Reversion of Steatosis by SREBP-1c Antisense Oligonucleotide did not Improve Hepatic Insulin Action in Diet-induced Obesity Mice

Authors

M. F. Vitto[1], G. Luz[1], T. F. Luciano[1], S. O. Marques[1], D. R. Souza[1], R. A. Pinho[1], F. S. Lira[1], D. E. Cintra[2], C. T. De Souza[1]

Affiliations

[1] Laboratory of Exercise Biochemistry and Physiology, Programa de Pós-Graduação em Ciências da Saúde, Unidade em Ciênicas da Saúde, Universidade do Extremo Sul Catarinense, Criciúma, SC, Brazil
[2] Faculty of Applied Sciences, University of Campinas – UNICAMP, Limeira, SP, Brazil

Key words
- SREBP-1c
- steatosis
- insulin resistance
- inflammation

Abstract

The literature has associated hepatic insulin action with NAFLD. In this sense, treatments to revert steatosis and improve hepatic insulin action become important. Our group has demonstrated that inhibition of Sterol Regulatory Element Binding Proteins-1c (SREBP-1c) reverses hepatic steatosis. However, insulin signals after NAFLD reversion require better investigation. Thus, in this study, we investigated if the reversal of NAFLD by SREBP-1c inhibitor results in improvement in the hepatic insulin signal in obesity mice. After installation/achievement of diet-induced obesity and insulin resistance, Swiss mice were divided into 3 groups: i) Lean, ii) D-IHS, diet-induced hepatic steatosis [no treatment with antisense oligonucleotide (ASO)], and iii) RD-IHS, reversion of diet-induced hepatic steatosis (treated with ASO). The mice were treated with ASO SREBP-1c as previously described by our group. After ASO treatment, one set of animals was anesthetized and used for in vivo test, and another mice set was anesthetized and used for histology and Western blot analysis. Reversion of diet-induced hepatic steatosis did not change blood glucose, glucose decay constant (k_{ITT}), body weight, or serum insulin levels. In addition, results showed that the protocol did not improve insulin pathway signaling, as confirmed by the absence of changes in IR, IRS1, Akt and Foxo1 phosphorylation in hepatic tissue. In parallel, no alterations were observed in proinflammatory molecules. Thus, our results suggest that the inhibition of SREBP-1c reverts steatosis, but without improving insulin hepatic resistance.

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Correspondence
C. T. de Souza, PhD
Laboratório de Fisiologia e Bioquímica do Exercício
Programa de Pós-Graduação em Ciências da Saúde
Universidade do Extremo Sul Catarinense
88806-000 Criciúma, SC
Brazil
Tel.: +55/48/3431 2773
Fax: +55/48/3431 2500
ctsouza@unesc.net

Introduction

Nonalcoholic fatty liver disease (NAFLD) is an expanding clinical entity and is considered the most common liver disorder in Western countries [1]. The spectrum of the disease covers steatosis to steatohepatitis that can evolve to cirrhosis, the end-stage liver disease. NAFLD is associated with genetics and environmental factors, such as sedentarism, high fat diet, and obesity. In the liver, the transcription factor SREBP-1c, the crucial transcription factor responsible for lipogenesis, regulates the production of lipids, inducing the transcription of almost all the genes that encode enzymes involved in fatty acid synthesis and esterification of fatty acids to triglycerides [2]. Previous studies by our group demonstrated that the blocking of SREBP-1c by antisense oligonucleotide (ASO) decreased protein levels of lipogenic enzymes, reducing total liver fat accumulation and, finally, reversing hepatic steatosis in mice [3].

NAFLD is associated with insulin resistance and hyperinsulinemia. Considering the fact that NAFLD patients often have a feature of metabolic syndrome such as insulin resistance and diabetes [4], this dysfunction deserves to be better investigated. The insulin receptor (IR) is a protein with endogenous tyrosine kinase activity that, following activation by insulin, undergoes rapid autophosphorylation and, subsequently, phosphorylates intracellular protein substrates, including insulin receptor substrate (IRS) 1 and 2 [5,6]. After stimulation by insulin, IRS1 and IRS2 associate with several proteins, including phosphatidylinositol 3-kinase (PI3K). Downstream from PI3K, the serine threonine kinase, Akt, is activated and plays a pivotal role in the regulation of various biological processes [7,8]. There is a current consensus that insulin resistance is caused by defects in intracellular insulin signaling, and several causes have been proposed to justify how the insulin signaling defects are present in NAFLD subjects [9]. Intake of high fat diet and accumulation of hepatocellular lipids activate the 2 main inflammatory

pathways that impair the action of insulin; JNK and IKK/NFκB. Together, JNK and IKKβ might serve as an inhibitor of insulin signaling, by inducing inhibitory serine 307 (Ser307) phosphorylation of IRS1 [10]. IKK is a serine kinase, and its activation phosphorylates the inhibitor of kappaB (IκB). After phosphorylation, IκB is ubiquitinated and degraded in the proteaosome, releasing nuclear factor κB (NFκB) for the translocation to the nucleus and activation of gene expression [11]. It has been proposed that increased NFκB activation may play an important role in the pathogenesis of insulin resistance [12].

Hepatic lipid accumulation leads to insulin resistance. However, the reduction in liver lipids to result in improved hepatic insulin resistance needs further investigation. Based on the premises that accumulation of hepatocellular lipids is linked with insulin resistance in NAFLD, we hypothesized that blocking the SREBP-1c reverse steatosis may improve the molecular insulin pathway and reduce hepatic insulin resistance. In this sense, the aim of the present study was to evaluate whether a reversion of diet-induced hepatic steatosis by ASO SREBP-1c improves hepatic insulin resistance in obese mice.

Materials and Methods

Animals and diet

Male, 4-week-old Swiss mice were obtained from our breeding colony (UNESC) and maintained on a 12-h artificial light-dark cycle and housed in individual cages. The investigation followed the University guidelines for the use of animals in experimental studies. After the acclimatizing period (3 days), the animals were randomly divided into 2 groups; control mice (Lean) fed on standard rodent chow and obese mice, fed on a high-fat diet for 2 months (DIO). Eight weeks of fat-rich diet feeding was set as the time by which all mice should have developed obesity [13]. After installation/achievement of diet-induced obesity, mice were divided in 2 groups: D-IHS, diet-induced hepatic steatosis (no treatment with ASO), and RD-IHS, reversion of diet-induced hepatic steatosis (treated with ASO). The mice were treated intraperitoneally with ASO SREBP-1c (3 nmol/day) for 14 days concurrently with a high-fat diet, as described by Frederico and colleagues [3]. One set of animals was anesthetized and used for in vivo test, and another mice set was anesthetized and used for histology and Western blot analysis.

Hormone and biochemical measurements

Serum insulin and TNFα were measured by ELISA kit (Crystal Chem Inc., Chicago, IL). Blood glucose was measured by glucosimeter.

Intraperitoneal insulin tolerance test (ipITT)

Insulin (2 U/kg body weight) was injected via i.p. and blood samples were collected from the tail at 0, 5, 10, 15, 20, 25, and 30 min for blood glucose determination. The constant rate for glucose disappearance (k_{ITT}) was calculated using the formula $0.693/t_{1/2}$. Glucose $t_{1/2}$ was calculated from the slope of the least-square analysis of the plasma glucose concentrations during the linear decay phase [13].

Protein analysis by immunoblotting

As soon as anesthesia was assured by the loss of pedal and corneal reflexes, the abdominal cavity was opened. For analysis of the insulin pathway, mice were opened and the animals received an injection of insulin (+) (100 µl, 10^{-6} mol/l) or saline (−) (100 µl) through the cava vein. Afterwards, fragments of hepatic tissue were excised. For proinflammatory analysis insulin was not injected. The tissues were homogenized immediately in extraction buffer at 4 °C with a Polytron MR 2100 (Kinematica, Switzerland), operated at maximum speed for 30 s. The extracts were centrifuged at 11 000 rpm and 4 °C (5804R) (Eppendorf AG, Hamburg, Germany) for 40 min to remove insoluble materials, and the supernatants were used for protein quantification, using the Bradford method [14] and proteins were denaturated by boiling in Laemmli [15] sample buffer containing 100 mM DTT. Then, 0.2 mg of protein extract obtained from each sample was separated by SDS-PAGE, transferred to nitrocellulose membranes and blotted with anti-IR, anti-IRS1, anti-Akt, anti-phospho[Ser473]Akt, anti-Foxo1, anti-β-actin, anti-phospho -JNK, anti-JNK, anti-TLR2, anti-MyD88, anti-NFκB, and anti-IKKβ antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA); and anti-phospho-Foxo1, anti-phospho-IR, anti-phospho-IKKβ, anti-TNFα, anti-IL-1β antibodies (Cell Signaling Technology, Beverly, MA, USA). The original membrane was stripped and reblotted for total protein levels and β-actin loading protein. Chemiluminescent detection was performed with horseradish peroxidase-conjugate secondary antibodies. Autoradiographs of membranes were taken for visualization of protein bands. The results of blots are presented as direct comparisons of bands in autoradiographs and quantified by densitometry using the Scion Image software.

Histology

Hydrated, 5.0 µm sections of paraformaldehyde-fixed, paraffin-embedded liver fragments of 4 mice were stained with hematoxylin/eosin and photodocumented using an Olympus BX60 microscope.

Statistical analysis

Results are expressed as the means ± SEM. Differences between the groups were evaluated using one-way analysis of variance (ANOVA). When the ANOVA indicated significance, a Bonferroni post hoc test was performed. A probability of less than 0.05 was considered significant. The software used for analysis of the data was the Statistical Package for the Social Sciences (SPSS) version 17.0 for Windows.

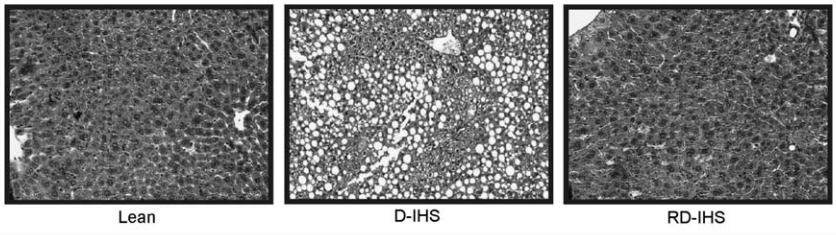

Fig. 1 Hematoxylin/eosin staining in the liver of Lean, D-IHS, and RD-IHS mice. Hematoxylin/eosin staining of 5.0 µm sections of livers (n = 4) was performed to confirm efficiency of diet for induce hepatic steatosis (D-IHS) and ASO treatment to revert hepatic steatosis (RD-IHS).

Results

Diet-induced hepatic steatosis was used in the present study, since studies performed by our group have demonstrated it earlier [3,16,17]. On the other hand, our group has proved the reversion of steatosis using the ASO technique anti-SREBP-1c [3]. Thus, in the present study we used ASO SREBP-1c for reversion of diet-induced hepatic steatosis (**Fig. 1**).

The next step was to evaluate the effect of reversion of steatosis on metabolic and physiological parameters. **Table 1** shows comparative data regarding lean, D-IHS, and RD-IHS mice. The D-IHS mice had a higher body weight, epididymal fat, serum insulin, blood glucose, serum TNFα, and a lower glucose disappearance than age-matched controls. In RD-IHS mice these parameters were not altered (**Table 1**). The results of serum insulin, blood glucose and k_{ITT} (**Table 1**) suggested that the reversion of diet-induced hepatic steatosis model (**Fig. 1**) is not sufficient to alter the insulin resistance. There was no significant difference in food intake in the group treated with antisense SREBP-1c (data not shown).

The insulin signaling was examined in the liver of D-IHS mice. High-fat diet reduced IR and IRS1 phosphorylation (**Fig. 2a, b**, respectively). Reversion of steatosis did not result in significant changes in IR and IRS1 phosphorylation. In addition, D-IHS mice demonstrated reduced Akt^{ser473} phosphorylation, when compared with the lean mice (**Fig. 2c**). Results of equal magnitude occur in the phosphorylation of Foxo1 (**Fig. 2d**). As a result of the sequence of the state of insulin resistance described above (k_{ITT}), in the livers of the RD-IHS group, Akt^{ser473} and Foxo1 phosphorylation did not change (**Fig. 2c, d**). No differences were observed in the basal (no insulin) levels of either Akt^{ser473} or Foxo1 phosphorylation between the groups. The IR, IRS1, Akt, and Foxo1 protein levels did not differ between the groups (**Fig. 2a–d**, lower panels).

To verify why the fatty liver does not alter resistance to insulin, we analyzed inflammatory protein expression. The high-fat diet induced an increase the cytokine TNFα and IL-1β, when compared with Lean group. The use of SREBP-1c inhibitor reverts steatosis but not alter the protein levels of these cytokines (**Fig. 3a, b**). Toll like receptor 2 (TLR2) is a crucial receptor activated to inflammatory signal and saturated fatty acids through their molecule adaptator MyD88. Livers of the obese mice showed increased protein levels of TLR2 and MyD88, when compared with Lean group (**Fig. 3c** upper and lower panels, respectively). Steatosis reversion does not change protein levels of TLR2 and MyD88. The high-fat diet increased both IKKβ phosphorylation and NFκB protein levels, when compared to the lean diet (**Fig. 3d, e**). Reversion of diet-induced hepatic steatosis does not cause significant changes in either IKKβ phosphorylation or NFκB protein levels, when compared with respective D-IHS mice (**Fig. 3d, e**). The high-fat diet induced an increase JNK phosphorylation in the liver of D-IHS mice, when compared with lean mice (**Fig. 3f**). In RD-IHS mice significant changes in JNK phosphorylation were observed (**Fig. 3f**).

Table 1 Metabolic characteristics.

	Lean	D-IHS	RD-IHS
Body weight (g)	39.3 ± 2.0	53.4 ± 1.9*	51.6 ± 1.7*
Epididymal fat (%/b.w.)	1.0 ± 0.2	4.3 ± 0.3*	4.1 ± 0.5*
Insulin (ng/ml)	2.0 ± 0.6	12.3 ± 2.5*	11.9 ± 3.1*
Blood glucose (mmol/l)	5.8 ± 0.4	11.1 ± 0.7*	10.7 ± 0.7*
k_{ITT} (%/min)	4.0 ± 0.9	2.3 ± 0.4*	2.6 ± 0.6*
TNFα (pg/ml)	4.0 ± 1.5	31.8 ± 6.1*	29.7 ± 7.2*

Symbols represent statistical significance. n = 8, *p < 0.05 D-IHS and RD-IHS groups when compared with Lean mice

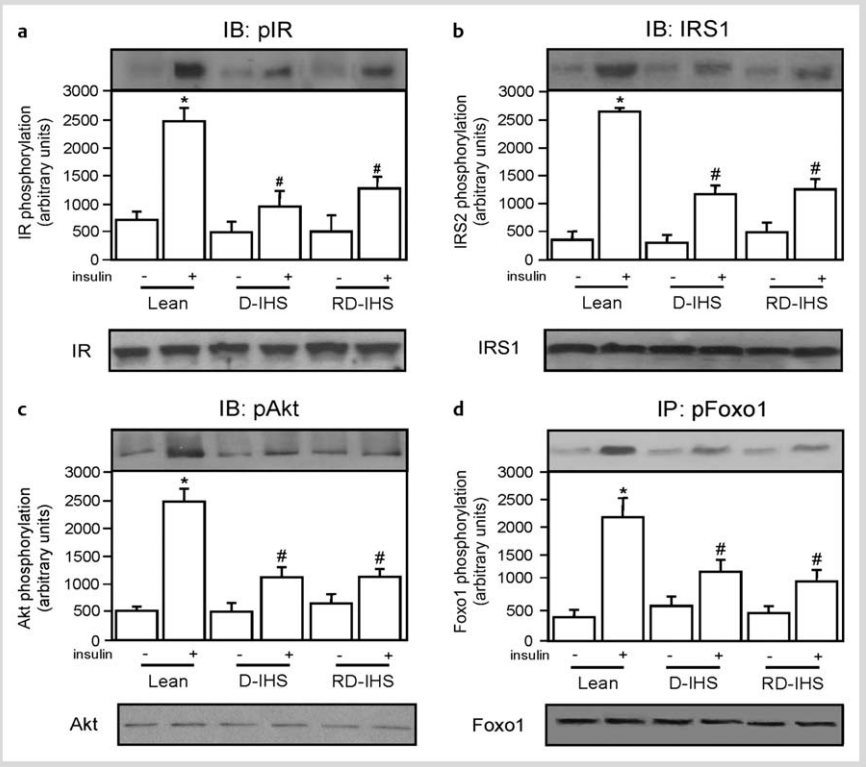

Fig. 2 *p < 0.05 Lean group with insulin injection (+) when compared with Lean mice (−), and #p < 0.05; RD-IHS and D-IHS groups (+) when compared with Lean group (+).

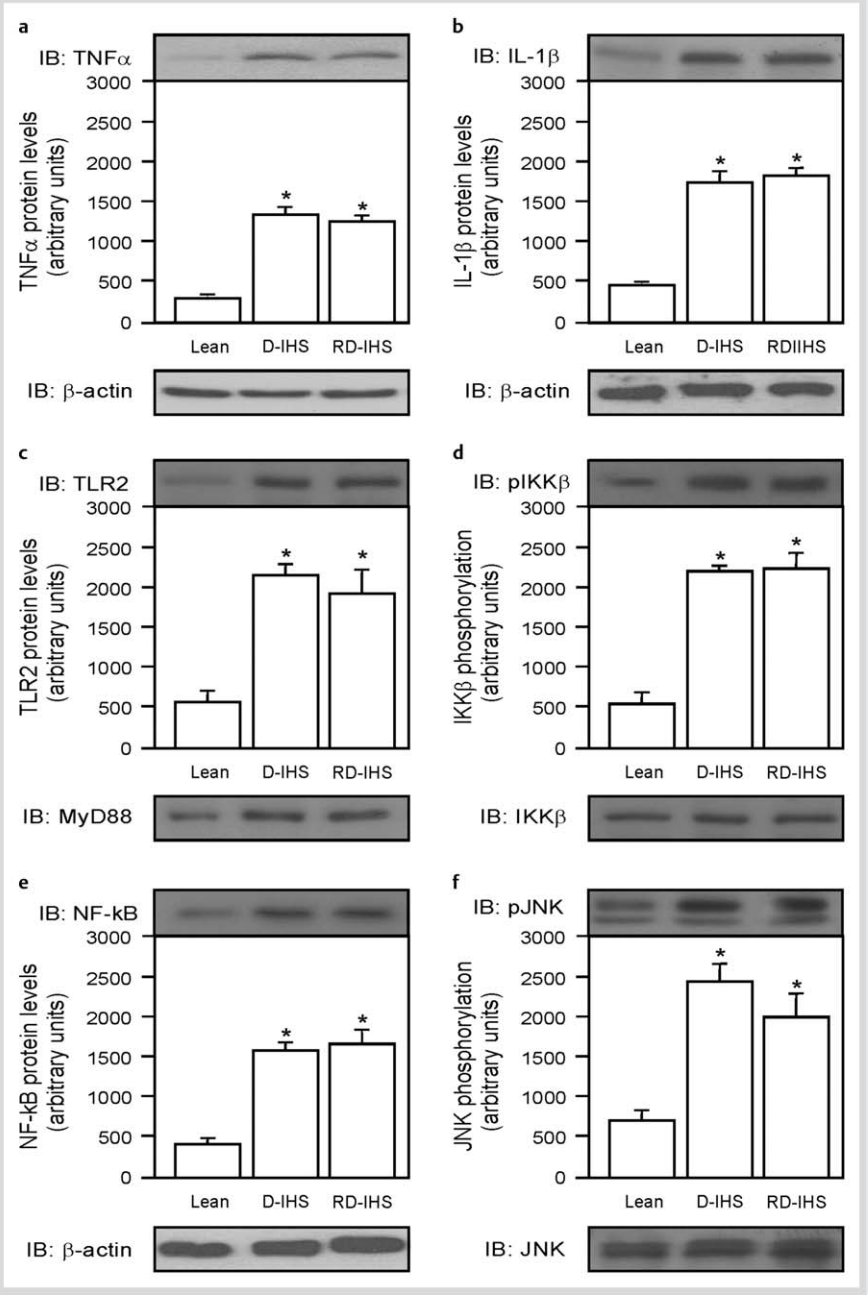

Fig. 3 *p<0.05; RD-IHS and D-IHS groups when compared with Lean group.

Discussion

Hepatic insulin resistance is associated with NAFLD and is a major factor in the pathogenesis of type 2 diabetes mellitus and metabolic syndrome [18,19]. In NAFLD, fat accumulation in the liver may occur as a result of increased fat deposition, increased fat synthesis and reduced fatty acid oxidation [20]. Scientific advances have identified possible molecular mechanisms related to the onset of steatosis. In this sense, some studies have shown that the accumulation of fat in the liver is associated with high levels of SREBP-1c protein [20–22]. Our group has previously demonstrated that inhibition of SREBP-1c by ASO results in decreased protein levels of the lipogenic enzymes, reduction of 69% in total liver fat and, finally, a reversion of hepatic steatosis in obese mice [3]. However, hepatic insulin action in this model of reversion steatosis has not been described. With this in mind, we evaluated whether the reversion of steatosis by SREBP-1c inhibitor improves hepatic insulin action in obese mice.

In the present study, we observed that high-fat diet leads to reduction in whole body insulin sensitivity (k_{ITT}), elevated blood glucose levels, serum insulin, epididymal fat, and serum TNFα. On the other hand, the use of ASO reverted steatosis in RD-IHS group, but no improvement in systemic insulin sensitivity; demonstrated by the absence of changes in disappearance of glucose, blood glucose, and serum insulin. In addition, no change was observed in body weight and epididymal fat. Thus, our data indicate that the reduction in intrahepatic lipids alone was not an effective treatment of systemic insulin resistance. More recently, Moon et al. [23] have found similar data; the authors also showed that elimination of nuclear SREBPs in liver ameliorates pathologic triglycerides accumulation in different experimental models, but they reported that the reduced hepatic fatty acid overproduction is not required for the development of systemic insulin resistance in mice, since hepatic resistance to insulin action was not recovered. Taken together, our results suggest that other alterations are necessary for improvement of insulin resistance, mainly such as reduced adiposity and inflammation.

Next, we analyzed the hepatic insulin action, through IR, IRS, Akt, and Foxo1 phosphorylation, in obese mice treated with ASO SREBP-1c. Hepatic tissue of obese mice showed reduced IR, IRS1, Akt, and Foxo1 phosphorylation. The treatment with ASO SREBP-1c reverses hepatic steatosis, but not altered the phosphorylation of these molecules. We suggest that the reversion of hepatic steatosis by ASO SREBP-1c did not reverse hepatic insulin action because it does not reverse TNFα and proinflammatory molecules.

The excess fat affects the integration of metabolic and immune response pathways. Recently, several studies have demonstrated that obesity is associated with low-grade chronic inflammation that is causally involved in the development of insulin resistance [24]. The negative impact of increased fat accumulation in sensitivity to insulin can be clearly demonstrated in most subjects, as well as reduction in insulin resistance observed with weight loss. Inflammation is markedly evident in a number of human and mouse models of obesity, as determined by increased of inflammatory cytokines [24]. The cytokine TNFα was the first indication that inflammatory mediators are associated with obesity [25]. Moreover, the TNFα have been implicated in the development of insulin resistance [26]. Our study showed that the use of ASO reverses steatosis, but it does not reduce adiposity and serum levels of TNFα.

One mechanism by which cytokines proinflammatory-induced by lipids induce insulin resistance is through active proinflammatory molecules, including JNK and NFκB/IKK. Suppression of the JNK pathway in liver ameliorates insulin resistance status and glucose tolerance in both genetic and dietary models of diabetes [27]. On the other hand, studies indicate that lipid accumulation in the liver leads to hepatic inflammation through NFκB activation and downstream cytokine production; this causes insulin resistance both in liver and systemically [11,28,29]. Our results show that animals submitted to high-fat diet has pronounced increase of IKKβ phosphorylation and NFκB protein levels and, after treatment with ASO, no changes were observed these parameters.

In the present study, we also examined the contribution of the toll like receptors (TRL2) upon inflammatory response. The inflammatory response in part, is mediated by toll like receptors, following by activation NFκB pathway. Studies have shown increased expression of key components of the innate immune cascade in states of obesity and diabetes mellitus type 2, in particular TLR2 and TLR4 [30–32]. In the present study, it was observed that TLR2 protein levels exhibited the same pattern of the other proteins involved in inflammatory pathway.

In summary, our results demonstrate that antisense oligonucleotide treatment attenuates the incidence of fatty liver, but not obesity or insulin resistance in DIO mice. The inhibition with ASO may represent a pharmacological treatment in humans after the establishment of disease, but when the objective is to reverse steatosis and also to reduce hepatic insulin resistance, other drugs should be associated.

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Conflict of Interest

The authors report no conflicts of interest.

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