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*Deficient NNT Reduces Mitochondrial Mass and Cholesterol Trafficking in the Adrenal Cortex, Accumulating ROS and Impairing Steroids Production*

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**Background:** The mitochondrial enzyme nicotinamide nucleotide transhydrogenase (NNT) is essential in the antioxidant defense mechanisms. In addition, adrenal cortex cells...
appear to require NNT for normal steroidogenesis. However, to date, the mechanisms involved in NNT regulation of steroidogenesis are not fully understood. **Aim:** To assess how NNT regulates steroidogenesis by characterizing how a loss-of-function NNT mutation impairs antioxidants mechanisms in adrenal cells resulting in FGD. **Methods:** Genomic DNA of a boy with FGD was evaluated by whole-exome sequencing (WES). Candidate variants were analyzed in silico and confirmed by Sanger sequencing. Genotype-phenotype association of a new homozygous loss-of-function NNT variant with FGD was assessed in vitro. We built a stable knockdown model of the NNT gene in the H295R adrenal cell line using CRISPR Cas9 technology. We evaluated the effect of alterations in NNT on Cholesterol Trafficking (NBD-Cholesterol) and mitochondrial parameters (DCFDA and Mitotracker) as well as on adrenal steroidogenesis by cortisol (RIA) and aldosterone (ELISA) production. In this NNT stable knockdown model, we inserted the commercial plasmid NNT HaloTag human ORF in pFN21A (FHCO1572) containing the wild type (WT) sequence of the NNT gene and the direct site mutagenesis. **Results:** WES revealed the NNT p. G866D pathogenic variant in the affected patient. The familial pedigree analysis confirmed the segregation of this variant with FGD in homozygosis. The clone selected after NNT modification by the CRISPR- Cas9 system showed a 75% reduction in NNT protein expression. The NNT-deficient cell clone increased reactive oxygen species (ROS) production (p<0.0001), decreased mitochondrial mass (p<0.0001), and decreased density (p=0.03) of cholesterol lipid droplets as well as cortisol (p<0.0001) and aldosterone (p=0.006) secretion. Reexpression of NNT in an NNT-deficient cell clone restored the ROS levels (p<0.0001). Compared to wild-type (WT), both under basal and oxidative stress conditions after stimulation of 10μM forskolin, homozygous p. G866D NNT exhibited increased ROS production (p=0.0002; p<0.0001, respectively). **Conclusions:** Deficient NNT decreases mitochondrial mass and impairs cholesterol trafficking by reducing cholesterol droplets mass, leading to the loss of antioxidants mechanisms and accumulation of ROS. NNT impairment in adrenal cells decreases cortisol and aldosterone secretion. Altogether, these data enlighten the mechanisms by which NNT mutations result in primary adrenal insufficiency.

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