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# Impaired glucose homeostasis after a transient intermittent hypoxic exposure in neonatal rats ☆

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ABSTRACT

This initial report presents a neonatal rat model with exposure to a transient intermittent hypoxia (IH), which results in a persisting diabetes-like condition in the young rats. Twenty-five male pups were treated at postnatal day 1 with IH exposure by alternating the level of oxygen between 10.3% and 20.8% for 5 h. The treated animals were then maintained in normal ambient oxygen condition for 3 week and compared to age-matched controls. The IH treated animals exhibited a significantly higher fasting glucose level than the control animals (237.00 ± 19.66 mg/dL vs. 167.25 ± 2.95 mg/dL; $P = 0.003$); and a significantly lower insulin level than the control (807.0 ± 72.5 pg/mL vs. 1839.8 ± 377.6 pg/mL; $P = 0.023$). There was no difference in the mass or the number of insulin producing beta cells as well as no indicative of inflammatory changes; however, glucose tolerance tests showed a significantly disturbed glucose homeostasis. In addition, the amount of C-peptide secreted from the islets harvested from the IH animals were decreased significantly (from 914 pM in control to 809 pM in IH; $P = 0.0006$) as well. These observations demonstrate that the neonatal exposure to the IH regimen initiates the development of deregulation in glucose homeostasis without infiltration of inflammatory cells.



## 1. Introduction

Increasing evidence suggests that free radical mediated oxidative stress could induce diabetes mellitus *via* an epigenetic etiologic pathway [1,2]. An excessive accumulation of reactive oxygen species can elevate antioxidant enzymes, and then impair beta cell function [3]. Exposure to intermittent hypoxia (IH), a typical sign of obstructive sleep apnea, can result in oxidative stress and subsequent diabetes [4–6]. For instance, pregnant women with sleep disordered breathing resulting in gestational diabetes [4,5]. Diabetes is one of the prevalent diseases affected by prenatal distresses such as hypoxia [5,6]. Such relationships are suggestive of an association of perinatal exposure to IH and the initiation of glucose–insulin dysfunction leading to the subsequent development of diabetes. However, the identification of the mechanism and factors involved in this process requires an animal model of perinatal IH induction of diabetic symptoms.

Understanding the diabetogenic mechanism may be a crucial step to clarify the enigma as to how diabetes mellitus initiates. Autoimmune reaction has been understood as a pathogenic step for loosing beta cells in type 1 diabetes. However, there has been no direct evidence of increased beta cell destruction, decreased population of beta cells, or accompanied inflammation in the early stage of type 1 diabetes because such defects are noted only after diabetic symptoms have sufficiently advanced. In addition, presence of pro-inflammatory cytokines is often considered as a preceding indicator for infiltration of immune cells in relation to diabetes [7–9]; yet, there has been no report whether pro-inflammatory changes such as an increased expression of interleukin-1 or -6 (IL-1/6) precedes inflammation in neonatal stage after short-term IH challenge. As we focused our research goals strictly on neonates, we performed *in vivo* experiments and *in vitro* analyses to characterize the IH induced perinatal diabetes in which the hypoinsulinemia is the result of beta cell dysfunctional under production of insulin rather than destruction of beta-cells or increased insulin resistance.

*Abbreviations:* IH, intermittent hypoxia; IL, interleukin; GAD65, glutamate decarboxylase65.



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## 2. Materials and methods

### 2.1. Preparation of animals

Ten near end-term pregnant Sprague–Dawley rats were maintained until parturition. One day after birth, five dams along with their respective offspring were randomly selected and designated the control group. Number of pups per each colony was controlled. The other five dams with offspring were designated the experimental group. The two groups included a total of fifty-one male pups. Female pups were excluded from the study. The animals were housed in commercially designed chambers with food and water accessible *ad libitum*, as previously described [10]. The experimental animals were maintained at oxygen concentrations that alternated between room air, 20.8% and 10.3% every 420 s for 5 h; and the control animals were maintained in room air oxygen concentration for 5 h. Each 5 h preparation was carried out once per animal. The pups spent approximately 20–30 min every day with experimenter(s) to minimize stress during the time of procedures. This study was performed in strict accordance with the recommendations in the guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The experimental protocol was approved by the Institutional Animal Review Committee (ARC #2007–008-02) at UCLA and the entire process was conducted by the highest principles of animal welfare which did not conflict with EU Directive 2010/63/EU.

### 2.2. Measurements of blood glucose and insulin

After 2 h food deprivation as described by Durham and Truett [11], the animals were anesthetized with pentobarbital (100 mg/kg, i.p.), the chest was opened, and at least 1.5 mL of blood was drawn from the left ventricle of the heart into serum collection tubes. Separated serum was transferred to 2 mL cryotubes and immediately frozen at −20 °C. Glucose and insulin assays were out-sourced to the Pathology and Laboratory Medicine Services, UCLA. Insulin was measured using the MILLPLEX X-Map magnetic bead-based immunoassay kit by Millipore using Luminex technology. Glucose in blood was determined by ACE Glucose reagent (Alfa Wassermann Diagnostic Technologies, NJ, USA) and the VetACE Clinical Chemistry System.

### 2.3. Euthanasia and tissue procurement

Pups were fasted for 2–2.5 h prior to being anesthetized with pentobarbital (100 mg/kg, i.p.) and perfused with 30 ml of 0.1 M phosphate-buffered saline. The pancreas was rapidly harvested from each animal, weighed and divided into two halves; with one half immediately stored in liquid nitrogen (−80 °C), and the other half fixed in cold 10% neutral buffered formalin for paraffin embedding.

### 2.4. Immunohistochemistry (IHC) for fixed and frozen tissues

Paraffin embedded tissues were sectioned at 10 μm thickness and mounted on slides. The slides with anti-insulin primary antibody (guinea pig anti-insulin, Zymed, Invitrogen) at 1:100 dilution in Tris-buffered saline were used in accordance with manufacturer's guideline. For assessing beta cell mass, the red outline depicted the total pancreas area and the yellow areas were estimated for beta cells (Fig. 1A). Total beta-cell mass was calculated by multiplying total pancreatic wet weight by the fraction of the area occupied by insulin-containing cells with respect to the total area measured by Image-Pro plus 4.1 software (DataCell Ltd). At least 10 images per rat were scanned.

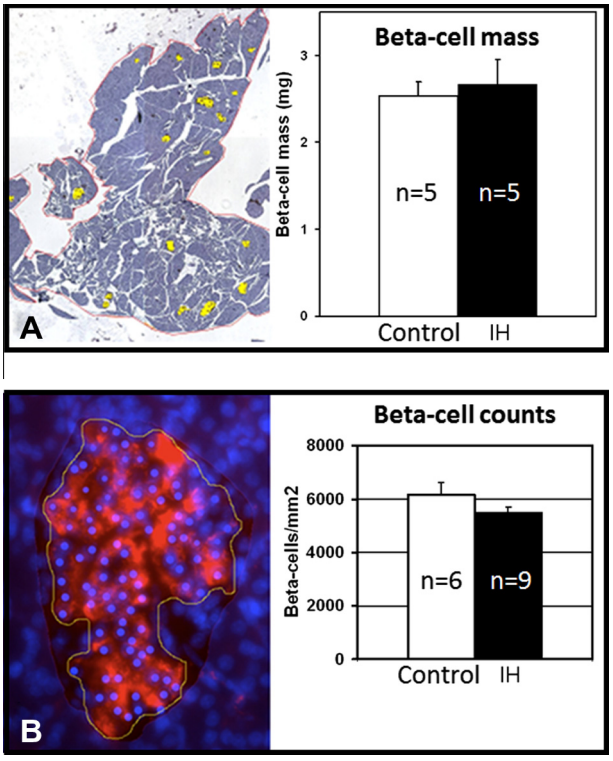


**Fig. 1.** Beta cell mass and counts in IH-treated and control animals. In picture (A), islets are identified by yellow stain. The difference in beta cell mass was not statistically significant. As shown in (B), the number of beta cells was counted in an islet demarcated by orange line. The difference in beta cell count was not statistically significant.

Antibody GAD65 isoform was used to stain with insulin for identifying insulin-expressing beta cells on frozen sections (Fig. 1B). Cells were counted manually using the counter tool in Adobe photoshop. Image J software was used for measuring islet area and circumference. Digital images of a micrometer with known measurements were used to set the scale in Image J. Lastly, H & E staining was performed to examine infiltration of inflammatory cells and followed by IHC using IL-1β (Rabbit, Santa Cruz, USA) and IL-6 (Rabbit, Abcam) polyclonal antibodies to assess pro-inflammatory activity in the islets of paraffin-embedded pancreata. All sections were visualized with the diaminobenzidine reaction and counterstained with hematoxylin. All staining processes were outsourced.

### 2.5. Glucose tolerance tests

Glucose tolerance tests (GTT) were performed on a separate day on control ($n = 7$) and experimental IH animals ($n = 6$) without anesthesia or sedation. The pups were separated from mothers and deprived of food 2 h prior to the test. Glucose (1.0 g/kg) was injected i.p. and blood was sampled from the tip of tails at each time point. A glucometer (Bionime, GM550) and GS550 strips measured the level of glucose at baseline, 2, 5, 10, 15, 30 and 60 min.

### 2.6. Islets isolation and ELISA assays for C-peptide

Islet isolation from pancreata harvested from 3 control and 3 IH treated pups was performed as described by Carter et al. [21]. Immediate after sacrifice, cold collagenase solution was injected into the pancreas through the common bile duct. The removed pancreas was placed into conical tube for digestion at 37 °C for 8 min in collagenase, followed by two-times washing using