

Effects of GGsTop® on Collagen and Glutathione in the Oral Mucosa Using a Rat Model of 5-Fluorouracil-Induced Oral Mucositis

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Abstract. *Background/Aim:* To evaluate the usefulness of GGsTop® for oral mucositis, a quantitative study focusing on oral mucosal tissues is necessary. In this study, we aimed to quantify collagen and glutathione using a rat model of 5-fluorouracil-induced oral mucositis. *Materials and Methods:* Changes in ulcer area and erythrocyte count were measured to confirm the usefulness of GGsTop® for oral mucositis. The effect of GGsTop on collagen was evaluated by observing oral mucosal tissue sections and measuring the collagen concentration in the tissues. The total glutathione concentration and the oxidized glutathione concentration were measured, and the concentration of the reduced form was calculated. *Results:* GGsTop® shortened the treatment period for oral mucositis without affecting the white blood cell count. In addition, GGsTop® promoted collagen production and alleviated oxidative stress conditions. *Conclusion:* GGsTop affects collagen and glutathione in the treatment of oral mucositis.

The occurrence of oral mucositis is a major problem in cancer treatment, however, it is difficult to use existing drugs, which mainly comprise steroidal anti-inflammatory drugs (1). Therefore, there is a need for new therapeutic agents, and in the previous study, we reported the therapeutic effect of GGsTop®, a newly developed selective γ -glutamyl transpeptidase (GGT) inhibitor, on a mouse model of 5-fluorouracil (5-FU)-induced oral mucositis (2). GGT is an enzyme that hydrolyzes the γ -glutamyl bond of glutathione, which is a tripeptide consisting of glutamic acid, cysteine, and

glycine. Glutathione normally exists in the reduced form (GSH) in the body, and GSH is converted into an oxidized form [glutathione disulfide (GSSG)] by stimulation, such as oxidative stress. GSH is known to have many physiological actions such as a radical scavenger action (3), action as a coenzyme (4), and detoxification action by conjugation reaction to foreign substances (5), and plays an important role in the biological defense mechanism. GGT is known to play a central role in mediating the redox balance of cells, and the detoxification of xenobiotics and reactive oxygen species (ROS) through glutathione metabolism (6-8). GGT is considered to be involved in many diseases triggered by oxidative stress, and an epidemiological study on cardiovascular diseases such as atherosclerosis, myocardial infarction, and angina has reported that elevated GGT activity is a risk factor (9). GGT supplies intracellular cysteine from extracellular GSH, and cysteinylglycine, a by-product, is a highly reactive thiol compound that produces ROS by reducing oxygen during the process of oxidizing Fe^{3+} to Fe^{2+} under physiological conditions (10, 11). Therefore, increased GGT activity causes increased oxidative stress. Oral mucositis due to side effects of anticancer agents is caused by tissue destruction due to ROS production (12). GGT inhibitors, which suppress ROS and induce the production of collagen and elastin, which are the basis of mucosal tissues (13), are promising as new therapeutic agents for oral mucositis. Acivicin is widely used as a GGT inhibitor because its inhibitory effect is irreversible, inexpensive and easily available (11, 13). However, acivicin irreversibly inhibits various glutamine amidotransferases and inactivates many biosynthetic enzymes, resulting in potent cytotoxicity and central nervous system toxicity (14-18). In addition to GGT inhibitors, rebamipide is drawing attention in the treatment of oral mucositis, however, there is a problem with retention time in the oral cavity (19, 20).

GGsTop® has a simple structure, high water solubility, and chemical stability. It was reported that its GGT inhibitory activity greatly exceeded acivicin and it does not have inhibitory activity against glutamine amidotransferase, which is a problem

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of acivicin (21, 22). Furthermore, it was reported that GGsTop[®] did not show toxicity even at high concentrations and was negative in a mutagenicity test using microorganisms. In the previous study, we investigated the therapeutic effect of GGsTop on oral mucositis and confirmed shortening of treatment period and promotion of collagen production (2). This result showed the effectiveness of GGsTop for treating oral mucositis, however, the effect of promoting collagen production was limited to qualitative results by observing the oral mucosal tissue section, due to the insufficient amount of oral mucosal tissue obtained from animal disease models (23).

In this study, we planned to quantitatively evaluate the effect of GGsTop[®] on collagen and glutathione by using oral mucositis model rats (23). The therapeutic efficacy of GGsTop in treating oral mucositis using this animal model was evaluated by changes in ulcer area and white blood cell (WBC) count (2). The effect on collagen production was evaluated by observing the oral mucosal tissue section and measuring the concentration in the oral mucosal tissue. In addition, the concentrations of GSH and GSSG in oral mucosal tissues were determined.

Materials and Methods

Materials. 5-FU (C₄H₃FN₂O₂, purity >98.5%) was purchased from Fujifilm Wako Pure Chemical Corp. (Osaka, Japan). Isoflurane for the animal was purchased from Mylan Inc. (Pittsburgh, PA, USA). Medetomidine hydrochloride (Domitol[®]), which is an alpha 2 adrenoceptor agonist, was purchased from Nippon Zenyaku Kogyo Co., Ltd. (Koriyama, Japan). Midazolam, which is a benzodiazepine derivative, was purchased from Teva Takeda Pharma Ltd. (Nagoya, Japan). Butorphanol tartrate (Vetorphale[®]) was purchased from Meiji Seika Pharma Co., Ltd. (Tokyo, Japan). 5-Sulfosalicylic acid dehydrate (C₆H₃(OH)(SO₃H)COOH·2H₂O, purity >99.0%) was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). GGsTop[®] was kindly donated by Nahls. Co., Ltd. (Kyoto, Japan). Other chemicals were of the highest reagent grade commercially available.

Therapeutic effect of GGsTop[®] in the treatment of oral mucositis. Nine-week-old male Wistar rats were purchased from Japan SLC Inc. (Tokyo, Japan). All animal care was conducted under the Guidelines for Animal Experimentation of Tokyo University of Science, which are based on the Guidelines for Animal Experimentation of the Japanese Association for Laboratory Animal Science. Rats were housed in stainless-steel cages under standard environmental conditions (23±1 °C, 55%±5% humidity and a 12/12 h light/dark cycle) and maintained with free access to water and a standard laboratory diet (carbohydrates 30%; proteins 22%; lipids 12%; vitamins 3%) *ad libitum* (Nihon Nosan Kogyo Co., Yokohama, Japan).

A rat model of 5-FU-induced oral mucositis was produced in the same manner as in the previous study (24). Briefly, 8.0 mg/ml of 5-FU solution was prepared by dissolving 360 mg of 5-FU in 45 ml of physiological saline. On days -5, -3, and -1 of the experiment, rats were administered the 5-FU solution at a dose of 40 mg/kg body weight under isoflurane anesthesia. On day 0, they were anesthetized by intraperitoneal administration of a combination of

anesthetics, which was prepared with 0.3 mg/kg of medetomidine hydrochloride, 4.0 mg/kg of midazolam, and 5.0 mg/kg of butorphanol, under isoflurane anesthesia (24), and then 25.0 µl of 25% acetic acid aqueous solution was injected into the right buccal mucosa. Then, 33.1 mg of GGsTop[®] bulk powder was mixed with 966.9 mg of lactose for the purpose of adjusting the amount of GGsTop[®] administered to rats to 1.0 µmol. From day 1, the physical mixture of GGsTop[®] and lactose was applied to the ulcer of the right buccal mucosa of rats once a day under the mixed anesthesia. After the treatment, the rats were allowed to sleep for several hours until the effects of anesthesia ceased. Then, changes in WBC counts and ulcer area were measured. Ulcer area measurements were performed using an image analysis software (Image J, National Institutes of Health, Bethesda, MD, USA) (2, 19, 23, 25). The results were compared to the group without treatment and the group in which lactose alone was applied to the ulcer.

Effect of GGsTop[®] on collagen in oral mucosal tissues. Histological evaluation of oral mucosal tissues was performed to confirm the effect of GGsTop[®] on collagen production of oral mucositis model rats. The oral mucositis model rats were divided into two groups: a group in which a mixture of GGsTop[®] and lactose was applied to the ulcer and a group in which PBS was dropped on the ulcer (no-treatment group). On days 3, 7, and 12 of the experiment, their tissue sections were produced. Preparation of frozen tissue sections was performed based on the method of Kawamoto (26); the obtained tissue sections were compared after staining collagen using collagen stain kit (K-61, Collagen Research Center, Kiyose, Japan). This kit stains collagen and non-collagen proteins as red and green, respectively. Staining and quantification of collagen were performed based on the instruction manual of the kit. Two hundred microliters of the staining solution was added dropwise to the obtained sample, and 30 min later, it was washed with purified water and then sealed. Tissue sections were observed using a fluorescence microscope (BZ-9000, Keyence Corp., Osaka, Japan). Next, 200 µl of the extraction solution was added dropwise to the stained sections and collected. This operation was repeated five times. The optical densities (O.D.) of the collected solutions at wavelengths of 530 and 605 nm were measured using an ultraviolet-visible spectrophotometer. The amount of collagen and the amount of non-collagen proteins were calculated using the following formulas:

$$\begin{aligned} \text{Collagen (mg)} &= \text{O.D. at 530 nm} - 0.254 \times \text{O.D. at 605 nm} \times 40.8 \\ \text{Non collagen proteins (mg)} &= \text{O.D. at 605 nm} \times 2.04 \end{aligned}$$

For comparison, collagen staining and collagen quantification of oral mucosal tissue sections of healthy rats were performed.

Effect of GGsTop[®] on GSH and GSSG in oral mucosal tissues. GSH and GSSG in oral mucosal tissues were quantified using GSSG/GSH quantification kit (G257, Dojindo Laboratories, Mashiki, Japan). The quantitative measurement was performed based on the instruction manual of the kit using 5-sulfosalicylic acid dehydrate. As a control group, normal and healthy rats were used.

Results

Figure 1 shows changes in the ulcer area of oral mucositis. On day 2 of the experiments, when the measurement of the

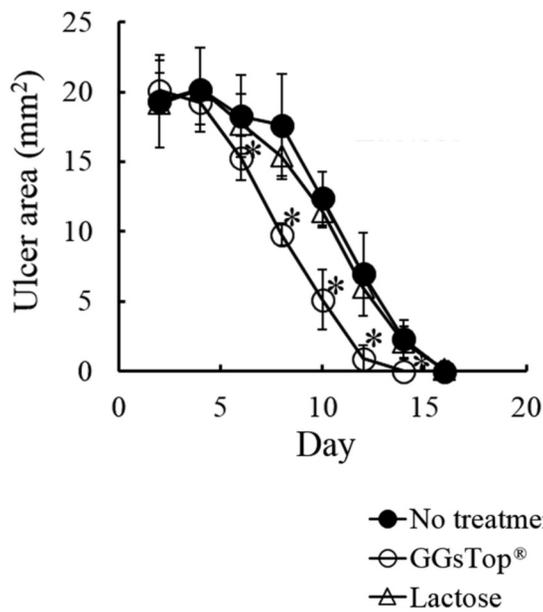


Figure 1. Changes in the ulcer area in the group without treatment (no treatment), GGsTop®-treated group (GGsTop®), and lactose-treated group (lactose) (mean±S.D., n=5). *Significantly different at $p<0.05$ compared to the no-treatment group (Dunnett's test).

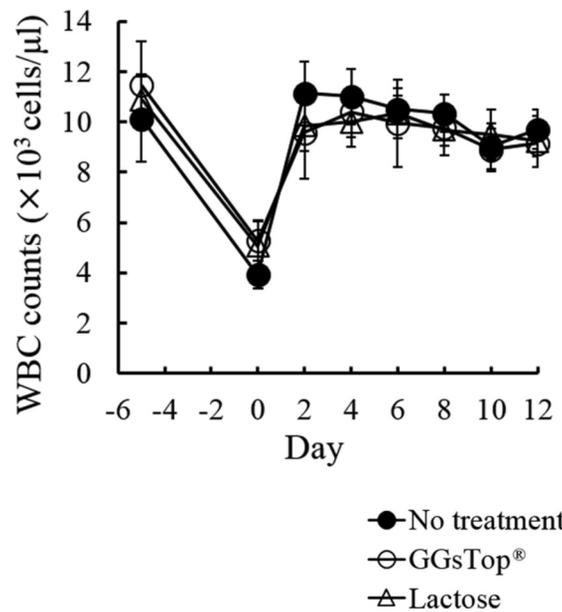


Figure 2. Changes in white blood cell count in the group without treatment (no treatment), the GGsTop®-treated group (GGsTop®), and the lactose-treated group (lactose) (mean±S.D., n=5).

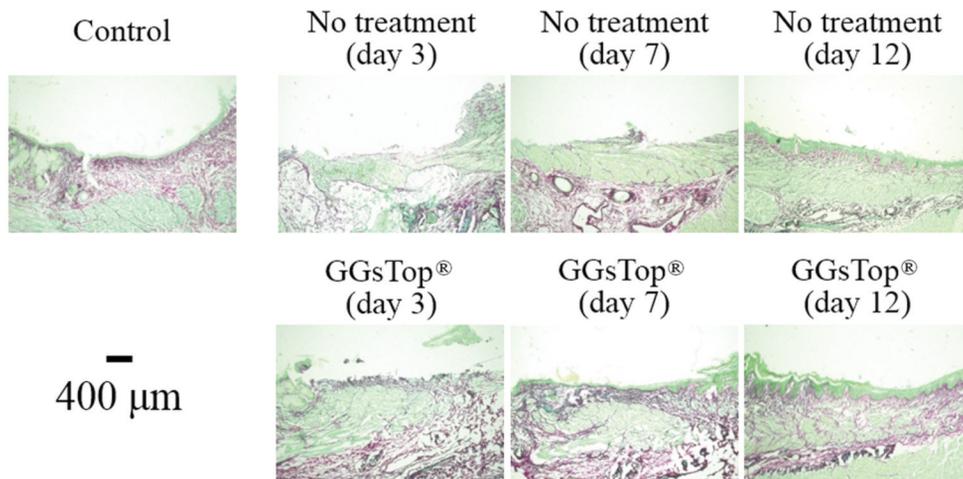


Figure 3. Collagen-stained oral mucosal tissues in the group without treatment (no treatment) and the GGsTop®-treated group (GGsTop®). As a control group, normal healthy rats were used. Collagen and non-collagen proteins were stained red and green, respectively.

ulcer area was started, it was confirmed that the ulcer areas of all groups were similar. From day 6 onward, the ulcer area was significantly reduced in the GGsTop®-treated group, and the period until healing was shortened by 2 days compared with the no-treatment group and the lactose-treated group. Figure 2 shows the effect of treatment of GGsTop® to ulcers on the WBC count in rats. No significant difference was found between the three groups.

Figure 3 shows images of the oral mucosal tissue section. On day 3 immediately after the ulcer occurred, it was confirmed that the tissue on the mucosal surface was destroyed and the tissue under the mucosa was hollowed out. From day 7 onward, it was observed that collagen was increased in the GGsTop®-treated group compared with the no-treatment group. As shown in Figure 4, on days 7 and 12, the GGsTop®-treated group had a significantly increased

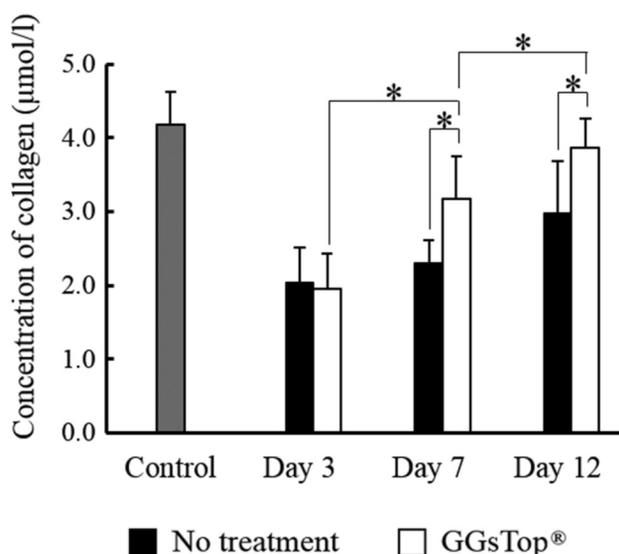


Figure 4. Changes in collagen concentration in oral mucosal tissues in group without treatment (no treatment) and GGsTop[®]-treated group (GGsTop[®]). As a control group, normal healthy rats were used (mean±S.D., n=3, *p<0.05, Tukey's test).

collagen concentration in the oral mucosa tissue as compared with the no-treatment group. On day 12, the GGsTop[®]-treated group approached the value of the control group, and it was confirmed that the ulcer had almost healed in terms of collagen concentration.

Figure 5 shows the quantitative results of GSH and GSSG. It was revealed that the GSH concentration was significantly increased by the treatment with GGsTop[®] and recovered to the same level as the control group on day 12. The sum of GSH concentration and GSSG concentration shows the concentration of total glutathione. Although the GSSG concentrations were not significantly different between the three groups, on day 5, total glutathione levels were decreased in the GGsTop[®]-treated group and no-treatment groups. The results show that the ratio of GSSG concentration to total glutathione concentration was increased by the occurrence of oral mucositis. In addition, on days 5 and 12, it was confirmed that in the GGsTop[®]-treated group, the ratio of the GSSG concentration to the total glutathione concentration was smaller than in the no-treatment group.

Discussion

Treatments with triamcinolone acetonide (Kenalog[®]) caused a decrease in WBC count (23, 25), whereas GGsTop[®] shortened the treatment period for oral mucositis without affecting WBC count (Figures 1 and 2). This result supports the previous study using mice, and suggests that GGsTop[®] can be used in combination with an anticancer drug that causes an

immunosuppressive effect (2). The results in Figures 3 and 4 indicate that treatment with GGsTop[®] induces expression of collagen and elastin synthesis by inhibiting GGT (27). We considered that the mucosa damaged by inflammation was repaired as the production of collagen was promoted. The decrease in the concentration of total glutathione due to the occurrence of oral mucositis in Figure 5 was considered to be caused by overproduction of ROS. Overproduction of ROS in the early stages of 5-FU-induced oral mucositis may induce redox imbalance due to consumption of antioxidants, including glutathione (28). The ratio of GSSG concentration to total glutathione concentration is an indicator of oxidative stress. Therefore, the change in the ratio of the GSSG concentration indicates that the tissue in which the oral mucositis has occurred was in the oxidative stress state, and that the state was alleviated by the treatment with GGsTop[®]. There are several reports on the antioxidant effect of GGsTop[®]. It was reported that GGsTop[®] reduced oxidative stress and suppressed asthma attacks by increasing the amount of glutathione (29). Furthermore, it was reported that GGsTop[®] protected hepatic ischemia-reperfusion injury in rats by inhibiting GGT activity and ROS production (14).

Conclusion

In this study, the usefulness of GGsTop[®] in the treatment of oral mucositis induced by an anti-cancer drug was shown. The measurement of collagen in the oral mucosal tissue revealed that GGsTop[®] promoted the induction of collagen synthesis and accelerated the recovery of mucosal tissue. From the quantitative measurement of glutathione, it was confirmed that GGsTop[®] reduced oxidative stress due to oral mucositis. These results also indicate that a rat model of 5-FU-induced oral mucositis can be used for quantitative studies.

Conflicts of Interest

The Authors declare that they have no potential conflicts of interest regarding this study.

Authors' Contributions

Takeuchi and R. Kawamata designed the study, and wrote the initial draft of the manuscript. K. Makino contributed to analysis and interpretation of data, and assisted in the preparation of the manuscript. All Authors approved the final version of the manuscript, and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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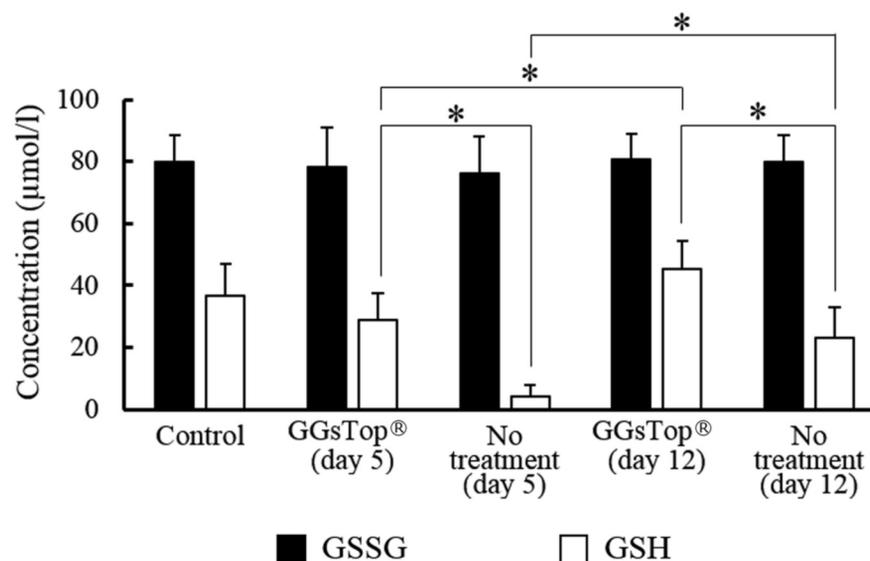


Figure 5. Changes in GSH and GSSG concentrations in oral mucosal tissues in group without treatment (no treatment) and GGsTop®-treated group (GGsTop®). As a control group, normal healthy rats were used (mean±S.D., n=3, *p<0.05, Tukey's test).

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