

CHAPTER 4: Diethylstilbestrol (DES)-Induced Fetal Thymic Atrophy in C57BL/6

Mice: Inhibited Thymocyte Differentiation and Increased Apoptotic Cell Death

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4.1 Abstract

Treatment of pregnant C57Bl/6 mice with 48 µg/kg DES on gestation days (gd) 14 and 16 resulted in both decreased day 18 fetal thymic cellularity as well as alterations in thymocyte phenotype. Histopathologic examination of gd 18 fetal thymi from DES-exposed dams demonstrated a decrease in thymic size and cellularity and an increase in pyknotic nuclei, indicative of apoptosis, relative to control thymi. Thymic architecture was also altered by DES treatment with a decrease in the distinction between the cortical and medullary regions. Flow cytometric staining of day 18 thymocyte suspensions with the apoptotic marker 7-AAD showed a decrease in thymocyte viability after DES, and a concomitant increase of thymocytes in early apoptosis. When thymocytes were co-identified by CD4 and CD8 cell surface antigen expression, trends toward increased apoptosis were present in the CD4⁺CD8⁺ and CD4⁺CD8⁻ subpopulations, and significantly increased apoptosis occurred in the CD4⁻CD8⁻ and CD4⁻CD8⁺ subpopulations. These histopathologic and flow cytometric findings support enhanced apoptosis of thymocytes as a contributing factor to fetal thymic atrophy caused by DES.

Keywords: DES, developmental, immune, murine, thymus

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4.2 Introduction

Pharmacologic or supra-pharmacologic exposure to diethylstilbestrol (DES) causes immune suppression in the laboratory rodent (Luster et al. 1984a; Nilsson and Carlsten 1994). Deliberate, but not efficacious, administration of this agent as a hormone for the maintenance of pregnancy led to numerous developmental human exposures (Bamigboye and Morris 2003). Immune modulation has been reported among those who were exposed during fetal development (Vingerhoets et al. 1998). For instance, *in utero*-exposed women show alterations in T-cell mediated immunity when compared to age-matched unexposed controls (Burke et al. 2001). Although DES is no longer prescribed for the maintenance of pregnancy, its current therapeutic uses include the treatment of prostate cancer in men and inclusion in hormone replacement formulations for women (Chodak et al. 2002; Gangemi et al. 1995). In addition, environmental contaminants identified as endocrine disrupters may act upon the estrogen receptors to evoke immune system responses similar to DES (reviewed by Ahmed 2000).

The thymus is a remarkably sensitive target of estrogenic compounds. Estrogen receptors are present on both the thymocytes as well as cells of the thymic stroma (Mor et al. 2001), suggesting multiple targets of estrogenic exposure that may contribute to altered T cell development. Identification of sex hormone receptor-containing cells via immunoelectron microscopy demonstrated the highest number of these cells are found in

the subcapsular and medullary regions of the thymus (reviewed by Sieki and Sakabe 1997).

Thymic atrophy, decreased thymic cellularity, alterations in thymocyte cell surface marker expression, and impaired cell-mediated immune responses have all been reported in rodents treated with DES (Barnes et al. 1983; Holladay et al. 1993; Holsapple et al. 1983). Multiple mechanisms have been proposed by which DES may reduce thymocyte number and thymic size. These include maturation arrests in the thymocyte populations (Rihjsinghani et al. 1997), inhibition of thymocyte proliferation (Gould et al. 2000), and targeting of the hemopoietic progenitors that colonize the thymus (Holladay et al. 1993; Luster et al. 1984b). Enhanced apoptotic cell death may also play a role in estrogen-induced thymic atrophy (Mor et al. 2001; Do et al. 2002; Yoa and Hou 2004), however definitive *in vivo* demonstration of such has been elusive. Because DES readily crosses the rodent placenta (McLachlan 1979; Shah and McLachlan 1976) and large numbers of thymocytes migrate through the thymus during the perinatal development of this organ, we evaluated gestational exposure as a model for detecting an *in vivo* effect of DES on thymocyte apoptosis.

4.3 Materials and Methods

Animal Model. Eight week old C57Bl/6 timed pregnant female mice were obtained from Harlan Sprague-Dawley (Indianapolis, IN) on the morning of gestation day (gd) 14. These mice were arbitrarily assigned to control and treatment groups. Insufficient time-pregnant mice were available on any of the experimental days to produce the target group

size of five for both treatment and control groups. Therefore, five pregnant mice were used for each of two control experiments (total of 10 mice), and five pregnant mice were used for each of two treatment experiments (total of 10 mice), with the data from each group of five mice collected on four separate experimental days. Data from the groups were then pooled for evaluation. The mice were housed from gestation days 14-16 in groups of 5 per cage and provided with Harlan 2018 Teklab Global 18% Protein Rodent Diet (Harlan) and distilled water ad libitum. A constant temperature of 21 ± 2 °C was maintained, and a 14.5/9.5 hour light/dark cycle used in the facility. On day 16, mice were weighed prior to final dosing and then housed individually under similar conditions until time of sacrifice on gestation day 18. All procedures were reviewed and approved by the Virginia Tech Animal Care and Use Committee prior to onset of experiments.

Chemical Exposure. DES (Sigma Aldrich, St. Louis, MO) was dissolved in corn oil (Sigma Aldrich, St. Louis, MO) to concentrations of 14.5 µg/mL (48 µg/kg exposure) and administered in a volume of 120 – 200 µL by oral gavage. Doses were administered on gd 14 and 16 based on the body weight of the mice. Control mice were administered comparable volumes of plain corn oil by oral gavage on the same dates.

Tissue Collection. On gd 18, mice were sacrificed by cervical dislocation and feti removed. Total fetal weight per litter as well as total fetal number per litter were recorded. Fetal thymi were removed and placed in pre-weighed culture dishes containing 2 mL RPMI (Sigma Aldrich, St. Louis, MO). Dishes were re-weighed (Mettler Toledo PB303, Carlton Scale, Roanoke, VA), and then stored in the refrigerator until all thymi

were collected from all fetal mice. Two thymi from each litter were then removed from culture dishes and submersed in 10% neutral buffered formalin for histopathologic evaluation.

Cell Enumeration and Flow Cytometry. Thymocytes from remaining thymi of each litter were released by mechanical disruption using curved forceps on steel sieve screens (Sigma Aldrich) and the cells collected into 2 mL of RPMI. One mL of RPMI was added and the cells washed at 200 x g for 5 minutes (IEC Centra GP8R, International Equipment Company, Needham Heights, MA). Cells were washed once more in 3 mL of RPMI and then resuspended in Hanks Buffered Salt Solution (Cambrex BioScience Walkersville, Inc., Walkersville, MD) for counting on a CASY-1 cell counter (Cell Tools Inc., San Francisco, CA). After enumeration, cells were resuspended at a concentration of 5×10^6 cells per mL. One hundred μL of cells (0.5×10^6) from each sample were placed into individual tubes and anti-mouse CD4 antibodies labeled with phycoerythrin (PE; 0.2 $\mu\text{g}/\text{tube}$ diluted in 100 μL HBSS) and anti-mouse CD8 antibody labeled with fluorescein isothiocyanate (FITC; 1 $\mu\text{g}/\text{tube}$ diluted in 100 μL HBSS) were incubated for 30 minutes on ice in the dark. Two mL of HBSS were then added after incubation and the cells washed and resuspended in 400 μL of HBSS. Two and one half μL 7-AAD diluted in 100 μL HBSS were then added to each tube, and tubes were incubated for 15 minutes in the dark on ice. Cells were then evaluated by flow cytometry (Coulter Epics XL, Miami, FL).

Histopathology. Tissues were fixed in 10% neutral buffered formalin for a minimum of 24 hours prior to processing, embedded in paraffin, sectioned at 6 microns, and stained with hematoxylin and eosin. Tissues were then evaluated microscopically.

Thymi were assessed for alterations in architecture or cellularity at both 100x and 600x magnification, as well as nuclear condensation (pyknosis) indicative of apoptosis.

Mitotic indices in the different cell populations were assessed. Evaluation of the surface epithelial layer as well as the underlying cortex and medulla was performed separately.

Proportion of the cortex to the medulla was evaluated as well as the maintenance of architecture including the distinct cortical/medullary regions. A minimum of four (4) fields of 600x magnification were used to evaluate mitotic indices, pyknotic cells, and cellular density.

For each thymus, size was determined as the portion of the field of view filled by the tissue samples on the slide and was assessed at 100x magnification and based on a sampling of all sections on the slide. The quality of tissue preparation and staining may affect interpretation, therefore those sections considered of poor quality due to fractures in the tissue, folds of tissue, autolysis, or inadequate staining were excluded when evaluating architecture and other indices.

Statistics

The statistical unit for the flow cytometric data, cellularity data, and weight data is the dam. Individual thymus weights were calculated by dividing the total weight measured by the number of feti in the litter. Individual thymic cellularity was similarly calculated by dividing the total cellularity by the number of thymi used to determine total cellularity (subtract 2 for histopathology from the total number of feti in the litter). No statistically

significant differences were noted between the number of control feti per litter and the number of DES-exposed feti per litter (control = 7.5 ± 2.1 , DES-exposed = 8.6 ± 1.2 , $p < 0.05$)

The two-tailed Student's T-test with $p \leq 0.05$ was applied to flow cytometric data, cellularity data, weight data, and data involving litter size, and histopathologic findings that were numerically evaluated. Some observations, such as alterations of cellular density were not enumerated. Such findings were assessed as being present or absent and thus described.

4.4 Results

Organ Weights and Cellularity. Fetal thymic weights were diminished by DES treatment as was cellularity of the fetal thymus (Table 4.1). Significant differences in the average individual fetal weights were also present in DES treated feti when compared with controls animals. When comparing pooled thymic weights with pooled fetal weights by litter, DES treatment also caused a significant decrease in the fetal thymic weight/fetal body weight ratio (Table 4.2). The pooled fetal weights were decreased by an average of 10.8% while the pooled thymic weights were decreased by more than 33% as a result of treatment with DES.

Fetal Thymocyte Expression of CD4 and CD8 antigens and 7-AAD fluorescence: In addition to fetal thymocyte depletion, DES treatment caused altered patterns of

intrathymic differentiation as defined by the percentage of thymocytes expressing surface antigens CD4 and CD8 (Table 4.3). Percentages of cells in the CD4⁻8⁻ and CD4⁺8⁺ populations were increased in the DES exposed thymi, while percentages of CD4⁺8⁺ cells were decreased. Absolute numbers of thymocytes in each phenotype, calculated as total cellularity x percentage of cells in each phenotype, also changed after DES. DES significantly decreased cell numbers in the CD4⁺8⁻ and CD4⁺8⁺ phenotypes, did not change the CD4⁻CD8⁻ phenotype, and increased cell number in the CD4⁻8⁺ phenotype (Table 4.4).

Thymocyte 7-AAD fluorescence demonstrated a small increase of apoptosis in total fetal thymocytes caused by DES (Fig. 1). Further characterization of the apoptotic cells by CD4 and CD8 surface antigen expression in concert with 7-AAD staining showed decreased viability in the CD4⁺8⁺, CD4⁻8⁺, and CD4⁻8⁻ phenotypes, numeric but non-significant trends toward increased apoptosis in the CD4⁺8⁺ and CD4⁻8⁺ phenotypes, and significantly increased early apoptosis in CD4⁻8⁺ and CD4⁻8⁻ phenotypes. A small increase in late apoptosis/necrosis was also detected in the CD4⁻8⁺ subpopulation (Table 4.5).

Histopathology: Control fetal thymi were densely cellular organs within a thin connective tissue capsule (Figs. 4.2A, 4.3A, and 4.4A). A thin subcapsular population of large polygonal epithelial cells was present and formed the lobules of this tissue. Mitotic figures were found at the rate of approximately 2 per 600x magnification field. This epithelial cell layer surrounded a predominant cortical region which was densely cellular and consisted of deeply staining thymocytes. Nucleoli were present in approximately

half of these cells and mitoses were noted at the rate of about 1-2 per 600x magnification field. Pyknotic nuclei, characteristic of apoptosis, were also present at approximately the same rate. A few pale histiocytic cells were found in the cortical region as were scattered vascular channels. The medullary region was much smaller and less cellular and consisted of pale staining histiocytic cells with fewer numbers of thymocytes. Widely scattered large epithelial cells with increased cytoplasmic eosinophilia were noted in the medulla (Hassall's corpuscles).

In the DES treated thymi, the size of the organ was visibly smaller than control thymi (Figure 4.2B). Less distinction between the cortical and medullary regions was noted after DES treatment (Figure 4.3B). A decreased density of cells was noted in all areas, but was most apparent in the cortical region. Increased numbers of pyknotic nuclei were seen with an average of 4 per 600x magnification field (Figure 4.4B). Decreased mitoses were found in all areas with an average of <1 mitotic figure per field found in the cortical or epithelial layers. No inflammatory response was noted within the organ.

4.5 Discussion

The literature has been divided regarding the potential contribution of increased thymocyte apoptosis to estrogen-induced thymic atrophy. Silverstone et al. (1994) examined mechanisms of action leading to thymic hypocellularity after dexamethasone (DEX), β -estradiol, or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) administration in 25 day-old female BALB/cJ mice. While all three chemicals caused thymic atrophy, only thymocytes from DEX treated mice displayed increased apoptosis within 24 hours of

administration. Thymocytes collected as many as twelve days after chemical treatment did not show enhancement of apoptotic activity in the β -estradiol treated animals even though maximal thymic atrophy occurred within this time frame.

Forsberg (1996) suggested an age dependent response to DES in mice, with neonatal exposure causing the most profound thymic atrophy. A transient arrest of the cell cycle was noted in the DES exposed thymocytes, while the level of apoptosis in thymocytes from the DES-treated mice was not different from controls. Staples et al. (1998) used transgenic *lck^{pr}bcl-2* mice to evaluate the possibility of protection against thymocyte depletion in DES-treated mice by this anti-apoptotic gene. These authors reported that *bcl-2* over-expression did not abate the thymic atrophy induced by DES, again suggesting lack of contribution from estrogen-enhanced apoptosis. Donner et al. (1999), using *in vitro* DES thymocyte exposures, detected control-level apoptosis at multiple time points out to 48 hours. The positive apoptosis control, DEX, significantly enhanced thymocyte apoptosis beginning 4 hours after exposure onset.

In contrast to the above reports, Zajchowski and Hoffman-Goetz (2000) exposed adult female C57Bl/6 mice to supraphysiologic levels (71 μ g/mouse/day) of 17 β -estradiol (E2) for 14 consecutive days and, using annexin V and flow cytometry, observed a decrease in thymocyte viability and increased apoptosis. Specifically, apoptosis increased from about 36% in thymocytes from control mice to about 42% in thymocytes from estrogen-dosed mice. These results in control thymocytes differ, however, from previous reports of limited apoptosis in freshly harvested murine thymocytes (Jiang et al. 1999; Small and Kraal 2003) and from the present observation of non-detected apoptosis in control thymocytes. Lai et al. (2000) examined fetal thymic

organ culture (FTOC) of *bcl-2* transgenic mice (TG+), and found reduced thymocyte depletion caused by DES in the *bcl-2* TG+ FTOC compared to the transgenic negative (TG-) or wild type FTOC. Calemine et al. (2002) dosed adult mice for 1 week with low levels of DES and, using 7-AAD and flow cytometry, demonstrated dose-related increased apoptosis in the CD4⁺CD8⁺, CD4⁺CD8⁻, and CD4⁻CD8⁺ thymocyte subsets. This observation occurred, however, without a concurrent decrease in thymic cell counts.

The Fas/Fas ligand system is a common pathway of T cell apoptosis, thus estrogen-induced upregulation of this pathway in the thymus may support apoptosis as a mechanism contributing to thymocyte hypocellularity. Mor et al. (2001) reported decreased thymus size and corresponding increased FasL expression in female rats after DES exposure. These authors also detected estrogen receptor expression, both α and β , in the rat thymocytes and thymic stromal cells. Yao and Hou (2004) used pharmacologic levels of β -estradiol in rats to produce thymic atrophy. Then, using TUNEL assay, these authors detected increased apoptosis in the estrogen-exposed total thymi, as well as an increased Fas/FasL mRNA levels by RT-PCR. Do et al. (2002) similarly observed decreased thymic cellularity in wild type C57Bl/6 mice, C57Bl/6 Fas-deficient (*lpr/lpr*) and C57Bl/6 FasL-deficient (*gld/gld*) mice exposed to β -estradiol. However, the level of thymic atrophy in the *lpr/lpr* and *gld/gld* mice was less than that of the wild type mice, and decreased apoptosis occurred in the Fas-deficient and FasL-deficient strains compared to wild type mice. Thus, collective recent data support enhanced apoptosis as a mechanism that may contribute to estrogen-induced thymic atrophy.

Approximately 98% of thymocytes fail intrathymic selection for self antigen recognition or self antigen-presenting (MHC) molecule recognition and are eliminated by

apoptosis (Elgert 1996). As a consequence, this organ has well developed phagocytic function for eliminating apoptotic cells, which may make chemical enhancement of increased thymocyte death difficult to detection (Kamath et al. 1997). Large numbers of thymocytes seed the fetal thymus during the time of organ development, whereas phagocytic removal of apoptotic cells may not yet be developed to adult levels (Holladay and Smialowicz 2000). For these reasons, we hypothesized that an *in vivo* enhancement of thymocyte apoptosis by pharmacologic or suprapharmacologic estrogenic exposure may be detectable, if present, in the perinatal thymus as compared to the adult thymus. Decreased fetal thymic cellularity and atrophy of the fetal thymus were caused by DES in the present experiments. Flow cytometry and 7-AAD fluorescence detected a small increase in thymocyte apoptosis, both in total thymocytes and within two of four phenotypes defined by CD4 and CD8 antigens. Histopathologic evaluation of the fetal thymi supported the cytometric data, providing visual evidence of increased apoptotic cells. While in both cases the increase in observed apoptotic cells was small, it should be considered that such increased cell death, in an organ with a highly effective removal system for apoptotic cells, may impact total organ cellularity over time more than initial interpretation might suggest. These results in the developing thymus support previous suggestions of estrogen-related enhanced thymocyte apoptosis, and support the idea that thymic atrophy production by DES is a multifactoral process.

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TABLE 4.1

The effect of gestational exposure to DES on gd 18 fetal thymic weight and cellularity.

Weights and cellularity are presented on a per organ basis (mean $\pm$ SEM), i.e., data represent total thymic weights or cellularity per litter divided by the number of feti in the litter.

	Weights (μ g)	Cellularity ($\times 10^6$ cells)
Control	2.75 ± 0.65	5.92 ± 2.14
48 μ g/kg DES	$1.55 \pm 0.58^*$	$2.91 \pm 0.97^*$

Total number of control litters: N=10; total number of feti/litter for weight measurement: 7.5 ± 2.1 ; total number of thymi/litter for cellularity: 5.5 ± 2.1

Total number of DES-exposed litters: N=10; total number of feti/litter for weight measurement: 8.6 ± 1.2 ; total number of thymi/litter for cellularity: 6.6 ± 1.2

*Significantly different from control, $p < .05$ by Student's t-test.

TABLE 4.2

The effect of late gestational exposure to DES on gd 18 fetal weight, thymic weight, and thymic weight/fetal weight ratio

	Fetal Weights (μ g)	Thymic weight	Thymic wt/fetal wt
Control	8.96 ± 2.10	0.0203 ± 0.006	0.00226 ± 0.00046
48 μ g/kg DES	$7.99 \pm 2.01^*$	$0.0134 \pm 0.006^*$	$0.00163 \pm 0.00047^*$

Total number of control litters: N=10; total number of feti/litter for weight measurement: 7.5 ± 2.1

Total number of DES-exposed litters: N=10; total number of feti/litter for weight measurement: 8.6 ± 1.2

*Significantly different from control, $p < .05$ by Student's t-test.

TABLE 4.3

The effect of gestational exposure to DES on gd 18 thymocyte populations defined by CD4 and CD8 cell surface antigens. Numbers are percentages (mean $\pm$ SEM) of cells within each phenotype

Phenotype	CD4 ⁻ 8 ⁻	CD4 ⁻ 8 ⁺	CD4 ⁺ 8 ⁻	CD4 ⁺ 8 ⁺
Control	8.99 $\pm$ 3.52	1.65 $\pm$ 0.30	1.85 $\pm$ 0.97	87.51 $\pm$ 3.78
48 μ g/kg DES	16.79 $\pm$ 6.57*	3.92 $\pm$ 1.16*	1.61 $\pm$ 0.90	77.69 $\pm$ 6.91*

Total number of litters for control data: N=10

Total number of litters for DES-exposed data: N=10

*Significantly different from control, $p < .05$ by Student's t-test.

TABLE 4.4

Cell number in gd 18 fetal thymocyte phenotypes defined by CD4 and CD8 expression, calculated as total cellularity multiplied by the percentage of cells within each phenotype. Numbers are $\times 10^6$ cells per thymus.

Phenotype	CD4 ⁻ 8 ⁻	CD4 ⁻ 8 ⁺	CD4 ⁺ 8 ⁻	CD4 ⁺ 8 ⁺
Control	0.47 $\pm$ 0.19	0.09 $\pm$ 0.02	0.10 $\pm$ 0.05	4.62 $\pm$ 0.20
48 μ g/kg DES	0.49 $\pm$ 0.19	0.11 $\pm$ 0.03*	0.05 $\pm$ 0.03*	2.26 $\pm$ 0.20*

Total number of litters for control data: N=10

Total number of litters for DES-exposed data: N=10

*Significantly different from control, $p < .05$ by Student's t-test.

TABLE 4.5

Effects of DES on gd 18 fetal thymocyte viability and apoptosis. Results, shown as percentages, are for total thymocytes as well as subpopulations defined by CD4 and CD8 cell surface markers.

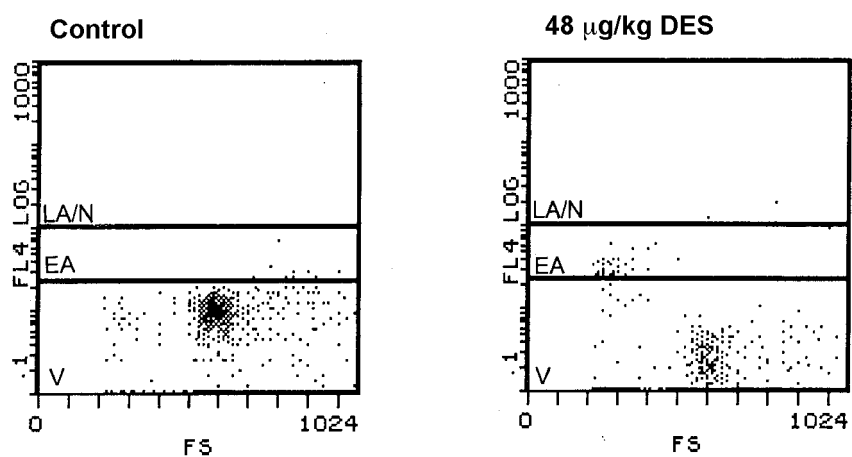
	Viable cells	Early apoptosis	Late apoptosis/ Necrosis
Control			
Total thymocytes	98.4 ± 0.6	1.38 ± 0.5	0.24 ± 0.1
CD4+CD8-	98.8 ± 1.2	1.2 ± 1.2	0.0
CD4+CD8+	97.4 ± 1.1	2.2 ± 1.0	0.4 ± 0.1
CD4-CD8+	99.1 ± 1.2	0.9 ± 1.2	0.0
CD4-CD8-	99.4 ± 0.7	0.6 ± 0.7	0.0
DES exposed			
Total thymocytes	95.7 ± 3.4*	4.10 ± 3.4*	0.24 ± 0.1
CD4+CD8-	96.6 ± 4.3	3.4 ± 4.3	0.0
CD4+CD8+	94.8 ± 3.6*	4.8 ± 3.5	0.4 ± 0.1
CD4-CD8+	91.3 ± 6.7*	7.7 ± 7.1*	1.0 ± 0.2*
CD4-CD8-	96.7 ± 2.8*	3.2 ± 2.8*	0.1 ± 0.1

Total number of litters for control data: N=10

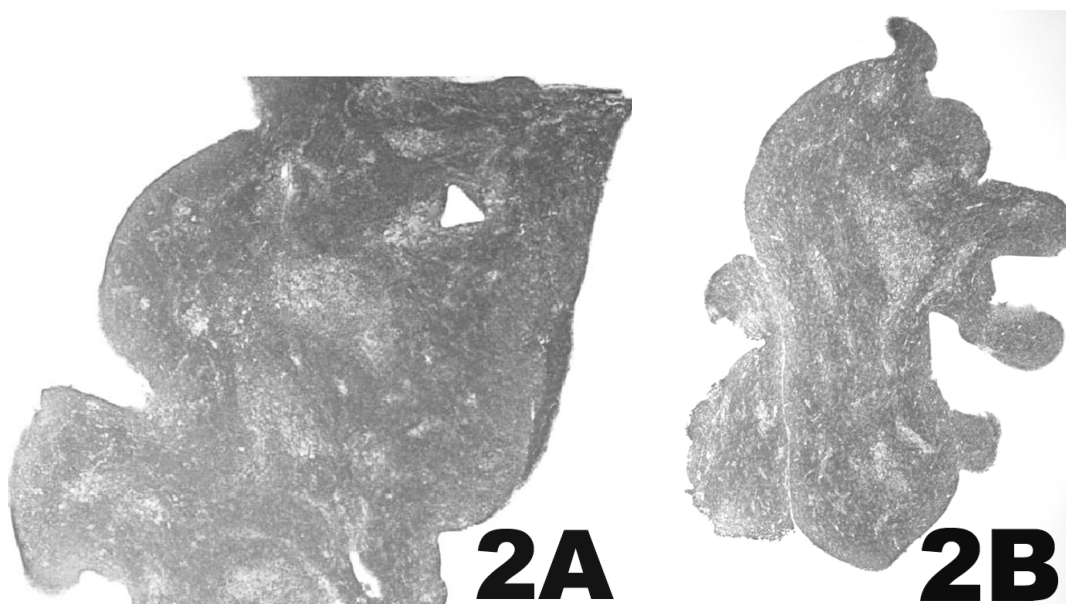
Total number of litters for DES-exposed data: N=10

*Significantly different from control, $p < .05$ by Student's t-test.

FIGURE 4.1

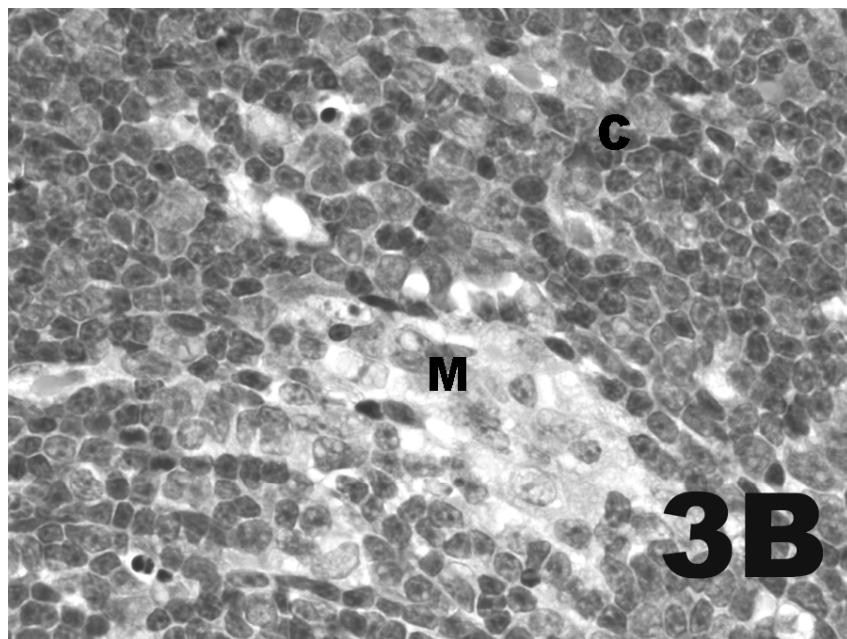
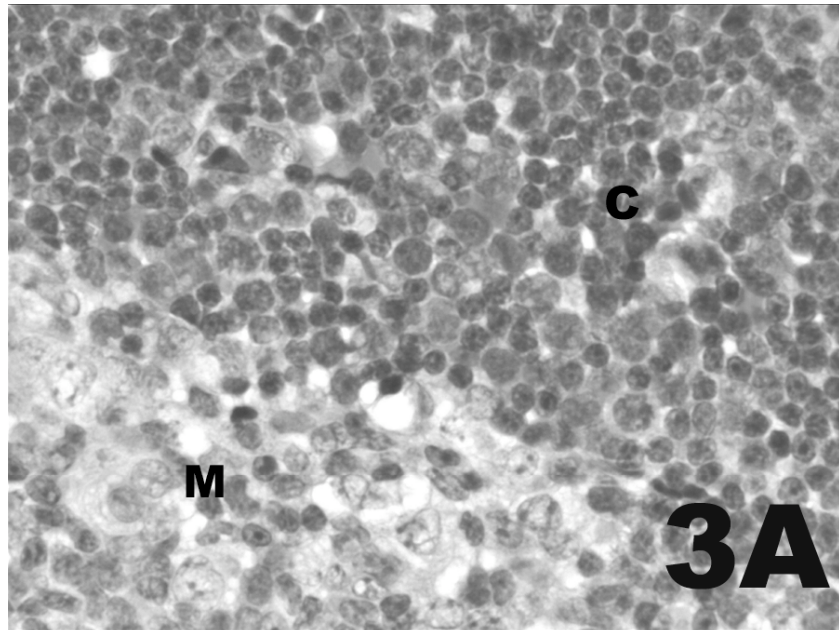


Representative histograms from control and DES-exposed gestation day 18 mice, showing 7-AAD fetal thymocyte fluorescence. Control histogram is on the left, DES-exposed is on the right. Abbreviations: V- viable cells; EA- early apoptotic cells; LA/N- late apoptotic/necrotic cells.



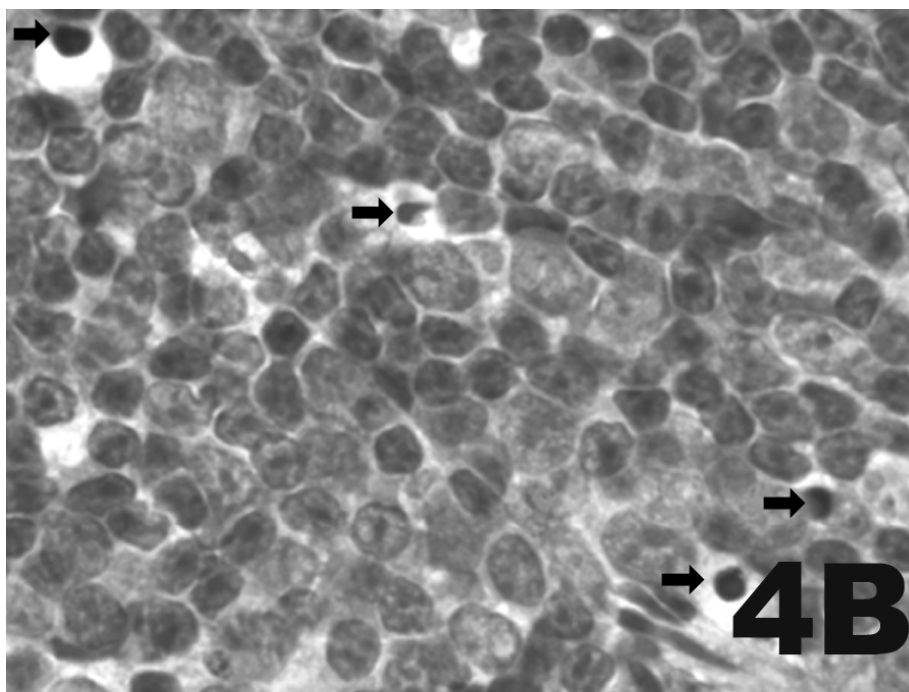
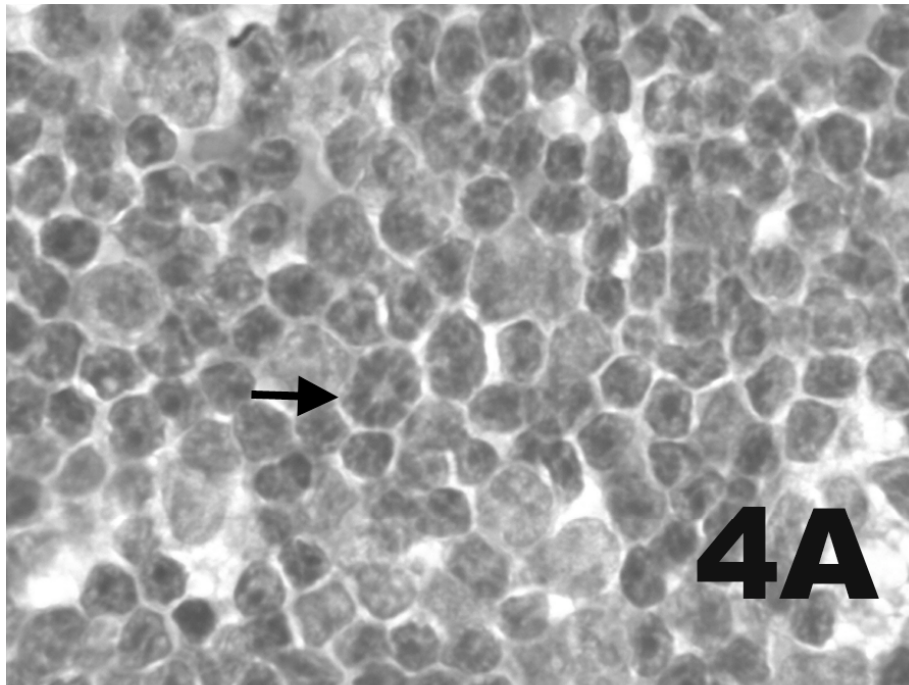
FIGURES 4.2A AND 4.2B

Representative gestation Day 18 Fetal Thymi, 40x magnification. Figure 4.2A is a control thymus, Figure 4.2B is a DES-exposed thymus. Note the decreased cellularity and size of the DES-exposed thymus.



FIGURES 4.3A and 4.3B

Gestation Day 18 Fetal thymus at 100x magnification. Figure 4.3A is the control thymus, Figure 4.3B is the DES-exposed thymus. The cortex is labeled C and the medulla is labeled M. Note the clear demarcation of the cortical/medullary junction for the control thymus, while the DES thymus lacks clear organization.



FIGURES 4.4A and 4.4B

Gestation Day 18 Fetal Thymus, 500x magnification. Figure 4.4A is the control thymus, Figure 4.4B is the DES-exposed thymus. The thin arrows indicate mitotic figures; short thick arrows indicate pyknotic nuclei.