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Quantitation of malondialdehyde in gingival crevicular fluid by a high-performance liquid chromatography-based method

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ABSTRACT

Lipid peroxidation (LPO) has been associated with periodontal disease, and the evaluation of malondialdehyde (MDA) in the gingival crevicular fluid (GCF), an inflammatory exudate from the surrounding tissue of the periodontium, may be useful to clarify the role of LPO in the pathogenesis of periodontal disease. We describe the validation of a method to measure MDA in the GCF using high-performance liquid chromatography. MDA calibration curves were prepared with phosphate-buffered solution spiked with increasing known concentrations of MDA. Healthy and diseased GCF samples were collected from the same patient to avoid interindividual variability. MDA response was linear in the range measured, and excellent agreement was observed between added and detected concentrations of MDA. Samples' intra- and interday coefficients of variation were below 6.3% and 12.4%, respectively. The limit of quantitation (signal/noise = 5) was 0.03 μM. When the validated method was applied to the GCF, excellent agreement was observed in the MDA quantitation from healthy and diseased sites, and diseased sites presented more MDA than healthy sites ($P < 0.05$). In this study, a validated method for MDA quantitation in GCF was established with satisfactory sensitivity, precision, and accuracy.

Reactive oxygen species (ROS)[1] are produced as an integral feature of normal cellular metabolism under physiological conditions [1]. The generation of ROS is an important mechanism during phagocytosis as part of the bactericidal reaction [2–4]. However, it has been well established that overproduction of ROS occurs at sites of chronic inflammation [4], and uncontrolled production of lipid peroxides can result in oxidative stress. These conditions can contribute to injury of the host tissue [5] and significant damage to cell integrity [6,7] by different mechanisms such as DNA damage, oxidation of important enzymes, lipid peroxidation, and stimulation of proinflammatory cytokine release [8,9].

Lipid peroxidation (LPO) is thought to play an important role in aging, atherosclerosis [4], late complications of diabetes mellitus [10] such as micro- and macrovascular alterations [11,12], rheumatoid arthritis [13], chronic obstructive pulmonary disease [14], and periodontitis [3,15–18].

Periodontitis is an infectious inflammatory disease involving the connective tissue and bone that support the teeth, and its primary etiological factor is the biofilm constituted by several pathogenic bacteria. The severity of periodontal disease is determined by the interactions between host defense and pathogens and can lead to periodontal destruction and lost teeth [19,20]. There have been many investigations regarding the systemic conditions and modifier factors that can be involved in the pathogenesis of periodontitis. Recently, more studies have focused on the roles of antioxidant activity, ROS, and LPO products in the pathogenesis of periodontitis [18,21].

It has been demonstrated that patients with periodontitis have increased levels of LPO in plasma [17,18], saliva [3,22,23], gingival tissue [24], and gingival crevicular fluid (GCF) [16–18]. In addition, these levels have been correlated with periodontal parameters such as gingival index, probing pocket depth, and GCF volume [16,17]. Interventional studies have demonstrated that conventional periodontal treatment resulted in decreased levels of LPO in GCF, saliva [16,18], and plasma [25], suggesting important roles for ROS and LPO in the pathogenesis of periodontal disease.

GCF is considered as a serum transudate or, more commonly, as an inflammatory exudate from the surrounding tissue of the periodontium [26]. GCF contains substances from the host such

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[1] *Abbreviations used:* ROS, reactive oxygen species; LPO, lipid peroxidation; GCF, gingival crevicular fluid; MDA, malondialdehyde; TBA, thiobarbituric acid; TBARS, thiobarbituric acid-reactive substance; HPLC, high-performance liquid chromatography; PBS, phosphate-buffered solution; DAD, diode array detector; BHT, butylated hydroxytoluene; CV, coefficient of variation; LOQ, limit of quantitation.

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as inflammatory cells and serum-derived factors as well as from microorganisms in the subgingival and supragingival plaque [26,27]. The potential diagnostic value of the GCF has been well recognized since early studies reported that the composition of gingival fluid seemed to be promising as a potential medium for the detection of early changes that could indicate the onset of disease [28]. The major interest in GCF as a diagnostic marker is due to the site-specific nature of the sample [29]. Furthermore, it can be collected from the gingival sulcus surrounding the teeth [26] by a noninvasive procedure, serving as an expedient biological source of patient information [30].

Numerous markers have been investigated for monitoring the production of ROS. malondialdehyde (MDA) is the most-studied product of polyunsaturated fatty acid peroxidation and can be an indicator of oxidative stress increase [7,31]. Several methods have been described in the literature to measure the levels of the adduct formed between MDA and two molecules of thiobarbituric acid (TBA), namely MDA–$(TBA)_2$ (Fig. 1) [7,16,32–34]. However, spectrophotometer or spectrofluorimeter determination of thiobarbituric acid-reactive substances (TBARSs) has been criticized for a lack of specificity in the precise evaluation of MDA without the interference of other molecules [21,35].

Of the studies that measured MDA in GCF, some used a method to evaluate TBARS or colorimetric assays [16], and two recent studies described an LPO assay by a high-performance liquid chromatography (HPLC)-based method [17,18]. However, this is the first study to demonstrate the measurement and validation of MDA in healthy and diseased GCF, including modifications in the assay described by the above-mentioned authors, as well as to optimize chromatographic conditions, eliminate interferents that could influence the results of the assay, and improve the method sensitivity.

Considering the importance of having a reliable and validated method to identify and quantify a specific product of the LPO process in human GCF and the possibility to use this measurement as a risk marker of disease, we describe here the validation of a method to quantitate MDA in this matrix using HPLC with photo diode array detection.

Materials and methods

The current study was approved by the ethics in human research committee of the Araraquara School of Dentistry at the Universidade Estadual Paulista (UNESP, Araraquara, São Paulo, Brazil; protocol No. 50/06).

Sample selection and assessment of periodontal disease

The clinical measurements were performed by a single calibrated examiner (kappa = 0.89) using a Williams periodontal probe PCPUNC15-6 (Hu-Friedy, Chicago, IL, USA). The periodontal status was evaluated by probing depth (distance from the gingival margin to the most apical penetration of the probe), clinical attachment level (distance from the cementoenamel junction to the most apical penetration of the probe), and bleeding on probing. All measurements were evaluated at six sites per tooth. In the second clinical session, the GCF was collected to avoid stimulation of the fluid or bleeding during the probing, which would interfere with the sample collection process.

The selected healthy sites had probing depth $\leqslant 3$ mm without clinical attachment loss and without bleeding on probing. The sites with periodontal disease had probing depth $\geqslant 4$ mm, clinical attachment loss $\geqslant 4$ mm, and bleeding on probing.

GCF collection

The GCF samples were collected with standardized paper strips (Periopaper, Oraflow, Smithtown, NY, USA), and the volume of GCF in each strip was measured with specific precalibrated equipment (Periotron 8000, Oraflow). After supragingival plaque was removed, the sites were isolated with cotton rolls to avoid saliva contamination and gently air-dried. GCF was collected by means of a paper strip inserted into each sulcus/pocket and left in place for 1 min. The GCF collection was performed in different nonadjacent teeth. Samples contaminated with blood or saliva were discarded.

Samples from four different healthy sites were collected and pooled in a microtube with 300 μl of phosphate-buffered solution (PBS), and the same procedure was performed in four different diseased sites. This procedure was repeated four times with new paper strips and the same methodology in the same sites with 5-min intervals. In total, 32 samples were collected, resulting in 16 samples from four healthy sites (4 samples at each site) and 16 samples from four diseased sites (4 samples at each site) of the same patient to avoid interindividual variability. Samples from the same site were pooled in the same PBS, resulting in 4 different healthy site solutions and 4 different diseased site solutions for MDA quantitation.

Samples were eluted for 40 min on ice and centrifuged at 3000*g* for 5 min. The supernatants were transferred to new microtubes and stored at −80 °C until the MDA analysis.

MDA assay

Chemicals and reagents

All chemicals used here were of the highest grade purity commercially available. Chromatography-grade methanol was obtained from Merck (Darmstadt, Germany). All other reagents used were acquired from Sigma–Aldrich (St. Louis, MO, USA). Water was purified in a Milli-Q system (Millipore, Billerica, MA, USA).

Chromatographic conditions

HPLC analyses were carried out with a Shimadzu system (Kyoto, Japan) equipped with two LC-20AT pumps, a photo diode array detector (PDA-20AV), an autoinjector (Proeminence SIL-20AC), and a column oven (CTO-10AS/VP) controlled by a CBM-20A communication module and LC-Solution software. The elution system was as follows. A Luna C18 (2) column (150 × 4.6 mm i.d., 5 μm,

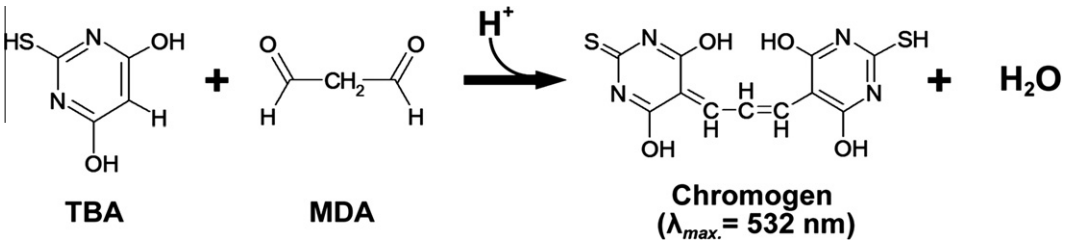

Fig.1. Scheme of the adduct MDA–$(TBA)_2$.

Phenomenex, Torrance, CA, USA) with a C18 SecurityGuard cartridge (4.0 × 3.0 mm (Phenomenex) was eluted in isocratic mode with a mobile phase consisting of 35% CH_3OH and 65% potassium phosphate buffer (50 mM, pH 7.0) filtered through a Millipore membrane filter (0.22 μm, Millipore) at a flow rate of 1 ml/min and 30 °C. The diode array detector (DAD) was set at 532 nm for detection of the adduct TBA–MDA–TBA obtained from the reaction.

Preparation of MDA stock solution

A solution was prepared with 22 μl of 1,1,3,3-tetramethoxypropane added to 10 ml of H_2SO_4 (1%), and it was left to stand at room temperature for 2 h in a place protected from light to generate MDA from acid hydrolysis of the standard. Then, 10 μl of the MDA stock solution were added to 3 ml of H_2SO_4 (1%). We determined the concentration of the MDA stock solution by reading the absorbance at 245 nm in the spectrophotometer (ε_{245nm} = 13700 M^{-1} cm^{-1}).

Calibration curves

MDA calibration curves were prepared with PBSs spiked with known concentrations of MDA. Increasing concentrations of MDA were used (0, 0.05, 0.1, 0.5, and 1.0 μM), and these curves were included in parallel with all samples. These solutions were prepared on the same day of the experiment.

Sample preparation

MDA levels were determined by the method of Hong and coworkers [36], with slight modification to measure the MDA in the GCF. The tubes containing 300 μl of the GCF in PBS were vortexed, and a volume of 250 μl of these solutions was added to 36 μl of 0.2% BHT (butylated hydroxytoluene) and 100 μl of H_3PO_4 (0.44 M). The tubes were vortexed and left to stand at room temperature for 10 min. The tubes used in this experiment had screws to avoid inadvertent opening during the incubation period. After this, 150 μl of a solution containing 0.6% TBA in H_3PO_4 (0.44 M) was added to all tubes, vortexed, and heated in a dry bath for 45 min at 90 °C. After the incubation period, the tubes were cooled at room temperature, and the extraction with 150 μl of *n*-butanol was carried out by vortex mixing for 1 min, followed by centrifugation at 3000*g* for 10 min. A volume of 100 μl of the butanol layer was transferred to a vial, and 40 μl was injected into the HPLC system.

Results

A typical calibration curve in the range of 0.05 to 1 μM is presented in Fig. 2. The DAD response was linear in the range measured. Method accuracy was determined by the addition of MDA standard to the PBS to achieve different samples within this concentration range. Samples were then processed in two different days as described above, and 40 μl of the butanol layer was injected into the HPLC system. Representative chromatograms are shown in Fig. 3. Excellent agreement was observed between added and detected concentrations of MDA (Table 1).

Method precision was determined by quadruplicate analysis of the same samples used for method accuracy determination. Intra- and interday coefficients of variation (CVs) were below 6.3 and 12.4%, respectively (Table 1). The limit of quantitation (LOQ, signal/noise = 5) was 0.03 μM.

The validated method was then applied to GCF in PBS. The periodontal clinical characteristics of the sites from which GCF was collected are presented in Table 2. In total, 4 healthy and 4 diseased samples from the same person were processed. As presented in Table 3 and Fig. 4, a significant increase in MDA concentration was observed in the periodontally diseased GCF when compared with healthy fluid samples ($P < 0.05$). Similar MDA concentrations were also observed for different sites within each group, showing the method precision when applied to real samples (Table 3).

Discussion

It has been postulated that the levels of oxidative markers are increased in periodontally diseased sites [16,33,37,38]. Furthermore, several studies have shown that increased levels of MDA in GCF significantly correlate with clinical parameters of periodontal disease [16–18]. Investigators have attempted to identify the role of oxidative stress products in the inflammation and destruction of the periodontium in periodontal disease. It has been reported that this destruction can be caused or enhanced by ROS and active proteases released during inflammatory and host immune responses to bacterial challenge [3,18,30,39].

However, it has not been well established whether the increased levels of LPO products in the periodontal region are the cause or a result of periodontitis. Some authors believe that LPO products in GCF can enhance the inflammatory reaction in periodontitis, but it is also possible that local inflammatory responses in the gingival tissue can generate LPO products [18,30]. Nevertheless, whatever the case, an appropriate measurement of LPO markers is essential to give accurate and reproducible results. In this study, we described a reliable and validated method to identify and quantify a specific product of LPO in human GCF by HPLC. By applying this method to real samples, we demonstrated that, in fact, sites with periodontal disease have increased MDA concentrations when compared with healthy sites.

For many years, the analysis of MDA has been widely employed for the assessment of LPO in biological systems [31,40]. The

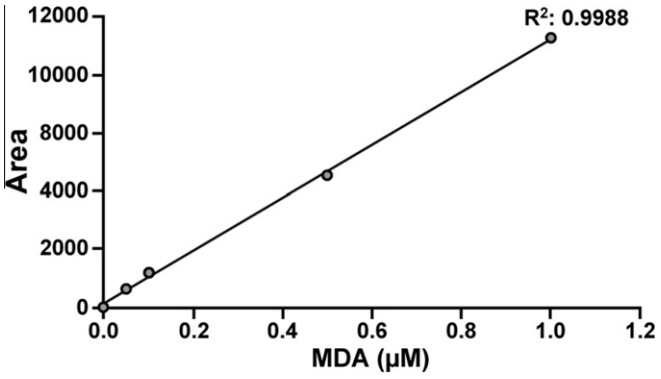

Fig.2. A typical calibration curve in the range of 0.05 to 1 μM with regression line $y = 18191x + 275.48$ ($r^2 = 0.9988$).

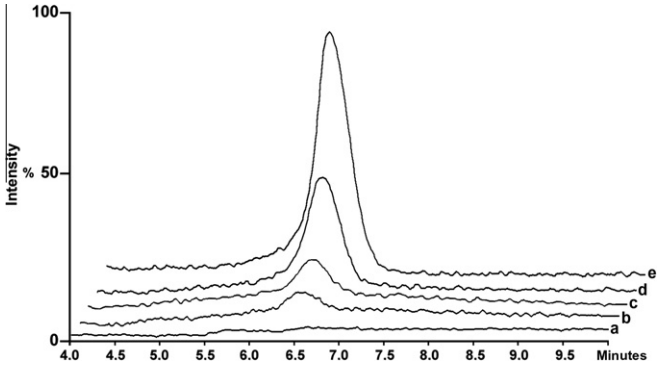

Fig.3. Typical sample chromatograms showing the elution of MDA–$(TBA)_2$ at 6 min in the concentration range tested: (a), blank; (b), 0.05 μM; (c), 0.1 μM; (d), 0.5 μM; (e), 1.0 μM.

Table 1
Precision and accuracy of method for MDA quantitation.

MDA added (μM)	MDA (day 1) detected (μM) ($n = 4$)	MDA (day 2) detected (μM) ($n = 4$)	Intraday precision (day 1) CV (%) ($n = 4$)	Interday precision CV (%) ($n = 8$)	Accuracy (day 1) (%) ($n = 4$)	Accuracy (day 2) (%) ($n = 4$)
0.05	0.0520 ± 0.0033	0.0568 ± 0.0089	6.3	12.4	104	114
0.1	0.1329 ± 0.0045	0.1178 ± 0.0111	3.4	9.0	133	118
0.5	0.4922 ± 0.0244	0.4853 ± 0.0253	5.0	4.8	98	97
1	1.0005 ± 0.0122	1.0052 ± 0.0264	1.2	1.9	100	101

Note. Samples consisted of PBS buffer contaminated with the indicated concentrations of MDA.

Table 2
Characteristics of sampled sites.

	Probing depth (mm)	Clinical attachment level (mm)	Bleeding on probing (% sites)
Healthy sites ($n = 16$)	2.5 ± 0.5	2.5 ± 0.5	0
Periodontal diseased sites ($n = 16$)	5.75 ± 0.5*	5.75 ± 0.5*	100*

* $P < 0.05$, Mann–Whitney test.

Table 3
MDA quantitation in GCF from healthy and diseased sites.

Sampled sites		MDA concentration (μM)	Mean MDA concentration (μM)	MDA (pmol/min) in each site	Intersample precision (CV, %)
GCF of healthy sites ($n = 4$)	1	0.0482		3.6141	
	2	0.0407	0.0415 ± 0.0082	3.0534	19.8
	3	0.0469		3.5193	
	4	0.0302		2.2655	
GCF of periodontal diseased sites	5	0.0873		6.5497	
($n = 4$	6	0.0863	0.0877 ± 0.0015*	6.4755	1.8
	7	0.0872		6.5373	
	8	0.0899		6.7439	

* $P < 0.05$, Mann–Whitney test.

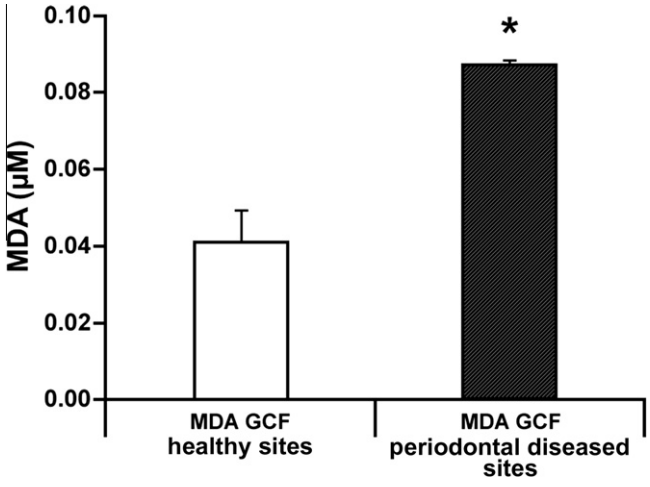

Fig.4. MDA concentrations in GCF collected from healthy and periodontally diseased sites. *$P < 0.05$, Mann–Whitney test.

measurement of TBARSs has been commonly used as an index of MDA generation and consists of a spectrophotometric or spectrofluorimetric assay of the products generated when the sample is heated under acidic conditions to form the adduct MDA–$(TBA)_2$. However, aldehydes other than MDA can react with TBA, and several other pigments may interfere, usually giving an overestimation of the MDA concentrations. For these reasons, the specificity of the TBARS assay has been open to question [35,36,41].

In this way, HPLC has been introduced as a means of improving the specificity and reliability of the measurement [42] because the authentic MDA–TBA_2 chromogen is efficiently separated from other chromogens [3,36,43–46]. Several HPLC methods have been validated for determination of MDA in plasma [36,41,46]. By these methods, it has been postulated that systemic conditions such as glomerular disease [41], male infertility [47], hyperlipidemia [44], and diabetes mellitus [48–50] correlate with increased MDA levels in plasma.

In evaluating LPO markers in patients with periodontitis, studies have demonstrated the measurements of these products in saliva [3,22,23], plasma [17,18], gingival tissue [24], and GCF [16–18]. Methods used to measure MDA in GCF are TBARS or colorimetric assays [16], and only two recent studies have evaluated LPO using HPLC-based methods [17,18]. However, the method described in the current study for MDA analysis consists of GCF collection, TBA reaction, and HPLC/PDA quantitation of the MDA–TBA adduct. The approach described here has not been used previously. Of the few studies that have detected MDA in GCF, none described the sensitivity and validation of the method or determined the quantitation of MDA in healthy sites.

In addition, the method was directed at GCF, making it distinct from other methods used for MDA measurement in other biological samples such as saliva and plasma. In this study, the samples were treated with BHT, which is a chain-breaking antioxidant added to suppress peroxidation during the assay and eliminate artifacts due to events taking place during the assay itself [44]. Furthermore, a sample extraction step with *n*-butanol before injection into the chromatographic system was added to improve the method. The extraction removed interference and extended the lifetime of the column by preventing contaminants from the incubation mixture to be injected [36,46].

The chromatographic conditions we used in the current study are very similar to those previously used for MDA analysis in

plasma [36]. However, the matrix used here for MDA determination is much less complex, consisting of a few microliters of GCF diluted in 300 μl of PBS buffer, which diminishes the possibility of interference. In addition, the chromatographic peak corresponding to MDA–$(TBA)_2$ in the samples presented the same retention time and absorbance spectrum of the MDA standard added to the buffer and subsequently reacted with TBA. To test for interference from the reagents, we processed blank samples without GCF in parallel with each set of analyses, and the peak due to MDA–$(TBA)_2$ was not present.

Furthermore, the reaction volume we used for MDA analysis in GCF was decreased approximately 4-fold, improving method sensitivity. For comparison, a 0.5-μM MDA standard processed by the method described here gave an average peak area of 9104, whereas the average peak area when the same standard was processed by the method described by Hong and coworkers [36] was 2069. Method sensitivity allowed for MDA quantitation with excellent accuracy and precision. The sensitivity improvement of the current method (LOQ = 0.03 μM) was necessary for MDA quantitation in concentrations as low as 0.04 to 0.05 μM. This was of fundamental importance for the quantitation of MDA in GCF from healthy sites, which has not been measured previously. The method used for GCF collection, with the improvement in sensitivity, allowed for the determination of MDA levels with high reproducibility within healthy or diseased sites.

GCF samples collected from 4 different healthy sites and 4 different diseased sites presented similar MDA concentrations within each group (healthy or diseased), demonstrating the precision of the method when applied to real GCF samples. Furthermore, a significant increase in MDA concentrations was observed in GCF from periodontally diseased sites compared with samples from healthy sites. These findings are in accordance with those of Tsai and coworkers [16], who also detected higher MDA levels in sites with periodontal disease in comparison with healthy sites. However, this is the first study to use HPLC to detect MDA in the GCF of healthy sites. This implies not only a reliable measurement of MDA, even in low concentrations in these biological samples, but also the possibility for use of this measurement as a risk marker of periodontal disease.

In this study, using an HPLC-based assay, an improved method for MDA quantitation in GCF was established with satisfactory sensitivity, precision, and accuracy. Such a reliable and validated method to measure MDA in human GCF, collected noninvasively, may be very useful in clarifying the role of LPO in the pathogenesis of periodontal disease.

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