CHAPTER 3

PREPARATION AND EVALUATION OF MESALAMINE COLLAGEN IN SITU RECTAL GEL: A NOVEL THERAPEUTIC APPROACH FOR TREATING ULCERATIVE COLITIS

3.1 INTRODUCTION

Ulcerative colitis (UC) is a chronic inflammatory disease that primarily affects the colonic mucosa. The annual incidence of UC in western European countries and the USA is estimated to be six to eight cases per 100,000 individuals, with the prevalence of UC reaching 70–150 cases per 100,000 individuals (Ardizzone 2012). UC is classified based on the location of the disease and includes proctitis, which is confined to the rectum; left-sided colitis, which occurs up to the splenic flexure; and extensive colitis/pancolitis, which occurs up to the hepatic flexure or affects the entire colon (Travis et al 2008). Left-sided colitis is the most prevalent subtype of UC that accounts for 60-85% of all ulcerative colitis cases at diagnosis (Andres & Friedman 1999). The most common clinical symptoms are mucosal ulceration, rectal bleeding, diarrhoea, and abdominal pain.

As far as the management of UC is concerned, mesalamine chemically 5-amino salicylic acid (5-ASA, Table 3.1) remains the first line drug for treating mild to moderate UC. In distal colitis and extensive cases, a combination of oral and rectal mesalamine appears to be more effective than either alone (Marteau et al 2005). Rectally administered mesalamine
formulations, which include suspensions, suppositories, gels and foams, offer the advantage of delivering a known amount of mesalamine topically to the distal colon. Earlier reported studies suggest that topical preparations result in better response and earlier improvement in mild-to-moderate distal UC when compared with oral therapy (Marshall & Irvine 1995; Gionchetti et al 1998; Cohen et al 2000). The current practice guidelines of the American College of Gastroenterology emphasize the superiority of topical therapy, either alone or in combination with oral 5-ASA agents for the treatment of distal UC (Kornbluth & Sachar 2010).

Collagen, the wound healing protein can be moulded into various forms of delivery systems. As mentioned in earlier chapters due to its excellent biocompatibility and safety, the use of collagen in biomedical applications has been rapidly growing and widely expanding to bioengineering areas (Lee et al 2001; Adhirajan et al 2009; Teles et al 2010; Groning et al 2007; Natarajan et al 2012). Patho-physiologically, ulceration in the mucosal and submucosal areas of patients with UC is due to excessive degradation of extracellular matrix (Wang & Mao 2007). The degradation of collagen which is the major components of damaged mucosa in ulcerative colitis is regulated by a cascade of matrix metalloproteinases (MMPs). In particular, excessive expression of MMP-1 in the diseased colonic mucosa of UC patients causes excessive hydrolysis of the collagen and ulceration (Wang & Yan 2006). Baugh and co-workers demonstrated an increase in metalloproteinase activity in inflamed mucosa in patients with UC (Baugh et al 1999). Therefore, we hypothesize that the administration of collagen with the mesalamine therapy may act as a synergistic combination for effective management of UC. To attain this, delivering mesalamine in the form of pH/temperature sensitive hydrogel using collagen may emerge as a suitable delivery system for treating distal colitis. Drug delivery systems based on pH stimuli are effective for localised treatment and used in various
drug delivery applications (Bos et al. 2004; Lee et al. 2008). Rosenblatt and co-workers developed injectable pH sensitive collagen hydrogel for biomedical application (Rosenblatt et al. 1994). The sol-gel transition of collagen is a very well established phenomenon. Acid soluble collagen solution, when adjusted to physiological pH and temperature forms a translucent gel. Therefore, the aim of the present study is to formulate a mesalamine \textit{in situ} hydrogel using collagen and evaluate its efficacy for the treatment of ulcerative colitis.

**Table 3.1 Mesalamine drug profile** (Adopted from DrugBank)

<table>
<thead>
<tr>
<th></th>
<th>Mesalmine Drug Profile</th>
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<tbody>
<tr>
<td>1</td>
<td>Generic Name</td>
</tr>
<tr>
<td>2</td>
<td>Brand Name</td>
</tr>
<tr>
<td>3</td>
<td>Structure</td>
</tr>
<tr>
<td>4</td>
<td>IUPAC Name</td>
</tr>
<tr>
<td>5</td>
<td>Molecular Weight</td>
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<td>6</td>
<td>Mechanism of action</td>
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Table 3.1 (Continued)

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<td><strong>Indication</strong></td>
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<tr>
<td>8</td>
<td><strong>Absorption</strong></td>
</tr>
<tr>
<td>9</td>
<td><strong>Metabolism</strong></td>
</tr>
<tr>
<td>10</td>
<td><strong>Protein Binding</strong></td>
</tr>
<tr>
<td>11</td>
<td><strong>Route of elimination</strong></td>
</tr>
<tr>
<td>12</td>
<td><strong>Half life</strong></td>
</tr>
<tr>
<td>13</td>
<td><strong>Toxicity</strong></td>
</tr>
</tbody>
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### 3.2 MATERIALS AND METHODS

#### 3.2.1 Materials

Pepsin treated Type I collagen was extracted from rat tail tendon as per the earlier reported procedure (Tanaka et al 1988). The detailed extraction procedure of Type I collagen is given in appendix 1. Mesalamine standard was provided in Kind by Glenmark pharmaceuticals limited (Mumbai, India). Deionised water from a Milli-Q water purification system was used to prepare the aqueous solutions. Dextran sodium sulphate (MW: 36000-50000) was
purchased from MP Biomedicals Pvt (India) Ltd. All other chemicals used were of reagent grade.

3.2.2 Preparation of in-situ Mesalamine Collagen Hydrogel System

First, 5 mg of lyophilised collagen was dissolved in 1 ml of 20 mM acetic acid under continuous stirring at temperature < 10 °C until a homogenised solution was obtained. Then, 20 mg of mesalamine was added to 1 ml of phosphate buffer saline (pH 7.4) and vortexed for 2 min. The mesalamine suspension was added drop-wise to the collagen solution at temperature < 10 °C under stirring and the resulting mixture showed uniform white suspension. The system was further evaluated for in situ pH and temperature responsive gelling procedure. In order to compare the therapeutic efficacy, the mesalamine suspension of same potency was prepared using 0.3 % carbopol 934.

3.2.3 Characterisation of Collagen Mesalamine in-situ Gelling System

3.2.3.1 Tube inversion test

Two ml of the mesalamine collagen in situ gelling system was transferred into a 5 mL sample tube. The system pH was raised to physiological pH by adding 20 μL of 1 M NaOH followed by incubation at 37 °C. The sol–gel phase behaviour was monitored by inverting the tube at 2 min time of interval after incubation. Separately, 2 ml of the gelling system was dialysed against 1X Phosphate buffer saline pH 7.4 in dialysis tube at 37 °C for 30 min. The system was monitored every 2 min to observe the gelation.
3.2.3.2 Rheological study

The oscillatory rheological experiment was performed on Anton Paar Physica MCR 301 stress controlled rheometer using cone and plate geometry (1° cone angle and 25 mm cone diameter). The mechanical spectra, namely storage G’ and loss, G” was recorded versus frequency at constant strain of 5%. The frequency sweep experiment for sol and pH adjusted gel was carried out in the frequency range of 0.1 to 500 rad/s and the preheated plate maintained at 37°C.

3.2.3.3 FTIR characterization of hydrogels

FTIR spectra were taken to investigate the chemical interactions between the mesalamine and collagen in the formulation. IR pellets of mesalamine, collagen and freeze dried formulation were prepared using KBr and spectra was recorded in the transmittance mode with 25 scans acquired at 2 cm\(^{-1}\) resolution, between 4000 and 600 cm\(^{-1}\) using Nicolet 360 FTIR Spectrometer.

3.2.4 In vitro Release of Mesalamine from Collagen-mesalamine in situ Gel

In vitro release of mesalamine from formulations (gel and suspension) was monitored by the USP basket method at a rotating speed of 100 rpm in 1000 ml phosphate buffer medium (pH 7.4) at 37°C. Two millilitre of formulation was introduced into a dialysis tubing (MW CO 12,000 D, 16 mm diameter, Himedia Laboratories, Mumbai, India) after one end was tightly tied. Then, the other end was also tied to obtain a bag which was immediately put into a basket. Three bags were prepared for each formulation, maintaining sink conditions during the study. Five ml of medium
was withdrawn at appropriate intervals (0.08, 0.25, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0 and 12 h) and replaced by same volume of fresh medium. Mesalamine release was determined by spectrophotometric method measured at the $\lambda_{\text{max}}$ of 302 nm.

3.2.5 Stability Studies of Collagen-mesalamine in-situ Gel

Mesalamine, a structural analogue of p-amino phenol easily undergoes oxidation to form quinoneimine. Therefore, mesalamine stability in the in situ gelling system was evaluated periodically for drug content, sedimentation, colour and pH. The formulation was stored at 2-8 °C for 28 days and the drug content was determined at regular intervals using spectrophotometric method.

3.2.6 In vivo Study of Collagen Mesalamine in-situ Gel

Female, 6 to 8 week old BALB/c mice weighing 20 to 22 g were used in the in vivo study. They were fed standard feed and drinking water ad libitum. The animals were acclimatized under standard animal laboratory condition for 7 days before they were used in experiments. All experiments were approved by the institutional Animal Ethics Committee (Sri Ramachandra University, IEAC No: IEAC/XXIII/SRU/180/2011) and were in total agreement with the guidelines for proper use of animals in biomedical research. DSS induced colitis model (Loher et al 2003) was selected to evaluate the efficacy of the formulation. Animals were divided into four groups, 1) Normal control, 2) DSS control, 3) collagen mesalamine gel (Test group) and 4) mesalamine suspension (Reference group). All groups excluding normal control were fed 3.5% DSS dissolved in sterile, drinking water ad libitum from day 1 to 5 followed by treatment (Therapeutic model). In situ mesalamine collagen gel and reference mesalamine suspension (20
mg/kg b.w) were administered intra rectally from day 6 to 12 while DSS control group received PBS during this period. Both induction and treatment progress were assessed by 1) clinical score evaluation, and 2) histological analysis. Clinical score was evaluated daily using parameters such as body weight, rectal bleeding, and stool consistency. The scoring was performed by two investigators blinded to the experimental design and scoring is presented in Table 3.2. Six mice were sacrificed by cervical dislocation under isoflurane anesthesia in all the groups at day 9 and day 13 to monitor the treatment progress. The distal colon was isolated and subjected to histological evaluation.

Table 3.2  Clinical activity grading score for rectal bleeding and stool consistency

<table>
<thead>
<tr>
<th>Clinical score</th>
<th>Stool consistency</th>
<th>Rectal bleeding</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Normal</td>
<td>Nil</td>
</tr>
<tr>
<td>1</td>
<td>Pasty</td>
<td>Mild</td>
</tr>
<tr>
<td>2</td>
<td>loose stools</td>
<td>Moderate</td>
</tr>
<tr>
<td>3</td>
<td>Diarrhoea</td>
<td>Severe</td>
</tr>
</tbody>
</table>

3.2.7  Real Time PCR Analysis

Total mRNA from colon tissues were extracted and reverse transcribed into cDNA using Gent Bio RT-PCR Kit (Biobase, Germany). Individual mRNA levels were relatively quantified using Real-Time PCR (Agilent Startagene, Germany). Each reaction contains 2 μL cDNA sample, 1 μL forward primers (10 ng), 1 μL reverse primers (10 ng), 10 μL SYBR Green (2x green star qPCR master mix, K6251) and 6 μL water in a total
volume of 20 μL per sample. The primers are listed in Table 2.1 in the previous chapter.

All measurements were performed in triplicate. Real-time RT-PCR data were represented as Ct values, the Ct or threshold value of the target sequence is directly proportional to the absolute concentration when compared with the threshold value for reference genes. The relative expression level of target gene were plotted as fold change compared to control and determined by the $2^{-\Delta\Delta ct}$ method (Rosenblatt et al 1994), a relative quantification algorithm. The factor X by which the amount of the changed gene can be calculated with the formula: $X=2^{-\Delta\Delta ct}$. where $\Delta\Delta ct=(Ct \text{ of target}) - (Ct, \text{of target} \times \beta$-actin) control- (Ct, of target x $\beta$-actin) sample.

3.2.8 Histology Analysis

Sections of distal colon were fixed in 10 % neutral buffered formaldehyde and embedded in paraffin before staining with hematoxylin and eosin. The histological scoring of colitis was performed by pathologist as treatment-blinded assessments. The histological scoring was based on the observation of mucosal damage and inflammatory cell infiltrate. In the mucosal damage assessment, intact mucosa was scored as 0, discrete lympho-epithelial lesions were taken as 1, surface mucosal erosions were 2 and the score for extensive mucosal damage was given as 3.

For inflammatory cells infiltrate, rare inflammatory cells in the lamina propria were scored 0; increased numbers of inflammatory cells in the lamina propria was given a score of 1, confluence of inflammatory cells, extending into the submucosa as 2 and a score of 3 was given for transmural extension of the infiltrate.
For CD 34 immunohistochemical analysis (Mikalsen et al 2011), deparaffinised sections were microwaved in Tris/EDTA (pH 9.0), followed by 5 min treatment with 0.03 % hydrogen peroxidase. The sections were incubated with anti-CD4 QBEND-10 (Biogenex, Netherlands) against CD34 at room temperature for 30 min, then with peroxidase-labelled polymer conjugated to goat antimouse antibody for 30 min, and finally with 3-3'-diaminobenzidine tetrahydrochloride for 10 min. Counterstaining was performed using haematoxylin. Appropriate negative and positive controls were included.

3.2.9 Statistical Analysis

Data are expressed as means ± SEM. Statistical significance was determined by one way ANOVA using Dunnetts multiple comparison test. Differences were considered statistically significant for $p < 0.05$. Statistical analyses were performed using GraphPad Prism 4.01 software.

3.3 RESULTS AND DISCUSSIONS

3.3.1 Characterization of Collagen-mesalamine in situ Gel

3.3.1.1 Tube inversion test

The sol-gel transition of the in situ gel system was visually observed by inverting the sample tube. The sol-gel transition occurred at 2 min interval and is shown in Figure 3.1. In contrast, the gelation was observed at 10 min interval in the dialysis method and it is more precise as per the in vivo condition. This gelation phenomenon is due to the formation of collagen fibril arrangement at physiological pH and temperature. However, gel uniformity is influenced by ionic strength of the solution. The uniform gel
formation occurred at 1X PBS concentration and the gelation is not cohesive upon increasing the ionic strength.

Figure 3.1  Tube inversion test of mesalamine in situ gel at a) pH 4.75, 4-8°C and b) pH 7.4, 37°C

3.3.1.2  Rheological study

Sol–gel transition of in situ gel accompanies with a significant change in storage/loss modulus. Figure 3.2 shows the change in storage modulus (G’) and loss modulus (G’’) of in situ mesalamine gel. The logarithmic plot of G’ and G’’ of pH adjusted gel showed no cross over zone throughout the frequency, which confirms both collagen and collagen-mesalamine system to be in gel form. In addition, drug loaded gel showed slight increase in G’ and G’’ value indication of stable gel structure and it is clearly evidenced that the drug loading did not affect the gel integrity. The increase of frequency brought about the increase of both G’ and G’’ gradually.
Figure 3.2 Storage modulus ($G'$) and loss modulus of ($G''$) a) collagen gel and b) Mesalamine loaded collagen gel formed under in situ condition (pH 7.4 and 37°C)
3.3.1.3 FTIR Characterization of collagen, mesalamine and collagen-mesalamine formulation

In the FTIR analysis (Figure 3.3), mesalamine shows characteristic IR bands at 1651 cm\(^{-1}\) corresponds to the CO stretching, 1621 cm\(^{-1}\) to NH bending and 1355 cm\(^{-1}\) to CN stretching. The collagen also has characteristic IR band at 1652 cm\(^{-1}\) attributed to amide I CO stretching, 1545 cm\(^{-1}\) by amide II NH bending and 1238 cm\(^{-1}\) for amide III CN stretching. In addition, it shows broader peak at 3417 cm\(^{-1}\) corresponding to amide functionality. Freeze dried formulation of collagen mesalamine showed all major peaks corresponding to collagen and mesalamine without significant shifts in the peaks, which is indicative of weak interaction existing between collagen and mesalamine.

![FTIR spectra of mesalamine, collagen and mesalamine collagen gel](image-url)

**Figure 3.3** FTIR spectra of mesalamine, collagen and mesalamine collagen gel
3.3.2 Stability of Collagen-mesalamine in situ Gel

The decomposition of mesalamine in solution occurs rapidly under conditions promoting oxidation and is most stable under conditions tending to inhibit oxidation (Palmeier et al 1992). In addition, mesalamine is sensitive to light and higher pH and degrades faster under such conditions. In order to assess the stability of mesalamine, drug content was measured at given intervals (Day 0, 3, 7, 14, 21, 28) using spectrophotometric method. The initial colour of the in situ collagen-mesalamine gel has been observed to be milky white, which did not change for the period of 28 days when they were refrigerated at 4 °C (Table 3.3). Though slight changes in the pH of the formulation and mild sedimentation were observed with increasing time of storage, the stability of mesalamine did not alter significantly. Hence the formulation of collagen mesalamine sol may not affect the efficacy of mesalamine for the storage period employed in the study. However adequate care should be taken to avoid the exposure of the formulation to light in order to prevent the oxidation of mesalamine, and temperature of the formulation has to be maintained below 10 °C to control the fibrillogensis of collagen.

Table 3.3 Stability study indicating the drug content, sedimentation, colour and pH change upon storage of mesalamine in-situ gel

<table>
<thead>
<tr>
<th>Sampling</th>
<th>% Stability ± SD</th>
<th>Colour of the formulation</th>
<th>pH of the formulation</th>
<th>Sedimentation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>99.33 ± 0.12</td>
<td>Milky white (Initial colour)</td>
<td>4.76</td>
<td>No</td>
</tr>
<tr>
<td>Day 3</td>
<td>99.27 ± 0.20</td>
<td>No change</td>
<td>4.80</td>
<td>No</td>
</tr>
<tr>
<td>Day 7</td>
<td>98.78 ± 0.56</td>
<td>No change</td>
<td>4.72</td>
<td>No</td>
</tr>
<tr>
<td>Day 14</td>
<td>98.89 ± 0.31</td>
<td>No change</td>
<td>4.78</td>
<td>Mild</td>
</tr>
<tr>
<td>Day 21</td>
<td>98.91 ± 0.57</td>
<td>No change</td>
<td>4.73</td>
<td>Mild</td>
</tr>
<tr>
<td>Day 28</td>
<td>98.02 ± 0.72</td>
<td>No change</td>
<td>4.71</td>
<td>Mild</td>
</tr>
</tbody>
</table>
3.3.3 *In vitro* Release of Mesalamine from Collagen-Mesalamine *in situ* Gel

Mesalamine release profile obtained from *in situ* gel and suspension is shown in the Figure 3.4. Drug release was significantly delayed in the *in situ* gel system (with collagen) when compared to the suspension form. There was a burst release observed in initial sampling hours for the *in situ* gel. Within 30 min, 25 % and 46 % of drug was released from the gel and suspension formulations, respectively. As evidenced from this, the sol to gel transition phase delayed the drug release during the initial period. After 3 hours, 90 % of the drug was released from suspension but in contrast, only 60 % was released from *in situ* gel. The DT 50 and DT 80 for the gel were found to be 1.74 hr and 5.64 hr. The release from the gel is significantly prolonged when compared to the suspension form. In addition, the *in vitro* release mechanism of mesalamine from this gel was further evaluated. The results show that the mesalamine release from *in situ* gel follows Higuchi square root law, i.e amount of drug released is proportional to the square root of the time (Higuchi 1962). The percentage release plotted against the square root of time is shown in Figure 3.4. A linear correlation ($r^2 = 0.98$) observed between percentage drug release and the square root of time indicates that the drug release from the collagen hydrogel delivery system followed a matrix diffusion controlled mechanism.
3.3.4 In Vivo Efficacy of Collagen-mesalamine in situ Gel

Mice fed with 3.5 % DSS in drinking water developed clinical signs of colitis (body weight loss, rectal bleeding, stool consistency) from Day 3 and showed severe clinical signs on day 6. Mice produced loose stools or diarrhoea, gross rectal bleeding and significant body weight loss. After DSS induction, the efficacy of the in situ gel and reference mesalamine suspension was assessed through intra-rectal administration. Three clinical symptoms assessed were beneficially influenced by the treatment which is shown in Figures 3.5a and 3.5b. There was a non-significant trend observed in
case of body weight for both treatment groups until day 11. From day 12, test group started showing an increasing trend with respect to the reference group. Similarly, the clinical score for stool consistency had shown significant reduction on day 13 for both treatment groups (p < 0.01) compared to the DSS control. The rectal bleeding of mice when compared to the reference group (p < 0.05) there was a significant reduction in clinical score for the test group (p < 0.01). This observation could be due to the collagen healing property. Also, as collagen constitutes as an abundant protein of mucosal layer, collagen formulation helps in the faster regeneration of colonic mucosa. The clinical signs have shown significant improvement for the treatment group over DSS control group. Interestingly, the test groups have shown very significant reduction in the rectal bleeding score compared to the reference group.

Figure 3.5a  Body weight changes observation for normal, DSS Control, mesalamine suspension (reference group) and collagen-mesalamine in situ gel (Test group)
Figure 3.5b *In vivo* efficacy of mesalamine *in situ* gel on DSS induced ulcerative colitis mice model

Clinical score activity a) Stool consistence and b) Rectal bleeding for DSS control, mesalamine suspension (reference group) and collagen-mesalamine *in situ* gel (Test group)

The histological section image of DSS control, test group and reference group are shown in Figure 3.6. Histological examination of distal colon sections from normal control mice showed no signs of inflammation and mucosal damage. Histology of colon sections of DSS-treated mice
revealed extensive mucosal damage and dense inflammatory cell infiltrate extending to sub-mucosal layer. The distal colon was isolated on day 6, 10 and 13, the histological score assessing the extent of infiltration of inflammatory cells and mucosal damage were evaluated. After 5 days of continuous DSS induction, histological score revealed severe mucosal damage (2.83 ± 0.16) and dense inflammatory infiltrate (3.00 ± 0.00) in all the groups (Table 3.4). In addition, the histological score showing minimal variation confirmed uniform disease induction. On day 10, the sections showed partially regenerated colonic mucosa and mucosal glands with moderate inflammatory infiltrate in both the treatment groups, but interestingly, the score for mucosal damage (1.33 ± 0.21) in the test group was significantly decreased (p< 0.05) compared to the reference group (2.16 ± 0.17) showing faster ulcer regeneration. On day 13, there was a notable reduction in inflammatory cells (0.33 ± 0.21) in the both the treatment groups and test group showing significant regeneration of colonic mucosa (0.17 ± 0.17) compared to the reference group (0.83± 0.17). In contrast, DSS group showed severe mucosal damage and inflammatory infiltrate indicated by abnormal clinical score. Pathologically, UC is characterized by moderate to severe ulceration in the mucosa, sub-mucosal area and degradation of extracellular matrix. The over expression of MMP 1, an intestinal collagenase which degrades collagen and plays a major role in extracellular matrix degradation. It has been reported that the expression of MMP 1 is increased by 230 fold in colonic mucosa of UC patients compared to the normal control (Marshall & Irvine 1995). The mesalamine collagen in situ gel treated test group showed faster regeneration of mucosal damage and significant reduction in the rectal bleeding score. The presence of collagen had served as a matrix for damaged mucosa and aided in faster regeneration of diseased mucosa. Overall the in vivo experiments clearly demonstrate that collagen-mesalamine in situ gel is very effective for the treatment of UC compared to mesalamine suspension.
Table 3.4 Histological evaluation of distal colonic mucosa for mucosal damage and inflammatory infiltrate observation during treatment at day 6, 10 and 13

| Animal Groups | Histology score | Mucosal damage | | Inflammatory infiltrate | | Day 6 | Day 10 | Day 13 | | Day 6 | Day 10 | Day 13 |
|---------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
|               | Score          | SEM            | Score          | SEM            | Score          | SEM            | Score          | SEM            | Score          | SEM            | Score          | SEM            |
| Normal control| 0.00           | 0.00           | 0.00           | 0.00           | 0.00           | 0.00           | 0.00           | 0.00           | 0.00           | 0.00           | 0.00           | 0.00           |
| DSS control   | 2.83           | 0.16           | 2.67           | 0.52           | 2.33           | 0.52           | 3.00           | 0.00           | 2.83           | 0.16           | 2.16           | 0.17           |
| Reference     | 3.00           | 0.00           | 2.16           | 0.17           | 0.83           | 0.17           | 3.00           | 0.00           | 2.16           | 0.17           | 0.33           | 0.21           |
| Test          | 3.00           | 0.00           | 1.33           | 0.21           | 0.17           | 0.17           | 3.00           | 0.00           | 2.00           | 0.00           | 0.33           | 0.21           |
Figure 3.6  Histology observation of distal colonic mucosa for mucosal damage and inflammatory infiltrate for the treatment group

a) DSS control group  
b) mesalamine suspension group  
c) collagen-mesalamine  in situ gel group  

during treatment at day 6, 10 and 13
Immunohistochemical analysis revealed CD 34 expression was observed in all tissue specimens in the area of colonic mucosa and the capillaries of lamina propria. Collagen mesalamine in situ gel treatment (58.5 ± 5.4) showed abundant CD 34 expression compared to the mesalamine (32.6 ± 3.5 %) and the control (24.4 ± 2.5 %) group (Figure 3.7). No significant change in the expression of CD 34 is exist between the collagen mesalamine and the positive control mesalamine group. All the treatment group showed higher expression compared to the vehicle control.

![Figure 3.7 CD 34 immunohistochemical analysis](image)

**Figure 3.7 CD 34 immunohistochemical analysis**
Representative cross sectional images of vehicle control, mesalamine positive control, and collagen mesalamine treatment. (Magnification: 400X)

### 3.3.5 RT-PCR of Inflammatory Markers and VEGF

In UC, TNF-α, IL-1β and IL-6 are proinflammatory cytokines that have been reported to be up regulated during mucosal damage (Kusugami et al 1995; MacDonald et al 1990; Ligumsky et al 1990). In agreement with previous findings, all DSS-induced groups showed significant upregulation of TNF-α, IL-1β and IL-6 mRNA levels and the vehicle treated group did not show any reduction in upregulation of inflammatory markers throughout the course of the treatment. Mesalamine, a first line drug used to treat mild to moderate UC, is known to bring about a significant suppression in the expression on the levels of all three proinflammatory cytokines. In our study, it has been observed (Figure 3.8A) that collagen mesalamine treatment was
very effective in down-regulating TNF-α expression by day 10 & 15, followed by mesalamine and vehicle treatment (Day 10, Collagen mesalamine, 1.347 ± 0.04, Mesalamine, 1.82 ± 0.08 and vehicle, 8.16 ± 0.30, Day 15, Collagen mesalamine, 0.42 ± 0.04, Mesalamine, 1.10 ± 0.08 and vehicle, 9.09 ± 0.34). Also, Collagen mesalamine showed significant reduction in the expression levels of both IL-1β (Figure 3.8 B) and IL-6 (Figure 3.8 C) compared to the mesalamine positive control (IL-1β, Collagen mesalamine, 0.73 ± 0.02, Mesalamine, 1.52 ± 0.04, vehicle, 5.41 ± 0.19 on day 10 and Collagen mesalamine, -0.88 ± 0.04, Mesalamine, -0.62 ± 0.02, vehicle, 6.70 ± 0.17 on day 15 and IL-6, Collagen mesalamine, 0.88 ± 0.01, Mesalamine, 1.27 ± 0.01, vehicle, 4.02 ± 0.14 on day 10 and Collagen mesalamine, 0.25 ± 0.02, Mesalamine, 0.54 ± 0.07, vehicle, 6.56 ± 0.23 on day 15).

There are a lot of evidences that demonstrate the upregulated serum and tissue levels of VEGF in patients with active ulcerative colitis (UC) and in animal models of UC (Tolstanova et al 2009; Griga et al 1998a; Griga et al 1998b; Kanazawa et al 2001; Sandor et al 2006).

All groups that were induced by DSS showed significantly increased levels of VEGF, which is an indication of disease severity (Figure 3.9). Among the groups that underwent therapy, collagen mesalamine in-situ gel group (1.22 ± 0.07) showed marked reduction in VEGF expression by day 10, followed by mesalamine (2.65 ± 0.33) and vehicle group (4.17 ± 0.15).
Figure 3.8 Effect of collagen mesalamine in-situ gel on the mRNA expression levels of pro-inflammatory cytokines (A) IL-1β, (B) IL-6 and (C) TNF-α. Each column represents the mean ± S.E.M. of 6 mice. **P<0.01 and ***P<0.001, compared with the vehicle group. Collagen mesalamine in-situ gel treatment was very effective in decreasing the expression levels of IL-1β, IL-6 and TNF-α by day 10 compared to mesalamine treatment. Each column represents the mean ± S.E.M. of 6 mice. **P<0.01 and ***P<0.001, compared with the vehicle group. (Vehicle, mesalamine, collagen mesalamine in-situ gel)
Figure 3.9 Effect of collagen mesalamine in-situ gel on the VEGF mRNA expression in DSS induced ulcerative colitis in mice

VEGF levels in colonic tissue were determined by real time-PCR. All the treatment group caused significant increase in VEGF mRNA levels followed by DSS induction by day 5. Collagen mesalamine in-situ gel treatment showed marked reduction in VEGF expression levels followed by mesalamine and vehicle treatment on both day 10 and 15. Each column represents the mean ± S.E.M. of 3 samples. ***P<0.001, compared with the vehicle group. ( Vehicle, □ mesalamine, ■ collagen mesalamine in-situ gel)

3.4 CONCLUSION

The gelation behaviour of collagen at physiological pH and temperature is effectively tapped for formulation of in situ mesalamine gel using a simple procedure and the same had showed excellent efficacy in treating DSS induced ulcerative colitis in mice model. The in situ gel showed sustained release for a time period of 12h. Interestingly, the histological score for mucosal damage and clinical score for rectal bleeding was significantly reduced for the collagen-mesalamine in situ gel in comparison to mesalamine suspension. The novel therapeutic strategy of utilizing the sol – gel behaviour
of collagen in enhancing the availability of mesalamine for longer period and synergistic efficacy of collagen and mesalamine combination had augured very well for the treatment of UC. Similar strategy can be employed for the management for several other diseases or disorders where site specific treatment is critical.