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# A Rat Model for Oral Mucositis Induced by a Single Administration of 5-Fluorouracil

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**Abstract.** *Background/Aim: This study aimed to develop a reliable chemotherapy-induced oral mucositis (CIOM) rat model by intraperitoneally administering a single dosage of 5-fluorouracil (5-FU) combined with a chemical stimulus. Materials and Methods: The 5-FU dosage for CIOM development was determined by the survival rate of rats administrated 160 mg/kg, 200 mg/kg, and 240 mg/kg of 5-FU. Thirty rats were assigned to normal control (NC) and three experimental groups: i) ulcer formation without 5-FU administration (PBS/U+), ii) 5-FU administration without ulcer formation (5-FU/U–), and iii) ulcer formation after 5-FU administration (5-FU/U+). White blood cell count and weight were measured at the day of 5-FU administration (D0), ulcer formation (D2), and two days after ulcer formation (D4). The oral mucosa for histologic evaluations was obtained two (D4) and five days (D7) after ulcer formation. Results: The 5-FU dosage for CIOM development was 200 mg/kg. White blood cell count (WBC) counts and weight of rats were significantly lower in 5-FU/U– (WBC, p<0.001; weight, p=0.002) and 5-FU/U+ (WBC, p<0.001; weight, p<0.001) groups compared to those in the NC group at D4. The number of Ki-67 positive cells in the oral epithelium was lower in 5-FU/U+ group compared to that in NC (p<0.001) and PBS/U+ (p=0.047) groups at D7. Conclusion: Single administration of 200 mg/kg of 5-FU combined with a chemical stimulus can lead to an immune-suppressive status, failure of weight gain, and impairment of epithelium regeneration as observed in a CIOM rat model.*

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*Key Words:* Oral ulcer, fluorouracil, rats, antineoplastic agents, mucositis.



Conventional chemotherapeutic drugs have non-specific toxicities on both malignant cells and rapidly multiplying cells, causing various unintended side effects (1, 2). Among various side effects of chemotherapeutic drugs, oral mucositis is a relatively common side effect that occurs within a certain period of time after administrating chemotherapeutic drugs and causes considerable pain to patients (3, 4). Although most patients undergoing combined chemo-radiation therapy for head and neck cancer experience severe oral mucositis (5), chemotherapy alone can also lead to oral mucositis with adverse impact on treatment outcomes (3). Incidence and severity of chemotherapy-induced oral mucositis (CIOM) may vary depending on the type of chemotherapeutic drug (6). 5-Fluorouracil (5-FU) is still widely used as a major drug for treating various solid tumors. Although CIOM is a relatively predictable side effect, effective prevention and treatment strategies for CIOM have not been established yet (3, 6).

Understandings of development, progression, and healing of CIOM are expanding using various animal models that can allow preclinical studies of various treatments (7). However, the development of oral mucositis in murine is difficult when administrating chemotherapeutic drugs alone without radiation exposure (8). In addition, rats have a stratified squamous epithelium in the oral cavity, which plays a role as a mechanical barrier (9). Therefore, an additional mechanical or chemical trauma on oral mucosa is generally required to develop CIOM unlike intestinal mucositis, which can be induced by chemotherapeutic drug administration

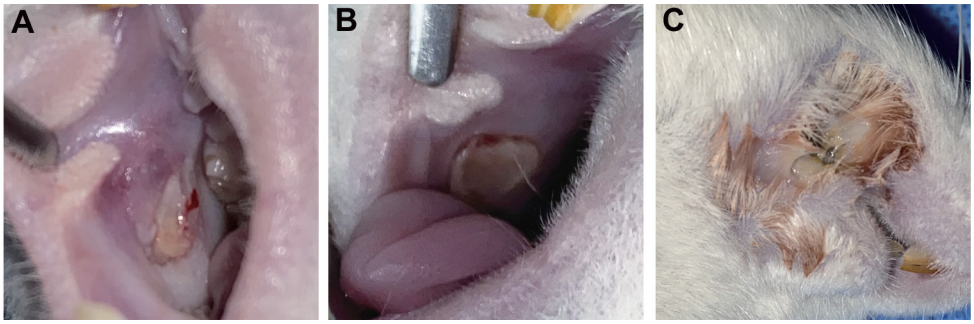


Figure 1. *Development of oral ulcers in rats using acetic acid. (A) Ulcer with granulation observed at two days (D4) after ulcer formation using 25% acetic acid at a volume of 0.15 μl. (B) Ulcer lasting for five days (D7) after ulcer formation. (C) Cheek necrosis after injecting 25% acetic acid at a volume of 0.25 μl into the buccal mucosa.*

alone (10, 11). Intraperitoneal (IP) administration with a single high dose or multiple low doses of 5-FU is the most widely used and simple method for inducing CIOM (11, 12). Myelosuppression and CIOM-related distress following IP administration of chemotherapeutic drugs are parameters that can evaluate the reliability of a CIOM animal model (7, 10).

Much more complex mechanisms that are difficult to explain simply by direct toxicity of 5-FU to the epithelium are involved in CIOM development (6). Since apoptosis and impairment of proliferation of basal epithelial cells are remarkably observed in CIOM, they are factors that can be used to quantitatively evaluate therapeutic response of CIOM to drugs (12, 13). Higher amounts of specimens that allow quantitative analysis of CIOM can be obtained from rats than from mice (10). The aim of this study was to develop a reliable chemotherapy-induced oral mucositis (CIOM) rat model through intraperitoneal administration of a single dosage of 5-fluorouracil (5-FU) in combination with a chemical stimulus.

## Materials and Methods

*Animals, materials, and study design*. This study was approved by the Institutional Animal Care and Use Committee (IACUC) of our Institution (IACUC No. 2021-05218). This study was designed to have two steps. After determining a 5-FU dosage for developing CIOM by an explorative study, systemic and local effects of 5-FU were quantitatively evaluated. Six-week-old male Sprague-Dawley rats (Orient Bio, Republic of Korea) were purchased and housed for a week in a pathogen-free animal facility under controlled conditions (12-h light-dark cycle, 21-23˚C, and 40-60% relative humidity). Rats were allowed free access to food pellets and water. 5-FU ($C_4H_3FN_2O_2$) with purity of 99% was purchased from Alfa Aesar (Ward Hill, MA, USA) for ulcer formation. Isoflurane (Hana Pharm Co, Republic of Korea) was used for anesthesia. 5-FU was prepared at a concentration of 10 mg/ml mixture with phosphate-buffered saline (PBS) in consideration of its solubility of 12.5 mg/ml in water at room temperature (14). After IP administration of 5-FU (D0), a chemical stimulus for ulcer formation was applied at two days after 5-FU administration (D2). After euthanizing rats, the buccal mucosa including the ulcer and underlying muscle was obtained at four (D4) and seven (D7) days after 5-FU administration. All procedures were performed after anesthetizing rats using isoflurane.

*Determination of 5-FU dose for inducing CIOM*. Twelve rats were randomly assigned to three groups, with four rats in each group. A higher dose of 5-FU was administrated to induce CIOM than the dose used to induce intestinal mucositis in murine (13). Since a single IP injection of 150 mg/kg of 5-FU successfully induced intestinal mucositis in a previous study (15), we IP injected 5-FU with a higher single dosage of 160 mg/kg, 200 mg/kg, or 240 mg/kg after anesthetizing rats using isoflurane. The maximum volume of IP injection in rats is known to be 20 ml/kg (16, 17). Therefore, approval for IP injection of 5-FU was obtained from IACUC. Chemical stimulus for ulcer formation was identically applied to all rats at D2. Acetic acid at a concentration of 25% and a volume of 25.0 μl was applied to the buccal mucosa for generating ulcers based on results of a previous study (10). The ulcer was visually monitored at D4 and D7. Among the three different dosages of 5-FU administrated in rats, the maximum dosage of 5-FU to induce CIOM was the dosage at which rats survived and ulcers lasted until D7.

*Systemic effect of 5-FU*. The single dosage of 5-FU for inducing CIOM in rats was determined to be 200 mg/kg. After IP administration of 200 mg/kg of 5-FU at D0, 25% acetic acid at a volume of 0.15 μl into the buccal mucosa was gently injected at D2. A total of 30 rats were randomly assigned into four groups, with six rats in the normal control (NC) group and eight rats in each experimental group. There were three experimental groups: 1) ulcer formation without 5-FU administration (PBS/U+), 2) 5-FU administration without ulcer formation (5-FU/U–), and 3) ulcer formation after 5-FU administration (5-FU/U+). White blood cells (WBC) counts and weights of rats were parameters used for evaluating myelosuppression status and CIOM induced-distress in rats, respectively (10, 18). WBC counts and weights were measured at D0, D2, and D4.

*Histologic evaluations*. Three rats in the NC group and four rats in each experimental group were euthanized to harvest the buccal mucosa at D4 and D7. After fixing the obtained specimens with

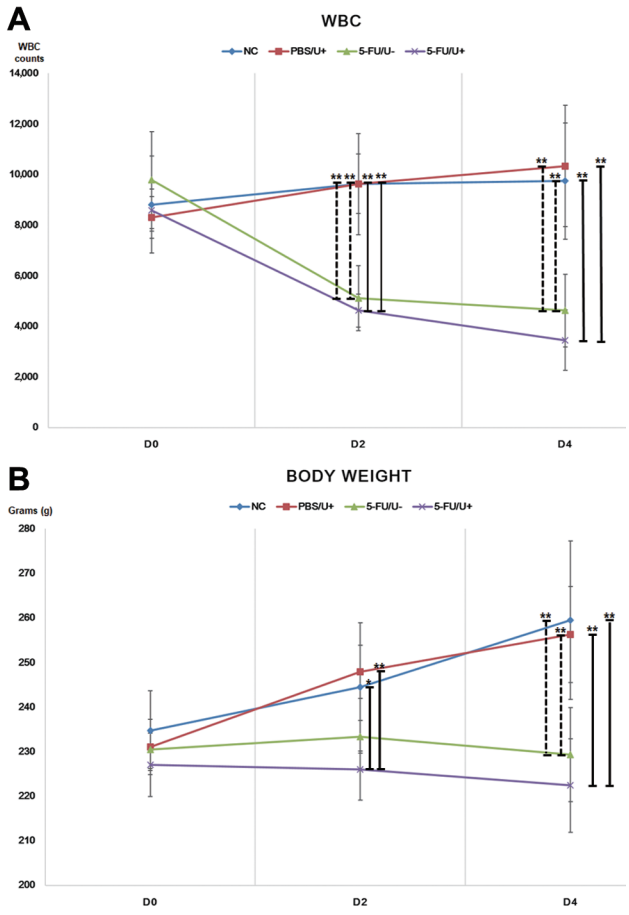


Figure 2. *Changes in white blood cell (WBC) counts and weights of rats from the day of administration of 5-FU (D0) to four days after 5-FU administration (D4). (A) The relative decrease in WBC counts in both 5-FU/U– and 5-FU/U+ groups compared to those in NC (5-FU/U–, p<0.001; 5-FU/U+, p<0.001) and PBS/U+ (5-FU/U–, p<0.001; 5-FU/U+, p<0.001) groups lasted until D4. (B) Weights of rats in both 5-FU/U– and 5-FU/U+ groups at D4 were lower than those in NC (5-FU/U–, p<0.001; 5-FU/U+, p<0.001) and PBS/U+ (5-FU/U–, p<0.001; 5-FU/U+, p<0.001) groups. *p<0.05, **p<0.001.*

buffered 4% formalin for 24 h, specimens were embedded in paraffin and sectioned into 4 μm-thick specimens. Hematoxylin (Cat #ab220365, Abcam, Cambridge, UK) and eosin Y (Cat #ab246823, Abcam) staining was then performed. Immunohistochemical (IHC) staining for Ki-67 was performed to evaluate proliferation of the epithelium during the ulcer healing process. Deparaffinized slides were then incubated with a primary antibody against Ki-67 at 4˚C for 24 h. Contrast staining was conducted using 3, 3'-Diaminobenzidine (DAB) (Cat #K3468, Dako, Carpinteria, CA, USA). Apoptosis of basal epithelial cells was evaluated using TUNEL DAB Apoptosis detection kit (#VB-4005D, Vitro Vivo Biotech, Rockville, MD, USA) according to the manufacturer's instructions. Positive cell counts for Ki-67 and TUNEL were measured by two-blinded evaluators using four rats (three fields per sample and five measurements per field).

*Statistical analyses*. All statistical analyses were performed using IBM SPSS Statistics for Windows version 22.0 (IBM, Armonk, NY, USA). The null hypothesis of no difference was rejected if the *p*-value was less than 0.05. After evaluating the normality of data using Shaprio-Wilk test, analysis of variance (ANOVA) was used to determine the difference among groups at each time point. Differences between groups were subjected to post-hoc analysis.

## Results

*Determinations of 5-FU dosage*. All rats administrated with 5-FU at 160 mg/kg and 200 mg/kg survived until D7. Two rats (2/4, 50%) administrated with 240 mg/kg of 5-FU died at D2 and D3 each. Ulcer was observed in all survived rats at D7 regardless of the administration dosage of 5-FU (Figure 1A and B). However, cheek necrosis was observed in each rat administrated with 5-FU at 160 mg/kg and 200 mg/kg (Figure 1C). Therefore, 200 mg/kg of 5-FU was determined to be an effective dosage for inducing CIOM. The volume of acetic acid for generating ulcer was reduced from 25 μl to 15 μl.

*Systemic effect of 5-FU*. Although the WBC counts and weights of rats were measured at planned time points in a specific number of rats, each rat in the PBS/U+ and 5-FU/U+ groups was arbitrarily sacrificed at D4 for obtaining the buccal mucosa due to obvious decreases in activity and increases in efforts for breathing. The other rats were randomly sacrificed.

WBC counts were significantly decreased in both 5-FU/U– and 5-FU/U+ groups at D2 compared to those in NC (5-FU/U–, $p<0.001$; 5-FU/U+, $p<0.001$) and PBS/U+ (5-FU/U–, $p<0.001$; 5-FU/U+, $p<0.001$) groups. The relative decrease in WBC counts in both 5-FU/U– and 5-FU/U+ groups compared to those in NC (5-FU/U–, $p<0.001$; 5-FU/U+, $p<0.001$) and PBS/U+ (5-FU/U–, $p<0.001$; 5-FU/U+, $p<0.001$) groups lasted until D4 (Figure 2A). Weights of rats in 5-FU/U+ group at D2 were significantly lower than those in NC ($p=0.024$) and PBS/U+ ($p<0.001$) groups. Weights of rats in both 5-FU/U– and 5-FU/U+ groups at D4 were lower than those in NC (5-FU/U–, $p<0.001$; 5-FU/U+, $p<0.001$) and PBS/U+ (5-FU/U–, $p<0.001$; 5-FU/U+, $p<0.001$) groups (Figure 2B).

*Apoptosis and proliferation of the epithelium*. Large granulation with lymphocyte infiltration around the ulcer area was observed in PBS/U+ and 5-FU/U+ groups at D4 on H&E staining. In contrast, squamous epithelium was preserved in NC and 5-FU/U– groups (Figure 3A). Ulcers were under the healing process with greatly decreased granulation in PBS/U+ and 5-FU/U+ groups at D7. However, squamous epithelium was not fully recovered in 5-FU/U+ group at D7 (Figure 3B). Positive cells for TUNEL in the basal epithelium were only observed in the 5-FU/U+ group at D4. However, positive cell

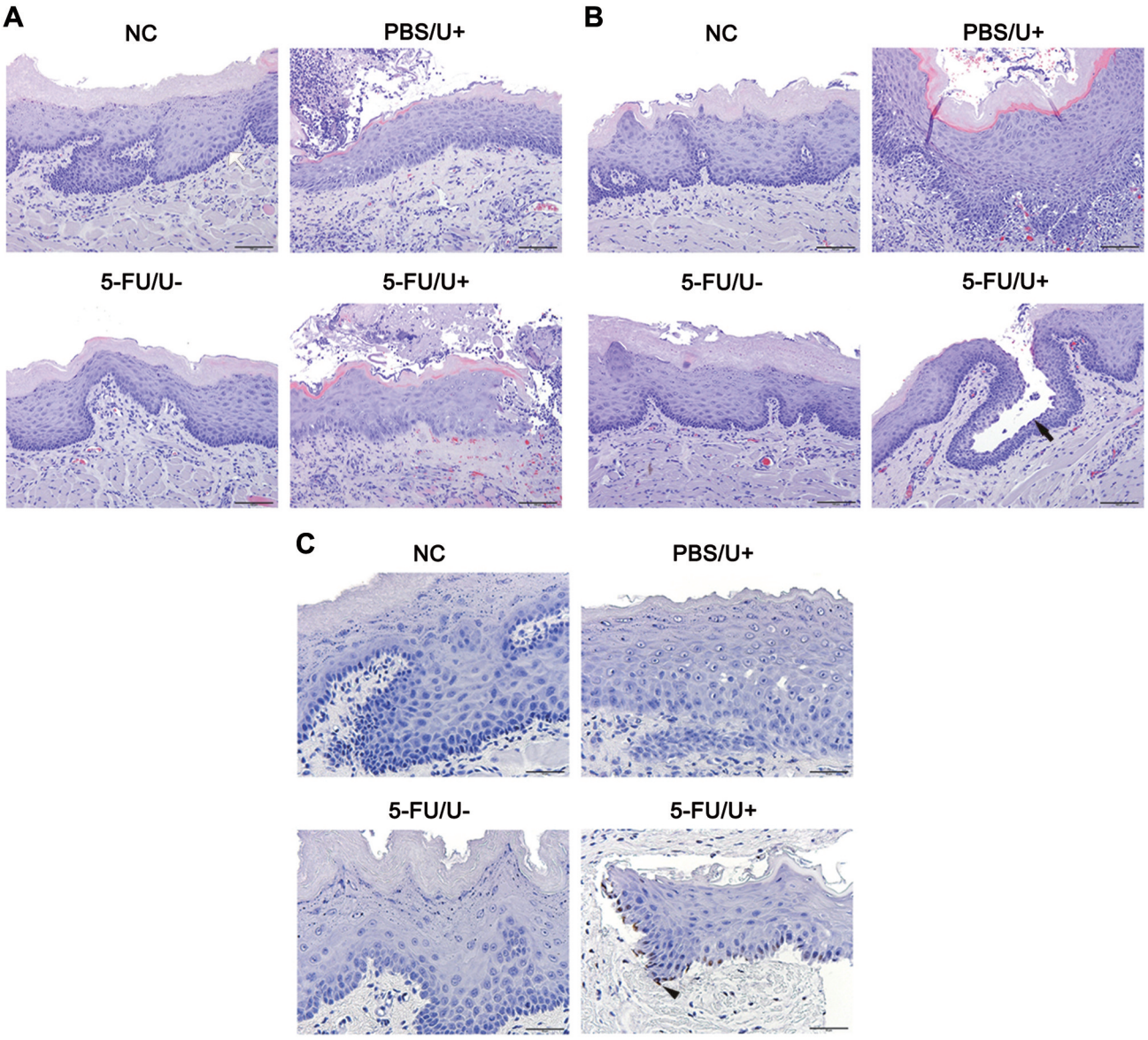


Figure 3. *Histologic evaluations. (A) Large granulation with lymphocyte infiltrations and breakdown of squamous epithelium were observed around the ulcer in PBS/U+ and 5-FU/U+ groups at D4 (H&E, ×200). (B) Squamous epithelium was not fully recovered (black allow) in 5-FU/U+ group at D7 (H&E, ×200). (C) Positive cells for TUNEL in the basal epithelium (black arrow head) were observed only in the 5-FU/U+ group at D4 (immunohistochemistry for TUNEL, ×400).*

numbers in the epithelium based on TUNEL assays were not significantly different between the 5-FU/U+ group and other groups (Figure 3C). The number of Ki-67 positive cells in the epithelium was significantly lower in 5-FU/U– ($p$=0.002) and 5-FU/U+ groups ($p$<0.001) than in the NC group at D4 (Figure 4A and B). The number of Ki-67 positive cells was also significantly lower in the 5-FU/U+ group than that in the PBS/U+ group ($p$=0.019). Decreased number of Ki-67 positive cells lasted until D7, showing the same pattern observed on D4 (Figure 4C and D). The number of Ki-67 positive cells was significantly lower in 5-FU/U– ($p$=0.004) and 5-FU/U+ groups ($p$<0.001) than that in the NC group. The number of Ki-67 positive cells was also significantly lower in the 5-FU/U+ group than that in the PBS/U+ group ($p$=0.047).

## Discussion

The severity and duration of CIOM are dose-dependent (3). Since administration of the highest possible dose of 5-FU can increase the severity and duration of CIOM, it is

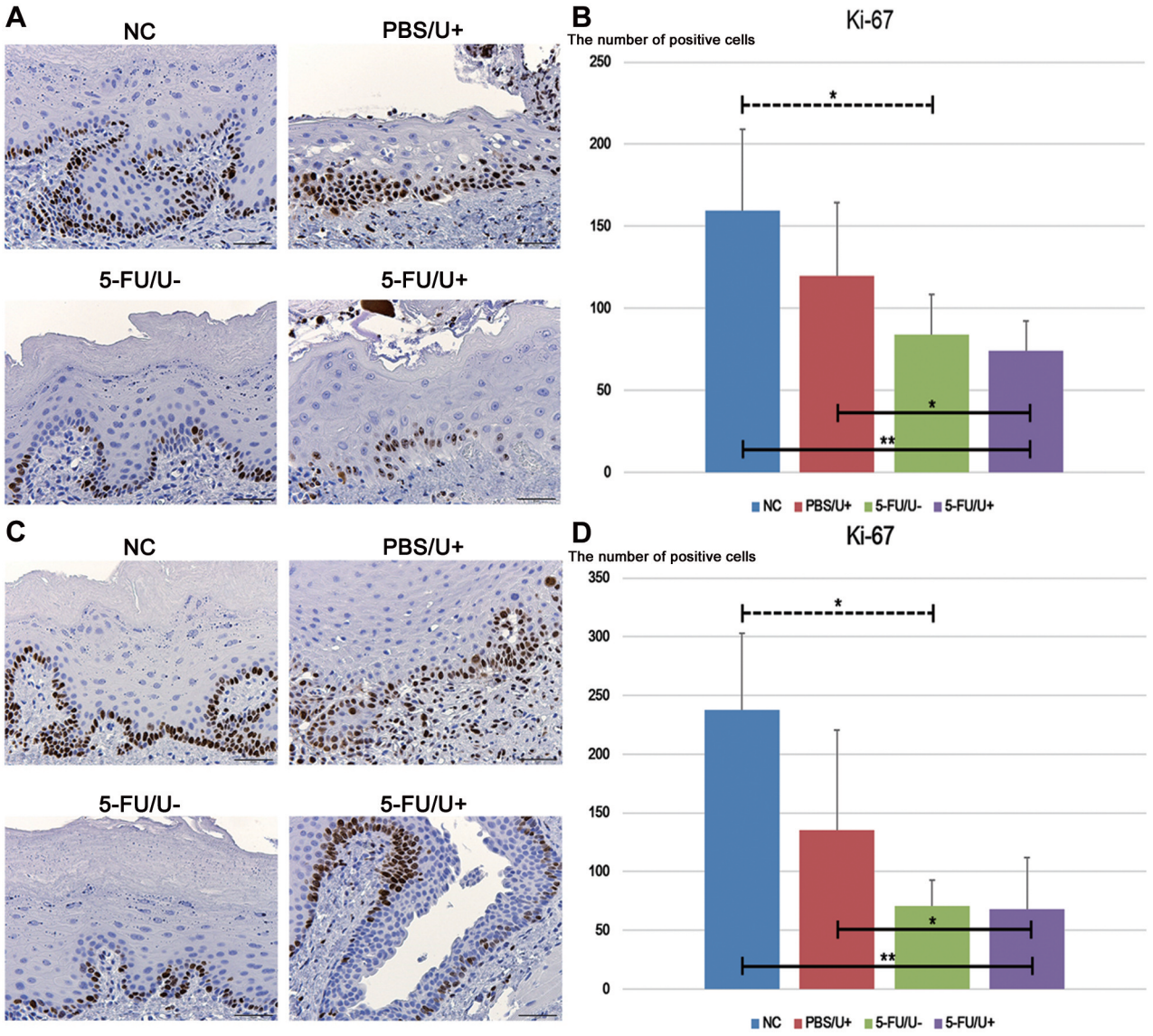


Figure 4. *Immunohistochemistry for Ki-67. (A) Positive cells for Ki-67 in basal epithelial cells at D4. (B) The number of Ki-67 positive cells in the epithelium was significantly lower in 5-FU/U– (p=0.002) and 5-FU/U+ groups (p<0.001) compared with that in the NC group at D4. (C) Ki-67 positive basal epithelial cells at D7. (D) Ki-67 positive cell number was significantly lower in 5-FU/U– (p=0.004) and 5-FU/U+ groups (p<0.001) than that in the NC group. *p<0.05; **p<0.001.*

advantageous for quantitative evaluation of the response to treatment. In the present study, IP administration of 5-FU at a single dose of 200 mg/kg decreased WBC counts and weights of rats and actually impaired proliferation of the epithelium following a chemical stimulus.

There are technical considerations when developing CIOM in murine. A high-dose IP administration of 5-FU ranging from 100 to 500 mg/kg has been used for inducing CIOM in murine (15). Since the solubility of 5-FU at room temperature is about 12 mg/ml in water (14), more than 3 ml of 5-FU mixture administration is generally required for inducing CIOM in rats when considering rat's weight in the 7$^{th}$ week (19). Although intravenous administration of 5-FU can more effectively induce CIOM in murine, it is technically difficult to inject a large volume of drugs or perform repeated injections through the tail vein. On the other hand, IP injection is technically easy. It can deliver a relatively larger amount of 5-FU up to 20 ml/kg compared to intravenous administration (17). Therefore, IP administration is an easy and feasible route for inducing CIOM using a single high

dose of 5-FU. In addition, we recommend a smaller volume of 25% acetic acid for chemical stimulus compared to the previous study to prevent cheek necrosis, which is clinically outside the general category of oral mucositis (10).

The 5-FU dose for inducing CIOM by single administration in rats has not been established yet. Administration of a total of 150 mg/kg of 5-FU in rats through three times of injection at 50 mg/kg each with at interval of two days resulted in a high mortality in a previous study (10). However, rats in this study survived after IP administration of 5-FU at a single dose of 200 mg/kg, in contrast to results of the previous study (10). Interestingly, another previous study has reported a rat model of intestinal mucositis produced by IP administration of 5-FU at a single dose of 400 mg/kg (12). A total dose of 5-FU used in the present study (200 mg/kg) was higher compared to that in previous study (150mg/kg) (10). A possible reason that could cause such mortality difference among studies might be a stressful situation due to multiple injections of 5-FU despite the lower dose of it in the previous study. Further research on the overall 5-FU metabolism from absorption to systemic circulation according to 5-FU administration methods is necessary in order to clarify the cause of the low mortality in this study unlike the previous study.

Induction of myelosuppression using 5-FU in rats is the first and essential step for inducing CIOM (10). Survived rats administrated with 5-FU at a single dose of 200 mg/kg in the present study actually showed significant reductions in WBC counts and weights, which continued until D4. These results indicated that myelosuppression and CIOM-induced distress were successfully achieved in rats (10, 18). Basal epithelial cells are responsible for regenerating the damaged epithelium (20). Direct toxicity of 5-FU to the epithelium, especially the basal and suprabasal cells, can cause cell death in the initiation phase of CIOM, which can activate various inflammatory pathways (3). Inflammation activated by complex biological pathways can impair the proliferation of basal epithelial cells, eventually resulting in prolonged ulcers (6). In our IP CIOM model in rats, proliferation of basal cells in the 5-FU/U+ group was reduced compared to those in NC and PBS/U+ groups until D7. This phenomenon indicates that that 5-FU can impair the normal healing process of ulcers. Although a statistical significance in the number of positive cells for TUNEL among groups failed to be proven in this study, apoptosis of basal cells was observed in rats administrated with 5-FU showing ulcer formation at D4. Since apoptosis concurrently occurs with 5-FU administration (3), statistical significance of apoptosis differences among groups could be seen if a histologic evaluation for apoptosis is conducted earlier than that in this study.

## Conclusion

Single administration of 5-FU at 200 mg/kg combined with a chemical stimulus can lead to an immune-suppressive status, failure of expected weight gain, and impairment of epithelium regeneration as seen in CIOM after administration of 5-FU. Therefore, we conclude that 200 mg/kg 5-FU is a sufficient dose to induce CIOM effectively and to maintain oral ulcer for a sufficient period of time to quantitatively evaluate the recovery process of ulcers in rats. This CIOM model of rats can be used to quantitatively evaluate preclinical responses to treatment.

## Conflicts of Interest

The Authors have no conflicts of interest relevant to this study to disclose.

## Authors' Contributions

Conception or design of this study: B.H.K; Data collection: D.H.K, and B.H.K; Data analysis and interpretation: J.E.C, J.H.P, H.J.H, Y.S.L, and C.G.C; Drafting of this manuscript: D.H.K, and B.H K; Critical revision of the manuscript: D.H.K, and B.H.K.

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