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The effect of acetaminophen on the expression of BCRP in trophoblast cells impairs the placental barrier to bile acids during maternal cholestasis

Alba G. Blazquez [a,d], Oscar Briz [a,d], Ester Gonzalez-Sanchez [a], Maria J. Perez [a,b,d], Carolina I. Ghanem [c], Jose J.G. Marin [a,d,*]

[a] Laboratory of Experimental Hepatology and Drug Targeting (HEVEFARM), IBSAL, University of Salamanca, Salamanca, Spain
[b] University Hospital of Salamanca, IECSCYL-IBSAL, Salamanca, Spain
[c] Instituto de Investigaciones Farmacologicas, Facultad de Farmacia y Bioquimica, CONICET, Universidad de Buenos Aires, Buenos Aires, Argentina
[d] CIBERehd, Instituto de Salud Carlos III, Madrid, Spain

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ABSTRACT

Acetaminophen is used as first-choice drug for pain relief during pregnancy. Here we have investigated the effect of acetaminophen at subtoxic doses on the expression of ABC export pumps in trophoblast cells and its functional repercussion on the placental barrier during maternal cholestasis. The incubation of human choriocarcinoma cells (JAr, JEG-3 and BeWo) with acetaminophen for 48 h resulted in no significant changes in the expression and/or activity of MDR1 and MRPs. In contrast, in JEG-3 cells, BCRP mRNA, protein, and transport activity were reduced. In rat placenta, collected at term, acetaminophen administration for the last three days of pregnancy resulted in enhanced mRNA, but not protein, levels of Mrp1 and Bcrp. In fact, a decrease in Bcrp protein was found. Using *in situ* perfused rat placenta, a reduction in the Bcrp-dependent fetal-to-maternal bile acid transport after treating the dams with acetaminophen was found. Complete biliary obstruction in pregnant rats induced a significant bile acid accumulation in fetal serum and tissues, which was further enhanced when the mothers were treated with acetaminophen. This drug induced increased ROS production in JEG-3 cells and decreased the total glutathione content in rat placenta. Moreover, the NRF2 pathway was activated in JEG-3 cells as shown by an increase in nuclear NRF2 levels and an up-regulation of NRF2 target genes, NQO1 and HMOX-1, which was not observed in rat placenta. In conclusion, acetaminophen induces in placenta oxidative stress and a down-regulation of BCRP/Bcrp, which may impair the placental barrier to bile acids during maternal cholestasis.

Introduction

The placenta expresses a large number of transport proteins responsible for the translocation of a broad spectrum of pharmacological agents and endogenous substances. Some of them are potentially toxic, such as bile acids. These are taken up by the trophoblast through anion-exchange mechanisms, probably mediated by members of the OATP family of transporters (Marin et al., 2003), and actively exported toward the maternal blood in an ATP-dependent manner (Bravo et al., 1995). In this context, the ATP-binding cassette (ABC) superfamily of proteins is particularly important since it is involved in both the removal of fetal metabolites and the placental barrier functions (Marin et al., 2004). Among the ABC pumps expressed in the placenta are the multidrug resistance protein-1 (MDR1, gene symbol *ABCB1*), several members of the multidrug resistance-associated proteins (MRPs, gene symbol *ABCCs*), and the breast cancer resistance protein (BCRP, gene symbol *ABCG2*) (Marin et al., 2004; St-Pierre et al., 2000). Placental MDR1 plays an important protective role that has been reviewed recently (Iqbal et al., 2012; Ni and Mao, 2011). The functional importance of MRP1, MRP3 and MRP4 in the placenta remains poorly understood. MRP1 and MRP3 are mainly localized in the basolateral membranes of polarized cells and in the endothelium of fetal vessels, although apical syncytiotrophoblast expression has also been identified (Ni and Mao, 2011). MRP4 has been detected in the placenta at the mRNA level (Langmann et al., 2003; Maher et al., 2005; Serrano et al., 2007), but its exact localization in this tissue remains to be elucidated.

Abbreviations: *ABCG2* and *Abcg2*, human and rat gene for breast cancer resistance protein (BCRP/Bcrp), respectively; calcein-AM, calcein acetoxymethyl ester; FTC, fumitremorgin C; GCA, glycocholic acid; Mdr/MDR, multidrug resistance protein; Mrp/MRP, multidrug resistance-associated protein; NAPQI, N-acetyl-p-benzoquinone-imine; PBS, phosphate-buffered saline; QPCR, real-time quantitative PCR; PI, propidium iodide; DAPI, 4′,6-diamidino-2-phenylindole; DTT, dithiothreitol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HMOX-1, hemeoxygenase-1; NQO1, NAD(P)H quinone oxido-reductase I; ROS, reactive oxygen species.

* Corresponding author at: Department of Physiology and Pharmacology, Campus Miguel de Unamuno, E.I.D., S-09, 37007 Salamanca, Spain. Fax: +34 923 294669.
E-mail addresses: albamgb@usal.es (A.G. Blazquez), obriz@usal.es (O. Briz), u60343@usal.es (E. Gonzalez-Sanchez), mjperez@usal.es (M.J. Perez), cghanem@ffyb.uba.ar (C.I. Ghanem), jjgmarin@usal.es (J.J.G. Marin).

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BCRP is one of the most important ABC pumps in placental barrier (Iqbal et al., 2012; Ni and Mao, 2011). This protein has recently been identified as a key player in the function of exporting bile acids from the fetus to the mother (Blazquez et al., 2012). Under pathological circumstances, such as maternal cholestasis, when these potentially toxic compounds become accumulated, BCRP may play an important role in the protection of the fetus against maternal hypercholanemia (Blazquez et al., 2012).

Acetaminophen is a drug widely used in pregnancy because at therapeutic doses it is considered safe for both the mother and the fetus. The drug is mainly metabolized in the liver by UDP-glucuronosyltransferases and sulfotransferases, generating harmless metabolites that are eliminated by renal and biliary excretion (James et al., 2003). In addition, a small proportion of acetaminophen, depending on the dose used but usually lower than 5%, is biotransformed by cytochrome P450 (CYP450) enzymes to the electrophilic highly reactive intermediate metabolite, N-acetyl-p-benzoquinone-imine (NAPQI) (Dahlin et al., 1984). Under normal conditions, this compound has a short half-life and is eliminated by conjugation with glutathione in a reaction mediated by glutathione S-transferases. The metabolite is further transformed to mercapturic acid and excreted into the urine (Beckett et al., 1985). In the case of acetaminophen overdose, the glucuronidation and sulfation pathways become saturated, and excessive amounts of acetaminophen undergo P450-mediated formation of NAPQI, which results in a reduction of intracellular glutathione levels (Potter et al., 1974). Accumulated NAPQI then binds to cellular macromolecules such as DNA and proteins, leading to structural and metabolic alterations and eventually to necrosis (Jollow et al., 1973). Some of the ABC export pumps are involved in the elimination of acetaminophen metabolites from the liver and kidney cells. Both *in vivo* drug disposition studies and *in vitro* functional transport experiments indicate that rodent Mrp2, Mrp3, Mrp4, and Bcrp have the ability to transport sulfate-, glucuronate- or glutathione-conjugated acetaminophen (Zamek-Gliszczynski et al., 2005, 2006). The induction of the expression of these transporters, and also of MDR1/Mdr1 and MRP1/Mrp1, has been reported in both human and rodent liver during the administration of high toxic doses of acetaminophen (Aleksunes et al., 2005, 2008; Barnes et al., 2007; Ghanem et al., 2004). However, the effect of non-toxic treatments has been less studied.

Since pregnant women are often treated with acetaminophen for pain relief, it is important to know the effect of exposure to this drug on placental ABC transporters and its functional repercussion on the placental barrier, in particular under pathological conditions that require enhanced protection of the fetus, such as maternal cholestasis. Thus, the aim of the present study was to evaluate the effect of acetaminophen on the expression of ABC proteins in the placenta and the integrity of the placental barrier against the overall transfer of maternal bile acids into the fetal compartment.

Materials and methods

Chemicals. Acetaminophen, dichlorofluorescein diacetate (DCFH-DA), fumitremorgin C (FTC), polyethylene glycol 400, probenecid, propidium iodide (PI), rhodamine 123, and verapamil were obtained from Sigma-Aldrich Quimica (Madrid, Spain). BODIPY FL-prazosin and calcein-acetoxymethyl ester (calcein-AM) were from Life Technologies (Madrid, Spain). [^{14}C]-glycocholic acid ([^{14}C]-GCA) (specific activity 56 mCi/mmol) was from GE Healthcare (Barcelona, Spain). According to the suppliers, the purity of these compounds was ≥97%. All other chemicals were of analytical grade.

Cell lines and culture conditions. The BeWo (CCL-98), JEG-3 (HTB-36), and JAr (HTB-144) cell lines from human choriocarcinoma were obtained from the American Type Culture Collection (LGC Standards, Barcelona, Spain). Cells were cultured as recommended by the supplier in a humidified atmosphere with 5% CO_2 at 37 °C. The cells were subcultured or used in the experiments at subconfluence. To determine the effect of acetaminophen on cell viability, the cells were incubated with increasing concentrations (from 20 μM to 40 mM) of acetaminophen for 48 h. Cell viability was measured using the CellTiter 96 AQueous non-radioactive cell proliferation assay (Promega, Madrid, Spain). From these studies, the subtoxic acetaminophen concentration was calculated as the highest one with no effect on cell viability (Supplementary Fig. 1). In further studies, cells were exposed to a subtoxic concentration of acetaminophen for 48 h before being detached with trypsin-EDTA, pelleted, rinsed with phosphate-buffered saline (PBS), and pelleted again to proceed with gene expression assays or with flow cytometry studies to determine the activity of ABC transporters and reactive oxygen species (ROS) generation.

In vivo experiments. Pregnant Wistar rats (day 18 of gestation) were provided by the University of Salamanca Animal House (Salamanca, Spain). The animals received humane care as outlined in the National Institutes of Health guidelines for the care and use of laboratory animals (http://grants.nih.gov/grants/olaw/Guidebook.doc). The experimental protocols were approved by the Ethical Committee for Laboratory Animals of the University of Salamanca. Acetaminophen dissolved in 45% polyethylene glycol 400 was administered (i.p.) to pregnant rats at a daily dose of 0.4 g/kg body weight before pregnancy (i.e., ≈200 g) for the last three consecutive days of pregnancy. The control group received only vehicle. The acetaminophen dose was selected from a previously described protocol (Ghanem et al., 2009) to achieve a non-toxic situation. Samples from anesthetized animals (sodium pentobarbital 50 mg/kg b.w., i.p., Nembutal N.R., Abbott, Madrid, Spain) were collected at term on day 21 of gestation, and were immediately immersed in the RNAlater RNA-stabilization reagent (Life Technologies) or frozen in liquid nitrogen and stored at −80 °C until measurement of gene/protein expression. To determine the effect of acetaminophen on the functionality of the placental barrier for bile acids two separate sets of experiments were carried out: i) *In situ* single-pass perfusion of one placenta per rat was performed as described elsewhere (Briz et al., 1998), with some modifications (Blazquez et al., 2012). Briefly, [^{14}C]-GCA (20 nmol) was single-pass-perfused (500 μl/min over 5 min) through the umbilical artery, alone or with FTC (50 nmol) to inhibit the Abcg2-mediated transport. The placenta was then perfused with heparinized saline solution for 30 min, followed by a second bolus of [^{14}C]-GCA (20 nmol) without Abcg2 inhibitor and heparinized solution along the rest of the experimental period. In order to make the model more robust we used the same placenta as its own control. As a reduction in bile acid transfer due to the perfusion of the ABCG2 inhibitor was expected, in order to prevent artifacts due to deterioration of perfused placenta preparation over time, test period preceded the control one. Bile samples were collected periodically to carry out analytical measurements. ii) Complete obstructive cholestasis was imposed in pregnant rats by bile duct ligation and section (BDL) on day 19 of gestation (Macias et al., 2000). Control animals underwent sham operation. The maternal bile acid pool was labeled on day 20 of gestation by i.p. administration of 22 μCi [^{14}C]-GCA (400 nmol). Bile acid concentrations in maternal serum were determined enzymatically to calculate the corrected specific radioactivity of [^{14}C]-GCA, which was used to determine bile acid levels in maternal and fetal sera and tissues and bile samples by radioactivity measurement as previously described (Vicens et al., 2007).

Determination of gene expression. Total RNA was isolated from cell or tissue lysates using RNeasy spin columns from GE Healthcare, treated with RNase-free DNase I (GE Healthcare), determined fluorometrically with the RiboGreen RNA-Quantitation kit (Life Technologies), and used for cDNA synthesis using random hexamers and avian myeloblastosis virus RT (Cloned AMV First-Strand cDNA Synthesis kit, Life Technologies). Real-time quantitative PCR (QPCR) was carried out using AmpliTaq Gold Polymerase (Life Technologies) in a 7300 Real-Time PCR System (Life Technologies). The thermal cycling conditions were as follows: a

single cycle at 50 °C for 2 min, and 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s and at 60 °C for 60 s. The oligonucleotide sequences of primers are shown in Supplementary Table 1. Detection of the amplification products was carried out using SYBR Green I (Life Technologies). The presence of non-specific amplified products, as examined by 2.5% agarose gel electrophoresis and melting temperature curves, was ruled out in all cases, except for human ABCC1 and ABCC3. In these cases, detection was carried out using TaqMan probes with the following sequences: 5′-ACCGTGCTGCTGTTTGTCACTGCC-3′ for ABCC1 and 5′-TGGCCGTGAAGA TGCGC-3′ for ABCC3. The results concerning mRNA abundance for the target genes in each sample were normalized on the basis of the 18S rRNA content of the samples, which was measured using the TaqMan Ribosomal RNA Control Reagents kit (Life Technologies). Inter-reaction variability was corrected using total RNA from human and rat liver, kidney or placenta as calibrators.

Export activity of ABC proteins, ROS generation and total glutathione content. The functionality of ABC proteins was determined by their ability to export specific substrates, i.e., rhodamine 123 for MDR1, calcein (administered to cells as calcein-AM) for MRPs, and BODIPY-prazosin for BCRP, and was measured by flow cytometry. In brief, the cell suspension was incubated in 100 μl of uptake medium (96 mM NaCl, 5.3 mM KCl, 1.1 mM KH_2PO_4, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, 11 mM glucose and 50 mM HEPES, pH 7.40), containing 1 μM of the substrate and 50 μg/ml PI, for 30 min at 37 °C. Then, 900 μl of uptake medium, containing or not a specific inhibitor of each pump (10 μM verapamil for MDR1, 500 μM probenecid for MRPs, and 5 μM FTC for BCRP) was added and cells were incubated for 30 min at 37 °C. To stop transport processes, ice-chilled uptake medium was added, after which intracellular fluorescence was determined with a FACSCalibur flow cytometer (BD Biosciences, Madrid, Spain). The reduction of intracellular fluorescence over time was assumed to be due to the efflux of the substrates in the absence or presence of the pump inhibitor. We have previously found that in our experimental setting, the presence of the fluorescent compound in the extracellular medium does not significantly interfere with the measurement (data not shown). The intracellular ROS levels were measured by flow cytometry using DCFH-DA as described previously (Perez et al., 2011). PI staining was used to exclude dead cells from the analysis. Total glutathione content was determined by an enzymatic method in trichloroacetic acid supernatants obtained from placental homogenates (Tietze, 1969).

Preparation of nuclear and cytoplasmic fractions. Nuclear extracts were prepared as described elsewhere (Perez et al., 2011), with minor modifications. All steps were carried out at approximately 4 °C. Cells were rinsed and scraped off into PBS and the pellets were suspended in 3 volumes of ice-cold hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM dithiothreitol (DTT) and protease inhibitors) and incubated on ice for 15 min. Igepal CA-630 (Sigma-Aldrich) was then added to a final concentration of 2% before vortexing for 10 s. After centrifugation (500 ×*g* for 30 s), the supernatant was collected and saved as the cytoplasmic fraction. The remaining crude nuclear pellet was washed with hypotonic buffer and resuspended in two-third volumes of ice-cold extraction buffer (20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 0.42 M NaCl, 0.2 mM EDTA, 25% (vol/vol) glycerol, 1 mM DTT and protease inhibitors). The sample was kept on ice for 30 min with gentle vortexing every 5–10 min. Samples were then centrifuged (5 min at 20 000 ×*g*) and the supernatant (nuclear extract) was collected. All samples were stored at −80 °C until use. To assess the purity of the nuclear and cytoplasmic fractions, immunoblot-based detection of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as a cytoplasmic marker, and Lamin B1, as a nuclear marker, was carried out.

Determination of protein expression. Immunoblotting analyses were carried out on placental homogenates, total cell lysates, and nuclear/cytoplasmic fractions using 7.5 or 8% sodium dodecyl sulfate-polyacrylamide gels, using 50 μg of protein per lane, and then transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The primary antibodies (working dilutions) used were as follows: mouse or rat monoclonal antibodies against human and rat BCRP/Bcrp (BXP21, Abcam, Cambridge, UK) (1:500 dilution), rat Mdr1 (ab3364, Abcam) (1:500 dilution), rat Mrp1 (Alexis, Exeter, UK) (1:500 dilution), rat Mrp2 (M2III5, Alexis) (1:250 dilution), and rat Mrp4 (ab15602, Abcam) (1:500 dilution), and rabbit polyclonal antibodies against rat Mrp3 (M0318, Sigma-Aldrich) (1:300 dilution), human Lamin B1 (Abcam) (1:500 dilution) and human NRF2 (sc-722, Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1:500 dilution). The protein load was normalized using an antibody against human or rat GAPDH/Gapdh (Santa Cruz Biotechnology) (1:1000 dilution). The appropriate horseradish peroxidase-linked secondary antibodies were from GE Healthcare. An enhanced chemiluminescence detection system (Hybond ECL; GE Healthcare) was used to visualize the bands. The relative abundance of proteins was determined by densitometric analysis of the bands using the Fujifilm MultiGauge software (TDI, Madrid, Spain). Immunofluorescence studies were carried out on fixed JEG-3 cells, using BXP-21 antibody (Abcam) (1:20 dilution) to detect human BCRP and Alexa Fluor 488 anti-mouse secondary antibody (Life Technologies) (1:1000 dilution). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Negative controls were obtained by omitting the primary antibody. Fluorescence staining was visualized using a Leica TCS SP2 confocal microscope.

Statistical analyses. Paired or unpaired t-tests were used, as appropriate, to calculate the statistical significance of differences between two means, whereas the Tukey HSD test was used as post-hoc test to carry out multiple range testing after ANOVA. Correlations were calculated by the least-squares method.

Results

Effect of subtoxic concentrations of acetaminophen on the expression and activity of ABC proteins in human trophoblast cells

Human trophoblast cells (BeWo, JAr and JEG-3) were incubated with a range of increasing concentrations of acetaminophen for 48 h at 37 °C (Supplementary Fig. 1). JAr and JEG-3 cells showed a lower sensitivity to the toxic effect of acetaminophen. Thus, 20 mM of this drug was required to reduce the viability of these cultures by 50% (Supplementary Fig. 1 and Supplementary Table 2). The subtoxic concentration for each cell line, i.e., 1 mM for BeWo and JAr cells, and 5 mM for JEG-3 cells was used in further studies.

Consistent with results previously reported by our group (Serrano et al., 2007), as compared with human tissues (liver, kidney and placenta) used as calibrators in each case, a higher expression of MRP1 and BCRP, but a poor or negligible expression of MDR1, MRP2, MRP3, and MRP4 was found in BeWo, JAr and JEG-3 cells (Table 1). Incubation with acetaminophen induced no significant changes in the expression levels of MDR1, MRP1, MRP3 and MRP4 mRNA (Table 1). However, although under basal conditions the abundance of MRP2 mRNA in JEG-3 cells was markedly lower than that in human liver, acetaminophen was able to enhance (1.8-fold) MRP2 expression in JEG-3 cells (Table 1). In contrast, a reduction in BCRP mRNA levels (1.5-fold) was found in JEG-3 cells (Table 1).

To investigate whether the acetaminophen-induced changes in the expression of ABC transporters in trophoblast cells had functional consequences, the activity of MDR1, MRP1-4 and BCRP was evaluated by their ability to export rhodamine-123 (a typical MDR1 substrate), calcein (a shared substrate of MRP1-4) and BODIPY-prazosin (a specific BCRP substrate) from preloaded cells in the absence or presence of specific inhibitors (Fig. 1). The results of functional studies were consistent

Table 1
Effect of incubation with subtoxic concentrations of acetaminophen for 48 h on the relative mRNA abundance of ABC proteins in human choriocarcinoma cell lines.

	Calibrator	Ct of calibrator	mRNA abundance (% of human tissue used as calibrator)					
			BeWo		JAr		JEG-3	
			Control	Acetaminophen	Control	Acetaminophen	Control	Acetaminophen
MDR1	Liver	28	ND	0.9 ± 0.9	1.1 ± 0.9	0.4 ± 0.3	ND	ND
MRP1	Placenta	30	465 ± 59	629 ± 136	662 ± 87	535 ± 34	1040 ± 105	980 ± 46
MRP2	Liver	28	2.1 ± 0.3	3.1 ± 1.5	19.1 ± 1.3	19.6 ± 1.5	3.6 ± 0.3	6.8 ± 0.3[a]
MRP3	Kidney	31	0.6 ± 0.5	0.6 ± 0.5	ND	ND	1.0 ± 0.3	0.6 ± 0.1
MRP4	Kidney	27	8.8 ± 2.3	11.2 ± 2.4	45.4 ± 6.2	64.8 ± 15	37.2 ± 6.7	41.0 ± 7.3
BCRP	Placenta	29	1537 ± 76	1444 ± 60	671 ± 51	583 ± 33	617 ± 34	411 ± 56[a]

Values (means ± SEM), determined by real time RT-QPCR in 3 cultures measured in triplicate for each data point, are expressed as percent of the calibrator used in each case. Ct, threshold cycle. ND, non-detected.
[a] $p < 0.05$, as compared with control untreated cells.

with those obtained in the gene expression analyses. These cells were not able to export rhodamine-123 or calcein in an inhibitor-sensitive manner. Only BCRP-mediated efflux, sensitive to inhibition by FTC, was observed in these cells (Fig. 1). After treatment with acetaminophen, a significant reduction in BCRP-mediated BODIPY-prazosin efflux from BeWo and JEG-3 cells was observed (Fig. 1).

The results of immunolocalization analyses revealed that incubation with acetaminophen resulted in a lower presence of BCRP at the plasma membrane of JEG-3 cells (Figs. 2A and B). The decreased abundance of BCRP protein was further confirmed by western blot analysis (Figs. 2C and D). To elucidate whether differential response was due to the fact that JEG-3 cells were treated with higher concentration (5 mM) of acetaminophen, incubation of JEG-3, as well as BeWo and JAr, cells with 0.1 and 1.0 mM acetaminophen were carried out (Supplementary Fig. 2). In BeWo and JAr cells no significant effect was found. In contrast, in JEG-3 cells although a trend was already observed at low concentrations, significant decrease in BCRP expression was seen at 1.0 mM acetaminophen. Intrinsic characteristics must hence account for the differential response of these cells to the drug. Next we investigated the rate of ROS production in response to different concentrations of acetaminophen in these three cell lines. At 1.0 mM acetaminophen, oxidative stress was found in BeWo and JAr, but not JEG-3 cells. In these cells enhanced ROS production was only seen at 5.0 mM acetaminophen (Supplementary Table 3 and Fig. 7A), a concentration that was toxic in BeWo and JAr, but not JEG-3, cells (Supplementary Fig. 1).

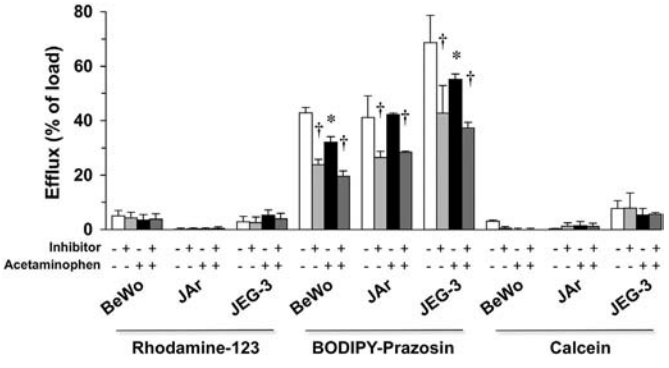

Fig. 1. Effect of acetaminophen on the efflux of rhodamine-123, BODIPY FL-prazosin and calcein from preloaded human placenta cells. Cells were incubated with a subtoxic concentration (1 or 5 mM) of acetaminophen for 48 h. After being loaded with the substrates at 37 °C for 30 min the cells were diluted 1:10 with substrate-free medium containing or not the specific ABC inhibitors – 10 μM verapamil (MDR1), 5 μM fumitremorgin C (BCRP) and 500 μM probenecid (MRPs) – and incubated at 37 °C for 30 min. Values (means ± SEM), determined by flow cytometry in 9 plates per condition from 3 different cultures, reflect changes in the mean fluorescence as compared with that found at the end of the loading period. *, $p < 0.05$, on comparing acetaminophen-treated with untreated Control cells. †, $p < 0.05$, as compared with the efflux in the absence of the inhibitor.

Effect of acetaminophen on ABC protein expression in rat placenta

The most abundantly expressed ABC transporters in rat placenta at term were Mdr1, Mrp1 (Fig. 3) and Bcrp (Fig. 4). The expression of Mrp2, Mrp3 and Mrp4 was also clearly detected in rat placenta although at lower levels than in the tissue used as calibrator in each case (kidney for Mrp3 and Mrp4, and liver for Mrp2) (Fig. 3). The treatment with acetaminophen during the last three days of pregnancy resulted in a moderate increase (1.5-fold) in the abundance of Mrp1 mRNA, which was not accompanied by a parallel increase in the abundance of Mrp1 protein (Fig. 3). The abundance of Bcrp mRNA was also increased (1.9-fold) by acetaminophen treatment (Fig. 4A). In contrast, western blot analyses revealed that Bcrp protein was decreased in the acetaminophen-treated group (Fig. 4B). The expression of the rest of ABC proteins investigated here was not markedly affected by the treatment with acetaminophen (Fig. 3).

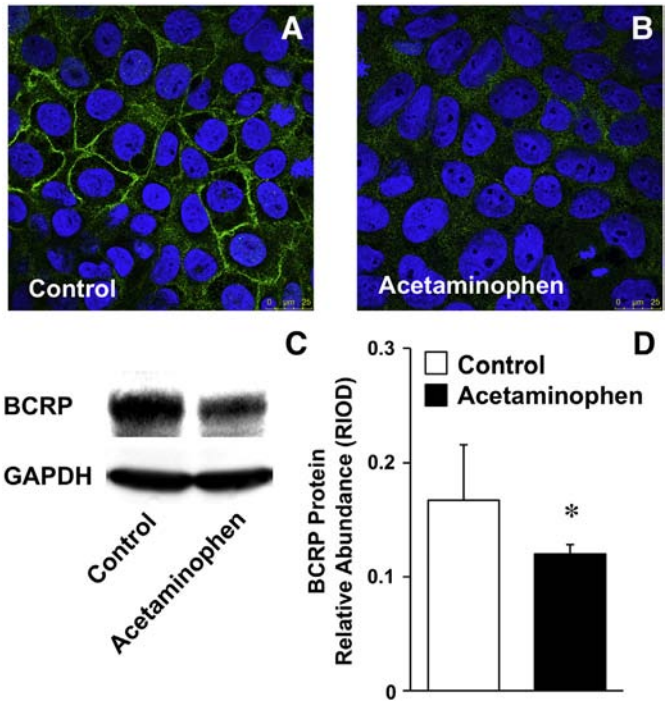

Fig. 2. Immunolocalization of BCRP (in green) in JEG-3 cells treated with a subtoxic concentration of acetaminophen for 48 h. Representative images obtained by laser confocal microscopy of BCRP (A and B). Nuclei were stained with DAPI. Representative western blot analysis of BCRP (C) and results from relative integrated optic density (RIOD) upon correcting the intensity of the target band with that of GAPDH (D). Values are means ± SEM from 3 cultures for each data point. *, $p < 0.05$, as compared with Control cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

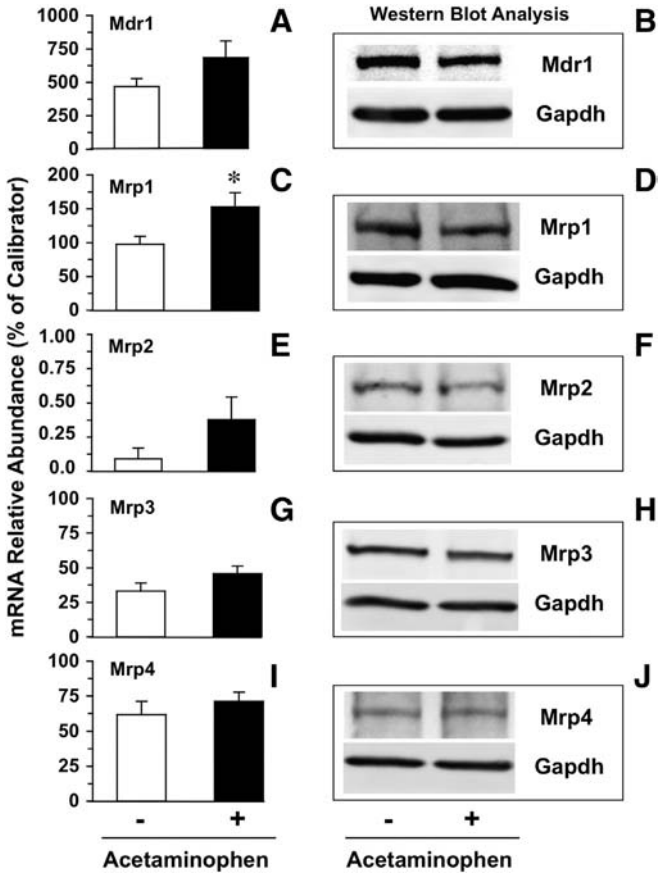

Fig. 3. Effect of acetaminophen on the expression of ABC transporters in rat placenta at term. Rats were treated with acetaminophen (0.4 g/kg b.w., i.p.) on days 18, 19 and 20 of pregnancy. The abundance of mRNA of Mdr1 (A), Mrp1 (C), Mrp2 (E), Mrp3 (G), and Mrp4 (I) in placentas (n = 9 per group) from treated and untreated Control animals was determined by RT-QPCR and expressed as the percentage of calibrator, which was rat liver (Mdr1 and Mrp2), kidney (Mrp3 and Mrp4) and placenta (Mrp1). Representative western blots of Mdr1 (B), Mrp1 (D), Mrp2 (F), Mrp3 (H) and Mrp4 (J) in rat placenta. These results were confirmed in 3 additional experiments. Values are expressed as means ± SEM. *, $p < 0.05$, as compared with Control untreated animals.

Effect acetaminophen on rat placental barrier in vivo

To evaluate the repercussion of changes in Bcrp expression on placental transfer of bile acids, *in situ* perfused rat placenta was used as previously described (Blazquez et al., 2012). In Control animals, when radiolabeled bile acid was co-administered with the Bcrp inhibitor FTC, a marked reduction in the placental transfer of GCA – and the subsequent secretion into bile – was observed (Fig. 5). In acetaminophen treated animals, the placental transfer of GCA was very low and could not be further reduced by co-administration of FTC (Fig. 5).

To evaluate the repercussion of these changes on the integrity of the placental barrier to bile acids, maternal BDL was performed on day 19 of gestation. As expected, cholestasis caused an increase in the levels of bile acids in maternal liver (Fig. 6A) and serum (Fig. 6B). Although the levels in fetal liver and placenta (Fig. 6A) and serum (Fig. 6B) were also increased as compared with the non-cholestatic group, the placental barrier limited the maternal-to-fetal transfer of bile acids, and hence prevented a similar accumulation of these compounds in the fetal compartment. The treatment with acetaminophen had no significant effect either on the maternal accumulation of bile acids (Fig. 6A) or on the basal levels of bile acids in fetal serum and tissues. In contrast, maternal treatment with acetaminophen resulted in a markedly higher bile acid accumulation in the fetal compartment during maternal cholestasis (Fig. 6).

Effect of acetaminophen on the redox state and activation of the Nrf2-mediated pathway

Acetaminophen-induced toxicity is characterized by alterations in the redox state due to enhanced oxidative stress and glutathione depletion. This was confirmed *in vitro* by determination of ROS generation by JEG-3 cells, which was enhanced (+150%) (Fig. 7A), and *in vivo* by measurement of the placental glutathione content, which was significantly reduced (−40%) (Fig. 8A) in acetaminophen-treated rats. Since the NRF2 pathway is involved in the cellular response to toxic/oxidative challenge, we investigated whether, under our experimental conditions, the expression of NRF2, and hence the sensitivity of this defense mechanism, could be changed by acetaminophen treatment. In BeWo and JAr cells, no change in NRF2 expression was detected. In contrast, in JEG-3 cells, a moderate but significant up-regulation was observed (Fig. 7B). This was accompanied by an increase in nuclear translocation on NRF2 (Fig. 7C) and up-regulation of its target genes, NAD(P)H quinone oxidoreductase I (NQO1) (Fig. 7D) and hemeoxygenase-1 (HMOX-1) (Fig. 7E). In the placenta of rats treated with acetaminophen, a mild but significant Nrf2 up-regulation was also found (Fig. 8B). Although no significant changes in the expression of Nqo1 (Fig. 8C) and Hmox-1 (Fig. 8D) were detected, a significant correlation was observed between the expression of Nrf2 and the abundance of Mrp1 (Fig. 8E) and Bcrp (Fig. 8F) mRNA.

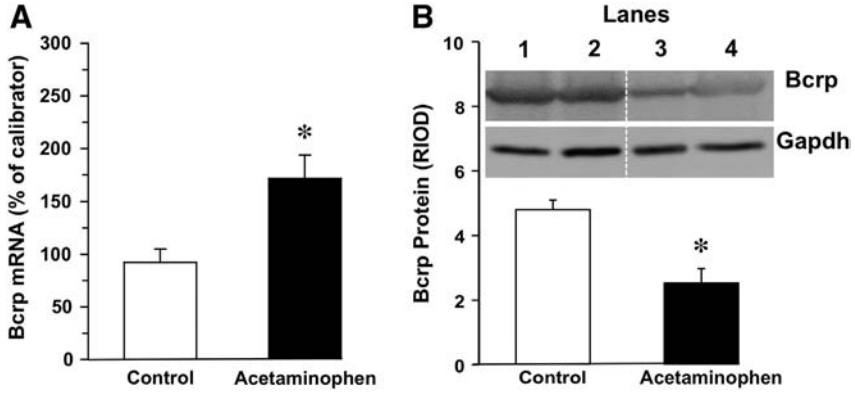

Fig. 4. Effect of acetaminophen on the expression of Bcrp in rat placenta at term. Rats were treated with acetaminophen (0.4 g/kg b.w., i.p.) on days 18, 19 and 20 of pregnancy. The abundance of Bcrp mRNA in placentas (n = 9 per group) from treated and untreated Control animals was determined by RT-QPCR and expressed as the percentage of calibrator, which was rat placenta (A). Representative western blot analysis of Bcrp in rat placenta and results from the determination of relative integrated optic density (RIOD) upon correcting the intensity of Bcrp with that of Gapdh (n = 6 per group) (B). Values are expressed as means ± SEM. *, $p < 0.05$, as compared with Control untreated animals.

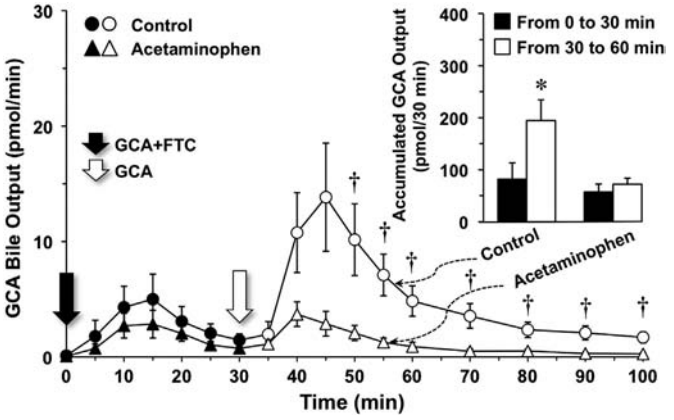

Fig. 5. Effect of acetaminophen on placental transfer of bile acids mediated by Bcrp. The animals were treated with a subtoxic dose of acetaminophen (0.4 g/kg b.w., i.p.) on days 18, 19 and 20 of pregnancy. Radiolabeled glycocholic acid ([^{14}C]-GCA) secretion into rat bile was measured when this bile acid was administered as a bolus alone or together with fumitremorgin C (FTC) through the umbilical artery of *in situ* perfused placentas. The cumulative output over 30 min periods after bolus administration is depicted in the insets. Values are means ± SD from 5 pregnant rats. *, $p < 0.05$ on comparing with and without FTC. †, $p < 0.05$ on comparing with and without treatment with acetaminophen. Circles; Control animals. Triangles; acetaminophen treated animals.

Discussion

Several ABC proteins are involved in the placental barrier that protects the fetus from noxious substances (Ni and Mao, 2011). The expression of these transporters is under the control of transcription factors able to respond, through complex signaling mechanisms, to exposure to potentially toxic compounds, including many drugs, such as acetaminophen. Thus, pharmacological treatment during pregnancy may modify the efficacy of the placental barrier. In addition, a large number of drugs administered to pregnant women are substrates of ABC proteins. Although acetaminophen is used as a first-choice drug for pain relief during pregnancy and although its ability to cross the placenta has been reported (Wilkes et al., 2005), the impact of this drug on the expression of placental ABC proteins and the functional repercussions of this was previously poorly understood. The present study provides important information because under pathological circumstances, such as maternal cholestasis, characterized by an accumulation of bile acids, whose transfer from the maternal-to-fetal compartment across the placenta is potentially toxic, impairment of the placental barrier due to prolonged or high doses of acetaminophen administration may have serious consequences. BCRP/Bcrp activity in the placental barrier to bile acids, in part due to the restricted location of this pump at the apical (maternal-facing) membrane of the trophoblast (Serrano et al., 2007), has been demonstrated previously (Blazquez et al., 2012). The results of the present study showed that even though acetaminophen induced an increase in placental BCRP/Bcrp mRNA, the amount of BCRP/Bcrp protein was decreased. In pregnant rats, this reduction was accompanied by a reduced ability of the placenta to carry out Bcrp-mediated bile acid transport, together with a decreased efficacy of the placental barrier to protect the fetus from maternal hypercholanemia. Up-regulation of mRNA in parallel with down-regulation of protein expression occurs with other proteins that are targeted for degradation via the proteasome system after ubiquitination whereas their mRNA remain at the same, or even in some cases, increased levels (Thapar and Denmon, 2013). The formation at the endoplasmic reticulum of an intramolecular disulfide bond in the BCRP protein is critical for the stability of this protein. Misfolded BCRP proteins are removed from the endoplasmic reticulum and degraded by the ubiquitin-proteasome system (Wakabayashi-Nakao et al., 2009). Endoplasmic reticulum stress and oxidative stress, such as that caused by acetaminophen, may enhance the risk of incorrect protein folding leading to removal of these proteins (Landau et al., 2013).

To investigate the cellular and molecular mechanisms underlying the effect of acetaminophen on the placental barrier, we used the human choriocarcinoma cell lines, BeWo, JAr and JEG-3. These have been widely used previously as *in vitro* experimental models to study different aspects of trophoblast physiology and pharmacology. A comparison of the expression profile of ABC transporters between these cell lines and human trophoblast cells freshly isolated from human placenta at term has been reported previously (Serrano et al., 2007). Regarding the expression of ABC transporters, several reports have shown the response of these cells to exposure to drugs included in the treatments of pregnant women. For instance, dexamethasone, which is used in pregnancies at risk of premature delivery, is able to increase MDR1 mRNA in placental cell lines (Pavek et al., 2007), but decreases BCRP expression in breast cancer cell lines (Elahian et al., 2009). Moreover, exposure to saquinavir, an HIV protease inhibitor used in antiretroviral therapy, results in an up-regulation of MDR1 in primary trophoblasts (Beghin et al., 2010). Regarding acetaminophen, BeWo cells have been used previously to investigate the ability of ABC transporters to export acetaminophen sulfate from trophoblasts (Mitra and Audus, 2010). Whether acetaminophen can affect the expression of placental ABC transporters remains unknown. Our results indicate that this indeed occurs and must be considered an important side-effect of acetaminophen, at least when used at high doses or in a prolonged way, because it may affect the role of BCRP/Bcrp in the placental barrier.

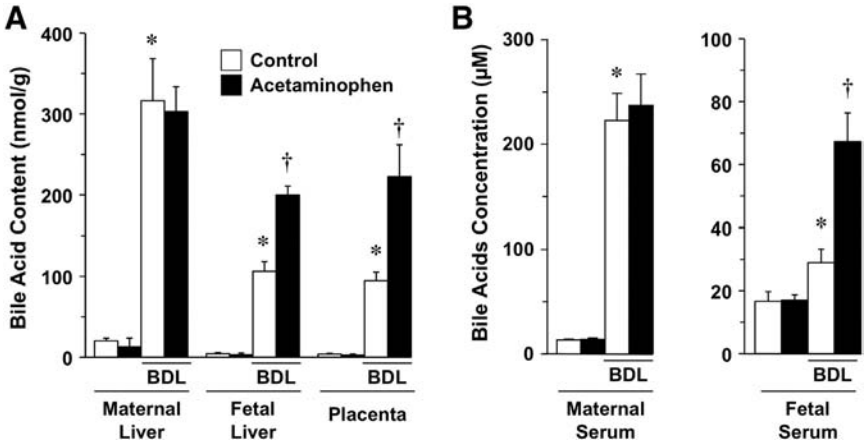

Fig. 6. Effect of acetaminophen on bile acid distribution in maternal and fetal tissues (A) and serum (B) from pregnant rats at term. The animals were treated with a subtoxic dose of acetaminophen (0.4 g/kg b.w., i.p.) on days 18, 19 and 20 of pregnancy. The maternal bile acid pool was labeled on day 20 of pregnancy by administering 400 nmol [^{14}C]-glycocholic acid. To induce complete obstructive cholestasis, maternal common bile duct ligation (BDL) was performed on day 19 of pregnancy. Control animals underwent sham operation. Values are means ±SEM from 4 pregnant rats and 12 fetuses per group. *, $p < 0.05$, as compared with Control non-BDL animals; †, $p < 0.05$ on comparing BDL with BDL plus acetaminophen treatment.

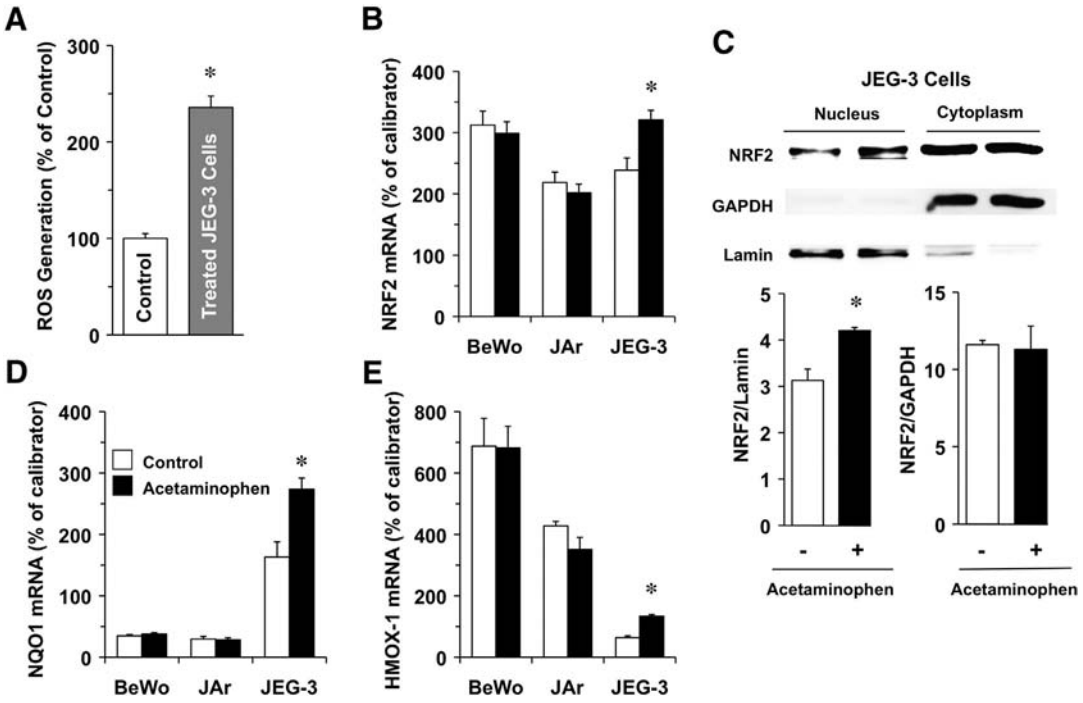

Fig. 7. Effect of acetaminophen on ROS production and on the activation of the NRF2-mediated pathway in human trophoblast cells. Cells were incubated with a subtoxic concentration of acetaminophen for 48 h. ROS production was determined by flow cytometry in JEG-3 cells (A). The relative mRNA abundance of NRF2 (B) and its target genes, NQO1 (D) and HMOX-1 (E), was determined by RT-QPCR and expressed as the percentage of the calibrator used in each case, which was human liver (NRF2 and HMOX-1) and kidney (NQO1). Values are means ± SEM from 3 cultures measured in triplicate for each data point. *, $p < 0.05$, as compared with untreated Control cells. Representative western blot of NRF2 translocation from the cytoplasm into the nucleus in JEG-3 cells and relative optic density versus Lamin as determined in four different experiments (C). Lamin B1 and GAPDH were used as nuclear and cytoplasmic markers, respectively.

Regarding the mechanism by which acetaminophen affects BCRP/Bcrp expression, two important aspects must be considered. On one hand, the conversion of acetaminophen by cytochrome P-450 to the highly reactive metabolite NAPQI is thought to account in part for the toxic effects of this drug (Dahlin et al., 1984). However, we found that in the three trophoblast cell lines used here, the expression of CYP2E1 and CYP3A4, the most important enzymes involved in NAPQI formation (McGill and Jaeschke, 2013), was negligible as compared with human

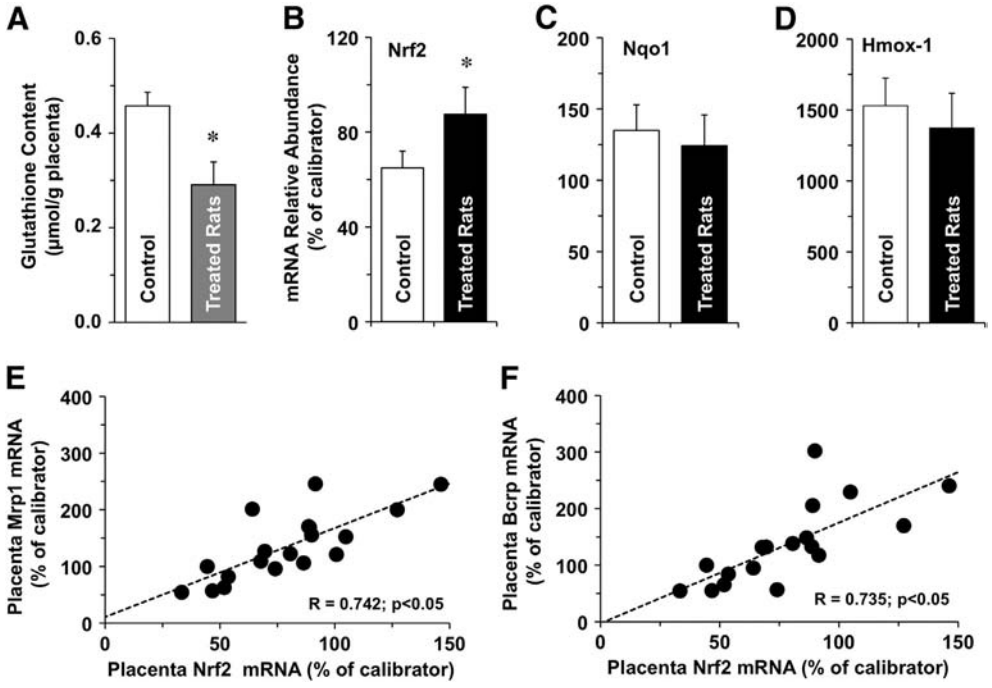

Fig. 8. Effect of acetaminophen on total glutathione content (A) and Nrf2-mediated pathway activation (B–F) in rat placenta at term. Rats were treated with acetaminophen (0.4 g/kg b.w., i.p.) on days 18, 19 and 20 of pregnancy. The relative mRNA abundance of Nrf2 (B) and its target genes, Nqo1 (C) and Hmox-1 (D), was determined by RT-QPCR and shown as the percentage of the calibrator (rat liver for Nrf2 and Hmox-1, and rat kidney for Nqo1). Values are means ± SEM from 9 placentas per group. *, $p < 0.05$, on comparing acetaminophen-treated and untreated groups. Relationship between the abundance of mRNA for Nrf2 and Mrp1 (E) or Nrf2 and Bcrp (F) determined by RT-QPCR and expressed as the percentage of the calibrator (placenta). Data from both untreated and treated animals were plotted together.

liver (data not shown). Nevertheless, there is evidence suggesting that non-metabolized acetaminophen may also induce oxidative damage and apoptosis directly (Boulares et al., 2002; Manov et al., 2006; Wiger et al., 1997). On the other hand, the role of nuclear receptors able to respond to chemical/oxidative stress must be taken into account. Thus, the acetaminophen-induced modulation of ABC protein expression may involve nuclear receptors such as CAR and PXR (Mottino and Catania, 2008). However, in rat placenta the expression of these nuclear receptors is very poor (St-Pierre et al., 2004). In contrast, Nrf2 is highly expressed in this organ. This transcription factor plays a key role in the mammalian response to chemical and oxidative stress upon its activation through two mechanisms: i) a direct modification of cysteine residues in Keap1 by NAPQI; and ii) a substantial depletion of glutathione (Copple et al., 2008). The findings of the present study indicate that both in human trophoblast cells and rat placenta the sensitivity to acetaminophen of the Nrf2-mediated cell defense system is not impaired but enhanced as suggested by the up-regulation of this transcription factor. Moreover, in JEG-3 cells an enhanced nuclear translocation and an up-regulation of target genes were observed. In rat placenta, no up-regulation of Nqo1 and Hmox-1 by acetaminophen was observed. In contrast, an elevation in the mRNA levels of Mrp1 and Bcrp was seen. Additionally, a significant correlation between the expression levels of Nrf2 and those of Mrp1 and Bcrp emerged, suggesting the existence of an NRF2-dependent expression of these transporters in placenta, as has been previously described for other tissues (Adachi et al., 2007; Singh et al., 2010).

As commented above, regardless the fact that in different experimental models acetaminophen was able to induce either decreased or enhanced abundance of mRNA for BCRP and Bcrp, respectively, the overall result was a decreased amount of BCRP and Bcrp proteins. This was presumably due to increased protein degradation, associated with the alteration in the cellular redox environment. In this respect, it has recently been reported that in human embryonic stem cells mild oxidative stress causes the internalization and subsequent lysosomal degradation of a fraction of the plasma membrane BCRP protein pool (Erdei et al., 2013). This process may not be specific for BCRP/Bcrp, because it has been shown that other ABC transporters also behave similarly under oxidative stress conditions and glutathione depletion (Perez et al., 2006; Sekine et al., 2008, 2012).

In conclusion, these results indicate that the treatment with subtoxic doses of acetaminophen during pregnancy may induce oxidative stress and alter the efficacy of the placental barrier, which may reduce the protection of the fetus against xenobiotics and potentially toxic maternal metabolites. In particular, the acetaminophen-induced reduction in BCRP/Bcrp function may impair the placental barrier to bile acids, which results in a lower protection of the fetus against the maternal hypercholanemia accompanying cholestatic liver diseases.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.taap.2014.02.019.

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Conflict of interest statement

The authors disclose that they do not have any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the work submitted that could inappropriately influence their work.

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