Applied Physiology, Nutrition, and Metabolism

Switching to normal diet reverses kwashiorkor-induced salivary impairments via increased nitric oxide level and expression of aquaporin 5 in the submandibular glands of male Wistar rats

Journal:	<i>Applied Physiology, Nutrition, and Metabolism</i>
Manuscript ID	apnm-2018-0282.R1
Manuscript Type:	Article
Date Submitted by the Author:	06-Jul-2018
Complete List of Authors:	Lasisi, Taye; University of Ibadan, Physiology; University College Hospital, Oral Pathology Shittu, Shehu-tijani; University of Ibadan, Physiology Alada, Akinola; University of Ibadan, Physiology
Keyword:	Kwashiorkor, Aquaporin 5, Leptin, Ghrelin, Salivary secretion, Malnutrition
Is the invited manuscript for consideration in a Special Issue? :	Not applicable (regular submission)

SCHOLARONE™
Manuscripts

1 **Title:** Switching to normal diet reverses kwashiorkor-induced salivary impairments via
2 increased nitric oxide level and expression of aquaporin 5 in the submandibular
3 glands of male Wistar rats

4
5 **Authors:** Taye Jemilat Lasisi^{1,2}, Shehu Tijani Shittu¹, Akinola Rashid Alada¹

6
7 **Affiliation:** Departments of ¹Physiology and ²Oral Pathology
8 College of Medicine, University of Ibadan
9 Ibadan, Nigeria

10
11 **Corresponding Author:** Dr. Taye J. Lasisi
12 Department of Physiology
13 College of Medicine, University of Ibadan
14 Ibadan, Nigeria.

15
16 **Postal Address:** P. O. Box 22040
17 University of Ibadan Post Office
18 Ibadan, Nigeria

19
20 **E mail:** jameelahlasisi@yahoo.com

Abstract

Kwashiorkor, a form of malnutrition has been shown to cause impaired salivary secretion. However, there is dearth of information on mechanism that underlies this complication. Also, whether returning to normal-diet after kwashiorkor will reverse these complications or not is yet to be discerned. Thus, this study aimed at assessing the mechanisms that underlie kwashiorkor-induced salivary impairments and to evaluate the effects of switching back to normal-diet on kwashiorkor-induced salivary impairments. Weaning rats were randomly divided into 3 groups (control, kwashiorkor, re-fed kwashiorkor) of 7 rats each. The control had standard rat-chow while the kwashiorkor group (KG) and re-fed kwashiorkor group (RKG) were fed 2% protein-diet for 6 weeks to induce kwashiorkor. The RKG had their diet changed to standard rat-chow for another 6 weeks. Blood and stimulated saliva samples were collected for the analysis of total protein, electrolytes, amylase, IgA secretion rate, leptin and ghrelin. Tissue total protein, nitric oxide level, expressions of Na^+/K^+ -ATPase, muscarinic (M3) receptor and aquaporin5 in the submandibular glands were also determined. Data were presented as mean $\pm$ SEM and compared using ANOVA with Tukey's post-hoc test. RKG showed improved salivary function evidenced by reduced salivary lag-time, potassium, with increased, flow rate, sodium, amylase, IgA secretion rate, leptin, submandibular nitric oxide level and aquaporin5 expression compared with KG. This study for the first time has demonstrated that kwashiorkor caused significant reduction in salivary secretion through reduction of nitric oxide level and aquaporin5 expression in submandibular salivary glands. Normal-diet re-feeding after kwashiorkor returned salivary secretion to normal.

Key words: Kwashiorkor, Aquaporin 5, Leptin, Ghrelin, Salivary secretion

44 Introduction

45 Saliva is the physiologic fluid that maintains the homeostasis of the oral tissues. The constituents
46 of saliva are important factors responsible for its physiological functions (De Almeida et al.
47 2008). Salivary secretion is mainly through parasympathetic nerve-mediated stimuli and the
48 release of acetylcholine that activates muscarinic receptors in the salivary glands (Proctor and
49 Carpenter 2007). Also, the secretion is facilitated by increased salivary glandular blood flow via
50 cholinergic, peptidergic and nitric oxide mediated vasodilatation (Anderson and Garrett 1998).
51 Ion channels and transporters expressed at the apical and basolateral membranes of the secretory
52 cells in salivary glands play a key role in fluid secretion. The Na^+/K^+ -ATPase (among others that
53 include $\text{Na}^+/\text{K}^+/\text{Cl}^-$ co-transporter, Na^+/H^+ and $\text{Cl}^-/\text{HCO}_3^-$ exchangers) is highly expressed in
54 the basolateral membrane of secretory cells, and maintains an inward-directed Na^+
55 electrochemical gradient (Catalan et al. 2009). The osmotic gradient established upon
56 electrolytes secretion facilitates water secretion through aquaporin5 (AQP5), the major water
57 channel expressed in the apical membrane of secretory acinar cells in the salivary glands
58 (Delporte and Steinfeld 2006).

59 Various salivary functions including lubrication, cleansing action, antibacterial activity,
60 buffering action, maintenance of tooth integrity, taste sensation and digestion may be disturbed
61 by changes in salivary flow and biochemical properties. Impaired salivary functions could be as
62 a result of changes in the parameters which can be caused by many factors including diet.
63 Metabolic alterations have been documented to influence synthesis, composition and secretion of
64 saliva (Elverdin et al. 2006). Inadequate nutrition has been shown to cause various salivary
65 impairments. Rats fed a nutritionally adequate but liquid diet showed atrophied salivary glands
66 (Hall and Schneyer, 1964) while moderate reduction in food intake caused hypertrophy of the rat

parotid gland, which was attributable to increased storage of secretory proteins (Sreebny and Johnson 1968). Although, Johansson et al. (1985) reported higher parotid saliva protein concentration in malnourished rats compared with control, chronic protein insufficiency was earlier shown to cause significant reduction in the total activity of salivary amylase and total protein in rats (Watson 1980). Recently, we reported prolonged salivary lag time, reduced salivary flow rate and sodium concentration, as well as elevated potassium and bicarbonate in kwashiorkor rats, a form of protein insufficiency (Lasisi and Alada 2015). In humans, stimulated and unstimulated salivary flow rates were reduced in individuals who had experienced severe malnutrition in their early childhood (Psoter et al. 2008). In an Indian population, Johansson et al. (1994) found that children with moderate to severe protein energy malnutrition had a reduced salivary secretion rate, reduced buffering capacity, lower calcium, and lower protein secretion in stimulated saliva than children with no or mild protein energy malnutrition. The reports on the presence of leptin (Groschl et al. 2001) and ghrelin (Groschl et al. 2005) which are appetite hormones in the saliva are indicators that the nutritional status of the body may have bearing on salivary functions.

In spite of the reports on the effects of malnutrition on salivary secretion, there is dearth of information on mechanism that underlies these effects. Also whether returning to normal diet after kwashiorkor will reverse the effects or not is yet to be discerned. Thus, this study aimed at assessing the mechanisms that underlie kwashiorkor-induced salivary impairments and also to evaluate the effects of switching back to normal diet on kwashiorkor-induced salivary impairments.

90 **Methodology**

91 **Experimental animals:** Twenty one rats weighing between 55 and 65 g obtained from the
92 Central Animal House, College of Medicine, University of Ibadan were used for the
93 experiments. They were housed and acclimatized for one week in the Animal house of the
94 Department of Physiology, College of Medicine, University of Ibadan. Animals were housed
95 under a standard temperature (23-25°C), humidity (35-55%), and natural photoperiod of 12 hours
96 light/dark cycle with free access to water. They were fed on standard rat pellet diet purchased
97 from Ladokun feeds for the period of acclimatization. All experimental protocols and handling
98 were carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and
99 Canadian Council on Animal Care (CCAC).

100 **Animals grouping:** After one week of acclimatization, the rats were randomly divided into 3
101 groups of 7 rats each. The groups were normal diet group (control), kwashiorkor group (KG) and
102 re-fed kwashiorkor group (RKG). The dietary compositions were as previously described
103 (Olowookere et al. 1991; Lasisi and Alada 2015). The control animals were fed with standard
104 normal feed while the animals in the KG and RKG were fed with low protein diet (2% protein)
105 for 6 weeks ad libitum. In addition, the RKG group had their feed changed to the normal diet for
106 another 6 weeks. The low-protein and standard rat chow diets were standardized by the Animal
107 Nutrition Laboratory of the Department of Animal Science of the Institution. The animals in all
108 the groups were fed ad libitum and had free access to water.

109 **Saliva collection:** On the day of experiment after overnight fasting, each rat was weighed,
110 anesthetized using ketamine (75 mg/kg i.p.) and xylazine (0.5 mg/kg body weight). After
111 anesthesia, each rat was positioned laterally after saliva stimulation with pilocarpine
112 hydrochloride (10 mg/kg, i.p.). Saliva was allowed to drop inside a graduated plain tube adapted

to the mouth of the rat for a period of 10 minutes. Saliva was collected between 8 am and 10 am for all the groups in order to reduce the effects of diurnal variation. Saliva samples collected were stored at -20°C until laboratory analysis. Saliva samples were defrosted at room temperature and then centrifuged at $1500 \times g$ for 10 minutes before analysis.

Blood biochemical analysis: Blood samples were taken from cardiac puncture (after) saliva collection into lithium heparin and EDTA sample tubes. Samples in the EDTA tubes were used to determine packed cell volume, hemoglobin concentration and albumin concentrations using standard methods. Samples in the lithium heparin sample tubes were centrifuged at 3000 rpm for 15 minutes to obtain plasma which was used for the analysis of sodium, potassium and bicarbonate concentrations.

Surgical removal of salivary glands and tissue processing: The tissue harvesting was done immediately after saliva and blood collection before euthanasia by manual cervical dislocation in the anaesthetized rats. Removal of salivary glands was performed as previously described (Lasisi and Alada 2015). Because of the light weights of the glands, they were carefully weighed using a semi-micro analytical balance (Ming Heng Electronic digital scale, India). Each of the submandibular gland was put in either PBS (pH 7.4) or 10% formosaline for histopathologic assessment and immunohistochemical analysis. The glands in PBS were homogenized and the supernatant was used for the assessment of the nitric oxide and tissue total protein levels using the Greiss and Biuret methods respectively with AGAPPE kits (Switzerland). Expressions of muscarinic (M3) receptor, Na^+/K^+ -ATPase and aquaporin 5 were assessed immunohistochemically using CHRM3 clonal Antibody (A1602) from Abclonal, USA, Anti- Na^+/K^+ -ATPase antibody from St John's Laboratory, UK and AQP5 (D-7): sc-514022 from Santa Cruz Biotechnology,

Inc. USA kits, respectively. All analyses were done in accordance with the manufacturers' instructions.

Photomicrographs: Photomicrographs of the slides were taken with a model Canon Power Shot A650 IS, at focal length 25mm, width – 4000 pixels, height – 3000 pixels mounted on a Carl Zeiss Am Scope - A1 light microscope set at X100, 200 and 400 mm magnifications. The Am Scope was connected to the computer to save all the sections taken. Each H & E stained slide was reviewed by two pathologists independently to determine the histologic changes in the salivary glands.

Quantification of expression of Na⁺/K⁺-ATPase, aquaporin 5 and M3 receptor in the tissues: Each stained slide was reviewed by 2 assessors independently to determine the intensity of expression of the proteins (Na⁺/K⁺-ATPase, AQP5 and M3 receptor). Also, photomicrographs of the slides were copied to the ImageJ software for an objective quantitative analysis of the protein expressions.

Determination of salivary lag time and flow rate: Salivary lag time was determined according to the method previously described (Choi et al. 2013). Salivary 'lag time' was defined as the interval between pilocarpine administration and beginning of salivary secretion. Salivation was determined by visual observation of pool of saliva in the floor of the mouth, and 'lag times' were measured in seconds using a stop watch. Salivary flow rate (ml/min) was calculated as total saliva volume divided by the collection time.

Determination of total protein and electrolytes concentrations: Total protein concentrations in the samples were determined using the Biuret method. Sodium and potassium levels were determined using spectrophotometry while estimation of calcium was done using indirect colorimetric method. Concentrations of chloride and bicarbonate were determined using

colorimetric method using mercuric nitrate. Concentration of phosphate was determined using the ammonium molybdate method. Analyses of total protein, sodium, potassium, calcium, chloride and phosphate were done using their respective kits from AGAPPE DIAGNOSTICS, Switzerland, while analysis of bicarbonate was done using kits from TECO DIAGNOSTICS, India.

Determination of amylase, IgA, leptin and ghrelin concentrations: The determination of concentrations of alpha amylase and IgA in saliva was performed using Enzyme Linked Immunosorbent (ELISA) kits (Elabscience, China). The determination of the concentration of ghrelin in saliva and serum was performed using Enzyme Immunoassay kit (RayBiotech, USA). The determination of the concentration of leptin in saliva and serum was performed using an in-vitro Enzyme-linked Immunosorbent assay (ELISA) kit (RayBiotech, USA). All analyses were done according to the manufacturers' instructions.

Data analysis: Data are presented as mean $\pm$ SEM. Variables were compared using independent sample t test (for two groups) and ANOVA with Tukey's HSD post hoc test (for more than two groups). Level of significance was set at $p < 0.05$ for all analyses. All statistical analyses were performed using SPSS version 22.

Results

There were significant improvements in the body and salivary glands weights of the kwashiorkor rats after re-feeding with normal diet (Table 1). The percentage recovery of body weight was 93.4% with reference to the normal diet group. Also, there were significant improvements in the weights of right and left submandibular glands, right and left parotid glands from the RKG after

six weeks of normal diet. The percentage recovery was 94.1%, 100%, 89.7% and 89.3% (for right and left submandibular glands, right and left parotid glands, respectively) with reference to the normal diet group.

In the re-fed kwashiorkor group, there were significant increases in the levels of PCV, Hb concentration, plasma albumin, plasma total protein and sodium after re-feeding with normal diet. The levels of these parameters were not significantly different when compared with similar parameters in the normal diet group (Table 2). In the re-fed kwashiorkor group, the percentage recovery was 94.1%, 100%, 89.7%, 66% and 89.3% (for PCV, Hb concentration, plasma albumin, total protein and sodium, respectively) with reference to the normal diet group.

There was significant reduction in the salivary lag time and significant increase in the salivary flow rate in the rats fed normal diet after kwashiorkor when compared with the kwashiorkor group (Table 3). Also, the salivary lag time and low rate were not significantly different when compared with the values recorded in the normal diet group. In the re-fed kwashiorkor group, the percentage recovery in lag time and flow rate was 88.3% and 116.2%, respectively with reference to the normal diet group.

Levels of salivary potassium and phosphate were significantly reduced, while levels of salivary sodium, amylase, secretion rates of IgA and ghrelin were significantly elevated in the re-fed kwashiorkor group when compared with the kwashiorkor group. However, there were no improvements in the levels of salivary sodium (Table 3) and leptin (Table 4).

Levels of tissue total protein and nitric oxide in the submandibular salivary glands were significantly increased in the re-fed kwashiorkor group compared with the kwashiorkor group (Figure 1). The reduced expression of AQP5 in the submandibular salivary gland of the kwashiorkor group was significantly improved in the re-fed group compared with kwashiorkor

group (Figure 2). However, there was no significant difference in the relative expressions of Na^+/K^+ -ATPase (Figure 3) and M3 receptor (Figure 4) in the submandibular salivary glands of the kwashiorkor group compared with control.

Discussion

Nutritional status has been documented to alter salivary functions (Watson 1980; Johansson et al. 1985; Lasisi and Alada 2015); accordingly salivary secretion was impaired in kwashiorkor rats in the present study which was reversed following re-feeding with normal diet. The observed reversal of impaired salivary parameters in the re-fed kwashiorkor rats could be associated with improvement in the body weight and salivary gland weights of the animals. Many studies have shown regeneration of salivary gland following atrophy due to duct ligation (Burgess and Dardick 1998; Takahashi et al. 2004; Nagai et al. 2014), or denervation (Zhang et al. 2014) in rodents. Nagai et al. (2014) reported that in the duct ligated portion of the submandibular gland in a mouse that underwent atrophy, newly formed acinar cells were observed arising from the tubular structures after the release of the duct obstruction. Thus, it was suggested that the tubular structures localized in an atrophic gland are the source of acinar cell regeneration of the salivary gland (Nagai et al. 2014).

The observed reduction in the levels of submandibular tissue total protein and expression of aquaporin 5 in the submandibular glands of the kwashiorkor rats in this study could also be linked to the observed impaired salivary secretion. Aquaporin 5, which is predominantly found in the glandular tissues, is present in the luminal surface membrane of salivary gland acinar cells and provides a transcellular water transfer pathway for salivary fluid secretion (Matzusaki et al. 1999). Functionally, among the various aquaporins previously identified in the salivary glands in

rodents and human, AQP5 appears to be the only salivary aquaporin clearly playing a major role in the salivary secretion process (Delporte and Steinfeld 2006). In the acinar cells, AQP5 participates in water movement to the lumen leading to the primary salivary secretion (Delporte and Steinfeld 2006). Agonist-stimulated saliva secretion requires AQP5 trafficking from intracellular vesicles or secretory granules to the apical plasma membrane of acinar cells. The translocation of AQP5 between intracellular and apical membranes from rat parotid gland (acini and ducts) occurs after M3 receptor (Murdiastuti et al. 2002) or $\alpha 1$ -adrenergic receptor (Ishikawa et al. 1998) stimulation. This is thought to be due to an elevation in intracellular calcium concentration involving the stimulation of the nitric oxide/cyclic GMP pathway (Ishikawa et al. 1999). In the parotid glands (acini and ducts) from senescent rats, decreased AQP5 translocation to the apical membrane in response to acetylcholine, but not to epinephrine, was not due to decreased M3 receptor and/or Gq protein expression, but rather to decreased nitric oxide synthase activity (NOS) (Inoue et al. 2003). Although NOS activity was not assessed in this study, the assayed nitric oxide level in the submandibular gland may be a reflection of the NOS activity. Nitric oxide level has been reported to indicate the NOS activity (Lomniczi et al. 1998) in salivary glands. In addition, NOS activity has been shown to be involved in AQP5 translocation (Ishikawa et al. 2002). Thus, the reduced expression of AQP5 in the submandibular gland of kwashiorkor rats may be related to reduction in NOS activity as evidenced by the lower level of nitric oxide in this group of animals. Generally, expression of AQP5 in rats' salivary glands are reported to be reduced in conditions that induce salivary hypo-function such as three days fasting (Susa et al. 2013), irradiation (Han et al. 2015) and botulinum toxin (Xu et al. 2015). Impairment in the vasodilatory effect of nitric oxide could have also contributed to the observed hyposecretion of saliva in the kwashiorkor rat since nitric oxide level was significantly reduced

in the submandibular glands of the animals. Such reduction in nitric oxide level had been earlier observed in cadmium- and nifedipine-induced salivary hypo-function in rats (Abdollahi et al. 2000; Rezaie et al. 2005). Nitric oxide is an important tissue factor with multifaceted cellular effects. Nitric oxide has profound effects on Ca^{2+} homeostasis. Its effects include the regulation of voltage-dependent Ca^{2+} channels and voltage-independent, store-operated Ca^{2+} channels, modulation of IP_3 -induced intracellular Ca^{2+} release, Ca^{2+} release from ryanodine stores, regulation of Ca^{2+} influx, and IP_3 and cyclic ADP-ribose generation (Clementi et al. 1995). Many of these actions appear to be mediated via cGMP through activation of a G-kinase or phosphodiesterase. Parasympathetic stimulation leading to muscarinic cholinergic receptor activation is linked to formation of inositol triphosphate and diacylglycerol and subsequent rises in intracellular calcium which open membrane ion channels, leading to fluid secretion (Baum and Wellner 1999).

The lack of difference in the expression of M3 receptors as well as the Na^+/K^+ -ATPase in the submandibular glands of the kwashiorkor rats suggests that mechanisms that involve M3 receptors and Na^+/K^+ -ATPase locally are not implicated in the impaired salivary secretion associated with kwashiorkor in the present study. Hence, other mechanisms which were not assessed in this study like involvement of M1 receptors as well as other transport mechanisms such as the $\text{Na}/\text{K}/\text{Cl}$ co-transporter could be affected in the kwashiorkor rats.

The increased salivary flow rate observed in the re-fed kwashiorkor group compared with the kwashiorkor group in this study might have contributed to the reversal of the salivary electrolytes (sodium and potassium) concentrations. Increased salivary flow rate causes higher levels of sodium with decreased concentration of potassium due to reduced ability of the ducts to absorb sodium as well as to secrete potassium (Dawes 1970). Similarly, the increased secretion rate of

272 salivary IgA in the re-fed kwashiorkor group is explained by the increased salivary flow rate.
273 Salivary immunoglobulin A levels are affected by flow rates, with concentrations normally
274 decreasing as flow rates increase (Bishop and Gleeson 2009). However, the secretion rate
275 determines the physiologic function and the higher the secretion rate the better the immune
276 function. Thus, the improved salivary flow rate, electrolytes concentration as well as the
277 secretion rate of IgA in the re-fed kwashiorkor group may indicate better salivary function which
278 is vital to good oral health.

279 The present study is the first to report the effect of kwashiorkor on salivary appetite hormones
280 (ghrelin and leptin) in rats. The reduced salivary leptin and ghrelin level in the kwashiorkor rats
281 observed in this study suggests suppression of these hormones by malnutrition which was
282 returned to normal in the re-fed kwashiorkor rats. Soliman et al. (2012) reported that leptin is an
283 important signal in the process of metabolic/endocrine adaptation to prolonged nutritional
284 deprivation (Soliman et al. 2012). Low leptin levels decrease leptin inhibition on neuropeptide Y
285 (NPY) that affects the regulation of pituitary growth and pituitary adrenal axes (Soliman et al.
286 2012). Thus, the decreased leptin concentrations in response to food deprivation are responsible
287 for the starvation-induced suppression of the hypothalamic-pituitary-gonadal axes as well as the
288 malfunction of several other neuroendocrine axes (Sainsbury et al. 2002). Salivary ghrelin and
289 leptin have been implicated in the growth and immuno-modulatory mechanisms in the oral
290 tissues. Ohta et al. (2011) reported that ghrelin produced in the oral cavity appears to play a
291 regulatory role in innate immune responses to inflammatory infection. It has been shown that
292 ghrelin increased intracellular calcium mobilization and cAMP levels in oral epithelial cells,
293 suggesting that ghrelin acts on epithelial cells to induce cell signaling (Ohta et al. 2011). Also, it
294 was reported that synthetic ghrelin inhibited the production of interleukin 8 (IL-8) from tumor

necrosis factor alpha (TNF- α) or lipopolysaccharide (LPS)-stimulated oral epithelial cells (Ohta et al. 2011). Bohlender et al. (2003) reported the influence of leptin on the growth of rodent salivary glands by demonstrating the presence and distribution of leptin and its receptor suggesting an autocrine role of salivary leptin within the glands. Hence, the lower levels of salivary leptin and ghrelin in kwashiorkor rats observed in this study may contribute to oral infections as well as delayed wound healing. However, higher levels of the hormones in the re-fed kwashiorkor rats suggest that normal diet re-feeding following kwashiorkor could restore the functions salivary ghrelin and leptin.

In conclusion, the present study for the first time has demonstrated that kwashiorkor caused significant reduction in salivary secretion through reduction of nitric oxide level and AQP5 expression but not through reduced expression of M3 receptor and Na⁺/K⁺-ATPase in submandibular salivary glands. Normal diet re-feeding after kwashiorkor returned salivary flow rate, salivary electrolytes, salivary IgA secretion rate as well as salivary appetite hormones to normal. Thus, the present study has demonstrated also, that change of diet may be a form of treatment or control of impaired physiological parameters of salivary secretion following diet-induced kwashiorkor in rats.

Conflict of interest: The authors have no conflicts of interest to report.

317 **References**

- 318 Abdollahi, M., Dehpour, A., and Kazemian, P. 2000. Alteration by cadmium of rat
319 submandibular gland secretory function and the role of the l-arginine/nitric oxide pathway.
320 *Pharmacol. Res.* **42**(6): 591-597. DOI:10.1006/phrs.2000.0731. PMID:11058413
- 321 Anderson, L.C., and Garrett, J.R. 1998. Neural regulation of blood flow in the rat submandibular
322 gland. *Eur. J. Morphol.* **36** Suppl: 213-218. PMID:9825925
- 323 Baum, B.J. and Wellner, R.B. 1999. Receptors in salivary glands. In: Garrett, J.R., Ekstrom, J.
324 and Anderson, L.C. (eds) *Neural Mechanisms of Salivary Secretion* 1999, pp. 44-58. Karger,
325 Basel.
- 326 Bishop, N.C. and Gleeson, M. 2009. Acute and chronic effects of exercise on markers of
327 mucosal immunity. *Front. Biosci.* **14**: 4444-4456. PMID:19273362
- 328 Bohlender, J., Rauh, M., Zenk, J., and Gröschl, M. 2003. Differential distribution and expression
329 of leptin and the functional leptin receptor in major salivary glands of humans. *J. Endocrinol.*
330 **178**(2): 217-223. PMID:12904169
- 331 Burgess, K.L. and Dardick, I. 1998. Cell population changes during atrophy and regeneration of
332 rat parotid gland. *Oral. Surg. Oral. Med. Oral. Pathol. Oral. Radiol. Endod.* **85**(6): 699-706.
333 PMID:9638704
- 334 Catalán, M.A., Nakamoto, T., and Melvin, J.E. 2009. The salivary gland fluid secretion
335 mechanism. *J. Med. Invest.* **56** Suppl: 192-196. PMID:20224180
- 336 Choi, J.S., Park, I.S., Kim, S.K., Lim, J.Y. and Kim, Y.M. 2013. Analysis of age related changes
337 in the functional morphologies of salivary glands in mice. *Arch. Oral. Biol.* **58**: 1635-1642.
338 DOI:10.1016/j.archoralbio.2013.07.008. PMID:24112729

- 339 Clementi, E., Vecchio, I., Sciorati, C., and Nisticò, G. 1995. Nitric oxide modulation of agonist-
340 evoked intracellular Ca^{2+} release in neurosecretory PC-12 cells: inhibition of phospholipase C
341 activity via cyclic GMP-dependent protein kinase I. *Mol. Pharmacol.* **47**(3): 517-524.
- 342 Dawes, C. 1970. Effects of diet on salivary secretion and composition. *J. Dent. Res.* **49**(6): 1263-
343 1273. PMID:8848007
- 344 de Almeida, P. V., Grégio, A. M., Machado, M. A., de Lima, A. A., and Azevedo, L. R. 2008.
345 Saliva composition and functions: a comprehensive review. *J. Contemp. Dent. Pract.* **9**(3): 72-80.
346 PMID:18335122
- 347 Delporte, C., and Steinfeld, S. 2006. Distribution and roles of aquaporins in salivary glands.
348 *Biochimica. et Biophysica. Acta.* **1758**: 1061-1070. DOI:10.1016/j.bbamem.2006.01.022.
349 PMID:16537077
- 350 Elverdin, J.C., Chiarenza, A., and Luchelli, M.A. 2006. Protein free diet feeding: effects on
351 sympathetic activity and salivary evoked secretion in the submandibular gland of rat. *Arch. Oral.*
352 *Biol.* **51**: 621-628. DOI:10.1016/j.archoralbio.2006.02.001. PMID:16600171
- 353 Gröschl, M., Rauh, M., Wagner, R., Neuhuber, W., Metzler, M., Tamgüney, G., et al. 2001.
354 Identification of leptin in human saliva. *J. Clin. Endocrinol. Metab.* **86**(11): 5234-5239.
355 DOI:10.1210/jcem.86.11.7998. PMID:11701683
- 356 Gröschl, M., Topf, H.G., Bohlender, J., Zenk, J., Klusmann, S., Dötsch J., et al. 2005.
357 Identification of ghrelin in human saliva: production by the salivary glands and potential role in
358 proliferation of oral keratinocytes. *Clin. Chem.* **51**(6): 997-1006.
359 DOI:10.1373/clinchem.2004.040667. PMID:15790755

- 360 Hall, H.D., and Schneyer, C.A. 1964. Salivary gland atrophy in rat induced by liquid diet. Proc.
361 Soc. Exp. Biol. Med. **117**: 789-793. PMID:14244956
- 362 Han, L., Wang, L., Zhang, F., Liu, K.J., and Xiang, B. 2015. Effect of phenylephrine
363 pretreatment on the expressions of Aquaporin 5 and c-Jun N-Terminal Kinase in irradiated
364 submandibular gland. Radiat. Res. **183**(6): 693-700. DOI:10.1667/RR13890.1. PMID:25950818
- 365 Inoue, N., Iida, H., Yuan, Z., Ishikawa, Y., and Ishida, H. 2003. Age-related decreases in the
366 response of aquaporin-5 to acetylcholine in rat parotid glands. J. Dent. Res. **82**: 476-480.
367 DOI:10.1177/154405910308200614. PMID:12766202
- 368 Ishikawa, Y., Eguchi, T., Skowronski, M.T., and Ishida, H. 1998. Acetylcholine acts on M3
369 muscarinic receptors and induces the translocation of aquaporin5 water channel via cytosolic
370 Ca²⁺ elevation in rat parotid glands. Biochem. Biophys. Res. Commun. **245**: 835-840.
371 DOI:10.1006/bbrc.1998.8395. PMID:9588201
- 372 Ishikawa, Y., Iida, H., and Ishida, H. 2002. The muscarinic acetylcholine receptor stimulated
373 increase in aquaporin-5 levels in the apical plasma membrane in rat parotid acinar cells is
374 coupled with activation of nitric oxide/cGMP signal transduction. Mol. Pharmacol. **61**: 1423-
375 1434. PMID:12021404
- 376 Ishikawa, Y., Skowronski, M.T., Inoue, N., Ishida, H. 1999. $\alpha(1)$ -adrenoceptor induced
377 trafficking of aquaporin-5 to the apical plasma membrane of rat parotid cells, Biochem. Biophys.
378 Res. Commun. **265**: 94-100. DOI:10.1006/bbrc.1999.1630. PMID:10548496
- 379 Johansson, I., Ericson, T., Bowen, W., and Cole, M. 1985. The effect of malnutrition on caries
380 development and saliva composition in the rat. J. Dent. Res. **64**(1): 37-43. PMID:3855418.
381 DOI:10.1177/00220345850640010701

- 382 Johansson, I., Lenander-Limikari, M., and Saellstrom, A.K. 1994. Saliva composition in Indian
383 children with chronic protein energy malnutrition. *J. Dent. Res.* **73**: 11-19.
384 DOI:10.1177/00220345940730010101. PMID:8294612
- 385 Lasisi, T.J., and Alada, A.R. 2015. Effects of induced-kwashiorkor on salivary parameters in
386 Wistar rat. *Afr. J. Med. Sci.* **44**(2): 125-132. PMID:26937525
- 387 Lomniczi, A., Suburo, A.M., Elverdin, J.C., Mastronardi, C.A., Diaz, S., Rettori, V., et al. 1998.
388 Role of nitric oxide in salivary secretion. *Neuroimmunomodulation.* **5**(5): 226-233.
389 DOI:10.1159/000026342 PMID:9730690
- 390 Matsuzaki, T., Suzuki, T., Koyama, H., Tanaka, S., and Takata, K. 1999. Aquaporin-5 (AQP5), a
391 water channel protein, in the rat salivary and lacrimal glands: immunolocalization and effect of
392 secretory stimulation. *Cell. Tissue. Res.* **295**(3): 513-521. PMID:10022971
- 393 Murdiastuti, K., Miki, O., Yao, C., Parvin, M.N., Kosugi-Tanaka, C., Akamatsu, T., et al. 2002.
394 Divergent expression and localization of aquaporin 5, an exocrine-type water channel, in the
395 submandibular gland of Sprague-Dawley rats. *Pflugers. Arch.* **445**: 405-412.
396 DOI:10.1007/s00424-002-0940-8. PMID:12466944
- 397 Nagai, K., Arai, H., Okudera, M., Yamamura, T., Oki, H., and Komiyama, K. 2014. Epiregulin is
398 critical for the acinar cell regeneration of the submandibular gland in a mouse duct ligation
399 model. *J. Oral. Pathol. Med.* **43**(5): 378-387. DOI:10.1111/jop.12145. PMID:24354788
- 400 Ohta, K., Laborde, N.J., Kajiya, M., Shin, J., Zhu, T., Thondukolam, A.K., et al. 2011.
401 Expression and possible immune-regulatory function of ghrelin in oral epithelium. *J. Dent. Res.*
402 **90**(11): 1286-1292. DOI:10.1177/0022034511420431. PMID:21865591

- 403 Olowookere, J.O., Konji, V.N., Makawiti, D.W., Kiaira, J.K., Kamau, J.M., and Omwandho,
404 C.A. 1991. Defects in resting metabolic rates and mitochondrial respiration in Kwashiorkor and
405 dietary obese rats. *J. Comp. Physiol. B.* **161**(3): 319-322. PMID:1939742
- 406 Proctor, G.B., and Carpenter, G.H. 2007. Regulation of salivary gland function by autonomic
407 nerves. *Auton. Neurosci.* **133**(1): 3-18. DOI:10.1016/j.autneu.2006.10.006. PMID:17157080
- 408 Psoter, W.J., Spielman, A.L., Gebrian, B., St Jean, R., and Katz, R.V. 2008. Effects of childhood
409 malnutrition on salivary flow and pH. *Arch. Oral. Biol.* **53**(3): 231-237.
410 DOI:10.1016/j.archoralbio.2007.09.007. PMID:17983611
- 411 Rezaie, S., Rezaie, A., Minaiee, B., Khorasani, R., and Abdollahi, M. 2005. On the relation of
412 nitric oxide to nifedipine-induced gingival hyperplasia and impaired submandibular glands
413 function in rats in vivo. *Fundam. Clin. Pharmacol.* **19**(1): 65-71. DOI:10.1111/j.1472-
414 8206.2004.00313.x. PMID:15660961
- 415 Sainsbury, A., Cooney, G.J., and Herzog, H. 2002. Hypothalamic regulation of energy
416 homeostasis. *Best. Pract. Res. Clin. Endocrinol. Metab.* **16**: 623-627.
- 417 Soliman, A.T., Yasin, M., and Kassem, A. 2012. Leptin in pediatrics: A hormone from adipocyte
418 that wheels several functions in children. *Indian. J. Endocrinol. Metab.* **16** (Suppl 3): S577-S587.
- 419 Sreebny, L.M., and Johnson, D.A. 1968. Effect of food consistency and decreased food intake on
420 rat parotid and pancreas. *Am. J. Physiol.* **215**(2): 455-460. PMID:12468411
- 421 Susa, T., Sawai, N., Aoki, T., Iizuka-Kogo, A., Kogo, H., Negishi, A., et al. 2013. Effects of
422 repeated administration of pilocarpine and isoproterenol on aquaporin-5 expression in rat
423 salivary glands. *Acta. Histochem. Cytochem.* **46**(6): 187-197. DOI:10.1267/ahc.13037.
424 PMID:24610966

- 425 Takahashi, S., Shinzato, K., Nakamura, S., Domon, T., Yamamoto, T., and Wakita, M. 2004.
 426 Cell death and cell proliferation in the regeneration of atrophied rat submandibular glands after
 427 duct ligation. *J. Oral. Pathol. Med.* **33**(1): 23-29. PMID:14675137
- 428 Watson, R.R., and Antal, M. 1980. Effect of moderate chronic protein deficiency on rat salivary
 429 components. *J. Nutr.* **110**: 771-777. DOI:10.1093/jn/110.4.771. PMID:6154135
- 430 Xu, H., Shan, X.F., Cong, X., Yang, N.Y., Wu, L.L., Yu, G.Y., et al. 2015. Pre- and Post-
 431 synaptic effects of Botulinum Toxin A on submandibular glands. *J. Dent. Res.* **94**(10): 1454-
 432 1462. DOI:10.1177/0022034515590087. PMID:26078423
- 433 Zhang, S.E., Su, Y.X., Zheng, G.S., Liang, Y.J., and Liao, G.Q. 2014. Reinnervated nerves
 434 contribute to the secretion function and regeneration of denervated submandibular glands in
 435 rabbits. *Eur. J. Oral. Sci.* **122**(6): 372-381. DOI: 10.1111/eos.12154. PMID:25363784

437 **Figure Legends**

438 Figure 1: Submandibular tissue total protein (A) and nitric oxide levels (B) in control,
 439 kwashiorkor and re-fed kwashiorkor groups

440 Figure 2: Expression of Aquaporin 5 in the submandibular glands from control (A), kwashiorkor
 441 (B) and re-fed kwashiorkor (C) rats X200. Figure 2D shows the quantitative analysis of the
 442 expressions of Aquaporin 5 among the groups.

443 Figure 3: Expression of M3 receptor in the submandibular glands from control (A), kwashiorkor
 444 (B) and re-fed kwashiorkor (C) rats X200. Figure 3D shows the quantitative analysis of the
 445 expressions of M3 receptor among the groups.

446 Figure 4: Expression of Na^+/K^+ -ATPase in the submandibular glands from control (A),
447 kwashiorkor (B) and re-fed kwashiorkor (C) rats X200. Figure 4D shows the quantitative
448 analysis of the expressions of Na^+/K^+ -ATPase among the groups.

Draft

Table 1: Body and salivary glands weights (g) in control, kwashiorkor and re-fed kwashiorkor groups

	Control	Kwashiorkor group	Re-fed kwashiorkor group	P value
Body weight (g)	174.17 ± 6.99 ^a	65.2 ± 3.26 ^b	162.67 ± 7.51 ^a	< 0.001
Right SMG (g)	0.17 ± 0.02 ^a	0.07 ± 0.03 ^b	0.16 ± 0.01 ^a	< 0.001
Left SMG (g)	0.16 ± 0.02 ^a	0.07 ± 0.01 ^b	0.15 ± 0.01 ^a	< 0.001
Right PG (g)	0.07 ± 0.02 ^a	0.02 ± 0.01 ^b	0.05 ± 0.01 ^a	< 0.001
Left PG (g)	0.06 ± 0.01 ^a	0.02 ± 0.01 ^b	0.05 ± 0.01 ^a	< 0.001

SMG = Submandibular gland, PG = parotid gland. Values are mean ± standard error of mean. Values with different superscript letters represent significant difference between means. Mean comparisons were performed using One Way ANOVA and Turkey Post Hoc tests.

Table 2: Levels of PCV, hemoglobin, albumin, total protein and sodium in control, kwashiorkor and re-fed kwashiorkor groups

	Control	Kwashiorkor group	Re-fed kwashiorkor group	P value
PCV (%)	44.14 ± 1.6 ^a	20.8 ± 1.93 ^b	44.6 ± 2.41 ^a	< 0.001
Hb conc. (g/dL)	14.71 ± 0.53 ^a	6.93 ± 0.64 ^b	14.94 ± 0.73 ^a	< 0.001
Albumin (mg/dL)	3.11 ± 0.08 ^a	2.54 ± 0.21 ^b	3.20 ± 0.14 ^a	0.01
Total protein (g/dl)	8.16 ± 0.18 ^a	6.37 ± 0.23 ^b	7.08 ± 0.14 ^a	0.03
Sodium (mEq/L)	144.38 ± 2.32 ^a	134.86 ± 2.32 ^b	140.83 ± 1.05 ^a	0.01

PCV = Packed cell volume, Hb = hemoglobin. Values are mean ± standard error of mean. Values with different superscript letters represent significant difference between means. Mean comparisons were performed using One Way ANOVA and Turkey Post Hoc tests.

Table 3: Salivary lag time, flow rate, levels of sodium, potassium, and phosphate in control, kwashiorkor and re-fed kwashiorkor groups

	Control	Kwashiorkor group	Re-fed kwashiorkor group	P value
Lag time (seconds)	3 ± 0.24^a	4.88 ± 0.13^b	2.57 ± 0.22^a	0.01
Flow rate (ml/min)	0.06 ± 0.01^a	0.01 ± 0.004^b	0.09 ± 0.01^a	< 0.001
Sodium (mEq/L)	61.7 ± 2.63^a	49.04 ± 4.64^b	51.17 ± 5.5^b	0.01
Potassium (mEq/L)	39.85 ± 1.54^a	55.36 ± 2.72^b	33.9 ± 0.99^a	< 0.001
Phosphate (mg/dL)	1.16 ± 0.1^a	1.95 ± 0.41^b	1.05 ± 0.13^a	0.03

Values are mean $\pm$ standard error of mean. Values with different superscript letters represent significant difference between means. Mean comparisons were performed using One Way ANOVA and Turkey Post Hoc tests.

Table 4: Salivary levels of amylase, leptin, ghrelin, and secretion rates of IgA in control, kwashiorkor and re-fed kwashiorkor groups

	Conrol	Kwashiorkor group	Re-fed kwashiorkor group	P value
IgA (ng/mL)	847.28 ± 13.92 ^a	926.73 ± 36.53 ^b	547.13 ± 25.23 ^c	0.01
IgA SR (ng/mL/min)	37.93 ± 3.78 ^a	11.86 ± 2.72 ^b	53.5 ± 4.25 ^c	0.01
Amylase (ng/mL)	158.1 ± 14.65 ^a	71.05 ± 12.48 ^b	156.65 ± 12.56 ^a	0.01
Leptin (pg/mL)	1304.67 ± 122.21 ^a	1039.67 ± 80.95 ^b	1102 ± 143.33 ^b	0.02
Ghrelin (ng/mL)	194.83 ± 35.02 ^a	137.65 ± 11.63 ^b	255.08 ± 54.10 ^c	0.01

IgA SR = Immunoglobulin A secretion rate. Values are mean ± standard error of mean. Values with different superscript letters represent significant difference between means. Mean comparisons were performed using One Way ANOVA and Turkey Post Hoc tests.