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ORIGINAL ARTICLE

Temporal Expression Pattern of Lipid Metabolism-related Genes and Reactive Oxygen Species (ROS) Level in Grass Carp (Ctenopharyngodon idellus) L8824 Cell Line during Hepatic Steatosis

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Abstract: Objective: To investigate the mechanism of grass carp hepatic steatosis and the role of ROS in this process, grass carp liver cell line L8824 is used to establish the hepatic steatosis model by induction with oleic acid. Methods: The CCK8 kit and alanine transaminase (ALT) and aspartate transaminase (AST) were used to evaluate the cell variability and cell damage. Real time-PCR was used to detect the key genes of lipid metabolism (PPAR, SREBP-1c, ACC, SCD-1, CPT-1 and MTTP). DCFH-DA probe was used to detect ROS. The results showed that treatment with oleic acid can continuously reduce the cell viability and exacerbate cell damage. The fatty acid de novo synthesis, β-oxidation a temporal expression pattern of lipid metabolism-related genes and Reactive Oxygen Species (ROS) level in grass carp (Ctenopharyngodon idellus) L8824 cell line during hepatic steatosisnd VLDL assemble were enhanced during the treatment from 0-24h, and then all the aspects were impaired by continuous induction. The ROS was maintained at a low level during 0-24h, then significantly increased at 36 h, then decreased to a second high level. Conclusion: The lipogenesis, β-oxidation, and triglyceride (TG) transportation are all heightened during nutritional steatosis until the hepatocytes fully loaded with fat, and the ROS levels perform in an opposite trend.

Keywords: Aquaculture; Hepatic Steatosis; Lipid Metabolism Related Genes; Reactive Oxygen Species (ROS)

Introduction

Non-alcoholic fatty liver disease (NAFLD) is a common disease in cultured fish, which can cause acquired metabolic stress liver injury. The main causes of this disease are the large use of high-fat feed, high-density breeding and unbalanced nutrition. This kind of problem is especially prominent in grass carp breeding. As China's largest aquaculture output.

For large aquatic species, grass carp breeding generally adopts the intensive feeding mode of compound feed[1]. In this mode, the breeding population often suffers from symptoms such as excessive fat accumulation and liver fat metabolism disorder, which will lead to the decline of meat quality and even cause a large number of deaths of cultured fish in high temperature seasons.

To explore the formation mechanism and pathological process of fatty liver in grass carp, it is necessary to select appropriate cell models to reduce the complexity of the study. Lu Ronghua *et al.*[2] and Li xuexian *et al.*[3] established fatty degeneration models of grass carp hepatocytes induced by fatty milk and oleic acid respectively.

Lipid metabolism in normal liver is always in a dynamic equilibrium state. On the one hand, carbohydrate is used as raw material for de novo fatty acid synthesis (de novo lipogenesis, DNL), and fatty acid esterification is carried out at the same time to generate Triglyceride (triglyceride, TG), i.e. fat. on the other hand, fat is continuously transported to the outside of liver cells through ultra-low density lipoprotein (vldl), while hepatocyte mitochondria are also continuously oxidized to provide energy for the body. Therefore, the formation of fatty liver is the result of this balance being broken

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and moving towards one side. Statistics show that in patients with hepatic steatosis, the fat accumulated in the liver mainly comes from Free fatty acids brought by the circulatory system.

FFAs is re-esterified, and a small portion (26%) comes from de novo lipogenesis (DNL)[5]. In addition, intracellular triglyceride deposition will increase mitochondrial oxidative compensation and generate a large amount of reactive oxygen species (ROS). Reactive oxygen species will oxidize organelle membranes such as mitochondria, causing mitochondrial dysfunction, and will further lead to liver cell inflammation and eventually develop towards liver fibrosis. At present, the source of triglyceride and the change rule of ROS in the formation of fatty liver in fish have not been reported.

In this study, the fatty degeneration model of grass carp liver cells induced by oleic acid was taken as the object, and the lipid metabolism related genes and active oxygen content of grass carp liver fine cells at different stages of fatty degeneration were detected to explain the possible mechanism of fatty degeneration of grass carp liver cells and the related oxidative damage, thus laying a theoretical foundation for prevention and nutrition regulation of grass carp fatty liver.

1. Materials and methods

1.1 Test material

Grass carp hepatocyte line (L8824) is purchased from China Center for Type Culture Collection, CCTCC for short.

1.2 Main instruments and reagents

Olympus IX71 inverted fluorescence microscope, Tecan Infinite F200 microplate reader, Tatungci-160-A Co 2 culture. The incubator, PCRmax ECO48 real-time quantitative PCR instrument, fetal bovine serum (Gibco), oleic acid (Sigma), oil red o (mclean), trypsin (mclean), Triglyceride, TG) detection kit (blue sky), CCK-8 Cell Activity Test Kit (Jiman Biology), Alanine Amino Transfer (ALT)/aspartate aminotransferase, AST) Test Kit (Nanjing Built), DCFH-DA Active Oxygen Test Kit (Blue Sky").

1.3 Establishment of fatty degeneration model of grass varp hepatocytes

L8824 cells were cultured in MEM medium (Gibco) containing 10% FBS V/V, Sijiqing and Sigma each 100000 U/L. The cells were placed in a 280C, 5%CO2 cell incubator. When the cells grew to 70%-80% fusion, the cells were digested and passaged with 0.25% trypsin. Using oleic acid to induce lipid-filling of grass carp hepatocytes, refer to Li Xuexian *et al.*[3].

1.4 Detection of cell viability and hepatocyte damage

Liver cell injury was detected by ALT and AST content indexes in cell culture solution. After cell induction 0h, 12h, 24h, 36h and 48h, respectively, ALT and AST are detected by taking cell culture solution and using ALT and AST detection kits, and the operation steps are strictly carried out according to the operation specifications of the kits.

1.5 Determination of triglyceride content

Methods Refer to Xiao Peizhen et al[7]. The steps are as follows: oleic acid is used to induce cells to 0h, 12h, 24h, 36h and 48h, then the culture solution is carefully discarded, PBS is used for cleaning 3 times, 10% neutral formaldehyde is added for fixing 45 min ,and then PBS is used for cleaning 3 times. Oil red O dyeing 45 min, washing several times with triple distilled water, thoroughly washing suspended cells and residual oil red O dye, adding 60% isopropyl alcohol extraction and measuring absorbance at 510 nm.

2. Results

2.1 Effect of leic acid induction on triglyceride (TG) content in hepatocytes

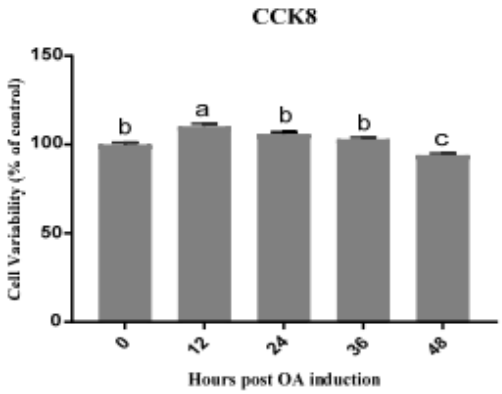
Cell induction was carried out with 400 μmol/L oleic acid. the content of triglyceride in cells was detected during induction of 0h, 12h, 24h, 36h and 48h. from the beginning to 24h, the content of triglyceride increased significantly ($p<0.05$), 24h reached saturation state, i.e. full of fat, 24-36h triglyceride content did not change significantly, 48h began to show significant decline

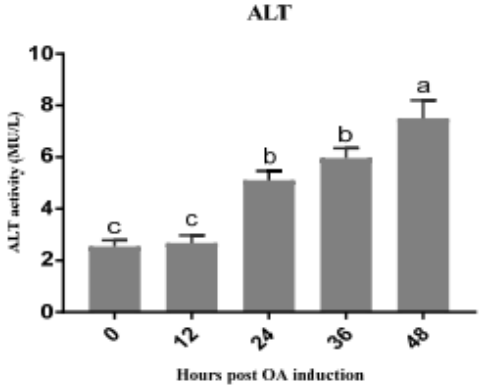
2.2 Effect of oleic acid induction on liver cell viability and liver injury index of grass carp (Ctenopharyngodon idellus)

The liver cell activity of grass carp significantly increased after 12h induced by oleic acid ($p<0.05$), and significantly decreased at the highest point, 24h ($p<0.05$) to the pre-induction level (0h). the cell activity decreased after 24-36 h but did not reach a significant level ($p>0.05$). the cell activity at 48 h significantly decreased and was significantly lower than the initial level (Figure2, $p<0.05$). Glutamic pyruvate transaminase activity showed an upward trend in the process of oleic acid induction, and showed a significant increase at 24h ($p< 0.05$), 36h continued to increase but did not reach a significant level ($p>0.05$), 48 h the enzyme activity level rose again significantly (figure 2, $p<0.05$), glutamic pyruvic transaminase activity also showed an upward trend during oleic acid induction, but only showed a significant increase at 48 h (figure 2, $p<0.05$).

2.3 Changes of lipid metabolism gene expression in adipose degenerated hepatocytes induced by oleic acid

After the grass carp liver cells were treated with 400 μmol/L oleic acid, SREBP-1c gene transcription level expression significantly increased at 12h and 24h ($p<0.05$) and reached the highest at 24 h, significantly decreased at 36h ($p<0.05$) and did not reach a significant level at 48 h (Figure3A, $p>0.05$); The transcriptional expression of PPAR gene increased significantly at the first 36 h ($p<0.05$), reached the highest at 24h ,decreased significantly at 48h and was equivalent to the gene expression level at 24h (Figure 3B, $p<0.05$); The expression level of ACC gene increased significantly at 12h and 24h ($p<0.05$), reached the highest at 24h, decreased significantly at 36h ($p<0.05$), decreased slightly at 48h but did not reach a significant level (Figure 3C, $p>0.05$). The expression level of SCD-1 gene increased significantly at 12h and 24h ($p<0.05$) and reached the highest at 24h. it decreased significantly at 36h ($p<0.05$). Although it decreased slightly at 48h, it did not reach the significant level (Figure 3D, $P > 0.05$). The expression level of CPT-1 gene showed an upward trend during 0-36h and reached the highest level at 36h, but reached a significant level at 24h and 36h ($p<0.05$); while the level rose at 12h but did not reach a significant level. The $p>0.05$ and 48h levels decreased but did not reach significant levels (Figure3E, $p<0.05$). The expression level of MTTP 155 gene was significantly increased at 12h and 24h ($p<0.05$), and reached the highest at 24h and significantly decreased at 36h. Decrease ($p<0.05$); decrease at 48h but did not reach significant level (Figure 3F, $p>0.05$).





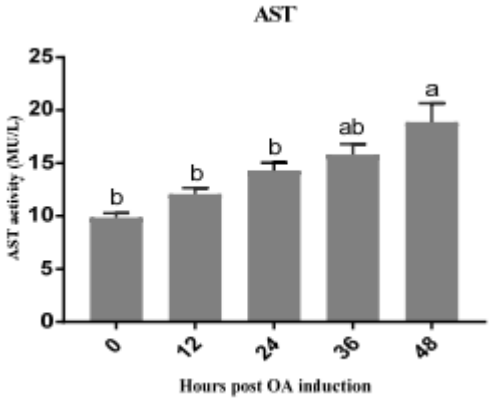


Figure 2. Effects of oleic acid on cell viability and ALT/AST level in grass carp hepatocytes.

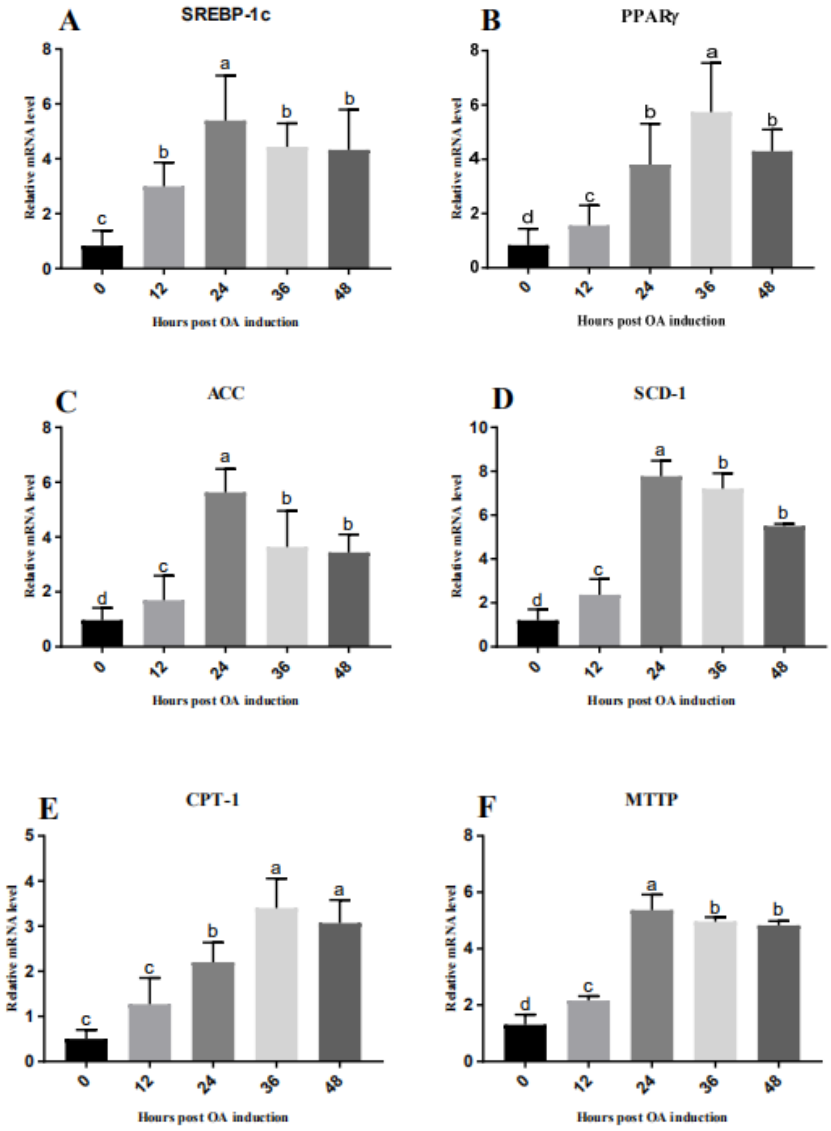


Figure 3. The relative expression level of lipid metabolism-related genes in Ctenopharyngodon idellus hepatocytes after oleic acid induction

2.4 Detection of ROS in fatty liver cells of grass carp induced by oleic acid

During oleic acid-induced lipid-filling of grass carp hepatocytes, the overall level of ROS showed a slight decrease and then a sudden upward trend. The content of active oxygen in the first 24h did not change significantly ($p>0.05$), and

the active oxygen levels in the 36h and 48h increased significantly (Figure 4, $p<0.05$).

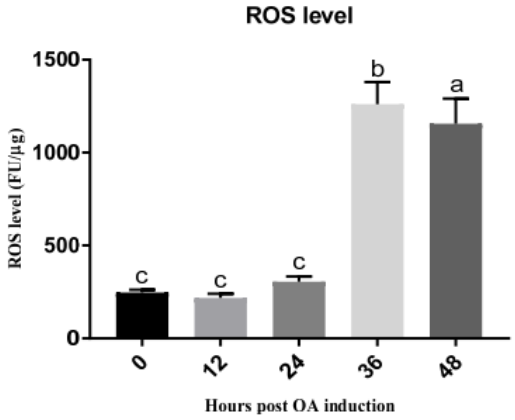


Figure 4. The content of reactive oxygen species in Ctenopharyngodon idellus hepatocytes after inducing with oleic acid.

3. Discussion

Accompanied by an increase in reactive oxygen species and a series of metabolic disorders[8]. In order to simulate this process, this study continued to treat cells with oleic acid to 48 h after the cells reached saturation (24h). Cell viability analysis showed that grass carp hepatocytes showed a significant decrease in cell viability after oleic acid treatment for 48h, while the levels of ALT and AST in cell culture fluid showed an upward trend, indicating continuous oleic acid treatment.

Peroxisome proliferator activated receptor (PPAR) and sterol regulatory element binding protein (SREBP-1c) are two important transcription factors related to lipid metabolism. Studies in mammals have shown that overexpression of SREBP-1c and PPAR will increase the fat content in the liver[9] while treatment with inhibitors of PPAR or deletion of PPAR gene with adenovirus-mediated vector can alleviate this situation[10]. The results of the study on SREBP-1c are similar. reducing the expression of this gene can also effectively alleviate the degree of hepatic steatosis[11]. In fish, after feeding medaka with high-fat diet, the expression of srebp-1c increased, while the expression of the corresponding lipolytic gene (CPT-1) decreased[12].

Acetyl coenzyme A carboxylase (Acetyl CoA carboxylase) catalyzes the first step of fatty acid de novo synthesis and is the rate-limiting enzyme[13] for aliphatic acid synthesis. Carnitine palmitoyl transferase -1 (Carnitine palmitoyl transferase 1, CPT-1) is located on the outer membrane of mitochondria and catalyzes the synthesis of fatty acyl carnitine from long-chain fatty acyl coenzyme A and carnitine. It is the first rate-limiting reaction in the oxidation process of mitochondrial fatty acid, and its expression level can reflect the strength of mitochondrial oxidation. In this study, the expression of CPT-1 showed an upward trend 0-36 h after induction, and then began to decline, indicating that the oxidation of grass carp hepatocytes has been increasing from the beginning of lipid denaturation to the saturation level, and the decrease after h may be due to apoptosis caused by lipid toxicity.

4. Conclusion

This study found that oleic acid induces fatty degeneration of grass carp hepatocytes 48h and its lipid metabolism related gene expression profile and reactive oxygen species production can be divided into two stages: 0-24h. The triglyceride content increased rapidly, the cell activity did not change significantly, the hepatocyte damage increased, the fatty acid synthesis, mitochondrial oxidation and triglyceride transport related gene expression were up-regulated, and the reactive oxygen species content remained at a low level. 24-48h, the cell is fully lipid-filled, triglyceride content is stable and moderate, cell vitality is reduced, and liver cell injury is advanced.

With the increase of step, the expression of genes related to fatty acid synthesis and triglyceride transfer began to decline, and the expression of mitochondrial oxidation-related genes also began to decline after reaching a high point 36 h, and the content of active oxygen increased and maintained at a high level. The results of this study can lay a foundation for further research on the pathogenesis and pathological process of fatty liver in fish.

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