Hyaluronic Acid Increases Anti-Inflammatory Efficacy of Rectal 5-Amino Salicylic Acid Administration in a Murine Colitis Model

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Abstract

5-amino salicylic acid (5-ASA) is a standard therapy for the treatment of mild to moderate forms of inflammatory bowel diseases (IBD) whereas more severe forms involve the use of steroids and immunosuppressive drugs. Hyaluronic acid (HA) is a naturally occurring non-sulfated glycosaminoglycan that has shown epithelium protective effects in experimental colitis recently. In this study, both 5-ASA (30 mg/kg) and HA (15 mg/kg or 30 mg/kg) were administered rectally and investigated for their potential complementary therapeutic effects in moderate or severe murine colitis models. Intrarectal treatment of moderate and severe colitis with 5-ASA alone or HA alone at a dose of 30 mg/kg led to a significant decrease in clinical activity and histology scores, myeloperoxidase activity (MPO), TNF-α, IL-6 and IL-1β in colitis mice compared to untreated animals. The combination of HA (30 mg/kg) and 5-ASA in severe colitis led to a significant improvement of colitis compared to 5-ASA alone. Combined rectal therapy with HA and 5-ASA could be a treatment alternative for severe cases of IBD as it was the only treatment tested that was not significantly different from the healthy control group. This study further underlines the benefit of searching for yet unexplored drug combinations that show therapeutic potential in IBD without the need of designing completely new drug entities.

Key Words: Hyaluronic acid, Inflammatory bowel disease, Colitis, Inflammation, 5-amino salicylic acid

INTRODUCTION

Inflammatory bowel diseases (IBD) are chronic and recurring diseases of the gastrointestinal tract that are characterized by abdominal pain, diarrhoea, rectal bleeding and weight loss. Ulcerative Colitis and Crohn's Disease are the two most common types of IBD, where patients either experience acute attacks lasting weeks to months followed by periods of remission or relapse, or chronic persistent inflammation. Choice of conventional IBD therapies depend on the severity of the disease and aim to relieve symptoms or induce and maintain remission in order to improve the quality of life of the patient (Danese and Fiocchi, 2011). The treatment with anti-inflammatory drugs, such as salicylates e.g. sulfasalazine, balsala-zide and olsalazine, which are prodrugs of 5-aminosalicylic acid (5-ASA), is usually the primary choice in mild-moderate courses of the disease (Travis *et al.*, 2008). In such cases, 5-ASA preparations are administered as oral or rectal formulations and a combination of both formulations is considered to be more effective in mild to moderate colitis (Marteau *et al.*, 2005). However, in severe cases, 5-ASA often proves to be less effective.

Glycosaminoglycans (GAGs) such as hyaluronic acid (HA) are biomacromolecules that are extensively present in the human body namely in biological fluids and connective tissues (Volpi *et al.*, 2009). HA is a linear, non-sulphated GAG consisting of disaccharide units made up of D-glucuronic acid and *N*-acetyl-D-glucosamine linked by glycosidic β-1,3 bond. It is

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synthesized by synoviocytes, fibroblasts and chondrocytes. In addition, HA is a component of the extracellular matrix of cartilage and synovial fluid in which it exerts a viscosity-enhancing and lubricating effect that makes it useful in the treatment of patients with osteoarthritis (Altman and Moskowitz, 1998). In IBD, disruption of the mucosal epithelium occurs as a result of various immune triggers from the inflammatory cascade (Sturm and Dignass, 2008). Subsequently, a loss of GAGs from the sub-epithelial basal lamina is thought to result in the leakage of proteins and fluids leading to edema formation (Murch *et al*., 1993). Although the topical application of GAGs such as HA in IBD has been reported to mitigate edema formation and assist in the reconstruction of the mucosal barrier (Fiorino *et al*., 2014), there is no in-depth information on the effects and mechanisms of local therapy with GAGs on the inflammatory cascade in IBD. First indicators led to the conclusion that HA administration in murine colitis may result in an anti-inflammatory response involving Toll-like receptors, TLR-2/TLR-4 and COX-2 (O'Neill, 2009; Zheng *et al*., 2009).

In view of a local anti-inflammatory dual drug strategy, we studied HA-5-ASA combinations at different doses after intrarectal administration in a murine model of moderate and severe colitis and analyzed the general clinical outcome as well as the levels of relevant cytokines in the inflamed tissues.

MATERIALS AND METHODS

Materials

5-aminosalicylic acid and fluorescein hyaluronic acid were purchased from Sigma-Aldrich Chemie GmbH, Steinheim, Germany. Hyaluronic acid was a donation from Bio-Technology General Israel Ltd (Kiryat Malachi, Israel). All other chemicals used for the *in vivo* studies were obtained from Sigma Aldrich (Deisenhofen, Germany).

Animal treatment

Experiments were conducted using male Swiss/CD-1 mice (4-6 weeks, average weight=25 g) purchased from JANVIER (Saint-Berthevin, France). All animal experiments were performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences, Washington DC, USA). Experiments were conducted at the University of Franche-Comté in Besançon, France in compliance with the French legislation on animal experimentation under the experimentation authorization no. A-25-48. The 2,4,6-trinitrobenzenesulfonic acid (TNBS) colitis model was used as it is a reproducible and reliable model that induces colitis at an exact location (Moulari *et al*., 2014).

The mice (all groups n=6) were acclimatized to laboratory conditions for one week preceding the start of the experiments with food and water *ad libitum*. Food was withheld from the animals 24 h before the start of the experiment although water was provided. The mice were lightly sedated using isoflurane prior to intrarectal catheterization (4 cm) to insert 100 μL of TNBS in 50% ethanol at a dose of 90 mg (moderate colitis) or 120 mg (severe colitis) per kg body weight. One group received no TNBS treatment and served as a healthy control. All mice were kept for 24 h without any treatment in order to allow the full development of colitis. Subsequently, the mice

were treated with either 15 (moderate colitis only) or 30 mg (moderate and severe colitis) kg per body weight of HA solution alone or in combination with 30 mg per kg body weight of 5-ASA. A reference treatment with 5-ASA alone was run at 30 mg/kg. The treatment was also administered using intrarectal catheterization (4 cm) in a volume of 100 μL for three consecutive days. The colitis control group received saline solution for three consecutive days after induction of colitis with TNBS. The mice were sacrificed 24 h after the last treatment and the colon was resected and washed with 1 mL cold PBS prior to storage in an appropriate buffer solution. Groups were denominated as healthy control, colitis control, HA15, HA30, 5ASA, HA15+5ASA, HA30+5ASA, respectively.

Clinical activity score, histological score and therapeutic index

A clinical activity score (CAS) was used to determine the extent of inflammation in the animals from the assessment of the body weight, stool consistency and presence of rectal bleeding (Lamprecht *et al*., 2001b). The percentage weight loss was obtained from the loss in baseline body weight and allocated a score. No weight loss was scored as 0 points, 1% to 5% as 1 point, 5% to 10% as 2 points, 10% to 20% as 3 points, and >20% as 4 points. The presence of blood in stools and diarrhea was assessed using a scoring system. For stool consistency, 0 points were assigned for well-formed pellets, 2 points for pasty and semisolid stools that did not stick to the anus, and 4 points for liquid stools that stuck to the anus. Bleeding was graded with 0 points for no blood, 2 points for a positive detection, and 4 points for gross bleeding. The mean of these 3 parameters formed the clinical activity score which ranged from 0 (healthy) to 6 (maximum colitis).

Histological assessment was conducted from H&E-stained microscopic images of colons in colitis as described previously (Chinen *et al*., 2011). A score of 0-3 was assigned after observing the microscopic image for the severity of colitis in each animal (n=3) and an average was calculated. The score was allocated according to criteria such as the loss of goblet cells, presence of crypt abscesses, hyperaemia in the mucosa and cellular infiltration in the lamina propria as well as the elongation of the colonic mucosa and the occurrence of epithelial erosion.

The colon weight/length index (CWL) was calculated as the ratio of weight of the inflamed colon to the total length of the colon. The CWL allows a more accurate assessment of inflammation-related morphological changes through normalization and also improves comparability between different laboratories.

Assessment of the inflammatory biomarkers

Distal colonic tissues including the macroscopically visible inflamed gut regions were minced in 1 mL buffer solutions and subjected to homogenization using the Ultra-Turrax® (IKA, Staufen, Germany) at 10,000 rpm for one min and this procedure was conducted for three freeze-thaw cycles. The homogenates were centrifuged at 10,000 rpm at 4°C for 10 min and the supernatant was collected and used for the analysis of the inflammatory biomarkers.

Chronic inflammation of colon is marked by the migration of lymphocytes and plasma cells, while acute inflammation results in the infiltration of granulocytes, local aggregation of neutrophils, eosinophilic and chemotactic mediators (Krawisz

et al., 1984). As these neutrophil granulocytes consist of multiple enzymes such as myeloperoxidase (MPO), which on release from the granulocytes confers local immunity or assists in combatting infections. The MPO activity derived from the solubilized inflamed tissue can be used as a direct measure of neutrophils count (Krawisz *et al.*, 1984). The myeloperoxidase (MPO) was solubilized with hexadecyltrimethylammonium bromide and MPO activity was measured with a dianisidine-H202 assay according to a colorimetric standard method (Krawisz *et al.*, 1984). The concentrations of pro-inflammatory cytokines, TNF-α, IL-6 and IL-1β were determined from the homogenates using a commercial ELISA kit (Mouse Ready-Set-Go®, eBioscience, Vienna, Austria) according to the manufacturer's instructions. The total mouse Nuclear factor-kappa B (NF-κB) p65 levels in the colonic homogenates were measured using a commercial ELISA kit engineered for a fast analysis of samples, NF-κB p65 (Total/Phospho) Human InstantOne™ ELISA Kit (eBioscience) according to the manufacturer's instructions. The results of the NF-κB assay were presented by normalizing each group to the healthy control both in moderate and severe colitis.

A scoring system was devised to include the dead animals after development of severe colitis in the measurement of cytokines, from which the colon could not be resected and therefore was not analyzed. Dead animals received a score of 12. Compared to the untreated colitis control group, a reduction in cytokine secretion of 0-20% was attributed a score of 10, 20-40% was attributed a score of 8, 40-60% was attributed a score of 6, 60-80% was attributed a score of 4, 80-100% was attributed a score of 2.

In order to evaluate the overall benefit of the different treatment groups a Normalized Inflammatory Index (NNI) was calculated. This was achieved by normalizing the measured cytokine values (TNF-α, IL-6, IL-1β and NF-κB) and the CWL to the colitis control. Through addition of the individual values, a new index was formed, which represents all measured results equally and summarized.

Bioadhesion studies

100 μL of the fluorescein HA solution was prepared as an aqueous solution (1 mg/mL) as per the manufacturer's recommendations and administered intrarectally. The colon was then resected 24 h later and stored at –20°C. Cryosections of a thickness of 13 μm were prepared using a cryomicrotome (Slee, Mainz, Germany) prior to the visualization of the sample using Confocal Laser Scanning Microscopy (CLSM) (Nikon Instruments Europe B.V., Amsterdam, Netherlands). Images obtained from the CLSM were analyzed by setting a threshold using the thresholding tool (Jensen, 2013). Fluorescence intensity was measured using imaging software ImageJ (NIH, MD, USA) (n=3) as described earlier (McCloy *et al.*, 2014). Pictures were taken using the same settings and the pixel density of the green fluorescence was measured relative to the pixel density of the whole image. The corrected total fluorescence (CTF) was calculated as the difference between integrated density and (area of selected cell or tissue×mean fluorescence of background readings).

Statistical analysis

Statistical analysis was conducted using Graphpad Prism 8 Software (GraphPad Software Inc., San Diego, CA, USA). Statistical difference was determined using ordinary one-way
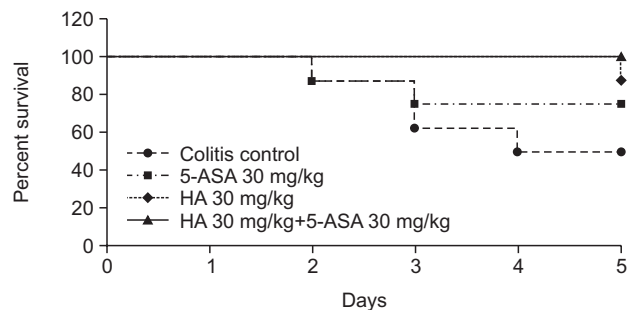

Fig. 1. Kaplan-Meier plot showing the survival rate of animals over experimental period (5 days) determined for n=8 animals after treatment of severe colitis (120 mg/kg TNBS) with HA30, 5ASA and their combination.

ANOVA and followed by multiple comparisons using the Dunnett's test. The data was expressed as mean ± SD and treatments were considered significantly different if $p<0.05$.

RESULTS

Kaplan-Meier plots demonstrated that treatment of animals with moderate colitis did not lead to measurable differences in survival (100% survival in all groups), however, differences were observed in severe colitis (Fig. 1). Compared to the untreated colitis control group, the treatment groups showed a significantly higher survival rate at day 5 ranging between 88% to 100% for HA30 formulations. Interestingly, the combination of HA30+5ASA resulted in no mortality throughout the study period and the combination groups showed better survival in severe colitis than the groups treated with 5ASA alone.

Treatment with HA alone and in combination with 5ASA was found to lower the clinical activity score (CAS) compared to the untreated control group that received saline solution after induction of colitis (Fig. 2A). In both moderate and severe colitis, a slight but non-significant improvement in clinical activity score was observed when HA was present in the formulations at a dose of 30 mg/kg compared to the HA+5ASA combination (Fig. 2B). As expected, mice treated with TNBS showed weight loss of 5-10 % accompanied by the softening of stools starting from 24 h after colitis induction compared with the healthy control mice (i.e., no TNBS treatment). Mice treated with TNBS continued to lose weight till day 5 and a maximum weight loss of 20% in body mass was observed compared to the untreated healthy control and treated groups. However, mice treated with 5-ASA alone, HA alone and HA+5ASA combinations displayed a divergent pattern of significant gradual weight gain as from day 2 of treatment compared to the colitis control group, thereby allowing the mice to eat and move normally again and decreasing the CAS to healthy levels. Administration of HA with 5-ASA during induction of severe colitis significantly ameliorated body weight loss and clinical activity scores compared to the colitis control in both moderate and severe colitis ($p≥0.05$) (Fig. 2). A significant reduction in CAS could be seen from day 1 to 5 in the groups treated with HA alone and HA-5ASA in moderate colitis whereas in severe colitis, a significant improvement in these two groups could be noted as from day 4 to 5.

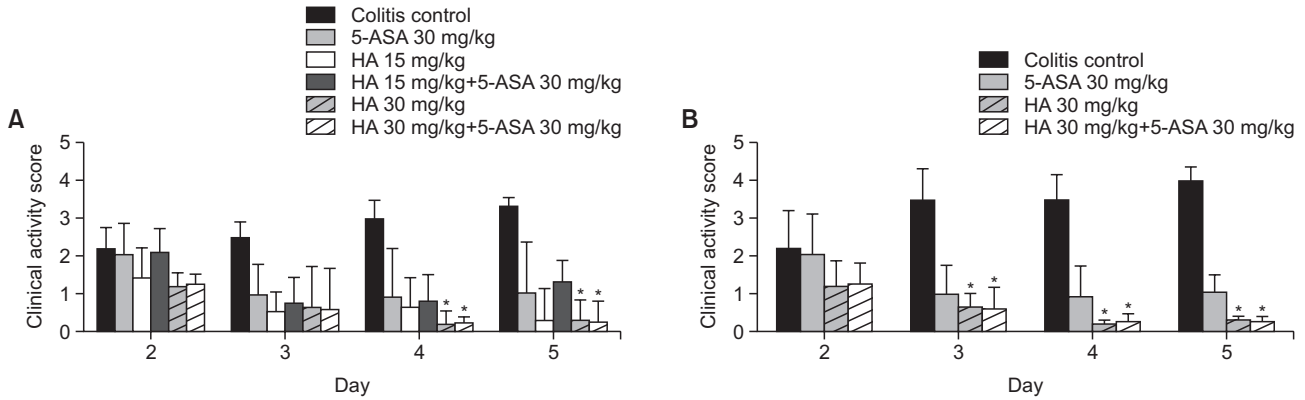A morphologic characteristic of IBD is the thickening of the

Fig. 2. Clinical activity scores (5 days) during treatment of moderate colitis (A; 90 mg/kg TNBS; n=6) with HA at doses of 15 or 30 mg/kg and 5-ASA or severe colitis (B; 120 mg/kg TNBS; n=8) with HA at a dose of 30 mg/kg and 5-ASA (Mean ± SD; *p<0.05, compared to colitis control on the respective day, ANOVA+Dunnett's multiple comparison test.

Fig. 4. Photographs representative of the mouse colon show tissue sections from the healthy group (I), untreated colitis control group (II), group treated with 30 mg/kg 5-ASA (III), 30 mg/kg HA alone (IV) and 30 mg/kg HA and 30 mg/kg 5-ASA (IIV) in severe colitis (120 mg/kg TNBS).
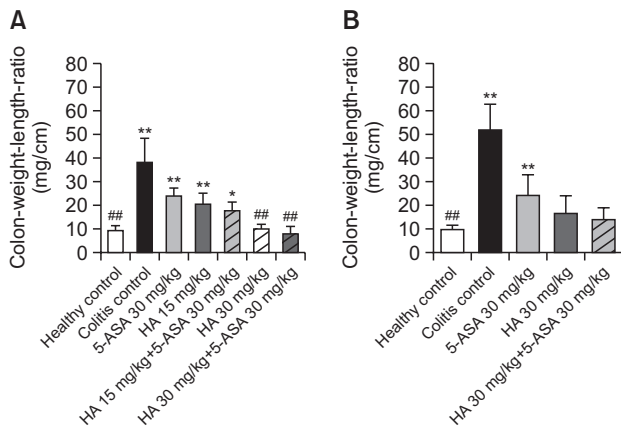
Fig. 3. Determination of colon/body weight ratio after treatment of moderate colitis (A) with HA at doses of 15 or 30 mg/kg and 5-ASA at a dose of 30 mg/kg or severe colitis (B) with HA at a dose of 30 mg/kg and 5-ASA at a dose of 30 mg/kg. All treatments were significantly different from colitis control (Mean ± SD; n=6; *p<0.05, compared to healthy control; **p<0.01 compared to healthy control, ##p<0.01 compared to 5-ASA alone, ANOVA+Dunnett's multiple comparison test).

large bowel which can be measured as an increase in the colon weight/length ratio. In both moderate and severe colitis, there was a significant reduction in CWL in the treatment groups that received HA30 or HA30+5ASA compared to 5ASA alone (Fig. 3). However, HA15 showed no significant contribution in therapeutic benefit over 5ASA (p=0.5908). Increasing the dose of HA to 30 mg/kg was found to significantly suppress the CWL compared to 5ASA (p≤0.0001 for HA30 and HA30+5ASA, respectively). In severe colitis, no significant differences were noted between the groups treated with 5ASA or HA alone or with HA30+5ASA (Fig. 3B).

Macroscopic images of the colon tissue sections in severe colitis reveal the benefit of the HA+5ASA combination as no ulcerated tissue and minimal swelling of the colon was observed in group V with its integrity and length preserved after 5 days, hence suggesting less inflammation (Fig. 4). The group treated with 30 mg/kg 5ASA and 30 mg/kg HA sacrificed on
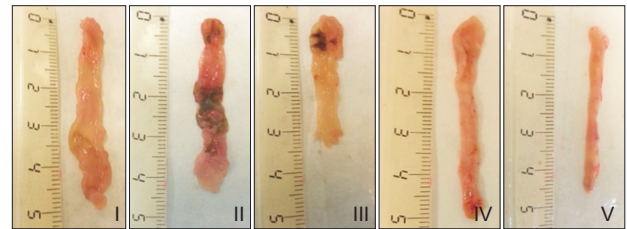
day 5 revealed considerably longer colons with no swelling compared with control mice, indicating that the combination ameliorates the symptoms of TNBS-induced acute colitis in mice.

Fig. 5A-5E depict the H&E images of sections of the colon tissue after treatment saline solution (healthy), treatment with TNBS, 5-ASA, HA alone and HA-5ASA in severe colitis (n=3). In the treatment groups that received HA-5ASA, there was an absence of epithelial damage and hyperplasia and an intact epithelium as well as a lack of inflammatory cell infiltrates could be seen, which confirms the anti-inflammatory effect of HA. A histological score was allocated to the samples as depicted in Fig. 5F. The lowest histological score was obtained with the HA-5ASA combination. Compared to the colitis control, all three treatment groups showed a significantly lower histological score. However, no significant difference was noted amongst the treatment groups. These results confirm the anti-inflammatory effects of HA and the beneficial effects of combining HA and 5ASA on tissue repair and regeneration. The histopathological assessment of TNBS-colitis mouse revealed features such ulceration, mucous cell depletion, inflammatory cell infiltration and severe structural edematous changes (Fig. 5B). The marked reduction in aforementioned histopathological signs and partial or complete restoration of the normal state confirmed the therapeutic benefits of HA alone and HA-5ASA combinations.
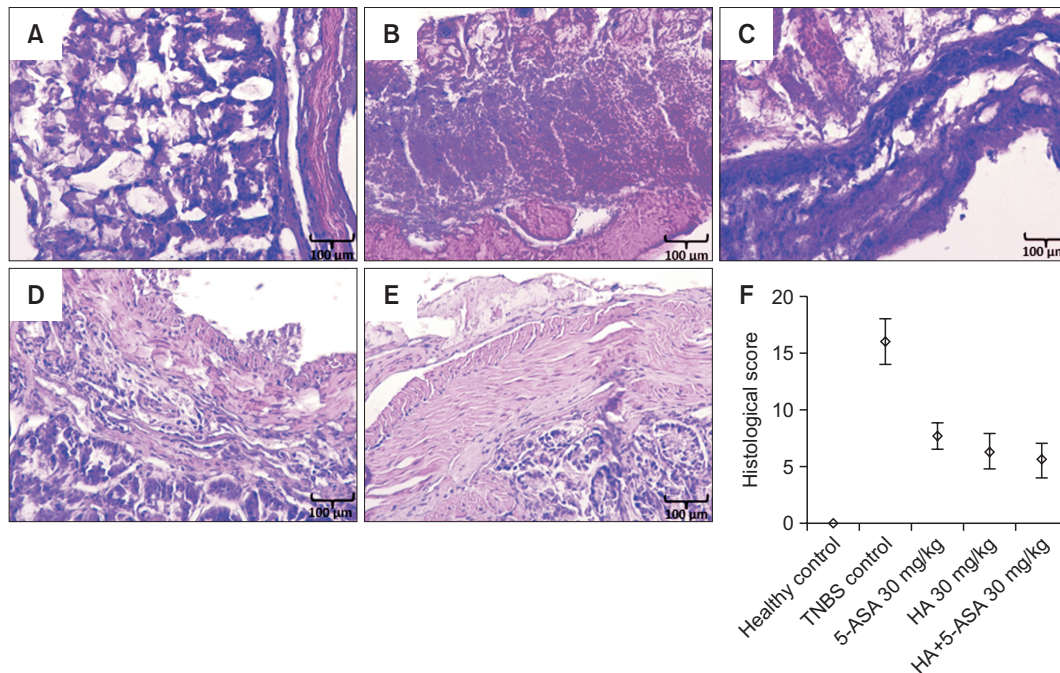
Reduced MPO activity was observed for all treatment

Fig. 5. H&E-stained sections of colon tissue (×20) after intrarectal treatment of mice with (A) saline solution (healthy control), (B) TNBS (colitis control), (C) 5-ASA 30 mg/kg, (D) HA 30 mg/kg alone, (E) HA 30 mg/kg and 5-ASA 30 mg/kg. (F) Histology scoring for each treatment group (n=3) in severe colitis (120 mg/kg TNBS). Hematoxylin and eosin-stained tissues were imaged on a Leica DM 2700M light microscope attached to a camera Q-capture Pro 7 using a 20× objective.
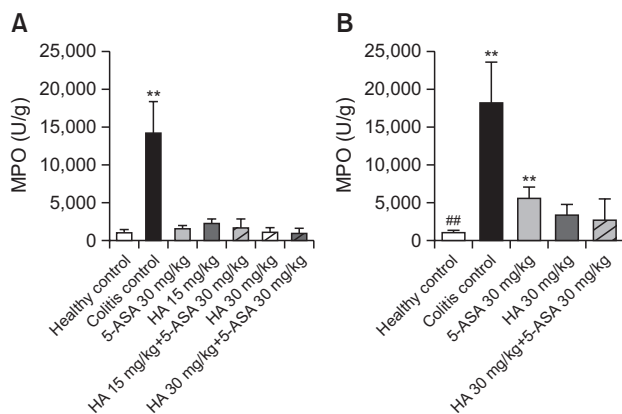

Fig. 6. MPO activity after treatment of moderate colitis (A) with HA at a dose of 15 or 30 mg/kg and 5-ASA at a dose of 30 mg/kg and MPO activity after treatment of severe colitis (B) with HA at a dose of 30 mg/kg and 5ASA. All treatments were significantly different from colitis control (Mean ± SD; n=6; **$p<0.01$ compared to healthy control, ##$p<0.01$ compared to 5ASA alone, ANOVA+Dunnett's multiple comparison test).

groups in moderate colitis, which reflects the diminished neutrophils counts present in the tissue and shows the therapeutic-anti-inflammatory effect of 5-ASA and HA and its combination. However, differences between treatment groups were minor, such as the non-significant difference between HA15 and HA30 treated groups ($p=0.7797$, Fig. 6A). It is worth noting that no significant differences were found between groups treated with HA30 and HA30+5ASA combination compared to 5ASA alone. Moreover, none of the treatment groups significantly differed from the inflammation level of healthy controls.

Similarly, in severe colitis, a significant decrease in MPO activity was observed for all treatment groups compared to the colitis group (Fig. 6B) although only a trend and no statistically significant differences were noted between groups treated with HA or 5ASA alone and HA+5ASA.

Pro-inflammatory cytokines were quantified in the mice colonic tissue homogenates. Cytokines that are pro-inflammatory markers such as IL-6 and TNF-α are thought to determine the extent of inflammation in IBD patients. The mean TNF-α scores in our study were significantly reduced compared to HA alone and the healthy control. Treatment of TNBS-induced colitis in experimental mice with 5-ASA or HA greatly reduced the levels of pro-inflammatory cytokine production compared to the colitis group. Similar trends in anti-inflammatory efficiency as for MPO activity were observed from the IL-1β, TNF-α and IL-6 levels in moderate colitis as HA15 or HA30 alone and combinations thereof with 5ASA were found to effectively suppress the secretion of most cytokines compared to untreated controls (Fig. 7). However, there was no significant improvement between the treated groups compared to 5ASA alone.

A significant reduction in IL-1β, IL-6, and TNF-α scores was noted for all treatments compared to the colitis control group in severe colitis (Fig. 8). In addition, the TNF-α score was significantly reduced for the HA+5ASA compared to HA30 alone ($p<0.0001$). Overall, it is worth noting that only the HA30+5ASA group was not significantly different form the healthy controls for all cytokines ($p<0.05$).

A similar trend was also observed in the normalized NF-κB levels in tissue homogenates in both moderate and severe colitis (Fig. 9). In all cases, the treatment groups showed a sig-
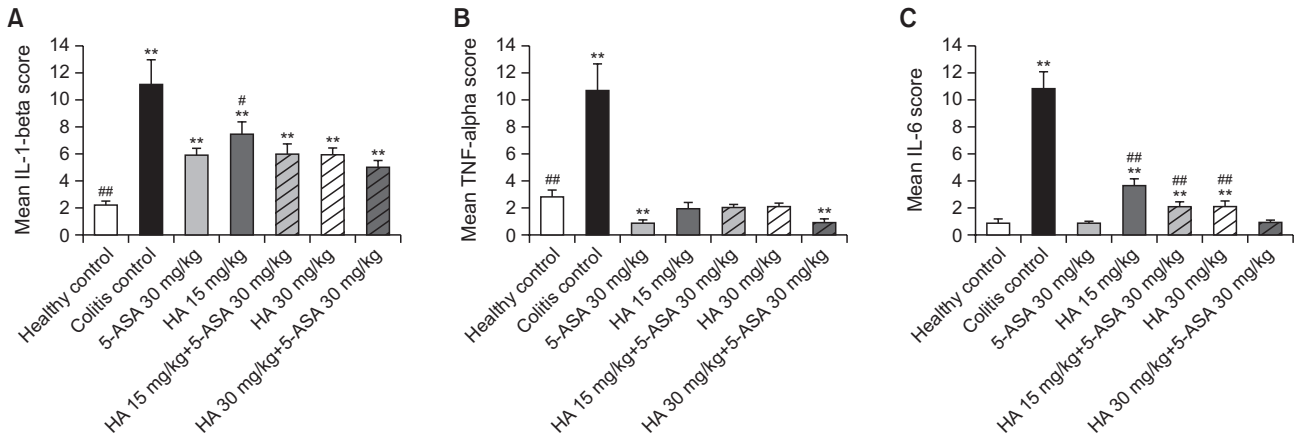
Fig. 7. IL-1β (A), TNF-α (B) and IL-6 (C) secretion in colonic tissue homogenates after treatment of moderate colitis (90 mg/kg TNBS) with HA at a dose of 15 or 30 mg/kg in combination with 5-ASA at a dose of 30 mg/kg. All treatments were significantly different from colitis control (Mean ± SD; n=6; **$p<0.01$ compared to healthy control, #$p<0.05$ compared to 5-ASA 30 mg/kg, ##$p<0.01$ compared to 5-ASA 30 mg/kg, ANOVA+Dunnett's multiple comparison test).
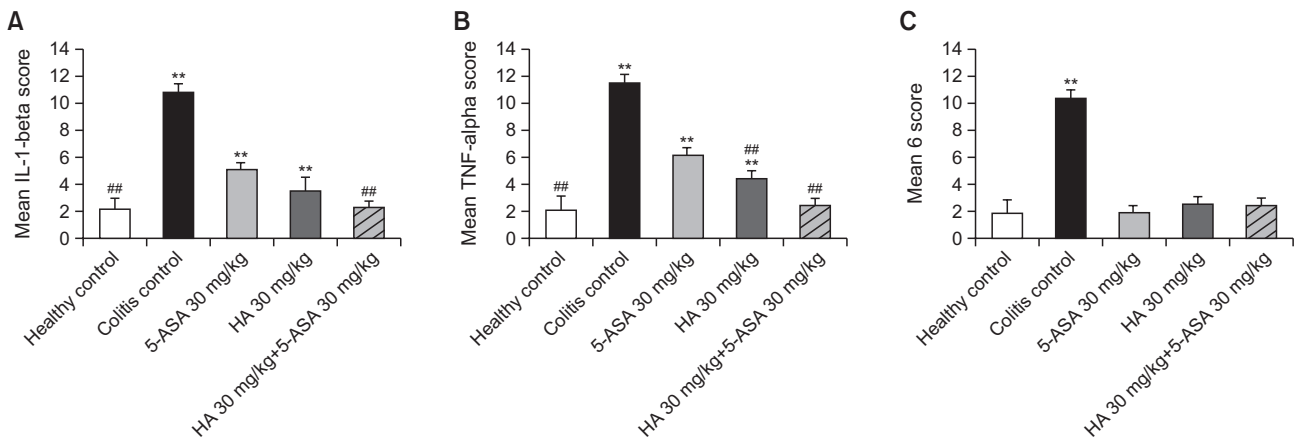

Fig. 8. IL-1β (A), TNF-α (B) and IL-6 (C) scores in colonic tissue homogenates after treatment of severe colitis (120 mg/kg TNBS) with HA at a dose of 30 mg/kg in combination with 5-ASA at a dose of 30 mg/kg. All treatments were significantly different from colitis control (Mean ± SD; n=6; **$p<0.01$ compared to healthy control, ##$p<0.01$ compared to 5-ASA 30 mg/kg, ANOVA+Dunnett's multiple comparison test).

nificantly lower NF-κB activity compared to the colitis group. In moderate colitis, the combinations of HA+5-ASA at both 15 mg/kg and 30 mg/kg led to significant reduction in NF-κB activity compared to HA alone ($p=0.0292$ for HA15 and $p=0.0279$ for HA30, respectively). Besides, the combination groups HA15+5ASA and HA30+5ASA were non-significantly different from healthy control ($p<0.05$). The combination of HA 30 mg/ kg and 30 mg/kg 5-ASA led to a significant reduction in NF-κB activity in severe colitis compared to moderate colitis, which confirms the *in vivo* anti-inflammatory activity of HA even in severe cases of experimental colitis.

In severe colitis, the HA+5ASA exhibited a significantly lower NF-κB activity when compared to the 5ASA30 reference group ($p=0.0440$). The combination HA30+5ASA was non-significantly different from healthy control ($p<0.05$).

This is also reflected in the results of the NNI for the combined evaluation of the anti-inflammatory potency of the different treatments (Fig. 10). In moderate colitis, the combination of HA 30 mg/kg and 5ASA is the only treatment is to perform significantly better than 5-ASA alone ($p<0.05$). The result is more evident in the severe form of colitis, where again only the combination HA 30 mg/kg and 5ASA has a significantly stronger anti-inflammatory effect than 5ASA ($p<0.001$). Moreover, NNI of the HA 30 mg/kg and 5ASA combination was the only treatment that was not significantly different from healthy control ($p=0.6103$). CLSM studies showed qualitative and semi-quantitative differences in the bioadhesive properties of HA in colonic tissues (Fig. 11A, 11B). FITC-labelled HA rectally administered to healthy or colitis mice led to a nearly 30-times higher bioadhesion to inflamed colons compared to healthy tissues (Fig. 11C).

DISCUSSION

IBD is a chronic disease in which pharmacotherapy typically requires continuous long-term drug therapy. Therefore, adverse effects of these anti-inflammatory drugs play an im-
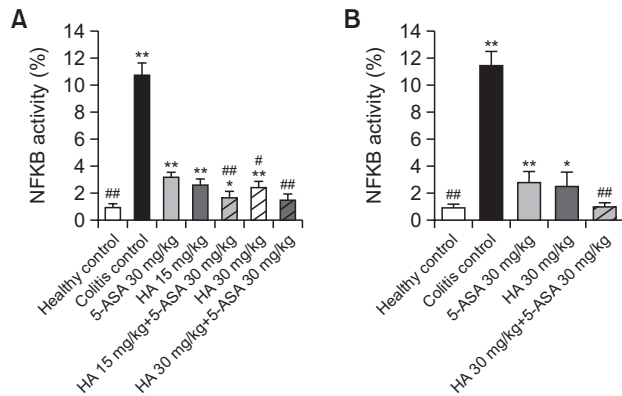
Fig. 9. NF-κB activity in colonic tissue homogenates after treatment of moderate (A; 90 mg/kg TNBS) and severe colitis (B; 120 mg/kg TNBS) with HA and 5-ASA combinations. NF-κB activity was normalized to the healthy control in both moderate and severe colitis. All treatments were significantly different from colitis control (Mean ± SD; n=6; *$p<0.05$, compared to healthy control; **$p<0.01$ compared to healthy control, #$p<0.05$ compared to 5-ASA 30 mg/kg, ##$p<0.01$ compared to 5-ASA 30 mg/kg, ANOVA+Dunnett's multiple comparison test).
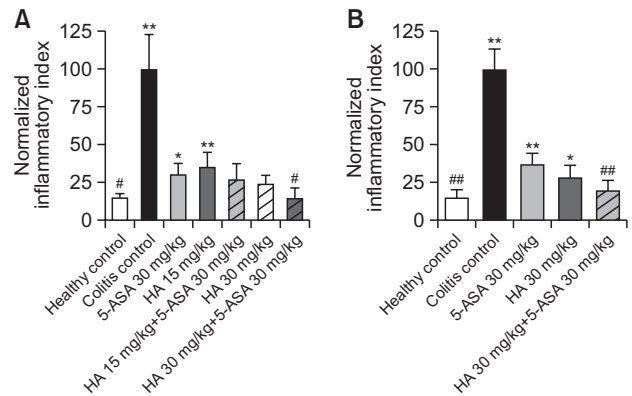
Fig. 10. Normalized Inflammatory Index combining the cytokine measurements and the CWL to allow an overall analysis of anti-inflammatory effect after treatment of moderate (A; 90 mg/kg TNBS) and severe colitis (B; 120 mg/kg TNBS) with HA and 5-ASA combinations. All treatments were significantly different from colitis control (Mean ± SD; n=8; *$p<0.05$, compared to healthy control; **$p<0.01$ compared to healthy control, #$p<0.05$ compared to 5-ASA 30 mg/kg, ##$p<0.01$ compared to 5-ASA 30 mg/kg, ANOVA+Dunnett's multiple comparison test).

portant role in maintaining the patient's quality of life. 5ASA combined with HA is a promising drug combination, especially since 5ASA is rather well-tolerated (Chiu *et al*., 2017). However, its efficiency in severe cases of colitis can be limited and our data suggest the combination of rectally administered HA and 5ASA therapy might provide an increased benefit in the treatment of severe colitis. In this study, we found that HA+5ASA combinations showed a trend towards an enhanced effect compared to HA and 5ASA alone on inflammation markers such as MPO and disease activity indices such as the CAS and CWL. Similarly, the measurement of NF-κB and cytokines such as TNF-α from colonic tissues after treatment with HA+5ASA showed an improved therapeutic effect. It has been reported that the expression levels of IL-6 and TNF-α increase as the severity of ulcerative colitis increases, hence suggesting a positive correlation between cytokine expression and severity of IBD (Akazawa *et al*., 2002; Indaram *et al*., 2002). As shown with the Normalized Inflammatory Index, the use of combinations of HA and 5ASA resulted in an overall additive mitigating effect, which was more evident in severe colitis as compared to moderate colitis. This combination may offer a more tolerable alternative to corticosteroids and immunosuppressive agents especially since HA acts locally and is not absorbed from the gastrointestinal tract due to its macromolecular structure.

It has been established earlier that HA reduces cellular damage by impeding NF-κB DNA binding and inhibiting the production of reactive oxygen species (ROS). The anti-oxidant activity of HA is mediated by its ability to directly neutralize ROS as a result of its chemical interaction with the OH· radical and ability to form chelates with transition metal ions (Campo *et al*., 2008). Interestingly, the potency of HA has been found to be dependent on its molecular weight (Safrankova *et al*., 2010) and in a state of continuous inflammation, HA undergoes extensive degradation to low-molecular-weight (LMW) fragments, which are believed to be pro-inflammatory and angiogenic whilst high-molecular-weight HA is known to exhibit
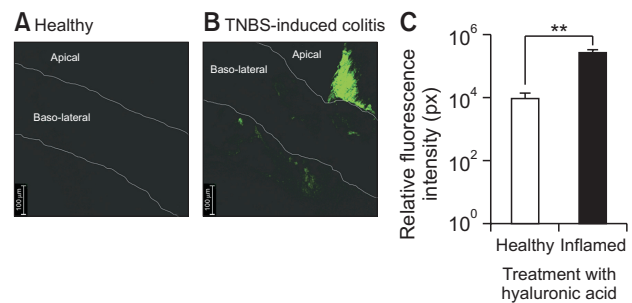

Fig. 11. CLSM images after administration of fluorescently labelled HA to healthy (A) and inflamed colonic tissue (B). (C) Semi-quantitative image analyses of bioadhesion in healthy and inflamed tissues, **$p<0.01$ compared to healthy tissues.

anti-angiogenic, anti-inflammatory and immunosuppressive properties (Laurent *et al*., 1986; Tesar *et al*., 2006). Our data suggest that HA in itself has suppressive effects in this colitis model which is in line with the abovementioned observations as well as other recent findings from other inflammatory diseases (Chen *et al*., 2019). In this case, high molecular weight HA was used which may have led to the described anti-inflammatory effects. A potential degradation down to monomers or low-molecular weight oligomers in the rectum is unlikely especially to a degree that would impact the therapeutic benefit. This finding is in line with more than 95% of unfragmented radiolabeled high-molecular-weight HA excreted in the faeces after oral administration to rats and dogs (Balogh *et al*., 2008), which confirms no degradation. As the uptake of HA into epithelial cells is dependent on its molecular size it can be concluded that HA derived effects emerge from the lumen of the colon (Kim and de la Motte, 2020). However, an in-depth study on the impact of molecular weight of HA in such drug combination would require additional investigation to determine to which extent the biodegradation of HA after rectal administra-

tion leads to resorbable components and subsequent changes in therapeutic outcome. This is especially true in view of recent findings that also indicated relatively low molecular weight fractions (10 to 60 kDa) still show anti-inflammatory effects via the cell-surface-mediated toll-like receptor 4 signaling pathway (You *et al.*, 2020) in addition to the long-established understanding that high molecular weight HA inhibits advanced glycation end product-induced NF-κB activation and cytokine expression (Neumann *et al.*, 1999).

Inflammatory diseases such as IBD result in a loss of glycosaminoglycans in the mucosa due to a degradation process attributed to reactive oxygen and nitrogen species (Murch *et al.*, 1993; Ade-Ajayi *et al.*, 1996; Hassan *et al.*, 1998). To better understand the anti-inflammatory effects observed in these studies, it is important to elucidate their distinct structural and physicochemical characteristics. HA is negatively charged at physiological pH and may show enhanced adhesion to inflamed tissues, as ulcerated tissues consist of a high number of positively charged proteins (Lamprecht *et al.*, 2001a). Consequently, the differences in structure and viscosities of HA affect the stacking of the polymer chains and polymer conformation within the mucus layer, which in turns influences the ability of the polymer to form hydrogen bonds or electrostatic interactions with the mucin proteins. HA is non-sulphated and has a high molecular weight and water-binding capacity. HA exists in a flexible, coiled conformation that retains approximately 1,000 times its weight in water, hence enabling it to control tissue hydration and maintain extracellular space. The balance between degradation and regeneration of matrix constituents, such as HA, maintains the integrity of tissue function and regulates wound healing (Bhattacharya *et al.*, 1989; Jiang *et al.*, 2005). It has been reported that HA induces clinical and endoscopic remission in distal ulcerative colitis due to its ability to promote epithelial restitution and form a protective hydrating coat on the colonic mucosa thereby protecting the colon from further toxins and bacteria (Dicker *et al.*, 2014; Fiorino *et al.*, 2014). In addition, HA is able to reorganize the cytoskeleton, facilitate the migration of adjacent epithelial cells into the wound in order to cover the open lesions, facilitate the proliferation of epithelial cells to replace the damaged cells and promote differentiation which sustains the structural activities of the mucosal epithelium (Sturm and Dignass, 2008). HA also has a moderating effect on the migration of leucocytes into the site of inflammation (Dillon *et al.*, 1994). In addition, it has been postulated that the administration of exogenous HA to the colon in murine colitis might lead to the enhancement of synthesis of endogenous HA via HA synthase, which propagates the anti-inflammatory response within the colonic epithelium (O'Neill, 2009). This would subsequently complementary to the mechanism of action of 5ASA alone.

Therefore, local delivery of HA in conjunction with 5ASA to the site of inflammation may also lead to beneficial therapeutic effects on IBD due to the mucoprotective effects, ability to promote healing via epithelial restitution in the lumen and bioadhesive effects that prolongs the retention of 5ASA at the site of inflammation and thus increases the contact time with the intestinal epithelium and consequently the absorption and effect of 5-ASA.

The CLSM data in this study suggest that the local proximity of HA due to its remarkably high mucoadhesion might increase the residence time of 5ASA within the inflamed tissue. In addition, the possible penetration of HA within the mucus layer might be the mechanism of delivery that leads to its therapeutic effect observed in the *in vivo* studies. The retention within the healthy tissue is limited in duration and therefore only slight bioadhesion of the polymer was observed after 24 h. Further analyses on how the interaction of HA with mucus exactly modulates 5ASA's local availability and anti-inflammatory efficacy might reveal exciting answers on an optimal design of the final dosage form.

It should be underlined that the 5ASA-HA combination forms an adhesive high viscosity-based gel-forming properties of HA, which may intrinsically lead to the longer retention of the incorporated 5ASA at the site of inflammation, resulting in an adhesive sustained drug release formulation. Although this effect is purely mechanic it potentially goes beyond the simple pharmacological combination of the two active components. These results warrant further investigation of the combination HA and 5ASA in a larger animal model i.e. rabbits or pigs that can more reliably mirror the impact of the viscosity of the lavage and give potential insights into the efficacy of this combination in humans as an anti-inflammatory treatment for colitis.

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