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Effect of echinacoside as a palliative for irinotecan-induced intestinal mucositis

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Abstract. Irinotecan, an anticancer drug, causes severe delayed diarrhea due to its active metabolite, SN-38, which damages the intestinal mucosa. This diarrhea may lead to the discontinuation of anticancer therapy in clinical practice. Therefore, in this study, we aimed to elucidate the pathogenesis of irinotecan-induced intestinal inflammation. We examined the effects of echinacoside, which has been reported to reduce LPS (lipopolysaccharide)-induced apoptosis and inflammation. We administered irinotecan (75 mg/kg) to seven-week-old male BALB/c mice intraperitoneally once daily for 4 d; a daily decrease in body weight, and no diarrhea was observed. Necropsies were performed 24 and 72 h after the last dose. Irinotecan caused cellular damage in the small intestine, particularly the ileum. After 72 h, a significant increase in myeloperoxidase activity was observed in the ileum. Concomitant oral administration of echinacoside (500 and 1000 mg/kg) with irinotecan significantly prevented weight loss and cellular damage in the ileal region. These results suggested the role of intestinal bacteria as previously reported with 5-FU-induced enteritis. The increased rate of deconjugation by β-glucuronidase may have increased the direct damage caused by SN-38. Additionally, irinotecan caused less histological damage to the large intestine than to the small intestine, possibly explaining the clinical absence of diarrhea. In conclusion, concomitant administration of echinacoside significantly inhibited the severity of irinotecan-induced intestinal inflammation, indicating their usefulness against irinotecan-induced enteritis.

Keywords: irinotecan, anticancer, intestinal inflammation, echinacosides, diarrhea

Влияние эхинакозида на течение мукозита на фоне противоопухолевой терапии иринотеканом

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Финансирование: Исследование частично было поддержано Программой содействия исследованиям для получения грантов на научные исследования Университета Рицумейкан. Приглашенный исследователь Нахла Хамуда имел финансовую поддержку в рамках полной стипендии [PR-216] Министерства высшего образования Арабской Республики Египет.

Аннотация. Противоопухолевый препарат иринотекан метаболизируется с образованием SN-38. Последний повреждает слизистую оболочку кишечника и может вызывать тяжелую отсроченную диарею, что, как показывает клиническая практика, может стать причиной прекращения противоопухолевой терапии. Исследование ставило целью выяснить патогенез кишечного воспаления, возникающего на фоне терапии иринотеканом. Также было изучено действие эхинакозида, который, согласно опубликованным данным, уменьшает апоптоз и воспаление, вызванное липополисахаридами. Иринотекан (75 мг/кг) вводили семинедельным самцам мышей BALB/c внутрибрюшинно один раз в день в течение четырех дней. При этом наблюдалось ежедневное снижение массы тела и отсутствие диареи. Некропсии проводили через 24 и 72 ч после введения последней дозы. Было обнаружено клеточное повреждение в тонком кишечнике, особенно выраженное в подвздошной кишке. Через 72 ч в подвздошной кишке наблюдалось значительное повышение активности миелопероксидазы. Одновременное с иринотеканом пероральное введение эхинакозида (500 и 1000 мг/кг) значимо предотвращало потерю веса и повреждение клеток в подвздошной кишке. Это указывает на участие кишечных бактерий в развитии воспаления, что коррелирует с ранее описанными случаями энтерита на фоне терапии флюороурацилилом (5-FU). Увеличение скорости деконъюгации бета-глюкуронидазой могло усилить повреждение тканей, вызванное непосредственно SN-38. Кроме того, было обнаружено, что иринотекан вызывает меньшее повреждение тканей толстой, нежели тонкой кишки, что, потенциально, объясняет клиническое отсутствие диареи. В заключение следует отметить, что одновременное применение эхинакозида на фоне терапии иринотеканом значительно подавляет выраженность кишечного воспаления, что говорит о пользе применения эхинокозида при энтеритах, вызванных приемом иринотекана.

Ключевые слова: иринотекан, противоопухолевые препараты, воспаление кишечника, эхинакозид, диарея

Introduction

Chemotherapeutic agents target rapidly dividing cells and thus damage the intestinal epithelial cells, causing gastrointestinal symptoms. Among these adverse effects, diarrhea is particularly problematic in clinical practice (Andreyev et al. 2014). Chemotherapy-induced diarrhea has been reported to occur in 89% of patients treated with the FOLFIRI regimen and 50% of patients treated with the FOLFOX regimen for colorectal cancer (Keefe et al. 2014). If diarrhea occurs, reducing the dose or discontinuing chemotherapy is necessary, minimizing the likelihood of cancer remission. In addition, complications resulting from cancer- or chemotherapy-induced bowel injury increase the

medical financial burden and mortality (Boeing et al. 2020). Despite the prevalence and clinical significance of chemotherapy-induced bowel toxicity, the exact mechanism remains unclear and treatment options for patients are limited (Lalla et al. 2014).

Irinotecan (CPT 11) is a semisynthetic derivative of the natural alkaloid camptothecin used to treat colorectal cancer, and its pharmacological action is attributed to inhibiting DNA synthesis by topoisomerase I inhibition (Dancey, Eisenhauer 1996). Combining anticancer drugs with irinotecan significantly increases patient survival rates but causes intestinal mucositis with severe diarrhea (Boeing et al. 2020). Hepatic carboxylesterases primarily metabolize irinotecan to produce the active metabolite SN-38, which is subsequently metabolized by uridine diphosphate glucuronosyltransferase 1A1 (UGT1A1) to SN-38-glucuronide (SN-38G) (Iyer et al. 1998). SN-38G is secreted into the intestine and then reactivated to SN-38 by β-glucuronidase secreted by intestinal bacteria. Irinotecan-induced diarrhea is thought to occur when SN-38 damages the intestinal mucosal epithelial cells (Ribeiro et al. 2016). No standard treatment exists for this side effect. Therefore, there is an urgent need for preventive and therapeutic methods against irinotecan-induced enteritis.

Echinacoside (ECH) is the major component of phenylethanoid glycosides (PhG). More than 50% of ECH is present in extracts of Chinese juniper (*Cistanche deserticola*) (Cai et al. 2010). Additionally, it is a component of Echinacea, one of the best-selling herbal medicines in the West; it has long been used as an immunostimulant (Bauer 1998; Brevoort 1998). Echinacoside is a natural phenol, possessing various activities, including antioxidant (Zhang et al. 2017), anti-inflammatory (Wang et al. 2015), and antitumor (Dong et al. 2015) effects. Additionally, it has been reported to promote cell proliferation in mouse intestinal epithelial cells, improve cell viability by inhibiting cellular apoptosis (Jia et al. 2012), and inhibit lipopolysaccharide-induced apoptosis and inflammation in rat intestinal epithelial cells (Li et al. 2018), suggesting that it may be effective in treating gastrointestinal diseases. However, the effect of echinacoside on irinotecan-induced enteritis remains unknown.

In this study, we analyzed the pathogenesis of irinotecan-induced enteritis and created a pathological model of the disease in mice. In addition, we investigated the effects of echinacoside on irinotecan-induced enteritis.

Materials and methods

Animals

Six-week-old male BALB/c mice (Japan SLC, Shizuoka, Japan) were housed for one week under a 12-h light/dark cycle and used at seven weeks of age. All experiments were performed using five to six mice per group.

Drugs

Irinotecan hydrochloride (Sun Pharma Co., Ltd., Tokyo, Japan) was diluted in physiological saline. Echinacoside (provided by the Laboratory of Pharmacognosy, Kyoto Pharmaceutical University) was dissolved in sterile water. Irinotecan HCl and echinacoside were prepared immediately before use and administered intraperitoneally (ip) at 75 mg/kg and orally at 10 mL/kg body weight, respectively.

Preparation of irinotecan-induced enteritis

Mice were randomly divided into three experimental groups. The vehicle group received intraperitoneal injection of physiological saline, while the other two groups received intraperitoneally injection of 75 mg/kg irinotecan hydrochloride for 4 d and were necropsied 24 and 72 h after the last dose to remove the small and large intestines. Body weight and fecal changes were measured during the study period. Fecal matter was scored on a 5-point scale: 0, normal stool; 1, soft stool; 2, mild diarrhea; 3, severe diarrhea; and 4, watery diarrhea.

Preparation of irinotecan-induced enteritis treated by echinacoside

Mice were randomly divided into four experimental groups. The vehicle group received intraperitoneal injection of physiological saline once daily for 4 days and oral administration of sterile water once daily for 6 days. The control group received intraperitoneal injection of 75 mg/kg irinotecan hydrochloride once daily for 4 days, and oral administration of sterile water, once daily for 6 days. Two combination groups received intraperitoneal injection of 75 mg/kg irinotecan hydrochloride once daily for 4 days and oral administration of echinacoside at doses of 500 mg/kg and 1,000 mg/kg, respectively, once daily for 6 days. After 6 days, all mice were necropsied to remove the small and large intestines. Body weight and fecal changes were measured during the study period. Fecal matter was scored on a 5-point scale: 0, normal stool; 1, soft stool; 2, mild diarrhea; 3, severe diarrhea; and 4, watery diarrhea.

Hematoxylin and Eosin staining

The mice were euthanized using carbon dioxide or cervical dislocation, and their intestines were removed and washed with saline. The intestine was incised along the opposite side of the mesentery and fixed overnight in 10% formalin. After the intestine was dehydrated with a tissue dehydration solution (Nacalai Tesque Co., Ltd., Kyoto, Japan), xylene (Nacalai Tesque Co., Ltd.) was used as an intermediate agent to replace alcohol in the tissue, and paraffin (Sigma-Aldrich, St. Louis, MO, USA) was permeated into the tissue. Prepared paraffin blocks were thinly sliced at 4 μm thickness using a microtome (LEICA RM2245). Hematoxylin and eosin staining (hematoxylin: Sigma-Aldrich, 0.5% eosin Y ethanol solution: Fujifilm Wako Pure Chemicals Corporation, Osaka, Japan) was performed; the whole intestine was observed under an optical microscope (Olympus CX43) to measure villus length and the number of glandular fossae in the ileal region.

Measurement of meloperoxidase (MPO) activity

Excised small and large intestines were washed with ice-cold saline. Tissues were homogenized in 0.5% HTAB extraction buffer (hexadecyltrimethylammonium bromide, FUJIFILM Wako Pure Chemicals), frozen and thawed three times, and centrifuged at 20 °C, 3,000 rpm for 10 min. Ninety-six-well plate was filled with the supernatant, phosphate buffer (pH 6, 10 mM), and hydrogen peroxide/o-dianisidine reaction solution (0.3% hydrogen peroxide (Santoku Chemical Industry Co., Ltd., Tokyo, Japan). O-dianisidine (Sigma-Aldrich) = 1:200) (20 mM) was added immediately and incubated in a microplate reader (Corona SH-9500Lab) to measure the change in absorbance at a wavelength of 450 nm. The amount of protein in the samples was determined using the Pierce™ BCA Protein Assay Kit (Thermo Scientific, Waltham, MA, USA). MPO levels were calculated using the following formula:

MPO activity (nmol H_2O_2/min/mg protein) = (OD/min)/(OD min H_2O_2 x mg protein)

Immunohistochemical staining

The excised small intestine was washed with ice-cold saline. The intestine was incised along the opposite side of the intestinal mesentery and fixed overnight in 4% paraformaldehyde in phosphate buffer (Nacalai Tesque). The water in the tissue was replaced with 10%, 20%, or 30% sucrose and embedded in an Optimal Cutting Temperature Compound (Sakura Finetek Japan Co., Ltd.). Frozen sections were prepared by thin sectioning at 15 μm thickness using a cryostat (Leica CM1860). Histological localization of apoptotic cells was detected by the TUNEL method (TdT-mediateddUTPnickendlabeling) using the *in situ* Apoptosis Detection Kit (Takara, Shiga, Japan) and observed under a fluorescence microscope.

Statistical analysis

All data are represented as means ± standard error of 4–6 animals per group. Statistical significance was determined using the Student's t-test and was considered significant when the p-value was < 0.05.

Results

Irinotecan-induced enterocolitis model

Body weight and fecal changes

Administration of irinotecan to mice decreased body weight over time, with a significant reduction beginning on day 1. Twenty-four hours after the end of day four of irinotecan administration, the body weight was 93.3 ± 0.9% of the pre-dose level, and at 72 h, the body weight was 83.5 ± 2.1% (Fig. 1A).

No diarrhea was observed during irinotecan administration or 24 h after the last dose. Watery diarrhea was observed in 1 of 6 mice after 48 h of the last dose, with a mean value of 0.7 ± 0.7 at 72 h (Fig. 1B).

Histological study (small intestine)

In the ileum, the length of the villi in the vehicle group was 172.4 ± 10.7 μm. The villus length at 24 h after the last irinotecan administration was 182.9 ± 7.2 μm, showing no significant difference compared to that in the vehicle group. In contrast, villus length at 72 h after the last irinotecan administration was 107.6 ± 15.2 μm, which was significantly shorter than that of the vehicle group and the irinotecan group at 24 h after the last irinotecan administration (Figs. 2A, 2B).

The number of glandular foci in 1 mm of the ileal region in the vehicle region was 13.4 ± 0.6 crypts/mm. The number of crypts per mm 24 h after the last irinotecan administration was 7.8 ± 1.4 crypts/mm, significantly lower than that in the vehicle group. The number of crypts/mm at 72 h after the last dose was 4.2 ± 1.6 crypts/mm, even lower than that at 24 h after the last dose (Fig. 2C).

No significant damage was observed in the duodenum or jejunum in the whole small intestine 72 h after the last irinotecan administration. In contrast, cellular damage was observed in the ileum, including reduction of the glandular foci, vacuolation, and enlargement of the cells (Fig. 2D).

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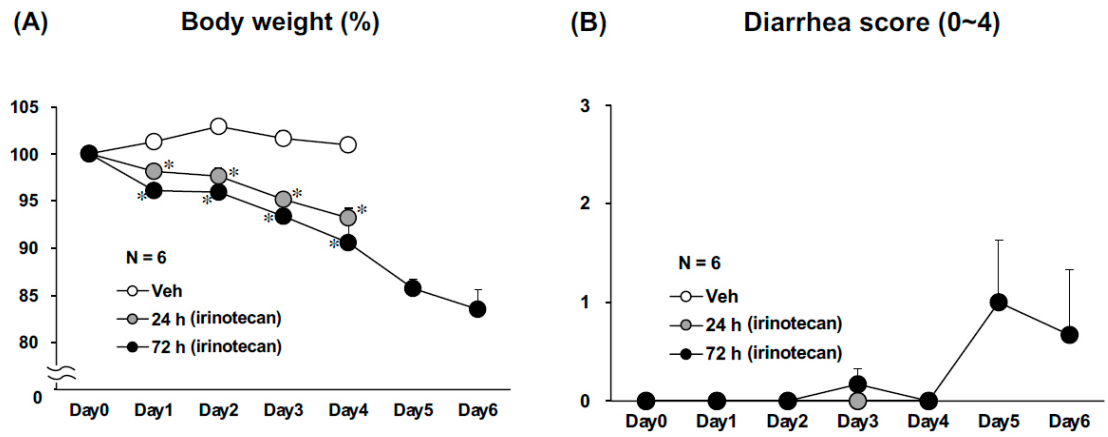

Fig. 1. Changes in body weight and diarrhea score during irinotecan treatment. Irinotecan (75 mg/kg) was injected i. p. once daily for four days (days 0–3). Body weight was measured daily (A), while diarrhea was scored daily using a 5-point scale (0–4) as described in Materials and Methods (B). Data are presented as mean ± SEM: * — $p < 0.05$ from vehicle (not treated with irinotecan, n = 6)

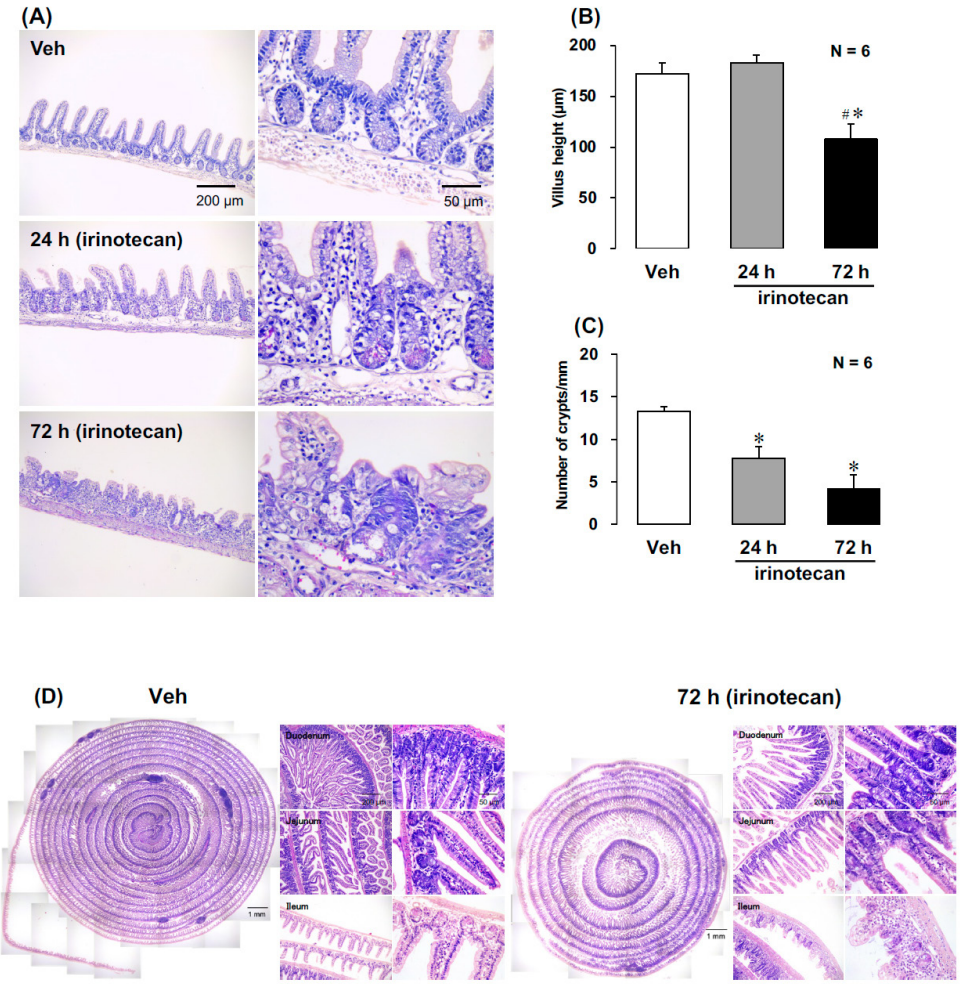

Fig. 2. Histopathological analysis of mice small intestines. Irinotecan (75 mg/kg) was injected i. p. once daily for 4 d (days 0–3). Ileum tissues were collected 24 h (day 4) or 72 h (day 6) after the last dose of irinotecan. Microscopical observations by H&E staining are shown in (A) at 100× (left panels) and 400× (right panels). The height from the top of the villus-crypt junction (B), and the number of crypts per mm (C) were measured under light microscopy. Whole small intestine tissues collected on day 6 (shown in (D)) were stained and imaged at 40× (left panels), 100× (middle panels), and 400× (right panels). Data are represented as mean ± SEM: * — $p < 0.05$ from vehicle (not treated with irinotecan, n = 6)

Histological studies (large intestine)

In the large intestine, no obvious damage was observed in the tissue 72 h after the last irinotecan administration in the upper or lower colon, compared to that in the vehicle group (Figs. 3A, 3B). In contrast, crypt length increased in the lower colon (Fig. 3A).

Changes in MPO activity

MPO activity in the vehicle group in the ileal region was 83.0 ± 7.3 nmol H_2O_2/min/mg protein. The MPO activity at 24 h after the last irinotecan administration was 123.9 ± 16.7 nmol H_2O_2/min/mg protein, significantly higher compared to that in the vehicle group. The MPO activity at 72 h after the last dose was 140.9 ± 24.4 nmol H_2O_2/min/mg protein, which was even higher than that at 24 h after the last dose (Fig. 4A). MPO activity in the vehicle group in the upper colon was 35.3 ± 4.3 nmol H_2O_2/min/mg protein. The MPO activity 24 h after the last irinotecan administration was 33.7 ± 3.7 nmol H_2O_2/min/mg protein, showing no significant difference compared to that in the vehicle group. In contrast, the MPO activity at 72 h after the last dose was 59.1 ± 8.0 nmol H_2O_2/min/mg protein, which was significantly higher compared to that in the vehicle group and the group 24 h after the last dose (Fig. 4B).

MPO activity in the vehicle group in the lower colon was 29.1 ± 1.3 nmol H_2O_2/min/mg protein. The MPO activity 24 h after the last irinotecan administration was 31.9 ± 6.3 nmol H_2O_2/min/mg

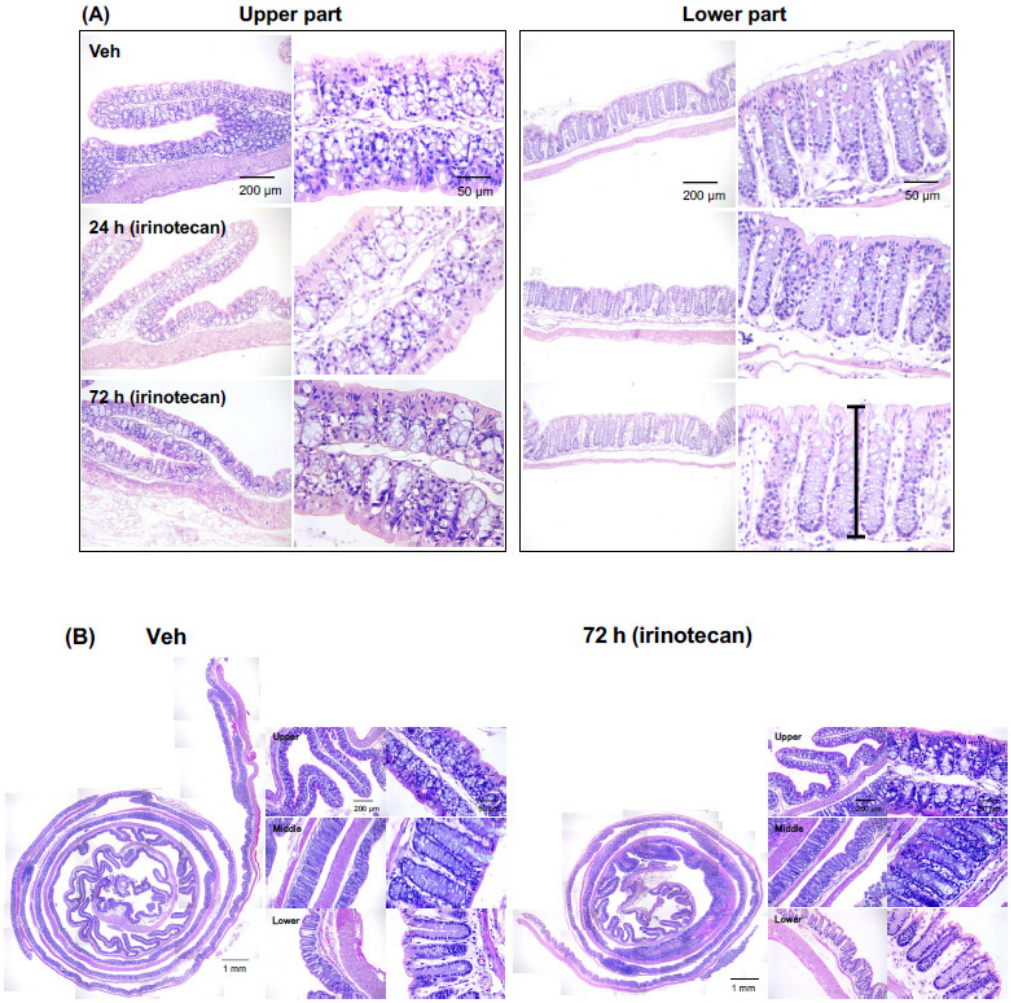

Fig. 3. Histopathological analysis of mice large intestines. Irinotecan (75 mg/kg) was injected i. p. once daily for 4 d (days 0–3). Colon tissues were collected 24 h (day 4) or 72 h (day 6) after the last dose of irinotecan. Microscopical observations by H&E staining are shown in (A) at 100× (left panels) and 400× (right panels). Whole large intestine tissues collected on day 6 (shown in (B)) were stained and imaged at 40× (left panels), 100× (middle panels), and 400× (right panels). Data are represented as mean ± SEM:
* — $p < 0.05$ from vehicle (not treated with irinotecan, n = 6)

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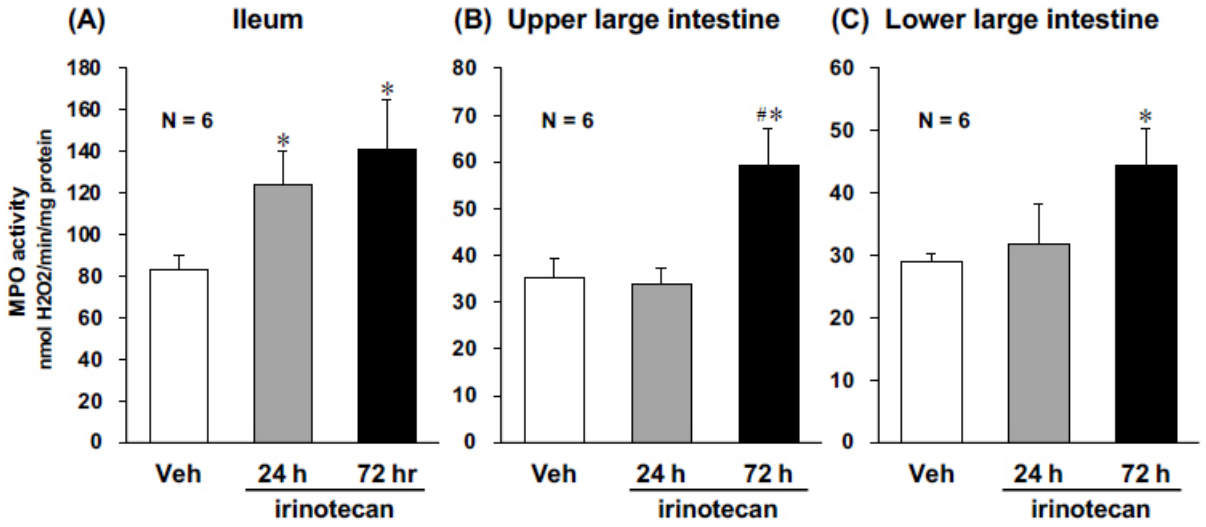

Fig. 4. MPO activity in the mice small and large intestines with or without irinotecan. Irinotecan (75 mg/kg) was injected i. p. once daily for 4 d (days 0–3). Small and large intestinal tissues were collected 24 h (day 4) or 72 h (day 6) after the last dose of irinotecan. MPO activity in the ileum (A), upper large intestine (B), and lower large intestine (C) was determined using o-dianisidine. Data are represented as mean ± SEM: * — $p < 0.05$ from vehicle (not treated with irinotecan, n = 6); #—$p < 0.05$ from animals killed 24 h after the last dose (treated with irinotecan, n = 6)

protein, showing an increasing trend, although there was no statistically significant difference compared to that in the vehicle group. The MPO activity at 72 h after the final administration was 44.6 ± 5.8 nmol H_2O_2/min/mg protein, significantly higher compared to that in the vehicle group (Fig. 4C).

Effect of echinacoside on irinotecan-induced enterocolitis

Body weight and fecal changes

Body weight decreased daily with irinotecan administration in the control group, with a significant decrease compared to that in the vehicle group beginning on day 4 (Fig. 5A). At 72 h after the last irinotecan dose, body weight was 89.4 ± 2.9% of the pre-dose level. The concomitant administration of 500 mg/kg echinacoside prevented irinotecan-induced weight loss. Although significant weight loss was observed 24 h after the last dose of irinotecan compared to that in the vehicle group, a recovery trend was observed after 48 h. At 72 h after the last irinotecan administration, the body weight of mice in the 500 mg/kg echinacoside group was 98.6 ± 1.9% of the pre-dose level. The concomitant administration of 1,000 mg/kg echinacoside resulted in a weight loss similar to that in the control group up to day 4 of treatment, and the weight loss was suppressed 24 h after the last dose of irinotecan. In the echinacoside 1,000 mg/kg combination group, body weight 72 h after the last dose of irinotecan was 93.6 ± 2.8% of the pre-dose level. No delayed diarrhea was observed during this period (Fig. 5B).

Histological study (small intestine)

In the ileum, the length of the villi in the vehicle group was 235.3 ± 10.8 μm. The villus length at 72 h after the last irinotecan administration in the control group was 168.0 ± 11.3 μm, significantly shorter than that in the vehicle group. In the echinacoside 500 mg/kg and 1,000 mg/kg combination groups, the shortening of villus length was significantly suppressed, with villus lengths of 250.8 ± 22.0 and 225 ± 8.1 μm, respectively (Fig. 6B).

The number of glandular foci in 1 mm in the vehicle group was 14.3 ± 1.2 crypts/mm. In the control group, the number of glands was 10.0 ± 0.9 crypts/mm at 72 h after the last dose of irinotecan, which is significantly lower than that in the vehicle group. The combined echinacoside 500 mg/kg and 1,000 mg/kg groups showed a tendency to suppress the irinotecan-induced decrease in glandular foci counts, which were 13.4 ± 1.3 and 11.0 ± 0.7 crypts/mm, respectively (Fig. 6C).

Cytotoxic effects, such as cell vacuolation and hypertrophy, were observed more frequently in the control group and less frequently in the echinacoside combination group (Fig. 6A).

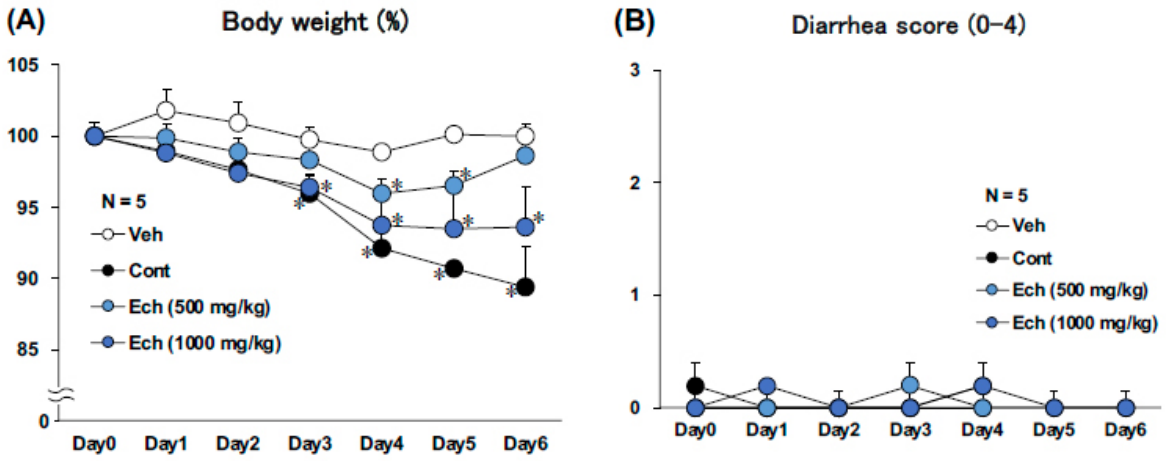

Fig. 5. Effect of echinacoside on changes in body weight and diarrhea during irinotecan treatment. Irinotecan (75 mg/kg) was injected i. p. once daily for 4 d (days 0–3). Echinacoside at 500 and 1,000 mg/kg (n = 5) was co-administered once daily for 6 d (days 0–5). Body weight was measured daily (A), while diarrhea was scored daily using a 5-point scale (0–4) as described in Materials and Methods (B). Data are represented as mean ± SEM: * — $p < 0.05$ from vehicle (not treated with irinotecan, n = 5)

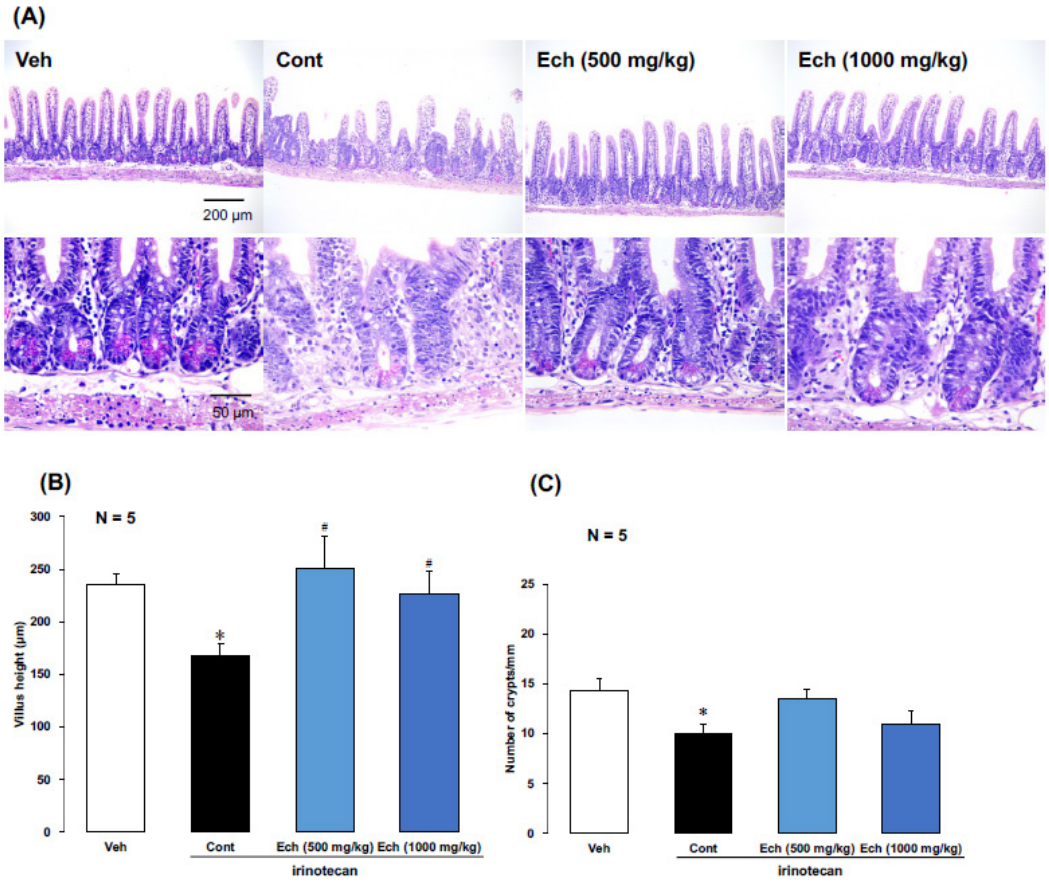

Fig. 6. Effect of echinacoside on histopathological changes in mice small intestines. Irinotecan (75 mg/kg) was injected i. p. once daily for 4 d (days 0–3). Echinacoside at 500 and 1,000 mg/kg (n = 5) was co-administered once daily for 6 d (days 0–5). Microscopical observations by H&E staining are shown in (A) at 100× (upper panels) and 400× (lower panels). The height from the top of the villus-crypt junction (B), and the number of crypts per mm (C) were measured under light microscopy. Data are represented as mean ± SEM: * — $p < 0.05$ from vehicle (not treated with irinotecan, n = 5); # — $p < 0.05$ versus animals killed 24 h after the last dose (treated with irinotecan n = 5)

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Immunohistochemistry (TUNEL-method)

In the ileal region, the number of TUNEL-positive cells in the vehicle group was 1.3 ± 0.1 counts/0.01 mm^2. In the control group, the number of TUNEL-positive cells at 72 h after the last irinotecan administration was significantly higher than that in the vehicle group, 3.6 ± 0.5 counts/0.01 mm^2. The number of TUNEL-positive cells in the echinacoside 500 mg/kg combination group was significantly suppressed compared to that in the control group at 1.5 ± 0.1 counts/0.01 mm^2 at 72 h after the last dose of irinotecan. The number of apoptotic cells in the echinacoside 1,000 mg/kg group was 1.7 ± 0.1 counts/0.01 mm^2 at 72 h after the last dose of irinotecan, which was significantly lower than that in the echinacoside 500 mg/kg group (Fig. 7).

Discussion

The administration of irinotecan to mice has been reported to induce significant weight loss and diarrhea (Lian et al. 2017). In this study, irinotecan administration in mice resulted in a daily decrease in body weight. In contrast, delayed diarrhea was observed in approximately 20% of mice after the last 48 h of irinotecan administration. Delayed diarrhea induced by irinotecan is supposed to be caused by SN-38-induced intestinal mucositis.

In this study, MPO activity in the ileal region significantly increased 24 h after the last administration of irinotecan and further increased at 72 h. The result suggests that irinotecan-induced small-intestine inflammation is exacerbated over time after the completion of irinotecan administration. In the large intestine, neither upper nor lower MPO activity increased significantly at 24 h after the last irinotecan administration, but increased significantly at 72 h. This suggests that in irinotecan-induced intestinal mucosal damage, inflammation is delayed in both the small and large intestines. The small intestine undergoes inflammation at an earlier stage than the large intestine.

In the ileal portion of the small intestine, no shortening of the villus length was observed

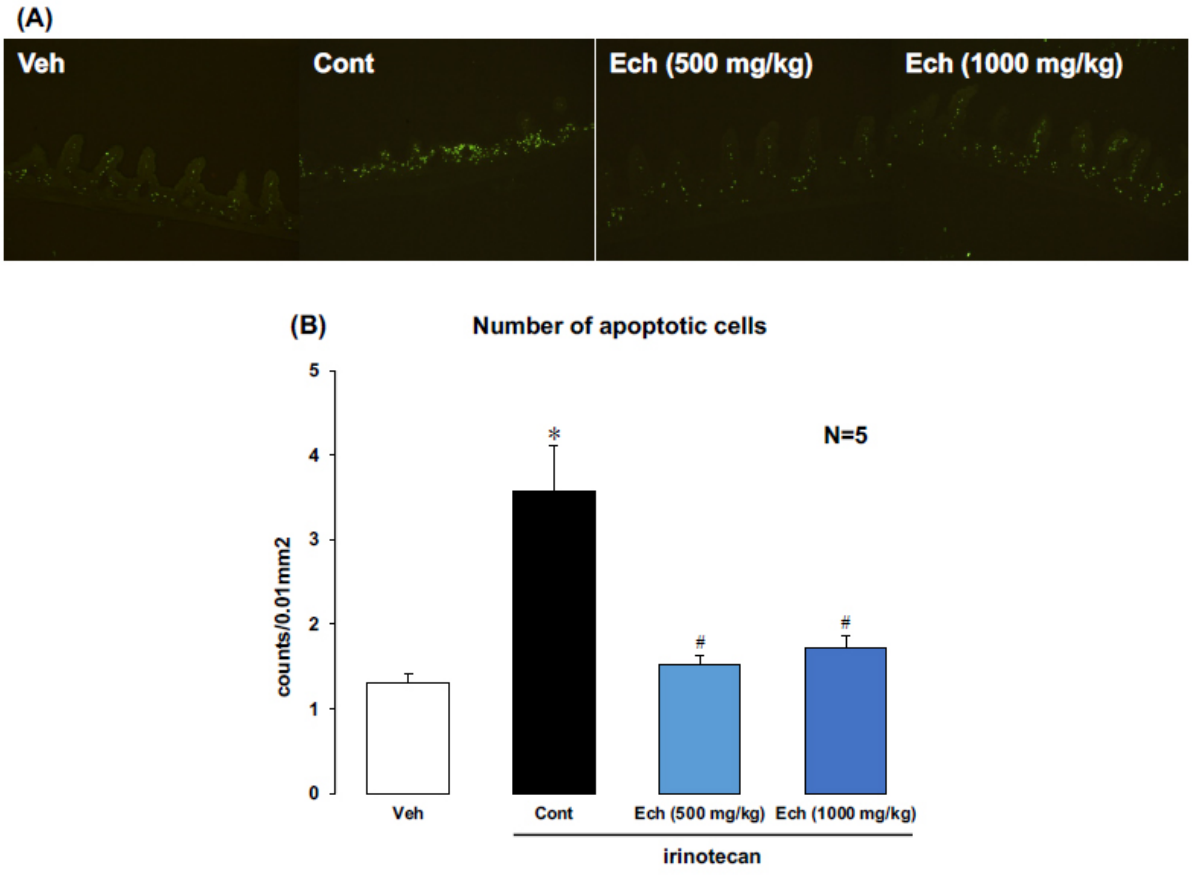

Fig. 7. Effect of echinacoside on mice small intestine tunnel-positive cells. Irinotecan (75 mg/kg) was injected i. p. once daily for 4 d (days 0–3). Echinacoside at 500 and 1,000 mg/kg (n = 5) was co-administered once daily for 6 d (days 0–5). Apoptosis in intestinal crypts was assessed at 72 h (day 6) after the last dose of irinotecan using the TUNEL assay (A, 100×) and quantified under a light microscope (B). Data are represented as mean ± SEM: * — $p < 0.05$ from vehicle (not treated with irinotecan, n = 5); # — $p < 0.05$ from animals killed 24 h after the last dose (treated with irinotecan, n = 5)

24 h after the last administration of irinotecan; however, at 72 h after the last administration, the villus length significantly shortened. In addition, the number of glandular foci significantly reduced 24 h after the last irinotecan administration compared with that in the vehicle group, and the number of glandular foci further reduced 72 h after the last irinotecan administration. These findings suggest that both inflammation and cellular damage occur in intestinal mucosal structures in a delayed manner. This study indicates that intestinal mucosal damage worsens slowly from the end of irinotecan administration and that enteritis occurs more slowly in the large intestine than in the small intestine, suggesting that a longer rest period after the end of irinotecan administration may bring us closer to the pathophysiology of diarrhea.

The lower part of the small intestine is more severely affected than the entire small intestine. This is because the lower part of the small intestine is more susceptible to the effects of intestinal bacteria that are abundant in the cecum and large intestine. Anticancer treatment with 5-fluorouracil shows that enteritis mainly occurs in the ileum, which contains more intestinal bacteria (Andrade et al. 2023; Jonan et al. 2022). In this study, the damage was more pronounced in the ileal region because of the same effect of the intestinal bacteria. Antibiotic administration markedly improves irinotecan-induced diarrhea (Takasuna et al. 1996). Reactivation of SN-38G to SN-38 by β-glucuronidase-positive Enterobacteriaceae (e. g., *Escherichia coli, Staphylococcus* spp. and *Clostridium* spp.) is an important factor in causing intestinal mucositis (Roberts et al. 2013; Sezer et al. 2009; Stringer et al. 2008; Takasuna et al. 1996). This suggests that SN-38G is particularly susceptible to conversion into SN-38 in the lower part of the small intestine, where there is a high concentration of intestinal bacteria, leading to increased cellular damage of the intestinal mucosa.

The cytotoxicity was less severe in the large intestine than in the small intestine. One possible reason may be that the sensitivity to SN-38 is weaker in the colon than in the small intestine. Bcl-2, an anti-apoptotic protein, is strongly expressed in the crypts of the large intestine, whereas it is weakly expressed in the crypts of the small intestine. Bax, an apoptotic protein, is strongly expressed in the crypts of the small intestine, while it is scarcely expressed in the crypts of the colon (Bowen et al. 2006). In addition, pharmacological studies have shown differences in sensitivity to apoptotic stimuli in the small and large intestines, with apoptosis observed more frequently in the small intestine than in the large intestine (Gauthier et al. 2001a; 2001b). The greater sensitivity of crypt cells in the small intestine to apoptosis than that of crypt cells in the colon suggests that the same may be true for irinotecan and SN-38.

SN-38 is reabsorbed in the small intestine by enterohepatic circulation, resulting in less SN-38 exposure in the colon than in the small intestine. Irinotecan and SN-38 have lactone and carboxylate forms, and their interconversion is reversible and driven by pH (Fassberg, Stella 1992), leaning toward the lactone form at low pH and the carboxylate form at high pH. The lactone form is taken up significantly faster than the carboxylate form in cells of the intestinal tract, with the uptake rate increasing with decreasing pH, as does the cytotoxicity (Kobayashi et al. 1999). The effect of the physiological pH of the intestinal lumen on the initial uptake rate of CPT-11 and SN-38 (6.2–8.0) is decreased by approximately 65% at pH 6.8 or higher. The fact that the pH of the small intestine is approximately 6.8 and that of the large intestine is approximately 7.3 (Ringel-Kulka et al. 2015) suggests that the higher pH in the large intestine compared to the small intestine may have weakened the toxicity and reduced the degree of damage.

Concomitant administration of 500 mg/kg echinacoside suppressed irinotecan-induced weight loss. Although significant weight loss was observed 24 h after the last dose of irinotecan compared to that in the vehicle group, a recovery trend was observed after 48 h. In the combination treatment with 1,000 mg/kg echinacoside, body weight loss was similar to that in the control group up to the fourth day of treatment and was suppressed 24 h after the last dose of irinotecan. Thus, echinacoside suppresses weight loss, a side effect of irinotecan, and accelerates recovery.

Irinotecan-induced intestinal injury is characterized by increased apoptosis of the crypts and the loss of villi (Bastos et al. 2016). In this study, irinotecan administration to mice resulted in a significant shortening of the villus length and a significant reduction of the glandular fossae in the ileal region. The combination treatment with 500 mg/kg and 1,000 mg/kg echinacoside significantly suppressed villus shortening. However, it did not significantly reduce the number of glandular foci compared to that in the control group. Cell damage such as vacuolation and hypertrophy, frequently observed in the control group, were less frequent in the echinacoside combination groups. In addition, combination treatment with 500 and 1,000 mg/kg echinacoside significantly suppressed the number of apoptotic cells, which was significantly higher in the control group. These findings suggest that echinacoside may be effective against irinotecan-induced enteritis.

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In addition, the body weight, chorionic villus length, number of glandular foci, and number of apoptotic cells showed fewer pathological changes induced by irinotecan at 500 mg/kg than at 1,000 mg/kg of echinacoside, indicating that an appropriately selected dosage may be effective in preventing the adverse effects of irinotecan on the gastrointestinal tract. This indicates that echinacoside is useful in preventing the adverse gastrointestinal effects of irinotecan at appropriate doses.

Conflict of Interest

The authors declare no conflict of interest, either existing or potential.

Ethics Approval

All procedures involving animals were approved by the Committees for Animal Research of the Ritsumeikan University Institutional Animal Care and Use Committee.

Author Contributions

a. Hikaru Otsuki — conducted experiments, contributed to the interpretation of the results, and drafted the original manuscript;
b. Shizuka Jonan — conducted experiments, contributed to the interpretation of the results, drafted the original manuscript, reviewed the manuscript draft, and critically revised its intellectual content;
c. Taisei Tsujii — conducted experiments;
d. Nahla Hamouda — conceived the study's idea, reviewed the manuscript draft, and critically revised its intellectual content;
e. Kikuko Amagase — conceived the study's idea, supervised its conduct, reviewed the manuscript draft, and critically revised its intellectual content.

References

Andrade, M. E. R., Trindade, L. M., Leocádio, P. C. L. et al. (2023) Association of fructo-oligosaccharides and arginine improves severity of mucositis and modulate the intestinal microbiota. *Probiotics and Antimicrobial Proteins*, vol. 15, no. 2, pp. 424–440. https://www.doi.org/10.1007/s12602-022-10032-8 (In English)

Andreyev, J., Ross, P., Donnellan, C. et al. (2014) Guidance on the management of diarrhoea during cancer chemotherapy. *Lancet Oncology*, vol. 15, no. 10, pp. e447–e460. https://www.doi.org/10.1016/S1470-2045(14)70006-3 (In English)

Bastos, R. W., Pedroso, S. H., Vieira, A. T. et al. (2016) *Saccharomyces cerevisiae* UFMG A-905 treatment reduces intestinal damage in a murine model of irinotecan-induced mucositis. *Beneficial Microbes*, vol. 7, no. 4, pp. 549–557. https://www.doi.org/10.3920/BM2015.0190 (In English)

Bauer, R. (1998) Echinacea: Biological effects and active principles. In: L. D. Lawson, R. Bauer (eds.). *Phytomedicines of Europe: Chemistry and Biological Activity*. Washington: American Chemical Society Publ., pp. 140–157. (In English)

Boeing, T., de Souza, P., Speca, S. et al. (2020) Luteolin prevents irinotecan-induced intestinal mucositis in mice through antioxidant and anti-inflammatory properties. *British Journal of Pharmacology*, vol. 177, no. 10, pp. 2393–2408. https://www.doi.org/10.1111/bph.14987 (In English)

Bowen, J. M., Gibson, R. J., Cummins, A. G. et al. (2006) Intestinal mucositis: The role of the Bcl-2 family, p53 and caspases in chemotherapy-induced damage. *Support Care in Cancer*, vol. 14, no. 7, pp. 713–731. https://www.doi.org/10.1007/s00520-005-0004-7 (In English)

Brevoort, P. (1998) The booming U.S. botanical market: A new overview. *HerbalGram*, vol. 44, pp. 33–46. (In English)

Cai, R.-L., Yang, M.-H., Shi, Y. et al. (2010) Antifatigue activity of phenylethanoid-rich extract from *Cistanche deserticola*. *Phytotherapy Research*, vol. 24, no. 2, pp. 313–315. https://www.doi.org/10.1002/ptr.2927 (In English)

Dancey, J., Eisenhauer, E. A. (1996) Current perspectives on camptothecins in cancer treatment. *British Journal of Cancer*, vol. 74, no. 3, pp. 327–338. https://www.doi.org/10.1038/bjc.1996.362 (In English)

Dong, L., Wang, H., Niu, J. et al. (2015) Echinacoside induces apoptotic cancer cell death by inhibiting the nucleotide pool sanitizing enzyme MTH1. *OncoTargets and Therapy*, vol. 8, pp. 3649–3664. https://www.doi.org/10.2147/OTT.S94513 (In English)

Fassberg, J., Stella, V. J. (1992) A kinetic and mechanistic study of the hydrolysis of camptothecin and some analogues. *Journal of Pharmaceutical Sciences*, vol. 81, no. 7, pp. 676–684. https://www.doi.org/10.1002/jps.2600810718 (In English)

Gauthier, R., Harnois, C., Drolet, J. F. et al. (2001a) Human intestinal epithelial cell survival: Differentiation state-specific control mechanisms. *American Journal of Physiology. Cell Physiology*, vol. 280, no. 6, pp. C1540–C1554. PMID: 11350749 (In English)

Gauthier, R., Laprise, P., Cardin, É. et al. (2001b) Differential sensitivity to apoptosis between the human small and large intestinal mucosae: Linkage with segment-specific regulation of Bcl-2 homologs and involvement of signaling pathways. *Journal of Cellular Biochemistry*, vol. 82, no. 2, pp. 339–355. https://www.doi.org/10.1002/jcb.1172 (In English)

Iyer, L., King, C. D., Whitington, P. F. et al. (1998) Genetic predisposition to the metabolism of irinotecan (CPT-11). Role of uridine diphosphate glucuronosyltransferase isoform 1A1 in the glucuronidation of its active metabolite (SN-38) in human liver microsomes. *Journal of Clinical Investigation*, vol. 101, no. 4, pp. 847–854. https://www.doi.org/10.1172/JCI915 (In English)

Jia, Y., Guan, Q., Guo, Y., Du, C. (2012) Echinacoside stimulates cell proliferation and prevents cell apoptosis in intestinal epithelial MODE-K cells by up-regulation of transforming growth factor-β1 expression. *Journal of Pharmacological Sciences*, vol. 118, no. 1, pp. 99–108. https://www.doi.org/10.1254/jphs.11186FP (In English)

Jonan, S., Hamouda, N., Fujiwara, A. et al. (2022) Alleviative effects of glutamate against chemotherapeutic agent-induced intestinal mucositis. *Journal of Physiology and Pharmacology*, vol. 73, no. 4, pp. 539–546. https://www.doi.org/10.26402/jpp.2022.4.07 (In English)

Keefe, D. M., Elting, L. S., Nguyen, H. T. et al. (2014) Risk and outcomes of chemotherapy-induced diarrhea (CID) among patients with colorectal cancer receiving multi-cycle chemotherapy. *Cancer Chemotherapy and Pharmacology*, vol. 74, no. 4, pp. 675–680. https://www.doi.org/10.1007/s00280-014-2526-5 (In English)

Kobayashi, K., Bouscarel, B., Matsuzaki, Y. et al. (1999) pH-dependent uptake of irinotecan and its active metabolite, SN-38, by intestinal cells. *International Journal of Cancer*, vol. 83, no. 4, pp. 491–496. https://doi.org/10.1002/(sici)1097-0215(19991112)83:4%3C491::aid-ijc10%3E3.0.co;2-m (In English)

Lalla, R. V., Bowen, J., Barasch, A. et al. (2014) MASCC/ISOO clinical practice guidelines for the management of mucositis secondary to cancer therapy. *Cancer*, vol. 120, no. 10, pp. 1453–1461. https://doi.org/10.1002/cncr.28592 (In English)

Li, L., Wan, G., Han, B. et al. (2018) Echinacoside alleviated LPS-induced cell apoptosis and inflammation in rat intestine epithelial cells by inhibiting the mTOR/STAT3 pathway. *Biomedicine & Pharmacotherapy*, vol. 104, pp. 622–628. https://doi.org/10.1016/j.biopha.2018.05.072 (In English)

Lian, Q., Xu, J., Yan, S. et al. (2017) Chemotherapy-induced intestinal inflammatory responses are mediated by exosome secretion of double-strand DNA via AIM2 inflammasome activation. *Cell Research*, vol. 27, no. 6, pp. 784–800. https://doi.org/10.1038/cr.2017.54 (In English)

Ribeiro, R. A., Wanderley, C. W. S, Wong, D. V. T. et al. (2016) Irinotecan- and 5-fluorouracil-induced intestinal mucositis: Insights into pathogenesis and therapeutic perspectives. *Cancer Chemotherapy and Pharmacology*, vol. 78, no. 5, pp. 881–893. https://doi.org/10.1007/s00280-016-3139-y (In English)

Ringel-Kulka, T., Choi, C. H., Temas, D. et al. (2015) Altered colonic bacterial fermentation as a potential pathophysiological factor in irritable bowel syndrome. *American Journal of Gastroenterology*, vol. 110, no. 9, pp. 1339–1346. https://doi.org/10.1038/ajg.2015.220 (In English)

Roberts, A. B., Wallace, B. D., Venkatesh, M. K. et al. (2013) Molecular insights into microbial β-glucuronidase inhibition to abrogate CPT-11 toxicity. *Molecular Pharmacology*, vol. 84, no. 2, pp. 208–217. https://doi.org/10.1124/mol.113.085852 (In English)

Sezer, A., Usta, U., Cicin, I. (2009) The effect of *Saccharomyces boulardii* on reducing irinotecan-induced intestinal mucositis and diarrhea. *Medical Oncology*, vol. 26, no. 3, pp. 350–357. https://doi.org/10.1007/s12032-008-9128-1 (In English)

Stringer, A. M., Gibson, R. J., Logan, R. M. et al. (2008) Faecal microflora and beta-glucuronidase expression are altered in an irinotecan-induced diarrhea model in rats. *Cancer Biology & Therapy*, vol. 7, no. 12, pp. 1919–1925. https://doi.org/10.4161/cbt.7.12.6940 (In English)

Takasuna, K., Hagiwara, T., Hirohashi, M. et al. (1996) Involvement of beta-glucuronidase in intestinal microflora in the intestinal toxicity of the antitumor camptothecin derivative irinotecan hydrochloride (CPT-11) in rats. *Cancer Research*, vol. 56, no. 16, pp. 3752–3757. PMID: 8706020 (In English)

Wang, Y.-H., Xuan, Z.-H., Tian, S. et al. (2015) Echinacoside protects against 6-hydroxydopamine-induced mitochondrial dysfunction and inflammatory responses in PC12 cells via reducing ROS production. *Evidence-Based Complementary and Alternative Medicine*, vol. 2015, article 189239. https://doi.org/10.1155/2015/189239 (In English)

Zhang, D., Lu, C., Yu, Z. et al. (2017) Echinacoside alleviates UVB irradiation-mediated skin damage via inhibition of oxidative stress, DNA damage, and apoptosis. *Oxidative Medicine and Cellular Longevity*, vol. 2017, article 6851464. https://doi.org/10.1155/2017/6851464 (In English)