Effects of activated carbon \(N\)-acetylcysteine sustained-release microcapsule on dipeptidyl peptidase IV expression in young rats with non-alcoholic fatty liver disease

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Abstract. Non-alcoholic fatty liver disease (NAFLD) in children has become the most common liver disease influencing adolescent health and one of the most influencing chronic liver diseases among children in Chinese wealthy families, particularly in coastal regions. However, the medicine available for the treatment of NAFLD is deficient. In order to solve this problem, our team studied the activated carbon \(N\)-acetylcysteine (NAC) sustained-release microcapsule, which improves the oxidation resistance, bioavailability and drug stability of acetylcysteine and reduces toxic and side effects. In addition, it accords with the characteristics of medication in infants and children. The present study mainly discusses whether the activated carbon NAC sustained-release microcapsule has effects on dipeptidyl peptidase IV (DPPIV) activity and protein in young rats with NAFLD, and whether it has the effect of an DPPIV inhibitor, hoping to provide new thoughts and methods with respect of basic studies on young rats with NAFLD/non-alcoholic steatohepatitis.

Introduction

With the globalization and younger-age trend of obesity, non-alcoholic fatty liver disease (NAFLD) has become one of the most prevalent chronic liver diseases among children in developed countries and wealthy Chinese families, particularly in coastal regions (1-5). Dipeptidyl peptidase IV (DPPIV) is a type of transmembrane serine protease, T cell activation protein and adenosine deaminase binding protein, which is also known as cluster of differentiation 26. Besides the hydrolytic activity of peptidase, it also has regulating effects on fat metabolism, immunity and inflammation (6). The results of previous studies have revealed that the activity of DPPIV is associated with chronic liver diseases, including NAFLD (7-9). Previously, Machado et al (10) and Qu et al (11) effectively combined the powerful antioxidant \(N\)-acetylcysteine (NAC) and activated carbon for medicine with good adsorption properties and biocompatibility, and prepared an NAC-activated carbon sustained-release microcapsule (ACNAC). ACNAC reduced various side effects of NAC and enhanced the half-life of drugs and bioavailability. Based on previous studies (12-15), the present study aimed to further explore the effects of ACNAC on young rats with NAFLD by establishing a model of young rats with NAFLD in order to detect the serum DPPIV activity level and the expression of DPPIV protein in the liver. The present study discusses whether ACNAC is a DPPIV inhibitor, and whether it is able to protect young rats with NAFLD.

Materials and methods

Preparation of non-alcoholic fatty liver model and sample collection. A total of 64 healthy, clean and weaned Sprague-Dawley male rats (weight, 51.93±4.28 g; 21 days old) were purchased from the Animal Center of Zhejiang Academy of Medical Sciences [Hangzhou, China; animal license number: SCXK (Zhe) 2014-0001]. The rats had \textit{ad libitum} access to a standard commercial diet and water, with the exception of preoperative fasting and were kept in rooms maintained at 22±1℃, 40-60% relative humidity with a 12 h light/dark cycle throughout the experiments. All experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and ethical approval was granted by the Animal Care Committee of Xixi Hospital Affiliated to Zhejiang University of Traditional Chinese Medicine (Hangzhou, China). Following 1 week of.
acclimatization to these conditions, rats were randomly divided into the following groups (n=8 per group): Normal group, fed standard diets; model group; fed high-fat diets (69% basic feed, 10% lard oil, 2% cholesterol, 5% sugar, 0.5% cholate, 10% yolk powder, 3% yeast powder and 0.5% decavitamin); polyene phosphatidyl choline group; administered 60 mg/kg PPC (Sanofi-aventis; Beijing, China) by gastric perfusion daily and fed high-fat diets; NAC group, administered 60 mg/kg NAC (Whu hoyo Co., Ltd, Wuhan, Hubei) by gastric perfusion daily and fed high-fat diets; activated carbon release microcapsule group, administered 60 mg/kg activated carbon release microcapsule (Zhejiang Hangzhou Wood industry Co., Ltd., Huzhou, Zhejiang) and fed high-fat diets; and ACNAC low-, medium- and high-dose groups, fed high-fat diets and administered 15, 30 and 60 mg/kg of ACNAC [made in-house as described previously (12-15)], respectively by gastric perfusion daily. Following 7 weeks of these treatments, the young rats were sacrificed, and their blood and livers were collected and stored at -80˚C for future use.

Calculation of liver index. The liver-wet weight and the body weight of the young rats were calculated. The liver index was calculated according to the following formula: Liver index (%) = liver-wet weight / body weight x 100.

Observation of the degree of fatty degeneration in the liver tissue of young rats by hematoxylin and eosin (HE) staining. Fresh liver tissues from rats of each group were treated in 4% paraformaldehyde for 24 h at room temperature, dehydrated with alcohol prepared according to a set gradient (75, 85, 90, 95 and 100%) and then embedded into paraaffin blocks. A total of 5 blocks were selected at random and each one was cut into three sections on a paraaffin microtome with a thickness of 4-μm. Sections were deparaffinaged in dimethylbenzene, embedded in xylene I for 20 min, xylene II for 20 min, absolute ethyl alcohol I for 10 min, absolute ethyl alcohol II for 10 min, 95% alcohol for 5 min, 80% alcohol for 5 min and 70% alcohol for 5 min successively prior to washing with water. Nuclei staining with hematoxylin: Sections were stained with hematoxylin for 3-8 min and eosin 1-3 min. The liver tissue structures were observed with a light microscope (magnification, x400; Nikou Corporation; Tokyo, Japan).

Blood from celiac vein was used to determine the biochemical indicators. A Hitachi 7060 automatic biochemical analyzer (Hitachi, Ltd., Tokyo, Japan) was used to detect serum alanine transaminase (ALT; Wako Pure Chemical Industries, Ltd., Osaka, Japan), aspartate transaminase (AST; Wako Pure Chemical Industries, Ltd., Osaka, Japan), total cholesterol (TC; Beijing Homa Biological Engineering Co., Ltd., Beijing, China), total triglycerides (TG; Beijing Homa Biological Engineering Co., Ltd.), high-density lipoprotein cholesterol (HDL-C; Medicalsystem Biotechnology Co., Ltd., Ningbo, Zhejiang), low-density lipoprotein cholesterol (LDL-C; Medicalsystem Biotechnology Co., Ltd.), fasting blood glucose (FBG; Autec Diagnostic; Baden-Württemberg, Germany). An abbott i2000 (Abbott Molecular Inc., Chicago, IL, USA) was used to detect serum fasting insulin (FINS; Abbott Molecular Inc.).

**Detection of the activity of DPPIV by a Gly-Pro-7-aminio-4-methylcoumarin (AMC) fluorescence method.** The operations were conducted according to the manufacturer's instructions of a DPPIV kit (AAT Bioquest Inc., California, USA). A total of 5 mg of Gly-Pro-AMC (a sensitive fluorogenic substrate; molecular weight, 443.37) was introduced into 563.5 µl of dimethyl sulfoxide (DMSO) to prepare 20 mM substrate DMSO stock solution. The substrate DMSO stock solution were diluted into 100 µM substrate DMSO stock solution with 50 mM Tris-HCl, and mixed with rat serum (150 µl:150 µl) for 1 h at room temperature. The fluorescence intensity was measured with an enzyme-labeled instrument (Biotek Corporation; Broadview, IL, USA) at Ex/Em=380/500 nm. The activity inhibition rates of DPPIV were calculated with the following equation (16): Activity inhibition rate of DPPIV = (fluorescence intensity of negative control group - fluorescence intensity of each administration group) / fluorescence intensity of negative control group x 100%.

Detection of DPPIV protein expression by an immunohistochemical staining method. Liver tissue were fixed with formaldehyde and hydrated with gradient ethanol as described in HE staining. Following citrate buffer antigen retrieval, slices were incubated with 3% deionized water for 20 min and then washed with PBS for 5 min, 3 times. Following blocking with 5-10% normal goat serum (Boster Biological Technology Co., Ltd.) for 25 min at room temperature, 4 μm thick slices were incubated with DPPIV primary antibody (cat. no. 10940_1_ap; 1:100; Proteintechn group, Inc., Wuhan, Hubei) at 4˚C overnight. Subsequently, samples were incubated with goat anti-rabbit secondary antibody (cat. no. GB23303; 1:200; Wuhan Goodbio Technology Co., Ltd., Wuhan, Hubei) for 50 min at room temperature. Following color development with 3,3'-diaminobenzidine reagent for 5 min at room temperature, the sections were mounted and observed under a light microscope (magnification, x400; Nikou Corporation), and the appearance of a brown-yellow liver cell membrane indicated positive expression. A total of five non-overlapping fields of view were selected and the immunohistochemical scores (IHS) method (17) was used to score samples according to the percentage of positive cells and their staining intensity, and the results were analyzed with Image Pro-Plus version 6.0 (Media Cybernetics, Rockville, Maryland, USA).

Detection of the expression of DPPIV protein in liver tissues by western blot analysis. Total protein was extracted from the liver tissue. The total sample protein was extracted using the holoprotein extraction kit (Nanjing KeyGEN Biotech Co., Ltd., Nanjing, China). Liver tissue (100 mg) was placed in a petri dish and cut into blocks (~3x3 mm), 0.5-1 ml of 0-4˚C Lysis Buffer were added, which contained 5 µl of phosphatase inhibitor, 1 µl of protease inhibitor and 5 µl 100 mM phenylmethylsulfonyl fluoride. The mixture was homogenized 15 times under 4˚C prior to centrifugation at 10,625 x g for 15 min at 4˚C. The supernatants were collected and protein quantification were performed using the BCA method; and the absorbance was read at 562 nm with a ultraviolet spectrophotometer. In total, 20 µg protein was loaded into each well and subjected to 5% SDS-PAGE at 60 mA. The proteins were then transferred onto a polyvinylidene difluoride membrane at 100 V constant.
voltage for 65 min, and incubated using 5% skimmed milk powder overnight at 4°C. The PVDF membrane was incubated with DPPIV and β-actin primary antibody (1:500, Abcam; Cambridge, UK) for 2 h at room temperature, and then washed three times for 10 min with phosphate buffered saline with Tween 20 (PBST). The PVDF membrane was incubated with horseradish peroxidase labeled goat anti-rabbit secondary antibody (cat. no. ab97200; 1:1,000; Abcam, Cambridge, England) for 1 h at room temperature and washed three times for 10 min with PBST. The dianinobenzidine developer were prepared before use. The washed PVDF membrane was put into the developer for 3 min, and then the dianinobenzidine reaction was terminated with water. The membrane was imaged with a full-automatic digital gel image analysis system (Shanghai Tanon Technology Co., Ltd., Shanghai, China) and the results were analyzed with Image J version 1.48 (National Institutes of Health, Bethesda, USA).

Statistical methods. Data are presented as the mean ± standard deviation. Comparison among groups was performed using one-way analysis of variance and the least significant difference method was used for pairwise comparison among groups when the variances were equal. In addition, the Games-Howell method was used for pairwise comparison among groups when the variances were not equal. SPSS version 18.0 software (SPSS, Inc., Chicago, IL, USA) was used for the statistical analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

Liver index results of young rats. Compared with the normal group, the liver index in the model group increased significantly (P<0.05). Compared with the model group, the liver index in the ACNAC groups were significantly decreased (P<0.05), and the most marked decrease was observed in the high-dose group. No significant difference was observed in liver index between the ACNAC high-dose and PPC groups (Fig. 1).

Detection of DPPIV protein expression by immunohistochemical staining. Positive expression of DPPIV is indicated by a brown-yellow liver cell membrane. Compared with the percentage of DPPIV-positive cells of the liver cell membrane in the normal group (89.19±6.23%) the expression of positive cells in the model group (43.48±4.99%) was significantly decreased (P<0.05). Compared with the model group, a significantly higher percentage of DPPIV-positive cells were detected in the liver cell membranes of the ACNAC groups (P<0.05), and the positive expression of DPPIV increased with the decrease of fatty degeneration of liver tissue, particularly in the high-dose ACNAC group in which the percentage (68.57±6.48%) of positive cells was most markedly increased. In comparison with the PPC group, which had an inhibition rate of 46.42±5.88%, the ACNAC high-dose group had an increased inhibition rate of DPPIV activity in the serum and the intergroup differences, however, this was not statistically significant (P>0.05; Fig. 1).

Results of DPPIV protein expression detection by western blot analysis. In comparison with the normal group, the DPPIV protein expression in the model group was significantly decreased (P<0.05). Compared with the model group, DPPIV protein expression in the low-, medium- and high-dose ACNAC groups significantly increased in a dose-dependent manner (P<0.05; Fig. 6).

Discussion

NAFLD has become the most common liver disease influencing the health of children and adolescents (18-20). According to a previous study, NAFLD patients most likely account for 30% among 70 million individuals in the USA and up to 8% among children and adolescents between 2 and 19 years old (20). The pathogenesis in children may be different from adults; however, the overall incidence trend is simple fatty liver, fatty liver hepatitis, liver fibrosis and even liver cirrhosis (21). Consequently, it is necessary to screen...
the children with such a disease risk as soon as possible and to study the pathogenesis (22,23). For obese children with NAFLD, any unhealthy dietary and lifestyle habits should be corrected (24-26), aerobic exercise encouraged, and their weight should be controlled to reduce insulin resistance so that liver damage may be reversed at an early stage (27-29). If necessary, drug intervention with low levels of toxicity and side effects, and evident curative effects may be used to effectively reduce the occurrence of NAFLD and other chronic metabolic diseases in children (30,31).

At present, the main issues facing medication for children are imperfect drug safety information and the lack of medicinal varieties (32), specification and special formulations, which results in adulteration of medicine in children and various disadvantages (33). In order to solve these problems, China has increased the input of oral sustained-release therapies, controlled release preparations, orally disintegrating tablets and transdermal absorption preparations, with the aim of developing preparations that conform to the characteristics of medication in infants and children and that have a low toxicity, few side effects and a high bioavailability (34). For the present study, the advantage of adsorptive behavior and in vitro release properties of activated carbon for medicine were considered, and activated carbon NAC sustained-release microcapsules were studied in order to improve in oxidizability and bioavailability of NAC, reduce the dose in clinical use, increase drug stability and elucidate the ideal pharmaceutical preparation for infants and children.

DPPIV is widely found in organisms and its expression is predominantly in epithelial cells, lymphocytes, endothelial cells and fibroblasts. Previous studies have demonstrated that DPPIV has a multiple-effect on biological activity (35,36), particularly the protease in known DPPIV affects fat metabolism, predominantly lipid metabolism of an organism through neuropeptide Y and inactivation of other polypeptides. The present study used a high-fat diet to induce a model of young rats with NAFLD and combines children’s dietary structure and habits in order to establish a young animal model with abdominal obesity, lipid metabolism disorder and elevated liver enzyme levels that are similar to children with NAFLD. The results demonstrated that compared with the normal group, the model group exhibited weight gain, liver index increase, visceral fat deposition, evident liver volume increase, and the livers were grayish-yellow color and had a greasy surface area in young rats. Additionally, different degrees of fatty changes and ballooning degeneration were observed via HE pathological staining. In addition, vacuoles of different sizes were filled in the cells, and various areas were accompanied by inflammatory cell infiltration and occasional focal necrosis. Furthermore, the activity of ALT, AST, TC, TG, LDL-C, FBG, FINS and DPPIV in the serum was evidently increased and the expression of HDL-C and
DPPIV protein in the liver cell membrane were markedly decreased. A previous study by Balaban et al (37) demonstrated that the activity of DPPIV in the serum in patients with
non-alcoholic steatohepatitis was markedly higher than those in the normal group. Furthermore, the degree of pathological change of liver tissue and degree of fat change of liver cells of NAFLD were associated with the serum DPPIV activity and the intensity of protein expression. It is thought that DPPIV may be closely associated with the pathogenesis of
NAFLD (38). It has previously been revealed that the DPPIV inhibitor may improve fatty degeneration of the liver in rats (39). Furthermore, a previous clinical experiment demonstrated that the DPPIV inhibitor may also improve transaminase levels and ballooning degeneration of liver cells in patients with NAFLD (40). The present experimental results demonstrated that, compared with the model group, the ACNAC groups (particularly the high-dose group) exhibited different degrees of improvement in the indexes detailed above, which reduced the activity of ALT, AST and DPPIV and the content of TC, TG, LDL-C, FBG and FINS, increased the content of HDL-C, (particularly the high-dose group) exhibited different degrees that, compared with the model group, the ACNAC groups, ACNAC2, ACNAC3, ACNAC4, N-acetylcysteine in nonalcoholic steatohepatitis: A preclinical study in a dietary mouse model. Dig Dis Sci 61: 137-148, 2016.

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