

Variability of thiobarbituric acid reacting substances in saliva

Michal Behuliak^{a,b}, Roland Pálffy^{a,b}, Roman Gardlík^{a,b}, Július Hodosy^{a,c}, Lukáč Halčák^d and Peter Celec^{a,b,e,*}

^a*BiomeD Research & Publishing Group*

^b*Institute of Pathophysiology, Faculty of Medicine, Comenius University, Bratislava, Slovak republic*

^c*Institute of Physiology, Faculty of Medicine, Comenius University, Bratislava, Slovak republic*

^d*Institute of Medical Chemistry, Biochemistry and Clinical Biochemistry, Faculty of Medicine, Comenius University, Bratislava, Slovak republic*

^e*Department of Molecular Biology, Faculty of Natural Sciences, Comenius University, Bratislava, Slovak republic*

Abstract. *Introduction:* Salivary TBARS are a potential marker of oxidative stress in the oral cavity. Previous studies have found increased levels of salivary TBARS in various diseases. The aim of this study was to assess the variability of salivary TBARS in both genders.

Subjects & Methods: Saliva samples from thirty-eight healthy volunteers (18F & 20M) were collected every day during 30 day period. TBARS levels were measured spectrophotometrically using a high-throughput 96-well plate method. Time series analysis was performed using standard statistical methods.

Results: Repeated measures ANOVA showed a significant variation of salivary TBARS within day and subjects ($p < 0.001$). The dynamics did not differ between genders. Intraindividual variability was very high in both genders with coefficients of variation of more than 60%. Interindividual variability was higher in men than in women (73% vs. 46%; $p < 0.01$).

Discussion: The relatively high intraindividual variability indicates that the use of salivary TBARS will be limited to research on a population level, although some informative value might be gained by repeated samplings. Factors influencing the biological variability of salivary TBARS should be identified in further studies.

Keywords: Thiobarbituric acid reacting substances, malondialdehyde, saliva, interindividual and intraindividual variability

1. Introduction

Thiobarbituric acid reacting substances (TBARS) are produced during the oxidative stress-induced damage of lipids, i.e. lipoperoxidation. They represent a heterogeneous group of compounds. Malondialdehyde (MDA) is the best known TBARS compound. TBARS are widely used as a marker of oxidative stress in the plasma [25] and different types of tissues [8]. Urinary MDA is considerably affected by exogenous factors in-

cluding diet and air pollution and, thus, is rarely used in research [12,18].

In a previous study, we have shown that TBARS are measurable in saliva [10] and that their levels are higher in patients with gingivitis [14]. In addition, the results from our studies indicate that levels of TBARS in plasma and saliva do not correlate, suggesting that salivary TBARS can represent a marker specific for local (oral) disorders and could be useful for assessing oral status and efficacy of treatment oral diseases. However, in some studies increased salivary TBARS levels were found in patients suffering from disease primarily affecting extraoral tissues – such as osteoporosis [31], Crohn's disease [16] and ulcerative colitis [23].

Other authors have confirmed our results with find-

*Corresponding author: Peter Celec, MD, Dipl. Ing., MSc, PhD, MPH, Institute of Pathophysiology, Department of Molecular Biology, Comenius University, Sasinkova 4, 811 08 Bratislava, Slovak republic. E-mail: petercelec@gmail.com.

ings of increased salivary MDA levels, but similar plasma MDA levels have not been found in patients with chronic periodontitis compared to healthy controls [3].

The origin and the composition of TBARS in saliva remain unclear. One of the key issues is to identify the cause of increased TBARS levels. There are already known factors influencing salivary TBARS levels beyond diseases. It has been repeatedly shown that smoking increases salivary MDA levels, at least in dental patients [11,13]. Cadmium and some drugs induce lipoperoxidation directly in the salivary gland [1,2]. Moreover, TBARS levels in saliva are affected by the time of sampling, tooth-brushing and ascorbic acid pre-treatment [14]. Details of interindividual (among individuals) and intraindividual (among days of sampling within one individual subject) variability of TBARS levels in saliva are unknown. The aim of our study was to analyze the interindividual and intraindividual variability of salivary TBARS in young healthy volunteers and to describe potential gender differences.

2. Subjects and methods

Samples of saliva were collected from 18 young healthy female volunteers with an average age of 23.4 ± 3.0 years and 20 young healthy male volunteers with an average age of 25.4 ± 3.1 years. The volunteers were instructed to collect whole saliva samples without the use of any stimulants, daily in the morning during a period of 30 consecutive days. Behavioural habits which may influence the salivary TBARS levels were monitored using a questionnaire. Sampling was carried out 10 minutes after tooth-brushing and the volunteers were instructed not to eat before sampling. Collected saliva samples were immediately frozen at -20°C until measurement.

Salivary TBARS were determined by the spectrophotometric method after derivatization with 0.67 % thiobarbituric acid in 1.5 mol/l acidic medium of acetic acid (95°C , 45 min). Before derivatization, the samples in collection tubes were centrifuged (3000 g, 10 min) and supernatants (0.5 ml) were transferred to the deep-well plates. After derivatization, the colored product was extracted with 0.5 ml of n-butanol, centrifuged (2250 g, 10 min), transferred 0.25 ml to the spectrophotometric 96 well plate and measured ($\lambda_{\text{ex.}} = 515 \text{ nm}$, $\lambda_{\text{em.}} = 535 \text{ nm}$, Safire 2, Tecan). TBARS concentration was expressed as $\mu\text{mol/l}$ of saliva on the basis of the calibration curve of 1,1,3,3-tetramethoxypropan standard. All 30 samples of each volunteer were measured in separate

plates. Calibration curve was prepared for each plate in triplicates. For evaluation of intra- and inter-plate TBARS variability caused by methodological bias the plate assay reliability was determined using coefficient of variation.

The data was analysed by repeated measured ANOVA (analyzed factors were time of sampling – day as within factor, subject as subject variable and gender as between factor) and F-test for the comparison of variability between genders. The level of significance was set to 0.05. All statistical analyses were performed using NCSS 2007 and Origin 6 software.

3. Results

Salivary TBARS levels did not differ between genders (Fig. 1). Repeated measured ANOVA showed significant influence of sampling day as a factor affecting the variability of salivary TBARS ($F = 2.85$; $p < 0.001$). Intraindividual variability was very high in both genders with coefficients of variation (CV) of more than 60% (Fig. 2). Salivary TBARS levels varied between the subjects considerably ($F = 14.8$; $p < 0.001$). The interindividual variability was higher in men than in women (73% vs. 46%; $p < 0.01$) (Fig. 3). The inter-plate TBARS variability was 4.23 % and the intra-plate TBARS variability expressed by CV was 3.79 %. These inter- and intra-plate CV values are sufficiently low to use the microplate method for high-throughput measurement of TBARS levels in saliva.

4. Discussion

Salivary markers of oxidative stress (MDA, TBARS or protein carbonyls) and antioxidants have been analyzed in clinical studies involving subjects with various conditions, including diabetes [5,17,24], aphtous ulceration [28], Alzheimer disease [30] and periodontitis [11,20]. Further studies are running. There is a clear need for a biochemical evaluation of these salivary parameters. This is the first study dealing with the variability of a salivary marker of oxidative stress. TBARS are widely used, although sometimes reported as MDA. We have decided to follow the consensus to use the term TBARS for colorimetric measurements and MDA for chromatographic methods. Based on our previous results, it seems that more than half of the salivary TBARS is MDA, but a detailed targeted analysis is needed.

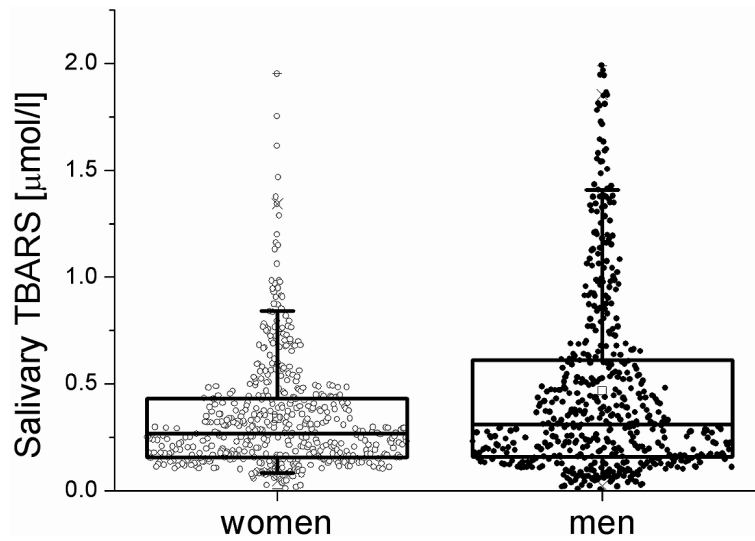


Fig. 1. Salivary TBARS levels in men and women. All analyzed samples are plotted.

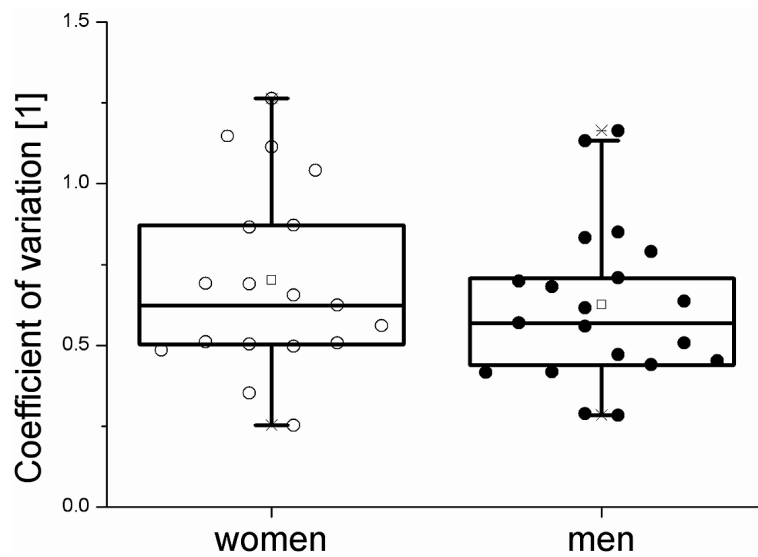


Fig. 2. Intraindividual variability of TBARS levels among 30 days of sampling. No gender difference has been found.

Jens Lykkesfeldt has pointed out problems with the interpretation of MDA levels in a recent review [19]. A major issue is the analytical method, as the results seem to vary extremely between laboratories and are actually not comparable. Adhering to standard analytical methods and especially, the correct reporting on the used methods in the manuscripts is extremely important for the reproducibility and comparability of results. The method used in this study is described in detail and can be easily reproduced. Furthermore, the microplate-based analysis using pipetting robots and microplate spectrophotometer enables a high-throughput approach

and decreases the analytical variability to a satisfactory low level.

In this study we have found an extremely high intraindividual and interindividual variability which makes the interpretation of individual values difficult, if not impossible. Such high variance may lead to false negative results in experiments and clinical trials analyzing salivary TBARS, which are then often not reported in the literature due to publication bias. A previously published analysis of plasma MDA levels revealed an average CV of 18% (intraindividual) and 24% (interindividual) with a conclusion that the use of this

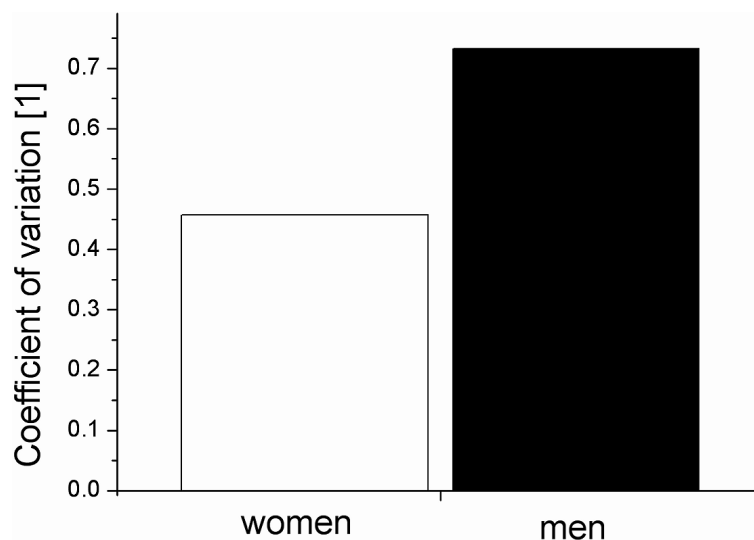


Fig. 3. Interindividual variability of TBARS levels among 30 days of sampling. The variability in men was significantly higher than in women.

marker for individual diagnostics is questionable [21]. Although a comparison is biased due to different cohorts (age 20–79 years vs. age 18–28 years) and other analytical methods (HPLC vs. spectrophotometry), it seems that the biological variability in saliva is even higher than in plasma.

Principally, there are two potential ways how to cope with biological variability – repeated sampling and identification of sources of variability. Only the latter will, however, enable a wider use of salivary TBARS in research. Previous studies have shown that the levels of TBARS/MDA undergo temporal changes including potentially endogenous diurnal [9,14,27] and seasonal variations probably induced by changes of nutritional composition [4,6,29]. In our study the effects of seasonal and diurnal variations can be excluded, as the sampling was done in the same time and same season in all subjects.

The dynamics of salivary TBARS levels did not differ between genders. However, the data was not synchronized, and thus, gender differences in endogenous dynamics cannot be ruled out. Notably, as plasma MDA levels were found to be affected by gender in a recent study [7] and estradiol decreases lipoperoxidation as found previously [22].

Analysis of disease markers in saliva represents a valuable tool for modern diagnostic approaches, mainly due to the non-invasive nature of sample collection and the possibility of long-time monitoring [26]. The production of saliva (or oral fluid) affects the salivary concentrations of biomarkers to a highly variable extent, depending on the origin and the molecular size,

structure and characteristics of the biomarker [15]. As the composition and source of salivary TBARS are currently unknown, it is difficult to estimate the proportion of variability that might be explained. A consensus on a normalization factor like creatinine in the urine or total proteins in tissue homogenates in the future is needed.

The high intraindividual variability indicates that repeated samplings might improve the informative value of salivary TBARS. However, the use of this marker will probably be limited to research on population level. Gender differences and interindividual variability should be taken into account in clinical studies on patients with oral diseases. Other factors influencing the variability of salivary TBARS including diet, composition of microbial flora and genetic polymorphisms should be uncovered by further studies.

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