

available at www.sciencedirect.comjournal homepage: <http://www.elsevier.com/locate/aob>

Expression of ghrelin in human salivary glands and its levels in saliva and serum in Chinese obese children and adolescents

Bin-Bin Li^{a,f}, Zhi-Bin Chen^{b,f}, Bo-Chun Li^c, Qin Lin^d, Xiao-Xin Li^d, Sheng-Lin Li^c, Chong Ding^e, Li-Ling Wu^{e,*}, Guang-Yan Yu^{c,**}

^a Department of Oral Pathology, Peking University School and Hospital of Stomatology, Beijing, PR China

^b Department of Periodontics, Peking University School and Hospital of Stomatology, Beijing, PR China

^c Department of Oral and Maxillofacial Surgery, Peking University School and Hospital of Stomatology, Beijing 100081, PR China

^d Clinical Laboratory, Peking University School and Hospital of Stomatology, Beijing, PR China

^e Department of Physiology and Pathophysiology, Peking University Health Science Center, Beijing 100191, PR China

ARTICLE INFO

Article history:

Accepted 18 October 2010

Keywords:

Ghrelin
Saliva
Children
Obesity

ABSTRACT

Objective: The aim of the present study was to reveal the expression characteristics of ghrelin in human three major salivary glands and to investigate saliva and serum ghrelin level and the relation with weight and lipid indices in Chinese children.

Design: Expression and distribution of ghrelin in parotid, submandibular, and sublingual glands were measured by reverse transcription-polymerase chain reaction and immunohistochemistry. Saliva and serum samples were collected from 194 Chinese children and adolescents (mean age 12.98 years). Ghrelin levels were tested by enzyme-linked immunosorbent assay. Significant differences were estimated by one-way ANOVA.

Results: Ghrelin mRNA was expressed in parotid and submandibular glands, but was not detectable in sublingual glands. Ghrelin proteins were widespread in the cytoplasm of striated, intercalated and excretory ducts, as well as in serous acini of parotid and submandibular glands, but not in mucous acinar cells of sublingual glands. Saliva and serum ghrelin levels were increased along with BMI. There was positive correlation between saliva and serum ghrelin levels ($r = 0.534$, $P < 0.01$). Serum and saliva ghrelin levels were both significantly correlated with BMI ($r = 0.523$, $r = 0.374$, $P < 0.01$, respectively), but not with blood lipid levels.

Conclusions: Parotid and submandibular glands were primary sources of ghrelin produced and released in saliva. Although whether salivary ghrelin could be useful in the diagnosis of obesity remains to be determined, salivary ghrelin might be a possible alternative to serum ghrelin for predicting obesity.

© 2010 Published by Elsevier Ltd.

* Corresponding author. Tel.: +86 10 82802403; fax: +86 10 82802403.

** Corresponding author. Tel.: +86 10 62191099; fax: +86 10 62173402.

E-mail addresses: pathophy@bjmu.edu.cn (L.-L. Wu), gyyu@263.net (G.-Y. Yu).

^f These authors contributed equally to this work.

0003-9969/\$ – see front matter © 2010 Published by Elsevier Ltd.

doi:10.1016/j.archoralbio.2010.10.014

1. Introduction

Overweight or obesity is a serious health issue, especially for children and adolescents. These age groups may be at greater risk of metabolic and cardiovascular complications, as well as increased mortality. The conditions are prevalent in economically developed as well as developing countries, such as China.¹⁻³

Ghrelin is a human growth-hormone-releasing peptide with a stimulatory effect on food intake, energy expenditure and fat accumulation, and thereby contribute to the control of body weight gain.^{4,5} Synthesis of ghrelin occurs predominantly in epithelial cells lining the fundus of the stomach, with smaller amounts produced in the placenta, kidney, pituitary and hypothalamus.^{6,7} Human salivary glands such as the parotid, submandibular, and sublingual glands can also produce and release ghrelin.^{8,9} Most reports showed the close association of serum or plasma ghrelin levels and obesity and lipid metabolism disorders.¹⁰⁻¹⁵ Ghrelin levels are decreased because of increased calorie intake in patients with obesity and increased during the fasting state and in patients with anorexia nervosa.¹⁰ Ghrelin administration was found to improve food intake and weight gain.^{11,12} Ghrelin injections in rats had a positive and cumulative effect on food intake, body weight, and retroperitoneal adipose tissue but not blood total cholesterol (TC), which suggests the positive association of ghrelin level and obesity.¹³ However, the association of ghrelin level and obesity is a matter of debate. Nieminen and Mustonen¹⁴ found ghrelin levels correlated with the plasma level of high-density lipoprotein (HDL) in voles. Fagerberg et al.¹⁵ showed that although ghrelin level was positively related to levels of HDL, the association did not remain after adjustment for body fat. Park et al.¹⁶ reported that fasting plasma ghrelin levels were negatively correlated with weight, body mass index (BMI), percent body fat, waist circumference, hip circumference, and triglyceride (TG) level and positively correlated with HDL level. *In vitro*, ghrelin was found to suppress an adipogenic effect.¹⁷ Wang and Zhang¹⁸ found plasma ghrelin level negatively related to weight, BMI and TC level.

Saliva is often used as a testing source.¹⁹ Saliva testing does not injure blood vessels of patients and avoids cross-infection. Therefore, saliva testing is preferred over blood testing, especially for children, adolescents and their families. Some reports suggest an association of ghrelin level in saliva and serum, in terms of the feasibility of saliva-testing replacing blood samples. Gröschl et al.⁸ found concentrations of ghrelin lower in saliva than in serum. However, Aydin et al.⁹ reported salivary ghrelin levels higher than plasma ghrelin levels, which is consistent with the work of Cetinkaya et al.²⁰ Despite the above differences, saliva might be a possible alternative to serum for ghrelin testing because of the possible close relationship in levels. However, the results of these studies have been limited by the small sample sizes.

In the present study, we aimed to explore the expression pattern of ghrelin in 3 major salivary glands. We also aimed to investigate the association of salivary and serum ghrelin levels and the relation with body weight and lipid indices in a large sample of Chinese children and adolescents with different weights.

2. Materials and methods

2.1. Reverse transcription-polymerase chain reaction (RT-PCR)

Parotid, submandibular and sublingual glands excised during tumour surgery were used as the source of salivary gland tissue for this study. As malignant tissue is dissected with sufficient safety margins, some normal healthy tissue is included. Gland tissue confirmed to be histologically normal was selected for this study. Total RNA from glands was purified with the use of Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA preparations were treated with DNase I to remove contamination by genomic DNA before RT-PCR. cDNA was prepared from 4 µg of total RNA with use the of M-MLV reverse transcriptase (Promega, Madison, WI) and primed with oligo dT. PCR amplification involved 1 cycle at 94 °C for 5 min, then 40 cycles of 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 45 s. The sense and antisense primers for ghrelin (NM_000738), and β-actin were 5'-GCCACCTGTCTGCAACC-CAGCTGA-3' and 5'-CTGGACTCTCTGGTTCAGG-3', 5'-TCCTCCCTGGAGAAGAGCTA-3' and 5'-TCAGGAGGAGCAAT-GATCTTG-3', respectively. β-actin was used as an internal standard. The amplification products were visualized on 1.5% agarose gel with ethidium bromide and sequenced to confirm their identities.²¹

2.2. Immunohistochemistry

Glandular tissue samples dissected from 10 human parotid, 10 submandibular, and 10 sublingual glands as described in Section 2.1 above were fixed in 10% (v/v) neutral buffered formalin, then dehydrated in a graded series of ethanol solutions and embedded in paraffin. The paraffin-embedded samples were cut into 4 µm sections. The sections were deparaffinised in xylenes two times for 30 min each, hydrated gradually through graded series of alcohols (100% ethanol two times for 5 min each, then 95%, 85%, 75% ethanol for 5 min each time), and rinsed in distilled water for 1 min. Deparaffinised sections were treated with 3% H₂O₂ for 10 min at 37 °C and then rinsed in phosphate balanced solution (PBS) three times for 5 min each time. Then the slides were incubated with normal serum for 1.5 h at 37 °C to block non-specific binding of antibody. After the sections were rinsed with buffer, rabbit anti-human ghrelin antiserum (Phoenix Pharmaceuticals, USA) was added and incubated for 2 h at room temperature in a humid box. After a buffer rinse, rhodamine red-tagged goat anti-rabbit IgG and peroxidase complex were applied. For the diaminobenzidine staining step, the slides were coloured for 40 s, and then the nucleus were counterstained with haematoxylin for 6 s. After a final washing step, the sections were put on coverslips and observed under a light microscope (Olympus CX31). For negative controls, the primary antibody was replaced with PBS.

2.3. Study population and protocol

We included 194 healthy volunteers (range 7–18 years). They visited our hospital for orthodontic treatment. Standing height

and weight were measured, and BMI was calculated as $BMI = kg/m^2$. All measurements were obtained by the same experienced staff member. Subjects were not receiving regular medication and were systemically healthy without metabolic syndrome. Levels of alanine aminotransferase, serum total protein, serum albumin, and ratio of leukocytes to erythrocytes were within normal ranges, and subjects were negative for hepatitis B and C antigens. Subjects with secondary obesity induced by endocrine disease or with abnormal liver function or exposure to smoking were excluded. The research protocol was approved by Ethics Committee of Peking University Health Science Centre and all parents of volunteers signed an informed consent documents to participate in the study.

The BMI range varies with the age and sex of children and adolescents. According to the World Health Organization criteria for BMI,²² subjects were divided into 4 groups: lean ($n = 79$), normal ($n = 33$), overweight ($n = 37$) and obesity ($n = 45$).

2.4. Saliva collection

Serum and saliva samples were collected simultaneously between 8:30 and 10:00 am. Subjects could not eat, drink, or smoke before the collection of saliva. Blood samples (2 ml) were collected into plastic EDTA- Na_2 tubes and centrifuged for 10 min at 5000 rpm at 4 °C, and then serum samples were removed and transferred to new tubes.

Non-stimulated whole saliva was collected as routine. After subjects were advised to rinse their mouths with water thoroughly, they bent their heads forward for 10 min to allow saliva to flow into disposable cups. The saliva samples were immediately transferred into new tubes, centrifuged for 15 min at 1300 rpm at 4 °C; the supernatant was removed and transferred to new tubes. The storage time in the 4 °C environment was limited to 2 h. Each supernatant was divided into 4 aliquots, and all samples were stored at -80 °C until analysis.

2.5. Enzyme-linked immunosorbent assay (ELISA) for ghrelin

The ghrelin level was measured with a commercial ELISA kit according to the manufacturer's instructions (USCN Life Science & Technology, USA) and the report of Germain et al.²³ Saliva and serum samples for each subject were measured simultaneously in the same experimental set. The minimum detectable dose of ghrelin for this assay was typically less than 7.8 pg/ml. The sensitivity of this assay was defined as the lowest detectable concentration that could be differentiated from zero. The detection range of this kit was 31.2–2000 pg/ml. The standard curve concentrations used for the ELISA were 2000, 1000, 500, 250, 125, 62.5, and 31.2 pg/ml. The linear results indicated the reliability of salivary and serum measurements.

2.6. Measurement of blood lipid levels

Levels of lipid metabolism indicators, such as TC, TG, HDL and LDL cholesterol, were all tested by use of the automatic analyzer HITACHI 7180 (Japan). TC and TG levels were measured by enzymatic colorimetry. HDL and LDL levels were determined by

the enzymatic clearance method. The reference values of TC, TG, HDL and LDL for healthy Chinese were 3.10–5.70, 0.45–1.70, 0.90–1.68 and 2.08–3.64 mmol/L, respectively.

2.7. Statistical analysis

Statistical analysis involved use of SPSS v13.0 for Windows (SPSS Inc., Chicago, IL). All values were reported as means \pm SD. Significant differences were estimated by one-way ANOVA followed by the Student-Newman-Keuls test. LSD post hoc assay was used when equal variances were assumed, otherwise Dunnett's T3 test was used. Correlations were estimated by bivariate correlation analysis. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Expression and distribution of ghrelin

Expression of ghrelin mRNA was investigated in 3 human major salivary glands. As shown in Fig. 1, ghrelin mRNA was identified at the expected size of 137 bp in parotid and submandibular glands, whereas expression of ghrelin mRNA was not detectable in sublingual glands.

Immunohistochemistry demonstrated ghrelin distribution in the cytoplasm of striated and intercalated ducts of glands, as well as excretory ducts and some serous acini. In parotid gland (Fig. 2A), ghrelin was expressed strongly in the striated and intercalated ducts, with weak staining in serous acini. This expression pattern was obvious in submandibular gland, cells throughout the entire duct system showed strong staining. However, in the structure of mixed acini, serous acinar cells were weak staining, whereas mucous acinar cells were not stained (Fig. 2B). In sublingual gland, staining in the striated ducts was weaker than those in parotid and submandibular glands, whereas mucous acini were not stained (Fig. 2C).

3.2. Demographic characteristics and blood lipid levels

The demographic data for the 194 subjects are shown in Table 1. The BMI values ranged from 11.53 to 33.98 kg/m². The subjects were divided into 4 groups according to the World Health Organization criteria for BMI.²² The lipid profiles were given in Table 2. TC level in overweight group was higher than lean and normal-weight groups, however, the difference was not statistically significant. TC level of

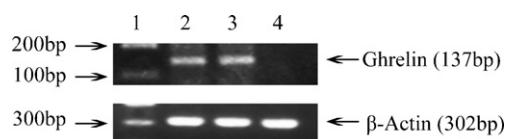


Fig. 1 – RT-PCR analysis of mRNA expression of ghrelin in human salivary glands. Ghrelin mRNA was observed at the expected size of 137 bp in parotid and submandibular glands. Lane 1, DNA marker; lane 2, parotid gland; lane 3, submandibular gland; lane 4, sublingual gland.

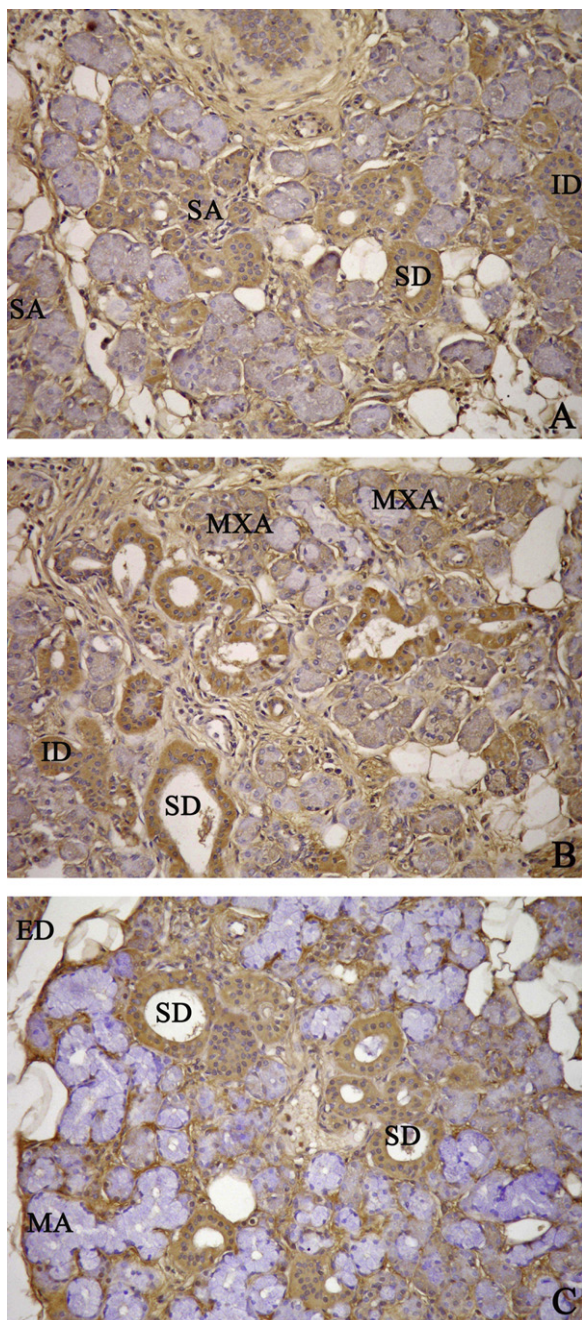


Fig. 2 – Ghrelin expression in parotid (A), submandibular (B) and sublingual (C) glands. Ghrelin protein was strongly positively stained in the striated ducts (SD), intercalated ducts (ID) and excretory ducts (ED), whilst weakly stained in serous acini (SA) and no stain in mucous acini (MA). The contrast of SA and MA was obvious in the structure of mixed acini (MXA) in submandibular glands. Magnification 200 \times .

obese subjects was higher than lean and normal subjects ($P < 0.05$). TG level was significantly higher for overweight than lean and normal subjects ($P < 0.05$), and further increased in obese subjects ($P < 0.01$). Moreover, the obese subjects had lower HDL cholesterol levels and higher LDL cholesterol levels than those of lean subjects ($P < 0.05$ or

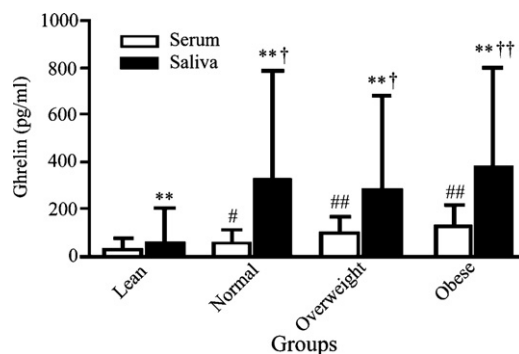


Fig. 3 – Ghrelin levels in serum and saliva of subjects. Values are mean \pm SD. ** $P < 0.01$ compared with serum ghrelin levels between same BMI groups. # $P < 0.05$ and ## $P < 0.01$ compared with serum ghrelin level of lean group. † $P < 0.05$ and †† $P < 0.01$ compared with saliva ghrelin level of lean group (one-way ANOVA followed by the Student–Newman–Keuls test).

$P < 0.01$). HDL and LDL cholesterol levels did not differ significantly between normal and overweight groups.

3.3. Serum and saliva ghrelin levels

Concentrations of serum ghrelin ranged from 0.87 to 434.28 pg/ml and concentrations of salivary ghrelin ranged from 0.06 to 1602.97 pg/ml amongst 194 subjects. The ghrelin level in serum was significantly lower than that in saliva (67.98 ± 75.90 vs. 211.61 ± 356.21 pg/ml, $P < 0.01$), but the variance of the latter was also larger than that of the former. When the subjects were divided into groups according to their BMI, the results showed that serum ghrelin level for lean subjects was significantly lower than those in normal-weight ($P < 0.05$), overweight ($P < 0.01$), and obese subjects ($P < 0.01$) (Fig. 3). The saliva ghrelin level was also lower for lean subjects than for the other 3 groups ($P < 0.05$, $P < 0.05$, and $P < 0.01$, respectively). There was a significant linear correlation between salivary and serum ghrelin ($r = 0.534$, $P < 0.01$). Additionally, both serum and saliva ghrelin levels were significantly correlated with BMI ($r = 0.523$ or $r = 0.374$, $P < 0.01$, respectively). However, serum and salivary ghrelin levels were not associated with levels of lipid metabolism markers.

4. Discussion

It has been reported that expressions of ghrelin mRNA and protein were detected in human parotid, submandibular, and sublingual glands.⁸ In the present study, we confirmed that ghrelin was mainly expressed in human parotid and submandibular gland, whereas expression of ghrelin was much weak or even not detectable in sublingual glands. Parotid gland is composed of serous acini, submandibular gland contains mixed acini, whereas sublingual gland is composed of mucous acini. Immunohistochemistry further demonstrated that ghrelin was widespread in striated, intercalated, and excretory ducts, as well as serous acinar

Table 1 – Demographic characteristics of subjects.

Parameters	Lean (n = 79)	Normal (n = 33)	Overweight (n = 37)	Obese (n = 45)	Total (n = 194)
Gender (no. boys/girls)	29/50	14/19	23/14	38/7	104/90
Age (yrs)	12.51 ± 2.18	13.06 ± 2.12	13.49 ± 2.10	13.36 ± 2.41	12.98 ± 2.23
Weight (kg)	42.00 ± 9.66	54.44 ± 10.32	65.83 ± 10.65	78.83 ± 15.25	57.17 ± 18.65
Height (m)	1.56 ± 0.11	1.60 ± 0.10	1.63 ± 0.10	1.65 ± 0.12	1.60 ± 0.11
BMI (kg/m ²)	16.94 ± 2.03	21.17 ± 1.88	24.62 ± 1.97	28.63 ± 2.73	21.82 ± 5.17

Data are means ± SD unless indicated. BMI: body mass index. The grouping of subjects was according to the World Health Organization criteria for BMI.²²

Table 2 – Lipid metabolism indicators.

Indicators	Lean (n = 18)	Normal (n = 19)	Overweight (n = 28)	Obese (n = 41)
TC (mmol/L)	3.83 ± 0.56	3.84 ± 0.60	4.10 ± 0.44	4.19 ± 0.77 ^{*,†}
TG (mmol/L)	1.22 ± 0.32	1.17 ± 0.38	1.52 ± 0.45 [†]	1.73 ± 0.82 ^{*,††}
HDL (mmol/L)	1.44 ± 0.27	1.34 ± 0.27	1.34 ± 0.18	1.30 ± 0.23 [†]
LDL (mmol/L)	1.93 ± 0.38	2.22 ± 0.62	2.28 ± 0.54	2.42 ± 0.67 ^{**}

TC: total cholesterol; TG: triglyceride; HDL: high-density lipoprotein; LDL: low-density lipoprotein. The grouping of subjects was according to the World Health Organization criteria for BMI.²² Values are means ± SD.

* P < 0.05.

** P < 0.01 compared with lean group.

† P < 0.05.

†† P < 0.01 compared with normal group (one-way ANOVA followed by the Student–Newman–Keuls test).

cells, but not mucous acinar cells, suggesting that parotid and submandibular glands were primary sources of ghrelin produced and released in saliva.

The values of serum and saliva ghrelin are varied in the literature. The present data showed that serum ghrelin (67.98 ± 75.90 pg/ml) and saliva ghrelin (211.61 ± 356.21 pg/ml) levels in Chinese children and adolescents were similar to those from other published reports,^{9,20,24} and saliva ghrelin level was higher than serum level. Gröschl et al.⁸ reported that the concentration of total ghrelin in saliva ranged from 10 to 198 pg/ml, which was lower than that in serum. However, another study indicated that saliva ghrelin levels (190.3 ± 80.2 pg/ml) were higher than plasma levels (120.4 ± 35.7 pg/ml) in young males.⁹ These discrepancies may be associated with a multitude of factors such as different treatments of the samples, storage time, reagents, or the selection of subject population.

Studies have shown lower ghrelin concentrations in obese than lean subjects.^{8,25,26} Aydin et al. found that saliva ghrelin level was lower in obese and non-obese subjects with type II diabetes than in controls.²⁷ Fagerberg et al.¹⁵ reported that ghrelin level negatively correlated with body fat and waist circumference. Body fatness was the strongest determinant of circulating ghrelin level. BMI does not indicate body fat directly, but research has shown that BMI is correlated with direct measures of body fat.²⁸ BMI is a reliable indicator of body fatness for most children and adolescents. Additionally, BMI is an inexpensive and easy-to-perform method of screening for weight categories that may lead to health problems. The pathogenesis of obesity has not yet been fully elucidated. In the present study, salivary and serum ghrelin levels were significantly correlated with BMI but not TC, TG, HDL, or LDL levels. Saliva has been increasingly used as a diagnostic fluid and in predictions of disease progression in the last decade.²⁹ Measurement of ghrelin in saliva is non-invasive, simple, and in general preferred by patients. The

results showed that serum and saliva ghrelin levels were correlated with BMI, suggesting salivary collection may be an acceptable alternative to serum sampling. However, saliva ghrelin values were subject to variation, so the stability of saliva data should be improved.

5. Conclusion

Our data demonstrate that parotid and submandibular glands were primary sources of ghrelin produced and released in saliva. Ghrelin was mainly located in the entire duct system and in serous acinar cells, but not in mucous acinar cells. Saliva ghrelin level was correlated with serum ghrelin level and BMI, suggesting salivary ghrelin might be a possible alternative to serum ghrelin for predicting obesity.

Funding

This study was supported by grants from the National Natural Science Foundation of China (No. 30370102) and Ministry of Education (20050001113).

Competing interests

The authors have no conflicts of interest to disclose.

Ethical approval

The study was approved by Ethics Committee of Peking University Health Science Center and all parents of volunteers signed an informed consent documents.

REFERENCES

1. Weigel C, Kokocinski K, Lederer P, Dotsch J, Rascher W, Knerr I. Childhood obesity: concept, feasibility, and interim results of a local group-based, long-term treatment program. *J Nutr Educ Behav* 2008;**40**:369–73.
2. Adair LS. Child and adolescent obesity: epidemiology, and developmental perspectives. *Physiol Behav* 2008;**94**:8–16.
3. Wu YF, Huxley R, Li M, Ma J. The growing burden of overweight and obesity in contemporary China. *CVD Prev Control* 2009;**4**:19–26.
4. Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* 1999;**402**:656–60.
5. Horvath TL, Diano S, Sotonyi P, Heiman M, Tschöp M. Minireview: ghrelin and the regulation of energy balance—a hypothalamic perspective. *Endocrinology* 2001;**142**:4163–9.
6. Ghelardoni S, Carnicelli V, Frascarelli S, Ronca-Testoni S, Zucchi R. Ghrelin tissue distribution: comparison between gene and protein expression. *J Endocrinol Invest* 2006;**29**:115–21.
7. Date Y, Kojima M, Hosoda H, Sawaguchi A, Mondal MS, Suganuma T, et al. Ghrelin, a novel growth hormone-releasing acylated peptide, is synthesized in a distinct endocrine cell type in the gastrointestinal tracts of rats and humans. *Endocrinology* 2000;**141**:4255–61.
8. Gröschl M, Topf HG, Bohlender J, Zenk J, Klussmann S, Dötsch J, et al. Identification of ghrelin in human saliva: production by the salivary glands and potential role in proliferation of oral keratinocytes. *Clin Chem* 2005;**51**:997–1006.
9. Aydin S, Halifeoglu I, Ozercan IH, Erman F, Kilic N, Aydin S, et al. A comparison of leptin and ghrelin levels in plasma and saliva of young healthy subjects. *Peptides* 2005;**26**:647–52.
10. Nakazato M, Murakami N, Date Y, Kojima M, Matsuo H, Kanganak K, et al. A role for ghrelin in the central regulation of feeding. *Nature* 2001;**409**:194–8.
11. DeVriese C, Delporte C. Ghrelin: a new peptide regulating growth hormone release food and intake. *Int J Biochem Cell Biol* 2008;**40**:1420–4.
12. DeBoer MD, Zhu XX, Levasseur P, Meguid MM, Suzuki S, Inui A, et al. Ghrelin treatment causes increased food intake and retention of lean body mass in a rat model of cancer cachexia. *Endocrinology* 2007;**148**:3004–12.
13. Stevanovic D, Nestic D, Miloseric V, Starcevic V, Severs WB. Consummatory behavior and metabolic indicators after central ghrelin injections in rats. *Regul Pept* 2008;**147**:52–9.
14. Nieminen P, Mustonen AM. Effects of peripheral ghrelin on the carbohydrate and lipid metabolism of the tundra vole (*Microtus oeconomus*). *Gen Comp Endocrinol* 2004;**138**:182–7.
15. Fagerberg B, Hulthen LM, Hulthe J. Plasma ghrelin, body fat, insulin resistance, and smoking in clinically healthy men: the atherosclerosis and insulin resistance study. *Metabolism* 2003;**52**:1460–3.
16. Park HS, Lee KU, Kim YS, Park CY. Relationships between fasting plasma ghrelin levels and metabolic parameters in children and adolescents. *Metabolism* 2005;**54**:925–9.
17. Zhang W, Zhao L, Lin TR, Chai B, Fan Y, Gantz I, et al. Inhibition of adipogenesis by ghrelin. *Mol Biol Cell* 2004;**15**:2484–91.
18. Wang Z, Zhang Q. The relationship between plasma ghrelin levels and lipid metabolism of child and adolescent obesity. *Med J Commun* 2008;**22**:365–6.
19. Miller SM. Saliva testing—a nontraditional diagnostic tool. *Clin Lab Sci* 1994;**7**:39–44.
20. Cetinkaya Z, Aydin S, Cerrahoglu YZ, Ayten R, Erman F, Aygen E. Changes in appetite hormone (ghrelin) levels of saliva and serum in acute appendicitis cases before and after operation. *Appetite* 2009;**52**:104–7.
21. Zhang Y, Xiang B, Li YM, Wang Y, Wang X, Wang YN, et al. Expression and characteristics of vanilloid receptor 1 in the rabbit submandibular gland. *Biochem Biophys Res Commun* 2006;**345**:467–73.
22. World Health Organization Expert Committee. Physical status, the use and interpretation of anthropometry. WHO Tech Rep Ser 1995;No 854.
23. Germain N, Galusca B, Grouselle D, Frere D, Tolle V, Zizzari P, et al. Ghrelin/obestatin ratio in two populations with low bodyweight: constitutional thinness and anorexia nervosa. *Psychoneuroendocrinology* 2009;**34**:413–9.
24. Whatmore AJ, Hall CM, Jones J, Westwood M, Clayton PE. Ghrelin concentrations in healthy children and adolescents. *Clin Endocrinol (Oxf)* 2003;**59**:649–54.
25. English PJ, Ghatei MA, Malik IA, Bloom SR, Wilding JP. Food fails to suppress ghrelin levels in obese humans. *J Clin Endocrinol Metab* 2002;**87**:2984.
26. Shiiya T, Nakazato M, Mizuta M, Date Y, Mondal MS, Tanaka M, et al. Plasma ghrelin levels in lean and obese humans and the effect of glucose on ghrelin secretion. *J Chin Endocrinol Metab* 2002;**87**:240–4.
27. Aydin S. A comparison of ghrelin, glucose, alpha-amylase and protein levels in saliva from diabetics. *J Biochem Mol Biol* 2007;**40**:29–35.
28. Mei Z, Grummer-Strawn LM, Pietrobelli A, Goulding A, Goran MI, Dietz WH. Validity of body mass index compared with other body-composition screening indexes for the assessment of body fatness in children and adolescents. *Am J Clin Nutr* 2002;**75**:978–85.
29. Srivastava S, Krueger KE. In: Wong, editor. *Salivary diagnostics*. Iowa: Wiley-Blackwell Press; 2008. p. 94–103.