Available online at www.sciencedirect.com

SciVerse ScienceDirect

journal homepage: www.elsevier.com/locate/jobcr

Original Article

Salivary defense system alters in vegetarian

Nour Amirmozafari^a, Houra Pourghafar^a, Reyhaneh Sariri^{b,*}^a Department of Microbiology, Lahijan Branch, Islamic Azad University, Lahijan, Iran^b Professor, Department of Microbiology, Lahijan Branch, Islamic Azad University, Lahijan, Iran

ARTICLE INFO

Article history:

Received 29 March 2013

Accepted 20 May 2013

Keywords:

Saliva

Antimicrobial activity

Antioxidant enzymes

Vegetarian

ABSTRACT

Purpose: The aim of this research was investigating antimicrobial and enzymatic antioxidant activities in salivary fluids of vegetarians as compared to normal subjects.**Material & Methods:** Antimicrobial activity of the saliva samples was evaluated against four clinically important bacteria. The biological activities of three of the main antioxidant enzymes of saliva were measured using appropriate methods of enzyme assay in both groups.**Results:** According to the results, saliva obtained from vegetarians showed a reduced inhibitory effect on growth of *Staphylococcus aureus*, *Klebsiella oxytoca*, *Pseudomonas aeruginosa* and *Escherichia coli* as compared to those obtained from the non-vegetarian subjects. The activity of salivary peroxidase, catalase and superoxide dismutase showed a statistically marked decrease in vegetarian group.**Conclusions:** According to our literature survey, this is the first report on the antibacterial and antioxidant capacity in saliva of vegetarians. Results obtained from the present study have opened a new line of research with the basis of saliva as a research tool.

Copyright © 2013, Craniofacial Research Foundation. All rights reserved.

1. Introduction

Vegetarianism is referred to using plant-based diets excluding meat and including or excluding dairy products, eggs and honey. There are a number of aims including health, religious, political, environmental, cultural, esthetic, economic and ethical reasons that encourage people to become vegetarian. In recent years, scientific research has slightly shifted from concerns about nutritional adequacy to investigating health benefits and disease prevention. It has been shown that at all stages of life, a vegetarian diet is healthful, nutritionally adequate, and provides health benefits in the prevention and treatment of certain diseases. Large-scale studies have shown that mortality from ischemic heart disease was 30% lower among vegetarian men and 20% lower among vegetarian

women than in non-vegetarians.¹ It is known that most of necessary nutrients, proteins, and amino acids can be found in vegetables, grains, nuts, soymilk, eggs and dairy.² However, as with many long-term non-usual habit, vegetarianism may result in side effects such as reduction in antioxidant and antibacterial activity of various body fluids leading to a decrease in overall defense activity. Saliva is a special body fluid with a rich biochemistry which may be used in various diagnostic investigations.³ It can protect oral cavity against detritus agents such as microorganisms, toxins and various oxidants.⁴ In some special circumstances, the antioxidant capacity and reducing power of saliva is altered.⁵ It has been shown that *in vitro* exposure to cigarette smoke could significantly decrease biological activity of some enzymes, both in plasma and in saliva.^{6,7} Saliva is secreted by three pair of

* Corresponding author. Tel./fax: +98 1313233647.

E-mail addresses: sariri@guilan.ac.ir, sariri2@yahoo.co.uk (R. Sariri).

2212-4268/\$ – see front matter Copyright © 2013, Craniofacial Research Foundation. All rights reserved.

<http://dx.doi.org/10.1016/j.jobcr.2013.05.004>

major salivary glands as well as by hundreds of minor glands located below the mucosal surfaces of the mouth.^{3,8} It is composed of complex mixture of substances, similar to other body fluids.⁹ Biochemistry of saliva is composed mostly of the locally produced proteins and enzymes together with some other biochemicals including antioxidants, electrolytes, epithelial and immune cells, microorganisms, and bronchial products.³ Besides, other external substances with varied concentrations are also found in salivary fluid.¹⁰ Having a non-invasive type of sampling, the use of saliva as a source of important biomarkers has recently attracted attention of some researchers.¹¹ It has been shown that biochemical composition can also reflect the causes of some systemic disease.¹² Human saliva can reflect the relationship between oral hygiene and some chronic systemic diseases¹³ as well as intensity of exercise.¹⁴

2. Materials and methods

2.1. Saliva collection, flow rate and storage

Twenty-five female university students aged 22–25 years who had become vegetarian from 4 to 8 years ago together with 25 non-vegetarians volunteered entered into our study. The subjects did not suffer from internal or genetic diseases and they had healthy teeth and gums. They agreed to donate their saliva according to the instruction given to them and signed a consent. They were asked to fill a form about their health background and various aspects of life and history of becoming vegetarian. Their un-stimulated saliva samples (3 ml) were collected in clean and dry sterile pre-weighted tubes after 8 h of fast and rinsing their mouth with distilled water.¹⁴ The time in minutes for collecting one ml of saliva was taken as flow rate. The collected samples were immediately centrifuged at $800\times g$ for 10 min at 4 °C to remove squamous cells and cell debris. The resulting supernatant was stored at –18 °C until examination for antimicrobial and enzyme activity.

2.2. Selection of microorganisms and preparation of growth medium

Antimicrobial studies were carried out using four microorganisms which included one Gram-positive: *Staphylococcus aureus* (PTCC 1133) and three Gram-negatives: *Klebsiella oxytoca* (PTCC 1402), *Escherichia coli* (PTCC 1553), and *Pseudomonas aeruginosa* (PTCC 1558). All strains were obtained from Persian Type Culture Collection (PTCC) centre in Tehran and stored frozen at –80 °C. The bacterial vials were opened as recommended by PTCC and the strains were grown in nutrient broth followed by incubation at 37 °C for 4 h. Disk diffusion method was used in order to evaluate *in vitro* antibacterial activity of the saliva samples with a few known antibiotics. In practice, broth subcultures were prepared by inoculating, with one single colony from a plate, a test tube containing 5 ml of sterile nutrient broth. The tubes were then incubated at 37 °C for at least 24 h.

The bacterial culture was allowed to reach a concentration of 10^8 CFU/ml. Each suspension was spread on Muller Hinton Agar medium by sterile swabs. Whitman filter paper disks

(diameter 6 mm) dipped in saliva samples and two known antibiotics, i.e. gentamicin and ampicillin (Padtan Teb™, Iran) were placed on the agar surface. The diameter of inhibition zone was measured after incubation of all plates at 37 °C for 24 h. The growth inhibition zone is referred to as the diameter of the clear zone surrounding the filter paper disks which also includes the size of disk itself. The inhibition zones (including disk diameter) less than 10 mm were negative. Zone calculation was the average of three measurements. The t-test was used with the GraphPad PRISM® software (GraphPad Software, Inc., San Diego, CA, USA) and $p \leq 0.05$ differences were considered as significant.

2.3. Peroxidase assay

The biological activity of enzyme on 4-amino antipyrine was measured spectrophotometrically. The oxidation of 4-amino antipyrine was measured at 25 °C in 3 ml of 0.3 M phosphate buffer, pH 7.4, containing 0.0010 M hydrogen peroxide, 0.002 M 4-amino antipyrine and 0.15 M phenol. Forty μ l of saliva samples was then added and the change in absorption at 510 nm ($\Delta A/\text{min}$) was recorded. The change in absorption at 510 nm is due to the formation of a chromogen product with a λ_{max} at 510 nm. One unit of activity was defined as the amount of enzyme that caused an absorbance change of 0.001 per min under standard conditions.

2.4. Catalase assay

Salivary catalase activity was measured using EnzyChrom™ Catalase Assay Kit (ECAT-100) designed for direct assay of catalase in serum, saliva and urine. The method was based on degradation of H_2O_2 by catalase using a redox dye. The change in color intensity at 570 nm, proportional to the catalase activity in the sample, was measured spectrophotometrically. One unit of catalase activity was the amount of catalase that decomposes 1 mol of H_2O_2 per min at pH 7.0 and room temperature. The biological activity of the enzyme was expressed as U/ml.

2.5. Superoxide dismutase (SOD) assay

The activity was measured using a special superoxide dismutase kit made by R&D Systems Europe, Ltd. for research purposes, Catalog number: 7500-100-K. In this assay, superoxide ions (O_2^-), generated by xanthine oxidase (XOD) conversion of xanthine to uric acid and hydrogen peroxide, converts NBT to NBT-diformazan, which absorbs light at 560 nm. SOD reduces the superoxide ion concentration and thereby lowers the rate of NBT-diformazan formation. The extent of reduction in the appearance of NBT-diformazan is a measure of SOD activity present in a saliva sample. One unit of SOD inhibits the rate of increase in absorbance at 550 nm by 50% under the conditions of the assay. The percent inhibition of the saliva sample correlated with SOD activity using an SOD standard curve.

2.6. Statistical analysis

Each assay was repeated at least in duplicate and the results were presented as mean $\pm$ SD values. Statistical difference

between individuals was compared by un-paired t-test, p values less than 0.05 were retained as significant. Significant differences between means were determined by Duncan's multiple range tests. p values less than 0.05 were considered statistically significant.

3. Results and discussions

3.1. Salivary flow rate

Table 1 has compared the results of flow rates in vegetarian and non-vegetarian subjects. Salivary flow rates were expressed as volume of un-stimulated saliva (ml) collected per minute. Statistically significant changes in saliva flow rate were observed between smokers and non-vegetarian. Saliva flow rate ranged from 0.8 to 1.60 ml min⁻¹ in the non-vegetarians with a significant increase to (0.9–1.7 ml.min⁻¹) among the vegetarians ($p < 0.05$). It has been shown that the flow rate of any biological fluid such as tears^{15,16} and saliva^{17,18} depends on various external and internal factors. In a previous study, it was found that the salivary flow rate does not significantly alter due to exercise intensity,¹⁴ but decreases sharply in smokers and passive smokers. The increase in vegetarian's salivary volume and flow rate in comparison with those from meat eaters may be due to the slightly alkaline pH of most vegetable derived foods. This can be considered as an advantage for older ages, as they sometime complain from dry mouth.

3.2. In vitro antimicrobial characteristics

Table 1 shows the results of antimicrobial studies. As no antibacterial activity was observed in salivary fluid of the vegetarians; therefore, the table represents the abundance of each inhibition of growth observed within the whole saliva samples. It is known that antibacterial characteristic of saliva is not only important to protection of oral cavity, gums and teeth, but it also plays an important role in protection of body environment. Saliva is the first body fluid which encounters any type of incoming pathogen and plays protection duty for the body as whole. Therefore, the antioxidant and antibacterial power of salivary fluid is determinant in protecting all type of damages to oral and gastrointestinal tissues.

Table 1 – Mean diameter (mm) of growth inhibition zone for various bacteria in the presence of saliva samples of vegetarian compared to non-vegetarian groups ($p < 0.05$ significant).

Bacterial abundance (No. in the zone)	Growth inhibition zone (mm) $\pm$ 0.005							
	<i>S. aureus</i>		<i>E. coli</i>		<i>K. oxytoca</i>		<i>P. aeruginosa</i>	
	Non-veg.	Veg.	Non-veg.	Veg.	Non-veg.	Veg.	Non-veg.	Veg.
7–12	18	7	14	3.6	19	8	21	9
13–18	12	1.8	11	–	14	3	15	4
19–24	11	–	11	–	12	1	13	2
24–30	11	–	10	–	11	–	12	1
Average	13	2.2	11.5	0.9	14	3	15.2	4

Table 2 – The effect of salivary fluid of both vegetarian and non-vegetarian groups on Gram-positive and Gram-negative bacteria compared to some known antibiotics ($p < 0.05$ significant).

Growth inhibition zone $\pm$ 0.2 (mm)	<i>S. aureus</i>	<i>E. coli</i>	<i>K. oxytoca</i>	<i>P. aeruginosa</i>
Veg. group (average)	2.2	0.9	3.0	4.0
Non. veg. (average)	13.0	11.5	14.0	15.2
Gentamicine	23.0	23.0	24.0	20.0
Ampiciline	16.0	13.0	12.0	7.0
Average	11.60	10.66	18.04	14.62

In Table 2, the antibacterial effect of saliva has been compared with known antibiotics. The salivary fluid of the non-vegetarians posed a strong antibacterial effect on both types of Gram staining bacteria; in some cases, comparable to strong antibiotics. As noted in this table, the saliva antibacterial activity is almost lost in vegetarian cases. Care must be taken in choosing a special type of diet, as many side effects are not yet investigated.¹ Salivary antioxidant and antibacterial power could help to prevent many internal diseases as well as oral ulcers.^{12,19}

3.3. Peroxidase activity

Fig. 1 shows peroxidase activity in salivary fluid of vegetarian and non-vegetarian subjects. Peroxidase is the most important antioxidant enzyme in human saliva.²⁰ It can scavenge free radicals produced by different events in the body environment or present in various foods. Reduction in the activity of the enzyme leaves the oral environment almost unprotected against danger of free radicals.¹⁴ It was previously investigated that some external factors such as exercise could help to increase the activity of peroxidase in saliva.¹⁴ It is tempting to suggest that people who choose to eat vegetables should consider exercise to counter the reduction in the peroxidase activity of their saliva.

3.4. Catalase activity

Fig. 2 shows the activity of catalase in saliva samples of both groups. It can be seen that the activity of this enzyme is also

Fig. 1 – The biological activity of peroxidase in saliva samples of vegetarian compared to non-vegetarian group ($p < 0.05$ significant).

Fig. 2 – Comparison of catalase activity in salivary fluid of vegetarian compared to non-vegetarian group ($p < 0.05$ significant).

reduced in subjects taking only vegetables. Although catalase has not been extensively studied in saliva according to our literature survey, the activity of this enzyme is also important in the defense system of saliva. Therefore, its reduction could be compensated by altering other styles of life, i.e. increasing exercise.¹⁴

3.5. Superoxide dismutase activity

Fig. 3 shows the activity of superoxide dismutase in saliva samples of both groups. The activity of this enzyme showed significant reduction in saliva samples of the vegetarian subjects. Combining these results with reduced antibacterial effect, would cause vegetarian's saliva at risk of low defense power. It has been shown that ascorbic acid plays an essential role in antioxidant activity of human saliva.²¹ Therefore, taking into account the strong antioxidant power of ascorbic acid, supplementation of vegetarian diet with vitamin C as well as increase exercise¹⁴ could be beneficial to compensate for the decreased antioxidant power.²¹

Fig. 3 – Superoxide dismutase activity in salivary fluid of vegetarian compared to non-vegetarian group ($p < 0.05$ significant).

4. Conclusions

In this study the antibacterial activity of saliva in two groups of human subjects were compared. The quantitative data from both antibacterial and antioxidant studies indicated a significant reduction in protecting ability of saliva in vegetarian subjects when compared to subjects who eat meat and vegetables. Therefore, this investigation suggested that remaining on vegetarian diet for a long period could gradually reduce the ability of saliva as a barrier to free radicals and various bacterial contaminations present in food. This may lead to various health problems, especially in the oral cavity.

Conflicts of interest

All authors have none to declare.

Acknowledgments

The authors highly acknowledge the financial support provided by the Lahijan Branch of Islamic Azad University.

REFERENCES

1. Key TJ, Fraser GE, Thorogood M, et al. Mortality in vegetarians and non-vegetarians: detailed findings from a collaborative analysis of 5 prospective studies. *Am J Clin Nutr.* 1999;70:516–520.
2. Soy milk at soyfoods.com.
3. Herrera JL, Lyons IIMF, Johnson LF. Saliva: its role in health and disease. *J Clin Gastroenterol.* 1998;10:569–578.
4. Tabak LA, Levine MJ, Mandel ID, Ellison SA. Role of salivary mucin in protection of the oral cavity. *J Oral Pathol.* 1982;11:1–7.
5. Kohen R, Tirosh O, Kopolovich K. The reductive capacity index of saliva obtained from donors of various ages. *Exp Gerontol.* 1992;27:161–168.
6. Nagler R, Lischinsky S, Diamond E. Effect of cigarette smoke on salivary proteins and enzyme activities. *Arch Biochem Biophys.* 2000;379:229–236.
7. Zappacosta B, Persichilli S, Mordente A, et al. Inhibition of salivary enzymes by cigarette smoke and protective role of glutathione. *Hum Exp Toxicol.* 2002;21(1):7–11.
8. Mandel ID. The functions of saliva. *J Dent Res.* 1987;66:623–627.
9. Bardow A, Madsen J, Nauntofte B. The bicarbonate concentration in human saliva does not exceed plasma level. *Clin Oral Invest.* 2000;4:245–253.
10. Rezaei A, Sariri R. Salivary diagnosis of periodontal status. A Review. *Pharmacologyonline.* 2011;1:428–444.
11. Wong DT. Salivary diagnostics powered by nanotechnologies, proteomics and genomics. *J Am Dental Assoc.* 2006;137:313–320.
12. Porter SR, Leao JC. Review article: oral ulcers and its relevance to systemic disorders. *Aliment Pharmacol Therapeut.* 2005;21:295–306.
13. Novak MJ, Potter RM, Blodgett J, Ebersoll JA. Periodontal disease in Hispanic Americans with type 2 diabetes. *J Periodont.* 2008;79:629–636.

14. Damirchi A, Sariri R, Kiani M. Response of salivary peroxidase to exercise intensity. *Eur J Appl Physiol*. 2010;108:1233–1237.
15. Gaffney EA, Tiffany JM, Yokoi N. A mass and solute balance model for tear volume and osmolarity in the normal and the dry eye. *Prog Retin Eye Res*. 2010;29:59–78.
16. The epidemiology of dry eye disease. A report of the Epidemiology Subcommittee of the International Dry Eye Workshop. *Ocul Surf*. 2007;5:93–107.
17. Nederfors T, Naunton B, Twetman S. Effects of furosemide and bendroflumethiazide on saliva flow rate and composition. *Arch Oral Biol*. 2004;49:507–513.
18. Simi R, Walter L, Siqueira J. A study of the influence of mouth-breathing in some parameters of unstimulated and stimulated whole saliva of adolescents. *Int J Pediat Otorhinolary*. 2006;70:799–805.
19. Moee S, Calder KAC, Miller NJ. Antioxidant activity of saliva and periodontal disease. *Free Radic*. 1994;21:417–425.
20. Sariri R, Varasteh A, Erfani Karimzadeh A. Inhibition of salivary peroxidase by cigarette smoke health. *Pharmacologyonline*. 2010;2:347–351.
21. Mäkilä E, Kirveskari P. A study of ascorbic acid in human saliva. *Arch Oral Biol*. 1969;14:1285–1292.