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Authors

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Histopathologic changes in the rabbit submandibular gland after 5-fluorouracil chemotherapy

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Aim: To determine the early-stage effects of 5-fluorouracil (5-FU) on the histology of the submandibular gland.

Materials and methods: The study included 30 New Zealand rabbits. They were divided into 3 groups. No drugs were administered to group 1 (control group), and 5-FU was administered for 5 days to group 2 and for 10 days to group 3. Submandibular gland excision was then performed. Histopathological examination was performed with hematoxylin-eosin staining, apoptosis was determined based on TUNEL staining, and cell proliferation was examined using the PCNA antibody.

Results: While no granular change was seen in group 1, granular change was observed in the acinar cell cytoplasm in 5 rabbits in group 2 and in 5 rabbits in group 3 ($P < 0.05$). The rate of apoptosis in the acinar and ductal cells was higher in group 3 than in groups 1 and 2, according to TUNEL staining ($P < 0.05$). While no cellular proliferation in the acinar cells was observed with PCNA staining in any of the groups, there was a statistically significant difference in group 3 in ductal cellular proliferation, as compared to groups 1 and 2 ($P < 0.05$).

Conclusion: Toxic effects of 5-FU were observed after 10 days. We suggest that a standard animal model can be developed based on group 3 results. We also think that drug trials concerned with protecting the salivary gland from the effects of chemotherapy can be conducted based on this model.

Key words: 5-Fluorouracil, mucositis, apoptosis, chemotherapy

5-Florourasil ile tedavi sonrası tavşan submandibuler bezinde gözlenen histopatolojik değişiklikler

Amaç: Bu çalışmanın amacı 5-fluorouracil (5-FU)'in submandibuler gland histolojisi üzerindeki erken dönem etkilerini değerlendirmektir.

Yöntem ve gereç: 30 adet Yeni Zelanda tipi tavşan üç gruba bölündü. 1. gruba (kontrol) ilaç verilmedi. 2. gruba 5 gün, 3. gruba 10 gün 5-FU verildi. Tavşanlara submandibuler bez eksizyonu yapıldı. Hematoksilen-eozin boyama ile histopatolojik muayene, TUNEL tekniğiyle apoptozis ve PCNA antikoruna ile hücre proliferasyonu yapıldı.

Bulgular: 1. grupta hiçbir granüler değişiklik gözlenmezken, 2. ve 3. gruptaki 5 tavşanda asinüs hücre sitoplazmalarında granüler değişiklik görüldü ($P < 0,05$). 3. grupta 1. ve 2. gruba göre TUNEL boyamayla asiner ve duktal hücrelerde daha fazla apoptozis görüldü ($P < 0,05$). Tüm gruplarda PCNA boyamayla asiner hücrelerde hücre proliferasyonu gözlenmezken, 3. grupta duktal hücre proliferasyonu istatistik olarak anlamlı derecede farklıydı ($P < 0,05$).

Sonuç: Bu çalışmada 5-FU'nun tükürük bezi üzerine toksik etkilerinin 10. günden sonra başladığını saptadık. Bu çalışmada olduğu gibi bir hayvan modeli geliştirilebileceğini ve bu modelle kemoterapi etkilerinden tükürük bezlerini koruyabilmek için ilaç denemeleri yapılabileceğini önermekteyiz.

Anahtar sözcükler: 5-Fluorouracil, mukozit, apoptozis, kemoterapi

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Introduction

The mucositis status (stomatitis, esophagopharyngitis, and diarrhea) secondary to the stomatotoxic effects of antineoplastic drugs increases the morbidity and cost of treatment in cancer patients. This complication also significantly affects the duration of hospitalization and survival rates. Oropharyngeal pain, eating and speech disorders, weight loss, oral infections, bacteremia, and sepsis can be seen in patients with mucositis. The risk of septicemia is 4-fold higher in neutropenic patients with mucositis (1). Mortality is as high as 40% in cases of severe ulceration (2). It was suggested that 5-fluorouracil (5-FU) leads to mucositis in 4%-74% of head and neck cancer cases, while the rate of mucositis was reported to be 40% in patients treated with chemotherapy (3).

In order to maintain oral hygiene, salivary glands should have normal histology and physiology. While the mucin glycoproteins in saliva decrease the permeability of the oral mucosa, they also help phonation, chewing, and swallowing by providing lubrication. Antibacterial-affecting products in saliva, such as lysozymes, lactoperoxidases, immunoglobulins, lactoferrins, and histatins, provide for the continuity of healthy oral mucosa. An effective agent or a gold standard therapy for the prophylaxis of oral mucositis is still not available (4).

5-FU is a fluorinated pyrimidine analogue used as an antineoplastic drug (5). It was synthesized after the observation that some tumor cells utilize uracil, a pyrimidine base suitable for normal body cells. It achieves its activity in the body by first transforming into fluorouridylate (FUMP), and then transforming into a type of deoxynucleotide—fluorodeoxyuridylate (5-fluoro-2'-deoxyuridine 5'-monophosphate)—in other words FdUMP (6). This active metabolite forms the trio-complex, combining with thymidylate synthase and N⁵⁻¹⁰ methylene tetrahydrofolate, a cofactor with folate, and inhibits the thymidylate synthase enzyme. When thymidylate production decreases in a cell, thymine synthesis is disrupted and DNA synthesis stops. Moreover, FUMP—formed from fluorouracil in the body—plays a role in the structure of RNA and thus disrupts RNA functions and protein synthesis (7,8); however, the most important antitumoral effect of 5-FU is the inhibition of thymidylate synthase (9).

5-FU sensitizes tissues to the effects of radiation; therefore, it can be used simultaneously with

radiotherapy in the primary treatment of head and neck, esophageal, lung, and rectal tumors (10,11). The primary side effects of 5-FU are anorexia, gastrointestinal disturbances, such as nausea and vomiting, and bone marrow depression. Dose-limiting side effects are mucositis, such as stomatitis, esophagopharyngitis, and diarrhea.

The aim of the present study was to determine the early-stage effects of 5-FU on the histology of the submandibular gland. Based on the obtained results, a standard animal model could be developed in order to monitor the histopathological changes following chemotherapy. This standard animal model could be used as a method to compare the efficacy of different drugs in the prophylaxis of these types of complications.

Materials and methods

Histopathological examination was performed with hematoxylin-eosin (H&E) staining, apoptosis was determined with TdT-mediated dUTP nick end labeling (TUNEL), and cell proliferation was determined using the proliferating cell nuclear antigen (PCNA) antibody.

Tissue samples

The study included 30 healthy white New Zealand rabbits weighing between 1800 and 2200 g. During the study the rabbits were divided into 3 groups and were supervised in a 15-m² closed balcony system illuminated by a night lamp. Mean temperature was maintained at 22 ± 3 °C. Appropriate nutrition was provided; the main food sources were green vegetables, carrots, and tap water. The Afyon Kocatepe University Animal Ethics Committee approved the study protocol. Each animal that underwent surgery was anesthetized with 25 mg kg⁻¹ IM ketamine hydrochloride (Ketalar[®], Eczacıbaşı Parke-Davis, İstanbul, Turkey) and 5 mg kg⁻¹ IM xylazine HCl (Alfazyne[®], Alfasan International B.V. Woerden, Holland). Submandibular gland excision was achieved surgically.

The 30 rabbits were divided into 3 groups, each containing 10 rabbits. No drugs were administered to group 1. Bilateral submandibular gland excision was performed on these control group rabbits under anesthesia (Table).

Table. The study design.

Group	Number of rabbits	Drug administration protocol (dose, duration)	Date of submandibular gland excision
1 (control)	10	–	day 5
2	10	4 mg kg ⁻¹ day ⁻¹ , 5 days	day 11
3	10	4 mg kg ⁻¹ day ⁻¹ , (5 + 5 days)	day 16

Group 2 was administered 4 mg kg⁻¹ 5-FU IM (5-FU®, Choongwae Pharma Corporation Seoul, Korea) for 5 days. From day 6 to 11 no drugs were administered to group 2. Bilateral submandibular gland excision was performed in group 2 rabbits under anesthesia on day 11.

Group 3 was administered 4 mg kg⁻¹ 5-FU IM for 5 days. Between day 5 and 11 no drugs were administered to group 3. On day 11, 4 mg kg⁻¹ 5-FU IM was again administered for another 5 days. Bilateral submandibular gland excision was performed on group 3 rabbits under anesthesia on day 16.

Two tissues were sampled from both right and left submandibular gland material. Routine tissue processing was performed after the sampled tissues were fixed for 24 h in buffered formalin solution. Then, 4-µm thick cross-sections were taken from paraffin-embedded tissues and were examined under a light microscope after H&E staining. During microscopic examination irregularity and hypertrophy in cytoplasmic granules, inflammatory cell infiltration, and fibrosis formation in acinar cells were noted.

Immunohistochemical staining

In order to examine cellular proliferation in the cross-sections placed on microslides with poly-L-lysine, immunohistochemical staining was performed according to the following procedure: the cross-sections were made step by step:

1. Cross-sections were deparaffinized and dehydrated.
2. They were boiled twice for 10 min in a 90-W microwave oven in citrate buffer (pH 6).
3. They were washed with Tris buffered saline.

4. To stop endogenous peroxide activity, they were incubated with 1% H₂O₂ for 5 min.
5. They were washed with Tris buffered saline.
6. They were incubated with PCNA antibody for 30 min (1:200 dilution, Neomarkers, USA) to evaluate cell proliferation.
7. They were washed with Tris buffered saline.
8. They were incubated in biotinized horseradish peroxidase (Labvision, USA) solution for 10 min.
9. They were washed with Tris buffered saline.
10. They were left in a 3-amino-9-ethylcarbazole solution, as a chromogen, for 5-15 min while the staining concentration was monitored.
11. They were processed with distilled water.
12. Mayer's hematoxylin (10 min) was used for contrast staining.
13. They were washed with distilled water.
14. They were assembled after drying.

The stained preparations were then examined under a light microscope.

Immunohistochemical evaluation

In total, 500 acinar and ductal cells were counted, and the nuclei that stained positively with PCNA antibody were noted in every cross-section. The number of positive cells in each group was calculated and the mean values for each group were determined. Mean group values were statistically compared.

TUNEL staining for apoptosis

The TUNEL (TdT-mediated dUTP nick end labeling) technique was used to determine which cells led to apoptosis in the tissues. Cross-sections 2-µm

thick were placed on microslides with poly-L-lysine for the staining procedure. The staining procedure was performed according to the manufacturer's instructions (Roche-Applied-Science Cat no: 1 684 817); however, some modifications were made in order to obtain optimum staining. The cross-sections were treated as follows:

1. They were deparaffinized and dehydrated.
2. They were washed with PBS (phosphate buffered saline).
3. They were incubated with 0.1% trypsin for 10 min at 37 °C.
4. They were boiled twice for 10 min in a 350-W microwave oven.
5. They were washed with PBS.
6. They waited in 50 µL of the TUNEL mixture solution at 37 °C for 60 min in a dark, moist medium.
7. They were washed with PBS.
8. They were incubated in 50 µL of converter-POD (peroxidase) solution at 37 °C for 120 min in a moist medium.
9. They were washed with PBS.
10. They were incubated in 50-100 µL of diaminobenzidine solution for 15-25 min.
11. They were washed with PBS.
12. Contrast staining was performed with methylene green with 5 min of waiting at room temperature.
13. They were washed with distilled water.
14. They were dried and assembled with glycerin.

Evaluation of apoptotic cells

Acinar and ductal cells that stained brown with diaminobenzidine chromogen were counted in 10 different areas in every cross-section under a light microscope at 400× magnification. The number of apoptotic cells in 10 different microscopy areas was calculated. The mean value of acinar and ductal apoptotic cell groups was obtained for every salivary gland, and these mean apoptotic cell group values were compared.

Statistical analysis

The chi-square test was used to compare the results of H&E staining. The Kruskal-Wallis test was used to compare the results of PCNA and TUNEL staining.

Results

Histopathological examination of the submandibular glands that were excised from the rabbits in all 3 groups showed no evidence of fibrosis or an increase in inflammatory cells. No granular change in the cytoplasm of acinar cells was observed in group 1 (control group) (Figures 1 and 2). Granular changes were observed in 5 rabbits (50%) in group 2 and in 5 rabbits (50%) in group 3 (Figure 3). While there was a statistically significant difference between the control group and the other 2 groups ($P < 0.05$), the difference between groups 2 and 3 was not significant ($P < 0.05$).

The rate of apoptosis in submandibular gland acinar cells was higher in group 3 than in groups 1 and 2, based on histopathological examination with TUNEL staining (Figure 4). While there was a statistically significant difference between group 3 and the other 2 groups in terms of the rate of apoptosis ($P < 0.05$), a statistically significant difference was not observed between groups 1 and 2 ($P < 0.05$).

The rate of apoptosis in submandibular gland ductal cells was higher in group 3 than in groups 1 and 2, based on histopathological examination with TUNEL staining (Figure 5). While there was a statistically significant difference between group 3 and the other 2 groups, in terms of the rate of apoptosis ($P < 0.05$), a statistically significant difference was not observed between groups 1 and 2 ($P < 0.05$).

Cellular proliferation in the acinar cells was not observed in any of the groups with PCNA staining. However, while there was a statistically significant difference between group 3 and the other 2 groups, in terms of cellular proliferation in ductal cells ($P < 0.05$) (Figure 6), a significant difference was not observed between groups 1 and 2 ($P < 0.05$). There was marked hair loss, localized alopecia, malnutrition, and weight loss in groups 2 and 3.

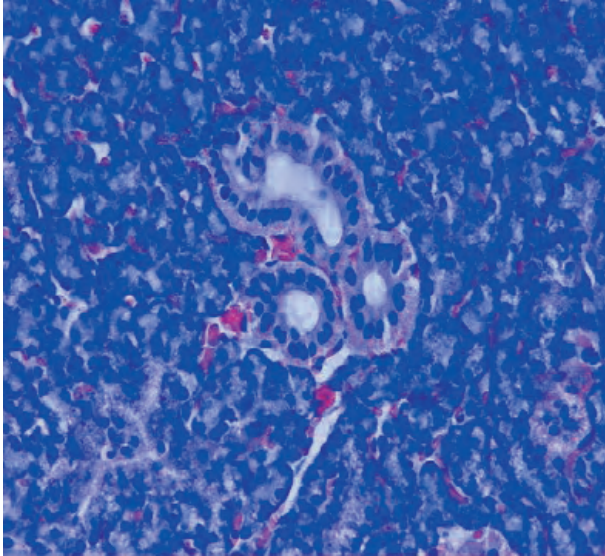


Figure 1. Normal salivary gland; regular structured acini and ducts. One-layered cubic epithelium is laid on the ducts (H&E $\times 400$).

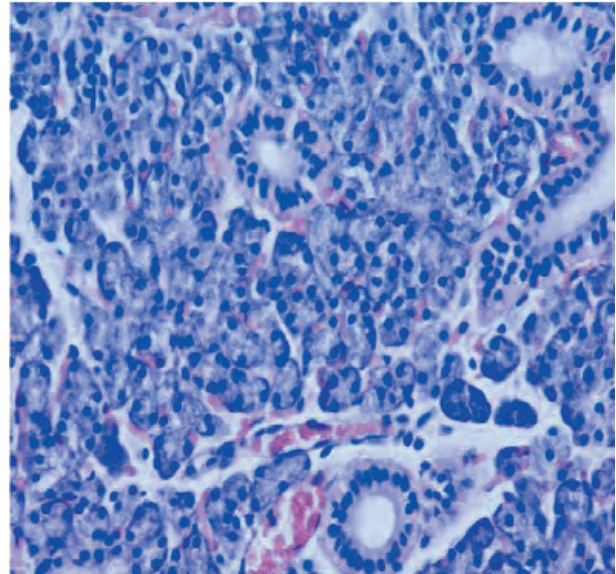


Figure 2. Normal salivary gland; regular structured acini and ducts. Acini have thin granular cytoplasm (H&E $\times 400$).

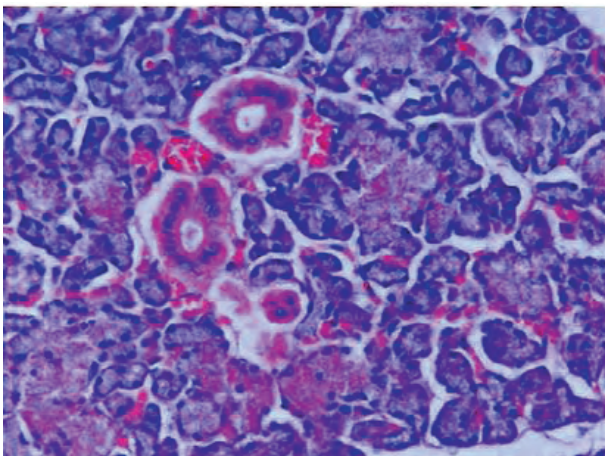


Figure 3. Chemotherapy effects in the acinus; the granules in the acinus cell cytoplasm are irregular and large (H&E $\times 400$).

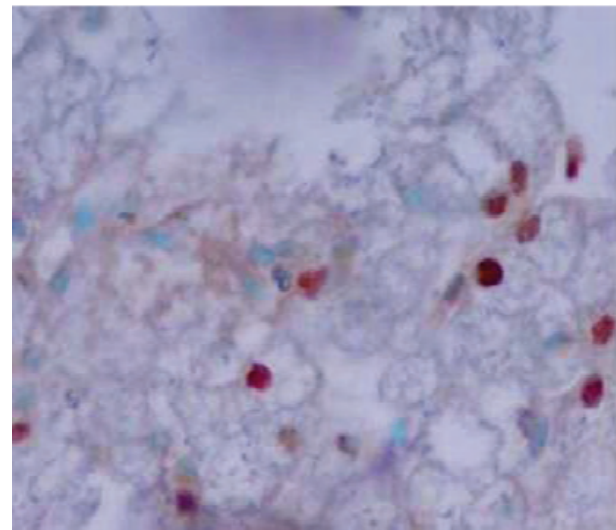


Figure 4. Apoptotic cells that are stained brown in the acinus cells (TUNEL $\times 400$).

Discussion

Oral mucositis is a serious complication that increases morbidity and the cost of treatment in cancer patients treated with radiotherapy and chemotherapy. 5-FU is one of the most popular and effective antineoplastic drugs used in the treatment of head and neck cancers—including salivary gland

cancers (12). Chemotherapeutic agents, such as 5-FU, methotrexate, etoposide, and irinotecan, are mucotoxic and increase the incidence of mucositis (4). Not only do these stomatotoxic conditions negatively affect the quality of life and nutrition of patients, they delay implementation of treatment protocols and dose reduction.

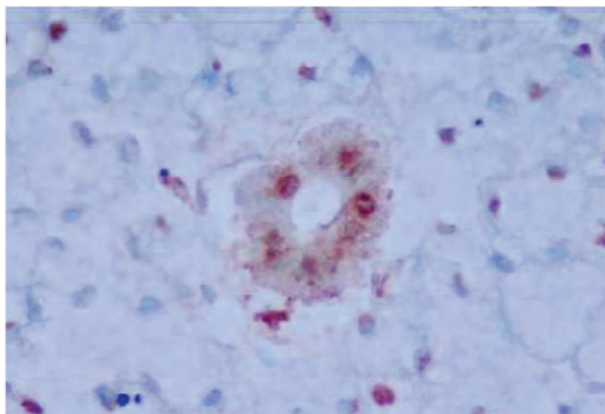


Figure 5. Apoptotic ductal cells that are stained brown in the ductus (TUNEL $\times 400$).

Reduced saliva secretion is one of the factors that increase patient susceptibility to mucotoxic effects. McCarthy et al. reported that 46% of patients had oral mucositis during a 2-month follow-up of 63 gastrointestinal system cancer patients treated with 5-FU, and that xerostomia increased their susceptibility to mucositis (13). They also reported that the saliva secretion rate is an important marker for oral mucositis and highlighted the necessity of routine follow-up of basal saliva secretion in patients that receive stomatotoxic treatment. In contrast, Jensen et al. suggested that such factors as the lack of standard research methods, scarcity of research subjects, short-term studies, and different treatment protocols prevent a clear understanding of some of the effects of chemotherapeutic agents on salivary gland functions (14).

After 1 dose of methotrexate IP injection given to rats, acinar and ductal cell vacuolization and swelling, apoptosis in the acinar cells with pyknosis in the nuclei, and a reduction in secretion granules were reported as early pathological findings within the first 7 days, based electron microscopic examination (15). The same authors also reported that the severity and prevalence of pathological changes increased as chemotherapeutic doses increased. McBride and Siegel reported that the number of beta-adrenergic receptors increased in the submandibular glands of rats injected with methotrexate $15 \text{ mg kg}^{-1} \text{ day}^{-1}$ IP for 3 days (16).

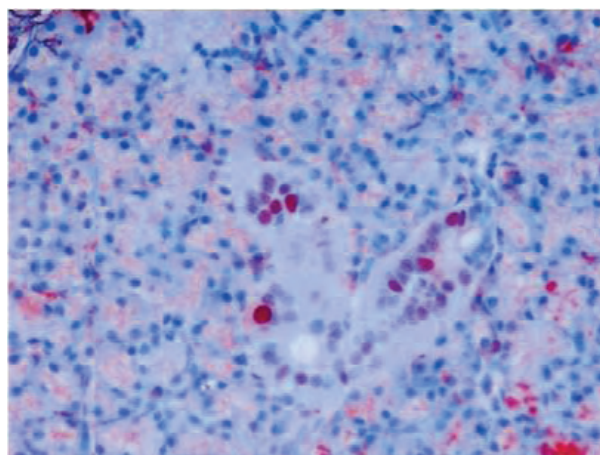


Figure 6. Proliferative cells that are stained red in the nuclei of ductal epithelial cells by PCNA antibodies (Immunohistochemical staining $\times 400$).

In the present study no inflammatory cell increase or fibrosis was observed in the submandibular gland cross-sections in any of the 3 groups upon light microscopic examination. Granular changes were observed in the cytoplasm of acinar cells in the submandibular gland of the rabbits in groups 2 and 3; the difference between the control group (group 1), and groups 2 and 3 was statistically significant ($P < 0.05$). This condensation in the granules indicates non-specific cytoplasmic degeneration and shows reversible cellular damage (17). The secretory granules, especially those seen in the serous cells, include many proteolytic enzymes and heavy metals (18). Normal rabbit submandibular gland histology shows a mixed type, including serous tubules proximally and seromucous acinar cells distally (19).

While apoptosis was not observed in groups 1 and 2 in the present study, it was observed in the acinar and ductal cells in group 3. These results show that the initial changes in normal salivary glands in rabbits treated with 5-FU were observed in the cytoplasmic granules. It was suggested that serous cells lead to autolysis as a result of such granular damage, as well as secretion of proteolytic enzymes in the granules into the cytoplasm (20). Cellular infiltration towards the subepithelial tissue, vascular dilatation, and leukocyte margination were observed in oral mucosa biopsies of rats treated with 5-FU and bleomycin as early as 24 h after drug administration (4).

We think that salivary gland damage does not play a primary role in the development of mucositis, based on the fact that granular changes were observed in 50% of the rabbits in groups 2 and 3 following 5-FU administration for 5 and 10 days, respectively. Apoptosis, as observed in the acinar and ductal cells in group 3, shows that salivary gland damage increased as the dose increased. Aota et al. reported that 5-FU, which is often used in the treatment of salivary gland cancer, induces apoptosis in salivary gland cancer cells and that suppression of NF- κ B activity may be a mechanism involved in the development of apoptosis (21). P53, c-jun, and interferon-regulating factor 1 are other known transcription factors associated with apoptosis (22).

Salivary gland malignancies are reported to be resistant to both radiotherapy and chemotherapy (23). A lifespan for salivary gland cells of between 60 and 120 days, low mitotic activity, and frequent G₀ period of the cell status are among the factors that account for the resistance of tumors (24). In the present study the development of apoptosis and cytoplasmic granular changes in the acinar and ductal cells after 10 days of 5-FU administration shows that normal salivary glands were affected by 5-FU.

In the present study PCNA antibody positivity, which indicates proliferation in ductal cells, was significantly higher in group 3 than in groups 1 and 2. No proliferation was observed in the acinar cells in any of the 3 groups. This finding suggests that regeneration in the ductal cells after cellular damage occurred earlier than in the acinar cells.

Conclusion

The present study determined that the first sign of the effect of 5-FU on acinar cells in the submandibular gland was changes in cytoplasmic secretory granules, according to histopathological examination with a light microscope. The damage caused by these cytoplasmic secretory granules should be considered among the mechanisms that lead to cell apoptosis.

It is known that the signs of mucositis appear within the first 24-48 h of the start of chemotherapy. In contrast, it can be understood that the negative effects of 5-FU on the salivary gland are not primary factors in the development of mucositis, as a statistically significant difference in the rate of apoptosis was not observed between the control group and group 3, based on cross-sections that were examined after 11 days of 5-FU administration. It is more plausible to consider that there is an association between salivary gland functions and the deterioration of late-stage mucositis.

We suggest that a standard animal model can be developed based on group 3 results. We also think that drug trials concerned with protecting the salivary gland from the effects of chemotherapy can be conducted according to this model.

The fact that proliferation occurred earlier in the ductal cells is proof that regeneration or amelioration began earlier than in the acinar cells. Nonetheless, with the administration of a drug that protects salivary gland functions, the appearance of such regeneration in the acinar cells can be used as a criterion.

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