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Lipid peroxidation: a possible role in the induction and progression of chronic periodontitis

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Objectives: Reactive oxygen species (ROS) are implicated in the destruction of the periodontium during inflammatory periodontal diseases. The imbalance in oxidant/antioxidant activity may be a key factor in the damaging effects of ROS. This study aimed to determine the lipid peroxidation levels in gingival crevicular fluid and saliva, and glutathione (GSH) and glutathione peroxidase (GPx) in saliva in patients with chronic periodontitis.

Methods: Gingival crevicular fluid and saliva were collected from 13 patients and 9 healthy control subjects during the preliminary study, and from 21 patients during the subsequent study. Lipid peroxidation level, GSH level and GPx activity were determined by spectrophotometric assay.

Results: The preliminary study found that when comparing patients to healthy controls, the gingival crevicular fluid samples produced the following results, respectively: higher lipid peroxidation concentration (μM) (by sites: 167.55 vs. 53.71, $p < 0.0001$; by subjects: 151.99 vs. 50.66, $p < 0.005$) and total amount (pmol) (by sites: 93.02 vs. 8.47, $p < 0.0001$, by subjects: 80.44 vs. 7.84, $p < 0.0005$). In saliva samples, lower GSH concentration (μM) (373.04 vs. 606.67, $p < 0.05$), higher lipid peroxidation concentration (μM) (0.66 vs. 0.13, $p < 0.0005$), and no difference in GPx activity were found in patients than in those of healthy controls. The subsequent study showed statistically significant ($p < 0.05$) improvement of clinical periodontal parameters (plaque index, gingival index, probing attachment level, probing pocket depth and gingival crevicular fluid volume), decreases in gingival crevicular fluid lipid peroxidation levels (concentration and total amount) at the sites after the completion of phase 1 periodontal treatment. Similarly, the periodontal treatment resulted in a significant decrease of lipid peroxidation concentrations ($p < 0.05$), increase in GSH concentration ($p < 0.001$), and no change in GPx activity in saliva samples.

Conclusion: The increased levels of lipid peroxidation may play a role in the inflammation and destruction of the periodontium in periodontitis.

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Human gingivitis and periodontitis are the results of complex interactions between pathogenic bacteria and the host's immunoinflammatory responses (1). The destruction of periodontal tissue is thought to be mainly due to host-derived mediators (2–4). Reactive oxygen species (ROS) have been implicated in the pathogenesis of many diseases, including rheumatoid arthritis (5), chronic obstructive pulmonary disease (6), AIDS (7), atherosclerosis (8) and more recently periodontal disease (9, 10). Different mechanisms, including DNA damage, lipid peroxi-

dation, protein damage, oxidation of important enzymes and stimulation of proinflammatory cytokine release, have been implicated for the cause of tissue damage by ROS (11–18). Polyunsaturated fatty acids are highly susceptible targets for the attack of ROS (19). Such reaction leads to lipid peroxidation, which can be measured by a specific, sensitive colorimetric assay, based on the measurement of malondialdehyde (MDA) and 4-hydroxyalkenals (4-HDA) (20–24). The antioxidants including glutathione (GSH) and glutathione peroxidase (GPx) are important to ROS detoxification (25–29). GPx is an important peroxidized product including lipid peroxidation degradation enzyme (30, 31). The imbalance between oxidative stress induced by ROS and the concentrations (or activity) of the antioxidant may result in tissue damage (30, 32).

The main purpose of this research was to investigate the lipid peroxidation levels in gingival crevicular fluid and whole saliva samples of chronic periodontitis patients.

Material and methods

Subject selection

Twenty-two subjects (15 males and seven females) in the preliminary study and 21 chronic periodontitis patients (13 males and eight females) in the subsequent study were selected from those being treated at the Department of Periodontology, Dental Clinic, Kaohsiung Medical University, Taiwan. The enrolment criteria were at least 20 teeth, no antibiotic usage within 3 months, and not a regular user of non-steroidal anti-inflammatory drugs. Additional exclusion criteria were pregnancy or lactation, diabetes, HIV infection, bleeding disorders, immunosuppressive chemotherapy, and any condition necessitating antibiotic premedication for dental appointment. This study was approved by the Institutional Review Board at Kaohsiung Medical University. Informed consent was obtained from all subjects. In addition to patients with periodontitis, nine subjects without evidence of periodontitis (healthy) were included in this study. Smokers were excluded from this study.

Gingival crevicular fluid collection and clinical parameters

Each clinical evaluation was preceded by collection of gingival crevicular fluid from the mesiobuccal surfaces of maxillary teeth to avoid saliva contamination. Briefly, teeth were air dried and isolated with cotton rolls, supragingival plaque was gently removed, and gingival crevicular fluid samples (132 sites in the preliminary study, 102 sites in the subsequent study) were collected with Periopaper® gingival crevicular fluid collection strips (Proflow, Amityville, NY, USA) for 30 s. Paper strips were measured for fluid volume with a calibrated Periotron® 8000 (Oraflow Inc., Plainview, NY, USA), then removed to separate microcentrifuge tubes containing 100 µl physiologic saline−0.1% Tween 20. The tubes were stored at −70°C until eluted. Following elution, each gingival crevicular fluid sample was analyzed separately.

Clinical measurement was performed immediately after the gingival crevicular fluid collection. Probing pocket depth is the distance in mm from the most coronal margin of the free gingiva to the most apical penetration of the probe. Probing attachment level is the distance in mm from the cementoenamel junction to the most apical penetration of the probe. Probing pocket depth and probing attachment level were measured using a Williams Periodontal probe (Hu-Friedy™) at 6 sites per tooth. The gingival index (33) and the plaque index (34) were recorded dichotomously during gingival crevicular fluid collection.

Values were then pooled to give a single mean value for each patient, and for diseased sites or healthy sites.

The sample sites were categorized according to Alpagot *et al*. (35). The diseased sites (including those with gingival recession and pseudo-pockets) had probing pocket depth ≥ 3 mm, probing attachment level ≥ 2 mm and gingival index ≥ 1. The healthy sites had probing pocket depth ≤ 3 mm, probing attachment level ≤ 1 mm and gingival index = 0, were not bleeding on probing and did not show any signs of bone loss. A diagnosis of patients with periodontal disease was based upon the presence of at least one site with the criteria of diseased sites. The healthy persons were required to have all sites with the criteria of healthy sites.

Collection of whole saliva

Unstimulated whole saliva samples were collected by expectoration (36). Subjects had no food intake, drink or oral rinsing 2 h prior to sample collection. The collected saliva samples were centrifuged (4000 $g \times 5$ min). The supernatants were stored at −70°C until further analysis.

Analysis of glutathione, glutathione peroxidase and lipid peroxidation

In the preliminary study GSH, GPx and lipid peroxidation were determined by spectrophotometric assay using commercial kits (Calbiochem®, San Diego CA, USA). For the subsequent study, GSH and lipid peroxidation assay kits were purchased from BIOXYTECH® (OxisResearch™, Portland, OR, USA), GPx assay kits were from Calbiochem. The total GSH (glutathione) assay is based on the reaction of GSH with a chromogenic reagent R1 (12 mM solution of chromogenic reagent in 0.2 N HCl) and metaphosphoric acid (R2 reagent) at 25°C. The final absorbance was measured at 400 nm. GPx activity was measured by the reaction of GPx in the assay buffer (Tris-EDTA) and NADPH reagent (GSH + NADPH + GSH reductase) with tert-butyl hydroperoxide (70% aqueous solution). The absorbance at 340 nm was recorded every 30 s for 30 min. The measurement of MDA and 4-HAD is used as an index of lipid peroxidation. The lipid peroxidation assay is based on the reaction of a chromogenic reagent R1 (*N*-methyl-2-phenylindole in acetonitrile) with MDA and 4-HAD at 45°C. The stable chromophore with maximal absorbance at 586 nm was recorded.

Statistics

All data analysis was done in JMP and SAS (SAS Institute, Cary, NC, USA). Data are presented as means and standard deviations (SD). Significance of mean differences between healthy and diseased subjects (sites) were tested by (paired) *t*-test, assigning significance for two-tail $p < 0.05$. Relationships among mean clinical periodontal parameters and gingival crevicular fluid lipid peroxidation were tested by analysis of variance (ANOVA) followed by a *post hoc* comparison using Tukey–Kramer paired *t*-test. The associations between the levels of various gingival crevicular fluid/saliva substances and clinical parameters were calculated using Pearson's correlation and expressed by Pearson's correlation coefficient.

Results

Clinical parameters and lipid peroxidation level in gingival crevicular fluid

Small amount of GSH in limited volumes of gingival crevicular fluid, and the elution of gingival crevicular fluid from paper strips with physiologic saline–0.1% Tween 20 made it impossible for us to analyze the GSH and GPx in gingival crevicular fluid. Clinical parameters and gingival crevicular fluid lipid peroxidation level of the individual sites in the preliminary study (mean ± SD) are provided in Table 1. Significant differences ($p < 0.0001$) in the concentration (μM) and total amount (pmol/site) of lipid peroxidation and gingival crevicular fluid volume (μl) between diseased and healthy sites were observed. The total amount of gingival crevicular fluid lipid peroxidation was positively correlated with the four clinical parameters ($p < 0.0001$); however, lipid peroxidation concentration was only correlated with gingival index, probing pocket depth and probing attachment level (Table 2).

When comparing the mean total patient gingival crevicular fluid lipid peroxidation levels (Table 3), we found a significant difference in the concentration and the total amount of lipid peroxidation and gingival crevicular fluid volume between periodontally diseased and healthy subjects ($p < 0.0001$).

Table 1. Characteristics of sites selected to participate in preliminary study sites

	n	Mean ± SD
Diseased sites	66	
PLI		1.71 ± 0.76
GI		1.44 ± 0.53
PD		5.39 ± 1.61
PAL		5.59 ± 1.78
LPO (μM)[(1)]		167.55 ± 150.86[a]
LPO (pmol/site)[(2)]		93.02 ± 81.71[b]
GCF (μl)[(3)]		0.62 ± 0.28[c]
Healthy sites	66	
LPO (μM)[(1)]		53.71 ± 92.70[a]
LPO (pmol/site)[(2)]		8.47 ± 15.90[b]
GCF (μl)[(3)]		0.13 ± 0.08[c]

[(1)]LPO (μM): concentration of LPO.
[(2)]LPO (pmol/site): total amount of LPO.
[(3)]GCF (μl): GCF volume.
[a–c]Significantly different from diseased and healthy sites ($p < 0.0001$).
PLI, plaque index; GI, gingival index; PD, probing pocket depth; PAL, probing attachment level; LPO, lipid peroxidation; GCF, gingival crevicular fluid.

Table 2. Person's correlation coefficients between clinical parameters and various substances in gingival crevicular fluid of preliminary study sites

	LPO concentration $n = 132$	LPO total amount $n = 132$
PLI	0.0537	0.3271*
GI	0.3099*	0.5487*
PD	0.2728*	0.4984*
PAL	0.2457*	0.4812*

*Significant correlation ($p < 0.0001$).
LPO, lipid peroxidation; PLI, plaque index; GI, gingival index; PD, probing pocket depth; PAL, probing attachment level.

Table 3. Summary statistics for lipid peroxidation level in gingival crevicular fluid of preliminary study subjects

	n	Mean ± SD
Diseased subjects	13	
LPO (μM)		151.99 ± 77.65[a]
LPO (pmol/site)		80.44 ± 53.32[b]
GCF (μl)		0.55 ± 0.22[c]
Healthy subjects	9	
LPO (μM)		50.66 ± 37.22[a]
LPO (pmol/site)		7.84 ± 6.65[b]
GCF (μl)		0.12 ± 0.03[c]

[a]Significantly different from diseased and healthy subjects ($p < 0.005$).
[b]Significantly different from diseased and healthy subjects ($p < 0.0005$).
[c]Significantly different from diseased and healthy subjects ($p < 0.0001$).
LPO, lipid peroxidation; GCF, gingival crevicular fluid.

Table 4. Summary statistics for the glutathione, lipid peroxidation concentrations and the glutathione peroxidase activity in whole saliva of preliminary study subjects

	n	Mean ± SD
Diseased subjects	13	
GSH (μM)		373.04 ± 287.42[a]
GPx (mU/ml)		92.99 ± 74.40
LPO (μM)		0.66 ± 0.36[b]
Healthy subjects	9	
GSH (μM)		606.67 ± 191.02[a]
GPx (mU/ml)		92.90 ± 58.58
LPO (μM)		0.13 ± 0.08[b]

[a]Significantly different from diseased and healthy subjects ($p < 0.05$).
[b]Significantly different from diseased and healthy subjects ($p < 0.0005$).
GSH, glutathione; GPx, glutathione peroxidase; LPO, lipid peroxidation.

Glutathione, glutathione peroxidase and lipid peroxidation in whole saliva

The mean saliva GSH, GPx and lipid peroxidation values are shown in Table 4. Significant difference in the concentration of GSH between diseased and healthy subjects was noted ($p < 0.05$). The difference in the concentration of lipid peroxidation between the diseased and healthy subjects was also statistically significant ($p < 0.0005$). Non-significant difference in saliva GPx activities between periodontally diseased and healthy subjects was found ($p > 0.05$).

Correlation between gingival crevicular fluid lipid peroxidation and saliva lipid peroxidation

The concentration of lipid peroxidation in gingival crevicular fluid correlated with the saliva lipid peroxidation concentration (Fig. 1). The saliva lipid peroxidation concentration is

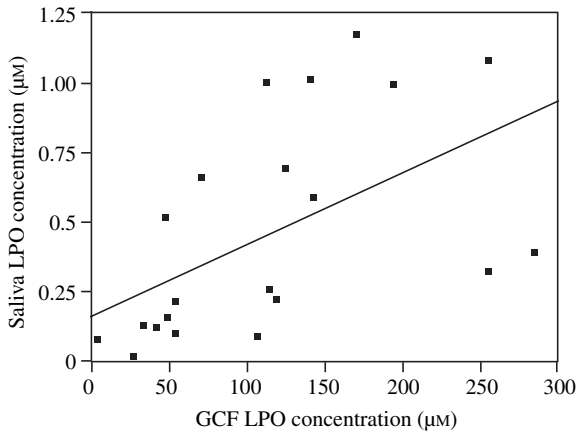

Fig. 1. Relationship in between levels of gingival crevicular fluid lipid peroxidation and saliva lipid peroxidation of 22 subjects in preliminary study. The regression line in saliva LPO concentration = 0.1588471 + 0.0025673 × GCF LPO concentration; $r = 0.6378$, $p < 0.005$. GCF, gingival crevicular fluid; LPO, lipid peroxidation.

correlated with the gingival crevicular fluid lipid peroxidation total amount ($r = 0.6378$, $p < 0.005$).

The effect of phase 1 periodontal treatment on gingival crevicular fluid lipid peroxidation levels and saliva glutathione, glutathione peroxidase and lipid peroxidation levels

Gingival crevicular fluid and saliva samples were collected from 21 patients with chronic periodontitis prior to and 1 month after the completion of phase 1 periodontal treatment. A total of 102 sites with gingival index > 0, probing pocket depth > 3 mm and probing attachment level > 3 mm were subjected to scaling and root planing. A significant correlation was found between probing attachment level and lipid peroxidation concentration ($p < 0.05$) in the untreated sites (data not shown). A significant difference in the concentration ($p < 0.005$) and total amount ($p < 0.0001$) of lipid peroxidation between periodontally diseased sites of prior to and after treatment was found (Table 5). However, no correlation ($p > 0.05$) between clinical parameters and lipid peroxidation levels was found in treated sites (data not shown).

The mean saliva concentrations of GSH, GPx and lipid peroxidation are shown in Table 6. No significant correlation among saliva GSH, GPx and lipid peroxidation levels in the untreated patients was found (data not shown). Significant differences were noted in the concentrations of saliva GSH and lipid peroxidation between prior to and after periodontal treatment ($p < 0.001$ and $p < 0.05$, respectively). Non-significant differences in patients' saliva GPx activities between prior to and after treatment were noted ($p > 0.05$). A negative correlation ($p < 0.05$) between saliva GSH concentration and lipid peroxidation concentration was noted in the patients after treatment (Fig. 2).

Discussion

Several studies indicated that there are increases of nitric oxide synthesis (37), superoxide anion levels and myeloperoxidase activity (38) in the inflamed periodontium. ROS play important roles in physiological and immunoinflammatory reactions. In the human body, there is an antioxidant mechanism to maintain the balance of oxidation–reduction (9). The breakdown of this balance (i.e. increased ROS) could lead to increased damage directly by ROS. Indeed, several diseases have been correlated to an imbalance of oxidation–reduction or oxidative stress (6, 8, 39, 40). There are two possible causes for the imbalance of oxidative stress: increased ROS with no concomitant or less increased antioxidant, or decreased antioxidant with no marked change of ROS. Enhanced superoxide anion production with no change of the antioxidant activity in gingival fluid of patients with chronic adult periodontitis, presumably due to bacterial stimuli, has been reported (41, 42). More recently, Brock *et al.* (43, 44) reported that antioxidant defense is reduced, and non-surgical therapy with improvements in clinical parameters can increase the antioxidant defense in chronic periodontitis patients. Similar results were reported in the present study. Therefore, it is suggested that periodontal disease patients are more susceptible to an imbalance of antioxidant–oxidative stress situations. However, ROS has a very short life, so it is not easy to detect the presence of ROS. The ROS-related tissue destruction could be measured by the final

Table 5. Summary statistics for the clinical parameters and lipid peroxidation in gingival crevicular fluid of subsequent study sites

	Plaque index	Gingival index	Probing attachment level	Probing pocket depth	Gingival crevicular fluid volume (μl)	Lipid peroxidation (μM) (concentration)	Lipid peroxidation (pmol) (total amount)
Untreated sites ($n = 102$)	1.64 (0.69)	1.96 (0.54)	5.54 (1.68)	4.92 (1.48)	0.91 (0.50)	78.08 (116.84)	43.17 (47.52)
Treated sites ($n = 102$)	0.60 (0.72)	0.76 (0.83)	4.44 (1.93)	2.87 (1.37)	0.45 (0.40)	43.36 (65.29)	12.68 (18.23)
p-value	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.005	< 0.0001

Table 6. Summary statistics for the glutathione, lipid peroxidation concentration and the glutathione peroxidase activity in whole saliva of subsequent study patients

	GSH (μM)	GPx (U/ml)	LPO (μM)
Untreated patient	353.59 (141.93)	96.50 (35.14)	0.63 (0.49)
Treated patient	602.92 (170.15)	99.34 (45.72)	0.41 (0.26)
p-value	< 0.001	< 0.05	< 0.05

GSH, glutathione; GPx, glutathione peroxidase; LPO, lipid peroxidation.

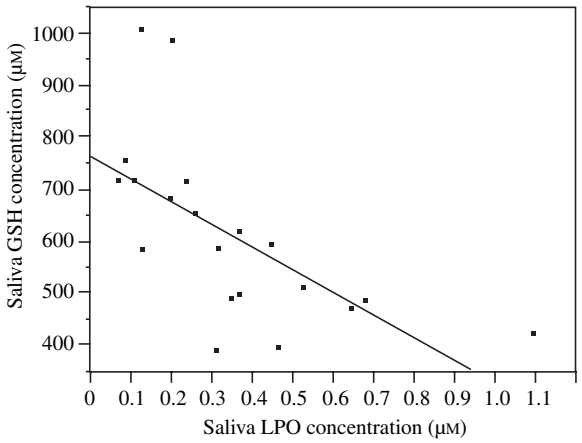

Fig. 2. Relationship between saliva glutathione and saliva lipid peroxidation of 21 patients after periodontal treatment. Each point represents a single subject. The regression line is salivary GSH concentration = 762.99243 − 434.29601 LPO concentration, $p < 0.05$. GSH, glutathione; LPO, lipid peroxidation.

product of lipid peroxidation, such as the 4-hydroxyalkenal (4-HDA) and malondialdehyde (MDA).

MDA has important pathophysiological effects (20, 24). 4-HDA can inactivate enzymes including glucose-6-phosphate dehydrogenase, glutathione *S*-transferase and glutathione reductase, can denature protein, inhibit protein synthesis, damage DNA, cause apoptosis and loss of cell replication (22, 23, 45). 4-HDA has been associated with atherosclerosis, cancer, Alzheimer syndrome, Parkinson disease and aging (45–50). Marton *et al*. (51) reported that the MDA contents of chronic apical periodontitis tissues are higher than in healthy tissue of the same individuals. The studies of Sobaniec and Sobaniec-Lotowska (52) indicated that periodontitis rats have higher blood lipid peroxidation concentrations than periodontally healthy ones. Lipid peroxidation caused by oxygen radicals from *Fusobacterium*-stimulated neutrophils has been suggested as a possible model for the emergence of periodontitis (53). These experiments investigated the cellular or blood MDA and 4-HDA levels to understand the situations of lipid peroxidation. Our present study analyzed the gingival crevicular fluid lipid peroxidation content (MDA + 4-HDA). We reported that the lipid peroxidation concentration is higher in diseased sites (167.55 ± 150.86 μM) than in healthy sites (53.71 ± 92.70 μM) ($p < 0.005$). The lipid peroxidation total amount in diseased sites (93.02 ± 81.71 pmol) is also higher than in healthy sites (8.47 ± 15.90 pmol) ($p < 0.0005$). Our results were in agreement with others and indicated that lipid peroxidation concentration is markedly increased in periodontal inflammation. In other words, the balance between oxidative stress and antioxidant level was perturbed, and more cells and tissues were under attack by ROS in inflammatory periodontal diseases. The lipid peroxidation concentration and total amount were significantly correlated with the clinical parameters ($p < 0.005$), indicating that the more severe the inflammation of the periodontal tissue the higher the level of lipid peroxidation. The increased gingival crevicular fluid lipid peroxidation levels not only reflect the increased ROS damage to the periodontal tissue, but also periodontal inflammation. It is possible, as suggested by Sheikhi *et al*. (53), that the increased gingival crevicular fluid lipid peroxidation concentration and total amount could occur through the mechanism of superoxide anion production during the interaction of periodontopathogens or their products and neutrophils within periodontal tissues or pockets. In addition, the periodontitis patients had significantly higher gingival crevicular fluid lipid peroxidation concentration and total amount than healthy controls (Table 3, $p < 0.005$). The whole saliva of periodontitis subjects also had significantly higher mean lipid peroxidation concentration than healthy ones (Table 4, $p < 0.0005$). This could be associated with increased percentage of gingival crevicular fluid in saliva in periodontitis (54) and suggests that saliva lipid peroxidation level might be used as an indicator of periodontal damage by ROS. Glutathione (GSH) and glutathione peroxidase (GPx) are two important antioxidants, but little information relevant to periodontal disease is available. The small amount of GSH in limited volumes of gingival crevicular fluid made it impossible for us to analyze the GSH in gingival crevicular fluid. We investigated the saliva and found that GSH concentration of healthy subjects is higher than in periodontitis patients (Tables 4, $p < 0.05$). Previous studies have shown that saliva GSH of asthmatic patients is significantly lower than those of healthy individuals (55). The blood GSH level of arthritis patients was about 50% of healthy controls (56). This data suggests that a large amount of GSH was consumed during the ROS generation leading to a deficiency of antioxidant.

In addition, many bacteria in the oral cavity and periodontal pockets

could also consume GSH. Carlsson *et al.* (57) reported that *Peptostreptococcus micros* uses GSH and produces H_2S. Chu *et al.* (58) indicated that *Treponema denticola* can metabolize GSH, which is also necessary for virulence expression. The periodontitis patients had higher plaque indices and higher percentages of subgingival periodontopathogens than healthy controls (59). It is conceivable that many microbiota metabolized GSH leading to lower levels of saliva and/or gingival crevicular fluid GSH.

Because of the increased level of ROS, GSH was continuously consumed and became greatly insufficient. The reduction of glutathione disulfide (GSSG) to become GSH, and the speed of synthesis of GSH from amino acids could not match the consumption of GSH. The subsequent increase in ROS could cause periodontal tissue damage. This is evident by the higher level of lipid peroxidation in the inflamed sites. In addition, the change of oxidation-reduction condition could activate nuclear factor-kB. Nuclear factor-kB triggers the production and activation of proinflammatory mediators, i.e. intercellular adhesion molecules and cytokines (60, 61). As our present and other studies (42, 43, 62) indicate an imbalance of ROS and antioxidants, the administration of antioxidant as an adjunct therapy for periodontitis warrants further study.

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