Effects of denture adhesives and mouth moisturizers to human oral fibroblast and human keratinocyte cells using direct and indirect cell culture systems

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The purpose of this study was to evaluate the effects of commercialized denture adhesives and mouth moisturizers using direct and indirect cell cultures for in vitro examinations with human fibroblast and epithelial cells. Denture adhesives (Faston, Poligrip Powder, New Poligrip Free, Tafugurippu Kurimu, Polident Adhesive, Tafugurippu Tomei) and mouth moisturizers (Concool Mouth Gel, Biotene Oral Balance Gel) were subjected to live and dead detection and pH level determination. The mouth moisturizers showed higher cytotoxicity effects comparing with control on every cell cultures and cells, and pH level did not show any significant differences. However, there was no correlation of type of denture adhesive or mouth moisturer with cytotoxicity. We concluded that cytotoxicity affects human cells regardless of type of material, though some dependence was noted.

Keywords: Denture adhesives, Mouth moisturizers, Cytotoxicity, Fibroblasts, Keratinocytes

INTRODUCTION

With the increasing aging of society in recent years, there has been an increase in number of denture wearers, even though dental implants have become popular in place of conventional removable dentures. The unique “8020-campaign” in Japan has promoted keeping more than 20 natural teeth in individuals older than 80 years. In fact, a survey performed in 2016 found that 51.2% of individuals at least 80 years old had more than 20 teeth remaining, a percentage that has been increasing since 1987 according to a survey of dental diseases by the Ministry of Health. Therefore, it is likely that complete denture wearers are decreasing along with the increase in remaining teeth. Furthermore, failure or recovery of fixed prosthetic treatment is found in severe cases, which must be supported by removable denture treatments. For such intractable conditions, patients must rely on denture adhesives. According to a study by Love, an increase in the number of remaining teeth results in a pH level below the critical point of hydroxyapatite in aqueous solutions provided by some denture adhesives. Several reports have noted that denture adhesives are often used as an over-the-counter approach for improving denture retention and stability, though patients sometimes have difficulties with improper usage or without specialist advice.

Denture adhesives can be classified as glue type, available in powder, cream, sheet, and tape forms, and liner type. The Japan Prosthodontic Society recommends cream and powder-form denture adhesives for short-term use under guidance from a dentist. Furthermore, they suggest that patients who use denture adhesives should be basically limited to those with severely resorbed alveolar bone, lack of neuromuscular control (e.g., stroke, Parkinsonism), xerostomia, or maxillofacial defects caused by an accident and/or tumor. Such adhesives may be quite helpful for individuals with xerostomia, as they also frequently use mouth moisturizers to improve dehydration. On the other hand, patients requested by dentists to use denture adhesives sometimes note that the treatment lacks quality and/or use of a prosthesis is unacceptable. In addition, those who routinely swim or exercise sometimes utilize denture adhesives for support. Recent studies have revealed that continued use of denture adhesives with ill-fitting dentures increases vertical dimension, enhances alveolar bone resorption, and triggers hypersensitivity reactions, which has been shown to cause cytotoxicity effects on cultured cells in vitro. In contrast, use of denture adhesives with correctly adapted dentures can improve retention and stability, increase bite force, and reduce the frequency of adjustments.

A previous study found that denture adhesives in the form of a zinc-containing cream can induce hypocupremia, with the possibility of serious neurologic consequences. On the other hand, rapid improvements in biomechanical properties have recently been attained, though additional information regarding the biocompatibility of denture adhesives is still needed. The cytotoxic effects of commercially available denture adhesives have been widely studied using agar diffusion and filter diffusion tests, as well as MTT assays with mono-layered cell culture systems. As previously noted, denture adhesives are used not only for well- or ill-fitting dentures, but may also assist with retention, and have beneficial effects towards fibroblast and epithelial cells during healing, such as following a surgical procedure.

Although the biocompatibility of mouth moisturizers has rarely been investigated, their usage has been increasing along with the aging of society, especially...
in facilities used for nursing or those providing care-giver support. A recent study revealed that some of the ingredients in those materials should be carefully used by older adults, instead of a bactericidal approach\textsuperscript{21-23}).

Since oral care materials are used by patients receiving nursing care, it is important to elucidate their effects on oral mucosa. Thus, the aim of this study was to evaluate the effects of commercialized denture adhesives, as well as mouth moisturizers under direct and indirect cell culture for \textit{in vitro} testing with human fibroblast and epithelial cells. We speculated as hypothesis that the effects to fibroblast and epithelial cells would depend to the type of materials used in this study.

**MATERIALS AND METHODS**

\textit{Denture adhesives and mouth moisturizers}

We examined 6 different types of denture adhesives and 2 mouth moisturizers, which are listed along with their manufacturers, compositions, and batch numbers in Table 1. Specifically, 4 types of glue-type denture adhesives (2 powder form, 2 cream form), 2 types of liner-type denture adhesives, and 2 different non-alcoholic mouth moisturizers were examined. All specimens were used at a concentration of 10\% (w/v) for testing.

\textit{Cell cultures}

Human epidermal keratinocyte (NHEK; Lonza Walkersville, MD, USA, 18 passages) cells and human oral fibroblast (NHDF; ATCC, Manassas, VA, USA, 16 passages) cells were used.

NHEK cells were grown in T-75 flasks (Falcon, New York, NY, USA) in a total 15 mL of Humedia-KG2 (KURABO, Osaka, Japan) as well as a proliferating agent, Humedia-KG (KURABO) (denoted as Humedia-KG plus hereafter) at 37°C in a humidified incubator with an atmosphere of 5\% CO\textsubscript{2}/95\% air. The medium was changed every 2 or 3 days until the cells reached 80\% confluence. Thereafter, 1 mL of 0.05\% trypsin in 0.02\% ethylenediaminetetraacetic acid (Life Technologies, Carlsbad, CA, USA) was added to the culture wells, with aspiration of the culture medium. The cells were then further incubated at 37°C for 1 min, then the trypsin reaction was stopped by addition of 9 mL of Humedia-KG plus. Prior to performing subcultures, cells were centrifuged at 223\times g for 5 min to avoid a trypsin reaction and the supernatant was aspirated, then Humedia-KG plus at 10 mL was added as the proliferating agent for subculturing.

NHDF cells were grown in 90-mm diameter dishes (Falcon) in DMEM (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10\% fetal bovine serum (SFBM-0500, Equitech-bio, Kerrville, TX, USA) and 1\% antibiotic-antimycotic (Life Technologies, Carlsbad, CA, USA) (denoted as DMEM(S+) hereafter) at 37°C in a humidified incubator with an atmosphere of 5\% CO\textsubscript{2}/95\% air. The medium was changed every 2 or 3 days until the cells reached 80\% confluence. Thereafter, 1 mL of 0.05\% trypsin in 0.02\% ethylenediaminetetraacetic acid (Life Technologies) was added to the culture wells.

| Material          | Code | Type                        | Manufacturer                          | Composition*                                                                 |
|-------------------|------|-----------------------------|---------------------------------------|-------------------------------------------------------------------------------|
| Faston FA         | FA   | Powder type denture adhesive| Lion, Tokyo, Japan                    | Karaya gum, menthol, ethanol                                                  |
| Poligrip Powder   | PGP  | Powder type denture adhesive| GlaxoSmithKline, Tokyo, Japan          | Methoxyethylene/maleic anhydride copolymer, sodium carboxymethylcellulose     |
| New Poligrip Free | NPF  | Cream type denture adhesive | GlaxoSmithKline                       | Methoxyethylene/maleic anhydride copolymer, sodium carboxymethylcellulose, liquid paraffin, petrolatum |
| Tafugurippu Kurimu| TGK  | Cream type denture adhesive | Kobayashi Pharmaceutical, Osaka, Japan | Methoxyethylene/maleic anhydride copolymer, petrolatum, sodium carboxymethyl cellulose, propyl parahydroxybenzoate, liquid paraffin |
| Polident Adhesive | PA   | Home reliner                | Earth Chemical, Tokyo, Japan           | Polyvinyl acetate, propylene glycol, polyethylene glycol 400, precipitated calcium carbonate, acetylglycerol, fatty acid ester |
| Tafugurippu Tomei | TGT  | Home reliner                | Kobayashi Pharmaceutical               | Polyvinyl acetate, methylmethacrylate, dehydrated ethanol                      |
| Concool Mouth Gel | CM   | Mouth moisturizer           | Weltec, Osaka, Japan                  | Water, maltitol, sorbitol, glycerin, PG, xylitol, milk protein, carboxomer, sodium polyacrylate, dextrin, mannitol, lactoferrin, human oligopeptide-1, aloe vera gel, glycyrrhizin ammonium |
| Biotene Oral Balance Gel | BO | Mouth moisturizer | GlaxoSmithKline | Glycerin, water, sorbitol, xylitol, carboxomer, hydroxyethyl cellulose, sodium hydroxide |

\*Provided by the manufacturer, also noted in reference 3.
with aspiration of the medium for subcultures. The cells were then further incubated at 37°C for 1 min, then the trypsin reaction was stopped by addition of 9 mL of DMEM (S+).

Both types of cells underwent 5 to 8 subcultures then used in the experiments.

**Indirect contact culture**

Cell suspensions were centrifuged at 223× g for 5 min and re-plated at a density of 1×10⁵ cells/well into a 24-well Cell Culture Insert Tissue Cultured Treated Plate (BD, NJ, USA) for the experiments. DMEM (S+) and Humedia-KG plus were used as culture medium for NHDF and NHEK, respectively.

After incubating the cell culture plates for 24 h, the materials noted above were added using a cell culture insert (Fig. 1) and culturing was performed for 24 h. The materials were not directly in contact with the NHDF or NHEK cells, but rather isolated by a membrane with 8-μm pores that formed the bottom of the cell culture insert, allowing the medium or eluate of the materials to pass through the membrane. After culturing for 24 h, those materials were removed from the insert, then the wells were gently washed twice with 1 mL of PBS for the following procedures.

**Direct contact culture**

NHDF and NHEK (1.0×10⁵/well) cells were separately plated in 24-well plates (Sigma-Aldrich), and cultured in the appropriate culture medium, as noted above. After 24 h of incubation, the test material was directly added as 10% of a total liquid volume and the medium was changed to fresh. Following another 24 h of incubation with the tested material, the cells were washed with PBS at least twice to remove the denture adhesive as much as possible before undergoing an assay (Fig. 2).

Cytotoxicity was examined using determination of live and dead cells was performed a fluorescence microscope. Those procedures are detailed following.

**Determination of live and dead cells**

NHDF and NHEK cells were cultured at a density 1×10⁵ cells/well in 24-well cell culture plates (BD) for 24 h (n=3) using appropriate culture medium. Direct and indirect assays were individually performed with the cells. All medium and floating cells were collected, then adhered cells were trypsinized with 0.05% trypsin in 0.02% ethylenediaminetetraacetic acid and centrifugated in a micro-tube for 5 min at 223× g. FBS was used for pausing trypsin validity. After the supernatant was aspirated, 50 μL of FBS along with 10 μL of Propidium Iodide solution (MKBV9923V, Sigma) and 10 μL of Hoechst solution (33342, Sigma) were added to each tube, and kept in the dark for 60 min. Thereafter, 20 μL was collected from each tube, then dropped and rolled into a thin layer on object glass, and air dried. Analyses of the dried smears were performed using a fluorescence microscope (Olympus BX51, Tokyo, Japan) equipped with a 100× oil-immersion objective, excitation filter (BP400–440), dichromatic mirror (DM455), and emission filter (LP475) (U-MWBV2). A hundred cells were counted in each smear, and classified as dead or viable. Cells in which red or blue stain was evenly distributed were classified as dead or viable, respectively. Cells that were broken during the procedure or somehow not counted were considered as dead cells. The counting cells were run into 3 times at each smear randomly.

**pH measurement**

pH levels were determined before, and then 2 and 24 h after immersion of the test material in DMEM (S+) without cells, using a pH meter (sensION™, Hach, New York, NY, USA) in the same manner as in the WST cytotoxicity assay (n=5). These determinations were promptly performed as soon as the samples were retrieved from the controlled incubator (5% CO₂/95% air atmosphere). Control samples were incubated in medium alone with an empty cell culture insert.
Statistical analysis
Three experiments were performed for each cytotoxicity test and material. pH was measured 5 times for each material. Determinations of percent or value as compared to the control and each type of material obtained by live or dead counting and pH measurement results were subjected to one-way analysis of variance (ANOVA), then mean values were compared using Tukey’s method, with a 5% difference used to denote significance (alphabet letters). In all experiments, results for each type of material were separately subjected to one-way ANOVA, then those mean values were compared using Tukey’s method, with a 5% difference used to denote significance (*). We analyzed denture adhesives and mouth moisturizers separately, because of their different forms. Values obtained for the control and each mouth moisturizer obtained by live or dead counting assay results were subjected to one-way ANOVA, then those mean values were compared using Tukey’s method, with a 5% difference used to denote significance (**). For all statistical analysis, the SPSS Statistics software package, version 17.0 (SPSS, Chicago, IL, USA) was used.

RESULTS
To assess the numbers of viable and dead cells, propidium iodide and Hoechst staining were performed using a fluorescence microscope. PI and Hoechst staining were used for identifying dead cells and live cells respectively in a population of cells. The numbers of living cells are shown in Fig. 3. Indirect assay findings performed with NHEK cells and FA, PGP, NPF, TGK, CM, and BO showed significantly lower numbers of living cells as compared to the control (Fig. 3A). Indirect assay findings with NHDF and FA, TGT, CM, and BO showed a significantly lower number of living cells as compared to the control. Furthermore, TGT showed a significantly lower number of living cells than PA (Fig. 3B). Findings of direct assays with NHEK and PA, TGT, CM, and BO also showed a significantly lower number of living cells as compared to the control, and that number was also significantly lower with FA than PGP (Fig. 3C). In direct assay findings of NHDF, CM and BO showed significantly lower number of living cells as compared to the control, while PA also showed a significantly lower number as compared to TGT (Fig. 3D).

Finally, there were no significant differences in regard to pH values of the specimens 0, 2, and 24 h after immersion in medium among any of the tested materials (Fig. 4).

DISCUSSION
The present study performed 3 types of cytotoxicity assays to assess the effects of denture adhesives and mouth moisturizers on NHEK and NHDF in this study. We speculated that the effects on both types of cells would be dependent on the categorized type of materials.
used, though that was not supported by the present findings. In other words, the effects of the materials on both types of cells were not corresponded to the types or forms of materials.

The International Organization for Standardization (ISO)\textsuperscript{24} has provided recommendations regarding protocols used for cytotoxicity effects by the dental materials, such as direct or indirect methods. For clinical application, cytotoxic effects towards monolayers of cultured cells might be much stronger as compared to materials with a 3D tissue organization and extracellular matrix, because of the presence of several protective factors such as oral mucosa and fluids. The present results are limited by the in vitro setting and the experiments did not reflect all factors related to oral condition, such as saliva, or those related to the vascular and immune systems. Furthermore, different types of medium were used in the culture systems, as noted in the MATERIALS AND METHODS section. Notably, FBS is well known to increase the activity of cells to profusely produce an extracellular matrix, which provides a protective effect to cell layers. In the present study, FBS was used for culturing NHDF but not NHEK cells. Therefore, it was difficult to compare cytotoxicity effects between cells cultured in medium either with or without serum. Furthermore, NHEK cells were derived from human epidermal tissue, while NHDF cells were derived from human oral tissue. It would be possible to use NHEK derived from oral tissue, though we used NHEK cells to assess reactivity variations, as that is the first barrier against ambient harmful agents\textsuperscript{25}. However, among the denture adhesives and mouth moisturizers examined, there were no correlations seen between types or forms. Although significant differences were found between some forms and types, there was no correlation between any two different cells or culture types. Thus, definitive conclusions may be difficult based on our findings.

To assess the cytotoxic effects on the materials used in this study, Hoechst and Propidium Iodide solutions were used to stain live and dead cells respectively\textsuperscript{26}. As a regulation of this study, the denture adhesives and mouth moisturizers thought to be separated to compare with control. One of the reason was the differences of properties, for instance forms and solubility to the medium. The results suggested that no correlation between the types and forms of denture adhesives or mouth moisturizers (Fig. 3). However, the moisturizers showed a significantly higher level of cytotoxicity than the control. The mouth moisturizers used in this study were classified into gel and liquid type, and are commonly used as symptomatic treatment for dry mouth\textsuperscript{27-29}. Additionally, salivary gland massage, as well as facial muscle and tongue exercises have been reported useful to improve saliva secretion in patients with dry mouth\textsuperscript{28,30}. There are no specific recent findings available regarding the cytotoxicity of mouth moisturizers, though several studies of antifungal effects have been presented\textsuperscript{31}. Since the denture adhesives and mouth moisturizers consisted in different forms, this study was unable to compare them under the same conditions. This study showed that the mouth moisturizers had a high level of cytotoxicity towards every cell culture subjected to microscopic observations for live and dead detection. The exposure time used in this study may have been too long for the mouth moisturizers, though additional experiments are needed.

Information regarding potential cytotoxicity of all the denture adhesives tested in the present study is available, though no precise differences among the types have been presented\textsuperscript{7,8,13,16,19}, with the degree of cytotoxicity varying and dependent on the conditions. Such cytotoxicity might have been caused by the components of the denture adhesives, though low pH is also a possibility\textsuperscript{25}. It is known that any solution with a pH ranging from below 5.5 to 6.5 will dissolve a hydroxyapatite structure. In the present study conditions, there were no significant differences as compared to the control (i.e., medium alone) after 24 h of incubation in DMEM medium (Fig. 4). It is possible that pH value did not have an influence on cytotoxic effects in the present cell culture systems.

Exposure time and the concentration of denture adhesives also have great influence on detecting cytotoxicity. In this study, a 10% concentration (w/v) and 24-h exposure time were used, which may have been relatively excessive as compared to actual clinical situations and conditions used in other studies\textsuperscript{16}. This study chose a higher concentration for the denture adhesives used in order to simulate oral conditions, while exposure time was used to simulate patients receiving nursing care. Ideally, a denture adhesive should be used for every meal, as well as when going out or participating in sports, though patients often use it for an extended time period. Thus, a 24-h exposure period was chosen for this study. Also, this study performed both direct and indirect cell cultures for cytotoxicity testing to simulate clinical conditions as much as possible. However, limitations of this study include duration and concentration. Additional experiments that utilize longer and shorter time durations, as well as various
concentrations may be necessary.

Findings for the 6 denture adhesives and 2 mouth moisturizers evaluated in this study suggested cytotoxicity towards human NHDF and NHEK in both direct and indirect cell culture systems. There are no definite standards for cytotoxicity based on type of materials, and this study showed different effects depending on the material and culture system. Since these materials demonstrated cytotoxicity towards cells, clinicians should advise their patients to be careful regarding overuse of denture adhesives in terms of amount and duration.

CONCLUSIONS

Within the limitations of this in vitro study, we concluded the following.

1. The degree of cytotoxicity was influenced by the material, cell type, and culture system.
2. Mouth moisturizers showed significantly higher cytotoxicity than the control by the live and dead detection assays.
3. None of the tested materials had an effect on pH level of the medium.

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