International Journal of Pharmaceutics 416 (2011) 145–154

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

N-Succinyl-chitosan systems for 5-aminosalicylic acid colon delivery: *In vivo* study with TNBS-induced colitis model in rats

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ARTICLE INFO

Article history:
Received 23 March 2011
Received in revised form 14 June 2011
Accepted 16 June 2011
Available online 23 June 2011

Keywords:
N-Succinyl-chitosan systems
5-Aminosalicylic acid
Colon delivery, TNBS induced colitis

ABSTRACT

5-Aminosalicylic acid (5-ASA) loaded *N*-Succinyl-chitosan (SucCH) microparticle and freeze-dried system were prepared as potential delivery systems to the colon. Physicochemical characterization and *in vitro* release and swelling studies were previously assessed and showed that the two formulations appeared to be good candidates to deliver the drug to the colon. In this work the effectiveness of these two systems in the treatment of inflammatory bowel disease was evaluated. *In vitro* mucoadhesive studies showed excellent mucoadhesive properties of both the systems to the inflamed colonic mucosa. Experimental colitis was induced by rectal instillation of 2,4,6-trinitrobenzene sulfonic acid (TNBS) into male Wistar rats. Colon/body weight ratio, clinical activity score system, myeloperoxidase activity and histological evaluation were determined as inflammatory indices. The two formulations were compared with drug suspension and SucCH suspension. The results showed that the loading of 5-ASA into SucCH polymer markedly improved efficacy in the healing of induced colitis in rats.

1. Introduction

Colon-targeting drug delivery systems have applications in several therapeutic areas (Chourasia and Jain, 2003; Kumar and Mishra, 2008; Sinha and Kumria, 2001; Yang et al., 2002). These include topical treatment of colon diseases and systemic delivery of therapeutic peptide and proteins (that are normally degraded in the upper gastrointestinal tract). Additionally, colonic delivery of drugs may be extremely useful when a delay in drug absorption is required from a therapeutic point of view, e.g. in case of diurnal asthma, angina pectoris and arthritis. One of the important therapeutic applications of colon targeting delivery systems is the treatment of large intestine disorders, such as irritable bowel syndrome, ulcerative colitis, Crohn's disease, colon cancer and amebiasis. Chron's disease and ulcerative colitis are two related but distinct chronic inflammatory disorders of the gastrointestinal tract, commonly denoted as inflammatory bowel disease (IBD). The exact causes remain uncertain but thus far, IBD is thought to be the result of an appropriate and ongoing activation of the mucosal immune system driven by the normal luminal flora in a genetically susceptible host (Baumgart and Carding, 2007; Sartor, 2006). This paradigm has emerged, to a great extent, from studies in animal models of mucosal inflammation (Borm and Bouma, 2004). Several animal models of intestinal inflammation have been developed and can be divided into four categories: spontaneous colitis, inducible colitis, genetically engineered and adoptive transfer models (Hibi et al., 2002). Among them trinitrobenzene sulfonic acid (TNBS)-induced colitis have been widely used (Crcarevska et al., 2009; Jung et al., 2006; Tozaki et al., 2002). TNBS is thought to be hapten, because it is presumed to bind endogenous proteins in the colonic mucosa and to induce a local immunologic response through macrophage and T cell activation (Ishiguro et al., 2010).

5-Aminosalycilic acid (5-ASA) is an anti-inflammatory drug largely used to treat inflammatory bowel disease (Sands, 2000). 5-ASA oral administration is limited due to its rapid absorption in the upper part of the gastrointestinal (GI) tract (Zhou et al., 1999) and there is a little localization of the drug in the site of action (colon). The efficacy of treatment depends on providing the therapeutical concentration of the drug at the site of inflammation. For this reason three methods have been widely used for 5-ASA targeting: a prodrug concept, enteric coating and/or prolonged release of

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doi:10.1016/j.ijpharm.2011.06.025

the drug through semipermeable membrane (Qureshi and Cohen, 2005). Controlled release preparation are specifically designed to minimize systemic absorption and to achieve optimum delivery of the biologically active 5-ASA to the distal small intestine and the colon. Thus relatively high concentrations of free 5-ASA can be achieved in the intestinal lumen without producing systemic exposure and subsequent toxicity.

Chitosan and some of its various synthetic derivatives have recently attracted great interest for colon delivery. Chitosan (CH) is a polycationic polysaccharide derived from naturally occurring chitin by alkaline deacetylation. Chemically, it is formed by β-(1–4))-linked D-glucosamine (deacetylated unit) and *N*-acetyl-D-glucosamine (acetylated unit) (Kumar, 2000). It has favorable biological properties but it rapidly dissolves in the gastric cavity. Different strategies have been developed to prevent its solubility at acidic pH and thus obtain a chitosan-based colon specific delivery system; some examples are: enteric coated chitosan capsules and microparticles (Lorenzo-Lamosa et al., 1998; Tozaki et al., 1997), pH-sensitive based chitosan hydrogels systems (Jain et al., 2007) and chitosan polyelectrolyte complexes (Bigucci et al., 2008) as well as chitosan salts (Orienti et al., 2002) and derivative (Aiedeh and Taha, 1999).

N-Succinyl-chitosan (SucCH) is a derivative of chitosan that could be obtained by introducing succinyl groups into *N*-terminals of chitosan glucosamine units (Hirano and Moriyasu, 1981; Yamaguchi et al., 1981; Yan et al., 2006). It is reported to have favorable drug carrier properties such as biocompatibility and low toxicity (Rekha and Sharma, 2008). Due to the presence of carboxyl groups it exhibits pH-dependent swelling behavior and it is insoluble at acidic pH value. It possesses mucoadhesive properties because of its hydrophilicity leading to hydrogen bond formation, swelling characteristics, and sufficient chain flexibility. Moreover, it is a negatively charged polymer and it adheres more easily to the inflamed tissues due to the presence in the ulcerative tissues of a high number of positively charged proteins (Jubeh et al., 2004). All these advantages endowed this material with huge potential for the application as site-specific or controlled-release drug delivery systems.

Recently, we have developed 5-ASA loaded *N*-Succinyl-chitosan microparticles (MP) and freeze-dried systems (FD) as a targeted colon delivery system against IBD. Physicochemical characterizations, including FTIR, DSC, X-ray diffraction studies, zeta potential, drug loading and microparticles size were performed. *In vitro* swelling and release studies were also carried out and the obtained results from *in vitro* characterization showed that the two systems could be suitable candidate for colon delivery of 5-ASA.

In the present studies initially *ex vivo* mucoadhesion studies were performed and then the therapeutic efficiency of these drug carrier systems was evaluated using experimental TNBS colitis rat model. To demonstrate the efficiency of the systems, rats were also treated with 5-ASA suspension, as well as SucCH suspension. The efficacy of all formulations was determined by the colon/body weight ratio, clinical activity score system, myeloperoxidase activity, histological evaluation and CD3 and CD20 lymphocytes distribution.

2. Materials and methods

2.1. Materials

Chitosan of medium molecular weight, succinic anhydride and 5-ASA were obtained from Sigma–Aldrich (Milan, Italy). 2,4,6-Trinitrobenzenesulfonic acid (TNBS), hexadecyltrimethylammonium bromide (HTAB), 3,3′,5,5′-tetramethylbenzidine (TMB), hydrogen peroxide 30% and peroxidase from horseradish were purchased form Sigma–Aldrich (Spain). Spectra-por® dialysis membrane (MWCO 12,000–14,000 Da, regenerated cellulose) was purchased from Spectrum Lab (Inc, USA). All the products and solvents were of analytical grade.

2.2. Preparation of N-Succinyl-chitosan and 5-ASA loaded SucCH systems

Chitosan medium molecular weight was succinylated according to the method reported previously (Mura et al., 2011).

5-ASA/SucCH system (FD) was prepared at 1:1 molar ratio using freeze-drying technique (Freeze-dryer Criotecnica, MM Cota, Rome, Italy).

5-ASA loaded SucCH microparticles (MP) were obtained using spray-drying method, (Minispray Dryer, Büchi B-290, Switzerland) at 1:1.25 ratio (5-ASA:SucCH). The conditions of the spray-drying process were: nozzle diameter 0.7 mm, aspiration: 80%, inlet temperature: 150 °C, outlet temperature: 100 °C. Blank SucCH microparticles were prepared using the same conditions as for the drug-loaded particles.

2.3. Physicochemical characterization of MP and FD

FTIR, DSC and X-ray diffractogram spectra were performed for 5-ASA, chitosan, SucCH, FD and MP. FTIR measurements were taken at an ambient temperature using Bruker Equinox 55 (Germany).

DSC studies were performed using a DSC Mettler Toledo model 821e (Switzerland).

X-ray diffractograms pattern were recorded with Bragg–Brentano geometry on a Bruker AXS D5005 (DRXP, Germany).

Shape and surface morphology of the empty and 5-ASA loaded SucCH microparticles were examined using a scanning electron microscope (SEM, S-4100 Hitachi, Madrid, Spain).

Measurement of the particle size and diameter of the microparticles was carried out with Analysette 22 Micro tec plus (Fritsch, Germany). The average particle size was expressed as the volume surface diameter, d_{vs} (μm).

The zeta potential of the systems was recorded using Malvern Zeta Sizer apparatus (model Zen 3600, Malvern, UK).

5-ASA content in the two systems was assayed by HPLC as described elsewhere (Hussain et al., 1998).

2.4. In vitro swelling and drug release studies

Swelling and drug release studies were carried out in three different solutions in order to simulate the gastro-intestinal tract pH values: stomach (pH = 2.0), small intestine (pH = 5.5) and large intestine (pH = 7.4). The *in vitro* release studies were performed under sink conditions and the amount of 5-ASA released was assayed by HPLC as described elsewhere (Hussain et al., 1998).

2.5. Preparation of GI tissues and mucoadhesive test

Wistar rats (13-week old) had been fasted for 24 h. The fasted conditions were set to minimize the contents in the GI tract, which disturbed the washing process for the following use. The tissues (i.e. duodenum and colon) were excised from rats that were sacrificed by intraperitoneal administration of 60 mg/kg of dolethal® (sodium pentobarbital). Each section of tissues was then slowly washed with a large amount of normal saline solution. Then, the tissues (duodenum, healthy colon and inflamed colon) were immediately used for this study. Inflamed colon was obtained by inducing

the model of chronic inflammation in the rat colon as described below. Samples were placed in a closed flat bottom tube with 1 ml of buffer and in a thermostated bath at 37.0 °C. pH = 5.5 solution was used for the mucoadhesion in the duodenum and pH = 7.4 was used for the mucoadhesion in the colon. At scheduled time intervals the mucoadhesion studies were done using the different intestine tissue sections. The mucoadhesion study was done using a universal tensile tester (Lloyd Instruments, LR 50K model, UK). The stainless steel plate (L-shape) was fitted by one of its side into the upper and lower jaws of the instrument so as the other surfaces of the plates were facing each other. The rat tissue (rectangle shape, 9 mm × 7 mm) was stuck at the upper plate surface with the glue (Super Glue-3, Loctite®, Henkel, Barcelona) while the sample was placed on the lower plate. Then the upper jaw with tissue stuck on the plate was lowered slowly so that it just touched the sample surface. No external force was applied. The sample was kept in contact with the tissue for 5 min and then the upper jaw was slowly moved upward at the speed of 3 mm/min (Escobar-Chavez et al., 2011). All the experiments were done in triplicate. The maximum detachment force (F_{max}), i.e. the force required for separating the sample from the tissue surface was obtained directly from NimaST518.vi software (Nima Technology Ltd., Coventry, England) and the total amount of forces involved in the probe withdrawal from the tissue (work of adhesion, W_{ad}) was then calculated from the area under the force versus distance curve. These parameters were used to compare the different formulations tested.

2.6. Induction of colonic inflammation

The studies reported here adhere to the Principles of Laboratory Animal Care and were approved by the institutional ethics commitee of the University of Valencia (Spain) according to RD 1201/2005.

These studies were carried out on Wistar male rats aged 8–12 weeks and weighing 230–250 g. Animals were housed in an air-conditioned room at 22 ± 3 °C, 55 ± 5% humidity, 12 h light/dark cycles and allowed free access to water and laboratory chow for the duration of the studies. To induce the model of chronic inflammation in the rat colon, the method described by Morris et al. (1989) was followed with some slight modifications. Briefly, rats were arbitrarily separated into treatment groups, fasted for 48 h with free access to water and then anaesthetized with isoflurane. A graduated rubber canula was inserted rectally into the colon such that the tip was 8 cm proximal to the anus. 0.5 ml of a solution of TNBS (81 mg/kg body weight) dissolved in 50% ethanol (v/v) was instilled into the lumen of the colon through the rubber probe (total volume 0.5 ml solution). A control group received 0.5 ml 50% ethanol (v/v) administered as before. The induction and development of inflammation were monitored every day during 13 days. Rats (in groups of 5) were sacrificed with an overdose of anesthesia at days 3, 5, 7, 9 and 13 after TNBS administration. The development of inflammation was evaluated with respect to colon/body weight ratio, clinical activity score, myeloperoxidase activity and histological changes.

2.7. Treatment studies design

Rats were divided into 4 groups: to group one was administered SucCH suspension, group two received 5-ASA suspension, group three received 5-ASA loaded freeze-dried system (FD) and finally to group four microparticles (MP) were administered. A dose of 120 mg/kg/day of 5-ASA calculated from the dose from humans (70 kg) (Sandborn and Hanauer, 2003) were administered by oral gavage once a day for three days in the period of the most intensive inflammation (days 3, 4 and 5 after TNBS administration).

2.8. Assesment of colonic injury and inflammation

The rats were killed with an overdose of anesthesia (dolethal®) then the abdomen was opened and the distal colon was removed. The samples of inflamed tissue were excised to measure the ratios of distal colon weight to body weight (C/B ratio), the criteria for scoring the gross morphologic damage (clinical activity score system), the myeloperoxidase activities and histological evaluation.

2.8.1. Determination of colon/body weight ratio

The rats were killed with an overdose of anesthesia, then the abdomen was opened and the distal colon was rapidly excised and opened longitudinally along the mesenteric edge. The colon was washed with 0.9% (w/v) saline and placed with the mucosal surface upward over a glass plate chilled with ice and then weighted (Mladenovska et al., 2007). The ratio of the 8 cm segment distal colon weight was calculated as an index of colonic tissue edema.

2.8.2. Clinical activity score system

Colitis activity was quantified with a clinical score assessing weight loss, stool consistency and rectal bleeding (Hartmann et al., 2000; Lamprecht et al., 2001). No weight loss was counted as 0 point, 1–5% as 1 point, 5–10% as 2 points, 10–20% as 3 points and >20% as 4 points. For stool consistency, 0 point was given for well-formed pellets, 2 points for pasty and semiformed stools that did not stick to the anus and 4 points were given for liquid stools that stick to the anus. Bleeding was scored as 0 point for no blood, 2 points for positive finding and 4 points for gross bleeding. The sum of these scores was forming the clinical score ranging from 0 (healthy) to 12 (maximal activity of colitis).

2.8.3. Myeloperoxidase activity

Myeloperoxidase activity was measured as index of inflammation; it is a peroxidase enzyme most abundantly present in activated neutrophils into the inflamed tissue. Activity was analyzed according to established method (De Young et al., 1989; Sato et al., 2004). Briefly, colon specimen was added to 750 μl of HTAB buffer (0.5% in 80 mM phosphate buffer pH = 5.4) on ice and homogenized. The homogenate was centrifuged (Heraeus Fresco 17 Centrifuge, Thermo Electron Corporation, Spain) at −4 °C and at 10,000 rpm for 15 min (Eppendorf AG 22331, Germany). Myeloperoxidase activity in the supernatant was measured spectrophotometrically. Supernatant (25 μl) was incubated with 75 μl of phosphate buffer pH = 7.4 and 10 μl of phosphate buffer pH = 5.4 and 0.026% hydrogen peroxide (10 μl) at 37 °C for 5 min. Then 20 μl of TMB 18 mM (dissolved in 8% DMF) were added to the previous mixture and incubated for 10 min. Finally the reaction was stopped by the adding of 15 μl of sodium acetate 1.5 M (pH = 3.0) and the absorbance was measured at 620 nm.

2.8.4. Histological evaluation

Two tissue samples (3 cm samples distal and proximal) were excised from each colon and maintained in formaldehyde (10%, v/v) for microscopic studies. These tissue samples were processed routinely and embedded in paraffin. Longitudinal sections (5 μm) were stained with haemotoxylin and eosin. Microscopic assessment by light microscope was performed blind on coded slices.

2.8.5. Distribution of CD3 T and CD20 B lymphocytes

Tissue samples of colon were formalin fixed and embedded in paraffin wax. The immunohistochemical (ABC) method was performed by using policlonal antibody CD3 T lymphocytes (DAKO

policlonal) in 1:50 dilution and monoclonal (clone L:26, DAKO) antibody CD20 B lymphocytes was used in 1:50 dilution.

2.9. Statistical analysis

Analysis of variance (ANOVA) and Bartlet's test for homogeneity of variance were performed using SPSS version 17.0 for Windows (SPSS Inc., USA). Post hoc testing ($P < 0.05$) of the multiple comparisons was performed by the Scheffe test.

3. Results and discussion

5-ASA loaded MP and FD were prepared according to the previously stated formulations and subjected to further characterization.

3.1. Physicochemical characterization of the systems

N-Succinyl-chitosan was successfully synthesized according to the methods reported in literature. FTIR, DSC and X-ray diffractograms spectra revealed the derivatization of chitosan and confirmed the loading of the drug into 5-ASA/SucCH systems, denoting that some interaction between the polymer and the drug are present.

Negatively charged particles with a d_{vs} (volume surface diameter) of 5.1 ± 2.2 μm were prepared. No remarkable differences were found in particle size and distribution between blank- and drug-loaded microparticles, indicating that 5-ASA loading in the microparticles substantially did not influence their size.

Zeta potentials of blank SucCH microparticles and 5-ASA loaded SucCH microparticles and freeze-dried system were -44.2 ± 3.9 mV, -20.7 ± 4.9 mV and -11.3 ± 3.9 mV, respectively.

By imaging with SEM, an acceptable spherical morphology was observed. The surface appeared mostly smooth with some roughness.

The amount of 5-ASA present in FD and MP were 50% and 49.2%, respectively.

3.2. In vitro swelling and drug release studies

As shown previously, the two systems showed the highest swelling at pH = 7.4, while the swelling at acidic pH was considerably smaller as a consequence of the physico-chemical properties of the SucCH.

The *in vitro* release studies were in accordance with the swelling studies. They showed that drug release rate from FD and MP increased with the increase of pH. The maximum drug release percentage was reached at pH = 7.4, as expected. Drug release from FD was slower than that from microparticles due to the different specific surface area. The higher specific surface area speeded up the microparticles swelling and helped the solvent penetration into the polymer matrix causing a faster drug release. As results show, FD and MP could be useful for the preparation of new 5-ASA formulation.

3.3. Mucoadhesion studies

Mucoadhesive polymers are used to immobilize a drug delivery device on a specific site for targeted release and optimal drug delivery due to intimacy and duration of contact. It has been proposed that the interaction between the mucus and mucoadhesive polymers is a result of physical entanglement and secondary bonding, mainly H-bonding and van der Waals attraction. These forces are related to the chemical structure of the polymers and chemical groups of mucoadhesive polymers that contribute to mucoadhesion include hydroxyl, carboxyl, amine, and amide groups in the structure. Polymer characteristics that are necessary for mucoadhesion are: strong H-bonding groups, strong anionic charges, high molecular weight, sufficient chain flexibility, and surface energy properties favoring spreading onto mucus (Thirawong et al., 2007).

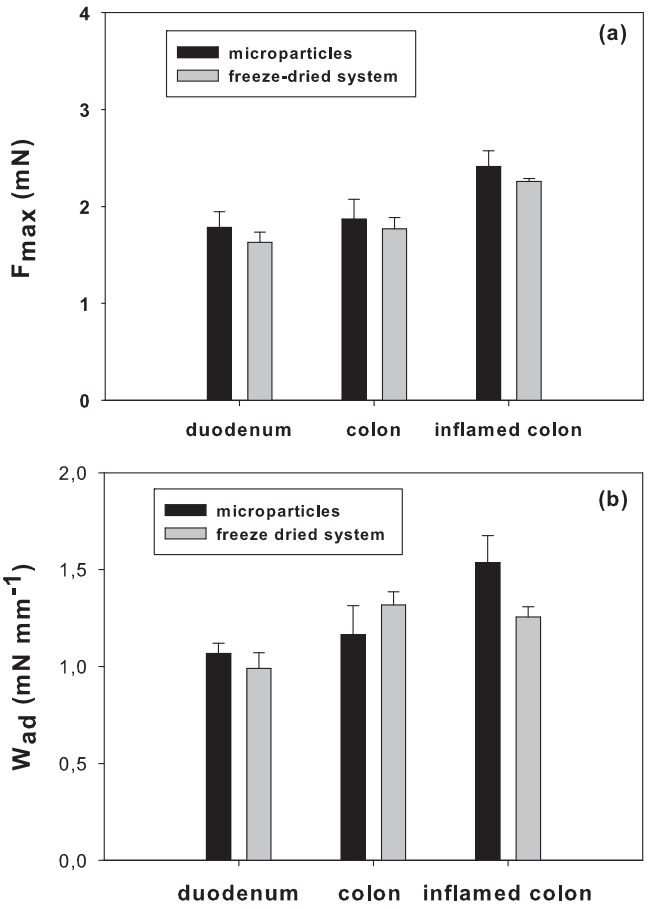

Fig. 1. *Ex vivo* mucoadhesive performance of 5-ASA systems. Effect of GI mucosa on (a) maximum detachment force (F_{max}) and (b) work of adhesion (W_{ad}). Error bars represent standard deviation, $n = 3$.

N-Succinyl-chitosan presents all of these properties, and therefore it could be a good mucoadhesive polymer. The *in vitro* mucoadhesive properties of 5-ASA loaded SucCH systems were studied using rat's duodenum, healthy and inflamed colon (induced by TNBS administration as seen before). The maximum detachment force (F_{max}), i.e. the force required for separating the system from the tissue surface and the total amount of forces involved in the probe withdrawal from the tissue (work of adhesion, W_{ad}) of both formulations on different GI mucosa are shown in Fig. 1. Large intestinal mucosa showed a stronger mucoadhesion than small intestinal mucosa. This is probably due to the fact that there is a difference in the functional histology of epithelia of small and large intestinal mucosa. The absence of villi in large intestine, at the tissue level, may be a benefit for the mucoadhesion as the adhesion between the sample and mucosa or epithelia can occur easily. Moreover, at cellular level, the ratio of goblet cells in large intestine is higher than in other parts of GI tract resulting in higher mucin level, and thus in higher mucoadhesion onto the large intestinal mucosa (Thirawong et al., 2007).

Mucoadhesion of the two systems to the inflamed colonic mucosa resulted higher than healthy mucosa; it has been previously suggested by other authors that a possible cause for the affinity of negatively charged systems to the ulcerated mucosa of the rat was the high concentration of positively charged proteins in the inflamed regions (Jubeh et al., 2004).

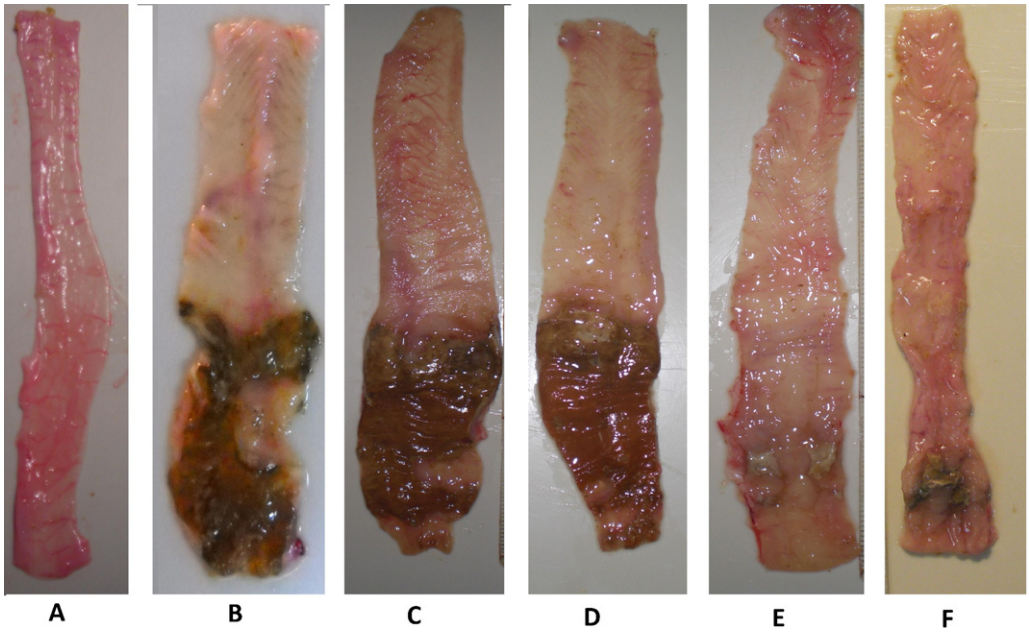

Fig. 2. Photographs of the colon of rat after the induction of colitis with TNBS sacrificed on day 9: A control (ethanol receiving group), B (untreated TNBS group), C (SucCH treated group 1) D (5-ASA treated group 2) E (freeze-dried system treated group 3) and F (microparticles treated group 4).

No significant mucoadhesion differences between microparticles and freeze-dried systems were observed.

3.4. Induction of colonic inflammation

TNBS was chosen as a model of inflammatory bowel disease; IBD can be induced by the administration of an enema containing the contact sensitizing allergen trinitrobenzene sulfonic acid (TNBS) in ethanol 50%. The main advantages of this model were: simplicity, reproducibility and time and dose related development of inflammation (Tozaki et al., 1999, 2002).

First of all, to select an optimal schedule to induce the inflammation by TNBS, we examined the development of the inflammation process at different days after intracolonic administration of TNBS in comparison to the control group that received ethanol 50% (v/v).

The development of the inflammation was monitored daily; rats suffered from diarrhea and weight-loss and during the first days of TNBS administration, rats suffered from rectal bleeding. Fig. 2A and B shows respectively opened colon of control rats that received ethanol 50% (v/v) and of rats after induction of colitis with TNBS, killed on day 9. The group of animals sacrificed 9th day after colonic administration of TNBS showed necrotic changes and presented thick and rigid bowel.

Figs. 3 and 4 show respectively colon/body (C/B) weight ratio and clinical activity score system in TNBS induced colitis on days 3, 5, 7, 9 and 13. All of the three experiments had a maximum at day 9 after the administration of TNBS, and recovered on the day 13. Myeloperoxidase activity (not shown) gave the same results.

3.5. Treatment studies design

Afterward, the efficacy assessment of the 5-ASA formulations in the treatment of TNBS induced colitis in rats was compared to 5-ASA suspension and SucCH suspension, as well as induced TNBS colitis untreated rats and ethanol receiving control rats. For this purpose C/B ratio, clinical activity score and myeloperoxidase activity were determined.

In Fig. 2(C–F) opened colons of groups 1, 2, 3 and 4 are presented, respectively. Colons from groups 1 (Fig. 2C) and 2 (Fig. 2D) showed the presence of necrotic zone and thick and rigid bowel. Colons from groups 3 (Fig. 2E) and 4 (Fig. 2F) present a little necrotic tissue, but generally they appeared healthy.

After FD and MP administration, rats from group 3 and 4 started to gain weight and have normal stool without the presence of bleeding; on the contrary after 5-ASA or SucCH administration rats from group 1 and 2 continued to lose weight and had diarrhea during all the experiment long.

The effects of 5-ASA, SucCH and the two 5-ASA formulations on the C/B ratio and clinical activity score after oral administration are shown in Figs. 5 and 6, respectively. The colon body weight ratio significantly decreased in rats of group 3 and 4 compared to the control that received only intracolonic TNBS (untreated rats). On

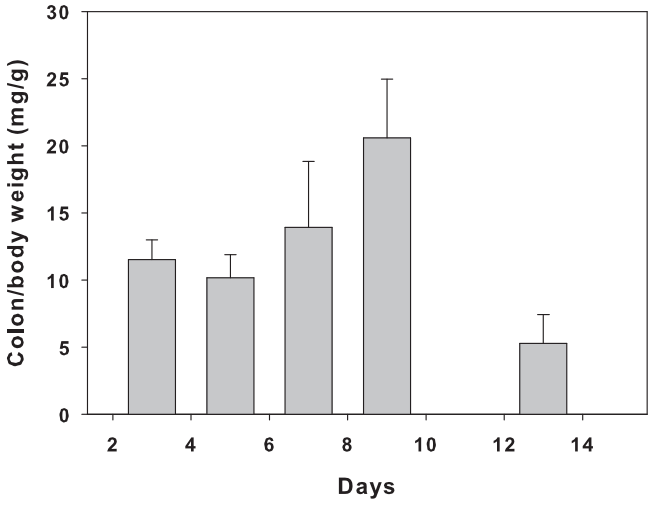

Fig. 3. Index of colonic tissue edema (colon/body weight ratio) of animals with TNBS induced colitis. Each bar is an average value ± S.D. of five animals.

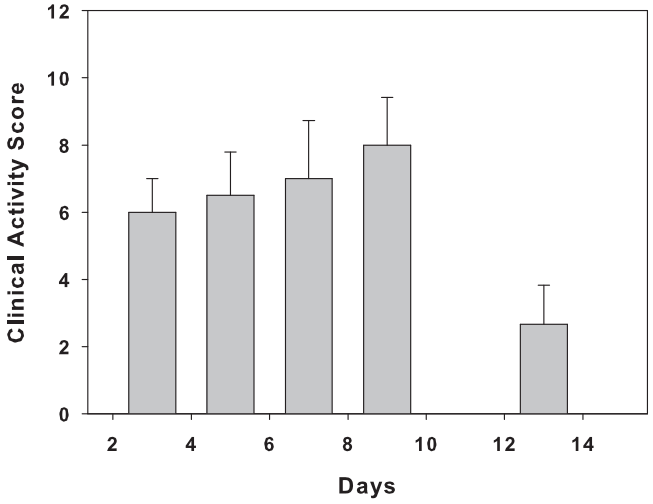

Fig. 4. Clinical activity score system of animals with TNBS induced colitis. Each bar is an average value ± S.D. of five animals.

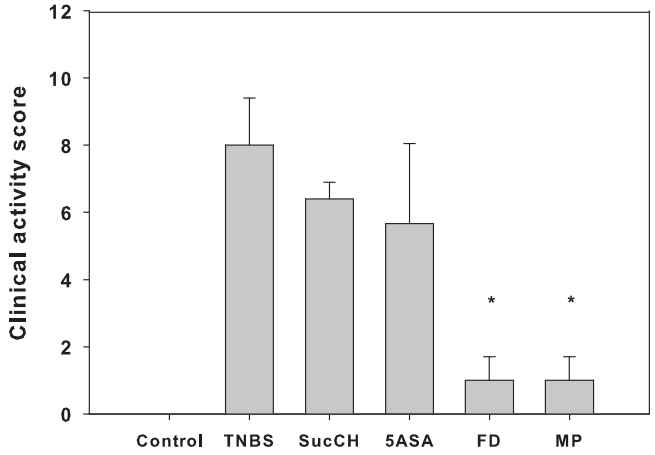

Fig. 6. Clinical activity score of animals with TNBS induced colitis after treatment with SucCH (group 1), 5-ASA (group 2), FD (group 3), MP (group 4) and compared with induced TNBS colitis untreated rats (TNBS) and healthy (ethanol receiving rats, Control). Each bar is an average value ± S.D. of five animals (*statistical significant difference ($P < 0.05$) compared to TNBS).

the other hand no marked effect was observed on the C/B ratio of the rats of group 1 and 2 (Fig. 5). It confirms that 5-ASA alone when taken orally is almost completely absorbed before it reaches the site of action. SucCH did not have any therapeutic effect for IBD.

Similar results were observed in the experiments of clinical activity score systems (Fig. 6) and MPO activity (Fig. 7). Myeloperoxidase activity markedly decreased after oral administration of FD and MP in the animal groups 3 and 4, confirming that the inflammation decreased after the oral administration of the two formulations.

3.6. Histological evaluation

Histological examination was made for the 50% ethanol receiving rats (healthy control), untreated TNBS induced colitis rats and treated group 1, 2, 3, and 4 rats. All the samples were taken from rats sacrificed on the 9th day after administration of TNBS.

The control group shows normal colon structure: healthy mucosa with both enterocytes and goblet cells and between them connective tissue (lamina propria, Fig. 8A), muscularis mucosae and normal submucosa and muscularis externa.

Untreated animals showed necrosis, loss of the necrotic mucosa and substitution with granulation tissue. A strong inflammatory process was present in the lamina propria, submucosa and muscularis externa. As can be seen in Fig. 8B process of ulceration with fibrinoid necrosis of the mucosal surface and granulation tissue below the necrotic tissue were observed.

Animals treated with SucCH suspension showed necrosis, loss of the mucosa, intense transmural inflammation and granulation tissue appearance. Histological findings indicated also the presence of ulceration with fibrinoid material on the surface of the mucosa and granulation tissue that involves the whole submucosa (Fig. 8C). Moreover the muscularis propria presented chronic inflammation and fibrosis.

Animals treated with 5-ASA suspension presented superficial erosion, thinning of the mucosa accompanied by thickening of the muscularis mucosae, and a cronic inflammatory process that affects the mucosa and submucosa with early development of lymphoid follicles. A few parts with normal mucosa structure but presence of strong follicular hyperplasia in the muscularis externa

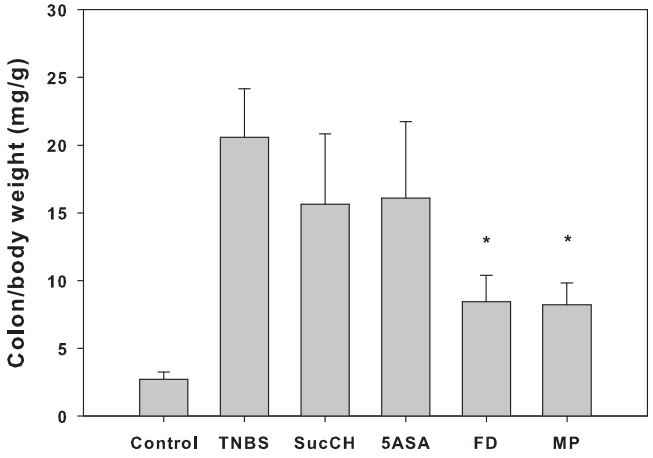

Fig. 5. Colon/body weight ratio of animals with TNBS induced colitis after treatment with SucCH (group 1), 5-ASA (group 2), FD (group 3), MP (group 4) and compared with induced TNBS colitis untreated rats (TNBS) and healthy (ethanol receiving rats, Control). Each bar is an average value ± S.D. of five animals (*statistical significant difference ($P < 0.05$) compared to TNBS).

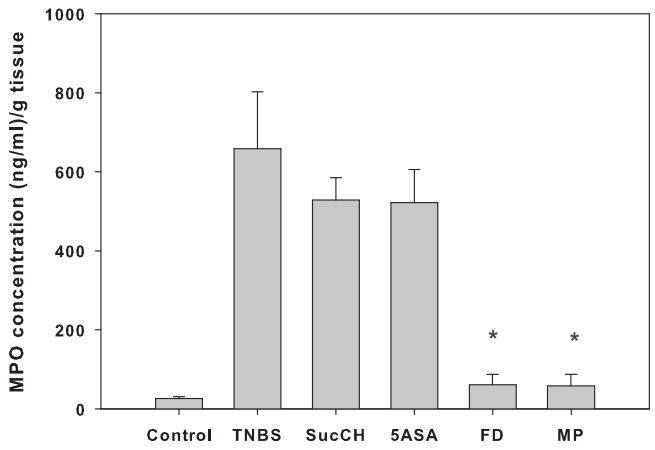

Fig. 7. Myeloperoxidase activity of animals with TNBS induced colitis after treatment with SucCH (group 1), 5-ASA (group 2), FD (group 3), MP (group 4) and compared with induced TNBS colitis untreated rats (TNBS) and healthy (ethanol receiving rats, Control). Each bar is an average value ± S.D. of five animals (*statistical significant difference ($P < 0.05$) compared to TNBS).

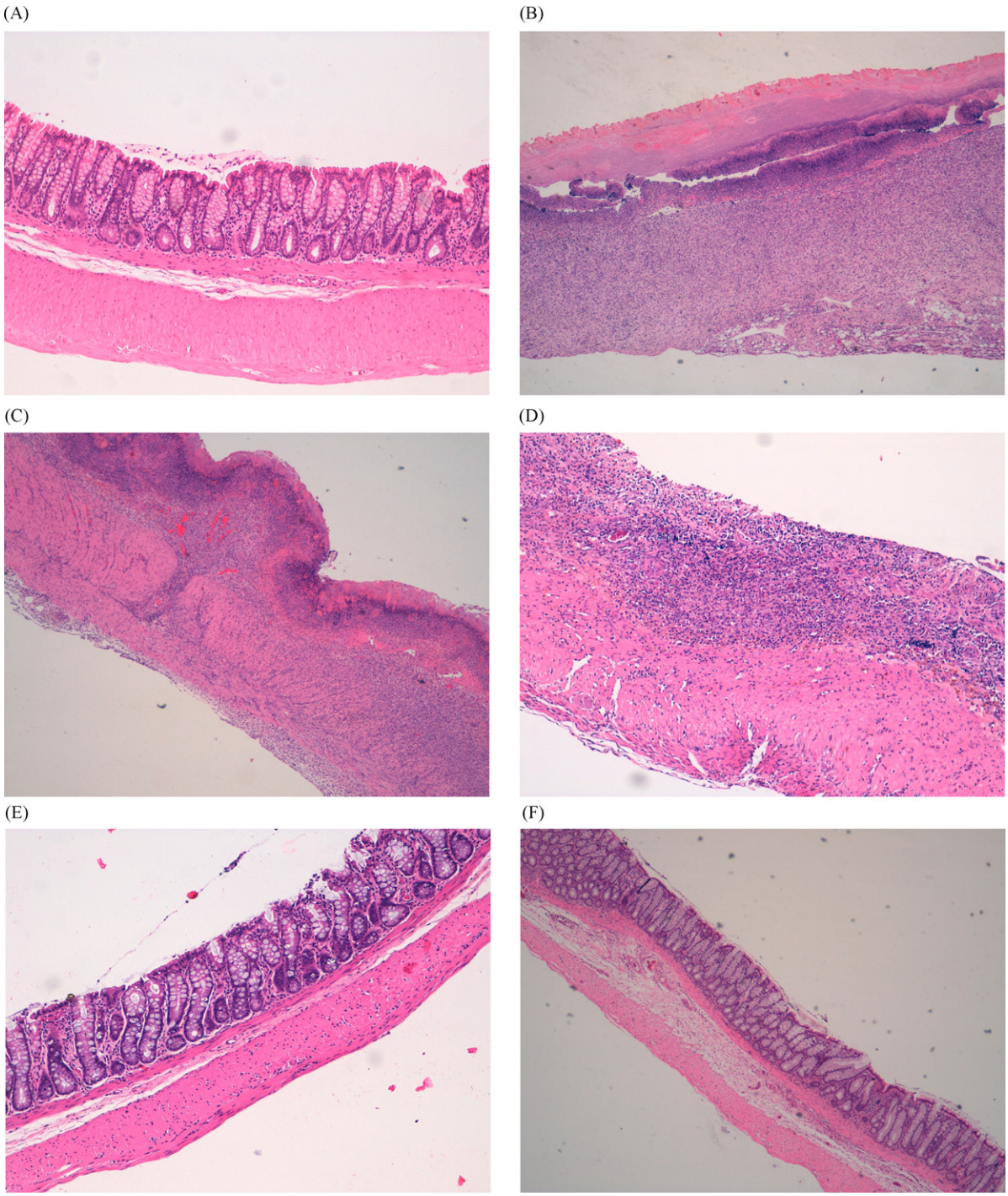

Fig. 8. Histology of a representative colon specimen of a rat after the induction of colitis with TNBS sacrificed on day 9: A control (ethanol receiving group), B (untreated TNBS group), C (SucCH treated; group 1), D (5-ASA treated; group 2), E (FD treated; group 3) and F (MP treated; group 4).

and parts with necrosis, loss of mucosa and substitution with granulation tissue and inflammation process were also observed (Fig. 8D).

Animals treated with FD showed substantially normal mucosal structure with slight presence of chronic inflammation in the muscularis propria (Fig. 8E).

Animals treated with MP showed normal mucosa structure (Fig. 8F).

Histological findings indicated that untreated TNBS group showed presence of strong inflammation accompanied with necrosis and loss of the mucosa. Similar results were also obtained with SucCH suspension receiving group and 5-ASA suspension receiving group. Indeed histological findings FD and MP showed decreasing of inflammation followed by intensive regeneration and normal mucosal structure.

Histological evaluation provides additional information in addition to these obtained by the clinical activity score, colon/body weight ratio, and myeloperoxydase activity, thus confirming the usefulness of the two 5-ASA/SucCH systems in the treatment of inflammatory bowel disease.

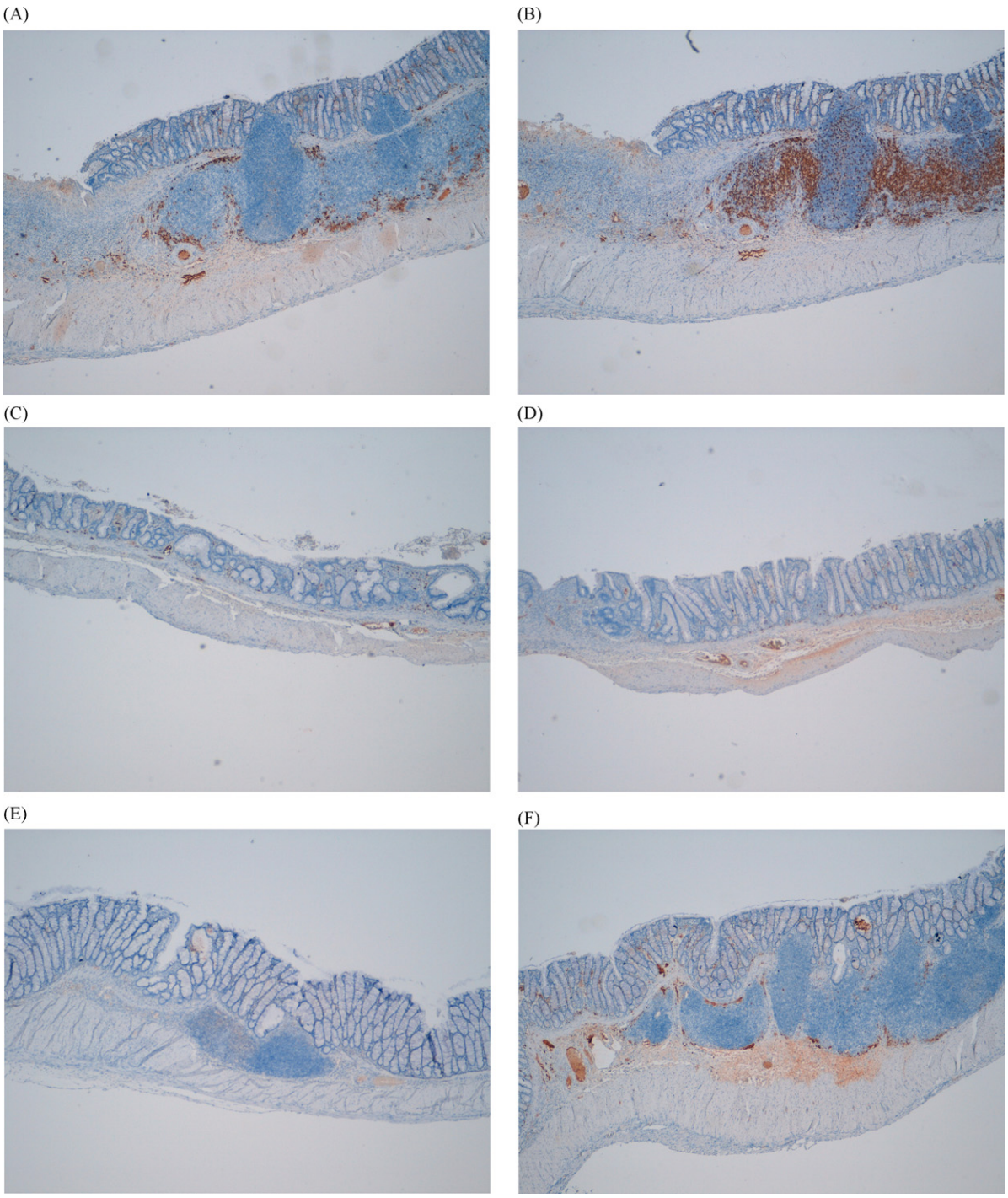

Fig. 9. CD3 and CD20 lymphocytes distribution in colon specimens of rat after the induction of colitis with TNBS and after 5-ASA suspension treatment (Fig. 8A and B, respectively), FD treatment (Fig. 8C and D, respectively) and MP treatment (Fig. 8E and F, respectively). The brown stain represents CD3 or CD20 lymphocytes.

3.7. Distribution of CD3 T and CD20 B lymphocytes

CD3 T and CD20 B lymphocytes distribution was made for the 50% ethanol receiving rats (control), untreated TNBS induced colitis rats and treated group 1, 2, 3, and 4 rats. All the samples were taken from rats sacrificed on the 9th day after administration of TNBS.

In Table 1 content and distribution of CD3 T and CD20 B lymphocytes are shown. The content was represented with "+", ranging from 0 (no lymphocyte) to +++ (maximal quantity of lymphocytes).

Generally CD3 concentration was higher than CD20.

The control group showed small presence of both CD3 and CD20 lymphocytes just in the mucosa.

TNBS untreated animals showed higher concentration of CD3 and CD20 in the submucosa (mucosa was not present due to the necrosis).

SucCH and 5-ASA treated animals presented very high concentration CD3 and smaller CD20 quantity distributed in the whole colon structure: mucosa (when it was present), submucosa, muscularis and in some cases even in the serosa. In Fig. 9A and B CD3 and CD20 distribution in colon specimens of rat after the induction

Table 1
Content and distribution site of CD3 and CD20 lymphocytes in colon specimen of rats after the induction of colitis with TNBS and after treatment of SucCH suspension, 5-ASA suspension, FD (freeze-dried system) and MP (microparticles) and compared with induced TNBS colitis untreated rats (TNBS) and healthy (ethanol receiving rats, Control).

	Control	TNBS	SucCH	5ASA	FD	MP
CD3	+	++	+++	++	+	+
Distribution site	Mucosa	Submucosa	Mucosa Submucosa Muscularis	Submucosa Muscularis Serosa	Mucosa Submucosa	Mucosa Submucosa
CD20	+	++	++	+	+	+
Distribution site	Mucosa	Submucosa	Mucosa Submucosa Serosa	Submucosa Muscularis Serosa	Mucosa Submucosa	Mucosa Submucosa

+: no/or small presence of lymphocyte (<5% of total lymphocytes); ++: intermediate presence of lymphocytes (10–50% of total lymphocytes); +++: big presence of lymphocytes (>50% of total lymphocytes).

of colitis with TNBS and after 5-ASA suspension treatment is shown.

FD (Fig. 9C and D) and MP (Fig. 9E and F) systems showed fair concentration of both CD3 and CD20 restricted to the mucosa and submucosa.

4. Conclusion

Two new systems for the controlled delivery of 5-aminosalicylic acid were prepared and their efficacy in the treatment of IBD was evaluated with TNBS colitis rat model. In our previous work, 5-ASA loaded microparticles and freeze-dried system were subjected to physicochemical characterization and *in vitro* swelling and release studies. Physical properties of the evaluated systems were in favor of drug accumulation in the site of action. In this work, the two formulations showed good mucoadhesion properties. The TNBS model confirmed that both formulations could serve as new drug delivery systems for 5-ASA. Colon/body weight ratio, clinical activity score systems, myeloperoxidase activity, and histology evaluation showed that the animals treated with the two formulations had an improvement in the pathology.

5-ASA loaded SucCH MP and FD compared with 5-ASA and SucCH suspension markedly improved efficacy of 5-ASA in the healing of induced colitis in rats.

In conclusion, we demonstrated that 5-ASA can be specifically delivered to the site of action and that the described systems may be useful for the treatment of inflammatory bowel disease.

Conflict of interest statement

The authors report no conflict of interest.

Acknowledgements

This work was partially supported by MIUR grants (PRIN 2008, Prot. N. 2008HTJLN2_002; Azioni Integrate Italia-Spagna 2009).

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