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Abstract

Background: The biochemical bone turnover markers for residual ridge resorption (RRR) are unclear. Therefore, the present study aimed to determine the biochemical bone turnover markers associated with RRR by comparing proteomics between the compressed mucosa of denture wearers and the non-compressed mucosa of non-denture wearers.

Methods: The mucosal specimens of 11 complete-denture wearers were obtained from the alveolar ridge during surgical implant exposure for implant-retained overdentures. All denture wearers had been edentulous and worn dentures for at least 5 years. The tissues of 11 non-denture wearers were taken from the ridge during minor preprosthetic surgery. The mucosal proteins were extracted, purified, precipitated, and subsequently separated by two-dimensional gel electrophoresis for comparative proteomics. Differentially expressed proteins between the groups were analyzed by ANOVA using Progenesis SameSpots software.

Results: Comparative proteomics analysis showed significant upregulation of 78 kDa glucose-regulated protein (GRP78; +2.2 fold, p = 0.015) and lumican (+1.8 fold, p = 0.005), as well as significant downregulation of heat shock protein 27 (HSP27; −1.9 fold, p = 0.029) in the denture group.

Conclusions: Differential expression of the biochemical bone turnover markers of GRP78, lumican, and HSP27 may occur as a result of denture pressure on the mucosa. These markers may play important roles in RRR.

Keywords: bone resorption, dentures, mouth mucosa, proteomics

INTRODUCTION

A removable denture is a successful form of treatment and will continue to be the mainstay of prosthodontic care for partially dentate and edentulous patients as the proportion of the elderly population increases worldwide.[1] Despite their clear benefits, however, dentures resting on the mucosa are often associated with increased rates of residual ridge resorption (RRR).[2–4] The biomechanics of how pressure from denture compression on the mucosa causes increased hydrostatic pressure, which, in turn, leads to hypoxia and subsequent RRR, is quite clear.[5–8] However, the biological reactions induced by mechanical stresses and the mechanobiology related to RRR are less well understood in comparison with the mechanobiology of periodontal bone resorption[9] or orthodontic tooth movement with the accompanying bone resorption and deposition.[10–12] The mechanical stimulus provided by the denture base has been shown to increase localized bone metabolism,[13–14] but the expression of the corresponding biochemical bone turnover markers has not been adequately studied.[15–17] Knowledge of the biomarkers involved in RRR is important to enhance the understanding of the mechanism of RRR and facilitate its diagnosis, improve RRR risk assessment and treatment strategies to minimize RRR, reduce the frequency of denture relines, and improve treatment outcomes.

Puri *et al*.[17] reported a significant correlation between the frequency of complete-denture relines and the concentration of serum bone turnover biomarkers of C-terminal telopeptide and osteocalcin. The authors thus proposed that serum bone turnover markers may predict individuals at risk of frequent complete-denture relines because of rapid RRR. Because RRR is essentially a

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localized phenomenon, the mucosa underneath the denture could be an excellent source of bone turnover markers. Cells in the underlying mucosa have been shown to secrete heat shock protein 70 (HSP70),[15] vascular endothelial growth factor (VEGF),[15] and prostaglandins in response to hypoxic stress caused by increased hydrostatic pressure from denture compression in animal models.[16] Increases in hydrostatic pressure could also cause irreversible damage to the osteocytes or a disturbance in the composition of the interstitial fluid, which, in turn, could affect osteoblastic and osteoclastic functions.[18,19] Hydrostatic pressure has been correlated with RRR,[4,6] but little is known about the differentially expressed proteins or proteomic changes resulting from RRR induced by denture compression on the mucosa.

This study was undertaken to identify differentially expressed proteins between the compressed and non-compressed mucosa of denture and non-denture wearers. This study used two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and matrix-assisted laser desorption-ionization time-of-flight mass spectrophotometry (MALDI-ToF/ToF MS) peptide mass fingerprinting for protein identification. We hypothesized that the pressure exerted by the denture on the mucosa would stimulate localized bone metabolism and cause proteins related to bone resorption and deposition to be differentially expressed. Our null hypothesis is that no difference in protein expression would be observed between compressed and non-compressed mucosa. This study could provide preliminary insights into the proteomic changes related to RRR induced by denture wearing, which may potentially be used as biomarkers for RRR.

METHODS

Ethics statement
This study was approved by the Research Ethics Committee of Universiti Teknologi MARA, Malaysia (600-RMI [5/1/6] 30 Nov. 2015). The clinical study was conducted at the Faculty of Dentistry, and the proteomic work was performed at the Institute for Medical Molecular Biotechnology, Faculty of Medicine. The participants were informed of the objectives of the study, and written consent was obtained.

Participant recruitment
In this case-control study, 11 complete-denture patients undergoing implant treatment for implant-retained overdentures were recruited as the test group. This sample size represents the total number of eligible edentulous patients attending the implant clinic within a 6-month recruitment period. The exclusion criteria were uncontrolled diabetes, irradiated jaw, medical conditions that may contraindicate surgical procedures, smoking, psychiatric treatment, or a history of substance abuse. All edentulous patients had had multiple sets of dentures

and had been edentulous for at least 5 years. The control group comprised 11 partially edentulous patients who had not worn any dentures to replace their missing teeth and had come for preprosthetic surgery to remove either multiple non-restorable teeth, an impacted tooth, buried or fractured roots, a torus, or bony spicules on the ridge. Patients in this group consented to tissue specimen collection within the same recruitment period.

For the test group, mucosal tissues measuring approximately 4 mm in diameter and 1 mm in thickness were obtained from the ridge crest with a tissue punch (Tissue Punch RP, Nobel Biocare, Kloten, Switzerland)) during the surgical exposure of the implants. For the control group, mucosal tissues of a similar size were obtained from the edentulous ridge at the surgical site. The tissues were kept in mammalian protein extraction reagent (MPER) buffer in microcentrifuge tubes and stored at −80 °C until use.

Protein preparation
Proteins from the mucosal tissues were extracted using a grinder (ReadyPrep Mini Grinder, Bio-Rad Laboratories), and ultrasonically homogenized (Omni-Ruptor 4000, Omni International Inc.) in MPER buffer in an ultrasonic homogenizer. Proteins were extracted by sonication for over 4 h at 4 °C. The samples were centrifuged at ~16000 ×g for 30 min at 4 °C.

The protein concentration in the clear extract was measured by using a bicinchoninic acid protein assay kit (Thermo Scientific). First-dimension isoelectric focusing (IEF) was then performed to separate the proteins according to their isoelectric point difference by using precast 7 cm-long immobilized pH gradient (IPG) strips (Ready Strip IPG Strip 3-10 NL, Bio-Rad Laboratories). The protein specimens were loaded onto focusing trays (PROTEAN IEF Focusing Tray, Bio-Rad Laboratories) containing 300 μL of a mixture of rehydration buffer (7M urea, 2M thiourea, 4% CHAPS, 100 mM DTT, 0.2% carrier ampholyte [pH 3–10], trace of bromophenol blue), and sample buffer containing 160 μg of protein. After in-gel rehydration at 20 °C for 12 h, the proteins were focused at 250 V with a linear ramp for 20 mins, 4000 V with a linear ramp for 2 h, 8000 V with a rapid ramp for 2 h, and 10000 V with a rapid ramp for 6 h. Immediately after IEF, the IPG strips were equilibrated in the first equilibration buffer (6 M urea, 2% SDS, 375 mM Tris-HCl [pH 8.8], 20% glycerol, 2% [w/v] DTT) for 15 min and then in the second equilibration buffer (6 M urea, 2% SDS, 375 mM Tris-HCl [pH 8.8], 20% glycerol, 2.5% [w/v] iodoacetamide) for another 15 min. After rinsing with 1× Tris–glycine–SDS running buffer, the IPG strips were placed on 12% SDS-PAGE gels and sealed with 1% agarose (low-melt agarose, Sigma-Aldrich).

Second-dimension SDS-PAGE was conducted in a Mini-PROTEAN cell at 100 V for 0.5 h, followed by

electrophoresis at 200 V until the bromophenol blue front reached the bottom of the gels. The protein spots on the gels were fixed and visualized by staining with Coomassie blue R-250 and scanned using a Molecular Imager GS 800 Calibrated Densitometer (Bio-Rad Laboratories) with PD Quest software (Bio-Rad Laboratories). The gel images were analyzed using Progenesis SameSpots software (Nonlinear Dynamics). The gels of each group were pooled, and patterns reflecting differential expression were determined by spot-matching and assessed by principal component and correlation analyses. Mean differences were considered statistically significant at $P <$ 0.05 and ≥ 1.5-fold variation. Significantly different protein spots were then subjected to tryptic digestion according to the protocols described by Shevchenko *et al.*[20] The gel spots were destained overnight via incubation in 50% acetonitrile and 50 mM ammonium bicarbonate. Each gel spot was then reduced using 10 mM dithiothreitol and alkylated using 55 mM iodoacetamide in 100 mM ammonium bicarbonate. Following trypsin (Promega, Thermo Fisher Scientific) digestion for 18 h at 37 °C, the peptides were recovered and extracted from the sliced gels using 5% formic acid and 50% acetonitrile. After extraction, the peptides were dehydrated with 100% acetonitrile and dried in a vacuum concentrator (SpeedVac, Thermo Scientific, Savant DNA 120) for 3 h. Trypsin (final concentration, 7 ng/μL) in 50 mM ammonium bicarbonate was digested at 37 °C for 18 h. The peptides were subsequently recovered and extracted from the sliced gels by using 5% formic acid and 50% acetonitrile. The peptides were then solubilized with 10–20 μL of 0.1% formic acid, desalted with ZipTip C18 (Millipore), and then stored at −80 °C until MALDI-ToF/ToF MS for comparative proteomics.

Comparative proteomics by MALDI-ToF/ToF MS

Each of the excised gel plugs, which represent an individual protein from the gels, was analyed by MALDI-ToF/ToF MS on a 5800 System (ABSciex, Framingham, USA). A matrix consisting of a saturated α-cyano-4-hydroxycinnamic acid solution (Sigma-Aldrich, Malaysia) prepared from 50% acetonitrile/0.1% trifluoroacetic acid was mixed with peptide specimens at a 1:1 ratio. Subsequently, 0.7 μL of each specimen was spotted onto the target plates. The specimens spread and evaporated rapidly. The mass spectra of the peptides were acquired in positive-ion reflector mode, and default peak calibration was implemented for the MS/MS spectra. The precursor ion was selected from the mass spectra. Fragmentation was subsequently performed for the top 20 most abundant precursor ions using high-energy collision induced dissociation (CID). The collision energy was set to 1 keV, and air was used as the collision gas. The criterion for precursor selection was a minimum signal-to-noise ratio of 5. Mass accuracy was within 50 ppm for the mass measurements and within 0.1 Da for the CID experiments.

The list of peptide masses obtained was matched with the Swiss-Prot protein database. Peaks from trypsin auto-proteolysis and known contaminants, such as keratin, were discarded in the database searches. Global Protein Server Explorer 3.6 software (Applied Biosystems), which uses an internal MASCOT (Matrix Science) program to match MS and MS/MS data against database information, was used to process and analyze the peaks generated from the protein spectra. MS profiles were used by the search engines to identify proteins from the primary sequence databases, and the data were screened against the latest human databases, which were downloaded from the Swiss-Prot/TrEMBL homepage (http://www.expasy.ch/sprot).

RESULTS

Demographic data

The participants in the denture group consisted of four males and seven females with ages ranging from 52 years to 79 years (mean, 62 years). These patients had been wearing complete dentures for at least 3 months, and some have had multiple sets of dentures. The control group comprised three males and eight females, with ages ranging from 30 years to 62 years (mean, 49 years).

Mucosal protein profile analysis

Differentially expressed protein spots were visualized by Coomassie brilliant blue staining. Figure 1 shows the difference in 2D gel maps obtained between the denture and non-denture groups. Data analysis was performed using Progenesis SameSpots software. Proteins showing statistically significant differential expression with p ≤ 0.05 (ANOVA) as the significance threshold and minimum fold-change ≥1.5 fold were selected for in-gel trypsin digestion. MALDI-ToF/ToF MS was then conducted for protein identification. A total of 11 gels from each group were analyzed, and 1 gel was selected as the representative gel.

Comparison of the results of the control and test groups yielded a total of seven statistically significant spots: three upregulated spots and four downregulated spots. These seven spots were excised, analyzed using MALDI-ToF/ToF and identified using the MASCOT search engine against the entries of *Homo sapiens* in the Swiss-Prot database. The spots indicated by black circles in the 2D master map obtained from the analysis were developed as shown in Figure 2. In the denture group, lumican (+1.7 fold, P = 0.026), 78 kDa glucose-related protein (GRP78; +2.1 fold, P = 0.024), and serum albumin (+1.8 fold, P = 0.028) showed significant upregulation in the denture group (Table 1). Downregulation of the expression of hemoglobin subunit beta (HBB; +1.9 fold, P = 0.010), (HSP27; +1.9 fold, P = 0.016), and Ig gamma-1 chain C region (IGHG1) (+1.9 fold, P = 0.036 and +3.9 fold, P = 0.042) was also observed in the denture group. The two IGHG1 proteins identified in this study represent IGHG1 isoforms with different isoelectric

points and molecular masses. Lumican, GRP78, and HSP27 are known proteins associated with bone remodeling. Serum albumin, HBB, and IGHG1 are common proteins found in abundance in serum and non-target proteins from the biomarker perspective.[21]

DISCUSSION

This study compared differentially expressed proteins between the compressed and non-compressed mucosa of denture and non-denture wearers. The null hypothesis was rejected. The results revealed significant upregulation of lumican and GRP78, which are proteins involved in bone resorption, as well as significant downregulation of HSP27, a protein involved in bone deposition, in the denture group. This finding supports the hypothesis that the pressure exerted by the denture on the mucosa could cause differential expression in some proteins related to bone resorption and deposition. However, this finding should be interpreted with caution because the number of patients involved is quite small and the age groups were not similar. The dissimilarity in age groups in this study is due to the difficulty of recruiting healthy edentulous participants suitable for implant overdenture treatment. Diabetes is highly prevalent in the Malaysian population,[22] and uncontrolled diabetes is a contraindication for dental implants.[23] Difficulties in recruiting age-matched participants with healthy periodontia for the control group (diabetes is also associated with periodontitis) were also encountered;[24] thus, participants in the control group were generally younger than those in the test group. Patients with periodontitis were excluded to avoid false-positive results because some biomarkers related to bone remodeling induced by mechanical pressure are also inflammatory cytokines, such as IL-6 and TNF-α, which are similarly expressed in periodontitis.[7,25–27]
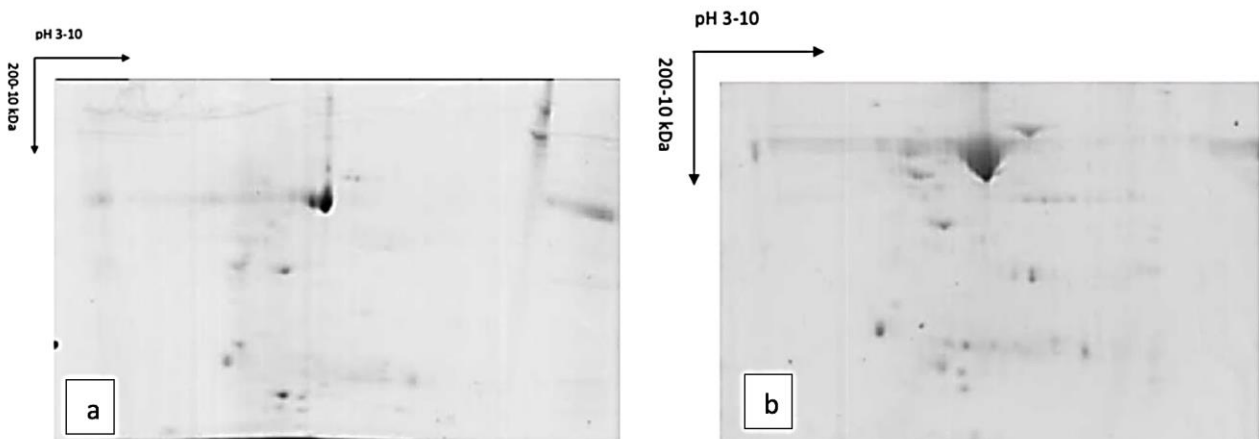
FIGURE 1. Representative 2D gels (pH 3–11) of the non-denture (a) and denture (b) groups. Approximately 160 µg of proteins was initially separated by a linear pH of 3–10, followed by separation on SDS-PAGE gels (12%) and Coomassie blue staining

FIGURE 2. Representative 2-DE gel map of tissue proteins for spot excision and protein identification by using Progenesis SameSpots software. Black circles highlight protein spots reflecting statistically significant differences

TABLE 1. Differentially expressed tissue proteins observed among complete-denture wearers

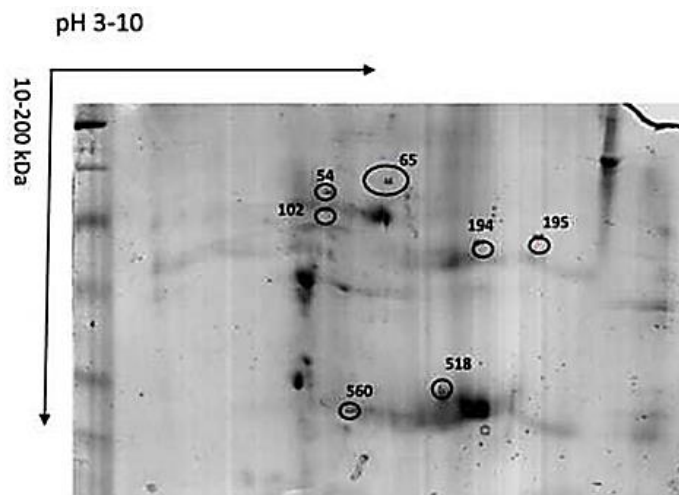| Spot No	Identified Protein	Accession Number	Calculated pI value	Nominal Mass (Mr)	Number of Matched Peptides	Sequence Coverage (%)	Fold Change	Mascot Score	p	General Function
518	Hemoglobin subunit beta	HBB_HUMAN	6.75	16102	11	62	1.9	471	0.010	Oxygen transport
560	Heat Shock Protein 27	HSPB1_HUMAN	5.98	22826	11	34	1.9	323	0.016	Molecular chaperone
54	78 kDa glucose-related protein	GRP78_HUMAN	5.07	72402	21	21	2.1	355	0.024	Protein folding
102	Lumican	LUM_HUMAN	6.16	38747	4	6	1.7	104	0.026	Collagen binding
65	Serum albumin	ALBU_HUMAN	5.92	71317	7	6	1.8	208	0.028	Blood colloidal osmotic pressure regulation
194	Ig gamma-1 chain C region	IGHG1_HUMAN	8.46	36596	6	10	1.9	128	0.036	Immune response
195	Ig gamma-1 chain C region	IGHG1_HUMAN	8.46	36596	2	3	3.9	73	0.041	Immune response

Lumican belongs to the family of small leucine-rich proteoglycans known to regulate collagen fibril organization to promote tissue healing,[28] maintain extracellular bone matrix homeostasis, and enrich bone mineralization.[29] Lumican promotes collagen organization when induced by pressure[30] and stimulates the expression of transforming growth factor-β, which has been shown to be highly expressed under mechanical loading.[31,32] We believe that a similar phenomenon occurs in the mucosal tissue underneath the denture due to pressure from denture loading, resulting in the high expression of lumican observed in the current study. Lumican has been shown to mediate cartilage destruction and upregulate macrophages and inflammation;[33] it is also known to be highly expressed in degenerative changes of the temporomandibular joint.[34] The protein has been reported to play an osteoprotective role during bone metabolism and represents a dual-action therapeutic target for osteoporosis.[35] Lumican inhibits osteoclast differentiation and in vitro bone resorption and could affect most stages of osteoclastogenesis by suppressing Akt activity. In our study, lumican was upregulated in denture-wearing patients. We thus hypothesize that changes in lumican may represent a form of feedback to increases in bone formation and decreases in bone resorption.

GRP78 belongs to the family of high-molecular weight HSP70. HSP70 is also known as endoplasmic reticulum (ER) chaperone binding immunoglobulin protein (BiP) and heat shock 70 kDa protein 5 (HSPA5). GRP78/BiP is a major

Ca^{2+}-binding protein in the ER, modulates the unfolding protein response (UPR), facilitates protein assembly in the ER, regulates calcium homeostasis, and protects cells from ER stress.[36–40] ER stress is a condition caused by the accumulation of unfolded proteins in the ER lumen as a result of endogenous and exogenous factors, such as hypoxia, starvation, oxidative stress, and protein synthesis overload.[41] The increased GRP78 expression observed in the current study may be attributed to hypoxia resulting from tissue compression underneath the denture, which could cause ER stress. Sensing stress, ER activates UPR through the activation of transcription factor 6, pancreatic ER kinase, and serine/threonine-protein kinase/endoribonuclease inositol-requiring protein-1α pathways.[42,43] These pathways upregulate GRP78, induce RANKL, and activate osteoclastogenesis.[44] Bone destruction that occurs during periodontitis could be caused by the excessive activation of osteoclasts or osteoclastogenesis. Osteoclastogenesis is regulated by RANKL-produced osteoblasts and osteocytes, and activation of this process causes bone resorption, as observed by the upregulated expression of GRP78 in denture wearers in this study.

HSPs are major proteins expressed in various tissues and organs as a result of mechanical load[15] and cytotoxic stress.[45] HSP's are induced not only by heat shock but also by various pathological changes, such as ischemia, infection, and inflammation.[46,47] HSP's are involved in the regulation of cell function and defense and responses to cell injury.[48,49] HSP27, HSP70, and HSP90 have been

reported to induce bone resorption, but their mechanisms of action have yet to be established.[15,50,51] During orthodontic treatment, significant expression of HSP27 may be triggered on the tension side after induction of a mechanical load; the protein then acts as a molecular chaperone for osteoblastic activation. Bone is formed on the tension side in the presence of active osteoblasts and resorbed on the compressive side where osteoblasts are unstimulated, thus creating progressive tooth movement.[12] These findings are in agreement with the downregulation of HSP27 observed in the present study. Because the tissue underneath dentures is in a compressive state, the pressure exerted by the prosthetic may lead to the resorption of bones observed in the patients.

Other than HSP27, which belongs to the same family of HSP70 that was previously found in the compressed mucosa of a rat model,[15] other proteins previously reported in bone resorption associated with denture wear, such as VEGF,[15] prostaglandin,[16] osteocalcin,[17] and C-terminal telopeptide,[17] were not observed to be differentially expressed in this work. This finding may be the result of differences in the specimens used for analysis (e.g., tissues versus serum) and sampling time points, which may represent different stages of inflammation. In addition, because pooled specimens were used in this study, dilution of low-abundance proteins in the specimens may have occurred. Validation of the identified proteins and specificity/sensitivity analyses could not be performed because of the limited amount of specimens collected from each patient. Future studies may collect larger amounts of specimens and perform individual analysis to allow identification of specific proteins as biomarkers. Specificity and sensitivity tests and receiver operating characteristic curves should also be analyzed to ensure the accuracy and replicability of the method used in the current study.

CONCLUSIONS

The results of our proteomics analyses suggest that soft-tissue proteomic profiling may potentially differentiate between non-compressed and compressed tissues; therefore, studying tissue proteins based on these profiles may provide some insights into the bone resorption mechanism of denture wear. The observation of differentially expressed proteins, such as lumican, GRP78, and HSP27, in the mucosa is likely a result of hypoxia and ER stress originating from tissue compression due to wearing mucosal-borne removable dentures.

CONFLICT OF INTEREST

None declared.

FUNDING

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