Journal of Diabetes and Metabolism
ISSN: 2155-6156

Garcia et al., J Diabetes Metab 2015, 6:4
DOI: 10.4172/2155-6156.1000527

Short Communication Open Access

Preventive Effect of a *Fraxinus Excelsior* L Seeds/Fruits Extract on Hepatic Steatosis in Obese Type 2 Diabetic Mice

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Abstract

Background: Non-alcoholic fatty liver is recognized as one of harmful consequences of the metabolic syndrome and hepatocytes steatosis is well connected with loss of insulin sensitivity, impaired glucose tolerance and can lead to impaired fasting glucose and type2 diabetes mellitus. *Fraxinus excelsior* L. seed extract has been used as traditional folk medicine by Mediterranean population and Glucevia®, a natural extract of *Fraxinus excelsior* L. derived from seeds/fruits of the plant and standardized to 10% Nuzhenide and GI3, has been previously reported to regulate glucose homeostasis in healthy overweight people.

Methods: The effect of seven-month administration of Glucevia® on liver parameters was investigated in a diabetic mouse strain (BKS ++Lepr db (db/db)). The severity of fatty change and grading of hepatic steatosis were determined by estimating the fat hepatocytes contain in animals fed with a control diet or with a control diet supplemented with 0.07 % (w/w) of the extract.

Results: Glucevia® was shown to significantly reduce fatty liver in diabetics mice (-54%; $p<0.05$). A concomitant improvement in alkaline phosphatase (ALP) levels and in aspartate aminotransferase/alanine aminotransferase (AST/ALT) ratio were observed between groups ($p<0.05$). A significant decrease in insulin plasma level (-22%; $p<0.05$) was measured in Glucevia® group leading to an improvement of HOMA-IR between groups ($p<0.001$) while no significant change of fasting blood glucose was observed between group.

Conclusion: The results observed supports the potential hepatoprotective function of Glucevia®, which seems to prevent fatty liver formation in type 2 diabetes mice model.

Keywords: *Fraxinus excelsior* L; Fatty liver; hepatic Steatosis; Type 2 Diabetes

Introduction

Among the medicinal plants used for the treatment of diabetes, the seeds of the common European ash (*Fraxinus excelsior* L.) have been identified in ethnobotanical surveys as having anti-diabetic properties [1]. Oral administration of an aqueous extract of *Fraxinus excelsior* L. was found to inhibit renal glucose reabsorption with hypoglycaemic activity in normal and diabetic rats [2], and the hypoglycaemic effect in mice with type 1 diabetes mellitus has been reported to be independent of insulin secretion [3]. Glucevia®, a natural extract from the seeds/fruits of *Fraxinus excelsior* L. standardized in the secoiridoid glucosides nuzhenide and Gl3 (US 8293292), belongs to the class of herbal insulin sensitizer. Indeed, the effects of a single dose of Glucevia® have been evaluated on postprandial glycaemia and insulin secretion in non-diabetic healthy individuals and exhibited a significant reduction in the mean area under the plasma time-concentration curve (AUC) for glucose levels without significantly altering insulin secretion [4]. This natural insulin sensitizer property has been checked in a randomized, crossover, double-blind and placebo-controlled 7-week nutritional intervention in non-diabetic overweight/obese healthy subjects [5]. Three weeks Glucevia® administration resulted in a 2-h blood glucose values reduction following an oral glucose tolerance test (OGTT), while no significant changes were found in the control period. A significant lower incremental glucose area under the curve has been shown with the extract while treatments were not able to induce significant changes in insulin levels. More interesting, administration of Glucevia® significantly decreased fat mass compared to vehicle and increased the adiponectin:leptin ratio suggesting its wide range of targets. *In vitro*, isolated secoiridoid glucosides including nuzhenide, Gl3 and Gl5 from *Fraxinus excelsior* L. extract were found to activate peroxisome proliferator-activated receptor alpha [6]. Moreover, the differentiation of 3T3-L1 mouse embryonic fibroblasts into adipocytes was shown to be inhibited by isolated secoiridoid glucosides of Glucevia®. Chronic administration of the extract (0.5% of the diet) to mice fed a high fat diet resulted in significantly lower liver weight by 63.6% and reduction of the incidence of fatty livers by 66.7% compared to mice fed a high-fat diet alone, and authors have first suggested that Glucevia® could prevent against obesity-related hepatic steatosis [7].

Lipid deposition in the hepatocytes is a common pathology observed in overweight individuals and fatty liver can lead to Nonalcoholic Steatohepatitis (NASH) and turn into fibrosis, a common severely complication among type 2 diabetic patients with a high prevalence of CVD [8,9]. According to the World Gastroenterology Organisation,

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Received February 10, 2015; **Accepted** March 19, 2015; **Published** March 21, 2015

Citation: Garcia FG, Flanagan J, Molina OG, Vivo VV, Carrillo NG, et al. (2015) Preventive Effect of a *Fraxinus Excelsior* L Seeds/Fruits Extract on Hepatic Steatosis in Obese Type 2 Diabetic Mice. J Diabetes Metab 6: 527. doi:10.4172/2155-6156.1000527

nonalcoholic fatty liver disease (NAFLD) is a condition defined by excessive fat accumulation in the form of triglycerides (steatosis) in the liver [10]. The main therapeutic treatments for type 2 diabetes with clinical evidence of benefits are: (i) bodyweight management with caloric restriction and exercise, (ii) insulin sensitization with different classes of API and (iii) lipid lowering API. In parallel, to prevent healthy population against diabetes and fatty liver, dietary intervention policy plans have been set, especially to manage Impaired Glucose Tolerance (IGT) and Impaired Fasting blood Glucose (IFG) [11].

The broad-ranging effects of Glucevia® were studied in two animal models which are representative of metabolic disorders: spontaneously hypertensive rats (SHR) and obese Zucker rats [12]. A significant reduction in plasma levels of triglycerides accompanied by a reduction in fasting plasma glucose and body weight were found in Zucker rats fed with the extract for 5 weeks. No studies to date have focused on the direct benefits of the extract for prevention of fatty liver. In the present study, we therefore evaluated the effect of a long-term administration diet supplemented with Glucevia® on the development of NAFLD in diabetic BKS (db/db) mice. For this end, histological hepatic steatosis and biomarkers of liver injury were studied in addition to body weight management, glycaemic and dyslipidemia biomarkers.

Materials and Methods

Materials

Glucevia® (product code EA149251) is a natural extract of *Fraxinus excelsior* L. derived from seeds/fruits standardized to 10% Nuzhenide and Gl3 (US 8293292). The extract was supplied by Naturex S.A. (Avignon, France).

Animal study design

This study was carried out at the Laboratory Animals Service of the SAI (Servicio de Apoyo a la Investigación/Research Support Service, with license number REGAES 300305440012 at the University of Murcia) in compliance with European Union norms for the protection of animals used in experimentation (2010/63/UE). All experiments were approved by the Bioethics Committee of the University. Animals were housed in cages (480×270×200 cm), in a controlled 12/12 h light/darkness cycle room at 22°C and received food and water *ad libitum*.

Twenty diabetic mutant female mice BKS. BKS ++Lepr db (db/db) from Harlan Laboratories (Barcelona, Spain), aged 5 weeks, with mean body weight 33 ± 3g, were randomly assigned to the following groups: (1) a control group that received standard chow (control, n=10) and (2) a Glucevia® treated group (n=10) that received a standard chow with 0.07% (w/w) Glucevia®. Diet and tap water were administered ad libitum. The standard chow (PanLab, Barcelona) had the following composition: 14.3% protein, 4.0% fat, 48.0% carbohydrate, 4.1% crude fiber, 18.0% neutral detergent fiber and 4.7% ash; energy density, 2.9 kcal/g). The dose correspond to an initial 170 mg of extract per kg body weight per day, approximately 13,7 mg per kg body weight HED, similar to the dose used during the clinical trials conducted [4,5]. Dietary intervention lasted for 28 weeks. Body weight, food and water intake were recorded throughout the study. At the end of the experimental period, over-night fasted animals were euthanized by CO_2 chamber. Livers were taken and weighed prior to hepatic steatosis histological studies.

Biochemical analyses

Blood samples were taken from the superficial temporal vein of the BSK mice. All biochemical parameters were analyzed using the multi-channel auto-analyzer Olympus A400 (Olympus, Hamburg, Germany). Serum insulin was determined by enzyme-linked immunosorbent assay (mouse insulin ELISA kit supplied by Zeleste Diagnostic Etyca Research, Barcelona) and HOMA-IR was calculated as described by Matthews et al. [13]. The extent of liver injury was assessed by determination of alkaline phosphatase (ALP), total bilirubin, creatinine, serum alanine aminotransferase (ALT) and serum aspartate amino transferase (AST) using the multi-channel autoanalyzer Olympus A400.

Liver histopathology

The liver morphology was visualized by hematoxylin and eosin (H&E) staining. Liver samples were rapidly fixed in 10% formaldehyde and embedded in paraffin. Paraffin sections were cut at 3 μm and stained with H&E, using standard procedures. For the detection of lipid deposition in liver, liver section were prepared from frozen liver and stained with oil red O as previously reported [14]. Digitized H&E and oil red O-stained slides were analyzed with image analysis software to obtain a quantitative histologic measurement of steatosis using a Leica DM 600B microscope, with Leica DFC280 camera and a Leica image analysis program supplied by Leica Microsystems AG (Solms, Germany). Six random images at x40 magnification for each liver biopsy were taken to ensure a representative sample for each specimen. A histogram of pixel intensity was generated from the image, the area was measured and the results were expressed as fat percentage by area. Liver steatosis was blindly evaluated by two expert liver pathologists according to the Kleiner and Brunt classification [15]: Grade 0 (<5%), 1 (5-33%), 2 (34-66%), 3 (>67%). In addition, the size of the fat droplets was calculated to identify the percentage of microvesicular and macrovesicular steatosis. Microvesicular steatosis was defined as a diameter ≤ 15 μm [16].

Statistical analysis

Data were analyzed using the SPSS version 19.0 statistical package (SPSS® Inc., Chicago, IL, USA). A descriptive study was made of each variable. All results were expressed as mean ± standard error of the mean. Statistical comparisons of groups were conducted using the Student´s t-test or one way ANOVA with Bonferroni post-hoc analysis. Statistical significance was set at $p<0.05$.

Results

Growth parameters

The two groups of BSK db/db mice did not differ in their initial body weight. Food intake was significantly higher ($p<0.05$) in animals treated with Glucevia® than in their control counterparts and remained constant during the study (7.09 ± 0.53 vs. 4.99 ± 0.72 g/mouse/day). The global health status of animals treated with Glucevia® was better than in control group; the mice were more active and were eating more. However, after 28 weeks of administration, animals treated with Glucevia® gained less weight than control group (Table 1). Total body weight gain were respectively 17.91 g vs 13.1 g for control group and Glucevia® group ($p=0.07$). There were no significant differences in the consumption of water between the control and the treated groups (3.23 ± 0.41 vs. 3.49 ± 0.27 mL/mouse/day).

Plasmatic markers of type 2 diabetes

A decrease in fasting blood glucose (FBG) by 9% was observed in animals treated with Glucevia® compared with placebo after 7 months, without statistically significance. Nevertheless, at the end of the experiment, Glucevia® reduced fasting plasma insulin levels by 22%

	Control	Glucevia®
Initial Body Weight (g)	28.59 ± 1.83	28.69 ± 2.00
Final Body weight (g)	46.50 ± 6.69	41.82 ± 8,63
Initial Food intake (g/mouse/day)	5.37 ± 1.01	7.05 ± 1.87*
Final Food intake (g/mouse/day)	4.18 ± 0.97	6.79 ±1.52*

The value are expressed as mean ± SE for 10 mice
*Significant ($p<0.05$) difference between the control and Glucevia® groups

Table 1: The effect of Glucevia® on growth parameter in BKS db/db mice.

compared with control group mice ($p<0.05$) equivalent a 46% decrease of the HOMA-IR parameter in treated animals (Table 2).

Total-cholesterol and HDL-C serum levels were not found to be different between the control and Glucevia® groups at the end of the study (143.63 ± 24.77 and 88.00 ± 21.07 $mg.dl^{-1}$ vs. 133.12 ± 36.43 and 88.81 ± 35.05 $mg.dl^{-1}$ respectively). However, significant changes in plasmatic triglycerides levels were observed at end point depending on the treatment (264.83 ± 49.36 vs. 162.72 ± 73.90 for control and Glucevia® respectively), which corresponds to a 33.4% reduction induced by Glucevia® treatment ($p<0.05$). Chronic administration of the extract did not modify adiponectin plasma levels (13.14 µg/ml vs. 10.67 µg/m in Glucevia® and control groups respectively).

Markers of NAFLD

The relative liver weight was found to be 18% lower in the Glucevia® treated db/db mice than in the control mice (Table 3). To estimate liver injury, ALP, AST and ALT biomarkers, total bilirubin and creatinine were compared between groups at the end of the experiment. The chronic administration of Glucevia® led to decrease plasma level of ALP compared to control group (116.33 ± 34.12 U/l vs. 87.38 ± 30.93 U/l; $p<0.05$) while no difference in total bilirubin and creatinine was observed. Furthermore, the AST/ALT ratio was significantly lower in the Glucevia®-treated group compared to control group mice (1.56 ± 0.52 vs. 2.91 ± 1.85; $p<0.05$). Neither inflammation nor fibrosis were detected in either groups of mice. Only a slight lobular inflammation and a diffuse hepatocytes ballooning were observed in the livers of the control group. Ballooning degeneration was less pronounced and lobular inflammation was almost absent in treated animals, while microvesicular steatosis was observed and had a closed prevalent disease grade 2 (28%) according to Kleiner and Brunt classification [15]. In Glucevia® treated mice, steatosis was markedly reduced by 54% in hepatocyte fat content ($p<0.05$) compared to controls (Figure 1).

Discussion

In the current study, we provide for the first time evidence supporting the beneficial effect of Glucevia® to prevent hepatic steatosis in a diabetic animal model. The liver plays a key role in metabolic homeostasis, not only on gluconeogenesis and glycogen storage, but also thanks to its ability to control massive amounts of lipogenesis and cholesterol synthesis and secretion [17]. The genesis of steatosis is closely related to the development of obesity and particularly insulin resistance (IR), a near universal finding in patients with NAFLD [18,19]. Genetic leptin-resistant BKS (db/db) mice were choose to conduct this study because they suffer from hyperphagia and develop multiple metabolic and hormonal disorders, including NAFLD, and shares many features with human metabolic syndrome [20,21]. It is characteristic for individuals with type 2 diabetes to manifest a dyslipidemia of elevated triglyceride, reduced HDL-C, and small, dense LDL-C composition, a pattern that has also been related to obesity and IR [22]. In our study, although total cholesterol and HDL-C levels were found similar between the two groups at the end of the study, serum triglyceride concentration was statistically decreased in Glucevia® treated animals. This result in concomitant with our previous finding in Zucker rats fed with the same extract [12]. Steatosis occurs when the rate of import or synthesis of fatty acids by hepatocytes exceeds the rate of export or catabolism [23]. Dyslipidemia as a predictor of NAFLD is unclear, but triglyceride level of 1.7 mmol/L (equivalent to 150 mg/dl) or greater have been found to be a significant predictors of septal fibrosis in overweight subjects [24]. Final triglycerides levels in treated mice (162 mg/dl) were quite closed to this predictive value while animals control exhibited 1.8 times more triglycerides levels (265 mg/dl). Consistent with the observed reduction of triglycerides, hepatomegaly was lower (18%) in the treated group compared to the

	Control	Glucevia®
Initial Fasting Blood Glucose (mg/dl)	202.17 ± 28.39	202.00 ±56.20
Final Fasting Blood Glucose (mg/dl)	791.25 ± 140.44	726.31 ± 127.63
Insulin (mU/L)	5.60 ± 0.82	4.39 ± 1.50*
HOMA-IR	9.55 ± 1.33	5.36 ± 2.53*
Total cholesterol (mg/dl)	143.63 ± 24.77	133.12 ± 36.43
HDL-C (mg/dl)	88.00 ± 21.07	88.81 ± 35.05
Triglycerides (mg/dl)	264.83 ± 49.35	162.72 ± 73.90*
Adiponectin (µg/ml)	10.67 ± 1.79	13.14 ± 0.06

The value are expressed as mean ± SE for 10 mice
*Significant ($p<0.05$) difference between the control and Glucevia® groups

Table 2: The effect of Glucevia® on plasmatic markers in BKS db/db mice.

	Control	Glucevia®
Liver weight (g)	2.94 ± 0.86	2.43 ± 0.35
Liver weight (g/100 g bw)	6.35 ± 0,57	6.35 ± 1.23
ALP (U/l)	116.33 ± 34.12	87.38 ± 30.93*
Total Bilirubin (mg/dl)	0.28 ± 0.04	0.29 ± 0.04
Creatinine (mg/dl)	0.25 ± 0.05	0.24 ± 0.05
AST/ALT quotient	2.91 ± 1.85	1.56 ± 0.52*
Steatosis (%)	28.19 ± 13.65	12.59 ± 6.81*

The value are expressed as mean ± SE for 10 mice
*Significant ($p<0.05$) difference between the control and Glucevia® groups

Table 3: The effect of Glucevia® on NAFLD in BKS db/db mice.

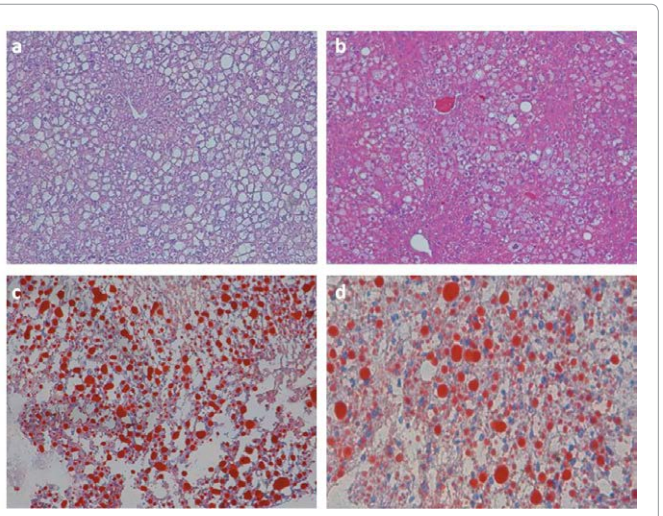

Figure 1: a) H&E stained slide from control group showing intense macrovesicular steatosis; b) H&E stained slide from Glucevia® treated mouse showing steatosis; Oil red O-stained slides from c) control group and d) treated animals.

control group. In non-diabetic overweight/obese healthy subjects, the administration of Glucevia® for 3 weeks resulted in fat mass decrease and an improvement of adiposity related markers as adiponectin:leptin ratio, but no change was observed for plasmatic triglycerides between groups [5]. Extract supplementation over a long period should be required to lead to an improvement of plasma triglycerides level. Due to imbalance in energy consumption, impairment in fatty acid metabolism is closely related to fatty liver. Nuzhenide and GI3, the principal bioactive compounds present in Glucevia®, have been shown to activate the peroxisome proliferator-activated receptor PPARα that serves a critical role in the gene regulatory control of cellular lipid metabolic pathways [25]. Isolated secoiridoid glucosides, excelside A and excelside B (compounds isolated from a methanolic extract of *Fraxinus excelsior* L.) were also found to inhibit differentiation of 3T3-L1 mouse embryonic fibroblasts into adipocytes [6]. This agrees with a recent study where associated-lipogenic transcriptional factors and numerous lipogenic enzymes were shown to be suppressed by mulberry leaf polyphenol extracts, leading to reduced hepatic lipid accumulation in hepatocytes culture [26]. It is important to point out that our study was conducted over 7 months and that Glucevia® not only prevented hepatosteatosis but also showed healthy liver improvement. Elevated transaminases levels are commonly observed in NAFLD stage and an AST:ALT ratio - a standard biomarkers of a healthy liver - higher than 1 predicts the presence of fibrosis [27]. The magnitude of the ASL:ALT ratio was found twice as high in control group compared with treated mice. Interestingly, the plasma level of ALP was shown to be improved in Glucevia® treated mice compared with control mice. In a previous study, a *Fraxinus excelsior* extract was described to increase bile secretion and the detoxifying function of the liver [28]. Our results show that total bilirubin and creatinine were similar in the two groups of mice and plasma levels suggests an early stage of fatty liver disease, in agreement with the lack of fibrobis observed in tissue sample. A human evaluation of Glucevia® was done in a double-blind, placebo-controlled parallel study of 100 healthy volunteers and all the liver variables assessed - AST, ALT, gamma-glutamyltransferase (GGT), alkaline phosphatase, total bilirubin, creatinine - revealed the safety of the extract on the liver [29]. Although we have not observed notable signs of inflammation and fibrosis in the liver samples, our histopathological study along with the liver safety biomarkers clearly indicate a more advanced stage of NAFLD in control animals versus treated animals.

The effect of Glucevia® on FBG remains unclear as no significant reduction in FBG was observed between groups. However, the insulin sensitizer capacity of the extract is once again illustrated. Our results showed an insulin response disproportionally increased in control group *vs.* treated animals for similar FBG. These effect could be due to enhanced glucose uptake in the liver and skeletal muscle as was previously described for another iridoid glycoside extracted from the roots of *Rehmannia glutinosa* [30]. Ibarra et al. [7] reported that mice fed a high-fat diet and administered Glucevia® also decreased significantly fasting insulin levels at the end of the 16-week study compared to mice fed a high-fat diet alone. Furthermore, the extract was found to maintain a similar insulin area under the curve, concomitant with a better glucose tolerance after an OGTT in acute clinical trial on healthy subjects [4]. In the current study, the HOMA-IR parameter was significantly lower in the treated animals, implying reduced insulin-resistance. Alteration in the insulin signaling pathway lead to accumulation of fatty acids and lipid metabolites as suggested by Zhang et al. [31]. The improvement of HOMA-IR in treated animals may delay the imbalance between fatty acid uptake and oxidation responsible for lipid accumulation in the liver. In accordance with this founding, the administration of iridoid glucoside isolated from Vitex negundo leaves has recently been shown to improve glucose uptake as well as fatty liver incidence in a type 2 diabetic-induced mouse model [32].

The processes by which steatohepatitis evolves from hepatic steatosis are not fully understood, but the initiation and perpetuation of cell injury in NAFLD is associated with the increase of free radicals and the depletion of endogenous anti-oxidant defense both in human and rodents [33]. Developing effective therapies for treating steatosis is necessary, and discovering nutrients that can reduce the risk of NAFLD would be useful [34]. In conclusion, this study suggests that long term administration Glucevia® prevents hepatic steatosis in db/db mice, probably mediated by glucose homeostasis and insulin resistance improvements. Further studies are necessary to generate mechanistic insight on steatosis prevention and to identify companion biomarkers before investigating the use of Glucevia® to prevent NAFLD in patients suffering for diabetes.

Acknowledgments

This work was supported by the CENIT Programme from the Spanish Government (SENIFOOD project).

Naturex is involved in the research/development and marketing/sales of Glucevia® as an ingredient for the food, cosmetic, and nutraceutical industries.

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