Proteomic analysis of salivary proteins and metagenomic analyses of oral and intestinal microflora in a rat model of chronic restraint stress

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Introduction:

Psychological stress is an important factor in the etiology of various oral diseases, such as burning mouth syndrome (BMS), atypical odontalgia, lichen planus, and recurrent aphthous stomatitis. The pharmacotherapeutic outcomes of psychotropic agents in BMS have been recently demonstrated (Paudel et al. Oral Diseases 24, 2020). The outcomes were found to vary among individuals, which may be due to differences in the psychological backgrounds and stress levels. Alterations in salivary proteins and the oral microflora as a result of chronic psychological stress might be associated with the etiopathogenesis of these diseases. However, there is little information on this association so far. Recently, it was demonstrated that chronic psychological stress elevates IL-1β levels in the saliva and submandibular glands of mouse (Paudel et al., Medical Molecular Morphology 53 (4), 2020). IL-1β might be involved in stress-related diseases and can act as a potential biomarker in response to chronic stress. Global analyses are required to fully understand the associations between stress and both salivary proteins and the oral microflora. Therefore, proteomic analysis of salivary proteins and metagenomic analyses of oral and intestinal microflora were carried out using a rat model of chronic restraint stress.

Materials and Methods:

A. Stress protocol and saliva collection

Six-week-old Sprague Dawley rats were randomly divided into a control group and a stress group. The rats in the stress group (n = 10) were maintained under restraint stress for 1 month following an established protocol (Yun et al., Journal of Neurochemistry, 2010). Briefly, the rats were enclosed in a plastic tube with the ends covered by a wire mesh thereby restricting movement for 4 hours daily. After a month, behavior analysis (elevated plus maze test) was done to confirm the stress level. The mice were anesthetized with pentobarbital (50 mg/kg, intraperitoneal) and pilocarpine (5 mg/kg, intraperitoneal) was injected to stimulate the saliva, which was collected in a 1.5 ml centrifuge tube using a pipette.

B. *Identification of altered proteins*

i. Gel electrophoresis and identification of protein spots

Two-dimensional gel electrophoresis was performed for each sample to determine the alterations in the salivary proteins. The salivary proteins were focused into two dimensions: the isoelectric point with an IpG strip (Bio-Rad, USA) and the molecular weight with sodium dodecyl sulfate-polyacrylamide gel. The protein spots on the gels were recorded using the ProExpress 2D Proteomic Imaging System (PerkinElmer Inc.,USA). The expression levels of the protein spots were quantified using the Progenesis SameSpot software (Nonlinear Dynamics Ltd, UK), and differences in expression among samples were analyzed statistically using the analysis of variance (ANOVA test). A p-value of < 0.05 was considered significant.

ii. Mass spectrometry for protein identification

The significantly altered spots were subjected to LTQ linear ion trap mass spectrometry (ThermoElectron, San Jose, USA) equipped with a nano-electrospray ion source (AMR, Japan) for protein identification. The Mascot software (v2.3.01; Matrix Science, UK) was used to search for peptide mass ion peaks against the SWISS-PROT database.

iii. Western blotting for protein confirmation

The candidate proteins were subjected to Western blotting for confirmation. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed in precast gels, which were then transferred onto polyvinylidene difluoride membranes. The membrane was then stained with Ponceau S stain for total protein normalization. The membrane was blocked with 5% skimmed milk and incubated with primary and secondary antibodies.

C. *Identification of altered oral and intestinal microflora*

i. Sample preparation and sequencing

Oral swabs were collected using a polystyrene swab. Genomic DNA was extracted from the swabs using the DNeasy Blood and Tissue kit (Qiagen, Germany). Stool was collected from

the intestine and genomic DNA was extracted using the DNeasy Powersoil Kit (Qiagen). The DNA was then used for 16S metagenomic sequencing library preparation for the Illumina MiSeq System. The V3-V4 regions of the bacterial ribosomal RNA were targeted using a two-step polymerase chain reaction (PCR) process.

ii. Data analysis

The obtained data were analyzed with Quantitative Insights into Microbial Ecology. Bacterial taxonomy, alpha diversity, and beta diversity were evaluated in the saliva and stool; significant differences in alpha diversity, beta diversity and relative abundances were assessed using the Kruskal-Wallis test, Permutational multivariate ANOVA and Analysis of Composition of Microbiomes (ANCOM), respectively.

Results and Discussion:

1. Stress markers

The mean body weight of the stress group rats was significantly lower than that of the controls ($p < 0.05$). The number of entries and time spent in the open arm of the elevated plus maze were significantly lower in the stress group compared to the controls ($p < 0.05$). This confirmed that rats were sufficiently stressed after 1 month of the stress protocol.

2. Alterations in the salivary proteins

The Progenesis SameSpot software detected 33 significantly altered protein spots on the gel. The gels were further stained with a silver stain from which eight spots were visible and cut out for mass spectrometry. Proteins such as the BPI fold-containing family A member 2 (BPIFA2; 2.6-fold change), von Ebner's gland protein (VEGP: 1.9-fold change), α-amylase (1.8-fold and 1.5-fold change), carbonic anhydrase 6 (1.6-fold change), common salivary protein (1.7-fold change) and cystatin D (1.6-fold change) were identified. The two most significantly altered proteins, BPIFA2 and VEGP, were confirmed by Western blotting.

BPIFA2 is a parotid secretory protein known to colonize bacteria and *Candida albicans* in the oral cavity. Recently, it has been demonstrated to play a physiological role in maintaining the surface tension of the saliva. VEGP belongs to the Lipocalin family and is known to play a role in bitter taste perception. The results suggest that BPIFA2 and VEGP affect the physical properties of saliva, taste perception, and bacterial and fungal colonization under stress conditions.

3. Alteration in oral and intestinal microflora

Metagenomic analysis of the oral microbiome revealed significant reductions in alpha diversity (observed operational taxonomic units) in the stress group when compared to the controls (Kruskal-Wallis test; $p < 0.05$). Metagenomic analysis of the intestinal microbiome demonstrated results that were consistent with those of previous studies, thus validating our stress protocol.

Conclusion:

This study identified novel salivary proteins and reduced alpha diversity of the oral microbiome that was caused by psychological stress in an animal model. These findings may help identify potential biomarkers for psychological stress and aid in understanding the pathogenesis of stress-related oral diseases.