RESEARCH COMMUNICATION

Effect of Dietary Intake on the Levels of Biliary Unsaturated Free Fatty Acids Having Inhibitory Activity on Mutagens

Iván Serra[1], Yasuo Tsuchiya[2]*, Yasushi Hori[3], Kazutoshi Nakamura[2], Rodrigo Villegas[4], Mari Oyama[2], Tomoo Makiguchi[5], Masaharu Yamamoto[2]

Abstract

Unsaturated free fatty acids (FFAs), such as palmitoleic, oleic, linoleic, linolenic and arachidonic acids, have inhibitory actions on mutagenesis. These FFAs in bile may play a role in preventing cholecystopathy and their levels may be influenced by diet. However, the effects of dietary intake on biliary FFAs levels is not known. In order to examine possible associations between dietary habits and biliary FFAs levels, bile samples were collected from resected gallbladders of 114 Chilean female patients with gallstones, and FFAs were measured with an HPLC system. The long-term dietary intake of the patients was investigated through a semi-quantitative food frequency questionnaire. A high intake of vegetables was negatively correlated with the total FFA level (r = -0.264, P = 0.010). Positive correlations were found between fruit consumption and the lauric acid level (r = 0.200, P = 0.041), fish consumption and the levels of oleic (r = 0.370, P <0.0001), linolenic (r = 0.197, P = 0.038) and arachidonic (r = 0.200, P =0.035) acids, and consumption of foods fried in vegetable oil and the linoleic acid level (r = 0.269, P =0.004). Linoleic, linolenic, and arachidonic acids which may have an inhibitory effect on actions of unknown mutagens in bile appear to be increased by consumption of high levels of fish and fried foods.

Key Words: Diet - fish - fried food - free fatty acids - bile

Asian Pacific J Cancer Prev, **10**, 899-902

Introduction

A previous *in vitro* study showed that unsaturated free fatty acids (FFAs) of carbon numbers 16-24, such as palmitoleic, oleic, linoleic, linolenic, and arachidonic acids, have an inhibitory effect on mutagenesis (Hayatsu et al., 1988). Although these FFAs in gallbladder bile are normally detected in very small amounts (van Berge Henegouwen et al. 1987; Mingrone et al. 1988; Schöne et al. 2000), they may play a role in the inhibitory effect *in vivo* similar to the *in vitro* experiment. Since some mutagenic and other unidentified substances in gallbladder bile have been demonstrated (Manabe and Wada, 1990; Mano et al. 1993), these FFAs in gallbladder bile may produce inhibitory effects expected from the above.

In fact, our ecologic studies have shown significant associations between the decreased unsaturated FFAs levels and gallbladder cancer (GBC) incidence (Hori et al., 1999; Tsuchiya et al., 2006). Since intake of foods rich in fats and sugar has been associated with the changes of blood FFAs level (Baker and Rostami, 1969; Pannall et al., 1977), biliary FFAs may be influenced by dietary intake. To the best of our knowledge, however, no study has examined the association between dietary intake and the biliary FFAs levels.

An association between dietary intake and GBC risk has been shown in many studies (Kato et al., 1989; Zatonski et al., 1997; Lowenfels et al., 1999; Serra et al., 2002; Matsuba et al., 2005; Rai et al., 2006; Barclay et al., 2008). Although these epidemiological studies have investigated various dietary factors (fat, dairy products, fruits, vegetables, fish, meat, chili pepper, sweetened drink, dessert, and so on) for the development of GBC, and have demonstrated the risk or preventive factors, the relation between dietary intake and the development of GBC is complex. The consumption of diets high in fats and calories has been associated with an increased risk of GBC (Lowenfels et al. 1999). A significant relationship between total carbohydrate intake and GBC was observed in both men and women (Zatonski et al., 1997). On the other hand, fruit and fish consumption showed inverse association with the risk of GBC (Matsuba et al., 2005; Rai et al. 2006). Therefore, to know whether these FFAs in bile are influenced by dietary habits will be helpful to determine the etiology of GBC.

Although the mechanism causing GBC has not been understood yet, evidence from the view point of the development or prevention of GBC may be obtained by

*[1]Ministry of Health, Sótero del Río Hospital, Santiago, Chile,[2]Department of Community Preventive Medicine, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan ,[3]Department of Hospital Pharmacy, Niigata City General Hospital, Niigata, Japan,[4]Center for Research in Environmental Epidemiology (CREAL), Barcelona, Spain, and School of Public Health, University of Chile, Santiago, Chile, [5]Department of Structural Pathology, Niigata University Graduate School of Medical and Dental Sciences, *For Correspondence: troof@med.niigata-u.ac.jp*

clarifying the association between dietary intake and biliary FFAs levels, especially unsaturated FFAs levels. We therefore determined whether dietary intake in the patients with GS is associated with biliary FFAs levels. Main reasons why we collected the bile from GS patients are that we are seeking for risk or preventive factors of GBC working together with the presence of GS under the multi-factorial causation hypothesis, and it is very difficult to collect samples from GBC patients because a large proportion of them are at advanced stage.

Materials and Methods

Study subjects

A total of randomly selected 114 female patients were sampled at the beginning of an elective cholecystectomy at the Sótero del Río university hospital in the South Orient Health Area, attending 1.5 million people, almost one tenth of the total population in Chile (16 millions). This health area is considered representative of Chilean people having mostly median and low median socio economic strata. All patients gave their written informed consent for the study at the time of hospital admission. The study protocol was approved by the Ethics Committee at Niigata University School of Medicine.

Bile sampling

Gallbladder bile was collected by needle aspiration during cholecystectomy into a glass or plastic tube and stored as aliquots at -80ºC in an ultra-deep freezer. The samples collected in Chile were sent to Japan in a frozen condition until analysis of the FFAs concentrations.

Measurement of biliary FFAs

The bile samples were analyzed with a high-performance liquid chromatography system (HPLC: LC10AS, Shimadzu Corporation, Kyoto, Japan) equipped with a spectrofluorometric detector (RF-550 GLP, Shimadzu Corporation) and an Eclipse XDB-C8 column (Dimensions: 4.6 x 250 mm; Particle size: 5 μm; Rockland Technologies, Inc., USA). A total of 9 biliary FFAs, namely lauric acid, myristic acid, palmitoleic acid, palmitic acid, linolenic acid, linoleic acid, oleic acid, stearic acid, and arachidonic acid, were identified following a procedure described previously (Hori et al. 1998). Of the detected 9 FFAs, palmitoleic, oleic, linoleic, linolenic, and arachidonic acids are categorized as unsaturated FFAs. The mean recovery rate for FFAs was 98% (range 92-104%), and the detection limit of the assay was 100 pg. The calibration curves for all FFAs were linear over the range of 0.5-400 ng.

Measurement of dietary intake

A food frequency questionnaire which was discussed with nutritionist and validated in our previous studies (Endoh et al. 1997; Serra et al. 2002) was used in the present study. The patients were asked to report their long-term frequency intake of vegetables, chili pepper, fruits, beef, pork, chicken, fish, and foods fried in vegetable oil (fried foods). A trained interviewer asked the participants how often they had consumed a standard portion of each food over the previous year, assuming that this would reflect long-term intake. Food intake was grouped into 5 categories: (1) never; (2) 1-3 times/month or less; (3) 1-3 times/week; (4) 4-6 times/week; and (5) everyday.

Statistical evaluation

Significant differences between the dietary intake of each food item and the biliary FFAs levels were assessed, following logarithmic conversion of data, by Pearson's correlation coefficient analysis. Correlations that showed a p-value of less than 0.05 were regarded as statistically significant. Statistical analyses were performed using Statistical Analysis System software (SAS Institute Inc., Cary, NC, USA).

Results

Data on dietary habits and biliary FFAs were obtained from the 114 patients participating in the study. The mean age was 48.4 years , with a range of 23 to 84. Nearly half weighed more than 70 kg, although 55% of them were 1.50-1.59 meters tall. Two-thirds of the patients were overweight (43.4%), obese (30.1%), or morbidly obese (1.8%).

The frequency of consumption of vegetables and fruits was very high compared to the other food groups studied, with over two-thirds of the participants reporting daily intake of regular portions. Over two-thirds of the participants reported eating green or red chili peppers at least once a month. Nearly all the patients reported eating beef or chicken at least once a week, while pork was less often consumed. A weekly intake of fish was reported by over 50%. Almost 60% of the patients consumed fried foods 1-3 times per week (Table 1).

As expected, the total FFAs level was higher than the total inhibitory FFAs level, which accounted for approximately 52% of the total FFAs level. The major FFAs in the bile were linoleic, palmitic, and oleic acids. The mean compositions of the palmitic, linoleic, and oleic acids accounted for over 70% of the total FFAs level (Table 2).

A statistically significant inverse association between vegetable consumption and the total FFA level was observed (r =-0.264, P = 0.010). Fish consumption was

Table 1. Food Intake Frequencies of Chilean Females with Gallstones

Item	Mean frequency categories ± SD	Frequency of food intake (%) (1)	(2)	(3)	(4)	(5)
Vegetables	4.53 ± 0.94	2.1	1.1	14.9	5.3	76.6
Red chili[1]	2.50 ± 1.38	33.6	17.3	28.2	7.3	13.6
Green chili[1]	2.73 ± 1.47	31.2	10.1	33.0	5.5	20.2
Fruits	4.08 ± 1.23	3.8	7.6	25.7	2.9	60.0
Beef	3.20 ± 0.62	0	5.4	74.8	14.4	5.4
Pork	2.61 ± 0.71	8.1	27.3	61.6	2.0	1.0
Chicken	3.09 ± 0.51	0.9	4.5	81.1	11.7	1.8
Fish	2.50 ± 0.63	6.4	38.2	54.5	0.9	0
Fried foods[2]	3.10 ± 0.87	4.5	12.5	58.9	17.0	7.1

[1]Pepper; [2]Food intake frequencies were grouped into 5 categories: (1) never; (2) l-3 times/month or less; (3) 1-3 times/ week; (4) 4-6 times/week, and (5) every day

Table 2. Biliary Free Fatty Acids Concentrations or Compositions in Chilean Females with Gallstones

	Concentration (mmol/l)*	Composition (%)
Total FFA	6.91 ± 8.31	
Total IFFA	4.11 ± 5.96	52.1 ±17.9
Lauric acid	0.07 ± 0.06	2.6 ± 4.1
Myristic acid	0.41 ± 0.87	4.6 ± 6.5
Palmitic acid	1.86 ± 1.81	31.1 ±14.2
Stearic acid	0.46 ± 0.39	9.7 ± 5.4
Palmitoleic acid	0.22 ± 0.55	2.2 ± 4.4
Oleic acid	1.31 ± 1.62	20.9 ±13.6
Linoleic acid	2.12 ± 3.95	21.7 ±13.8
Linolenic acid	0.14 ± 0.31	2.0 ± 6.4
Arachidonic acid	0.32 ± 0.64	6.0 ± 6.1

*values are mean ± SD; FFA, free fatty acid; IFFA, inhibitory FFA

Table 3. Significant Correlations Between Dietary Intake and Biliary Free Fatty Acids Levels in Chilean Females with Gallstones

	Vegetable	p value	Fish	p value	FF	p value
Total FFA	-0.264	0.010	0.084	0.380	0.111	0.242
Oleic[1]	-0.164	0.115	0.370	<0.001	-0.032	0.736
Linoleic[1]	-0.012	0.912	-0.163	0.088	0.269	0.004
Linolenic[1]	0.030	0.773	0.197	0.038	0.162	0.088
Arachidonic[1]	-0.036	0.728	0.200	0.035	-0.198	0.036

[1]acid; FFA, free fatty acid; FF, fried foods

significantly associated with the unsaturated FFAs levels. Positive correlations were found between fish consumption and the levels of oleic (r = 0.370, P < 0.0001), linolenic (r = 0.197, P = 0.038) and arachidonic (r = 0.200, P = 0.035) acids. Fried food consumption was positively associated with the linoleic acid level (r = 0.269, P = 0.004), while it was inversely correlated with the arachidonic acid level (Table 3). Consumption of chili pepper, fruit, beef, pork, and chicken was not associated with the total FFAs level or the unsaturated FFAs levels, but a positive correlation was found between fruit consumption and the lauric acid level (r = 0.200, P = 0.041).

Discussion

We found that a high consumption of vegetables, fish, and fried foods was significantly associated with the total FFA level or the unsaturated FFAs levels in Chilean female patients with GS. To our knowledge, this is the first analysis of the association between the long-term reported intake of foods and the biliary FFAs levels.

A significant inverse correlation was found between the consumption of vegetables and the total FFA level. Previous studies have examined the association between vegetable consumption and GBC risk (Moerman et al., 1995; Pandey and Shukla, 2002, Serra et al., 2002; Rai et al., 2006). A significant inverse association was reported not only in Chilean people (Endoh et al., 1997; Serra et al., 2002) but also in Indian (Panday et al., 1995) and Netherland populations (Moerman et al., 1995). Vegetables include many anticarcinogenic agents such as dithiolthiones, isothiocyanates, indole-3-carbinol, allium compounds, isoflavones, protease inhibitors, saponins, phytosterols, inositol hexaphosphate, vitamin C, d-limonene, lutein, folic acid, beta carotene, lycopene, selenium, vitamin E, flavonoids, and dietary fiber (Nguyen, 1999). They have the capacity to inhibit, block, or suppress carcinogenic processes and therefore may play a central role in the protective effects of vegetables. However, a beneficial effect may also be explained by the favorable effects of the change in the total FFA level. Previous researchers suggested that high levels of biliary FFAs may produce epithelial injury to gallbladder mucosa (Mingrone et al. 1988). Here vegetable consumption was inversely associated with the total FFA level.

A significant positive correlation between fish consumption and the biliary oleic, linolenic, and arachidonic acids levels was observed in this study. A previous study has shown that high intake of fish has a significant preventive relationship with GBC in women (Matsuba et al. 2005). Researchers also reported that broiled fish consumption is a protective factor for GBC (Kato et al. 1989). The biliary FFAs levels increased by a high consumption of fish in the present study could enhance inhibitory effects on mutagens in gallbladder bile. Because a previous in vitro study has shown that unsaturated FFAs of carbon numbers 16-24, such as palmitoleic, oleic, linolenic, and arachidonic acids, have an inhibitory effect on mutagens (Hayatsu et al. 1988). Furthermore, the decrease in the palmitoleic and linolenic acids levels were associated with the high incidence of GBC in our previous studies (Hori et al. 1999; Tsuchiya et al. 2006). The increase in the biliary FFAs levels caused by a high consumption of fish may play an important role in the prevention of GBC.

Fried food consumption may increase the biliary linoleic acid level because of the large amount of linoleic acid in the cooking oil. Palm oil and soybean oil are the two most commonly used cooking oils in the world (Landes et al., 1997). In Chile, both vegetable and animal fats and oils are frequently used for cooking. It has been established that soybean oil is the most important contributor to the dietary fat supply in Chile (Valenzuela and Uauy, 1999). Soybeans are usually solvent-extracted with commercial hexane and the oil is then refined, blended for different applications and sometimes hydrogenated. This oil is normally sold as “vegetable oil” and because of the treatment it undergoes, it is one of the cheapest of the non-animal oils. It contains about 75% soybean oil, 25% sunflower oil, and a small amount of hydrogenated oil (Moreno and Bouchon, 2008). Because the chief fatty acid in soybean oil and sunflower oil is linoleic acid, high fried food consumption may contribute to the increase in the biliary linoleic acid level. Researchers reported that intake of fried foods, mainly cooked with vegetable oil, was a low risk factor for GBC (Kato et al., 1989). This mechanism may be explained by effects of biliary linoleic acid increased by vegetable oil consumption.

Some potential limitations of this study require discussion. We used a brief food frequency questionnaire including 8 food groups and items that are not only commonly consumed by Chilean adults, but were also potentially relevant in the study of gallbladder disease. Although we did not include individual foods that could have a relation with specific FFAs, we believe our results provide an initial understanding of how long-term food intake and biliary FFAs correlate. We did not collect the bile samples from the patients with GBC because the clinical diagnosis of GBC is yusually performed by its pathological examination after cholecystectomy. As a result, we collected the bile samples only from patients with GS who received cholecystectomy. To clarify associations more clearly, we should use the samples collected from GBC patients.

In summary, we observed positive associations between fruit consumption and the lauric acid level, between fish consumption and the oleic, linolenic, or arachidonic acids levels, and between fried food consumption and the linoleic acid level. Of these diets, fish consumption was particularly evident in the positive association with biliary unsaturated FFAs level. High consumption of fish which is a preventive factor for GBC proposed by previous researchers (Matsuba et al. 2005) may be associated with an increase in inhibitory effect to be caused by biliary linolenic acid. While our findings need further confirmation, they provide evidence that consumption of high levels of fish and fried food increase the levels of biliary unsaturated FFAs which may have an inhibitory effect on mutagens.

Acknowledgements

This study was supported in part by a Grant-in-Aid for Scientific Research (C) (2004, #16590473) from the Japanese Ministry of Education, Science, Sports and Culture.

References

Baker N, Rostami H (1969). Effect of glucose feeding on net transport of plasma free fatty acids. *J Lipid Res*, **10**, 83-90.

Barclay AW, Petocz P, McMillan-Price J, et al (2008). Glycemic index, glycemic load, and chronic disease risk--a meta-analysis of observational studies. *Am* J *Clin Nutr*, **87**, 627-37.

Endoh K, Nakadaira H, Yamazaki O, et al (1997). Risk factors for gallbladder cancer in Chilean females. *Nippon Koshu Eisei Zasshi*, **44**, 113-22 (in Japanese).

Hayatsu H, Arimoto S, Negishi T (1988). Dietary inhibitors of mutagenesis and carcinogenesis. *Mutat Res*, **202**, 429-46.

Hori Y, Nakamura K, Yamamoto M, et al (1998). Determination of free fatty acids in human bile by high-performance liquid chromatography. *Ann Clin Biochem*, **35**, 279-82.

Hori Y, Nakamura K, Yamamoto M, et al (1999). Geographical variations in the concentration of biliary free fatty acids with anti-mutagenic action. *Mutat Res*, **444**, 41-7.

Kato K, Akai S, Tominaga S, Kato I (1989). A case-control study of biliary tract cancer in Niigata Prefecture, Japan. *Jpn J Cancer Res*, **80**, 932-8.

Landes R, Westcott P, Wainio J (1997). International Agricultural Baseline Projections to 2005: Oilseeds and Products. Agricultural Economic Report: *USDA Economic Research Service;* . Report No. 750.

Lowenfels AB, Maisonneuve P, Boyle P, Zatonski WA (1999). Epidemiology of gallbladder cancer. *Hepato-gastroenterology*, **46**, 1529-32.

Manabe S, Wada O (1990). Identification of carcinogenic tryptophan pyrolysis products in human bile by high-performance liquid chromatography. *Environ Mol Mutagen*, **15**, 229-35.

Mano H, Yamamoto M, Araya JC, et al (1993). Mutagenicity of blue rayon extracts of human bile in the Ames test. *Mutat Res*, **290**, 303-9.

Matsuba T, Qiu D, Kurosawa M, et al (2005). Overview of epidemiology of bile duct and gallbladder cancer focusing on the JACC Study. *J Epidemiol*, **15**, S150-6.

Matsuyama T, Shoji K, Takase H, et al (2007). Effects of phytosterols in diacylglycerol as part of diet therapy on hyperlipidemia in children. *Asia Pac J Clin Nutr*, **16**, 40-8.

Mattson FH, Grundy SM, Crouse JR (1982). Optimizing the effect of plant sterols on cholesterol absorption in man. *Am J Clin Nutr*, **35**, 697-700.

Miettinen TA, Vanhanen H (1994). Dietary sitostanol related to absorption, synthesis and serum level of cholesterol in different apolipoprotein E phenotypes. *Atherosclerosis*, **105**, 217-26.

Mingrone G, Greco AV, Finotti E, Passi S (1988). Free fatty acids: a stimulus for mucin hypersecretion in cholesterol gallstone biles. *Biochim Biophys Acta*, **958**, 52-9.

Moerman CJ, Bueno de Mesquita HB, Smeets FW, Runia S (1995). Consumption of foods and micronutrients and the risk of cancer of the biliary tract. *Prev Med*, **24**, 591-602.

Moghadasian MH, Frohlich JJ (1999). Effects of dietary phytosterols on cholesterol metabolism and atherosclerosis: clinical and experimental evidence. *Am J Med*, **107**, 588-94.

Moreno MC, Bouchon P (2008). A different perspective to study the effect of freeze, air, and osmotic drying on oil absorption during potato frying. *J Food Sci*, **73**, E122-8.

Nguyen TT (1999). The cholesterol-lowering action of plant stanol esters. *J Nutr*, **129**, 2109-12.

Pandey M, Shukla VK (2002). Diet and gallbladder cancer: a case-control study. *Eur J Cancer Prev*, **11**, 365-8.

Pannall PR, Swanepoel E, Bennett JM, Pauw FH (1977). Increased concentrations of serum free fatty acids falsely increase serum thyroxine as determined by competitive protein-binding. *Clin Chem*, **23**, 990-3.

Rai A, Mohapatra SC, Shukla HS (2006). Correlates between vegetable consumption and gallbladder cancer. *Eur J Cancer Prev*, **15**, 134-7.

Serra I, Yamamoto M, Calvo A, et al (2002). Association of chili pepper consumption, low socioeconomic status and longstanding gallstones with gallbladder cancer in a Chilean population. *Int J Cancer*, **102**, 407-11.

Schöne A, Jüngst D, Meyer G, Hernandez-Richter T, Fischer S (2000). Effects of phospholipase A2, free fatty acids and 2-lysolecithin on the crystallization of cholesterol in gallbladder bile. *Eur J Clin Invest*, **30**, 715-21.

Tsuchiya Y, Serra I, Hori Y, et al (2006). Free fatty acid concentrations in gallbladder bile collected from Chilean patients with gallstones. *Clin Biochem*, **39**, 410-3.

van Berge Henegouwen GP, van der Werf SD, Ruben AT (1987). Fatty acid composition of phospholipids in bile in man: promoting effect of deoxycholate on arachidonate. *Clin Chim Acta*, **165**, 27-37.

Valenzuela A, Uauy R (1999). Consumption pattern of dietary fats in Chile: n-6 and n-3 fatty acids. *Int J Food Sci Nutr*, **50**, 127-33.

Zatonski WA, Lowenfels AB, Boyle P, et al (1997). Epidemiologic aspects of gallbladder cancer: a case-control study of the SEARCH Program of the International Agency for Research on Cancer. *J Natl Cancer Inst*, **89**, 1132-8.