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# Reversion of down-regulation of intestinal multidrug resistance-associated protein 2 in fructose-fed rats by geraniol and vitamin C. Potential role of inflammatory response and oxidative stress

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**Running title:** Down-regulation of intestinal Mrp2 is reverted by geraniol and vitamin C in a metabolic syndrome model.

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**ABSTRACT**

Intestinal multidrug resistance-associated protein 2 is an ABC transporter that limits the absorption of xenobiotics ingested orally, thus acting as essential component of the intestinal biochemical barrier. Metabolic Syndrome (MetS) is a pathological condition characterized by dyslipidemia, hyperinsulinemia, insulin resistance, chronic inflammation, and oxidative stress (OS). In a previous study we demonstrated that MetS-like conditions induced by fructose in drinking water (10% v/v, during 21 days), significantly reduced the expression and activity of intestinal Mrp2 in rats. We here evaluated the potential beneficial effect of geraniol or vitamin C supplementation, natural compounds with anti-inflammatory and anti-oxidant properties, in reverse fructose-induced Mrp2 alterations. After MetS-like conditions were induced (21 days), animals were cotreated with geraniol or vitamin C or vehicle for another 14 days. Decreased expression of Mrp2 protein and mRNA due to fructose administration was reversed by geraniol and by vitamin C, consistent with restoration of Mrp2 activity evaluated in everted intestinal sacs. Concomitantly, increased intestinal IL-1β and IL-6 levels induced by fructose were totally and partially counterbalanced, respectively, by geraniol administration. The intestinal redox unbalance generated by fructose was improved by geraniol and vitamin C, as evidenced by decreasing lipid peroxidation products and activity of Superoxide Dismutase and by normalizing glutathione reduced/oxidized glutathione ratio. The restoration effects exhibited by geraniol and vitamin C suggest that local inflammatory response and OS generated under MetS-like conditions represent important mediators of the intestinal Mrp2 down-regulation. Additionally, both agents could be considered of potential therapeutic value to preserve Mrp2 function under MetS conditions

**Key words:** fructose-rich diet, metabolic syndrome, insulin resistance, intestine, Mrp2, oxidative stress, inflammation, geraniol, vitamin C.

**Abbreviations:** ABC, ATP-Binding Cassette; AUC, area under the curve; BBM, brush border membrane; b.wt., body weight; CAT, Catalase; CDNB, 1-chloro-2,4-dinitrobenzene; C, control; DNP-SG, dinitrophenyl-S-glutathione; GSH, reduced glutathione; GSSG, oxidized glutathione; IPGTT, intraperitoneal glucose tolerance test;

ITT, insulin tolerance test; HOMA, homoeostasis model assessment; IL-1β, Interleukin-1β; IL-6, Interleukin-6; IR, insulin resistance; LPO, lipid peroxidation; Mrp2, multidrug resistance-associated protein 2; OS, oxidative stress; SOD, Superoxide Dismutase; TAG, triacylglycerol; TBARS, thiobarbituric acid reactive substances.

## INTRODUCTION

Metabolic syndrome (MetS) is a contemporary condition defined by a cluster of physiological interconnected biochemical, clinical, and metabolic factors consisting of elevated blood pressure, hypertriglyceridemia, reduced serum high-density lipoprotein-cholesterol, glucose intolerance, hyperinsulinemia, and insulin resistance. These factors increase the risk of individuals to develop cardiovascular disease and Type 2 Diabetes [1-3]. MetS and its associated complications have become one of major health problems worldwide, with an increase incidence due to improper nutrition and sedentary lifestyle [4]. In the last decades a marked increase in average fructose intake in the way of sweeteners used by food industry has been documented [5]; at the same time, there is growing evidence indicating that excessive fructose consumption is causally linked to MetS prevalence [6-8]. Rats fed with a fructose-rich diet constitute a valid model of diet-induced insulin resistance, associated with hyperinsulinemia, glucose intolerance, hypertriglyceridemia, as well as inflammation and oxidative stress (OS) in different tissues [9-11]. Although the precise molecular mechanism by which fructose-rich diet induces MetS remains in discussion, it has been postulated that an increase in OS plays a key role in its pathogenesis and subsequent complications [12-14]. In support to this assumption, epidemiologic studies show that anti-oxidants (e.g., vitamin A, C, and E) are associated with reduced risk of MetS [15,16], consistent with their effects in reducing main alterations induced by fructose-rich diet [17,18].

Interestingly, high fructose consumption correlates with increased intestinal permeability, as a significant component of fructose-induced MetS [19,20]. Consumption of fructose raises fructokinase-C expression in small intestine [21]; consequently, the acceleration of intestinal fructolysis originates local OS and inflammation and reduces the expression of relevant tight junction proteins, ultimately resulting in increased paracellular permeability [22]. Regarding the transcellular component of intestinal barrier function, we have recently reported a significant reduction in Multidrug resistance protein 2 (Mrp2, ABCC2) expression in normal rats fed with a fructose-rich diet for 21 days [23], resulting in impaired activity and consequently, increased absorption of Mrp2 substrates. Mrp2 is a drug efflux pump belonging to the ATP-Binding Cassette (ABC) transporter superfamily and is constitutively expressed in the apical membrane of enterocytes, mainly from proximal jejunum, where it acts in concert with biotransformation enzymes to prevent absorption

of food contaminants and drugs incorporated orally [24, 25]. Thus, any alteration in the intestinal Mrp2 activity will increase the toxicity of xenobiotics as well as it will affect the bioavailability and eventually the safety of therapeutic drugs.

The specific mechanisms underlying the reduction of intestinal Mrp2 under MetS conditions are still unknown. However, it is tempting to speculate that OS and inflammation may play a significant role as they occurred in simultaneous with Mrp2 down-regulation induced by fructose administration [26]. Both factors were previously shown to be important regulators of Mrp2 expression under other experimental pathological conditions such as LPS-induced endotoxemia and obstructive cholestasis [27-29]. Therefore, administration of anti-oxidant and anti-inflammatory agents to rats with MetS could be a useful tool to support our hypothesis. Geraniol, is an important monoterpene alcohol naturally found in the essential oils of lemon, rose, ginger, orange, among others [30]. Pharmacological studies involving geraniol have shown that it possesses potent anti-oxidant and anti-inflammatory properties [31, 32]. While the antidiabetic, anti-oxidant and anti-inflammatory effect of this monoterpene in experimental MetS-rats model has previously been well documented [33], studies regarding its efficacy in reversing intestinal Mrp2 alterations have not been performed until the present.

On the other hand, given that OS has a central role in the etiology of MetS and that MetS patients have a deficient endogenous anti-oxidant capacity [34], it was also our interest to evaluate the effect of vitamin C on Mrp2 down-regulation in our experimental model of MetS. Vitamin C constitutes an important non-enzymatic anti-oxidative defense and its supplementation was proven effective to decrease the incidence of MetS in patients [35, 36]. Accordingly, the aim of this study was to validate the efficacy of geraniol (as anti-oxidant and anti-inflammatory agent) and vitamin C (as anti-oxidant agent) in revert intestinal Mrp2 down-regulation in an insulin resistant rat model that mimics aspects of MetS, with the final purpose of identifying the possible components of this pathological condition responsible for Mrp2 alteration.

## 2. MATERIALS AND METHODS

### 2.1. Chemicals

Fructose was obtained from Laboratorio Cicarelli (Bs. As, Argentina). Geraniol, vitamin C, leupeptin, phenylmethylsulfonyl fluoride, pepstatin A, 3-isobutyl-1-methylxanthine, glutathione, dithiothreitol, 1-chloro-2,4-dinitrobenzene (CDNB), 2-thiobarbituric acid, nitroblue tetrazolium, and hydrogen peroxide ($H_2O_2$) were obtained from Sigma-Aldrich (St. Louis, USA). 2-vinylpyridine was obtained from Fluka Chemical Corp (Milwaukee, USA). All other chemicals and reagents used were commercial products of analytical-grade purity.

### 2.2. Animals and treatments

Adult male Wistar rats (220–250 g b.wt.; 70-day old) were purchased from Centro de Medicina Comparada-Instituto de Cs. Veterinarias del Litoral (UNL-CONICET, Esperanza, Argentina). Animals received standard commercial diet *ad libitum* and either tap water (C group), or tap water with 10% (w/v) fructose (FRU group), for 21 days to induce MetS-like conditions [23,36,37] (Fig.1). Animals were grouped (two animals per cage) and kept under controlled conditions (23 ± 2°C) with a fixed 12-h light–dark cycle (06:00–18:00 h). Installation of MetS was verified by plasma TAG (triacylglycerol) and immunoreactive insulin levels and by the intraperitoneal glucose tolerance test (IPGTT) (data not shown). At day 21, either MetS or control rats were randomly divided into 8 subgroups as shown in Fig. 1: four groups belonging to the protocol of reversion by geraniol and four groups belonging to the protocol of reversion by vitamin C. In the first protocol (Fig. 1A), control and MetS rats were daily administered with 1% Tween 80 (C and FRU groups) or geraniol in 1% Tween 80 (250 mg/kg/day, p.o [37]; GO and FRU+GO groups). In the second protocol (Fig. 1B), control and MetS rats were daily administered with tap water (C and FRU groups) or with vitamin C (100 mg/kg/day, p.o [38]; VitC and FRU+VitC groups).

Rats of all experimental groups were subjected to the IPGTT and to the insulin tolerance test (ITT) at the end of the treatments, after overnight and 6 hours fasting respectively. Liquid intake was monitored every other day and body weight once a week throughout the treatment. Total drinking volume of animals housed together was averaged and considered as a single data. All the experimental protocols were performed according to the Regulation for the Care and Use of Laboratory Animals

(Expedient 6109/012 E.C. Resolution 267/02) and were approved by the Institutional Animal Use Committee of the National University of Rosario, Argentina.

### 2.3. Specimen collection

Animals were fasted 12 h before sacrifice, performed under intraperitoneal anesthesia [ketamine: 100 mg (0.42 mmol)/kg b.wt.; xylazine: 15 mg (0.07 mmol)/kg b.wt.] between 08:00 and 09:00 h. After an abdominal incision, blood samples were taken through cardiac puncture and placed into heparinized tubes to measure plasma glucose, triacylglycerol (TAG) and immunoreactive insulin levels. For collection of jejunum specimens, the first 15 cm starting from the pyloric valve and corresponding to the duodenum were excluded and the following 30 cm were taken and considered as the proximal jejunum. This segment was carefully rinsed with ice-cold saline and dried with filter paper. For western blot studies, the jejunum was immediately opened lengthwise, the mucus layer was carefully removed, and the mucosa was obtained by scraping, weighed, and used for brush border membrane (BBM) preparation. For total RNA isolation, small rings were cut from this same region of the intestine, frozen in liquid nitrogen and kept at -70°C until isolation by using TRIzol reagent (Invitrogen, Carlsbad, USA) following the manufacturer's protocol. For Mrp2 transport studies *in vitro*, 3-cm segments of the proximal jejunum were immediately used in preparation of everted sacs. Aliquots of proximal jejunum were homogenized in saline (1:2) for assessment of glutathione content, lipid peroxidation and anti-oxidant enzyme activities, or in phosphate-buffered saline (pH: 7.40) (1:2) for assessment of IL-1β and IL-6 when it corresponds.

### 2.4. Biochemical assays

Plasma glucose and TAG levels were determined spectrophotometrically using commercial kits (Wiener Laboratorios, Rosario, Argentina). Plasma insulin levels were measured by RIA using a commercial kit (Rat insulin, Millipore Corporation, Billerica, USA).

The IPGTT was performed 3 days before the animals were sacrificed as described previously [23]. For the calculation of the area under the curve (AUC) the GraphPad Prism 5 software was used and the values were expressed in mM/120 min.

For the ITT, 2 days before the experiments, the rats were fasted for 6 hours and then a blood sample was taken from the tail vein (basal glycemia, 0 min). The animals were immediately injected i.p. with 0.75 units/kg human recombinant insulin (Denver Farma S.A, Buenos Aires, Argentina) [39] and additional blood samples were taken at 30, 60, and 90 min also from the tail vein. For the calculation of the area under the curve (AUC) the GraphPad Prism 5 software was used and the values were expressed in mM/90 min.

Serum insulin and fasting blood glucose values were used to estimate IR (insulin resistance) by HOMA (homoeostatic model assessment)-IR index, using the equation: serum insulin (μUI/ml)×fasting blood glucose (mmol/l)/22.5 [40]. When the value of HOMA-IR increases, IR augments, thus indicating a decrease in insulin sensitivity.

### 2.5. Western blot studies

BBM were prepared from mucosa samples as described previously [24]. Protein concentration was measured by using bovine serum albumin as standard [41]. Aliquots of the BBM preparations were kept on ice and used the same day in western blot studies. Mrp2 was detected in BBMs as described previously [24]. Equal loading and transference of protein was systematically checked by both detection of β-actin and staining of the membranes with Ponceau S. Primary antibodies used were anti-MRP2 (M2 III-6, Alexis Laboratories, San Diego, USA) and anti-β-actin (A-2228, Sigma–Aldrich). Immunoreactive bands were quantified with Gel-Pro Analyzer software (Media Cybernetics, Inc., Bethesda, USA).

### 2.6. Real-Time Polymerase Chain Reaction (PCR) studies

Quantitative real-time PCR studies of *ABCC2* mRNA were performed as described previously [42] using the following primer pairs: forward, 5'-accttccacgtagtgatcct-3' and reverse, 5'-acctgctaagatggacggtc-3 for *ABCC2* and forward, 5'-gtaacccgttgaaccccatt-3' and reverse, 5'-ccatccaatcggtagtagcg-3' for 18S rRNA (housekeeping gene).

### 2.7. Assessment of Mrp2 activity *in vitro*

To characterize the effect of the different treatments on intestinal Mrp2 efflux activity, the *in vitro* model of everted sacs was chosen. The everted sacs, prepared from

proximal jejunum, were incubated for 30 min in the presence of 100 µM CDNB in the mucosal compartment as described previously [43]. After diffusion of CDNB into the enterocyte, and further endogenous conjugation with glutathione, the product dinitrophenyl-S-glutathione (DNP-SG) was detected by high-performance liquid chromatography in the same mucosal compartment as described [43].

### 2.8. Determination of intestinal glutathione content and lipid peroxidation (LPO)

Total glutathione [reduced (GSH) + oxidized (GSSG)] and GSSG were assessed as described previously [44], in supernatants of proximal jejunum homogenates prepared as it was previously described [23]. GSH values were determined from total glutathione and GSSG concentrations and oxidative stress index was calculated from the GSH/GSSG ratio and expressed as % of controls.

LPO was estimated by measuring thiobarbituric acid reactive substances (TBARS) using the procedure of Ohkawa et al. [45] in intestinal homogenates as described previously [23].

### 2.9. Assessment of anti-oxidant enzyme activities

Enzyme activities were assessed in supernatants of jejunum homogenates prepared as was previously described [23]. Protein concentration was measured by using bovine serum albumin as standard [41]. Superoxide Dismutase (SOD) assay was based on the method of Beauchchamp and Fridovich [46] with modifications of Donahue et al. [47], which measures the SOD inhibition of photochemical reduction of nitroblue tetrazolium. Catalase (CAT) activity was determined by monitoring the rate of decomposition of $H_2O_2$ as a function of decrease in absorbance at 240 nm for 2 min [48]. Both enzyme activities were expressed as % of controls.

### 2.10. Assessment of interleukins

IL-1β and IL-6 levels were determined in jejunum homogenates as described previously [23] and expressed as % of controls.

### 2.11. Statistical Analysis

Data are presented as mean ± S.D. All statistical analyses were performed using one-way ANOVA followed by Newman-Keuls test. Values of $P<0.05$ were considered statistically significant**.**

# 3 RESULTS

## 3.1. Protocol of reversion by geraniol administration

### 3.1.1. Effect of geraniol on body weight and food/water intake

Following 35 days of treatment, body weight gain did not differ among the different experimental groups (93±21, 111±11, 93±9, 96±21 g for C, FRU, GO and FRU+GO, respectively). Liquid consumption was higher for FRU animals than for C (50.23±0.64 *vs*. 38.00±0.04 ml/day, respectively; $P<0.05$), whereas coadministration of geraniol did not change water intake respect to administration of fructose alone (46.72±4.90 *vs*. 50.23±0.64 ml/day for FRU+GO and FRU, respectively). Food intake was significantly lower in FRU, GO and FRU+GO groups respect to C (15.23±0.27, 16.31±0.50 and 14.70±0.27 *vs* 22.20±0.72 g/day, respectively; $P<0.05$). In contrast, calories consumption was significantly higher in FRU and FRU+GO groups respect to C and GO groups (47.20±0.90 and 44.80±0.90 *vs* 39.50±5.40 and 29.04±0.90 kcal/day, respectively; $P<0.05$).

### 3.1.2. Effect of geraniol on altered glucose homeostasis and plasma TAG level induced by fructose administration

Plasma biochemical determinations as well as IPGTT and ITT curves of experimental groups are shown in Table 1 and in Figure 2, respectively. Concerning the fasting level of blood glucose, there were no significant differences among the four groups studied (Table 1). However, FRU group displayed an impaired glucose tolerance as indicated by increased AUC value in the IPGTT measure (Fig. 2A). ITT analysis also revealed an impaired insulin sensitivity in FRU *vs.* C rats (a greater AUC value, Fig. 2B), which together with elevated fasting insulin levels and HOMA-IR index indicated the establishment of IR in these rats. Treatment with geraniol significantly improved the response to glucose overload and insulin administration and restored the HOMA-IR index in fructose fed rats as well, consistent with reversion of the IR condition (Table 1). Also, the increase of TAG plasma levels induced by fructose administration, were completely reverted by coadministration with geraniol (Table 1).

### 3.1.3. Effect of geraniol on Mrp2 down-regulation induced by fructose administration

Figure 3A shows that Mrp2 expression, detected in BBM, was significantly decreased in FRU group with respect to C (77% decrease), as we previously described [26]. Cotreatment with geraniol was able to reverse Mrp2 down-regulation to the extent that densitometry revealed no difference with respect to C (Fig. 3A). Geraniol administered alone did not affect Mrp2 expression with respect to control rats.

We performed real-time PCR studies to evaluate the involvement of a transcriptional mechanism and found that fructose administration indeed reduced *ABCC2* mRNA levels respect to C (40% decrease) and that geraniol reverted such effect (Fig. 3B).

Mrp2 activity was evaluated by the luminal secretion of DNP-SG, a typical Mrp2 substrate, using the *in vitro* model of everted intestinal sacs. Consistent with western blot studies, the amount of DNP-SG accumulated in the mucosal compartment decreased in FRU group with respect to C (43% decrease) and returned to the normal value in the cotreated group (Fig. 3C).

### 3.1.4. Effect of geraniol on altered intestinal redox balance and anti-oxidant defenses induced by fructose administration

To evaluate the anti-oxidant properties of geraniol, we tested GSH/GSSG ratio and TBARS levels. Figure 4A shows that reduction of the GSH/GSSG ratio induced by fructose (38% decrease) was completely restored by cotreatment with geraniol. At the same time, GSSG intestinal content was increased in FRU group (58% increase) and preserved in FRU+GO when compared to C, while GSH content remained unchanged irrespective of the treatment (data not shown). Fructose administration increased TBARS levels respect to C (60% increase), whereas geraniol coadministration lowered such measure under control value (27% decrease) (Fig. 4B). Also, cotreatment with geraniol counteracted the increase in SOD activity observed for FRU group (61% increase) (Fig. 4C), whereas CAT activity did not vary among the different experimental groups (Fig. 4D). Geraniol administration alone did not affect GSH/GSSG ratio, TBARS levels or SOD activity.

### 3.1.5. Effect of geraniol on increased intestinal proinflammatory cytokine levels induced by fructose administration

Panels A and B from Figure 5 show that both IL-1β and IL-6 levels increased significantly in proximal jejunum from FRU group when compared to control group (58% and 123% increases, respectively). Cotreatment with geraniol completely restored IL-1β levels and only partially those of IL-6.

### 3.2. Protocol of reversion by vitamin C administration

#### 3.2.1. Effect of vitamin C on body weight and food/water intake

After 35 days of treatment, body weight gain did not differ between experimental groups (83±8, 87±3, 77±9 and 87±3 g for C, FRU, VitC and FRU+VitC, respectively). Liquid consumption was higher for FRU animals than for C (55.60±1.30 *vs*. 34.40±1.70 ml/day, respectively; $P<0.05$), whereas this measure was similar between FRU+VitC and FRU groups (54.08±6.50 *vs*. 55.60±1.30 ml/day, respectively). Food consumption was significantly lower in FRU group respect to C (22.33±1.82 *vs.* 26.07±0.50 g/day, respectively; $P<0.05$), while in FRU+ VitC group this parameter remained unchanged respect to FRU group (22.25±1.13 *vs.* 22.33±1.82g/day, respectively). Calories consumption were significantly higher in FRU and FRU+VitC groups respect to C and VitC groups (46.01±2.80 and 45.50±0.43 *vs* 40.12±0.60 and 41.25±0.35 kcal/day, respectively; $P<0.05$).

#### 3.2.2. Effect of vitamin C on altered glucose homeostasis and plasma TAG levels induced by fructose administration

Biochemical measures are shown in Table 2. There were no significant differences in basal blood glucose levels among groups. Plasma TAG and insulin levels were significantly increased by fructose administration and completely reverted by vitamin C. In addition, key parameters of glucose homeostasis were assessed in all experimental groups and are shown in Figure 6. The highest glycemia levels in the IPGTT and ITT curves (Fig. 6), demonstrated by a significantly elevated AUC values (inserts in Fig. 6), as well as in the HOMA-IR value (Table 2) found in FRU group confirmed development of IR. Rats that underwent vitamin C cotreatment demonstrated better glucose tolerance (Fig. 6A and Table 2) and insulin sensitivity (Fig. 6B) than those receiving only fructose. Cotreatment with vitamin C also normalized the altered insulin sensitivity index (Table 2), whereas administration with vitamin C alone did not affect any of the parameters studied.

### 3.2.3. Effect of vitamin C on Mrp2 down-regulation induced by fructose administration

Fructose administration caused a significant decrease in Mrp2 protein expression with respect to C (75% decrease) in proximal jejunum. However, the decreased levels of Mrp2 protein were significantly attenuated in rats cotreated with vitamin C (Fig. 7A). Likewise, detection of mRNA levels revealed that fructose administration decreased Mrp2 expression with respect to C (56% decrease), whereas this measure returned to normal values in the FRU+ VitC group (Fig. 7B). Figure 7C shows that variations in Mrp2 activity in response to fructose and/or vitamin C administration were consistent with those of mRNA, indicating a 42% decrease in FRU group respect to C and restoration under cotreatment conditions. Vitamin C administered alone did not affect Mrp2 expression or activity with respect to control rats.

### 3.2.4. Effect of vitamin C on altered intestinal redox balance and anti-oxidant defenses induced by fructose administration

Figure 8A shows that reduction of the GSH/GSSG ratio induced by fructose (36% decrease) was completely restored by cotreatment with vitamin C. At the same time, GSSG intestinal content was higher in FRU group (60% increase) and was normalized in FRU+VitC as compared to C, while GSH content remained unchanged (data not shown). Similarly, the increase of TBARS levels induced by fructose (65% increase) returned to normal value after vitamin C administration, as shown in Figure 8B. Regarding the anti-oxidant defense, SOD activity was increased in FRU group (63% increase) and restored in FRU+VitC rats (Fig. 8C). CAT activity was not affected by fructose administration but significantly reduced in both VitC and FRU+VitC groups when compared to control animals (37 and 38% decrease respectively) (Fig. 8D).

## DISCUSSION

Fructose is a sugar widely found in natural foods, as fruits and vegetables, and is also used as a supplement in a variety of processed foods. Excessive fructose consumption is an important risk factor contributing to development of MetS, with associated dysfunctions in multiple tissues and organs including liver, adipose, pancreatic islet, skeletal muscle and intestine, among others [49, 50]. Although the exact pathogenesis of MetS is still under debate, it is firmly postulated that primary metabolites of fructose, produced in organs where fructokinase-C is expressed, play a central role initially inducing systemic and local insulin resistance, as well as triggering overproduction of reactive oxygen species. These primary alterations subsequently lead to exacerbated secretion of inflammatory cytokines, adiponectin, endotoxin and leptin, which cause systemic or local inflammation response, lipid accumulation, and endothelial dysfunction, in addition to hyperphagia [50]. Clearly, OS is a key event contributing to the etiopathogenesis of MetS.

Evidence from animal models as well as human studies indicate that insulin resistance associated with overconsumption of fructose induces important alterations in the intestinal mucosa, possibly due to fructose metabolism in the intestinal wall since this tissue also expresses fructokinase-C [51, 21]. Some of these alterations are increased intestinal permeability, consequence of local inflammation and reduction of expression of tight junction proteins such as occludin and zone occludes-1 [52]. In line with this, we recently demonstrated a significant down-regulation of the drug transporter Mrp2 in proximal jejunum of fructose-fed rats, not associated with erosive alterations of the intestinal epithelium, but rather with a specific regulation, at least in part, at transcriptional level [23]. Interestingly, such regulation occurred in parallel with alteration of the redox balance and increased inflammatory response in intestinal tissue [23]. Down-regulation of Mrp2 protein by the systemic pro-inflammatory cytokine IL-1β was previously observed in the duodenum of extrahepatic cholestasic rats, with concomitant reduction of its mRNA levels resulting from its transcriptional regulation [28]. Similarly, IL-1β appears to be the major regulator of Mrp2 expression under endotoxemic conditions, since its blockade led to a complete preservation of the transporter expression (protein and mRNA) [53]. On the other hand, exposure to OS was found to produce a marked decrease in hepatic Mrp2 protein expression, mainly through a post-transcriptional regulation [27,54]. Thus, it is possible that IL-1β (as a

main mediator of inflammatory response) and/or OS mediate down-regulation of intestinal Mrp2 under MetS conditions induced by fructose administration. In order to confirm this supposition, we evaluated the effect of coadministration with geraniol or vitamin C.

Geraniol is an acyclic monoterpene alcohol found in medicinal plants and used traditionally for several medical purposes including diabetes [55]. Geraniol possesses pharmacological effects such as anti-oxidant, anticancer and anti-inflammatory properties [56,57,58]. Under the present experimental conditions, geraniol *per se* did not affect body weight gain, plasma glucose, TGA or insulin levels. However, under MetS-like conditions, geraniol reduced plasma TGA levels to normal values and also restored insulin sensitivity, indicated by reduction of HOMA-IR index and by a better response to both the ITT and IPGTT, as was previously reported [32]. As expected, OS indicators were also reversed by geraniol supplementation, agreeing well with previous studies demonstrating the anti-oxidant potential of geraniol *in vivo* [59]. Similarly, geraniol totally counteracted the increase in IL-1β production and partially neutralized the increase of IL-6 induced by fructose administration, consistent with its anti-inflammatory action. More importantly, geraniol treatment totally reversed loss of Mrp2 expression and activity. A direct action of geraniol on expression of Mrp2 seems unlikely since GO group exhibited similar levels of protein and mRNA when compared to control rats. Therefore, it is possible to postulate that the modulation of both OS and inflammatory response by geraniol contributed to ameliorate Mrp2 down-regulation under MetS conditions.

Vitamin C is a recognized natural anti-oxidant of therapeutic application [15]. Our data indicate that vitamin C restored the intestinal redox unbalance induced by fructose, as evidenced by reduction of LPO and concomitant normalization of GSH/GSSG ratio and SOD activity. Catalase activity was reduced in both VitC and FRU+VitC groups respect to control rats. This is likely due to formation of semidehydroascorbate, an intermediate in vitamin C oxidation, which exhibits inhibitory action on catalase activity [60]. Our results demonstrate for the first time that administration of vitamin C alleviates MetS in rat model, essentially by reducing insulin resistance, as demonstrated by reduction of HOMA-IR index as well as by a better response to both the ITT and IPGTT. This is in accordance with previous epidemiological studies demonstrating that high intake of vitamin A and C were

associated with lower risk of MetS in women and that MetS patients had considerably lower plasma levels of vitamin A, C and E compared to healthy subjects [35,16]. Significantly, vitamin C administration was also able to reverse intestinal Mrp2 down-regulation, restoring its activity as well as protein and mRNA levels. Consequently, it is possible to postulate that OS plays a central role in the regulation of intestinal Mrp2 expression under MetS conditions. Whether vitamin C corrected also, directly or indirectly, the inflammatory response was not explored in this study. However, this possibility should not be ruled out since, as was mentioned above, the sequential link between a fructose-rich diet, OS and inflammatory response has been largely demonstrated [61-63]. Even more, recent studies had revealed anti-inflammatory properties of vitamin C, whose mechanism have been attributed to its ability to modulate NF-κB-DNA binding activity and down-regulate hepatic mRNA expression of TNF-α and IL-6, among other actions [64-66]. Clearly, more direct studies are necessary to elucidate to what extent OS or inflammation is responsible for down-regulation of intestinal Mrp2.

In conclusion, we demonstrate for the first time that geraniol or vitamin C administration can completely revert down-regulation of intestinal Mrp2 in fructose-fed rats, strongly suggesting that OS and the inflammatory response are critical factors leading to the impairment of Mrp2 function. Our study suggests that modulation of Mrp2, as a relevant member of the intestinal transcellular barrier that conditions drug bioavailability and xenobiotics toxicity, represent an additional beneficial effect of geraniol and vitamin C as natural therapeutic agents for the treatment of MetS.

## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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## FIGURE LEGENDS

**Fig. 1: Schematic representation of treatment protocols**

Two treatment protocols were used, named: **A,** Protocol of reversion by geraniol (Groups: C, FRU, GO, and FRU+GO; n=6-8), and **B,** Protocol of reversion by vitamin C (Groups: C, FRU, GO, and FRU+VitC; n=6-12). The duration of both protocols was 35 days in total. The initial 21 days correspond to the installation of Mets, which was verified by plasma TAG (triacylclicerol) and inmunoreactive insulin levels, and by the intraperitoneal glucose tolerance test (IPGTT). Treatment with geraniol and vitamin C was initiated at day 22, while fructose administration was maintained. Animals were fasted 12 hours before sample collection.

**Fig. 2. Effect of geraniol on glucose homeostasis**

Response curves during the intraperitoneal glucose tolerance (IPGTT) **(A)** and insulin tolerance (ITT) **(B)** tests. Serum glucose concentrations under IPGTT and ITT conditions are shown in mM (mmol/L). Inserts depict quantification of cumulative glucose clearance in the IPGTT and the ITT by integration of the area under the curve (AUC), in mM/120 min or mM/90 min respectively.

Data are expressed as means±S.D. of 6 rats per group.

*****, significantly different from C, GO and FRU+GO, $P < 0.05$.

**Fig. 3: Effect of geraniol on Mrp2 down-regulation induced by fructose administration**

**A,** Western blot analysis of Mrp2 in BBM vesicles from proximal jejunum. Equal amounts of total protein (20 µg) were loaded in all lanes. Uniformity of loading and transfer from gel to nitrocellulose membrane was controlled with Ponceau S and detection of β-actin. Densitometry data were related to β-actin and presented as % of controls (C). **B**, Quantitative real-time PCR assessment of *ABCC2* mRNA levels in proximal jejunum. Results were referred to 18s rRNA and expressed as % of controls (C). **C,** Cumulative content of DNP-SG in the mucosal compartment of everted intestinal sacs at 30 min of incubation. Results were expressed as % of controls (C).

Data are expressed as means±S.D. of 6 to 8 rats per group.

*****, significantly different from C, GO and FRU+GO, $P < 0.05$.

**Fig. 4: Effect of geraniol on altered intestinal redox balance and anti-oxidant defenses induced by fructose administration**

GSH/GSSG ratio **(A)**, thiobarbituric acid reactive substances (TBARS) **(B)**, **s**uperoxide dismutase (SOD) activity **(C),** and catalase (CAT) activity **(D)** were determined in intestinal homogenate of rats from C, FRU, GO and FRU + GO groups. All results were expressed as % of controls (C).

Data are expressed as means±S.D. of 6 to 8 rats per group.

*****, significantly different from C, GO and FRU+GO, $P< 0.05$.

**&**, significantly different from C, $P<0.05$.

**Fig. 5: Effect of geraniol on increased intestinal proinflammatory cytokine levels induced by fructose administration**

Interleukin1β (IL-1β) **(A)** and interleukin 6 (IL-6) **(B)** were determined in intestinal homogenate of rats from C, FRU, GO and FRU + GO groups. All results were expressed as % of controls (C).

Data are expressed as means±S.D. of 6 to 8 rats per group.

**ω**, significantly different from C and FRU+GO, $P< 0.05$.

**&**, significantly different from C, $P<0.05$.

**Fig. 6. Effect of vitamin C on glucose homeostasis**

Response curves during the intraperitoneal glucose tolerance (IPGTT) **(A)** and insulin tolerance (ITT) **(B)** tests. Serum glucose concentrations under IPGTT and ITT conditions are shown in mM (mmol/L). Inserts depict quantification of cumulative glucose clearance in the IPGTT and the ITT by integration of the area under the curve (AUC), in mM/120 min or mM/90 min respectively.

Data are expressed as means±S.D. of 6 rats per group.

**#**, significantly different from C, VitC and FRU+VitC, $P < 0.05$.

**Fig.7: Effect of vitamin C on Mrp2 down-regulation induced by fructose administration**

**A,** Western blot analysis of Mrp2 in BBM vesicles from proximal jejunum. Equal amounts of total protein (20 µg) were loaded in all lanes. Uniformity of loading and

transfer from gel to nitrocellulose membrane was controlled with Ponceau S and detection of β-actin. Densitometry data were related to β-actin and presented as % of controls (C). **B,** Quantitative real-time PCR assessment of Mrp2 mRNA levels in proximal jejunum. Results were referred to 18s rRNA and expressed as % of controls (C). **C,** Cumulative content of DNP-SG in the mucosal compartment of everted intestinal sacs after 30 min of incubation. Results were expressed as % of controls (C).
Data are expressed as means±S.D. of 10 to 12 rats per group.
[#], significantly different from C, VitC and FRU+VitC, $P < 0.05$.

**Fig. 8: Effect of vitamin C on altered intestinal redox balance and anti-oxidant defenses induced by fructose administration**

GSH/GSSG ratio **(A)**, thiobarbituric acid reactive substances (TBARS) **(B)**, **s**uperoxide dismutase (SOD) activity **(C),** and catalase (CAT) activity **(D)** were determined in intestinal homogenate of rats from C, FRU, VitC and FRU + VitC groups. All results were expressed as % of controls (C).
Data are expressed as means±S.D. of 10 to 12 rats per group.
[#], significantly different from C, VitC and FRU+VitC, $P< 0.05$.
[&], significantly different from C, $P<0.05$.

**TABLES**

**Table 1.** Serum markers of MetS

| | C | FRU | GO | FRU+GO |
|---|---|---|---|---|
| **Plasma glucose** (mmol/l) | 5,71 ± 0,44 | 6,21 ± 0,50 | 5,27± 0,72 | 5,44± 0,11 |
| **Plasma triglycerides**(mmol/l) | 0,98 ± 0,09 | 1,40 ± 0,08***** | 0,72± 0,17 | 0,94± 0,06 |
| **Plasma insulin** (ng/ml) | 1,24 ± 0,12 | 2,58 ± 0,37***** | 1,16± 0,36 | 1,21 ± 0,12 |
| **HOMA-IR** | 5,85 ± 3,82 | 14,81 ± 6,67***** | 6,60 ± 1,19 | 6,78± 0,92 |

The HOMA-IR was calculated with the formula: serum insulin (µUI/ml)×fasting blood glucose (mmol/l)/22.5. An increment in the HOMA–IR index indicates an insulin resistance state in FRU rats.

Data are expressed as means±S.D. of 6 to 8 rats per group.

*****, significantly different from C, GO and FRU+GO, $P < 0.05$.

**Table 2.** Serum markers of MetS

| | C | FRU | VitC | FRU+VitC |
|---|---|---|---|---|
| **Plasma glucose** (mmol/l) | 5,38 ± 0,77 | 5,51 ± 0,19 | 5,15 ± 0,16 | 5,35 ± 0,09 |
| **Plasma triglycerides**(mmol/l) | 0,95 ± 0,11 | 1,34 ± 0,29$^{\#}$ | 0,64 ± 0,04 | 0,91 ± 0,09 |
| **Plasma insulin** (ng/ml) | 1,58 ± 0,18 | 3,10 ± 0,34$^{\#}$ | 1,58 ± 0,12 | 1,78 ± 0,18 |
| **HOMA-IR** | 5,46 ± 1,20 | 9,20 ± 1,78$^{\#}$ | 4,79 ± 0,48 | 6,30 ± 1,28 |

The HOMA-IR was calculated with the formula: serum insulin (µUI/ml)×fasting blood glucose (mmol/l)/22.5. An increment in the HOMA–IR index indicates an insulin resistance state in FRU rats.

Data are expressed as means±S.D. of 6 to 8 rats per group.

$^{\#}$, significantly different from C, VitC and FRU+VitC, $P < 0.05$.

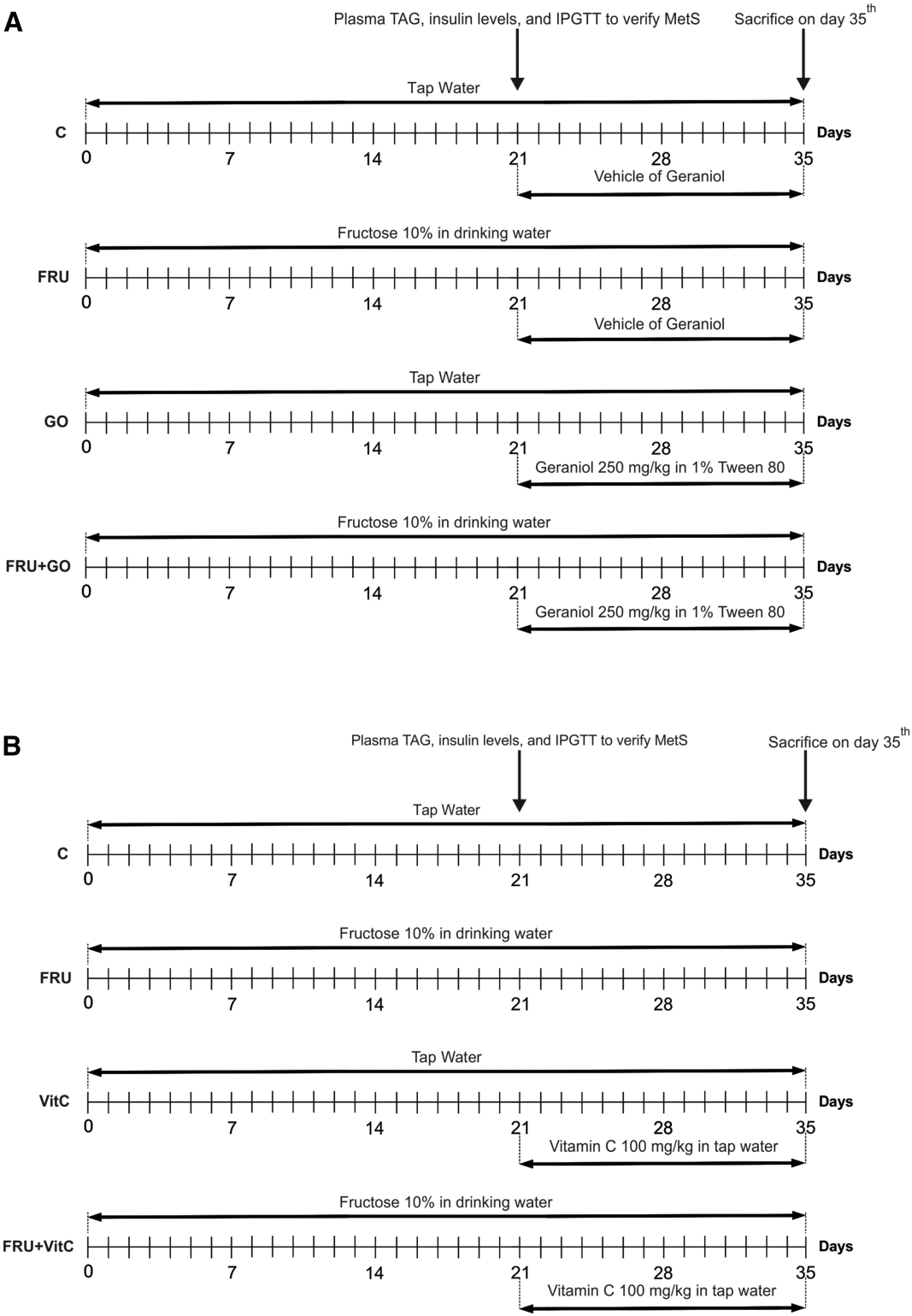
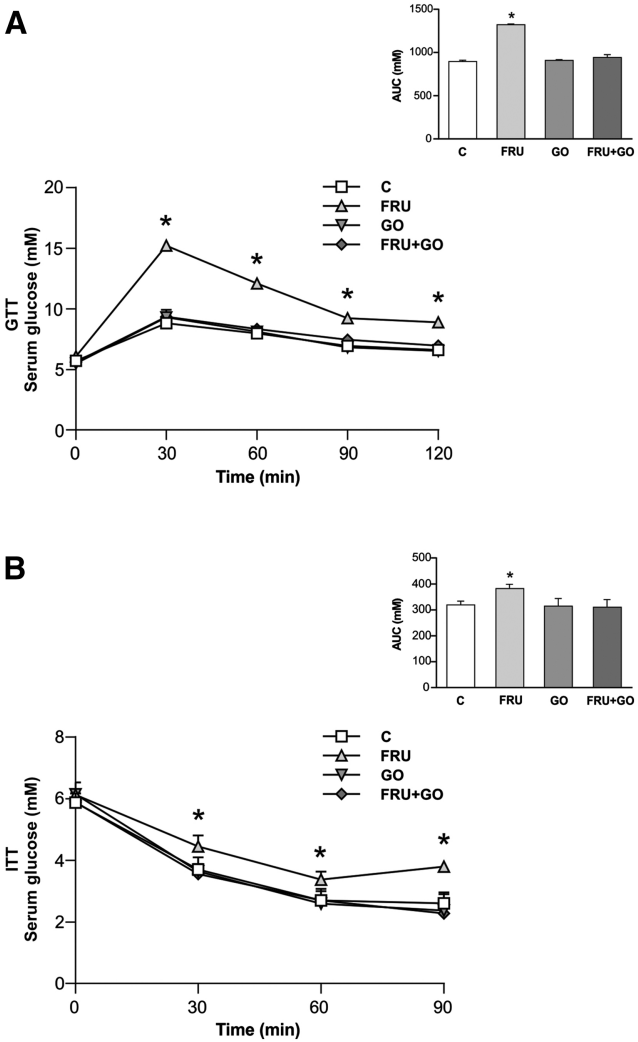

A
Plasma TAG, insulin levels, and IPGTT to verify MetS
Sacrifice on day 35th
Tap Water
C
Days
0
7
14
21
28
35
Vehicle of Geraniol
Fructose 10% in drinking water
FRU
Days
0
7
14
21
28
35
Vehicle of Geraniol
Tap Water
GO
Days
0
7
14
21
28
35
Geraniol 250 mg/kg in 1% Tween 80
Fructose 10% in drinking water
FRU+GO
Days
0
7
14
21
28
35
Geraniol 250 mg/kg in 1% Tween 80
B
Plasma TAG, insulin levels, and IPGTT to verify MetS
Sacrifice on day 35th
Tap Water
C
Days
0
7
14
21
28
35
Fructose 10% in drinking water
FRU
Days
0
7
14
21
28
35
Tap Water
VitC
Days
0
7
14
21
28
35
Vitamin C 100 mg/kg in tap water
Fructose 10% in drinking water
FRU+VitC
Days
0
7
14
21
28
35
Vitamin C 100 mg/kg in tap water

Figure 1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AUC (mM)
1500
1000
500
0
*
C
FRU
GO
FRU+GO
GTT
Serum glucose (mM)
20
15
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5
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C
FRU
GO
FRU+GO
*
*
*
*
0
30
60
90
120
Time (min)
B
AUC (mM)
500
400
300
200
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C
FRU
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FRU+GO
ITT
Serum glucose (mM)
8
6
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C
FRU
GO
FRU+GO
*
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30
60
90
Time (min)

Figure 2

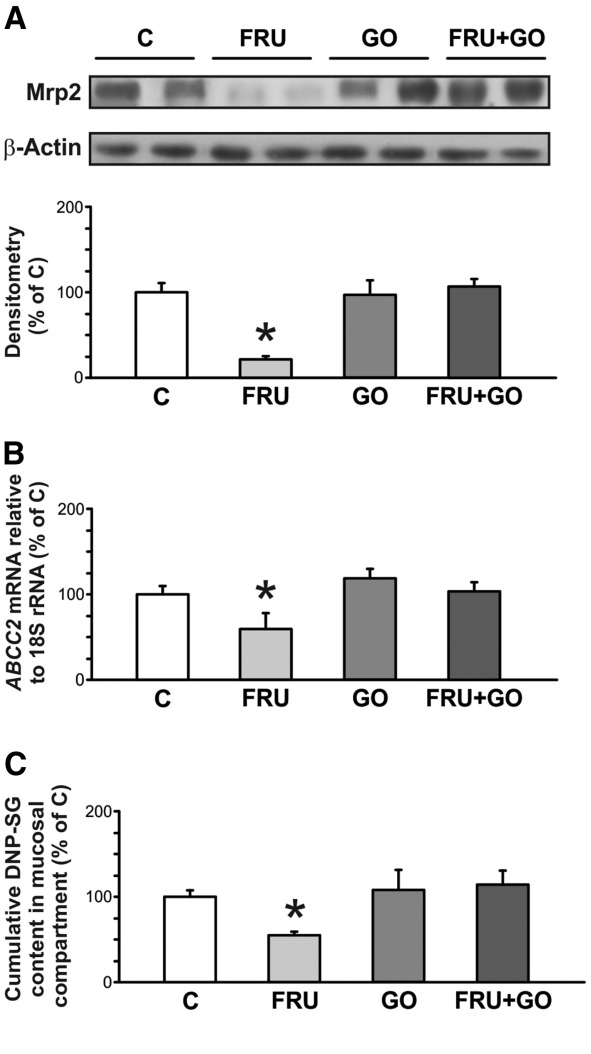
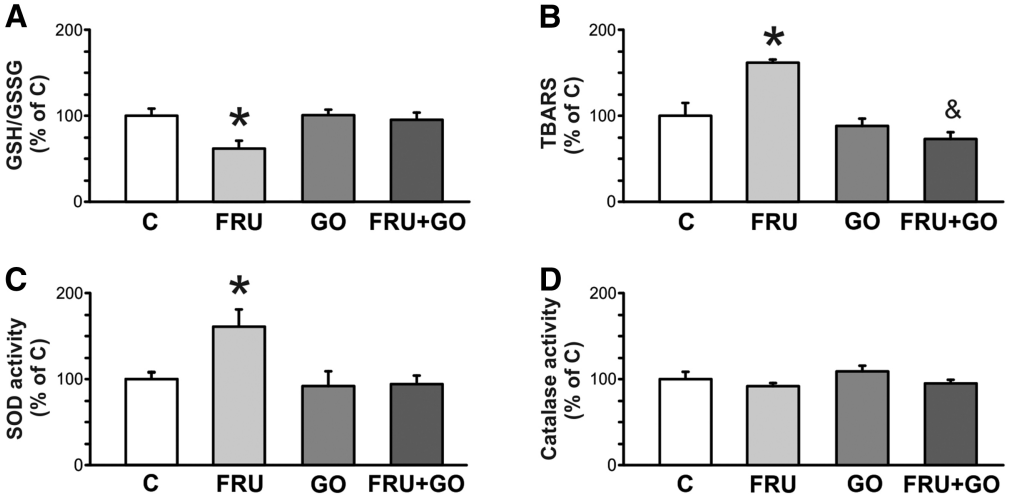

A
C
FRU
GO
FRU+GO
Mrp2
β-Actin
Densitometry (% of C)
200
100
0
*
C
FRU
GO
FRU+GO
B
ABCC2 mRNA relative to 18S rRNA (% of C)
200
100
0
*
C
FRU
GO
FRU+GO
C
Cumulative DNP-SG content in mucosal compartment (% of C)
200
100
0
*
C
FRU
GO
FRU+GO

Figure 3


A
GSH/GSSG (% of C)
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C
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GO
FRU+GO
B
TBARS (% of C)
200
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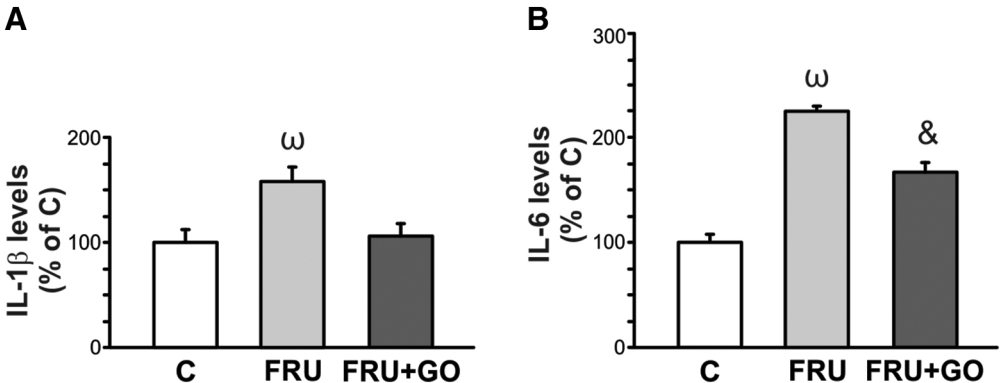
*
&
C
FRU
GO
FRU+GO
C
SOD activity (% of C)
200
100
0
*
C
FRU
GO
FRU+GO
D
Catalase activity (% of C)
200
100
0
C
FRU
GO
FRU+GO

Figure 4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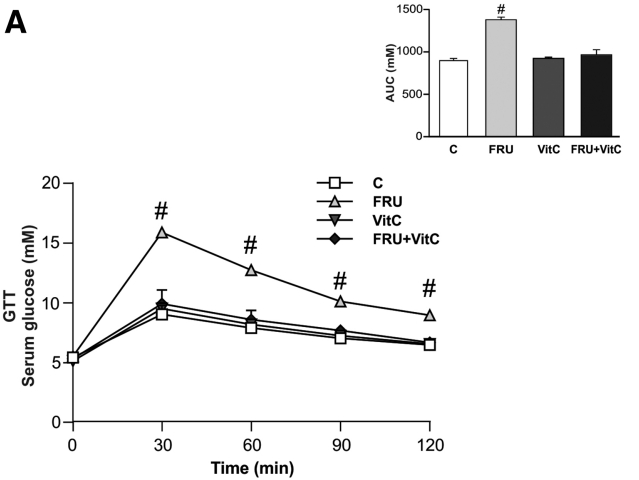
IL-1β levels
(% of C)
200
100
0
ω
C
FRU
FRU+GO
B
IL-6 levels
(% of C)
300
200
100
0
ω
&
C
FRU
FRU+GO

Figure 5


A
AUC (mM)
1500
1000
500
0
#
C
FRU
VitC
FRU+VitC
GTT
Serum glucose (mM)
20
15
10
5
0
#
#
#
#
C
FRU
VitC
FRU+VitC
0
30
60
90
120
Time (min)

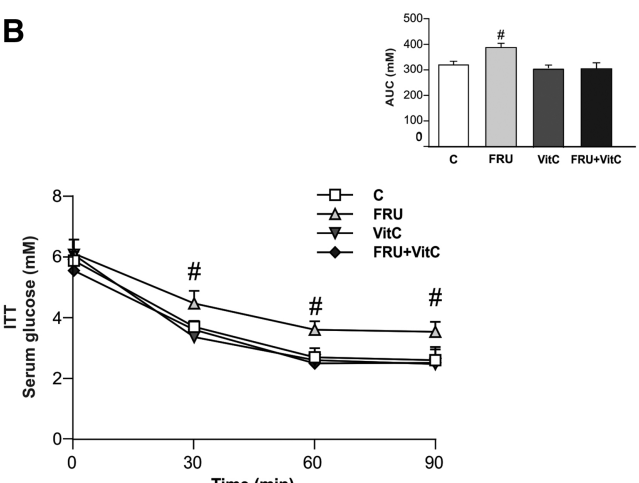

B
AUC (mM)
500
400
300
200
100
0
#
C
FRU
VitC
FRU+VitC
ITT
Serum glucose (mM)
8
6
4
2
0
#
#
#
C
FRU
VitC
FRU+VitC
0
30
60
90
Time (min)

Figure 6

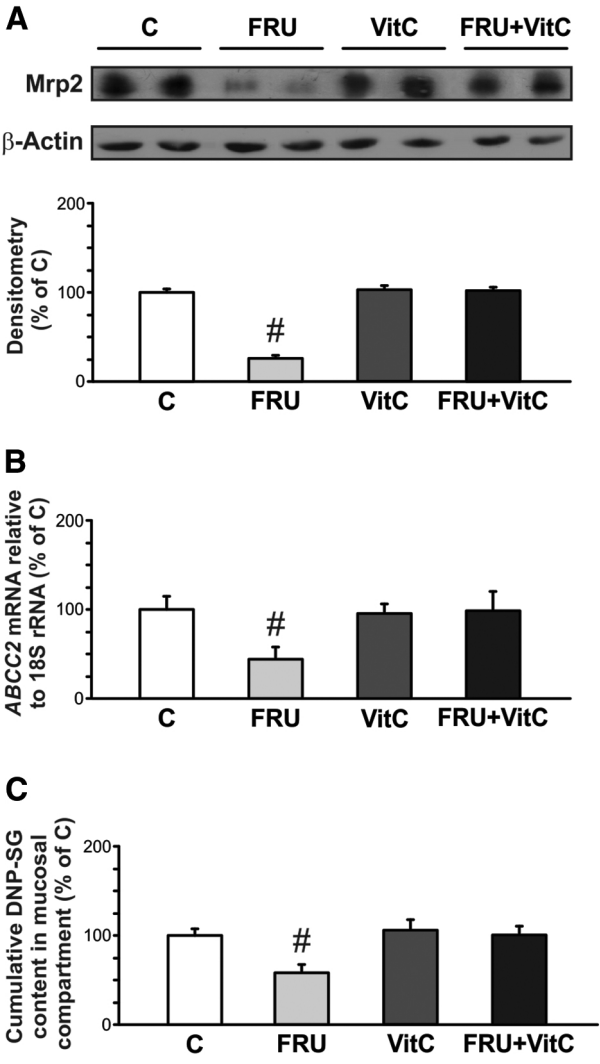

A
C
FRU
VitC
FRU+VitC
Mrp2
β-Actin
Densitometry (% of C)
200
100
0
#
C
FRU
VitC
FRU+VitC
B
ABCC2 mRNA relative to 18S rRNA (% of C)
200
100
0
#
C
FRU
VitC
FRU+VitC
C
Cumulative DNP-SG content in mucosal compartment (% of C)
200
100
0
#
C
FRU
VitC
FRU+VitC

Figure 7

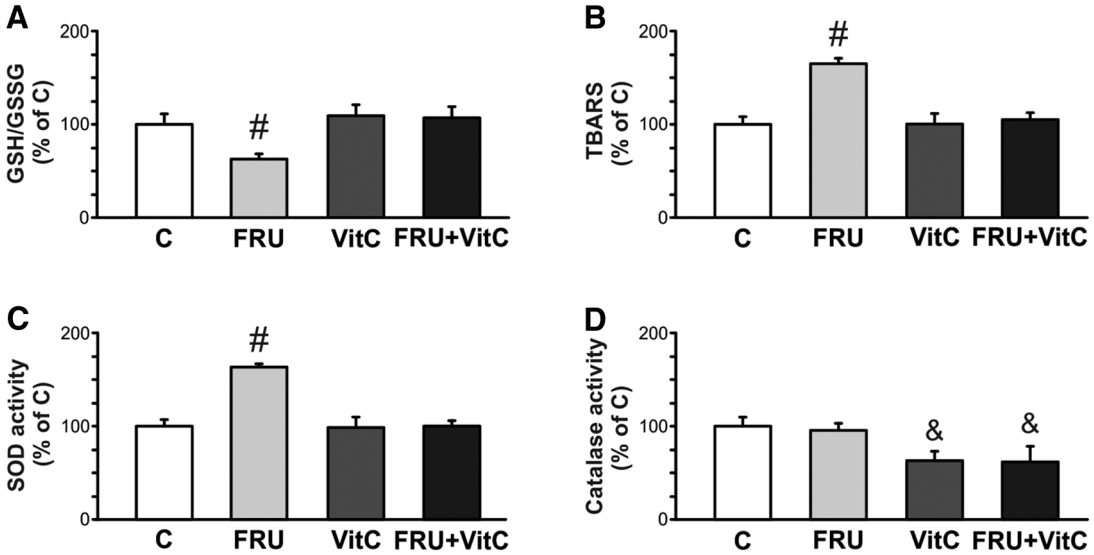

A
GSH/GSSG (% of C)
200
100
0
#
C
FRU
VitC
FRU+VitC
B
TBARS (% of C)
200
100
0
#
C
FRU
VitC
FRU+VitC
C
SOD activity (% of C)
200
100
0
#
C
FRU
VitC
FRU+VitC
D
Catalase activity (% of C)
200
100
0
&
&
C
FRU
VitC
FRU+VitC

Figure 8