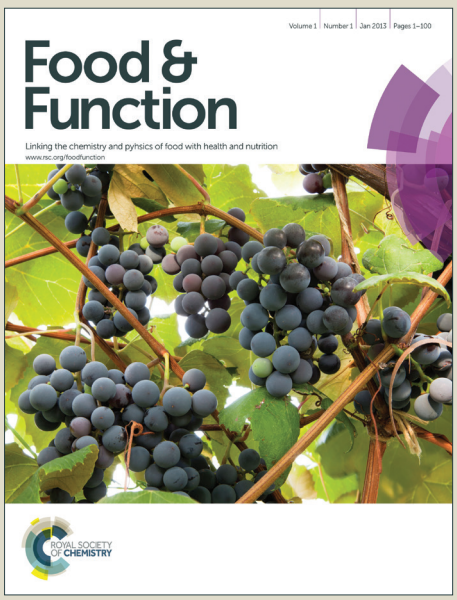
Food & Function

Accepted Manuscript



This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/foodfunction

Action of an extract from the seeds of *Fraxinus excelsior* L., on metabolic disorders in hypertensive and obese animal models.

Fermí MONTÓ[*], Cristina ARCE[*], Maria Antonia NOGUERA[*], Maria Dolores IVORRA[*], John FLANAGAN[†], Marc ROLLER[†], Nicolas ISSALY,[§] and Pilar D´Ocon[*1]

* Departamento de Farmacología, Facultat de Farmàcia, Universitat de València, Avda. Vicent Andrés Estelles s/n, Burjassot, 46100 Valencia, Spain.

[†] Naturex SA, Site d'Agroparc BP 1218, 84911 Avignon Cedex 9, France.

[§] Naturex Spain SL, Autovia A3, salida 343, Camino de Torrent s/n, 46930 Quart de Poblet (Valencia) Spain.

[1]**Corresponding author**: Dr. Pilar D´OCON. Departamento de Farmacología, Facultat de Farmàcia, Universitat de València, Avda. Vicent Andrés Estelles s/n, Burjassot, 46100 Valencia, Spain

Phone: 34 96 3544828

Email address: m.pilar.docon@uv.es

ABSTRACT

Nuzhenide and GI3, the principal secoiridoids of an extract obtained from the seeds of *Fraxinus excelsior* L. (FXE) are believed to be the active compounds responsible for the previously reported hypoglycemic effects of this extract. In this study, the effects of FXE were studied in two animal models which are representative of metabolic disorders: spontaneously hypertensive rats (SHR) and obese Zucker rats. SHR were acutely treated (oral gavage) with different doses of FXE. In addition, SHR and Zucker rats were chronically fed (20 or 5 weeks, respectively) with standard chow supplemented with FXE. Acute treatment with FXE (200 mg/kg body weight) decreased systolic blood pressure as did captopril (50 mg/kg body weight). Chronic treatment with FXE at 100 mg/kg body weight/day, a dose equivalent to that showing hypoglycemic activity in humans, resulted in a significant decreased in glycemia (-16.3%), triglyceridemia (-33.4%) and body weight (-8.1%) in Zucker rats as well as a significant decrease in SBP in SHR (-6.7%), with a concomitant improvement in endothelial function in both strains. The broad-ranging effects of FXE may be due to a unique compositional profile that could be useful to prevent metabolic syndrome, characterized by obesity, insulin resistance, glucose intolerance, hypertriglyceridemia and elevated blood pressure.

INTRODUCTION

Iridoids and secoiridoid glucosides are the major phenolic compounds of *Fraxinus excelsior* L., (1) an Oleaceae family tree more commonly known as "common ash" or "European ash" in the countries of temperate Asia and Europe (2). Like other Oleaceae-derived products (eg. olive oil), iridoids and secoiridoids share some characteristics with medicinal plant-sourced flavonoids and polyphenols and are associated with antioxidant activity (3-5) and with lower incidence of atherosclerosis and cardiovascular disease (6,7).

Fraxinus excelsior L. is normally found in temperate European climates, and also in the Tafilalet region of Morocco where the seeds of this tree have been administered in a tisane for the traditional treatment of diabetes (8). FraxiPure® (FXE), a natural extract produced from the seeds of *Fraxinus excelsior* L., contains secoiridoids, primarily nuzhenide and GI3 as its active ingredients (US 8293292). Nuzhenide and GI3 were found to activate peroxisome proliferator-activated receptor alpha (PPARα) in vitro and inhibit differentiation of 3T3-L1 mouse embryonic fibroblasts into adipocytes (9).

Ibarra *et al*. (10) found that mice fed a high-fat diet and administered FXE (0.5% of the diet) had significantly lower fasting insulin levels at the end of the 16-week study compared to mice fed a high-fat diet alone, and displayed significantly reduced fasting blood glucose levels from week 5 and throughout the remainder of the study.

The effects of FXE on postprandial glycaemia and insulin secretion have been evaluated in healthy human volunteers in a randomized, double-blind, placebo-controlled, crossover study (11). The results showed that acute administration of FXE (1000 mg) and glucose (50 g) to 16 non-diabetic, healthy volunteers could significantly reduce the mean area under the plasma time-concentration curve for glucose levels compared to placebo ($p=0.02$). There was no significant difference noted in the mean insulin AUC values between the extract and placebo groups, suggesting that acute consumption of FXE may produce a slight reduction in glucose levels in non-diabetic, healthy individuals without significantly altering insulin secretion.

Finally, the safety of FXE *in vitro*, *in vivo* and in human volunteers has been evaluated and results have clearly demonstrated that the extract is safe and is well tolerated in healthy subjects (12).

Other *Fraxinus excelsior* L. seed extracts have also been reported to have positive health benefits. Oral administration of an aqueous extract of the seeds of *Fraxinus excelsior* L. inhibited renal glucose reabsorption and concomitantly reduced glycemia in normal and diabetic rats (8). Maghrani *et al.* (13) reported no effects on insulin levels following the single or repeated administration in mice of an *F. excelsior L*. extract. Further studies from the same group demonstrated a potential hypotensive action of *Fraxinus excelsior* seeds in hypertensive rats (14). More recently, Lopez-Carreras *et al.* (15) substantiated this study in hypertensive SHR rats but without providing further answers on the direct link between the extract and hypertension.

Research to date on FXE has indicated that it can play a role in controlling glucose homeostasis in normal and diabetic animals (8) and healthy humans (11). Nevertheless, FXE may also have an effect on the global metabolic state and could, based on previous research, also provide cardiovascular health benefits. The purpose of this study is to elucidate the effect of FXE in metabolic parameters and vascular reactivity in various animal models which are representative of the diseased cardiovascular state. To achieve this objective, and in keeping with the objectives of the SENIFOOD project (a collaborative research project devoted to designing food products for a suitable and balanced diet in elderly Spanish people) the authors have evaluated both acute and chronic administration of FXE to spontaneously hypertensive (SHR) and obese Zucker rats. For this purpose rats were treated with FXE and blood pressure, cardiac hypertrophy, vascular reactivity, lipid profile, liquid and solid intake, body weight, glycemia and plasma levels of insulin and adiponectin were studied.

RESULTS

1. Acute treatment

Acute administration of 200 mg/kg body weight FXE significantly reduced SBP compared to the control and resulted in a time-response curve similar to that obtained with captopril, although the time to maximum effect was longer for FXE compared to captopril (Figure 1A). The lowest (100 mg/kg body weight) and the highest dose of FXE assayed (400 mg/kg body weight) did not significantly modify SBP compared to control (Figure 1B).

2. Chronic treatment

The range of doses was chosen according to the dose of 100 mg/kg body weight/day (FXE100) which is equivalent, according to Reagan-Shaw et al. (16), to the dose used in humans during the clinical trial conducted by Visen *et al.* (11). In addition, a lower (20 mg/kg body weight/day, FXE20) and a higher (400 mg/kg body weight/day, FXE400) dose were also chronically administered in Zucker. According to the results obtained with the acute treatment, the dose of 20 mg/kg body weight/day was not tested in SHR rats.

All animals tolerated their respective chronic treatment included in the diet and no adverse effects were observed during the treatment period.

2.1. Systolic blood pressure

SBP was attenuated in the SHR animals treated during 20 weeks administered captopril 50 mg/kg body weight/day, as well as in the group receiving FXE 100 mg/kg body weight/day (FXE100, Figure 2A and Table 1). No significant changes in SBP were observed in the group receiving FXE 400 mg/kg body weight/day (FXE400, Table 1). A similar discrepancy between lower and higher doses of *Fraxinus excelsior* extracts on SBP has been described in SHR animals (15) indicating that the range of effective doses is a resultant of the complex composition of the extract, and at higher concentrations, the effect of a given component of the extract on SBP could be undermined by others. In Zucker animals, which are not in a hypertensive state, a slight but not significant decrease in SBP values was also observed only in the group treated with FXE100 (Table 2)

After the 20-week treatment with captopril or extracts, a subgroup of SHR animals was submitted to a washout period of 4 weeks without any treatment. A significant increase in SBP was observed in the SHR groups treated with captopril, FXE100 and FXE400 when the antihypertensive treatment was removed (Figure 2B).

2.2. Body weight

Body weight gain was monitored weekly in all groups. Control animals grew regularly throughout the entire study. Treatment with FXE100 and metformin significantly diminishes body weight evolution in the Zucker rats (Figure 3A). A non-significant reduction in body weight was observed following administration of FXE20 and FXE400 in Zucker strain (Table 2). In SHR animals, no significant decrease in body weight was observed with FXE or captopril treatment (Table 1).

2.3. Solid and liquid intake

Solid and liquid intake and the ratio between solid or liquid intake and body weight were higher in Zucker than SHR animals, independent of the treatment (Tables 1 and 2). Except in the case of metformin which reduced food intake in Zucker rats, treatments did not significantly change food intake nor the food intake/ body weight ratio, compared to their respective controls (Tables 1 and 2). However, liquid intake and especially liquid intake/ body weight ratio, were significantly increased by captopril, and FXE400 in the SHR model (Table 1). This increase was reverted after the 4 weeks washout period without treatment (Figure 3B). In Zucker rats, an increase in the liquid intake was also observed with FXE100 and FXE400 (Table 2).

2.4. Cardiac hypertrophy

No changes were observed in heart weight after treatments, but a significant decrease was found in the heart/body weight ratio in the group of SHR animals receiving captopril (Table 1). An increase in this ratio was observed in SHR rats treated with the highest dose of FXE (FXE400, Table 1) and in Zucker rats treated with metformin (Table 2).

2.5. Fasting plasma glucose

The SHR (4 weeks old) group had normal glycemia before treatment (89.9±7.2 mg/dL). Table 1 summarizes the levels of fasting plasma glucose at the end of the 20 week treatment in each group. Higher glucose levels were observed in 24 weeks old SHR animals, except in the captopril group which exhibited glucose levels similar to young animals and significantly lower levels compared to the 24 week-old control group (Table 1).

In Zucker rats, fasting blood glucose levels were recorded weekly throughout the experiment. Figure 4A shows the evolution of plasma glucose during the experiment in the control group and those untreated with FXE100 or metformin. In this case, treatments decreased fasting blood glucose levels progressively from the beginning of the study until week 5 of treatment, whereas in the control group, plasma glucose levels increased during the same period. As reported in Table 2, at the end of the study, a

significant decrease in glucose levels was observed in animals treated with metformin or all three doses of FXE.

2.6. Lipid Profile

Plasma cholesterol, HDL and triglyceride levels were determined in SHR and Zucker rats and, as expected, lipid levels were higher in the Zucker strain. In SHR animals, the lipid profile was determined at the beginning and the end of treatment (weeks 0 and 20) and also in a subgroup of animals submitted to the washout period without treatment. In Zucker animals, the evolution of lipid levels were followed during the treatment (weeks 0, 2, 4 and 5). No significant changes were observed in total cholesterol or HDL levels in either SHR or Zucker models (Table 1).

However, significant changes in triglycerides profile were observed depending on the treatment. As Figure 4B shows, plasma levels of triglycerides increased progressively in the control group and this effect was also observed in the group treated with metformin in the Zucker rat model. Compared to the control group, significant decreases in triglycerides were observed following treatment with FXE100 (Figure 4B) - with a -33.4% reduction. A similar decrease was observed with FXE400 (Table 2). No significant changes were found in the FXE20 group compared to control (Table 2).

In SHR animals, administration of captopril and the two doses of FXE (FXE100 and FXE400) significantly reduced plasma triglyceride after 20 weeks of treatment with a decrease of 22% observed following treatment with both concentrations of FXE (Table 1). Furthermore, as Figure 7 shows, significantly decreased levels of triglycerides were found *vs* control after 20 week's intervention but the differences between triglycerides concentrations was no longer significant when FXE treatment was removed for 4 weeks (FXE Washout, Figure 4C).

2.7.Insulin, adiponectin and HOMA-IR index

Table 2 summarizes the plasma insulin and adiponectin levels at the end of the study. HOMA-IR was calculated and values were also included in Table 2. No significant changes were observed in groups treated with metformin or FXE *vs* the control group..

2.8. Vascular reactivity

Vascular reactivity was determined in isolated aorta from SHR and Zucker animals at the end of each treatment (20 and 5 weeks, respectively) and in the subgroup of SHR animals that were maintained for 4 weeks in a washout period without any treatment.

CRC of vasodilatation were performed by addition of increasing concentrations of acetylcholine (Ach) on aortas pre-contracted with 1 μM phenylephrine (PHE). The relaxant response to Ach, mediated by activation of endothelial eNOS and endogenous NO-release, is an indicator of endothelial function. In this case, in aortas from SHR, administration of captopril, FXE100 and FXE400 significantly increased the vasodilatory potency of Ach (Figure 5A and Table 3), a beneficial effect that disappeared after the washout period without treatment (results not shown). A significantly increased potency of Ach was also found in Zucker rats treated with FXE20 and FXE100 although treatment with metformin or FXE400 did not result in any significant change (Figure 5B and Table 3).

Addition of increasing concentrations of sodium nitroprusside to aortas pre-contracted with 1 μM PHE elicited a concentration-dependent vasodilatation mediated by liberation of NO. In SHR animals, treatment with captopril and FXE at different doses significantly increased the vasorelaxant potency of nitroprusside (Figure 6 and Table 3) an effect that disappeared after the washout period without treatment (results not shown). No changes in the vasorelaxant response to nitroprusside were observed in Zucker rats (Table 3 and Figure 6B).

To avoid involvement of endogenous nitric oxide release, aortas were previously treated with the NOS inhibitor L-NAME, and contractile response of vessels performing CRCs of contraction by PHE, an α_1-adrenoceptor agonist, were measured. Chronic treatment with captopril and FXE100 significantly increased the force of maximal contraction induced by PHE without modifying the pEC_{50} (Figure 7 and Table 4). As with other vascular reactivity markers, this effect disappeared after the washout period without treatment (results not shown). In Zucker animals, normal responses to PHE in presence of L-NAME were observed, and treatments did not significantly modify these responses (Table 4).

DISCUSSION

The present article highlights the potential health benefits of both acute and chronic consumption of FXE, a well-characterized extract from the seeds of *Fraxinus excelsior* L., on systolic blood pressure in SHR rats as well as its ability to improve glucose homeostasis, , dyslipidemia and body weight in Zucker rats. For the first time, vascular reactivity was determined on isolated aorta from both strains, and significant improvement in endothelial function was evidenced.

Acute administration of FXE to SHR resulted in a time-response BP decrease similar to that obtained with captopril, although the point of maximum reduction took longer with FXE administration. Nevertheless, this hypotensive effect was not observed at higher concentrations. Longer-term administration of FXE to SHR reinforced this finding, with the dose of 100 mg/kg body weight/day, equivalent to that showing hypoglycemic activity in humans (11), significantly reducing SBP compared to placebo, although the effect was not as strong as that induced by captopril. Interestingly, SBP significantly increased in all treatment groups following the washout period, clearly indicating the effectiveness and the reversibility of the treatments.

Eddouks *et al* (14) previously described the hypotensive actions of a daily oral administration of an aqueous *Fraxinus excelsior* extract to SHR for 3 weeks, results substantiated more recently by Lopez-Carreras *et al.* (15) Surprisingly, in the previous studies, the dose of *Fraxinus excelsior* extract was five times lower than the dose of FXE used in the present study, although the magnitude of the antihypertensive effect is comparable.. Differences in the preparation, manipulation and administration of the extracts could be invoked to justify this divergence.

In previous studies, increased urinary excretion was observed in conscious SHR treated with *Fraxinus excelsior* seed extracts (14,15) but not in Wistar rats treated with leaf extracts (17). The increased urinary excretion was interpreted as a diuretic activity and was used to explain the hypotensive effect of the extracts. The present study did not address the diuretic action of FXE although increased liquid intake observed in treated *vs* untreated animals could be related to the increased urinary excretion as reported by other authors.

Finally, an important finding in the current study is the increased potency of acetylcholine as a vasodilator of isolated rat aorta in both SHR and Zucker rats treated

with FXE. Impaired vascular function, manifested by an altered ability of the endothelium to release endothelium-derived relaxing factors and endothelium-derived contracting factors, is consistently reported in Zucker (18) and SHR strains (19,20) and is considered the first step in the progression of cardiovascular diseases (21,22). Determination of acetylcholine-induced endothelium-dependent vasorelaxation is commonly used as an indicator to test the endothelial function and, in the aorta, this response is mainly mediated by NO release (23, 24). In fact, in SHR and Zucker rats an impaired acetylcholine-mediated vasodilatory response has been described (18, 25). In this regard, the increased potency of acetylcholine observed in SHR and Zucker rats chronically treated with FXE could be interpreted as an improvement of endothelial function which was also observed with captopril but not metformin treatment. According to this, metformin, which restores endothelial function in aorta of non-obese diabetic rats (26) did not exhibit the same activity in obese Zucker rats.

The protective effect of FXE on endothelial function, was accompanied by an improvement of nitroprusside mediated vasodilatation after a 20-week treatment with FXE 100 and 400 mg/kg body weight/day in SHR, but not by a 5-week treatment in Zucker rats. This increased potency of nitroprusside, a NO-releasing drug (27), after FXE treatment could be related to changes in the activity of the NO/soluble guanylate cyclase/cGMP pathway. Future work must be performed to clarify this point.

The beneficial effects of FXE on endothelial function were accompanied by an improvement of contractile activity of rat aorta, pathologically altered in SHR rats and not previously evidenced with other extracts rich in polyphenolic compounds (28). In fact, contractile responses to depolarization and α_1-adrenoceptor activation were impaired in SHR aortas (29) an effect prevented by captopril treatment (30) that could be attributed to the arterial remodeling caused by hypertension. As shown in the current results, the chronic administration of FXE 100mg/kg body weight/day or Captopril 50 mg/kg body weight/day improved these responses and the improvement was reverted after 4 weeks without treatment. Therefore, we can postulate that the deleterious effect of hypertension on the contractile response of aorta was avoided in animals treated with FXE.

Administration of FXE, 100 mg/kg body weight/day, to obese Zucker rats significantly reduced glycemia as well as body weight gain. The magnitude of the

reduction (-16.3 %) was similar to that observed in the group treated with metformin (-15.8 %), and was not due to increased insulin secretion nor to reduced dietary intake as treatment with FXE did not modify insulinemia nor the average daily food intake or the solid intake/body weight ratio. The same dose of FXE produced additional beneficial effects not observed with metformin treatment: a significant reduction in plasma levels of triglycerides accompanied by an ameliorated endothelial function which was detected in isolated rat aortas. The results also show that chronic administration of FXE did not modify adiponectin plasma levels in Zucker rats, a result not previously reported.

The results presented herein concur with previous studies on the effect of administration of *Fraxinus excelsior* L. seed extracts on glucose homeostasis. Oral administration of an aqueous extract of *Fraxinus excelsior* L. inhibits renal glucose reabsorption with hypoglycaemic activity in normal and diabetic rats (8, 13), reduces hyperglycemia and decreases body weight gain in obese mice (10), and diabetic rats (13). The effect of nuzhenide and GI3, the principal secoiridois of the *Fraxinus excelsior* L. seed extracts, on fasting blood glucose could be due to enhanced glucose uptake in the liver and skeletal muscle (11) as was previously described for catalpol, an iridoid glycoside from the roots of *Rehmannia glutinosa* (31). The effects of *F. excelsior* L. extract on fasting insulin levels are not as consistent as its effects on fasting blood glucose levels. Maghrani *et al.* (13) reported no effect on insulin levels following single or repeated administration (15 days) of 20 mg/kg body weight of an *F. excelsior L.* extract in mice. In contrast, Ibarra *et al.*, (10) found that mice fed a high-fat diet and administered FXE (0.5% of the diet) had significantly lower fasting insulin levels at the end of the 16-week study compared to mice fed a high-fat diet alone. The differing effects on fasting insulin levels reported in these studies could be due to differences in extract composition, dose, study duration, or background diet.

The ability of FXE to reduce plasma triglycerides was outstanding compared with metformin action. Metformin has been shown to improve lipid profile and to decrease level of triglycerides (32) but no reduction in these parameters were observed during ours studies. Administration of FXE resulted in a reduction of triglycerides by 22.4% in the SHR strain, while in the Zucker strain, triglycerides were reduced by up to 36.6 % were observed. As this effect was found in both Zucker and SHR strains, it implies that lipid-regulating activity takes place independently of the pathological state. Furthermore, the significant reduction in triglycerides observed following 20-week

treatment in the SHR model was negated following 4 weeks of washout, confirming the activity of FXE.

A significant decrease in triglyceridemia was also observed after captopril treatment and had been previously described by other authors (33) who related this effect to the modulation of angiotensin (1-7) production.

Previous evidence indicates that iridoids from *Fraxinus excelsior* inhibit adipocyte differentiation and activate PPARα-mediated pathways (9), two mechanisms that could be involved in the control of weight gain observed in Zucker rats as well as in the decreased plasma levels of triglycerides found in our study. PPARα pathways are known to be involved in lipid homeostasis and inflammation (34-36). In fact, fibrates, which activate PPARα receptors, also lower plasma triglycerides and VLDL particles and increase HDL cholesterol, effects that are associated with its cardiovascular benefit (37). In future works, chronic treatment of Zucker and SHR rats with fibrates could confirm this proposal.

Additionally, an ethanolic extract from *Fraxinus rhynchophylla* barks also inhibited adipocyte differentiation (38 and the secoiridoids content of this extract, while different from those in FXE, not only inhibited pancreatic lipase (39) but also inhibited the early stage of adipocyte differentiation and diminished triglyceride content in differentiated 3T3-L1 cells (40), an activity that could be related to the decrease in triglycerides level after chronic treatment with FXE.

Metabolic syndrome frequently precedes type 2 diabetes and atherosclerosis and in most cases requires treatment with antihypertensive drugs, metformin and statins or fibrates. Among the antihypertensives, thiazide diuretics and β-adrenergic antagonists have slightly adverse effects, long-acting calcium channel antagonists have inconsistent effects whereas α_1-adrenergic antagonists and angiotensin-converting enzyme inhibitors have positive effects on glucose and lipid homeostasis. On the other hand, metformin or statins/fibrates act specifically by controlling glucose or lipid profile respectively, without any effect on the blood pressure, although recent results appear to indicate a correlation between intake of some types of statins and risk of Type 2 diabetes (41). Only diuretics and metformin contribute to control body weight gain. Therefore, instead of, or in addition to, these habitual treatments, the inclusion of FXE in the diet could be

an efficacious strategy to prevent or control metabolic syndrome and its inherent cardiovascular risk, or to reduce the risk of development of Type-2 diabetes in subjects under medication for CV risk factors.

Although the results of present work, obtained in animal models, should not be extrapolated to humans, the results of a clinical assay determining the effects of an equivalent dose of FXE in postprandial glycemia and insulin secretion on healthy volunteers (11) supports this assumption. In fact, the FXE benefits on glucose homeostasis shown in rodents models are currently being studied in a longitudinal, randomized, crossover, double-blinded and placebo controlled, 7 weeks nutritional intervention study with elderly overweight/obese subjects.

In conclusion, treatment with FXE (100 mg/kg body weight/day), a dose equivalent to that showing hypoglycemic activity in humans, resulted in significantly decreased glycemia, triglyceridemia, body weight gain and systolic blood pressure in SHR and Zucker rats, and these effects were accompanied by an improvement in endothelial function and NO/sGC pathway. The broad-ranging effects of FXE represent a unique pharmacological profile that could be more extensively assayed in humans to analyze its usefulness to prevent the metabolic syndrome, characterized by obesity, insulin resistance or glucose intolerance, hypertriglyceridemia and elevated blood pressure.

EXPERIMENTAL

This study was carried out at the Experimental Animal Facility of the University of Valencia (Spain) in accordance with the recommendations of the European Union regarding animal experimentation (Directive of the European Council 86/609/EC). The protocols were approved by the Animal Ethics Committee of the University of Valencia. Rats were housed 3–4 to a cage in a room with controlled temperature (23ºC), and a 12 h light-dark cycle. They were fed with a standard chow (PanLab) for 1 week before the start of the experiments (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).

FraxiPure® (product code EA149251) now commercially marketed as Glucevia, was supplied by Naturex S.A. (Avignon, France). Captopril and Metformin were supplied by Sigma-Aldrich Química S.L. (Madrid Spain).

Acetylcholine chloride, (R)-(-) phenylephrine hydrochloride, L-NAME (Nω-Nitro-L-Arginine Methyl Ester) and sodium nitroprusside were supplied by Sigma-Aldrich, (St Louis, MO, USA). All the drugs were prepared in distilled water.

1. Experimental procedures

1.1.Acute treatment

Eighteen SHR male rats aged 17 weeks were purchased from Janvier Laboratoires (France) and randomly assigned to the following groups: a control group that received vehicle by oral gavage (control, n = 6) and two treated groups (n = 6 each one) which received an oral dose of captopril 50 mg/kg body weight or FXE 100 mg/kg body weight. Systolic arterial pressure was recorded before and 2, 4, 8, 24 h and 7 days after oral administration of vehicle or drugs. After a week of washout, an oral dose of vehicle, captopril 50 mg/kg body weight or FXE 200 mg/kg body weight was administered to each group and arterial pressure was newly recorded at the same intervals as previously described. Finally, after a week of washout, a third oral administration of vehicle, captopril 50 mg/kg body weight or FXE 400 mg/kg body weight was accompanied by periodic measurements of arterial pressure. At the end of the experimental period, over-night fasted animals were anaesthetized by isoflurane (IsoFlo® 100% p/p. Esteve) and sacrificed by heart puncture exsanguination. All efforts were made to minimize suffering. The range of doses of FXE used (100, 200 and 400 mg/kg/day) was chosen in agreement with the Human Equivalent Dose (HED) definition proposed by Reagan-Shaw *et al.,* (16). The dose of 100 mg/kg body weight is approximately 16.2 mg/kg body weight HED, similar to the dose used during the clinical trial conducted by Visen *et al.* (11) wherein FXE significantly reduced postprandial glycemia. The dose of captopril has been previously assayed in SHR animals (42).

1.2.Chronic treatment

Thirty-two SHR male rats aged 3 weeks (63.8 ± 5.4 g) were purchased from Janvier Laboratoires (France) and randomly assigned to the following groups: a group

fed with the standard chow alone (control, n = 8), and three treated groups (n = 8 each one) fed with the standard chow supplemented with different treatments: i) Captopril 50 mg/kg body weight/day as positive control (Cap), ii) FXE 100 mg/kg/day (FXE100) and iii) FXE 400 mg/kg body weight/day (F 400). Diet and tap water were administered ad libitum. Dietary intervention lasted for 20 weeks. At this time, four animals from each group were sacrificed, and the remaining four animals were returned to a standard chow for 4 weeks. Systolic arterial pressure, body weight, food and water intake were recorded weekly throughout the study.

Thirty male Zucker rats aged 8 weeks (332.6 ± 5.06 g) all purchased from Charles River Laboratories (Spain) were used in the study. Animals were randomly assigned to two experimental groups: a control group (Control, n= 6) fed with the standard chow alone, which was used as control for normal values of this rat strain, and four treated groups (n = 6 each) fed with the standard chow supplemented with i) metformin 300 mg/kg body weight/day as positive control (Met), ii) FXE 20 mg/kg body weight/day (F20), iii) FXE 100 mg/kg body weight/day (F100) and iv) FXE 400 mg/kg body weight/day (F400). The dose of metformin was chosen based on previous data (43). Diet and tap water were administered *ad libitum*. Dietary intervention lasted for 5 weeks. At the end of the experimental period, over-night fasted animals were anaesthetized by isoflurane and sacrificed by heart puncture exsanguination.

Systolic arterial pressure, body weight, food and water intake were recorded throughout the study. Biochemical determinations were performed throughout the treatment in Zucker and at the end of treatment in Zucker and SHR. Functional experiments to analyze the vascular reactivity were performed at the end of treatment.

2. Systolic blood pressure determination

Systolic blood pressure (SBP) was measured from the tail of unanesthetized rats with a plethysmographic method (NIPREM 645; Cibertec, Madrid, Spain) as previously described (44). An average of six SBP readings was recorded for each determination.

3. Biochemical analysis

Blood samples were taken in Zucker rats by tail incision before and during the study (2 and 4 weeks after the treatment initiation), and by cardiac puncture in Zucker and SHR animals at the end of the study. Samples were placed in heparinized tubes

centrifuged at 1500 x *g* at room temperature for 30 min in an Eppendorf Centrifuge 5804-R (Hamburg, Germany) to obtain plasma which was immediately frozen at -80ºC prior to analysis of biochemical parameters. Levels of glucose, total cholesterol, HDL-cholesterol (HDL-c), and triglycerides (TGs), were measured using an autoanalyzer (Gernonstar®, Ansasia, Bombay, India). Insulin and adiponectin concentrations were quantified using solid phase two-side enzyme immunoassay. An ultrasensitive rat insulin enzyme immunoassay kit (Mercodia AB, Uppsala, Sweden) and an ultrasensitive rat adiponectin enzyme immunoassay kit (Mediagnost®, Reutlingen, Germany) were used for these determinations. Results were analyzed with a 450 nm filter in a microplate reader (Perkin Elmer 2030 Multilabel Reader. VICTORTMX3. Massachusetts, USA).

4. Functional studies

Thoracic aortas were obtained as previously described (45). Aortas were removed, cleaned from adipose tissue, placed into Krebs solution (mM): NaCl 118; KCl, 4.75; $CaCl_2$, 1.8; $MgCl_2$, 1.2; $NaHCO_3$, 25; KH_2PO_4, 1.2; glucose, 11 (pH=7.4) and cut in 4 mm rings which were set-up in an isometric organ bath, filled with Krebs solution at 37ºC and gassed with 95% O_2 and 5% CO_2. After a 1 hour stabilization period, all vessels were contracted with a depolarizing solution (80 mM KCl-Krebs obtained by an isotonic replacement of NaCl by KCl) to check the vessels' functionality.

To study the influence of treatment on contractile responses induced by α_1-adrenergic stimulation, cumulative concentration-response curves (CRCs) of contraction by phenyleprine (PHE) were performed by addition of cumulative concentrations of PHE (1 nM-1 μM) to the bath in presence of a nitric oxide synthase inhibitor (L-NAME, 100 μM) added to the bath 30 min before and during PHE addition, in order to avoid the vasorelaxant effect mediated by PHE-induced NO release (45). To analyze whether the response to PHE is partially NO-dependent, CRCs to PHE (1 nM-1 μM) were also performed in the absence of L-NAME.

Vasodilatation induced by nitric oxide (NO) endogenously released by the endothelium and by exogenous NO were determined by cumulative CRCs of relaxation to acetylcholine (ACh, 10 nM-100 μM) during sustained PHE 1 μM induced contraction. To analyze whether the response to ACh was fully or partially dependent of

endogenously released NO, CRCs to ACh (10 nM-100 μM) were also performed in the presence of L-NAME. The relaxations elicited by sodium nitroprusside were also studied in precontracted PHE (1 μM) vessels in the presence of L-NAME (100 μM).

5. Statistical analysis

The results are expressed as mean values ± SEM. Sequential data were analyzed by two-way ANOVA using GraphPad Prism 4 software. Differences between control and treated groups were assessed by Student's *t* test, or by one way ANOVA followed by Dunnett's test, if two or more groups were compared. Differences between the means were considered to be significant when $p < 0.05$. CRC of contraction by PHE were expressed as a percentage of initial KCl-induced contraction. CRC of relaxation by ACh and SNP were expressed as a percentage of the previous contraction induced by PHE. Data were plotted using the Graph Pad Software version 4.0 (San Diego, CA, USA), with sigmoid curve fitting performed by non-linear regression; these curves were used to derive Emax (the maximal relaxant response), pEC50 (-log of the agonist concentration needed to produce 50% of Emax) and statistical analysis of differences in these parameters

CONCLUSION

Treatment with FXE (100 mg/kg body weight/day), a dose equivalent to that showing hypoglycemic activity in humans, resulted in significantly decreased glycemia, triglyceridemia, body weight gain and systolic blood pressure, and these effects were accompanied by an improvement in endothelial function in SHR and Zucker rats. The broad-ranging effects of FXE represent a unique pharmacological profile that could be useful to prevent the metabolic syndrome, characterized by obesity, insulin resistance or glucose intolerance, hypertriglyceridemia and elevated blood pressure. Future assays in humans will allow confirm this assumption.

ACKNOWLEDGEMENTS

This work was supported by the CENIT Programme from the Spanish Government (SENIFOOD project). We thank Dr. Antoine Bily (Naturex Inc.) for FXE extract sample preparation and acknowledge the assistance of Dr. Pierre Gourdy (CHU de Toulouse and I2MC/INSERM U1048, Toulouse) for critical reading of this manuscript.- Naturex is involved in the research/development and marketing/sales of FraxiPure® as an ingredient for the food, cosmetic, and nutraceutical industries. Therefore, Naturex has a commercial interest in this publication.

REFERENCES

1. I. Kostova and T. Iossifova. Chemical components of *Fraxinus* species. *Fitoterapia,* 2007, 78, 85-106

2. S. Damtoft, H. Franzyk and S.R. Jensen. Excelsioside, a secoiridoid glucoside from *Fraxinus excelsior*. *Phytochemistry*, 1992, 31, 4197– 4201

3. V. Lavelli and L. Bondesan. Secoiridoids, tocopherols, and antioxidant activity of monovarietal extra virgin olive oils extracted from destined fruits. *J. Agric. Food Chem.,* 2005, 53, 1102-1107

4. H.K.Obied, P. Karuso, P.D. Prenzler and K. Robards. Novel secoiridoids with antioxidant activity from Australian olive mill waste. *J. Agric. Food Chem,* 2007, 55, 2848-2853

5. P. Rosignoli, R. Fuccelli, R. Fabiani, M. Servili and G. Morozzi. Effect of olive oil phenols on the production of inflammatory mediators in freshly isolated human monocytes. *J Nutr Biochem,* 2013, 24(8), 1513-1519..

6. B. Dinda, S. Debnath and R. Banik Naturally occurring iridoids and secoiridoids. An updated review, part 4. *Chem Pharm Bull,* 2011, 59(7), 803-33.

7. E.L. Ghisalberti. Biological and pharmacological activity of naturally occurring iridoids and secoiridoids. *Phytomedicine,* 1998, 5(2), 147-163.

8. M. Eddouks and M. Maghrani. Phlorizin-like effect of *Fraxinus excelsior* in normal and diabetic rats. *J Ethnopharmacol,* 2004, 94, 149–154.

9. N. Bai, K. He, A. Ibarra, A. Bily, M. Roller, X. Chen and R. Ruhl. Iridoids from *Fraxinus excelsior* with adipocyte differentiation-inhibitory and PPAR-alpha activation activity. *J Nat Prod,* 2010, 73, 2-6.

10. A. Ibarra, N. Bai, K. He, A. Bily, J. Cases, M. Roller, M. and S. Sang. *Fraxinus excelsior* seed extract FraxiPure™ limits weight gains and hyperglycemia in high-fat diet-induced obese mice. *Phytomedicine,* 2011, 18, 479-485.

11. P. Visen, B. Saraswat, A. Visen, M. Roller, A. Bily, C. Mermet, K. He, N. Bai, B. Lemaire, S. Lafay and A. Ibarra. Acute effects of *Fraxinus excelsior* L. seed

extract on postprandial glycemia and insulin secretion on healthy volunteers. *J Ethnopharmacol,* 2009, 126, 226-232.

12. J. Flanagan, M. Meyer, M.A. Pasamar, A. Ibarra, M. Roller, I. Alvarez, N. Genoher, S. Leiva, F. Gomez-García, M. Alcaraz, A. Martínez-Carrasco and V. Vicente. Safety evaluation and nutritional composition of a *Fraxinus excelsior* seed extract, FraxiPure™. *Food Chem Toxicol,* 2013, 53, 10-7.

13. M. Maghrani, N.A. Zeggwagha, A. Lemhadria, M. El Amraouia, J.B. Michel and M. Eddouks. Study of the hypoglycaemic activity of *Fraxinus excelsior* and *Silybum marianum* in an animal model of type 1 diabetes mellitus. *J Ethnopharmacol,* 2004, 91(2–3), 309–316.

14. M. Eddouks, M. Maghrani, N.A. Zeggwagh, M. Haloui and J.B. Michel. *Fraxinus excelsior* L. evokes a hypotensive action in normal and spontaneously hypertensive rats. *J Ethnopharmacol,* 2005, 99, 49–54.

15. N. Lopez-Carreras, S. Fernandez-Vallinas, R. Hernández, M. Miguel and A. Aleixandre. Short-term effect of aqueous *Fraxinus excelsior* L. seed extract in spontaneously hypertensive rats. *Food Res Intern,* 2013, 53, 81-87.

16. S. Reagan-Shaw, M. Nihal and N. Ahmad. Dose translation from animal to human studies revisited. *FASEB Journal,* 2007, 22, 659–661.

17. J. Casadebaig, M. Jacob, G. Cassanas, D. Gaudy, G. Baylac and A. Puech. Physicochemical and pharmacological properties of spray-dried powders from *Fraxinus excelsior* leaf extracts. *J Ethnopharmacol*, 1989, 26(2), 211-216.

18. N.S. Lobato, F.P. Filgueira, R. Prakash, F.R. Giachini, A. Ergul, M.H. Carvalho, R.C. Webb, R.C. Tostes and Z.B. Fortes. Reduced endothelium-dependent relaxation to anandamide in mesenteric arteries from young obese Zucker rats. *PLoS One,* 2013, 8(5), e63449.

19. M. Félétou, T.J. Verbeuren and P.M. Vanhoutte. Endothelium-dependent contractions in SHR: a tale of prostanoid TP and IP receptors. *Br J Pharmacol,* 2009, 156, 563-574.

20. P.M. Vanhoutte, H. Shimokawa, E.H. Tang and M. Félétou. Endothelial dysfunction and vascular disease. *Acta Physiol (Oxf),* 2009,196 (2), 193-222.

21. N. Gokce, J.F. Keaney, Jr, L.M. Hunter, M.T. Watkins, Z.S. Nedeljkovic, J.O. Menzoian and J.A.Vita. Predictive value of noninvasively determined endothelial dysfunction for long-term cardiovascular events in patients with peripheral vascular disease. *J Am Coll Cardiol,* 2003, 41(10), 1769–1775.

22. A. Lerman and A.M. Zeiher. Endothelial function: cardiac events. *Circulation,* 2005, 111(3), 363–68.

23. S. Moncada, R.M. Palmer and E.A. Higgs Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol Rev,* 1991, 43, 109-142.

24. T. Chataigneau, M. Félétou, P.L. Huang, M.C. Fishman, J Duhault and P.M. Vanhoutte. Acetylcholine-induced relaxation in blood vessels from endothelial nitric oxide synthase knockout mice. *Br J Pharmacol,* 1999, 126, 219–226.

25. S. Ulker, D. McMaster, P.P. McKeown and U. Bayraktutan. Impaired activities of antioxidant enzymes elicit endothelial dysfunction in spontaneous hypertensive rats despite enhanced vascular nitric oxide generation *Cardiovasc Res,* 2003, 59 (2), 488-500.

26. C.M. Sena, P. Matafome, T. Louro, E. Nunes, R. Fernandes. and R.M. Seiça. Metformin restores endothelial function in aorta of diabetic rats. *Br J Pharmacol,* 2011, 163 (2), 424-437.

27. C. Napoli, L.J. Ignarro. Nitric oxide-releasing drugs. *Annu Rev Pharmacol Toxicol*. 2003, 43, 97-123.

28. A.Z. Kalea, K. Clark, D.A. Schuschke, A.S. Kristo and D.J. Klimis-Zacas. Dietary enrichment with wild blueberries (Vaccinium angustifolium) affects the vascular reactivity in the aorta of young spontaneously hypertensive rats. J Nutr Biochem, 2010, 21 (1), 14-22

29. E. Oliver, D. Martí, F. Montó, N. Flacco, L. Moreno, D. Barettino, M.D. Ivorra and P. D'Ocon. The impact of α1-adrenoceptors up-regulation accompanied by

the impairment of beta-adrenergic vasodilatation in hypertension. *J Pharmacol Exp Ther,* 2009, 328(3), 982-990.

30. L.B. Tan, C. Brilla and K.T. Weber. Prevention of structural changes in the heart in hypertension by angiotensin converting enzyme inhibition. *J Hypertension,*1992, Suppl. 10(1), S31-34

31. J.P. Shieh, K.C. Cheng, H.H. Chung, Y.F. Kerh, C.H. Yeh and J.T. Cheng. Plasma glucose lowering mechanisms of catalpol, an active principle from roots of Rehmannia glutinosa, in streptozotocin-induced diabetic rats. *J. Agric. Food Chem,* 2011, 59, 3747-3753.

32. D. Kirpichnikov, S. McFarlane. and J.R. Sowers. Metformin: An Update. *Ann Intern Med,* 2002, 137, 25-33.

33. J.F. Giani, M.C. Muñoz, R.A. Pons, G. Cao, J.E. Toblli, D. Turyn, F.P. Dominici. Angiotensin-(1-7) reduces proteinuria and diminishes structural damage in renal tissue of stroke-prone spontaneously hypertensive rats. *Am J Physiol Renal Physiol*. 2011, 300(1): F272-82.

34. S.A. Kliewer, S.S. Sundseth, S.A. Jones, P.J. Brown, G.B. Wisely, C.S. Koble, P. Devchand, W. Wahli, T.M. Willson, J.M. Lenhard. and J.M. Lehmann. Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors alpha and gamma. *Proc Nat Acad Sci USA,*1997, 94, 4318–4323

35. R. Kostadinova, W. Wahli and L. Michalik. PPARs in diseases: control mechanisms of inflammation. *Currt Med Chem,* 2005, 12, 2995–3009.

36. L. Széles, D. Töröcsik and L. Nagy. PPAR-gamma in immunity and inflammation: cell types and diseases. *Biochim Biophys Acta,* 2007, 1771(8):1014-1030.

37. B. Staels and J.C. J.C. Fruchart. Therapeutic roles of peroxisome proliferator-activated receptor agonists. *Diabetes,* 2005, 54(8), 2460-70.

38. E. Shin, K-M. Choi, H-S. Yoo, C-K. Lee, B.Y. Hwang and M.K. Lee. Inhibitory effects of coumarins from the stem barks of *Fraxinus rhynchophylla* on adipocyte differentiation in 3T3-L1 cells. *Biol Pharm Bull,* 2010, 33, 1610-1614.

39. J.H. Ahn, E. Shin, Q. Liu, S.B. Kim, K.M. Choi, H.S. Yoo, B.Y. Hwang and M.K. Lee. Secoiridoids from the stem barks of *Fraxinus rhynchophylla* with pancreatic lipase inhibitory ctivity. *Nat Prod Res,* 2012, 27(12), 1132-1135.

40. K.M. Choi, E. Shin, Q. Liu, H.S. Yoo, Y.C. Kim, S.H. Sung, B.Y. Hwang and M.K. Lee. Hydroxyframoside B, a secoiridoid of *Fraxinus rhynchophylla*, inhibits adipocyte differentiation in 3T3-L1 cells. *Planta Medica,* 2011, 77(10), 1020-1023.

41. K. Ray. Statin diabetogenicity: guidance for clinicians. *Cardiovasc Diabetol,* 2013, 12(S1):S3.

42. R. Miquel, R. Gisbert, E. Serna, F. Perez-Vizcaino, E. Anselmi, M. A. Noguera, M. D. Ivorra, and M. P. D'Ocon. Acute and chronic captopril, but not prazosin or nifedipine, normalize alterations in adrenergic intracellular Ca^{2+} handling observed in the mesenteric arterial tree of spontaneously hypertensive rats. *J Pharmacol Exp Ther,* 2005, 313: 359-367.

43. N.S. Farrar, N.J. Chambers, A.R. Carlsson, G. Denyer, G.A. Johnston. Effect of a series of novel sulphonylthioureas on glucose tolerance in the obese fa/fa Zucker rat. *Clin Exp Pharmacol Physiol*, 2001, 28: 386-91.

44. R. Gisbert, Y. Madrero, V. Sabino, M.A. Noguera, M.D. Ivorra and P. D'Ocon. Functional characterization of α1-adrenoceptor subtypes in vascular tissues using different experimental approaches: a comparative study. *Br J Pharmacol,* 2003, 138(2), 359-368.

45. V.M. Victor, C. Nuñez, P. D'Ocon, C.T. Taylor, J.V. Esplugues and S. Moncada. Regulation of oxygen distribution in tissues by endothelial nitric oxide. *Circulation Res,* 2009, 104(10), 1178-1183.

.

Figure 1. Changes in systolic blood pressure (SBP) after oral administration of a single dose of (A) 200 mg/kg body weight FraxiPure® (FXE200), (B) 400 mg/kg body weight FraxiPure® (FXE400), captopril (50 mg/kg body weight) or vehicle (control) in SHR rats. Values are mean ± SEM for n=6 rats. Two-way ANOVA indicated that treatment with captopril and FXE200 significantly changed SBP (***$P < 0.001$ and **$P < 0.01$, respectively). One way ANOVA followed by Dunnet's test vs control was applied to determine significant changes in SBP at different time-point during captopril or FXE100 treatments ($^{\omega}P < 0.05$, $^{\omega\omega}P < 0.01$).

Figure 2. (A) Changes in systolic blood pressure (SBP) during the last 10 weeks of a 20-week chronic treatment of SHR rats with 100 mg/kg body weight/day FraxiPure® (FXE100) or vehicle (control). Values are mean ± SEM for n=8 rats. Two-way ANOVA indicated that treatment significantly changed SBP (** $P < 0.01$). Student's *t* test vs control was applied to determine significant changes in SBP at different time-point during FXE100 treatments ($^{\omega}P < 0.05$, $^{\omega\omega}P < 0.01$).

(B) Systolic blood pressure (SBP) values after chronic treatment (20 weeks), (black bars) in the groups of animals receiving vehicle (control), captopril (50 mg/kg body weight/day), 100 (FXE100) or 400 mg/kg body weight/day FraxiPure® (FXE400), and at the end of the washout period of 4 weeks without treatment (white bars). Values are mean ± SEM for n=8 rats (treatment) or n=4 (treatment washout). * $P< 0.05$, *** $P < 0.001$ *vs* the treatment period (Student's *t* test).

Figure 3. (A) Changes in body weight in Zucker animals over 5 weeks treatment with metformin (300 mg/kg body weight/day) or 100 mg/kg/day FraxiPure® (FXE100). Two way ANOVA indicates that treatment significantly changes body weight in Zucker rats (* $P < 0.05$).

(B) Liquid intake/ Body weight ratio following 20-week chronic treatment (Treated) with captopril (50 mg/kg body weight/day), 100 (FXE100) and 400 mg/kg body weight/day FraxiPure® (FXE400) and after a 4-week washout period without treatment (FXE Washout) in SHR. Values are mean ± SEM for n = 4-8 rats. Significance was calculated by one way ANOVA followed by Dunnett's test *vs* control; ** $P < 0.01$

Figure 4. Changes in fasting blood glucose levels (A) or triglycerides levels (B) in Zucker rats during 5 weeks treatment with vehicle (Control), metformin (300 mg/kg body weight/day), or 100 mg/kg body weight/day FraxiPure® (FXE100). Values presented are differences relative to baseline and are mean ± SEM for n = 6 rats. Two way ANOVA indicated significant changes (**$P < 0.01$, *** $P < 0.001$).
(C) Plasma levels of triglycerides in SHR model following 20 week chronic treatment with 100 (FXE100) and 400 mg/kg body weight/day FraxiPure® (FXE400), and after a 4-week washout period (FXE Washout) without treatment. Values are mean ± SEM for n = 4-8 rats. Significance was calculated by one way ANOVA followed by Dunnett's test *vs* control, ** $P < 0.01$.

Figure 5. Concentration-response curves of relaxation induced by acetylcholine in aortas previously contracted with 1 µM phenylephrine. Aortas had been obtained from (A) SHR rats previously treated for 20 weeks with captopril (50 mg/kg body weight/day), or (B) Zucker rats previously treated for 5 weeks with metformin (300 mg/kg body weight/day), in addition to treatments with 20 (FXE20), 100 (FXE100) and 400 mg/kg body weight/day FraxiPure® (FXE400). Values are expressed as mean ± SEM for n = 4 rats.

Figure 6. Concentration-response curves of relaxation induced by sodium nitroprusside in aortas previously contracted with 1 µM phenylephrine. Aortas had been obtained from (A) SHR rats previously treated for 20 weeks with captopril (50 mg/kg body weight/day), or (B) Zucker rats previously treated for 5 weeks with metformin (300 mg/kg body weight/day), in addition to treatments with 20 (FXE20), 100 (FXE100) and 400 mg/kg body weight/day FraxiPure® (FXE400). Values are expressed as mean ± SEM for n = 4 rats.

Figure 7. Concentration-response curves of contraction induced by Phenylephrine in aortas from SHR or Zucker rats in presence of the nitric oxide synthase inhibitor L-NAME 100 µM. Aortas had been obtained from (A) SHR rats previously treated for 20 weeks with captopril (50 mg/kg body weight/day), or (B) Zucker rats previously treated for 5 weeks with metformin (300 mg/kg body weight/day), in addition to treatments with 20 (FXE20), 100 (FXE100) and 400 mg/kg body weight/day FraxiPure® (FXE400). Values are expressed as mean ± SEM for n = 4 rats

Table 1. Systolic blood pressure, body and heart weight, food intake and metabolic markers in SHR following 20 weeks chronic treatment with a standard chow diet (Control) supplemented to achieve 50 mg/kg body weight captopril (Captopril), 100 (FXE100) or 400 mg/kg body weight/day FraxiPure® (FXE400).

SHR	Control	Captopril	FXE100	FXE400
SBP (mm Hg)	219.3 ± 2.8	154.1 ± 2.1 **	204.6 ± 0.9 **	214.6 ± 3.4
Body weight (g)	347.6 ± 5.0	326.6 ± 9.3	333.5 ± 8.2	334.5 ± 5.1
Heart weight (g)	1.27 ± 0.02	1.08 ± 0.03 *	1.24 ± 0.04	1.31 ± 0.03
Heart weight/ Body weight	0.373 ± 0.005	0.332 ± 0.007 **	0.373 ± 0.007	0.392 ± 0.001 *
Solid Intake[1] (g/rat/day)	18.80 ± 0.23	17.16 ± 0.11	18.04 ± 0.17	18.97 ± 0.20
Solid intake/ Body weight [1]	5.55±0.08	5.41± 0.07	5.51± 0.06	5.74± 0.05
Liquid intake [1] (ml/rat/day)	19.11 ± 0.36	20.62 ± 0.44 *	18.94 ± 0.38	23.21 ± 0.28 **
Liquid intake/ Body weight[1]	5.51 ± 0.09	6.50 ± 0.17 **	5.88 ± 0.09	6.90 ± 0.09 **
Glucose (mg/dL)	137.8 ± 9.5	97.2 ±10.4 **	132.7 ± 6.7	132.0 ± 5.8
Cholesterol (mg/dL)	44.7 ± 1.4	41.0 ± 1.2	45.0 ± 3.3	44.3 ± 2.6
HDL (mg/dL)	19.2 ± 0.7	20.2 ± 1.2	20.5 ± 1.7	19.7 ± 0.9
Triglycerides (mg/dL)	44.7 ± 1.4	36.7 ± 2.6 **	34.7 ± 1.1 **	34.7 ± 0.7 **

Data represents mean ± S.E.M. of n = 8 animals (n = 4, heart weight)

*P < 0.05, ** P < =.01, *** P < 0.001 vs Control, one way ANOVA followed by Dunnett's test

[1]Calculated as mean from weeks 16 to 20 of treatment

Table 2. Systolic blood pressure, body and heart weight, food intake and metabolic markers in Zucker rats following 5 weeks chronic treatment with a standard chow diet (Control) supplemented to achieve 300 mg/kg body weight metformin (metformin), 20 (FXE20), 100 (FXE100) or 400 mg/kg body weight/day FraxiPure® (FXE400).

ZUCKER	Control	Metformin	FXE20	FXE100	FXE400
SBP (mmHg)	127.4 ± 7.0	117.5 ± 9.2	120.3 ± 6.8	113.2 ± 4.7	127.6 ± 7.3
Body weight (g)	520.2± 14.6	450.5±21.7 *	481.0±13.5	478.2±9.0	508.0±15.9
Heart weight (g)	1.15±0.04	1.37±0.15	1.08±0.03	1.08±0.02	1.18±0.02
Heart weight/ Body weigth	0.223±0.003	0.258±0.007 **	0.223±0.003	0.219±0.003	0.233±0.007
Solid Intake[1] (g/rat/day)	38.03±0,62	33.07±1.62 **	37.40±0.76	34.70±0.49	38.15±0.65
Solid intake/Body weight[1]	7.42±0.10	7.35±0.20	7.82±0.21	7.20±0.089	7.53±0.21
Liquid intake[1] (ml/rat/day)	36.10± 0.88	34.40±1.13	37.50±0.63	42.70±1.39 **	42.75±0.47 **
Liquid intake/body weight[1]	7.05±0.18	7.73±0.55	7.82±0.21	8.85±0.21 **	8.43±0.24 *
Glucose (mg/dL)	137.4 ± 7.1	115.7 ± 5.1 *	118.2 ± 3.0 *	115.0 ± 4.6 *	114.2 ± 5.4 *
Cholesterol (mg/dL)	166.2 ± 5.9	173.9 ± 6.5	175.2 ± 5.9	171.1 ± 6.2	172.9 ± 5.6
HDL (mg/dL)	54.17 ± 2.1	54.8 ± 1.9	50.9 ± 1.4	55.4 ± 1.5	56.3 ± 1.6
Triglycerides (mg/dL)	406.0±53.2	517.0±79.2	312.8±40.3	270.3±33.7	257.5±28.97
Insulin (ng/mL)	1.44±0.05	1.47±0.01	1.35±0.06	1.50±0.01	1.53±0.01
HOMA-IR	5.04±0.05	4.75±0.13	4.67±0.22	4.78±0.12	4.84±0.10
Adiponectin (ng/mL)	4.25±0.23	3.26±0.33	2.89±0.54	3.88±0.38	3.97±0.36

Data represents mean ± S.E.M. of n = 6 animals
HOMA-IR homeostatic model assessment-insulin resistance
*P < 0.05, ** P < =.01, *** P < 0.001 vs Control, one way ANOVA followed by Dunnett's test
[1]Calculated as mean from 5 weeks of treatment

Table 3. Changes in the pEC_{50} and Emax of the concentration-response curves of relaxation of acetylcholine and sodium nitroprusside in pre-contracted aortas from SHR treated for 20 weeks and Zucker rats treated for 5 weeks with captopril (50 mg/kg body weight/day), metformin (300 mg/kg body weight/day), or FraxiPure® at doses of 20 (FXE20), 100 (FXE100) and 400 mg/kg body weight body weight/day (FXE400) .

	SHR			
	Acetylcholine		**Nitroprusside**	
	pEC50	***Emax***	***pEC50***	***Emax***
Control	5.91±0.15	67.0±3.8	7.95±0.05	100.0±3.1
Captopril	6.67±0.05 ***	57.4±2.0	8.29±0.04 ***	100.0±2.3
FXE100	6.52±0.10 ***	67.7±3.7	8.10±0.04 *	100.0±3.0
FXE400	6.88±0.16 ***	58.3±4.2	8.18±0.03 ***	100.0±1.7
	Zucker			
	Acetylcholine		**Nitroprusside**	
	pEC50	***Emax***	***pEC50***	***Emax***
Control	5.99±0.23	85.9±8.3	7.71±0.03	100.0±2.7
Metformin	6.03±0.26	83.0±8.6	7.74±0.04	100.0±1.4
FXE20	6.81±0.14 **	70.0±4.6	7.78±0.08	100.0±4.2
FXE100	6.49±0.1 *	69.6±4.3	7.79±0.07	100.0±2.3
FXE400	5.96±0.20	72.8±6.1	7.86±0.04	100.0±3.3

Emax was expressed as % of relaxation vs the maximal PHE-induced contraction
Data represent mean ± S.E.M. of n= 4- 6 experiments
*P < 0.05, ** P < 0.01, *** P < 0.001 vs Control
Data were plotted using the Graph Pad Software version 4.0 (San Diego, CA, USA), with sigmoid curve fitting performed by non-linear regression; these curves were used to derive Emax (the maximal relaxant response), pEC50 (-log of the agonist concentration needed to produce 50% of Emax) and statistical analysis of differences in these parameters

Table 4. Changes in the Emax and pEC_{50} of the concentration-response curves of phenylephrine in the presence of the nitric oxide synthase inhibitor L-NAME 100 mM, in aortas obtained from SHR treated for 20 weeks and Zucker rats treated for 5 weeks with captopril (50 mg/kg body weight/day), metformin (300 mg/kg body weight/day), or FraxiPure® at doses of 20 (FXE20), 100 (FXE100) and 400 mg/kg body weight/day (FXE400).

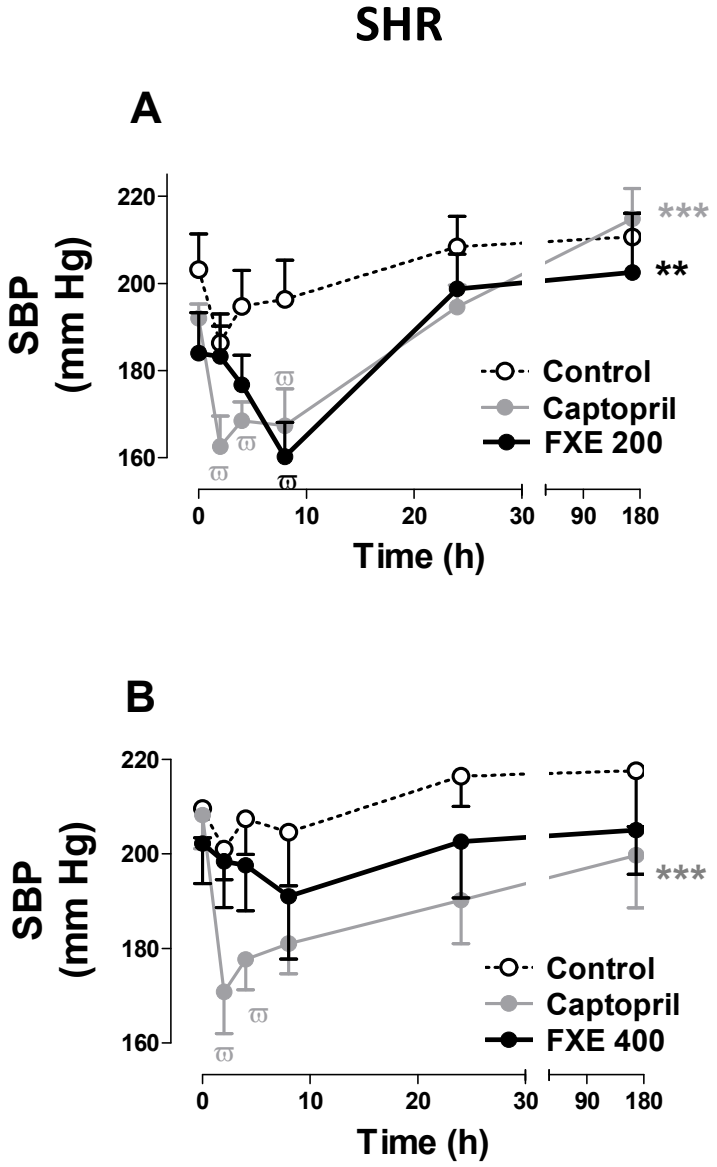
	Phenylephrine			
	SHR		**Zucker**	
	pEC50	***Emax***	***pEC50***	***Emax***
Control	7.48±0.11	109.0±7.9	7.34±0.06	120.1±5.2
Metformin			7.40±0.05	123.5±4.1
Captopril	7.53±0.05	135.6±5.2 *		
FXE20			7.28±0.05	135.1±5.5
FXE100	7.49±0.04	132.8±3.3 *	7.23±0.04	128.4±3.7
FXE400	7.43±0.05	104.3±3.3	7.57±0.04	121.0±3.4

Emax was expressed as % of the maximal KCl-induced contraction
Data represent mean ± S.E.M. of n= 4-6 experiments
*P < 0.05 vs Control
Data were plotted using the Graph Pad Software version 4.0 (San Diego, CA, USA), with sigmoid curve fitting performed by non-linear regression; these curves were used to derive Emax (the maximal relaxant response), pEC50 (-log of the agonist concentration needed to produce 50% of Emax) and statistical analysis of differences in these parameters

.

1

Figure 1

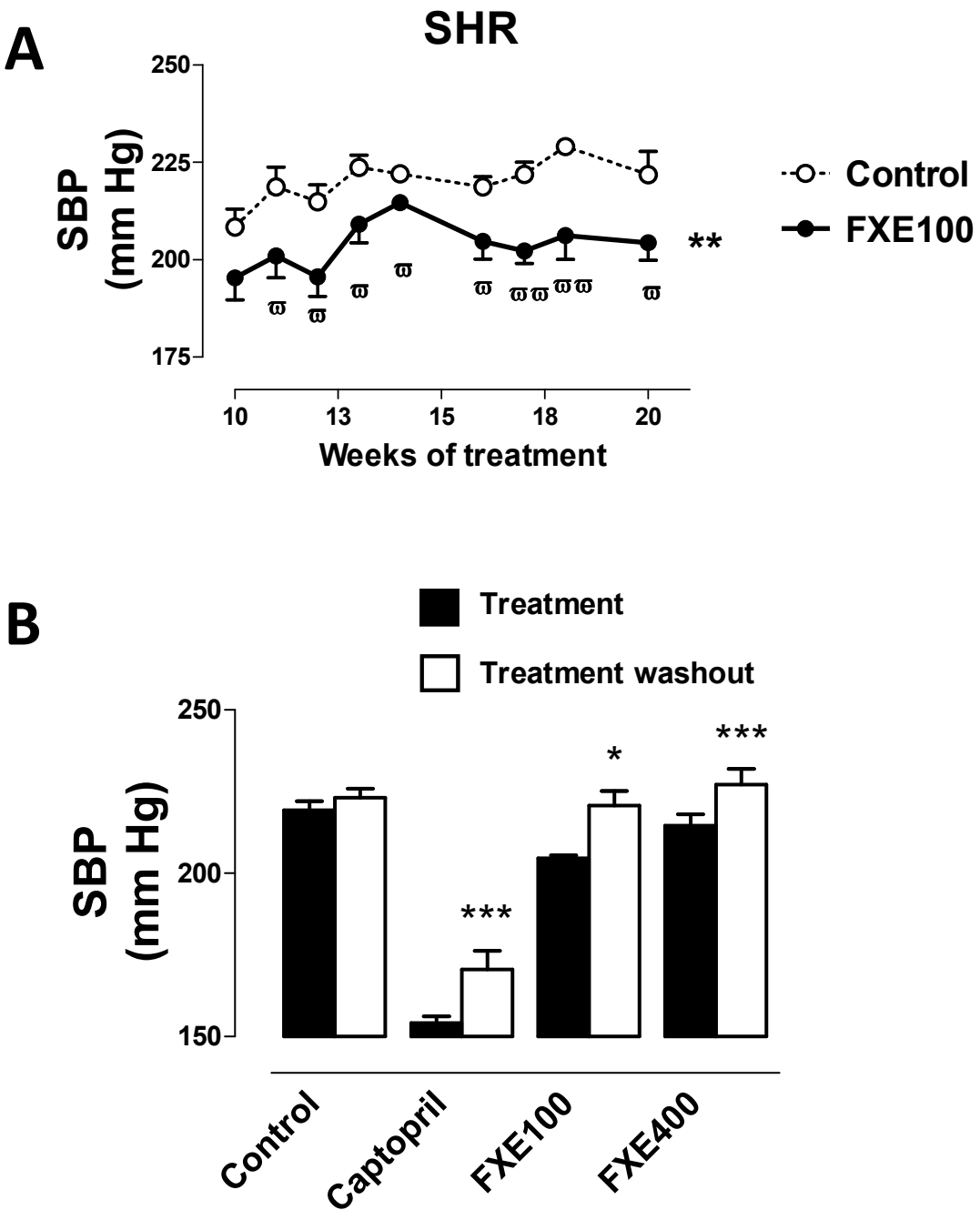


2
3

4

1

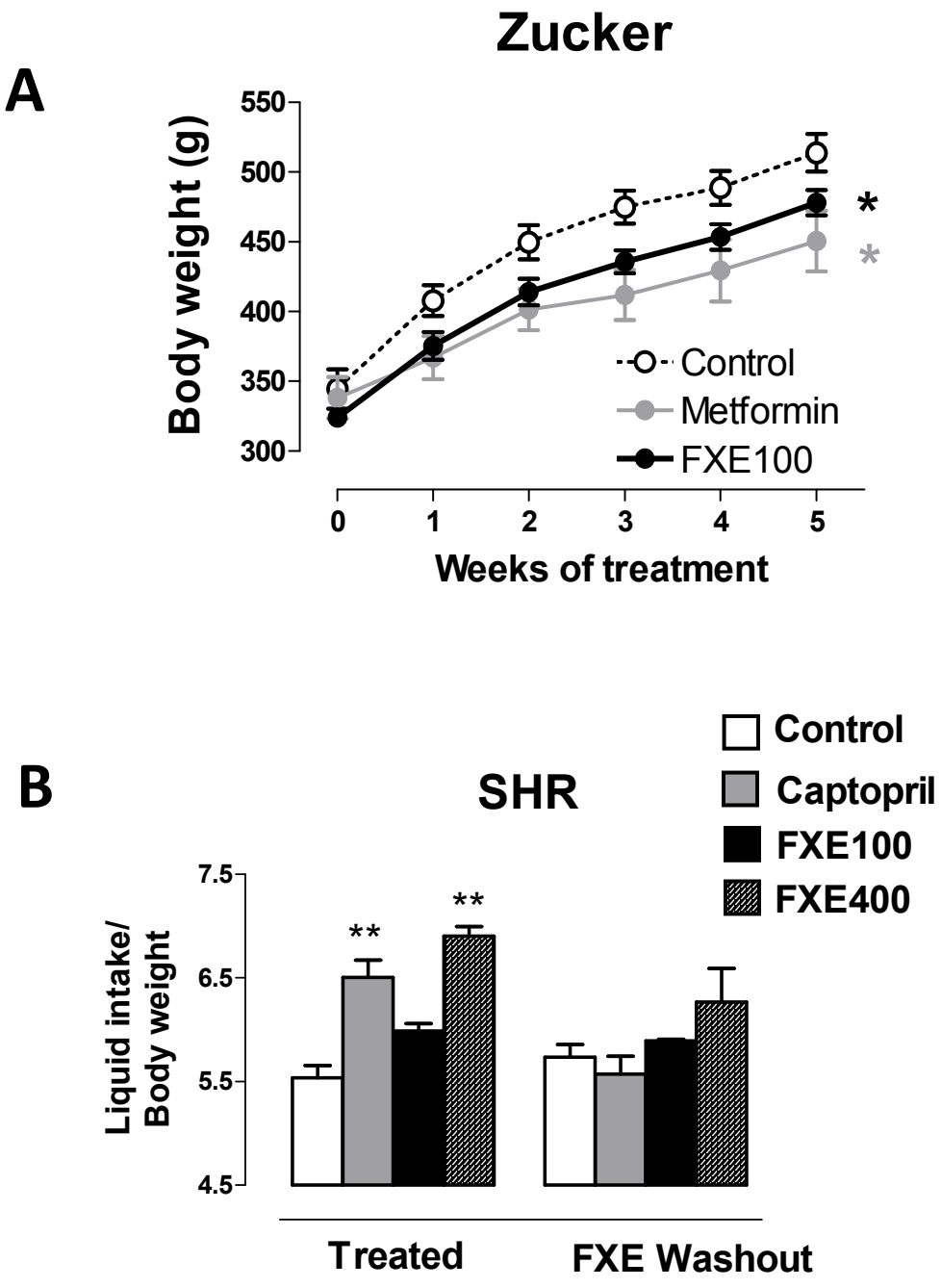
Figure 2



2
3

1

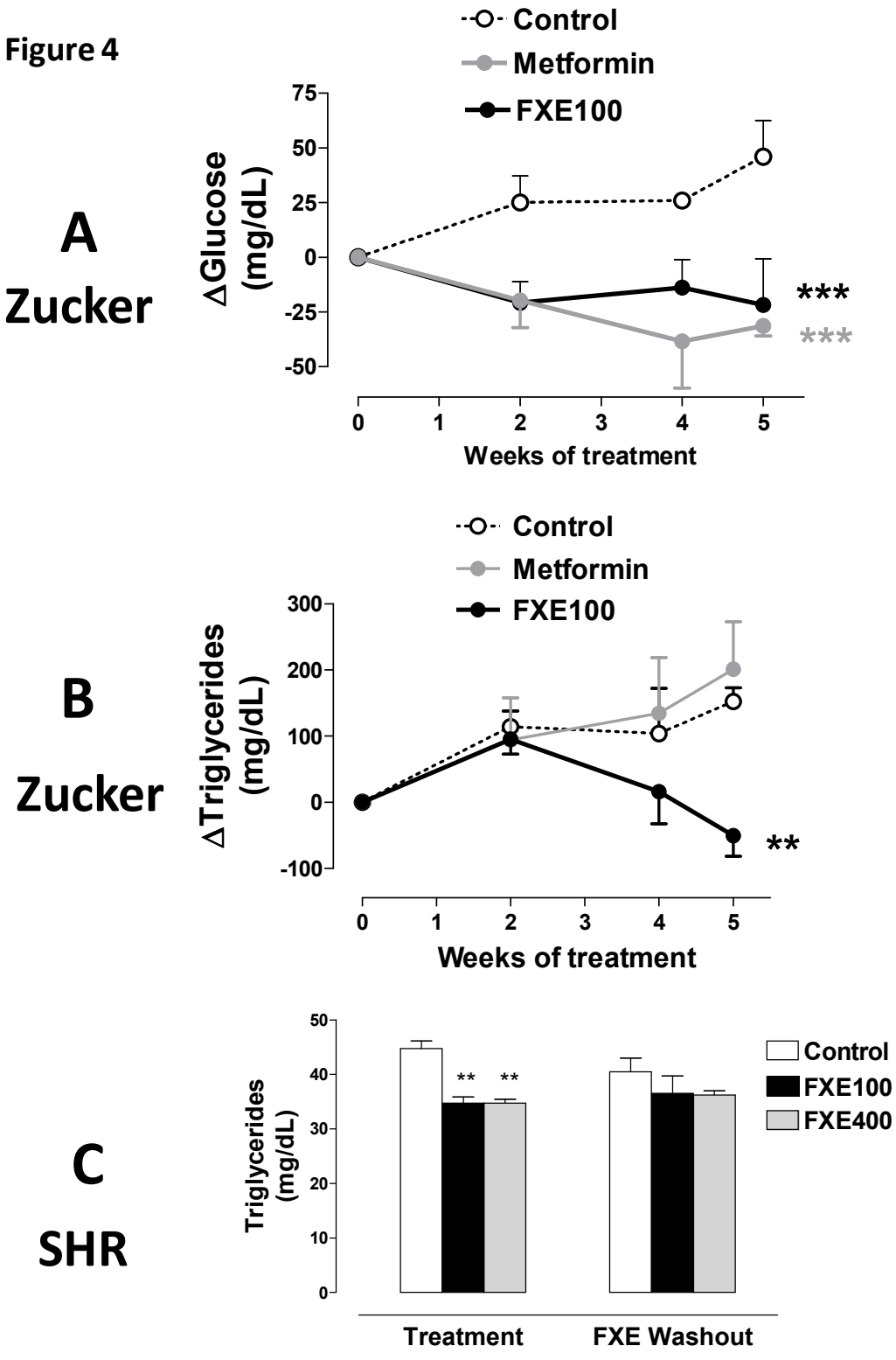
Figure 3



2
3

1

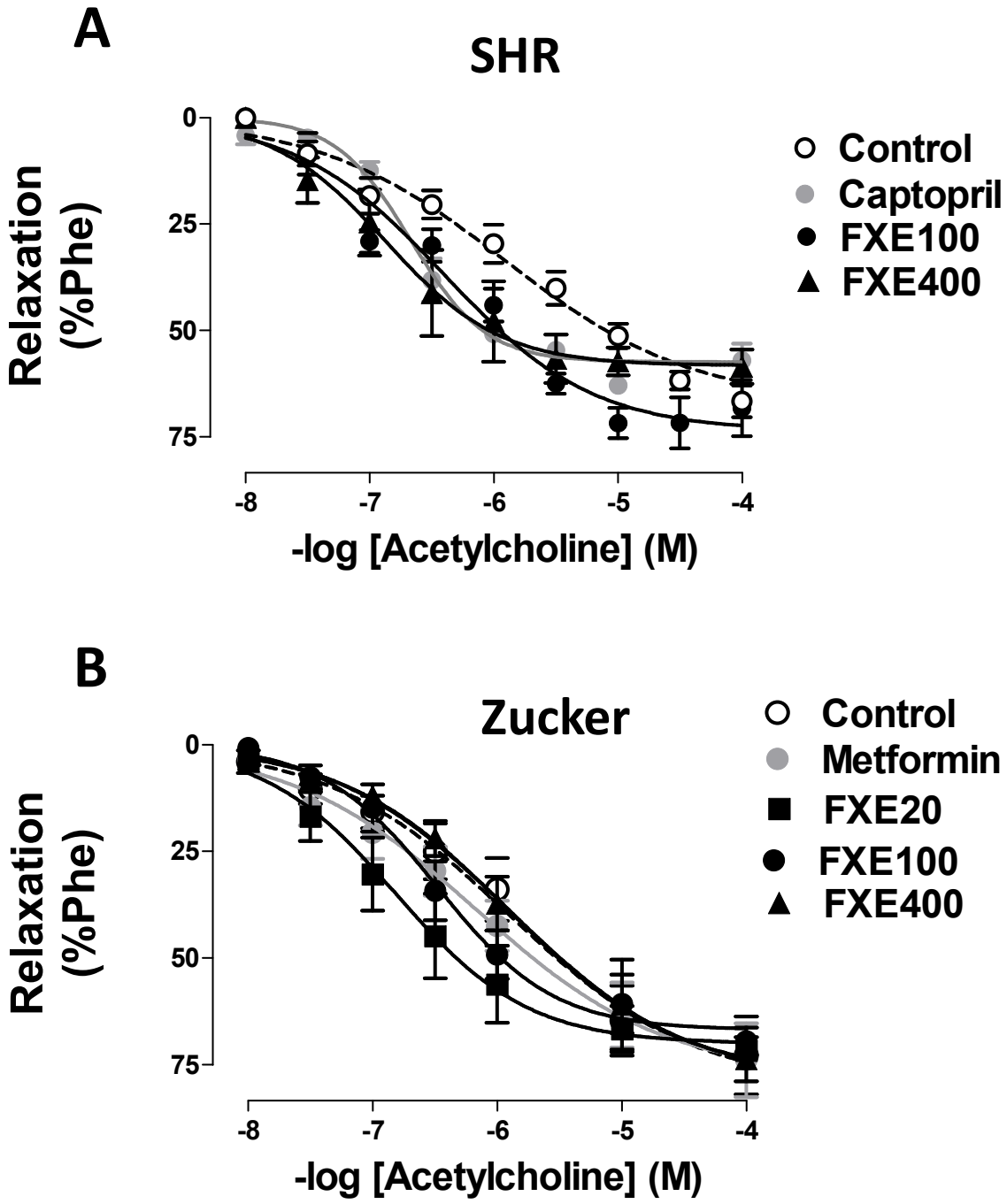
Figure 4



2
3

1

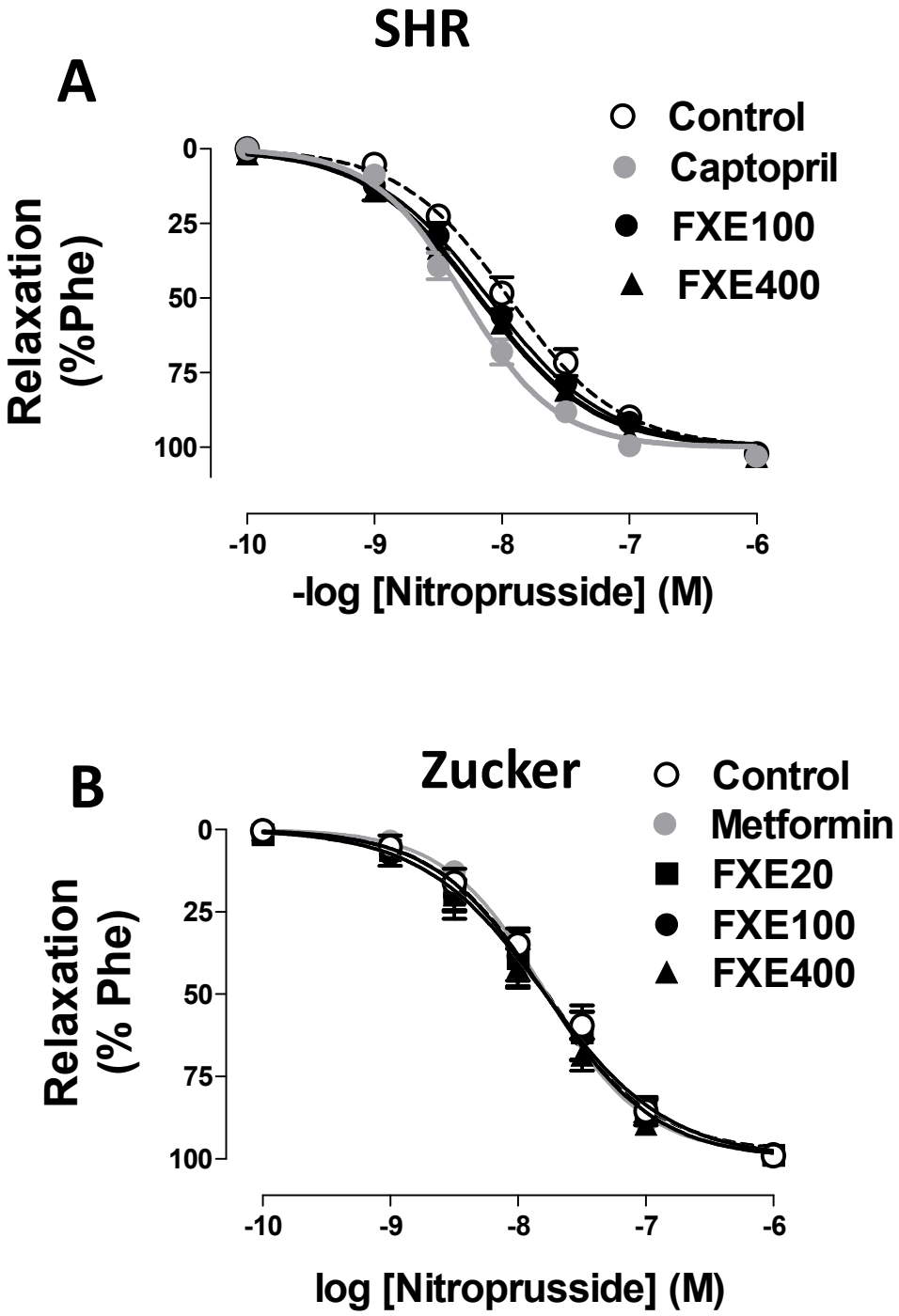
Figure 5



2
3

1

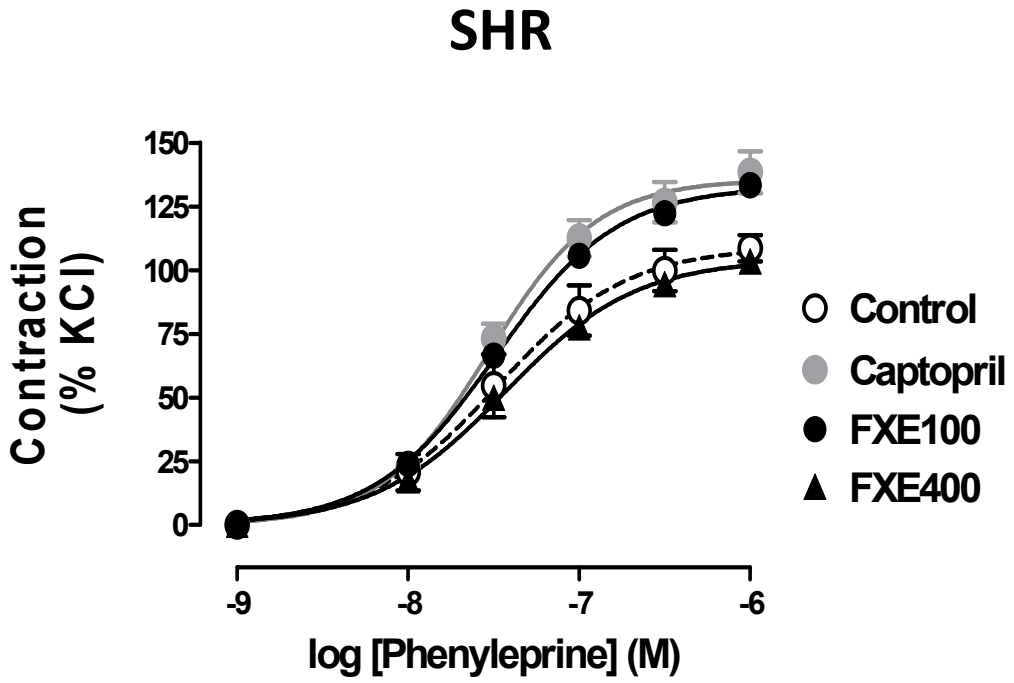
Figure 6



2
3

1

Figure 7

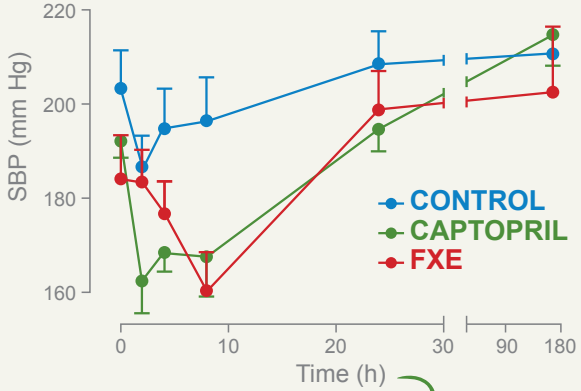


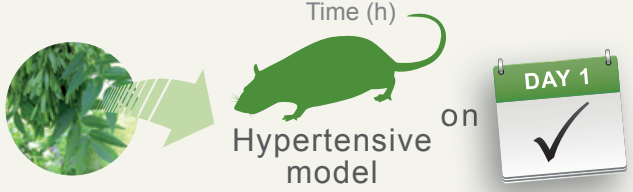
2
3

FRAXIPURE® ATTENUATES METABOLIC DISORDERS

Food & Function

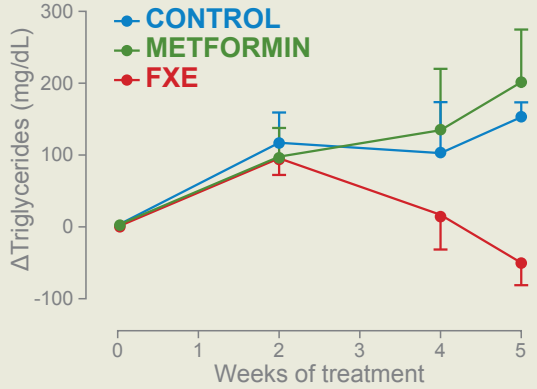
Reduced **blood pressure**





Also effective during 20-week supplementation.

Reduced **triglyceridemia** **-33.4%**



Reduced **glycemia** **-16.3%**