Study on the Mechanism of Erucamide Mediating Anti-stress Liver Injury in Rats by Inhibiting Glycine Cleavage

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Abstract

Objectives: In order to research the erucamide effect on Stress-induced rats liver injury and potential mechanism of the protect effect.

Methods: Stress-induced liver injury in SD rats was used as an in vivo model. Adult female SD rats were differentiated into three groups: Normal group, SLI(Stress-induced liver injury)-group and [SLI+Erucamide (95.4uM/Kg)]-treated group. In addition, the transcriptomes from 9 liver tissues were sequenced using an Illumina sequencing platform, screened the differentially expressed genes and related cell signal transduction pathways. Gene expression was measured by RT-qPCR. Furthermore, observe the pathological changes of rats liver tissues by HE staining , and levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured by microplate method. Human LO2 cells was used for in vitro model, the cells in the logarithmic growth phase were differentiated into normal group, corticosterone groups, erucamide, and glycine intervention groups. Corticosterone (300uM) was used to induce LO2 cell damage in vitro. And using tetrazolium (MTT) colorimetric method to determine and calculate cells viability. Hoechst 33342 staining was used to observe the morphology of hepatocyte apoptosis.

Result: This study showed that erucamide has a significant effect on Stress-induced liver injury in rats, which is proved by HE staining test and ALT / AST detection experiment ($p<0.05$). The results of transcriptomes indicated that erucamide could inhibit the cleavage of glycine to prevent stress-induced liver injury in rats. Moreover, RT-qPCR results demonstrated that erucamide reduced the expression of DLD and AMT($p<0.05$) genes in glycine cleavage system, thereby reducing glycine cleavage. Compared with the SLI group, erucamide inhibited UNC5B gene expression($p<0.05$), which is related to cell apoptosis, indicating that erucamide can inhibit cell apoptosis. In addition, erucamide inhibited the expression of GPSM3 ($p<0.05$) and IGSF11 that are associated with inflammation. In addition, both erucamide and glycine could prevent the damage of LO2 hepatocytes induced by CORT. Erucamide and Glycine were effective in preventing Crot-induced LO2 cell damage.

Conclusions: In this research, erucamide inhibits the cleavage of glycine to anti-stress liver injury in rats.

1. Introduction

Stress is defined as a state in which the living organism is in a process of imbalance in dynamic balance and re-establishing balance [1]. Perturbation of an organism's homeostasis by stress can trigger biological or behavioral adaptation and accelerate onset and course of several diseases [2]. Corticotropin-releasing hormone (CRH) is a key controller of the stress reaction. This peptide controls the hypothalamic-pituitary-adrenal (HPA) axis as well as a variety of behavioral and autonomic stress responses. The glucocorticoid finally secreted by the HPA axis is corticosterone (CORT) [3]. Stress-related hypothalamic-pituitary-adrenal (HPA) axis disorders are associated with increased serum corticosterone (CORT) [4]. Long-term excessive exposure to stress cause CORT continue to rise, leading to a wide range of disease symptoms, for instance anxiety, insomnia, depression, metabolic syndrome, cardiovascular disease, etc [5]. The liver will experience varying degrees of liver damage because of stress, called “Stress-induced liver injury”, for example stress-induced metabolic disease, for instance non-alcoholic fat disease, and the incidence is increasing year by year. However, lacking of consistent evidence, it is still difficult to date clear safe and effective drug treatment plan [6]. Therefore, screening anti-stress liver injury drugs is imminent.

Erucamide (Era) is a bioactive fatty acid amide, which is like to the classical endocannabinoid analogue oleoylethanolamide (OEA) [7]. In addition, erucamide is an endogenous EA-amide derivative that regulates

angiogenesis and water balance, and exerts antidepressant and anxiolytic effects in mice [8]. The ECS signalling has long been known to regulate the hypothalamic-pituitary-adrenocortical (HPA) axis, which is the major neuroendocrine stress response system of mammals, and stress may be related to the occurrence of mental illness, for instance anxiety, insomnia, depression [9–10]. Animal experiments have shown that erucamide has anti-depressant and anxiety disorders, but there is no report of erucamide on stress liver injury. Therefore, this article mainly studies the mechanism of anti-stress liver injury of erucamide.

Transcriptomics is one of the most developed fields in the post-genomic era. Transcriptome is the intact set of RNA transcripts in a concrete cell type or tissue at a certain developmental stage and/or under a specific physiological condition. Transcriptomics pay attention to the gene expression at the RNA level and provides the genome-wide information of gene structure and function so as to reveal the molecular mechanisms involved in respective biological processes [11–12]. This article utilized transcriptomics technology to study the effect of erucamide on stress liver injury.

Glycine is a non-essential amino acid with a wide range of anti-inflammatory and cell protection [13–14]. Generally, glycine is synthesized from choline, serine, hydroxyproline, and threonine through interorgan metabolism in which kidneys and liver are the primarily involved [15]. It is reported that glycine can inhibit organ damage caused by oxidative stress [16–18]. The body produces a large amount of oxygen free radicals under Stress. Excessive oxygen free radicals triggered lipid peroxidation, destroy cell membrane function, damage normal physiological functions of cells, and cause organic and functional lesions in liver and other tissues. The brain of rats treated with alcohol showed edema, edema is significantly reduced after treatment with glycine [19–21]. The results of the KEGG and GO enrichment pathway of the transcriptome of stress liver injury showed that the cleavage of glycine in the carbon metabolism pathway of the SLI group increased, resulting in a decrease in the level of glycine in the body. In contrast the erucamide group reduces the cleavage of glycine. The glycine cleavage system consists of four proteins: three enzymes and one carrier protein [22–23]. These enzymes are: glycine decarboxylase (GLDC), aminomethyltransferase (AMT) and dihydrolipidamide dehydrogenase (DLD), the carrier protein is called H protein (GCSH, a lipoic acid-containing protein) [24]. When glycine is cleaved too much, the decrease of glycine level in the body will cause diseases [25]. Therefore, the erucamide in this article may protect the stress liver injury by inhibiting the cleavage of glycine.

2. Materials And Methods

2.1 Animal culture and reagents

All animal experiments were approved by the Animal Ethics Committees of Guangdong Pharmaceutical University. SD rats (female, 180–220 g) were used and preserved in the Animal Housing Unit in an environment with controlled temperature (22 ± 2 °C) and lighting (12 h light-dark cycle). Standard laboratory food and drinking water were offered at discretion. A period of at least 3 days was permited for the animals to acclimatize before performing any experimental procedures. Erucamide (CAS number 112-84-5) obtained from Macklin; ALT kit and AST kit (Nanjing Jiancheng Bioengineering Institute) ; Hoechst 33342 (Biyuntian Biotechnology Company)

2.2 Establishment of stress liver injury model and drug effect

Female SD rats were randomly divided into three groups: Normal group, SLI(Stress-induced liver injury)-group and [SLI + Erucamide (95.4uM/Kg)]-treated group, each with 8 rats. After continuous intraperitoneal injection of erucamide every morning and evening for 3 days. Except for the normal group, the rats in the other groups were

restrained under the cold water (21℃) for 48 hours. Rats fasted under restraint stress in cold water (21℃). After the experiment, all rats were anesthetized with ether, blood was collected from the abdominal aorta, and liver tissue was quickly isolated on an ice tray.

2.3 Morphological examination

The liver tissues of the rats were fixed with 4% paraformaldehyde, embedded with paraffin, sliced, stained with hematoxylin-eosin (HE) and inspected under a light microscope.

2.4 Determination of biochemical indexes and its methods

After the experiment, the rats were anesthetized with 10% chloral hydrate, and blood samples were collected from their abdominal aorta and centrifuged to obtain serum. ALT and AST activities in the serum were detected in accordance with the kit instructions. The liver tissue were washed with cold normal saline and then prepared into 10% liver homogenates; the homogenates were centrifuged to obtain supernatant fraction. Determine ALT and AST activity in accordance with the kit instructions.

2.5 The rat liver tissue transcription experiment

The experimental process is shown in the Fig. 1

The rat liver tissue transcription experiment commissioned by Gideo(Guangzhou, China)

2.6 Quantitative real-time RT-PCR

For a quantitative comparison of the AMT, DLD, UNC5B, GPSM3 and IGSF11 mRNA levels, RT-qPCR was performed using SYBR Green fluorescence. Total RNA was extracted with TRIzol reagent (Takara, Japan) from frozen liver tissues according to the manufacturer's instructions. The primers for the AMT, DLD, UNC5B, GPSM3 and IGSF11 sequences are listed in Table 1 (Shenggong Biological Engineering Co., Ltd., Shanghai, China). The RNA concentration and purity were determinated by Micro spectrophotometer (NanoDrop™ One, Thermo, USA). Total RNA from each original sample was converted into cDNA, and RT-qPCR was performed in a Lightcycler 480 Thermo Cycler (Roche, Basel, Switzerland) according to the manufacturer's protocols. The amplification was performed in a 10μL reaction mixture that consisted of the following: 5 μL of qPCR Master Mix (Accurate Biology, China), 0.5 μL of cDNA, 0.2 μL of 10 μmol/L forward primers and 0.2 μL of 10 μmol/L reverse primers and 4.1 μL of nuclease-free water. Each assay was performed in triplicate with GAPDH mRNA as the endogenous control. The RT-qPCR thermal cycling parameters were an initial denaturation for 30 s at 95 °C that was followed by 40 cycles at 95 °C for 5 s and 52 °C for 30 s. Melting curves were performed to investigate the specificity of the PCR reaction. Relative quantification (RQ) of each gene expression was calculated according to the comparative Ct method using the formula: $RQ = 2^{-\Delta\Delta Ct}$

Table 1
Primer pairs for real-time PCR.

Gene name	Forward primers (5'-3')	Reverse primers(5'-3')
GAPDH	ACAGCAACAGGGTGGTGGAC	TTTGAGGGTGCAGCGAACTTACATTGGACCAGCAACCACATCTC
DLD	ACGGCCCTTTACGCAGAATTTGG	CGGCAGCCAGCATTGGACACTAC
AMT	AGTGGTTGGAGACATTGCGGAAC	GGATCTTGTCGGCAGAGTCC
UNC5B	GTTTCCACCCCGTCAACTTC	TCACCTGTTCACCATCTTCTTCC
GPSM3	CTTCCTGTGTTGTAGCTAGTTCC	GTGTGAAATGCTTTGGCAGAAG
IGSF11	ACCTGTCTTCTGGATCTCCAG	

2.7 Cell culture and reagents

Human LO2 cell (Gift from Professor Zhou Yuan, Guangzhou University of Chinese Medicine) were cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin in a 5% CO2 atmosphere at 37 °C. Erucamide (CAS number 112-84-5) and glycine (CAS number 56-40-6) obtained from Macklin and MTT (3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-H-tetrazolium bromide) were obtained from Sigma-Aldrich. Corticosterone obtained from aladdin.

2.8 MTT assay

The cells were seeded at 8×10^3 cells/well in a 96-well plate for 24 hours, and treated with different concentrations of erucamide and glycine. After 1 hour, except for the Normal, corticosterone (300 uM) was added to each well, and co-cultivation for 24 hours. In addition, add 100 μl MTT (0.5 mg/ml) solution to each well and incubate at 37 °C for 4 hours. Then, the supernatant was absorbed and 100 μl DMSO was added to dissolve the MTT-formazan crystals. The absorption was measured by the microplate at 570 nm.

2.9 Determination and method of biochemical index

The cells were cleaned with cold normal saline and then prepared into 10% cells homogenates; the homogenates were centrifuged to get the supernatant. Determine ALT and AST activity in the light of the kit instructions.

2.10 Hoechst 33342 stain

LO2 cells were inoculated in a 12-well plate, the cells are divided into normal group, Cort group (300 μmol/L), Gly (600 μmol/L) and Era (10 μmol/L) drugs group. Discard the culture medium, the drugs Gly and Era were added in a 12-well plate, one hour later add Cort and continue to incubate for 24 hours, discard the culture medium, wash 2 times with PBS, fix with fixative for 10 min, discard fixative, wash 3 times with PBS, add 10 μg/ml Hoechst 33342 staining solution, stain for 10 minutes at room temperature and avoid light, discard the staining solution and wash twice with PBS, aspirate the liquid, and observe the morphological changes of apoptotic cells under a fluorescence microscope.

2.11 Statistical analysis

All data were presented as mean ± SD (standard deviation). The comparison between groups was assessed by analysis of variance. $p < 0.05$ was considered statistically significant. Statistical analysis was performed with

SPSS 19.0 software (IBM, Armonk, NY, USA).

3. Result

3.1 Erucamide effects on pathological changes in the liver tissue of rats with Stress-induced liver injury

The pathological changes of liver tissue were inspected under a light microscopy. The results demonstrated that liver cells were actually arranged with distinct nucleoli and involved plentiful cytoplasm with a red color and round nuclei. The nuclear chromatin appeared blue. No unusual pathological changes in liver tissues in the Normal group were inspected (Fig. 2A). In the SLI group, liver cells were obviously injured, showing distinct liver cell swelling, with aberrant nucleus size, hepatic cord mistake, and large area of vesicular degeneration of liver cells (Fig. 2B). In the SLI + Era group, cells with hydropic degeneration were inspected in liver tissues,but a lesser extent than in the SLI group, and liver cells were mildly dropsical (Fig. 2C)

3.2 Effects of erucamide on AST and ALT in serum of rats with Stress-induced liver injury

Compared with those in the Normal group, serum ALT and AST activities of rats in the SLI group were obviously increased ($P < 0.001$). In contrast to those in the SLI group, serum AST activities of rats in the SLI + Era groups were unsignificantly reduced, and ALT activities of rats in the SLI + Era groups were obviously reduced ($P < 0.001$), see Fig. 3.

3.3 Effects of erucamide on AST and ALT in liver tissues of rats with Stress-induced liver injury

AST and ALT activities of liver tissues in the SLI group significantly distinct from those in the Normal group ($P < 0.01$).In contrast to those in the SLI group, AST and ALT activity was significantly reduced ($P < 0.05$) in the SLI + Era group, as shown in Fig. 4.

3.4 Screening of differentially expressed genes by mRNA expression chip

The consequence of distinctively expressed genes screened by mRNA expression microarrays illustrated that there were 5432 distinctively expressed genes in the Normal group contrast to the SLI group, among which the expression of 3139 (57.79%) genes was obviously up-regulated, and the expression of 2293(42.21%) genes was obviously down-regulated (see Fig. 5A). In contrast to the SLI group, there were 319 distinctively expressed genes in the SLI + Era group,among which the expression of 132 (41.38%) genes was obviously up-regulated, and the expression of 187(58.62%) genes was obviously down-regulated (see Fig. 5B).

3.5 Functional enrichment analysis of differentially expressed gene KEGG

From metabolic analysis, the mechanism of erucamide protectingrats from Stress-induced liver injury is mainly related to homeostasis, endocrine metabolism and neurosecretory metabolism, among which the regulation of

carbon metabolism and amino acid metabolism is the main potential mechanism(see Fig. 6 and Fig. 7).

From the analysis of the organism's organic system, the mechanism of erucamide protecting rats from Stress-induced liver injury is mainly related to the immune system, endocrine system and nervous system(see Fig. 6 and Fig. 7).

From the analysis of human diseases, the mechanism of erucamide protecting rats from Stress-induced liver injury is highly related to immune diseases, metabolic diseases and neurological diseases(see Fig. 6 and Fig. 7).

From the results of the KEGG enriched carbon metabolism pathway analysis, it can be seen that compared with the Normal group, the catabolism of glycine was increased in the SLI group increased. On the contrary, in contrast to the SLI group, the SLI + Era group reduced the metabolism of glycine(see Fig. 8 and Fig. 9:Black frame). In the central nervous system, glycine is an inhibitory neurotransmitter. Glycine and glutamate are both agonists in the central nervous system, regulating the nervous system and related neurological diseases. Therefore, erucamide may prevent stress liver injury by inhibiting the cleavage of glycine.

3.6 Quantitative real-time RT-PCR

The DLD, AMT, UNC5B, GPSM3 and IGSF11 genes were choosed for real-time quantitative PCR. (Fig. 10). The consequence demonstrated that the DLD, AMT, UNC5B, GPSM3 and IGSF11 genes played an important part in the Stress-induced rats liver injury. Among them, DLD and AMT are the key enzymes of the glycine cleavage system. In contrast to the Norma group, the gene expression of DLD and AMT in the SLI group has a significant increase (###P < 0.001), and Erucamide can inhibit its expression. In addition, the main biological function of UNC5B gene is to participate in cell apoptosis [26]. Compared with the SLI group, Erucamide can inhibit its expression, indicating that erucamide can inhibit cell apoptosis. In addition, the abnormal expression of GPSM3 [27] and IGSF11(an immunoglobulin (Ig) superfamily member) [28] genes is associated with inflammation. Experimental results clarified that Erucamide can also regulate inflammation.

3.7 Assessment of beneficial effects of erucamide and glycine in protecting Crot-induced LO2 cell damage

In this study, in contrast to the Normal group,Crot (300uM) treatment significantly decreased the viability of LO2 cell (P < 0.001). On contrary，erucamide at 2.5μM、5μM、10 μM concentrations also prevented Crot-induced LO2 cell damage, and erucamide at 10 μM concentrations significantly prevented Crot-induced LO2 cell damage(P < 0.001,see Fig. 11A). In addition, glycineat 1.5mM、3mM、6 mM concentrations also prevented Crot-induced LO2 cell damage ; and glycine at 6 mM concentrations Significantly prevented Crot-induced LO2 cell damage(P < 0.05, see Fig. 11B). Overall, these data suggested that Erucamide and Glycine were effective in preventing Crot-induced LO2 cell damage.

3.8 Effects of erucamide on AST and ALT in LO2 cell with Crot-induced LO2 cell damage

AST and ALT activities in the Cort group obviously differed from those in the Normal group (P < 0.01). In contrast to those in the Cort group, AST activity was obviously reduced (P < 0.05) in the erucamide group, as shown in Fig. 12.

3.9 Effects of glycine on AST and ALT in LO2 cell with Crot-induced LO2 cell damage

AST and ALT activities of LO2 cell in the Cort group obviously differed from those in the Normal group ($P < 0.01$,$P < 0.05$). In contrast to those in the Cort group, AST and ALT activity was obviously reduced ($P < 0.001$ $P < 0.05$) in the glycine group, as shown in Fig. 13

3.10 Hoechst 33342 staining to observe the results of cell apoptosis

The chromatin distribution of LO2 cells in the normal group was uniformly diffused low-intensity fluorescence (see Fig. 14A), and normal organelles and intact cell membranes were visible. After adding 300 μmol/L corticosterone for 24 h, a large number of nuclei appeared densely stained with dense condensed morphology or granular fluorescence (see Fig. 14B), indicating that the action of 300 μmol/L corticosterone for 24 h caused obvious apoptosis of LO2 cells. Compared with corticosterone model group, glycine (see Fig. 14C) and erucamide (See Fig. 14D) The drug can reduce the apoptosis induced by corticosterone.

4. Discussion

The best of our knowledge, cold-restraint stress (CRS) induced hepatic dysfunction in rats [29–30]. Liver damage cause damage to the stability and integrity of various biofilms and increase their permeability, leading to the outflow of enzymes in the cytoplasm into the blood, such as ALT and AST. Therefore, the resolution of ALT and AST activities in the serum is a significant index for assessing liver injury [31]. In the current experiment, the activity of serum and liver tissues ALT and AST in the SLI group was obviously higher than that in the Normal group. In contrast, the activity of serumand liver tissues ALT and AST in the SLI + Era group was obviously lower than that in the SLI group, recommending that erucamide may exert a conservatory effect on Stress-induced liver injury. CORT-induced cellular stress injury model is a classic model, that is frequently used to study the effect and mechanism of anti-cell stress injury drugs. In cell experiments, the results showed that erucamide and glycine were effective in preventing Crot-induced LO2 cell damage. In addition, the transcriptome and real-time fluorescent quantitative RT-PCR results expounded that erucamide inhibits stress-induced liver injury by regulating the glycine lysis system, cell apoptosis and immune system.

In the study, the liver injury in rats was caused by Stress-induced liver injury. It is known that liver injury is carefully associated with apoptosis of liver cells and that the promotion of liver cell apoptosis can cause liver injury. According to research reported that glycine can inhibit liver cell apoptosis [32–33]. Therefore, erucamide inhibits the cleavage of glycine, and increases the glycine content in the body, which inhibits the apoptosis of liver cells, inhibiting stress-induced liver injury in rats.

The human liver is classically perceived as a non-immunological organ, required for metabolic activities, nutrient storage and detoxification[34]. Transcriptome results testified that the mechanism of erucamide protecting rats from stress liver injury is highly related to immune diseases, metabolic diseases and neurological diseases. Stress is an ever-present part of modern life. The "stress response" forms an organism's mechanism for deal with a given stress, which is mediated via the release of glucocorticoids and catecholamines. Giving consideration to the broad impact glucocorticoids and catecholamines have on immune cell function, it is very likely that stress has a great impact on the hepatic inflammatory response [35].Glycine played an important role in immunity, which treated metabolic disorders in obesity, diabetes, cardiovascular disease [36]. In the vitro experiment of this research, glycine and erucamide can prevent glucocorticoid Crot-induced LO2 cell damage.

In conclusion, 95.4uM/Kg erucamide injected 3 days prior to induction of low-temperature restraint stress liver injury ameliorated the disease by inhibiting glycine cleavage, inhibiting cell apoptosis, and regulating immunity. Thus erucamide and glycine provides a precious lead for the treatment of Stress-induced lievr injury.

Declarations

Competing Interests:

The authors have declared that no competing interests exist.

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Figures

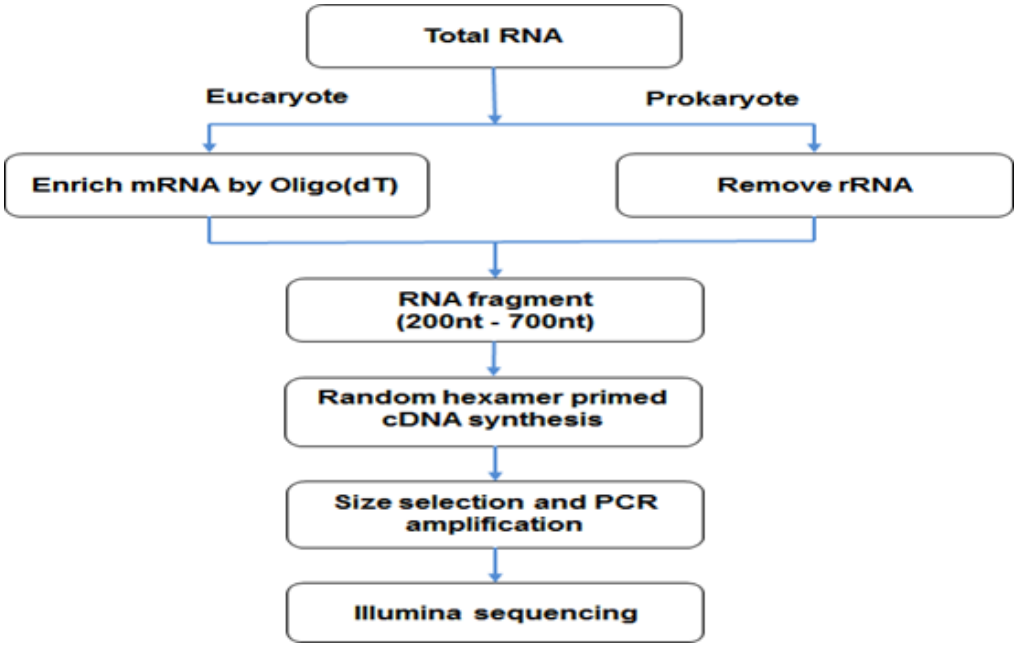

Figure 1

Flow chart of transcriptome experiments

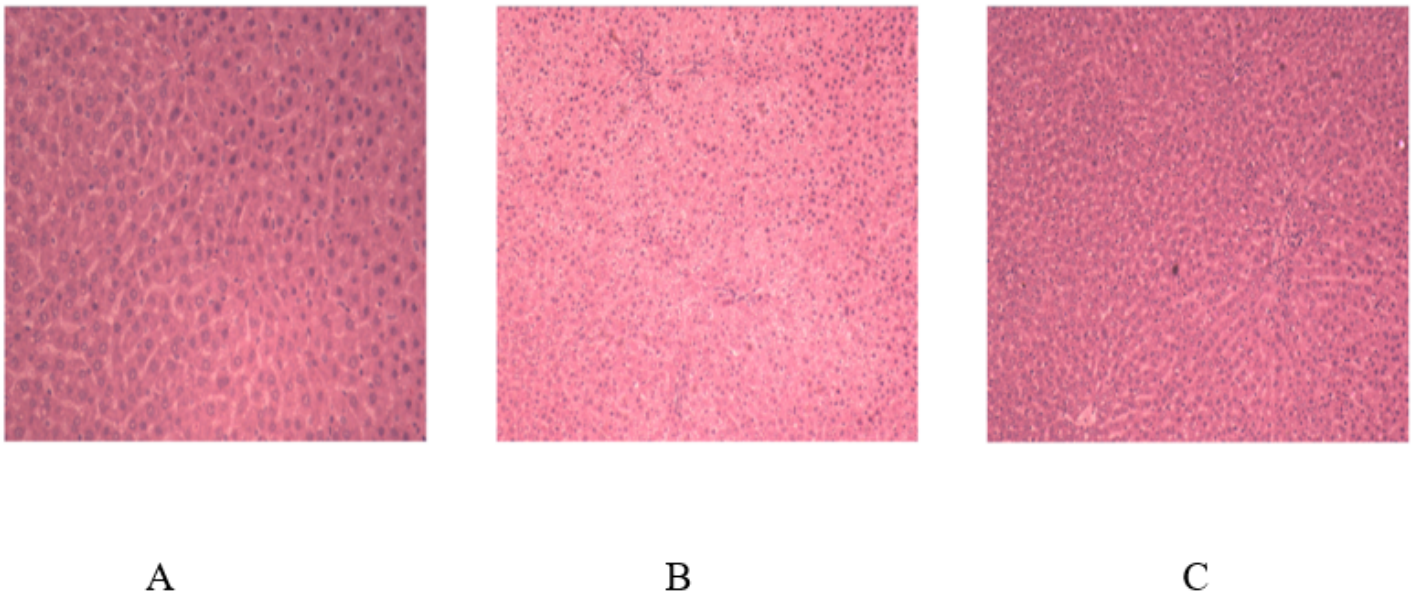

Figure 2

HE staining of the liver cells of rats (HE100×)

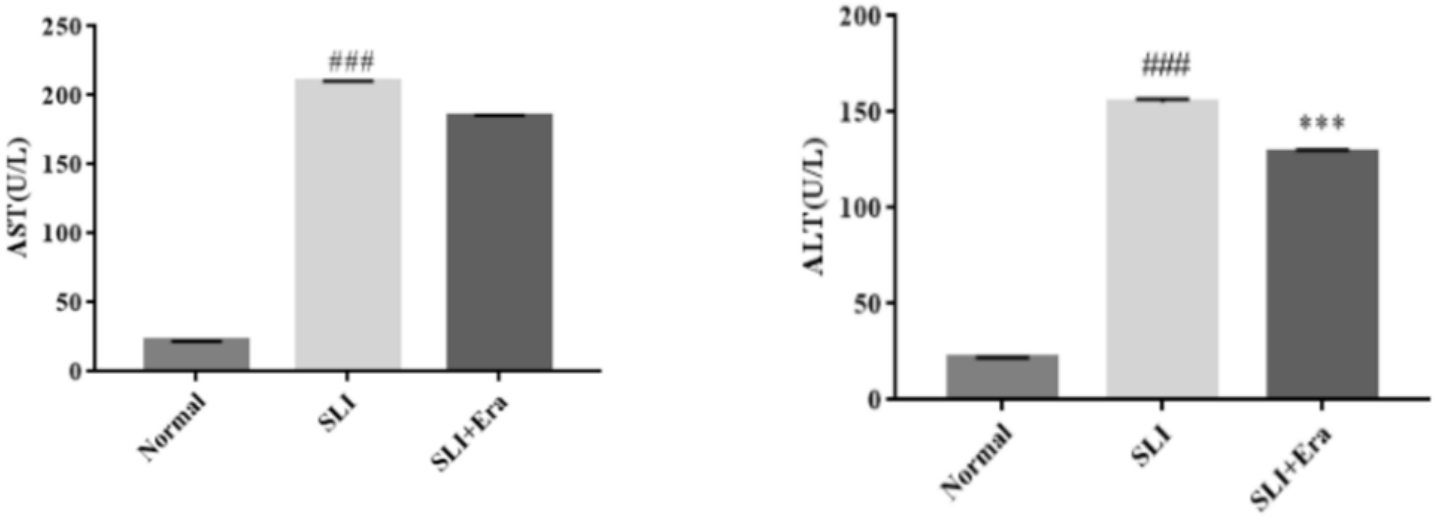

Figure 3

Effects of erucamide on AST and ALT in serum of rats with Stress-induced liver injury (AST: SLI vs Normal group ###P<0.001. ALT:SLI vs Normal group ###P<0.01 ; SLI+Era vs SLI ***P<0.001)

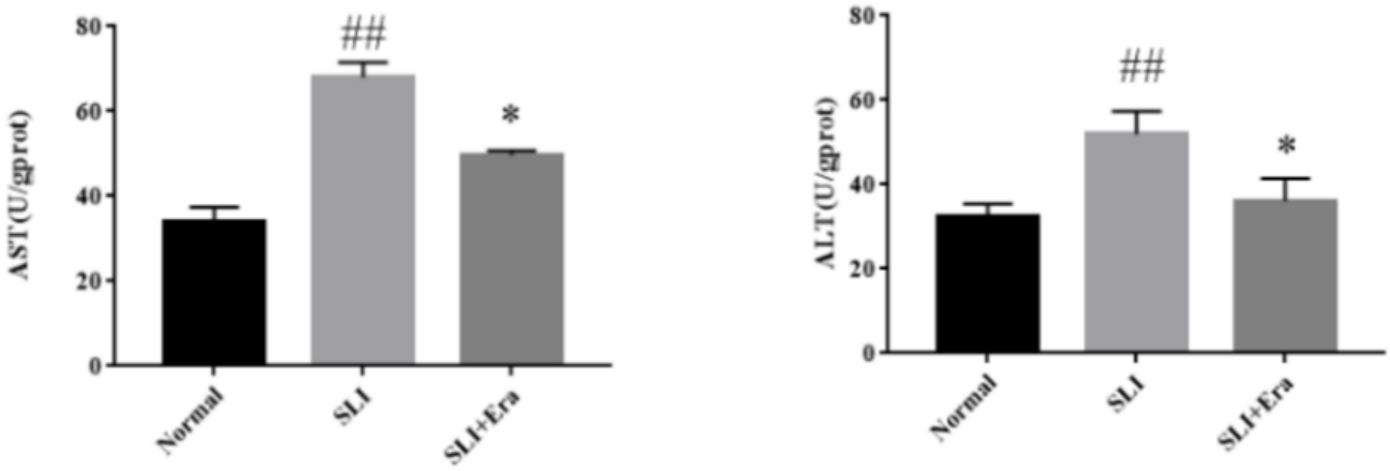

Figure 4

Effects of Erucamide on AST and ALT in liver tissues of rats with Stress-induced liver injury (AST: SLI vs Normal group ##P<0.01; SLI+Era vs SLI*P<0.05. ALT:SLI vs Normal group ##P<0.01 ;SLI+Era vs SLI *P<0.05)

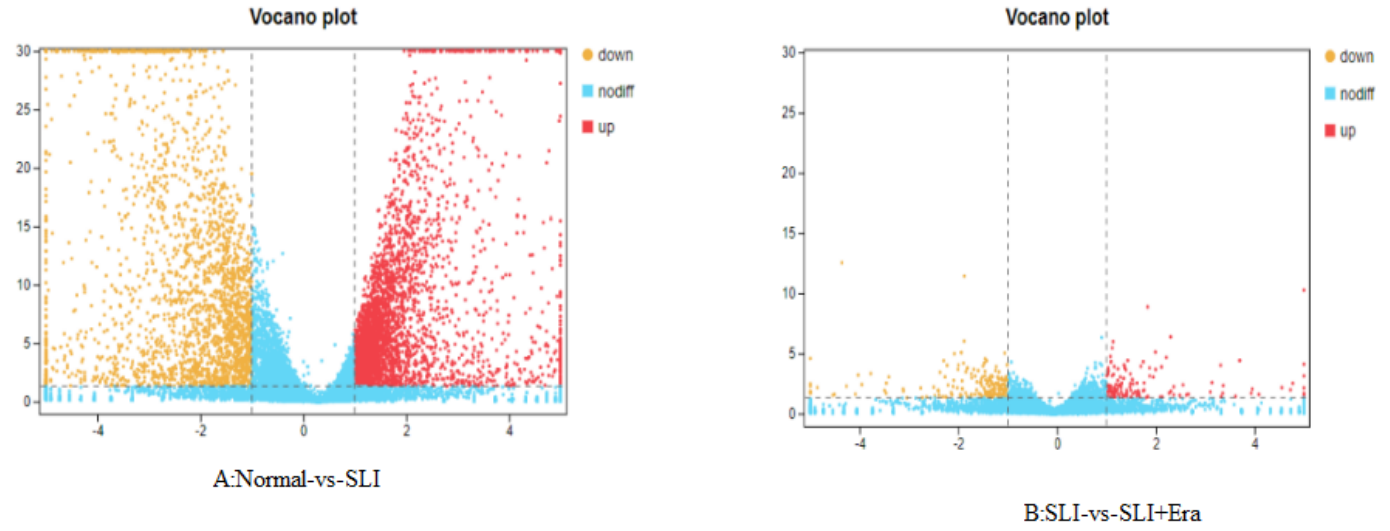

Figure 5

Volcano map of differentially expressed genes in rats liver tissue

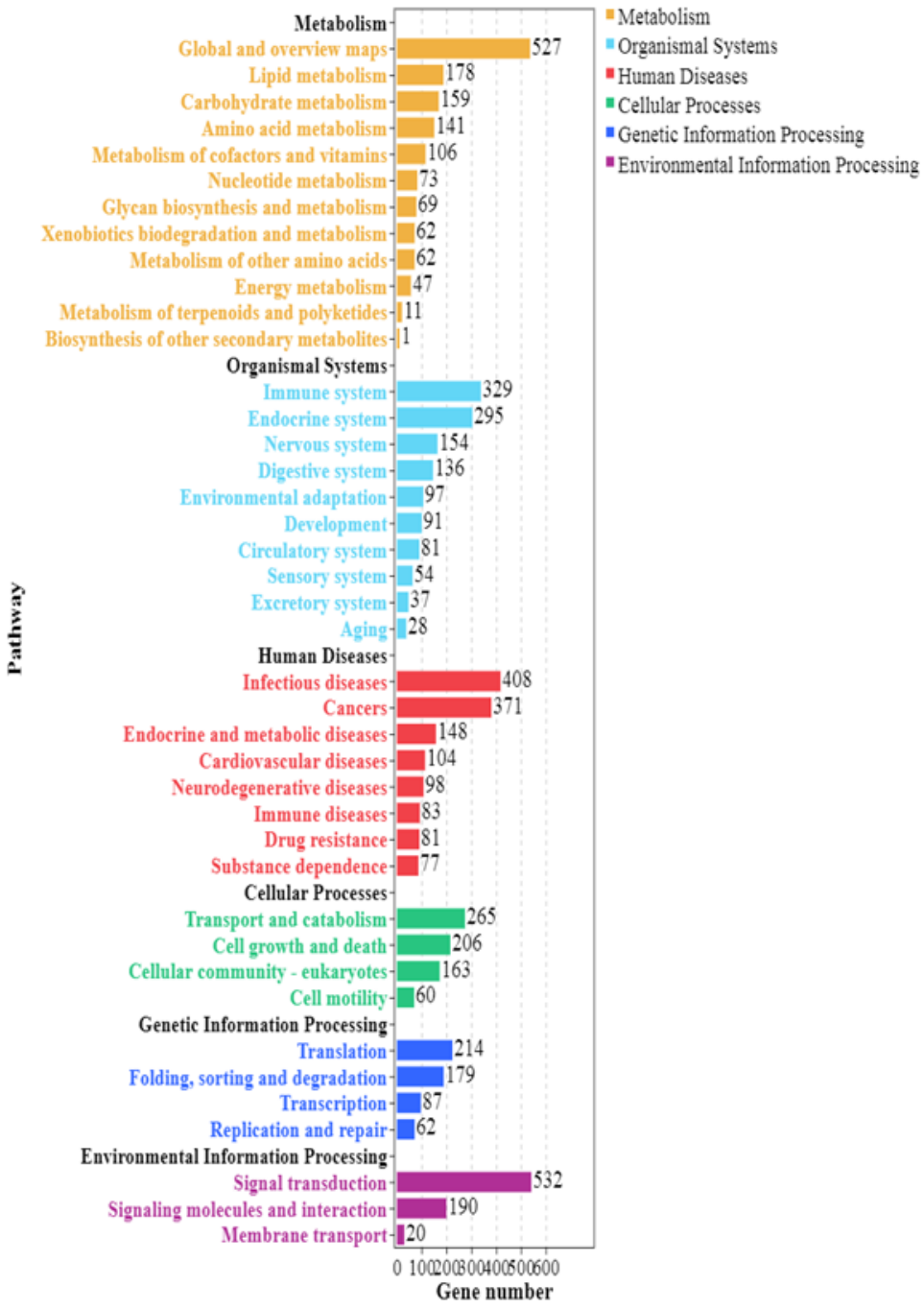

Figure 6

KEGG map of differentially expressed genes in rats liver tissue (SLI vs Normal group)

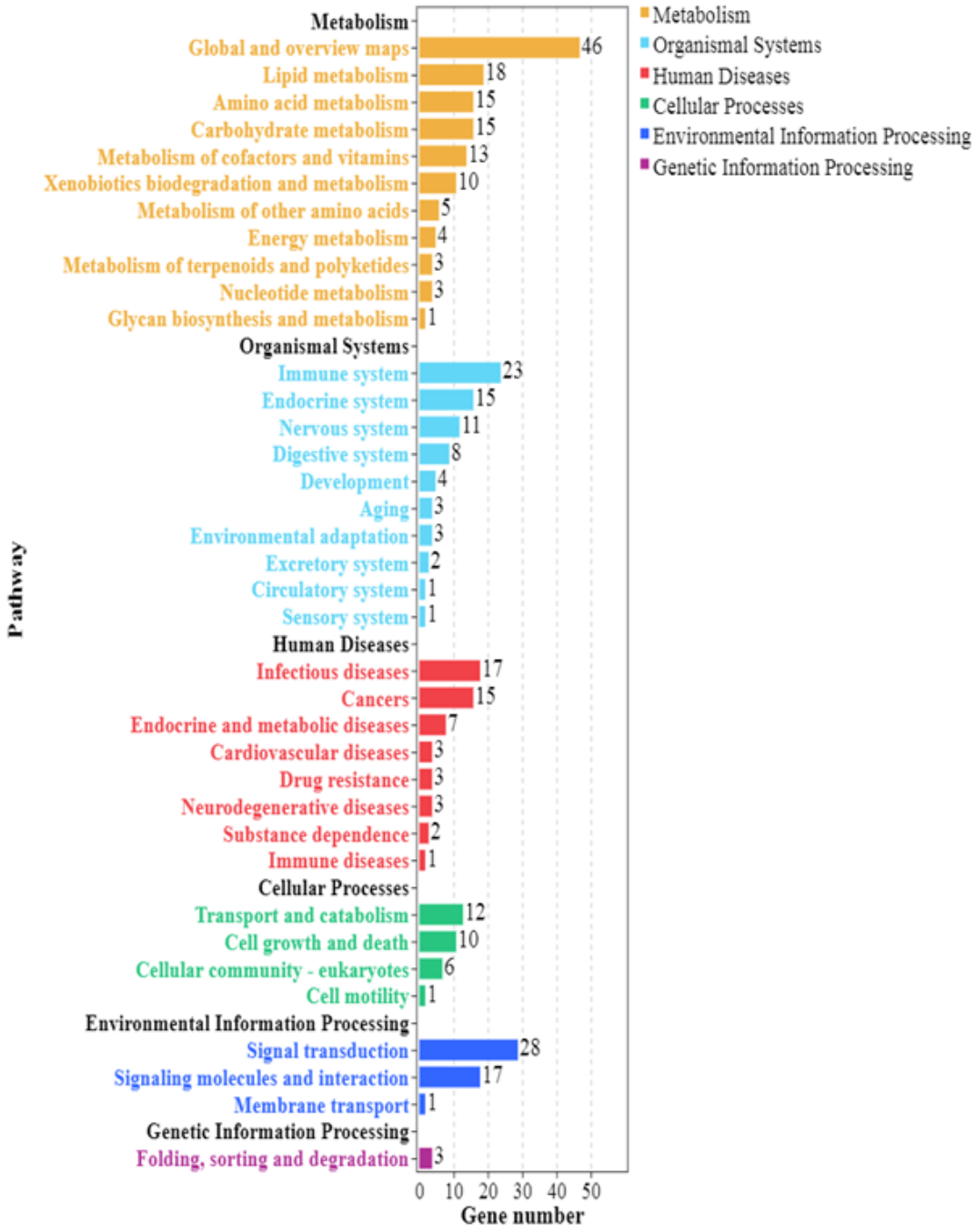

Figure 7

KEGG map of differentially expressed genes in rats liver tissue (SLI+Era vs SLI group)

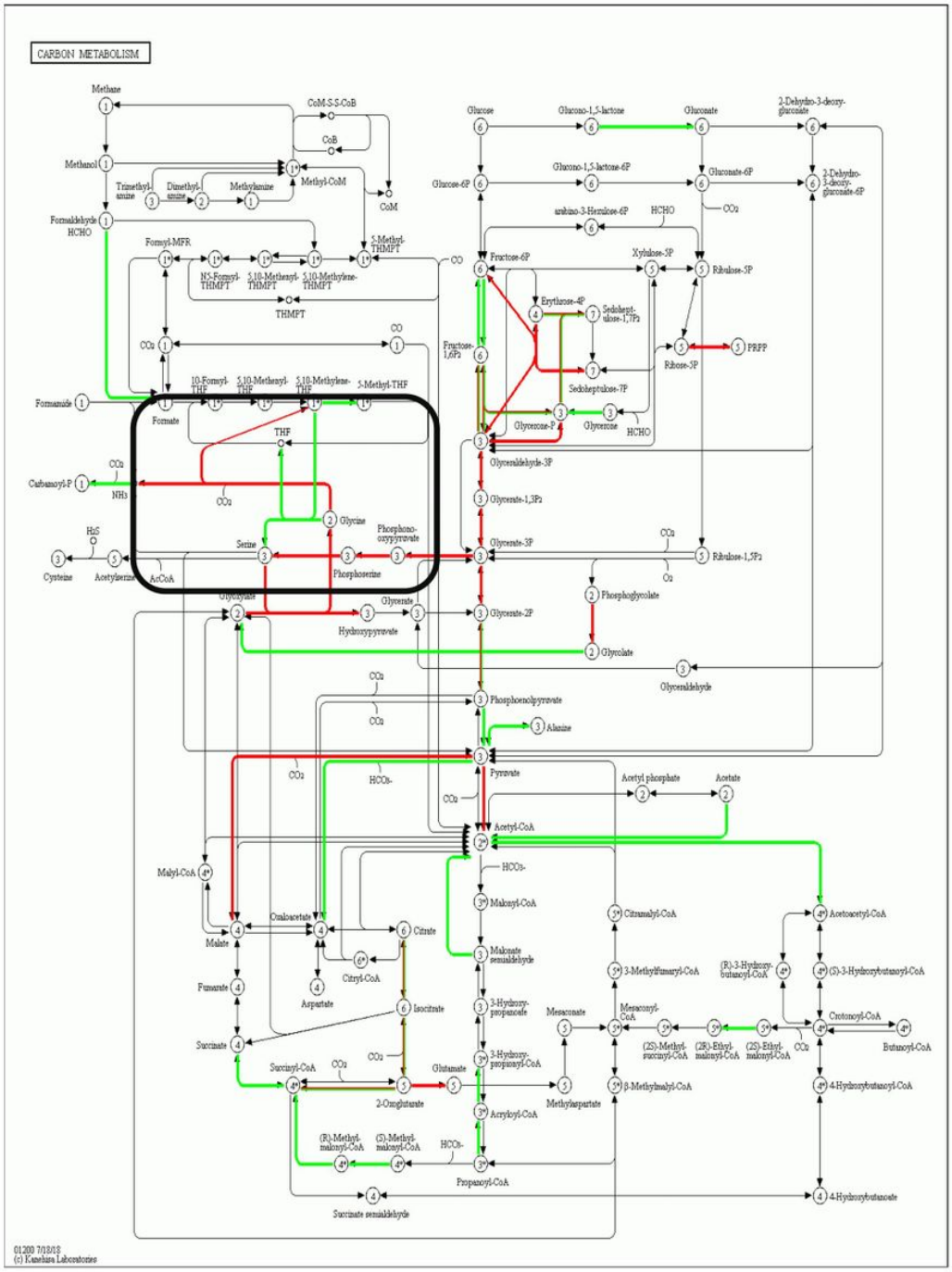

Figure 8

Analysis of carbon metabolism pathway of differentially expressed gene KEGG in rats liver tissue (Red means up regulation, green means down regulation, SLI vs Normal groups)

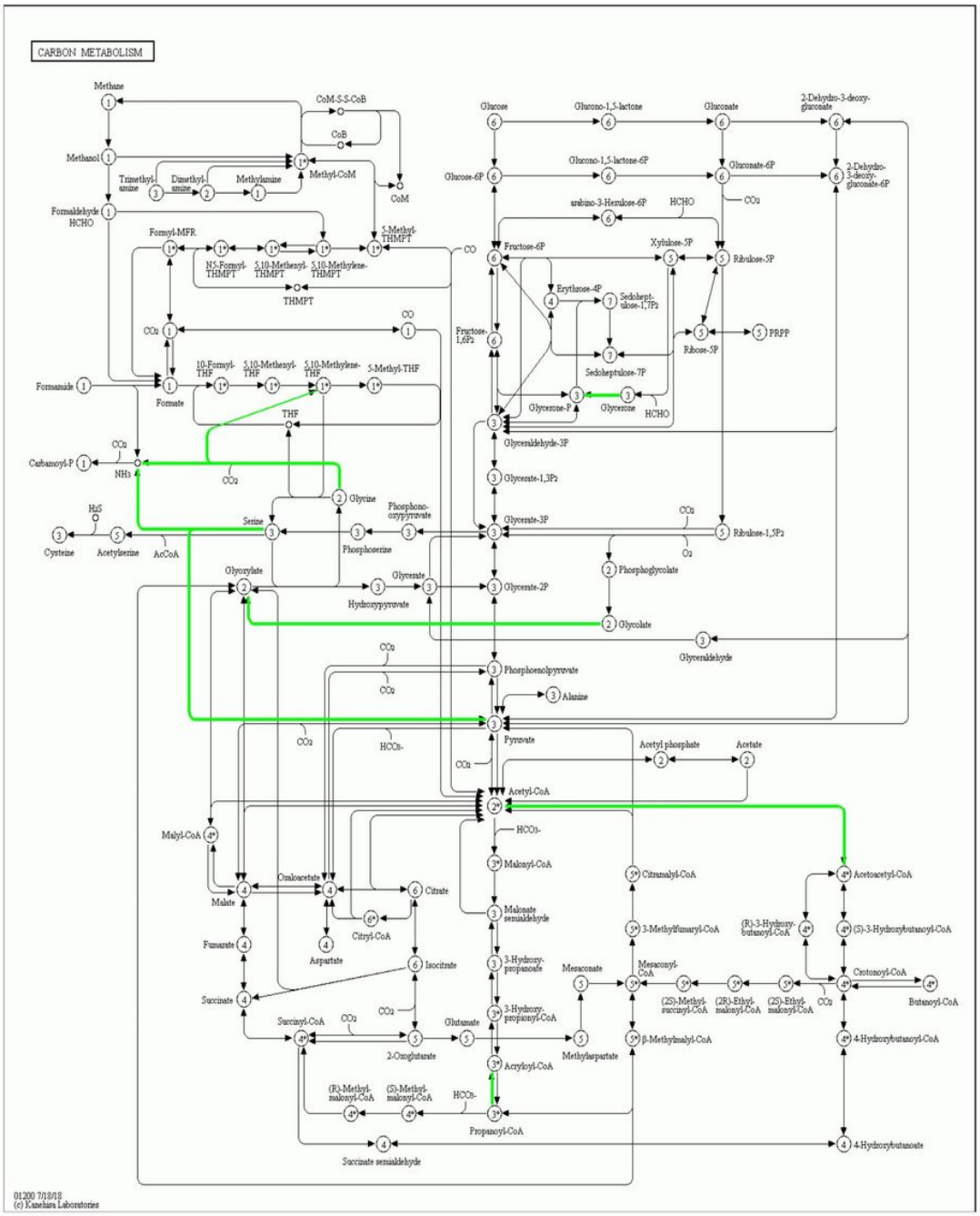

Figure 9

Analysis of carbon metabolism pathway of differentially expressed gene KEGG in rats liver tissue (Red means up regulation, green means down regulation, SLI + Era vs SLI groups)

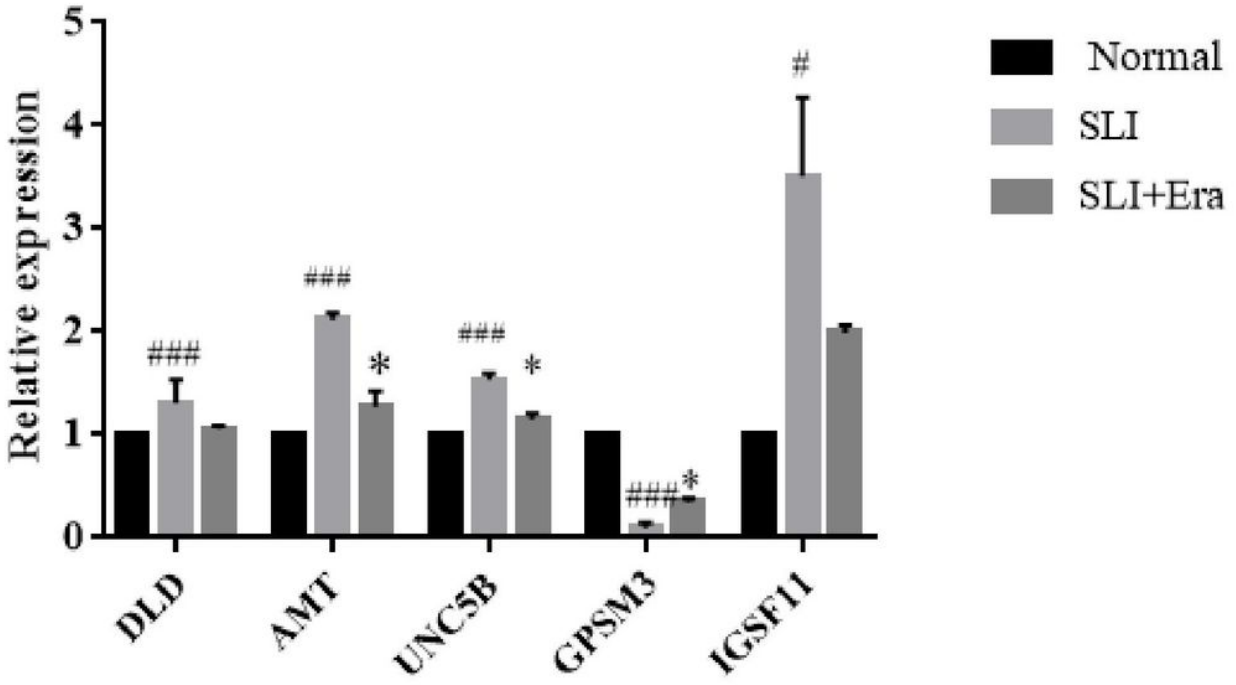

Figure 10

Real-time quantitative PCR validation of the DLD,AMT , UNC5B, GPSM3 and IGSF11 genes(SLI vs Normal ###P<0.001, #P<0.05; Era+SLI vs SLI *P<0.05)

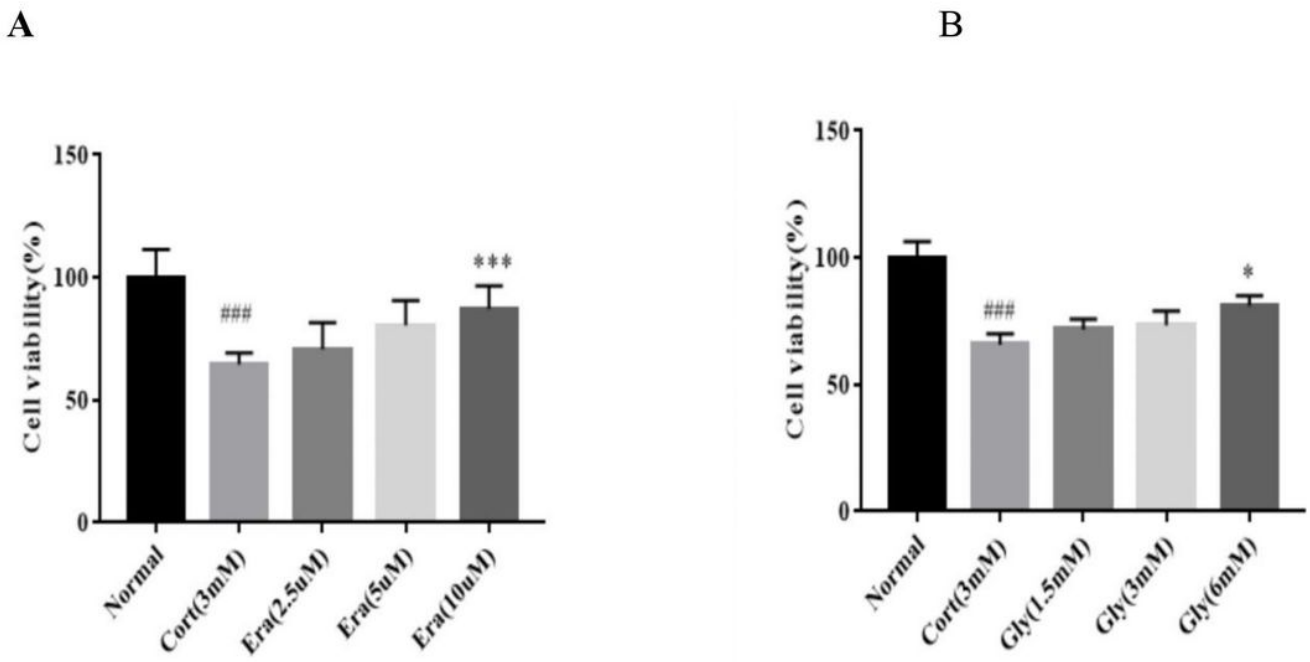

Figure 11

Assessment of beneficial effects of erucamide and glycine in protecting Crot-induced LO2 cell damage A: Cort vs normal ### P<0.001, Era vs Cort *** P<0.001; B: Cort vs normal ### P<0.001,Gly vs Cort*P<0.05)

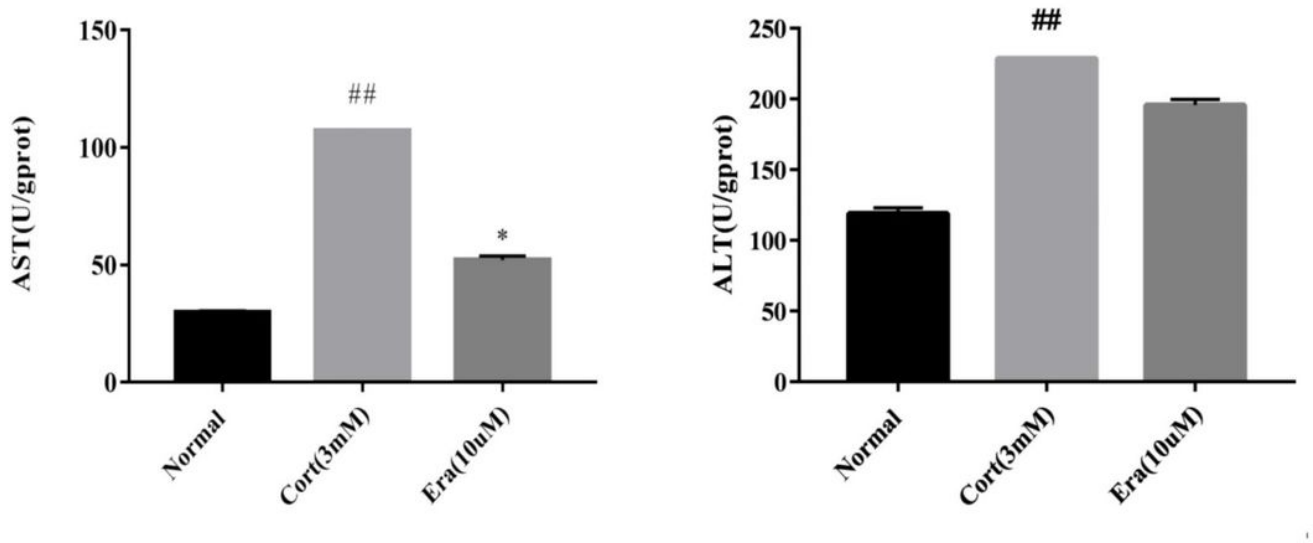

Figure 12

Effects of Erucamide on AST and ALT in LO2 cell with Crot-induced LO2 cell damage(AST: Cort vs Normal ##P<0.01; Era vs Crot *P<0.05. ALT:Cort vs Normal ##P<0.01)

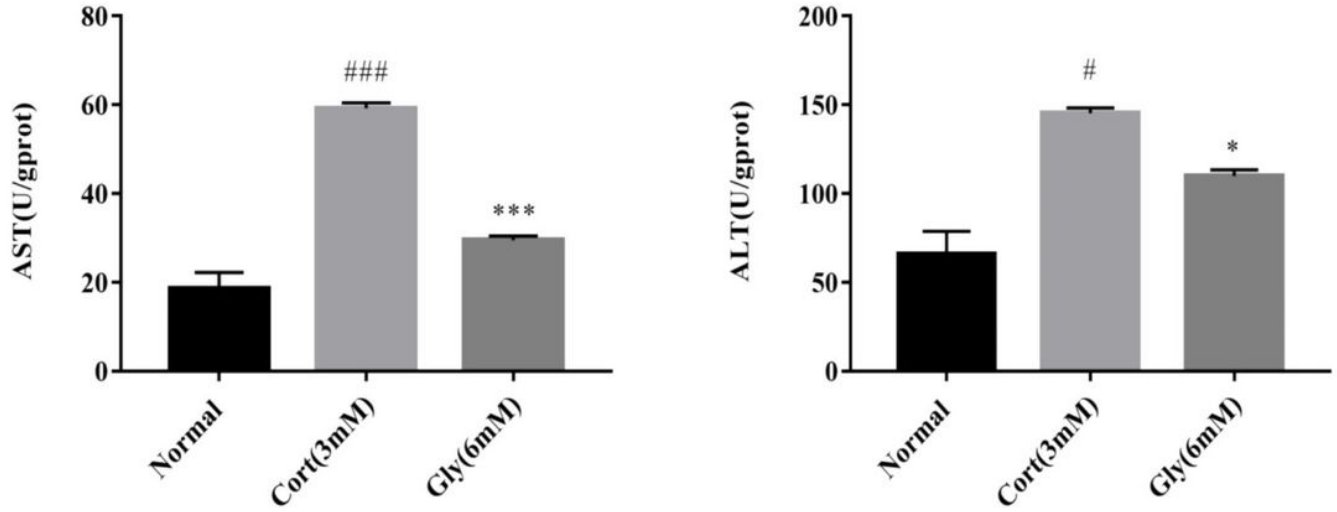

Figure 13

Effects of Glycine on AST and ALT in LO2 cell with Crot-induced LO2 cell damage(AST: Cort vs Normal ###P<0.001; Gly vs Crot ***P<0.001. ALT:Cort vs Normal #P<0.05, Gly vs Crot *P<0.05)

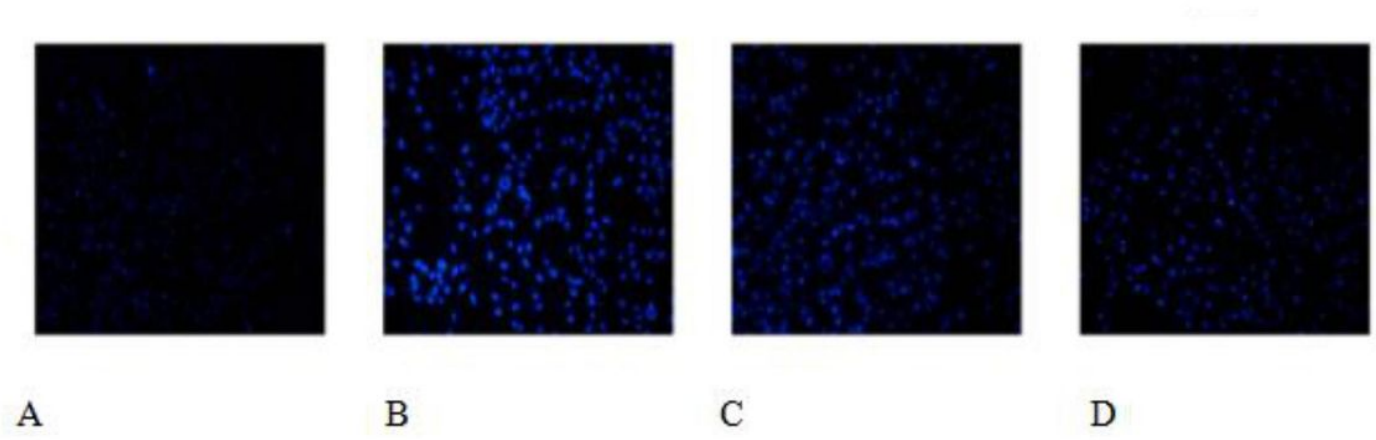

Figure 14

Hoechst 33342 staining to observe the morphological observation of erucamide and glycine on the damage of corticosterone-induced LO2 cells（100X)