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A Pilot Study of a New Chicken Model of Alcohol-Induced Cardiomyopathy

Nancy Morris, Catherine S. Kim, Angelia A. Doye, Roger J. Hajjar, Nancy Laste, and Judith K. Gwathmey

From the Angell Memorial Animal Hospital (NM, NL), Boston, Massachusetts; Whitaker Cardiovascular Research Institute (CSK, AAD, JKG), Department of Medicine, Boston University School of Medicine. Boston, Massachusetts; Gwathmey Inc (CSK, AAD, NM, JKG), Cambridge, Massachusetts; Cardiology Division (RJH), Massachusetts General Hospital, Boston, Massachusetts.

Abstract

Background—Excessive alcohol consumption is recognized as a common cause of left ventricular (LV) dysfunction. It is currently thought that 36% of all cases of dilated Cardiomyopathy are due to excessive alcohol intake. Suitable animal models are needed to study the pathogenic mechanisms of ethanol-induced LV dysfunction. We have therefore created a new model of ethanol-induced LV dysfunction in the chicken.

Methods—For 12 weeks, adult chickens were given, twice a day, by gavage, 73% of their total calculated daily water intake containing a 20% ethanol concentration. Twenty percent ethanol also was placed in the water and provided ad libitum. Control chickens received the same volume of water by gavage twice a day without ethanol. Water without ethanol was given ad libitum to control birds.

Results—Our study shows that after a relatively short duration of ethanol ingestion, chickens developed LV dilatation and LV dysfunction. The serum concentrations of ethanol attained in this new model were similar to those reported in humans. Furthermore, unlike other currently available animal models of ethanol-induced cardiac disease, this model demonstrates myocyte hypertrophy, interstitial fibrosis, and myocytolysis, similar to observations in human ethanol-induced cardiac dysfunction.

Conclusions—We conclude that this new avian model should provide a useful tool for investigating the mechanism(s) and pathophysiology of ethanol-induced dilated cardiomyopathy and heart failure.

Keywords

Chicken; Ethanol; Cardiac Dysfunction

EXCESSIVE ALCOHOL (ethanol) consumption is now recognized as a common cause of dilated cardiomyopathy in Western industrialized countries (Fernandez-Sola et al., 1994; Fuster et al., 1981). It is currently thought that 36% of all cases of dilated cardiomyopathy are due to excessive ethanol intake (Dancy et al., 1985, Song and Rubin, 1972). Although the mechanism by which ethanol induces cardiac dysfunction has not been fully elucidated, a direct correlation between ethanol consumption and depression of cardiac ejection fraction has been reported in populations of chronic alcoholics (Patel et al., 1997, Urbano-Marquez et al., 1989). According to the American Heart Association's Annual Report for 1992, heart disease

continues to be the number one killer of Americans, and approximately \$109 billion is spent each year on heart disease. As cultural drinking patterns and drinking by American youth have increased dramatically, it is likely that heart disease due to chronic ethanol consumption will continue to increase. The need for investigative research using suitable animal models also will increase.

Ethanol-induced heart muscle disease is defined as that occurring in patients in whom the sole causative agent is excessive ethanol consumption (Richardson and Wodak, 1983, Richardson et al., 1986). Chronic ethanol abuse has been shown to have a dose-related effect on cardiac muscle independent of the presence of malnutrition or of vitamin deficiencies (Estruch et al., 1993, Urbano-Marquez et al., 1989). Heart disease is characterized by decreased contractility and cardiomegaly (i.e., dilated cardiomyopathy). Histological changes include myocyte loss, myocyte hypertrophy, fibrosis (Ferrans et al., 1965; Preedy et al., 1996), and inflammatory infiltrates (Ferrans et al., 1965; Hibbs et al., 1965).

Animal models for ethanol cardiomyopathy are based on ethanol consumption and often do not represent the human situation (Regan and Moval, 1987). Methods of ethanol delivery on an individual basis in rats and mice can be labor-intensive or costly and require individual housing. These barriers have forced many investigators to resort to general water addition and ad libitum liquid diets as opposed to individual dosing. We have developed a model system that allows dosing on an individual basis by an oral route that is not labor-intensive. The size of the avian species in combination with our expertise in handling the model makes this possible.

In rats, after over 8 months of ethanol feeding with ethanol accounting for about 30% of total calories, it has been reported that there is no significant cardiac dilatation, global cardiac dysfunction, or significant arrhythmias (Capasso et al., 1992). Larger animal models, e.g., canine and monkey, do not demonstrate global cardiac dysfunction despite the administration of high ethanol concentrations for prolonged periods (Bing et al., 1974; Fahimi et al., 1979; Pachinger et al., 1973; Vasdev et al., 1975). It also is true that many studies have shown no ethanol-induced depression in contractile parameters such as peak systolic pressure, rate of pressure development, and ejection fraction (Chan and Sutter, 1982; Thomas et al., 1994). These observations in commonly used models may have been detrimental to clinical investigations and delayed recognition of ethanol-associated cardiomyopathy in humans. We have, therefore, developed a new avian model of ethanol-induced cardiac dysfunction in adult chickens. Preliminary studies have convinced us that our model of ethanol-associated heart disease has many similarities to the human condition, warranting further investigation.

METHODS

Animal Model Development

Adult chickens (age 2 months) were randomized into experimental groups using a random number generator. Each bird was marked by color for ease of identification. Chickens for each experimental group were group housed. At the time of randomization, each bird was weighed and baseline echocardiograms obtained. Chicken feed (Blue Seal Layer Pellets, Blue Seal Feeds inc., Londonderry, NH) and water with or without ethanol were provided ad libitum to both experimental groups. Echocardiograms were repeated at eight weeks. The Institutional Animal Care and Use Committee (IACUC) of Gwathmey Inc. approved the experimental protocol.

For 12 weeks, twice a day, adult chickens ($n = 6$) were given by gavage 73% of their total calculated daily water intake (Sturkie, 1986) containing a 20% ethanol concentration. Daily water intake was calculated as 5.5% of body weight, e.g., $2000 \text{ g of body weight} \times 5.5\% = 110$

g for daily water intake. The amount of ethanol was then calculated based on that result: 20% of 110 g = 22 g of ethanol to be administered twice daily. Twenty percent ethanol also was placed in the water and provided ad libitum. Chickens were group housed, and individual water intake was not recorded. Watering devices were filled three times a day for both experimental groups. Three chickens that received the same volume of water by gavage twice a day (73% of daily calculated water intake [Sturkie, 1986]) without ethanol served as controls. Water without ethanol was given ad libitum to control birds.

We first performed a standard level pharmacokinetic study in the chicken to determine serum concentrations of ethanol. This allowed us to compare blood levels with those reported in humans and other animal models. After oral gavage of 20% ethanol, blood samples were collected in nonsedated birds resting quietly at 20 min, 1 hr, 2 hr, and 4 hr. Blood samples were frozen for later analysis of ethanol concentration (Analytics Inc., Gaithersburg, MD). Coefficient of variation between tests was 0.002%.

Prior to euthanasia at 12 weeks, animal body weights were recorded. Birds were then euthanized by an intravenous injection of pentobarbital and the heart quickly removed. The atria were removed, and the left ventricle was arrested in diastole and filled with Krebs solution at 41°C (body temperature of chickens) and an in vitro heart volume determined. The hearts were blotted dry and weighed. The right ventricle was then removed and the remaining left ventricle (LV) weighed. Hearts were then sectioned longitudinally. Left ventricular free-wall thickness and left ventricular luminal diameter were recorded at right angles to the long axis of the heart just below the level of the mitral valve. Left ventricular luminal length also was measured.

Echocardiography

Echocardiographic windows on the left and right sides caudal to the ribs as well as short-axis views were obtained using a 5.0-MHz transducer using InterSpex Cardioscan Model 2096 on nonsedated, hooded, and quietly resting birds (Gruver et al., 1993; Hajjar et al., 1993). Several cardiac cycles were recorded on videotape, and the two-dimensional images subsequently were played back for analysis on a Panasonic 63300 videocassette recorder. Fractional shortening (FS%) was used as an index of in vivo contractile performance (Gruver et al., 1993; Hajjar et al., 1993; Henry et al., 1980; Sahn et al., 1978), FS% was defined as $(LVIDd - LVIDs) / LVIDd \times 100$.

Histopathology

A section of the left ventricle was placed in 10% buffered formalin. Sections 5 μ m thick were prepared in paraffin, stained with hemoxylincosin for myocyte fiber diameter measurements, and counterstained with trichrome for connective tissue content determinations as previously described (Gwathmey, 1983, 1991). Briefly, fiber diameter was measured using a linear ocular scale at the level of a centrally located nucleus in longitudinally cut fibers (Gwathmey, 1983). A point counting technique was used to quantitate connective tissue content.

For semiquantitative evaluation of myocytolysis, sections were graded 1+ to 4+, depending on the extent of the finding. Grade 0 signifies not present; 1+ indicates 25% of the area affected; 2+ is 50% of the area affected; 3+ represents 75% of the area affected; and 4+ indicates 100% of the area affected. For the point counting technique, a Zeiss Integrated phase 11 grid measuring $3 \times 10^2 \mu\text{m}$ ($9 \times 10^4 \mu\text{m}^2/\text{area}$) was used at a magnification of $\times 320$ with a Zeiss microscope (Gwathmey, 1983).

Statistical Analysis

Results are presented as mean $\pm$ SD. Statistical significance was determined by Student's *t*-test. The level of statistical significance was set at a probability of 0.05.

RESULTS

Serum Concentrations of Ethanol

Blood ethanol levels were $0.07 \pm 0.02\%$ at 20 minutes, $0.2 \pm 0.05\%$ at 1 hr ($p = 0.07$ compared to 20 min), $0.1 \pm 0.01\%$ at 2 hr ($p = 0.133$ compared to 1 hr), and $0.08 \pm 0.03\%$ at 4 hr ($p = 0.55$ compared to 2 hr). The p value for the effect of time as a variable was >0.05 . There were no significant differences in ethanol blood concentrations at any of the time points sampled (i.e., 20 min to 4 hr). These data indicate that after gavage, serum ethanol concentration is stable for up to 4 hours within values reported in humans abusing alcohol and reported as intoxicated (about 0.15%). For the first 2 weeks, the birds behaved as if "drunk." They demonstrated a wobbly gait, staggering, and poor balance. After this time point, the birds tolerated the ethanol quite well with no outward behavioral signs of "drunkenness" or diminished motor coordination and mobility.

Body Weight

At initiation of the study, control chickens weighed significantly more than did chickens selected to receive ethanol (3.17 ± 0.08 vs. 2.7 ± 0.11 kg, respectively; $p = 0.02$). However, body weights were 3.8 ± 0.06 kg for control ($n = 3$) and 3.2 ± 0.27 kg for ethanol-treated birds ($n = 6$; $p = 0.2$) at 8 weeks and 4.75 ± 0.07 kg vs. 4.52 ± 0.41 kg ($p = 0.5$) at the end of the study (i.e., 12 weeks). These data suggest that ethanol treatment did not impact nutritional status or growth in the birds.

Echocardiography

Figure 1 shows echocardiographic images from two birds. A short-axis view with M-mode is demonstrated. Eight weeks after ethanol treatment, the following ECHO data were obtained. Fractional shortening was $49 \pm 12\%$ vs. $36 \pm 9\%$ for control ($n = 3$) and ethanol-treated chickens ($n = 6$), respectively. LV end-diastolic dimensions were 1.8 ± 0.3 cm ($n = 3$) vs. 2.2 ± 0.3 cm ($n = 6$), and LV end-systolic dimensions were 0.91 ± 0.16 cm vs. 1.4 ± 0.28 cm ($p = 0.02$) for control and ethanol-treated chickens. Although the numbers are not significantly different except for LV end-systolic dimension at the 8-week time point, they clearly show a trend toward LV dilatation and LV dysfunction. LV wall thickness was 0.42 ± 0.04 cm vs. 0.46 ± 0.03 cm for control and ethanol-treated chickens.

Gross Morphology

After 12 weeks, the chickens were sacrificed and the gross heart morphology documented and gross appearance of the liver noted. There were no visible signs of liver damage, e.g., discoloration, signs of congestion, nodules, or enlargement noted in the ethanol-treated chickens. Heart weight was 13.7 ± 1.1 g vs. 13.9 ± 2.7 g ($p = 0.9$). LV weight was 9.4 ± 0.4 g vs. 10.7 ± 0.9 g ($p = 0.053$), and LV volumes were 1 ± 0 ml vs. 2.9 ± 0.6 ml ($p = 0.008$) for control and ethanol-treated chickens. LV length was 2.5 ± 0.28 cm vs. 3.38 ± 0.38 cm ($p = 0.03$) and LV width 2.0 ± 0 cm vs. 2.6 ± 0.26 cm ($p = 0.03$) for control and ethanol-treated chickens, respectively. LV wall thickness was 0.6 ± 0 cm vs. 0.46 ± 0.11 cm for control and ethanol-treated birds, respectively ($p = 0.07$). Although wall thickness was not different, there was a trend toward left ventricular wall thinning with chronic ethanol treatment.

Histopathology

Myocyte fiber diameter was $6.5 \pm 0.89 \mu\text{m}$ vs. $13.2 \pm 2.19 \mu\text{m}$ ($p < 0.001$) for control and ethanol-treated chickens, respectively. Connective tissue content was $2 \pm 0.1\%$ vs. $4 \pm 0.1\%$ in control and ethanol-treated chickens ($p < 0.001$). In addition, the left ventricular myocardium of birds given ethanol demonstrated myocytolysis (+1 for ethanol treated birds, 0 for control birds). Therefore, after only 8 to 12 weeks, chickens show cardiac dilatation. These data again support the utility of this model, which demonstrates myocyte hypertrophy (unlike the rat model), myocytolysis, and increased connective tissue content, as reported in human myocardium from patients with ethanol-induced cardiomyopathy. Interestingly, one chicken developed an arrhythmia similar to findings in humans.

Eggs

All chickens continued to lay eggs while on ethanol. We noticed that eggs were significantly smaller from chickens receiving ethanol ($62.9 \pm 2.4 \text{ g}$ vs. $50.7 \pm 2.0 \text{ g}$ for control and ethanol-treated birds ($n = 10$ eggs per group; $p < 0.001$). These observations might suggest an interesting use and development of this model to study the effects of ethanol on in ovo fetal development.

DISCUSSION

It is clear that new animal models should be developed that more closely mimic the functional, morphologic, and histopathological changes identified in human ethanol-induced cardiomyopathy. Rubin and Urbano-Marquez have pointed out that before one can adequately study the clinical presentation and outcomes that are relevant to humans, the clinical presentation and syndrome of ethanol-induced cardiomyopathy must be induced in an appropriate animal model (Rubin and Urbano-Marquez, 1996). A clear example of the lack of consistency in experimental findings with the rodent model is demonstrated in two publications from Capasso et al. (1991, 1992). In one paper, they state that ethanol-induced cardiomyopathy in rats typically is characterized by the absence of reactive hypertrophy at the cellular level. This is of great concern because myocyte hypertrophy is characteristic of heart failure and cardiomyopathy in humans and has been implicated in the pathogenesis of the disease. They concluded that their experimental findings had the “ingredients for the development of cardiac failure of alcoholic origin.” They were not able to induce heart failure, however.

Ethanol-induced heart muscle disease is defined as that occurring in patients in whom the sole causative agent is excessive ethanol consumption (Richardson and Wodak, 1983; Richardson et al. 1986). Chronic ethanol abuse has been shown to have a dose-related effect on cardiac muscle independent of the presence of malnutrition or of vitamin deficiencies (Estruch et al. 1993; Urbano-Marquez et al., 1989). In our avian model, we did not notice signs of malnutrition. Body weights were not different in the two experimental groups at the conclusion of our study.

A variety of functional abnormalities have been measured in attempts to assess the abnormalities of myocardial cell function. To interpret these experimental studies in a meaningful way, careful staging and hemodynamic assessment of a representative model of dilated cardiomyopathy is needed. The size of the chicken, as well as the ability to perform longitudinal, noninvasive studies on cardiac function, can ensure careful study of the disease progression. The biochemical and molecular mechanisms underlying the pathology, pathophysiology, or pathogenesis of ethanol-induced cardiomyopathy can then be studied in a better-controlled setting.

Larger animal models afford the investigator with sufficient tissue and blood samples for multiple assays and studies. Furthermore, cardiac regulatory proteins in the avian species are

similar to those seen in humans (Chapados et al., 1992; Genao et al., 1996; Glass et al., 1993; Gruver et al., 1993; Gwathmey and Hajjar, 1992; Hajjar et al., 1993), and large animal models do not demonstrate developmental myosin isoform switching in the heart as seen in rodent models. Rodents demonstrate multiple developmental isoform switching of key calcium regulatory proteins and undergo developmental changes in myosin isoforms switching from V1 to V3 with age and cardiac disease. Our avian model has all the advantages of larger animal models, e.g., V3 myosin isoforms, regulatory proteins similar to humans, and similar functional characteristics. Furthermore, both noninvasive and invasive studies are much easier to perform in our avian model (Chapados et al., 1992; Genao et al., 1996; Glass et al., 1993; Gruver et al., 1993; Gwathmey and Hajjar, 1992; Hajjar et al., 1993). We have been able to obtain echocardiograms noninvasively and without chemical restraint while the bird is quietly resting. The lack of statistical significance in our echocardiographic findings most likely reflects the small number of birds used in this pilot study.

It has become clear that impairment in myocardial contractility in the cardiomyopathic human heart is associated with alterations in Ca^{2+} mobilization and a negative force-interval relationship (Gwathmey et al., 1990; Mulieri et al., 1992). Chronic ethanol consumption in rodent models is not associated with a negative force interval relationship; however, it is important to note that rodents naturally have a negative force interval relationship. For this reason, we have developed a new larger animal model of ethanol-induced cardiac dysfunction in a species that has a positive force interval relationship, thereby affording investigators the ability to address clinical and functional questions related to the human condition.

Ethanol-induced cardiomyopathy can be caused by feeding ethanol to turkeys and is manifested physiologically by cardiac dysfunction, decreased norepinephrine content, and myofibrillar lysis (Czarnecki et al., 1985; Edes et al., 1987; Noren et al., 1983; Pierpont et al., 1992). Many of the ethanol studies in turkeys have been performed on immature birds (i.e. turkey poults) (Czarnecki et al., 1985; Noren et al., 1983; Pierpont et al., 1992). This approach then confounds developmental changes with ethanol-induced changes on myocardial function and subcellular adaptive changes. This scenario typically is not reflective of the adult human condition and, furthermore, is not ideal for studying the developmental influences of ethanol on fetal growth. Cardiac dysfunction at 2 months of age in birds fed 5% ethanol has been shown to be the same order of magnitude as that in 25-week-old turkeys fed 15% ethanol for 16 weeks. However, although these studies report cardiac dysfunction with increased end-systolic and end-diastolic diameter, fractional shortening was not significantly different from that in control animals. Heart weight also was not increased.

In conclusion, we report for the first time a new adult avian model of ethanol-induced cardiac dysfunction. Unlike other models, this model presents myocyte hypertrophy, fibrosis, and myocytolysis. The greater versatility of this model should make it attractive to scientific investigators. Furthermore, it is thought that longer durations of ethanol administration in this model might result in congestive dilated cardiomyopathy. We plan further development and characterization of the model by investigating the effects of differing concentrations and durations of ethanol administration thereby optimizing animal maintenance costs and durations of experimental protocols for investigators.

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Fig. 1. Echocardiographic images of chicken hearts. (A) Control chicken heart. M-mode tracing through the left ventricle. (B) Control chicken. Short axis view of the left ventricle in diastole.