An elemental diet protects mouse salivary glands from 5-fluorouracil-induced atrophy

KOJI HARADA[1], TARANNUM FERDOUS[1], RIEKO FUJIWARA[1], KENJI WATANABE[2], YOICHI MIZUKAMI[2] and KATSUAKI MISHIMA[1]

[1]Department of Oral and Maxillofacial Surgery, Yamaguchi University Graduate School of Medicine; [2]Center for Gene Research, Yamaguchi University, Ube, Yamaguchi 755-8505, Japan

Received October 2, 2021; Accepted February 14, 2022

DOI: 10.3892/ol.2022.13298

Abstract. An elemental diet (ED) reduces adverse effects of chemotherapy, including oral mucositis, in patients with cancer. However, the detailed mechanism(s) of the healing effects of an ED remains unclear. In the present study, the protective effects of the ED, Elental®, were examined against 5-fluorouracil (5-FU)-induced oral mucositis and salivary gland atrophy in mice. Mucositis was induced in female ICR mice by injection of 5-FU. The mice were orally administered Elental® (ED group) or saline (control group). After treatment, the mice body weight, salivary gland weight and the histological changes in the salivary gland granular duct area were monitored. The mice body weight remained stable in the ED group, but was significantly decreased in the control group. Moreover, the salivary gland weight was higher in the ED group compared with the control group. In addition, the salivary gland granular duct area cells were larger in the ED group compared with the control group. Whole transcriptome analysis and network analysis were conducted to understand the mechanisms of action of Elental® against oral mucositis. Whole transcriptome analysis and Ingenuity Pathways Analysis data suggested that Elental® contributed to the recovery of mitochondrial function in 5-FU-damaged salivary glands. Immunohistochemical analysis of salivary gland tissue demonstrated that the expression of cytochrome c oxidase subunit 4 and epidermal growth factor were higher in the ED group compared with the control group. Next, the rate of apoptosis in the salivary glands was examined using terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) assays. The number of TUNEL-positive cells in the salivary glands was lower in the ED group compared with the control group. These findings suggested that Elental® may protect mouse salivary glands from 5-FU-induced atrophic changes, which suggests that ED treatment may improve xerostomia and alleviate oral mucositis in patients with cancer receiving 5-FU-based chemotherapy.

Correspondence to: Dr Koji Harada, Department of Oral and Maxillofacial Surgery, Yamaguchi University Graduate School of Medicine, 1-1-1 Minamikogushi, Ube, Yamaguchi 755-8505, Japan
E-mail: harako@yamaguchi-u.ac.jp

Key words: elemental diet, 5-fluorouracil, salivary gland, granular duct, whole transcriptome analysis, network analysis

Introduction

Chemotherapy may induce both acute toxicities (mucositis, infection, bleeding) and late toxicities (atrophy, xerostomia) in patients with cancer (1). Oral mucositis is one of the most common oral complications resulting from cancer chemotherapy. According to an article published in 2020, globally 30-40% of patients with cancer treated with chemotherapy develop oral or gastro-intestinal mucositis (2). In patients with head and neck cancer (HNC) treated with both chemo- and radiotherapy, 90% of the patients developed mucositis (3). Oral mucositis is often accompanied with acute oral pain and a compromised nutritional status, in addition to reduced kidney, liver and salivary gland function (1,4). To the best of our knowledge, the mechanism of chemotherapy-induced mucositis is unknown and likely involves a multistep process, which may be associated with several factors. Chemotherapy can have adverse effects on any healthy rapidly multiplying cells including the mucosal lining of the mouth and gastro-intestinal tract. This may lead to the loss of the renewal capacity of the epithelium, which ultimately results in atrophy and ulcers (5-7). Mucositis is not limited to the epithelium; it might involve all the mucosal tissues. The severity of mucositis depends not only on anticancer regimens but also on patients' characteristics. Female patients, elderly patients and patients with a dihydropyrimidine dehydrogenase deficiency may develop severe 5-FU-induced mucositis (8). There are very few therapeutic options available for mucositis prevention or treatment, and unfortunately their effectiveness is poor or negligible (7,9-14). Therefore, novel strategies to alleviate mucositis in patients with cancer are urgently needed.

Elental® (Ajinomoto Co., Inc.) is a widely used nutritional supplement in Japan for patients suffering from malnutrition. This elemental diet (ED) has a special formula consisting of 18 amino acids with high L-glutamine (2,415 mg/100 gm) and L-leucine (1,124 mg/100 gm) content. It also contains eight minerals, 14 vitamins and dextrin as the major energy source (Table I) (3). Several reports have demonstrated that

ED with glutamine has beneficial effects against Crohn's disease and chemotherapy-induced mucositis in patients with cancer (15-20). Moreover, weight loss is a common side effect of advanced cancer and leucine can stimulate muscle protein synthesis (16). In our recent clinical studies, we successfully used Elental® to treat malnutrition as well as chemotherapy-induced oral mucositis and dermatitis in patients with HNC (21,22). Tanaka *et al* (3) reported Elental® can reduce adverse events in patients with esophageal cancer receiving docetaxel/cisplatin/5-fluorouracil in multicenter study of a phase III randomized controlled clinical trial. These observations led to the hypothesis that Elental® might be useful against 5-fluorouracil (5-FU)-induced atrophic changes in salivary glands.

Cytochrome c (Cytc) and Cytc oxidase (COX) catalyze the terminal reaction of themitochondrial electron transport chain (ETC), which is important in the production of cellular energy (23). Among numerous mitochondrial marker genes, COX subunit 4 (COX IV) has a pivotal role in the regulation of cellular energy metabolism, mitochondrial function and oxidative phosphorylation (24-26). Malfunction or reduced activity of COX is caused by mitochondrial defects, which might be associated with a number of human diseases and disorders, including stroke, heart or liver diseases, and cancer (23,26). Under these stressful conditions, the ETC produces reactive oxygen species, which may trigger cell death processes and tissue damage (23,26). Moreover, downregulation of COX IV may result in mitochondrial dysfunction and oxidative stress, which may inhibit epithelial repair processes and wound healing (25,27,28).

In patients with cancer undergoing chemotherapy and radiotherapy, a high incidence of mucositis is often correlated with decreased saliva secretion (29,30). Salivary gland hypofunction may induce xerostomia, which negatively affects oral health as well as the nutritional status of patients (30). Saliva, a mixture of water, ions and proteins, is mainly produced by three pairs of major salivary glands: The parotid, submandibular and sublingual glands. Saliva is rich in immunoglobulin A (IgA) and epidermal growth factor (EGF) (31). IgA plays an important role in mucosal immunity and EGF is required for tissue repair in salivary glands (24,32-34). It has been suggested that reduction in both saliva and EGF may be associated with the severity of oral mucositis (32,35,36). It is also generally accepted that salivary EGF may promote wound healing and may be an effective treatment for gut ulcers (30).

In the present study, an animal model was used to evaluate the efficacy of the ED, Elental®, for treating oral mucositis and salivary gland atrophy associated with cancer chemotherapy. The present examined the effect of Elental® treatment on the body weight and salivary gland weight of 5-FU-treated mice, as well as its beneficial effects against 5-FU-induced oral mucositis. Furthermore, the underlying mechanisms of the healing effects of Elental® were investigated.

Materials and methods

Animals. A total of 6 ICR female mice at 10 weeks of age (average, weight, 31.5; range, 30-33 g) were purchased from CLEA Japan Inc. They were housed in a temperature-controlled (20-25˚C) and pathogen free environment with ~69.31% humidity and a 12 h light/dark cycle. The mice were provided with a commercial diet (CRF-1; Oriental Yeast Co., Ltd.) and sterilized water *ad libitum*. Surgical procedures and animal treatments were conducted in accordance with the Guidelines for Animal Experimentation of Yamaguchi University (Ube, Japan). All *in vivo* experiments were approved by the Institutional Animal Care and Use Committee of Yamaguchi University (approval no. 55-017).

Oral mucositis induction and ED treatment in mice. After a 7 day habituation period, mice were divided into two groups: Control group (n=3) and ED group (n=3) with similar mean body weights. In order to induce atrophic changes in the salivary glands, all mice were injected intraperitoneally with 20 mg/kg/day of 5-FU (Kyowa Kirin Co., Ltd.) twice per week for 3 weeks. Simultaneously, the ED group received 1.6 kcal/0.8 ml/day of Elental® (Ajinomoto Co., Inc.) orally (twice/day; 5 times/week for 3 weeks) and the control group received the same amount of saline administered orally (twice/day, 5 times/week for 3 weeks). The duration of the experiment was 21 days. The body weight and health of the mice were checked every other day until the end of the experiment (day 21). The humane end point of the experiment was decided as ≥40% loss of body weight, severe diarrhea or loss of appetite. On day 21, the experiment was terminated, and all six mice were sacrificed via cervical dislocation. Mortality was confirmed by checking the loss of respiratory movement and discoloration of the ocular bulb. The salivary glands (the submandibular gland and sublingual gland) were collected and weighed. Next, half of each samples were kept at -80˚C in RNA-later (Thermo Fisher Scientific, Inc.) for whole transcriptome analysis, and the other half of the samples were fixed with 10% neutral buffered formalin (Mildform® 10N; FUJIFILM Wako Pure Chemical Corporation) at room temperature overnight and were paraffin-embedded. The tissue sections were then subjected to hematoxylin and eosin (HE) staining, immunohistochemical (IHC) staining, and terminal deoxynucleotidyl transferase (TdT)-mediated nick end labeling (TUNEL) assays as described below.

HE staining and observation. Salivary glands were fixed in 10% neutral buffered formalin at 4˚C for 24 h, and then were embedded in paraffin. The 4 μm-thick tissue sections were subjected to HE staining. The entire procedure of HE staining was carried out at room temperature. The tissue sections were immersed in xylene followed by rehydration in graded ethanol series (100-70%). After washing with tap water, the tissue sections were immersed in hematoxylin for 10 min, 1% acid ethanol for 30 sec and 1% ammonia water for 30 sec. The sections were washed with running tap water again and then treated with eosin for 1 min, followed by graded ethanol (70-100%) and xylene. After clearing the section with Histo-clear (National Diagnostics), the slides were mounted with glass coverslips using DPX mounting medium (Sigma-Aldrich; Merck KGaA). The stained sections were observed at x200 magnification using a BX51 fluorescence microscope (Olympus Corporation).

Whole transcriptome analysis. Total RNA was isolated from salivary gland samples using the RNeasy Mini kit (Qiagen

Table I. Composition of Elental® (one package=80 g).

Composition	Amount
Energy (kcal)	300
Carbohydrate (g)	
Dextrin	63.41
Fat (g)	
Soy bean oil	0.51
Amino acid total (g)	14.1
Amino acid (mg)	
L-Isoleucine	642
L-Leucine	899
Lysine hydrochloride	888
L-Methionine	648
L-Phenylalanine	871
L-Threonine	523
L-Tryptophan	151
L-Valine	701
L-Histidine hydrochloride monohydrate	501
L-Arginine hydrochloride	1,125
L-Alanine	899
L-Aspartic acid magnesium potassium	1,036
L-Aspartic acid sodium monohydrate	867
L-Glutamine	1,932
Aminoacetic acid	505
L-Proline	630
L-Serine	1,159
L-Tyrosine	110
Branched-chain amino acids or BCAA (mg)	2,242

Information was obtained from the study by Tanaka *et al* (3).

GmbH). The quality of RNA was examined with an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.) using RNA 6000 Nano kit (Agilent Technologies, Inc.) after the concentrations were determined by Qubit (Thermo Fisher Scientific, Inc.). The RNA Integrity Number values were >7 in all samples, indicating that the samples contained high-quality RNA. The expression libraries were produced using the NEBNext ultra II RNA library prep kit (cat. no. E7770L; New England BioLabs, Inc.) and NEBNext Multiplex Oligos for Illumina (cat. no. E7335S; New England BioLabs, Inc.). According to the manufacturer's protocol, total RNA (500 ng) extracted from each sample was reverse-transcribed into cDNA with the NEBNext Ultra II RNA First Strand Synthesis Module (cat. no. E7771; New England BioLabs, Inc.), and then the index sequences were inserted during PCR amplification. Following initial denaturation at 98˚C for 30 sec, amplification was performed for 9 cycles of a denaturation at 98˚C for 10 sec, annealing at 65˚C for 75 sec and extension at 65˚C for 75 sec with a final extension at 65˚C for 5 min. Primers were as follows: NEBNext Index 6 Primer for Illumina, 5'-CAAGCAGAAGACGGCATACGAGATATTGGCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-3'; NEBNext Index 7, 5'-CAAGCAGAAGACGGCATACGAGATGATCTGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-3'; NEBNext Index 8, 5'-CAAGCAGAAGACGGCATACGAGATTCAAGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-3'; NEBNext Index 9, 5'-CAAGCAGAAGACGGCATACGAGATCTGATCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-3'; NEBNext Index 10, 5'-CAAGCAGAAGACGGCATACGAGATAAGCTAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-3'; NEBNext Index 11, 5'-CAAGCAGAAGACGGCATACGAGATGTAGCCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-3' and NEBNext Universal PCR Primer, 5'-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT-3'. The quality of the library was examined with a Bioanalyzer (Agilent Technologies, Inc.) after purification using AMPure XP beads (cat. no. A63882; Beckman Coulter, Inc.). The libraries were sequenced on an Illumina Next-seq sequencer (Illumina, Inc.) with an Illumina NextSeq High Output 150 bp pair-end cycle sequencing kit (cat. no. 20024907; Illumina, Inc.). More than 30 million reads in each sample were detected in the reaction and were trimmed and mapped with the mouse reference genome GRCm38 release-92 using CLC Genomics Workbench software (ver.8.01; Qiagen GmbH); and the mapping ratio was 98%.

Principal component analysis (PCA). PCA is a type of data dimension reduction algorithm which was used to verify the quality of the gene expression data. The analyses of differential gene expression profiles were conducted using JMP Pro software (ver.15.0.0; SAS Institute, Inc.) and then dimension reduction analysis was performed. The principal component 1 (PC1) was plotted on the x-axis and the principal component 2 (PC2) was plotted on the y-axis of the scatter diagram. Each data point represents a sample. The differential gene expression pattern based on the control (C) and ED (E) groups were analyzed using PCA.

Ingenuity network analysis. Co-relation analysis was conducted with Prism (ver.9.0.3; GraphPad Software, Inc.). The gene sets that demonstrated significantly increased or decreased expression in the co-relation analysis were then examined by network analysis using the IPA software (version 8.6, Qiagen GmbH). The IPA software revealed the molecular and cellular functions of the dataset members as well as the canonical pathways represented in the dataset. The IPA software is derived from a vast amount of molecular interactions reported in the literature and the software is updated weekly (37). The IPA uses a Fisher's exact test to determine whether the differentially expressed genes are significantly related to pathways compared with the whole ingenuity knowledge base.

Volcano plot. A volcano plot was constructed to identify the differentially expressed gene between the ED group and the control group. The horizontal and vertical coordinates denoted the average expression value of each gene between these two groups. The screening criteria cutoff comprised a log2 fold change of >3 or <-3 with P<0.01 using JMP Pro software (ver.15.0.0; SAS Institute, Inc.). The significantly upregulated genes are shown in red, and the significantly downregulated genes are shown in green.

IHC staining. The expression profiles of COX IV and EGF in mice salivary glands were detected using IHC analyses. Paraffin-embedded 4 μm-thick tissue sections were immersed in xylene at room temperature and then rehyraded in graded ethanol (100-70%). Next, the sections were washed with phosphate buffered saline (PBS) at room temperature, immersed in a Target Retrieval Solution (pH 9; Agilent Technologies, Inc.) and heated in a microwave for 10 min. Endogenous peroxidase activity was quenched with a 0.3% hydrogen peroxide/methanol mixture for 20 min. Next, the sections were rinsed in PBS and incubated with Dako REAL™ peroxidase-blocking solution (Agilent Technologies, Inc.) at room temperature for 30 min, followed by an overnight incubation at 4˚C with anti-COX IV rabbit polyclonal antibody (1:200; cat. no. 11242-1-AP; ProteinTech Group, Inc.) and anti-EGF rabbit polyclonal antibody (1:200; cat. no. AP12878c; Abcepta). After rinsing in PBS for 10 min, the tissue sections were incubated with a secondary antibody (undiluted; Dako REAL™ EnVision™ Detection system, HRP conjugated; cat. no. K5007; Agilent Technologies, Inc.) at room temperature for 30 min, and the expression of COX IV and EGF were detected using the Dako REAL™ EnVision™ detection system (Agilent Technologies, Inc.) according to the manufacturer's instructions. Lastly, the tissues were rinsed in tap water, and then counterstained with hematoxylin for 1-2 min at room temperature. They were subsequently dehydrated in graded ethanol (70-100%) and xylene, cleared with Histo-clear (National Diagnostics) and mounted with glass coverslips using DPX mounting medium (Sigma-Aldrich; Merck KGaA). The tissue sections were then observed at x200 magnification using a BX51 fluorescence microscope (Olympus Corporation).

TUNEL assay. In order to detect apoptotic cells in control and ED-treated salivary glands, a TUNEL assay was performed with 4 μm-thick paraffin sections from biopsy tissues using the DeadEnd™ Colorimetric TUNEL System (Promega Corporation) according to the manufacturer's instructions. Briefly, the tissue sections were deparaffinized by xylene and rehydrated in graded ethanol (100-50%) at room temperature. The tissues were then incubated in 0.85% NaCl for 5 min, fixed in 4% paraformaldehyde solution for 15 min at room temperature and washed with PBS. Next, they were treated with 20 μg/ml proteinase K at room temperature for 15 min and were immersed in 4% paraformaldehyde solution again at room temperature for 5 min followed by a PBS wash. The tissues were incubated in a 3% hydrogen peroxide solution and then in an equilibration buffer (0.05 M phosphate buffer containing 0.145 M NaCl, pH 7.4). Next, the rTdT reaction mix (rTdT enzyme and biotinylated nucleotide mix dissolved in equilibration buffer) was added to the samples and they were incubated in a humidified chamber at 37˚C for 60 min. The enzymatic reaction was stopped by immersing the tissues into a stop wash buffer for 10 min at room temperature. After a PBS wash, the tissue sections were incubated with anti-digoxigenin-peroxidase conjugate for 30 min at room temperature, and then with diaminobenzidine for 5 min at room temperature. Hematoxylin was used as a counterstain at room temperature for 1 min. In three random fields of each tissue section, at least 100 cells were counted under x100 magnification using a BX51 fluorescence microscope (Olympus Corporation). The number of apoptotic cells (TUNEL-positive cells) was calculated by dividing the number of TUNEL-positive cells by the total number of counted cells, and the result was expressed as a percentage (TUNEL-positive score).

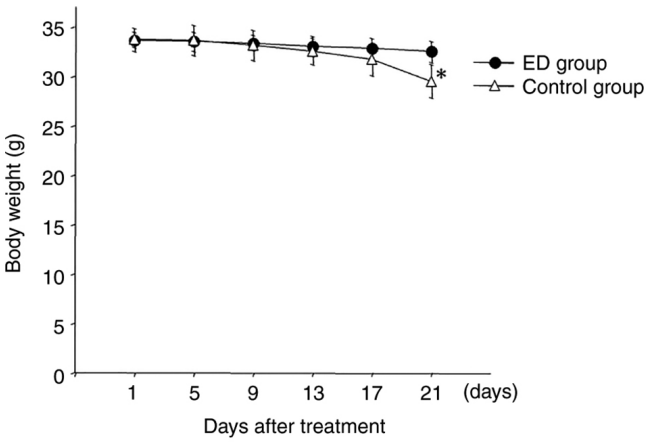

Figure 1. Effect of an ED on body weight of mice injected with 5-FU. Saline-treated (control) mice and Elental®-treated (ED) mice were injected with 5-FU. The body weight of ED-treated mice remained stable during the treatment period. The body weight of the control group mice gradually decreased over time, and a significant difference was observed on day 21 between the ED group and the control group. n=3 mice per group. *P<0.01 vs. control group. ED, elemental diet; 5-FU, 5-fluorouracil.

Statistical analysis. All data are indicated as the mean ± standard deviation. The mice body weight data and the TUNEL data from the ED group were compared with the control group using Mann-Whitney U tests. P<0.05 was considered to indicate a statistically significant difference. StatView software (version 5.0J; SAS Institute, Inc.) was used for statistical calculations.

Results

Body weights and health of the control and ED group members. Fig. 1 presents the body weight changes in mice during the treatment period. The body weight of mice in the ED group remained stable even after 5-FU administration throughout the treatment period. However, the body weight gradually decreased in the control group after day 9. At the end of the experiment (day 21), a significant difference in body weight was observed between the ED group and the control group. During the experiment, all mice were healthy until they were sacrificed on day 21. These data demonstrated that Elental® helped to maintain the body weight of 5-FU-treated mice.

Salivary gland weight and histology. The weight and histology of murine salivary glands were compared between the control and ED groups. The salivary gland weight was higher in the ED group (0.212±0.190 g) compared with the control group (0.140±0.027 g) (Fig. 2). The histology of the salivary glands is presented in Fig. 2. A higher level of atrophy was observed in the salivary acini cells from control group mice compared with ED group mice. Moreover, histological changes in the granular duct area of the salivary glands were more prominent in the ED group mice compared with the control group mice

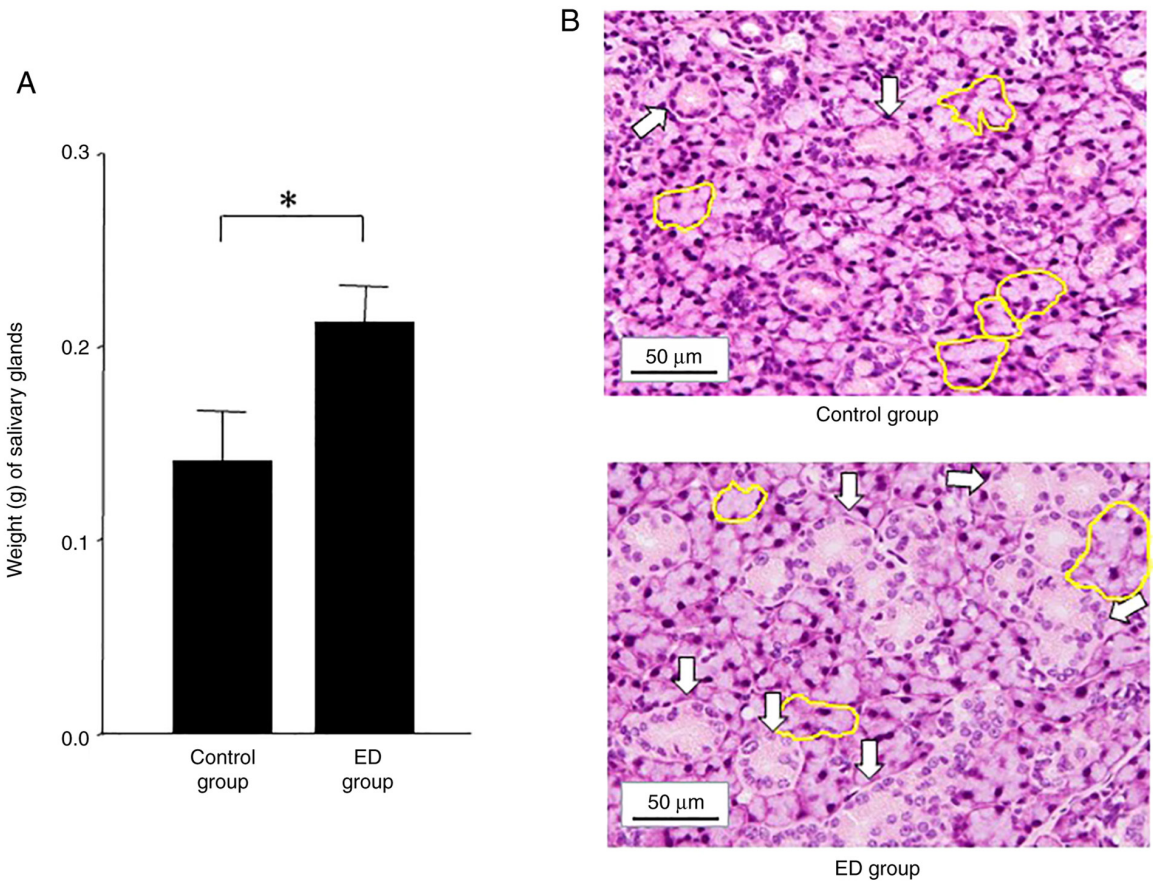

Figure 2. Effects of ED on salivary glands of mice injected with 5-FU. Saline-treated (control) mice and Elental®-treated (ED) mice were injected with 5-FU. (A) Salivary gland weight was measured and was significantly higher in the ED group compared with the control group. (B) Histological changes in salivary glands of mice observed by HE staining. In control tissue, salivary acini cells (yellow line) and granular duct cells (arrow) atrophied upon treatment with 5-FU (magnification, x200; scale bar, 50 μm). By contrast, mice in the ED group appeared to recover from 5-FU-induced atrophy. n=3 mice per group. *P<0.01 vs. control group. ED, elemental diet; 5-FU, 5-fluorouracil; HE, hematoxylin and eosin.

(Fig. 2). In addition, the granular duct cells in the ED group appeared larger compared with the control group. In brief, ED treatment could heal 5-FU-damaged salivary glands in nude mice.

ED mechanism of action in the salivary gland. The ED mechanism of action was investigated by whole transcriptome analysis, which identified differentially expressed genes between the salivary glands from the ED group and control group of mice. These differentially expressed genes were subjected to IPA in order to assign functions to the genes as well as identify any pathways represented in the differentially expressed gene dataset. PCA was used to verify the data quality (Fig. 3A). The PCA data demonstrated that the distance between the samples in the control group (C) was small, indicating that the gene expression patterns were similar. However, the distance between samples from the ED group (E) was relatively large; therefore, the gene expression patterns of E were different compared with that of C (Fig. 3A). A volcano map was created which represents the average value of each expressed gene between the ED group and control group. Significantly upregulated genes are presented in red, and significantly downregulated genes are presented in green (Fig. 3B). IPA analysis of these upregulated genes (>1.5-fold) revealed that the 'oxidative phosphorylation' pathway was the top canonical pathway related to these differentially expressed genes. The second pathway among the top canonical pathways was the 'mitochondrial dysfunction'-related pathway (Fig. 3B). The data obtained from IPA were also used to understand the relationships and functional interaction network among these differentially expressed genes. Fig. 3C presents the oxidative phosphorylation pathway-related network and Fig. 3D indicates the mitochondrial function-related network associated with these differentially expressed genes (Control vs. ED). These data demonstrated that a number of the upregulated genes in the salivary glands of the ED group were associated wih mitochondrial functions. This suggested that the healing function of Elental® might be associated with the recovery of mitochondrial function in 5-FU-damaged cells.

Expression of COX IV and EGF in salivary glands of 5-FU-treated mice. The expression of COX IV in the salivary glands of control and ED mice were examined by IHC because it is an important mitochondrial marker. IHC analysis was also used to detect the expression of EGF in salivary gland tissues. The expression of both COX IV (Fig. 4A) and EGF (Fig. 4B) in the salivary glands were higher in the ED group compared with the control group. These data indicated that ED might heal 5-FU-damaged salivary gland tissues by upregulating EGF and by recovering mitochondrial functions in cells.

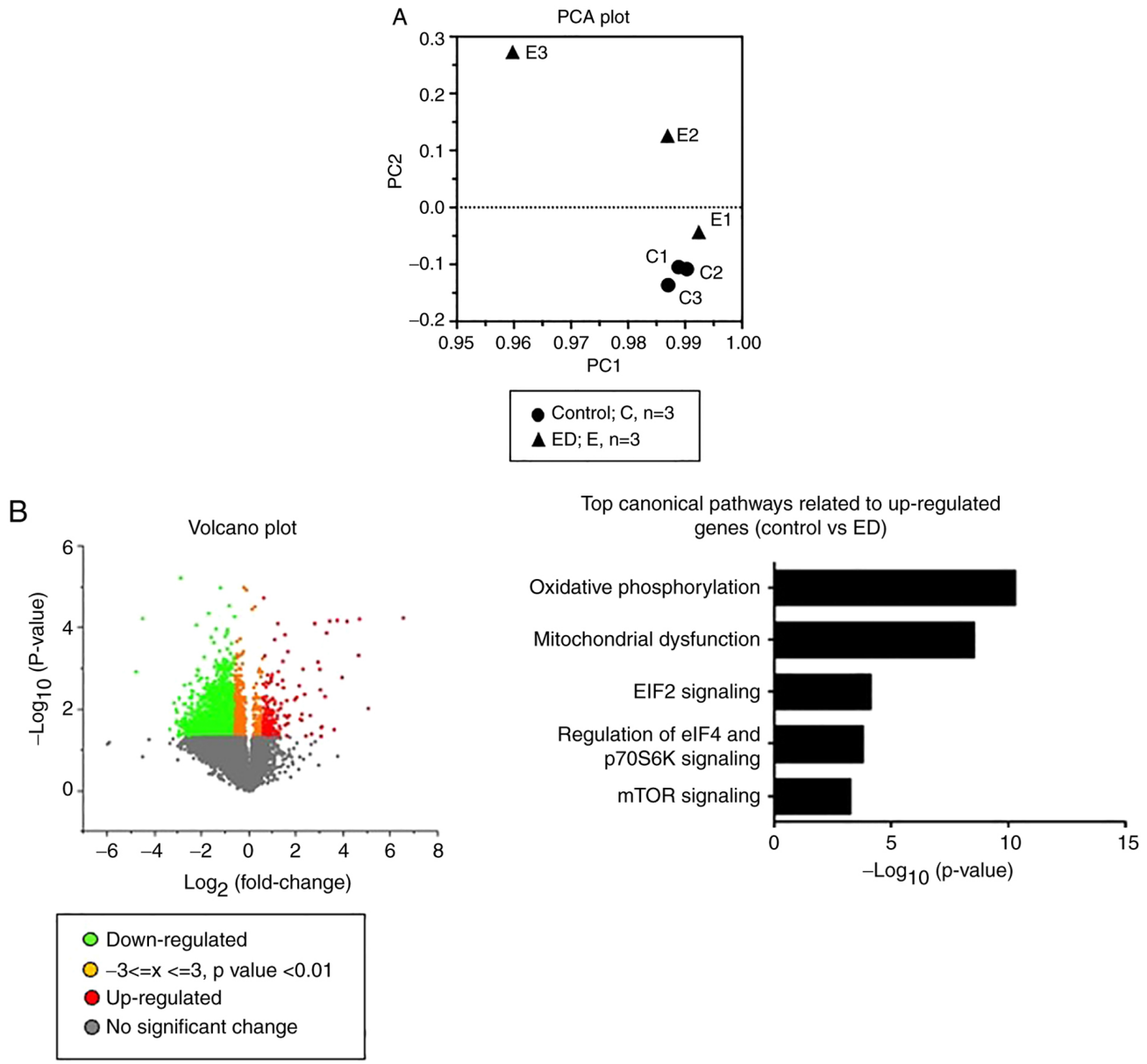

Figure 3. Continued.

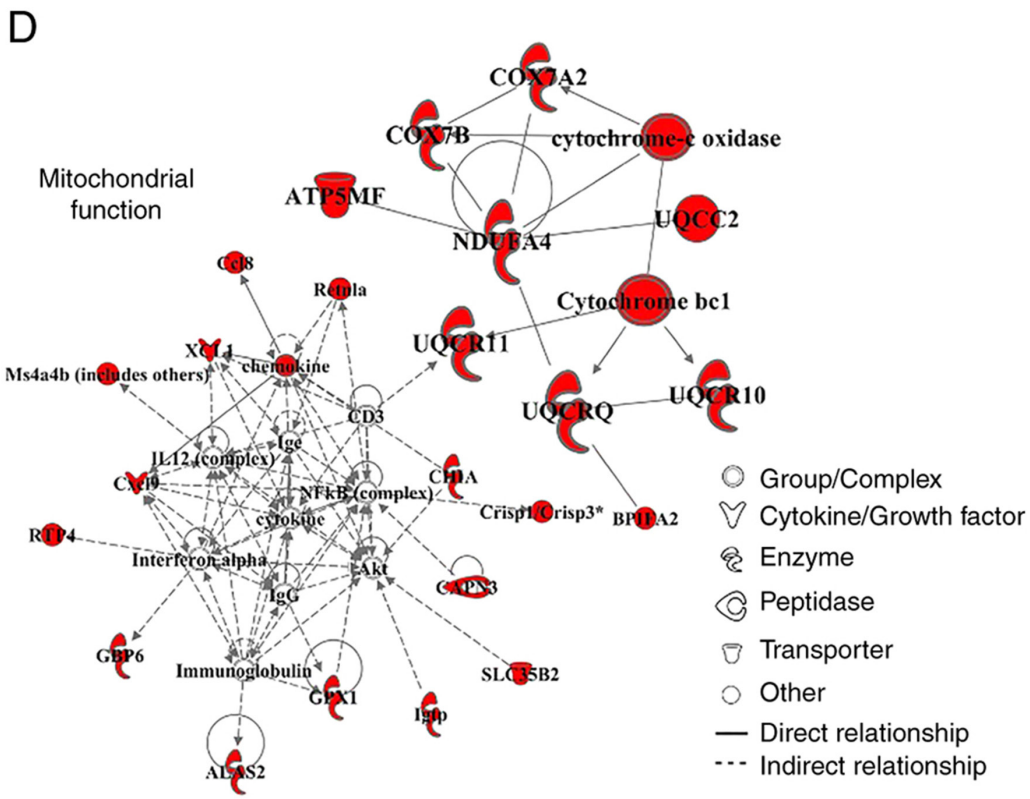

Figure 3. Data obtained from whole transcriptome analysis, PCA and IPA of 5-FU-injected ED) mice and saline-treated (control) mice. On day 21 of treatment, the mice were sacrificed and the salivary glands were collected for whole transcriptome analysis to identify the differentially expressed genes in the salivary glands from the ED group compared with the control group. (A) PCA was used to verify the data quality. PC1 and PC2 were plotted on the x-axis and y-axis, respectively, to draw the scatter diagram, where each point represents a sample. E1, E2 and E3 represent the ED group; and C1, C2 and C3 represent the control group. (B) Left panel: A volcano plot showing each differentially expressed gene in the ED group compared with the control group. The significantly upregulated genes are shown in red, and the significantly downregulated genes are shown in green. Right panel: IPA analysis of the upregulated (>1.5-fold) genes identified with the volcano plot indicated that these genes were mostly associated with oxidative phosphorylation and mitochondrial dysfunction pathways. (C) IPA analysis of the differentially expressed genes in the salivary glands from the ED group compared with the control group, which were associated with the oxidative phosphorylation pathway-related network. (D) Mitochondrial function-related network constructed from the IPA data of the differentially expressed genes in the salivary glands from the ED group compared with the control group (large block letters refer to the upregulated genes). PCA, principal component analysis; IPA, Ingenuity Pathways Analysis; ED, elemental diet; 5-FU, 5-fluorouracil; PC, principal component; E, ED group; C, control group.

TUNEL-positive cells in salivary glands of 5-FU-treated mice. The rate of apoptosis in the salivary glands of mice was analyzed to evaluate the tissue damage caused by 5-FU as well as the protective and recovery effects of ED against that damage. The number of TUNEL-positive cells in the salivary glands of the control group was significantly higher compared with the ED group (Fig. 5). The TUNEL data demonstrated that ED could protect salivary glands from tissue damage caused by 5-FU.

Discussion

Oral mucositis is a life-threatening disease and one of the most common side effects of chemotherapy and radiotherapy. Oral mucositis and salivary gland dysfunction caused by 5-FU-based chemotherapy may result in a decreased quality of life and an increased treatment cost (1). Until now, very few agents exhibit protective and healing effects against mucositis (7,9,11,14,38). Elental® is an easily digestible nutritional supplement or ED which is composed of free amino acids and dextrin (3). In Japan, Elental® is administered to malnourished high-risk patients for the maintenance and improvement of their nutritional status (3,16,18). Elental® and other EDs were reported to be effective in the treatment of inflammatory bowel diseases such as Crohn's disease and chemotherapy-induced oral mucositis (17-22). However, the detailed mechanisms contributing to these beneficial effects of EDs are not clear. In the present study, the efficacy of an ED, Elental®, against 5-FU-induced salivary gland atrophy and dysfunction was examined.

Antineoplastic drugs reportedly injure salivary gland tissues and decrease saliva secretion volume (25,39). McCarthy *et al* (35) reported that the incidence of 5-FU-induced oral mucositis is higher in patients with lower saliva secretion volumes. Notably, Kawashima *et al* (39) reported that 5-FU treatment (40 mg/kg/day) decreases the volume of saliva in mice, and then a 7-day treatment of Elental® recovers the saliva volume and increases mouse body weight. In addition, this study indicated that Elental® may protect the submandibular acini against 5-FU-induced atrophic changes (40). In our previous study, we used an experimental protocol (a 7-day treatment of Elental®) similar to the one developed by Kawashima *et al* to understand the protective effects of Elental® on salivary glands of 5-FU-administered mice (39). Additionally, this study also investigated which components of Elental® are most effective against the 5-FU-induced

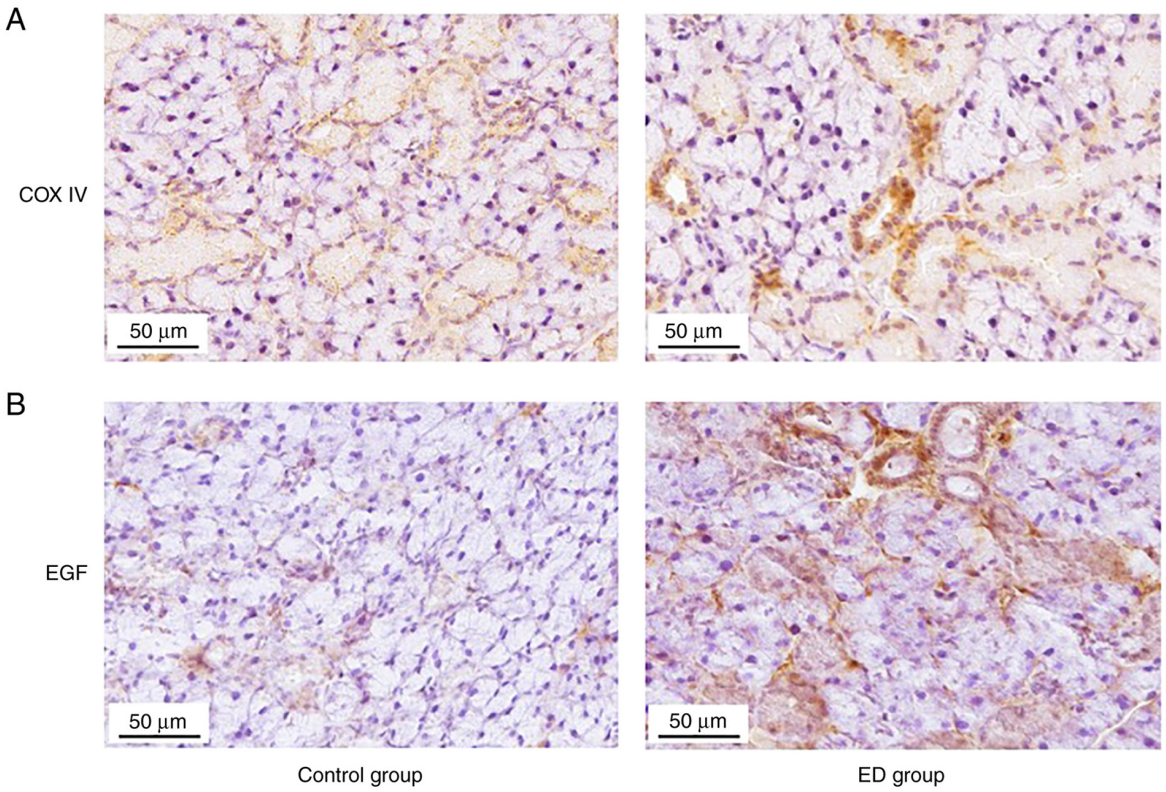

Figure 4. Effect of an ED on the expression of COX IV and EGF in salivary glands of ED- or saline-treated (control) mice injected with 5-FU as observed by immunohistochemical staining. (A) Expression of COX IV was higher in the salivary glands from ED mice compared with control mice. (B) EGF expression was higher in the salivary glands from ED mice compared with the control mice. Magnification, x200. Scale bar, 50 μm. ED, elemental diet; COX IV, COX subunit 4; EGF, epidermal growth factor.

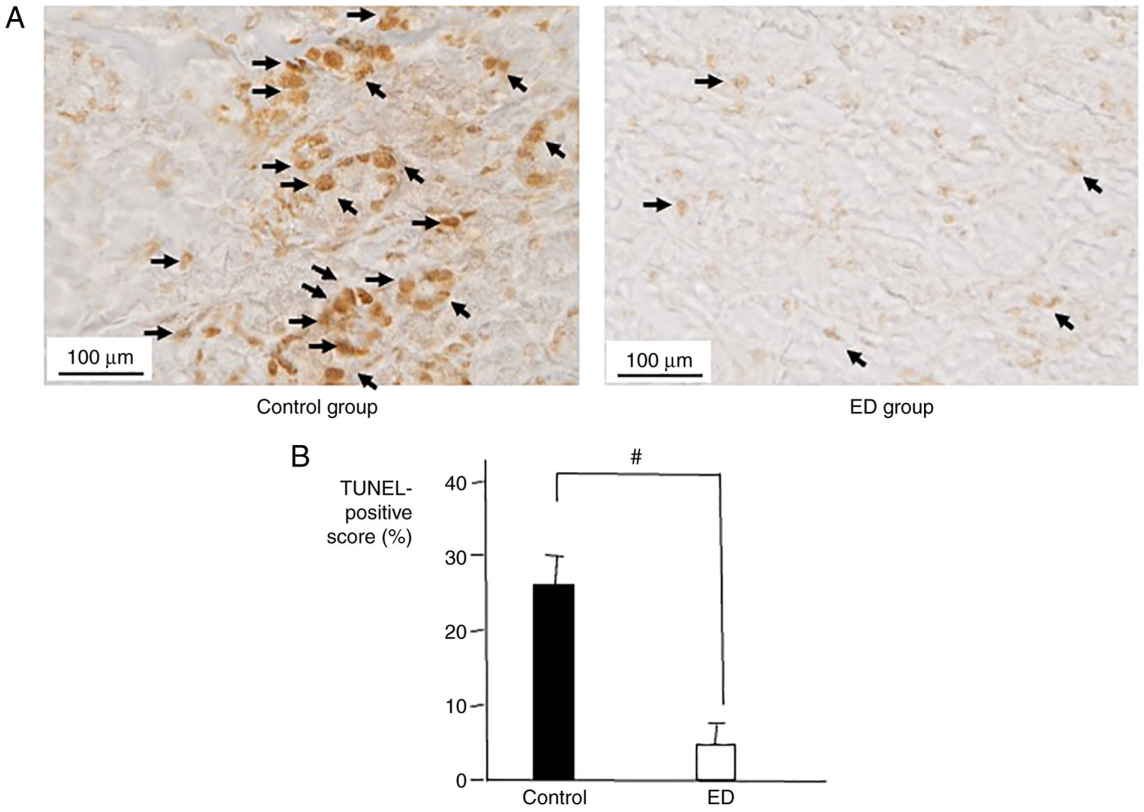

Figure 5. Effect of an ED on salivary gland apoptosis in ED- or saline-treated (control) mice injected with 5-FU. (A) A TUNEL assay was performed to detect apoptotic cells in control and ED-treated salivary glands. Arrows indicate apoptotic cells (magnification, x100; scale bars, 100 μm. (B) TUNEL-positive cells in the salivary glands of the control group mice were significantly higher compared with those from the ED group mice. n=3 mice per group. $^{\#}$P<0.01 vs. control group. ED, elemental diet; 5-FU, 5-fluorouracil.

damage of the salivary glands. The data indicated that among all the components of Elental®, amino acids exert the highest ameliorating effects against 5-FU-induced atrophic changes in salivary glands (41). However, both of these studies do not focus on the underlying mechanism of action of Elental® responsible for its protective effects (40,41). In our present study, a different treatment protocol was used compared with our previous study. In the present study, mice were treated with 20 mg/kg/day 5-FU (twice per week for 3 weeks). Elental® was administered for 21 days or 3 weeks (5 times a week) to understand its long-term effects. Moreover, this study did not focus on the different components of Elental® this time. The main focus was to examine the underlying mechanisms of the healing effects of Elental® by whole transcriptome analysis and IPA.

Over the past few years, the usefulness of EDs against the negative side effects of antineoplastic drugs has been observed in patients with cancer at Yamaguchi University Hospital. Elental® was effective against chemotherapy- and/or radiotherapy-induced mucositis and dermatitis in HNC patients, as well as *in vitro* and *in vivo* (21,22,42,43). Based on these results, it was hypothesized that Elental® may help to alleviate oral mucositis by preserving saliva secretion volume and improving the oral environment.

The current study demonstrated that Elental® helped to maintain the body weight of 5-FU-treated mice. Moreover, the weight of 5-FU-damaged salivary glands and the size of granular duct cells in salivary glands were increased in the ED group compared with the control group. These findings suggested a possible healing effect of Elental® against 5-FU-induced salivary gland atrophy and oral mucositis.

In previous clinical trials, oral squamous cell carcinoma patients with oral mucositis are advised to swish Elental® suspension in their mouth and swallow it. None of the patients complained of any irritation, pain or difficulty during oral administration (22). Moreover, murine back skin wounds are healed by topical application of Elental®, which suggests that it may have direct soothing effects on the wounded areas upon contact (43). Therefore, it was assumed that Elental® is suitable for patients with severe oral mucositis who are unable to eat solid foods.

A reduced saliva secretion volume hampers the salivary clearance ability and decreases the concentrations of secretory IgA, which possess antibacterial activity (33). In addition, EGF is required during tissue repair in salivary glands (32,34). The present study demonstrated that Elental® upregulated the expression of EGF. Notably, the present whole transcriptome analysis and IPA data revealed that a number of the upregulated genes in the salivary glands of the ED group of mice are associated with mitochondrial functions. This indicated that the healing function of Elental® may be associated with the functional recovery of mitochondria in 5-FU-damaged cells.

COX IV is a typical mitochondrial marker and is essential for the regulation of cellular energy metabolism and mitochondrial function. It is also a highly regulated enzyme, which controls the oxidative phosphorylation pathway (24,26). Decreased expression of COX IV results in impaired COX activity followed by mitochondrial dysfunction and sensitization of cells to apoptosis, which may hamper the normal functions of cells and tissues including damage repair mechanisms (25,26,44). The present data indicated upregulated expression of COX IV in the salivary gland tissues from the ED group, which suggested that Elental® might facilitate the recovery of 5-FU-damaged mitochondrial function. Moreover, increased EGF expression and reduced apoptosis were observed in the salivary glands of the ED group compared with the control group, which also suggested that Elental® may protect salivary glands from 5-FU-induced atrophic changes. These data supported the hypothesis that the Elental®-mediated repair of 5-FU-induced damage of the salivary glands promotes adequate saliva secretion and adjustment of oral bacterial flora. Therefore, Elental® may be used to treat xerostomia and oral mucositis in patients with cancer.

The present *in vivo* experiments were performed twice (n=3 per group) and the findings were almost similar. However, there are some imitations of the present study. The sample size was small (n=3) per group for each experiment and no true control group (without 5-FU) was included. Therefore, future studies will include a true control group and a larger sample size (n=5-10 mice per group) to generate more reliable data.

In conclusion, the present study demonstrated that ED Elental® may be able to alleviate adverse effects of 5-FU-based chemotherapy including oral mucositis and atrophic changes in the salivary glands. In order to further investigate the healing mechanisms of EDs, the effects of EDs on genes involved in oxidative phosphorylation and mitochondrial dysfunction will be examined further. In addition, further studies are required to clarify the mechanisms of the healing and protective effects of EDs in a clinical setting.

Acknowledgements

Not applicable.

Funding

The present study was supported in part by a Grant-in-Aid from the Japanese Ministry of Education, Science and Culture (grant no. 55-017).

Availability of data and materials

The data generated in the present study are openly available at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE196407 [accession no. GSE196407 (access code: knabcecwrhuhpcr)].

Authors' contributions

KH designed the study. KH, RF and TF performed the experiments. KH, KW and YM analyzed and interpreted the data. KH, TF, KW and YM wrote and revised the manuscript. YM and KH confirmed the authenticity of all the raw data. KM assisted in data interpretation, revised the manuscript and provided valuable suggestions during the study. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

All *in vivo* experiments were approved by the Institutional Animal Care and Use Committee of Yamaguchi University (approval no. 55-017; Ube, Japan).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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