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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.

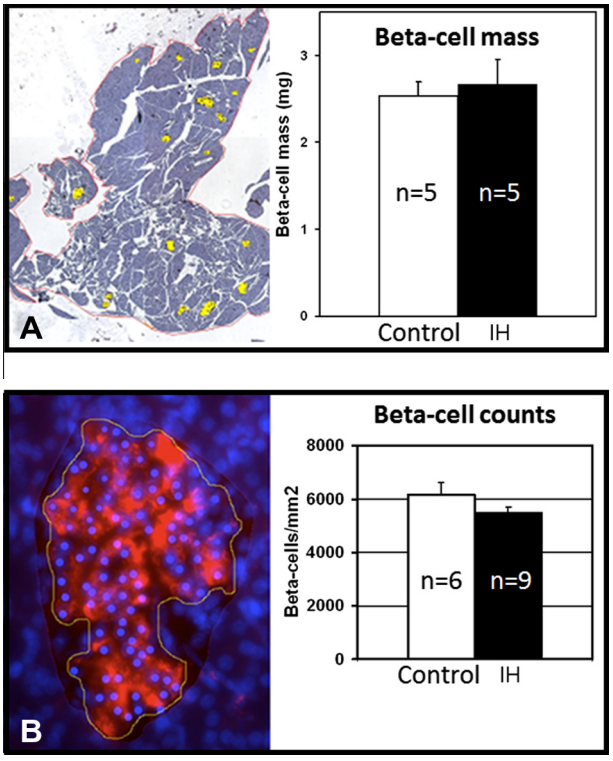

Fig. 1. Beta cell mass and counts in IH-treated and control animals. In picture (A), islets are identified by yellow stain. The difference in beta cell mass was not statistically significant. As shown in (B), the number of beta cells was counted in an islet demarcated by orange line. The difference in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

G-solution (1% BSA containing Hank's balanced salt solution) which dilutes collagenase to slowdown digestive process. Then, the tissue was filtered through a Netwell Insert 500 μm Polyester Mesh (Cat# 3480, Corning Inc.). The flow-through was centrifuged for 2 min at 12,000 rpm and the pellet was re-suspended with Histopaque 1100 solution (Cat# 10771, 11191, Sigma–Aldrich) for gradient separation by centrifuging for 20 min at 12,000 rpm. The supernatant was transferred into a new tube and washed with G-solution twice. The pellet was resuspended in RPMI 1640 media, supplemented with 10% FBS and 1% Penicillin–Streptomycin mixture and cultured at 37 °C and 5% CO_2 incubator for 4 h. After centrifuged for 5 min at 3000 rpm, 20 μl of each supernatant was taken from control and IH islets in new tubes for C-peptide assay. Pellets were washed with 1× phosphate-buffered saline. Each pellet was incubated with RIPA buffer (Sigma–Aldrich, Inc.) containing protease inhibitor cocktail (Roche Applied Science) for 15 min on ice to extract whole cell lysate, and centrifuged at 13,000 rpm for 15 min. 10 μg of cell lysate was used to estimate the amount of C-peptide produced.

C-peptide were quantified using an ELISA Kit (Cat# EZRMI-13 K, EMD Millipore Corporation) in accordance with the manufacturer's protocol. Samples were incubated on the monoclonal anti-insulin coated plate for 2 h, followed by incubation with a horse radish peroxidase-conjugated anti-insulin antibody. TMB (3,3′,5,5′-tetramethylbenzidine) substrate and the stop solution were added for the reaction for color changes. Absorbance was measured at 450 nm in a spectrophotometer.

2.7. Statistics

For group comparisons of the insulin and glucose levels, two tailed *t*-tests were performed. Beta cell counts were compared using a non-parametric, Kruskal–Wallis test (SPSS vs. 12.0). Intra-rater reliability assessment was performed on numbers of beta cells. Cell counts were performed by the same operator at least 3 weeks apart using the same method. On the results of ELISA, *t*-tests were used to assess differences between the groups.

3. Results

No visible difference in the activity and behavior were noted between the control and IH treated pups and mothers. There was no difference in food and water intake of the control and treated mothers. Weight was compared between 12 control and 12 IH treated male pups. No statistical difference was found between the groups (61.4 ± 5.62 g for control; 62.5 ± 7.19 g for IH treated).

3.1. Blood glucose and insulin measurements in 3 week old rats

The blood glucose level in the IH-treated animals was significantly increased by 42% compared to the control animals (237 ± 20 vs. 167 ± 3 mg/dL; $P = 0.003$). The level of blood insulin was decreased by 56% in the IH treated animals compared to the control animals (807 ± 72 vs. 1839 ± 377 pg/mL, $P = 0.023$) (Fig. 2).

3.2. Measurements of beta-cell mass and beta-cell counts

Beta cell mass was measured in islet tissues harvested from 5 controls and 5 IH animals (see Fig. 1A). The wet weight of pancreas in the IH treated and control animals did not differ (328 ± 20 vs. 304 ± 20 mg); and the beta cell mass was unchanged (2.66 ± 0.29 vs. 2.53 ± 0.16 mg). There was no statistical difference in the beta cell count per unit area of islets for IH treated vs. control animals (5520 ± 191 and 6157 ± 458), which coincided with the results of beta cell mass comparisons (Fig. 1B). These collective results

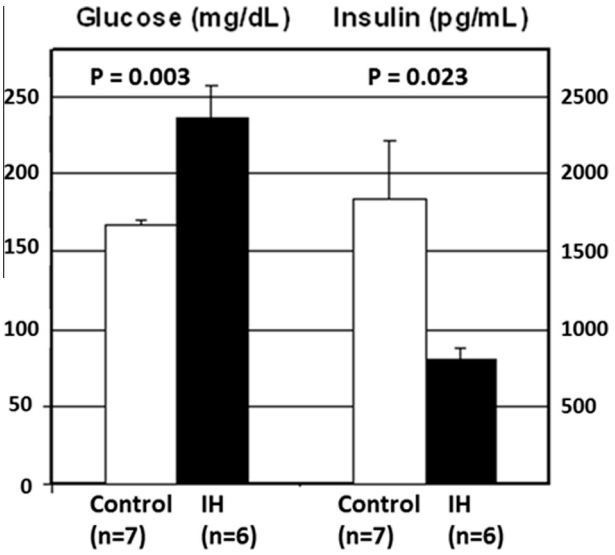

Fig. 2. Comparison of serum glucose and insulin levels of IH treated and control animals. The differences of both blood and insulin are statistically significant.

demonstrate that the decrease in plasma insulin in the IH treated pups may not be due to a decrease in the beta cell population.

3.3. Assessing inflammation and pro-inflammation in the islets

An alternative explanation for the decreased serum insulin level may be impaired beta cell functions in synthesis or/and secretion of insulin due to inflammation in the islets by IH stress [7,8]. As shown in Fig. 3A, there is no histological evidence of islet inflammation or any other apparent histopathology in either group of animals. A mild activity of IL-6 in some islets harvested from the IH treated animals was presented occasionally in contrast to controls (Fig. 3B). Yet, IL-1β activity showed no difference between the control and IH treated islets. Both IL-1 and -6 are known to be increased by boosted activity of monocytes which are not shown anywhere in our samples [12]. Positive control pancreata obtained from streptozotocin (STZ) injected adult mice donated other lab show dark stained IL-1β and IL-6 antibody positive beta cells.

3.4. Assessing function of beta cells

The glucose tolerance tests revealed that the IH treated animals exhibited blood glucose increased at a faster rate, which peaked at a significantly higher level, and with a more prolonged return toward the initial glucose level (Fig. 4A). These conditions typify an impaired glucose tolerance response; and thus, the IH treated pups exhibited abnormal blood glucose regulation.

When ELISA assays were performed to compare functions of islets obtained from control vs. IH treated animals, the level of secreted C-peptide (914.46 pM in control vs. 809.41 pM in IH) measured in media (i.e., which is secreted C-peptide) was significantly different at $P = 0.0006$, while produced C-peptide protein measured in cell lysates (312.65 pM/ng in control vs. 313.60 pM/ng in IH) showed no difference (Fig. 4B).

4. Discussion

Type 1 diabetes (T1D) has been characterized as an autoimmune form of diabetes, which results in destruction of pancreatic beta cells; and is often accompanied with inflammation of the

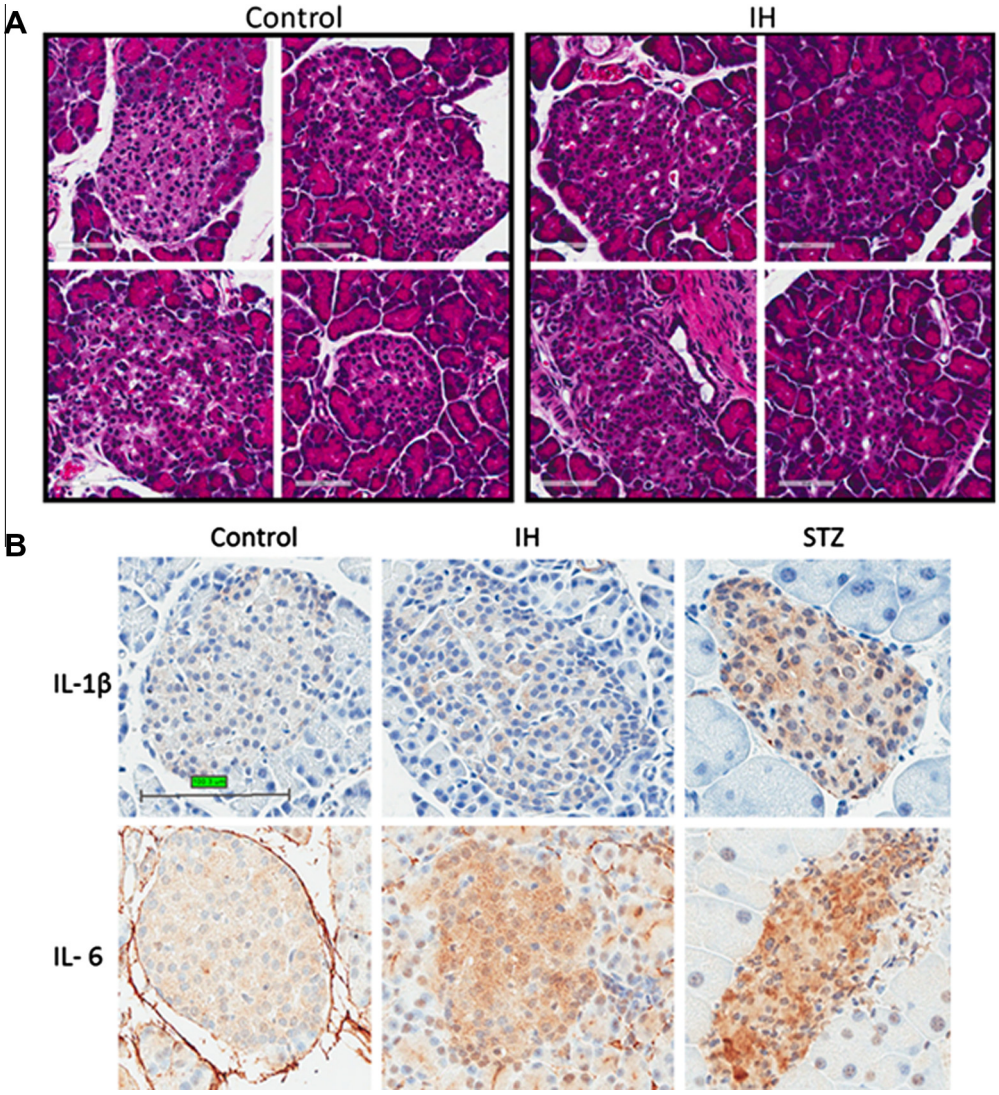

Fig. 3. (A) Histological H & E staining of pancreatic tissue obtained from 3 week-old IH-treated and control animals. Neither control nor IH treated animals exhibit any histological evidence of inflammation or other apparent histopathology. The white scale bars in the pictures indicate 50 μm. (B) Immunohistochemistry staining for IL-1β and IL-6 activities. IH treated pancreas obtained from 3 week old rats exhibit a sign of IL-6 activities in brown color (DAB) that appears milder than the signals in the pancreatic islets harvested from streptozotocin (STZ) treated mice, positive controls. Control islets show no color changes. Pictures were taken by Aperio ScanScope AT in ×20 magnification. The scale bar indicates 100.3 μm. (For interpretation of color in this Figure, the reader is referred to the web version of this article.)

islets usually identified in the advanced stage of disease progression [13]. In this scenario the destruction of the beta cells is considered as the cause of the hypoinsulinemia and hyperglycemia. In our animal model, no evidence exists for involvement of decrease or destruction of beta cells, nor any histological evidence of inflammation in the islets associated with the IH induced T1D. A mild, but not significant, sign of pro-inflammatory cytokines was observed occasionally in islets of IH treated pups, but not yet showed any potential for full development. Despite the intact anatomy of the IH treated islets, glucose regulation was apparently impaired; however, the graphical depiction of glucose tolerance tests shown in Fig. 4A does not represent a typical shape of either type 1 or 2 diabetes.

The light signals of IL-6 in some IH treated islets alluded above are unlikely associated with autoimmune-related inflammation, this finding parallels to the beta cell dysfunction in the absence of beta cell destruction associated with the development of T1D observed in monogenic diabetes [14,15]. It brings into question the validity of a rigid definition of T1D as resulting from an autoimmune condition; particularly in the early initial stage of T1D. There are likely numerous etiological conditions and polygenic factors that can initiate the manifestation of T1D symptoms. It is also probable that the insulin insufficiency and hyperglycemia could initially arise as an early event; which is then followed by 'autoimmune-like' inflammatory involvement as the condition progresses. Notably, in the recent excellent review of T1D, Rowe et al. [16] also challenge the justification and rigidity of the autoimmune disease requirement for T1D. This view has recently received a wider support in the field of autoimmunity [17,18]. A barrier to elucidation of this issue has been the absence of an 'appropriate' animal model for studying of initiating events in the development of T1D, particularly in the absence of the inflammation in T1D. The animal model that we describe here may provide these criteria.

Both *in vivo* and *in vitro* analyses on our model indicate that the off-balance in glucose-insulin homeostasis in our IH model appears not due to anatomical but to functional defects in pancreatic beta cells after IH stimulation. We measured the level of C-peptide rather than insulin to assess total proinsulin production. The decreased C-peptide secretion from the islets harvested from IH treated animals indicates a decrease of the total insulin secretion in

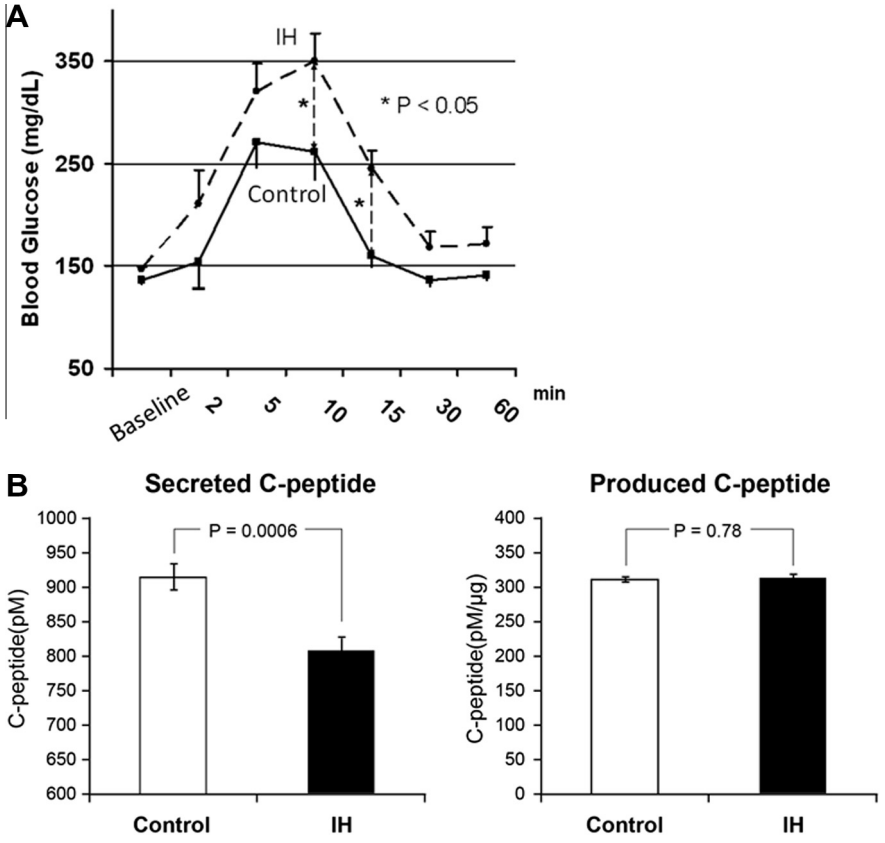

Fig. 4. Glucose tolerance tests (GTT) and secretion of C-peptide. (A) GTT results show a significant difference at 10 and 15 min points. At 30 min point, the glucose level returns to the baseline in control pups. (B) C-peptide concentrations measured from each medium significantly differed between control and IH treated groups, whereas the measurements obtained from cell lysates remain the same.

blood. However, no change in C-peptide concentration in cell lysates suggests no change in proinsulin production in the islets after IH treatment. Therefore, we could assume some impairment in cleaving or releasing process of insulin along the manufacturing system after IH challenge [19]. We also can conclude that this dysfunction is not likely associated with type 2 diabetes which is often characterized by hyperproinsulinemia [20]. Plausible underlying reasons could be damaged endoplasmic reticulum (ER) by reactive oxygen species (ROS) since depleting ER calcium due to stress leads to protein misfolding [21] or an elevated mitochondrial ROS which down regulates the conversion of proinsulin to insulin [22]. However, again, we did not observe any evidence of C-peptide increase which approximates proinsulin accumulation in beta cells in our short term study.

Animal studies reported on metabolic effects of spontaneous neonatal IH exposure are rare [23]. A recent study by McPherson et al. [24] showed that postnatal maternal separation may be a significant additive causal factor in developing metabolic syndrome in later age. Our animal protocol which successfully minimized potential stress in pups by accompanying their mothers throughout the experiment and acclimatizing them by spending 20–30 min with an experimenter daily for 3 weeks; thus, we were able to rule out, or at least minimize, any potential influence of stress from parental separation on glucose and insulin levels. We report this based on preliminary observations; therefore, we do not know whether the current form of diabetic onset belongs to T1D, T2D or double diabetes which is an emerging question of the field [25] or simply, a clinical expression of temporary mismatch between beta cell function and beta cell mass [26]. We do not know whether this dysfunction in glucose homeostasis would progress as the pups age because the dysfunction could be due to a remnant "toxicity" from IH stimulation. As discussed by many others [27], assuming diabetes onset based on beta cell mass is an ongoing controversial topic. In this report, we have described an animal model for the study of the implications of IH in the early development of diabetes. The model permits the identification of hypoinsulinemia and accompanying hyperglycemia in the absence of autoimmune-like inflammatory conditions that accompany the later progression of diabetes. We recognize that additional studies on the underlying mechanism including genetic tests are necessary to establish further the characteristics of this model.

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