Evaluating the mucoprotective effects of glycyrrhizic acid-loaded polymeric nanoparticles in a murine model of 5-fluorouracil-induced intestinal mucositis via suppression of inflammatory mediators and oxidative stress

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Abstract

Objectives 5-Fluorouracil (5-FU), a chemotherapeutic drug, has severe deteriorating effects on the intestine, leading to mucositis. Glycyrrhizic acid is a compound derived from a common herbal plant Glycyrrhiza glabra, with mucoprotective, antioxidant and anti-inflammatory actions, however, associated with poor pharmacokinetics. Owing to the remarkable therapeutic action of glycyrrhizic acid-loaded polymeric nanocarriers in inflammatory bowel disease, we explored their activity against 5-FU-induced intestinal mucositis in mice. Polymeric nanocarriers have proven to be efficient drug delivery vehicles for the long-term treatment of inflammatory diseases, but have not yet been explored for 5-FU-induced mucositis. Therefore, this study aimed to produce glycyrrhizic acid-loaded polylactic-co-glycolic acid (GA-PLGA) nanoparticles to evaluate their protective and therapeutic effects in a 5-FU-induced mucositis model.

Methods GA-PLGA nanoparticles were prepared using a modified double emulsion method, physicochemically characterized, and tested for in vitro drug release. Thereafter, mucositis was induced by 5-FU (50 mg/kg; IP) administration to the mice for the first 3 days (day 0, 1, 2), and mice were treated orally with GA-PLGA nanoparticles for 7 days (day 0–6).

Results GA-PLGA nanoparticles significantly reduced mucositis severity measured by body weight, diarrhea score, distress, and anorexia. Further, 5-FU induced intestinal histopathological damage, altered villi-crypt length, reduced goblet cell count, elevated pro-inflammatory mediators, and suppressed antioxidant enzymes, all of which were reversed by GA-PLGA nanoparticles.

Conclusion Morphological, behavioral, histological, and biochemical results suggested that GA-PLGA nanoparticles were efficient, biocompatible, targeted, and sustained release drug delivery nano-vehicle for enhanced mucoprotective, anti-inflammatory, and antioxidant effects in 5-FU-induced intestinal mucositis.

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Introduction

Intestinal mucositis is clinically characterized by severe abdominal pain, nausea, vomiting, bloating, diarrhea, and abdominal cramps, caused by mucosal inflammation and loss of the intestinal epithelial lining that culminates in mucosal ulceration of the gastrointestinal tract (Blijlevens et al. 2000; Logan et al. 2009). Intestinal mucositis is a common side effect of cancer chemotherapy (van Vliet et al. 2010), affecting almost 40% of patients receiving standard doses and 80–100% of patients who receive higher doses (Keefe et al. 1997; Lalla et al. 2014). Chemotherapeutic agents, in general, interfere with rapidly dividing cells, therefore, rapidly proliferating mucosal cells are more susceptible to these agents (Duncan and Grant 2003). Intestinal mucositis is an indication to halt the chemotherapeutic regimen, altering the dosing schedule, and thereby contributing to higher mortality in cancer patients (Naidu et al. 2004).

Recently, research has focused on the resolution of intestinal injury caused by chemotherapeutic agents. 5-Fluorouracil (5-FU) is a common anti-cancer drug, used to treat various tumors such as head and neck, gastrointestinal, and breast tumors. 5-FU, a fluorinated pyrimidine analog, exerts its chemotherapeutic action through inhibition of DNA synthesis. However, it can induce intestinal mucositis by disrupting the intestinal epithelium and by its indiscriminate action against rapidly proliferating intestinal mucosal cells (Soares et al. 2008; Wright et al. 2009). Although the complete pathogenesis of 5-FU-induced intestinal mucositis is not fully understood, the main stages are well-characterized. Initially, there is an inflammatory phase when DNA strand breakage occurs, characterized by the production of reactive oxygen species (ROS) with minimal disruption to the epithelial lining. The second stage involves an excessive burst of pro-inflammatory cytokines (IL-1, IL-6, TNF-α, IFN-γ) and activation of a transcription factor (NF-κB). This leads to the epithelial phase where cell proliferation terminates and cell death begins, progressing to an ulcerative phase consisting of cell necrosis and ulceration, followed by a healing phase that involves repair of the damaged epithelial lining (Basile et al. 2019; Blijlevens et al. 2000). This process heavily distresses mucosal cells and causes intestinal villi-crypt atrophy and damage by facilitating apoptosis and increasing the cellular burden of oxidative and pro-inflammatory molecules (Atiq et al. 2019; Basile et al. 2019).

Unfortunately, currently available therapies for 5-FU-induced mucositis are associated with deleterious side effects (Atiq et al. 2019). Currently, research has been directed towards exploring the medicinal uses of herbal products for a range of ailments to avoid unnecessary effects associated
with synthetic compounds (Yuan et al. 2016). In this context, glycyrrhizic acid, a triterpene glycoside, obtained from the roots of the licorice plant, Glycyrrhiza glabra, is of particular interest because of its various potential medicinal benefits including anti-inflammatory, anti-diabetic, antioxidant, anti-tumor, antimicrobial, and anti-viral effects (Ming and Yin 2013). Recently, it has been suggested that glycyrrhizic acid possesses anti-inflammatory activity through inhibition of cyclooxygenase-2 (COX-2) and prostaglandin E2 (PGE-2) (Wang et al. 2011, 2017). Moreover, it can suppress the inflammatory response by blocking the NF-κB pathway, which is responsible for the production of pro-inflammatory cytokines and chemokines (Cheng et al. 2006; Wang et al. 2017). Furthermore, it has been found to possess anti-cancer properties, enabling its dual use for the treatment of chemotherapy-induced mucosal damage and proliferation of tumorous lesions (Khan et al. 2013; Su et al. 2017). Glycyrrhizic acid reduced tumorous mass development, prevented infiltration of mast cells, attenuated production of TNF-α, and prevented mucosal layer disruptions (Khan et al. 2013; Khan et al. 2018). Furthermore, it was demonstrated that glycyrrhizic acid, owing to its anti-inflammatory and anti-oxidant properties has the potential to serve as a chemopreventive agent (Bode and Dong 2015). Therefore, in the present study, glycyrrhizic acid was explored in a mouse model of 5-FU-induced intestinal mucositis.

Oral route is the most convenient route to target the inner lumen of the intestine. However, conventional delivery of glycyrrhizic acid via the oral route is limited by a significant first-pass effect resulting in decreased bioavailability and reduced therapeutic efficiency in medical conditions like intestinal mucositis (Ploeger et al. 2000). Therefore, a drug delivery system is required to deliver the therapeutic agent to the inflamed intestines for an adequate duration without being metabolized in the gut. In this context, nanoparticle-based drug delivery systems may be able to overcome these limitations due to their nanometer-scale size that ultimately results in improved accumulation and enhanced residence time at the inflamed tissue via the enhanced epithelial permeability and retention effect (eEPR) (Boisseau and Louba-ton 2011; Zeeshan et al. 2019a). Previously, nanoparticles smaller than 500 nm were shown to be efficiently taken up by infiltrated immune cells (Zeeshan et al. 2019a), which increases the time span for the drug to be released and therapeutic efficacy at the inflamed tissues while preventing rapid diarrhea-associated clearance from the intestine. A large range of polymers can be employed for nanoparticle-mediated drug delivery; however, polymers such as polylactic-co-glycolic acid (PLGA) are of particular interest due to their biodegradability, biocompatibility, and their ability to sustain drug release over a long period to achieve better therapeutic outcomes (Danhier et al. 2012). In our previous work, PLGA nanoparticles were shown to have therapeutic efficacy in an ulcerative colitis model (Ali et al. 2016; Zeeshan et al. 2019b).

To date, PLGA nanocarriers have been investigated in models of oral mucositis (Takeuchi et al. 2018); however, no evidence is available on their efficacy for 5-FU-induced intestinal mucositis. In the present study, intestinal mucositis was induced with 5-FU in BALB/c mice and treated with both glycyrrhizic acid free drug and glycyrrhizic acid-loaded PLGA (GA-PLGA) nanoparticles by oral administration to investigate the therapeutic efficacy of the drug with or without the nanocarrier. Intestinal toxicity was evaluated using a variety of morphological, behavioral, biochemical, and histological analyses.

Materials and methods

Preparation of GA-PLGA nanoparticles

GA-PLGA nanoparticles were prepared by a modified double W/O/W emulsification–evaporation method, as reported previously (Zeeshan et al. 2019b). Glycyrrhizic acid (mono-ammonium salt), purchased from Sigma-Aldrich, Germany, was dissolved in a concentration of 10 mg per 5 mL of distilled water and added dropwise to 5 mL of organic solution, containing 100 mg PLGA (Resomer RG502, 50:50, Evonik, Germany) in 5 mL ethyl acetate. The resultant primary emulsion was probe sonicated and added into an external aqueous phase, consisting of 5 mL of 2% polyvinyl alcohol (PVA) solution (Sigma-Aldrich, Germany) in 5 mL ethyl acetate. The resultant primary emulsion was probe sonicated and added into an external aqueous phase, consisting of 5 mL of 2% polyvinyl alcohol (PVA) solution (Sigma-Aldrich, Germany). The obtained W/O/W emulsion was stirred to evaporate the organic solvent, centrifuged, and freeze-dried to obtain lyophilized GA-PLGA nanoparticles.

Physicochemical characterization

Average particle diameter was determined by dynamic light scattering (Brookhaven 90Plus Instrument, USA) in triplicate, with results expressed as mean ± standard deviation (SD). The zeta potential of the nanoparticles was analyzed in triplicate using a Malvern zeta sizer 2000 HS (Malvern instrument, UK), with results expressed as mean ± SD. The percentage yield of the freeze-dried nanoparticles was obtained by weighing freeze-dried nanoparticles and calculated using the formula:

\[
\text{% Yield} = \frac{\text{weight of freeze-dried nanoparticles}}{\text{weight of polymer + drug}} \times 100.
\]

(1)

The surface morphology of GA-PLGA nanoparticles was analyzed by scanning electron microscopy (SEM) (Vega 3 TESCAN, Czech Republic).
Encapsulation efficiency (EE)

Drug entrapment within PLGA nanoparticles was estimated by spectroscopic determination of free drug content in the supernatant collected after centrifugation of the nano-formulation at a wavelength ($\lambda_{\text{max}}$) of 252 nm (Ultraviolet–Visible Spectrophotometer, Agilent Technologies, US). %EE was calculated using the formula:

$$\% \text{ Encapsulation} = \left( \frac{\text{total drug} - \text{free drug}}{\text{total drug}} \right) \times 100. \quad (2)$$

The loaded drug content was determined spectroscopically at 252 nm by the method previously described (Zeeshan et al. 2019b). % Drug content was calculated from the formula:

$$\% \text{Drug content} = \left( \frac{\text{actual drug content in nanoparticles}}{\text{theoretical drug content used in nano-formulation}} \right) \times 100. \quad (3)$$

Fourier transformed infrared spectroscopy (FTIR)

FTIR analysis of glycyrrhizic acid, PLGA, and GA-PLGA nanoparticles was performed using the potassium bromide disc technique. FTIR spectra were observed in the region of 400–4000 cm$^{-1}$ wave number against % transmittance using a Perkin-Elmer100 FTIR spectrometer (USA).

In vitro drug release studies

In vitro drug release was tested at pH 1.2 and 6.8, corresponding to the physiological pH of gastrointestinal tract (GIT) organs. 50 mg of the GA-PLGA nanoparticles were suspended in a simulated gastric fluid (SGF), prepared according to the United States Pharmacopeia. The release profile was observed for 2 h, after which Na$_2$HPO$_4$.2H$_2$O and KH$_2$PO$_4$ were added and pH was adjusted to 6.8 with 0.1 N NaOH solution. The release profile was continued for 48 h, and samples were collected at pre-determined intervals from the buffers and analyzed by UV–Vis spectrophotometry to obtain drug release estimates.

Animal studies

Experimental animals

Experiments were performed on 3–4 weeks old BALB/c mice obtained from the National Institute of Health (NIH, Islamabad, Pakistan) and kept under standard laboratory conditions (20–25 °C, 55±5% humidity) in stainless steel cages. Mice were acclimatized to the environment for a period of one week before experimentation. All animal experiments were conducted according to the bioethical committee protocols of Quaid-i-Azam University, Islamabad for the care and use of lab animals (Approval no. BES-fbs-QAU2018-75). Mice were given ad libitum access to food and drinking water.

Induction and evaluation of mucositis

Mice weighing 25–30 g were randomly divided into four groups ($n$ = 5 mice/group). The number of mice in each group was decided keeping in mind the ethical condition of minimal harm and applying the resource equation to generate sound statistical data. Group one received vehicle only (drinking water) and served as a normal control group. Groups 2, 3, and 4 received 5-FU (Sigma-Aldrich, Germany) through intraperitoneal (IP) injection at a dose of 50 mg/kg daily for three consecutive days to induce experimental intestinal mucositis (Atiq et al. 2019). In addition, groups 3 and 4 were treated with the glycyrrhizic acid free drug and GA-PLGA nanoparticles, respectively, through oral gavage, at a drug dose equivalent to 10 mg/kg body weight daily for 7 days. The dose was optimized for anti-inflammatory activity in an ulcerative colitis model in our previous study (Zeeshan et al. 2019b). For the first 3 days, treatment either in the form of free drug or nanoparticles was given 30 min before 5-FU IP administration. On the 7th day, mice were euthanized, and tissue samples were removed for further testing (Fig. 1).

Assessment of intestinal mucositis severity through morphological and behavioral parameters

Mice were monitored to assess 5-FU-induced mucositis severity daily. It was manifested through bodyweight loss, stool consistency, distress scores, anorexia, and mortality rate (Basile et al. 2019). Mice from all four groups were weighed daily to measure body weight loss. The results were expressed as percent mean body weight loss ± SD. Similarly, stools were examined for diarrheal indication and scored using Bowen’s score system (Leocádio et al. 2015; Yeung et al. 2015).

Daily distress scores were calculated based on cumulative average scores of fur/coat condition, change in temperament, and reluctance to movement. Fur/coat was characterized as 0, shiny and smooth; 1, coat raised around the neck; 2, coat raised around neck and belly; 3, coat raised around the whole body with or without coat loss. Change in temperament was scored as 0, normal; 1, agitated behavior; 2, stress marks on body parts; 3, stress marks with hunching. Movement reluctance of the mice was scored as 0, normal activity; 1, movement on the placement of a near object; 2, movement only after lifting mice up; 3, no movement even on handling (Atiq et al. 2019).
Evaluation of food consumption and survival analysis

Consumption of food on each day was recorded to assess the food intake pattern of the mice in different groups. Anorexia is a common symptom of chemotherapy-induced mucositis (Basile et al. 2019). Therefore, reductions in daily food intake were taken as a demonstration of the development of 5-FU-induced mucositis and the effect of therapy. Survival analysis was performed by observing the death rate of mice in each group daily.

Effect of 5-FU-induced mucositis on organs

At the end of the experiment, mice were euthanized, and the entire small intestine and colon were excised. The weight and length of the small intestine and the colon were recorded after the removal of feces and adjacent fats. The tissues were fixed in 10% formalin and frozen at –80 °C for further analysis. Spleen weight was evaluated as an inflammation index. In addition, the liver and kidney were resected and weigh to evaluate the effect of chemotherapy and treatment on these vital organs.

Histopathological evaluation

For histological analysis, a 1 cm excised tissue sample of the small intestine (jejunum) and large intestine from each mouse group was fixed in 10% buffered formalin and embedded in paraffin wax. 5 μm sections were taken from each sample, mounted on glass slides and stained with hematoxylin and eosin (H & E). The prepared samples were observed microscopically at different magnifications (4 ×, 10 ×, and 40 ×) to assess the severity of intestinal damage. The histomorphometric parameters for the small intestine include measurement of small intestinal villus height (top of the villus to a villus-crypt junction; \( n = 20 \) villi), crypt depth (adjacent villi intussusceptions; \( n = 20 \)), and calculating the villi to crypt ratio index to estimate villi-crypt damage using TCapture imaging software (Tucsen Photonics Co. Ltd.) and a light microscope equipped with a high-resolution camera (ISH 500, Tucsen CMOS USB 2.0). The villi-crypt measurements were performed using ImageJ software (NIH, USA). While, the large intestine histological score was based on inflammation indices including sum scores of surface epithelial loss, crypt destruction, and inflammatory cell infiltration according to a pre-determined scale (Erben et al. 2014).

Goblet cells evaluation

Similarly, 1 cm thin sections from each harvested small intestine (jejunum) were fixed in 10% formalin and embedded in paraffin wax. 5 μm sections were taken and stained with periodic acid Schiff-Alcian blue (PAS-Alcian blue) to visualize goblet cells and mucin content in the jejunum samples of each group (Ali et al. 2019; Stringer et al. 2009). The stained images were captured as described above and processed to calculate the number of intact goblet cells per villus, mucin-stained area containing MUC-2 protein, and intensity of the blue-colored stain using ImageJ software. Results were expressed as mean ± SD.

Enzyme-linked immunosorbent assay (ELISA) for cytokine profiling

The severity of intestinal inflammation was determined by measuring the expression of pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) in the jejunum sections excised from each group using ELISA kits (eBioscience, Inc., San Diego, CA, USA). All assays were conducted according to the manufacturer’s instructions.
Biochemical assays to determine the intestinal antioxidant protection level

To assess antioxidant enzymatic activity in the intestine, 1 cm jejunum tissue was excised at the end of the experiment from all mice in all groups. The tissue was immediately immersed in phosphate-buffered saline (PBS) (1 mL) and homogenized at 10,000 rpm until the single-cell suspension was obtained, then centrifuged (489 g for 10 min). Biochemical assays were performed using the supernatants to estimate levels of reduced glutathione (GSH), glutathione sulfotransferase (GST), and catalase antioxidant enzymes.

GSH levels were determined using Ellman’s reagent method (Arruda et al. 2013; Moron et al. 1979) with a UV/Vis spectrophotometer at 412 nm. PBS was used as a blank. The GSH values were expressed as nmol/g tissue ± SD.

GST assay was conducted to determine the intestinal tissue antioxidant capacity. The assay endpoint was a spectroscopic measurement at 340 nm using potassium phosphate buffer as a blank. Assay mixture without supernatant was used as a control to determine non-specific binding of the substrates (GSH and 1-chloro-2,4-dinitrobenzene (CDNB)) (Arruda et al. 2013; Habig et al. 1974). GST activity was expressed as nmol of CDNB conjugated/g tissue.

Catalase activity was determined by combining 3 ml of H2O2 and potassium phosphate buffer (0.6 M) mixture to 40 μl of supernatant (Hadwan and Abed 2016). Absorbance was measured spectrophotometrically at 240 nm. The same assay mixture without H2O2 was used as a blank. Results were stated as enzyme units/min/mg.

Statistical analysis

Statistical analysis to compare between the groups was conducted using one-way ANOVA or t test. The significance level was denoted as *(α = 0.05), **(α = 0.01) or ***(α = 0.001).

Results

Physicochemical characterization of the prepared GA-PLGA nanoparticles

GA-PLGA nanoparticles were successfully prepared with an average particle size of 190.46 ± 7.49 nm, the zeta potential of -11.7 ± 2.5 mV, and polydispersity index (PDI) lesser than 0.3 (0.123 ± 0.112). Particles smaller than 200 nm can easily accumulate and reside at the targeted site (Collnot et al. 2012; Zeeshan et al. 2019a). Moreover, nanoparticles were fabricated with a good percentage yield (78.00 ± 2.64%) and drug entrapment (67.7 ± 2.5%) (Table 1).

FTIR investigations

FTIR was used to analyze the functional groups within the chemical structures of glycyrrhizic acid, PLGA polymer, and GA-PLGA nanoparticles to ascertain drug–polymer interactions and drug entrapment (Fig. 2a–c). The FTIR spectrum of glycyrrhizic acid showed a broad OH peak at 3391 cm⁻¹, sharp C=CH₂ peaks at 2926 cm⁻¹ and 2855 cm⁻¹, C=O at 1745 cm⁻¹ and 1634 cm⁻¹, CH₃ at 1458 cm⁻¹, OH in-plane bend at 1279 cm⁻¹, an acetate peak at 1209 cm⁻¹, esteric C=O stretch at 1163 cm⁻¹, characteristic secondary cyclic alcohol C–O stretch at 1032 cm⁻¹, C–C peaks at 979 cm⁻¹ and 700 cm⁻¹, and NH out-of-plane bend at 615 cm⁻¹. Similarly, the PLGA FTIR spectrum (Fig. 2) demonstrated functional groups of free OH at 3517 cm⁻¹ with low intensity, C=CH₂ peaks at 2926 cm⁻¹ and 2855 cm⁻¹, C=O at 1746 cm⁻¹ and 1629 cm⁻¹, other stretches of CH₃ at 1456 cm⁻¹, C–OH in-plane bend at 1427 cm⁻¹, CH₃ at 1381 cm⁻¹, esteric C–O stretch at 1163 cm⁻¹, primary alcohol C–O stretch at 1088 cm⁻¹ and CH₂ at 719 cm⁻¹. Next, the FTIR spectrum of GA-PLGA nanoparticles (Fig. 2c) showed a broadened OH peak at 3383 cm⁻¹, secondary

| Formulations       | Particle size (nm) | PDI    | % Encapsulation efficiency | Zeta potential (mV) | % Yield |
|---------------------|--------------------|--------|---------------------------|---------------------|---------|
| Blank PLGA NPs     | 157.6 ± 7.9        | 0.229 ± 0.15 | N/A                       | -9.33 ± 3.21       | 75.33 ± 2.51 |
| GA-PLGA NPs        | 190.46 ± 7.49      | 0.123 ± 0.112 | 67.7 ± 2.5                | -11.7 ± 2.5        | 78.0 ± 2.64 |

PDI polidispersity index
cyclic alcohol C–O stretch at 1037 cm⁻¹, and OH in-plane bend at 1276 cm⁻¹ which demonstrated drug entrapment. Other representative peaks of both drug and PLGA are C–CH₂ at 2928 cm⁻¹ and 2856 cm⁻¹, C=O at 1745 cm⁻¹ and 1643 cm⁻¹, CH₃ at 1453 cm⁻¹, and esteric C–O at 1161 cm⁻¹. Other peaks in the spectra are for C–C vibrations at 980 cm⁻¹, C–C at 702 cm⁻¹, and NH out-of-plane bend at 617 cm⁻¹. The fingerprint regions of both drug and polymer were retained in the spectra which confirmed drug–polymer compatibility and excluded the possibility of chemical interaction or bond formation. Drug entrapment was confirmed by a relative change in the intensity of transmittance at wave-numbers corresponding to functional groups present in both drug and polymer (Fig. 2c).

**Morphological analysis**

SEM analysis confirmed that the particle size was within the required size range (<200 nm). Further, SEM revealed spherical shape nanoparticles with a smooth surface (Fig. 3).
In vitro drug release study

Drug release from the GA-PLGA nanoparticles was conducted in SGF (pH 1.2) for the first 2 h, followed by 46 h in PBS (pH 6.8) at 37 °C. The pattern obtained for the drug release at pre-determined time points indicated an initial burst release, followed by sustained release up to 48 h, accounting for 77.67 ± 1.15% of drug released till 48 h (Fig. 3). The drug release behavior is the typical pattern of drug release from PLGA nanoparticles (Ali et al. 2016).

Assessment of 5-FU mucositis induction and treatment through morphological and behavioral parameters

5-FU induction decreased bodyweight, measured by daily weighing. This is a common symptom of intestinal inflammation. Treatment with either glycyrrhizic acid or GA-PLGA nanoparticles restored body weight (Fig. 4a). However, the highest recovery in body weight was noticed with GA-PLGA nanoparticle administration to the 5-FU induced mice (p < 0.001). The two treatment groups, glycyrrhizic acid, and GA-PLGA nanoparticles differ by a factor of four, in terms of % body weight recovery (Fig. 4a). Furthermore, mice from all groups were assessed for stool consistency; initially softened stools were observed in all groups. After the third day, mice injected with 5-FU suffered from pronounced diarrhea. Treatment relieved diarrheal symptoms somewhat; GA-PLGA nanoparticles had a statistically significant effect (p < 0.001) (Fig. 4b).

Induction of mucositis by 5-FU caused distress, manifested as behavioral signs like difficulty to move, anxious behavior, and poor fur condition (score = 3; p < 0.001) compared to the normal healthy mice. Glycyrrhizic acid and GA-PLGA nanoparticles showed significantly reduced distress in the 5-FU mice. At the end of the experiment, glycyrrhizic acid decreased the distress score to 1.67 ± 0.57 points (p < 0.05) and GA-PLGA reduced the distress score to 1.25 ± 0.50 points (p < 0.001; Fig. 4c).

![Graphs showing body weight, stool consistency, stress score, and percentage survival](image-url)
Evaluation of daily food consumption and survival analysis

The mortality rate was assessed in all groups. The percentage survival rate was lowest for the 5-FU group (20%); in comparison to the normal mice group (100%). Free glycyrrhizic drug improved survival rate to 40%, whereas GA-PLGA nanoparticles increased the survival rate to 60% at the end of the experiment (Fig. 4d).

Anorexia is a common side effect of chemotherapy (Basile et al. 2019); therefore, food intake was assessed in the mice of all four groups daily. After 3 days of intraperitoneal 5-FU injection, a sharp decline in food consumption was observed ($p<0.001$), compared to the control mice. At the end of the experiment, food intake in the 5-FU group was reduced to 31% of the initial food intake. Treatment with glycyrrhizic acid or GA-PLGA nanoparticles significantly reduced anorexia ($p<0.001$), with food intake increased to 59% and 78%, respectively, at the end of the experiment (Fig. 5a).

Effect of 5-FU-induced mucositis and GA-PLGA nanoparticles effect on the organs

The effect of 5-FU on the spleen, liver, kidney, small intestine, and large intestine (colon) was investigated (Song et al. 2013). 5-FU significantly decreased the weight of the spleen ($p<0.01$) and kidney ($p<0.05$), but did not affect the liver (Fig. 5b). Likewise, shortening of the small intestine ($p<0.05$) and colon ($p<0.001$) was observed to a significant extent (Fig. 5c, d). Glycyrrhizic acid or GA-PLGA nanoparticles administration to the 5-FU-induced mice increased the spleen weight significantly ($p<0.01$). Moreover, glycyrrhizic acid ($p<0.05$) and GA-PLGA nanoparticles ($p<0.01$) significantly restored kidney weight. Glycyrrhizic acid had minimal effect on the restoration of the length of the small intestine; however, resumed the colon length ($p<0.01$). While GA-PLGA nanoparticles have significantly recovered the length of both the small intestine ($p<0.05$) and the colon ($p<0.001$) (Fig. 5c, d).
Histopathological evaluation

Histological microscopy revealed intact epithelial integrity, well-oriented villi, aligned crypt cells, and lack of inflammatory infiltrates in normal small intestine (jejunum) and large intestine (colon), whereas marked injury to both small and large intestine was observed after 5-FU, as shown in Fig. 6a–e. 5-FU-induced small intestine histomorphological damage is evident from disrupted epithelium, shortened and distorted villi, vacuolated crypt cells, necrosis, and immune cell infiltration. A marked decrease in villi length \( (p < 0.001) \) was observed with slightly enlarged crypts \( (p < 0.01) \), which altered the villi to crypt ratio \( (p < 0.001) \), compared to the normal intestine (Fig. 6a, c–e). Similarly, the large intestine had pronounced deformation with epithelium loss, villi–crypt morphological destruction, and immune cell infiltration (Fig. 6a, b). Treatment with glycyrrhizic acid or GA-PLGA nanoparticles restored the normal histomorphological features. Small intestine villi-crypt length and ratio and large intestine histopathological scores were improved by glycyrrhizic acid treatment of 5-FU mice. Likewise, GA-PLGA nanoparticles recovered small intestine villi length \( (p < 0.001) \), crypt depth \( (p < 0.01) \), and villi to crypt ratio \( (p < 0.001) \) in the 5-FU mice significantly (Fig. 6a, c–e). In addition, large intestine histology was improved by glycyrrhizic acid \( (p < 0.05) \) and more substantial through GA-PLGA nanoparticles treatment \( (p < 0.001) \) (Fig. 6a,b).

Exploration of goblet cell count and mucin content

5-FU-mediated inflammation resulted in the decline and distortion of goblet cells in the jejunum, thus reducing the mucin content in the intestinal tissue. Treatment with glycyrrhizic acid restored the goblet cell count to some extent \( (p < 0.01) \), while GA-PLGA nanoparticles increased the goblet cell count and architecture significantly \( (p < 0.001) \) (Fig. 7a, b). The MUC-2 protein is mainly responsible for mucin formation in the intestine (Tadesse et al. 2017). The protein was indirectly assessed through PAS-Alcian blue staining followed by quantification of the mucin area and staining intensity within goblet cells using ImageJ software. The mucin-stained area \( (p < 0.01) \) and average staining intensity \( (p < 0.001) \) were found to be significantly altered following 5-FU induction, compared to controls (Fig. 7c, d). Glycyrrhizic acid caused a limited increase in the overall mucin area; however, enhanced staining intensity.

![Histopathology of the excised small and large intestines from normal mice, 5-FU induced mucositis group, Glycyrrhizic acid and GA-PLGA nanoparticles treated groups](image-url)
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GA-PLGA nanoparticles markedly increased the stained area (p < 0.05) and intensity (p < 0.001), compared to the 5-FU control group (Fig. 7c, d).

Enzyme-linked immunosorbent assay (ELISA) for cytokines profiling

5-FU-induced mucositis is associated with increases in pro-inflammatory cytokines including TNF-α, IL-1β, and IL-6 (Chang et al. 2012; Song et al. 2013). To assess the anti-inflammatory activity of the treatments, pro-inflammatory cytokines were quantified in the excised jejunum tissues from all groups. There was a marked elevation in pro-inflammatory cytokines levels in the 5-FU-induced mucositis group compared to the normal control group. Treatment, either with glycyrrhizic acid or GA-PLGA nanoparticles, significantly decreased TNF-α, IL-1β, and IL-6 levels, thus demonstrating their activity against inflammation in the disease model (Fig. 8a–c).

Biochemical assays to determine intestinal protection level

5-FU-associated intestinal inflammation leads to the generation of excessive reactive oxygen species (ROS), whose overproduction results in a diminished activity of antioxidants (GSH, GST, catalase) (Chang et al. 2012; Gelen et al. 2018). In this study, 5-FU reduced GSH concentration from $81.20 \pm 0.75$ to $3.5 \pm 0.5$ nmol/g (p < 0.001), reduced GST concentration from $845.22 \pm 9.14$ to $125.0 \pm 5.0$ nmol/g (p < 0.001), and reduced catalase activity from $0.527 \pm 0.078$ to $0.318 \pm 0.035$ U/min/mg (p < 0.05), compared to control normal mice. Mice treated with glycyrrhizic acid had elevated GSH (p < 0.01) and GST levels (p < 0.001) compared to the 5-FU mice but had a non-significant effect on catalase activity. GA-PLGA nanoparticles had a more pronounced effect in levelling-up antioxidant protection in 5-FU induced mice, increasing GSH concentration to $34.00 \pm 1.43$ nmol/g (p < 0.001), GST concentration to $600.33 \pm 5.51$ nmol/g (p < 0.001).
Discussion

5-FU, an anti-pyrimidine and antimetabolite drug, is widely used for the chemotherapeutic purpose to treat malignant colorectal and breast tumors (Chang et al. 2012; Chen et al. 2020). However, the continuous use of 5-FU is associated with intestinal mucositis leading to the destruction of the intestinal microstructure like villi, crypts, epithelial cells, and goblet cells, leading to clinical symptoms like body-weight loss, heavy diarrhea, anorexia, and anxiety (Song et al. 2013). 5-FU-induced mucositis severely compromises patients’ compliance with anti-neoplastic chemotherapy regimens, and cancer treatment often has to be discontinued due to severe mucositis.

Glycyrrhizic acid is herbal medicine, which has been used to treat several ailments for centuries. Recently, it was found effective in the treatment of methotrexate-induced enteritis in rats (Wang and Du 2016). Owing to its anti-inflammatory, anti-ulcer, mucoprotective, immunomodulatory, antibacterial, antioxidant, and anti-cancerous actions (Ming and Yin 2013), it is an obvious candidate to protect the intestine from injury. However, its protective effect is limited in the intestine because of rapid elimination from the gut resulting from heavy diarrhea, and degradation by endogenous enzymes. Therefore, a sustained therapeutic effect can be achieved by encapsulating glycyrrhizic acid inside a biocompatible nanocarrier. In our previous research, glycyrrhizic acid-loaded polymeric nanocarriers proved to be efficient in ameliorating dextran sulfate sodium (DSS)-induced ulcerative colitis in murine mice model (Zeeshan et al. 2019b). However, there is no scientific evidence for the use of glycyrrhizic acid and its encapsulation into a nanocarrier for the treatment of 5-FU-induced intestinal mucositis, as per our knowledge. PLGA is a widely used biocompatible, biodegradable, and well-tolerated polymeric carrier. Therefore, PLGA was used to prepare glycyrrhizic acid-loaded PLGA nanoparticles using a double emulsion method in this study. Previously, it was found to be a sustained drug release carrier in the treatment of oral mucositis (Takeuchi et al. 2018). Thus, PLGA would be an ideal carrier to be explored for 5-FU-induced mucositis of the intestines.

The synthesized nanoparticles had an optimal size of < 200 nm and low PDI, indicating a monodispersed system. The drug had a suitable encapsulation efficiency, and
the nanoparticles had a spherical morphology (Table 1, Fig. 3). Particles with a size lesser than 200 nm accumulate in inflamed tissues through the eEPR effect and are prone to rapid uptake by immune cells recruited as a consequence of inflammation (Zeeshan et al. 2019a). In vitro drug release studies indicated an initial burst release of the drug, followed by sustained release drug behavior over 48 h (Fig. 3), consistent with previous findings (Zeeshan et al. 2019b).

In the mice, dosing range to develop 5-FU induced intestinal mucositis vary in the literature, from 25 to 250 mg/kg (dos Filho et al. 2016; Li et al. 2017; Zhang et al. 2017, 2018). In a recent attempt, researchers inferred that the appropriate mice dose to establish 5-FU-induced intestinal mucositis is 50–100 mg/kg (Zhang et al. 2018), that can be correlated to human mg/kg dose range. Therefore, to explore the preventive and therapeutic use of GA-PLGA nanoparticles against chemotherapy-induced intestinal injury, intestinal mucositis was induced in mice by intraperitoneal injection of 5-FU (50 mg/kg), according to the mentioned protocol (Fig. 1) (Ali et al. 2019; Atiq et al. 2019). The features of our experimental animal model were similar to that reported in previous studies (Gelen et al. 2018; Zhang et al. 2017b), including diarrhea, loss in body weight, reduced food intake, histological architecture destruction (reduced villi and crypt lengths), mucin depletion and decreased goblet cell count. In addition to 5-FU IP injection, treatment groups received a once-daily intake of glycyrrhizic acid or GA-PLGA nanoparticles through the oral route. The oral route is preferred in this condition since it provides a natural route to the intestines. Both glycyrrhizic acid and GA-PLGA nanoparticles alleviated the clinical symptoms and reduced the severity of diarrhea, weight loss, anorexia, and distress (Figs. 4 and 5). Aggressive behavior, manifested as distress, was very prominent in the untreated mucositis mice. Similarly, the 5-FU-induced mucositis increased the mortality rate, which was reduced by the glycyrrhizic acid or GA-PLGA nanoparticle treatment (Fig. 4). Although the free drug proved to be effective in alleviating symptoms and increasing survival, however, the GA-PLGA nanoparticles were even more effective, paving a way for PLGA and other polymeric nanocarrier systems to be further explored for the management of intestinal mucositis.

In previous studies, 5-FU significantly affected the weight of the spleen, kidney, and to a lesser extent, the liver (Gelen et al. 2018; Whittaker et al. 2016; Yang et al. 2017). In our experiments, 5-FU reduced spleen weight by 66% and kidney weight by 32% (Fig. 5) but did not significantly alter liver weight. The weight reduction was possibly driven by a 5-FU-induced excessive inflammatory response, or by immunosuppressive action of 5-FU that decreased spleen weight (Whittaker et al. 2016). Further, 5-FU shortened the length of the small intestine and large intestine (colon) (Fig. 5). Glycyrrhizic acid restored colon length \( p < 0.01 \), but had a little effect on small intestine length recovery (Fig. 5), whereas GA-PLGA nanoparticles had significantly restored both small intestine \( p < 0.05 \) and colon length \( p < 0.001 \).

Moreover, intestinal mucosal and epithelial layer destruction by 5-FU was efficiently reversed by glycyrrhizic acid or GA-PLGA nanoparticles. Our findings indicated that 5-FU-induced histomorphological damage, as measured by shortened villi and crypt lengths, disintegrated epithelium, immune cell infiltration, and goblet cell loss was alleviated by glycyrrhizic acid or GA-PLGA nanoparticles. Constantly, GA-PLGA nanoparticles had superior mucoprotective effects compared to free drug alone (Fig. 6). These results suggest that the recovery of intestinal epithelia and alleviation of mucosal damage could be the reason for the reduction in diarrhea that we observed (Zhang et al. 2019). Additionally, the histopathological findings were supported by the goblet cell counts and measurements of mucin content. Consistent with previous data, 5-FU disrupted barrier function through loss of goblet cells and disintegration of goblet cell structure leading to mucin discharge and depletion (Ali et al. 2019; Stringer et al. 2009). Again, the treatments elevated goblet cell count and MUC-2 protein-containing mucin content in the intestine, with a larger improvement in the GA-PLGA nanoparticle group (Fig. 7).

To date, the pathogenesis of 5-FU-induced intestinal mucositis is incompletely understood. However, several of the underlying elements that are responsible for mucositis damage DNA and cellular and sub-cellular structures because of overproduction of ROS, and uncontrolled inflammation that perturbs intestinal homeostasis and alters the natural microbiome (Yan et al. 2020). Excessive ROS production activates inflammatory pathways and the production of pro-inflammatory cytokines (Gelen et al. 2018; Yan et al. 2020). Among many others, some transcription proteins activated by ROS are NF-κB and MAPK that increase the production of pro-inflammatory cytokines, including TNF-α, IL-6, and IL-1β (Chang et al. 2012; Song et al. 2013; Zhang et al. 2017b). Elevation of pro-inflammatory cytokines activates a series of acute reactions to damage cells and tissues, thus causing mucosal injury and mucositis. Interestingly, glycyrrhizic acid has been explored to mediate its action through inhibition of NF-κB and MAPK pathways (Wang and Du 2016), thus reducing pro-inflammatory cytokine production and the resulting inflammation. In this study, glycyrrhizic acid as a free drug or encapsulated within nanoparticles reduced the expression of TNF-α, IL-6, and IL-1β, thus protecting intestinal mucosa from 5-FU-induced damage (Fig. 8).

5-FU-induced inflammation and ROS overproduction weaken the body’s defensive antioxidant system (Yan et al. 2020). Thus, the protective antioxidant enzymes are unable to detoxify excessive ROS, leading to increased oxidative...
stress and cellular damage. In the present study, 5-FU depleted antioxidants including GSH, GST, and catalase (Fig. 8) and caused mucosal damage. Glycyrrhizic acid has been shown to have anti-inflammatory and antioxidant effects (Ming and Yin 2013), and reduces oxidative stress in DSS-induced colitis through increasing antioxidants levels (Zeeshan et al. 2019b). Consistent with previous findings, both free drug and encapsulated drug increased mucosal protection through an increase in GSH and GST concentrations and catalase activity, with GA-PLGA nanoparticles exerting a larger effect (Fig. 8), because of sustained drug release at the targeted intestinal tissues with more localized therapeutic action.

Hence, glycyrrhizic acid proved to be an effective candidate for alleviating intestinal mucositis, with its pharmacokinetics improved by encapsulating it in a PLGA nanocarrier. PLGA nanoparticles successfully delivered the drug to the inflamed intestinal tissues and enhanced therapeutic action by facilitating sustained drug release and accumulation at the target site and minimizing drug clearance due to diarrhea.

Conclusion

We conclude that GA-PLGA nanoparticles with appropriate physicochemical properties and drug release behavior could be a promising approach to protect small and large intestines against 5-FU-induced intestinal mucositis. GA-PLGA nanoparticles effectively reversed 5-FU-induced damage, improved clinical symptoms, behavioral manifestations, histomorphological architecture, reduced distress-related behaviors, and regulated intestinal homeostasis by minimizing oxidative stress and pro-inflammatory cytokines. Together, GA-PLGA nanoparticles could serve as a protective and therapeutic option to alleviate 5-FU induced mucositis and further investigated into clinics for large-scale benefits.

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Data availability Manuscript has no associate data. Raw data will be available on a suitable request.

Declarations

Conflict of interest Authors declared no conflict of interests.

Ethics approval All animal studies were approved and performed according to the bioethical committee protocols of Quaid-i-Azam University, Islamabad for the care and use of laboratory animals (Approval no. BES-fbs-QUA2018-75).

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