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ORIGINAL ARTICLE

Autonomic dysfunction in patients with irritable bowel syndrome evidenced by alterations of salivary alpha-amylase secretion

Ling He [1], Mingjun Xie[1], Hong Zhang[2], Linfeng Meng[2], Xiaoping Zhang[1]

1 The Affiliated Hospital of Jiangxi University of Traditional Chinese Medicine, Nanchang, Jiangxi, China
2 Jiangxi University of Traditional Chinese Medicine, Nanchang, Jiangxi, China

Correspondence to: Ling He
Department of Gastroenterology, The Affiliated Hospital of Jiangxi University of Traditional Chinese Medicine
No. 445, Bayi Avenue, Nanchang 330006, Jiangxi, China
TEL.: 86-0791-86361147; E-MAIL: heling118@126.com

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Abstract

BACKGROUND: Patients with irritable bowel syndrome (IBS) frequently present with alterations of autonomic activity, especially higher sympathetic activity. Salivary alpha-amylase (sAA) has been implicated as a non-invasive biomarker to reflect the sympathetic activity. Thus, the current study aimed to determine if alterations of sAA secretion could be addressed in IBS patients.

METHODS: We recruited twenty-five IBS patients as well as twenty-four age- and sex-matched healthy controls (HCs). Basal and stimulated (by gustatory stimulation with citric acid) saliva samples were collected from each participant, with respective salivary flow rate (SFR) calculated accordingly. Western blotting (WB) was applied to determine the sAA amount by introducing human sAA protein of known quantity. Then the sAA amount ratio was calculated, as expressed by the stimulated sAA amount to basal sAA amount.

RESULTS: We observed high variability of the basal and stimulated sAA amount in both groups. An apparently higher prevalence of psychiatric disorders was detected in the IBS group, which was consistent with previous studies. Interestingly, we found elevated basal sAA amount in the IBS patients relative to HCs, which implicated higher sympathetic activities in IBS population. Moreover, we observed blunted sAA response to the gustatory stimulation in the IBS patients, which might be of pathophysiological importance for IBS.

CONCLUSION: This is the first attempt to associate sAA secretion with the pathophysiology of IBS. Our results suggest an autonomic dysfunction in IBS patients.

Abbreaviations:

IBS	- irritable bowel syndrome;	NE	- noradrenaline;
sAA	- Salivary alpha-amylase;	BAI	- Beck Anxiety Inventory;
HC	- healthy control;	BDI	- Beck Depression Inventory;
SFR	- salivary flow rate;	SD	- standard deviation
WB	- western blotting;		

INTRODUCTION

Irritable bowel syndrome (IBS) is a common functional gastrointestinal disorder characterized by recurrent abdominal pain and altered bowel patterns (Longstreth *et al.* 2006). The typical IBS symptoms include recurrent abdominal pain, discomfort, bloating, and altered bowel habits such as constipation and diarrhea. The disease affects up to 15 % of the adult population (Camilleri & Choi 1997). Despite the huge amount of studies conducted, the pathophysiology of IBS remains yet to be explored.

IBS patients have high prevalence of psychiatric disorders, including anxiety and major depression (Lydiard *et al.* 1993; Walker *et al.* 1990). Alteration in noradrenergic signaling has been implicated in the pathophysiology of IBS (Berman *et al.* 2012), and adrenergic receptors have been demonstrated as potential drug targets (Camilleri *et al.* 2003). Amazingly, Orr and colleagues found that IBS patients presented autonomic abnormalities as evidenced by greater sympathetic activity during waking and greater overall sympathetic dominance during rapid eye movement sleep in IBS patients (Orr *et al.* 2000). The results were further supported by a follow-up study by the same research team, in which the greater sympathetic dominance was evidenced by elevated low-frequency/light-frequency band ratio during rapid eye movement sleep (Thompson *et al.* 2002). Interestingly, a different research group also found an excess sympathetic activity in IBS patients as evaluated by fingertip blood flow (Tanaka *et al.* 2008). Mechanistically, the higher plasma noradrenaline (NE) might be able to explain the higher sympathetic activity of IBS patients (Berman *et al.* 2012).

Salivary alpha amylase (sAA) is one of the most abundant proteins in human saliva (Rohleder & Nater 2009). It's been well documented that sAA secretion is predominantly controlled by sympathetic nervous system via the release of NE from sympathetic neurons (Nater & Rohleder 2009). Recently, sAA has been proposed as a sensitive and non-invasive biomarker to reflect activity of the sympathetic nervous system, and an accumulating body of research further supports the validity and reliability of this parameter (Nater & Rohleder 2009). Because the literature implicates an elevated sympathetic activity in IBS patients as aforementioned, we speculate that alterations of sAA secretion might be detected among the IBS population. If so, sAA might serve as a non-invasive biomarker for IBS patients. Based on the consideration, we thus recruited IBS patients (and healthy controls) to investigate whether alterations of the sAA secretion could be addressed in IBS populations.

MATERIALS AND METHODS

Ethics statement

The current study was performed adhering to the Declaration of Helsinki. All procedures involving human subjects were reviewed and approved by the Academic Ethics Committee of Jiangxi University of Traditional Chinese Medicine. A written informed consent was collected from all participants.

Participants

Twenty-five IBS patients meeting Rome II criteria and twenty-four age- and sex-matched healthy controls (HCs) recruited from June 2015 to November 2016 completed the current study. Because IBS patients frequently present with psychiatric disorders, especially anxiety and major depression (Lydiard 2001), that may probably modify the saliva secretion and even its contents, thus we used Beck Anxiety Inventory (BAI) (Beck *et al.* 1988) and Beck Depression Inventory (BDI) (Beck *et al.* 1961) to evaluate the two psychiatric disorders among the IBS patients. Structured clinical interviews were performed by one of the author, L. He, and subjects were excluded if they used psychotropic substances or painkillers within one year. Participants with alcohol/substance abuse or suffered from recent respiratory/oral diseases were also excluded. Because caffeine ingestion can alter salivary secretion, we thus excluded those who reported acute caffeine consumption. All participants were free of sever somatic diseases.

Collection of the basal and stimulated saliva

Saliva collections were performed before breakfast in the morning. Participants were instructed not to eat or drink or take exercise 2 hours before saliva collection because these factors apparently affect salivary contents (Rohleder & Nater 2009). For collection of basal/unstimulated saliva, the passive drooling method was introduced as described by Navazesh (Navazesh 1993). Then we used citric acid to collect stimulated saliva according to the method developed by Chen et al (Chen *et al.* 2015). After collections, one freeze-thaw cycle was applied for all saliva samples to break down mucopolysaccharides that can interfere with pipetting. Upon thawing at room temperature, the samples were centrifuged at 13000 g for 15 minutes, followed by collection of supernatant for subsequent measurements of the basal and stimulated sAA amount.

Determination of sAA amount

We applied western blotting (WB) to determine the basal and stimulated sAA amount (μg/ml). Briefly, total protein of the processed saliva sample was measured by BCA Protein Assay Kit (Beyotime Biotechnology, Shanghai, China). Then the samples of equal quantity of total protein (basal saliva: 4 μg; stimulated saliva: 2 μg) were mixed and solubilized with SDS-PAGE loading buffer (Beyotime Biotechnology), followed by boiling for 10 minutes. For quantification purpose, a human sAA sample (Sigma-Aldrich, Shanghai, China) of known quantity was also loaded and run. Proteins were separated by SDS-PAGE and transferred onto a nitrocellulose membrane, followed by block-

ing of the membrane at room temperature for 2 hours. Then the membranes were incubated with rabbit anti-salivary alpha amylase antibody (Abcam, Hongkong) at room temperature for 2 hours. After thorough washing, the membranes were further incubated with goat anti-rabbit IgG-horseradish peroxidase conjugate (R&D Systems, USA) at room temperature for 1 hour. Then the membranes were exposed to 3,3'-diaminobenzidine substrate (Beyotime Biotechnology) for detection of the sAA protein band (glycosylated and non-glycosylated). The sAA amount (μg/ml) of the test samples were estimated by comparing with the respective standard sAA protein of known quantity.

Statistical analyses

STATISTICA version 10 (Dell, USA) was used to conduct the statistical analyses. Graph preparations were carried out by GraphPad Prism 5 (GraphPad Software, USA) and Microsoft Office Excel 2007. Data was expressed as mean±standard deviation (SD), otherwise indicated. Student's *t* test was applied to compare means between groups (IBS vs. healthy control), otherwise indicated. A *p* value of less than 0.05 was considered as statistically significant.

RESULTS

Participant characteristics

Table 1 summarizes the main characteristics of the two study groups, IBS patients and healthy controls. No significant difference was detected for age, sex, basal salivary flow rate (SFR), or stimulated SFR. It's noteworthy that we detected a high prevalence of psychiatric disorders in the current IBS patients, of whom 48 % (12/25) and 28 % (7/25) were recognized as presenting anxiety and major depression, respectively. Besides, we observed that participants secreted apparently more saliva after gustatory stimulation with citric acid (HC: 1.81±0.56 ml/min vs. 0.36±0.12 ml/min; IBS: 2.02±0.65 ml/min vs. 0.29±0.16 ml/min).

Tab. 1. Characteristics of the IBS patients and healthy controls

Characteristics	HC	IBS	*p*
no. of participant	26	25	n.s.
age, yr	36±6.8	38±8.2	n.s.
sex (female, male)	16,8	18,7	n.s.
duration of IBS, yr	–	5.3±2.6	–
psychiatric disorders			
anxiety, %	0	48	***
major depression, %	0	28	***
basal SFR, ml/min	0.36±0.12	0.29±0.16	n.s.
stimulated SFR, ml/min	1.81±0.56	2.02±0.65	n.s.

***$p < 0.001$ (*chi*-square test); HC, healthy control; SFR, salivary flow rate.

Elevated basal sAA secretion in IBS patients

Two dominant forms of sAA can be spontaneously detected in human saliva, i.e. the glycosylated and non-glycosylated sAA, of which the molecular weight is 62 kD and 56 kD, respectively. The sAA amount is a sum of amount of the two portions. In the current study, a standard human sAA protein was introduced to quantify the sAA amount in the test samples. We detected significant variations of sAA amount, ranging from 30 μg/ml to 215 μg/ml and from 47 μg/ml to 436 μg/ml in the healthy volunteers and IBS subjects, respectively, which was consistent with previous studies (Mandel *et al.* 2010; Chen *et al.* 2015). Interestingly, we observed that IBS patients had higher basal sAA amount relative to the healthy controls ($p < 0.05$), as shown in Figure 1A. Considering that the two study groups did not differ in the basal SFR, it could be inferred that the IBS patients probably secreted more basal sAA than that of the control group. Figure 1B is a representative WB image showing that the sAA protein is comprised of glycosylated and non-glycosylated portions and that IBS patients possessed higher amount of basal sAA relative to the healthy controls.

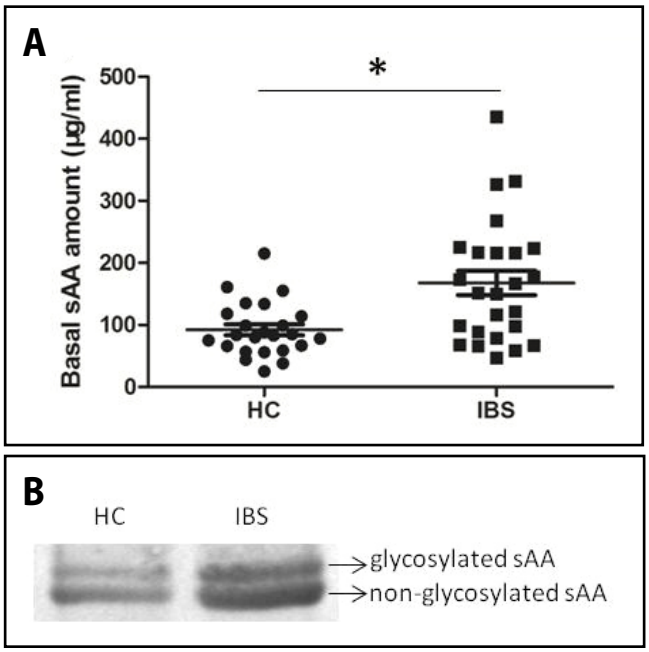

Fig. 1. Elevated basal sAA amount in IBS patients relative to the healthy controls. A: Comparison of the basal sAA amount (μg/ml) between IBS patients (IBS, n=25) and the healthy control (HC, n=24). Values are means, with S.E.M. represented by vertical bars. B: Representative WB image of sAA protein consisting glycosylated and non-glycosylated portion, as indicated. *$p<0.05$, IBS vs. HC.

Comparable stimulated sAA secretion between IBS patients and healthy controls

Saliva secretion responds to various factors, including gustatory stimuli. Because IBS patients were observed to secrete more basal sAA, next we determined whether

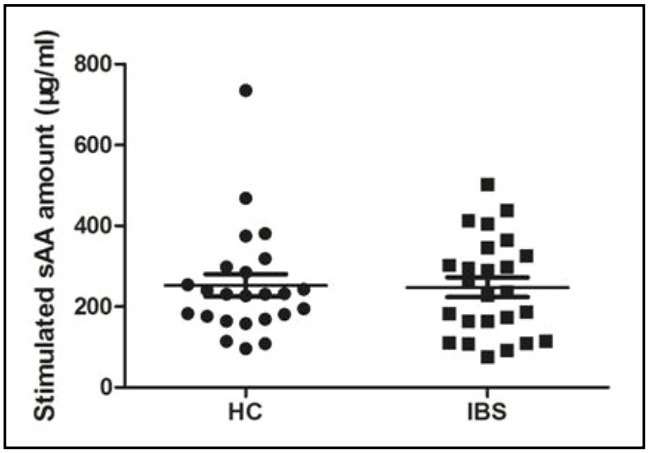

Fig. 2. Comparable level of stimulated sAA amount between IBS patients and the healthy controls. Comparison of the stimulated sAA amount (μg/ml) between IBS patients (IBS, n=25) and the healthy control (HC, n=24). Values are means, with S.E.M. represented by vertical bars.

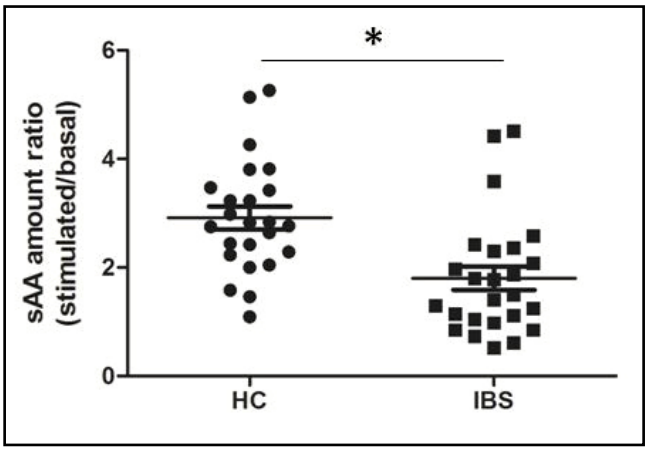

Fig. 3. Blunted sAA response to gustatory stimulus in IBS patients. Comparison of the sAA amount ration (stimulated sAA amount/ basal sAA amount) between IBS patients (IBS, n=25) and the healthy control (HC, n=24). Values are means, with S.E.M. represented by vertical bars. *p<0.05, IBS vs. HC.

IBS patients could be able to secret more sAA towards to gustatory stimulus by citric acid. Again, we detected high variations of stimulated sAA amount, ranging from 96 μg/ml to 735 μg/ml and from 76 μg/ml to 503 μg/ml in the healthy volunteers and IBS subjects, respectively. Unlike the basal sAA secretion, we did not detect difference in the stimulated sAA amount between the two study groups ($p > 0.05$), as shown in Figure 2.

Blunted sAA response to gustatory stimulus in IBS patients

sAA ratio, as measured by the stimulated sAA amount to basal sAA amount, might reflect individual's response pattern that associated with their physical conditions (Chen *et al.* 2015). Thus, we next compared the sAA amount ratio between IBS patients and the healthy subjects. To our surprise, we found that IBS patients presented blunted sAA response to citric acid stimulation when compared with that of the healthy controls ($p < 0.05$), as shown in Figure 3. Together with the elevated basal sAA amount, the blunted sAA response in the IBS group might reflect an autonomic dysfunction for IBS population.

DISCUSSION

In the present study, we detected elevated basal sAA secretion while attenuated sAA response to gustatory stimulus in IBS patients relative to healthy population. Our results suggest an autonomic dysfunction in IBS population. To our knowledge, this is the first attempt to associate sAA secretion with the pathophysiology of IBS.

As aforementioned, sAA secretion is mainly dominated by sympathetic nervous system. The components of saliva are produced primarily by acinar cells of the salivary glands. Anatomically, the acinar cells are innervated by both the sympathetic and the parasympathetic branches of the autonomic nervous system (Emmelin 1987). NE release from the sympathetic neurons binds to both alpha- and beta-adrenergic receptors on the acinar cells, and beta-receptor activation leads to elevation of intracellular cyclic adenosine monophosphate (cAMP), which is linked to the secretion of salivary proteins, including sAA, that are stored in secretory granules (Castle & Castle 1998).

Recently, couple systematic reviews and meta-analyses have been conducted to reveal the high prevalence of psychiatric disorders, including anxiety and major depression, in IBS patients (Fond *et al.* 2014; Hausteiner-Wiehle & Henningsen 2014). Clear associations of the two psychiatric disorders with autonomic dysfunction have been widely addressed in a myriad of medical conditions (Haj Kheder *et al.* 2018; Koschke *et al.* 2009; Tolentino & Schmidt 2016; Kim *et al.* 2016; Bajko *et al.* 2012). Because of the high prevalence of psychiatric disorders in IBS patients, an autonomic dysfunction in the population should be expected. No wonder that IBS patients were showed to have higher sympathetic activities than healthy controls (Berman *et al.* 2012; Orr *et al.* 2000; Thompson *et al.* 2002; Tanaka *et al.* 2008). In the present study, we found a relatively high rate of psychiatric disorders in the collected IBS patients, of which 48 % and 28% was of anxiety and major depression, respectively (Table 1). We speculated that the current IBS population might probably have higher sympathetic activities than the healthy subjects. Then we sought to address this by evaluating the sAA secretion. To our surprise, we found an elevated level of basal sAA in IBS patients relative to the healthy control, despite of the high variability of the sAA level across individuals (Figure 1). Our result might implicate higher sympathetic activities in IBS patients, which was consistent with previous studies (Orr *et al.* 2000;

Thompson *et al.* 2002; Tanaka *et al.* 2008). Although we did not observe any difference of the stimulated sAA level after gustatory stimulation with citric acid between IBS patients and healthy controls, we observed a blunted sAA increase as evidenced by the lower sAA amount ratio in the IBS population (Figure 3), which might be of pathophysiological importance for clinical manipulation of the IBS patients.

Although we found significant results, the study has two potential limitations. First, the current sample size is relatively small that may affect the current statistical power. Secondly, although detailed instructions were made before and during the saliva collection, various unpredictable factors can also influence saliva secretion that may disrupt the subsequent tests of the present study. Thus, findings of the current study should be considered as preliminary until replicated tests with larger sample size are conducted in the future.

Taken together, we, for the first time, detected an elevated basal sAA level while a blunted sAA response to gustatory stimulation in IBS population, which might be of pathophysiological importance for IBS patients.

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