Alcohol exposure during late gestation adversely affects myocardial development with implications for postnatal cardiac function

Joanna M. Goh, 1 Jonathan G. Bensley, 1 Kelly Kenna, 1 Foula Sozo, 1 Alan D. Bocking, 2 James Brien, 3 David Walker, 4 Richard Harding, 1* and M. Jane Black 1*

1 Department of Anatomy and Developmental Biology, Monash University, Clayton, Victoria, Australia; 2 Department of Obstetrics and Gynaecology, University of Toronto, Ontario, Canada; 3 Department of Pharmacology and Toxicology, Queen’s University, Kingston, Ontario, Canada; and * Monash Institute of Medical Research, Clayton, Victoria, Australia

Submitted 12 July 2010; accepted in final form 8 November 2010

Goh JM, Bensley JG, Kenna K, Sozo F, Bocking AD, Brien J, Walker D, Harding R, Black MJ. Alcohol exposure during late gestation adversely affects myocardial development with implications for postnatal cardiac function. Am J Physiol Heart Circ Physiol 300: H645–H651, 2011. First published November 12, 2010; doi:10.1152/ajpheart.00689.2010.—Prenatal exposure to high levels of ethanol is associated with cardiac malformations, but the effects of lower levels of exposure on the heart are unclear. Our aim was to investigate the effects of daily exposure to ethanol during late gestation, when cardiomyocytes are undergoing maturation, on the developing myocardium. Pregnant ewes were infused with either ethanol (0.75 g/kg) or saline for 1 h each day from gestational days 95 to 133 (term ~145 days); tissues were collected at 134 days. In sheep, cardiomyocytes mature during late gestation as in humans. Within the left ventricle (LV), cardiomyocyte number was determined using unbiased stereology and cardiomyocyte size and nuclearity determined using confocal microscopy. Collagen deposition was quantified using image analysis. Genes relating to cardiomyocyte proliferation and apoptosis were examined using quantitative real-time PCR. Fetal plasma ethanol concentration reached 0.11 g/dL after EtOH infusions. Ethanol exposure induced significant increases in relative heart weight, relative LV wall volume, and cardiomyocyte cross-sectional area. Ethanol exposure advanced LV maturation in that the proportion of binucleated cardiomyocytes increased by 12%, and the number of mononucleated cardiomyocytes was decreased by a similar amount. Apoptotic gene expression increased in the ethanol-exposed hearts, although there were no significant differences between groups in total cardiomyocyte number or interstitial collagen. Daily exposure to a moderate dose of ethanol in late gestation accelerates the maturation of cardiomyocytes and increases cardiomyocyte and LV tissue volume in the fetal heart. These effects on cardiomyocyte growth may program for long-term cardiac vulnerability.

cardiomyocyte; heart; pregnancy; maturation

MANY WOMEN CONTINUE TO CONSUME alcohol (ethanol, EtOH) during pregnancy (8, 16). It is well established that exposure to high levels of EtOH during pregnancy can lead to congenital cardiac defects such as atrial and septal defects (22, 24, 37); furthermore, cardiac function may be affected in the absence of structural abnormalities (21). However, the effects of moderate levels of EtOH exposure on cardiac muscle development during gestation are not well understood; they are difficult to ascertain in the human infant due to the many confounding factors including exposure at multiple time points and uncertainties regarding the level of exposure. To address this ques-

* R. Harding and M. J. Black are co-senior authors.

Address for reprint requests and other correspondence: M. J. Black, Dept. of Anatomy and Developmental Biology, Monash Univ., Clayton Campus, Bldg. 76, Victoria 3800 Australia (e-mail: Jane.Black@monash.edu).
performed at 134 DGA when some of the hearts were perfusion fixed (EtOH group, n = 8; saline group, n = 6), and in others the myocardium was sampled and snap frozen (EtOH group, n = 5; saline group, n = 6).

**Perfusion fixation of the heart.** At necropsy, fetal hearts were perfusion fixed via the aorta with 4% formaldehyde in 0.1 M phosphate buffer. Before fixation, the cardiac vasculature was cleared of blood using saline and maximally dilated with papaverine hydrochloride (DBL Pharmaceuticals, Australia); the cardiomyocytes were relaxed with potassium chloride. The fixed hearts were stored in 10% buffered formalin before tissue sampling.

**Heart muscle preparation and sampling.** Fat and connective tissue were removed from the fixed hearts, and the hearts weighed. The atria were separated from the ventricles. The right ventricle (RV) was then separated from the left ventricle plus septum (LV + S). The ventricles were cut into slices 3 mm thick, and the wall volumes of the RV and LV + S were determined using the Cavalieri principle (25). Subsequent sampling of the LV + S for morphological and stereological analyses was performed using a smooth fractionator approach (32); the selected samples were then embedded in either glycolmethacrylate or paraffin.

**Interstitial collagen quantification.** Paraffin-embedded samples of LV + S were sectioned at 5 μm and stained with 0.001% picrosirius red. The sections were uniformly, systematically sampled and the percentage of collagen within the tissue was quantified using image analysis (Image-Pro Plus Version 6.0, Media Cybernetics) (2, 32).

**Estimation of cardiomyocyte number.** Glycolmethacrylate-embedded samples of LV + S were serially sectioned at 20 μm, and every 30th section was stained with Harris’s Haematoxylin in a 1,000-watt microwave oven set at 50% power. Sections were uniformly, systematically sampled, and the number of cardiomyocyte nuclei per unit volume of tissue was determined using an optical disector stereological approach (2, 32). The total number of nuclei in the LV + S wall was calculated by multiplying the number of nuclei per unit volume of tissue by the total LV + S tissue volume. Total cardiomyocyte number in the LV + S was then determined following correction for binucleation (see below) (11).

**Cardiomyocyte nuclearity.** The nuclearity of cardiomyocytes within the LV + S (i.e., the proportions of mononucleated and binucleated cells) was examined using confocal microscopy in thick paraffin sections stained with wheat germ agglutinin-Alexa Fluor 488 conjugate (Invitrogen) to stain cell boundaries and 4’6-diamidino-2-phenylindole, dihydrochloride (DAPI) to stain cell nuclei (Invitrogen) (2). Sections were systematically sampled, and at least 200 cardiomyocytes per fetus were examined. Cardiomyocytes were recognized by the appearance of striations and the appearance of cardiomyocyte nuclei (long, round ended and dense nucleoli) (see Fig. 3).

**Analyzing cell size.** LV + S sections stained with wheat germ agglutinin-Alexa Fluor 488 conjugate, and DAPI (see above) were systematically sampled. Each field of view with cardiomyocytes seen in cross-section was analyzed for cardiomyocyte cross-sectional area (Fig. 1). The boundaries of the cardiomyocytes were traced and

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**Table 1. Primer sequences**

<table>
<thead>
<tr>
<th>Gene of Interest</th>
<th>Accession Number</th>
<th>Forward</th>
<th>Reverse</th>
<th>Primer, μM</th>
<th>cDNA, ng/μl</th>
</tr>
</thead>
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<tr>
<td>18S rRNA</td>
<td>X01117</td>
<td>GTC TGC GAT GCC CTT AGA TGT C</td>
<td>AAG CTT ATG ACC CGC ACT TAC</td>
<td>10</td>
<td>200</td>
</tr>
<tr>
<td>c-Myc</td>
<td>NM_001009426</td>
<td>CAT ACA TGC TTG CGG TCC AA</td>
<td>CAA CTG TTG TGG CCT CTT CC</td>
<td>10</td>
<td>200</td>
</tr>
<tr>
<td>IGF-1</td>
<td>DQ152962</td>
<td>TGG GTG GAT GCT CCT CAG TCC</td>
<td>AGG AGG ACT CAT CGA GGA TTT</td>
<td>10</td>
<td>500</td>
</tr>
<tr>
<td>IGF-2</td>
<td>M89789</td>
<td>GCT TCT TGC CCT CTT GCC CTT</td>
<td>TGG GTT TAT GCG GCT GGA T</td>
<td>10</td>
<td>62.5</td>
</tr>
<tr>
<td>IGF-1R</td>
<td>AY162434</td>
<td>AAG AAC CAT GGC TGC AGA AAG</td>
<td>GGA TGC TCC GGT TCC GGC CAT T</td>
<td>4</td>
<td>500</td>
</tr>
<tr>
<td>BAX</td>
<td>AF163774</td>
<td>TGG CTG AAG CGG ATT GGA GAT</td>
<td>AGG GCC TTG AGC ACC AGT TT</td>
<td>4</td>
<td>500</td>
</tr>
<tr>
<td>Caspase 3</td>
<td>AF068837</td>
<td>CGA CGC TCC TGC ATG T</td>
<td>GTC TGC CTG AAC TGG TAT TTT CTG A</td>
<td>10</td>
<td>500</td>
</tr>
</tbody>
</table>

Sequences for each forward and reverse primer (5′-3′) were used to amplify each gene of interest. Primer sequences were designed based on the nucleotide sequence that corresponds to the listed Genbank accession number. The starting primer and cDNA concentrations used for the amplification of each gene during quantitative PCR are also shown. The annealing temperature used for all primers was 60°C. IGF, insulin-like growth factor.

**Fig. 1.** Representative confocal image of left ventricle plus septum (LV + S; from a control lamb) stained with wheat germ agglutinin-Alexa Fluor 488 conjugate (to stain the cell boundaries; appears green) and TO-PRO-3 (Invitrogen; to stain the cell nuclei; appears blue). In cardiomyocytes cut in cross-section and nuclei centrally located, the boundaries of the cardiomyocytes were traced and cross-sectional area was determined. Two cardiomyocytes are delineated in white. Scale bar is 10 μm.
Effects of ethanol exposure on fetal blood gas status, arterial pressure, and heart rate. Between 130 DGA and 132 DGA, there were no significant differences between control and EtOH fetuses in fetal PaO₂ (23.7 ± 1.6 mmHg vs. 23.1 ± 0.8 mmHg), PaCO₂ (49.3 ± 1.4 mmHg vs. 49.1 ± 2.0 mmHg), arterial pH (7.364 ± 0.006 vs. 7.355 ± 0.003), SaO₂ (66.2 ± 1.8% vs. 63.0 ± 2.5%), mean arterial pressure (39 ± 0.5 mmHg vs. 40 ± 0.4 mmHg), and heart rate (154 ± 4 beats/min vs. 164 ± 5 beats/min).

Effects of EtOH exposure on body and heart growth, as shown in Table 2 and Fig. 2. There were no significant differences between the EtOH-exposed and control fetuses in body weight and absolute heart weight, RV weight, or LV + S weight; similarly, the absolute volume of wall tissue in the ventricles did not differ between groups. However, when heart weights and combined ventricular weights were expressed in relation to body weight, values in EtOH fetuses were significantly greater than in controls; a similar trend was seen for LV + S weight (P = 0.06).

Fig. 2. The weight (A) and volume (vol; B) of the LV + S, adjusted for body weight (body wt), of ethanol exposed fetuses and controls. *P < 0.05.

Table 2. Body weight and heart dimensions in ethanol-exposed and control fetuses

<table>
<thead>
<tr>
<th></th>
<th>Ethanol Exposed</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>Body weight, kg</td>
<td>3.84 ± 0.26</td>
<td>4.02 ± 0.16</td>
</tr>
<tr>
<td>Heart weight, g</td>
<td>34.1 ± 2.6</td>
<td>28.8 ± 1.7</td>
</tr>
<tr>
<td>Heart weight/body weight, g/kg</td>
<td>9.0 ± 0.7*</td>
<td>7.2 ± 0.5</td>
</tr>
<tr>
<td>Right ventricle weight, g</td>
<td>6.7 ± 0.6</td>
<td>6.7 ± 0.3</td>
</tr>
<tr>
<td>Right ventricle weight/body weight, g/kg</td>
<td>1.7 ± 0.1</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td>Left ventricle + septum weight, g</td>
<td>12.8 ± 0.9</td>
<td>12.2 ± 0.6</td>
</tr>
<tr>
<td>Left ventricle + septum weight/body weight, g/kg</td>
<td>3.4 ± 0.1</td>
<td>3.0 ± 0.1</td>
</tr>
<tr>
<td>Right ventricle wall volume, mm³/kg</td>
<td>7.58 ± 0.68</td>
<td>7.49 ± 0.49</td>
</tr>
<tr>
<td>Right ventricle wall volume/body weight, mm³/kg</td>
<td>1.96 ± 0.06</td>
<td>1.86 ± 0.09</td>
</tr>
<tr>
<td>Left ventricle + septum wall volume, ×10⁶ mm³</td>
<td>14.15 ± 0.95</td>
<td>13.57 ± 0.80</td>
</tr>
<tr>
<td>Left ventricle + septum wall volume/body weight, mm³/kg</td>
<td>3.71 ± 0.11*</td>
<td>3.37 ± 0.10</td>
</tr>
</tbody>
</table>

Values are means ± SE. For heart weight/body weight in ethanol-exposed group, P = 0.049. For left ventricle + septum weight/body weight in ethanol-exposed group, P = 0.06. For left ventricle + septum wall volume/body weight in ethanol-exposed group, P = 0.04. *Denotes significantly different from control.
In accordance with differences in relative tissue weights, the relative tissue volumes of the combined ventricles and LV + S of ethanol exposed and control fetal hearts were significantly greater in EtOH fetuses than in controls.

**Interstitial fibrosis.** The levels of interstitial fibrosis were low in the LV + S of all fetal hearts. There was no significant difference between levels of fibrosis in the LV + S of EtOH fetuses and controls (1.96 ± 0.16% vs. 2.19 ± 0.27%).

**Cardiomyocyte nuclearity and maturation, as shown in Fig. 3.** The percentage of mononucleated cardiomyocytes in the LV + S of EtOH fetuses was significantly lower than in controls (23.6 ± 1.0% vs. 36.0 ± 1.5%; P < 0.0001). Accordingly, there was a significantly higher percentage of binucleated cardiomyocytes in EtOH-exposed hearts than in controls (76.4 ± 1.0% vs. 64.1 ± 1.5%; P < 0.0001).

**Cardiomyocyte number and size, as shown in Fig. 4.** The total number of cardiomyocytes in the LV + S was not significantly different between EtOH fetuses and controls \((4.08 \times 10^9 \pm 3.0 \times 10^9 \text{ vs. } 4.38 \times 10^9 \pm 2.6 \times 10^9; P = 0.5)\). The mean cross-sectional area of LV + S cardiomyocytes was significantly greater in EtOH fetuses compared with controls \((48.7 \pm 2.1 \mu m^2 \text{ vs. } 42.1 \pm 0.9 \mu m^2; P = 0.02)\).

**Myocardial cell proliferation.** The rate of cell proliferation as measured by Ki-67 immunohistochemistry (brightfield and fluorescence), in the control and ethanol-treated hearts, was too low to quantify (<1 per 5,000 nuclei positively stained). The relative expression of the proliferation marker c-Myc within the myocardium was not different between the EtOH and control groups (1.07 ± 0.10 vs. 1.00 ± 0.13).
Growth factor gene expression, as shown in Fig. 5A. There was significantly greater relative expression of IGF-1 in the ventricles of EtOH fetuses compared with controls (2.21 ± 0.39 vs. 1.00 ± 0.18; \( P = 0.02 \)). There were no significant differences in ventricular relative IGF-2 or IGF-1R mRNA levels between EtOH and control fetuses.

Apoptotic marker gene expression, as shown in Fig. 5, B and C. There was a significant upregulation of relative caspase 3 mRNA levels in the ventricles of EtOH fetuses compared with controls (1.91 ± 0.12 vs. 1.00 ± 0.22; \( P = 0.007 \)). Similarly, there was a trend for relative BAX mRNA levels in the ventricles of EtOH fetuses to be greater than in the controls (2.00 ± 0.36 vs. 1.00 ± 0.29; \( P = 0.057 \)).

DISCUSSION

Our study clearly demonstrates that the fetal myocardium is structurally altered as a result of exposure to a moderate dose of EtOH during late gestation; in particular, the volume of the LV wall was increased together with accelerated maturation and an increase in cardiomyocyte size. Hence, although it is well recognized that exposure to high doses of EtOH during early pregnancy can lead to overt cardiac malformations, the findings of the present study indicate that the heart remains highly vulnerable to EtOH exposure at later time points in gestation, after the heart is fully formed. The changes in cardiomyocyte maturation may persist into postnatal life and may therefore have adverse programming effects on cardiac function.

Of concern, the findings of this study are highly relevant to the substantial number of individuals who have been exposed to moderate levels of EtOH during gestation. Until recently, women were often advised that moderate consumption of alcohol was not detrimental during pregnancy and many pregnant women continue to consume alcohol (8). In our animal model the maximal blood alcohol concentration of the ewes is approximately equivalent to that achieved by women 1 h after the consumption of 3 to 4 standard drinks (26); similar blood levels have been measured in young women who have been drinking alcohol socially (28).

In general, exposure to high levels of EtOH during gestation leads to reduced heart weight in parallel with reductions in body weight and in the weights of other organs (18, 33). However, in the present study we showed that, when the EtOH exposure is more moderate and restricted to a window late in gestation, there is induction of LV hypertrophy in the offspring. Whether this LV hypertrophy persists postnatally is yet to be elucidated, but if so, it is likely to lead to elevated cardiovascular risk (23). In support of our findings, an apparent increase in relative heart weight has also been described in fetal sheep following acute EtOH exposure on days 116, 117, and 118 of gestation; however, in that study heart weight was preserved in the presence of a significant reduction in fetal body weight (14).

Abnormal growth of the heart is often associated with increased deposition of interstitial collagen (cardiac fibrosis) (35, 36). Increased collagen deposition leads to stiffening of the ventricular wall (35, 36) and impaired myocardial conductivity and contractility (3, 5). We therefore considered it important to measure interstitial fibrosis in the fetal heart exposed to EtOH, because in the adult, chronic alcohol consumption typically leads to fibrosis (19, 29). However, we found that interstitial fibrosis was not induced in the fetal hearts exposed to EtOH above that found in controls, possibly because the degree of EtOH exposure was not severe.

Although the observed increase in relative LV size (weight and wall volume) in the EtOH-exposed fetuses cannot be attributed to increased deposition of collagen or number of cardiomyocytes, it is likely due to the observed increase in cardiomyocyte size. Indeed, the increase in cardiomyocyte size in the EtOH-exposed fetuses is in accordance with the acceler-
erated maturation of cardiomyocytes within these hearts; in general, mature binucleated cardiomyocytes are larger than immature mononucleated cardiomyocytes (4, 32).

In this regard a number of in vitro studies have shown that EtOH exposure can lead to alterations in the cell cycle and in cellular differentiation and maturation (10, 27). Of particular relevance, Adickes et al. (1) found that exposure to EtOH causes cardiomyocytes to become committed to the mature form prematurely. In their study, when cardiomyocytes from neonatal rat pups were exposed to different concentrations of EtOH in vitro it was found that EtOH-treated cultures contained a higher percentage of cells in the G0/G1 compartment (indicative of mature cells that have exited the cell cycle) and fewer cells in the S+G2/M compartment (actively proliferating cells). These findings of accelerated maturation as a result of EtOH exposure are in accordance with our present findings; at 134 days of gestation, the EtOH-exposed myocardium contained 64% binucleated cardiomyocytes, whereas the control myocardium contained 64% binucleated cardiomyocytes. These findings strongly suggest that the EtOH-exposed cardiomyocytes have matured earlier in gestation than in the controls.

A number of in vivo and in vitro studies have reported induction of apoptosis in cardiomyocytes in the presence of EtOH (7, 30). In support of these findings, we found marked increases in the relative mRNA levels of the proapoptotic genes caspase 3 (P = 0.007) and BAX (P = 0.057) in the myocardium of EtOH-exposed fetuses, but there were no differences in the proliferative gene c-Myc. Hence, as we initially hypothesized, there appeared to be increased apoptotic activity as well as accelerated maturation of cardiomyocytes in the EtOH-exposed hearts. However, the total number of cardiomyocytes was not significantly reduced; there was a trend in this direction (7% reduction) but it was not significant. Given our findings, it is likely that, if the EtOH exposure had been more prolonged and/or if the dose had been greater, overt reductions in total cardiomyocyte number would have become apparent; however, further studies are required to confirm this.

Interestingly, there was a significant upregulation of relative IGF-1 mRNA expression in the EtOH-exposed hearts. This is contrary to our initial hypothesis, in which we predicted reduced relative IGF-1 expression. The observed increase in relative IGF-1 expression in the present study may account for the increase in cardiomyocyte size in the EtOH-exposed hearts and may be a compensatory response of the myocardium to the increased apoptosis. Indeed, compensatory upregulation of IGF-1 gene expression has been described in other tissues undergoing apoptosis (9, 17). It is also possible that ethanol exposure results in altered relative IGF-1 gene expression via a direct effect on transcription of the gene or the rate at which the transcript is degraded.

In conclusion, using a clinically relevant model of heart development, we have shown that daily exposure to a moderate dose of EtOH in late gestation leads to left ventricular hypertrophy in parallel with alterations in cardiomyocyte growth. These adverse effects on cardiac growth may not affect cardiac function in the short term but could program for long-term cardiac vulnerability.

ACKNOWLEDGMENTS
We thank Edwin Yan and Stephen Gray for assistance.

GRANTS
This work is supported by Australian National Health and Medical Research Council and the Canadian Institutes of Health Research.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

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