

Original article

The Effects of Caffeic Acid Phenethyl Ester (CAPE) on Acetic Acid Induced Colitis in Rats

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ÖZET

Kafeik asit fenetil esterin ratlarda asetik asitle indüklenmiş kolite etkileri "

Kafeik asit fenetil ester (KAPE), iskemi-reperfüzyon ve toksik hasarda oksidatif stres yoluyla ortaya çıkan reaktif oksijen ürünlerinden dokuyu koruyan, propolis ekstresinin aktif bir komponentidir. İnflamatuvar barsak hastalığının (İBH) patogenezinde anormal oksidatif metabolizma önemli olduğundan, İBH'da serbest radikallerin rolü üzerine dikkat çekilmiştir. Bu çalışmanın amacı ratlarda asetik asitin (AA) neden olduğu kolitte KAPE'nin antioksidan parametrelere ve kolit gelişimi üzerine etkilerini değerlendirmektir. 21 adet 150-200 g ağırlığında Wistar-albino dişi rat kullanıldı. Ratlar rastgele 3 gruba ayrıldı: sırasıyla kontrol grubu (6), kolit grubu (8) ve KAPE grubu (7). %4'lük AA kolon içine lavmanla verilerek kolit oluşturuldu. AA lavmanı ile kolit oluşturulduktan sonra 3 gün süreyle KAPE verilerek kolit üzerine KAPE'nin sonraki etkileri değerlendirildi. Kolit grubunda artmış olan kolonik malondialdehit ve nitrik oksit düzeyi KAPE tedavisiyle (10 µmol/kg) azaldı (sırasıyla p=0.043, p=0.006). Kolit grubunda azalmış olan glutatyon aktivitesi KAPE tedavisiyle arttı (p=0.008). KAPE makroskopik olarak kolit grubunun lezyon skorunda azalmaya neden olmadı (p>0.05). Bizim verilerimiz KAPE tedavisinin biyokimyasal parametrelerde pozitif etkisini gösterirken, istatistiksel olarak anlamlı bir histolojik iyileşme oluşturmadığını göstermiştir. KAPE, antioksidan ve antiinflamatuvar etkileriyle ratlarda AA ile oluşturulan kolit modelinde yararlı gibi görünmektedir. Biz daha uzun süreli ve daha yüksek doz KAPE ile yapılan ilave, daha anlaşılır deneysel çalışmalarla KAPE'nin etkilerinin değerlendirilmesi gerektiğine inanıyoruz.

Anahtar Kelimeler: Asetik asit, kafeik asit fenetil ester, kolit, antioksidan, antiinflamatuvar etki

ABSTRACT

Caffeic acid phenethyl ester (CAPE), an active component of propolis extract, protects tissues from reactive oxygen species mediated oxidative stress in ischemia-reperfusion and toxic injuries. Since abnormal oxidative metabolism is important in the pathogenesis of inflammatory bowel disease (IBD), increased attention has been focused on the role of free radicals in IBD. The aim of this study was to investigate the effect of CAPE on the development of colitis and antioxidant parameters of rats subjected to acetic acid (AA) induced colitis. Twenty one female Wistar-albino rats weighing 150-200 g were used. The rats were randomly divided into three groups: control group (n=6), colitis group (n=8), CAPE group (n=7), respectively. Colitis was induced by intracolonic enema with 4% AA. Colitis was induced using an enema of AA following which CAPE was administered for 3 days to induce colitis and effect of CAPE was subsequently evaluated. The increase in colonic malondialdehyde and nitric oxide level at the colitis group was reduced by CAPE (10 µmol/kg) treatment (p=0.043, p=0.006, respectively). Reduced glutathione activity in the colitis group was increased by CAPE treatment (p=0.008). Treatment with CAPE did not reduce the lesion score of the colitis group at macroscopic level (p>0.05). Our data showed its positive effects on biochemical parameters, although there was not statistically significant histologic improvement with CAPE treatment. CAPE seemed to be beneficial in an AA-induced rat colitis model through the antioxidant and anti-inflammatory effect. We believe further studies such as longer CAPE treatment and higher dosages are needed to investigate the effects of CAPE more clearly in experimental colitis.

Key Words: Acetic acid, caffeic acid phenethyl ester, colitis, antioxidant, anti-inflammatory effect

INTRODUCTION

Inflammatory bowel disease (IBD) classically includes two forms, ulcerative colitis and Crohn's disease, each representing different patterns of chronic spontaneously remitting and relapsing disorders of the gastrointestinal tract¹. Although the pathophysiology of IBD is not known with certainty, immunological processes and reactive oxygen species (ROS), such as peroxide anion, hydrogen peroxide (H₂O₂), and hypochloric acid

have been proposed to contribute considerably in development of tissue injury^{2,3}. Many other inflammatory mediators have been also related; tumour necrosis factor-α (TNF-α) plays a prominent role and the neutralization of this cytokine is accompanied by a remarkable clinical response in patients with IBD⁴. Toxic oxidants are capable of destroying tissue if their rate of production exceeds the capacity of endogenous

antioxidant defense mechanisms^{5,6}. Under normal physiological conditions, antioxidant defense mechanisms protect tissues from ROS. Defense mechanisms consist of several radical scavenger and enzymes, including superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH) and peroxidases. However, the gut is potentially vulnerable to oxidant injury due to a low concentration of antioxidant enzymes, which are mainly localized in epithelial cells⁷.

Caffeic acid phenethyl ester, a flavonoid like compound, is one of the major components of honeybee propolis. CAPE, have possibly no harmful effects on normal cells⁸. It has been used in traditional medicine to treat a number of diseases. The biological activities of CAPE have been reported to include antioxidant activity^{9,10}, vasorelaxant effects¹¹, antiinflammatory effects¹², anticarcinogenic activities¹³, and immunomodulatory effects¹⁴. The antioxidant activity of CAPE is due to the presence of two hydroxyl groups in its structure. CAPE treatments have been shown to protect tissues from ROS-mediated oxidative stress and reduce lipid peroxidation in ischemia and toxic injuries^{15,16}.

To date there have been only one report on the possible use of CAPE as a therapeutic drug in (bilateral ovariectomized) rats subjected to trinitrobenzene sulfonic acid (TNBS)-induced colitis. The aim of the present study was to evaluate the effects of CAPE supplementation in tissue malondialdehyde (MDA) levels, NO levels, SOD activity, GSH activity and CAT activity in AA-induced IBD model in female rats.

MATERIAL AND METHODS

Caffeic acid phenethyl ester was synthesized in the Physico-Chemistry Laboratory using the technique described by Grunberger. A solution of CAPE was prepared (25 $\mu\text{mol/ml}$)¹⁷. Twenty one, female, Wistar albino rats (age 8-12 weeks), weighing 150-200 g, obtained from Laboratory Animal Production Unit of Fatih University were used in the experiment. The animals were procured, maintained and used in accordance with the "Animal welfare act and the guide for the care and use of laboratory animals" prepared by the Fatih University Animal Ethics Committee. Rats were placed in a temperature (22-28°C) and humidity (65-70%) controlled room, in which a 12-hour light/dark cycle was maintained for one week before the start of the experiment. A commercially balanced diet (Hasyem Ltd. Ankara Turkey) and tap water were provided ad libitum. The rats were randomly divided into three groups: Group I, control group (n=6); Group II, intrarectal

AA group (n=8); Group III, intrarectal AA + intraperitoneal CAPE group (n=7). After fasting the animals overnight, following intraperitoneal anesthesia with ketamine (50 mg/kg) inflammation was induced in the colon by the intrarectal administration of 1 ml 4% AA solution using 8-cm-soft 6F pediatric catheter. Group 3 rats were treated intraperitoneally (ip) with CAPE (10 $\mu\text{mol/kg}$) for 3 days following the induction of colitis. Control rats received 1ml 0.9% saline infusion instead of 4% AA. Under general anesthesia, all rats were sacrificed by cervical dislocation and then laparotomy was performed. The colon mucosa was sampled for a variety of determinations and observations after the animals were sacrificed.

Histopathological examination

The colon segment taken from 10 cm proximal to anus of the sacrificed rats was excised longitudinally, rinsed by saline buffer and fixed on a wax block. For histopathological examination colonic specimens were fixed in 10% buffered formalin and four to six colon rings were obtained from each colon segment. Then tissues were processed routinely, embedded in paraffin and cut into 4 μm sections. Paraffin sections were deparaffinized with xylene, hydrated and stained with hematoxylin and eosin. Then the sections were evaluated by light microscopy by a pathologist unaware of the experiments being performed. Histological scoring was performed as a combined score of inflammatory cell infiltration (score 0-3) and tissue damage (score 0-3)¹⁸. The presence of occasional inflammatory cells in the lamina propria was scored as 0, increased numbers of inflammatory cells in the lamina propria was scored as 1, confluence of inflammatory cells extending into the submucosa was scored as 2, and transmural extension of the infiltrate was scored as 3. For tissue damage; no mucosal damage was scored as 0, lymphoepithelial lesions were scored as 1, surface mucosal erosion or focal ulceration was scored as 2, and extensive mucosal damage and extension into deeper structures of the bowel wall were scored as 3. The combined histological score ranged from 0 (no changes) to 6 (extensive cell infiltration and tissue damage).

Biochemical study

All rat colon tissue samples were stored at -80 °C until biochemical analyses. MDA and NO levels, GSH, SOD, and CAT activities were measured in tissue extracts of the dissected colon.

Malondialdehyde level measurement

The breakdown product of lipid peroxidation, TBARS, was measured using the method of¹⁹ Buege and Aust briefly, the stock solution

contained equal volumes of 15% (w/v) trichloroacetic acid in 0.25 N hydrochloric acid and 0.37% (w/v) 2-thiobarbituric acid in 0.25 N hydrochloric acid. One volume of the sample and two volumes of stock reagent were mixed in a tube, vortexed, and heated for 15 min in a boiling water bath. After cooling on ice, the precipitate was removed by centrifugation at 1000 g for 15 min, and absorbance was measured at 532 nm against a blank containing all of the reagents except the test sample. Results were expressed in terms of nmol/g protein.

Superoxide dismutase activity measurement

The principle of the total (Cu-Zn and Mn) superoxide dismutase (t-SOD) enzyme activity method is based, briefly, on the inhibition of nitroblue tetrazolium (NBT) reduction by O_2^- generated by the xanthine/XO system^{20,21}. Activity was assessed in the ethanol phase of the supernatant from colon tissue after 1.0 ml ethanol/chloroform mixture (5/3, v/v) was added to the same volume supernatant and centrifuged. One unit of SOD was defined as the enzyme amount causing 50% inhibition in the NBT reduction rate. Colon tissue SOD activity was also expressed as units per m.gram protein (U/mg protein).

Glutathione activity measurement

Glutathione activity was measured by the method of Paglia and Valentine²². The enzymatic reaction in the tube that contained reduced nicotinamide adenine dinucleotide phosphate, reduced GSH, and sodium azide and glutathione reductase was initiated by the addition of H_2O_2 and the change in absorbance at 340 nm was monitored by a spectrophotometer. Activity was given in units per mg protein (U/mg protein).

Catalase activity measurement

Colon tissue CAT activity was measurement according to Aebi's method²³. The essential of the method was based on the determination of the rate constant k (s⁻¹, k) of the H_2O_2 decomposition rate at 240 nm. Results were expressed as rate constant per gram protein; k/g protein.

Nitric oxide level measurement

Nitric oxide has a half-life of only a few seconds because it is readily oxidized to nitrite (NO_2^-) and subsequently to nitrate (NO_3^-) which serve as index parameters of NO production. The method for nitrite and nitrate levels was based on the Griess reaction²⁴. Samples were initially deproteinized with Somogyi reagent. Total nitrite (nitrite+nitrate) was measured by spectrophotometry at 545 nm after conversion of nitrate to nitrite by copperized cadmium granules. A standard curve was established with a set of serial

dilutions (10^{-8} – 10^{-3} mol l⁻¹) of sodium nitrite. Linear regression was done by using the peak area from nitrite standards. The resulting equation was used to calculate the unknown sample concentrations. Results were expressed as μ mol/g protein.

Statistical analysis

All data were presented as means $\pm$ standard deviation (S.D.). Statistical analyses were carried out using the SPSS ver. 13 statistical program (SPSS, Chicago, IL). Distribution of the groups was analyzed with one sample Kolmogorov-Smirnov test. As all groups showed normal distribution, group differences were analyzed using parametric statistical methods, independent samples t test following one-way ANOVA. A P-value of <0.05 was considered as statistically significant.

RESULTS

The results of the histological analyses of microscopic damage scores revealed that there were no difference between the AA-induced colitis and the CAPE treated groups ($p>0.05$) (Figure 1 and 2).

Figure 1. Light microscopic examination of histological appearance of rat colonic mucosa in acetic acid-induced colitis; extensive mucosal damage was generally accompanied by diffuse submucosal oedema, hemorrhage and inflammatory cell infiltration which was predominated by polymorphonuclear leukocytes

Figure 2. Light microscopic examination of histological appearance of rat colonic mucosa in acetic acid-induced colitis and the effects of caffeic acid phenethyl ester (CAPE, 10 μ mol/kg) on colon injury. Significant histologic improvement was not observed in the colon specimens of CAPE treated rats compared to the rats received only acetic acid

Significant histologic improvement was not observed in the colon specimens of the CAPE treated rats compared to the rats received only acetic acid. In colon specimens of some rats treated with CAPE, smaller ulcers covered with regenerated epithelium were observed (Figure 3).

Figure 3. In some colon specimens of rats treated with CAPE smaller ulcers covered with regenerated epithelium were observed. Original magnification was 10x (figure 1,2,3) on hematoxylin and eosin stained preparations

In those rats treatment with CAPE did not show a significantly reduced microscopic damage score compared to rats with AA-induced colitis ($P>0.05$, Figure 2). For the biochemical analyses, the levels of MDA and NO level in the colitis group were higher than the control group, but did not show significant difference between colitis and control groups [(0.064±0.016, 0.049±0.012 nmol/g protein Figure 4) and (2.76±0.97, 2.07±0.76 µmol/g protein Figure 5), $p>$, respectively)]. Rats treated with CAPE have significantly reduced levels of MDA (0.047±0.006 nmol/g protein) and NO (1.39±0.232 µmol/g protein) compared to the rats with AA-induced colitis ($p=0.043$, $p=0.006$, respectively) (Figure 4, 5); however there was no difference between control and CAPE treated group ($p>0.05$) (Figure 4,5).

Figure 4. Effects of CAPE (10 µmol/kg) administered intraperitoneally for 3 consecutive days after the induction of colitis Malondialdehyde (MDA) levels in rat colonic mucosa (group 1; control, group 2; acetic acid, group 3; acetic acid and CAPE) $p=0.043$; group 2 compared with the group 3).

Figure 5. Effects of CAPE (10 µmol/kg) administered intraperitoneally for 3 consecutive days after the induction of colitis Nitric oxide (NO) levels in rat colonic mucosa (group 1; control, group 2; acetic acid, group 3; acetic acid and CAPE) ($p=0.006$; group 2 compared with the group 3)

The activity of GSH in the colitis group was lower than control group but was not significant statistically (4.2±2, 4.6±1.2 U/mg protein, respectively). CAPE administration significantly increased the levels of tissue GSH in comparison with colitis and control [(8.1±2.6 U/mg protein, 4.2±2.1 U/mg protein, 4.6±1.2 U/mg protein, respectively) ($p=0.008$, $p=0.01$), respectively)] (Figure 6).

Figure 6. Effects of CAPE (10 µmol/kg) administered intraperitoneally for 3 consecutive days after the induction of colitis. Glutation (GSH) activities in rat colonic mucosa (group 1; control, group 2; acetic acid, group 3; acetic acid and CAPE) ($p=0.008$; group 2 compared with the group 3).

Acetic acid administration significantly increased the tissue CAT activity in comparison with the controls [(0.054±0.015, 0.034±0.015) k/g protein ($p=0.046$)]. The CAT activity in rats receiving CAPE was lower than the rats with AA-induced colitis, but it did not show significant difference (0.044±0.012, 0.05±0.15 k/g protein, $p=0.469$) (Figure 7).

Superoxide dismutase activity was increased in the AA-induced colitis group compared to control group, but there was no significant difference (143.5±40.4, 137.7±27.2 U/mg protein, respectively). Also SOD activity in the CAPE treated group was

higher than the AA-induced colitis group; however there was no significant difference (146 ± 18 , 143.5 ± 40.4 U/mg protein, respectively (Figure 8).

Figure 7,8. Effects of CAPE (10 μ mol/kg) administered intraperitoneally for 3 consecutive days after the induction of colitis. Catalase (CAT) and superoxide dismutase (SOD) activities in rat colonic mucosa (group 1; control, group 2; acetic acid, group 3; acetic acid and CAPE) there was no significant difference between three groups.

DISCUSSION

In our study, significant histologic improvement was not observed in the colon specimens of CAPE treated rats compared to the rats received only acetic acid. Ek et al. suggests that, treatment with CAPE 10 and 30 mg/kg, them respectively, caused a significant reduction in colon injury compared to that observed in rats with TNBS-colitis (colitis control) and vehicle-treated (intrarectal TNBS+ip ethanol) groups²⁵. Yildiz et al. suggests that, treatment with CAPE 30 mg/kg, was more efficient in preventing intestinal injury²⁶. Fitzpatrick showed that treatment with CAPE 30 μ M/ml, caused a significantly reduced colonic injury associated with chronic reactivation phase of peptidoglycan-polysaccharide-induced colitis²⁷. In our study although in colon specimens of some rats treated with CAPE smaller ulcers covered with regenerated epithelium were observed, there was not significant histologic improvement in CAPE treated rats. This may be due to low doses of CAPE in our study.

We showed that the levels of MDA and NO in colitis group were higher than the control group, but did not show significant difference between these groups. Rats treated with CAPE have significantly reduced levels of MDA compared to the rats with AA-induced colitis. Therapy with CAPE (10 μ mol/kg) for 3 days resulted in a marked decrease in MDA levels in colonic tissue, suggesting that CAPE successfully inhibited lipid peroxidation induced by acetic acid. Oxidative stress and its consequent lipid peroxidation are able to aggravate free radical chain reactions, disrupt the integrity of intestinal mucosa barrier and activate inflammatory mediators, resulting in

increased colonic MDA contents, as shown in both human and experimental animal studies^{25,28}. Colonic biopsy from specimens of patients with active IBD had enhanced levels of lipid peroxidation products. These findings suggest that chronic gut inflammation promotes an imbalance between prooxidant and antioxidant mechanisms, leading to the net accumulation of oxidatively modified proteins and lipids. A number of experiments have shown that the MDA level in patients with IBD decreases as a result of antioxidant and antiinflammatory agents²⁸. Harputluoğlu et al. reported that tissue and serum MDA levels in colitis group were significantly higher than control group. In their study, tissue MDA level in treated group was lower than colitis group, but the difference was not significant statistically²⁸. Kurutas et al. showed that plasma MDA levels were increased in colitis group and in this study, tissue MDA level in treated group was lower than colitis group³¹. Ek et al. reported that the levels of MDA in the colitis and vehicle control groups increased statistically in comparison with the level in the CAPE (30 mg/kg) group. Therapy with CAPE for 3 days resulted in a marked decrease in MDA levels in colonic tissue in a dose-dependent manner²⁵. In our study we found similar results with this study in respect of CAPE's improvement effect on increased MDA levels in colitis model. We also found a significant increase in NO level in the colitis group compared to the control group. We showed a significant decrease in tissue NO level in CAPE group compared to AA-induced colitis group. It may be considered quite definitive that NO is associated with the initiation and maintenance of inflammation in IBD and that the selective inhibition of inducible NO synthase (iNOS) reduces the tissue damage³². Data from human samples confirm this proposal because in mucosa biopsies from patients with inflammatory colitis the NO levels are greatly increased and a marked decrease is observed in those patients that respond to anti-inflammatory therapy³³. Meanwhile, excessive NO could dilate vasculature and enhance vasopermeability, as well as inactivate the activity of antioxidants such as SOD, CAT, and GSH by means of reacting with hydrosulfide group (-SH) in the enzymes. Our findings indicate a slightly lower GSH activity in the colitis group than control but it was not statistically significant. Moreover GSH activity in CAPE group was significantly increased than control and colitis groups. We believe because of its antioxidant property, CAPE increased GSH activity in this group. GSH is an important nonenzymatic antioxidant and, similar to other

sulfhydryl-containing products, it also has regulatory and protective roles in the body. Nieto et al. found that the level of GSH was lower in TNBS induced ulcerative colitis³⁴. Ek et al. found that a significant decrease in the activity of GSH in the colonic tissue of the colitis control group and that the administration of CAPE prevented this depletion.

The balance between oxidant and antioxidant systems is extremely important in the pathogenesis of IBD and in the progression of tissue injury. Enzymes such as SOD and CAT as well as nonenzymatic antioxidants such as GSH constitute the antioxidant system. SOD is an important protective system that accelerates the dismutation of superoxide anion radicals to hydrogen peroxide and acts as a primary defense. Results of the studies examining the status of the antioxidant enzyme SOD in experimental colitis are controversial³¹. CAT is also an enzyme responsible for the detoxification of H₂O₂ during the reaction catalyzed by SOD. However, the results of studies examining the status of antioxidant systems such as SOD and CAT in IBD are open to debate²⁵. Yildiz et al. reported that both CAPE doses (10 mg/kg and 30 mg/kg) successfully enhanced endogenous antioxidant enzyme (SOD, GSH-Px and CAT) activities and prevented lipid peroxidation in intestinal tissue²⁶. Our study indicates that SOD activity increased in AA-induced colitis group compared to control group. This result are consistent with the study reported by Yildiz et al. Also SOD activity in CAPE treated group was higher than AA-induced colitis group, but there was no significant difference among three groups. CAT activities significantly increased in AA-induced colitis group than control group. The CAT activities in rats receiving CAPE were lower than AA-induced colitis group, but there was no significant difference between these two groups. We believe these findings indicate an increase response to oxidative stress in colitis group. Kuralay et al. showed that tissue SOD levels were elevated in response to oxidative stress in experimental colitis model, they were decreased by antioxidant agents, but there was no change in CAT activities in an AA-induced colitis model³⁵. Kruidenier et al. demonstrated that colonic mucosa Cu/Zn-SOD and Mn-SOD levels were higher than the control levels in patients with inflammatory bowel disease³⁶. On the other hand, Ek et al. showed that marked decrease in activities of SOD and CAT in the colonic tissue of the colitis control and vehicle-treated animals and that the administration of CAPE increased the

activities of SOD and CAT in a dose dependent manner²⁵.

Some oxidants have been known to modulate the expression of a variety of genes that are involved in the immune and inflammatory responses, which lead to the apoptosis of intestinal epithelial cells, cascades of inflammatory response and the disruption of integrity and function of the intestinal mucosa^{37,38}. Since abnormal oxidative metabolism is important in the pathogenesis of IBD, increased attention has been focused on the role of free radicals in IBD³⁹.

Induction of colitis by AA in rats is the classical method to produce an experimental model of inflammatory bowel disease. Several major causative factors in the initiation of human IBD such as exorbitant oxidative stress, enhanced vasopermeability, prolonged neutrophils infiltration and increased production of inflammatory mediators were all involved in the induction of this animal model^{40,41}. It is therefore acknowledged nowadays that this experimental model is suitable for the investigation of IBD pathogenesis and evaluation the therapeutic agents of this disease⁴².

In many studies, it has been reported that antioxidants show beneficial effects on experimental colitis^{43,44}. Several antioxidant agents have been investigated as a means to prevent colitis due to oxidant exposure, including N-acetylcysteine^{31,43}, ascorbate⁴⁵, vitamin E and selenium^{31,46}. One of these agents is CAPE, which is derived from honeybee propolis and has attracted attention of researchers because of its antioxidant activity^{9,10}, antiinflammatory effects¹², and immunomodulatory effects¹⁴. Administration of CAPE completely blocks both the production of ROS in human neutrophils and the xanthine-xanthine oxidase system.

Our findings indicated that the administration of CAPE significantly decreased MDA and NO levels and increased GSH activities, demonstrating the antioxidant and anti-inflammatory effect in AA-induced colitis. In our study although in colon specimens of some rats treated with CAPE smaller ulcers covered with regenerated epithelium were observed, there was not significant histological improvement. These results may be due to short treatment period with CAPE or due to its inadequate dosage.

In summary, although there was not statistically significant histologic improvement with CAPE treatment, we found its positive effects on biochemical parameters. And our data showed that CAPE exerts protective effects in the acute colitis model induced by AA. To our knowledge, this is the second study to examine the effects of

CAPE histopathologically and biochemically in an experimental colitis model rats. We believe further studies with different protocols such as longer CAPE treatment and higher dosages are needed to

investigate the effects of CAPE more clearly in experimental colitis.

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