Deletion of murine choline dehydrogenase results in diminished sperm motility

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ABSTRACT **Choline dehydrogenase (CHDH) catalyzes the conversion of choline to betaine, an important methyl donor and organic osmolyte. We have previously identified single nucleotide polymorphisms (SNPs) in the human *CHDH* gene that, when present, seem to alter the activity of the CHDH enzyme. These SNPs occur frequently in humans. We created a *Chdh*$^{-/-}$ mouse to determine the functional effects of mutations that result in decreased CHDH activity. *Chdh* deletion did not affect fetal viability or alter growth or survival of these mice. Only one of eleven *Chdh*$^{-/-}$ males was able to reproduce. Loss of CHDH activity resulted in decreased testicular betaine and increased choline and PCho concentrations. *Chdh*$^{+/+}$ and *Chdh*$^{-/-}$ mice produced comparable amounts of sperm; the impaired fertility was due to diminished sperm motility in the *Chdh*$^{-/-}$ males. Transmission electron microscopy revealed abnormal mitochondrial morphology in *Chdh*$^{-/-}$ sperm. ATP content, total mitochondrial dehydrogenase activity and inner mitochondrial membrane polarization were all significantly reduced in sperm from *Chdh*$^{-/-}$ animals. Mitochondrial changes were also detected in liver, kidney, heart, and testis tissues. We suggest that men who have SNPs in *CHDH* that decrease the activity of the CHDH enzyme could have decreased sperm motility and fertility.—Johnson, A. R., Craciunescu, C. N., Guo, Z., Teng, Y.-W., Thresher, R. J., Blusztajn, J. K., Zeisel, S. H. Deletion of murine choline dehydrogenase results in diminished sperm motility. *FASEB J.* 24, 2752–2761 (2010). www.fasebj.org**

Key Words: betaine · mitochondria

BETAINE IS AN IMPORTANT methyl donor required for the conversion of homocysteine to methionine (1, 2), and it is an organic osmolyte that is needed for normal kidney glomerular function (3, 4). Betaine can be obtained from the diet (from wheat, shellfish, spinach, and sugar beets, for example) (5–7), and it is formed, in mammals and some microorganisms, *via* the oxidation of choline in 2 steps catalyzed by choline dehydrogenase (CHDH; E.C. 1.1.99.1) in the inner mitochondrial membrane (8–12) and betaine aldehyde dehydrogenase (BADH; EC 1.2.1.8) in the mitochondria and cytosol (13). Choline oxidase (CO; EC 1.1.3.17), also found in microorganisms and in some plants, is capable of catalyzing both oxidation reactions (14–17). The oxidation of choline is irreversible, committing the choline moiety to the methyl-donation pathway for use in one-carbon metabolism (18). Choline that is not used to form betaine can be acetylated to form acetylcholine, or phosphorylated to form phosphatidylcholine and sphingomyelin (18).

CHDH activity can influence tissue homocysteine (Hcy) concentrations because betaine donates a methyl group to homocysteine in a reaction catalyzed by betaine:homocysteine methyl transferase (BHMT; EC 2.1.1.5). The product of this reaction is methionine, which is the precursor for *S*-adenosylmethionine (AdoMet), the most important methyl donor in biochemical reactions (including DNA, RNA, protein, and phospholipid methylations). Dietary betaine supplementation is effective in lowering plasma total Hcy (tHcy) concentrations in humans (2); elevated plasma tHcy concentration is associated with increased risk of cardiovascular disease (19, 20).

Several single nucleotide polymorphisms (SNPs) in *CHDH* occur frequently in humans. One such SNP, rs12676, is located in the coding region of the *CHDH* gene, with 42–47% of the population having 1 allele and 9% being homozygous (21, 22). Eighty-three percent of premenopausal women who were heterozygous for the rs12676 allele developed organ dysfunction (liver or muscle) when fed a choline-deficient diet, compared to only 20% of women who were wild type (22), suggesting that this SNP alters CHDH function.

The functional effects of null mutations of the *CHDH* gene have not been further investigated, but it would be reasonable to predict that they would decrease betaine concentrations and increase choline and homocysteine concentrations in tissues, alter osmolyte-dependent functions of the renal glomerulus, and might perturb mitochondrial function (as the enzyme is located on the inner mitochondrial membrane and concentrations of betaine in the mitochondria are micromolar; ref. 23). To better characterize the role of

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CHDH, we created a $Chdh^{-/-}$ mouse and now present evidence that *Chdh* mutation contributes to impaired mitochondrial function in several tissues, with the most striking effects observed in sperm.

MATERIALS AND METHODS

Generation of $Chdh^{-/-}$ mice

Chdh chimeric mice were generated using a gene-targeting vector that removed exons 1 though 3 of the gene. The 5′ arm of homology was derived from the region of the gene immediately 5′ of exon 1 (~5.5 kb), and the 3′ arm was derived from the region 3′ of exon 3 (~1.5 kb) and encompassed exons 4 through 7 (**Fig. 1*A***). The vector, containing positive- and negative-selection cassettes (neo and TK, respectively), was electroporated into E14TG2a ES cells. PCR-positive clones were confirmed for homologous recombination by Southern hybridization. Targeted cells were injected into blastocysts derived from mouse strain C57BL/6 (B6) to create transmitting chimeras.

Chdh chimeric mice were bred to B6 mice. $Chdh^{+/-}$ breeding pairs were used to generate litters composed of $Chdh^{+/+}$, $Chdh^{+/-}$, and $Chdh^{-/-}$ mice, which were used in these experiments. These animals are on a mixed 129/B6 genetic background. Mice were maintained on AIN-76A diet containing 1.1 g/kg choline chloride (Dyets, Bethlehem, PA, USA). Genomic DNA was isolated from tail biopsies collected at weaning using phenol:chloroform:isoamylalcohol purification. Animals were genotyped by multiplexing PCR using TaKaRa Ex *Taq*DNA polymerase (TaKaRa Bio, Madison, WI, USA) and the following primer sequences: $Chdh^{+/+}$ 5′-AGGGCCACAAGTGTGGGCTGGCTGAAACTG-3′, *Chdh* common 5′-GCTAGCTTGAACCCTTTGAAGGGTCTTCTCAGACTC-3′ and *Chdh* neo 5′-ACGCGTCACCTTAATATGC-3′. The primer locations are illustrated in Fig. 1*A*. PCR conditions were as follows: 95°C for 3 min, 94°C for 30 s, 56°C for 30 s, 72°C for 3 min (repeated 35 times), and 72°C for 10 min. $Chdh^{+/+}$ reactions produced a product 2.3 kb in size. The *Chdh* neo product was 1.6 kb in size (Fig. 1*B*). The Institutional Animal Care and Use Committee of the University of North Carolina (UNC) at Chapel Hill approved all experimental protocols.

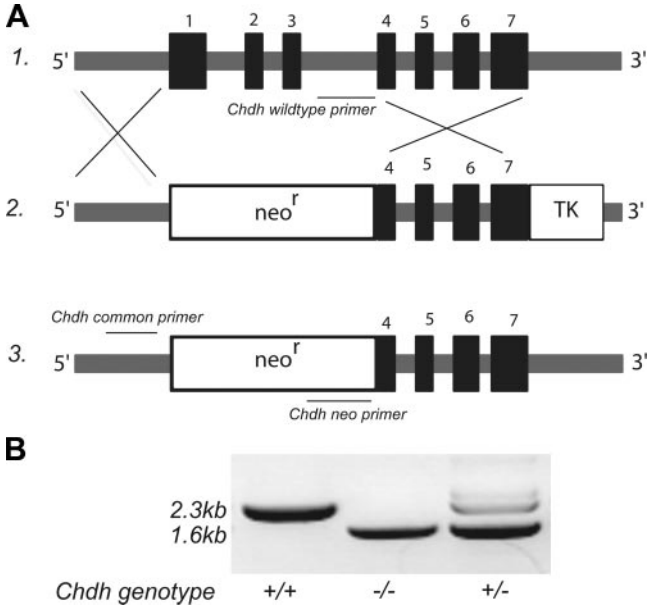


Figure 1. Mutation of *Chdh* gene in mice. *A*) Targeting vector used to generate $Chdh^{-/-}$ mice. *1*) Wild-type *Chdh* allele. *2*) Targeting vector. *3*) Targeted *Chdh* allele with exons 1 through 3 replaced with *neo*^{r} cassette. *B*) *Chdh* mouse genotyping. Tail biopsies were collected from mice at weaning. Tail tissue was digested in buffer containing proteinase K and genomic DNA purified by phenol:choloroform extraction. DNA was used for PCR-mediated genotyping of all animals. *neo*^{r}, neomycin resistance cassette; TK, thymidine kinase.

Body length, total body weight, fetal viability, and survival

Body length and total body weight of $Chdh^{+/+}$, $Chdh^{+/-}$, and $Chdh^{-/-}$ mice were measured at weaning [postnatal day (P)21], P42, and P63. Mice were weighed on a scale, and a ruler was used to measure body length (distance between the tip of the nose to the end of the buttock when the mouse is lying on its stomach). The genotype distribution of litters produced by 20 $Chdh^{+/-}$ mating pairs over a 3-yr period was recorded. Ten $Chdh^{-/-}$ mice were maintained over the course of 1 yr to determine whether *Chdh* mutation affected the 1-yr survival rates.

Measures of liver, kidney, and muscle function

Plasma alanine transaminase (ALT) activity, plasma total bilirubin concentration, blood urea nitrogen (BUN) concentration, and plasma creatine concentration were measured by the Animal Clinical Chemistry and Gene Expression Facility located at UNC-Chapel Hill, using an automatic chemical analyzer (VT250; Johnson and Johnson, Rochester, NY, USA). Plasma creatinine phosphokinase (CPK) activity was determined using a creatine kinase-SL assay kit (Diagnostic Chemicals Limited, Oxford, CT, USA), according to manufacturer's instructions. Urine collection and specific gravity measurement study were performed as described previously (24). Urine-specific gravity was measured using a refractometer (AO Instrument Company, Buffalo, NY, USA) by the UNC-Chapel Hill Department of Laboratory Animal Medicine Veterinary and Technical Services Facility.

CHDH enzymatic assay

$Chdh^{+/+}$ and $Chdh^{-/-}$ mice were anesthetized with halothane (Henry Schein, Melville, NY, USA) until they no longer responded to a hard pinch of their tail or foot. Tissues were harvested from animals and immediately snap-frozen in liquid nitrogen. Liver, kidney, testis, brain, skeletal muscle (vastus medialis), and heart were then pulverized in liquid nitrogen and stored at −80°C until CHDH activity was assayed.

Two hundred milligrams of liver or kidney was homogenized in 500 μl cold homogenization buffer (250 mM sucrose, 50 mM Tris, and 0.1 mM EDTA, pH 7.8) using a motorized tissue homogenizer (Talboys Engineering Corporation, Montrose, PA, USA). The entire brain, skeletal muscle, or heart was homogenized in 300 μl cold homogenization buffer. Both testes from one animal were homogenized together in 300 μl of homogenization buffer. Protein concentrations were measured by Lowry assay (25). CHDH activity was measured in $Chdh^{+/+}$ sperm that were treated with betaine *in vitro*. CHDH activity assay was measured, as described previously (26). The amount of betaine formed was determined by HPLC as described previously (27) using a Varian ProStar solvent delivery system (PS-210, Varian, Palo Alto, CA, USA), and a Pecosphere Silica column (3 μM, 4.6×83 mm; Perkin Elmer, Norwalk, CT, USA) with a Pelliguard LC-Si guard

column (Supelco, Bellefonte, PA, USA). Radiolabeled choline and betaine peaks were detected using a Berthold LB506 C-1 radiodetector (Berthold, Oak Ridge, TN, USA).

Targeted metabolomics

Choline metabolites

Liver, brain, kidney, skeletal muscle, heart, and testis tissues were collected from 7.5-wk-old *Chdh*$^{+/+}$ and *Chdh*$^{-/-}$ mice, quick frozen in liquid nitrogen, and then pulverized under liquid nitrogen. Mitochondria were isolated using a Percoll gradient following previously described methods (28–30). All procedures for mitochondria isolation were performed at 4°C. The concentration of choline metabolites [choline, glycerophosphocholine (GPCho), phosphocholine (PCho), phosphatidylcholine (PtdCho), and sphingomyelin (SM)] was measured by liquid chromatography-electrospray ionization-isotope dilution mass spectrometry (LC-ESI-IDMS), as described previously (31). Phosphatidylethanolamine (PtdEtn) concentrations were measured, after isolation by thin-layer chromatography, using an inorganic phosphorus assay (32).

Plasma tHcy

Blood from *Chdh*$^{+/+}$ and *Chdh*$^{-/-}$ anesthetized animals was collected *via* cardiac puncture. Plasma was isolated from other blood components by centrifugation at 400 *g* for 5 min at room temperature. Samples were derivatized using 7-fluorobenzofurazan-4-sulfonic acid (SBD-F), as described previously (33). tHcy concentration was measured by HPLC using a ProStar solvent delivery system (PS-210; Varian), a Microsorb-MV C18 (5 μm, 100 Å, 25 cm; Varian) and a fluorescence spectrophotometric detector (Varian Prostar model 360) with an excitation wavelength of 385 nm and an emission wavelength of 515 nm. Cysteamine (10 μM) was used as an internal standard.

AdoMet and AdoHcy

AdoMet and AdoHcy concentrations were measured in 50 mg of pulverized liver tissue using HPLC (34, 35). The assay was performed on a Varian ProStar HPLC system (PS-210) using a Beckman Ultrasphere ODS 5 μm C18 column, 4.6 mm × 25 cm (cat no. 235329; Beckman Coulter, Fullerton, CA, USA) at 55°C with an online Gilson 118 UV/VIS detector (Gilson, Middleton, WI, USA).

Tissue histology

Seven-and-a-half week-old *Chdh*$^{+/+}$ and *Chdh*$^{-/-}$ mice were anesthetized, and liver, kidney, skeletal muscle, heart, and brain were collected. The tissues were fixed in 4% paraformaldehyde/0.2% gluteraldehyde for 72 h, processed, paraffin embedded, and sectioned for hemotoxylin and eosin staining using standard techniques. Brain sections were stained with Luxol blue. Reproductive organs from adult (14–17 wk old) *Chdh*$^{+/+}$ and *Chdh*$^{-/-}$ male mice were fixed for 24 h in modified Davidson's fixative. Testis and epididymal sections were stained with hemotoxylin and eosin, as well as with periodic acid-Schiff stain (PAS) for histological analysis. A UNC-Chapel Hill Department of Laboratory Animal Medicine veterinary pathologist examined all tissues except brain. An expert in neuroanatomy examined brain sections.

Transmission electron microscopy (TEM)

Epididymal tissue was harvested from 8- to 10-wk-old *Chdh*$^{+/+}$ and *Chdh*$^{-/-}$ mice. The tissue was fixed in 2% paraformaldehyde, 2.5% gluteraldehyde, 0.2% picric acid in 0.1 M sodium cacodylate, pH 7.2. Liver, kidney, brain, skeletal muscle, heart, and testis tissues were harvested from animals perfused *via* cardiac puncture of the left ventricle using a gravity perfusion system (IV-140; Braintree Scientific, Braintree, MA, USA) with the same fixative. The UNC-Chapel Hill Microscopy Services Laboratory processed tissues for TEM according to standard techniques. TEM grids were observed and photographed using a Zeiss EM-10A transmission electron microscope (LEO Electron Microscopy, Thornwood, NY, USA) with an accelerating voltage of 60 kV.

Sperm count and motility

Sperm from the cauda epididymis was collected from 8- to 10-wk-old *Chdh*$^{+/+}$, *Chdh*$^{+/-}$, and *Chdh*$^{-/-}$ male mice into M16 medium (Sigma-Aldrich, St. Louis, MO, USA). Sperm count was determined by counting cells with a hemocytometer. Percentage progressive motility was determined by counting the number of progressively motile sperm per total number sperm present using a hemocytometer. "Progressively motile sperm" refers to sperm that swim forward in a somewhat straight line.

MTT assay

3-(4,5-Dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) acts as a hydrogen acceptor in oxidation reactions, and its conversion from a yellow substrate to a blue product measures total mitochondrial dehydrogenase activity. We assayed this conversion using a commercially available kit (CellTiter 96 nonradioactive cell proliferation assay; Promega, Madison, WI, USA) with some modifications. To assay MTT conversion in sperm, 100 μl of sperm/medium suspension was incubated in F10 medium (Life Technologies, Carlsbad, CA, USA) in a 96-well plate with 15 μl of MTT solution at 37°C/5% CO_2 for 2 h (36). Following solubilization, the absorbance of each sample was read at a wavelength of 562 nm using a BioTek plate reader (BioTek, Winooski, VT, USA). Absorbance was normalized to the number of sperm assayed.

Mitochondria from fresh liver, kidney, brain, and testis were isolated at 4°C using a Percoll gradient following previously described methods (28–30). An aliquot of each sample (~10–20 μg mitochondrial protein) was incubated in 100 μl of assay buffer (AB; 110 mM KCl, 10 mM ATP, 10 mM $MgCl_2$, 10 mM sodium succinate, 1 mM EGTA in 20 mM MOPS, pH 7.5), and 15 μl MTT solution (from kit) for 15 min at 37°C with 5% CO_2. Absorbance was normalized to the amount of mitochondrial protein assayed.

ATP assay

ATP concentration in sperm and isolated mitochondria was measured using an ATP bioluminescence assay kit CLS II (Roche Diagnostics, Mannheim, Germany), according to manufacturer's instructions. ATP concentration was normalized to either number of sperm or amount of mitochondrial protein assayed.

JC-1 assay (inner mitochondrial membrane potential, Ψ_m)

Membrane potential across the inner mitochondrial membrane was estimated using the fluorescent indicator dye JC-1

(Sigma-Aldrich). Sperm were incubated with JC-1 dissolved in DMSO at a final concentration of 10 μg/ml in F10 medium (Invitrogen, Carlsbad, CA, USA) at 37°C for 10 min. Sperm were pelleted at 800 *g* for 1 min. The supernatant was discarded, and sperm were resuspended in 37°C PBS. 20 μl of each stained sample was examined with a fluorescence microscope (BX50; Olympus, Center Valley, PA, USA) and large band epifluorescence filters. Cells that fluoresced red in the sperm midpiece were counted in 5 random fields of vision for each sample; results are expressed as a percentage of total cells in those fields.

Mitochondria (~100 μg protein) were incubated with 1.72 ml of AB and 18 μl of 0.2 mg/mL JC-1 dissolved in DMSO for 10 min at room temperature protected from light. A mitochondrial sample from $Chdh^{+/+}$ liver, treated with valinomycin (1:200 final dilution; Sigma-Aldrich) for 20 min, was used as a negative control. Fluorescence was measured using an excitation wavelength of 490 nm and an emission wavelength scan from 500 to 700 nm on a Hitachi F-2500 fluorescence spectrophotometer (Hitachi HTA, Pleasanton, CA, USA). A peak at 595 nm corresponds to red fluorescence of J-aggregates. Fluorescent units (FLU) were normalized to the amount of mitochondrial protein assayed.

Oral betaine supplementation

For betaine supplementation experiments, 8- to 10-wk-old $Chdh^{+/+}$, $Chdh^{+/-}$, and $Chdh^{-/-}$ mice were given free access to drinking water supplemented with betaine (Sigma-Aldrich) to a final concentration of 2% for 42 d. 2% betaine was chosen as it was previously shown to be an optimal concentration for treating animals with other fertility problems (37–39).

Statistics

Statistical differences were determined using JMP 6.0 software (SAS Institute, Cary, NC, USA) using ANOVA, Tukey-Kramer HSD, and Student's *t* test assuming equal variances. All tests were performed at $\alpha = 0.05$.

RESULTS

Chdh deletion has no effect on fetal viability, growth, or liver, kidney, or muscle function

The genotype distribution for litters born to $Chdh^{+/-}$ mating pairs (total 604 pups) was 23% $Chdh^{+/+}$, 51% $Chdh^{+/-}$, and 27% $Chdh^{-/-}$. Litter size for $Chdh^{+/-}$ mated pairs was 6–11 pups. Wild-type C57 mating pairs typically produce litters of similar size. Mating a $Chdh^{-/-}$ female with a male $Chdh^{+/-}$ mouse resulted in normal size litters (6–9 pups). However, 10 of 11 pairs of $Chdh^{-/-}$ males mated with female $Chdh^{-/-}$ mice had no litters over the course of 8 mo; during this time, 1 mating pair had a litter of 2 pups, both of which showed impaired growth.

There were no differences among $Chdh^{+/+}$, $Chdh^{+/-}$, and $Chdh^{-/-}$ mice in growth either in terms of body length or total body weight (data not shown). Selected tests of liver, kidney, and muscle function showed no changes in the $Chdh^{-/-}$ animals (Supplemental Table 1). $Chdh^{-/-}$ mice lived for ≥1 yr without any obvious serious health problems.

Chdh deletion results in loss of CHDH enzyme activity

In wild-type mice, kidney, liver, and testis had the highest activity of CHDH, with almost no activity detected in brain and skeletal muscle; CHDH activity was undetected in heart. Reduced CHDH activity was detected in tissues of $Chdh^{-/-}$ mice compared to $Chdh^{+/+}$ mice (**Fig. 2**). Liver samples from $Chdh^{+/-}$ mice had 37% of the CHDH activity measured in $Chdh^{+/+}$ samples (data not shown).

Choline metabolite profiles are changed in $Chdh^{-/-}$ testis and testis mitochondria

Loss of CHDH activity in testis tissue resulted in a 99% decrease in betaine concentration ($P>0.001$), a 97% increase in choline concentration ($P>0.001$), and a 20% increase in PCho concentration ($P>0.05$) compared to concentrations in $Chdh^{+/+}$ testis (**Table 1**). GPCho concentration, PtdCho concentration, and SM concentration did not change with genotype. In testis mitochondria, betaine concentration decreased to undetectable ($P>0.001$), choline concentration increased from 4 to 119 nmol/g ($P>0.001$), and PCho concentration increased by 86% ($P>0.001$). GPCho concentration, PtdCho concentration, and SM concentration in testis mitochondria did not change with genotype. There was no change in PtdEtn concentrations in isolated testicular mitochondria ($Chdh^{+/+}$: 2116±250 nmol/mg protein, $Chdh^{-/-}$: 2052±198 nmol/mg protein), and there was no change in the PtdCho/PtdEtn ratio in $Chdh^{-/-}$ testis mitochondria compare to wild-type.

Similar changes in choline metabolite profiles were measured in other tissues (Supplemental Tables 2 and 3).

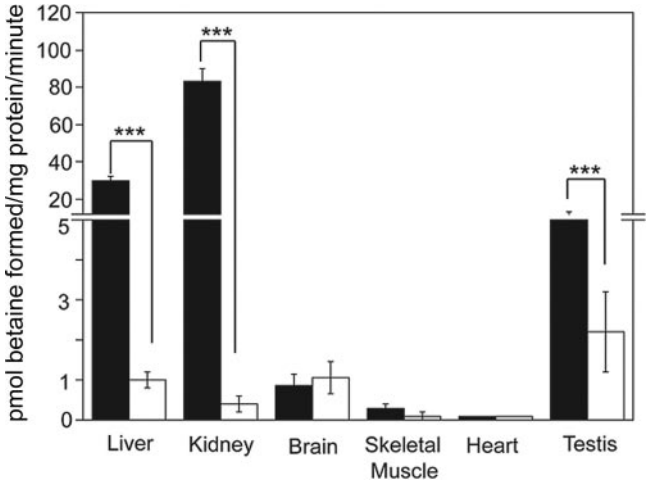


Figure 2. $Chdh^{-/-}$ animals have reduced CHDH enzyme activity. Liver, kidney, brain, skeletal muscle, heart, and testis were collected from $Chdh^{+/+}$ and $Chdh^{-/-}$ mice. CHDH activity was measured using a radioenzymatic assay. Solid bars, $Chdh^{+/+}$; open bars, $Chdh^{-/-}$. Data are presented as means ± SE. ***$P < 0.001$ *vs.* $Chdh^{+/+}$; ANOVA and Tukey-Kramer HSD test; $n = 6$ animals/group.

TABLE 1. *Choline metabolite concentrations in testis tissue and in purified mitochondria from testis*

Testis preparation	Metabolite					
	Betaine	Choline	GPCho	PCho	PtdCho	SM
Tissue						
Chdh$^{+/+}$	4589 ± 401	367 ± 30	816 ± 45	4030 ± 111	9176 ± 773	1633 ± 172
Chdh$^{-/-}$	31 ± 11†	722 ± 32†	836 ± 26	4861 ± 229*	9599 ± 819	1634 ± 137
Mitochondria						
Chdh$^{+/+}$	296 ± 33	4 ± 4	101 ± 35	332 ± 36	4452 ± 364	90 ± 20
Chdh$^{-/-}$	ND†	119 ± 10†	103 ± 44	617 ± 23†	5266 ± 346	89 ± 14

Testes were collected from *Chdh*$^{+/+}$ and *Chdh*$^{-/-}$ mice, and choline metabolites were measured using LC-ESI-IDMS. Testicular mitochondria were isolated as described in Materials and Methods. Data are presented as means ± SE (nmol/g for tissue; nmol/mg protein for purified mitochondria). *$P < 0.05$, $^{\dagger}P < 0.001$ *vs.* *Chdh*$^{+/+}$; Student's *t* test; $n = 5$ animals/group. GPCho, glycerophosphocholine; PCho, phosphocholine; PtdCho, phosphatidylcholine; SM, sphingomyelin; ND, not detected.

Chdh$^{-/-}$ mice have increased tHcy

Deletion of *Chdh* resulted in a significant increase in tHcy concentrations (from 6.1±0.9 μM in wild-type to 10.4±0.9 μM in knockout mice; $P<0.01$). Hepatic AdoMet concentrations were the same in *Chdh*$^{+/+}$ mice (73.4±6.2 pmol/mg liver), *Chdh*$^{+/-}$ mice (70.0±4.2 pmol/mg liver) and *Chdh*$^{-/-}$ mice (57.8±5.4 pmol/mg liver) ($P=0.11$, $n=6$/genotype). Hepatic AdoHcy concentrations did not change (*Chdh*$^{+/+}$, 51.2±6.9 pmol/mg liver; *Chdh*$^{+/-}$, 57.0±14 pmol/mg liver; *Chdh*$^{-/-}$, 54.0±11.7 pmol/mg liver; $P=0.95$, $n=6$/genotype). The AdoMet/AdoHcy ratio did not differ among genotypes ($P=0.49$, $n=6$/genotype).

Mitochondrial morphology is altered in *Chdh*$^{-/-}$ sperm

Gross anatomic and histopathologic examination at the light microscopy level of testis revealed no difference between *Chdh*$^{+/+}$ and *Chdh*$^{-/-}$ mice (**Fig. 3*A***). TEM examination of sperm ultrastructure revealed that mitochondria in the midpiece of the *Chdh*$^{-/-}$ sperm had malformed cristae and were enlarged/swollen compared to *Chdh*$^{+/+}$ mitochondria (Fig. 3*B*, cross-sectional view; *C*, longitudinal view). Skeletal muscle was the only other tissue examined that showed similar morphological changes in mitochondria (Supplemental Fig 1).

Chdh deletion resulted in decreased sperm motility and ATP content, which was partially reversed by dietary betaine supplementation

Although they display normal mating behavior, *Chdh*$^{-/-}$ males were largely unable to sire litters (as noted earlier, 1 of 11 *Chdh*$^{-/-}$ males fathered a litter of 2 pups). Female *Chdh*$^{-/-}$ mice had no reproductive impairment. There were no differences in sperm counts among *Chdh*$^{+/+}$, *Chdh*$^{+/-}$, and *Chdh*$^{-/-}$ males (**Fig. 4*A***). Normal sperm counts for C57/129 mixed mice are 21×10^6/ml (40). *Chdh*$^{-/-}$ males had significantly decreased sperm motility, with only 16% of sperm classified as being progressively motile (Fig. 4*B*). In contrast, wild-type and heterozygous males had 58% progressively motile sperm ($P<0.01$ different from *Chdh*$^{-/-}$) and bred successfully. Betaine supplementation did not have any effect on sperm concentration in

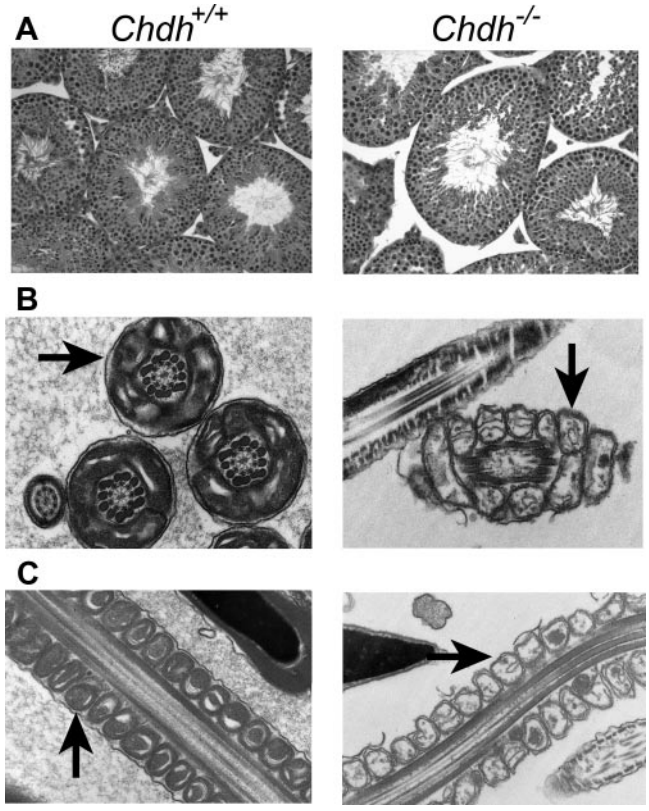
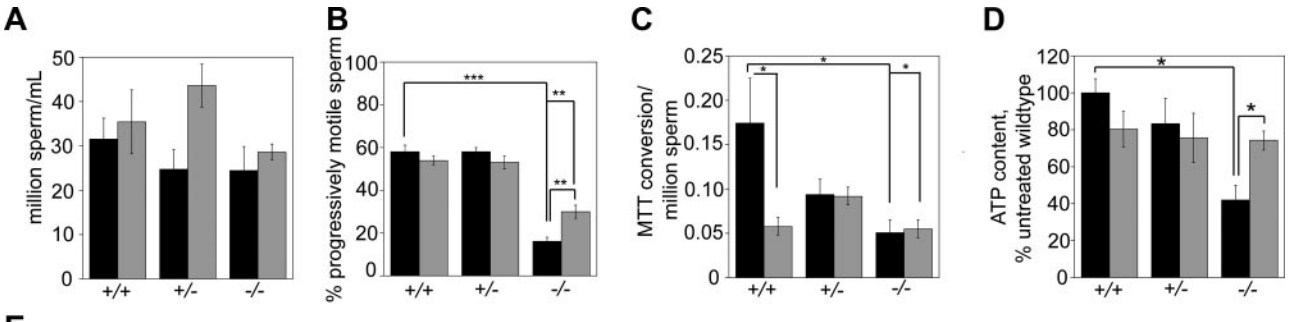


Figure 3. Testis histology and sperm transmission electron microscopy in *Chdh*$^{+/+}$ and *Chdh*$^{-/-}$ mice. *A*) Testes were harvested from 14- to 17-wk-old *Chdh*$^{+/+}$ and *Chdh*$^{-/-}$ males and fixed; 5-μm sections were stained with hematoxylin and eosin. (Periodic acid-Schiff stain showed similar results). Images are ×20 and are representative of $n = 4$ animals/group. *B*, *C*) Electron microscopy of sperm cross-sectional view (*B*) and longitudinal view (*C*). For TEM, cauda epididymides were harvested and processed as described in Materials and Methods. Ultrathin sections (70 nm) were analyzed with an electron microscope with an accelerating voltage of 60 kV. Images are ×50,000 and are representative of $n = 2$ animals/group. Arrows indicate typical mitochondria in sperm midpiece.



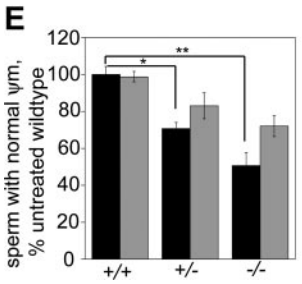


Figure 4. Sperm counts, motility, and mitochondria function. *A*) Counts. Sperm were harvested from 8- to 10-wk-old $CHDH^{+/+}$ (+/+), $Chdh^{+/-}$ (+/−), and $Chdh^{-/-}$ (−/−) mice. Sperm count was determined by counting cells using a hemocytometer. Solid bars, untreated animals; shaded bars, betaine-supplemented animals. Results are means ± SE; n = 11 ($Chdh^{+/+}$, untreated), 5 ($Chdh^{+/-}$, untreated), and 8 ($Chdh^{-/-}$, untreated) mice/group. For betaine supplementation study, n = 11 ($Chdh^{+/+}$), 5 ($Chdh^{+/-}$), 7 ($Chdh^{-/-}$) mice/group. *B*) Motility. Sperm were harvested from 8- to 10-wk-old $Chdh^{+/+}$ $Chdh^{+/-}$ and $Chdh^{-/-}$ mice. Motility assays were performed by counting the number of progressively motile sperm per total number of sperm in 5 squares of a hemocytometer. Results are means ± SE; n = 11 ($Chdh^{+/+}$, untreated), 5 ($Chdh^{+/-}$, untreated), and 8 ($Chdh^{-/-}$, untreated) mice/group. For betaine supplementation study; n = 11 ($Chdh^{+/+}$), 5 ($Chdh^{+/-}$), 7 ($Chdh^{-/-}$) mice/group. $^{**}P < 0.01$, $^{***}P < 0.001$; 2-factor ANOVA and Tukey-Kramer HSD. There is an interaction between genotype and treatment, $P = 0.0004$. *C*) MTT reduction. MTT reduction determined as described in Materials and Methods. Absorbance values were normalized to the number of sperm assayed. All assays were performed in duplicate. Value for each animal is the mean of 4 assays (2 cauda epididymides, each assayed in duplicate per animal). Results are means ± SE. $^{*}P < 0.05$; 2-factor ANOVA and Tukey-Kramer HSD. There is an interaction between genotype and treatment, $P = 0.02$; n = 5 mice/group. *D*) ATP content in sperm. Results are mean ± SE percentage of untreated $Chdh^{+/+}$. $^{*}P < 0.05$; Student's t test; n = 5 mice/group. *E*) Membrane potential. JC-1 staining determined as described in Materials and Methods. Number of cells that fluoresced red in the sperm midpiece was counted in 5 random fields of vision for each sample; results are expressed as a percentage of the total cells in those fields. Results are means ± SE. $^{*}P < 0.05$, $^{**}P < 0.01$; 2-factor ANOVA and Tukey-Kramer HSD. There is no interaction between genotype and treatment; n = 5 mice/group.

$Chdh^{+/+}$, $Chdh^{+/-}$, or $Chdh^{-/-}$ mice. Sperm motility, however, doubled in treated $Chdh^{-/-}$ males compared to untreated $Chdh^{-/-}$ mice, but this motility was still significantly lower than the motility of treated and untreated $Chdh^{+/+}$ and $Chdh^{+/-}$ males (Fig. 4*B*). There was an interaction between genotype and treatment (P=0.004).

MTT conversion (a measure of total dehydrogenase activity in mitochondria) was diminished in sperm from $Chdh^{-/-}$ mice compared to sperm from wild-type and heterozygous animals (P<0.05) (Fig. 4*C*). Betaine treatment decreased MTT conversion in $Chdh^{+/+}$ sperm by 67%, but it did not alter MTT conversion in $Chdh^{+/-}$ or $Chdh^{-/-}$ mice. There was an interaction between genotype and treatment (P=0.02) (Fig. 4*C*).

Sperm from $Chdh^{-/-}$ males had 55% less ATP than did $Chdh^{+/+}$ mice (P<0.05); there were no differences in ATP content between $Chdh^{+/+}$ and $Chdh^{+/-}$ sperm (Fig. 4*D*). Betaine supplementation restored ATP content of $Chdh^{-/-}$ sperm, but it had no effect on $Chdh^{+/+}$ or $Chdh^{+/-}$ sperm. There was no interaction between genotype and treatment. Significantly fewer sperm from $Chdh^{+/-}$ and $Chdh^{-/-}$ mice were able to maintain an Ψ_m of ≥−160 mV (Fig. 4*E*), and betaine treatment did not correct these differences.

Mitochondrial function is altered in $Chdh^{-/-}$ nontesticular tissues

Mitochondria from $Chdh^{-/-}$ liver had 62% lower ATP concentration than did $Chdh^{+/+}$ liver mitochondria (P<0.01), and $Chdh^{-/-}$ heart mitochondria had double the ATP concentration than did mitochondria from $Chdh^{+/+}$ heart (P<0.05) (**Fig. 5*A***). Ψ_m was increased in mitochondria samples from $Chdh^{-/-}$ testis as compared to $Chdh^{+/+}$ testis (Fig. 5*B*) (P<0.05). There were no genotype-specific differences in Ψ_m in the other tissues tested. Deletion of *Chdh* resulted in a decrease in the total mitochondrial dehydrogenase activity in liver and kidney (P<0.05, Fig. 1*B*). There were no differences in brain, skeletal muscle, or heart mitochondria for MTT conversion.

DISCUSSION

We report, for the first time, the successful creation of a mouse in which the *Chdh* gene has been deleted. Fetuses from heterozygous matings were viable, grew normally, and survived to at least 1 yr of age without presenting any obvious health problems. The most striking phenotype observed in these animals was severely impaired sperm motility in $Chdh^{-/-}$ males.

Deletion of *Chdh* greatly reduced CHDH activity in all tissues that normally express this gene (Fig. 2). The small residual activity detected probably reflects activity of other dehydrogenases that can use choline as a substrate. These were probably cytosolic enzymes, as mitochondrial betaine was not detectable in knockout mice (Table 1 and Supplemental Table 3). Liver samples from $Chdh^{+/-}$ mice had 37% of the CHDH activity measured in $Chdh^{+/+}$ samples, suggesting that there is

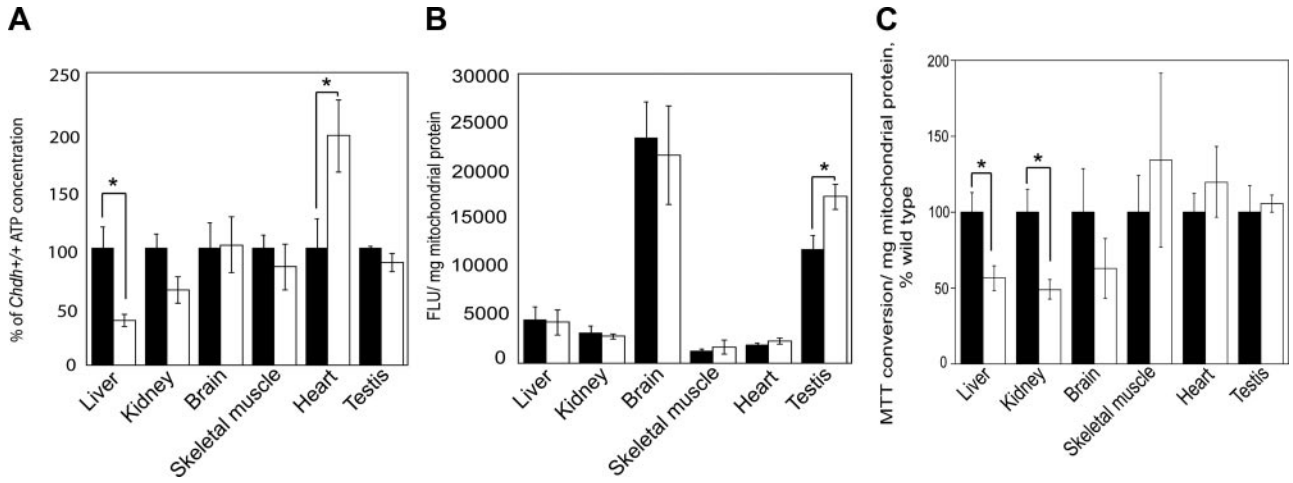


Figure 5. Functional assays of mitochondria isolated from liver, kidney, brain, skeletal muscle, heart, and testis. *A*) ATP concentration. Mitochondria were purified from fresh tissue as described previously. Values were normalized to amount of mitochondrial protein assayed. Results are presented as mean ± SE percentage of $Chdh^{+/+}$; $n = 5$ animals/group. *B*) Membrane potential. Mitochondria were purified from fresh tissues and JC-1 staining was determined as described in Materials and Methods. Values were normalized to amount of mitochondrial protein assayed. Results are means ± SE; $n = 5$ mice/group. *C*) MTT reduction. Mitochondria were purified from fresh liver, kidney, brain, skeletal muscle, heart, and testis tissues, and MTT reduction was determined as described in Materials and Methods. Values were normalized to amount of mitochondrial protein assayed. Results are means ± SE; $n = 9$–11 animals/group. Solid bars, $Chdh^{+/+}$; open bars, $Chdh^{-/-}$. $^*P < 0.05$ *vs.* $Chdh^{+/+}$; Student's *t* test.

one copy of the *Chdh* gene present in mice and that it is biallelically expressed.

$Chdh^{-/-}$ mice had significantly higher plasma tHcy concentrations than did their $Chdh^{+/+}$ littermates, again suggesting that *Chdh* deletion limited methyl-group availability for the methylation of homocysteine, which is removed by 3 pathways: it can be methylated using betaine as a methyl donor, or methylated using methyltetrahydrofolate as a methyl donor, or condensed with serine to form cystathionine (41). An accumulation of tHcy has been associated with an increased risk of cardiovascular disease (19, 20), and betaine has been employed as a treatment for elevated tHcy (2, 42–44). On the basis of the $Chdh^{-/-}$ mouse model, we would predict that individuals who harbor SNPs that decrease CHDH activity will have higher plasma tHcy concentrations and may be at greater risk for developing cardiovascular disease.

In all tissues studied, deletion of the gene resulted in extremely low concentrations of betaine; the small remaining amounts likely were derived from dietary betaine (AIN76A diet contains 70.4 nmol betaine/g diet) or from activity of cytosolic dehydrogenases. We note that testis, liver, and kidney are the tissues where CHDH activity was highest and where betaine and choline concentrations were most changed by the gene deletion (Fig. 2, Table 1, and Supplemental Table 2). Betaine concentrations were nanomoles per milligram protein in mitochondria from liver, kidney, and testes in wild-type animals (in agreement with previously published results for liver mitochondria; ref. 23), but in $Chdh^{-/-}$ mice, almost no betaine was detected in mitochondria (Table 1 and Supplemental Table 3). Assuming ~10% of a cell is protein, we estimate that betaine concentration in the mitochondria of wild-type mice is 2–7 times higher than is the concentration measured in the whole cell, depending on the tissue. The failure to convert choline to betaine resulted in the accumulation of choline in some tissues (in testis and liver, choline concentrations nearly doubled in $Chdh^{-/-}$ animals), while in others, PCho accumulated due to the activity of choline kinase (in kidney, brain, and testis whole tissue). Testicular betaine concentrations were the highest of all tissues studied—almost 10 times higher than concentrations found in the liver, the organ thought of as the primary site of choline metabolism. These data, in conjunction with the asthenospermic phenotype of the male $Chdh^{-/-}$ mice, suggest that betaine plays a critical role in testicular function.

Sperm function, though not viability (unpublished results), was severely compromised in $Chdh^{-/-}$ males. Homozygous males were largely infertile due to decreased motility of their sperm. We present evidence that a decrease in sperm ATP concentration contributes to the poor motility of these cells. $Chdh^{+/-}$ males did not demonstrate any impairment in sperm motility, likely because developing sperm share cytosolic components during spermatogenesis (45, 46) and the products of choline oxidation (reducing equivalents, betaine) could be shared between cells. Indeed, we routinely use $Chdh^{+/-}$ mice to generate animals used in our studies.

A constant supply of ATP, both from mitochondrial oxidative phosphorylation and glycolysis, is required for sperm motility (47–49). It is important to note that in sperm, the processes of oxidative phosphorylation and glycolysis are separated by permanent compartmentalization. Mitochondria are localized to the sperm midpiece, while glycolytic enzymes are found in the sperm principle piece. When CHDH, an enzyme localized to the inner mitochondrial membrane, is absent, mitochondria in the sperm midpiece appeared grossly

abnormal when examined by electron microscopy (Fig. 3*B*). In addition, these cells showed a decrease in mitochondrial dehydrogenase activity and ATP content and *Chdh*$^{-/-}$ sperm mitochondria were unable to maintain an electrochemical gradient across the inner mitochondrial membrane of ≥ -160 mV. Measuring the ability of mitochondria to reduce MTT is a measurement of the activity of the sum total of mitochondrial dehydrogenases; therefore, a decrease in the amount of MTT reduction in the *Chdh*$^{-/-}$ sperm reinforces the idea that CHDH activity represents a significant portion of the total mitochondrial reductive capacity. The JC-1 assay measures the polarity across the mitochondrial inner membrane Ψ_m, a mitochondrial characteristic not necessarily related to the activity of mitochondrial dehydrogenases. For example, inhibiting the electron transport chain will reduce the Ψ_m (50) but may not have an effect on mitochondrial dehydrogenase activity. Ψ_m is directly related to oxidation of NADH (51); therefore, a lack of NADH could result in a collapse of the inner membrane polarity. In this case, a smaller amount of protons will be transported into the inner membrane space and Ψ_m may be compromised. A decrease in either mitochondrial dehydrogenase activity or Ψ_m—or both —can ultimately result in a decrease in ATP production by the mitochondria. These results suggest that *Chdh*$^{-/-}$ sperm did not generate sufficient ATP through oxidative phosphorylation and that ATP generated by substrate-level phosphorylation during anaerobic glycolysis was inadequate to support sperm motility, although all necessary substrates were provided in the medium (M16 medium contains 1.0 g/L glucose, 0.036 g/L pyruvic acid and 4.35 g/L lactic acid).

The oxidation of choline to form betaine produces electron transport chain substrates. The addition of choline to isolated rat hepatic mitochondria increased resting state respiration (state II), accelerated ADP-stimulated respiration (state III), and slightly increased respiration in the presence of oligomycin (state IV) (B. Kristal, personal communication). Functional CHDH enzyme contains a flavin adenine dinucleotide (FAD) prosthetic group, which acts as an electron acceptor during the oxidation of choline to betaine aldehyde, producing $FADH_2$. Further oxidation of the betaine aldehyde intermediate to betaine, catalyzed by mitochondrial betaine aldehyde dehydrogenase, produces NADH (52). Therefore, oxidation of one choline molecule to betaine results in the generation of 5 ATP molecules by the mitochondrial electron transport chain (53). We present data showing that choline and betaine are highly concentrated in mitochondria, suggesting that choline flux through CHDH is high in this organelle and, therefore, choline is likely an important source of energy for the sperm.

When *Chdh*$^{+/+}$, *Chdh*$^{+/-}$, and *Chdh*$^{-/-}$ males were provided supplemental betaine *via* drinking water for 42 d, the asthenospermic phenotype of *Chdh*$^{-/-}$ males was improved but not fully rescued. In addition, the ATP content of the *Chdh*$^{-/-}$ sperm was increased with betaine supplementation. Although the proposed defect in mitochondrial ATP production mechanism described above is appealing, it does not explain why betaine supplementation restored normal sperm ATP concentrations (as no new oxidation of choline occurs due to this treatment). The conversion of choline to betaine cannot be the only defect in production of ATP, there must be another mechanism responsible for this effect.

As noted earlier, betaine is an important osmolyte, and we suggest that perturbation of betaine production in mitochondria resulted in fluid shifts that distorted the 3-dimensional architecture of the mitochondria in *Chdh*$^{-/-}$ mice. Treatment with betaine may allow mitochondria to reaccumulate betaine, establish osmotic balance, and restore function. It is possible that ionic effects of reduced betaine alter ATP-dependent ion pumps and increase utilization of ATP. ATP from the mitochondria may not diffuse out fast enough to sustain activity at the distal end of the sperm flagellum (49). Glycolytic enzymes are concentrated in the principal piece, and some are bound to the fibrous sheath of the flagellum (54), and glycolysis produces ATP adjacent to the site where it is required to support active sliding of the flagellar filaments (49). It is possible that betaine, *via* osmotic effects, alters glycolysis. When the maturing sperm leave the testis, they are nonmotile and their full maturation, including potential to display motility, takes place during transit through the epididymis (55). The maturational changes in sperm are caused by changes in the luminal ion concentration and the proteins secreted into the lumen by the epididymal epithelium (55). It is possible that betaine, *via* osmotic effects, alters sperm maturation. The volume of the entire sperm is modulated by intracellular osmolarity, and this too influences sperm motility and flagellar movement (56).

No improvement in MTT conversion was achieved because this measurement is a reflection of the mitochondrial dehydrogenases in these cells, as discussed above. Since CHDH was still absent from the *Chdh*$^{-/-}$ sperm, we would not expect a restoration of MTT conversion with betaine supplementation.

Unexpectedly, MTT conversion in sperm was decreased in betaine-supplemented *Chdh*$^{+/+}$ mice compared to untreated *Chdh*$^{+/+}$ mice. The simplest explanation would be that CHDH activity (which can catalyze MTT conversion to formazan) was inhibited by the reaction's product, betaine; however, we found that this is not the case when this hypothesis was tested *in vitro* (data not shown). Perhaps betaine suppresses the activity of other mitochondrial dehydrogenases or somehow interferes with uptake of the MTT by the mitochondria. Taken together, these results indicate that while sperm motility is partially dependent on the presence of betaine, full motility may require intact choline oxidation.

Mitochondrial abnormalities were not limited to sperm in *Chdh*$^{-/-}$ mice. MTT assays performed in isolated mitochondria from liver and kidney show that deletion of CHDH compromised mitochondrial dehydrogenase activity (Fig. 5*C*); however, unlike in sperm, Ψ_m was maintained in mitochondria from these tissues (Fig. 5*B*). Interestingly, compared to mitochondria from *Chdh*$^{+/+}$ testis, more mitochondria from *Chdh*$^{-/-}$ testis maintain Ψ_m. The testis has ~12 times higher

betaine concentration than does liver and ~3 times higher betaine concentration than does than kidney (Table 1 and Supplemental Table 2). Therefore, a loss of betaine *via Chdh* deletion would significantly reduce the amount of organic osmolyte available to the cells of the testis. This would create a hypoosmotic cytosol with regard to the mitochondrial matrix. Devin *et al.* (57) have reported that when mitochondria are studied in a hypoosmotic medium, there is an increase in state III respiration and in the Ψ_m across the inner mitochondrial membrane. The JC-1 assay only indicates whether the electrochemical gradient across the inner mitochondrial membrane is at least −160 mV and does not measure hyperpolarization; it is possible that all of the testis mitochondria are, in fact, hyperpolarized.

The decrease in liver mitochondrial dehydrogenase activity is associated with a decrease in ATP content in mitochondrial from this tissue; however, this was not true in kidney mitochondria. Interestingly, in the heart, $Chdh^{-/-}$ mitochondria had double the ATP concentration as compared to $Chdh^{+/+}$ mitochondria. Because CHDH activity was undetectable in this organ, the change in ATP content must result from a mechanism other than a direct effect of CHDH, perhaps metabolic changes in other tissues that affect energy metabolism in heart. Skeletal muscle mitochondria appeared abnormal in $Chdh^{-/-}$ mice when examined by TEM (Supplemental Fig. 1), although indicators of mitochondrial function in this tissue were not different than in $Chdh^{+/+}$ animals. It is possible that more extensive studies of mitochondrial function in this tissue will reveal more subtle abnormalities not detected by the assays performed thus far.

Our studies in the $Chdh^{-/-}$ mouse suggest that functional *CHDH* gene polymorphisms in humans may have important consequences, including elevated tHcy concentrations and male infertility. The rs12676 SNP, located in the coding region of the human *CHDH* gene, occurs at a high frequency in the human population (9% have two variant alleles; ref. 21), and it is associated with an increased risk of breast cancer and renders premenopausal women more susceptible to developing fatty liver when they are ingesting a choline-deficient diet (22). Several other *CHDH* SNPs have been identified in humans but have unknown functional consequences. It is estimated that ~20% of human couples worldwide are infertile; in 50% of these couples, the infertility was attributed to male factor infertility (58). Of these, asthenospermia was diagnosed in 15–17% of these men (59, 60). It would be interesting to explore whether these men have mutations in *CHDH*.

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