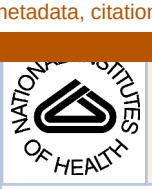

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Diethanolamine alters neurogenesis and induces apoptosis in fetal mouse hippocampus

Corneliu N. Craciunescu[*], **Renan Wu**[*], and **Steven H. Zeisel**[*,1]

* *Department of Nutrition, School of Public Health and Medicine, University of North Carolina, Chapel Hill, North Carolina, USA*

Abstract

Diethanolamine (DEA) is present in many consumer products such as shampoo. Dermal administration of DEA diminishes hepatic stores of the essential nutrient choline, and we previously reported that dietary choline deficiency during pregnancy reduces neurogenesis and increases apoptosis in the hippocampus of fetal rats and mice. Therefore, DEA could also alter brain development. Timed-pregnant C57BL/6 mice were dosed dermally from gestation day 7 through 17 with DEA at 0, 20, 80, 160, 320, and 640 mg/kg body/day. At doses of DEA > 80 mg/kg body/day, we observed decreased litter size. In fetuses (embryonic day 17) collected from dams treated dermally with 80 mg/kg body/day DEA, we observed decreased neural progenitor cell mitosis at the ventricular surface of the ventricular zone of the hippocampus [to 56±14% (SE) histone 3 (H3) phosphorylation as compared to controls; $P < 0.01$]. We also observed increased apoptosis in fetal hippocampus (to 170±10% of control measured using TUNEL and to 178±7% of control measured using activated caspase 3; $P < 0.01$). Thus, maternal exposure to DEA reduces the number of neural progenitor cells in hippocampus by two mechanisms, and this could permanently alter memory function in offspring of mothers exposed to this common ingredient of shampoos and soaps.—Craciunescu, C. N., Wu, R., Zeisel, S. H. Diethanolamine alters neurogenesis and induces apoptosis in fetal mouse hippocampus.

Keywords

choline; pregnancy; brain development

Diethanolamine (DEA; Chemical Abstracts Registration Number 111–42-2) is widely used as a chemical intermediate, as an anticorrosion agent in metalworking liquids, and as a surface-active agent in cosmetic formulations, pharmaceuticals, and agricultural products (1,2). Estimated annual production of DEA in the U.S. was 106,000 tons in 1995 (3). The most probable route of environmental exposure to DEA in humans is via dermal exposure to personal care products (i.e., soaps, shampoos, and cosmetics), detergents, and other surfactants that contain DEA; cosmetic formulations may have concentrations of DEA ranging from 1% to 25% (3). Occupational exposure to DEA is most likely through the use of lubricating liquids in various processes in machine building; DEA is a component of bulk cutting fluids, with concentrations ranging from 4% to 5% by weight (3). The National Institute for Occupational Safety and Health estimates that the number of workers potentially exposed to DEA is ~800,000/year (3).

Studies of the toxicity of DEA have focused on carcinogenicity; the International Agency for Research on Cancer concluded that DEA was not classifiable regarding its carcinogenicity in

[1] Correspondence: CB#7461, 2115A Michael Hooker Research Center, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7461, USA. E-mail: steven_zeisel@unc.edu.

humans (4). However, long-term toxicity studies found an association between an increase of liver and kidney tumors in F344/N rats and B6C3F1 mice and the application of DEA (5–7). The genotoxic activity of DEA has been extensively evaluated, and results have been consistently negative (8), with the noticeable exception of the genetic alteration in the *Catnb* gene in hepatocellular neoplasms of B6C3F1 mice after exposure to DEA for 2 years (9). Several excellent reviews exist that summarize the toxicology of DEA (8). In this study we examine, for the first time, whether DEA adversely affects brain development.

DEA is structurally similar to the essential nutrient choline, and DEA treatment in rodents perturbed choline metabolite concentrations in liver (10,11). DEA is metabolized by routes common to endogenous alkanolamines (ethanolamine and choline) and is incorporated into phospholipids in liver, kidney, spleen, and brain of mice and rats (12). Furthermore, DEA inhibits phosphatidylcholine synthesis in rat liver tissue *in vitro* (13) and *in vivo* (14). We previously reported that maternal choline deficiency during pregnancy results in diminished proliferation and increased apoptosis of neural progenitor cells in the fetal hippocampus (15, 16), and this results in lifelong changes in memory performance (17,18). Since DEA perturbs choline metabolism, it too might alter hippocampal development. Here we report that the developing mouse hippocampus is sensitive to dermally administered DEA during pregnancy.

MATERIALS AND METHODS

Timed-pregnant C57BL/6 mice used in all experiments were obtained from The Jackson Laboratories (Bar Harbor, ME, USA) on day 7 of gestation, maintained in a climate-controlled environment, and exposed to a 12 h light/dark cycle daily. All animal protocols were approved by the UNC Institutional Animal Use Committee. Pregnant female mice (n=6/group) were housed individually in stainless steel cages containing a feeder and water bottle equipped with a sipper tube. According to vendor specifications, the morning when the vaginal plug was observed after mating was considered embryonic day 0 (E0) of gestation. Mice were fed purified AIN-76A diet containing 1.1 g/kg (7.8 mmol/kg) choline chloride (Dyets, Bethlehem, PA, USA) and water *ad libitum* unless otherwise noted. The animals were dosed dermally with DEA (Sigma-Aldrich, St. Louis, MO, USA) dissolved in 95% ethanol at 0, 20, 80, 160, 320, and 640 mg/kg body wt from gestation day E7 through E17. Dermal application sites consisted of an ~ 2 cm^2 area running from the interscapular region to several centimeters posterior of this. For all the mice, including the untreated controls, the area was clipped free of fur, depilated using a commercial depilatory, rinsed with a wet paper towel, and blotted dry before initiation of dosing and subsequently depilated on an as-needed basis. Care was taken during the preparation of the site to avoid irritation of the skin. We used dosing methods similar to those described by the National Toxicology Program (5–7). Mice were dosed by spreading a known volume (1.78 μl/g × mouse wt, g) of test material/mouse over the prepared target site using a micropipette equipped with a blunted tip.

On gestational day 17, pregnant mice were anesthetized with a single injection of 0.03 cc ketamine and 0.02 cc xylazine (Henry Schein Inc., Melville, NY, USA) subcutaneously and the mice were kept on a heating pad to maintain body temperature. The uterine horns were exposed by a midline abdominal incision and fetuses were removed individually. The abdominal cavity of the dams was opened and the livers were collected, quick frozen in liquid nitrogen, and stored at −80°C. The fetuses were decapitated and the fetal skull was opened for fixation overnight. The heads were stored overnight in the perfusion fixative containing 4% formaldehyde and 0.2% glutaraldehyde (Polysciences, Inc., Warrington, PA, USA), then stored in 0.1 M phosphate buffer. The residual fetal bodies were kept on ice for later use for sex determination.

The fetal brains were embedded in paraffin and 5 μm coronal serial sections containing the brain regions of interest such as the hippocampus, the septum, the amygdala, and cortex were cut and applied on glass slides for histological and immunohistochemical assays. Since there is a posterior to anterior gradient of neurogenesis in fetal mouse brain, the paraffin sections were reviewed at the time of sectioning to ensure that they included anatomically reproducible areas of the hippocampus as defined by a standard atlas of the developing brain (19). In prenatal and early postnatal stages, neurogenesis occurs mostly within two relatively thin layers of tissue lining the primitive ventricular cavities, referred to as the ventricular zone (vz) and the subventricular zone (svz) (20). Each experiment was replicated.

Assessment of mitosis

The mitotic and synthetic zone of the ventricular zone adjacent to the lateral ventricle is the region of fetal hippocampus from which neuronal and glial type cells originate (21). Cells at the ventricular surface of the ventricular zone of the developing hippocampus include progenitor cells that have exited S phase and entered the mitotic phase of the cell cycle. To determine whether mitosis in fetal hippocampus was altered by maternal dermally administered DEA during pregnancy, coronal sections were probed with a rabbit polyclonal antibody (Ab) that recognizes phosphorylated histone H3 (phospho-H3, Upstate, Lake Placid, NY, USA). According to the manufacturer, this antibody recognizes histone H3, the core protein of the nucleosome, which becomes Ser-10 phosphorylated first in the G_2/M phase (initiating at pericentromeric heterochromatin), then progresses along the chromosomal arms until it spreads to the whole chromosome (22), an event that is essential for the maintenance of mitosis-associated chromosome condensation (23).

Slides were deparaffinized in fresh xylene for 3 steps of 20 min, then rehydrated in absolute ethanol for 15 min, 70% ethanol with 0.25% ammonium for 1 h (24), and 50% ethanol for 15 min. This was followed by three washes for 5 min in PBS with 0.1% Tween 20 (PBST; Sigma). Antigen retrieval was performed for 20 min in 20 μmol/l proteinase K (Sigma) and for 40 min in 10 mg/ml sodium borohydrate (Sigma). Nonspecific sites were blocked with 2% nonimmune goat serum (Chemicon, Temecula, CA, USA) in PBST. Brain sections were incubated in rabbit antiphospho-histone H3 Ab (Upstate) at a concentration of 2 μg/ml in blocking buffer overnight at 4°C. Sites of phosphorylated histone H3 expression were detected using a Cy3-conjugated goat anti-rabbit IgG (Chemicon) at 1:500 dilution, for 2 h at room temperature. 4′,6-Diamidino-2-phenylindole (4′,6′-diamidino-2-phenylidole (DAPI), Sigma) 0.1 μg/ml for 20 min was used to counterstain nuclear DNA. Sections were mounted using 80% Tris-buffered glycerol, pH 7.0 (25), and a No. 1 thickness coverglass. Images were acquired using a Zeiss Confocal Laser Scanning Microscope LSM 210 as described below. The incidence of phospho-histone H3 labeled cells was measured at the ventricular surface of the ventricular zone beginning at the junction of the hippocampus and choroid plexus (hippocampal wedge, ref 21) and extending toward the cortical ventricular zone. Cells were counted at a final magnification of 200× in four hippocampal hemispheres from two consecutive serial sections and the values were averaged to obtain a single value/hippocampus (region)/animal. Calibrated 50× magnification images of the same regions were used to measure the length of the hippocampal vz with an internal macro of NIH Image J program version 1.61. Analyses were replicated.

Assessment of apoptosis

We used a combination of activated caspase-3 immunoreactivity and terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP)-digoxigenin anti-digoxigenin fluorescein conjugate Ab nick end-labeling (TUNEL; S 7111 Apoptag® Plus Fluorescein *In Situ* Apoptosis Detection Kit, Serologicals, Norcross, GA, USA) to detect apoptotic cells in fetal hippocampus. For double-labeling of TUNEL and activated caspase-3,

DNA terminal labeling conducted according to the manufacturer's protocol was followed by overnight incubation with a primary Ab to cleaved (activated) caspase-3 (Asp175) (Cell Signaling Technology, Beverly, MA, USA). Then fluorescein-antidigoxigenin conjugate and goat Cy3-anti-rabbit IgG (Calbiochem, San Diego, CA, USA) were applied for 2 h at room temperature to display the TUNEL-positive nuclei and activated caspase-3. 4′,6-diamidino-2-phenylindole (DAPI, Sigma) 0.1 μg/ml for 20 min was used to counterstain nuclear DNA. The TUNEL and activated caspase-3 positive cells were identified and scored by a trained observer (C.N.C.), blinded to the animal's grouping, based on the presence of green fluorescent nuclear staining for TUNEL and red fluorescent staining for activated caspase-3. Stained nuclei were usually, but not always, condensed and intense blue fluorescent chromatin was often visible inside, consistent with fragmented DNA. Apoptotic indices for the fetal mouse brain hippocampus of the different animal groupings are presented as the number of apoptotic cells/section of the hippocampal hemispheres. Four hippocampal hemispheres from two consecutive serial sections were averaged to obtain single hemilateral value/hippocampal section/animal.

Image analysis

For the mitosis assessment, TUNEL, and activated caspase-3 evaluation, image analysis of fetal brain slices was performed using a Zeiss Confocal Laser Scanning Microscope LSM 210 (Carl Zeiss, Thornwood, NY, USA) equipped with an Optronics DEI 750 low light level integrating CCD camera (Optronics Engineering, Goleta, CA, USA) connected to an Apple Macintosh G3 computer utilizing a Scion CG7 image capture card for digital image capture of standard and epi-fluorescence images) and the public domain NIH Image program version 1.61. For replicated experiments, the apoptotic cells were detected hemilaterally in each of the selected sections after double-labeling for TUNEL and activated caspase-3 using a Nikon FXA microscope (Nikon, Garden City, NY, USA) equipped with an Optronics TEC-470 CCD Video Camera System (Optronics Engineering) and the public domain NIH Image program version 1.61. All images were captured by using 5, 20, or 40× objectives and fluorescent filters optimized for observing DAPI (blue), FITC (green), and Cy3 conjugates (red) signals, respectively. Images obtained from the same field with different fluorescent probes were subsequently overlapped or merged.

Analysis of choline compounds

Choline, betaine, phosphocholine (PCho), glycerophosphocholine (GPCho), phosphatidylcholine (PtdCho), and sphingomyelin (SM) were determined in maternal liver using a method of liquid chromatography-electrospray ionization-isotope dilution mass spectrometry (LC–ESI–IDMS; LCQ quadrupole ion trap mass spectrometer equipped with an API2 electrospray ionization source, ThermoQuest, San Jose, CA, USA) (26). Briefly, deuterium-labeled internal standards of choline, betaine, GPCho, PCho, SM, and PtdCho and 400 μl of methanol/chloroform (2:1, v/v) were added to 100 mg aliquots of tissue. Samples were vortexed vigorously and left at −20°C overnight. At the end of the extraction, samples were subjected to centrifugation at 1500 *g* for 5 min at room temperature. The supernatant was transferred to a new tube and the residue was re-extracted with 250 μl of methanol/chloroform/water (2:1:0.8, by vol). The supernatants from both extractions were combined. To the combined solution, 100 μl of chloroform, then 100 μl of water, were added to form two phases. After centrifugation at 1500 *g* for 5 min, the aqueous phase (which contained choline) was separated from the chloroform phase (which contained PtdCho and SM). A 10 μl aliquot of the lower organic phase was analyzed by LC-ESI-IDMS after 20-fold dilution with methanol. The aqueous phase was dried by vacuum centrifugation (Speed-Vac; Savant Instruments, Farmingdale, NY, USA) and redissolved in 20 μl of water. After addition of 200 μl of methanol, the aqueous phase was subjected to centrifugation at 1500 *g* for 5 min to remove the precipitated unknown compounds from the solution. A 10 μl aliquot of this solution was then analyzed by LC-ESI-IDMS.

Statistical analysis

To compare treatment groups with controls, we used 1-way ANOVA and the Tukey-Kramer test performed with JMP software (V 2, SAS Institute, Cary, NC, USA). Data are presented as mean ± SE.

RESULTS

Fetal wastage

At doses of DEA > 80 mg/kg body/day, we observed a dose-related decrease in litter size (Table 1), with number of fetuses reduced in the 640 mg/kg/day dose to a third of that in the control mice.

Choline metabolites in maternal liver

Treatment of pregnant mothers with 80 mg/kg body/day DEA resulted in decreased hepatic concentrations of choline and all of its metabolites (Table 2).

Mitosis

We measured the number of phospho-H3-labeled cells at the ventricular surface of the hippocampal ventricular zone adjacent to the fimbria (Fi), primordial dentate gyrus (DG), Ammon's horn (AH), and in the neocortical ventricular zone (Cx) surrounding the lateral ventricle toward the cortical plate (Fig. 1). In all regions studied, treatment with 80 mg/kg/day DEA diminished the proportion of cells that were in the mitotic phase to ~ 50% of control ($P < 0.01$ different from control; Fig. 2). DEA did not alter the length of the ventricular zone (inset Fig. 2).

Apoptosis

The number of apoptotic cells counted separately as TUNEL-positive and activated caspase-3 positive cells in the whole hippocampal area was > 70% higher in DEA treated animals compared to the controls ($P < 0.01$, Fig. 3).

DISCUSSION

We found that DEA, a common ingredient in commercial products, resulted in fetal wastage. This had not been appreciated before. It appears that pregnant mice are especially sensitive to these effects of DEA treatment, as the decreases in liver concentrations of choline and its metabolites were greater than those we earlier reported to occur in DEA-treated mice that were not pregnant (11) or that occurred in pregnant mice fed a choline-deficient diet alone (15). Since DEA administration perturbs choline metabolism in the liver (10,11), it is possible that a change in maternal delivery of choline to the fetus is responsible for the fetal wastage we observed. Choline is an essential nutrient (27), and we have reported that choline deficiency causes birth defects in mouse embryos in culture (28,29).

DEA treatment of pregnant mice also inhibited cell proliferation and increased apoptosis in fetal hippocampus progenitor cells *in vivo*. We previously reported that the availability of choline during gestation modulates cell proliferation and apoptosis in neural stem cells *in vivo* (15,30–33) and that DEA perturbs choline metabolite concentrations in rodent liver (10, 11). Thus, it is likely that the changes in fetal brain we observed after DEA administration to pregnant mice are secondary to diminished availability of choline to fetal brain. The effects of choline deficiency during pregnancy result in lifelong diminution of memory function (17, 18), and the same might be true for the effects of DEA exposure. A potential mechanism for the effect of choline, and perhaps DEA, on progenitor cell proliferation and apoptosis involves

abnormal methylation of promoter regions of genes that control cell cycling and apoptosis (34). Though the current studies focused on neural progenitor cells in the hippocampus, this mechanism would be applicable to all areas of the brain in which neural progenitor cells are proliferating. We plan future studies that will examine DEA effects on other regions of the developing brain.

As noted earlier, dermal exposure to DEA in personal care products is significant, with cosmetic formulations having concentrations of DEA ranging from 1% to 25% (3). If a 60 kg body wt human used 50 μl of a cosmetic solution containing 25% DEA daily, estimated exposure would be 12,000 mg/60 kg/day (200 mg/kg/day). If the cosmetic solution contained 1% DEA, exposure would be 8 mg/kg/day. We used 80 mg/kg/day DEA with observed effects in our mouse brain experiments and demonstrated effects on litter size at 160 mg/kg/day. These calculations do not correct for species differences in the capacity of DEA to penetrate skin. Absorption of DEA applied dermally in rats varied with dosage, ranging from 3 to 16% in 48 h at dosages of 2 and 28 mg/kg, respectively (12). In mice, absorption was more efficient: in doses ranging from 8 to 80 mg/kg the amount absorbed through the skin was 25–60% in 48 h. Skin penetration rates determined *in vitro* using full-thickness skin preparations also confirmed that dermal penetration of DEA in mice was much higher than that in rats (46.3 and 1.8 μg/cm^2/h, respectively) (35). Absorption of DEA across human skin is less efficient than in rats (36).

DEA is a commonly used ingredient in consumer products. Our work suggests that it may have adverse effects on pregnancy outcome and on brain development. Manufacturers sometimes substitute triethanolamine (TEA) for DEA in products. We found that TEA can also perturb choline metabolism (37), and suggest that it, too, should be evaluated for effects on pregnancy and brain development.

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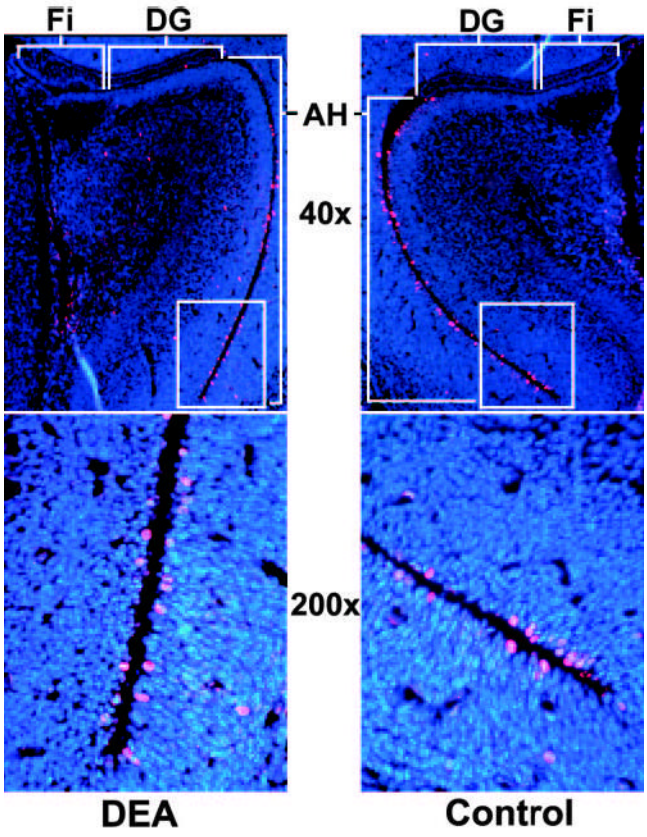

Figure 1.

DEA treatment during pregnancy reduces cell proliferation in progenitor cells of fetal embryonic day 17 mouse hippocampus. Pregnant mice were treated dermally with 80 mg/kg/day DEA (or vehicle control) from embryonic day 7 until they were killed on embryonic day 17. Coronal sections were prepared from the brains of fetuses from each group for the analysis of mitosis using the mitosis-specific marker phosphorylated histone H3 as described in text. Representative regions of fetal brain at 40× magnification are shown in the upper panels; the boxed area in the upper panels indicates regions shown at 200× magnification in the lower panels. The 4′,6-diamidino-2-phenylindole (DAPI) nuclear DNA counterstaining is blue, while the Cy3 conjugated secondary Ab bound to the antiphospho-histone H3 (Ser-10) primary Ab stains red and indicates mitotic cells at the ventricular surface of the hippocampal ventricular zone adjacent to fimbria (Fi), dentate gyrus (DG), and Ammon's Horn (AH) (each region indicated by white brackets.

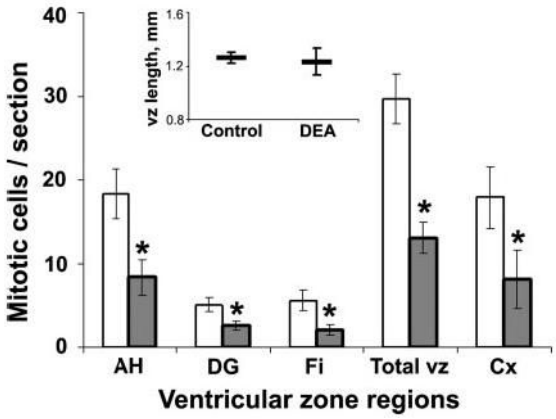

Figure 2.

Quantitative analysis of hippocampal sections from fetal mice at embryonic day 17. Pregnant mice were treated with DEA and fetal hippocampal sections prepared as in Fig. 1. Images were analyzed in an unbiased blinded manner as described in text. Two separate experiments were conducted with similar results. Pooled data from these replicate experiments are presented as mean number of mitotic cells (±SE) at the ventricular surface of the hippocampal ventricular zone adjacent to fimbria (Fi), dentate gyrus (DG), and Ammon's horn (AH), the calculated values for the whole hippocampal section length of ventricular zone (total vz) and for the neocortical ventricular zone (Cx) surrounding the lateral ventricle toward the cortical plate. Open bars = vehicle control; shaded bars = DEA treated. The inset shows mean total ventricular length (mm) (±SE) for treated and control groups. $n = 12$ animals per group; $^*P < 0.01$ different from control in same brain region by ANOVA and Tukey-Kramer test.

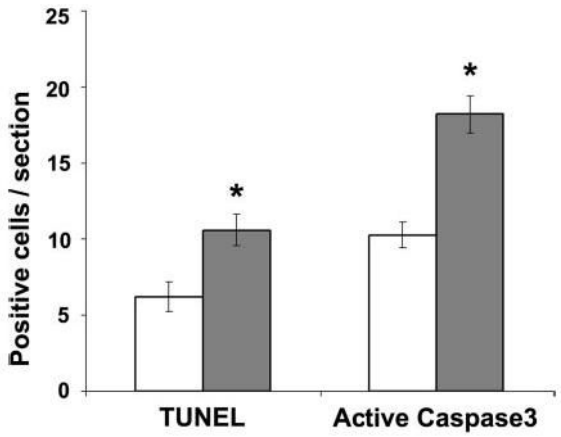

Figure 3.

Maternal DEA treatment increases apoptosis in fetal mouse hippocampus on embryonic day 17. Pregnant mice were treated with DEA and fetal hippocampal sections were prepared as in Fig. 1. These were analyzed using two measures of apoptosis-activated cleaved caspase-3 immunoreactivity (Casp3) and terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL), as described in text. Open bars = vehicle control; shaded bars = DEA treated. Two separate experiments were conducted with similar results. Pooled data from these replicate experiments are presented as mean number of positive cells ± SE; n = 8–10 animals per group. *P < 0.01 different from control by ANOVA and Tukey-Kramer test.

TABLE 1

Diethanolamine treatment during pregnancy reduces litter size[a]

DEA dose, mg/kg body wt/day	Number of live fetuses	Viable fetuses/litter
0	49	8.2 ± 0.4
20	48	8.0 ± 0.5
80	38	6.3 ± 0.6
160	29	4.8 ± 0.3*
320	20	3.3 ± 0.3*
640	16	2.7 ± 0.3*

[a] Pregnant mice (n=6/group) were treated dermally with DEA (or ethanol vehicle control) from embryonic day 7 until they were killed on embryonic day 17 and number of viable fetuses counted as described in Materials and Methods. One dam in the 640 mg/kg/d dose group died before gestational day 17. Data are expressed as mean ± SE; n = 6 dams/group.

* $P < 0.05$ different from control (ethanol, 0 DEA) by 1-way ANOVA and Tukey-Kramer test.

TABLE 2

Diethanolamine-induced decreases in concentrations of choline and metabolites in maternal liver[a]

	nmol/g liver					
	Choline	**PtdCho**	**SM**	**Betaine**	**GPCho**	**PCho**
Control	484 ± 60	15,057 ± 791	2483 ± 78	1067 ± 146	60 ± 11	664 ± 65
DEA	123 ± 9**	5050 ± 235**	1050 ± 61**	330 ± 31**	20 ± 4**	161 ± 34**

[a]Pregnant mice were treated with 80 mg/kg body/day DEA or vehicle control for 10 d as described in text. On pregnancy day 17 mothers were killed and liver collected. Choline and metabolites were assayed using liquid chromatography-electrospray ionization-isotope dilution mass spectrometry. Results represent the mean nmol/g liver ± SE n = 5–6 animals/group. PtdCho, phosphatidylcholine; SM, sphingomyelin; GPCho, glycerophosphocholine; PCho, phosphocholine.

**$P < 0.01$ DEA-treated group differs from the control group by ANOVA and Tukey-Kramer test.