ABSTRACT

AIM: Current investigations have focused on mechanisms of inflammation in inflammatory bowel disease (IBD). The gut bacteria play an important role in the pathogenesis of IBD, however due to the complexity of gut microflora some skepticisms still remain. Most of currently animal models of colitis are mainly based on bowel mucosal damage which has some limitation in terms of dissimilarity to human IBD. In the present study, a new model based on defined bacterial infections has been developed and tested.

METHODS: Seven groups of six rats were involved; normal, positive control which received trinitrobenzenesulfonic acid enema, Adjuvant + Ethanol 30% enema, Adjuvant + Ethanol 20% enema, Adjuvant + Ethanol 10% enema, Ethanol 30% enema and Adjuvant + Ethanol 30% enema which treated with 5/mg/kg/day infliximab. We administered two courses of Freund's complete adjuvant mixed with inactivated total enteric bacteria, then various percentages of ethanol enema used as a barrier breaker to expose the host immune system to its own flora. Colonic status was investigated two weeks after enemas. Macroscopic, histological and biochemical analyses were performed on samples.

RESULTS: Ethanol 30% enema in vaccinated rats caused histological damage and resulted in a significant rise of TNF-α, IL-1, IL-17, myeloperoxidase activity, and oxidative stress biomarkers in comparison to Sham.

CONCLUSION: This model is an immunogenic and reliable model, which demonstrates microscopic and macroscopic characteristics more similar to human IBD. These findings introduce a novel experimental IBD model and shed light on disease pathogenesis.

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Key words: Freund's adjuvant; Enteric bacteria; Immune based IBD model

INTRODUCTION

Inflammatory Bowel Disease (IBD) is a chronic, immune-mediated inflammatory condition with unclear mechanisms that affect statistically 7-10% of population worldwide[1]. IBD comprises two forms, including Crohn's disease (CD) and ulcerative colitis (UC). Colitis preferentially occurs in the colon and distal ileum, which contain the highest level of intestinal bacterial concentrations[2]. In addition, the majority of colitis models fail to develop colitis under germ-free conditions[3].

Detecting bacterial DNA and lipopolysaccharide (LPS) by the host enhances mucosal invasion and translocation of enteral bacteria[4]. Experimental studies have shown serological and aggressive T-cell responses to enteral microbial antigens[5].

It has been demonstrated that TNF-α, IL-1 and IL-17 play central role in the cytokine cascades, and induce the secretion of other cytokines, which result in inflammation and colitis[6-8]. After activation of TH1, TH2 and TH17 cells, they produce specific cytokines; some of these cytokines increase epithelial barrier permeability (e.g. IFN-γ) and...
some have destructive and apoptotic effects on mucosal cells, which eventually allow more antigens to pass and more agitation of immune cells amplifying the inflammatory cascade[12,14]. If anything alters the barrier function, lots of luminal antigens could pass to the submucosal layer leading to recruitment of neutrophils and macrophages. A number of studies have shown inflammatory cytokines like TNF-α and IFN-γ for their role in increasing enteric barrier permeability[12,13]. Furthermore, abundant evidence indicates increased gastroenteric permeability in IBD patients suggesting permanent stimulation of the mucosal immune system as the primary defect in IBD pathogenesis[14,15,17].

Most of extra-intestinal manifestations of IBD are mediated immunologically[18,19]. After an alteration in barrier function, various antigens pass to the interstitial space which finally activates T cells. In normal subjects, the response directed definitely against the specific epitope of antigens. Commensal organisms in the lumen have adhesive antigens (e.g. flagellar antigens) which adhere to the surface proteins of mucosal cells. If there are some predisposing factors, the APC process epitopes of antigens with a part of mucosal surface proteins would activate T lymphocytes against mucosal cells’ surface protein[20,19]. Another scenario happens when the response to the special epitopes of antigens is cross-reactive to auto-antigens. There is evidence demonstrating relations between precise HLA molecules and cross-reactive cellular antigens[20,18].

A long series of studies demonstrated that IBD patients possess auto-antibodies. Some of such autoantibodies like anti-lymphocytes, anti-globet cell, pancreatic autoantibodies, the autoantibody against tropomyosin isoform 5, and antibodies against RBC membrane antigens are indicators of IBD[19,22,23]. Although the presence of antibodies directed against microbial antigens has been illustrated in the serum of CD patients, a shared epitope among the host antigens has not been clearly defined. For example, 55% of CD patients have antibodies against outer membrane porin C (OmpC) of Escherichia coli and 50% have immune globulins that are reactive to a homologue of the bacterial membrane porin C (Omp C) of Escherichia coli and 50% have immune globulins that are reactive to a homologue of the bacterial transcription-factor families from a Pseudomonas fluorescens–associated sequence[25]. Around 30% of CD patients have serum reactivity to Cbi1, an immunodominant antigen of the enteric microbial flora. This antigen could strongly induce B cells and CD4+ T cell responses. Interestingly, transferring Cbi1-specific CD4+ TH1 T cells to C57/SCID mice generates a severe colitis dependent on exogenous expression of Cbi1 flagellin in the colon. In 60-70% of CD patients, anti-Saccharomyces cerevisiae antibodies (ASCA) can be found that recognizes mannose sequences in the cell wall of this commensal flora[25,24].

In the current study, we carried out a novel model of IBD and focused upon the impact of commensal microbiota according to our new idea published recently[27]. Although in vitro studies as well as the clinical trials improved our knowledge about IBD in recent years, the most important advances come from observing the pathogenesis of IBD in animal models[14,15]. Not all the experimental models can fully reflect human IBD, but they have provided important opportunities to examine the mechanisms of the disease[26]. In this novel model, the interaction between commensal microbiota and over activated host’s mucosal immune system causes chronic, spontaneously relapsing inflammation in distal ileum and colon.

**MATERIALS AND METHODS**

**Ethical approval**

All ethical cares on the use of animals in scientific studies were cautiously considered and the study protocol was approved by the institute review board.

**Animals**

Male Wistar-albino rats, weighing 200-250 g were used for the experiment. Animals were kept under standard conditions of temperature (23±1°C), relative humidity (55±10%), and 12/12 hours light/dark cycle and had free access to standard diet and tap water.

**Chemicals**

Thiobarbituric acid (TBA), TNBS, trichloroacetic acid (TCA), n-butanol, hexadecyltrimethyl ammonium bromide (HETAB), 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ), malondialdehyde (MDA), proteinase inhibitor, dinitrophenylhydrazine (DNP) from Merck (Germany), blood agar plates from Padtan Teb Co. (Iran), CFA from Razi Institute (Iran), Remicade® from Schering-Plough Pty Ltd. (Ireland), Triton X-100, rat specific ELISA kit for protein carbonyl enzyme, TNF-α, IL-1β and IL-17 ELISA kits from BioSource (Belgium) were used in this study.

**Preparation of mixed adjuvant-inactivated bacteria suspension**

A complete colon of a decapitated healthy rat with all its components was resected. The colon tissues were divided into several pieces. The pieces were moved into sterilized normal saline as quickly as possible. The suspension was inoculated into blood agar plates at 37°C for 72 hours.

For experimental procedure, two loops of the suspension were added to 2 mL normal saline and incubated at 70°C for 10 seconds[19]. Later 0.2 mL of the heated suspension was mixed with 0.2 mL of Complete Freund Adjuvant (CFA) to prepare each s.c. injection[12,18]. The booster dose was administrated 10 days later[26,27].

**Induction of colitis with TNBS**

In TNBS groups, after 36 hours fasting, rats received 10 mg TNBS dissolved in 30% ethanol intra-rectally. To follow procedure, the rats were slightly anesthetized with i.p. administration of 45 mg/kg pentobarbital sodium and the TNBS solution was administrated rectally using a rubber cannula when rats were on Trendelenburg position. It takes seven days to induce a complete colitis[20].

**Experimental design**

Seven groups of animals containing six rats in each group were used. The experimental groups included normal rats (Sham), positive control, which received TNBS enema (Control), ethanol 30% enema (E30), adjuvant+ethanol 10% enema (CFA+E10), adjuvant+ethanol 20% enema (CFA+E20), adjuvant+ethanol 30% enema (CFA+E30) and the last was another group of CFA+E30 which treated with infliximab (Infmab). Sham group received normal saline rectally. E30 group received 30% ethanol enema and Control group received TNBS dissolved in 30% ethanol. All the other four groups received the above-mentioned s.c. injection and the booster dose was injected 10 days later. Two weeks after the second dose, CFA+E10, CFA+E20 and CFA+E30 groups received 1 mL of various percentages of ethanol enema (30%, 20%, 10%), respectively using a rubber cannula. Infamb group received ethanol 30% enema followed by 5 mg/kg/day i.p. for five consecutive post observing diarrhea[27].

**Sample preparation**

Ten days after ethanol enema in CFA+E10, CFA+E20, CFA+E30 and Infamb groups, animals were sacrificed. Subsequently, the abdomens were dissected open and the colons were taken out, cut open in an
ice bath, cleansed gently with cold saline and then, divided into two pieces; one piece was weighted and homogenized in 10 volume ice cold potassium phosphate buffer (50 mM, pH=7.4), then 100 μL of the homogenates was taken for FRAP assay and kept in -80°C until analysis. The rest of sample was sonicated and centrifuged for 30 minutes at 3500 g. The plates were separated, and the supernatants were distributed into several microtubes kept in -80°C until final analyses. The second pieces were sent to a pathologic lab for macroscopic and microscopic scoring.

Macroscopic and microscopic assessment of colonic damage
The severity of colitis was assessed using the colon macroscopic scoring as described in table 1. For microscopic evaluation, the fixed segments in 10% formalin were embedded in paraffin and then 200 μm sections were prepared and stained with hematoxylin and eosin. Then, the sections were scored by a technician who was blind to treatment of experimental groups. Details are described in table 2.

<table>
<thead>
<tr>
<th>Table 1 Macroscopic scoring of colonic damage.</th>
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<tr>
<td><strong>Macroscopic features</strong></td>
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<tr>
<td>Normal appearance with no damage</td>
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<tr>
<td>Localized hyperemia without ulceration</td>
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<tr>
<td>Linear ulceration without significant inflammation</td>
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<tr>
<td>Linear ulceration with inflammation at one site</td>
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<td>Two or more sites of ulceration extending more than 1 cm along the length of colon</td>
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<tr>
<td>Damage extending more than 2 cm along the length of colon (score is increased by 1 for each increased cm of involvement)</td>
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<th>Table 2 Microscopic scoring of colonic damage.</th>
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<tr>
<td><strong>Microscopic criteria</strong></td>
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<tr>
<td>Score</td>
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<tr>
<td>No damage</td>
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<tr>
<td>Mild inflammation of mucosa</td>
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<tr>
<td>Moderate inflammation of mucosa and superficial sub-mucosa, focal superficial ulceration and granulation tissue formation, crypt distortions and crypt branching</td>
</tr>
<tr>
<td>Severe inflammation of mucosa, sub-mucosa and superficial muscular layer; Diffused ulceration and granulation</td>
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<td>Highly severe intense trans mural inflammation and/or diffuse necrosis</td>
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<th>Table 3 Macroscopic and microscopic assessment of colonic damage.</th>
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<td><strong>Groups</strong></td>
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<tr>
<td>Sham</td>
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<tr>
<td>Control</td>
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<td>Infmab</td>
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<td>CFA+E10</td>
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<td>CFA+E20</td>
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<td>CFA+E30</td>
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1Significantly different from normal group at p<0.05; 2Significantly different from control group at p<0.05; 3Significantly different from Infmab group at p<0.05; 4Significantly different from CFA+E10 group at p<0.05; 5Significantly different from CFA+E20 group at p<0.05; 6Significantly different from CFA+E30 group at p<0.05.

Biochemical Assays
TNF-α, IL-1β and IL-17
TNF-α, IL-1 and IL-17 were measured by use of rat specific ELISA kits according to the manufacturer’s protocols. To prepare ELISA samples from colon tissues, the colons were collected and homogenized with 0.5 mL of phosphate saline buffer containing 1% Triton X-100 with proteinase inhibitor cocktail. The amount of cytokines was assessed at the final step by measuring the absorbance of sample in 450 nm as the primary wave length and 620 nm as the reference by ELISA reader as described by kit user manual. Data were expressed as pg/mg of tissue for TNF-α, IL-1β and ng/mg protein for IL-17.<sup>29</sup>

Myeloperoxidase (MPO)
MPO activity was measured by observing the rate of changes in the absorbance of UV spectrophotometer for 3 minutes in 460 nm and was reported as u/mg of the tissue protein.<sup>29</sup>

Thiobarbituric Acid Reactive Substances (TBARS)
Lipid peroxidation process leads to variety of aldehydes, specifically MDA. This substance could react with TBA to produce a measurable pink color with maximum absorption in 532 nm. Data were reported as mmol/g protein of the tissue.<sup>28</sup>

Ferric Reducing Antioxidant Power (FRAP)
The reduction of Fe<sup>3+</sup> to Fe<sup>2+</sup> in complex with TPTZ produces a blue color that is measured at 593 nm by an UV spectrophotometer.<sup>31</sup> Data was expressed as mmol ferric ions reduced to ferrous per gram of colon tissue.

Protein carbonyl
Amount of protein carbonyl was determined by rate of conjugation with DNP that has an absorbance at 450 nm. Reference wave length was assigned in ELISA reader as instructed by the kit brochure. This method enabled us to measure carbonyls quantitatively with microgram quantities of protein.<sup>22</sup>

Total protein of colon homogenate
Total protein of tissue was measured according to Lowry’s method, and the standard curve was obtained from various concentration of BSA as the standard. Results were reported as mg/mL of homogenized tissue.<sup>31</sup>

Statistical analysis
Data were analyzed by one-way ANOVA followed by Tukey’s post-hoc test for multiple comparisons. P-values less than 0.05 were considered significant. Results were expressed as mean±S.E.M. Microscopic and macroscopic scores were analyzed by Kruskal-Wallis method followed by Nemenis post-hoc test for multiple comparisons. P-value less than 0.05 were considered significant and data were expressed as median, minimum and maximum scores. Stats Direct was used to analyze data.

RESULTS
Colonie TNF-α
The TNF-α increased in Control (p=0.001), CFA+E20 (p=0.01) and CFA+E30 (p<0.001) groups in comparison to Sham group. TNF-α was lower than that of Control group in Infmab, CFA+E10, CFA+E20 and E30 groups (p<0.001). There was no significant difference between CFA+E30 and Control groups. No difference was found between Infmab group and other groups, including CFA+E10, CFA+E20 and E30. TNF-α was increased in CFA+E30 group compared to CFA+E10, CFA+E20, E30 groups (p<0.001), and was lower in E30 group when compared to CFA+E20 group (p=0.01) (Figure 1).  

Colonie IL-1β
The IL-1β level was significantly increased in Control, CFA+E20 and CFA+E30 groups in comparison to Sham group (p<0.001). IL-1β was
lower than that of Control group in Infmab, CFA+E10, CFA+E20 and E30 groups (p<0.001), but there was no significant difference in IL-1β between CFA+E30 and Control groups. Infliximab significantly decreased IL-1β when compared to CFA+E30 (p<0.001) and CFA+E20 groups (p<0.01). Its level was significantly increased in CFA+E30 group compared to CFA+E20 group (p<0.001), and was increased in CFA+E20 group (p<0.001) when compared to E30 group (p<0.001) (Figure 2).

Colonic IL-17
The IL-17 was significantly increased in Control and CFA+E30 groups in comparison to Sham group (p<0.001), but there was no significant difference in IL-17 between CFA+E30 and Control groups. IL-17 was lower than that of CFA+E30 group in Infmab (p<0.01), CFA+E10 (p<0.05), CFA+E20 (p<0.05) and E30 groups (p<0.01). Infliximab decreased IL-17 significantly when compared to CFA+E30 (p<0.001), CFA+E20 (p<0.01) and E30 groups (p<0.01) (Figure 3).

Colonic MPO activity
The MPO activity in the Control and CFA+E30 groups were higher than that of Sham group (p<0.001). In addition, CFA+E20 group showed an increased MPO in comparison to Sham group (p<0.05). There was no significant difference in MPO activity between Infmab, CFA+E10, E30 and Sham groups. MPO activity was lesser than that of Control group in Infmab, CFA+E10, CFA+E20 and E30 groups (p<0.001), whereas MPO activity in CFA+E30 group was the same as Control. MPO activity in Infliximab treated group was significantly lesser than that of CFA+E30 (p<0.001) and CFA+E20 groups (p<0.05). MPO activity increased in CFA+E30 compared to CFA+E20 (p<0.001), and was lower in E30 when compared to CFA+E20 (p<0.05) (Figure 4).

Figure 1 Tumor necrosis factor-a (TNF-α) level in colon. Values are means±SEM. aSignificantly different from normal group at p<0.01. bSignificantly different from normal group at p<0.001. cSignificantly different from control group at p<0.01. dSignificantly different from Infmab group at p<0.01. eSignificantly different from CFA+E10 group at p<0.01. fSignificantly different from CFA+E20 group at p<0.01. gSignificantly different from CFA+E30 group at p<0.01.

Figure 2 Interleukin-1β (IL-1β) level in colon. Values are means±SEM. aSignificantly different from normal group at p<0.05. bSignificantly different from normal group at p<0.01. cSignificantly different from control group at p<0.01. dSignificantly different from Infmab group at p<0.01. eSignificantly different from CFA+E10 group at p<0.01. fSignificantly different from CFA+E20 group at p<0.01. gSignificantly different from CFA+E30 group at p<0.01.

Figure 3 Interleukin-17 (IL-17) level in colon. Values are means±SEM. aSignificantly different from normal group at p<0.005. bSignificantly different from normal group at p<0.001. cSignificantly different from control group at p<0.01. dSignificantly different from Infmab group at p<0.01. eSignificantly different from CFA+E10 group at p<0.01. fSignificantly different from CFA+E20 group at p<0.01. gSignificantly different from CFA+E30 group at p<0.01.

Figure 4 Myeloperoxidase (MPO) activity in colon. Values are means±SEM. aSignificantly different from normal group at p>0.05. bSignificantly different from normal group at p<0.001. cSignificantly different from control group at p<0.001. dSignificantly different from Infmab group at p<0.05. eSignificantly different from Infmab group at p<0.01. fSignificantly different from CFA+E10 group at p<0.01. gSignificantly different from CFA+E20 group at p<0.01. hSignificantly different from CFA+E30 group at p<0.01.
it was the same as Control in CFA+E30 group. FRAP in Infliximab treated group was significantly increased compared to CFA+E30 (p<0.001) and CFA+E20 (p<0.01). Its level was decreased in CFA+E30 group compared to CFA+E20 group (p<0.001) (Figure 6).

Colonic carbonyl proteins

Protein carbonyls in Control were significantly higher than Sham. Significant increase in CFA+E20 (p<0.05) and CFA+E30 (p<0.001) was observed in comparison to Sham. Protein carbonyls were lesser than that of Control in Inf, CFA+E10, CFA+E20 and E30 groups (p<0.001), and was the same as Control in CFA+E30. Infliximab caused a decrease in protein carbonyls amount in comparison to CFA+E30 (p<0.001) and CFA+E20 groups (p<0.05). Protein carbonyls increased in CFA+E30 group compared to CFA+E20 group (p<0.01) and were lower in E30 (p<0.01) and CFA+E10 (p<0.001) groups in comparison to CFA+E20 group (p<0.001) (Figure 7).

DISCUSSION

We activated immune system in rats by injecting a mixture of an approved adjuvant and mixture of heated enteric bacteria. Subsequently, we induced a calm disturbance in the colon’s epithelial cell barrier by ethanol enema. This mild breach caused more interactions between microflora and host mucosal immune system continued by immunologic responses.

In this model, the enteric bacteria have the fundamental role in developing the disease.

A recent study in mice showed that ethanol exposure reduces the distribution of tight junction proteins, but did not significantly affect the intestinal histopathology[35]. Other study showed that epithelial cells near the top of the intestinal villus were more affected with ethanol exposure[36].

According to previous findings, we used ethanol enema as an agent for disturbing the epithelial cells and tight junction proteins. We tried to create the same pathological condition which happens in IBD patients by enhancing the interactions between host immune system and microbiota with ethanol enema[34,36,37,38].

TNBS-induced colitis model has been extensively used in many experimental studies[39]. Although the caustic property of TNBS is partly responsible for the mucosal injury, this model shows inflammation and alteration in the colon with similar features to CD in humans.

Previous studies show that TNBS elevates IL-1β and TNF-α. In this study, a significantly higher amount of IL-β and TNF-α was
cytokine's patterns in IBD patients.

The chemotactic products of LPO provide positive feedback to accelerate the inflammatory oxidative process. Colonic mucosa may be overwhelmed during the active inflammatory response, and thus localized intestinal inflammation pursues. This may be due to the inability of the mucosa to ameliorate the generating stress[28, 41]. Measurement of MDA with TBA assay test for this study showed significantly higher amounts in Control, CFA+E30 ($p<0.001$), CFA+E20 ($p=0.01$) groups than that of Sham. The colonic injury and dysfunction observed in inflammatory bowel disease seem resulting from intensification of these reactive free radicals. Infliximab reduced MDA in CFA+E30 group. Infliximab binds to the human TNF-α, enhances leukocyte migration, activates neutrophils and eosinophils, and induces acute phase reactants and tissue degrading enzymes[27]. The pro-inflammatory cytokines are no longer induced after administration of Infliximab and it's binding to TNF-α.

A significantly increase of MPO in colon has been previously reported in IBD models that indicate the damage of neutrophil granules resulted from destructive action of autoantibodies. In our study, MPO increased in Control, CFA+E20, CFA+E20, and CFA+E30 groups. No significant difference between Control and CFA+E30 in MPO activity was found. We assume that neutrophil sequestration and infiltration into gastrointestinal tract happens in CFA+E30 group.

The antioxidant power of colonic tissue in CFA+E30 group was diminished most probably through action of autoantibodies against colonic tissue. Meanwhile no significant difference was observed in Infliximab treated group when compared to CFA+E30.

Protein carbonyl content is actually the most general indicator of protein oxidation generally used in evaluation of oxidative stress in IBD models. Significant increases in the carbonyl amount were seen in CFA+E20 and CFA+E30 in comparison to Sham. This biomarker did not significantly change in CFA+E30 when compared to Control. Infliximab decreased level of protein carbonyl in comparison to CFA+E30.

An ideal experimental model is one that mimics the complex pathology of a typical human IBD while covering all the characteristics of disease. Immunological and pathological assessment showed that the present model has such similar morbidity characteristics. In addition the present model has low cost, short duration of induction, simplicity, reproducibility, and minimal harm to the animal.

**CONCLUSION**

Recently we hypothesized that prior activation of adaptive immunity against microbial flora antigens could initiate an IBD-like chronic inflammation[8, 23]. Now the idea is confirmed by the present results and strengthen the possibility of subcutaneous vaccination with administration of all or specific enteric bacterial antigens. In the CFA+E30 group, the increase in neutrophil infiltration, TNF-α, IL-17, IL-1β and LPO and also reduction of antioxidant capacity in addition to increased microscopic and macroscopic scores in colonic tissue was observed. This model closely resembled the TNBS-induced colitis model. However, it seems to be more credible with macroscopic scores. Another benefit of this experimental model is that no mortality was observed while it is frequently seen in TNBS model. It is important to note that the caustic property of TNBS is partly responsible for the mucosal injury and induction of IBD, while in the present model, IBD develops in a way closely similar to typical human IBD in terms of microbial initiation and activation of immune system responses.
ACKNOWLEDGMENT

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CONFLICT OF INTERESTS

There are no conflicts of interest with regard to the present study.

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