

# Fetal Rat Hearts Do Not Display Acute Cardiotoxicity in Response to Maternal Doxorubicin Treatment<sup>§</sup>

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## ABSTRACT

Anthracyclines are used to treat cancers during the second and third trimester of pregnancy. The chemotherapeutic effect of anthracyclines is associated with a dose- and time-dependent cardiotoxicity that is well described for infants and adults. However, data regarding fetal anthracycline-related cardiotoxicity after administration of chemotherapeutics during pregnancy are limited. In this study, we analyzed the acute effect of doxorubicin, an anthracycline derivative, on fetal and maternal rat myocardium. We injected 10 or 20 mg/kg i.v. doxorubicin to pregnant Wistar rats at day 18 of pregnancy; age-matched pregnant rats injected with physiologic saline served as controls. Maternal echocardiography and fetal Doppler scanning were performed before the injection and before sacrifice. Cesarean

operation was performed at day 19 or 20, and maternal and fetal blood samples and heart biopsies were collected to measure apoptosis, the impact on cell proliferation, and structural cardiac damage. Acute maternal cardiotoxicity is associated with loss of body weight, moderately deteriorated left ventricular function, induction of apoptosis, and a decrease in cell turnover. Despite a 30% lower fetal body weight and elevated plasma B-type natriuretic peptide concentrations after doxorubicin administration, the fetal hearts had intact microstructure, an unaltered number of apoptotic cells, and preserved cell proliferation compared with controls. Our study suggests that acute treatment using anthracyclines during pregnancy impairs maternal cardiac function, whereas fetal hearts are protected.

## Introduction

Anthracyclines are widely used for hematologic cancers and for several solid tumors, including breast cancers. Beyond general cytotoxicity, chemotherapeutic agents containing derivatives of anthracyclines are associated with acute and chronic cardiotoxicity (Giantris et al., 1998). Although the exact mechanism of anthracycline-induced heart failure is currently unknown, inflammation, apoptosis, oxidative and mitochondrial DNA damage, impairment of calcium metabolism, and depletion of cardiac stem cells all may contribute to a variable extent to the deterioration of cardiac function (Minotti et al., 1996; Herman et al., 1998, 1999; Gianni et al., 2008; Sawyer et al., 2010; De Angelis et al., 2010). Cardiotoxic

effects are well described for children and adults, but to date, data are limited on the impact of chemotherapy used during pregnancy on the fetal heart (Gziri et al., 2012a). After a risk-benefit analysis, the application of anthracycline-based therapeutics may be considered after the first trimester of pregnancy. However, anthracyclines during the first trimester of pregnancy may induce dysmorphogenesis, including vertebral, anorectal, cardiac, tracheoesophageal, renal, and limb syndrome; intrauterine growth retardation; and prenatal mortality. Embryotoxicity and teratogenicity of anthracyclines observed from day 6 until day 17 of pregnancy in rats are dose- and gestational age-dependent and have already been reported in several studies (Damjanov and Celluzzi, 1980; Fantel et al., 1985; Kurebe, et al., 1986; Chung et al., 1995).

Placental transfer of anthracyclines was reported in vitro and in vivo in different animal models. In mice, the doxorubicin transplacental passage is  $5 \pm 0.2\%$ . In baboons, the fetal plasma concentrations reach  $7.5 \pm 3.2\%$  for doxorubicin and  $4 \pm 1.6\%$  for epirubicin compared with the maternal concentrations (Van Calsteren et al., 2010b, 2011). Transplacental passage of anthracyclines is lower when studied in vitro and

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**ABBREVIATIONS:** ANP, atrial natriuretic peptide; Bax, Bcl-2 associated X protein; Bcl-2, B-cell leukemia/ lymphoma-2; BNP, B-type natriuretic peptide; BrdU, 5-bromo-2-deoxyuridine; ELISA, enzyme-linked immunosorbent assay; FS, fractional shortening; MHC, myosin heavy chain; qPCR, quantitative polymerase chain reaction; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling.

has been evaluated at  $3 \pm 0.8\%$  for doxorubicin (Grohard et al., 1989) and  $3.7 \pm 1.1\%$  for epirubicin (Gaillard et al., 1995). Fetal cardiomyocytes differ from adult cardiomyocytes in size, nuclear structure, and number; in the number of sarcomeres per mass unit; and by their contractile protein isoforms (Rudolph, 2000; Siedner et al., 2003). The amount of anthracyclines passing the placenta may induce fetal cardiotoxicity. Therefore, we investigated the acute cardiotoxicity of anthracyclines in both the maternal and fetal heart after intravenous administration of doxorubicin to Wistar rats at a late phase of pregnancy.

## Materials and Methods

**Animals and Treatment.** The study protocols using Wistar rats ( $n = 43$ ; Janvier Breeding, Le Genest Saint Isle, France) were approved by the ethical committee of the Katholieke Universiteit Leuven and performed according to the *Guide for Care and Use of Laboratory Animals* of the National Institutes of Health. The animals were kept under standard laboratory conditions and supplemented with rat chow and water ad libitum. After 1 week of acclimatization, 80-day-old female rats were mated in separate cages overnight. Day 0.5 (E0.5) of pregnancy was established when spermatozoa were found in vaginal smears. Doxorubicin was administered after E17 to avoid embryotoxicity, and the highest doses were used to induce an eventual cardiotoxicity (E9–12). At E18.5, rats were randomized into six groups ( $n = 6$  to  $7$  for each group) as follows: doxorubicin-treated (10 mg) and killed after 24 (group I) and 48 hours (group II); doxorubicin-treated (20 mg) and killed after 24 (group III) and 48 hours (group IV); and saline-treated controls killed after 24 (group V) and 48 hours (group VI). Finally, the effect of higher doxorubicin dose on cellular proliferation was tested in additional rats (saline or doxorubicin 20 mg for 48 hours,  $n = 3$ , for each) using 5-bromo-2-deoxyuridine (BrdU; Sigma-Aldrich, St. Louis, MO) incorporation (Angert et al., 2011). Doxorubicin was administered as single bolus intravenous injection after ketamine (50 mg/kg; Parke-Davis, Zaventem, Belgium) and xylazine (10 mg/kg; Bayer, Leverkusen, Germany) anesthesia. Body weight, general health status, and mortality were recorded before the pregnancy and at E18.5, E19.5, and E20.5 of pregnancy. On the day of euthanasia, a median cesarean section was performed to remove all pups; ketamine or xylazine anesthesia was used. Fetal blood was withdrawn from the subclavian vein after axillary incision, and thoracotomy was performed to remove the heart. The maternal blood was collected by abdominal aorta cannulation followed by thoracotomy and removal of the heart. Blood samples collected into EDTA tubes were immediately centrifuged (300g, 10 minutes, 4°C), and plasma was stored at  $-80^\circ\text{C}$  for further analyses. All fetal and maternal hearts were perfused using physiologic saline and weighed. Heart samples were stored in 4% phosphate-buffered formalin, frozen in liquid nitrogen, and stored at  $-80^\circ\text{C}$ .

**Measurements of Plasma Concentrations of Doxorubicin.** High-performance liquid chromatography with fluorometric detection was used to analyze the fetal and maternal plasma levels of doxorubicin as described previously (Van Calsteren et al., 2009, 2010b). The transplacental passage of doxorubicin was evaluated by the ratio of fetal/maternal plasma doxorubicin concentrations.

**Echocardiography.** Maternal transthoracic echocardiography and fetal Doppler measurements were performed at E18.5 before the injection and at E20.5 before euthanasia. To determine maternal heart function, rats were anesthetized using ketamine and xylazine. Anterior chest and abdomen were shaved and scanned using a high-resolution ultrasound system (VeVo 770; Visualsonics, Inc., Toronto, ON, Canada) in the lateral decubitus position. An M-mode of the left ventricle was recorded; and left ventricular internal diameter, interventricular septum, and left posterior wall thickness were

measured during systole and diastole. Fractional shortening (FS) was calculated as  $\text{FS} (\%) = (\text{end diastolic diameter} - \text{end systolic diameter}) / \text{end diastolic diameter} \times 100$ . Three fetuses per mother were subjected to Doppler measurements to obtain waveforms representative of the umbilical artery and the ductus venosus (Nasu and Arishima, 2004). The color Doppler in a transverse or sagittal view of the fetal abdomen was used to detect the ductus venosus (Supplemental Fig. 1), and umbilical peak systolic velocity was evaluated. The ratio between ventricular systolic peak velocity (S) and the lowest velocity during atrial contraction (a) was calculated. A reverse a-wave was also recorded. All measurements were obtained from three consecutive cardiac cycles and averaged for each rat. Analyses of echocardiographic images were performed by two independent observers blinded to the treatment.

**Plasma Concentrations of Natriuretic Peptides.** Atrial and B-type natriuretic peptide (ANP and BNP) concentrations were determined in the maternal and fetal plasma using AssayMax Rat ANP ELISA Kit and AssayMax Rat BNP-45 ELISA Kit (ERA7010-1 and ERB1202-1; Gentaur Belgium BVBA, Kampenhout, Belgium) according to the manufacturer's instructions. ANP and BNP concentrations were expressed in nanograms per milliliter of plasma.

**Analysis of DNA Fragmentation.** To detect apoptotic cells, terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL) staining was performed on 5- $\mu\text{m}$  paraffin-embedded sections 1 and 2 days after doxorubicin injection using a CardioTacs Kit (4827-30-K; Gentaur) according to the manufacturer's instructions. Two independent observers blinded to the treatment evaluated the staining. The apoptotic index was expressed as a percentage of positive nuclei (blue cells) to total nuclei in left ventricles.

**Transcriptional Analysis.** Total RNA was extracted from fresh-frozen myocardium using the Tripure Isolation Reagent (Roche, Mannheim, Germany). Its concentration and quality were determined using spectrophotometry (ND-1000; Nanodrop, Wilmington, DE). RNA samples with acceptable purity (absorbance ratio  $A_{260/280 \text{ nm}} = 1.9\text{--}2.0$ ) were used for cDNA synthesis (QuantiTect Reverse Transcription kit 05311; Qiagen, Hamburg, Germany). Quantitative polymerase chain reaction (qPCR) assays were performed using the TaqMan PCR system with primers for proapoptotic Bax (assay ID: Rn02532081-g1) and antiapoptotic Bcl-2 (assay ID: Rn99999125-m1) factors, B-type natriuretic peptide BNP (assay ID: Rn00580641-m1),  $\beta$ -myosin heavy chain ( $\beta$ -MHC) (assay ID: Rn01488777\_g1), and embryonic myosin heavy chain (assay ID: Rn01332449\_m1). Expressions for each gene were represented as  $C_t$  differences ( $\Delta C_t$ ) relative to the housekeeping 18S rRNA gene (assay ID: 4310893E; Applied Biosystem, Lennik, Belgium). All experiments were performed in duplicate.

**Evaluation of DNA Turnover in Myocardial Cells.** Incorporation of BrdU was used to evaluate the effect of anthracyclines on cellular DNA turnover. A mini-osmotic pump (Alzet 2ml1; Alzet, San Francisco, CA) was filled with 2 ml of 50 mg/ml BrdU (dissolved in 50% deionized water and 50% dimethylsulfoxide), which delivered 14.4 mg/kg per day, implanted subcutaneously through a small incision in the interscapular space at E18.5, and removed on E20.5. After euthanasia of rats, the left ventricle was fixed in formaldehyde, paraffin-embedded, sectioned, and stained for BrdU positivity (BrdU detection kit; Roche). Cardiomyocytes were stained using mouse monoclonal anti- $\alpha$ -sarcomeric actin IgM (diluted 1:50, clone 5C5; Sigma-Aldrich) and visualized by Texas Red-conjugated donkey anti-mouse IgM (Strattech Scientific Ltd., Newmarket Suffolk, UK). Nuclei were counterstained using 4',6-diamidino-2-phenylindole (DAPI) included in the VECTASHIELD Mounting Medium (Vector Laboratories, Burlingame, CA). Unbiased 10 scans from maternal and 5 scans from fetal left ventricles were performed using LSM 510 META two-photon laser scanning confocal microscope (Carl Zeiss Belgium NV, Zaventem, Belgium). Images were exported in TIFF format and were not further modified. Proliferative capacity was expressed as percentage of BrdU-positive to total cell number.

**Statistics.** All data are expressed as mean  $\pm$  S.E.M. Different groups were compared using one-way analysis of variance with Dunnett's post hoc analysis, and a  $P < 0.05$  was considered significant. Statistical analyses were performed using the Prism 5 software (GraphPad Software, Inc., La Jolla, CA).

## Results

**Transplacental Passage of Doxorubicin.** To estimate most accurately the amount of chemotherapy targeting the fetal heart, we measured the amount of doxorubicin passing the placental barrier. The fetal plasmatic concentrations reached  $6.2 \pm 3.2\%$  of those measured in the maternal circulation. Doxorubicin was detectable in all plasma samples, including the lowest concentration (0.41 ng/ml), measured in the fetus derived from a mother exposed to 10 mg of doxorubicin for 48 hours. Plasma concentrations and transplacental passage ratios are summarized in Supplemental Table 1.

**Doxorubicin Decreases Fetal and Maternal Body Weight but Not Heart Weight.** All rats subjected to saline and 10-mg doxorubicin injections survived until euthanasia. One rat subjected to 20-mg doxorubicin injection died after 24 hours. After doxorubicin, we observed decreased appetite, immobility, and weakness, and these effects were more accentuated after a 20-mg injection. At E18.5, before the experiments, no significant difference was observed in body weight between the different groups. Decreased maternal weight was observed after treatment with 10 and 20 mg of doxorubicin for 24 and 48 hours, respectively (Fig. 1A,  $P < 0.05$  versus saline; Supplemental Table 2). These observations were more pronounced in rats injected with 20 mg of doxorubicin. The maternal heart/body weight ratio significantly increased after 20 mg of doxorubicin treatment for 48 hours (Supplemental Table 1). Decreased body weight was also observed for the fetuses after 48 hours after 10- and 20-mg doxorubicin treatment (Fig. 1B,  $P < 0.05$  versus saline; Supplemental Table 3). Observations using transmission electron microscopy revealed that the maternal and fetal cardiac muscular fiber structures, mitochondria, sarcomeres, and monoparticulated glycogen particles were similar in doxorubicin-treated and control groups (Supplemental Fig. 2, A–D).

**Maternal Cardiac Function and Fetal Doppler Analysis.** A decrease in heart rhythm is a phenomenon that occurs after doxorubicin administration. The greatest decrease (30 heartbeats per minute) in heart rhythm was observed with 10 mg of doxorubicin after 48 hours (Table 1). Dimensions of the maternal heart, measured using echocardiography, were comparable between the different groups subjected to saline or doxorubicin administration, although there was a trend for reduced end-diastolic dimensions after 20 mg of doxorubicin, possibly related to impaired fluid intake (Table 1). After administration of 20 mg of doxorubicin for 48 hours, the left posterior wall thickness in diastole was significantly decreased ( $P < 0.05$ ), whereas the left ventricular internal diameter and interventricular septum in diastole did not change significantly ( $P = 0.16$  and  $0.18$ ). Systolic thickening of the septum was markedly reduced (Table 1). Global myocardial function, measured as FS, was significantly decreased by 5–10% 24 and 48 hours after 20-mg bolus injection of doxorubicin (Fig. 1, C and D). The umbilical systolic peak velocity and the ductus venosus Doppler

measurements did not show significant differences between the groups (Fig. 1, E and F; Table 2).

**Acute Doxorubicin Treatment Alters BNP Biosynthesis but Has No Effect on Expression of ANP or Myosin Isoforms.** Maternal BNP transcript levels tended to decrease after doxorubicin treatment irrespective of dose or duration of treatment (Supplemental Fig. 3A). Transcriptional suppression was accompanied by significant time- and dose-dependent reductions in maternal plasma BNP concentrations in doxorubicin-treated rats (Fig. 2A). In fetal hearts, BNP mRNA levels were consistently higher compared with maternal hearts and were not affected by doxorubicin administration (Supplemental Fig. 3C). In contrast, administration of 20 mg of doxorubicin significantly increased plasma fetal BNP peptide levels after 48 hours (Fig. 2B). Plasma concentrations of ANP peptide were not affected by doxorubicin in maternal hearts and tended to rise by 10% in fetal groups after higher doses of chemotherapy (Fig. 2, C and D).

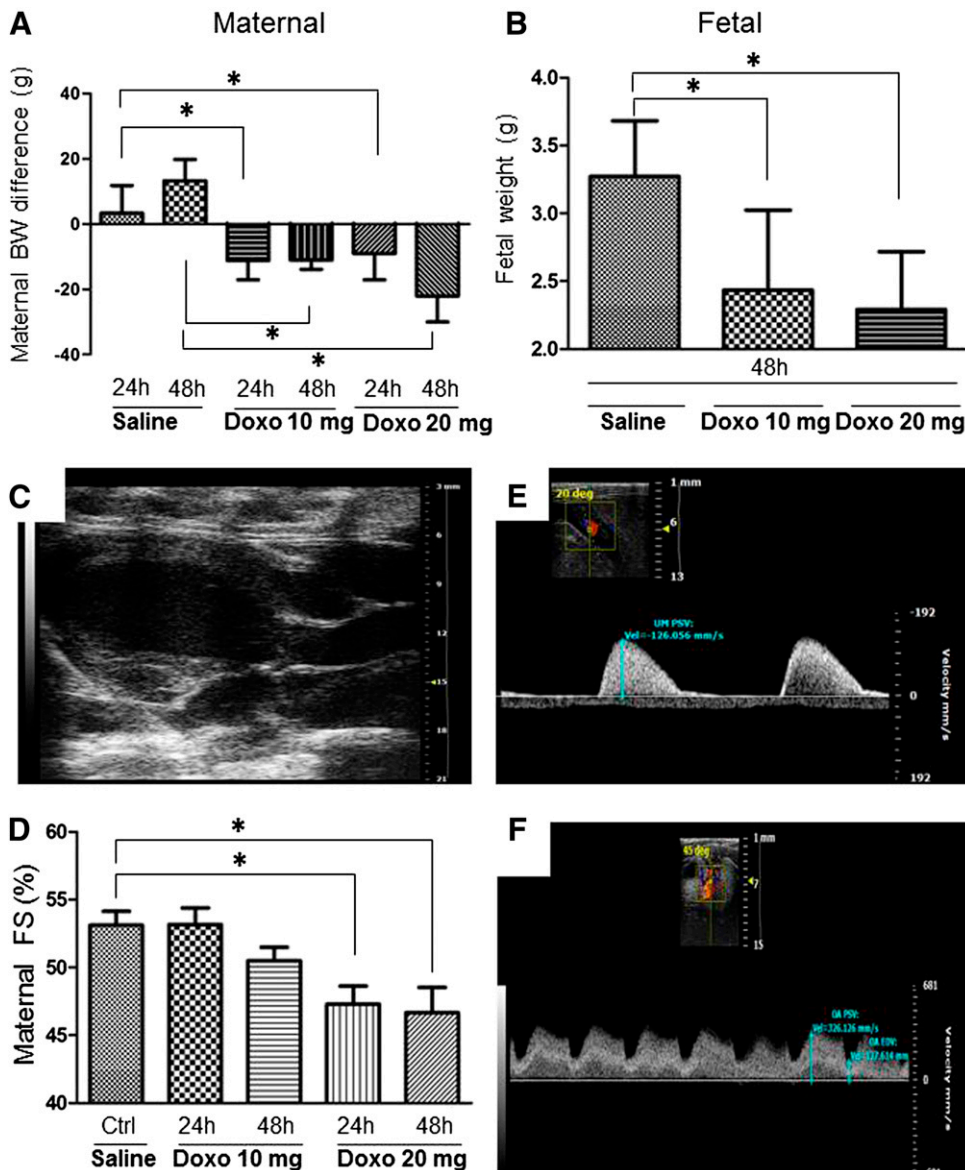
To investigate the regulation of myosin heavy chain isoforms during cardiac adaptation to increased toxic stress, we evaluated the transcript levels of embryonic and  $\beta$ -MHC. The levels of fetal and maternal ventricular expression of  $\beta$ -MHC were comparable between the different treatment groups (Supplemental Fig. 3, B–D). Embryonic myosin heavy chain mRNA was detectable only in fetal cardiac biopsies and was not influenced by exposure to doxorubicin (data not shown).

**Effect of Doxorubicin on Cellular DNA Turnover and Apoptosis.** Expression of proapoptotic and antiapoptotic factors was investigated using qPCR. Transcript levels of apoptosis-promoting Bax were higher in maternal hearts than in fetal hearts (Supplemental Fig. 4, A and C), whereas the expression of apoptosis-gating Bcl-2 was higher in fetal hearts (Supplemental Fig. 4, B and D). Expression of the proapoptotic factor Bax tends to increase, whereas expression of the antiapoptotic factor Bcl-2 tends to decrease in the maternal doxorubicin group (Supplemental Fig. 4, A and B). Expression of fetal pro- and antiapoptotic factors is not affected by doxorubicin. A significant increase in apoptotic cells in the maternal left ventricle was observed by immunohistochemistry (Fig. 3, A–C), whereas no increase was observed in the fetal heart (Fig. 3, C and D).

Anthracyclines alter the number of BrdU-positive cells in the maternal heart but not in the fetal heart. The ratio of BrdU-positive cells in the maternal left ventricle is  $0.5 \pm 0.3\%$  for the control group and decreased significantly 48 hours after 20 mg of doxorubicin administration to  $0.2 \pm 0.4\%$  (Fig. 4). In contrast, the ratio of BrdU-positive cells in fetal hearts is comparable between the control and doxorubicin-treated group ( $49.2 \pm 3.2$  vs.  $47.2 \pm 3.2\%$ , respectively) (Fig. 4).

## Discussion

Anthracycline chemotherapeutics induce cardiotoxic side effects. Doxorubicin administration during pregnancy damages not only the maternal heart but may hamper the fetal circulation and myocardial development. Early toxicity is the major risk factor of developing chronic cardiotoxicity (Grenier and Lipshultz, 1998). We report here for the first time the risk of acute doxorubicin-induced cardiotoxicity in a parallel functional and morphologic evaluation of both maternal and fetal hearts. We observed tiredness, weakness, and appetite reduction in pregnant rats after single-bolus intravenous



**Fig. 1.** Effect of doxorubicin treatment on Wistar rats body weight (BW) and cardiac global function. Maternal (A) and fetal (B) BWs significantly decreased after doxorubicin treatment ( $P < 0.05$  compared with respective control groups,  $n = 6$  for the maternal group,  $n = 76$  for the fetal control group,  $n = 73$  for the fetal group exposed to 10 mg of doxorubicin per 48 hours,  $n = 77$  for the fetal group exposed to 20 mg of doxorubicin per 48 hours). (C) High-resolution echocardiographic scans (a representative image of left ventricular long axis view) were used to calculate fractional shortening (FS) and evaluate maternal global heart function. (D) Significantly lower FS values were measured after 20 mg of doxorubicin treatment ( $n = 6$  per group). Doppler effect-based estimation of blood flow in umbilical artery with highlighted peak systolic velocity (blue arrow) (E) and flow pattern in ductus venosus (blue arrow) (F) indicated preserved fetal blood supply ( $n = 6$  per group). \* $P < 0.05$  versus the respective control.

doxorubicin administration, which resulted in a dose-dependent reduction of maternal body weight. Acute cardiotoxicity in maternal hearts was characterized by impaired fractional shortening measured by echocardiography, enhanced apoptotic

cell death, and reduced DNA turnover rate in the left ventricle. Doxorubicin-mediated effects on the maternal heart did not affect fetal blood flow. Despite detectable doxorubicin concentrations in the fetal circulation and in marked contrast

**TABLE 1**  
Transthoracic echocardiography of maternal hearts

|            | Saline ( $n = 12$ ) | Doxorubicin, 10 mg/kg |                  | Doxorubicin, 20 mg/kg |                  |
|------------|---------------------|-----------------------|------------------|-----------------------|------------------|
|            |                     | 24 h ( $n = 6$ )      | 48 h ( $n = 6$ ) | 24 h ( $n = 6$ )      | 48 h ( $n = 6$ ) |
| HR (BPM)   | 274 ± 3             | 264 ± 8               | 241 ± 6*         | 248 ± 11              | 260 ± 5          |
| LVIDd (mm) | 6.3 ± 0.5           | 6.5 ± 0.8             | 6.2 ± 1.2        | 6.1 ± 1.0             | 5.1 ± 1.8        |
| LVIDs (mm) | 2.8 ± 0.5           | 3.1 ± 0.4             | 3.1 ± 0.5        | 3.2 ± 0.6             | 2.7 ± 0.9        |
| IVSd (mm)  | 1.4 ± 0.3           | 1.5 ± 0.2             | 1.4 ± 0.2        | 1.4 ± 0.2             | 1.3 ± 0.1        |
| IVSs (mm)  | 2.0 ± 0.3           | 2.1 ± 0.4             | 1.8 ± 0.4        | 1.9 ± 0.2             | 1.8 ± 0.5        |
| LVPWd (mm) | 1.9 ± 0.2           | 1.7 ± 0.3             | 1.9 ± 0.3        | 1.9 ± 0.2             | 1.3 ± 0.5*       |
| LVPWs (mm) | 2.6 ± 0.4           | 2.5 ± 0.5             | 2.4 ± 0.5        | 2.6 ± 0.5             | 2.0 ± 0.9        |

BPM, beats per minute; HR, heart rate; IVSd, interventricular septum in diastole; IVSs, interventricular septum; LVIDd, left ventricular internal diameter in diastole; LVIDs, left ventricular internal diameter in systole, in systole; LVPWd, left ventricular posterior wall in diastole; LVPWs, left ventricular posterior wall in systole; data for saline-injected rats at 24- and 48-h time points were joined.

\* $P < 0.05$  vs. saline.

TABLE 2  
Doppler measurements of maternal fetal blood flow

|              | Saline       |              | Doxorubicin, 10 mg/kg |              | Doxorubicin, 20 mg/kg |              |
|--------------|--------------|--------------|-----------------------|--------------|-----------------------|--------------|
|              | 24 h (n = 6) | 48 h (n = 6) | 24 h (n = 6)          | 48 h (n = 6) | 24 h (n = 6)          | 48 h (n = 6) |
| UASPV (mm/s) | 117 ± 15     | 115 ± 5      | 123 ± 9               | 127 ± 11     | 115 ± 11              | 137 ± 9      |
| DV S/A ratio | 2.7 ± 0.4    | 2.4 ± 0.1    | 2.3 ± 0.2             | 2.4 ± 0.3    | 2.7 ± 0.1             | 2.9 ± 0.2    |

A, lowest velocity during atrial contraction; DV, ductus venosus; S, systolic peak velocity; UASPV, umbilical artery systolic peak velocity.

to the maternal heart, we did not observe significant acute cardiotoxicity in fetal rat hearts.

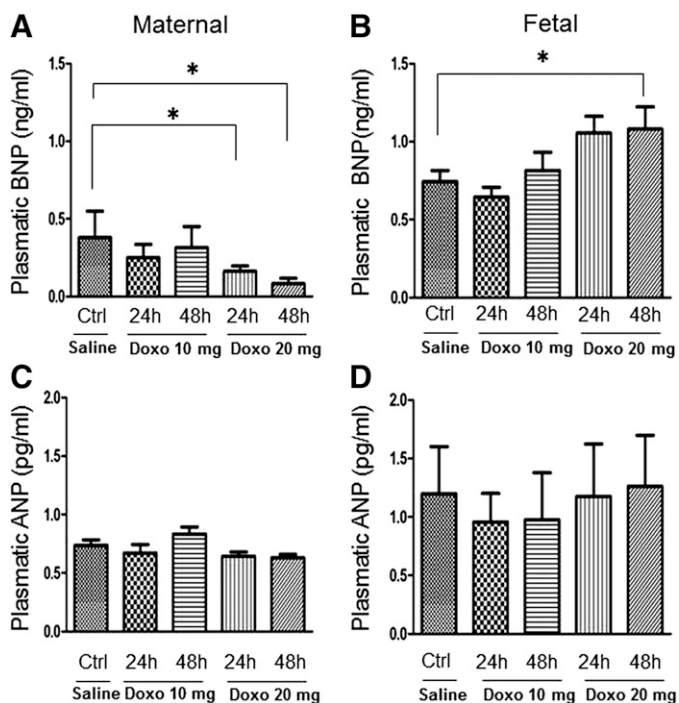
Doxorubicin is widely metabolized by the liver, and renal clearance is very low. Its plasma decay is triphasic, with the first half-time of 4.8 minutes, the second of 2.6 hours, and the last half-time of 48 hours (Danesi et al., 2002). In baboons, the transplacental transfer is  $7.5 \pm 3.2\%$ , and doxorubicin is not detectable after 24 hours (Van Calsteren et al., 2010b). In mice, transplacental transfer is  $5.1 \pm 0.6\%$  (Van Calsteren et al., 2011). In our pregnant rat model, we used established chromatographic techniques to quantify doxorubicin levels in maternal and fetal plasma 24 and 48 hours after administration and estimated transplacental passage of  $6.2 \pm 3.2\%$ .

In our experiments, tiredness, weakness, and appetite reduction were associated with a decrease in maternal body weight. Body weight loss may be partially explained by reduced food intake, but intestinal mucositis or doxorubicin's direct effect on the gastrointestinal tract causing excessive fluid loss should not be neglected (Danesi, et al., 1986;

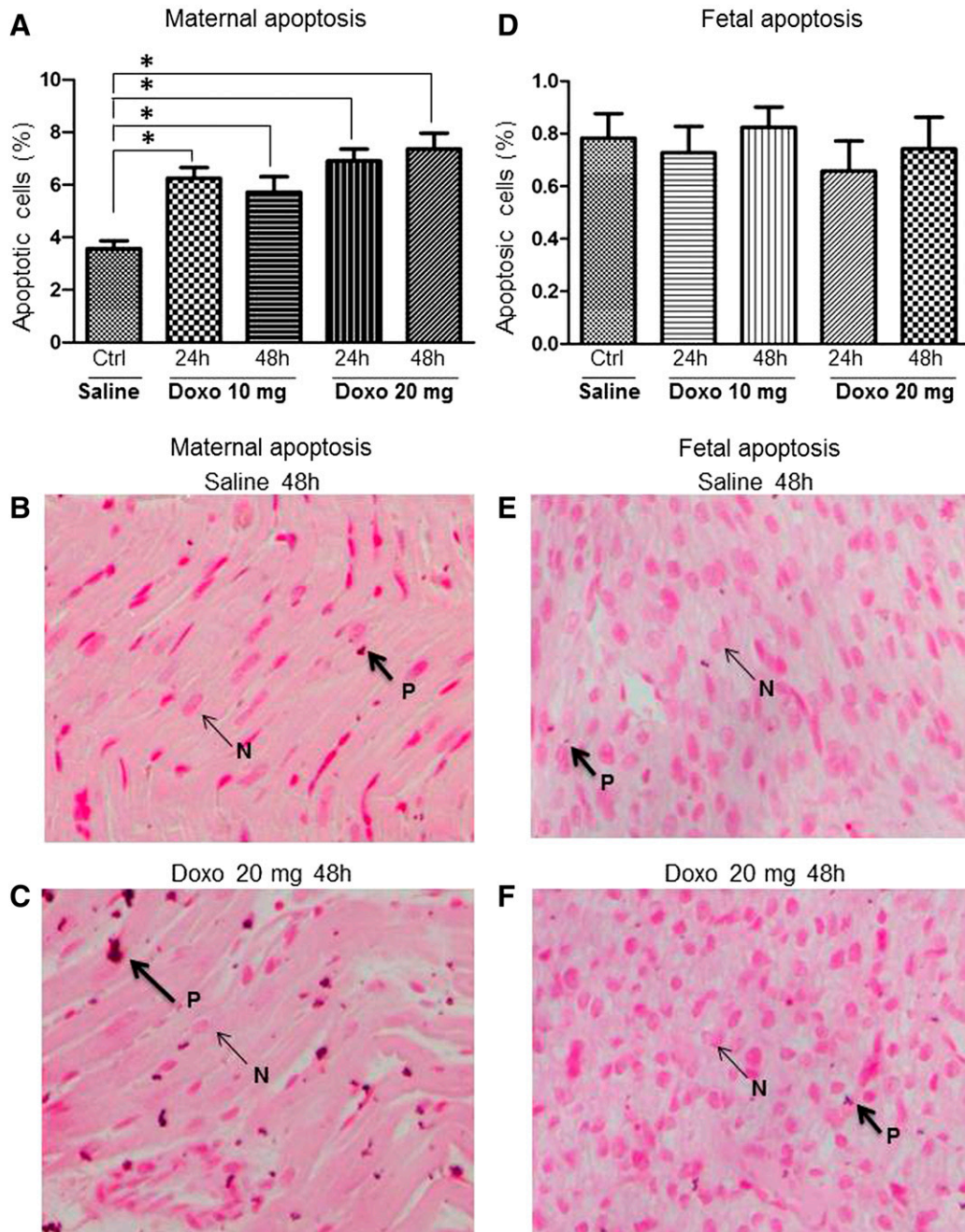
Hoekman et al., 1999; van Leeuwen et al., 2000). No fluid accumulation was observed in the pleural, peritoneal and pericardial cavities. Fetal rats subjected to circulating anthracycline also presented a reduced body weight after 48 hours. In patients, Cardonick, et al. (2010) reported no fetal growth restriction after chemotherapy, although this was described by Van Calsteren et al. (2010a) after maternal hematologic cancer treatments. Maternal malnutrition, placenta dysfunction, or abnormal fetal development can all explain the fetal growth restriction. To assess the fetoplacental hemodynamic function, the Doppler effect-based estimation of fetal blood supply is the only noninvasive method. For all fetuses, we confirmed normally preserved umbilical and ductus venosus flow after chemotherapy.

During the acute phase of treatment, the adult rodent's left ventricular dysfunction is not consistent and in general is moderate (Luo et al., 1997; Teraoka et al., 2000). Our recent study on maternal and fetal cardiac function showed no acute dysfunction after anthracycline administration during pregnancy (Gziri et al., 2012b). In our current experimental setting, we observed that acute high-dose doxorubicin administration impaired maternal global cardiac function. The decrease in FS was revealed by echocardiography and was in part attributable to a marked reduction in systolic septal thickening. Pregnant rats subjected to 10 mg of doxorubicin for 48 hours also showed a significant decrease in heart rate, as previously observed in rabbits (Yoshikawa et al., 1994) and in rats (Villani et al., 1990; Hazari et al., 2009). Anthracyclines may cause free radicals, which in turn may alter sympatho-neuronal activity. The high dose of doxorubicin (20 mg for 48 hours) also decreased the left ventricular posterior wall thickness, suggesting a direct cardiomyopathic effect. These results need to be confirmed in future experiments using larger subject numbers and refined imaging modalities, including magnetic resonance imaging and microcomputerized tomography.

Although functional deterioration is limited in the initial phase of chemotherapy, the generation of self-destructing cellular mechanisms may determine the later outcome. Therefore, we analyzed early cardiac markers of apoptosis, and in contrast to human studies, we could use the advantage of invasive methods such as left ventricular biopsies. Transmission electron microscopy failed to reveal maternal and fetal cardiac structural changes. By immunohistochemistry, an increased apoptotic cells was detected in the maternal heart. In addition, the upregulated synthesis of proapoptotic Bax and downregulated transcription of Bcl-2 in maternal hearts favors initiation of programmed cell death in doxorubicin-treated animals. These markers are involved in cytochrome *c* release by the mitochondria and activation of the caspase pathway (Ueno et al., 2006; Chandran et al.,



**Fig. 2.** Circulating concentrations of BNP 45 amino acid N-terminal fragment (A and B) and ANP levels (C and D) are indicated. Plasma BNP concentration in the maternal circulation (A) was significantly decreased, whereas in fetuses, concentration was elevated after administration of 20 mg of doxorubicin. Neither maternal nor fetal ANP levels (C and D) changed significantly after chemotherapeutic treatment ( $n = 6$  per group for each assay). \* $P < 0.05$  versus the control group.

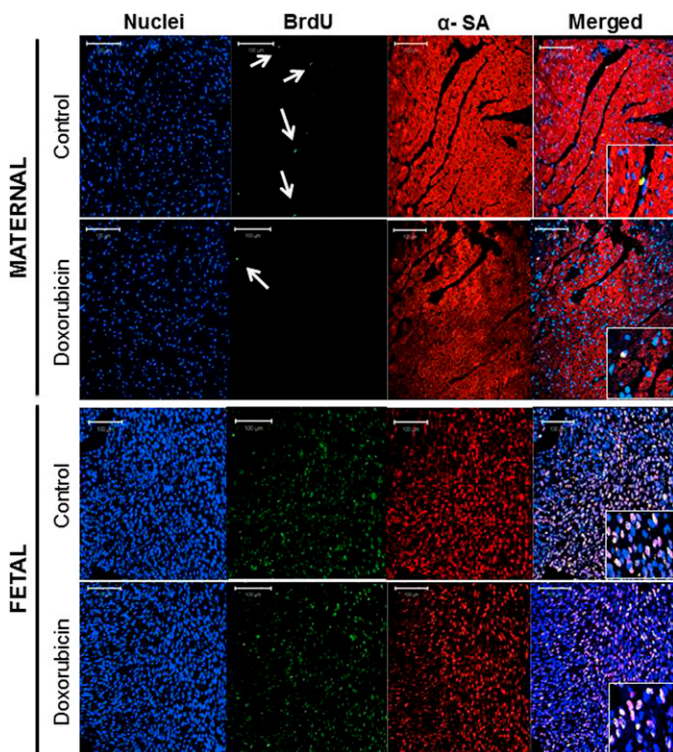


**Fig. 3.** Analysis of DNA fragmentation using TUNEL assay revealed 5–10% more apoptotic nuclei (blue-colored) in doxorubicin-treated maternal hearts (A), whereas fetal hearts (D) were protected. Representative microscopic images of cardiomyocyte apoptosis in maternal (B and C) and fetal hearts (E and F) after 20 mg of doxorubicin treatment for 48 hours with respective controls ( $n = 6$  per group). N, TUNEL-negative cardiomyocyte; P, apoptotic cardiomyocyte. \* $P < 0.05$  versus the control group.

2009). In agreement with previous reports, a significant decrease in BrdU-positive cells was measured in the maternal heart (De Angelis et al., 2010). Quantification of BrdU-positive cells revealed that the DNA turnover rate of fetal cardiac cells is not affected by the doxorubicin concentrations measured in fetal blood. These observations are consistent with low rates of apoptotic fetal cardiac cell death proven by DNA fragmentation or mitochondria-associated Bcl-2 and Bax gene expression levels.

The expression of atrial and B-type natriuretic peptides is activated during stretch-induced stress, hypoxia, inflammation,

hypertrophy, and fibrosis (Clerico et al., 2011). As a consequence, the impact of anthracyclines on ANP plasmatic release seems to be biphasic. In cultured neonatal rat myocytes, doxorubicin induces a decrease of ANP and BNP synthesis after 24 hours (Chen et al., 1999). According to previous studies using female Wistar rats, 10 mg/kg doxorubicin induces an initial ANP decrease after 3 and 6 hours, followed by increase after 21 and 31 days with continual treatment (Bernardini et al., 1992). In our experimental setup, we observed no differences in fetal transcript levels of ANP precursor and plasma ANP peptide concentrations. In



**Fig. 4.** Dual-photon microscopic analysis of cellular DNA turnover, inferred from BrdU incorporation, revealed that, in contrast to fetal myocardium, the percentage BrdU-positive nuclei was significantly reduced in maternal interstitial cardiac cells after doxorubicin treatment. Whereas in the maternal hearts BrdU-positive cells (arrows) were exclusively interstitial cells, in fetal hearts, a significant number of cardiomyocytes were actively undergoing DNA turnover (magnified on inset images) ( $n = 3$  per group). Nuclei, blue; BrdU-staining, green;  $\alpha$ -sarcomeric actin ( $\alpha$ -SA), red; BrdU-positive nuclei on merged images, white or purple. The scale bar represents  $100 \mu\text{m}$ . All pictures were taken with objective 20 and the insets are magnified  $2\times$ .

contrast, acute doxorubicin-treatment had a modest impact on BNP precursor transcript levels, but only in maternal hearts. Of note, significant differences were observed in the plasma levels of mature BNP-45 peptide. Maternal plasma showed significant decrease in BNP-45 concentrations 24 hours after 10 mg and 48 hours after 10 and 20 mg of doxorubicin administration, respectively. Fetal plasma concentrations of BNP-45 increased gradually in a dose- and time-dependent manner and reached significance 48 hours after 20 mg/kg doxorubicin injection. Similar concentration ranges have been described by Dilic et al. (2011) in acute myocardial infarction with impaired left ventricular ejection fraction, in which patients with a biphasic BNP release have a worse prognosis. The plasma half-life and the elimination of BNP are species-dependent (Thomas and Woods, 2003). Finally, we evaluated the expression of the  $\beta$ -MHC isoform, which correlates with contractile velocity. The expression ratio of  $\beta$ -MHC to  $\alpha$ -MHC-isoform changes during cardiac maturation and increases with cardiac dysfunction (Luther et al., 1997). We did not observe significant changes for the  $\beta$ -MHC transcripts, confirming the protected status of the fetal heart against the doxorubicin concentrations present in the fetal circulation/heart.

To confirm the potential protective, counter-regulatory effect of BNP in fetal rats is beyond the scope of this study.

Moreover, the doxorubicin-treated rats were not suffering from any oncologic disease, which is the main limitation of our study. The short period between doxorubicin administration and evaluation experiments represents an additional limitation. Nevertheless, the main aim of this study was to mimic the clinically relevant acute toxicity of anthracycline chemotherapeutics applied during the third trimester; in a rat model, we are limited by the 21 to 22 days of gravidity. Moreover, the period between days 6 and 17 in rats should be omitted because of proven teratogenicity and lethality of anthracycline compounds; therefore, we opted for a 48-hour period between 18 and 21 days (Damjanov and Celluzzi, 1980; Fantel et al., 1985; Kurebe et al., 1986; Chung et al., 1995). These two limitations may have an impact on the underlying molecular mechanisms, which deserve further research initiatives and a translational study with high relevance for clinical applications during pregnancy.

In conclusion, here we report for the first time the acute effect of anthracyclines on maternal and fetal hearts in a pregnant rat model. Experimental results showed a significant decrease of maternal body weight and plasma BNP concentrations. Doxorubicin dose- and time-dependently induced alterations in the pro- and antiapoptotic marker expression and tended to impair maternal left ventricular function. Despite significant variations in body weight and increased BNP plasma concentrations, the rodent fetuses were better preserved from the detrimental effects of doxorubicin treatment than their mothers.

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#### Authorship Contributions

*Participated in research design:* Gziri, Janssens, Amant.  
*Conducted experiments:* Gziri, Pokreisz.  
*Contributed new reagents or analytic tools:* Gziri, Pokreisz, De Vos, Verbeken, Janssens, Amant.  
*Performed data analysis:* Gziri, Pokreisz, Janssens, Amant.  
*Wrote or contributed to the writing of the manuscript:* Gziri, Pokreisz, Debiève, Mertens, Janssens, Amant.

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