Ameliorative effect of Silk Fibroin against 5-Fluorouracil (5-FU)-induced gastrointestinal damage in rats

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Abstract:

Objective: 5-Fluorouracil (5-FU), a widely administered anti-cancer drug, causes gastrointestinal damage through various mechanisms. This study aimed to investigate the ameliorative effect of silk fibroin (SF), which has anti-inflammatory and antioxidant properties, against 5 FU-induced gastrointestinal damage. Materials and methods: Wistar albino rats were divided into 3 groups; Control group, 5- FU group (30mg/kg/day on days 0, 2, 4), and 5-FU+SF (30 mg/kg/day 5-FU on days 0, 2, 4 + 600 mg/kg/day SF for 14 days). At the end of the experiment, stomach and intestinal tissue samples were collected and immunohistochemically iNOS, caspase-3, CD4+ and CD8+ were measured. Besides, structural damage was assessed with hematoxylin-eosin and caspase-3 and 9 activities were evaluated using western blot analysis. Results and Discussion: It was determined that iNOS, caspase-3, caspase-9 activities, CD4+ and CD8+ levels and structural damage significantly increased in both stomach and intestinal tissues. Furthermore, elevated markers were regressed almost to control levels in SF administrated group. Conclusion: SF may have an ameliorative effect, thus reducing the gastrointestinal damage caused by 5 FU. More clinical and scientific research should be conducted as the silk fibroin may be a suitable candidate against the adverse effects of chemotherapeutic agents.

Keywords: Silk Fibroin; 5-Fluorouracil; iNOS; CD4+; CD8+

Introduction:

Cancer is one of the most important causes of death worldwide 1. Besides, the adverse effects of chemotherapeutic drugs used in cancer treatment contribute significantly to quality of patients life and survival 2. Gastrointestinal mucositis is among the most important adverse effects of drugs used in cancer treatment 3. Adverse effects are frequently reported in the treatment of solid tumours with 5-fluorouracil (5-FU) 4,5. Although various agents have been used to eliminate mucositis, no radical solution has yet been found. Studies have shown that administration of 5-FU causes mucositis by inducing histopathological changes in the gastrointestinal region, with alterations in T-cell functions such as CD4+ and CD8+ and an increase in iNOS and caspase-3 activation 3,6.

Silk fibroin (SF) is obtained as a biomaterial from the silkworm. SF is used in regenerative medicine
for tissue repair and reconstruction. There is no evidence of cytotoxic properties and used in many different drug development systems. SF also has anti-inflammatory and antioxidant qualities that have been demonstrated in studies. Moreover, SF exhibits protective effects by preventing apoptosis and tissue damage its anti-apoptotic and anti-inflammatory effects.

The aim of the presented study was to investigate the role of CD4+, CD8+ cells, caspase-3, caspase-9 and iNOS activation of 5-FU, which is used in the treatment of various cancers but has a limiting treatment function due to mucositis, on gastrointestinal tissue damage and to determine the ameliorative effect of SF against gastrointestinal mucositis.

**Materials and methods:**

**Animals and ethics**

The Near East University Local Animal Ethics Committee approved the experimental procedures (No: 2018/21). In our study, 200-250 g Wistar albino rats of both sexes were used. The animals were acclimatised to laboratory conditions (20 °C ± 2, 12 h light/12 h dark) for two weeks before the experiment. Animals had access to feed and water ad libitum.

The rats were allocated into three groups, eight in each: Control, 5-FU and 5-FU+SF groups. Control group animals were not treated and only intra peritoneal (i.p.) saline was administered from day 1 once daily for 1 week. Animals in the 5-FU group were treated 3 times with 5-FU intramuscularly at a dose of 30 mg/kg/day on days 0, 2, 4 after the first day of saline (i.p.) administration. In the 5-FU+SF group, after the animals in this group were administered 600 mg/kg SF intraperitoneally on day 1, 5-Fluorouracil was administered 3 times intramuscularly at a dose of 30 mg/kg/day to rats on days 0, 2, 4. Animals were kept under observation for 14 days after the last dose. Besides, SF treatment at a dose of 600 mg/kg intraperitoneally and saline administration at a dose of once daily each were continued until the completion of the experiment. Animals were euthanized 14 days after the last dose, and stomach and intestinal tissue samples were collected.

**Method of histopathological and immunohistochemical analysis**

All samples were fixed in 10% formalin for 10 h at 24 ± 2 °C. After the processed tissues were embedded in paraffin, sections (4 µm) were taken on positively charged slides. Immunostaining was performed with an automated immunostainer (Ventana Medical Systems, Inc., Tucson, AZ, USA) using the CD4+ (Clone: SP35, dilution: 1:200, Ventana Medical Systems, Inc., Tucson, AZ, USA), CD8+ (Clone: SP57, dilution: 1:150, Ventana Medical Systems, Inc., Tucson, AZ, USA), Caspase-3 (Clone: Polyclonal, dilution: 1/100, ab4051, anti-caspase3 antibody, Abcam), iNOS (Clone: Polyclonal, dilution: 1/100, ab15323, anti-iNOS antibody, Abcam). Besides, paraffin sections of 5 µm thick were also stained using haematoxylin and eosin (H&E) and evaluated histopathologically. We used a 0–4 scoring system for immunohistochemical evaluation of structural damage with caspase-3, iNOS, CD4+ and CD8+ (0: None, 1: Rare, mild, 2: Moderate, 3: Frequent, 4: Abundant).

**Western blotting**

Dissected stomach and intestinal tissues were homogenized, centrifuged at 2000 x g for 15 min., and then treated for 60 min. with protease inhibitors. The protein concentration of each tissue was determined using Folin-Ciocalteu reagent. Samples prepared with 100 µg protein from each tissue were loaded on gel electrophoresis and then transferred to nitrocellulose membranes at 80 V for 75 min. All membranes were incubated with secondary antibodies for 1 h, after 14 h incubation with primary antibodies [β-actin (sc-130657), caspase-3 (casp-3; sc-56053), and caspase-9 (casp-9; sc-56076) (1:200 all antibody dilution)] at +4 ° C.

**Statistical Analysis**

Prism 9 was used to conduct the statistical analysis (Graphpad Software, CA, USA). Group comparison of iNOS, caspase-3 and caspase-9 activities, and CD4+ and CD8+ measured immunohistochemically in the stomach and intestinal tissues were examined using one-way analysis of variance (ANOVA), and Tukey’s test was used for pairwise comparisons. Cases with a P value less than 0.05 were considered significant.

**Ethical Clearance:**

The study has been approved by the Local Animal Ethics Committee of Near East University (Approval Number: 2018/21).

**Results:**

In the sections stained with H&E, inflammatory cells and apoptotic cells were not observed in the majority of the control group samples. Apoptotic body and inflammatory cell infiltration were seen in both
stomach and intestine samples that were administered 5-FU. In the immunohistochemical examination of caspase-3, iNOS, CD4+ and CD8+ (Figure 1-4, Table 1) were strongly stained in the stomach and intestinal tissues of the groups treated with 5-FU compared with the control group, while slightly stained in the SF-treated group (SF).

**Table 1.** Caspase-3, iNOS, CD4+ and CD8+ immunohistochemical scoring obtained from the stomach and intestine tissues of rats (n=8). Results are given as Mean ± SD.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control Group</th>
<th>5-FU Group</th>
<th>5-FU+SF Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caspase-3 Stomach</td>
<td>0.43 ± 0.20</td>
<td>2.50 ± 0.19***</td>
<td>1.43 ± 0.21**</td>
</tr>
<tr>
<td>Caspase-3 Intestine</td>
<td>0.25 ± 0.19</td>
<td>2.25 ± 0.16****</td>
<td>0.75 ± 0.16****</td>
</tr>
<tr>
<td>iNOS Stomach</td>
<td>0.50 ± 0.19</td>
<td>2.63 ± 0.18****</td>
<td>1.43 ± 0.20**,***</td>
</tr>
<tr>
<td>iNOS Intestine</td>
<td>0.29 ± 0.18</td>
<td>1.75 ± 0.16****</td>
<td>1 ± 0.22 +</td>
</tr>
<tr>
<td>CD4+ Stomach</td>
<td>0.56 ± 0.17</td>
<td>2.56 ± 0.18****</td>
<td>1.11 ± 0.26***</td>
</tr>
<tr>
<td>CD4+ Intestine</td>
<td>0.57 ± 0.20</td>
<td>2.37 ± 0.18****</td>
<td>1.29 ± 0.18**</td>
</tr>
<tr>
<td>CD8+ Stomach</td>
<td>0.40 ± 0.16</td>
<td>1.78 ± 0.22***</td>
<td>0.78 ± 0.22**</td>
</tr>
<tr>
<td>CD8+ Intestine</td>
<td>0.45± 0.15</td>
<td>1.91 ± 0.21***</td>
<td>0.82 ± 0.23***</td>
</tr>
</tbody>
</table>

** p<0.01, *** p<0.001, **** p<0.0001 compared to control group.
+ p<0.05, ++ p<0.01, +++ p<0.001, ++++ p<0.0001 compared to 5-FU group.

**Figure 1.** Immunohistochemical staining images obtained from the stomach and intestine tissues of rats treated with saline: (1A) H&E control group stomach, (1B) CD4+ control group stomach, (1C) H&E control group intestine, (1D) CD4+ control group intestine, (1E) H&E 5 FU group stomach, (1F) CD4+ 5 FU group stomach, (1G) H&E 5 FU group intestine, (1H) CD4+ 5 FU group intestine, (1I) H&E 5 FU+Silk group stomach, (1J) CD4+ 5 FU+Silk group stomach, (1K) H&E 5 FU+Silk group intestine, (1L) CD4+ 5 FU+Silk group intestine. All images were taken in X300 magnification.
**Figure 2.** Immunohistochemical staining images obtained from the stomach and intestine tissues of rats with 5-FU induced gastrointestinal mucositis: (2A) H&E control group stomach, (2B) CD8+ control group stomach, (2C) H&E control group intestine, (2D) CD8+ control group intestine, (2E) H&E 5 FU group stomach, (2F) CD8+ 5 FU group stomach, (2G) H&E 5 FU group intestine, (2H) CD8+ 5 FU group intestine, (2I) H&E 5 FU+Silk group stomach, (2J) CD8+ 5 FU+Silk group stomach, (2K) H&E 5 FU+Silk group intestine, (2L) CD8+ 5 FU+Silk group intestine. All images were taken in X300 magnification.

**Figure 3:** Immunohistochemical staining images obtained from the stomach and intestinal tissues of rats with 5-FU-induced gastrointestinal mucositis treated with silk fibroin: (3A) H&E control group stomach, (3B) INOS control group stomach, (3C) H&E control group intestine, (3D) INOS control group intestine, (3E) H&E 5 FU group stomach, (3F) INOS 5 FU group stomach, (3G) H&E 5 FU group intestine, (3H) INOS 5 FU group intestine, (3I) H&E 5 FU+Silk group stomach, (3J) INOS 5 FU+Silk group stomach, (3K) H&E 5 FU+Silk group intestine, (3L) INOS 5 FU+Silk group intestine. All images were taken in X300 magnification.
Figure 4: Immunohistochemical staining images of the stomach and intestinal tissues of rats with 5-FU-induced gastrointestinal mucositis treated with silk fibroin: (4A) H&E control group stomach, (4B) Caspase 3 control group stomach, (4C) H&E control group intestine, (4D) Caspase 3 control group intestine, (4E) H&E 5 FU group stomach, (4F) Caspase 3 5 FU group stomach, (4G) H&E 5 FU group intestine, (4H) Caspase 3 5 FU group intestine, (4I) H&E 5 FU+Silk group stomach, (4J) Caspase 3 5 FU+Silk group stomach, (4K) H&E 5 FU+Silk group intestine, (4L) Caspase 3 5 FU+Silk group intestine. All images were taken in X300 magnification.

Western blot assays were performed to assess the change in casp-3 and casp-9 levels in 5-FU-induced mucositis of rats, as well as the effect of SF therapy (Figure 5).

Figure 5. The representative images of membranes obtained from western blotting of the stomach and intestine tissues in saline, silk fibroin, 5-FU, and 5-FU+SF groups showing protein expressions of β-actin, caspase-3, and caspase-9.
Casp-9 expression in the stomach and intestine increased in the 5-FU-induced mucositis group compared to the control group (p<0.01, for both tissues; Fig. 6a and Fig. 6b). However, the SF treatment was found to lead decline in casp-9 expression compared to the 5-FU-induced mucositis group in both tissues (p<0.05; Figure 6a, b). In both tissues, the casp-3 expressions were significantly elevated in the 5-FU-induced mucositis group compared to the control group (p<0.05; Figure 6c and Fig. 6d).

**Figure 6:** The expression levels of caspase-3 and caspase-9, after saline, 5-FU or SF treatments in a mucositis rat model in stomach and intestine tissues. All membranes were normalized by using a β-actin antibody. * p<0.05 and **p<0.01 comparisons according to the control group, + p<0.05 comparisons according to the 5-FU-induced oral mucositis group. 5-FU: 5-fluorouracil, SF: silk fibroin, casp-3: caspase-3, casp-9: caspase-9
Discussion:
SF, which has no cytotoxic effect, is a silkworm-derived biomaterial used medically in both drug development and inflammation models \(^{14-15}\). Present study has demonstrated that SF reduce pathological damage caused by 5-FU. In our study, we investigated the mechanism of the protective effect of SF on the stomach and intestinal tissues and found that the administration of 5-FU prevented the increase in iNOS and caspase-3 activities as well as CD4+ and CD8+ levels \(^{16-17}\).

Apoptosis is regarded as a significant pathogenic element in 5-FU-induced gastrointestinal mucositis in addition to the inflammation. iNOS expression has been associated with gastrointestinal inflammation and apoptosis \(^{18}\). It has been reported that 5-FU induces iNOS expression in the mouse model of GIS mucositis \(^{19}\). Moreover, it is known from studies that the application of 5-FU triggers the activity of caspase-3 and causes apoptosis in tissues \(^{18-19}\). In our study, in accordance with the literature, it was shown that iNOS, caspase-3 and caspase-9 activities increased in both stomach and intestinal tissues when 5-FU was administered and this increase was prevented by administration of SF. iNOS is an enzyme that increases during inflammation and is known to cause apoptosis \(^{20}\). The decrease in iNOS and caspase-3 activities upon administration of SF in our study suggests that 5-FU may affect gastrointestinal mucositis. The evidence that SF has a reducing effect on iNOS, caspase-3 and caspase-9 activity in several studies supports this idea.

CD4+ and CD8+ are T cells that are crucial in the body’s defence mechanism in normal cells \(^{21}\). Overactivation of these cells has been shown to lead to inflammation and tissue damage \(^{22}\). Chemotherapeutic agent 5-FU has been shown to participate in the physiopathological mechanism of gastrointestinal mucositis. Activation of these cells causes structural damage \(^{23}\). Studies have shown that the administration of 5-FU causes damage by strong staining of CD4+ and CD8+ in tissues \(^{24-25}\). In our study, administration of 5-FU was found to increase CD4+ and CD8+ levels in the stomach and intestinal tissues immunohistochemically. These findings were also confirmed histopathologically and widespread damage was observed in the tissues. It has been shown that SF, the agent we use in treatment, pleiotropically regulates CD4+ and CD8+ levels \(^{26-27}\). In our study, slightly stained of CD4+ and CD8+ with SF in gastrointestinal mucositis caused by 5-FU is consistent with these studies. Two studies on the CD4+ and CD8+ levels of silk fibroin showed that it has a protective effect by regulating CD4+ and CD8+ levels in both buccal mucosa and spinal cord tissues \(^{26-27}\). The fact that structural damage was lower in the 5-FU group in which SF was applied is consistent with our results and the literature in pathological studies.

Conclusion:
Although 5-FU is a common antineoplastic agent for solid cancers, gastrointestinal damage is the most important factor limiting its use. In our study, immunohistochemical and pathological evidence of SF, a biomaterial to reduce gastrointestinal damage caused by 5-FU, suggests that the use of SF in the treatment of 5-FU may be beneficial in preventing clinical gastrointestinal side effects. Thus, SF is a good candidate for its ameliorative effect.

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Conflict of Interest: None

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Authors’ Contributions
Conception and design: AÖŞ, GS, SS
Analysis and interpretation of the data: AÖŞ, GS, SS; HÖ, AA
Drafting of the article: AÖŞ, SS
Critical revision of the article for important intellectual content: AÖŞ, GS, SS; HÖ, AA
Final approval of the article: AÖŞ, GS, SS; HÖ, AA
Provision of study materials: AÖŞ, GS, SS
Statistical expertise: AÖŞ
Obtaining of funding: GS
References:


