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Effect of lipoic acid on the expression of nonalcoholic fatty liver disease-associated genes in the liver of rats fed a hypercaloric choline-deficient diet

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ABSTRACT

Nonalcoholic fatty liver disease (NAFLD) is one of the most common chronic liver pathologies. Molecular mechanisms of NAFLD pathogenesis are complex and still require further clarification. Thus, this study aimed to investigate the impact of lipoic acid (LA), widely used as an antioxidant, on the development of different links of NAFLD, including *de novo* lipogenesis, antioxidant and xenobiotic-metabolizing enzymes by gene expression analysis. The experiment was carried out on three groups of male Wistar rats received control diet, hypercaloric choline-deficient diet (HCCD) or HCCD with LA (HCCD+LA). In the liver of rats the expression of acetyl-CoA carboxylase 1 (*Acaca*), fatty acid synthase (*Fasn*), stearoyl-CoA desaturase (*Scd*) and transcriptional regulators sterol-regulatory element-binding protein SREBP (*Srebf1*), carbohydrate response element-binding protein ChREBP (*Mlxipl*), peroxisome proliferator-activated receptor alpha PPARα (*Ppara*), heme oxygenase-1 HO-1 (*Hmox1*) and nuclear erythroid 2-related factor 2 Nrf2 (*Nrf2*), cytochrome P450 1A1 CYP1A1 (*CYP1A1*) and aryl hydrocarbon receptor AhR (*AhR*), cytochrome P450 2E1 CYP2E1 (*CYP2E1*) genes were evaluated. Supplementation of HCCD with LA led to an even greater than in HCCD group decrease in *Scd* gene expression by 88% ($p<0.05$), as well as a marked suppression of *Ppara* and *Mlxipl* by 37% ($p<0.05$) and 27% ($p<0.05$), to an 80% ($p<0.05$) elevation of *Hmox1* gene expression relative to the HCCD group, had no pronounced effect on *CYP1A1* and *AhR* gene expression but resulted in a 38% ($p<0.05$) suppression of *CYP2E1* expression compared with that slightly elevated by HCCD. In conclusion, the current study showed that LA reduces lipogenesis *de novo*, restores the expression of *Hmox1* gene of the antioxidant enzyme HO-1, diminished by the HCCD diet, and decreases HCCD-induced level of the expression of *CYP2E1* gene of CYP2E1 enzyme, a potential source of free radicals.

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is one of the most common chronic liver pathologies. Pathogenesis of NAFLD is represented by complex mechanisms including insulin resistance which promote fat accumulation in hepatocytes and increase the vulnerability of hepatocytes for many factors such as oxidative stress, inflammation, activation of Kupffer cells, fibrogenesis and other. These processes lead to endoplasmic stress, oxidative stress, hepatocyte apoptosis and fibrosis [1,2]. Molecular mechanisms of NAFLD pathogenesis are complex and still require further clarification.

High fructose intake affects the progression and severity of NAFLD by promoting *de novo* lipogenesis and increasing insulin resistance, oxidative stress, inflammation and fibrosis [3]. The regulation of lipogenesis occurs mainly at the level of transcription. Glucose metabolites produced during glycolysis activate the transcription factors SREBP and ChREBP [4], which bind to carbohydrate response elements in the promoters of target genes *Acaca*, *Fasn* and *Scd* [5], which catalyze, respectively, the conversion of acetyl-CoA to malonyl-CoA and further to acyl-CoA followed by the

formation of monounsaturated fatty acids. PPARα, which stimulates β-oxidation reactions of fatty acids, also plays an important role in lipid metabolism [6].

Fat accumulation causes metabolic abnormalities, leading to excessive mitochondrial reactive oxygen species (ROS) production and endoplasmic reticulum stress, which in turn can cause inflammation, cell damage and death. The main sources of ROS in the cells are mitochondria, peroxisomes, and cytochrome P450 [7, 8].

The antioxidant defense of the cell, regulated by the Nrf2/ARE pathway, counteracts the damage caused by oxidative stress by promoting the restoration of normal lipid metabolism [9]. High levels of ROS lead to the release of Nrf2 and its translocation into nucleus, which promotes the activation of transcription of antioxidant enzyme genes containing antioxidant responsive elements (ARE) in promoter region, one of such enzymes is HO-1 [10]. Oxidative stress markers are used to assess the pathological state and disease progression [11, 12].

Literature data indicate that lipoic acid (LA), a natural sulfur-containing compound, may have pronounced antioxidant activity [13]. This study aimed to investigate the impact of LA on the development of different links of NAFLD, including *de novo* lipogenesis, antioxidant and xenobiotic-metabolizing enzymes by gene expression analysis.

MATERIALS AND METHODS

Experimental protocol

All experimental procedures were performed according to the recommendations of the Council for International Organization of Medical Sciences and the International Council for Laboratory Animal Science, and adhered the standard principles described in "Guide for the Care and Use of Laboratory Animals" (8th edition). Ethical approval was obtained for this research from the Federal Research Centre of Nutrition and Biotechnology Ethics Committee (No. 7/24.06.2019).

Experimental design was described previously [14]. Study was carried out on three groups of male Wistar rats (n=8 of animals in each group). HCCD diet was used to simulate NAFLD. There was a control group fed a normal diet, HCCD group fed a HCCD diet and HCCD+LA group fed a HCCD diet supplemented with LA (Chem Impex International, Inc., Wood Dale, USA, Cat# 29862) in an estimated dose of 75 mg/kg of body weight. The average daily LA intake by animals calculated on the basis of the feed eaten throughout the experiment was 61 mg/kg body weight. The total duration of dietary feeding was 8 weeks. After 8 weeks of the experimental period, the animals were subjected to 16–18 h of fasting. After decapitation liver samples were collected and stored at -80°C before RNA isolation.

Gene expression analysis

In the liver of rats, the expression of the genes, *Acaca*, *Fasn*, *Scd*, SREBP-1c (*Srebf1*), ChREBP (*Mlxipl*), PPARα (*Ppara*), HO-1 (*Hmox1*), Nrf2 (*Nrf2*), CYP1A1 (*CYP1A1*), *AhR*, CYP2E1 (*CYP2E1*), β-actin (*Actb*) and GAPDH (glyceraldehydes-3 phosphate dehydrogenase) (*Gapdh*) were evaluated by real-time reverse transcription polymerase chain reaction (RT-PCR). The samples of total RNA from liver tissue were isolated using TRI Reagent (Sigma-Aldrich, St. Louis, USA), and synthesis of complementary DNA (cDNA) was performed using MMLV RT kit (Evrogen, Moscow, Russia) according to the manufacturer's protocol. The primers and probes for RT-PCR were

provided by DNA Synthesis (Moscow, Russia) (Table 1). The 25 µl PCR reaction mixture contained 2.5 µl of cDNA (dilution 1:10), prepared in the reverse transcription reaction from 2 µg of total RNA, 2.5 µl of 10×Taq Turbo buffer for HS Taq DNA polymerase (with 2.5 mM MgCl2) (Evrogen, Moscow, Russia), 1 µl (F+R) primers (10 µM), 0.5 µl of FAM probe (10 µM), 1.0 µl of dNTPs mixture (10 mM) (Evrogen, Moscow, Russia), 0.25 µl of HS Taq DNA polymerase (5 units/µl) (Evrogen, Moscow, Russia), 17.25 µl of nuclease-free water (Thermo Scientific, USA). Amplification was performed on a CFX96 device (Bio-Rad, USA, made in Singapore). Gene expression was assessed by threshold cycle value and normalized relative to reference genes *Actb* and *Gapdh* by $2^{-\Delta\Delta Ct}$ method [15].

Table 1. Primer and probe sequences in rats.

Gene Name	Primer and Probe Sequences
Acaca	Forward GTCAGGATCTTTGATGAAGTAATG Reverse TTCAGAATATGTATTGGTTCGTCC Probe FAM-CACCCCAAAGCCCCACATTCC-BHQ1
Fasn	Forward TGACATCGTGCATTCCTTTG Reverse CAATGATGCCATCAGGTTTC Probe FAM-CACCGCCATCCAGATTGCCCTC-BHQ1
Scd	Forward GTTCCAGAGGAGGTACTACAAGC Reverse AGGCTGTGCAGGAAAGTTTC Probe FAM-CCTGCTCATGTGCTTCATCCTGCC-BHQ1
Srebf1	Forward CCACTGGTAGAGCACATTCC Reverse GGCAGCCTTGAAGGAGTACA Probe FAM-GGAAACTGAGAGACCCCTTCCCA-BHQ1
Mlxipl	Forward GACTTGTCAGCACGCTCAGT Reverse GCTGTAGCATCAGGATGTACTC Probe FAM-CAGCCTCAAGGTGAGCAAAGCA-BHQ1
Ppara	Forward CTTAGGGAGCTGTCCAGGCT Reverse GGACTCATCTGTACTGGTGGG Probe FAM-CAGACACCCTCTCTCCAGCTTCCA-BHQ1
Hmox1	Forward CCAGCCTGAACTAGCCCA Reverse CCTTGGTGGCCTCCTTC Probe FAM-CCACAGCTCGACAGCATGTCC-BHQ1
Nrf2	Forward CACATCCAGACAGACACCAGT Reverse GAATGTCTCTGCCAAAAGC Probe FAM-CTCCCAGGTTGCCCACATTCCC-BHQ1
CYP1A1	Forward CAGCACCATAAGAGATACAAG Reverse CCGGAACTCGTTTGGATCA Probe FAM-GTTCCTGGTCATGGTTAACCTGCCA-BHQ1
Ahr	Forward CACAAGGAGTAGACGAGACTCAT Reverse CATCTGAAGCACCTCTCCA Probe FAM-CCCACAGCCAGCGGTCTACTACA-BHQ1
CYP2E1	Forward CCATGAAGCAACCAGAGATAC Reverse CTTCTCTGGATCTGGAAACTCA Probe FAM-CCCAAGGGTACAGTTGTGATTCC-BHQ1

Statistical analysis

To establish statistically significance of the differences ($p<0.05$) between animal groups we used Kruskal-Wallis test and Dann multiple comparison criterion as a post hoc test. The calculations were performed using the statistical package of SPSS® 23.0 and Microsoft Excel for Windows.

RESULTS

Effect of LA and HCCD on the gene expression of key *de novo* lipogenesis enzymes

As shown in Figure 1, the expression of the genes of the key *de novo* lipogenesis enzymes *Fasn* and *Acaca* did not differ significantly in the rats received HCCD compared to the control group, but a significant suppression by 79% ($p<0.05$) of the expression level of *Scd* gene responsible for monounsaturated fatty acid synthesis was observed.

There was also no statistically significant effect of the experimental diet on the mRNA level of the *Ppara* gene (Figure 2A), the *Srebf1* gene (Figure 2C) encoding the SREBP protein responsible for gene expression of *de novo* lipogenesis enzymes, the *Mlxipl* gene (Figure 2B) encoding the transcription factor ChREBP, which controls the gene expression of carbohydrate metabolism enzymes. However, supplementation of HCCD with LA led to an even greater decrease in *Scd* gene expression by 88% ($p<0.05$), as well as a marked suppression of *Ppara* and *Mlxipl* mRNA levels by 37% ($p<0.05$) and 27% ($p<0.05$), respectively, relative to the HCCD group.

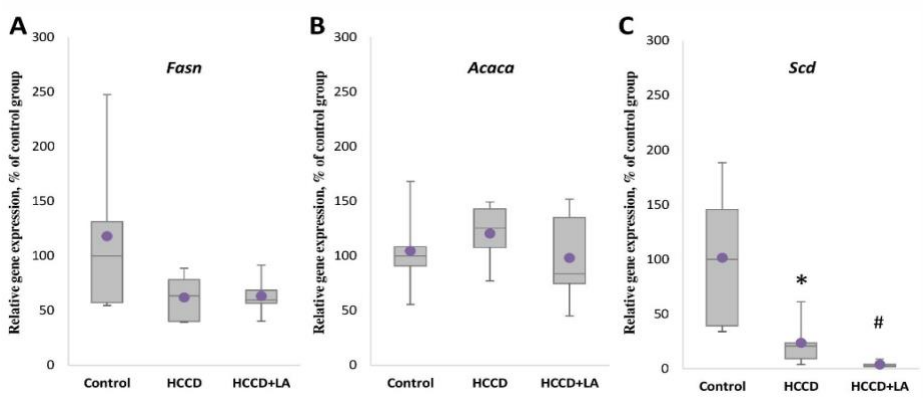

Figure 1. Effects of supplementation of hypercaloric choline-deficient diet (HCCD) with lipoic acid (LA) on: *Fasn* (A), *Acaca* (B), *Scd* (C) gene expression. Gene expression in all groups is normalized relative to the gene expression of housekeeping genes *Actb* and *Gapdh*. The data are presented in relative units, for each gene the values in the control group are taken as 100%. Results are expressed as median, interquartile range, maximum and minimum values. Designations: $p < 0.05$ vs. * Control; # HCCD+LA vs. HCCD.

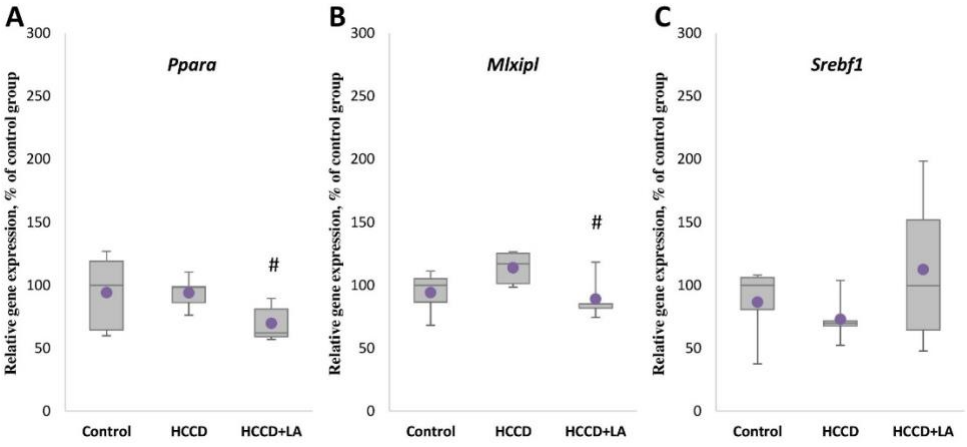

Figure 2. Effects of supplementation of hypercaloric choline-deficient diet (HCCD) with lipoic acid (LA) on: *Ppara* (A), *Mlxipl* (B), *Srebf1* (C) gene expression. Gene expression in all groups is normalized relative to the gene expression of housekeeping genes *Actb* and *Gapdh*. The data are presented in relative units, for each gene the values in the control group are taken as 100%. Results are expressed as median, interquartile range, maximum and minimum values. Designations: $p < 0.05$ vs. # HCCD+LA vs. HCCD.

Effect of LA and HCCD on the gene expression of antioxidant enzyme HO-1

Although not statistically significant a decrease by 40% in the mRNA level of *Hmox1* gene of the key antioxidant enzyme HO-1 (Figure 3A) was observed in animals receiving HCCD compared to the control group. The expression level of the transcription factor *Nrf2* gene (Figure 3B) in this group (HCCD) increased by 18% relative to the control group, but also not significantly. In turn, enrichment of the experimental diet with LA led to an 80% ($p<0.05$) elevation of *Hmox1* gene expression, relative to the HCCD group, and had no significant effect on *Nrf2* mRNA levels.

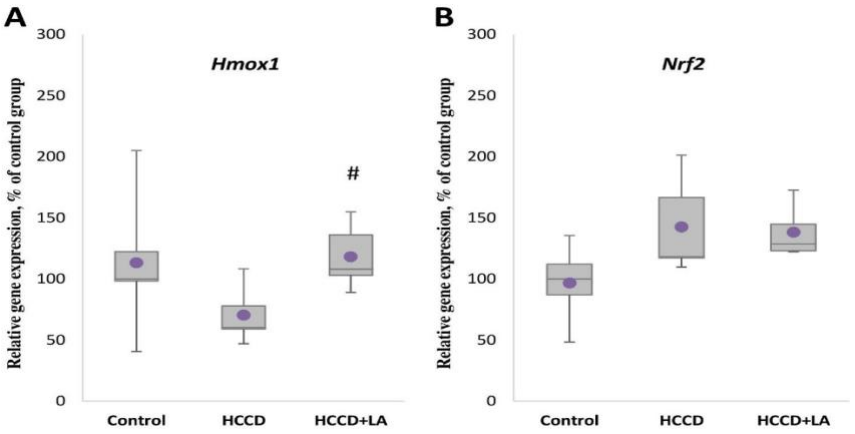

Figure 3. Effects of supplementation of hypercaloric choline-deficient diet (HCCD) with lipoic acid (LA) on: *Hmox1* (A), *Nrf2* (B) gene expression. Gene expression in all groups is normalized relative to the gene expression of housekeeping genes *Actb* and *Gapdh*. The data are presented in relative units, for each gene the values in the control group are taken as 100%. Results are expressed as median, interquartile range, maximum and minimum values. Designations: $p < 0.05$ vs. $^{\#}$ HCCD+LA vs. HCCD.

Effect of LA and HCCD on the gene expression of xenobiotic metabolizing enzymes

Analysis of the expression of the gene encoding the key xenobiotic metabolizing enzyme CYP1A1 showed that the level of *CYP1A1* mRNA (Figure 4A) significantly decreased in the HCCD group by 83% ($p<0.05$) relative to the control group, while no effect of experimental diet on the expression of the transcription factor *AhR* (Figure 4B) regulated the expression of this enzyme was observed. The mRNA level of the *CYP2E1* gene (Figure 4C) encoded the CYP2E1 enzyme, which is a potential source of free radicals, increased by 20% ($p>0.05$) in the group of animals receiving HCCD, although statistically insignificant. Supplementation of HCCD with LA had no pronounced effect on *CYP1A1* enzyme gene expression and *AhR* transcription factor expression compared to the HCCD group. Consumption of LA as part of HCCD diet resulted in a 38% ($p<0.05$) suppression of *CYP2E1* expression compared with that slightly elevated by HCCD.

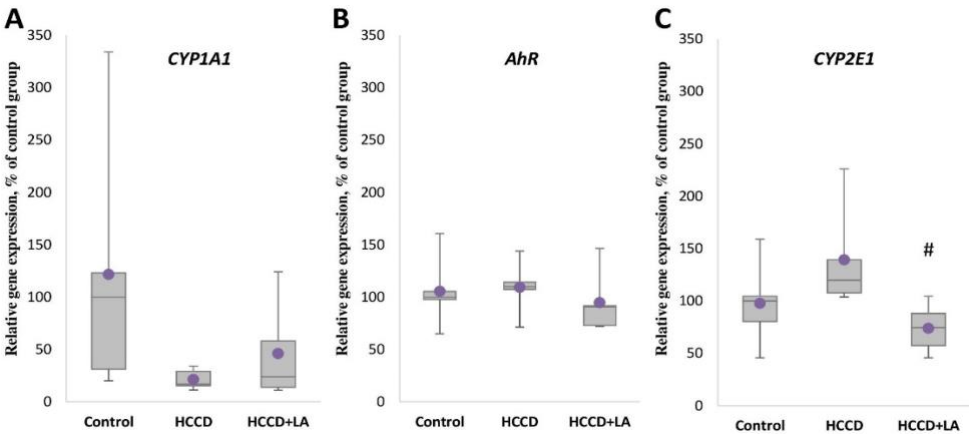

Figure 4. Effects of supplementation of hypercaloric choline-deficient diet (HCCD) with lipoic acid (LA) on: *CYP1A1* (A), *AhR* (B), *CYP2E1* (C) gene expression. Gene expression in all groups is normalized relative to the gene expression of housekeeping genes *Actb* and *Gapdh*. The data are presented in relative units, for each gene the values in the control group are taken as 100%. Results are expressed as median, interquartile range, maximum and minimum values. Designations: $p < 0.05$ vs. * Control; # HCCD+LA vs. HCCD.

DISCUSSION

The previously published results obtained in this work showed that feeding of rats with HCCD for 8 weeks led to an increase in final body weight and in liver weight, which was associated with the accumulation of lipids in the liver and the development of steatosis in Wistar rats, which is one of the manifestations of NAFLD [14].

Gene expression analysis conducted here in our study revealed that supplementation of HCCD with LA led to decreasing in *Scd* gene expression, as well as a marked suppression of *Ppara* and *Mlxipl* mRNA levels relative to the HCCD group. Similar results to ours have been demonstrated in a number of papers. Thus, a study using human abdominal subcutaneous fat adipocytes showed a decrease in the protein levels of FASN, SCD (SCD is a key enzyme involved in the control of lipogenesis *de novo*, catalyzing the limiting step of monounsaturated fatty acid synthesis) and ACC (ACACA), a key enzyme of the lipogenic pathway that mediates the initial stage of fatty acid synthesis [16]. According to Guo et al. addition of α-lipoic acid to the diet (0.2%) for 5 weeks reduced *Scd* expression in the liver but increased *Srebp1* expression at the mRNA level in Sprague-Dawley rats. In addition, supplementation with α-lipoic acid had no effect on hepatic *Fasn* mRNA levels but decreased hepatic FASN expression at the protein level in rats. The expression of carnitine-O-palmitoyltransferase 1a (*Cpt1α*) gene involved in fatty acid transport was unchanged [17]. Feeding of Zucker diabetic fatty (ZDF) rats with LA led to the suppression of the expression of the lipogenic genes *Acaca*, *Fasn* in liver and adipose tissue, reduced hepatic levels of ACC1/2, FASN proteins, and stimulated the expression of PPARa target genes, carnitine-O-palmitoyltransferase 1b (*Cpt1b*), but not *Cpt1a* [18]. In another study conducted on ZDF rats, feeding of ZDF rats with LA reduced the mRNA levels of *ACC1* and *FAS* by 66% and 74% respectively, compared to animals fed the paired diet. Nuclear ChREBP protein was decreased in LA-treated livers, whereas nuclear SREBP-1c protein was not significantly altered [19]. Enrichment of the experimental diet with LA led to an elevation of *Hmox1* gene expression, relative to the HCCD group, and had no significant effect on *Nrf2* mRNA levels. Same result was observed in the study on the male Otsuka Long-Evans Tokushima Fatty (OLETF) rats, LA increased HO-1 expression in the liver [20]. Consumption of LA as part of HCCD diet resulted in a suppression of *CYP2E1* expression compared with that slightly elevated by HCCD.

Changes in the activity of CYP2E1 enzyme were observed in our previously published paper, the activity of microsomal CYP2E1 in the liver of rats in the HCCD group exceeded that in the control group by 22%, noteworthy there was a 45% inhibition of CYP2E1 activity in the liver of rats treated with LA compared to HCCD group [14]. Similar results were obtained in a study on C57BL6 mice using a methionine choline deficient diet (MCD). Thus, *CYP2E1* expression in the liver of MCD mice was significantly higher than that of control mice, and LA inhibited the MCD diet-induced expression of *CYP2E1* at the mRNA and protein levels [21].

CONCLUSIONS

Thus, our study showed that LA reduces lipogenesis *de novo*, restores the expression of *Hmox1* gene of the antioxidant enzyme HO-1, diminished by the experimental diet (HCCD), and decreases HCCD-induced level of the expression of *CYP2E1* gene of CYP2E1 enzyme, a potential source of free radicals (Figure 5). More detailed investigations are needed for further clarification of impact of LA and another biologically active compounds on the development of different links of NAFLD including studies on skeletal muscle and white adipose tissue.

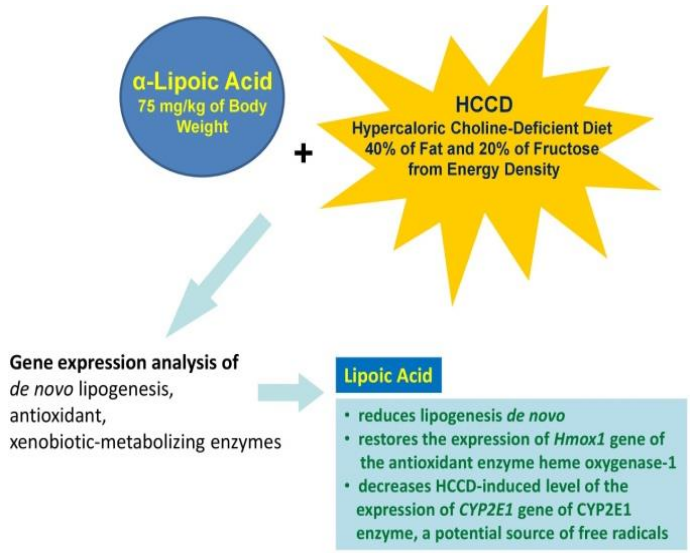

Figure 5. Summary of the effect of lipoic acid on the expression genes in the liver of rats fed a hypercaloric choline-deficient diet.

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AUTHOR CONTRIBUTIONS

AB: Data acquisition; data analysis/interpretation; statistical analysis; drafting manuscript. NT: Data acquisition; data analysis/interpretation; statistical analysis; critical revision of manuscript. VT: Concept and design; supervision; final approval. All authors read and approved of the final manuscript.

CONFLICTS OF INTEREST

There is no conflict of interest among the authors.

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