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Mitochondrial Damage Revealed by Morphometric and Semiquantitative Analysis of Mouse Pup Cardiomyocytes Following *in Utero* and Postnatal Exposure to Zidovudine and Lamivudine

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Zidovudine (ZDV), an antiretroviral drug used alone or in combination with other antiretroviral agents to treat HIV-infected pregnant women and their newborn infants, effectively reduces mother-to-child transmission of the virus. That myopathy and cardiomyopathy, related to mitochondrial damage, develop in some adults chronically treated with ZDV has long been known; recently, reports have suggested that similar adverse effects may occur in some infants exposed perinatally. Using a mouse model of human neonatal exposure, we treated pregnant CD-1 mice twice daily with doses of 75 mg/kg ZDV plus 37.5 mg/kg lamivudine throughout gestation and lactation; pups were exposed by direct gavage beginning postnatal day (PND) 4 and sacrificed on PND 28. Hearts were removed rapidly, and ventricles were processed for electron microscopy. Morphometric and semiquantitative morphological analyses were performed on three micrographs from each of three blocks from each of three females and three males from the control and treated groups. Treated mice showed significant increases in the mean area and decreases in the mean number of cardiomyocytic mitochondria compared to controls. We observed clusters of damaged mitochondria more frequently in treated animals than in controls; damage included fragmentation and loss of cristae. These results, demonstrating alterations in cardiomyocytic mitochondria of mice exposed *in utero* and postnatally, may model cardiac damage reported in human infants similarly exposed to ZDV. Critical insights derived from animal-model data like these may be used to mitigate risks to thousands of human infants receiving essential lifesaving therapy with antiretroviral drugs.

Key Words: **mitochondria; heart; AIDS drugs; transplacental exposure; mice; electron microscopy; morphometry.**

HIV-infected pregnant women are treated with antiretroviral nucleoside analogues to prevent mother-to-child transmission of the virus; administration of the nucleoside reverse transcriptase inhibitors (NRTIs) zidovudine (ZDV) and lamivudine (3TC), often in combination with additional antiretroviral agents, constitutes standard clinical practice in the United States (CDC, 2003; Cooper *et al*., 2002; Mofenson and McIntyre, 2000). Although ZDV alone has been shown to be effective in reducing perinatal transmission of HIV, monotherapy is now considered to be suboptimal in adults, and aggressive combination drug therapy regimens are considered the standard care. The recommended therapeutic regimen for pregnant women includes treatment of the mother with ZDV-containing combination therapy during at least the last trimester of pregnancy, intravenous ZDV during labor and delivery, and oral treatment of the infant with ZDV for 6 weeks after birth (CDC, 2003). Due to the success of ZDV therapy in reducing neonatal HIV infections, many of the approximately 7000 babies born to HIV-infected mothers annually in the United States are now exposed *in utero* and neonatally to ZDV and other nucleoside analogues (Poirier *et al*., 2003).

Most large-scale surveillance studies have found no persistent treatment-related adverse effects in ZDV-exposed children (Culnane *et al*., 1999; European Collaborative Study, 2003; Hanson *et al*., 1999; The Perinatal Safety Review Working Group, 2000; Tuomala *et al*., 2002). In HIV-infected adults, however, chronic ZDV therapy has long been associated with the development of myopathy, cardiomyopathy, neuropathies, and hepatic dysfunction resulting from mitochondrial toxicity and dysfunction (Dalakas *et al*., 1990; Gerard *et al*., 2000; Mhiri *et al*., 1991; Lewis and Dalakas, 1995). Whether these adverse effects are due to the treatment, the disease, or a combination of both has not always been clear.

Recent studies in laboratory animals and HIV-negative babies born to HIV-infected women have allowed better delineation between the effects of treatment and disease, and evidence for the potential for ZDV-induced genetic and mitochondrial damage in exposed infants is accumulating. Significant mitochondrial damage in heart muscle was demonstrated in 18-month-old mice prenatally exposed to ZDV during gestation days 12–18, a period in which the last 40% of *in utero* development occurs (Walker *et al*., in press). In addition, mitochondrial damage in

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cardiac and skeletal muscle was observed in fetal monkeys exposed to ZDV *in utero* (Gerschenson *et al*., 2000). Incorporation of ZDV into nuclear (Olivero *et al*., 1997, 1999, 2002) and mitochondrial (Olivero *et al*., 1997) DNA has been demonstrated in laboratory animals and human infants exposed *in utero*, and mitochondrial DNA depletion was observed in cord blood cells and peripheral leukocytes of HIV-negative infants and children exposed *in utero* to ZDV and 3TC (Poirier *et al*., 2003). Significant levels of genetic damage have been detected in erythrocytes of mouse pups treated with ZDV and other nucleoside analogues *in utero* or postnatally (Bishop *et al*., 2004; Von Tungeln *et al*., 2002). Reports of cardiopathology, neuromuscular disease, and mitochondrial damage in several infants in a large French cohort exposed perinatally to antiretroviral drugs have raised concern that short-term perinatal exposures to these powerful drugs may be sufficient to induce adverse health effects (Barret *et al*., 2003; Blanche *et al*., 1999).

In response to accumulating evidence that mitochondrial dysfunction and other adverse effects associated with ZDV treatment may not be limited to chronically exposed adult patients, we designed experiments using a mouse model of human perinatal antiretroviral therapy to explore further the relationship between *in utero* nucleoside analogue exposure and mitochondrial integrity in cardiomyocytes. We treated pregnant CD-1 mice throughout gestation with an antiretroviral drug combination of ZDV and 3TC; following birth, we treated pups directly using the same dosing regimen. Results of our analyses of the cardiomyocytic mitochondria in these treated pups further support the growing realization that therapeutic treatment of pregnant women with ZDV and other nucleoside analogues, although highly successful in reducing HIV infection rates in neonates and therefore a critical element in the anti-HIV arsenal, may also carry some risk for adverse consequences that may not become manifested until years after treatment has ended.

MATERIALS AND METHODS

Treatment of animals. Male and female CD-1 mice (Charles River Laboratories, Portage, MI) were administered two equally divided daily gavage doses of 0 or 150/75 mg/kg/day ZDV/3TC (ZDV Lot: FX2235; 3TC Lot: B20276 [Cipla, Mumbai, India]) beginning 2 weeks prior to cohabitation, and females were dosed throughout pregnancy and lactation. Direct treatment of pups using the same dosing regimen used for their parents began on postnatal day (PND) 4 and continued until PND 28. The dose volume was 10 ml/kg for dams and 5 ml/kg for pups. The stock drug concentration of ZDV/3TC for dams was 7.5/3.75 mg/ml and, for pups, 15/7.5 mg/ml. The solvent was a mixture of 0.1% po1ysorbate 80 and 0.2% methylcellulose.

Electron microscopy. On PND 28, three female and three male mice were selected randomly and anesthetized by intraperitoneal injection of pentobarbital; hearts were excised swiftly, and atrial and ventricular regions were transferred immediately into the fixative for electron microscopy, 3% glutaraldehyde (Ladd Research Industries, Inc., Burlington, VT) buffered to pH 7.2 with 0.1 M sodium cacodylate (Electron Microscopy Sciences, Fort Washington, PA). Small pieces approximately 1 mm^3 were stored in fixative at 4°C for 3 days, washed in 0.1 M sodium cacodylate buffer, postfixed in cacodylate-buffered 1% OsO_4 (Polysciences, Warrington, PA), rinsed in water, dehydrated through a series of graded ethanols, and embedded in Polybed 812 epoxy resin (Polysciences, Warrington, PA). Semithin (1/2 μm) sections of ventricle stained with 1% toluidine blue were examined by light microscopy to locate regions containing longitudinal fibers. Ultrathin (90 nm) sections were cut from these regions, stained on-grid with 5% uranyl acetate and Reynold's lead citrate, and examined in a Philips EM 400 electron microscope operated at 80 kV. Nine electron micrographs were taken for each animal (three blocks [regions]/animal, three random micrographs/block). Each micrograph was taken at 8350× and enlarged 3× to appear on the print at a magnification of 25,050× (Fig. 1).

Semiquantitative morphological and statistical morphometrical analyses. A semiquantitative evaluation of the 54 electron micrographs was independently conducted by four observers. The scoring method used in this evaluation was adapted from Walker *et al*. (in press), and included four grades as follows: Grade 0, no evidence of cellular pathology or early autolysis, or an occasional mitochondrion with minimal loss of cristae while the remainder of mitochondria appear normal; Grade 1, discontinuous cristal membranes and/or partial loss of cristae and matrix material in a few mitochondria; Grade 2, multiple disruptions of the cristal membrane and substantial loss of cristae and matrix in approximately half of the mitochondria; Grade 3, fragmented cristal membranes and effacement of central architecture in a majority of the mitochondria. After each observer analyzed the micrographs for degree of mitochondrial morphological pathology, the mean of the individual observations was calculated. The grading data were not subjected to statistical analysis.

Following visual assessment of mitochondrial damage in the micrographs, we conducted morphometric evaluations to quantitate the observed damage. All micrographs were scanned electronically. The area of ventricle analyzed for each micrograph was ~70 μm^2. After a pixel-to-micrometer conversion, the boundary of each mitochondrion was traced to measure the area using Image-Pro® Plus (Media Cybernetics, Inc., Silver Springs, MD). Mitochondria on the edge of micrographs were excluded from evaluation, since neither their boundaries nor area could be accurately determined. The numbers and areas of complete mitochondria were recorded. After converting these variables into a log scale, the mean area and mean number of mitochondria in the two groups (treated and control) were compared. The distribution of the raw data appeared to be extremely skewed with a very long righthand tail. Hence, we performed log transformation of the data prior to analysis, so that variances between the two groups were approximately equal. The resulting data were analyzed using nested mixed-effects analysis; animals within groups, blocks within animals within groups, and micrographs within blocks within animals within groups were treated as random effects. We used the statistical procedure PROC MIXED in SAS (version 8.2) to perform all analyses.

RESULTS

Semiquantitative Morphological Analysis

The mitochondrial damage observed in ZDV/3TC-treated animals was typified by fragmented and missing cristae, which rendered matrices electronlucent and increased the spaces between remaining cristae (Fig. 1B). Occasionally, unusual simple or whorled, lamellated membranes were observed within a mitochondrion, and the double bounding membrane was ruptured; rarely, fusion between mitochondria appeared to be occurring. Damaged mitochondria appeared swollen, displacing adjacent sarcomeres. The results of our semiquantitative evaluation of mitochondrial morphological pathology indicated that control animals (Fig. 1A) generally exhibited mitochondrial alterations that ranged from grade 0 to 1, whereas mitochondria in treated animals showed alterations generally in the 1-to-2-grade range. The number and severity of damaged mitochondria

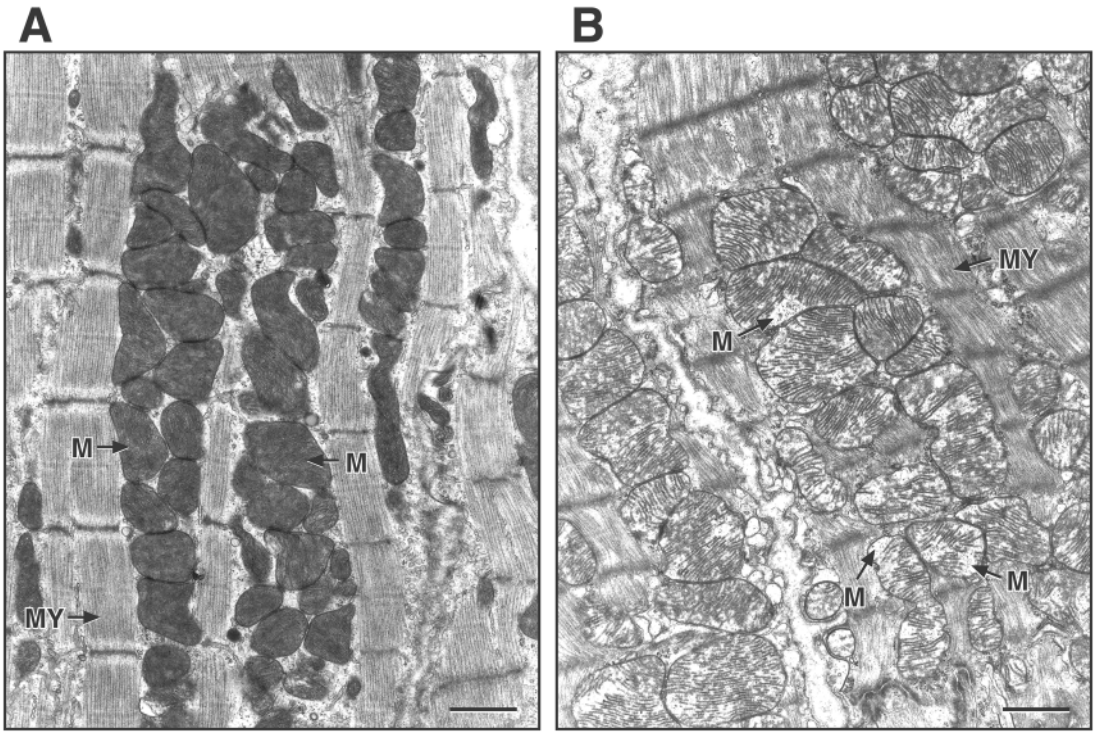

FIG. 1. Electron micrographs of mitochondria from hearts of 28-day-old control (A) and ZDV/3TC-treated pups (B). Each micrograph was taken at 8350× and enlarged 3× to appear on the study print at a magnification of 25,050×. M = mitochondrion, MY = myofibril; arrows indicate damage due to loss of cristae. Bar = 1 μm.

appeared to be greater in females than males. Since variability existed from region to region and micrograph to micrograph within an individual animal, grading reflected the overall assessment of damage for an animal.

Some minimal mitochondrial damage, consisting chiefly of cristal breakage and matrical electronlucency, was occasionally observed in control mice, and interanimal variability was observed in both treated and control groups. We therefore conducted morphometric evaluations to quantitate the damage more precisely (Table 1). Although the data were analyzed after log-transformation, for simplicity of presentation we summarized the means and standard errors in Table 1 using the untransformed data.

Morphometrical Statistical Analysis

Morphometry demonstrated that, for male and female mice combined, the mean area of mitochondria in the ZDV/3TC-treated group ($0.5862 \pm 0.0174\ \mu m^2$) was significantly greater than that of the control animals ($0.5111 \pm 0.0171\ \mu m^2$) (Table 1). The mean number of mitochondria in treated animals (39.63 ± 1.65 per picture) was significantly less than that in the controls (46.24 ± 1.65 per picture) (Table 1). The above numbers are means ± standard error obtained from the mixed effects model.

The fold-change in the mean area of mitochondria in ZDV/3TC-treated animals was 121% (21% increase) for females, with 95% confidence limits ranging from 102% to 143% (increase of 2% to 43%); for males the fold-change in mean area was 108% (modest 8% increase), with 95% confidence limits ranging from 84% to 139% (decrease of 16% to increase of 39%). For treated males and females combined (Fig. 2), the fold-change in mean area was 114% (14% increase), with 95% confidence limits ranging from 101% to 130% (1% to 30% increase).

The fold-change in the mean number of mitochondria in ZDV/3TC-treated animals was 83% (a 17% decrease) for females, with 95% confidence limits ranging from 67% to 103% (33% decrease to 3% increase); for males the fold-change in the mean number of mitochondria was 90% (decrease of 10%), with 95% confidence limits ranging from 72% to 111% (28% decrease to 11% increase). For males and females combined (Fig. 2) the fold-change in mean number was 86% (14% decrease), with the 95% confidence limits ranging from 76% to 97% (24% decrease to 3% decrease).

DISCUSSION

This study constitutes the first report of mitochondrial damage in cardiomyocytes using an animal model exposed to antiretroviral drugs both *in utero* and postnatally in a regimen similar to that used to prevent mother-to-child transmission of HIV in

TABLE 1
Morphometric Analysis of Mitochondria from 28-Day-Old Control and Zidovudine/Lamivudine-Treated Pups[a]

Group	Mitochondrial area (μm^2)			Number of mitochondria (per picture)		
	Males	Females	Combined	Males	Females	Combined
Control	3	3	6	3	3	6
	0.5112 ± 0.0286	0.5108 ± 0.0225	0.5111 ± 0.0171	47.11 ± 2.34	45.37 ± 2.65	46.24 ± 1.65
ZDV + 3TC[b]	3	3	6	3	3	6
	0.5625 ± 0.0288	0.6101 ± 0.0229	0.5862[c] ± 0.0174	42.96 ± 2.34	36.33 ± 2.65	39.63[c] ± 1.65

[a]Data presented as mean ± standard error derived from the mixed effects model; three males and three females in each group (treated and control). Combined groups consisted of three males plus three females.
[b]150 mg/kg ZDV + 75 mg/kg 3TC per day.
[c]$p < 0.05$ versus matched control; statistical analysis was performed only for the combined data.

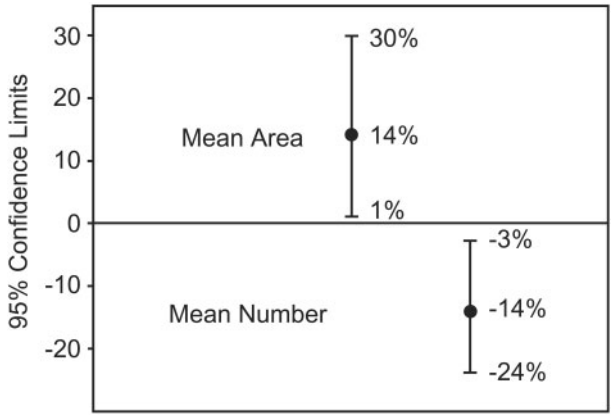

FIG. 2. Estimated percent change in mean area and in mean number of mitochondria in AZT/3TC-treated mice relative to controls. The estimated percentage of change in mean area of mitochondria for the treated mice relative to the control mice is 14%, with 95% confidence limits of 1% and 30%. The estimated percentage of change in mean number of mitochondria for the treated mice relative to the controls is −14%, with 95%, confidence limits of −24% and −3%. Because neither confidence interval line intersects zero, the estimated percentages of change in mean area and mean number are both statistically significant at $p = 0.05$.

humans. In addition, our study is the first to utilize quantitative morphometric techniques to analyze more precisely the morphological alterations induced by NRTIs in cardiomyocytic mitochondria. We clearly demonstrated a reduction in the number of cardiomyocytic mitochondria and an increase in the area of these mitochondria in male and female mice exposed to a combination of ZDV plus 3TC throughout gestation and for 28 days postnatally. Our data add to the growing body of experimental and clinical evidence for NRTI-induced damage to mitochondria in various animal and human tissues and indicate that not only chronic exposures, but also potentially short-term exposures during sensitive developmental periods, may be sufficient to induce adverse mitochondrial effects having possible long-term health consequences.

Our pilot study clearly indicated an effect of NRTI treatment on cardiomyocytic mitochondria, but three aspects of this study require further discussion. First, the investigation reported here was actually a substudy within a large comprehensive study designed to evaluate reproductive toxicity in mice dosed with ZDV plus 3TC; thus, the dosing of pups after birth consisted of the two-drug combination rather than ZDV alone. In the United States, the standard of care for human infants receiving prophylactic antiretroviral therapy is oral ZDV alone for 6 weeks, although variations in treatment may occur, and the ZDV/3TC drug combination is commonly administered as standard care to HIV-infected women during pregnancy (Fiscus *et al*., 2002). Second, this pilot study examined a small sample of animals available from the F_1 progeny produced in the initial round of matings in the reproductive study. Although a suggestion of a stronger effect of treatment in female pups was observed, our sample of three male and three female pups, while sufficient to detect treatment-related changes in mitochondrial number and size, was insufficient to determine possible gender-related differences. Finally, we did not assess mitochondrial or cardiac function, and heart weight measurements were not obtained. Additional investigations with more extensive parameters are ongoing and planned to elucidate these issues.

In contrast to our study, most investigations of ZDV-induced mitochondrial effects in humans and laboratory animals have targeted skeletal muscle tissue. One recent report, however, showed mitochondrial swelling, similar to that which we observed in cardiomyocytes of mouse pups, detected by electron microscopy of cardiomyocytes in adult rats exposed to 100 mg ZDV/kg/day for 240 days in drinking water (Ruga *et al*., 2003). Cardiovascular structural and functional alterations, including increases in systolic blood pressure and heart weight, hypertrophy of the interventricular septum, and changes in vascular smooth muscle responsiveness, were also noted in these rats at autopsy, but the relationship between the mitochondrial enlargement and the observed cardiovascular changes was not established.

The fate of initial limited damage induced by perinatal exposure to ZDV and other antiretroviral nucleosides is important to any determination of future risk. The mitochondrial damage observed in cardiomyocytes in our study was not sufficiently extensive or severe to cause overt clinical signs in pups at PND 28; however, mitochondrial damage in critical tissues such as the heart may not become symptomatic until levels of mitochondrial DNA (mtDNA) fall to less than 30% of normal (Haas, 2000). In HIV-infected adults undergoing antiretroviral therapy with nucleoside analogues, symptoms of mitochondrial damage often resolve soon after treatment with the causative agent is stopped (Brinkman *et al*., 1998). Recently, however, changes were reported (Walker *et al*., in press) in mitochondrial ultrastructural morphology and cardiac structure (heart enlargement) and function (increased cytochrome *c* oxidase activity) in both newborn and 18- to 24-month-old $B6C3F_1$ mice exposed transplacentally to ZDV/3TC during the last week of gestation. These data provide evidence that cardiac dysfunction in treated mice may persist into mid-to-late adulthood. In the 18-month-old mice exposed *in utero* to ZDV alone, mitochondria in damaged regions of cardiac muscle were swollen, reduced in number, and spatially disorganized; the occurrence of dose-related cardiomyopathy was more pronounced in female mice than in male mice. These latter observations in 18-month-old mice are in general agreement with our findings in 28-day-old pups. Interpreting their data, Walker and his colleagues hypothesized that, over time, regions of ZDV/3TC-altered mitochondria proliferated, creating expanded foci of damage. Other researchers have reported persistent clinical symptoms (including elevated plasma lactate levels, myopathy, neuropathy, seizures, and reduced respiratory chain activity) related to induced mitochondrial damage in human infants and children exposed to nucleoside antiretrovirals (Alimenti *et al*., 2003; Barret *et al*., 2003; Blanche *et al*., 1999; Domanski *et al*., 1995; Poirier *et al*., 2003). Thus, although in adults the symptoms of NRTI-induced mitochondrial damage appear to resolve rapidly after discontinuation of therapy, infants and children may respond differently following cessation of treatment.

Results of our experiments using a mouse model of human perinatal antiretroviral therapy have provided additional evidence that ZDV or the ZDV/3TC combination has the potential to induce adverse health effects in neonates exposed perinatally to antiretroviral therapy. Although clinical evidence of adverse effects in a few children within a large study cohort has been presented (Barret *et al*., 2003; Blanche *et al*., 1999), and elevated plasma lactate levels in a group of perinatally exposed infants were recently reported (Alimenti *et al*., 2003), other clinical assessments of ZDV-exposed HIV-negative children have not identified persistent treatment-related adverse effects during follow-up through approximately the first 6 years of life (Culnane *et al*., 1999; European Collaborative Study, 2003; Hanson *et al*., 1999; Lipshultz *et al*., 2000; The Perinatal Review Working Group, 2000). Symptoms of ZDV-induced mitochondrial damage in infants may remain subclinical for years, until triggered by a cardiac stressor such as strenuous physical activity or disease. Although the mouse pups in our study did not demonstrate overt clinical symptoms of cardiomyopathy, no specific tests were conducted to assay cardiovascular fitness or mitochondrial function. Additional studies in mice are currently underway in our laboratory to measure the progression or regression of perinatal ZDV-induced mitochondrial damage over time, after the cessation of exposure, and to evaluate other endpoints of mitochondrial activity. These data may help to clarify the potential long-term risk from NRTI-associated mitochondrial damage in exposed children.

Results of our experiments suggest that the CD-1 mouse might serve as a useful model for analysis of cardiac mitochondrial toxicity detected after *in utero* and postnatal antiretroviral drug therapy, a treatment regimen modeled after that typically used in humans to prevent mother-to-child transmission of HIV. Previous studies of antiretroviral-induced cardiac mitochondrial toxicity in animals have not modeled the human perinatal exposure regimen as completely. For example, using an *Erythrocebus patas* monkey model of *in utero* exposure, investigators (Gerschenson *et al*., 2000) demonstrated in full-term fetuses that transplacental exposure to ZDV at ~86% of the human daily dose for the second half of gestation resulted in structural alterations in mitochondria of skeletal muscle (including disrupted cristae), mtDNA depletion in cardiac and skeletal muscle, and other indicators of myopathy. Gerschenson *et al*. (2000) did not assess effects of postnatal dosing in juveniles. In rodent models, previously published studies of ZDV-induced heart damage have focused primarily on treatments of adult animals (Lewis *et al*., 1991, 2000, 2001; Masini *et al*., 1999; Ruga *et al*., 2003), which may respond differently to antiretrovirals than developing fetuses and neonates. Thus, as we continue to seek improved treatments to prevent mother-to-child transmission of HIV, we must take advantage of experimental systems such as this CD-1 mouse model to elucidate obscure biological side effects not detectable by standard clinical evaluation methods, which may substantially impact the long-term health of the exposed neonate.

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