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Hypercholesterolemia and hepatic steatosis in mice fed on low-cost high-fat diet

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ABSTRACT. To verify whether high-fat diet prepared from commercial diet plus chocolate, roasted peanuts and corn cookies induces hypercholesterolemia in mice and whether there is any hepatic involvement in this type of animal testing. Swiss mice received a high-fat diet for 15 and 30 days; plasma cholesterol, triglycerides and glucose rates were determined. Hepatic impairment was evaluated by histopathological analysis. Cholesterol levels increased 43% in animals treated with high-fat diet for 30 days. Further, histopathological analysis revealed that treatment of animals for 15 and 30 days produced hepatic steatosis and steatohepatitis, respectively. Experimental model is suitable for assessing the action of anti-hypercholesterolemia and the treatment of steatohepatitis.

Keywords: hypercholesterolemia, fatty liver, high-fat diet.

Hipercolesterolemia e esteatose hepática em camundongos submetidos a uma dieta hiperlipídica de baixo custo

RESUMO. Verificar se a dieta hiperlipídica preparada a partir de ração comercial acrescida de chocolate, amendoim torrado e bolacha de maisena é capaz de induzir hipercolesterolemia em camundongos e se há comprometimento hepático neste modelo de experimentação animal. Camundongos Swiss receberam a dieta hiperlipídica por 15 e 30 dias e após isso, foram realizadas dosagens plasmáticas de colesterol, glicose e triglicerídeos. O comprometimento hepático foi avaliado por análises histopatológicas. Os níveis de colesterol aumentaram em 43% nos animais após o tratamento com a dieta por 30 dias. Na análise do fígado, contatou-se esteatose e esteato-hepatite nos animais tratados com a dieta por 15 e 30 dias, respectivamente. Este modelo experimental é adequado para a avaliação da ação de fármacos anti-hipercolesterolêmicos e que auxiliem no tratamento da esteato-hepatite.

Palavras-chave: hipercolesterolemia, fígado gorduroso, dieta hiperlipídica.

Introduction

Cardiovascular diseases are currently a major cause of mortality in the world. Hypercholesterolemia, characterized by increased levels of blood cholesterol, is a major risk factor for developing cardiovascular diseases such as myocardial infarction and hypertension, atherosclerosis and its complications (GERHARDT; GALLO, 1998).

In humans, hypercholesterolemia may be installed via high-fat diet or in individuals with physiological changes, such as deficiencies in the number of functioning LDL-cholesterol receptors (BURTIS; ASHWOOD, 1998)

In addition to cardiovascular disease, there is a strong relationship between increased levels of cholesterol and non-alcoholic steatohepatitis, a chronic disease that affects the liver. Obese people often have high lipemic levels which trigger the onset of hepatic steatosis in 80% of cases (BUGIANESI; LEONE, 2002). Moreover, all obese patients with Type 2 diabetes mellitus have such liver disorders (WANLESS, LENTZ, 1990).

In the USA, approximately 70 million Americans have Non-Alcoholic Fatty Liver Disease (NAFLD). Since it is an asymptomatic disease, the liver abnormality may lead to various complications such as steatopathies (NASH), fibrosis, cirrhosis, portal hypertension and hepatocellular carcinoma, if the cause is not interrupted (BUGIANESI; LEONE, 2002; MATTEONI; YOUNOSSI, 1999; POWELL et al., 1990).

Owing to the importance of hypercholesterolemia in humans, a relentless pursuit for experimental animal models that induce this change has been undertaken to understand better its relation to

atherosclerosis and to establish studies on new types of treatment to reduce levels of blood cholesterol. Since the discovery that high cholesterol diet, with or without cholic acid, was per se atherogenic in mice, attention has focused on models based on dietary supplementation with purified cholesterol. However, due to its expensiveness, other experimental models are being developed. Estadella et al. (2004) concluded that it is possible to induce hypercholesterolemia in rats by low cost high-fat diet, even if the daily diet intake is not altered. In fact, the model comprising a diet supplemented with chocolate, peanuts and corn biscuits increased body weight of the animals and abdominal fat.

Current study determines whether the standardized diet proposed by Estadella et al. (2004) may also induce hypercholesterolemia in mice and whether the liver is damaged within the context of this animal model.

Material and methods

Population

The experimental protocol of this study followed the ethical principles for animal experimentation adopted by the Brazilian College of Animal Experimentation (COBEA) and was approved by the Ethics Committee on Animal Experiments of the Faculdade Integrado de Campo Mourão (EAEC), Campo Mourão, Paraná State, Brazil.

Male Swiss mice, weighing 30 ± 5 g, from Central Vivarium of the State University of Maringá, Maringá, Paraná State, Brazil, were used. The animals were acclimatized to the vivarium of the Faculdade Integrado de Campo Mourão for five days before the application of the respective treatments. The animals had free access to water and food and were maintained at a constant temperature of approximately 24°C, with a 12/12 h light/dark cycle. The animals, weighed at the start and end of the experiment, were divided into three groups (n = 6), denominated control group (DC) with normocaloric diet, hypercholesterolemic group (DH15) with high calorie diet for a period of 15 days and the hypercholesterolemic group (DH30) with high calorie diet for 30 days.

Preparation of diets

The control group received the commercial feed Nuvilab®, with 19% protein, 56% carbohydrate, 3.5% lipids, 4.5% cellulose, 5% vitamins and 3.78 kcal g^{-1} minerals. The other groups received a high calorie diet, standardized by Estadella et al. (2004), containing 19% protein, 47% carbohydrate, 16% lipids, 3% cellulose, 5% vitamins and 19.38 kcal g^{-1} minerals. The ingredients were ground and mixed in pellet form at the following ratio: 15 g of normocaloric feed Nuvilab (3.78 kcal g^{-1}), 10 g roasted peanuts (5.95 kcal g^{-1}), 10 g milk chocolate (5.4 kcal g^{-1}) and 5 g of corn starch biscuits (4.25 kcal g^{-1}).

Biochemical analyses

The mice were fed according to respective periods and made to fast for 15h. They were then killed and blood sampling was undertaken. Plasma levels of total cholesterol, glucose and triglycerides were determined by enzymatic colorimetric methods by commercial kits in Cobas XL. Blood glucose and plasma lipid rates were given in mg dL^{-1}.

Histology analyses

Analysis of hepatic damage was conducted by collecting the liver of animals and by initially analyzing the macroscopic aspect of the organ, such as color, weight and size. Slides were prepared for the microscopic analysis of the liver; one of the lobes of the organ was chosen and was cut at a maximum of 5 mm thickness. The organ was fixed in formalin 10% for 5h at a 1/20 ratio (liver in relation to formaldehyde) and then preserved in alcohol 70%. It should be emphasized that a properly labeled bottle with the relevant information was prepared for each animal. The embedment of the material followed, with several steps: first, the organ was dehydrated by a sequence of baths in ethanol 80% (1h), alcohol 95% (1h), alcohol 100% (1h), and another replacement by alcohol 100% (2.5h). When the organs were duly dried, they were diaphanized to make the translucent material, with three consecutive baths in xylene every 1.5h. The organs were then impregnated with paraffin making possible an extra thin cut for their attachment to the slide. The organs were bathed in previously melted paraffin and kept in an incubator at 60°C. The first bath lasted 1h and the second had a duration of 1h30 min. The samples were processed for 7 μM cuts from the material embedded in the paraffin and stained with hematoxylin and eosin.

Statistical Analysis

Data are given as mean ± standard error of mean (SEM). One-way ANOVA and Neumans-Keuls multiple comparison test at 5% significance was employed to determine the influence of diet on the significance of the model. Statistica (StatSoft Inc.) was used.

Results and discussion

Several hypercholesterolemia models with rats' intake of diets with different levels of lipids and carbohydrates are found in the literature. The most

widely used model is based on the administration of the purified AIN-93G diet (REEVES et al., 1993), modified by adding purified cholesterol (1%) and cholic acid (0.1%). The hypercholesterolemic effect may be often intensified by the substitution of vegetable oil by a saturated fat source, such as vegetal fat (MACHADO et al., 2003). However, purified cholesterol available on the market is expensive and thus its use in studies on experimental hypercholesterolemia is restricted. It is highly relevant to find more affordable methods for the installation of hypercholesterolemia in animals.

Current experiment used a diet supplemented with chocolate, peanuts and corn starch biscuits which provide increased lipid and caloric intake to conventional diets. In the first place, experiments were conducted to assess whether a high calorie diet induced hypercholesterolemia in mice and whether the same diet changed other biochemical parameters in the animals. Consequently, the animals received the high calorie diet for 15 or 30 days. Table 1 shows the plasma glucose, total cholesterol and triglycerides in the control group (DC), in the 15-day high calorie diet group (DH15) and in the 30-day high calorie diet group (DH30). A 12% glucose increase was reported in the DH15 group and a 26% glucose increase in the DH30 group when compared to DC group. However, no statistically significant difference was observed.

Table 1. Plasma concentration of glucose, total cholesterol and triglycerides from animals treated with normocaloric (DC), 15-day high calorie (DH15) and 30-day high caloric (DH30) diets.

	Glucose	Cholesterol	Triglycerides
DC	79.5 ± 21.47	81.2 ± 14.6	133.7 ± 45.3
DH15	89.0 ± 20.9	93.3 ± 19.9	136.2 ± 59.9
DH30	100.7 ± 21.4	116.2 ± 29.2 *	155.9 ± 44.6

*$p < 0.05$ when compared to DC group.

With regard to plasma cholesterol, high-calorie treatment induced statistically a significant increase of 43% when animals were treated for 30 days with a high calorie diet, in comparison to control group. Besides a higher caloric intake, an increase from 3.5 to 16% in the lipid contents of the diet, and a reduction from 4.5 to 3% in the fiber contents may explain this result. The increase in lipid supply to the liver not only by increased intake, but also by an increase in the intestine absorption capacity, enhanced by a decrease of soluble and insoluble fiber amounts in the diet, was able to influence various aspects of digestion, absorption and metabolism among them: a) an increase in intestinal transit time of food, b) a decrease in the rate of the intestinal absorption of glucose, c) an increase in the levels of blood cholesterol by a higher supply of triacylglycerides with a subsequent increase in beta oxidation free from fatty acids; d) an increase in the contents of calories digested. The proposed diet had a reduction in carbohydrate contents ranging from 56 to 47%, even though the capacity of organisms to convert these substrates into triglycerides and cholesterol by the limited capacity of glucose storage as glycogen should be taken into account. The group of animals that received the 15-day high-fat diet failed to have significantly different cholesterol levels from those of control. This may have been caused by insufficient time, proven by the induction of hypercholesterolemia in animals fed for 30 days with the diet.

Treatment with high calorie diet did not cause significant difference in plasma triglycerides at different treatment times and showed only a slight increase in triglyceride levels (16%) in animals treated for 30 days.

When the evolution of the animals' body weight during the experimental period is analyzed, it could be observed that high calorie diet did not cause significant increase in weight when compared to that of control animals that received a normocaloric (Figure 1).

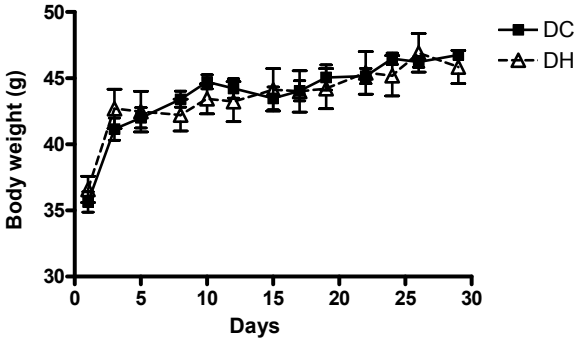

Figure 1. Weight evolution of animals treated with standard diet (DC) and animals treated with high calorie diet (DH) for 30 days. Rates are mean ± standard error of mean.

With regard to hepatic damage, macroscopic visualization did not show any changes in weight, color or texture of the liver among the groups of animals under analysis. However, the microscope analysis of histological cuts revealed micro-droplet hepatic steatosis (Figure 2) without inflammation in the liver's parenchymal cells in the 15-day high calorie diet group (DH15). Control animals with normal diet (DC) did not contract hepatic steatosis. Jaldin et al. (2006) studied the effect of a diet supplemented with egg yolk in the development of atherosclerosis in rabbits. This diet induced hypercholesterolemia when the animals were treated for a 30-day period and even caused the appearance of atherosclerotic lesions.

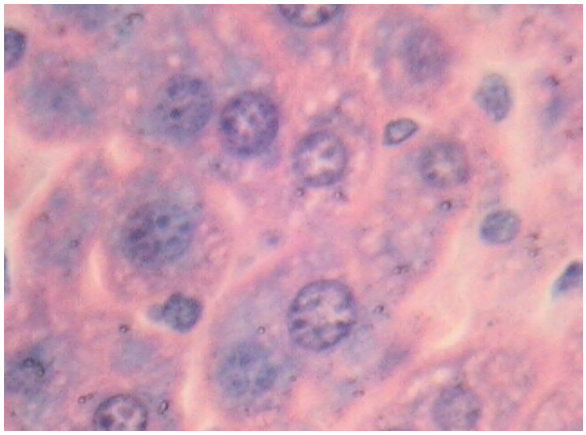

Figure 2. Arrows indicate accumulations of fat micro-droplets in the cytoplasm of hepatocytes.

The 30-day calorie diet animal group (DH30) contracted hepatic steatosis and also revealed a feature of steatosis evolution, known as steatohepatitis (NASH). The latter is an inflammatory process which may be perceived by the presence of leukocytes in the tissue (Figure 3). In fact, hyperlipidemia is a risk factor for changes in the liver, already demonstrated in several studies (SAITO et al., 2007).

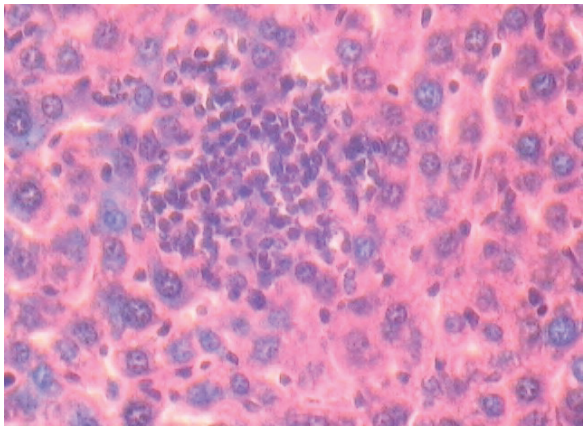

Figure 3. The arrow indicates inflammatory process in liver cells.

The amount of lipids in the liver is determined by the balance of several processes, such as the import of free fatty acids (FFA) from the adipose tissue, a renewed FFA synthesis in hepatocytes, beta oxidation of FFA, sterification of FFA in triacylglycerides and the export of triglycerides in very low density lipoproteins (VLDL). Hepatic steatosis is a consequence of the imbalance of one of these processes where the accumulation of triacylglycerides was harbored. Since high-fat diet is rich in triacylglycerides, an increased amount of FFA is transported to the liver and a condition of hepatic steatosis is installed.

When the cause of hepatic steatosis is not eliminated, a condition, such as steatohepatitis, may occur. In current analysis, such condition was observed in animals fed on high-fat diet for 30 days. FFA accumulation in hepatocytes stimulates the dependent NF-kB inflammatory cytokines such as TNF-alpha, IL-6 and IL-1 beta by Kupffer cells, which are specific macrophages found in the liver (FELDSTEIN et al., 2004), and trigger an inflammatory process in the liver. The evolution of this phase causes fibrosis, by which damage in hepatocytes and associated inflammation activates the hepatic stellate cells and the synthesis of extracellular matrix proteins (DUVNJAK et al., 2007).

Conclusion

Current study shows the efficiency of the model of hypercholesterolemia induced by high calorie diet, composed of Nuvilab feed, chocolate, peanuts and maize biscuits, with regard to an increase in plasma cholesterol levels of animals and the establishment of steatohepatitis. Due to its low costs, the experimental model is suitable for the evaluation of anti-hypercholesterolemic drugs and their activities in the treatment of steatohepatitis.

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