Microgravity Alters the Expression of Salivary Proteins

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Abstract

Spaceflight provides a unique opportunity to study how physiologic responses are influenced by the external environment. Microgravity has been shown to alter the function of a number of tissues and organ systems. Very little, however, is known about how microgravity affects the oral cavity. The rodent model is useful for study in that their salivary gland morphology and physiology is similar to that of humans. Useful also is the fact that saliva, a product of the salivary glands with a major role in maintaining oral health, can be easily collected in humans whereas the glands can be studied in experimental animals. Our working hypothesis is that expression of secretory proteins in saliva will respond to microgravity and will be indicative of the nature of physiologic reactions to travel in space. This study was designed to determine which components of the salivary proteome are altered in mice flown on the US space shuttle missions and to determine if a subset with predictive value can be identified using microscopy and biochemistry methods. The results showed that the expression of secretory proteins associated with beta-adrenergic hormone regulated responses and mediated *via* the cyclic AMP pathway was significantly altered, whereas that of a number of unrelated proteins was not. The findings are potentially applicable to designing a biochemical test system whereby specific salivary proteins can be biomarkers for stress associated with travel in space and eventually for monitoring responses to conditions on earth.

Background

Production and secretion of saliva, controlled by the body's sympathetic and parasympathetic autonomic nervous system, are essential for oral health and comfortable, daily function. The cleansing, buffering, and anti-microbial activities of saliva help to protect the teeth and the oral mucosa. Articulation, mastication and swallowing would become difficult without the mucous and serous secretions. The three major salivary glands, the parotid, submandibular and sublingual glands, are accompanied by numerous minor glands dispersed throughout most regions of the oral mucosa. Some of the major proteins in human saliva are amylase, lysozyme, peroxidase, proline-rich proteins, histatins, mucins and IgA [1-5]. Hundreds of others have been identified by proteomic analysis [6,7]. While some of the secretory proteins have specific function in the mouth, others can be indicators of the organism's systemic physiology or diagnostic of disease status [8,9]. Previously, the environmental effects of microgravity on rat parotid glands [10,11] were studied using animals flown on SpaceLab 3 and several Russian Cosmos missions, and the results compared with those of ground control animals. The present experiments were conducted to test the hypothesis that cyclic AMP (cAMP)-signaling pathways would be affected by time in space. Changes in protein expression would be found in catecholamine hormone regulated protein expression and longer exposure (to microgravity) has an additive effect on the expression of secretory proteins regulated by β-adrenergic stimulation in the parotid gland.

Cyclic AMP is a second messenger mediating the action of catecholamines and several peptide hormones. Extracellular stimuli such as stress, norepinephrine and numerous other hormones regulate the activity of other proteins acting downstream to cAMP signaling. Activation of the different receptors results in changes in local concentration of cAMP in cells that leads to different physiological responses. Numerous cellular processes affect cAMP signaling. These influences include other signaling pathways which are themselves variously regulated to organize the elements of the pathway, and subcellular targeting of components. Measurements solely of cAMP levels may minimize the complexity of the cAMP signals and the regulation of targets. Therefore, measuring subunit expression of cAMP-dependent protein kinase (protein kinase A, PKA) reflects a specific pathway that is affected by a particular stimulus and shows its physiologic response. Protein kinase A is an enzyme that regulates processes as diverse as growth, development, memory, and metabolism. The catalytic and regulatory subunits of PKA are highly dynamic signaling proteins but inactive in the holoenzyme form shown as R_2C_2 in the reaction formula below. The holoenzyme is dissociated by increased concentration of cAMP.

$$R_2C_2 \underset{}{\overset{[cAMP]}{\rightleftharpoons}} 2R + 2C$$

Type-II PKA associates with cellular structures and organelles; it is not a free-floating enzyme but is anchored to specific locations within the cell by specific proteins called A kinase-anchoring proteins (AKAPs). The structure of an AKAP docked to the dimerization/docking domain of the type II (RII alpha) isoform of PKA has been well characterized [12,13].

The discovery that the regulatory subunit of type II PKA (RII) is a pleiomorphic secretory protein has provided a direct means for studying cAMP regulated events using it as a biofluid marker protein [14-18]. Subsequent studies using the rat as a model have shown that measuring RII and several other secretory proteins in saliva reflected their expression as a consequence of altered gravity conditions [19,20]. Additionally, RII was affected in experimental diabetes [21,22], and similar results were found when human saliva was tested in response to mechanical and other types of stress and

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in salivary glands of human diabetics [16,23,24]. Measuring RII in specific disorders, in addition to standard clinical tests, may serve as a useful diagnostic biomarker.

Methods

A total of 15 adult female C57Bl/6J mice were flown for 15 and 13 days on STS-131 and -135, the Discovery and Atlantis space shuttle missions, respectively. The mice were housed in Animal Enclosure Modules (AEMs), which are enclosed rodent habitats that provide ventilation, lighting, waste collection, food and water for the rodents. Twenty-three ground control mice were housed in AEMs for the same length of time as the flight. Fifteen additional ground control mice housed under standard vivarium conditions were additional controls for the STS-135 mission. The flight and AEM ground control mice were fed the NASA semi-moist solid rodent food bar diet [25] *ad libitum*. Both flight and control mice were provided water *ad libitum*. Average body weights, food and water consumption of the flight and AEM ground control mice are given in *Table 1*. Within 5 hr of landing, parotid salivary glands were dissected, fixed for ultrastructural analysis and immunogold labeling, or frozen for Western blotting and microarray analyses. The glands were prepared for microscopic analyses as previously described [26]. Samples for Western blotting were frozen in buffer containing protease inhibitors; samples for RNA extraction and microarray analyses were frozen in RNAlater (Qiagen, Hilden, Germany).

Tissue morphology and immunocytochemistry were studied using light and transmission electron microscopy (TEM). Immunogold labeling experiments were carried out, which provide a method for quantitative analysis of relative changes in protein expression. After incubation of sections with the proper primary antibody and gold-labeled secondary antibody, the number of gold particles over cell organelles was counted in TEM images. Immunogold labeling is quantitatively comparable to biochemically derived protein expression data, electrophoresis and Western blotting. The proteins analyzed using immunogold labeling included: amylase, parotid secretory protein (PSP), proline rich protein (PRP), demilune cell and parotid protein (DCPP) and RII (type II regulatory subunit of PKA). Labeling densities recorded as gold particles/μm^2 were determined using data from randomized samples. A hierarchical linear model was used to determine statistically significant differences between flight and control animals. In this approach, the lowest unit of analysis was a single image, which was nested within each animal, while the highest unit of analysis, the animal, was nested within each condition of the independent variable, flight or control. This type of analysis accounts for the nesting of images within animals by assuming that each of the means and variances of cell images within a given animal are not necessarily identical across animals. This provides a more equitable assessment of differences while still utilizing the more conservative error degrees of freedom based on the number of animals as the units of analyses.

Amylase, RII and IgA were used for biochemical studies. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of proteins was carried out by a modified method of Towbin et al. [27], using 10% polyacrylamide gels. Identical amounts of protein were loaded per lane and a molecular size marker lane was included on each gel. Both flight and control samples were run on each gel for comparison consistency. The separated proteins were transferred to nitrocellulose membranes by electroblotting and the proteins were visualized by staining with Ponceau S, a washable dye [28]. The banding pattern was recorded digitally, the membrane cleared of dye, incubated with specific antibodies and the specific protein identified using peroxidase conjugated second antibody reaction. The resulting Western blot was digitized and quantified by densitometry. The ratio of specific protein to total protein per lane was calculated to indicate relative reactivity.

Microarray analyses (PhalanxBio Inc., San Diego CA) used isolated RNA from gland tissues in RNAlater, stored and shipped at -80˚C. Then a column-based or Trizol isolation, according to the company protocol, was used which included a DNase treatment. After extraction, an RNA Quality Control (QC) was performed prior to proceeding with microarray service. The QC steps included a measurement of 260, 280, 230 absorbances, running a gel, as well as the use of the Agilent Bioanalyzer to verify RNA quality. The analyzed data consisted of normalized intensities that are compared for fold-changes, and a p-value was calculated based on the replicate data. Biomarker candidates were selected based on p-value (<0.001) and changes in protein expression ($\geq$ 1.5-fold increase or decrease). Bioinformatics analyses were provided of salivary proteins in both flight and control samples and identified significant changes in expression due to space flight.

Results

Overall, the morphology of parotid acini and ducts of the flight animals was not extensively altered when compared to that of the AEM and vivarium ground controls. However, specific cellular modifications were observed in flight animals. These consisted of an apparent increase in the number of

Table 1. *Average Body Weights, Food and Water Consumption of Flight and AEM Control Mice.*

Mission Description[1]	Number of Mice	Average Weight (gm)		Average Daily Consumption	
		Cage Loading	Cage Unloading	Food (gm)	Water (ml)
STS-131 Flight	8	22.02	20.07	3.5	2.96
STS-131 Control	8	22.03	21.28	4.0	3.9
STS-135 Flight	7	20.74	18.43	4.1	2.73
STS-135 Control	15	20.69	19.4	4.1	3.38

[1]Data from shuttle missions STS-131 (age 16 weeks at launch) and STS-135 (age 9 weeks at launch).

autophagic vacuoles in acinar cells, and apoptotic acinar cells. Additionally, intercalated duct cells and occasionally striated duct cells of some flight animals contained large, electron dense vacuoles in their apical cytoplasm. These vacuoles were similar to those described in duct cells of diabetic rats and shown to contain acinar secretory proteins, presumably a result of endocytosis of luminal content [29,30]. These vacuoles were not observed in duct cells of ground control animals, implying a change in duct cell function during flight.

The molecular composition, shown by the distribution and expression of a number of specific cellular proteins, was altered in the flight animals. As determined by quantitative immunogold labeling, amylase ($p=0.002$) and PRPs ($p=0.003$) were significantly decreased in acinar cell secretory granules of flight animals compared to the AEM control mice. Labeling for PKA RII decreased in acinar cell cytoplasm ($p=0.04$) and was slightly reduced in secretory granules of flight animals, whereas RII labeling of nuclei was unchanged (*Figures 1 and 2, Table 2*) when compared to ground controls. Labeling of intercalated duct cell granules for DCPP was increased ($p=0.006$) in flight animals (*Table 2*). In contrast, no differences were seen in labeling for PSP ($p=0.63$). Thus, modifications in protein expression due to microgravity are cell type dependent indicating functional specificity.

Electrophoretically separated total protein banding patterns were not markedly altered in flight parotid glands compared to those of controls. However, the expression of α-amylase ($p<0.01$) and IgA ($p<0.05$) was significantly reduced. Proteins that govern β-adrenergic signal transduction appeared generally decreased during flight. Other secretory proteins were increased, some decreased and still others were unchanged after travel in space. These results indicate that the effect of microgravity is not a uniform disruption, but a specific response of individual pathways and the expression of corresponding protein components. The protein banding patterns of control and flight parotid gland soluble cell fractions are shown in *Figure 3A*, and the corresponding Western blot in *Figure 3B*. Densitometric analysis of the Western blot results is shown in *Table 3*. RI was absent in the parotid, but RII was significantly reduced in the flight animals when compared to that of controls.

Preliminary analysis of the microarray data showed consistent results for proteins tested by Western blotting (*Table 4*). The level of intracellular cAMP is regulated by the balance between the activities of two types of enzyme: adenylyl cyclase and cyclic nucleotide phosphodiesterase (PDE). Both cAMP specific isoforms of PDE, PDE 4A and PDE 4D, were significantly decreased by microgravity. Interestingly, the expression of Akap13, which binds to RIIα, was increased 1.5-fold in the flight animals.

The changes that occur during space flight may not necessarily be detrimental, but may serve to maintain basal levels of catecholamine hormone responses by selectively altering protein expression.

Discussion

The effects of gravity on biological systems are not well understood. Studying the effects of microgravity on cells and tissues can provide important information on how gravity affects a number of signaling and metabolic processes. The degree to which an organism resists perturbation or stressful influences depends on its resilience, *i.e.*, adaptability, or the capacity to change in response to stress in ways that maintain overall organismal integrity. These properties can be understood through studies of regulatory processes and structural properties.

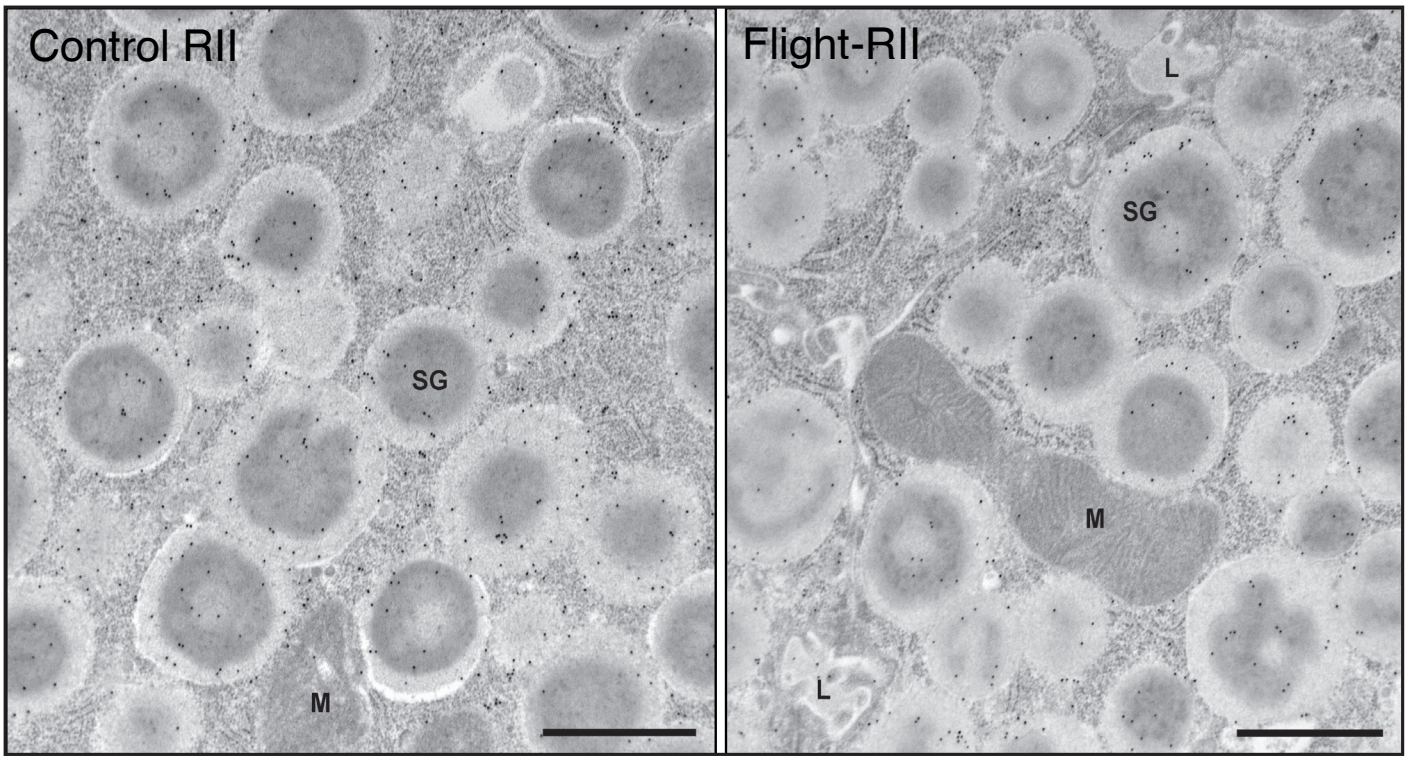

Figure 1. *Labeling of parotid acinar cells with anti-RII antibody. Label is present over secretory granules (SG) and the lumina (L), consistent with previous EM studies [15,17,21] and biochemical analyses [14,16,18]. Gold particles also are present over the cytoplasm, but mitochondria (M) are unlabeled. Scale bars = 1 μm.*

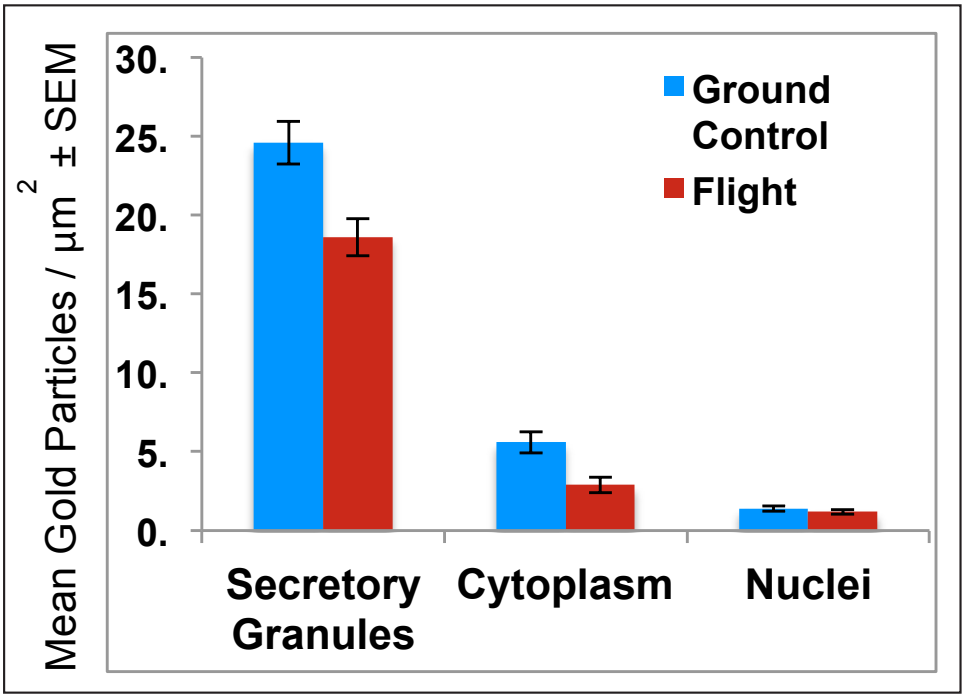

Figure 2. *Quantitative analysis of RII immunogold labeling of parotid acinar cells.*

Once activated the catalytic subunit of PKA can phosphorylate numerous substrates; the specificity of this process is determined by the cellular location of PKA through its association with AKAPs. The overall effect of PKA is to interact with co-activators and to facilitate the efficiency of signal transduction [31]. The activity of transcription factors, such as nuclear receptors and HMG (High Mobility Group)-containing proteins is modulated by PKA, influencing their dimerization or DNA-binding properties. In mammalian cells, including human cells, PKA also regulates a number of processes, including growth, development, memory, metabolism, and gene expression. Dysregulation of PKA can have disastrous consequences, including diseases such as cancer. Drugs based on altering PKA activity are under development for treating disease. Understanding, therefore, the consequences of changes in the expression of the components of this signaling pathway is important.

Salivary gland gene expression is modulated by numerous factors, including functional activity, neurohormonal stimulation and pharmacologic agents. Both the flight and AEM control mice were fed the same diet; therefore food consistency was not a factor contributing to the observed differences. Chronic β-adrenergic stimulation of rodent salivary glands leads to altered gene expression [2,32,33], including decreased amylase expression [33-35]. Stress due to microgravity exposure, acting *via* cAMP signaling pathways, coupled with decreased expression of PDE isoforms, could result in increased cAMP levels, PKA activation and alteration of gene expression. Although extended hypergravity conditions affect salivary protein expression [19], the transient hypergravity conditions occurring during launch and landing are not likely to alter the effects of 13-15 days of microgravity.

Rodent salivary glands are structurally and functionally similar to those of humans, and have been a widely used model for studies of salivary secretion. Exposing mice to microgravity results in changes in expression of specific

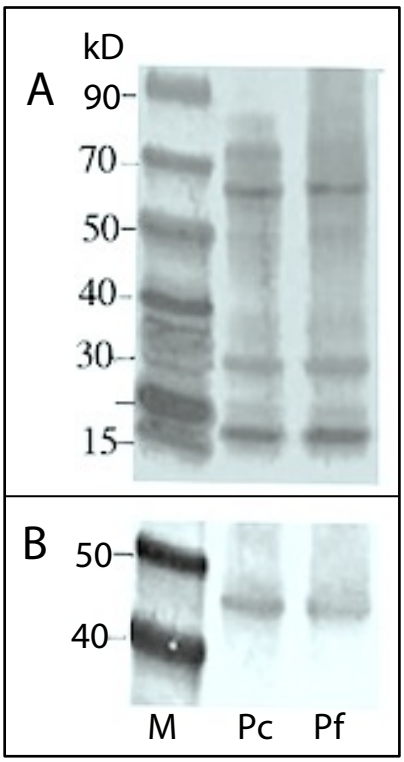

Figure 3. *PAGE, panel A and Western Blotting, panel B, of RII in flight and control parotid gland tissue extracts. Samples per lane were combined from 5 animals in each category. M is the kD marker lane, Pc and Pf are control and flight parotid, respectively.*

secretory proteins in their parotid glands compared to controls. Secretory granule proteins are released into saliva upon stimulation of the salivary glands. Changes in salivary proteins may serve as indices of physiological responses to travel in space by astronauts, as well as to environmental stressors in earth-based studies. Therefore employing saliva as a diagnostic test medium could be useful in measuring human responses to spaceflight and other stressful conditions, as well as having clinical applications.

Table 2. *Quantitative Analysis of Immunogold Labeling of Parotid Secretory Granules.*

Protein	Control	Flight	p Value
Amylase	21.49 ± 1.65	14.97 ± 1.09	0.002
PRP	41.44 ± 4.29	13.83 ± 1.48	0.003
RII	24.57 ± 1.35	18.60 ± 1.20	0.41
DCPP	52.04 ± 1.91	70.45 ± 4.31	0.006
PSP	34.49 ± 1.24	31.44 ± 1.42	0.63

Results expressed as mean gold particles/μm^2 ± SE are shown for a representative experiment.

Table 3. *Densitometric Analyses of RII Western Blot.*

	Integrated Density		Ratio
	Western Blot	Total Protein	
Control	10.05	60.35	0.17
Flight	9.63	67.98	0.14

Ratios, calculated as measured integrated density, in arbitrary units, of Western blot antibody absorbance to that of total protein per lane (each loaded with the same amount of total protein) show an approximately 18% decrease in the flight compared to that of control reactivity.

Table 4. *Microarray Analyses of the Effect of Spaceflight on Genes Involved in cAMP Signaling Pathways.*

Gene symbol	PF/PG log 2	PF/PG fold diff	p value
Prkar2a	-0.54	0.69	<0.001
Prkar2b	-0.001	0.999	0.987
Akap13	0.597	1.52	<0.001
Pde4a	-0.89	0.54	<0.001
Pde4d	-0.398	0.76	0.007

Ratios of samples from flight mice to AEM ground control mice (PF/PG) in microarray analysis. Significant differences were selected based on p-value (<0.001), changes in gene expression (≥ 1.5-fold increase or decrease). Gene symbols: Prkar2a, protein kinase, cAMP dependent regulatory, type II alpha; Prkar2ab, protein kinase, cAMP dependent regulatory, type II beta; Akap13, A kinase (PRKA) anchor protein 13; Pde4a, phosphodiesterase 4A, cAMP specific; Pde4d, phosphodiesterase 4D, cAMP specific.

No significant tissue damage was observed, therefore the molecular level differences due to travel in space may be homeostatic responses, adjustment to a changed environment. It will be important to determine if the values return to control levels after returning to earth, and if so within what time span. These results demonstrate specific environmental stress responses, measurable in an accessible body fluid, and may form a basis for developing analyses using saliva for measuring human responses to spaceflight. The specific purpose of these studies is to obtain data that can be used to develop a testing device for astronauts as well as have general clinical applications on Earth.

Acknowledgements

Support: CT Space Grant College Consortium, School of Dental Medicine Alumni Research Fellowship and the NASA Award Number, NNX09AP13G, entitled, "PROTEIN EXPRESSION IN SALIVARY GLANDS: EFFECTS OF EXTENDED SPACE FLIGHT". The authors also thank Ms. Maya Yankova for preparing sections for electron microscopy, and acknowledge use of the Central Electron Microscope Facility, University of Connecticut Health Center, and NIH Shared Instrumentation Grant 1S10RR24742-1A1.

References

1. Azen EA. Genetic protein polymorphism of human saliva; an interpretive review. *Biochemical Genetics*. 1978; **16**: 79-99.

2. Carlson DM, Zhou J, Wright PS. Molecular structure and transcriptional regulation of the salivary gland proline-rich protein multigene families. *Progress in Nucleic Acid Research*. 1991; **41**: 1-22.

3. Oppenheim FG, Hay DI, Franzblau C. Proline-rich proteins from human parotid saliva. I. Isolation and partial characterization. *Biochemistry*. 1971; **10**: 4233-4238.

4. Oppenheim FG, Xu T, McMillian FM, Levitz SM, Diamond RD, et al. Histatins, a novel family of histidine-rich proteins in human parotid secretion. Isolation, characterization, primary structure, and fungistatic effects on Candida albicans. *Journal of Biological Chemistry*. 1988; **263**: 7472-7477.

5. Offner GD, Troxler RF. Heterogeneity of high-molecular-weight human salivary mucins. *Advances in Dental Research*. 2000; **14**: 69-75.

6. Denny P, Hagen FK, Hardt M, Liao L, Yan W, et al. The proteomes of human parotid and submandibular/sublingual gland salivas collected as the ductal secretions. *Journal of Proteome Research*. 2008; **7**: 1994-2006.

7. Bandhakavi S, Stone MD, Onsongo G, Van Riper SK, Griffin TJ. A dynamic range compression and three-dimensional peptide fractionation analysis platform expands proteome coverage and the diagnostic potential of whole saliva. *Journal of Proteome Research*. 2008; **8**: 5590-8600.

8. Lima DP, Diniz DG, Moimaz SAS, Sumida DH, Okamoto AC. Saliva: reflection of the body. *International Journal of Infectious Diseases*. 2010; **14**: 184-188.

9. Miller CS, Foley JD, Bailey AL, Campell CL, Humphries RL, et al. Current developments in salivary diagnostics. *Biomarkers in Medicine*. 2010; **4**: 171-189.

10. Mednieks MI, Hand AR. Biochemical and morphological evaluation of the effects of space flight on rat salivary glands. *Physiologist*. 1985; **28**: S215-S216.

11. Mednieks MI, Hand AR. Salivary gland ultrastructure and cyclic AMP-dependent reactions in Spacelab 3 rats. American Journal of Physiology. 1987; **252**: R233-R239.

12. Burns LL, Canaves JM, Pennypacker JK, Blumenthal DK, Taylor SS. Isoform specific differences in binding of a dual-specificity A-kinase anchoring protein to type I and type II regulatory subunits of PKA. *Biochemistry*. 2003; **42**: 5754-5763.

13. Burns-Hamuro LL, Hamuro Y, Kim JS, Sigala P, Fayos R, et al. (2005) Distinct interaction modes of an AKAP bound to two regulatory subunit isoforms of protein kinase A revealed by amide hydrogen/deuterium exchange. *Protein Science*. 2005; **14**: 2982-2992.

14. Mednieks MI, Hand AR. Cyclic AMP binding proteins in saliva. *Experientia*. 1984; **40**: 945-947.

15. Hand AR, Mednieks MI. Regulatory subunits of cyclic AMP-dependent protein kinase: presence in granules and secretion by exocrine and endocrine cells. *Journal of Cell Science*. 1989; **93**: 675-681.

16. Mednieks MI, Epstein PM, Hachisu R, Hand AR, Bodner J, et al. Polymorphy of individual protein patterns and cyclic AMP reactions in human saliva. *Annals of the New York Academy of Sciences*. 1993; **694**: 299-301.

17. Piludu M, Mednieks M, Hand A. Cyclic AMP-receptor proteins in human salivary glands. *European Journal of Morphology*. 2003; **40**: 219-225.

18. Sivakumar T, Hand AR, Mednieks M. Secretory proteins in the saliva of children. *Journal of Oral Science*. 2009; **51**: 573-580.

19. Mednieks MI, Hand AR, Grindeland RE. Cyclic AMP-receptor responses to hypergravity. *Aviation, Space, and Environmental Medicine*. 1998; **69**: 28-36.

20. Mednieks MI, Burke JC, Sivakumar TP, Hand AR, Grindeland RE. Secretory proteins characteristic of environmental changes in cellular signal transduction: expression in oral fluid. *Proceedings of the International Forum on Biomedical Research on the International Space Station*. 2000; **504:** 433-455.

21. Szczepanski A, Mednieks MI, Hand AR. Expression and distribution of parotid secretory proteins in experimental diabetes. *European Journal of Morphology*. 1998; **36**: 240-246.

22. Mednieks MI, Szczepanski A, Clark B, Hand AR. Protein expression in salivary glands of rats with streptozotocin diabetes. *International Journal of Experimental Pathology*. 2009; **90**: 412-422.

23. Burke JC, Evans CA, Crosby TR, Mednieks MI. Expression of secretory proteins in oral fluid after orthodontic tooth movement. *American Journal of Orthodontics and Dentofacial Orthopedics*. 2002; **121**: 310-315.

24. Piras M, Hand AR, Mednieks MI, Piludu M. Amylase and cyclic AMP receptor protein expression in human diabetic parotid glands. *Journal of Oral Pathology & Medicine*. 2010; **39**: 715-721.

25. Sun G-S, Tou JC, Yu D, Girten BE, Cohen J. The past, present, and future of National Aeronautics and Space Administration spaceflight diet in support of microgravity rodent experiments. *Nutrition*. 2014; **30**: 125-130.

26. Hand AR. Electron microscopy. In: Introduction to Biophysical Methods for Protein and Nucleic Acid Research. Glasel JA, Deutscher M (Editors) New York: Academic Press. 1995, 205-260.

27. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proceedings of the National Academy of Sciences USA*. 1979; **76**: 4350-4354.

28. Correira H, Rivero J, Herrera F. Protein staining with Ponceau S during acid-urea-triton-polyacrylamide gel electrophoresis. *BioTechniques*. 1997; **22**: 846-848.

29. Hand AR, Weiss RE. Effects of streptozotocin-induced diabetes on the rat parotid gland. *Laboratory Investigation*. 1984; **51**: 429-440.

30. Lotti LV, Hand AR. Endocytosis of parotid salivary proteins by striated duct cells in streptozotocin-diabetic rats. *Anatomical Record*. 1988; **221**: 802-811.

31. Taylor SS, Kim C, Vigil D, Haste NM, Yang J, et al. Dynamics of signaling by PKA. *Biochimica et Biophysica Acta*. 2005; **1754**: 25-37.

32. Mednieks MI, Hand AR. Microheterogeneity of rat parotid gland proteins after chronic treatment with isoproterenol. *Journal of Dental Research*. 1984; **63**: 87-93.

33. Ten Hagen KG, Balys MM, Tabak LA, Melvin JE. Analysis of isoproterenol-induced changes in parotid gland gene expression. *Physiological Genomics*. 2002; **8**: 107-114.

34. Robinovitch MR, Keller PJ, Johnson DA, Iversen JM, Kauffman DL. Changes in rat parotid salivary proteins induced by chronic isoproterenol administration. *Journal of Dental Research*. 1977; **56**: 290-303.

35. Vugman I, Hand AR. Quantitative immunocytochemical study of secretory protein expression in parotid glands of rats chronically treated with isoproterenol. *Microscopy Research and Technique*. 1995; **31**: 106-117.