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DIETARY FOOD FORTIFIED WITH OROTIC ACID AND LIVER FUNCTION

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Abstract

The effects of dietary food fortified with orotic acid (1.0%) on liver function were studied in rats. The rats fed with orotic acid promoted liver triglyceride content markedly, that was 5-fold higher than that of the control. The liver malondialdehyde (MDA) content increased by 10%, but the gluthation peroxidase (GSH-Px) activity decreased by 50%. The serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities increased by 25% and 30%, respectively. Therefore, the decreased GSH-Px activity was associated with the promotions of AST, ALT, and the liver MDA levels. In conclusion: dietary orotic acid promotes lipid peroxidation but reduces the rate of the antioxidant enzyme. Therefore, dietary food fortified with orotic acid attenuates the liver function.

Keywords: animal model, liver function, steatohepatitis

1. Introduction

Liver is known as the central organ of metabolism providing numerous metabolic intermediates/precursors used in various enzymatic pathways throughout the body. The gluconeogenetic metabolism, for example, occurs in liver, but its precursors can come from extrahepatic tissue and its product, glucose, can be used to provide cellular energy metabolism throughout the body. Hence, the glucose, in the liver, can be synthesized from glucogenic amino acids from extra hepatic tissue, such as muscle. This mechanism is useful when glucose is the primary energy resource molecule. The energy needed by the some cells of human body, such as brain and erythrocytes, for example, comes only from glucose. Furthermore, in poison, the liver provides a specific metabolic pathway to detoxify the toxins known as glucuronidation pathway [1]. The glucuronidation pathway plays a role in removing non-polar molecules (toxins) and excreting them into the urine. The liver is also known as the main organ of lipid metabolism [2], mainly in anabolic metabolism. The liver converts glucose to fatty acid as well as to fat (triglyceride). Overall, liver is a visceral organ regulating metabolic functions throughout the body.

Most of the chemicals grouped as nutrients can enter and be known by the cellular metabolic pathways. Although each nutrient has major functions in particular metabolic reactions, most of those nutrients are interchangable. The glucose, for example, is the main source of energy for the metabolic system; however, it can also serve as structural molecule [1]. The nutrients can only be metabolized optimally in a certain quantity. Nutrients, therefore, can be categorized into macro- and micro-nutrients. The macronutrients are needed in large amounts by the metabolic systems. The micronutrients, however, are needed in small amounts. Several micronutrients, such as minerals, vitamins, the intermediary metabolic system, as well as orotic acid, play essential roles in homeostatic mechanisms.

The orotic acid is well known as an intermediate of pyrimidine nucleotides biosynthesis. Several studies have reported that dietary orotic acid tend to induce fatty acid biosynthesis accompanied with a promotion of triglyceride biosynthesis in liver cells [3-4]. The promotion of triglyceride biosynthesis which is not equivalent with the secretion and the degradation causes hepatic steatosis [3-5]. The appearance of the hepatic steatosis is often accompanied with an increase of free radical production that is not equivalent with their rate of use by the defense system. The latter condition can reduce the liver function. Biomarkers indicating decrease of liver function have been reported elsewhere [6-9]. Several of them are the final products of lipid peroxidation known as malondialdehyde (MDA), aspartate aminotransferase (AST), and alanine aminotransferase (ALT), and the gluthation peroxidase (GSH-Px). The AST and ALT are two enzymes found in serum, in which their increasing level as well as MDA indicate injury to the liver cells and vice versa. Our previous studies [3-5] found that dietary orotic acid induced fatty liver; however, the effects of liver function

induced by orotic acid have not been elucidated yet. Therefore, the present study was conducted to elucidate the effects of dietary orotic acid on liver function using Sprague-Dawley (SD) rats as animal model.

2. Methods

Animals, diets, and experimental design. All aspects of the experiment were conducted according to the guidelines provided by the ethical committee of experimental animal care at Saga University (Saga, Japan). Male SD rats aged 5 weeks were housed individually in an air-conditioned room (24 ^{o}C) with a 12-h light/dark cycle. After a 1-week adaptation period, rats were assigned to two groups (five rats each). Control diet (as control group) was prepared according to recommendations of the American Institute of Nutrition (AIN), containing (in weight %) 20 of casein, 10 of safflower oil, 1 of vitamin mixture (AIN-93), 3.5 of mineral mixture (AIN-93), 0.20 of choline bitartrate, 0.3 of DL-Methionine, 5 of cellulose, 15 of α-cornstarch, and sucrose to make 100. The orotic acid diet (as orotic acid group) was prepared by supplementing 1.0% orotic to the control diet at the expense of sucrose. The animals received the diets for 10 days. On day 11, rats were killed by decapitation after a 9-h starvation. Livers were excised immediately, and serum was separated from blood.

Analyses of liver and serum lipid contents. Liver lipids were extracted and the concentrations of triglyceride and phospholipids were measured by the methods used in our previous study [4]. The total cholesterol in liver tissues and the serum triglyceride, phospholipids, and cholesterol contents were measured using enzyme assay kits from Wako Pure Chemicals according to the manufacturer's instructions.

Assays of MDA contents. The MDA content of the liver tissue was determined according to method reported by Lykkesfeld [10]. The liver protein content of liver tissue was determined according to the Lowry's method as reported in previous study [4].

Assays of the AST and ALT enzymes. The AST and ALT activities were measured using enzyme assay kits according to the manufacturer's instructions.

Preparation of liver subcellular fractions and assay methods of GSH-Px. The mitochondria of liver subcellular fractions were prepared as previously reported by Nagao *et al*. [11]. The GSH-Px activities were determined according to the method reported by Torres *et al*. [12] with slight adaptation. The reagent mixture (reagent I) was 645 µL of phosphate buffer 50 mM containing 5mM EDTA and 1.125 M NaN_3 (pH 7.0); 25 µL of GSH 0.15 M (reagent II), and mitochondria which contained 0.24 mg of protein. These reagents were allowed to reach equilibrium at 20 ^{o}C. The reaction was started by the addition of 25 µL of H_2O_2 2.2 M and evaluated for 5 min at λ_{340nm}. The non-enzymatic oxidation of GSH was done by replacing mitochondrial protein with H_2O. The differences of the absorbance between the enzymatic and the non-enzymatic oxidation of GSH would indicate the rates of NADPH molecule oxidation.

Statistical analyses. Data were analyzed by one-way analysis of variance, and all differences were inspected by Duncan's new multiple-range test using SPSS statistical software.

3. Results and Discussion

Dietary food fortified with orotic acid reduces growth of body but promotes liver weight. The consequences of food intake generally induce growth; however, some disease might disturb this pathway. Growth means the individual organism growing organically, gaining weight of the body and weights of numerous organ of the body. The growth data is found by weighing the bodies as well as the organs, such as liver. The alterations of those growth factors indicate the effects of food intake.

In order to eliminate the deviations caused by the different quantities of food consumed, the differentiation in the amount of food intake is firstly canceled through a paired-fed procedure. The paired-fed food intake implicated similar quantities of macronutrients, vitamins, and minerals ingested. The replacement of a portion of dietary energy, sucrose (macronutrient), by orotic acid did not influence the homeostatic metabolism because the dietary energy prepared was in excess. Therefore, the effects of food quantity on the treatment have been firstly canceled in the dietary design.

Several growth parameters determined in present study are described in Table 1. The table shows that the average daily food intake between the groups were almost similar. However, it was found that the final body weight of the rats in orotic acid group decreased slightly. Hence, the decreased body weight as

Table 1. Dietary Orotic Acid Promotes Liver Weight

Groups	Control	Orotic acid
Initial BW* (g)	297.0 ±6.0	297.0± 9.0
Final BW* (g)	344.0±3.0	334.0±13.0
Food intake (g/day)	24.4±0.7	24.0± 1.0
Liver weight (g/100g BW)	4.4±0.2^{a}	5.8± 0.3^{b}

Values are expressed as mean± SEM of five rats. Clearly define a & b regarding difference of significance at $p < 0.05$. *BW, body weight

accompanied with an enhancement of the liver weight ($p < 0.05$). The weight of the liver was 32% higher than that of the control group. It can be concluded that orotic acid diet induces growth of the liver with a slight reduction of the body weight.

Dietary orotic acid promoted triglyceride content in liver. Liver cells, unlike the adipose, have a limited capacity for lipid storage. The lipid compositions contained in liver organ are reported as triglyceride, phospholipids, and cholesterol (Fig. 1). Both latter lipids are involved in structural lipids, the main lipids of cell membranes. The deposited lipids in liver tissue are mainly triglyceride and adipose tissue. The enlargement of liver weight induced by dietary orotic acid (Table 1) might indicate an excessive accumulation of triglyceride in that organ.

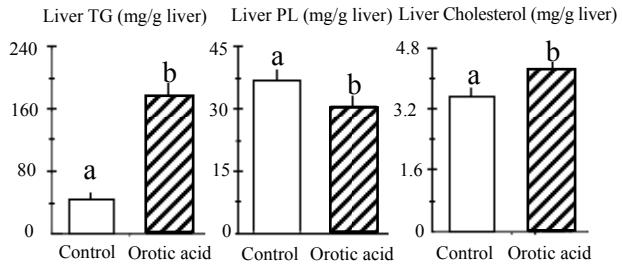
As shown in Fig. 1, the liver triglyceride levels of the orotic acid group were 5-fold higher than that of the control group. The liver triglyceride contents of the control and the orotic acid groups involved 7.5% and 17.9% of the liver weights, respectively. The liver cholesterol also increased, but it failed to reach significant levels ($p < 0.05$). The liver phospholipids, however, decreased significantly ($p < 0.05$). Because lipid storage in liver tissue contains of mainly triglyceride, the enlargement of the liver weight, therefore, might refer to their triglyceride contents in those organs. Sherlock and Dooley [13] report that the excess accumulation of lipids in the liver with a cut-off level exceeding 5-10% of the liver weight is known as simple fatty liver or hepatic steatosis. Based on this rule, therefore, dietary orotic acid develops hepatic steatosis.

Dietary orotic acid promotes lipid peroxidation accompanied with reduction of the antioxidant defense system. The fatty liver induced by orotic acid is included in the term of non-alcoholic fatty liver. Recently, the non-alcoholic fatty liver is the primary disease of the liver, known as the non-alcoholic fatty liver disease. The fatty liver at the steatosis level does not cause injury to the liver. However, at further stages, the steatosis generates steatohepatitis as well as non-alcoholic steatohepatitis (NASH). The NASH indicates the fatty liver accompanied with the inflammation of the tissues [15]. The enhancement of the NASH levels might induce apoptosis. This was because the triglyceride accumulation activates the mitochondrial apoptosis [14]. The swellings and pain are the types of responses of the healthy cells to the inflammation, a local case of results of injuries.

The inflammation caused by the steatosis is partially resulted by the excessive lipid accumulation in liver cells. The high lipid droplet level in cytosolic cellular fractions might interrupt its homeostatic cellular equilibrium and be possible to reduce space and pull the neighboring cells to induce inflammation. The increased degrees of inflammation are partially associated with a promotion of fatty acid peroxidation.

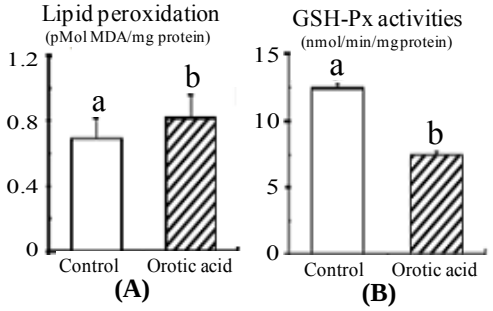
The peroxidation of hydrocarbon chains of the fatty acid on structural lipids can occur in overcapacity of antioxidant defense systems in consumption of the generated lipid peroxide. Therefore, the increase of uncontrolled lipid peroxidation of the tissues increases the inflammation. The uncontrolled inflammation of the liver tissue can cause fibrosis and even cirrhosis. Hence, the hepatic steatosis accompanied with the inflammation has two categories: alcoholic steatohepatitis and NASH. Several authors report that the steatosis that was developed to the steatohepatitis level can only cause reductions of the liver functions [11,15-16]. Therefore, the steatosis induced by orotic acid, if it reaches steatohepatitis level, is important in determining the risk factors of the inflammations. As shown before, the risk factors of inflammations include the increased magnitudes of MDA, reduced GSH-Px levels, and promoted AST and ALT levels.

The liver's MDA level slightly increased although it failed to reach significant levels ($p < 0.05$; Fig 2A). Because MDA is the final product of the lipid peroxidation, this result, therefore, indicates that the lipid peroxidation is promoted by the orotic acid.



Values are expressed as mean± SEM of five rats. They clearly define a & b regarding difference of significance at $p < 0.05$. Abbrev. TG, triglyceride; PL, phospholipids.

Fig.1. The Effects of Dietary Orotic Acid on Liver Lipid Levels



Values are expressed as mean± SEM of five rats. They clearly define a & b regarding difference of significance at $p < 0.05$

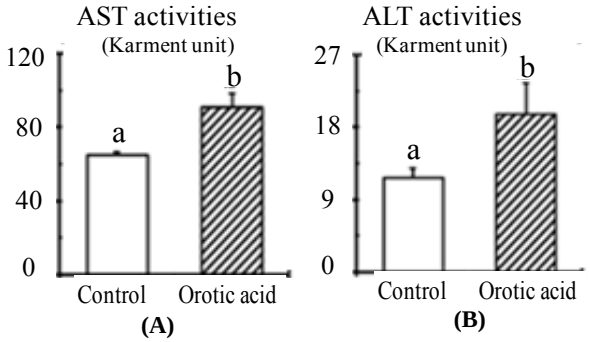
Fig. 2. The liver MDA Contents and GSH-Px activities

The increased liver MDA level indicates that rate of peroxidations on hydrocarbon chains of the fatty acids in metabolic system was stimulated by the orotic acid at present concentrations (1.0%). Presumably, the increased rate of the peroxidation onto the hydrocarbon chains of the fatty acids was related with the promotions of the fatty acid contents, which are substrates of the triglyceride biosynthesis, because the liver triglyceride content increased (Fig. 1). This is in agreement with the data reported in previous studies [3-5]. It is possible that in first stage of the peroxidation, both enzymatic and non-enzymatic reactions, the mono-dehydrogenation occurs first. The mono-dehydrogenation reactions deliver mono-radicals of the macromolecules. Secondly, the interactions between the two macromolecule mono radicals develop swelling as well as inflammation *in situ*. These events can induce the damaging of the macromolecules as well as the structural lipids and the functional proteins. The formation of the macromolecular mono-radicals, particularly on the protein enzymes, can cause enzyme malfunction.

One of the enzymes that play essential roles to attenuate the peroxidation is GSH-Px. This enzyme catalyzes conversion both of lipid peroxide (ROOH) into alcohol (ROH) and the hydrogen peroxide (H_2O_2) to generate H_2O [8,17]. The increased GSH-Px activity promotes levels of the healthy cells and vice versa. As shown in Fig. 2B, the GSH-Px activities decreased significantly ($p < 0.05$). This result indicates that the promotion of lipid peroxidation (Fig. 2A) is reasonable with the decreased GSH-Px activities. The decreased GSH-Px activities in orotic acid treated rats are in agreement with the report of Aoyama *et al*. [18] that dietary orotic acid reduces superoxide dismutase enzyme activities, in which the latter enzyme converses reactive oxygen species to generate O_2 and H_2O_2. Overall, the most important mechanism of liver cell injury by the orotic acid involves the formation of the reactive free radicals (GSH-Px decreased significantly) and subsequent lipid peroxidation (MDA increased slightly).

Dietary orotic acid promotes hepatotoxicities. The promotion of MDA level in liver tissue accompanied with the reduction of the GSH-Px activities indicates that dietary orotic acid induces lipid peroxidative degradation of biomembranes. Bruss *et al*. [16] report that the injuries of the liver cells induce secretions of AST. The AST is known as the enzyme involved in gluconeogenetic pathways, in which its activities are controlled by the glucocorticoid and glucagon hormones. The ALT is also one of the enzymes involved in injuries of the liver cells [6,11-12]. Both enzymes can be determined by their activities in bloodstream.

The activities of the AST and ALT found in serum increased significantly ($p < 0.05$; Fig.3). This result indicates that secretion of both protein enzymes into the bloodstream increased. This result is in agreement with the reports of Edgar *et al*. [7] that the AST and ALT levels in serum increased in stetatohepatis patients. Therefore, the changes of these AST and ALT levels (Fig. 3A-B) are reasonable with the alterations of the MDA and GSH-Px levels (Fig. 2A-B). In conclusion, these results indicate that the hepatosteatosis induced by orotic acid causes hepatotoxicity.



Values are expressed as mean ±SEM of five rats. Clearly define a & b regarding difference of significance at $p < 0.05$

Fig. 3. The AST and ALT Activities

4. Conclusion

The promotion of liver MDA levels accompanied with the reduction of GSH-Px activities, one of the antioxidant enzymes in defense system, suggests that dietary orotic acid causes inflammation and injuries to the liver. Although some other important factors failed to determine in present study (such as tumor necrosis factor-α, interleukin-1, and interleukin-6), the injuries of the liver tissue are reasonable because the AST and ALT levels in the serum increased. The inflammation of the liver cells, therefore, might reduce and even shift the liver function.

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