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Parameters of oxidative stress in saliva from diabetic and parenteral drug addict patients

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BACKGROUND: Oxidative stress constitutes the basis for many diseases and it may account for the severity of systemic and oral disease complications. The aim of this study was to assess whether saliva may be used to detect the body's oxidative stress level.

METHODS: Oxidative stress was determined in saliva from 14 diabetic patients and 10 heroin addicts; two different pathologic conditions related to free radical damage, and 21 healthy control subjects were included in the study. Glutathione peroxidase (GPx) and reductase (GRd) activities, and glutathione (GSH) and glutathione disulfide (GSSG) levels were analyzed in the saliva of all individuals. Other variables including salivary volume and the oral status were also analyzed.

RESULTS: Diabetic patients had GPx and GRd activities of 39.98 ± 1.61 and 6.19 ± 0.61 nmol/min/mg prot, respectively. These values were significantly higher ($P < 0.001$) than those obtained in control saliva (27.51 ± 0.86 and 3.44 ± 0.25 nmol/min/mg prot, respectively). Drug addicts showed significantly ($P < 0.001$) lower salivary GPx and GRd activities than controls. Both group of patients had significantly lower levels of GSH and higher of GSSG than controls ($P < 0.001$).

CONCLUSIONS: Changes in the antioxidant enzymes and glutathione levels in saliva from two different pathologic situations as those here studied suggest that this biologic fluid may be suitable for determining the prognosis and evolution of these diseases and its oral manifestations.

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Keywords: diabetes; heroin addicts; oxidative stress; prognosis; saliva

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Introduction

Free radicals, including reactive oxygen (ROS) and reactive nitrogen (RNS) species exist in normal cells at low but measurable concentrations (1). Their levels depend on the balance between their rates of production and their rates of clearance by the endogenous antioxidant systems, including superoxide dismutase (SOD), catalase, the glutathione redox cycling enzymes, glutathione peroxidase (GPx) and reductase (GRd), and glutathione itself. Under physiologic conditions, these systems tend to maintain a stable state called redox homeostasis (1, 2). When ROS/RNS production is increased in such form that the antioxidative response is unable to reset the system, the result is an oxidative stress status.

It is widely accepted that free radicals plays an important role in the onset and progression of many diseases (1, 2). Hyperglycemia is a hallmark of both non-insulin-dependent (DM-II) and insulin-dependent (DM-I) diabetes mellitus, and it is associated with increased production of ROS by several different mechanisms (3–8), leading to pro-oxidative shift of the glutathione redox state in the blood (9). High levels of free radicals and the simultaneous decline of antioxidant defense mechanisms can determine the development of the complications in the diabetes mellitus including worsening the insulin resistance (10–16). Both increased and reduced activities of GPx and GRd, one of the most important antioxidant defense systems in the body, were reported either increased or reduced in different tissues of diabetic animals (17–20). Several reports have shown a higher GSSG/GSH ratio in different fluids and tissues of diabetic patients compared with healthy subjects (21–25). Diabetic patients also present xerostomy and other oral manifestations derived from qualitative changes in salivary composition (26–28). Elevated GPx activity was reported in the parotid glands of diabetic rats, whereas reduced activity of the enzyme was found in submandibular glands (29).

In addition to diabetes, oxidative stress can also have important diagnotic and prognotic value in other situations or diseases such as in parenteral drug addiction. Most of the phenomena occurring in drug addicts, such as periodontal disease, abscesses, arthritis, other rheumatologic diseases and immune disorders are related to oxidative stress (30, 31). There are some indirect evidences showing the relationship between heroin addiction and free radical production. In heroin abusers, blood levels of lipoperoxides and nitric oxide are significantly increased (32). A single heroin administration increased both dopamine- and xanthine-dependent oxidative metabolism with further ROS production (33). In addition, heroin can also be metabolized to free radicals (34). On the other hand, a single morphine administration decreased endogenous intracellular GSH in brain and peripheral organs of rodents and in cerebrospinal fluid of patients (35).

Several independent strategies that ameliorate ROS production may prevent some of the secondary complications of diseases, including oral manifestations such as periodontal disease. Treatment with antioxidants ameliorates some of these complications in diseases such as diabetes (36–42). Thus, it would be of great interest to know the degree of the oxidative stress present in these situations to evaluate the efficacy of the antioxidant therapy. The determination of the oxidative stress requires sometimes invasive techniques such as blood samples. Exploring saliva for oxidative stress markers that accurately reflect the redox status of the body may have great clinical interest.

Accordingly, we considered it worthwhile to examine the salivary content of GPx and GRd activities, and GSSG/GSH ratio in two different types of diseases involving oxidative stress, such as diabetes and heroin addiction, looking for markers that accurately reflect the severity of the oxidative stress and permit to follow the disease's evolution.

Material and methods

Materials

Oxidized (GSSG) and reduced (GSH) glutathione, NADPH, GRd, cumene hydroperoxide, ophthalaldehyde, *N*-ethylmaleimide, and methanol were purchased from Sigma-Aldrich (Madrid, Spain). All other reagents were of highest purity available.

Study populations

The study was carried out in the School of Dentistry (Granada, Spain). A total of 21 male patients (11 patients with type II diabetes mellitus and 10 heroin addicts) from the Health Center of Pinos Puente (Granada, Spain) were included in the study. The protocol was approved by the University's Ethical Committee, which strictly adheres to the Code of Ethics as developed by the World Medical Association. The subjects were informed and consent to participate in the study was obtained from them.

All diabetic patients were insulin-dependent by the nature of their disease process. Their age (from 42 to 55 years), duration of diabetes mellitus, sex, average glucose level, and history of smoking were obtained from their medical records and personal interviews. Most of them were obese patients with associated complications (neuropathies, hypertension, cerebrovascular accidents, arrhythmias, and myocardial infarction), and under pharmacologic treatment for these complications in addition to insulin and hypoglycemic agents-like glybenclamide and metformin (Table 1).

Table 1 Main characteristics of the subjects included in the study

	Controls	*Diabetics*	*Heroin addicts*
Male	18	11	10
Age (years)	35 ± 2	49 ± 1.8	39 ± 2.3
Duration (years)	–	15 ± 2.3	11.1 ± 2.3
Smokers	2/18	4/11	10/10
CPI	0–1	2–3	3
Main treatment	–	Gybenclamide (4) Simvastatina/ Lovastatina (5) Amlodipine (3) Alprazolam (2) Atenolol (5) Metformin (3) NSAIDS (7)	Methadone (10) Rifampicine (2) Alprazolam (4) NSAIDS (5) Zolpidem (2) Famciclovir (2) Azitromicin (3)

The data are expressed as the mean ± SEM.
CPI, Community Periodontal Index; NSAID, non-steroidal anti-inflammatory drug.

Drug addict patients were heroin addicts from 6 to 20 years ago and they were 39 ± 2.3 years old. Seven of them had infection by Human Immunodeficiency Virus (HIV) with an average of 11 years of evolution and complications as pulmonary tuberculosis, pneumonia, hepatitis C. All the heroin addicts were under treatment with methadone and anxiolytics (Table 1). Eighteen male healthy individuals, aged 35 ± 2 years, free of medications and oral or systemic illness in the past 3 months, served as controls. Medical history of each participant was noted, and they were also under a dental examination according to the simplified WHO criteria, containing periodontal status following the Community Periodontal Index (CPI). For each patient, an oral cavity examination was performed and clinical data were registered by the same dentist.

Saliva collection

Participants (patients and controls) included in the study were under the same protocol. They come to the School of Dentistry of the University of Granada at 09:00 hours after 12-h overnight fast. After 15 min of rest, a sample of saliva was obtained from each individual. In order to stimulate saliva production, the participants chewed a piece of paraffin wax for 7 min. Saliva produced during the first 2 min was discarded. Then, saliva was collected during the following 5 min, after avoiding any possible contamination. The patients chewed the paraffin during the time of saliva collection. Samples of collected saliva were centrifuged at 3000 *g*, 4°C for 15 min, and the clear supernatant was frozen at −80°C until the assays were performed.

Determination of GPx and GRd activities

For GPx activity determination, 120 µl of saliva was incubated in a final volume of 3 ml with 100 mM phosphate buffer containing 1 mM EDTA-Na_2, pH 7.5, in the presence of 30 µl 0f 20 mM NADPH, 100 µl of 60 mM GSH, and 4 µl (1 IU) GRd for 5 min at room temperature. Then 100 µl of 36 mM cumene hydroperoxide solution was added and GPx activity was measured following the oxidation of NADPH for 3 min at 340 nm in a spectrophotometer (Shimadzu Deutschland GmbH, Duisburg, Germany; 43). GRd activity was measured in 35 µl saliva sample incubated in a final volume of 508.5 µl with 100 mM phosphate-EDTA-Na_2 buffer, pH 7.5, containing 2.5 mM GSSG for 5 min at room temperature. Then, 8.5 µl NADPH 12 mM was added and NADPH oxidation was followed for 3 min at 340 nm in an UV-spectrophotometer (Shimadzu Deutschland GmbH). In both cases, non-enzymatic NADPH oxidation was subtracted from overall rate. The activity of both enzymes is expressed in nmol/min/mg protein.

Determination of GH and GSSG

Both GSH and GSSG were measured by a fluorometric method (44) slightly modified by us. For GSH measurement, 10 µl saliva aliquots were incubated with 10 µl of an ethanol ophthalaldehyde solution (1 mg/ml) and 180 µl phosphate buffer (100 mM sodium phosphate, 2.5 mM EDTA-Na_2, pH 8.0) during 15 min at room temperature. Then, the fluorescence of the samples was measured in a plate-reader spectrofluorometer (Bio-Tek Instruments, Inc., Winooski, VT, USA). A standard curve of known GSH concentrations was prepared and processed with the samples.

For GSSG concentration measurement, 25 µl aliquots of saliva was pre-incubated with 10 µl *N*-ethylmaleimide solution (5 mg/ml in distilled water) for 40 min at room temperature, and then alkalinized with OHNa 0.1 N. Aliquots of 10 µl was incubated with 10 µl ophthalaldehyde solution and 180 µl OHNa 0.1 N for 15 min at room temperature. The fluorescence was then measured and the GSSG concentrations were calculated according to a standard curve prepared accordingly. The levels of GSH and GSSG are expressed in nmol/mg prot.

Statistical analysis

All data are expressed as mean ± SEM. A one-way ANOVA followed by Bonferroni *t*-test was used to compare the differences between groups (Table 2). A *P*-value of <0.05 was considered to be statistically significant.

Results

Figure 1 shows the activities of GPx and GRd in saliva of the patients. Diabetic patients show a significant increase in GPx (39.98 ± 1.61 nmol/min/mg prot) and GRd (6.19 ± 0.61 nmol/min/mg prot) activities whereas drug addicts show that these reduced (GPx, 17.58 ± 1.22; GRd, 1.93 ± 0.18 nmol/min/mg prot) compared with healthy subjects (GPx, 27.51 ± 1.22; GRd, 3.44 ± 0.25 nmol/min/mg prot, $P < 0.001$). The activity of these enzymes was significantly lower in heroin addicts than in diabetics ($P < 0.001$).

Figure 2 shows the levels of total glutathione, GSH, GSSG, and GSSG/GSH ratio in the saliva of the studied groups. Although diabetic (84.43 ± 3.12 nmol/min/mg prot) and drug addict (89.55 ± 3.74 nmol/min/mg prot) patients have similar levels of total glutathione than

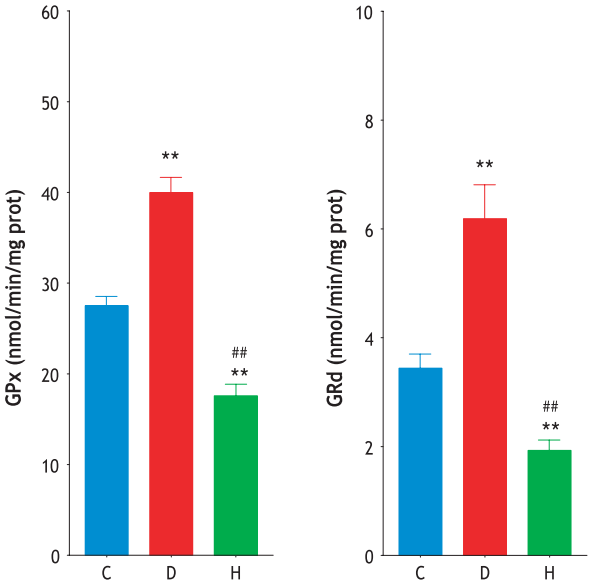

Figure 1 Glutathione peroxidase (GPx, left) and reductase (GRd, right) activities in saliva from healthy controls, diabetic patients, and heroin addicts. $^{**}P < 0.001$ vs. control; $^{\#\#}P < 0.001$ vs. diabetic group.

Table 2 Significant differences between controls, diabetic, and drug addict patients

Variable	*ANOVA*	*Bonferroni t-test*
Glutathione peroxidase (GPx)	$F = 31.22$, $P < 0.001$	Controls vs. diabetic and heroin group: $P < 0.001$ Diabetic vs. heroin group: $P < 0.001$
Glutathione reductase (GRd)	$F = 25.59$, $P < 0.001$	Controls vs. diabetic and heroin group: $P < 0.001$ Diabetic vs. heroin group: $P < 0.001$
Reduced glutathione (GSH)	$F = 63.77$, $P < 0.001$	Controls vs. diabetic and heroin group: $P < 0.001$ Diabetic vs. heroin group: $P < 0.05$
Oxidized glutathione (GSSG)	$F = 145.13$, $P < 0.001$	Controls vs. diabetic and heroin group: $P < 0.001$
Ratio GSSG/GSH	$F = 226.41$, $P < 0.001$	Controls vs. diabetic and heroin group: $P < 0.001$

The data are expressed as the mean ± SEM.

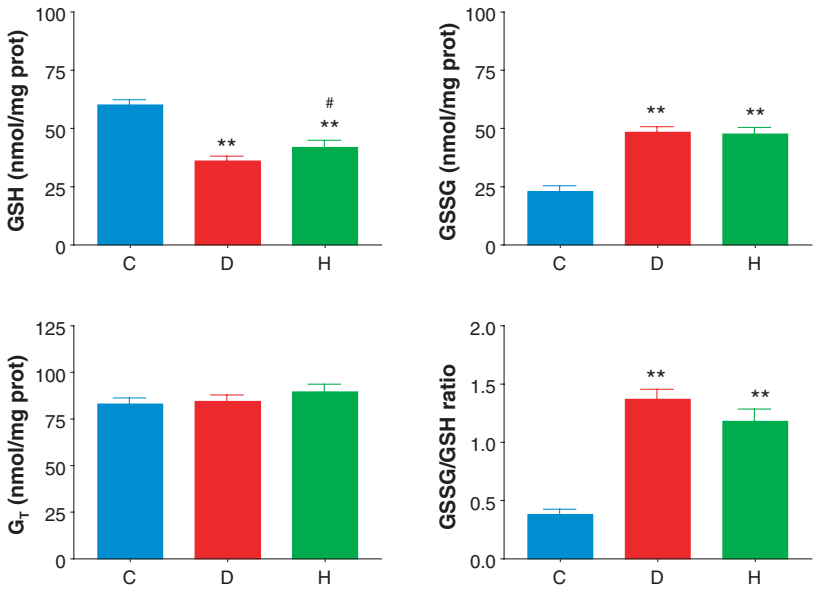

Figure 2 Glutathione (GSH, top left), disulfide glutathione (GSSG, top right), total glutathione (G_T, bottom left), and GSSG/GSH ratio (bottom right) in saliva from healthy controls, diabetic patients and heroin addicts. $^{**}P < 0.001$ vs. control; $^{\#}P < 0.05$ vs. diabetic group.

controls (82.96 ± 2.93 nmol/min/mg prot), the two groups of patients showed significant lower GSH levels than control group (diabetics, 36.04 ± 1.82; addicts, 41.89 ± 2.75; controls, 60.12 ± 1.89 nmol/min/mg prot, $P < 0.001$). Comparing between groups of patients, diabetics have lower GSH levels than heroin addicts ($P < 0.05$). GSSG levels show a similar increase in both groups of patients (diabetics, 48.38 ± 2.1; addicts, 47.65 ± 2.55; controls, 22.85 ± 2.3 nmol/min/mg prot, $P < 0.001$), and thus, GSSG/GSH ratio increases in these groups (diabetics, 1.37 ± 0.08; addicts, 1.18 ± 0.1; controls, 0.38 ± 0.04, $P < 0.001$) without differences between diabetic and addict groups.

The collected volume of saliva was similar in the two groups of patients (diabetics, 3.56 ± 0.57 mL, and heroin addicts, 3.55 ± 0.45 mL), and it was not significantly different from the control value (4.24 ± 0.44 mL). Our results did not show a correlation between the degree of salivary oxidative stress and the CPI, perhaps because most of the patients from both groups had a similar periodontal status (CPI among 2–3, data not shown).

Discussion

Increasing evidence over the last years confirms that the oxidative stress-dependent tissue injury may be involved in many diseases in humans (1, 2). The pathogenesis of these diseases, including diabetes and drug addiction, could be clarified with the use of biomarkers of oxidative stress in different body fluids. In accordance with the previous studies in other tissues or biologic fluids (8, 17, 31), the data of the present work support the existence of an unbalanced redox status in saliva of diabetic and drug addict patients. Our study is consistent with the observation that GSSG levels and GSSG/GSH ratio are higher in these patients than in healthy subjects (23, 24, 45), coinciding with a GSH decrease (35, 46). Thus, the hypothesis that chronic hyperglycemia leads to free radical generation and increased GSH oxidation, is further supported here by the salivary reduction in GSH (17, 47, 48). Moreover, the data also support the previous studies showing the presence of oxidative stress and lipid peroxidation levels in saliva from diabetic patients (49, 50). The GSSG/GSH ratio increase in the saliva of diabetic patients may depend on the increase in the GSSG derived from the parotid glands, as elsewhere reported (29). GSSG appears to be released from most cells as a consequence of oxidative stress so that an oxidation of the cellular pool could shift the balance of GSH and GSSG efflux and change the extracellular redox state (51). In fact, the observed differences in GSH concentration and altered redox status in saliva of these patients could reflect a generalized oxidative stress or a decline in antioxidant system in oral cavity. Changes in GSSG and GSH levels in saliva may also reflect changes in the GSH redox cycling enzymes (49). In diabetic patients, the activity of both enzymes, i.e. GPx and GRd is higher than in controls. GPx was previously reported to be increased in the parotid glands of diabetic animals (29), wheras data on salivary GRd activity are unclear. The increase in the activities of both enzymes herein reported possibly reflects a compensatory mechanism of the body to prevent oxidative damage (16). Diabetic patients expend an excess of NADPH through the polyalcohol pathway and probably, the availability of this coenzyme for GRd is reduced. In this situation, GRd is unable to recover the normal GSH pool from GSSG and, thus, GSSG/GSH ratio remained elevated in diabetic patients, reflecting the existence of a metabolic redox misbalance.

In the group of heroin addicts, the existence of an oxidative stress could also be detected in saliva. The activity of GPx and GRd was significantly reduced in

these individuals compared with controls. These changes were accompanied by an increase in GSSG/GSH ratio, reflecting a decrease in GSH and increase in GSSG levels. In this case, the high GSSG/GSH ratio may reflect the decreased GRd activity.

Both in the diabetics and in the drug addicts, the amount of total glutathione (GSH plus GSSG) remained unchanged compared with control values. The data suggest that the oxidative stress in these pathologies does not depend on a lost of glutathione or a lack of GSH synthesis, but a misbalance in the oxidation/reduction cycle of GSH. In fact, the lack of NADPH availability in diabetics and/or the decrease in the activities of both GPx and GRd in drug addicts supports this hypothesis (31, 32). Changes in these parameters seem not to be directly related to changes in the volume of saliva, because it was similar in all groups.

The overproduction of ROS and reduction of the antioxidant defense capability described herein in diabetics and parenteral drug addicts can contribute to complications in these patients including hypertension, neuropathies, and infections, in addition to a bad oral health state. Thus, accordingly with our results, we suggest that the occurrence of oxidative stress in poorly controlled diabetic and parenteral drug addict patients is associated with a poor prognosis and evolution of these diseases.

In conclusion, the assay of salivary oxidative stress parameters has brought substantial insight into the pathogenesis and evolution of diabetes and drug addiction. Measurement of salivary GPx and GRd activities and the GSSG/GSH ratio, and perhaps some other parameters such as 8-hydroxydeoxyguanosine (52), provide a non-invasive method to assess the degree of oxidative stress in pathophysiologic status, such as diabetes and drug addiction. The utility of these markers in determining the optimal pharmacologic therapy for the diseases involving free radicals remains to be addressed.

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