Oral Nitrate Reductase Activity Is Not Associated with Development of Non-Alcoholic Fatty Liver Disease (NAFLD) and Non-Alcoholic Steatohepatitis (NASH): A Pilot Study

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ABSTRACT

BACKGROUND

NAFLD/NASH is a manifestation of metabolic syndrome and is associated with obesity/overweight. Not all obese/overweight individuals develop NASH. Gastro-esophageal reflux disease (GERD) is considered a gastrointestinal manifestation of the metabolic syndrome and is associated with obesity/overweight. Again not all obese/overweight individuals develop GERD. Recent data show association of dietary nitrate content and oral nitrate reductase activity (NRA) with GERD. Nitrates need to be converted to nitrite (done in human beings by nitrate reductase of oral bacteria exclusively) to be active in metabolic pathways.

OBJECTIVE

To assess the relation between NASH/NAFLD and oral NRA.

METHODS

Oral NRA was measured in individuals with NASH (compatible abdominal ultrasound and two elevated ALT/AST levels over six months) and was compared with that of those without NASH. Oral NRA was measured according to a previously reported protocol.

RESULTS

Eleven NASH patients and twelve controls were enrolled. Mean oral NRA activity were 2.82 vs. 3.51 µg nitrite-N formed per person per minute for cases and controls respectively (p=0.46).

CONCLUSION

According to our data, oral nitrite production is not different between individuals with and without NASH.

KEYWORDS

NASH, NAFLD, GERD, Nitrate Reductase Activity, Oral Bacterial Flora

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) and its more severe form, non-alcoholic steatohepatitis (NASH), have become the metabolic epidemics of the modern world.[1] The same has occurred in developing countries as well.[2]

These have been attributed to the growing prevalence of obesity and insulin resistance in the population. Although plausible and applicable in many instances, but these associations are not universal. Roughly a third of obese people are prone to and develop NAFLD/NASH in their lifetime.[3] On the other hand, being obese is not a pre-requisite for NAFLD/NASH. This has been described extensively and we know that some lean people are prone to NAFLD/NASH as well.[4] Therefore, it is logical to think of other, yet unknown, factors triggering the process of fatty liver in the liver of obese and lean people.

The epidemic of obesity and NAFLD/NASH has occurred alongside economic development and adopting a more western type of diet in most parts of the world.[5] Meanwhile the metabolic syndrome has risen as a serious threat to the health of communities and a new burden on the health economy. The metabolic syndrome has various manifestations, NAFLD/NASH being one of them. Gastro-esophageal reflux disease (GERD) has also been considered as the gastro-intestinal manifestation of the metabolic syndrome.[6] Interestingly GERD is also on the rise in parallel with NAFLD/NASH and obesity is a risk factor for GERD too.[7,8] Again not all obese people develop GERD and many patients with GERD are not obese.[9] Having common epidemiologic backgrounds, it looks plausible that these conditions may have a common trigger or mechanism for their development. The exact pathophysiologic mechanisms causing GERD are not known, but recent, animal, ecologic, and human data have linked excess dietary nitrate to GERD.[10-12] Nitrate is an inert compound and can only take part in biochemical reactions after being reduced to nitrite by the nitrate reductase of oral bacteria residing on the dorsum of the tongue. This is the only known pathway which produces nitrite in human beings. The ingested nitrite then enters an enterosalivary circulation and is re-secreted into the mouth by salivary glands.[13] It has been recently shown that oral nitrate reductase activity (NRA) is increased in patients with erosive GERD.[14] Therefore, oral flora may play a role in development of erosive gastro-esophageal reflux disease. As GERD is considered a gastrointestinal (GI) manifestation of the metabolic syndrome which is in turn related to NASH/NAFLD, common pathways may exist.

Having these in mind, we hypothesized that oral NRA may have an effect on development of NAFLD/NASH. Therefore, we tested this hypothesis in a pilot study.

MATERIALS AND METHODS

Patients with clinical NASH and sex matched controls were enrolled in this study. NASH was defined as having at least grade-I fatty liver on abdominal ultrasound examination and either aspartate aminotransferase (AST) or alanine aminotransferase (ALT) more than 1.5 times the upper normal limit on two occasions during the six months before enrollment. Upper limit of normal for AST and ALT was considered 20 IU/L in females and 30 IU/L in males. All patients were checked for hepatitis B and C, autoimmune hepatitis, Wilson's disease, alpha-1 anti-trypsin deficiency, and hemochromatosis and required to be negative for all these. In addition, those who took herbal medicine or supplements for bodybuilding regularly were excluded from the study. Controls were selected from patients referring to one of the authors (SM) and were matched for age. Patients and controls were required not to have clinical evidence of GERD. Controls were also checked for NAFLD. The following data were obtained from each participant: height (in centimeters), weight (in kilograms), waist circumference (in centimeters), hip circumference (in centimeters), aspartate aminotransferase (AST, IU/L), alanine aminotransferase (ALT, IU/L), fasting blood sugar (FBS, mg/dl), total cholesterol (mg/dl), serum triglyceride (TG, mg/dl), high density lipoprotein (HDL, mg/dl)), low density lipoprotein (LDL, mg/dl), and fasting serum insulin level (µu/ml). Body mass index (BMI, kg/m^2) and homeostasis model assessment-Insulin resistance (HOMA-IR) were calculated, the latter using FBS and fasting serum insulin level. A HOMA-IR of two or more was considered as insulin resistance. Participants underwent abdominal ultrasound examination for assessment of fatty liver as well and their fat estimation reported as grades of fatty change. All participants were asked to report to the clinic after an overnight fast and avoiding food containing high nitrate (including lettuce, cabbage, celery, turnip, beetroot,

radish, and rhubarb) for at least eight hours. After filling in a questionnaire for demographic, clinical and paraclinical information, oral NRA was measured by the standardized quantitative mouth assay according to a previously described protocol. Briefly, particiapants rinsed their mouth with 22 ml of deionized, sterile water initially. Then he/she was asked to hold 22 ml of a 10 mg nitrate-N/L solution in the mouth for 3 min. The subject was instructed to mix the solution in the mouth at the beginning and every 1 min over the 3 min, and 1.5 ml of the solution incubated in the mouth was sampled with a sterile syringe after each mixing (i.e., at the beginning and at 1-min intervals). The rest of the solution was discarded. The samples were gathered in special tubes and the rest of the nitrate reductase in the samples was inactivated to prevent in vitro activity. The nitrite was then measured immediately according to the previously described method and the slope of nitrite concentration was used to determine the NRA accordingly and reported as µg nitrite-N formed per person per minute.

RESULTS

Twenty-three people (11 cases and 12 controls, all male) were enrolled. Baseline characteristics of participants are shown in table-1. The patients' heights were comparable but cases weighed significantly heavier than controls (Table-1). Total cholesterol, HDL and LDL were comparable in the two groups, but TG was significantly higher among cases than controls (187.5 vs. 118.1 mg/dl respectively, p=0.014). There was also a trend towards higher FBS among cases (91.0 vs. 83.7 mg/dl respectively, p=0.081). Two cases had grade-I, one had grade-III, and the rest had grade-II fatty liver on abdominal ultrasound examination. One control was reported to have a grade-I fatty liver with normal liver enzyme levels.

Mean oral NRA was not different between the two groups (2.82 vs. 3.51 µg nitrite-N formed per person per minute for cases and controls respectively, p=0.46). There were two outliers among controls (NRA level>8 µg nitrite-N formed per person per minute).

Table 1: Baseline characteristics

	Allocation	N	Mean	Std. Deviation	*p*-value
Age (years)	Cases	11	39.5	8.3	0.24
	Controls	12	35.4	8.0	
Height (cm)	Cases	11	175.4	7.1	0.79
	Controls	12	174.7	5.6	
Weight (kg)	Cases	11	93.4	15.4	<0.001
	Controls	12	72.0	6.7	
Waist Circ (cm)	Cases	11	101.4	9.7	<0.001
	Controls	12	88.0	5.3	
Hip Circ (cm)	Cases	11	110.6	7.9	0.002
	Controls	12	100.5	3.6	
BMI (kg/m^2)	Cases	11	30.3	4.5	<0.001
	Controls	12	23.5	1.9	
AST (IU/L)	Cases	11	39.3	26.1	0.017
	Controls	12	16.7	2.6	
ALT (IU/L)	Cases	11	66.9	26.9	<0.001
	Controls	12	19.1	5.6	
Total Cholesterol (mg/dl)	Cases	11	189.8	44.1	0.16
	Controls	12	166.0	31.9	
HDL (mg/dl)	Cases	11	49.0	5.4	0.828
	Controls	12	49.7	9.5	
LDL (mg/dl)	Cases	11	107.5	46.8	0.378
	Controls	12	92.7	28.4	
TG (mg/dl)	Cases	11	187.5	74.3	0.014
	Controls	12	118.1	34.4	
FBS (mg/dl)	Cases	11	91.0	10.5	0.082
	Controls	12	83.7	8.0	
Insulin Level (µu/ml)	Cases	11	12.4	4.6	0.009
	Controls	12	7.6	2.8	
HOMA-IR	Cases	11	2.7	0.99	0.003
	Controls	12	1.5	0.51	

DISCUSSION

NAFLD and NASH are among the common problems for which people seek medical advice and their prevalence is increasing worldwide. These conditions have been related to obesity and insulin resistance. Although frequency of NAFLD/NASH increases with increasing BMI, but most obese people never develop these conditions. Besides, lean people may develop NAFLD/NASH. Why some obese people develop NAFLD/NASH and others do not? Is it a genetic predisposition (which probably is) or are

there environmental factors affecting this? It is not possible to answer this question yet, but hypotheses can be generated.

The human microbiome has emerged as an important contributor to health and disease.[15] It is generally considered as a stable milieu within the human body during life. But recent data have shown that diet may change this microflora.[16] Considering the vast metabolic activities of these microbes and their extensive involvement in human physiology, it is plausible to hypothesize that alterations in this milieu may have effects on disease behavior in different patients. In other words, a given insult may have different consequences in individuals with different flora. Among these, the oral and GI flora may be more influential in metabolic diseases which involve diet and its constituents in some way. An important point in such studies is that it may not be a given microbe which affects the outcome, but the metabolic effect of the given microbe in a given milieu of other microbes and their topographical distribution in an environment determines the final effect. Therefore, despite the fact that looking for individual microbes is informative, but checking metabolic pathways may be more enlightening.

We tested the oral nitrate reductase activity as a marker of oral flora composition in individuals with and without NASH. NRA was selected as it has been recently shown that it may contribute to development of erosive reflux disease which itself is considered a GI manifestation of the metabolic syndrome just like NASH. According to our data, oral nitrite production is not different between people with NAFLD/NASH and age-matched controls. In other words, dietary nitrate is not handled differently between these two groups of individuals. Strengths of our study include that we measured NRA with an accurate and reproducible method in vivo. In addition, we selected our cases with rigorous criteria. But meanwhile we were not as meticulous with the controls. The number of cases and controls has been relatively small. Another point which may have affected our data is that NRA measurements for controls have been done in the colder months of the year. It is well known that oral NRA varies with ambient temperature. Furthermore, the number of outliers is more in the control group. All these make us to interpret our data with some caution. Another point which may have affected our results is that NASH patients in our study had significantly higher BMI levels. The question to be answered is that if BMI is associated with development of NASH, why some obese/overweight people develop NASH/NAFLD, while other people with similar BMI do not. For this to be answered, we need cases and controls to be comparable in their BMIs. In addition, controlling or matching for other confounders may in further studies may give us better insight to this concept. Therefore, it is still possible that oral NRA and excessive dietary nitrate can have a role in development of NAFLD/NASH. Hence, studies with higher numbers of cases and controls with a more meticulous methodology are warranted.

CONFLICT OF INTEREST

The authors declare no conflict of interest related to this work.

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