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Pulmonary Function and Pathology in Hydroxypropyl-beta-cyclodextin-treated and Untreated *Npc1*$^{-/-}$ Mice

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Abstract

Lung dysfunction is an important part of the pathology of the neurodegenerative disorder, Niemann-Pick C1 (NPC1). We have studied the pulmonary disease in the *Npc1*$^{NIH/NIH}$ mouse model. On histology, we find large numbers of alveolar foamy macrophages but no alveolar proteinosis. Lung weight as percent of body weight was markedly increased; using the flexiVent small animal ventilator (SCIREQ, Inc.), we find inspiratory capacity, elastance and hysterisivity to be increased while resistance was not changed. Cholesterol measurements show a doubling of lung cholesterol levels. Collagen is also increased. Treatment of *Npc1*$^{-/-}$ mice with hydroxypropyl-β-cyclodextrin (HPBCD), despite efficacious effects in brain and liver, results in little difference from age-matched controls (using a CNS-expressed transgene to extend the life expectancy of the *Npc1*$^{-/-}$ mice) for these variables.

Keywords

Niemann-Pick C1; intracellular cholesterol transport; foamy macrophages; small animal ventilator; hydroxypropyl-beta-cyclodextrins

Introduction

It has long been known that pulmonary disease, along with liver and spleen storage, are important aspects of NPC. In the period before Niemann-Pick A and B were distinguished from NPC, a variety of pulmonic x-ray descriptions were provided including micronodularity and diffuse changes similar to those seen in tuberculosis [1]. Even when the 3 types were distinguished, similarities of lung disease among NPCA, B, and C, with finding of interstitial lung pathology and foamy cells obtained by bronchoalveolar lavage, were confirmed [2]. Pulmonary disease in a case of the longer surviving NPCD (a milder mutant of *NPC1*) was well described and included decreased diffusion capacity for CO [3]. As NPC1 and NPC2 were distinguished, it became apparent that NPC2 has much earlier onset

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and showed more severe lung disease [4–5] which included pulmonary alveolar proteinosis [6]. The lung disease has been studied in the *Npc1*$^{NIH/NIH}$ mouse model by electron microscopy [7]. Lung endothelial cells and type I pneumocytes were heavily laden with stored material. Foamy macrophages were abundant in the alveolar spaces [7].

Nearly a decade ago, an efficacious effect of hydroxypropyl-beta-cyclodextrins (HPBCD) on slowing the neurodegeneration in the mouse model of Niemann-Pick C1 was shown [8]. HPBCDs are substituted rings of 7 glucoses and this same publication showed they do not cross the blood brain barrier (BBB). Perhaps because it seems counter-intuitive to treat a CNS disorder with a drug that does not cross the BBB, and perhaps because the effects are rather small when the drug is only started at the time of weaning, the treatment was not pursued. Interest was re-ignited when HPBCDs were used as a carrier for neurosteroids and injections were started at 7 days, when the BBB is still open in mice [9]. It was soon found that HPBCD alone was equally efficacious as HPBCD with the neurosteroid [10].

We have studied the pulmonary disease in the *Npc1*$^{NIH/NIH}$ model by histology, pulmonary function tests, and biochemical assays. We have also studied the effects of HPBCD therapy and find that it does not ameliorate the lung disease.

Materials and Methods

Mice

The *Npc1*$^{NIH/NIH}$ (*Npc1*$^{-/-}$) mutant is essentially a “natural” knockout: an active mouse retroposon inserted 1,100 bp and deleted 800 bp of the *Npc1* gene [11]. This also created a frameshift. Thus, only a small amount of the apparently truncated *Npc1* mRNA [12] and no protein is detectable in homozygous recessive mice. These mice are maintained on the BALB/cJ background and derived from heterozygous matings. Tail tips were routinely obtained at 14 days and DNA was typed by PCR according to Loftus, et al [11]. Only 17%, instead of the expected 25% *Npc1* −/− are weaned and untreated mice survive (16) 73 +/− 2 days.

We also used these mice carrying the glial fibrillary acidic protein promoter-promoted *Npc1* cDNA. These mice express Npc1 only in fibrillary astrocytes and have significantly prolonged survivals [13]. The transgene is not expected to have any significant influence on lung function, although it does influence the intestine because of the presence of glia in enteric ganglia [14]. Since there was likely to be progression of lung disease with age, we used these mice to provide age-matched controls for the cyclodextrin-treated mice. These are also on the BALB/cJ genetic background (N6 or greater).

We initially used 3 *Npc1*$^{+/+}$ or *Npc1*$^{+/-}$ as wild-type, age-matched controls, ages 44 to 47 days, for the untreated *Npc1*$^{-/-}$ mice, and 5, ages 94 to 112 days, as age-matched controls initially intended for treated mice. There were no statistical differences in any of the two wild-type age group measurements so they were combined as one group. We studied 8 untreated *Npc1*$^{-/-}$ mice (ages 72 to78 days) which were compared to the above controls. Five treated (ages 133 to 154 days) *Npc1*$^{-/-}$ mice were studied. As mentioned above, to provide age-matched *Npc1*$^{-/-}$ controls for the treated *Npc1*$^{-/-}$ mice, we studied 7 *Npc1*$^{-/-}$ GFAP tg mice (ages 106 to 116 days). Since not every study was successful on every mouse (e.g. some died on the flexiVent), actual numbers for each determination are provided in the figures of results. Treated mice that were not sacrificed for studies live to 150+ days.

Hydroxypropyl-beta-cyclodextrin treatment

(2-hydroxypropyl)-beta-cyclodextrin from Sigma, batch 094K1435, degree of substitution not provided, was dissolved in normal saline at 200 mg/ml. 0.02 c.c./g were injected

subcutaneously, to achieve a dose of 4g/kg. This was injected weekly starting at 7 days post-natal. All pups of a litter were injected at days 7 and 14, genotyping was performed as described above, and injections were continued on the *Npc1*$^{-/-}$ mice. Control mice for pulmonary studies were not injected. Some *Npc1*$^{+/+}$ received weekly injections of HPBCD for lung cholesterol and collagen determinations.

Pulmonary Function Tests

Mice were anaesthetized with 0.017–0.020 ml of 2.5% Avertin per g body weight and the trachea was dissected free and cannulated with a 20 or 21 gauge cannula (depending on the size of the trachea) which was kept in place with a single tie suture. The animals were then connected to a small animal ventilator (flexiVent, SCIREQ, Inc., Montreal, Quebec, Canada) and ventilated with a tidal volume of 10ml/kg, inspiratory/expiratory ratio of 66.67%, respiratory rate of 150/min and maximum pressure of 30 cm H_20. Positive end-expiratory pressures (PEEP) were maintained by submerging the expiratory limb from the ventilator into a water trap (3 cm H_20 pressure).

The flexiVent apparatus ventilates the mouse and then generates pressure-volume curves over an 8 sec. interval before continuing the respirations. The machine is calibrated for gas compressibility and resistive and accelerative losses in all the connections and calculates inspiratory capacity, elastance, hysterisivity, and resistance on the basis of the entered mouse weight.

Cholesterol determinations

Cholesterol was determined with the Infinity^{TM} reagent (ThermoFisherScientific, Inc., Middletown, VA) which measures total cholesterol. Briefly, tissue was homogenized in 20mmol. Tris, 2 mmol EDTA, 150 mmol NaCl with 1% Triton X-100, pH 8.0. An aliquot was taken for protein determination by the bicinchoninic acid technique (BCA Protein Assay, Pierce, Rockford, IL). Standards were dissolved in the same buffer. The homogenate was stirred for 5 min at RT, centrifuged at 18,000g for 5 min., up to 10 ul of supernatant incubated at 37 C for 5–10 min. with 0.3 ml.s of Infinity^{TM} reagent, and absorbance read at 500 nm.

Collagen determinations

Hydroxyproline was determined in lung samples after collagenase digestion of the sample. Briefly, tissue was homogenized in a citrate:acetate buffer (26 mmol citrate, 166 mmol acetate, pH 5.8) and an aliquot taken for protein determination by the bicinchonic acid technique. Collagenase (Clostridiopeptidase A, EC 3.4.24.3, Sigma) was added to 0.1% and the samples were incubated at 37 C with agitation for 16 hours. After digestion, the samples were centrifuged at 18,000g for 3 min. Hydroxyproline standards were diluted in the same homogenization buffer. Supernatants were incubated with one-half vol. of resolving buffer (freshly prepared 62mmol Chloramine T in 26% propanol and half-strength homogenization buffer) for 20 min at RT and then with another one-half vol. of colorimetric reagent (freshly prepared 116 mmol p-aminobenzaldehyde in 70% propanol and 18% percholoric acid) for 5 min. at 60 C. Absorption was measured at 562 nm.

Statistics

Paired t-tests were performed between *Npc1*$^{-/-}$ and controls and between GFAP tg *Npc1*$^{-/-}$ and HPBCD-treated *Npc1*$^{-/-}$ mice. Testing was done for differences between group variances and, if they differed, the t-test that did not assume equal variances was used.

Results

Npc1$^{NIH/NIH}$ compared to wild-type sibs

Histological staining of age-matched wild-type sibling mice shows normal pulmonary tissue (Fig 1A and E) while the affected mice show foamy macrophages but no alveolar proteinosis (Fig 1B and F). Lung weight as percent of body weight was increased (Fig 3A). When studied on the small animal ventilator, differences in pressure-volume loops are apparent (representative loops in Fig 2A).The calculations of the pulmonary function output from these loops showed increases in inspiratory capacity, elastance and hysterisivity (Fig 3) but no changes in resistance (not shown). Cholesterol and collagen were also increased (Fig 4).

HPBCD- treated Npc1$^{NIH/NIH}$ compared to Npc1$^{NIH/NIH}$, GFAP Npc1 (Npc1$^{-/-}$, GFAP transgenic) age-matched transgenics

Histological studies of HPBCD-treated *Npc1*$^{-/-}$ and similar-aged *Npc1*$^{-/-}$, GFAP transgenic controls showed marked increases in the number of alveolar foamy macrophages in both groups (Fig 1C, D, G and H). There was not much difference in the pressure volume loops (Fig 2B) or the values for lung weight as percent of body weight and elastance but hysterisivity was normal in the *Npc1*$^{-/-}$ GFAP transgenic (Fig 3) while the treated, age-matched controls had a small increase in inspiratory capacity. *Npc1*$^{+/+}$ mice treated with cyclodextrin showed lowered cholesterol ([3] 29.8±2.6, p≤0.0005) while collagen was not different ([3] 2.21±0.55, p=0.159). Cholesterol levels were not corrected in *Npc1*$^{-/-}$ mice, while there was a statistically insignificant trend for collagen to be decreased in them (Fig 4).

Discussion

Niemann-Pick disease type C (NPC) is an autosomal recessive, neurodegenerative disorder that usually presents in the first decade of life [15]. The classic presentation of NPC is a child of either sex developing coordination problems, dysarthria, and hepatosplenomegaly during early school-age years. The neurological progression of the disorder is relentless and is characterized by increasing severity of ataxia, developmental dystonia, and dementia, until death supervenes, usually during the second decade of life. Identification of the major gene responsible for the disorder, *NPC1/Npc1*, revealed one coding for a multipass transmembrane protein containing a sterol-sensing domain that shows homology to the genes for Patched, HMG CoA reductase, and SCAP (SREBP cleavage activating protein) [11,16] Analysis of NPC1 protein function suggest that it is involved in late endosomal lipid sorting/vesicular trafficking [17–19]. The fundamental role of NPC1 in intracellular lipid transport, and the severe visceral disease which sometimes causes death before the onset of neurodegeneration, established the idea that lipid accumulation is the primary defect in NPC and leads to secondary neurological impairment. Thus, the use of drugs to try and decrease cholesterol storage was an early goal for treatment of NPC1. However, classic cholesterol-lowering drugs did not have an effect on the neurodegeneration [20].

Beta-cyclodextrins have long been known to create inclusion complexes with cholesterol [21] and have been used to study cholesterol, and other steroid, metabolism in vitro [22–25] and in vivo [26–29], even in humans [30]. They appear to be non-toxic: even massive orally administered doses to pregnant rabbits resulted in no teratogenic or embryotoxic effects [31]. Much of the early literature is reviewed by Thompson [32] (337 references) and a critical discussion of beta-cyclodextrin modifications is provided by Blanchard and Proniuck [33].

HPBCDs are rapidly cleared by the kidney (90% in 4 hours); if pre-loaded with steroid, the steroid component is no longer present but has been transferred to serum proteins [34]. Uncomplexed HPBCD injected intracerebrally was rapidly cleared, cholesterol in such complexes was retained for at least 3 days. Uncomplexed HPBCDs given parentally to *Npc1*$^{-/-}$ mice result in significant clearance of cholesterol from liver [8, 10] but not from lung [10]. The sequestered cholesterol is secreted as bile acid [35]. The mechanism of cholesterol release from NPC1 cells involves endocytosis of the HPBCD [36] and corrects deficient transport of cholesterol from lysosomes to the endoplasmic reticulum [37]. Of interest, methyl-beta-cyclodextrin, which can lower brain cholesterol [38] and therefore, may be able to cross the BBB, was more effective in depleting cholesterol from NPC1 cells [36].

While Camargo, et al [8] found a modest effect with HPBCD injections at doses of 500mg/kg given 3 times a week starting at 28 days, Davidson, et al [39] found a doubling of life expectancy with every other day injections of 4,000mg/kg starting at 21 days. With this treatment, the authors found “a seemingly greater number of cholesterol positive cells present in the CD-treated lungs” , compared to age-matched untreated *Npc1*$^{-/-}$ mice. Using a similar treatment scheme, Ramirez, et al. [40] found a slight decrease in lung cholesterol levels but no decrease in CD68, CD11c, TNFα mRNA levels. Our histological results parallel those of Davidson, et al. [39] and Ramirez, et al. [40]: large numbers of foamy macrophages in the alveolar lining and alveolar spaces were found but no alveolar proteinosis was apparent. Trichrome staining suggested increased collagen which was confirmed by hydroxyproline assays.

We supplemented our histological results using quantitative measures of parameters of pulmonary function, cholesterol and collagen. Lung weight, inspiratory capacity, elastance and hysterisivity were increased in *Npc1*$^{-/-}$ as compared to *Npc1*$^{+/+}$ mice. Cholesterol and collagen were also increased. Thus, this mouse model mostly replicates the pulmonary disease seen in Niemann-Pick C patients although the alveolar proteinosis seen in NPC2 [6] was not seen. We also monitored the effects of HPBCD treatment on the pulmonary disease. We used *Npc1*$^{-/-}$, GFAP transgenics as age-matched controls. This GFAP-promoted Npc1 transgene expresses Npc1 in fibrilary astrocytes and would only affect lung neuronal activity by correcting astrocytes in their ganglia [41–42]. Interestingly, hysteresis (which may be influenced by muscle tone) was returned to normal in these mice but all other parameters were comparable to *Npc1*$^{-/-}$ mice at a younger age. HPBCD treatment did not alter these parameters although there was a trend towards decreased lung collagen.

While HPBCD’s decrease cholesterol in many tissues, including normal lung, they do not have this effect in diseased lung. Perhaps the large capillary bed of the lung results in transfer of cholesterol loaded into HPBCD from other organs to the capillary epithelia, and thus to the lung parenchyma. Possibly inhaled cyclodextrins would decrease the accumulation of cholesterol in the lung.

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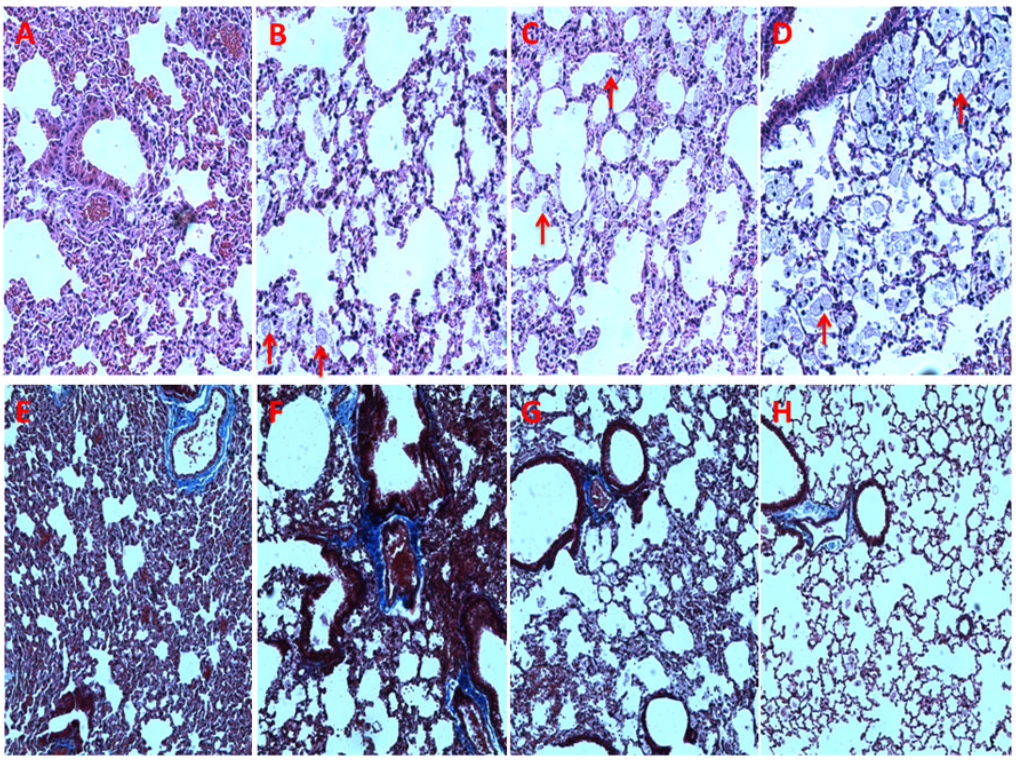

Figure 1.

Histological examination of lungs from control (16 weeks), *Npc1*$^{-/-}$ (11 weeks), GFAP transgenic (16 weeks), and HPBCD-treated *Npc1*$^{-/-}$ (18 weeks) mice. A. Hematoxylin and eosin (H&E) stained control lung. B. H&E stained *Npc1*$^{-/-}$ lung. C. H&E stained *Npc1*$^{-/-}$, GFAP tg lung. D. H&E stained HPBCD-treated *Npc1*$^{-/-}$ lung. E. Trichrome stained control lung. F. Trichrome stained *Npc1*$^{-/-}$ lung. G. Trichrome stained *Npc1*$^{-/-}$ GFAP tg lung. H. Trichrome stained HPBCD-treated *Npc1*$^{-/-}$ lung. Foamy macrophages indicated by arrows.

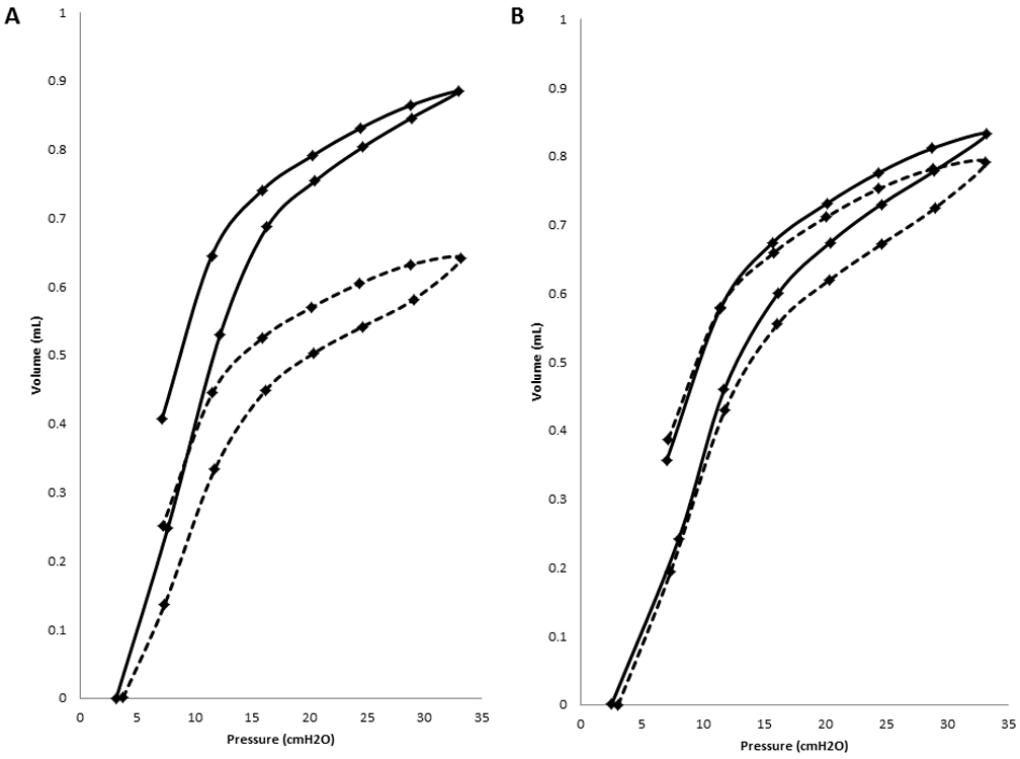

Figure 2.

Representative pressure-volume loops from the flexiVent small animal ventilator (statistical analyses of the values derived from multiple loops are provided in Fig 3). A. One *Npc1*$^{-/-}$ (dotted) compared to a wild-type *Npc1*$^{+/+}$ mouse (solid). B. *Npc1*$^{-/-}$ treated with HPBCDs (dotted) compared to age-matched *Npc1*$^{-/-}$, GFAP transgenic mouse (solid).

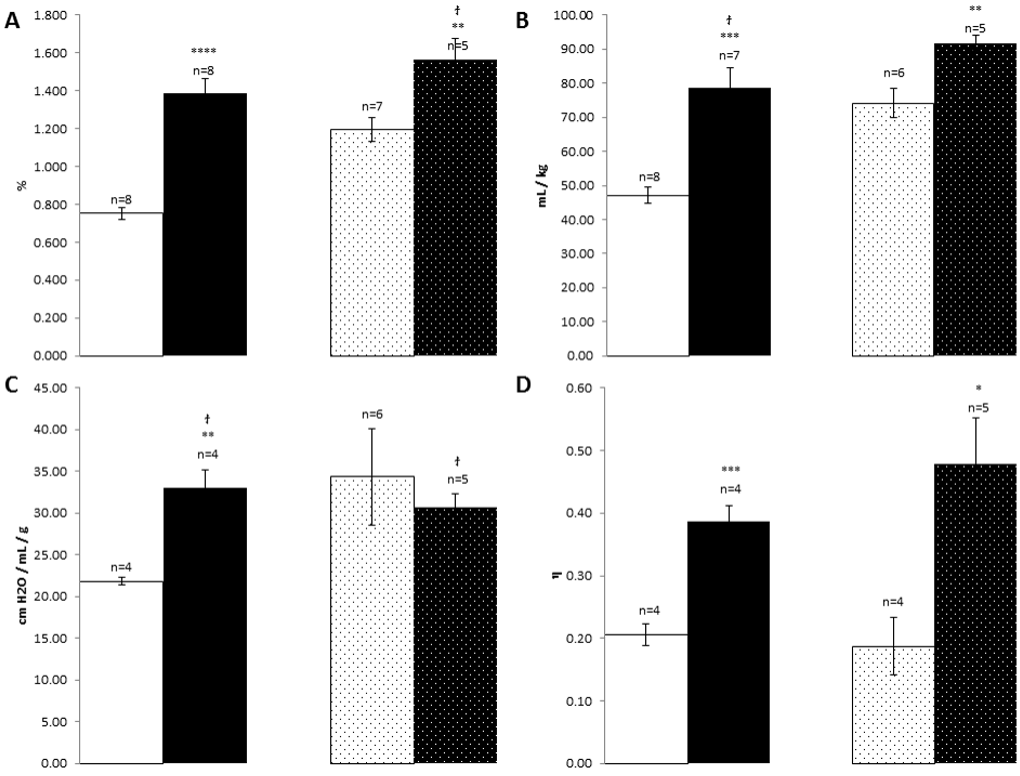

Figure 3.

Pulmonary function values for control mice (white), *Npc1*$^{-/-}$ (black), age-matched *Npc1*$^{-/-}$, GFAP transgenic mice (dotted white), and *Npc1*$^{-/-}$ treated with HPBCDs (dotted black). A. Lung weight as percent of body weight. B. Inspiratory capacity over body weight. C. Elastance. D. Hysterisivity. *p≤0.05; **p≤0.01; ***p≤0.005; ****p≤0.0005. group variances differed so the t-test that did not assume equal variances was used.

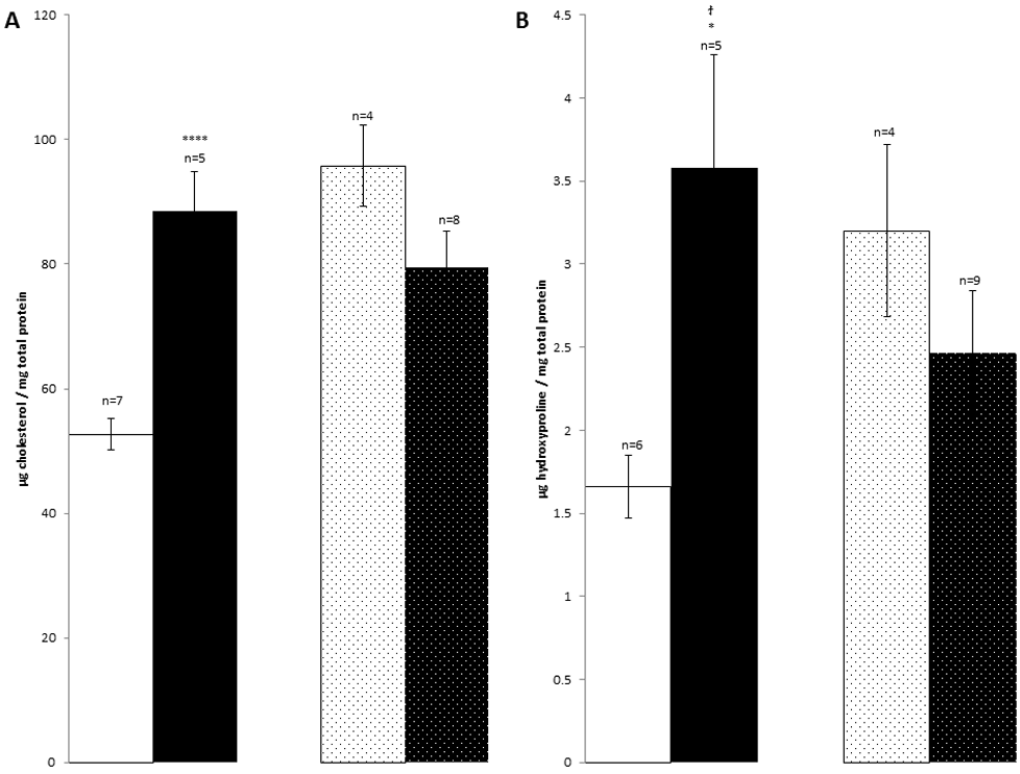

Figure 4.

Biochemical determinations in lungs for control mice (white), *Npc1*$^{-/-}$ (black), age-matched *Npc1*$^{-/-}$, GFAP transgenic mice (dotted white) and *Npc1*$^{-/-}$ treated with HPBCDs (dotted black). A. µg Cholesterol / mg protein. B. Collagen, determined as µg hydroxyproline / mg protein. *p≤0.05; ****p≤0.0005. group variances differed so the t-test that did not assume equal variances was used.