Age-related variations of protein carbonyls in human saliva and plasma: is saliva protein carbonyls an alternative biomarker of aging?

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Abstract Free radical hypothesis which is one of the most acknowledged aging theories was developed into oxidative stress hypothesis. Protein carbonylation is by far one of the most widely used markers of protein oxidation. We studied the role of age and gender in protein carbonyl content of saliva and plasma among 273 Chinese healthy subjects (137 females and 136 males aged between 20 and 79) and discussed the correlation between protein carbonyl content of saliva and plasma. Protein carbonyl content of saliva and plasma were, respectively, 2.391 ± 0.639 and 0.838 ± 0.274 nmol/mg. Variations of saliva and plasma different age groups all reached significant differences in both male and female (all $p < 0.05$) while both saliva and plasma protein carbonyls were found to be significantly correlated with age ($r = 0.6582$ and $r = 0.5176$, all $p < 0.001$). Gender was discovered to be unrelated to saliva and plasma protein carbonyl levels (all $p > 0.05$). Saliva and plasma protein carbonyls were positively related ($r = 0.4405$, $p < 0.001$). Surprisingly, saliva and

plasma protein carbonyls/ferric reducing ability of plasma (FRAP) ratios were proved to be significantly correlated with age ($r = 0.7796$ and $r = 0.6938$, all $p < 0.001$) while saliva protein carbonyls/FRAP ratio and plasma protein carbonyls/FRAP ratio were also correlated ($r = 0.5573$, $p < 0.001$). We concluded that saliva protein carbonyls seem to be an alternative biomarker of aging while the mechanisms of protein carbonylation and oxidative stress and the relationship between saliva protein carbonyls and diseases need to be further investigated.

Keywords Saliva · Plasma · Protein carbonylation · Free radicals · Oxidative stress · Aging

Introduction

Aging is defined as the degenerations which gradually reduce the level of fitness and the capacity to maintain homeostasis, simultaneously increasing the chance of death (Rajindar and William 2012). According to Harman, who raised the free radical theory, the deteriorative alterations above mainly arise from the accumulation of oxidative damage to cells and tissues caused by environmental and metabolically generated free radicals, random errors in biochemical reactions, and intake of nutrients (Harman 1956, 1972; Suresh 2006). It is widely acknowledged that reactive oxygen species (ROS) are normal by-products of aerobic metabolism while the maintenance of homeostasis can be affected by the imbalance between ROS caused damages and antioxidant defenses, leading to age-related macromolecular

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structural damages, diseases, aging, and death (Rajindar and William 2012; Harman 1956; Fridovich 1978). Oxidative modifications of cellular macromolecules such as DNA, RNA, carbohydrate, lipid, and proteins alone or in association with other biologic factors can lead to epigenetic changes, changes in both gene expression and DNA repair capacity, or mitochondrial and membrane dysfunction (Kimberly et al. 2013).

Among the various oxidatively modified macromolecules, protein is thought to be of vital importance, since proteins play the key part of receptors, transporters, enzymes, transcription factors, and cytoskeletal components. Furthermore, due to its abundance, proteins could probably be the major targets of ROS. Protein carbonylation is an irreversible, chemical stable oxidative modification, which may lead to inactivation, proteolysis, enfoldment, or formation of intra-/intermolecular cross-links (Rajindar and William 2012; Stadtman and Berlett 1997). Protein carbonyls are formed on protein side chains (especially of Pro, Arg, Lys, and Thr) when they are oxidized. Protein carbonyl derivatives can also be generated through oxidative cleavage of proteins by either the α -amidation pathway or by oxidation of glutamyl side chains, leading to formation of a peptide in which the N-terminal amino acid is blocked by an α -ketoacyl derivative (Berlett and Stadtman 1997). For its relative early formation, chemical stability, and uncomplicated detection method, protein carbonyl content has become by far one of the most widely used markers of protein oxidation (Berlett and Stadtman 1997; Chevion et al. 2000), and elevated amount of protein carbonyls has been detected in several human aging-related diseases including Alzheimer's disease, cancer, cardiovascular diseases, diabetes, and in plasma of elderly ones (Miriana and Denisa 2013; Mannello et al. 2013; Ian et al. 2013; Mediha et al. 2010; Pandey et al. 2010). Until recently, few research about saliva protein carbonyls have emerged while most focused on plasma or tissue protein carbonyls over the past decades (Pandey et al. 2010; Hensley et al. 1995; Su et al. 2008, 2009). Unlike plasma, saliva test which is currently promising has the advantage of no-pain, noninvasiveness, infection-free, and convenience, and the relevance of protein carbonyl content between saliva and plasma has never been explored.

The ferric reducing ability of plasma (FRAP) assay which is related to body antioxidant status and dietary intake of antioxidants has been widely used to evaluate antioxidant power. As body oxidative status is influenced by oxidants and antioxidants, it is indispensable

to detect both before assessing redox homeostasis (Pandey et al. 2010; Benzie and Strain 1996).

The rest of the paper focused on one question as the title: Is saliva protein carbonyls an alternative biomarker of aging?

Methods

Study population

The study was approved by the Medical Ethical Committee of the Chinese People's Liberation Army General Hospital (grant number s2014-114-01). In accordance with the random number table, the cross-sectional study enrolled 273 Chinese healthy subjects (137 females and 136 males) who were divided into young group (aged between 20 and 44, $n=104$), middle-aged group (aged between 45 and 59, $n=90$), and elderly group (aged between 60 and 79, $n=79$) according to the standards of the United Nations World Health Organization. All participants were recruited on the basis of a critical inclusion and exclusion criteria: (a) having physical examination in People's Liberation Army General Hospital (PLAGH) aged between 20 and 79; (b) no taking vitamins, minerals, or drugs affecting oxidation, immunity, metabolism, or salivary secretory since latest month; (c) no smokers or alcoholic drinkers; (d) good oral health status without oral mucosal diseases; (e) females not engaged in pregnancy or lactation; (f) elderly ones free of cognitive impairment; (g) no history or sign of systematic disorders including chronic inflammation, diabetes mellitus, cryptorrhea, renal diseases, liver diseases, tumor, digestive system disease, cardiovascular disease, or neurological disease in physical examination report (elderly ones with mild chronic illness which required no treatment or intervention were also included in our study); (h) exclusive of acute infectious disease or long-term mental stress. All volunteers were given written informed consent in our study (Pandey et al. 2010; Su et al. 2009, 2012; Zeng et al. 2012).

Sampling and preparation

Saliva Unstimulated whole saliva sample was collected from 8 a.m. to 10 a.m. in view of salivary diurnal fluctuations on condition that any food or liquid intake were forbidden 1 h before collection. Participants were required to rinse the mouth 10 min before sampling, and

saliva was collected by cylinder cotton swab from Salivette collection system (Sarstedt AG & Co., Germany). After collection, tubes with samples were kept at 4 °C while further process must be done in 1 h. The tubes were centrifuged at 4000 rpm for 20 min at 4 °C (Eppendorf Co., Hamburg, Germany), and the supernatants were gathered and stored in 1-mL aliquots for less than half a month ahead of detection. Samples mixed with blood or other visible impurities were discarded immediately, and each sample should not be frozen and thawed twice or more (Pandey et al. 2010; Su et al. 2008, 2009, 2012; Zeng et al. 2012).

Blood Five-milliliter venous blood was obtained under the circumstance mentioned above by venipuncture in sterile polystyrene vacuum tubes containing heparin, kept at 4 °C, and centrifuged at 4000 rpm for 10 min at 4 °C. Plasma was stored in 1-mL aliquots for less than half a month until detection and each sample was frozen and thawed only once (Pandey et al. 2010; Zeng et al. 2012).

Study parameters

Protein carbonyl assay Protein carbonyls in saliva and plasma were measured by an ELISA kit (Sigma-Aldrich Co. LLC., St. Louis, MO, USA) which is mainly dependent on Levine's method (Levine et al. 1990). Samples should be diluted with water to a protein concentration of 10 mg/mL. Each assay well requires 0.1 mL of sample containing 0.5–2.0 mg of protein per assay. Add 0.1 mL of water to a well to serve as a reagent background control. A total of 0.1 mL of samples (saliva and plasma) were treated with 0.1 mL of 2,4-dinitrophenylhydrazine (DNPH), vortexed, and incubated for 10 min at room temperature. Next, 30 μ L of 100 % trichloroacetic acid (TCA) was added, and the sample was vortexed and incubated on ice for 5 min. The tubes were then centrifuged at 13,000 rpm for 2 min to obtain the protein pellet. The precipitated proteins were subsequently washed twice with 500 μ L of ice-cold acetone to remove unreacted DNPH and lipid remnants. Each washing step was followed by centrifugation at 13,000 rpm for 2 min. The final protein pellet was dissolved in 0.2 mL of 6 M guanidine solution and incubated at 37 °C for 10 min. Transfer 0.1 mL of each sample to the 96-well plate. The carbonyl content was counted from peak absorption (375 nm). Transfer 5 μ L of sample to another set of wells and perform a protein assay to determine the amount of protein per sample.

Generate a protein standard curve according to assay protocols. Bovine standard albumin was used for the standard curve. The protein carbonyl content was expressed as nmol/mg protein.

FRAP assay FRAP in saliva and plasma was also measured by an ELISA kit (Arbor Assays LLC., Ann Arbor, MI, USA) which is mainly based on Benzie's method (Benzie and Strain 1996). A total of 20 μ L of samples, standards, or controls mixed with 75 μ L of the prepared FRAP Color Solution to each well was incubated at room temperature for 30 min. The absorbance was read at 560 nm.

Statistical analyses

One-way analysis of variance (one-way ANOVA) followed by SNK test, bivariate analyses using nonparametric (such as Wilcoxon rank-sum test) and parametric tests (such as Student's *t* test), and Pearson correlation test were used for analyzing. Age and gender were separately analyzed. Data are presented as mean $\pm$ SD. Pearson's linear correlation coefficient was utilized to evaluate the relevance between variables. All statistical analyses were finished by SPSS 21.0 software, and pictures were curved by PRISM 5 software (Graphpad Software Inc., San Diego, CA). $P<0.05$ was set as statistically significant.

Results

Basic clinical information of study population divided by age group is shown in Table 1. Comparison of saliva and plasma protein carbonyl content of different groups divided by age and sex is revealed in Table 2. Saliva protein carbonyl concentration was 2.391 ± 0.639 nmol/mg, and plasma protein carbonyl concentration was 0.838 ± 0.274 nmol/mg in 273 Chinese healthy subjects. Table 3 and Table 4 demonstrate the significantly positive effect of age factor on saliva and plasma protein carbonyls. Variations of saliva and plasma different age groups all reached significant differences in both male and female through one-way ANOVA and SNK test (all $p<0.05$ and most $p<0.01$). Gender was discovered to be unrelated to saliva and plasma protein carbonyl levels in Table 5 (all $p>0.05$).

Figure 1a–h displays all Pearson correlation analysis while the ratio of protein carbonyls/FRAP was introduced (Pandey et al. 2010; Rizvi and Rizvi 2007). Both saliva and plasma protein carbonyls

Table 1 Basic clinical information of study population divided by age group ($\bar{x}\pm s$)

	Young group (20–44 years)	Middle-aged group (45–59 years)	Elderly group (60–79 years)	Total
Age (years)	35.2 $\pm$ 6.1	51.2 $\pm$ 4.4	67.4 $\pm$ 5.3	49.0 $\pm$ 14.6
BMI (kg/m ²)	21.7 $\pm$ 1.5	24.1 $\pm$ 1.8	23.0 $\pm$ 2.1	22.8 $\pm$ 2.8
SBP (mmHg)	108.0 $\pm$ 6.5	116.3 $\pm$ 7.3	123.7 $\pm$ 10.2	115.2 $\pm$ 13.0
DBP (mmHg)	70.2 $\pm$ 4.2	73.0 $\pm$ 4.6	77.8 $\pm$ 5.5	73.3 $\pm$ 7.3
Glucose (mg/dL)	93.7 $\pm$ 2.4	98.3 $\pm$ 2.1	103.0 $\pm$ 3.7	98.1 $\pm$ 5.2
Hs-CRP (mg/L)	0.8 $\pm$ 0.3	0.7 $\pm$ 0.3	1.6 $\pm$ 0.4	0.9 $\pm$ 0.4
Leucocyte count ($\times 10^9$)	5.5 $\pm$ 0.8	5.7 $\pm$ 0.7	5.1 $\pm$ 1.1	5.4 $\pm$ 1.8
Uric acid (mg/dL)	3.5 $\pm$ 0.6	3.3 $\pm$ 0.5	3.7 $\pm$ 0.5	3.5 $\pm$ 0.9
BUN (mg/dL)	13.7 $\pm$ 4.4	15.0 $\pm$ 4.1	18.5 $\pm$ 3.7	15.4 $\pm$ 5.3
Creatinine clearance rate (mL/min)	100.8 $\pm$ 14.7	97.3 $\pm$ 13.2	82.0 $\pm$ 17.5	94.5 $\pm$ 20.2

were found to be significantly correlated with age ($r=0.6582$ and $r=0.5176$, all $p<0.001$, shown in Fig. 1a, b). Meanwhile, saliva protein carbonyls and plasma protein carbonyls were positively related ($r=0.4405$, $p<0.001$, shown in Fig. 1c). Figure 1d, e denies the age-dependent variations in saliva or plasma FRAP ($r=0.0604$ and $r=-0.0371$, $p=0.320$ and $p=0.542$). Surprisingly, saliva and plasma protein carbonyls/FRAP ratios were proved to be significantly correlated with age ($r=0.7796$ and $r=0.6938$, all $p<0.001$, shown in Fig. 1f, g) while saliva protein carbonyls/FRAP ratio and plasma protein carbonyls/FRAP ratio were also correlated ($r=0.5573$, $p<0.001$, shown in Fig. 1h).

Discussion

A large number of aging theories have emerged and been studied throughout centuries as human beings are

yearning for immortality and anti-aging since their appearance. To name a few, genome-controlled hypothesis, telomere hypothesis, DNA damage hypothesis, free radical hypothesis are among the most popular ones lately (Brutovska et al. 2013). Free radical hypothesis which is one of the most acknowledged theories was originally proposed by Harman and developed into oxidative stress hypothesis by Sies. Oxidative damage which could cause aging and aging-related diseases generate from exceeding oxidants rather than antioxidants. Recently, interference of free radicals on cell signaling and metabolic pathways was added to oxidative stress hypothesis (Harman 1956; Sies and Cadenas 1985; Sies 1997; Jones 2006). Free radicals primarily derived from normal endogenous cell metabolism of mitochondria and microsome and exogenous sources such as UV irradiation, X-rays, and pesticides can cause irreversible damage by attacking DNA, RNA, carbohydrate, lipid, and proteins. Internal antioxidant system (for example, SOD, glutathione reductase, and

Table 2 Comparison of saliva and plasma protein carbonyl content of different groups divided by age and sex ($\bar{x}\pm s$, nmol/mg)

Age groups	n, gender	Saliva protein carbonyls (nmol/mg)	Plasma protein carbonyls (nmol/mg)
Young group (20–44 years)	n=104 48F/56M	1.949 $\pm$ 0.549 1.921 $\pm$ 0.528/1.974 $\pm$ 0.570	0.706 $\pm$ 0.282 0.732 $\pm$ 0.302/0.684 $\pm$ 0.265
Middle-aged group (45–59 years)	n=90 55F/35M	2.492 $\pm$ 0.479 2.492 $\pm$ 0.511/2.491 $\pm$ 0.432	0.851 $\pm$ 0.204 0.858 $\pm$ 0.182/0.839 $\pm$ 0.237
Elderly group (60–79 years)	n=79 34F/45M	2.859 $\pm$ 0.5202 2.866 $\pm$ 0.528/2.853 $\pm$ 0.520	0.996 $\pm$ 0.247 0.980 $\pm$ 0.268/1.008 $\pm$ 0.232
Total	n=273 137F/136M	2.391 $\pm$ 0.639 2.385 $\pm$ 0.637/2.398 $\pm$ 0.643	0.838 $\pm$ 0.274 0.844 $\pm$ 0.267/0.831 $\pm$ 0.282

Table 3 Comparison of saliva and plasma protein carbonyls of male between different age group

Comparison group	q value	p value (no exact p value under SNK test)
Saliva protein carbonyls between young and middle-aged group	6.51	$p<0.01$
Saliva protein carbonyls between middle-aged and elderly group	4.36	$p<0.01$
Saliva protein carbonyls between young and elderly group	11.92	$p<0.01$
Plasma protein carbonyls between young and middle-aged group	4.12	$p<0.01$
Plasma protein carbonyls between middle-aged and elderly group	4.28	$p<0.01$
Plasma protein carbonyls between young and elderly group	9.25	$p<0.01$

ANOVA of saliva protein carbonyls $F=36.256$, $p<0.001$,
ANOVA of plasma protein carbonyls $F=21.429$, $p<0.001$

ascorbate) can sweep away some free radicals for maintaining homeostasis (Hohn et al. 2013). Although the hypothesis above remained controversial, it was widely acknowledged that oxidative stress should be an important risk factor of aging and age-related diseases at least. Protein carbonyls as one of the most commonly used biomarkers of oxidative stress was reported to increase in elderly ones and aging-related diseases while FRAP as one of the widely utilized signs of antioxidant defenses decreased among the senescence associated

Table 4 Comparison of saliva and plasma protein carbonyls of female between different age group

Comparison group	q value	p value (no exact p value under SNK test)
Saliva protein carbonyls between young and middle-aged group	7.85	$p<0.01$
Saliva protein carbonyls between middle-aged and elderly group	4.65	$p<0.01$
Saliva protein carbonyls between young and elderly group	11.45	$p<0.01$
Plasma protein carbonyls between young and middle-aged group	3.58	$p<0.05$
Plasma protein carbonyls between middle-aged and elderly group	3.16	$p<0.05$
Plasma protein carbonyls between young and elderly group	6.23	$p<0.01$

ANOVA of saliva protein carbonyls $F=34.720$, $p<0.001$,
ANOVA of plasma protein carbonyls $F=9.841$, $p<0.001$

Table 5 Comparison of saliva and plasma protein carbonyls of different age group between male and female

Comparison group	p value
Young group saliva protein carbonyls between male and female	$p=0.6263$
Middle-aged group saliva protein carbonyls between male and female	$p=0.9897$
Elderly group saliva protein carbonyls between male and female	$p=0.9115$
Young group plasma protein carbonyls between male and female	$p=0.3866$
Middle-aged group plasma protein carbonyls between male and female	$P=0.6744$
Elderly group plasma protein carbonyls between male and female	$p=0.6226$

disorders (Miriana and Denisa 2013; Mannello et al. 2013; Ian et al. 2013; Mediha et al. 2010; Pandey et al. 2010; Pulido et al. 2005).

Our study reported that saliva protein carbonyl content was 2.391 ± 0.639 nmol/mg, and plasma protein carbonyl content was 0.838 ± 0.274 nmol/mg. Plasma protein carbonyl results are in line with previous articles. We first discovered the age-dependent increase of saliva protein carbonyls and the positive correlation between them. Gender has no significant effect on saliva or plasma protein carbonyls. The results above are similar to some preceding research but also against others. Pandey observed an age-dependent increase in plasma protein carbonyl level ($r=0.768$) and protein carbonyls/FRAP ratio ($r=0.917$), and plasma protein carbonyl levels were between 20 and 70 nmol/L (Pandey et al. 2010). Su reported that the salivary protein carbonyl level among healthy adults was 1.80 ± 0.15 nmol/mg (Su et al. 2008), but he denied the relationship between levels of log protein carbonyls and age (Su et al. 2012). Baltacioglu discovered that the average levels of serum and gingival crevicular fluid protein carbonyls were 1.22 and 1.10 nmol/mg (Baltacioglu et al. 2008). Kamodyova' research showed that salivary FRAP was about 100–400 $\mu\text{mol/g}$ (Kamodyova et al. 2013). Differences may arise from race, laboratory, detection method, calculation method, or other factors. We brought in a ratio protein carbonyls/FRAP (Pandey et al. 2010; Rizvi and Rizvi 2007). Saliva and plasma FRAP had no age-dependent variation while saliva and plasma protein carbonyls/FRAP ratios surprisingly correlated with age. This tight correlation might be explained by the consideration of both oxidant and anti-oxidant state as the redox stress is affected by both

Fig. 1 **a** Relationship between saliva protein carbonyl concentrations and age. **b** Relationship between plasma protein carbonyl concentrations and age. **c** Relationship between plasma and saliva protein carbonyl concentrations. **d** Relationship between saliva

FRAP concentrations and age. **e** Relationship between plasma FRAP concentrations and age. **f** Relationship between saliva ratios and age. **g** Relationship between plasma ratios and age. **h** Relationship between plasma and saliva ratios

reactive oxygen species (ROS) production and antioxidant defenses (Rajindar and William 2012; Sohal and Allen 1990; Junqueira Virginia et al. 2004). The results and analysis above were indicating that protein carbonyls and the ratio of both saliva and plasma have the tendency of increasing during the aging process and saliva protein carbonyl content can generally reflect plasma protein carbonyl content.

It had been reported from human dermal fibroblasts, lens, and brain that protein carbonyl level can reflect cellular ROS generation and aging. The accumulation of protein carbonyls reflects chronological age when cells gradually become more vulnerable to oxidative stress during the aging process (Garland et al. 1988; Oliver et al. 1987; Smith et al. 1991). If we want to figure out the role of oxidative stress in aging and aging-related diseases, proper biomarkers should be well selected. Saliva is regarded as a prospective biofluid to supervise physical condition, aging, and diseases by its easy way of collecting useful information in the eyes of many researchers. For instance, saliva HIV test had been confirmed with high accuracy and approved by FDA. If useful data of saliva reflecting both oral and systemic health conditions could be unearthed, early detection of aging and diseases may be much easier and more convenient (Ai et al. 2012; Granade et al. 1995; Frerichs et al. 1992).

Conclusions

Our research reveals that age factor has significantly positive effect on saliva and plasma protein carbonyls while saliva protein carbonyls and plasma protein carbonyls are positively related. Saliva protein carbonyls seem to be an alternative biomarker of aging. Nevertheless, the mechanisms of protein carbonylation and oxidative stress affecting aging and aging-related diseases remain uncertain, and the relationship between saliva protein carbonyls and diseases needs to be further investigated.

Author contributions *Guarantor:* Yanyi Wang had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. *Study concept and design* Zhihui Wang, Yanyi Wang, Hongchen Liu.

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Conflict of interest None declared.

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