0.05), TBARS (0.2 ± 0.03 *vs* 0.49 ± 0.04 μg/mg protein, *P* < 0.01), MPO (15.32 ± 0.4 *vs* 27.24 ± 3.84 U/mg protein, *P* < 0.05), and elevation of FRAP (23.46 ± 1.2 *vs* 15.02 ± 2.37 μmol, *P* < 0.05) support the preference of combination in comparison to controls. Although the single therapies were aslo effective in improvement of colitis markers, the combination therapy was much better in reducing improvement of colon oxidative stress markers including FRAP, TBARS, and MPO.

**CONCLUSION:** The present combination is a suitable mixture in control of experimental colitis and should be trialed in clinic.

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**Key words:** Butyrate; L-Carnitine; Colitis; Oxidative stress; L. casei; Probiotic

**Core tip:** Inflammatory bowel disease (IBD) is among the common diseases in the globe that has no absolute cure yet. Although, corticosteroids, immunosuppressants, and aminosalicylates are conventionally used in management of IBD, their side effects reduce patients’ compliance. In this paper, we have shown that the combination of butyrate, L. casei, and, L-Carnitine reduce the amount of oxidative stress within the colon and provide significant anti-inflammatory effects. Optimistically, the proposed combination is from componenets with no serious side effect and is more economical to manufacture.

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**INTRODUCTION**

Inflammatory bowel disease (IBD), representing ulcerative colitis (UC) or Crohn’s disease (CD), with an increasing incidence, can be debilitating in the affected patients. Patients with IBD usually suffer from bloody diarrhea, abdominal pain, and also extra-gastrointestinal manifestations such as uveitis, arthritis, skin lesions, and hepatobiliary disease[1-3]. Although definite etiology of IBD remains debatable, the role of immune dysfunction, particularly over-activity of inflammatory factors including tumor necrosis factor-alpha (TNF-α), interlukin-1β (IL-1β), oxidants such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) have been defined along with other factors such as environment, genetics, and intestinal microbes[4,5]. Current protocol of IBD treatment consists of 5-aminosalcylic acid (5-ASA) derivatives, immuno-suppressive agents, corticosteroids, monoclonal antibodies, and some other complementary agents such as herbal medicines. However, increasing complications of conventional medicines besides decreased patients compliance have led scientists to focus on the safety alongside efficacy[6-9].

Butyrate, a type of short chain fatty acid, is produced naturally by bacterial fermentation of dietary fibers as a fuel in the colon. Previous reports emphasized its protective ability against oxidative stress and depletion of inflammatory markers including TNF- and IL-1β through interfering with Ikappa B kinase (IKK), resulting in down-regulation of nuclear factor-kappa B (NF-κB) which is responsible for generation of pro-inflammatory cytokines[10-13]. In addition, Lactobacillus casei (L. casei) as a probiotic, exhibits modulatory effects on immune response and oxidative stress via IKK or production of anti-oxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT). Several studies suggest that manipulation of normal flora content may have beneficial effects in IBD[14-16]. L-Carnitine (β hydroxyl-γ trimethyl amino butyrate) plays a significant role in fatty acid β-oxidation, glucose metabolism and general energy control in all types of cells including colonocytes. Production of SOD and inhibition of glutathione (GSH) reduction confirm its anti-oxidant feature and also protective effects against inflammation[17-20]. In summary, recent data revealed that butyrate, L-Carnitine, and L. casei have noticeable beneficial potential on experimental IBD models alone or in combination with other medicines[21-24]. Therefore, in the present experiment we evaluated the synergism effect of the combination of these three agents by assessment of inflammatory indicators and pathological markers.

**MATERIALS AND METHODS**

***Chemicals***

2,4,6-Trinitrobenzene sulphonic acid (TNBS), butyrate and L-Carnitine from Sigma-Aldrich Chemie (Gmbh Munich, Germany), trichloroacetic acid (TCA), thiobarbituric acid (TBA), 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ), N-butanol, hexadecyl tri-methyl ammonium bromide (HETAB), ethylene diamine tetra acetic acid (EDTA), malondialdehyde (MDA), hydrochloric acid (HCL), acetic acid, sodium acetate, hydrogen peroxide (H2O2), O-dianisidine hydrochloride, ferric chloride (FeCl3-6H2O), Coomassie reagent, bovine serum albumin (BSA), sodium sulphate (Na2SO4), sulphuric acid (H2SO4), phosphoric acid (H3PO4), potassium dihydrogen phosphate (KH2PO4), potassium hydrogen diphosphate (K2HPO4), sodium carbonate (Na2CO3), Na-K-tartarate and cupric sulphate (CuSO4-5H2O) from Merck (Darmstadt, Germany), whey powder from Shirpooyan-E-Yazd Co. (Tehran, Iran), powder of L. casei DN:114001 from Zist-Takhmir Co. (Tehran, Iran) and rat-specific tumor necrosis factor-α (TNF-α) and interlukin-1β (IL-1β) Enzyme-Linked ImmunoSorbaent Assay (ELISA) kits from (BenderMed Systems GmbH, Austria) were used in this study.

***Animals***

In this study, male Wistar rats weighing 180-200 g were selected according to regulations of the ethical committee of TUMS approved with code number of 91-03-33-19079. Animals, housed separately in standard polypropylene cages with a wire mesh top, kept under standard conditions including temperature (23 ± 1°C), relative humidity (55% ± 10%), and 12/12 h light/dark cycle, and feed of standard pellet diet and water ad libitum.

***Experimental design***

Animals were divided into seven groups containing seven rats in each group. Colitis was induced by injection of TNBS rectally in all groups except sham, which received normal saline. Groups receiving TNBS were divided into control (as an untreated groups), positive control (received 1 mg/kg dexamethasone dissolved in water), treatment groups containing butyrate (1 mL of 0.5% in which 0.5 g butyrate dissolved in 100 ml PBS), L-Carnitine (500 mg/kg in 1 mL), L. casei (1 ml of whey culture contains 108 Cfu L. casei), combination (0.5 mL butyrate, 0.5mL L-Carnitine, 1 ml L. casei).

Whey culture (10% w/v, 10 g whey powder in 100 mL distilled water) was prepared at 121°C for 20 min. Then, L.casei was added to it and incubated at 37°C for 48 h.

The day TNBS administered was assigned as the first day and all treatments started from the same day. During a 10-d treatment, the groups treated by gavage.

***Induction of colitis***

Prior to induction of colitis, rats were fasted for 36 h.They were anesthetized by 50 mg/kg pentobarbital sodium intraperitoneal injection[25]. Then, 0.3 mL of a mixture, comprising six volumes of 5% TNBS plus 4 volume of 99% ethanol, instilled through anus using a rubber cannula (8 cm long) into rats situated on their right side and at last the rats were held in a prone Trendelenburg position to stop the anal leakage of TNBS[26].

***Sample preparation***

On day eleven of treatment, animals were sacrificed and immediately, colonic tissues were separated. Isolated segments were rinsed with normal saline and then, they were stored in ice bath over procedure. Colonic tissue was divided into two major and minor pieces. First samples were weighed and kept in 10 mL formalin 10%, as a fixator for the purpose of histopathological evaluation. After weighing second samples, they were uniformed in 10 volume ice cold potassium phosphate buffer (50 mmol, pH = 7.4) and then stored at -20 ºC for 24 h till homogenization of samples. The samples were sonicated and centrifuged for 30 min at 3500 *g*. The supernatants were transferred to several microtubes. Then, they were kept at -80 C° as late as carry out of biomarker’s analyses.

***Macroscopic and microscopic assessments***

The following macroscopic scoring system used to evaluate the severity of colonic damage: 0-normal appearance with no damage; 1- localized hyperemia without ulcer; 2- localized hyperemia with ulcer; 3- linear ulcer with inflammation at one site; 4- two or more ulcers with damage extending 1-2 cm along the length of colon; and 5 to 8- damage extending more than 2 cm along the length of colon and the score was enhanced by 1 for each increased cm of involvement.

The microscopic scoring was done by a blind observer to the treated groups. Microscopic scores were determined as follow: 0- no damage; 1- focal epithelial edema and necrosis; 2- disperse swelling and necrosis of the villi; 3- necrosis with neutrophil infiltration in submocusa, and 4- wide spread necrosis with massive neutrophil infiltration and hemorrhage.

***Myeloperoxidase activity assessment***

Fifty ml of phosphate buffer containing 0.167 mg/mL O-dianisidine and 0.0005% H2O2 was blended with 0.1 mL of supernatant. The absorbance was measured for 3 min in 460 nm spectrophotometrically (Shimadzu 160A UV-VIS spectrophotometer) and expressed as unit per mg protein of colon tissue. One unite is equal to the change in absorbance per min at room temperature in the final reaction[27].

***Lipid peroxidation assessment***

Lipid peroxides as the end products of poly unsaturated fatty acid (PUFA) peroxidation are aldehydes that react with TBA named TBA reactive substance (TBARS) and form a complex which is detected in 532 nm by double beam spectrophotometer. Concentration of TBARS is recorded as µg/mg protein[28].

***Ferric reducing ability of plasma assessment***

Ferric-tripyridyltriazine (Fe3+-TPTZ) complex is reduced to bluish ferrous-tripyridyltriazine (Fe2+-TPTZ) with absorption at 593 nm. Values were reported as mM ferric ions reduced to ferrous per mg protein. Details have been described previously[29].

***Interlukin-1β (IL-1β) and tumor necrosis factor-alpha (TNF-α) assessment***

Upon basis of ELISA, quantity of IL-1β and TNF-α (pg/mg protein of tissue) were measured. Amount of blue complex resulting from conjugation of chromogenic substance with streptavidin-horseradish peroxidise (Streptavidin-HPR) were calculated in both 450 nm (primary wave length) and 620 nm (reference wave length). Details have been described previously[30].

***Total protein assessment***

Total protein was measured according to Bradford method using BSA as the standard and data were expressed as mg/ml of homogenized tissue at 540 nm[31].

***Statistical analysis***

One-way analysis of variance (ANOVA) followed by Tukey’s post-hoc tests were used for multiple comparisons of outcomes, shown as mean ± SD error of the mean (SEM). *P*-values less than 0.05 were considered significant. StatsDirect version 3.0.97 was used for statistical analysis.

**RESULTS**

***Macroscopic and microscopic evaluation of histological impairment (Table 1 and Figure 1)***

Histopathological examination of control group which received TNBS showed severe ulcer, diffused necrosis, edema, crypt destruction, and mucosal/submucosal polymorphonuclear (PMN) leukocyte infiltration that were significantly different (*P* < 0.001) from sham group that had normal histology and a regular mucosal layer with intact epithelial surface. In the L-Carnitine group, crypt destruction and abscess, submucosal inflammation and low PMN infiltration were observed. In the butyrate group mild infiltration, crypt destruction, edema, and disintegration of crypts were locally observed in some areas. Histopathological parameters in the L. casei group were most similar to butyrate group. A significant reduction in microscopic scores was observed in each group in terms of histological symptoms such as inflammation and/or diffuses necrosis hemorrhage and severe crypt destruction in comparison with control group (*P* < 0.001). In the combination group, crypt abscess and mild inflammation of submucosa with no PMN infiltration were observed. Although the histological scores decreased in combination group (Table 1) and histopathological symptoms including PMN infiltration and crypt destruction were more vivid in comparison with single groups, there was no significant difference between combination group and single groups statistically.

***Myeloperoxidase activity***

Myeloperoxidase (MPO) activity was increased in inflamed tissues of control group in comparison to sham group (*P* < 0.01). The group of animals receiving single therapies of butyrate, L-Carnitine, and L. casei showed reduction of MPO activity by 40.71%, 38.95%, and 39.86%, respectively in comparison with controls (*P* < 0.05). Dexamethasone decreased MPO by 60.82%, respectively in comparison with control group (*P* < 0.01). Also, in combination group, there was a notable reduction in MPO activity by 43.75% in comparison with control group (*P* < 0.05). In combination, butyrate, and L. casei groups, MPO increased by 17.07%, 20.11%, and 20.96%, respectively in comparison with dexamethasone group (*P* < 0.05). MPO increased by 21.87% in L-Carnitine group comparing with dexamethasone group (*P* < 0.01). Combination group showed more reduction of MPO by 4.80% rather than L-Carnitine group (*P* < 0.05) while there was no significant difference between combination group and single therapies of L. casei and butyrate (Figure 2).

***TNF-α level***

There was an increase in TNF-α level in controls comparing to sham (*P* < 0.001). In dexamethasone, TNF- reduced by 54.54% comparing with control group (*P* < 0.001). TNF- level decreased in combination, butyrate, and L-Carnitine groups by 33.25%, 28.07%, and 19.90%, respectively in comparison with control groups (*P* < 0.05). Dexamethason was more effective in reduction of TNF- than those of single therapies of butyrate by 26.46% (*P* < 0.05), L-Carnitine and L. casei groups by 34.63% and 46.32%, respectively (*P* < 0.01). There was not any notable difference when comparing combination therapy with single therapies (Figure 3).

***IL-1β level***

Control group showed a notable elevation in IL-1β in comparison with sham group (*P* < 0.001). In dexamethasone, combination, L-Carnitine, and L. casei groups, IL-1β diminished by 24.98%, 24.68%, 24.13%, and 24.22%, respectively in comparison with control group (*P* < 0.05). There was no significant change in combination group when compared to single therapies of butyrate, L-Carnitine, and L. casei (Figure 4).

***Anti-oxidant power as ferric reducing ability of plasma***

Anti-oxidant power decreased in control group as compard with sham group (*P* < 0.01). Ferric reducing ability of plasma (FRAP) value in dexamethasone group increased by 68.04% (*P* < 0.001) and in combination group by 56.19% when compared with controls (*P* < 0.05.( Single therapies of butyrate, L-Carnitine, and L. casei groups decreased FRAP by 59.92%, 50.73%, and 59.45%, respectively as compared to dexamethasone group (*P* < 0.01). The improvement of FRAP in single therapies of butyrate, L-Carnitine, and L. casei were 8.12%, 17.31%, and 8.58%, respectively that were all lower than that of combination group (56.19% , *P*  < 0.01)(Figure 5).

***Oxidative-stress as TBARS***

Elevation of TBARS was evident in the controls comparing with shams (*P* < 0.001). Dexamethasone (*P* < 0.001), combination and single therapies of butyrate, L-Carnitine, and L. casei groups (*P* < 0.01) restored TBARS by 61.22%, 59.18%, 38.77%, 32.65%, and 38.77%, respectively in comparison with controls. TBARS was significantly different in butyrate, L-Carnitine groups by 22.44%, 28.57% (*P* < 0.001), and L. casei group by 22.44%, respectively (*P* < 0.01) in comparison with dexamethasone group .Combination group showed more decrease in TBARS than butyrate and L. casei groups by 20.40% and 20.40%, respectively (*P* < 0.05). TBARS changed 32.65% in L. Carnitine group that was lower than that of combination group (59.18%, *P* < 0.01)(Figure 6).

**DISCUSSION**

Regarding overall results, the present study demonstrated the priority of combination of butyrate, L. casei and L-Carnitine in ameliorating the severity of colitis in comparison to single therapies. Macroscopic features including appearance of isolated tissue and histopathological scores such as presence of edema, necrosis, neutrophil infiltration, and biomarker of TNF-α, IL-1β, MPO, TBARS, and anti-oxidant power confirmed the beneficial effect of combination treatment in comparison to controls. Specifically, combination therapy was much better in reducing colon oxidative stress markers of FRAP, TBARS, and MPO.

Although, there are several reports on the positive effects of these three agents in inflammation or oxidative stress explained through various mechanisms[13,14,17], current study based on an original hypothesis[11] is the first one that confirms synergism between butyrate, L. casei and L-Carnitine in the immune-based model of colitis. TNBS-induced colitis is believed a preferential model since the colon’s barrier is broken by ethanol and then delayed-response hypersensitivity reaction by TNBS occur like that of human IBD[20,32-34]. This similarity is confirmed by the same pattern of changes in examined cytokines between experimental studies and those reported in human such as accumulation of MPO, TNF-α, IL-1β, ROS, and RNS[4,26,35-38]. Specifically, MPO, a hemo enzyme is released dramatically from neutrophils in order to eradicate pathogens. It facilitates production of cytotoxic agents like hypochlorite acid (HOCl) the stimulator of NF-ĸB inducing other inflammatory factors via hydrogen peroxide (H2O2)[39,40]. Results indicate that MPO as a neutrophils infiltration indicator and TBARS as indicator of lipid peroxidation are markedly up-regulated in colon tissues while these are restored by combination therapy. This is most likely due to their radical scavenging properties[3]. As shown schematically in the Figure 7, butyrate is able to lessen oxidants through suppressing IKK, which is responsible for dissociation of NF-κB from IKB-α, and then free-NF-κB fortifies oxidants via overexpression of inducible nitric oxide synthase (iNOS) gene[4]. In addition, probiotics have the same effect either directly by production of SOD and CAT against oxidants or blocking IKK, indirectly[15,16]. Likewise, L-Carnitine modifies activity of oxidative stress by elevation of SOD and prevention of decrease in GSH content[17-20] and eventually, combination group exerts well against oxidants with higher efficacy related to synergism of these three agents. In turn, observed changes in the FRAP test supports that idea. Regarding the inflammation, TNF-, a pro-inflammatory cytokine, is produced mainly by macrophages and activated T-lymphocytes. It is an active component in the apoptosis, stimulation of IL-1β secretion, and inflammation, in which re-induction of IKK occurs. This process is mediated through the cascade consisting of binding to TNF receptor (TNF-R) accompanied with dissociation of silencer of death domains (SODD) from TNF-R type1-associated death domain (TRADD). Further attachment of TRADD to death domain leads to activate the ribosome inactivating protein (RIP) and TNF-R associated factor 2 (TRAF2) which trigger IKK[11,13,41]. In addition, IL-1β has a crucial role in inflammation, proliferation, differentiation, and apoptosis secreted from macrophages, neutrophils, endothelial, and epithelial cells[42]. Results represent that treatment groups restrict abundant formation of IL-1β and TNF- in contrast to control group. This is explained by the drop in inflammation either through blocking IKK to activate NF-κB in overexpression of mentioned cytokines like that of butyrate and L. casei or L-Carnitine. However, obvious differences are evident between individual groups and the combination in amelioration of inflammation that originate from synergistic effects in the combination therapy.

In summary, high level of anti oxidative stress ability, anti-inflammation feature and positive interactions amplify their effects such as overexpression of sodium-coupled mono carboxylate transporter 1 (SMCT1) gene, an essential gene for butyrate’s absorption. Also, overencoding of L-Carnitine’s organic cation transporter number 2 (OCTN2) by probiotics and enhancement of butyrate’s β-oxidation by L-Carnitine show that this mixture is more remedial with less complications[11,18,20,33,42-47]. No toxicity following administration of each of them individually or in combination observed in this study certifies the safety of this mixture. Literature shows no severe complication in ordinary patients who take ordinary doses of L. casei or L-Carnitine[48,49,50]. However, more investigations are required to clarify the mixture’s safety in the clinic. According to the results, combination of these three agents is more effective than each alone in attenuation the inflammatory process.

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**COMMENTS**

***Background***

Inflammatory bowel disease (IBD) is divided into two major and idiopathic conditions including ulcerative colitis (UC) and Crohn′sdisease. Current choice in control of IBD is administration of aminisalicylates, corticosteroid, and immunosuppressants that are with side effects. Due to current conventional therapies side effects, scientists try to discover more potent and safer alternatives.We intended to demonstrate that combination of butyrate, L-Carnitine, and L. casei may be an effective and safe mixture in amelioration of colitis.

***Reaserch frontiers***

Several studies focused on major role of nuclear factor-kappa B (NF-kB) signaling which results in overproduction of inflammatory mediators and oxidants in progress of inflammation. In this study, we evaluated the combination’s capacity in healing the colitis and improvement of inflammatory cytokines.

***Innoviation and breackthroughs***

Present study indicated that the combination is able to heal colitis and down-regulate the production of inflammatory cytokines. Inhibition of NF-kB pathway either by suppression of IKK or production of enzymes against free radicals deemed the main mechanism of this mixture action.

***Applications***

Safety and effectiveness besides positive interactions of these three agents confirm that this combination can be more helpful in healing IBD and improving the patients’s quality of life.

***Peer review***

The main hypothesis of paper for management of IBD is valuable.

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**Table 1 Extent of colonic damage according to macroscopic and microscopic scores**

|  |  |  |
| --- | --- | --- |
| **Groups** | Macroscopic Score (Mean [±](http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2959222/) SEM); Median (Min-Max) | **Microscopic Score (Mean** [**±**](http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2959222/) **SEM);**  **Median (Min-Max)** |
| Sham | (0.0+ 0.0)  0(0.0 - 0.0) | (0.0+0.0)  0(0.0 - 0.0) |
| Control | (6.3+0.83)b  6(4.0 – 8.0) | (3.6+0.24)b  4(2.0 – 4.0) |
| Dexamethasone | (1.0+0.34)d  1(0.0 – 2.0) | (1.0 +0.31)b,d  2(1.0 – 3.0) |
| Combination | (1.00 – 0.51)d  1(0.0 – 2.0) | (1.2 – 0.21)d  2(1.0 – 3.0) |
| Butyrate | (1.6 +0.5)d  2(2.0 – 4.0) | (1.4 +0.24)d  2(1.0 – 4.0) |
| L-Carnitine | (2.44 +0.5)d  2(1.0 – 4.0) | (1.66 +0.26)b,d  3(2.0-4.0) |
| L.casei | (2.64 + 0.6)d  2(2.0 – 5.0) | (1.6 + 0.3)b,d  3(2.0-4.0) |

Significantly different from sham group (b*P* < 0.01 *vs* sham group); Significantly different from control group (d*P* < 0.01 *vs* control group).