Original Article

Investigating the Effect of Galbanic Acid on Lipin-1 and Lipin-2 Genes in Fatty Liver Cells with Palmitate

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Abstract

Background: Non-alcoholic fatty liver disease is related to lipid accumulation and inflammation. Considering the role of lipin-1 and lipin-2 in fat homeostasis and inflammation, this study aimed to explore the effect of galbanic acid (Gal) and resveratrol (RSV) on alterations in the gene expression levels and protein abundance of lipin-1 and lipin-2 in HepG2 liver cells lipid-enriched with palmitate (Pal).

Materials and Methods: HepG2 cells were subjected to different amounts of Gal and RSV for 24 hours in the presence of Pal to induce lipid accumulation. The RT-PCR method was employed to assess the expression of lipin-1 and lipin-2 genes, while protein levels were evaluated by western blot analysis. Lipid accumulation was determined qualitatively and semi-quantitatively using the oil-red staining technique.

Results: Gal treatment increased lipin-1 and lipin-2 gene expression ($P < 0.05$). In contrast, the groups treated with RSV did not show a substantial variance in the expression levels of the two genes ($P > 0.05$). In the groups treated with Gal/RSV, the intensity of lipin-2 protein bands was higher compared to the Pal group ($P > 0.01$); however, the intensity of lipin-1 protein bands was not significantly different ($P > 0.05$).

Conclusion: Gal, a coumarin compound, significantly increased the expression of lipin-1 and lipin-2 in HepG2 cells treated with Pal. Consequently, this research suggests gal as a novel strategy for regulating fat homeostasis in HepG2 cells.

Keywords: Galbanic acid, lipin-1, lipin-2, non-alcoholic fatty liver, resveratrol

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Introduction

Non-alcoholic fatty liver disease (NAFLD) is a prevalent global health issue characterized by excessive fat deposition in liver cells. Palmitate (Pal), a fundamental component of the high-fat Western diet, contributes to fat accumulation in liver cells.[1] A study by Luo X *et al*. explored the impact of Pal in the diet and its effect on pro-inflammatory cytokine production in macrophages through the stimulation of stimulator of interferon genes (STING).[2] A recent study uncovered the significant involvement of the death receptor TRAIL-R2 in inducing cell apoptosis in hepatocytes under the influence of Pal.[3] Recent findings have demonstrated that hepatocytes stimulated by Pal can release extracellular vesicles, leading to macrophage chemotaxis and the upregulation of proinflammatory cytokines, such as IL-6 and IL-1β, in an animal model of non-alcoholic steatohepatitis (NASH).[4,5] Lipins constitute a family of magnesium-dependent proteins with phosphatidate phosphatase activity, encompassing lipin-1,

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lipin-2, and lipin-3. These enzymes are essential in catalyzing the conversion of phosphatidic acid to diacylglycerol, a critical step in triglyceride synthesis. Lipins are crucial in metabolic regulation by contributing to lipid homeostasis and insulin sensitivity.[6] In liver tissue, lipin-1 and lipin-2 are the most common isoforms, and they regulate the expression of genes related to fatty acid and lipid metabolism. This is accomplished by directly influencing transcription factors such as HIF-a, PPAR, and SREBP.[7,8] Moreover, lipin-1 exists in two isoforms: alpha and beta. The alpha type is localized in the cytoplasm, while the beta type is found in the nucleus of the hepatocytes. In fatty liver conditions, the expression ratio of lipin1β to lipin1α tends to increase. This elevation in lipin1β expression has been linked to an exacerbation of alcohol's toxic effects on the liver.[9] Conversely, research indicates that lipin-1 possesses notable anti-inflammatory properties.[7,8] In contrast to previous findings, a recent study has uncovered an unexpected role for lipin-1. It appears that lipin-1 works as a mediator of pro-inflammatory activation in macrophages by stimulating toll-like receptor 4 (TLR4). This effect was observed in animal macrophages and human macrophages lacking lipin-1. Indeed, the study demonstrates that in the absence of lipin-1, there is a reduction in the production of pro-inflammatory cytokines, which are crucial in the inflammatory process.[9] Furthermore, alterations in the level of lipin-2 can affect the release of pro-inflammatory factors. A decrease in lipin-2 levels may lead to an increased expression of pro-inflammatory genes such as IL6, Ccl2, and TNF. The observed effect is determined by overstimulation of the JNK1/cJun pathway by fatty acids. Conversely, elevating the level of lipin-2 can reduce the release of pro-inflammatory factors.[10] Metabolically, the lack of lipin-2 leads to a decline in cellular triacylglycerol content in macrophages overloaded with saturated fatty acids. The development of potent anti-obesity drugs with minimal side effects has been a significant focus in recent times. As a result, herbal medicines have garnered considerable attention for their potential in this area.[11-15] According to previous studies, sesquiterpene coumarins, such as galbanic acid (Gal), significantly affect triglyceride and cholesterol levels in the blood, liver, and aorta. These compounds can potentially stop the rise in serum cholesterol and triglyceride concentration.[16] Given the pivotal role of lipin-1 and lipin-2 in fat homeostasis and inflammation, this study aimed to examine the impact of Gal and resveratrol (RSV) on the gene expression and protein levels of both in HepG2 liver cells treated with Pal to induce lipid accumulation.

Materials and Methods

Cell culture and treatment

HepG2 cells (Iran Center for Biological Resources, Tehran, Iran) were cultivated at 37°C with a 5% CO_2 in DMEM medium containing 10% FBS and 1% Penicillin-Streptomycin mixture (Pen/Strep) from Dena Bio Asia, Mashhad, Iran. The cells were treated when their density reached 70%–80%. To prepare the stock solution, Gal and RSV (GolEXIR, Mashhad, Iran) were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, USA). In the treatment, the maximum final concentration of DMSO was limited to 0.1% to minimize its impact on the cellular environment. The cells were then exposed to the treatment for 24 hours. In the cell culture experiment, the following subgroups were tested:

(1) Cells treated with 0.4 mM Pal. (2) Cells treated with a combination of 0.4 mM Pal and 300 μM Gal. (3) Cells treated with a combination of 0.4 mM Pal and 50 μM RSV. All experiments were performed in triplicate to ensure the reliability and reproducibility of the results.

Cell culture, treatment, oil red O staining, and measurement of TG content

The experiment began with the inoculation of HepG2 cells into 6-well plates. Once the cell density reached 80%–90%, different treatments were combined and applied. In the combined cases, the total cell fat content was assessed by conducting ORO staining using the final concentrations of Pal along with Gal or RSV. The cells were washed with 60% isopropanol after the initial wash with 1% paraformaldehyde solution and PBS. Subsequently, they were incubated for 30 minutes with a solution containing Oil Red O (ORO) mixed with 60% isopropanol. The staining technique used for the experiment involved preparing an ORO solution by dissolving 350 mg of ORO (Sigma Aldrich, USA) in 100 mL of 100% isopropanol, which was then distilled. After incubating the cells with ORO, they were washed with distilled water before the stained cells were imaged using an inverted microscope. This technique enabled the visualization and quantification of intracellular lipid droplets, offering valuable insights into the lipid content and accumulation within the cells under different treatment conditions.[16]

A cell pellet containing approximately 10 million cells was disrupted using a 5% solution of NP-40 to measure the triglyceride content. The mixture was gently heated to temperatures between 80°C and 100°C for intervals of 2–5 minutes, with the process being repeated twice to ensure complete dissolution of triglycerides. In the next step, the mixture was centrifuged at maximum speed for 2 minutes, and the supernatant was transferred to a fresh tube. Triglyceride of the cells was determined by a kit provided by Biovision (specifically the triglyceride quantification Colorimetric/Fluorimetric kit), adhering strictly to the guidelines provided by the manufacturer. The final step involved adjusting the triglyceride values to the total protein content, which was determined using the BCA technique, and the results were expressed in micrograms of triglyceride per milligram of protein.[17]

Lipin-1 and lipin-2 gene expression by RT-PCR

The RNA extraction from total cells was performed using Trizol reagent (GeneAll, Korea), following the specific kit's protocol. The quality and concentration of the total extracted RNA were assessed using the Nanodrop Spectrophotometer

1000 (Wilmington, DE, USA), with the optical density measured at A260/A280 ratio. To create cDNA from the total RNA samples, the researchers utilized a double-strand synthesis kit from GeneAll, Korea. The cDNA synthesis was conducted following the manufacturer's protocols. Finally, to assess the degree of changes in gene expression, reverse transcription PCR (RT-PCR) was carried out. The SYBR Green-based PCR Master Mix (Yekta Tejhiz, Iran) was used for the PCR reactions. The gene expression levels were assessed using a MIC system (Australia). The following PCR primer sequences were utilized for amplification [Table 1]. For the quantitative analysis of the PCR data, the ΔΔCt method was employed, and the efficiency correction was applied following the Pfaffl technique.

Western blot analysis

To lyse HepG2 cells, a protease/phosphatase inhibitor cocktail was used in combination with RIPA buffer. The RIPA buffer contained the following components: 150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 100 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM ethylenediaminetetraacetic acid (EDTA), 1% Nonidet P-40 (NP-40, a non-ionic detergent), 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS).

To quantify the total protein content in each sample, the researchers employed the BCA Protein Assay Kit (Thermo Fisher Scientific Inc., USA). After quantifying the total protein content in each sample, 40 µg of proteins from each sample was loaded onto a polyacrylamide gel using the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) technique. Following the separation on the gel, the proteins were transported from the gel onto the polyvinylidene difluoride (PVDF) membranes (Amersham, Buckinghamshire, England). In the next step, the membranes were blocked to prevent non-specific binding of antibodies. A 5% (w/v) bovine serum albumin (BSA) solution in Tris-buffered saline (TBS) was used for this purpose. After blocking, the membranes were incubated with primary antibodies (obtained from Santa Cruz, CA, USA) overnight at 4°C. After the overnight incubation with primary antibodies, the PVDF membranes were further incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies obtained from Santa Cruz, CA, USA, at room temperature for 1 hour. HRP-conjugated secondary antibodies bind specifically to the primary antibodies, allowing for the detection and visualization of the target proteins on the PVDF membrane. Imaging was done using a quantitative luminescence imaging system (Fusion FX, Vilber Lourmat) and ECL detection reagent. Finally, densitometric analysis was conducted by ImageJ software.

Statistical analysis

The results were offered as mean ± standard deviation. GraphPad Prism 8.0 software from San Diego, CA, USA, was utilized to analyze the data. The data underwent an analysis of variance (ANOVA) test and Tukey post-hoc test, and the significance level for the analysis was established at a *P* value of <0.05.

Results

The effect of Gal/RSV on intracellular total lipid content

In the experiments, the highest concentration of Gal tested was 300 µM, determined to be below the IC50. For comparison, 50 µM of RSV was chosen as a positive control in all the studies.[18] Qualitative and quantitative Oil Red O assay was performed for both RSV (50µM)/Gal (300 µM). Pal concentrations of 0.4 mM caused lipid accumulation in the cells. Microscopic examination revealed lower cell staining for both Gal and RSV treatments. The semi-quantitative Oil Red O results exhibited a significant decrease in lipid accumulation for both treatments with Gal ($P < 0.003$) and RSV ($P < 0.001$) in combination with Pal (0.4 mM).In addition, intracellular triglyceride levels were decreased by Gal ($P < 0.000$) and RSV ($P < 0.001$), similar to the Oil Red-O trend compared to the treatment groups with Pal alone [Figure 1].

The effect of Gal/RSV on the expression of lipin-1 and lipin-2 genes in steatosis

Figure 2 presents the expression levels of lipin-1 and lipin-2 genes in different treatment groups involving Gal and RSV combined with Pal compared to the group treated with Pal alone. The groups treated with Gal + Pal exhibited significantly higher expression levels of both lipin-1 and lipin-2 genes ($P < 0.01$) matched to the group treated with RSV + Pal and that treated only with Pal. In contrast, the groups treated with RSV and Pal did not show a significant difference in the expression levels of the two genes compared to the group treated with Pal alone [Figure 2].

Effect of Gal/RSV on lipin-1, and lipin-2 proteins in steatosis

Figure 3 shows the Western blotting results of lipin-1 and lipin-2 proteins. The intensities of lipin-1 and lipin-2 bands in each treatment group were compared to the beta-actin band, which served as the loading control. In the Gal/RSV treated groups, the intensity of lipin-2 protein ($P < 0.001$) bands was higher compared to the Pal group. On the contrary, in the Gal/RSV treatment group, the intensity of lipin-1 protein ($P > 0.05$) bands was no significant difference compared to the Pal group [Figure 3].

Table 1: Primer sequences for PCR amplification

Primers	Forward	Reverse
β-Actin	5'- AAACAGAGCCTCGCCTTTGC -3'	5'- ACACGATGGAGGGGAAGACG-3'
Lipin-1	5'- CAGTGTAGTCCAGACAGCA -3'	5'- AGGAAGGATCTCCGTTTTTG -3'
Lipin-2	5'-CGGTTTGGAAAGCTGGGAGTC-3'	5'-ATTGGTGAGGTGGCAAGGTAA-3'

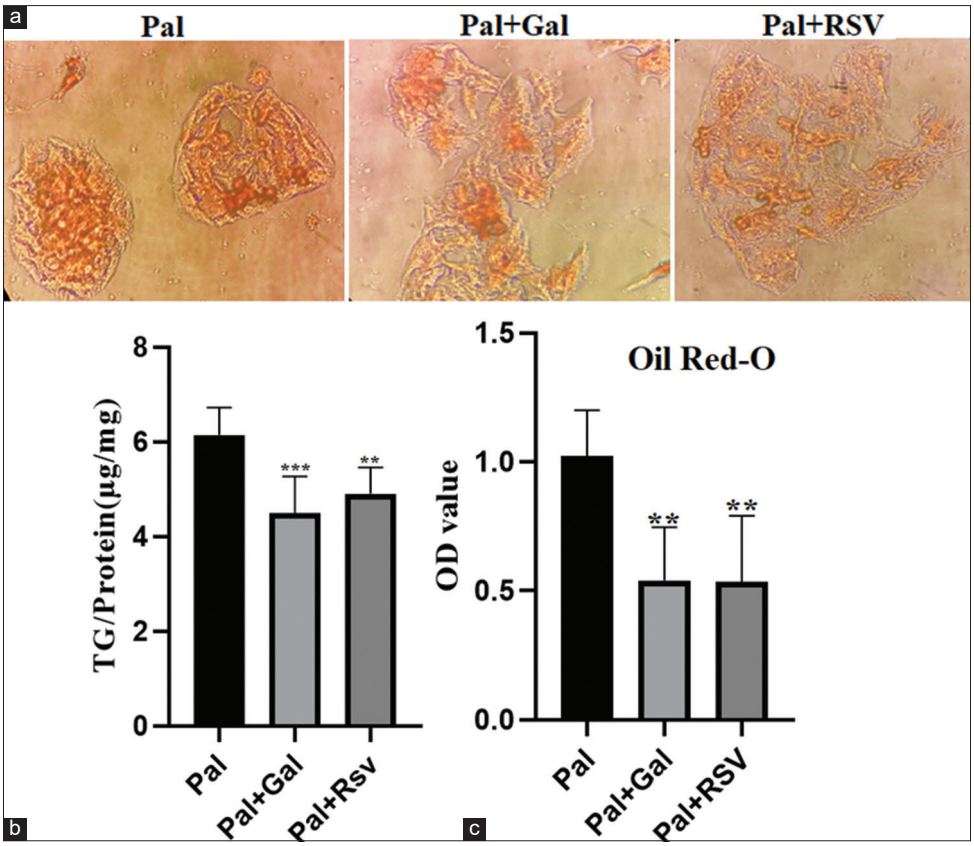

Figure 1: Effect of Gal/RSV on intracellular total lipid content in HepG2 cells (40× magnification). (a) The qualitative and (b)the semi-quantitative Oil Red O results in lipid accumulation, (c) measurement of intracellular triglyceride content. Study groups: HepG2 cells with 0.4 mM Pal alone, with a concentration of 300 μM Gal, and with a concentration of 50 μM RSV were treated with 0.4 mM Pal for 24 hours. *$P < 0.01$, **$P < 0.001$,***$P < 0.000$.

Discussion

Indeed, the accumulation of lipids in the liver is a critical factor in the pathology of NASH. NASH is identified by the buildup of fat in the liver, triggering inflammation and the possibility of liver damage. Researchers are actively exploring new treatment agents to prevent excessive lipogenesis and reduce lipid accumulation in the liver to address this health concern.[19] Indeed, Gal has garnered significant attention due to its diverse biological properties. Research has highlighted its potential as a natural agent with various therapeutic benefits, including anticancer, cancer chemo-preventive, anti-lipogenesis, and anticoagulant function. These diverse biological properties make Gal an intriguing candidate for further exploration in various biomedical applications.[20]

RSV is another fascinating natural compound that has gained significant attention due to its potential biological effects. It is a polyphenol found in various plant sources, including peanuts, grapes, and berries. RSV has been extensively studied for its health-promoting properties, and some of its potential effects include anti-inflammatory, antioxidant, cardiovascular benefits, anti-cancer, and anti-obesity.[21]

The study aimed to examine the effects of Gal and RSV on the expression of lipin-1 and lipin-2 genes and proteins in HepG2 liver cells treated with Pal. The findings revealed the following key observations:

The treatment groups with Gal + Pal demonstrated significantly higher expression levels of lipin-1 and lipin-2 genes compared to the group treated with RSV + Pal and that treated with Pal alone. This finding suggests that Gal may have a substantial stimulatory effect on the expression of lipin-1 and lipin-2 in the context of Pal treatment.

On the contrary, the groups treated with RSV along with Pal did not show a significant difference in the expression levels of lipin-1 and lipin-2 genes compared to the group treated with Pal alone. This conclusion demonstrates that RSV might not have a substantial influence on the expression of these genes under the given experimental conditions.

However, no significant difference was observed in the expression levels of lipin-1 and lipin-2 genes between the groups subjected to a combination of RSV and Pal treatments and the group treated with Pal alone. Bands of lipin-1 and lipin-2 were more prominent in the Gal treatment groups than in the Pal and RSV groups when it came to protein levels. In the RSV treatment group, the bands of lipin-1 and lipin-2 were less pronounced compared to both the Pal and Gal treatment groups. However, a previous study has shown an inhibitory effect of RSV on the gene expression level of

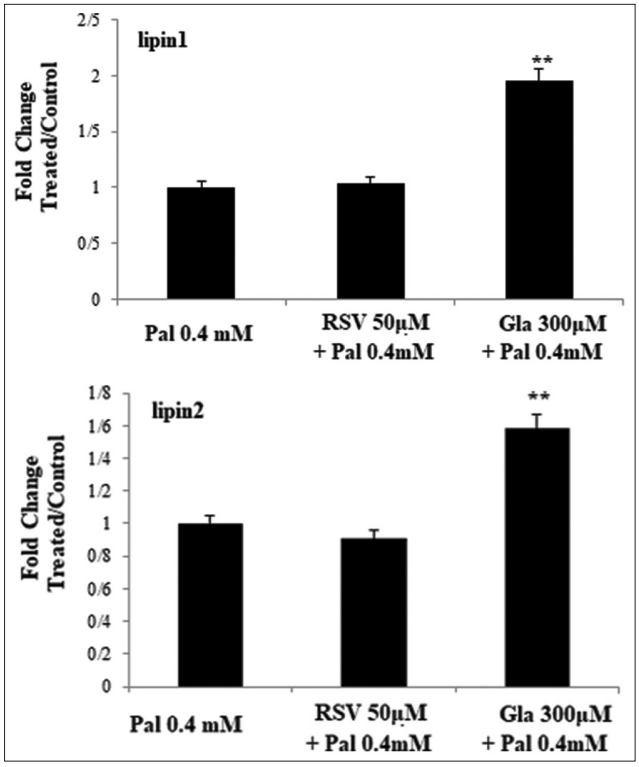

Figure 2: Effect of Gal (300 μM)/RSV (50 μM) on lipin-1 and lipin-2 gene expression in HepG2 cells were treated with 0.4 Pal mM compared to the Pal 0.4 mM alone for 24 hours. $^{**}P < 0.001$

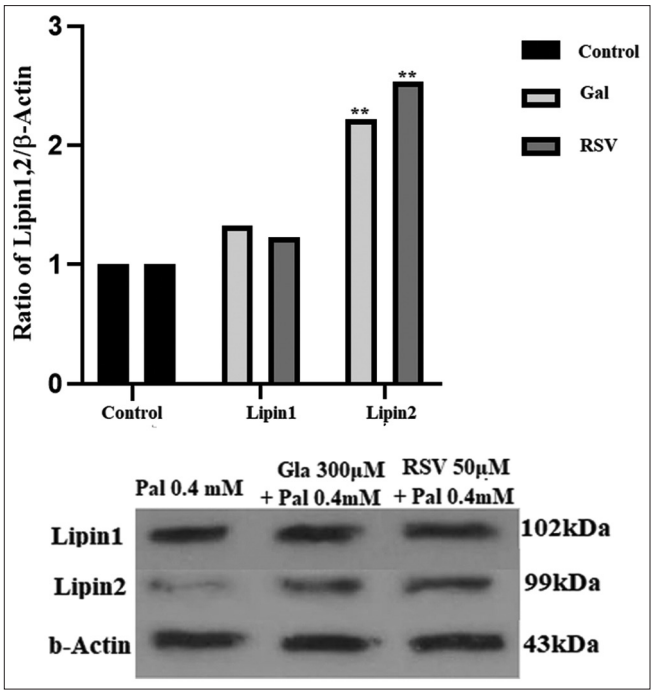

Figure 3: Effect of Gal (300 μM)/RSV (50 μM) on lipin-1 and lipin-2 proteins levels in HepG2 cells were treated with 0.4 Pal mM compared to the control group(Pal 0.4 mM alone) for 24 hours. $^{**}P < 0.001$.

lipin-1.[22] The different observations may be attributed to variations in study protocols; the current study used oleic acid and alcohol for 48 hours to stimulate superfluous lipid aggregation, while in our study, Pal treatment was employed. Lipin-1, known as $Mg2^{+}$-dependent phosphatidic acid phosphohydrolase, serves dual functions in cellular processes. On one hand, it plays a crucial role in the triglyceride synthesis pathway, facilitating the production of triglycerides. On the other hand, it functions as a transcriptional co-activator, promoting fat oxidation and inhibiting lipogenesis. These two distinct roles of lipin-1 contribute to the regulation of lipid metabolism and energy homeostasis within the organism.[23] Previously, our team demonstrated the beneficial effects of Gal on lipid accumulation in HepG2 cells. This finding was achieved through a reduction in the gene expression of enzymes associated with lipogenesis, thereby decreasing the synthesis of lipids within the cells. In addition, Gal treatment increased the levels of lipogenesis inhibitory enzymes, further promoting the inhibition of lipid formation.[18] The recent finding, in line with our previous research, reinforces the positive impact of Gal treatment on inhibiting lipogenesis and reducing related disorders in humans. The promising effect of Gal treatment has been reported in a variety of pathogenic conditions, such as cancer,[24,25] antibiotic resistance,[26] and hepatitis.[27] Furthermore, Huquan Yin*et al*. reported a noteworthy discovery in which the deletion of SIRT1 in an animal model disrupted lipin-1 signaling in alcoholic fatty liver, leading to lipid accumulation in hepatocytes. This result highlights the critical role of lipin-1 in regulating lipid metabolism and emphasizes the potential significance of targeting SIRT1 and lipin-1 pathways for managing lipid-related conditions, particularly fatty liver disease. The combined evidence from our study and that of Huquan Yin*et al*. supply beneficial understandings into possible medicinal approaches for addressing lipid-related disorders in both cellular and animal models.[28] Furthermore, it has been observed that obese individuals exhibit a reduction in lipin-1 expression compared to healthy controls. A decrease in lipin-1 expression has been attributed to insulin resistance, a situation in which cells fail to respond to insulin appropriately. In addition, it has been linked to increased rates of hepatic lipogenesis, contributing to the accumulation of fat in the liver.[29] The correlation between reduced lipin-1 expression and these metabolic abnormalities highlights the critical role of lipin-1 in regulating lipid metabolism and insulin sensitivity. It suggests that low levels of lipin-1 expression may contribute to the pathogenesis of insulin resistance and hepatic lipogenesis in the context of obesity.

Indeed, the effects of lipin-1 can be significantly influenced by its subcellular location and isoform expression. In cases of ethanol exposure, lipin-1 activity is impaired through the SIRT1-SFRS10-Lpin-1β/α axis. This process can promote triglyceride synthesis, as observed with overexpression of lipin-1β, correlating with the inhibition of the hepatic capacity for fatty acid oxidation.

Consequently, this results in decreased fatty acid oxidation and promotes lipid accumulation in the liver, ultimately contributing to the progress of alcoholic fatty liver disease (AFLD).[30] Thus, the presence of different lipin-1 isoforms is critical as they may have distinct roles and effects on lipid metabolism. Understanding the specific functions of these isoforms is crucial for comprehending their impact on various metabolic pathways, including triglyceride synthesis, fatty acid oxidation, and lipid accumulation.

Lipin-2, as a member of the lipin family, plays a crucial role in lipid biosynthesis within the body. Various studies have highlighted the significance of lipin-2, and it has been reported that mutations in the lipin-2 gene can lead to inflammatory-based disorders in humans. Based on the study by Azam Chahardoli *et al*., it appears that Gal could have a dual effect on the human body. The study reported anti-inflammatory effects of Gal, which reveals that Gal may help reduce inflammatory reactions in the body. Furthermore, the study suggests that Gal may influence lipin-2 gene and protein expression.[31]

Our findings show that Gal could have anti-inflammatory effects by increasing lipin-2 gene and protein expression. Based on our study results, Gal could regulate lipin-2 gene and protein expression, which could lead to a reduction in inflammatory reactions in the body. Although our study has provided an appreciated understanding, it is central to accept certain restrictions that require further investigation in future research. One of the significant aspects is the need to address specific molecular mechanisms that underlie the observed effects. Our study focused on lipin-1 and lipin-2, but there may be additional factors and pathways involved in the complex interactions that we have observed.

To fully validate and confirm the potential hepatoprotective effects of Gal and RSV, further research using animal models and clinical trials is necessary. Moreover, in this study, we investigated the lipid-1 and lipin-2 protein expression levels, and it would be better to investigate another molecule, such as phosphatidic acid and phosphorylation level plus JNK1/c-Jun pathway molecules.

Conclusion

In conclusion, our study highlighted the potential of Gal and RSV as promising candidates for regulating lipid metabolism and inflammation. Gal showed anti-inflammatory effects through an increase in lipin-2 gene and protein expression, suggesting a possible role in reducing inflammatory reactions in the body. In addition, the study identified the significance of lipin-1 and lipin-2 in lipid biosynthesis and their impact on metabolic health.

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Conflicts of interest

There are no conflicts of interest.

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