A prospective study on peptide mapping of human fatigue saliva markers based on magnetic beads

YANLI XU[1*], DI XIAO[2*], HUIFANG ZHANG[2], LIHUA HE[2], YIXIN GU[2], XIANHUI PENG[2], XIAOHUAN GAO[3], ZHIJUN LIU[1] and JIANZHONG ZHANG[2]

[1]Hebei University of Engineering, Affiliated Hospital, College of Medicine, Handan, Hebei 056002; [2]State Key Laboratory of Infectious Disease Prevention and Control, Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing 102206; [3]Beijing Huawei Tongke Medical Research Center, Beijing 100069, P.R. China

Received August 2, 2018; Accepted January 24, 2019

DOI: 10.3892/etm.2019.7293

Abstract. In order to explore convenient and stable fatigue markers, we studied various high-molecular-weight peptide fragments under fatigue state and non-fatigue state in the saliva using time of flight mass spectrometry. The saliva samples were collected from 10 healthy volunteers that were in the condition of fatigue and non-fatigue, respectively. Moreover, the time of flight mass spectrometry was conducted using two kinds of sample treatment methods, the magnetic beads enrichment (MB) and direct detection of stock solution. This was followed by modeling via the mass spectra of MB and supernatant (stock solution) directly collected after centrifugation. Both MB and direct sampling produced good spectrograms between 1,000 and 15,000 Da, while some peaks were lost in the enrichment. The spectrograms in the early and late period were different in each individual. Due to the limited sample size, 20 early and 20 late spectrograms were used for modeling analysis. Three different peptides were identified in the stock solution samples that can be detected in both fatigue and non-fatigue groups. The cross validity of MB model was 92.06%, while that of the stock solution model was 95.49%. The results showed that there were different peaks within the molecular weight of 2,000-15,000 Da, which provided a scientific basis for further realization of the convenient fatigue detection method based on the biosensor technique, with important theoretical and practical significance.

Correspondence to: Professor Jianzhong Zhang, State Key Laboratory of Infectious Disease Prevention and Control, Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, 155 Changbai Road, Changping, Beijing 102206, P.R. China
E-mail: uzz718@163.com; zhangjianzhong@icdc.cn

Professor Zhijun Liu, Hebei University of Engineering, Affiliated Hospital, College of Medicine, 83 Congtai Road, Congtai, Handan, Hebei 056002, P.R. China
E-mail: liuzhijun_2016@163.com

*Contributed equally

Key words: magnetic bead, fatigue, saliva, peptide mapping

Introduction

In April 2004, the world health organization (WHO) and the world bank released the road traffic report injury prevention, which predicted that by 2020, road traffic injuries will become the third global disease burden, among which fatigue driving is one of the causes (1). The direct and indirect total loss and social cost caused by fatigue are extensive (2). Currently, there is no convenient fatigue detection method, and several research groups are actively studying occupational fatigue detection. In Western developed countries, we see a substantial investment in this field (3). However, the objective data are very difficult to obtain, and the measuring methods and the evaluation indexes are very difficult to quantify (4,5). The sensitivity of existing fatigue detection methods is high; however, it is invasive and the signal paste electrode needs to be extracted.

The percentage of eyelid closure over the pupil over time (PERCLOS) has a high measurement accuracy, and its detection of behavioral characteristics is straightforward. Nevertheless, the detection and identification rules are complex, the pupil measurement information is difficult to extract, and the detection of viewing direction and mouth state is greatly affected by the individual, light and physiological conditions. Additionally, the method has poor reliability and anti-interference performance. Previous findings showed that the microarray technology developed in Japan and the vehicle-mounted module system (6) developed in the USA have improved detection efficiency. However, it is difficult for the method to be popularized due to the complex design and high-cost performance (7,8). Recently, Bhatti *et al* (9,10) reported that the level of DNA damage repair in individuals who work at night (work for 8 h at night and change shifts after 6:00 a.m. the next morning) is very low compared with

that in individuals who sleep at night. The former is only 20% of the latter. The problem associated with this method is the inconvenience associated with urine sampling and the fact that this method can be affected by renal function and drinking. Thus far, there has been no convenient, reliable, non-invasive and highly cost-effective fatigue detection method, and it is imperative that one or a group of clinical markers be identified. This is the present hotspot and difficulty in research worldwide, as well as a major public health problem faced by developing countries and developed countries (11).

The study on fatigue involves the physiology, molecular biomedicine, preventive medicine, computer technology, imaging and other fields. In this study, based on the previous disciplinary studies, the saliva that was clinically collected in a convenient and non-invasive manner was used (8-10). Compared with the innovated blood detection and urine detection affected by kidney and other factors, the saliva secreted by the glands is more stable and easy to be sampled. The prospect of saliva component analysis used for fatigue detection has attracted much attention in China as well as other countries (12). The determination of degree of fatigue via detecting saliva, developed jointly by the University of Toyama and enterprises, is simpler than the method of measuring brain waves. Studies have shown that the salivary gangliosides, cortisol and other small molecules show regular changes in the fatigue state, but it is difficult to establish a convenient detection system. The indices obtained in these studies are usually unstable small molecular substances, which are not conducive to the later promotion and application of fatigue detection (13). In this study, the mature saliva time of flight mass spectrometry at present was performed to identify the different high-molecular-weight peptide segments in the fatigue and non-fatigue states to provide an important theoretical basis for the study on more convenient and stable fatigue markers. The type of fatigue investigated in the current study was the fatigue caused by continuous work. After volunteers' continuous work, fatigue Theta waves were monitored, and the changes of the peptide spectrum was detected in saliva by MALDI-TOF-MS at different time-points. The fatigue wave of ECG was used as the diagnostic criterion to identify fatigue bio-markers. The current study investigated occupational fatigue markers caused by continuous work based on the diagnostic criteria of chronic fatigue syndrome previously described (23). Fukuda *et al* (14) developed the diagnostic standard of fatigue that was recognized as a golden standard by the international medical community, as well as EEG Theta wave, ECG HF and LF/HF ratio changes.

Materials and methods

Inclusion and exclusion criteria. Inclusion criteria were: normal healthy men and women aged 30-45 years, who signed the informed consent, and who were without organic diseases and chronic fatigue symptoms. Exclusion criteria were according to Breithaupt-Groegler *et al* (15): i) individuals with persistent or recurrent fatigue for >6 months; ii) individuals with sore throat; iii) individuals with neck or axillary lymph node swelling and pain; iv) individuals with muscle pain; v) individuals with multiple non-arthritic pain; vi) individuals with headache; vii) individuals with sleep disorders; viii) individuals with discomfort for >24 h after fatigue; ix) individuals with oral disease; x) individuals not taking drugs and dietary nutritional supplements in the last 3 months, and smokers and those who received tooth filling treatment during the week prior to the sampling date were excluded.

Participants. Only 10 healthy volunteers (5 males and 5 females) aged 30-45 years were enrolled due to the high experimental cost. The subjects volunteered to participate in this study and signed the informed consent. Saliva samples were collected from April 10, 2015 to April 12, 2015. The present study was approved by the Ethics Committee of Hebei University of Engineering, Affiliated Hospital, College of Medicine (Handan, China) on March 12, 2014, and informed consent was signed (Clinical Trial Registration no.: ChiCTR-DCD-14005746).

Prior to the study, the 10 subjects had good sleep and underwent an electroencephalogram (EEG) while awake. Since there was no fatigue wave, the subjects were considered to have no fatigue or MS/CFS. Subsequently, they began to work continuously to cause fatigue. Physiological responses were also measured using a fatigue scale. At different time-points, the fatigue response gradually increased and the subjects' saliva was collected. MALDI-TOF-MS peptide peak detection was performed at the end.

Saliva sample collection. i) Saliva sample collection: A Saliva collection tube (ISO9001 certified; Cryo.s™; Greiner Bio-One GmbH, Frickenhausen, Germany) was used. The samples were collected by physicians once prior to shifts commencing and once after at least 18 h. Before sampling, the mouth was rinsed three times with distilled water (30 ml NS for 1 min) and subjects sat quietly for 5 min in front of a mirror in an upright sitting position; the head slightly leaned forward and the eyes were kept open for masticatory movement to stimulate the secretion of saliva. When a certain amount of saliva was accumulated in the lower jaw, the tongue reached the upper jaw, the mouth was opened and the tongue was naturally lifted to form a V shape in the lower lips, allowing the saliva to flow into the saliva collection tube prepared in advance. Then 2 ml of saliva was collected and the interval between sampling and eating and the recipe characteristics were recorded. ii) Saliva sample storage: The sample was labeled by the science assistant, and stored at -70˚C. iii) Saliva sample detection was conducted by the National Key Laboratory of Institute of Infectious Diseases Prevention and Control of Chinese Center for Disease Control and Prevention (MALDI-TOF MS).

EEG detection and data collection. The EEG was obtained after at least 18 h to verify the presence of the fatigue wave (the slow wave increased and fast wave decreased; i.e., the quantities of delta, alpha and beta waves were compared). When the fatigue wave appeared, the saliva was immediately collected. EEG was monitored and obtained using the SOLAR-RTA and SOLAR-BFM brain function monitoring system (manufactured by SOLAR Electronic Technology Limited) for real-time analysis and comprehensive analysis of brain function.

Saliva flight mass spectrometry. Reagents and instruments: WCX magnetic beads kit; α-cyano-4-hydroxycinnamic

acid (HCCA) (both from Bruker, Billerica, MA, USA), mass concentration: 0.3 g/l; ethanol (chromatographic grade)/acetone (chromatographic grade) = 2/1, prepared freshly; AutoFlexIII type matrix assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometer (Bruker). WCX magnetic bead processing: WCX magnetic beads kit was taken from the refrigerator at 4˚C and treated according to the protocol. Finally, the eluted peptide sample solution was transferred into a clean 0.5 ml tube for mass spectrometric analysis. For sample application and mass spectrometric analysis, 1 μl peptide sample solution was separated by magnetic beads and 1 μl saliva was taken and dried at room temperature, followed by the addition of 1 μl HCCA matrix solution in the concentration of 3 g/l (dissolved in 50% acetonitrile and 2% trifluoroacetic acid). The prepared sample panels were placed on the MALDI-TOF mass spectrometer for analysis. Under the linear mode, the relative molecular weight between 1,000 and 10,000 Da with a laser energy of 20% was collected for a total of 400 shots. Beads enrichment (MB) saliva and saliva supernatant (stock solution) taken after centrifugation at 12,000 x g for 10 min at 4˚C were used for mass spectrometric analysis. A scan was performed 50 times, and the peptide mass fingerprint (PMF) was obtained.

Quality control: Before the test, the relevant group members were trained. Saliva was collected and the fatigue state was maintained. The subjects were informed about the details, and the chest cards were issued to the volunteers. The personnel responsible for saliva collection guidance and supervision were assigned. Each member of saliva sampling needed to accept the strict and unified training. The subjects could collect their own samples, and a specially-assigned person was responsible for EEG and supervision of sleep deprivation.

Statistical analysis. The molecular weight of collection ranged from 1 and 15,000 Da, and the standard product was selected for molecular weight correction. To establish the model and conduct the blind screening test, the genetic algorithm (GA) model was established and verified using the Clin Pro Tools (version 3.3.0) software provided by Bruker.

Results

Mass spectrograms of samples. Both MB and direct sampling can detect spectrograms between 1,000 and 15,000 Da (Fig. 1A), and some peaks which had little effect on the results were lost in the enrichment (Fig. 1B). The early and late spectrograms of each person were different (Fig. 1C), and the cross validation was not conducted due to the limited sample size, therefore two spectrograms were taken for each sample. Twenty early and 20 late spectrograms were used for modeling analysis. Three different peptides found in the two groups of stock solution samples have different expression in the fatigue and non-fatigue groups.

With the increase of fatigue, the number of peptides collected in saliva at different time-points increased significantly, especially in the range of high molecular weight (>5,000 Da). Under the same collecting parameters, when the molecular weight was >3,000, the peptide abundance in the fatigue population was significantly increased.

Model identification. The model was established via the mass spectra of MB and supernatant (stock solution) directly collected after centrifugation. A GA model was constructed to distinguish the fatigue and non-fatigue groups. The two parameters of cross-validation and recognition capability were calculated by ClinProTools (Bruker). For the GA typing model, the crossvalidation values, which reflect the model's ability to handle variability among test spectra, and the recognition capability value, which reflects the model's ability to correctly identify its component spectra, were all 100%. The cross validity of the MB model was 92.06%, while that of the stock solution model was 95.49% (Table I). The strain distribution map based on the GA classification model also showed that the fatigue and non-fatigue groups could be divided into one of two categories based on their PMFs (Fig. 2).

Discussion

The damage caused by fatigue every year accounts for 21.7% in the global occupational injuries, and 57% of traffic associated deaths can be linked to fatigue (16). In China, approximately 600,000 individuals lose their lives annually due to this type of accident (17). However, there is no non-invasive and convenient fatigue testing technology, similar to that for detecting drivers' alcohol level. In the Center for Health Studies of University of Sydney, the EEG signals collected were treated with artificial neural network and it was found that Theta wave was increased significantly under fatigue state, which provided the basis for the detection of fatigue (18). EEG is also known as the golden standard for testing fatigue, which can be used as the reference of diagnostic test (19). However, the signal must be extracted using the electrode in contact with the human body (20). At present, there is no golden standard for the diagnosis of fatigue, and most of the tools for diagnosing fatigue are fatigue scale, such as in literature (21-23). Fukuda *et al* (14) developed the diagnostic standard of fatigue that was recognized as a golden standard by the international medical community, as well as EEG Theta wave, ECG HF and LF/HF ratio changes. The fatigue diagnostic devices in the past were mainly the driving anti-sleep device (24), eye movement measurement system (25), automobile driver fatigue evaluation method (26) and high-speed image processing chip TMS320DM642 fatigue detection and early warning system (TI). The cost associated with these methods is high and its popularity is also limited (27). In the present study, the time-of-flight mass spectrometer was used for analysis of saliva samples obtained from a number of individuals under the limited fatigue state. Significant differences were found in the detected spectrograms, and these spectrograms were obtained within 2,000-15,000 Da by the MB and direct sampling. Several differential peptides were found and the saliva peptide mapping showed significant changes with a certain rule with the appearance of fatigue. These results proved the feasibility of fatigue identification via the analysis of saliva marker peptide. The model was established using the mass spectrums of MB and supernatant (stock solution) directly collected after centrifugation. The cross validity of MB model was 92.06%, while that of stock solution model was 95.49%.

In recent years, saliva proteomics research has attracted wide attention (28), and 309 different proteins have been

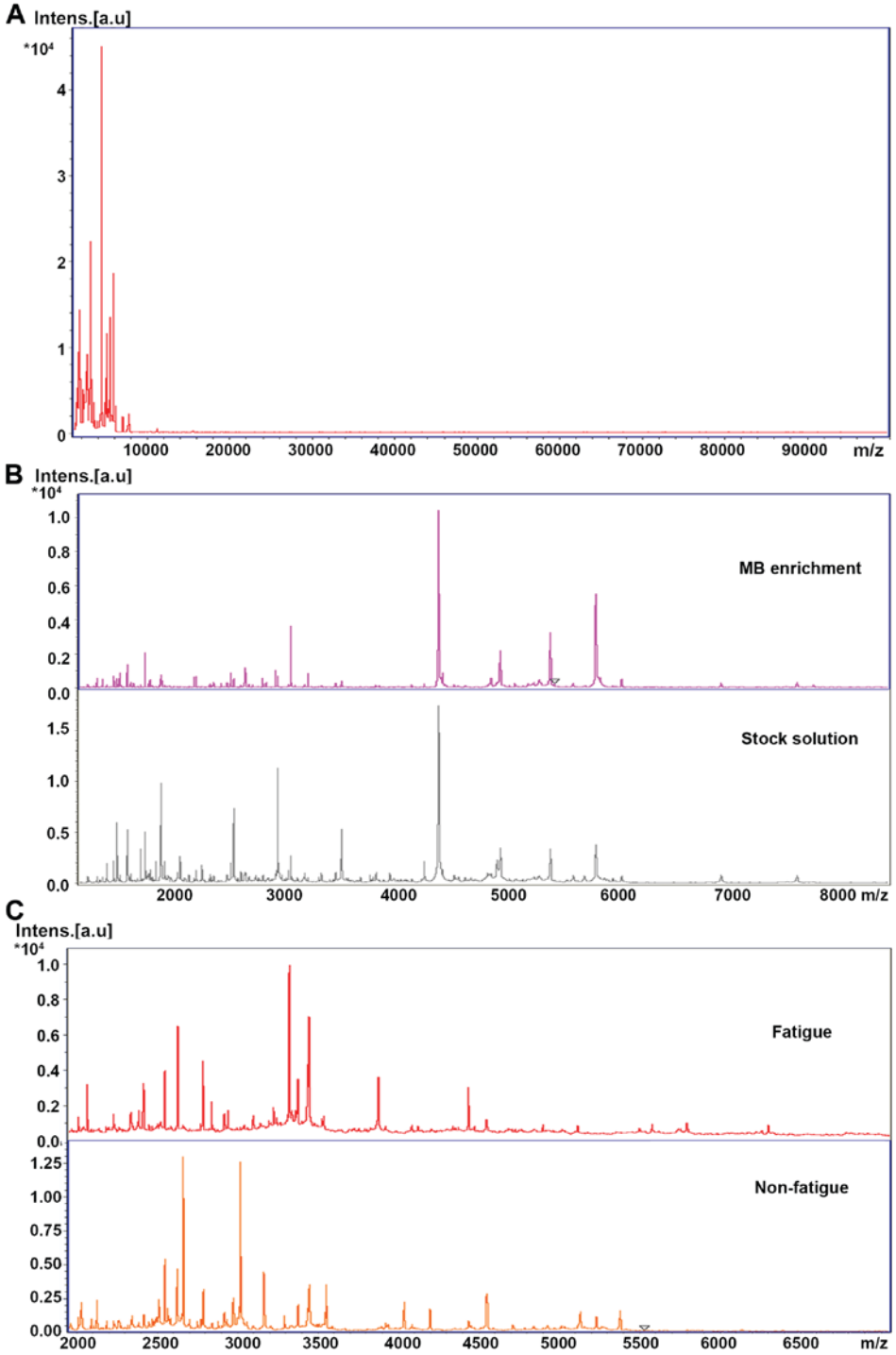

Figure 1. Mass spectrograms of samples. The horizontal coordinate indicates the intensity of the spectrum, and the longitudinal coordinate represents the mass to charge ratio. (A) Stock solution detection: almost no detection peak in the area exceeding 15,000 Da; (B) MB and stock solution (supernatant directly taken after centrifugation) saliva mass spectrogram; (C) Individual 1: comparison of early and late sample spectrograms. MB, bead enrichment.

identified successfully using the proteomics technique from normal human whole saliva. By dividing the functions of these proteins, proteins with unknown functions account for the most (28.7%), the immune-related proteins account for 21%, whereas proteins with functions linked to replication and repair accounted for 1.6%. In addition, proteins involved in the cell dynamics and those secretion-related account for 4.8%, transcription and ribose-related proteins account for 2.3%, the cell proliferation and cell cycle-related proteins account for 4.2%, signal transduction proteins for 9.7%, metabolism-related protein for 5.2%, and cytoskeleton and intima-related proteins for 7.1% (29). The unique and rich proteins in saliva, as a body fluid with a complex ingredient and a variety of biological functions, are undoubtedly the potential source for ideal biomarkers for tumors and other diseases. For a long time, the original technology limited the flux analysis and accurate determination of oral saliva protein; thus, the biological functions of most saliva proteins are unknown, and the potential role of saliva in the diagnosis and prognosis of human diseases is not apparent. With the

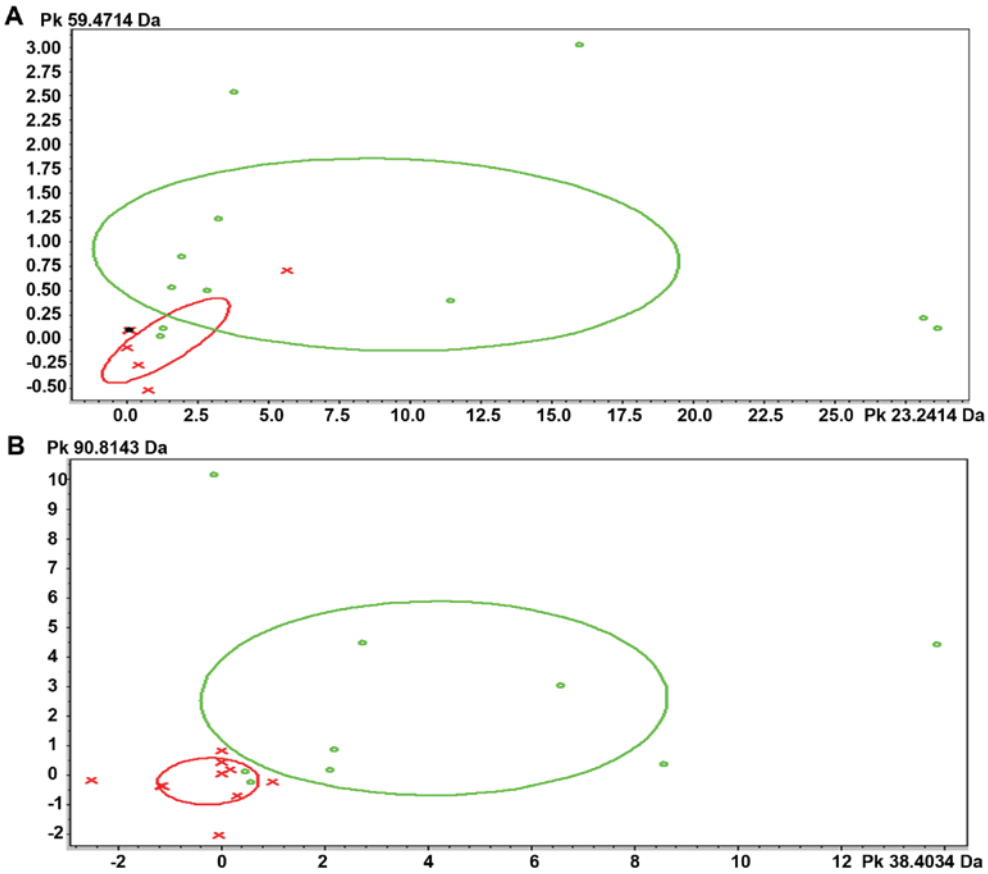

Figure 2. ClinProTools 2D peak distribution diagrams of two models: MB group (A), stock solution group (B). MB, bead enrichment.

Table I. Basic information, cross validity (%) and acceptability (%) of the two models.

Model name	Algorithm	Cross validation	Recognition capability
MB	GA	92.06%	100%
Stock solution	GA	95.49%	100%

MB, beads enrichment; GA, genetic algorithm.

application of high-throughput and high-precision proteomics, it has been possible to apply biomarkers of saliva proteins in the early diagnosis and prevention of different illnesses, bio-protein-targeted therapy and prognostic monitoring (30). This also served as the theoretical basis for selecting saliva in the present study.

Fatigue is a complex biochemical process, and a single biochemical index cannot accurately determine whether the body suffers from fatigue or not. The proteomics study of saliva provides a new direction for the detection of fatigue through saliva. Saliva contains a variety of bioactive ingredients, including some biomarkers that can be used for disease diagnosis (31). Compared with blood and urine, the collection process of saliva is simpler and it is completely non-invasive. The role of saliva, instead of blood and urine, in the non-invasive diagnosis of disease, has become increasingly apparent. Studies have shown that small molecules, such as salivary gangliosides (molecular weight: 1,563.85 Da) and cortisol (362.47 Da), are likely to show regular changes under fatigue state, but it is difficult to establish a convenient detection system (32). The prospect of saliva component analysis used for fatigue detection has begun to attract much attention in China as well as other countries. Compared with the method that uses brain waves for the determination of fatigue degree, saliva is simpler and less invasive. This method was originally developed by University of Toyama and enterprises.

The mechanism of fatigue is very complex. It has been argued that fatigue was the result of neuro-endocrine-immune network dysfunction caused by a variety of infections and stress, while recent studies have further found that the genetic and metabolic factors may also be involved (33). Many studies also investigated the changes in blood chemical composition during the long-term exercise, however the exact pathogenesis is unclear and biomarkers to be used for diagnosis, prevention and treatment has not been found yet. Currently, there is no universally accepted assessment criterion for fatigue. An ideal assessment method for fatigue degree determination can be used for real-time non-invasive assessment with high sensitivity and stability, and can effectively prevent and reduce risks associated with fatigue. Saliva can be obtained in a non-invasive manner. There are several reports on changes in saliva composition (34), most of which have been focused on the changes in the levels of metabolic products in the saliva of athletes and soldiers. Besides, the study on fatigue in the medical field is also a marginalized problem (35), and the emphasis lies on

the analysis of small molecular substances with the molecular weights below 1,000 Da (36-39). The fatigue-related indexes found in previous studies included cortisol, testosterone and dehydroepiandrosterone (40). Indexes obtained in those studies are small and unstable molecules, which were not conducive to the promotion and application of fatigue testing. For example, Kume *et al* (41) established the fatigue rat model and found that valine (molecular weight: 117.15 Da), leucine (131.18 Da) and isoleucine (131.17 Da) were significantly increased in the fatigue group compared with those in controlled feeding group, but the levels of citrulline and hydroxyproline were significantly reduced. The level of plasma total nitric oxide in fatigue group was increased, suggesting systemic oxidative stress. In addition, the plasma metabolites in fatigue group were involved in citric acid cycle, such as cis-aconitic acid and isocitrate (42). Mathematical model analysis was performed in several studies to screen the different indexes from the healthy control group from dozens of metabolites, such as cis-aconitate, socitrate, citrate and malate (43). Michael *et al* (44) found through metabolomics analysis that there were metabolites used as the fatigue markers in the saliva of athletes after three-day soccer game, namely 3-methylhistidine (short form: 3M-His), glucose-1-phosphate (short form: G-1-P), glucose 6-phosphate (short form: G-6-P) and some amino acid compound. However, the diagnostic efficacy of these markers was not further analyzed. Recently, Kataoka *et al* (45) measured the levels of testosterone (TES), cortisol (CRT) and dehydroepiandrosterone (DHEA) in saliva using the automatic in-tube solid-phase micro-extraction (SPME) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) and found that SPME and LC-MS/MS had a good linear relation ($R \geq 0.9998$), precision (intra-day and inter-day precision was 4.9 and 8.5%) and detection sensitivity (limit of quantitation was ~0.01, 0.03 and 0.29 ng/ml saliva) after the treatment of 40 μl samples. Furthermore, this method was also used to analyze the changes in the levels of TES, CRT and DHEA in saliva under pressure and fatigue, showing the advantages of mass-spectrometric technique.

Changes in such small molecules may be associated with fatigue, but these molecules are also susceptible to diets and other health conditions. At the same time, these small molecule markers often lack good antigenicity, and are not easy to detect using the simple methods, such as immunology or biosensor. In this study, the time-of-flight mass spectrometer was used and the results showed that there were significant differences in the spectrograms obtained by the MB and direct sampling within 2,000-15,000 Da. Several differential peptides were detected between groups and the saliva peptide mapping showed significant changes. These results proved the feasibility of fatigue identification via the analysis of saliva marker peptide. The molecular weight range of the markers obtained in this study laid a good foundation for finding the large molecular weight proteome in the later stage. Moreover, the large molecular weight indexes are stable, providing a theoretical basis for finding the fatigue detection indexes similar to the markers used in alcohol detection.

According to the 'Nihon Keizai Shimbun' (46), the number of special glucocorticoids in blood may increase during the fatigue, and saliva will secrete α-amylase. The so-called 'heart instrument' is equipped with a chip: The human saliva is placed on the chip, and then the chip is pasted to the instrument to detect the degree of fatigue. The saliva samples of Wiewelhove *et al* (47) were obtained from 9 cyclists who participated in a cross-study study aiming at simulating the long-term manual labor, military action and fire rescue during the rest period. The results of this study showed that the distribution difference in fatigue biomarker index (FBI) of subjects was decreased with the increase of the time of physical activity. The self-reported fatigue degree had a significantly positive correlation with the participants' CFS and serum leptin (16,000 Da) in healthy control, supporting our main hypothesis. The machine learning algorithm can distinguish the 78.3% low fatigue days in the high CFS group (48). Related studies found through the conventional evaluation of fatigue and recovery during the high-intensity interval training that creatine kinase (42,000 Da) had statistically significant difference during the fatigue and rest periods (47). The above research confirmed the correctness of results in this study indirectly.

Due to the high cost of this study, the sample size was not large. After receiving fund support, we aim to expand the sample size and carry out further study in different groups of individuals.

We concluded that there were different peaks within the molecular weight of 2,000-15,000 Da, which laid a good foundation for the later study on proteomics-based fatigue markers. The idea of fatigue-related biomarkers within 2,000-15,000 Da in this study was also characterized by the stable composition under test, less interference factors *in vivo* and easy conversion and popularization of the detection system. The exploration of fatigue-related biomarkers has theoretical significance and application prospects. In the future, the fatigue queue will be built to find the stable and specific biomarkers and its combinations under the fatigue state and establish the fatigue-related biomarker evaluation model using the mature mass spectrometry high-throughput sample analysis technique. Compared with the previous studies on saliva metabolomics, proteomics results are more stable. In further research, it is expected to systematically establish the saliva identification spectrum that can be used for fatigue identification, so as to provide a scientific basis for the further realization of convenient fatigue detection methods based on the biosensor technique, which has both important theoretical and practical significance.

Acknowledgements

Not applicable.

Funding

This research was supported by the National Natural Science Foundation of China (Project no. 81373095. 'Selection and Valuation of Fatigue Related Biological Marker in the Human Saliva'.

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

YX and DX drafted the manuscript. YX, DX, HZ and LH were mainly devoted to collecting and interpreting the data. YG and XP helped with electroencephalogram detection. XG, ZL and JZ were responsible for the saliva flight mass spectrometry analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Hebei University of Engineering, Affiliated Hospital, College of Medicine (Handan, China). Signed written informed consents were obtained from the volunteers.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Peden M, Scurfield R, Sleet D, Mohan D, Hyder AA, Jarawan E and Mathers C (Eds.): World report on road traffic injury prevention. World Health Organisation, Geneva, p217, 2004.
2. Sansó N, Galiana L, Oliver A, Pascual A, Sinclair S and Benito E: Palliative care professionals' inner life: Exploring the relationships among awareness, self-care, and compassion satisfaction and fatigue, burnout, and coping with death. J Pain Symptom Manage 50: 200-207, 2015.
3. Keltai M: Johnson JA, Kowey PR, Ried LD and Tueth M: INVEST substudies: Design and patient characteristics. Clin Cardiol 24 (Suppl 11): V9-V11, 2001.
4. Yang G, Lin Y and Bhattacharya P: A driver fatigue recognition model using fusion of multiple features. In: 2005 IEEE International Conference on Systems, Man and Cybernetics. Vol 2. IEEE Xplore, Waikoloa, pp1777-1784, 2006.
5. Ting PH, Hwang JR, Doong JL and Jeng MC: Driver fatigue and highway driving: A simulator study. Physiol Behav 94: 448-453, 2008.
6. Wishaw KJ, Munford BJ and Roby HP: The CareFlight Stretcher Bridge: A compact mobile intensive care unit. Anaesth Intensive Care 18: 234-238, 1990.
7. Sommer D and Golz M: Evaluation of PERCLOS based current fatigue monitoring technologies. Conf Proc IEEE Eng Med Biol Soc 2010: 4456-4459, 2010.
8. Daza IG, Bergasa LM, Bronte S, Yebes JJ, Almazán J and Arroyo R: Fusion of optimized indicators from Advanced Driver Assistance Systems (ADAS) for driver drowsiness detection. Sensors (Basel) 14: 1106-1131, 2014.
9. Bhatti P, Cushing-Haugen KL, Wicklund KG, Doherty JA and Rossing MA: Nightshift work and risk of ovarian cancer. Occup Environ Med 70: 231-237, 2013.
10. Bhatti P, Mirick DK, Randolph TW, Gong J, Buchanan DT, Zhang JJ and Davis S: Oxidative DNA damage during night shift work. Occup Environ Med 74: 680-683, 2017.
11. Malmir K, Olyaei GR, Talebian S, Jamshidi AA and Ganguie MA: Effects of peroneal muscles fatigue on dynamic stability following lateral hop landing: Time to stabilization vs. dynamic postural stability index. J Sport Rehabil: Sep 27, 2018 (Epub ahead of print).
12. Zanotti L, Paderno A, Piazza C, Pagan E, Bignotti E, Romani C, Bandiera E, Calza S, Del Bon F, Nicolai P, *et al*: Epidermal growth factor receptor detection in serum and saliva as a diagnostic and prognostic tool in oral cancer. Laryngoscope 127: E408-E414, 2017.
13. Zhang Q and Xiong ZY: The biologic mechanism of the central fatigue. J Phys Educ 10: 42-44, 2003 (In Chinese).
14. Fukuda K, Straus SE, Hickie I, Sharpe MC, Dobbins JG and Komaroff A; International Chronic Fatigue Syndrome Study Group: The chronic fatigue syndrome: A comprehensive approach to its definition and study. Ann Intern Med 121: 953-959, 1994.
15. Breithaupt-Groegler K, Coch C, Coenen M, Donath F, Erb-Zohar K, Francke K, Goehler K, Iovino M, Kammerer KP, Mikus G, *et al*: Who is a 'healthy subject'? - consensus results on pivotal eligibility criteria for clinical trials. Eur J Clin Pharmacol 73: 409-416, 2017.
16. Dasari D, Shou G and Ding L: ICA-derived EEG correlates to mental fatigue, effort, and workload in a realistically simulated air traffic control task. Front Neurosci 11: 297, 2017.
17. Polo-Kantola P, Laine A, Aromaa M, Rautava P, Markkula J, Vahlberg T and Sillanpää M: A population-based survey of sleep disturbances in middle-aged women--associations with health, health related quality of life and health behavior. Maturitas 77: 255-262, 2014.
18. Hu W, Li Y, Sun Y and Mosleh A: A model of BGA thermal fatigue life prediction considering load sequence effects. Materials (Basel) 9: E860, 2016.
19. Kappel SL, Looney D, Mandic DP and Kidmose P: Physiological artifacts in scalp EEG and ear-EEG. Biomed Eng Online 16: 103, 2017.
20. Rottoli M, La Gioia S, Frigeni B and Barcella V: Pathophysiology, assessment and management of multiple sclerosis fatigue: An update. Expert Rev Neurother 17: 373-379, 2017.
21. De Vries J, Michielsen H, Van Heck GL and Drent M: Measuring fatigue in sarcoidosis: the Fatigue Assessment Scale (FAS). Br J Health Psychol 9: 279-291, 2004.
22. Miao Y, Liu X, Liu W, Xie H and Deng G: Initial revision of the Chinese version of multidimensional fatigue inventory-20 in medical staff of military basic level. Chin Ment Health J 22: 658-660, 668, 2008 (In Chinese).
23. Schwartz JE, Jandorf L and Krupp LB: The measurement of fatigue: A new instrument. J Psychosom Res 37: 753-762, 1993.
24. King LM, Nguyen HT and Lal SK: Early driver fatigue detection from electroencephalography signals using artificial neural networks. Conf Proc IEEE Eng Med Biol Soc 1: 2187-2190, 2006.
25. Fu R and Wang H: Detection of driving fatigue by using noncontact EMG and ECG signals measurement system. Int J Neural Syst 24: 1450006, 2014.
26. Hostens I and Ramon H: Assessment of muscle fatigue in low level monotonous task performance during car driving. J Electromyogr Kinesiol 15: 266-274, 2005.
27. Jarrett MA, Gable PA, Rondon AT, Neal LB, Price HF and Hilton DC: An EEG Study of Children With and Without ADHD Symptoms: Between-group differences and associations with sluggish cognitive tempo symptoms. J Atten Disord: Aug 1, 2017 (Epub ahead of print).
28. Philip P, Sagaspe P, Taillard J, Moore N, Guilleminault C, Sanchez-Ortuno M, Akerstedt T and Bioulac B: Fatigue, sleep restriction, and performance in automobile drivers: A controlled study in a natural environment. Sleep 26: 277-280, 2003.
29. Xu YL, Gong YN, Xiao D, Zhao CX, Gao XH, Peng XH, Xi AP, He LH, Lu LP, Ding M, *et al*: Discovery and identification of fatigue-related biomarkers in human saliva. Eur Rev Med Pharmacol Sci 22: 8519-8536, 2018.
30. Lim SM and Chia SE: The prevalence of fatigue and associated health and safety risk factors among taxi drivers in Singapore. Singapore Med J 56: 92-97, 2015.
31. Korostoff A, Reder L, Masood R and Sinha UK: The role of salivary cytokine biomarkers in tongue cancer invasion and mortality. Oral Oncol 47: 282-287, 2011.
32. Francischetti IM, Ma D, Andersen JF and Ribeiro JM: Evidence for a lectin specific for sulfated glycans in the salivary gland of the malaria vector, Anopheles gambiae. PLoS One 9: e107295, 2014.
33. Singh VP, Khandelwal B and Sherpa NT: Psycho-neuro-endocrine-immune mechanisms of action of yoga in type II diabetes. Anc Sci Life 35: 12-17, 2015.
34. Sun W, Zhang X, Peeta S, He X, Li Y and Zhu S: A self-adaptive dynamic recognition model for fatigue driving based on multi-source information and two levels of fusion. Sensors (Basel) 15: 24191-24213, 2015.
35. Hu S, Loo JA and Wong DT: Human saliva proteome analysis and disease biomarker discovery. Expert Rev Proteomics 4: 531-538, 2007.
36. Castro M, Elias PC, Martinelli CE Jr, Antonini SR, Santiago L and Moreira AC: Salivary cortisol as a tool for physiological studies and diagnostic strategies. Braz J Med Biol Res 33: 1171-1175, 2000.

37. Makary MA and Daniel M: Medical error - the third leading cause of death in the US. BMJ 353: i2139, 2016.
38. You L, Ren J, Yang B, Regenstein J and Zhao M: Antifatigue activities of loach protein hydrolysates with different antioxidant activities. J Agric Food Chem 60: 12324-12331, 2012.
39. Wu CC, Liao MH, Chen SJ and Yen MH: Tetramethylpyradizine prevents inducible NO synthase expression and improves survival in rodent models of endotoxic shock. Naunyn Schmiedebergs Arch Pharmacol 360: 435-444, 1999.
40. Al-Turki HA: Dehydroepiandrosterone supplementation in women undergoing assisted reproductive technology with poor ovarian response. A prospective case-control study. J Int Med Res 46: 143-149, 2017.
41. Kume S, Yamato M, Tamura Y, Jin G, Nakano M, Miyashige Y, Eguchi A, Ogata Y, Goda N, Iwai K, *et al*: Potential biomarkers of fatigue identified by plasma metabolome analysis in rats. PLoS One 10: e0120106, 2015.
42. Jones RL, Owen LJ, Adaway JE and Keevil BG: Simultaneous analysis of cortisol and cortisone in saliva using XLC-MS/MS for fully automated online solid phase extraction. J Chromatogr B Analyt Technol Biomed Life Sci 881-882: 42-48, 2012.
43. Ra SG, Maeda S, Higashino R, Imai T and Miyakawa S: Metabolomics of salivary fatigue markers in soccer players after consecutive games. Appl Physiol Nutr Metab 39: 1120-1126, 2014.
44. Michael DJ, Valle B, Cox J, Kalns JE and Fogt DL: Salivary biomarkers of physical fatigue as markers of sleep deprivation. J Clin Sleep Med 9: 1325–1331, 2013.
45. Kataoka H, Ehara K, Yasuhara R and Saito K: Simultaneous determination of testosterone, cortisol, and dehydroepiandrosterone in saliva by stable isotope dilution on-line in-tube solid-phase microextraction coupled with liquid chromatography-tandem mass spectrometry. Anal Bioanal Chem 405: 331-340, 2013.
46. Michael DJ, Daugherty S, Santos A, Ruby BC and Kalns JE: Fatigue biomarker index: An objective salivary measure of fatigue level. Accid Anal Prev 45 (Suppl): 68-73, 2012.
47. Wiewelhove T, Raeder C, Meyer T, Kellmann M, Pfeiffer M and Ferrauti A: Markers for routine assessment of fatigue and recovery in male and female team sport athletes during high-intensity interval training. PLoS One 10: e0139801, 2015.
48. Stringer EA, Baker KS, Carroll IR, Montoya JG, Chu L, Maecker HT and Younger JW: Daily cytokine fluctuations, driven by leptin, are associated with fatigue severity in chronic fatigue syndrome: evidence of inflammatory pathology. J Transl Med 11: 93, 2013.