LIQUID CARBOHYDRATE INTAKE MODIFIES TRANSSULFURATION PATHWAY BOTH IN PREGNANT RATS AND IN THEIR MALE DESCENDANTS

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C.B. conceived and designed the study. S.R., E.F., R.A., P.O. and M.I.P. contributed reagents/materials/analysis tools for gene expression studies and parameter analysis. L.R. handled the animals. M.I.P. and J.J.A-M analyzed the data. C.B. and E.F. wrote the paper. None of the authors have any conflicts of interest to report.

Abstract

Introduction: Fructose, alone or in combination with glucose, has been used as a source of added sugars to manufacture sugary drinks and processed foods. High consumption of simple sugars, mainly fructose, has been demonstrated to be one of the causes of developing metabolic diseases. Maternal nutrition is a key factor in the health of the progeny when adult. However, ingestion of fructose-containing foods is still permitted during gestation. Hydrogen sulfide (H_2S) is a gasotransmitter produced in the transsulfuration pathway with proved beneficial effects to fight metabolic diseases.

Methods: Therefore, maternal intake of liquid carbohydrates (glucose or fructose) and its effect in H_2S production both in the offspring and in the response of the progeny to a fructose supplementation were studied.

Results: This study shows how in pregnancy, either a fructose-rich diet *per se* or situations that produce an impaired insulin sensitivity such an excessive intake of glucose, decrease hepatic and placental production of H_2S. Further, this effect was also observed in liver of male offspring (both in fetal and adult stages). Interestingly, when these adult descendants were subjected to a high fructose intake, diminutions in H_2S synthesis in liver and adipose tissue due to this fructose intake were maternal consumption dependent.

Conclusions: Given H_2S is a protective agent against diseases such as diabetes, obesity, cardiovascular diseases and metabolic syndrome, the fact that carbohydrate consumption reduces H_2S synthesis both in pregnancy and in their progeny would have clear and relevant clinical implications.

Resumen

La ingesta de carbohidratos en forma líquida modifica la ruta de la transulfuración tanto en ratas gestantes como en su descendencia macho

Introducción: La fructosa, sola o en combinación con glucosa, se usa como fuente de azúcares añadidos para elaborar bebidas azucaradas y comidas procesadas. El elevado consumo de azúcares simples, sobre todo fructosa, se ha mostrado como una de las causas del desarrollo de enfermedades metabólicas. La nutrición materna es un factor clave en la salud de la descendencia adulta. Sin embargo, el consumo de alimentos que contienen fructosa está todavía permitido durante la gestación. El sulfuro de hidrógeno (H_2S) es un gasotransmisor producido en la ruta de la transulfuration con probados beneficios para luchar contra las enfermedades metabólicas.

Métodos: Por lo tanto, estudiamos la ingestión de carbohidratos líquidos (glucosa o fructosa) y su efecto en la producción de H_2S tanto en la descendencia como en la respuesta de ésta a una sobrecarga de fructosa.

Resultados: Este estudio muestra cómo en la gestación, una dieta rica en fructosa *per se* o situaciones en las que se produce una empeorada sensibilidad a la insulina tal como un consumo excesivo de glucosa, disminuyen la producción hepática y placentaria de H_2S. Más aún, este efecto también fue observado en el hígado de la descendencia macho (tanto en el estado fetal como en la edad adulta). Es destacable que, cuando esta descendencia adulta era sometida a una ingesta elevada de fructosa, las disminuciones en la síntesis de H_2S en hígado y tejido adiposo debidas a dicho consumo de fructosa eran dependientes del consumo materno.

Conclusiones: Dado que el H_2S es un agente protector contra enfermedades tales como la diabetes, la obesidad, enfermedades cardiovasculares y el síndrome metabólico, el hecho de que el consumo de carbohidratos reduzca la síntesis de H_2S tanto en la gestación como en su descendencia tendría claras implicaciones clínicas.

Abbreviations

CC: male adult (261-day-old) offspring from control dams that consumed standard pellet and water without additives for their entire lives, including the last 21 days (from 240 to 261 days of age).

CF: male adult (261-day-old) progeny from control dams that consumed standard pellet and water without additives for their entire lives, except for the last 21 days when they ingested water containing 10% fructose.

FF: male adult (261-day-old) offspring from fructose-fed mothers that consumed standard pellet and water without additives for their entire lives, except for the last 21 days when they ingested water containing 10% fructose.

GF: male adult (261-day-old) offspring from glucose-fed mothers that consumed standard pellet and water without additives for their entire lives, except for the last 21 days when they ingested water containing 10% fructose

1. Introduction

Changes in food patterns and unhealthy diets are the main causes of the rising cases of metabolic syndrome (MetS), obesity and non-alcoholic fatty liver disease (NAFLD) all around the world, the excessive intake of sugar-sweetened beverages (SSB) being one the main sources (1). Fructose is used as a source of added sugar in the form of High Fructose Corn Syrup (HFCS) and sucrose and a close relationship between its consumption and higher risk of suffering cardiometabolic risk factors has been established (2). Moreover, according to Developmental Origins of Health and Disease (DOHaD) theory, the mother's diet during pregnancy plays a key role in the correct development of the offspring when adult, mainly due to epigenetic changes (3). Indeed, many studies (including some ours) have established an association between fructose intake during pregnancy and detrimental effects in the fetuses and adult descendants (4, 5). We have previously seen features of MetS in male, but not female, offspring from fructose fed mothers, such as an impaired insulin signaling, hypoadiponectinemia and steatosis (6). Thus, although a connection between a high consumption of fructose containing beverages and the global epidemic of obesity and metabolic syndrome could exist (5, 7), ingestion of these beverages and fruit juices is still permitted during gestation.

Metabolism is regulated by different chemical species, but only three of them are gasotransmitters: nitric oxide (NO), carbon monoxide (CO), and hydrogen sulfide (H_2S). They are produced enzymatically, are able to cross the cell membrane by simple diffusion and to modulate cellular function (8). Among them, H_2S has increased its relevance in the last years due to its emerging role in cardiovascular diseases, due to its anti-inflammatory, antioxidant and vasodilatory effects (9). This gasotrasmitter is synthesized in the transsulfuration pathway in many organs such as liver, kidney, heart and adipose tissue. There are three main enzymes involved in its formation: cystathionine beta-synthase (CBS), cystathionine gamma-lyase (CSE), which work in concert to transform Homocysteine (HCy) into H_2S, and 3-mercaptopyruvate sulfurtransferase (3-MST) which uses mercaptopyruvate to produce H_2S (8). Diminutions in H_2S levels and lower expression of transsulfuration enzymes have been linked to an impaired insulin sensitivity in adipose tissue (10). In the liver, H_2S donor administration

has been demonstrated to decrease liver steatosis by a reduction of lipid production and an activation of the antioxidant systems activity in mice treated with a High-Fat Diet (11). However, some studies have shown increases in CSE expression and H_2S production in insulin resistance animal models (12). Interestingly, H_2S has been proved to have beneficial effects in cardiovascular diseases (CVD). It works as a cardioprotective factor by reducing oxidative stress, inflammation, fibrosis and apoptosis (13, 14). Taking into account all these effects of H_2S, some authors have proposed it as a novel agent for therapy for hypertension, atherosclerosis, myocardial hypertrophy and heart failure by administering sulfur donors or increasing endogenous H_2S production (15).

Not many studies have evaluated the transsulfuration pathway after fructose intake and, even fewer, if the DOHaD theory could be involved. Therefore, we have investigated here whether maternal liquid fructose (10% wt/vol in drinking water) intake affects this pathway in pregnant rats and their fetuses, and if it differs from glucose consumption (10% wt/vol in drinking water) or water without additives (control). In addition, in order to study a possible fetal programming, the H_2S production in several tissues was measured in adult male offspring of mothers having consumed different carbohydrates. Subsequently, we wanted to know if males whose mothers had consumed water without additive, fructose or glucose during gestation maintained the same phenotype after themselves receiving fructose (10% wt/vol in drinking water) for three weeks, when adults.

2. Material and methods

2.1. Animals and experimental design

An animal model of maternal liquid fructose intake was developed as previously described (5, 6). Female Sprague-Dawley rats weighing 200-240 g were fed *ad libitum*, a standard rat chow diet (B&K Universal, Barcelona, Spain), and housed under controlled light and temperature conditions (12-h light-dark cycle; 22 ± 1ºC). The experimental protocol was approved by the Animal Research Committee of the University San Pablo-CEU, Madrid, Spain (ref. number 10/206458.9/13).

Pregnant animals were randomly separated into a control group, a fructose-supplemented group (Fructose), and a glucose-supplemented group (Glucose) (five to six rats per group). Fructose and glucose were supplied as a 10% (wt/vol) solution in drinking water throughout gestation. The concentration used here (10% w/v) is very close to that of sugar-sweetened beverages (SSB). Control animals received no supplementary sugar. The total amount of ingested energy did not differ between fructose-fed, glucose-supplemented and control rats, as described in (5) and the amount of total calories obtained from simple sugars was around 25% for the fructose-group and 35% for the glucose-group (16). On gestational day 21, food was removed at 8 a.m. and pregnant rats were sacrificed two hours later. Plasma was obtained by blood centrifugation in Na_2-EDTA tubes, aliquoted and stored at -80ºC until analysis. Liver and adipose tissue were obtained, placed in liquid nitrogen and kept at -80 ºC until analysis. The conceptus was dissected, and placentas and fetuses were collected. Placentas from the same litter were pooled and frozen. Fetuses (without being separated by gender) were decapitated. Blood from all pups of the same mother was collected and plasma was obtained in tubes containing Na_2-EDTA. The livers of the fetuses were obtained, and those coming from the same mother were pooled and placed in liquid nitrogen to be frozen until processed for further analysis (5, 16).

Other set of pregnant rats was allowed to deliver and on the day of birth, each suckling litter was reduced to nine pups per mother. After delivery, both mothers and their pups were maintained with water and food *ad libitum*. At 21 days of age, pups were separated by gender and kept fed on a standard rat chow diet (B&K Universal, Barcelona, Spain) and water without additives. Animals within each experimental group were born to different dams to minimize the “litter effects”. At 240 days of age, one half of the male progeny was randomly separated. When the progeny was 261-days-old, it was sacrificed and blood and tissues were collected. Remarkably, these animals had received no subsequent additive in the drinking water for their entire lives (17).

The other half of the male progeny was subjected to the next protocol: First, they were weighed and an aliquot of plasma was obtained from the tail in order to confirm that the values between the experimental groups both for body weight and for several analytes (glucose, triglycerides, non-esterified fatty acids, etc) were similar (18). Later, independently from the experimental group of mothers to which they had been born,

they were maintained on solid pellets and supplied with drinking water containing 10% (wt/vol) fructose. Thus, three experimental groups were formed: C/F, F/F, G/F, the first letter indicating whether the mothers had been supplied during pregnancy with tap water (C, control), or water containing a carbohydrate (F: fructose; G: glucose); and the second letter indicating the period with fructose (F), when they were adults. When the progeny was 261-days-old, they were decapitated at 10 a.m. and blood collected using tubes containing Na_2-EDTA. Prior to sacrifice, food was removed at 8 a.m. The period with fructose supplementation was selected to last 21 days (from 240 to 261 days of age) as previously described (18). Liver and lumbar and epididymal adipose tissues were immediately removed, placed in liquid nitrogen and kept at -80 ºC until analysis. Samples were then centrifuged, and plasma was stored at -80 ºC until processed for later determinations. In parallel, a fourth experimental group was used, C/C: male progeny from control mothers supplied with water without any additives when adult (18, 19). There was no difference in the total amount of ingested energy between fructose-fed rats and control (C/C) group (18) and, for the three groups of fructose-fed males (C/F; F/F, G/F), around 25% of the total amount of energy was acquired from fructose (18) and this is similar to the daily energy intake from simple sugars observed in heavy consumers of SSB in human populations (20-25%) (16).

2.2. Plasma determinations

Plasma H_2S levels were measured with the methylene blue method as previously described with modifications (20). Briefly, 200 μL of plasma were deproteinized with 150 μL 20% TCA. After centrifugation, 250 μL of supernatant were treated with 150 μL 1% zinc acetate, 100 μL 20 mM N1,N1- dimethylbenzene-1.4-diamine sulfate (Fluorochem, UK) in 7.2 M HCl and 133 μL 30 mM $FeCl_3$ (Panreac AppliChem, IL, USA) in 1.2 M HCl. Samples were then vortexed and incubated at room temperature for 20 minutes and absorbance measured at 630 nm. A standard curve from 1.56 to 200 μM of sodium hydrosulfide (NaHS, Fluorochem, UK) was performed following the same procedure as with the samples.

2.3. Determination of H_2S production in liver, adipose tissue and placenta

H_2S production in liver, lumbar and epididymal adipose tissue and placenta was evaluated following the Lead Sulfide method as previously described with modifications (21). Briefly, tissues were homogenized in Phosphate-buffered saline (PBS) and their protein levels measured with the BCA Assay Kit (Thermo Fisher, MA, USA). One hundred to 300 μg of protein were incubated at 37ºC in the presence of 10 mM Cys (Sigma-Aldrich, MO, USA) or 30 mM HCy (Fluorochem, UK), and 20 μM Pyridoxal 5'-phosphate (PLP, Sigma-Aldrich, MO, USA) (for livers) or 2 mM PLP (for adipose tissue and placenta) on 96 well plates covered with a lead acetate (II) membrane. Incubations were performed until dots of lead sulfide were detected but not saturated, and this occurred after 2 h for liver, 6 h for adipose tissue and 3 h for placenta. In order to prepare these membranes, Whatman nº 2 paper was soaked into 20 mM lead acetate (Sigma-Aldrich, MO, USA) and vacuum dried. Dots were densitometered (BioRad Densitomiter G-800, CA, USA) for quantification. A standard curve from 0 to 1000 mM NaHS was performed for each membrane.

2.4. RNA extraction and gene expression determination by qPCR

Total RNA was isolated from liver or placenta using Ribopure (Invitrogen, ThermoFisher Scientific, USA). Total RNA was subjected to DNase I treatment using Turbo DNA-free (Invitrogen, ThermoFisher Scientific, USA), and RNA integrity was confirmed by agarose gel electrophoresis. Afterwards, cDNA was synthesized by oligo(dT)-primed reverse transcription with Superscript II (Invitrogen, ThermoFisher Scientific, USA). qPCRs were performed using a Light Cycler 1.5 (Roche, Germany). The reaction solution was carried out in a volume of 20 μl, containing 10 pmol of both forward and reverse primers, 10x SYBR Premix Ex Taq (Takara Bio Inc., Japan) and the appropriate nanograms of the cDNA stock. Rps29 was used as a reference gene for qPCR. The primer sequences were obtained either from the Atlas RT-PCR Primer Sequences (Clontech, CA, USA) or designed using Primer3 software (University of Massachusetts Medical School, MA, USA) (22). Samples were analysed in duplicate on each assay. Amplification of non-specific targets was discarded using the melting curve analysis method for each amplicon. qPCR efficiency and linearity were assessed by optimization

of the standard curves for each target. The transcription was quantified with Light Cycler Software 4.05 (Roche, Germany) using the efficiency correction method (23).

2.5. Statistical Analysis

Results were expressed as means ± S.E. Treatment effects were analyzed by one-way analysis of variance (ANOVA). When treatment effects were significantly different ($P < 0.05$), means were tested by Tukey´s multiple range test, using the computer program SPSS (version 23). When the variance was not homogeneous, a *post hoc* Tamhane test was performed.

3. Results

3.1. Carbohydrate intake during pregnancy decreases H_2S production in liver and placenta, but not in lumbar adipose tissue.

Hydrogen sulfide is a gasotransmitter produced in the transsulfuration pathway with proved beneficial effects in many metabolic diseases. However, plasma H_2S levels hardly changed in pregnant rats after fructose or glucose consumption throughout gestation (11.90 $\pm$ 0.78; 11.84 $\pm$ 0.28; 10.94 $\pm$ 0.38 μM for control, fructose-, and glucose-fed mothers, respectively). Surprisingly, H_2S production in the liver presented a significant decrease in the two groups of rats that consumed any liquid carbohydrate during gestation in comparison to the control dams (Figure 1A). This organ is the main producer of H_2S in the organism (24). However, the expression of the three main enzymes involved in the transsulfuration pathway did not display significant differences (Table 1) in this tissue, although CSE gene expression did show a tendency to decrease in fructose-fed pregnant rats. The results obtained in plasma could be related to other tissues that are known to produce this gasotransmitter, although at a lower level, such as adipose tissue (10). In fact, H_2S production in lumbar adipose tissue (Figure 1B) did show a trend to be increased in the two carbohydrate-consuming groups, becoming almost significant when fructose-fed pregnants were compared to control rats ($P = 0.075$).

In order to evaluate the importance of the placenta in the production of H_2S during gestation, both synthesis of H_2S and transsulfuration enzymes gene expression were measured. In accordance with the findings observed in liver, placental H_2S production was also diminished in carbohydrate-fed dams (Figure 1C), becoming significant in fructose-fed pregnant rats and almost significant ($P = 0.093$) in glucose-fed mothers, in comparison to the control rats. As shown in Table 1, CBS gene expression presented an increase in placenta of fructose-fed pregnant rats and it was significant when compared to glucose-fed mothers. On the other hand, CSE and 3MST gene expression did not show significant changes between the three experimental groups (Table 1). Nevertheless, gene expression of the three enzymes of the transsulfuration pathway in placenta displayed lower levels than in liver.

As fructose is able to cross the placenta and it can also be produced there (25), all these changes observed after carbohydrate consumption during gestation could also be affecting the fetuses. Thus, H_2S production and transsulfuration enzymes gene expression were evaluated. In accordance with the findings found in maternal liver and placenta, H_2S production in the liver of fetuses from carbohydrate-fed mothers tended to be decreased when compared to those from control dams (Figure 1D), although without becoming significantly different. However, neither CBS nor CSE or 3MST gene expression presented changes in liver of fetuses from fructose-, glucose-fed or control mothers (Table 1).

3.2. Carbohydrate ingestion during gestation produces changes in the transsulfuration pathway in adult male offspring.

Bearing in mind the results observed in the liver of fetuses from fructose- or glucose-fed mothers, we wanted to evaluate if these changes were maintained in male offspring from carbohydrate-fed dams when adult. As shown in Figure 2A, H_2S production in the liver presented a non-significant decrease in males from fructose- or glucose-fed mothers, in consonance with the effects found in fetal liver (Figure 1D). Plasma H_2S levels were reduced in males from glucose-supplemented mothers in comparison to the other two groups (14.53 ± 2.11; 14.61 ± 0.21; 12.53 ± 0.55 μM for males from control, fructose- and glucose-fed dams, respectively; $P < 0.05$, glucose vs.

fructose). When gene expression was measured in the liver of male descendants, CBS did show a significant increase in male offspring from glucose-fed mothers compared to control and fructose groups, and 3MST displayed higher and significant gene expression in male progeny from carbohydrate-fed mothers versus control offspring (Table 2). This result could be in contrast with the findings observed for H_2S production in the liver but CSE, the main enzyme involved in transsulfuration pathway in the liver (26), did show a slight reduction in males from fructose-fed mothers, although this did not become significant (Table 2). On the other hand, although lumbar (Figure 2B) adipose tissue did not present significant changes in H_2S production between the three groups, epididymal fat pads displayed a non-significant trend to be increased in progeny from carbohydrate-fed rats in comparison to the control group (Figure 2C).

3.3. Fructose consumption in male adult rats causes a maternal-intake dependent reduction in H_2S production in liver and adipose tissue.

After evaluating the transsulfuration pathway in adult male descendants from carbohydrate-fed mothers, we wanted to discover if that phenotype was conserved by exposing male offspring from control, fructose- or glucose-fed mothers to a fructose consumption and to check if maternal diet influences the response to this fructose exposure.

Plasma H_2S levels were not affected by fructose ingestion, although they tended to be higher, but not significantly, independently of the maternal consumption (13.34 ± 0.63; 17.72 ± 3.08; 22.22 ± 2.81; 25.06 ± 5.42 μM for C/C, C/F, F/F and G/F, respectively). Surprisingly, the hepatic production of this gasotransmitter did seem to present the opposite result to that of plasma data, as shown in Figure 3A. H_2S production was diminished in the liver of male offspring from control, fructose- and glucose-fed mothers after fructose intake (Figure 3A), this reduction being more pronounced in F/F group. In fact, the effect became almost significant when F/F was compared to G/F group ($P = 0.065$). Moreover, these findings were corroborated when H_2S production was measured using HCy as a substrate. It is well known that CSE and CBS use Cys as substrate, whereas HCy is substrate only for CSE (26). Interestingly, the same results obtained for H_2S synthesis from Cys (Figure 3A) were observed when HCy was used as a substrate of the reaction (Figure

3B). In order to evaluate if these changes were caused by modifications in the trassulfuration pathway enzymes, CBS, CSE and 3-MST gene expression were measured. None of these enzymes presented significant changes in its gene expression (Table 3), although CSE, the main enzyme involved in H_2S production in the liver (26), tended to be decreased in liver from males that consumed fructose when adult and which mothers had ingested water (C/F) or liquid fructose (F/F) during gestation. Interestingly, H_2S production in adipose tissue responded to the fructose supplementation in a maternal-intake dependent manner. Thus, both lumbar (Figure 3C) and epididymal adipose tissues (Figure 3D) displayed a fructose-induced significant reduction in H_2S production in male descendants from fructose (F/F) and glucose (G/F) fed mothers, but not in C/F, when compared to C/C group.

4. Discussion

In the last decades, obesity, MetS, CVD and diabetes have escalated to epidemic proportions in many countries worldwide (27). The DOHaD theory has linked maternal nutrition to the risk of suffering metabolic diseases in descendants when adult. According to this, early life (pre- and post-natal) nutrition would produce molecular adaptations in pups that will predispose them to a higher risk of metabolic disturbances in later life (3, 28). Consumption of SSB and processed foods has increased in recent years and many studies have established a relationship between their consumption and the development of metabolic diseases (17, 29, 30).

H_2S is a gasotransmitter whose reduction in plasma has been related to obesity and type 2 diabetes and it seems to protect against the pathogenesis of atherosclerosis (31). Therefore, our goal in the present study is to investigate whether maternal fructose leads to changes in the transsulfuration pathway, which produces H_2S, in their pups and if these modifications, if any, are preserved later, when adult. In the present study, neither fructose- nor glucose-fed mothers presented changes in their H_2S plasma levels, however a significant reduction in the production of this gasotrasmitter was observed both in liver and in placenta. This discrepancy between H_2S plasma levels and its production in liver has been previously reported by Peh et al, but using a high fat diet (32), and they proposed that it could be caused by H_2S produced in non-hepatic tissues

(33), such as adipose tissue, kidney or gut. In fact, lumbar adipose tissue presented in our carbohydrate-fed mothers a non-significant increase in the H_2S production. In contrast to Peh et al (32), we did not observe significant changes in the gene expression of the transsulfuration pathway enzymes in neither liver nor placenta. Nevertheless, modifications in H_2S synthesis have been related not only with the gene expression of transsulfuration pathway but also with the activity of these enzymes (34). Moreover, H_2S has been inversely related to oxidative stress (34) and, in a previous report, we have showed an imbalanced redox status in these carbohydrate-fed pregnant rats (16).

We and others have demonstrated that maternal fructose produces profound molecular and metabolic changes in the fetuses (5, 16, 29), but there are no studies that evaluate its relationship with imbalances in the transsulfuration pathway in the offspring. Our findings indicate a slight reduction in H_2S production in the liver of fetuses from fructose- and glucose-fed mothers and, interestingly, this diminution induced by maternal carbohydrate intake was maintained in male descendants when adult. These results would indicate an example of fetal programming in the offspring. In fact, we have recently demonstrated that maternal fructose is able to modify the methylation state of gene promoters of key proteins in cholesterol metabolism in the adult progeny (35).

Bearing in mind that epigenetic modifications can cause different responses to environmental factors (36), we have demonstrated that the fructose-induced diminution in H_2S production observed here in liver and adipose tissue of male rats is a maternal nutrition-dependent effect. Beneficial effects of H_2S in cardiovascular events have been widely studied (37, 38), so a reduction in the gasotransmitter levels could be one of the links between fructose consumption and symptoms of CVD and MetS. In fact, we have previously reported that fructose intake provokes characteristic features of MetS, such as oxidative stress and lipid abnormalities, in the adult offspring and that these imbalances are influenced by maternal intake of carbohydrates (18, 19). However, H_2S plasma levels were not reduced by fructose consumption independently of that had been taken by their mothers. Therefore, more specific studies are needed to connect the diminution in H_2S production, H_2S plasma levels and characteristics of MetS observed in our rats. Nevertheless, as mentioned above, other tissues that can also produce H_2S could be involved (32). In addition, reductions in H_2S production have been related to insulin resistance in adipose tissue (10) and, interestingly, our animals which

were fed liquid fructose showed such a decrease. We were not able to detect systemic insulin resistance in these male rats (18), but it could possibly exist locally, in adipose tissue.

The administration of food containing 75% fructose in rats conduced to a reduction in CSE levels in thoracic aortas producing an impaired endothelium-dependent vasorelaxation (39) has been described and, further, a diet with 65% fructose did lead to oxidative stress and insulin resistance in rats with decreased levels of H_2S in plasma (40). In the present study, we reported similar alterations in the transsulfuration pathway both in liver and adipose tissue but using a much lower fructose amount (10% w/v). The reduction in H_2S production observed here in the liver could be due to a diminished CSE, the main transsulfuration enzyme (26), gene expression in C/F and F/F groups, but not in G/F. Nevertheless, since H_2S is generated from HCy (41), this reduction of H_2S production could possibly be related to lower plasma levels of HCy which we have found in the three groups that received fructose (data not shown). Furthermore, modifications described here in H_2S synthesis could also be due to changes in activity of the enzymes in the transsulfuration pathway, where fructose would be affecting it directly or indirectly through diverse factors such as calcium, oxidative stress, hormones or glucose (34). Interestingly, the effects observed here for H_2S in liver and adipose tissue are probably more relevant than if we had found them in plasma (32). Whereas plasma H_2S levels are the sum of production from many tissues, H_2S synthesis is key in the functioning of the tissue. This would be the situation for, among others, the aorta and relaxation of endothelium, the adipose tissue and insulin sensitivity, the kidney and blood pressure, and the placenta and preeclampsia (10, 39, 42, 43). In fact, although we did not see changes in plasma H_2S levels, the fructose-induced modifications in tissue production of H_2S found here would parallel the low levels of plasma urea (38.5 ± 0.42; 20.9 ± 0.90; 28.9 ± 2.1; 30.5 ± 2.4 mg/dL for C/C, C/F, F/F and G/F, respectively; $P < 0.05$, C/C vs. C/F and F/F; $P < 0.05$, C/F vs G/F). Since it is known that ammonia, produced along with the H_2S in the transsulfuration pathway, participates in the synthesis of urea, these results observed for urea are confirmatory of the reduced transsulfuration pathway after a fructose intake.

In summary, this study shows in pregnancy that a fructose-rich diet *per se* or situations producing an impaired insulin sensitivity such an excessive intake of glucose

(5) generate a clear diminution in the hepatic and placental production of H_2S. And this effect is observed not only in pregnant mothers, but also in male offspring (both in fetal and in adult stages) and, mainly, when their offspring are themselves subjected to a high fructose intake. Interestingly, these results correlate with others published where proatherogenic diets also caused H_2S depletion (32, 33, 40, 44). Given this gasotransmitter has been proposed as a possible treatment for CVD and as a protective factor in developing MetS alterations (37), the importance of these findings lies in the escalating worldwide intake of SSBs and its, increasingly clearer, relationship with non-communicable diseases (2, 45). Altogether, the clinical significance of the transsulfuration pathway studies and its connection with fructose intake should be taken into consideration.

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C.B. conceived and designed the study. S.R., E.F., R.A., P.O. and M.I.P. contributed reagents/materials/analysis tools for gene expression studies and parameter analysis. L.R. handled the animals. M.I.P. and J.J.A-M analyzed the data. C.B. and E.F. wrote the paper. None of the authors have any conflicts of interest to report.

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Figure Legends

Figure 1: Carbohydrate (fructose or glucose) consumption during pregnancy affects H_2S production in the liver and placenta of pregnant rats, but not adipose tissue. A) Hepatic H_2S production and membrane showing lead acetate precipitates formed in the assay. Darker precipitates indicate higher H_2S production in the tissue. B) Lumbar adipose, C) Placental and D) Fetal hepatic H_2S production of control (empty bar), fructose-fed (grey bar) or glucose-fed (black bar) pregnant rats. Data are means ± S.E. from 6-7 litters. Values not sharing a common letter are significantly different ($P < 0.05$).

Figure 2: Carbohydrate intake during pregnancy influences H_2S production in the liver and adipose tissue of male offspring. A) Hepatic, B) lumbar and C) epididymal adipose tissues H_2S production of 261-day-old male progeny from control (empty bar), fructose-fed (grey bar) and glucose-fed (black bar) pregnant rats. Data are means ± S.E. from 5-6 litters. Values not sharing a common letter are significantly different ($P < 0.05$).

Figure 3: Maternal carbohydrate intake differentially affects fructose-induced diminution of H_2S production in the progeny. A) Hepatic H_2S production from Cys and membrane showing lead acetate precipitates formed in the assay. Darker precipitates indicate higher H_2S production in the liver. B) Hepatic H_2S production from HCy. C) Lumbar and Epididymal adipose tissues H_2S production of control (CF, light grey bar), fructose- (FF, black bar), and glucose-supplemented (GF, dark grey bar) mothers. C/C: Control 261-day-old male offspring from control pregnant rats (empty bar, C/C). Data are means ± S.E. from 5-6 litters. Values not sharing a common letter are significantly different ($P < 0.05$). Cys: cysteine. HCy: homocysteine.

Figura 1- a color

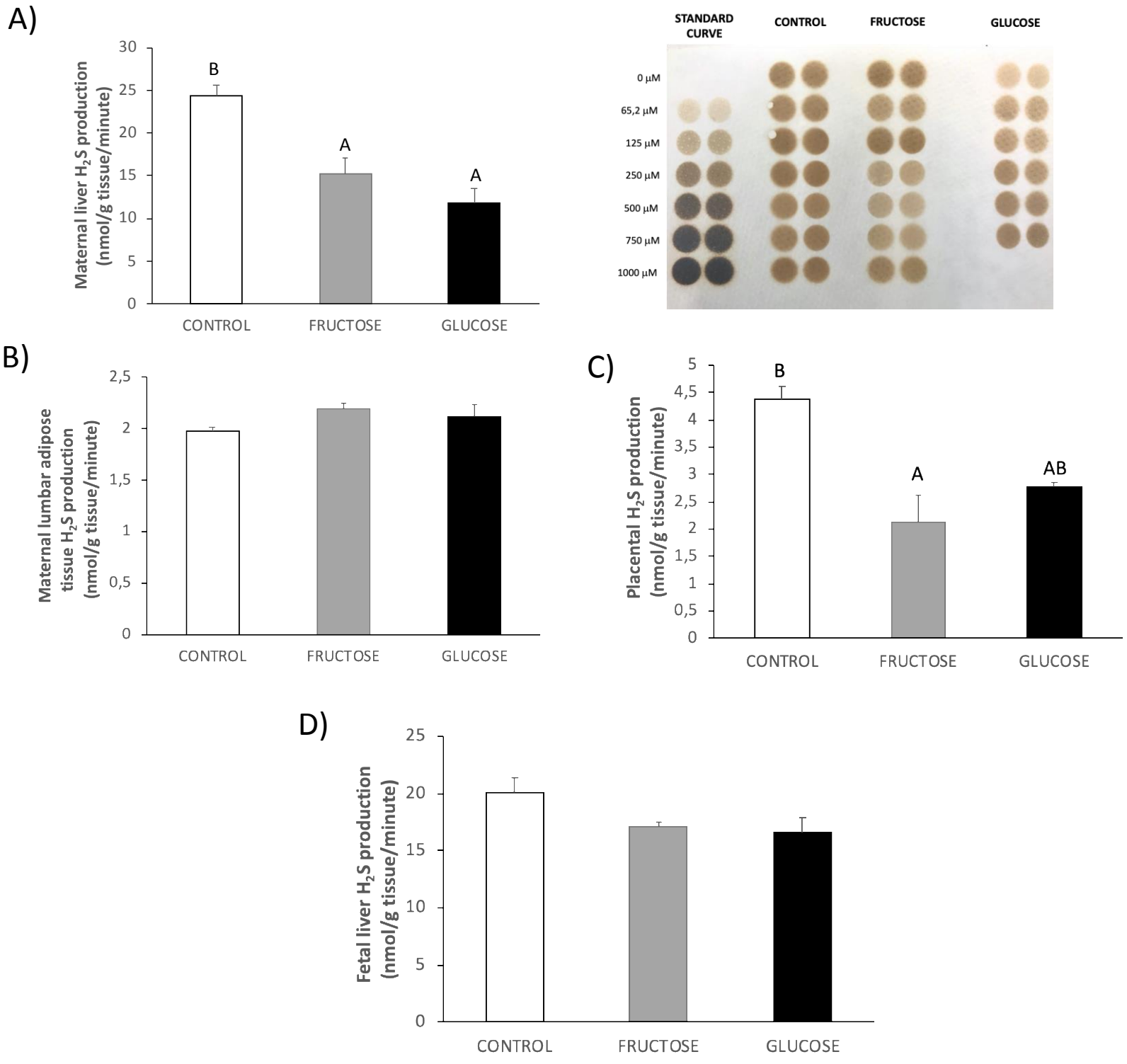
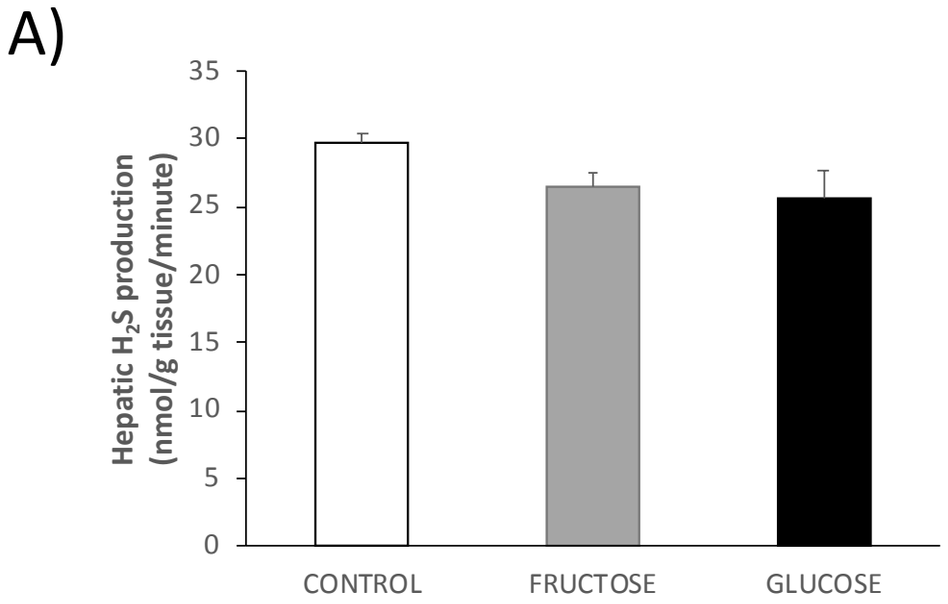
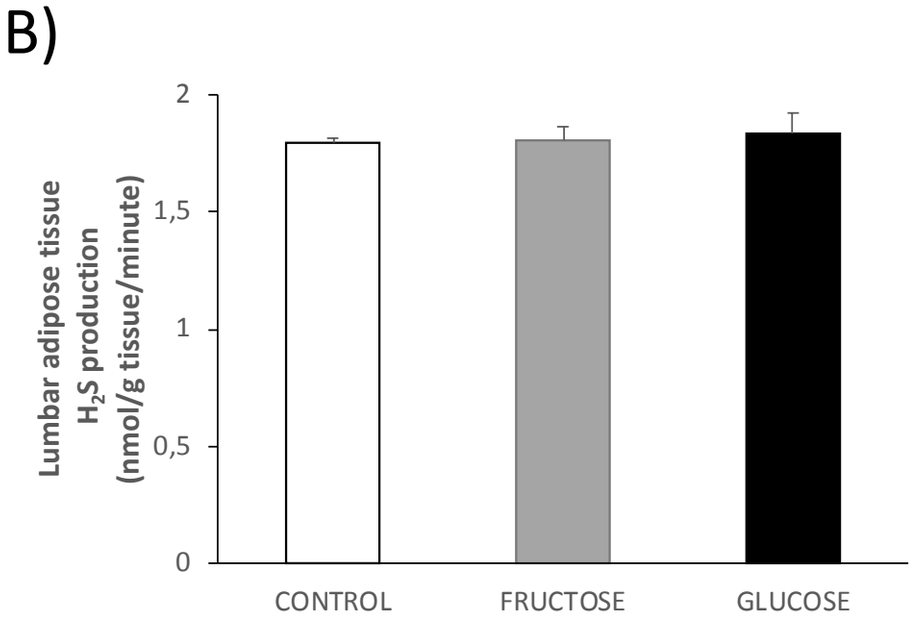


Figura 2


A)
Hepatic H_2S production (nmol/g tissue/minute)
35
30
25
20
15
10
5
0
CONTROL
FRUCTOSE
GLUCOSE


B)
Lumbar adipose tissue H_2S production (nmol/g tissue/minute)
2
1,5
1
0,5
0
CONTROL
FRUCTOSE
GLUCOSE

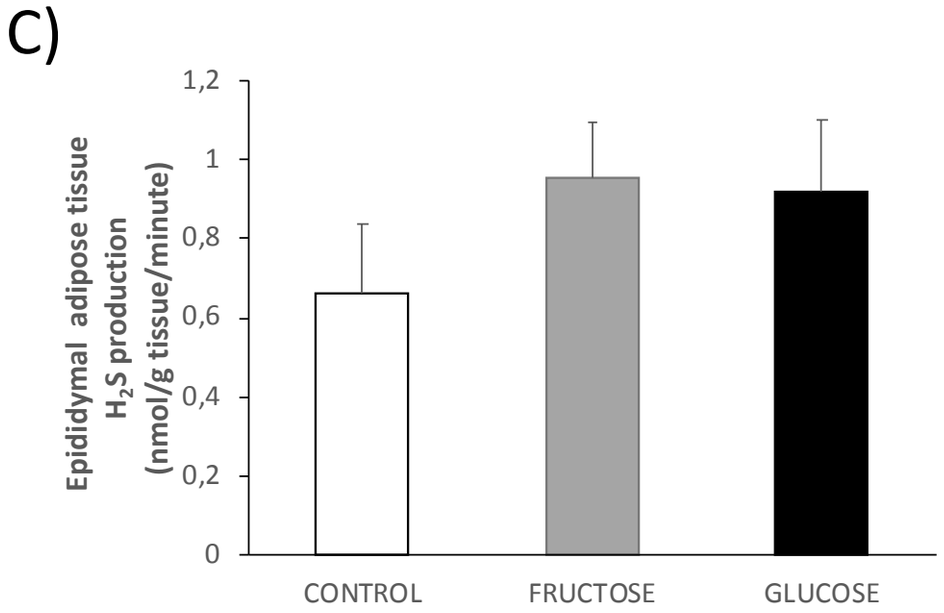

C)
Epididymal adipose tissue H_2S production (nmol/g tissue/minute)
1,2
1
0,8
0,6
0,4
0,2
0
CONTROL
FRUCTOSE
GLUCOSE

Figura 3- a color

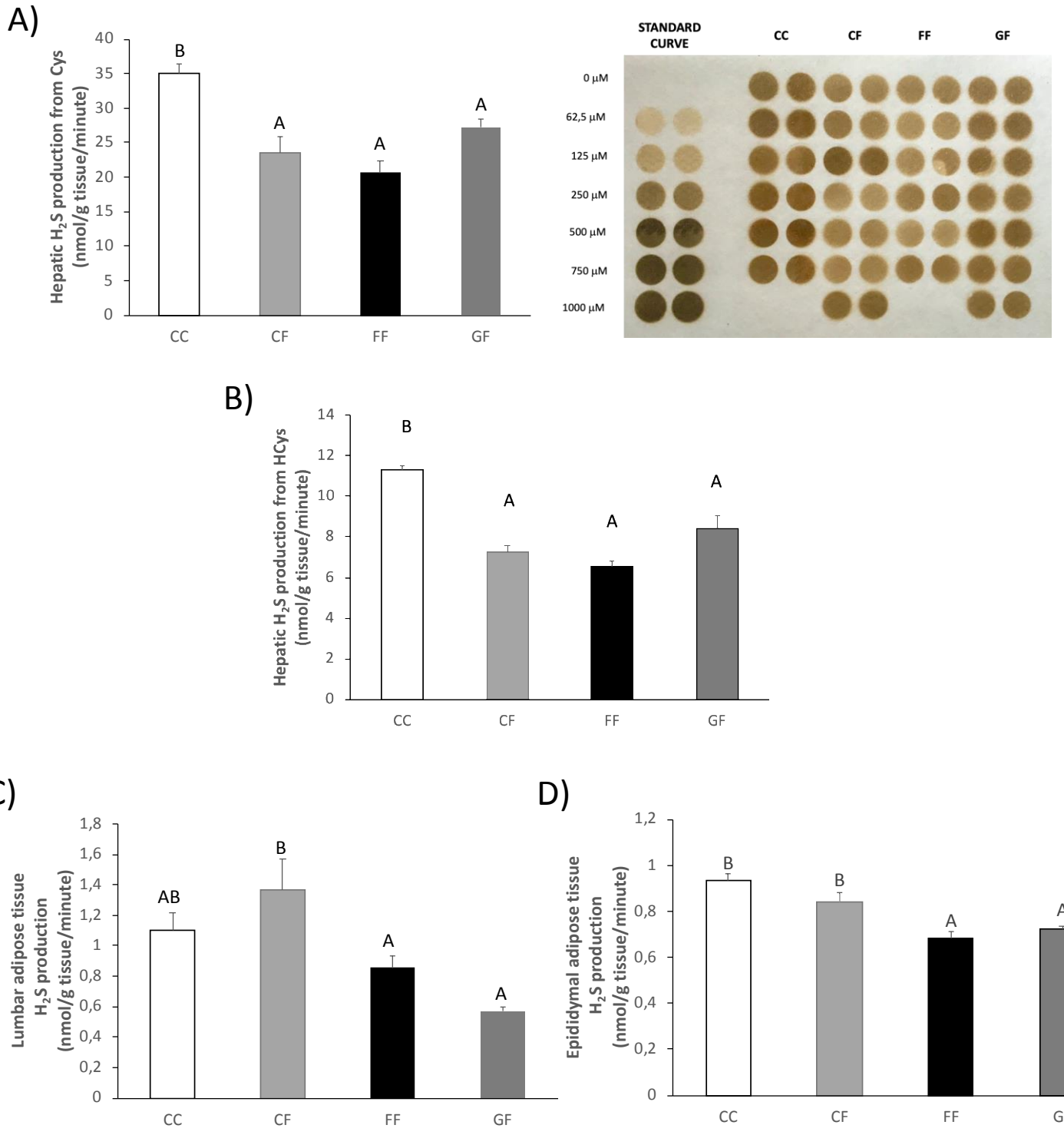


Table 1
Gene expression (mRNA) of the transsulfuration pathway in the liver and placenta of control, fructose- or glucose-supplemented pregnant rats and their fetuses.

	Control	**Fructose**	**Glucose**
Maternal liver expression			
CBS (a.u.)	3,92 ± 0,36	3,13 ± 0,53	3,06 ± 0,73
CSE (a.u.)	41,15 ± 3,80	31,50 ± 1,90	43,48 ± 8,17
3-MST (a.u.)	5,66 ± 0,11	6,11 ± 0,15	7,02 ± 0,57
Fetal liver expression			
CBS (a.u.)	0,504 ± 0,024	0,613 ± 0,100	0,383 ± 0,040
CSE (a.u.)	44,33 ± 6,16	49,10 ± 2,02	42,45 ± 3,84
3-MST (a.u.)	2,36 ± 0,16	2,41 ± 0,18	2,62 ± 0,16
Placental expression			
CBS (a.u.)	0,008 ± 0,001[a]	0,023 ± 0,008[ab]	0,005 ± 0,000[b]
CSE (a.u.)	0,069 ± 0,011	0,097 ± 0,013	0,099 ± 0,023
3-MST (a.u.)	0,715 ± 0,033	0,793 ± 0,092	0,672 ± 0,046

M ± SE, n = 5-6 pregnant rats; n = 5-6 liver pools of the fetuses of the same litter; n = 5-6 placental pools of the same litter. Different letters indicate significant differences between the groups ($P< 0.05$). Relative target gene mRNA levels were measured by Real Time PCR, as explained in Materials and Methods, and normalized to Rps29 levels and expressed in arbitrary units (a.u.). CBS: cystathionine beta-synthase; CSE: cystathionine gamma-lyase; 3-MST: 3-mercaptopyruvate sulfurtransferase.

Table 2
Liver gene expression (mRNA) of the transsulfuration pathway of 261-day-old male progeny from fructose- or glucose-supplemented and control mothers

	Control	**Fructose**	**Glucose**
CBS (a.u.)	$3,29 \pm 0,97^{a}$	$3,85 \pm 0,92^{a}$	$7,61 \pm 0,53^{b}$
CSE (a.u.)	$163,4 \pm 21,1$	$115,7 \pm 24,8$	$160,5 \pm 9,8$
3-MST (a.u.)	$6,70 \pm 0,66^{a}$	$9,24 \pm 0,54^{b}$	$10,24 \pm 0,13^{b}$

Data are M ± SE, male rats from 5-6 litters. Relative target gene mRNA levels were measured by real time PCR, as explained in Materials and Methods, and normalized to Rps29 levels and expressed in arbitrary units (a.u.). Different letters indicate significant differences between the groups ($P < 0.05$). CBS: cystathionine beta-synthase; CSE: cystathionine gamma-lyase; 3-MST: 3-mercaptopyruvate sulfurtransferase.

Table 3
Liver gene expression (mRNA) of the transsulfuration pathway of 261-day-old fructose-fed male progeny from fructose-fed (F/F) or glucose-supplemented (G/F) and control mothers (C/F)

	C/C	C/F	F/F	G/F
CBS (a.u.)	3,20 ± 0,60	2,06 ± 0,40	3,75 ± 1,29	2,35 ± 0,26
CSE (a.u.)	151,8 ± 15,8	125,1 ± 22,1	128,2 ± 20,3	168,9 ± 12,3
3-MST (a.u.)	6,70 ± 0,66	6,65 ± 0,89	7,44 ± 0,64	8,52 ± 0,41

Data are M ± SE, males from five to six litters. C/C: Control male offspring of control dams. Relative target gene mRNA levels were measured by real time PCR, as explained in Materials and Methods, and normalized to Rps29 levels and expressed in arbitrary units (a.u.). Different letters indicate significant differences between the groups ($P < 0.05$). CBS: cystathionine beta-synthase; CSE: cystathionine gamma-lyase; 3-MST: 3-mercaptopyruvate sulfurtransferase.