Short Communication

Measurement of Urea in the Saliva of Healthy Mice – a Pilot Study

(non-invasive sampling / salivary biomarkers / reference range / experimental animals / renal failure / technical variability)

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Abstract. Salivary urea is studied as a non-invasive alternative for screening and monitoring of renal diseases. Its high variability prevents a wider clinical use. Animal experiments are needed to identify factors affecting this marker. The aim of this study was to describe the inter-individual variability of salivary urea in healthy mice, establish reference intervals, and analyse the effects of sex, age and body weight. Plasma and saliva samples were obtained from 37 male and 41 female healthy adult CD1 mice aged 13–69 weeks (body weight 22–51 g). The reference interval for salivary urea in heathy mice based on our results is 2.7–8.4 mmol/l (CV = 23 %). Multivariate analysis did not show any significant effect of age, sex, or body weight. In addition, salivary urea did not correlate with its plasma concentrations. The high variability of the promising salivary marker of kidney function in healthy mice requires further research before its use to diagnose or monitor renal failure in animal models of kidney diseases. Other potential confounders should be analysed, including intra-individual and pre-analytical variability. In addition, a normalization factor such as total salivary proteins or salivation rate is likely needed.

Introduction

Saliva represents a unique body fluid with a diagnostic, prognostic, and monitoring potential. It could serve as a non-invasive, cheap, and easy replacement of blood that requires less effort, equipment, and trained personnel compared to blood collection. Saliva can be collected without needles and pain even at home – a great advantage for non-compliant patients such as children or aged people. Moreover, easy handling and storage increase its applicability in everyday clinical practice (Nunes et al., 2015).

Assessment of kidney functions is important for diagnosing and monitoring kidney diseases. Urea is a nitrogenous by-product of protein metabolism that is eliminated from the blood via glomerular filtration. A decline in renal function is associated with a rise of serum urea (Gowda et al., 2010). Repeated blood collections in patients with kidney diseases are associated with several complications including distress, pain, increased risk of vessel injury, infections, and anaemia (Liu and Duan, 2012). Therefore, the interest in saliva as an alternative to blood has rapidly increased due to easy and non-invasive sampling of its sufficient volume (Renda, 2017).

Concentrations of urea can be measured in the saliva of healthy individuals or patients with kidney disease using standard colorimetric methods (Celec et al., 2016). In healthy controls, concentrations of plasma urea are higher than in the saliva (Peng et al., 2013). More importantly, no correlation was found between plasma and salivary values (Suresh et al., 2014). However, an in-

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Abbreviations: CV – coefficient of variation, LOD – limit of detection, LOQ – limit of quantification.

crease in the plasma concentrations of urea in patients with kidney diseases increases their concentrations in the saliva, likely via passive diffusion following a concentration gradient (Kovalčíková et al., 2018). In uraemia, deterioration of acinar cell permeability contributes to the elevation of salivary concentrations of urea, suggesting that most of the salivary urea originate from the plasma (Ivanovski et al., 2012; Peng et al., 2013). Several studies evaluated and compared salivary concentrations of urea in healthy people and patients with kidney disease. In the study of Cardoso et al. (2009), the mean salivary urea in healthy individuals was 5.36 ± 1.43 mmol/l compared to 17.86 ± 5.98 mmol/l in patients with kidney disease. Salivary concentrations of urea varied between different stages of kidney disease (Tomás et al., 2008; Pham, 2017).

High biological and technical variability of salivary urea prevents the use of saliva in routine clinical practice. Animal experiments, in contrast to human studies, can be performed in well-defined, controlled conditions that allow identification of factors affecting salivary markers of the kidney function. According to our knowledge, no comprehensive study evaluating the effect of sex, age, or body weight on salivary urea as a marker of kidney functions has been published to date. Therefore, our study aimed to describe the inter-individual variability of salivary urea in healthy mice, establish reference intervals for this marker, and analyse the effects of sex, age and body weight.

Material and Methods

Design of the experiment

This study was approved by the Ethics Committee of the Institute of Pathophysiology, Faculty of Medicine, Comenius University, Bratislava, Slovakia. Adult female (N = 41) and male (N = 37) mice of CD1 strain were used (Anlab, Prague, Czech Republic). Animals were divided into groups of young adults (13–17 weeks), middle-age adults (18–43 weeks), and old adults (66–69 weeks). All animals were housed in standard cages, with free access to tap water and standard rodent chow, 12/12 light dark cycle, an ambient temperature of 22°C, and humidity of 40–50 %.

For collection of saliva, mice were anaesthetized with ketamine and xylazine (100 mg/kg and 10 mg/kg of the body weight, respectively) by intraperitoneal injection. Salivation was induced using pilocarpine (0.5 mg/kg of body weight; Unimed Pharma, Bratislava, Slovakia) by intraperitoneal injection. Saliva was collected for 15 min and centrifuged at 1,600 *g* for 10 min to remove the cell debris. Blood was collected using microvette tubes (Microvette 300 Lithium-heparin, Microvette 500 EDTA, Sarstedt, Nümbrecht, Germany) from retro-orbital plexus. Blood was centrifuged at 1,600 *g* for 10 min to obtain the plasma. Saliva and plasma were stored at −20 °C until analysis.

Biochemical analysis

Urea was measured using a commercial spectrophotometric kit (Urea Nitrogen, Colorimetric Detection Kit, Arbor Assays, Ann Arbor, MI). Technical, intra- and inter-individual coefficients of variation (CV) are 5 %, 13 % and 23 %, respectively. The limit of detection (LOD) and limit of quantification (LOQ) are 0.12 mmol/l and 0.35 mmol/l, respectively.

Statistical analysis

GraphPad Prism 6.01 (GraphPad Software, San Diego, CA) was used for statistical analysis. After testing the distribution of data with D'Agostino Pearson omnibus normality test, data were analysed using Student's *t*-test (sex differences of salivary urea) or Mann Whitney test (sex differences of plasma urea). Plasma and salivary urea within individual age groups were tested using one-way ANOVA and Tukey's multiple comparison test. Correlations between quantitative measures were assessed using the Spearman's or Pearson's correlation test. The correlation test was used for analysis of correlation. P values below 0.05 were considered statistically significant. Percentiles (2.5 and 97.5) are shown for each analysed parameter.

Multivariate analysis was conducted using the general linear model (SPSS 21, IBM, Chicago, IL). Age, body weight and sex were tested as determinants of plasma urea. Plasma urea was added to potential determinants in the models of salivary concentrations of urea. Eta^2 is reported as the measure of explained inter-individual variability.

Results and Discussion

Several studies have reported analyses of salivary urea both in healthy individuals and in patients with kidney disorders (Tomás et al., 2008; Pham, 2017). Age, sex, and body weight are known to affect plasma and partially also salivary urea in healthy humans (Peng et al., 2013). Salivary urea and plasma urea positively correlate in patients with kidney disease, especially in those with high plasma concentrations (Tomás et al., 2008; Renda, 2017). Cardoso et al. (2009) even found correlation between plasma and salivary concentrations of urea in healthy probands, and the same was evinced by Lasisi et al. (2016) and Pandya et al. (2016). It has been shown that plasma urea is influenced by gender and age (Gardner and Scott, 1980). Another study proclaimed that the variability of salivary concentrations of urea is related to its plasma concentrations and age but found no gender differences. Higher plasma and salivary concentrations of urea were found in older individuals (aged 45–65 years) in comparison to people aged 19–44 years (Peng et al, 2013).

To our knowledge, this is the first cross-sectional study describing the inter-individual variability of urea in the saliva of healthy mice. However, the results have shown high variability that complicates its clinical use

Table 1. Reference values of plasma and salivary urea in healthy mice. Values are expressed as median and 2.5–97.5 percentile.

Sex	Marker	Young adults	Middle age adults	Old adults
Females	Plasma urea [mmol/l]	9.81 (8.12–14.05)	9.76 (4.98–14.05)	9.98 (7.65–22.33)
	Salivary urea [mmol/l]	5.04 (2.81–7.08)	6.22 (3.61–8.03)	6.66 (4.33–8.20)
Males	Plasma urea [mmol/l]	10.58 (2.05–18.73)	9.71 (4.21–12.10)	10.45 (6.14–20.48)
	Salivary urea [mmol/l]	6.23 (3.17–7.42)	6.15 (2.00–9.32)	6.63 (3.52–7.87)

(Table 1). Animal experiments performed in well-defined and controlled conditions are superior to human studies in identifying sources of this variability. In our study on healthy mice, these associations were not confirmed. In the plasma urea, we did not find any significant differences between females and males regardless of the age of mice (U = 533.0, P = 0.62). Similarly, no differences between females and males in the salivary urea were observed (t_{70} = 0.33, P = 0.74). No correlation between the plasma concentrations of urea and age of mice (Spearman r = −0.21, P = 0.09; Fig. 1A) was found, and no correlation between the plasma urea and body weight of mice was observed (Spearman r = −0.06, P = 0.62; Fig. 1B). We found no correlation between the salivary concentrations of urea and age of mice (Spearman r = 0.14, P = 0.26; Fig. 1C), and no correlation between the salivary urea and body weight of mice was observed (Pearson r = 0.15, P = 0.22; Fig. 1D). This could be due to the high technical and biological variability of salivary urea, which is, however, in line with previously published studies in human population (Lasisi et al., 2016; Yajamanam et al., 2016).

As clinical studies on humans have confirmed the correlation between plasma and salivary urea, we ex-

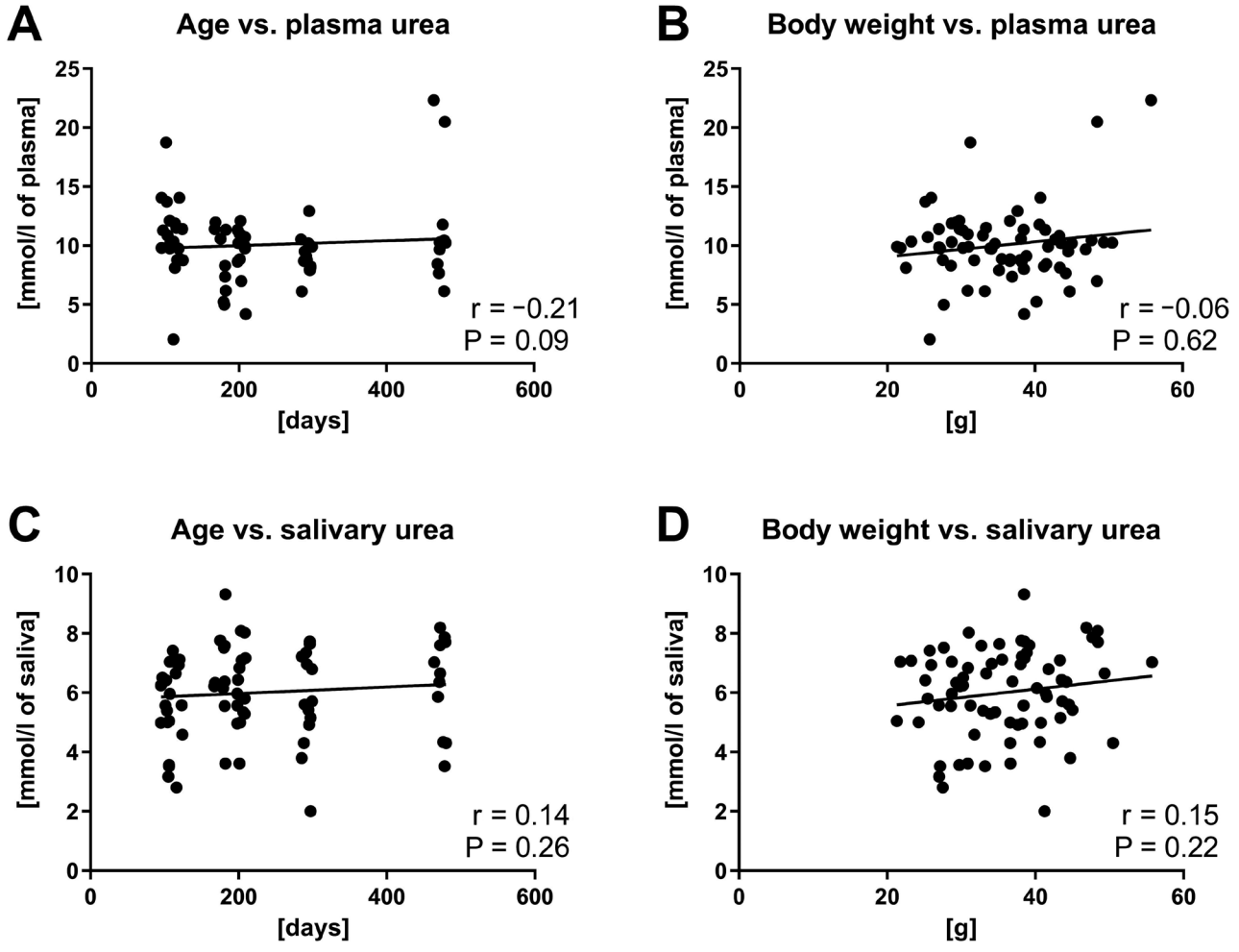

Fig. 1. Relation between **A**: plasma urea vs age, **B**: plasma urea vs weight, **C**: salivary urea vs age, **D**: salivary urea vs weight. Correlations were determined using Pearson's (**D**) or Spearman's (**A**, **B**, **C**) correlation analyses.

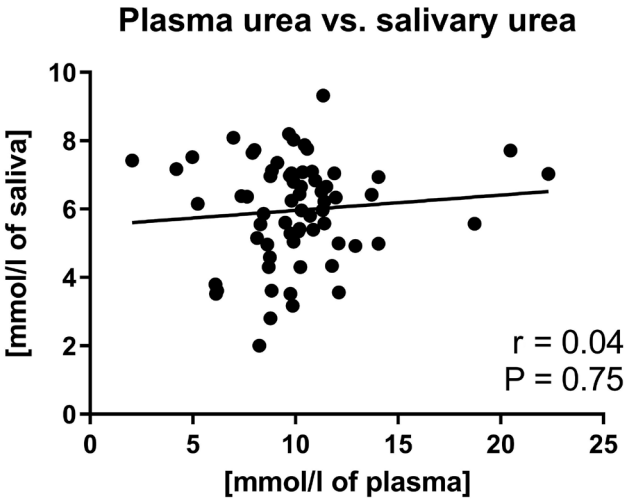

Fig. 2. Relation between plasma and salivary urea using Spearman's correlation analysis

pected the same results among healthy mice. In contrast, we found no correlations between the plasma and salivary concentrations of urea in healthy mice (Spearman $r = 0.04$, P = 0.75; Fig. 2). Our study is the first to analyse the effects of age and sex in experimental animals. Multivariate analysis revealed that urea in the saliva is not associated with age ($Eta^2 = 0.008$, P = 0.49), sex ($Eta^2 = 0.000$, P = 0.99), or body weight ($Eta^2 = 0.012$, P = 0.41) of healthy mice. In addition, the plasma concentrations were not identified as determinants of their salivary concentrations ($Eta^2 = 0.011$, P = 0.43; Table 2 and Table 3).

Table 2. Results of the general linear model analysis of urea concentrations in the saliva of healthy mice, with factors weight, age, sex and corresponding plasma concentrations

Source	Saliva	F	p	Partial Eta Squared
Corrected Model	Urea	0.82	0.54	0.067
Body weight	Urea	0.70	0.41	0.012
Age	Urea	0.49	0.49	0.008
Sex	Urea	0.00	0.99	0.000
Plasma concentrations	Urea	0.65	0.43	0.011

Table 3. Results of the general linear model analysis of urea concentrations in the plasma of healthy mice, with factors weight, age and sex

Source	Plasma	F	p	Partial Eta Squared
Corrected Model	Urea	0.88	0.45	0.040
Body weight	Urea	2.16	0.15	0.033
Age	Urea	0.58	0.45	0.009
Sex	Urea	0.80	0.36	0.013

One of the major limitations of our study is that salivation was induced using pilocarpine. Stimulated saliva has a slightly different composition than unstimulated saliva collected by passive drooling, at least in humans (de Almeida et al., 2008). However, this is the only currently available protocol to collect reasonable volumes of saliva from mice or rats. One way to avoid the bias from variable dilutions due to stimulated salivation would be to use a normalization factor. For saliva, such a factor has not been identified, although the total salivary proteins and salivation rate could be used. However, salivation measurements are highly imprecise and total proteins suboptimal for most salivary biomarkers. So, unsurprisingly, most studies analysing salivary urea, including this one, report total salivary concentrations without any attempt to normalize the values.

In conclusion, we found high variability in physiological reference concentrations of salivary and plasma urea. Additional studies are, however, needed to identify the determinants of variability of salivary urea in mice, as sex, age and body weight did not affect salivary urea in this first cross-sectional study in mice focusing on a salivary biomarker of renal function. Future studies will also focus on the intra-individual variability, which is currently unknown.

Conflicts of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

References

Cardoso, E. M., Arregger, A. L., Tumilasci, O. R., Elbert, A., Contreras, L. N. (2009) Assessment of salivary urea as a less invasive alternative to serum determinations. *Scand. J. Clin. Lab. Invest*. **69**, 330-334.

Celec, P., Tóthová, Ľ., Šebeková, K., Podracká, Ľ., Boor, P. (2016) Salivary markers of kidney function – potentials and limitations. *Clin. Chim. Acta* **453**, 28-37.

de Almeida, P. V., Grégio, A. M., Machado, M. A., de Lima, A. A., Azevedo, L. R. (2008) Saliva composition and functions: a comprehensive review. *J. Contemp. Dent. Pract*. **9**, 72-80.

Gardner, M. D., Scott, R. (1980) Age- and sex-related reference ranges for eight plasma constituents derived from randomly selected adults in a Scottish new town. *J. Clin. Pathol*. **33**, 380-385.

Gowda, S., Desai, P. B., Kulkarni, S. S., Hull, V. V., Math, A. A. K., Vernekar, S. N. (2010) Markers of renal function tests. *N. Am. J. Med. Sci*. **2**, 170-173.

Ivanovski, K., Naumovski, V., Kostadinova, M., Pesevska, S., Drijanska, K., Filipce, V. (2012) Xerostomia and salivary levels of glucose and urea in patients with diabetes. *Prilozi* **33**, 219-229.

Kovalčíková, A., Janšáková, K., Gyurászová, M., Podracká, Ľ., Šebeková, K., Celec, P., Tóthová, Ľ. (2018) Salivary creatinine and urea are higher in an experimental model of acute but not chronic renal disease. *PLoS One* **13**, e0200391.

Lasisi, T. J., Raji, Y. R., Salako, B. L. (2016) Salivary creatinine and urea analysis in patients with chronic kidney disease: a case control study. *BMC Nephrol*. **17**, 10.

Liu, J., Duan, Y. (2012) Saliva: a potential media for disease diagnostics and monitoring. *Oral Oncol*. **48**, 569-577.

Nunes, L. A., Mussavira, S., Bindhu, O. S. (2015) Clinical and diagnostic utility of saliva as a non-invasive diagnostic fluid: a systematic review. *Biochem. Med*. **5**, 177-192.

Pandya, D., Nagrajappa, A. K., Ravi, K. S. (2016) Assessment and correlation of urea and creatinine levels in saliva and serum of patients with chronic kidney disease, diabetes and hypertension – a research study. *J. Clin. Diagn. Res*. **10**, ZC58-ZC62.

Peng, C. H., Xia, Y. C., Wu, Y., Zhou, Z. F., Cheng, P., Xiao, P. (2013) Influencing factors for saliva urea and its application in chronic kidney disease. *Clin. Biochem*. **46**, 275-277.

Pham, T. A. V. (2017) Validation of the salivary urea and creatinine tests as screening methods of chronic kidney disease in Vietnamese patients. *Acta Odontol. Scand*. **75**, 551-556.

Renda, R. (2017) Can salivary creatinine and urea levels be used to diagnose chronic kidney disease in children as accurately as serum creatinine and urea levels? A case-control study. *Ren. Fail*. **39**, 452-457.

Suresh, G., Ravi Kiran, A., Samata, Y., Purnachandrarao Naik, N., Viiray Kuma, A. (2014) Analysis of blood and salivary urea levels in patients undergoing haemodialysis and kidney transplant. *J. Clin. Diagn. Res*. **8**, 18-20.

Tomás, I., Marinho, J. S., Limeres, J., Santos, M. J., Araújo, L., Diz, P. (2008) Changes in salivary composition in patients with renal failure. *Arch. Oral Biol*. **53**, 528-532.

Yajamanam, N., Vinapamula, K. S., Sivakumar, V., Bitla, A. R., Rao, P. V. (2016) Utility of saliva as a sample to assess renal function and estimated glomerular filtration rate. *Saudi J. Kidney Dis. Transplant.* **27**, 312-319.