Cytotoxic Compounds Generated in Heated Oil and Assimilation of Oil in Wistar Rats

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Abstract: We have previously suggested that gluten binds with decomposition products from thermally oxidized oil during frying, and that low-molecular-weight compounds bound to browned gluten damage the liver in rats. Ten-week-old male Wistar rats were fed for 11 weeks *ad libitum* a diet containing 7 wt% fresh frying oil and 0.1 wt% gluten heated in/without oil at 180°C for 10 h. Feces collected weekly and serum were subjected to lipid and hematological analyses, respectively. Values obtained in the analyses did not differ from those of the control group. The results show that thermally processed gluten does not influence the digestion, absorption, metabolism, and growth of rats, regardless of the cytotoxic low-molecular-weight compounds, and that ingested fresh oil was assimilated normally. Together with the previous results, the odor of thermally processed gluten stimulated the rats' appetite, and completely assimilable fresh oil and cytotoxic low-molecular-weight compounds bound to gluten were ingested, and thus organ damage and rapid body weight increase were observed. As commercial deep-fried products are often made with repeatedly used oil with periodically added fresh oil, similar to the present experimental diet, obesity and organ damage may also occur in humans.

Key words: gluten, cytotoxicity, diet, metabolic syndrome, feed efficiency, fecal analyses

1 INTRODUCTION

Deep-fried foods, such as tempura and pork cutlets are very popular among Japanese. In the last 20 years, the percentage of calories ingested from oil and fat has exceeded the value recommended for Japanese and metabolic syndrome has become more common, even in individuals in their late twenties. One of the reasons for this is the undesirable dining habits. Minihane *et al.*¹⁾ and Cohn²⁾ reported that chronic multifactorial diet-related diseases were a major cause of death and illness worldwide, and that the amount and composition of fat in the diet is an important determinant of the pathology of many of these conditions.

When the safety issues related to fried products are discussed, attention is focused on the oil contained in the product, and cutoff points have been established for the chemical properties of the oil³⁾. However, we found⁴⁾ that peroxide value (POV), carbonyl value (COV), contents of polar compounds (PC) and triacylglycerol (TG), and acid value (AV) has little to do with the cytotoxicity of heated

oil. We thus proposed that low-molecular-weight compounds formed during frying are responsible for the cytotoxicity of deteriorated oil. In addition, it was reported⁵⁾ that these low-molecular-weight compounds bound with gluten during heating, and the gluten complex resulted in severe organ damage in rats while oil filtered from a heated mixture of oil and gluten did not show any adverse effects on rats⁶⁾; diets containing oil heated with amino acids, wheat starch or sucrose showed cytotoxicity in animal experiments.

When foodstuffs are deep-fried, their surface shrinks due to sudden heating, and this results in exudation of juices containing various nutrients from the foodstuff^{7,8)}. Among these nutrients, proteins are possible to react/adsorb oxidized and decomposed oil at high temperatures. This suggests that the batter coating of fried products contains proteins bound to low-molecular-weight products. On the other hand, heated gluten can stimulate the appetite, and food ingestion and body weight in rats tends to increase⁵⁾.

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Fried products are often made with repeatedly used oil, and have batter coatings that contain high levels of oil⁹⁾ and low-molecular-weight compounds⁴⁾, and thus these products appear to be problematic for both men suffering from metabolic syndrome and healthy men. It is important to moderate appetite. Gluten heated without addition of oil did not result in cytotoxicity, but led to increased daily ingestion⁵⁾, thus suggesting that oil was not essential to maintain appetite.

In the present study, rats were fed a standard commercial diet (no fat) containing fresh oil and gluten heated in/without oil in order to investigate the effects of cytotoxic low-molecular-weight compounds bound to gluten on assimilation and fecal excretion of lipids.

2 EXPERIMENTAL

2.1 Materials

2.1.1 Oil

Commercial frying oil, The Nisshin Oil Group, LTD., Tokyo, Japan composed of myristic acid 0.1%; palmitic acid 9.4%; palmitoleic acid 0.1%; stearic acid 3.6%; oleic acid 32.6%; linoleic acid 44.1%; α -linolenic acid 6.3%; eicosenoic acid 0.5%; and others 3.3%, and gluten from wheat, Nacalai Tesque, Inc., Kyoto, Japan were employed for the heating⁷⁾.

2.1.2 Thermal treatment of oil^{10,11)}

One liter of fresh oil, commercial frying oil, was heated at 180°C for 10 h in a 2-L four-neck round-bottom flask under stirring with 1 wt% gluten from wheat (Nacalai Tesque, Inc., Kyoto, Japan). Heated oil was divided into gluten (gluten heated in oil, GHIO) and oil (filtered oil) by filtration with filter paper 2-55 mm, Advantec, Toyo Kaisha, Ltd., Tokyo, Japan: the chemical properties of the filtered oil were as follows, POV 1.4 mEq/kg, COV 30.6, PC 21.6%, TG 83.5%, AV 0.1¹⁷⁾. Fifteen grams of gluten was heated in a 20-mL glass tube at 180°C for 10 h (heated gluten, HG).

2.1.3 Diets^{12,13)}

A commercial pelleted diet (Labo MR Stock, Nihon Nosan Corporation, Yokohama, Japan) and a powdered fat-free diet (AIN93G, Clea Japan, Inc., Tokyo, Japan), were purchased. Using a blender, the latter was mixed uniformly with 0.1 wt% glutens (GHIO and HG) described above plus 7 wt% fresh oil, and 7 wt% fresh oil (control group), respectively. All the diets were used after radio-sterilization.

2.2 Animals^{14,15)}

Male Wistar rats aged 9 weeks were obtained from Japan SLC, Inc., Shizuoka, Japan, and were housed separately in wire cages at $24 \pm 2^\circ\text{C}$ and humidity $50 \pm 10\%$, with light from 7:00 to 19:00 at Japan SLC, Inc., Animal Experimental Center, Shizuoka, Japan. Animal care and handling were in

accordance with the Ethical Agreement Concerning Care and Use of Laboratory Animals for Research and Education, Kobe-Gakuin University.

2.3 Feeding regime and sample collection^{5,6)}

Twenty-four animals were maintained on Labo MR Stock for 1 week of adaptation, and divided into three groups (8 rats/group), then fed diets described in 2.1.3, respectively for 11 weeks. All the animals were allowed feed and water *ad libitum* throughout the experiment. Body weight was determined every Wednesday noon, and food intake of individual rat between every Wednesday noon – Thursday noon was measured for the calculation of feed efficiency ratio; the body weight gain in a week (from Wednesday to next Wednesday) was divided by (the food intake of the beginning of the week) and 7. Feces were collected once a week and subjected to oil extraction and determination of bile acids and cholesterol. After 11 weeks, the animals were sacrificed by the administration of anesthesia (a fasting period of 18 h), and blood was obtained from abdominal aorta followed by serum preparation, and stored at -80°C until the analyses of insulin and adiponectine. In addition, the retroperitoneal tissue was removed and stored at -80°C .

2.4 Oil analysis

Frozen retroperitoneal tissue and freeze-dried feces were ground into fine pieces with a mill mixer MR-280, Yamazen Corporation, Tokyo, Japan and subjected to lipid extraction using the chloroform/methanol (2:1 v/v) method¹⁶⁾. The solvent was then removed by a rotary evaporator from the samples and retroperitoneal tissue lipids were methyl-esterified for the fatty acid analysis by gas chromatography⁶⁾.

2.5 Analyses of insulin and adiponectine

Insulin and adiponectine were determined by Morinaga Insulin Measurement Kit, Morinaga Institute of Biological Science Inc., Tokyo, Japan and Mouse/Rat Adiponectine ELISA Kit, Otsuka Pharmaceuticals Corporation, Tokyo, Japan, respectively.

2.6 Steroid analyses¹⁷⁾

Analyses of bile acid and cholesterol were carried out with Total Bile Acid-Test Wako, and Cholesterol E-Test Wako (Wako Pure Chemical Industries, Osaka, Japan), respectively.

2.7 Statistical analysis

All the values obtained from animals are expressed as means $\pm$ SD. Data from 8 animals each for experimental and control groups were analyzed using one-way ANOVA and results were considered significant at $p < 0.05$.

3 RESULTS

3.1 Feed efficiency

The effects of diets containing processed glutens on feed efficiency (weight gain/food intake) are compared in Fig. 1. Feed efficiency ratio decreased suddenly at 14 weeks of age and decreased gradually thereafter. The presence of GHIO and HG had no significant effect on feed efficiency. Thus, thermally processed gluten appears to be unrelated with the efficiency, although it promoted slight increases in appetite and body weight⁵⁾.

3.2 Serum insulin and adiponectine

Figure 2 shows the concentrations of serum insulin and adiponectine. Each level in all the groups was not different statistically.

3.3 Analysis of retroperitoneal tissue

There were no significant differences in weight of retroperitoneal tissue, but the oil content tended to be lower in the HG group (Fig. 3).

Fatty acid composition of oil extracted from retroperitoneal tissue is shown in Fig. 4. Major component fatty acids were palmitic, oleic and linoleic acids, and no significant differences were found between the three groups.

3.4 Fecal oil analysis

3.4.1 Dietary oil excreted

The amount of oil excreted from the three groups tended to decrease as the amount of oil ingested also decreased⁵⁾ (Fig. 5). The value in the HG group remained comparatively high.

3.4.2 Dietary oil absorption

The effects of the experimental diets on oil absorption ((oil intake-fecal oil)/oil intake) are shown in Fig. 6. Oil absorption in all the groups were increased slightly throughout the study period (varying between 97-99%), and

the HG group often showed a tendency to low values.

3.4.3 Fecal lipid content

The effects of the experimental diets on fecal lipid content are shown in Fig. 7. The content of all groups increased slightly throughout the study period (approx. 3.5%), but there were no significant differences between them. The trend appears to be due to rapid decreases in fecal weight, despite the decreasing amount of fecal oil, as shown in Fig. 5.

Fig. 2 Determination of Serum Insulin and Adiponectine in Rats Fed Diets Containing Thermally Processed Gluten. Values are expressed as means $\pm$ SD (n=8).

Fig. 1 Feed Efficiency Ratios in Rats Fed Diets Containing Thermally Processed Gluten. Values are expressed as means $\pm$ SD (n=8).

Fig. 3 Oil Content of Rat Retroperitoneal Tissue. Values are expressed as means $\pm$ SD (n=8).

Fig. 4 Fatty Acid Composition of Oil Extracted from Rat Retroperitoneal Tissue. Values are expressed as means $\pm$ SD (n=8).

Fig. 7 Fecal Lipid Content in Rats Fed Diets Containing Thermally Processed Gluten. Values are expressed as means $\pm$ SD (n=8).

Fig. 5 Oil Excreted from Rats Fed Diets Containing Thermally Processed Gluten. Values are expressed as means $\pm$ SD (n=8).

Fig. 8 Fecal Bile Acids in Rats Fed Diets Containing Thermally Processed Gluten. Values are expressed as means $\pm$ SD (n=8).

Fig. 6 Oil Absorption in Rats Fed Diets Containing Thermally Processed Gluten. Values are expressed as means $\pm$ SD (n=8).

Fig. 9 Fecal Cholesterol in Rats Fed Diets Containing Thermally Processed Gluten. Values are expressed as means $\pm$ SD (n=8).

3.4.4 Fecal bile acid content

The effects of the experimental diets on fecal bile acid content are shown in Fig. 8. There were no significant differences among values in the groups.

3.4.5 Fecal cholesterol content

The effects of experimental diets on fecal cholesterol content are shown in Fig. 9. Cholesterol excretion in all the groups increased slowly but no significant differences were found.

4 DISCUSSION

Effects of used frying oil on living body have been investigated by giving the oil as is to animals^{18,19}. In accordance with the oxidation level of the oil, not only cytotoxicity but also gross symptoms, such as seborrhea, diarrhea, polyuria, excessive hair loss and so forth were observed. In general, used frying oil is composed of intact triacylglycerol, polymerized oil, oxidized oil, low-molecular- compounds generated from oxidized oil, etc. But toxic compounds of used frying oil are not always dissolved in the oil. A part of it evaporates from the oil¹¹ and the rest is bound to protein, if the oil contained an enough amount of protein exuded from frying foodstuffs in the practice^{5,6}. GHIO showed strong cytotoxicity against liver⁵, but it is another issue whether the cytotoxic compounds bound to gluten affect digestion, absorption, and metabolism of intact oil. This study clarified the point described above and gluten heated without oil was also employed as a reference.

Feed efficiency ratio (Fig. 1) of all the groups drastically decreased after 14 weeks of age, as body weights did not increase proportionally to the groups' food intake⁶. As all the groups showed no significant differences either in food intake⁶, or in serum insulin and adiponectine levels (Fig. 2), weights of retroperitoneal tissue, its oil contents (Fig. 3) and fatty acid compositions (Fig. 4), and body weight increases⁶ were similar. The HG group showed a tendency toward low oil absorption (Fig. 6) in the latter half of the experiment, while in oil excreted (Fig. 5) the HG group, often insignificantly high values. These tendencies had nothing to do with thermally processed oil. The weight-loss dieting effect by HG was expected but in vain because of the increased ingestion amount by stimulation of appetite⁵. Fecal lipid content (Fig. 7) and bile acid content (Fig. 8) were almost constant, but cholesterol content (Fig. 9) showed an increasing tendency. HG showed some different behavior in the excretion, but did not give any information to find the effect of GHIO on accumulation and fecal excretion of lipids.

As a whole, GHIO did not affect feed efficiency ratio (Fig. 1); retroperitoneal tissue weight (Fig. 3); fatty acid composition of oil extracted from retroperitoneal tissue (Fig. 4); serum levels of insulin, adiponectine (Fig. 2), glucose⁵, tri-

cylglycerol⁵, phospholipids⁵, free fatty acids⁵, and cholesterol⁵; oil absorption (Fig. 6); and excreted lipids (Fig. 5, Fig. 7-Fig. 9). Cytotoxic compounds bound to gluten were absorbed and damaged liver cells⁶, but there were no other influences on rats. Thus, when rats were administered a diet containing fresh oil and GHIO, growth was controlled by fresh oil, and cytotoxicity of GHIO had nothing to do with the digestion, absorption and metabolism of oil.

Koch *et al.*²⁰ and Eder²¹ showed that feeding thermally oxidized oils to rats caused a reduction in the concentrations of triacylglycerols and cholesterol in liver and plasma. They explain that the reduction of triacylglycerols may be due to stimulation of hepatic β -oxidation triggered by activation of peroxisome proliferators-activated receptor α (PPAR α) and reduced hepatic *de novo* fatty acid synthesis²²⁻²⁴. In our experiment⁶, thermally oxidized oil heated at 180°C for 10 h and the filtered oil significantly decreased levels of serum triacylglycerols, cholesterol, phospholipids and glucose in rats. The both oils had similar chemical properties as shown in 2.2.1 for the filtered oil and the latter, smelled mild. Thus, the decrease in hematological levels described above was probably due to the low absorption of thermally processed oil containing polymerized oil, which is not readily hydrolyzed by lipase. In fact, remarkably increased amounts of fecal oil were found in rats fed the diet containing the thermally oxidized and the filtered oils¹⁷, respectively. Many researchers seem to understand that the toxicity of practically used frying oil was derived from oxidized fatty acid moieties of the oil. But series of our studies^{4,5,6,17} showed that the toxicity was attributed to low-molecular-weight compounds generated by the decomposition of the oxidized oil. The chemical properties of the filtered oil (described in 2.2.1) reveal that the oil was composed of intact triacylglycerols and oxidized/polymerized oil. It is of interest that oxidized/polymerized oil has little to do with the toxicity⁶.

Some scientists have reported that protein is related to body-weight reduction. Tomotake *et al.*²⁵ found that consumption of high protein buckwheat flour for 10 days significantly suppressed adipose tissue weight when animals were fed experimental diets containing 20% casein, buckwheat protein extract, or high protein buckwheat flour. Bensaid *et al.*²⁶ reported that protein has a greater satiating effect than carbohydrate, and that the larger the proportion of protein in food, the larger the satiating effect; the quality of protein does not seem to play a significant role. Vazquez *et al.*²⁷ assessed the effects of carbohydrate and protein intakes during weight reduction by employing obese women to consume isoenergetic liquid diets for 28 days. They found that carbohydrate and protein have independent but additive protein-sparing effects during weight reduction. In the present study, however, processed gluten amounted to 0.1 wt% of the diet, and was browned and did not function as intact protein.

In the present study, fried gluten and fresh oil were added to feed instead of deteriorated oil¹²⁻¹⁴. Fried gluten increased appetite and the total amount of food ingestion during the experimental period⁵. In reality, deep-fried foods made in oil that is repeatedly used and periodically has fresh oil added contains cytotoxic low-molecular weight compounds^{4,5,6}, polymerized oil, and relatively fresh oil. Thus, both organ damage and obesity could result from consumption of such deep-fried foods.

In conclusion, it was found that cytotoxic low-molecular-weight compounds bound to fried gluten did not affect the digestion, absorption and metabolism of oil, but that fried gluten increased appetite possibly resulting in obesity.

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