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Effect of neonatal hypothyroidism on carbohydrate metabolism, insulin secretion, and pancreatic islets morphology of adult male offspring in rats

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ABSTRACT. ***Background:*** **Neonatal hypothyroidism has serious effects on growth, development, and metabolism.** ***Aim:*** **This study aims to investigate the effects of the neonatal hypothyroidism on carbohydrate metabolism, islet insulin secretion and morphology of the pancreatic islets in adult male offspring.** ***Materials/subjects and methods:*** **Lactating mothers of Wistar rats consumed 0.02% solution of 6-propyl-2-thiouracil during the weaning period (neonatal hypothyroid group), while mothers of the control group drank merely tap water. Body weight and survival of pups were followed up. Intravenous glucose tolerance test was performed in adult male offspring and 5-6 weeks later, glucose-stimulated insulin secretion (GSIS) was evaluated.** ***Results:*** **During the glucose tolerance test, plasma glucose level of the neonatal hypothyroid group (13.18±0.59 mmol/l) was significantly higher at 5 min compared to the control group (11.54±0.47 mmol/l), whereas plasma insulin concentrations and GSIS of the groups was not significantly different. Homeostasis model assessment of insulin resistance of adult male offspring of the hypothyroid group (9.1±1.0) was significantly higher as compared to the control group (4.5±0.6). Area (14,613.0±2646.3 μm^2) and the diameter of the islets (147±3.0 μm) of the neonatal hypothyroid group were significantly lower, as compared to the control group (32,886.3±4690.3 and 206.6±5.9 μm^2 and μm, respectively).** ***Conclusion:*** **Neonatal hypothyroidism can alter carbohydrate metabolism in euthyroid adult offspring, which may increase susceptibility to the development of glucose intolerance and occurrence of Type 2 diabetes later in life.**

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INTRODUCTION

Thyroid hormones (TH) modulate cellular signal transductions (1) and are critical for growth, development and metabolism throughout life (2, 3). Neonatal hypothyroidism affects energy metabolism, decreases glycerolipid synthesis in the liver and tampers with the glucose consumption of the neural tissue (1, 2). On the other hand, it has been shown that neonatal growth retardation causes irregular development of pancreatic tissue andleads to hyperglycemia in adult life (4).

In rats and humans, the fastest growth of β cells occurs in the later stages of the fetal life (5) and then goes through a period of apoptosis during early neonatal life, and proliferation resumes later on (5, 6). The cause for such sequence of apoptosis and proliferation of pancreatic islets is not exactly clear (7, 8), but it has been shown that IGF, as mediators of TH can considerably increase β cell growth in postnatal life (7, 9). Development of the key enzymes involved in carbohydrate metabolism including glucose-6-phosphotase, hexokinase, and glucokinase (GK) are regulated by TH during fetal and neonatal life (10). Throughout life, TH modulate glucose homeostasis by increasing intestinal glucose absorption, enhancing hepatic glycogenesis, altering oxidative and non-oxidative glucose metabolism, reducing dynamic insulin production, and increasing renal insulin clearance (11, 12). In the liver and skeletal muscle, glycolysis, gluconeogenesis and glycogenolysis are enhanced by these hormones (11, 13).

However, epidemiological and experimental studies show a strong relationship between early growth restriction, malnutrition during pre-natal, post-natal, and infancy and glucose intolerance, reduced glucose-stimulated insulin secretion (GSIS) and diabetes Type 2 in adulthood (14, 15), although the findings are controversial (16). We recently reported that the adult male rat offspring of hypothyroid pregnant rats show impaired glucose tolerance test, decreased GSIS and deteriorated morphology of their islets as well (17). Since TH are one of the most important factors in the development of tissues during neonatal life, in this study we aim to investigate the effect of neonatal hypothyroidism on carbohydrate metabolism, GSIS, and the morphology of the islets in their adult male rat offspring.

MATERIALS AND METHODS

Animals and diets

In-bred virgin female and male Wistar rats from the Research Institute for Endocrine Sciences were kept in Plexiglas cages with stainless coverings in standard conditions (22±4 C and a humidity of 25±8% with 12 h light/dark cycle) with free access to standard rat chow diet (Pars Co., Tehran) and water.

All procedures were performed in agreement with the accepted standards of the Ethics Committee of the Research Institute for Endocrine Sciences of the Shahid Beheshti University of Medical Sciences.

Key-words: Adulthood, carbohydrate metabolism, insulin secretion, langerhans islets morphology, neonatal hypothyroidism, rat.

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Induction of hypothyroidism in suckling rat offspring

To produce a sufficient number of adult male offspring for further experiments, after delivery, 42 lactating mothers and their weaning litters were randomly assigned to either the neonatal hypothyroid (NH) (no.=32) or the control (C) (no.=12) groups in separate cages. Mothers of the NH group consumed 0.02% 6-propyl-2-thiouracil (PTU) (gift from Iran-Hormone Co., Tehran) in the drinking water throughout lactation (18), whereas the mothers of the C group received only tap water. Weights of the litters were regularly recorded for 90 days. Blood samples of the neonates and their mothers were obtained on EDTA (0.005%) *via* tail cut at the end of the weaning time. Another blood sample was obtained from the neonates at the time of the glucose tolerance test.

Glucose and hormones measurements

Blood samples were centrifuged at 3000 × g for 10 min at 4 C and plasma was kept at –20 C for laboratory measurements. Plasma insulin was measured using the enzyme-linked immunosorbent assay (ELISA) method (Mercodia, Sweden), glucose was assessed by the glucose oxidase technique (Pars Azmoon Co., Iran), and T_3 and T_4 were evaluated by the ELISA method (PishtaztebZaman Co., Iran). Intra- and inter- assay coefficients of variations for insulin and glucose measurements were 5.4%, 9.3% and 3.2%, 9.3% and for T_4 and T_3, they were 4.5%, 5.7% and 3.2%, 4.8%, respectively.

Intravenous glucose tolerance test

After fasting for 14-16 h, anesthesia was induced in the adult (90 days old) male offspring (no.=12 for each group) by ip injection of pentobarbital (5 mg/100 g) and an anticoagulant coated catheter (24-G) was inserted into the femoral vein and artery in order to inject glucose solution and obtain arterial blood samples respectively. At the beginning (time zero), the first blood sample was obtained, then a 20% glucose solution (0.5 g/kg) was injected through the vein and blood samples (0.5 ml) were collected at 5, 10, 15, 20, 30, 60 min. To compensate for the blood removed, the same volume of normal saline was injected (17). At the end of the intravenous glucose tolerance test (ivGTT), the incised part was closed by stitching and the animals were returned to the facilities for later evaluation of the GSIS. Computation of the homeostasis model assessment of insulin resistance (HOMA-IR) index was performed by the formula; HOMA-IR = fasting insulin (μU/ml) × fasting glucose (mmol/l)/22.5 (19).

Isolated islets insulin secretion

In order to isolate the islets, the slightly modified method of Lacy and Kostianovsky (20) was used. In brief, about 5 to 6 weeks following recovery from ivGTT, the animals were anesthetized, the abdomen was exposed, a polyethylene catheter (20-G) was inserted into the common bile duct to inject 10 ml of cold Hank's balanced salt solution (HBSS) [pH,7.4; containing NaCl, 136; KCl, 5.36; $CaCl_2$, 1.26; $MgSO_4$ $7H_2O$, 0.8; Na_2HPO_4 $2H_2O$, 0.33; KH_2PO_4 0.44; $NaHCO_3$, 4.16 all mM (Merck, Germany) 0.5 mg/ml of collagens P (Roche, Cat.≠ 1213, Germany)] was injected. The extended pancreas was detached, transferred into a 50 ml falcon tube, and incubated in 37 C water bath for 17 min. After that, the cold HBSS the tube was shaken for 1 min, the suspension was conveyed into a glass container (7.5-cm diameter, 4.5-cm height placed on ice) through a 500-μm mesh. The yield mixture of islets and exocrine tissue was washed three times with cold HBSS and the islets were collected under a stereomicroscope by hand picking. Assessment of the sizes of the randomly picked islets was performed with a calibrated eye-piece graticule lens beneath a stereomicroscope (Steady, 400 X, England). To evaluate the insulin secretion of the isolated islets, four sets of eight islets from each animal were randomly picked and placed inside a plastic cup (5 animals in each group, overall 20 cupsfor each condition), and 1 ml of Krebs ringer solution [(pH,7.4); containing of mM: NaCl, 115; KCl, 5; $MgCl_2$ 6H2O, 1; $CaCl_2$, 2.5; $NaHCO_3$, 24 (Merck Germany); Hepes, 16 (Sigma, USA) all in mM], and BSA, 5 g/dl (Fluka, USA), with different glucose concentrations (5.6, 8.3,16.7 mM), was added into the cups and incubated for an hour in a 37 C water bath. The cups were gassed for 5 min with 95% O_2, and 5% CO_2 at the beginning of the incubation period. The supernatant from each cup was removed and stored at –20 C for later insulin measurements (17).

Pancreatic islet morphology

A number of the removed pancreata were placed in 10% formalin and the Gomorialdehyde-fuchsinstaining method was used to study the morphologyof the pancreatic islets under a light microscope (Leica, 400 X Germany), at least in 10 microscopic random fields (21). The area of the islets was calculated with Version 3.0 software of the UTHSCSA Image Tool (University of Texas Health Science Center, San Antonio, TX, USA).

Statistical analysis

To compare the findings, the independent t-test and one or two-way analysis of repeated measure were used. The survival of the litters was evaluated by analysis of Kaplan-Meier. Area under curve (AUC) was computed by Version 5 of the Graph Pad Prism software. All data are expressed as the mean±SEM and values below 0.05 were considered significant.

RESULTS

Plasma T_4 and T_3 levels

While plasma T_4 and T_3 levels were significantly lower in the NH mothers and neonates at the end of weaning period, in adult offspring of the NH group, hormone levels were not different from the adult offspring of the C

Table 1 - *Plasma T_3 and T_4 cocentration in the neonatal hypothyroid and control groups at weaning and adulthood and their mothers at the time of delivery.*

Hormones	Offspring				Mothers	
	End of weaning (no.=15)		Adulthood (no.=15)		End of weaning (no.=10)	
	C	NH	C	NH	C	NH
T_3 (nmol/l)	2.3±0.1	1.2±0.1[c]	2.7±0.3	2.5±0.3	2.7±0.4	1.2±0.1[a]
T_4 (nmol/l)	83.0±7.8	31.3±9.3[c]	84.8±5.4	77.1±8.6	81.7±4.8	56.7±6.5[b]

[a]$p<0.05$, [b]$p<0.01$, [c]$p<0.001$. Values are means±SE. C: control; NH: neonatal hypothyroid.

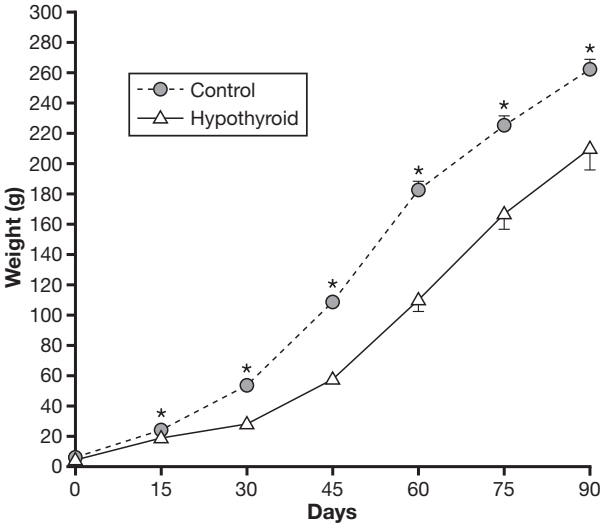

Fig. 1 - *Changes of body weight in the neonatal hypothyroid and control groups from delivery to 3 months. *p<0.01. Values are means±SE for 14-22 rats.*

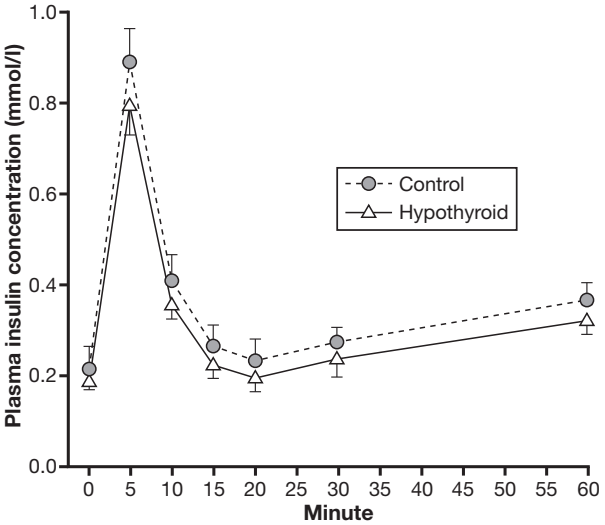

Fig. 3 - *Plasma insulin cocentration during intravenous glucose tolerance in the neonatal hypothyroid and control groups. Values are means±SE for 10 rats.*

group. Data for hormone measurements of NH group and C are expressed in Table 1.

Survival and weight gain of the animals

In the NH group, survival of the offspring from delivery to adulthood was 61.3%, significantly ($p<0.001$) lower than the C group (96%). In addition, weight gain of the offspring of the NH group throughout the recorded period was significantly ($p<0.001$) lower than that of the C group (Fig. 1).

Plasma glucose and insulin levels in ivGTT

During ivGTT assessment at different time points, mean plasma glucose levels of NH group (13.18±0.59 mmol/l) were significantly ($p<0.05$) higher than those of the C group (11.54±0.47 mmol/l) only at 5 min (Fig. 2). Plasma insulin levels of the NH group throughout the ivGTT and the AUC were not different from those found for the C group (Fig. 3). The HOMA-IR Index of offspring of the NH group at the time of adulthood (9.1±1.0) was significantly ($p<0.05$) higher compared to the C group (4.5±0.6).

Glucose-stimulated insulin secretion and morphology of the islets

At different glucose concentrations, the isolated islets of the NH group did not show significantly different GSIS patterns as compared to the C group (Fig. 4). Nevertheless

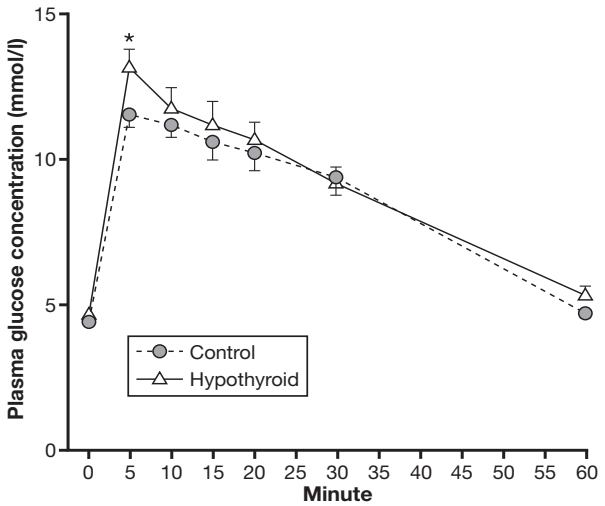

Fig. 2 - *Plasma glucose cocentration during intravenous glucose tolerance test in the neonatal hypothyroid and control groups. *p<0.05. Values are means±SE for 12 rats.*

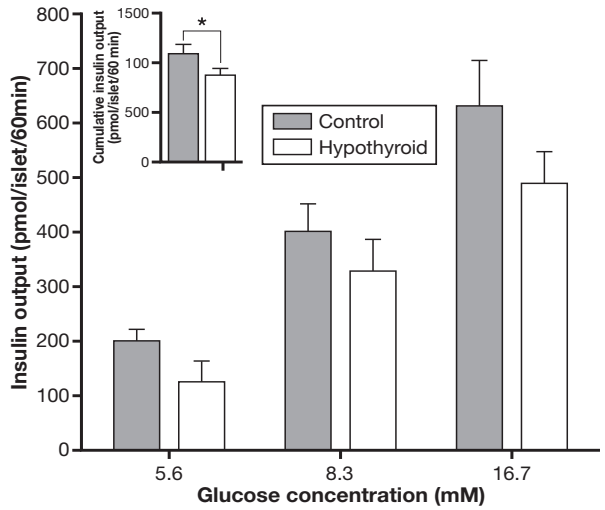

Fig. 4 - *Isolated islets glucose stimulated insulin secretion in the neonatal hypothyroid and control group; an inset shows the cumulative secretion of the islets. Values are means±SE for 20 measurements.*

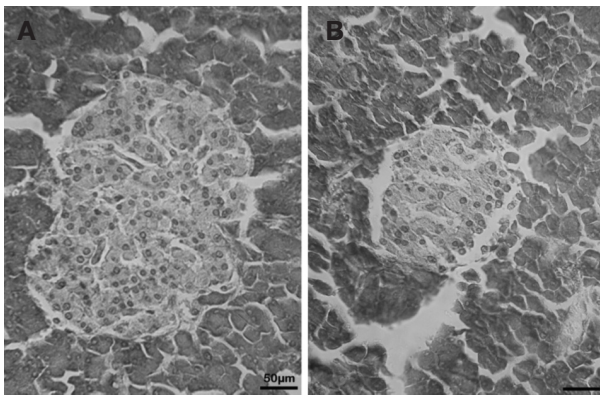

Fig. 5 - *Islets in the neonatal control (a) and hypothyroid (b) groups (Gomori staining, X 400).*

cumulative insulin secretion of the islets for three different glucose concentrations in the NH group (881.9±70.4 pmol/islet/60 min) was significantly ($p<0.05$) lower than the control group (1111.8±85.9 pmol/islet/60 min) (Inset, Fig. 4).

Islets of the NH group were significantly ($p<0.001$) smaller in size (147.0±3.07 µm) and had a lower number of cells (109.2±10.5), compared to the C group (206.6±5.9 µm) and (141.6±11.3), respectively. The area of the pancreatic islets of the NH group (14,613.0±2646.3 μm^2) was significantly lower than the C group (32,886.3±4690.3 μm^2) too (Fig. 5).

DISCUSSION

The results of the present study indicated that adult male offspring of hypothyroid lactating mothers showed relatively increased glucose intolerance, reduced insulin secretion capacity as well as reduction in size of pancreatic islets. We have previously shown that maternal hypothyroidism impairs carbohydrate metabolism and GSIS of the pancreatic islets of their adult male rat offspring and has deteriorating effect on pancreatic islets size (17). Considering the fact that B cell proliferation continues during certain periods of neonatal life (1), a result similar to our previous report (17) was expected, but it appears that the effect of TH on pancreatic islets development is more crucial during fetal life than the neonatal period. This study presents experimental data showing a relationship between neonatal hypothyroidism and carbohydrate metabolism during adult life which has not been tested before. In a recent report, Nader et al. have found that in euthyroid children, the increased TSH plasma levels was associated with IR, independent of age, sex, and adiposity (19). Although in this study the plasma level of TSH in adult male rat offspring was not measured, in a previous report from this laboratory it has been shown that the plasma TSH concentration in adult male offspring of the hypothyroid lactating rats was marginally high (22) and this increased TSH plasma level may explain the higher HOMA-IR index in these offspring.

Our results showed that the plasma glucose levels of ivGTT in offspring of the NH group were significantly higher at one time point (5 min) and this might be at least partly due to presence of IR considering the HOMA-IR index, since insulin secretion pattern is not different. Weight gain in the offspring of the NH group throughout the study period was lower as compared to the C group; similar to our finding, St-Pierre et al. have shown that the weight gain of the PTU (0.02%) treated rats (weaning period), during 90 days of follow-up, was significantly lower than the control group (18). Goldey et al. have shown similar results in hypothyroidism-induced rats during 6th day of gestation and throughout the weaning period (23). Neither in this study nor in the study by Goldey et al. the cause of retarded weight gain was explored; however, Fagundes et al. have suggested that lower weight gain in offspring of the lactating malnourished mothers is probably due to decrease in fat mass (24, 25). If the lesser weight gain of the animals in the neonatal hypothyroid offspring was due to lesser body fat mass, increased insulin sensitivity would be expected (26). Therefore, it appears that the observed IR must be due to a long-lasting effect of neonatal hypothyroidism on mechanisms, other than body weight retardation. González-Barrancoet al. have reported that independent of birth weight, adult men who have been malnourished during their first year of life, show a reduced glucose tolerance during oral GTT (27), a finding compatible with the results of our report. This suggests that TH deficiency during neonatal life can have a similar impact to malnourishment on carbohydrate metabolism. This effect too may be due the weight retardation, but this remains to be elucidated.

On the other hand, recent reports have shown that malnourishment of rats during pregnancy, lactation and even post-weaning, impairs glucose tolerance in adult male rat offspring, as a result of changing insulin sensitivity and secretion (28). Lopez-Soldado et al. have reported that in adult male and female rats offspring of malnourished lactating mothers, glucose tolerance is not altered, whereas GSIS is reduced (16). Berlezet al. have shown that a nutrition deficiency during post-natal life of female rat offspring increases both glucose tolerance and insulin sensitivity caused by increased glucose uptake by cells probably, through enhanced glucose transporter 4 in skeletal muscle fibers (29).

In this study, the GSIS of the islets from the NH group was less than the control group although the difference was not significant. When cumulative insulin secretion was compared, it appeared that a kind of reduced insulin secretion capacity was seen. This reduced insulin secretion is compatible with changes in the morphology of the islets. The alteration of the size of the islets might be due to hypothyroidism inducing an imbalance between apoptosis and regeneration of β cells (5). Considering the changes in the size of the islets in the NH group, alteration in the GSIS was expected, as we have reported in our previous study (17). Nevertheless changes in the morphology of the islets are much more remarkable in comparison to the reduction in the insulin secretion capacity. This might be explained by the fact that smaller islets secrete more insulin than large ones (30).

Latorraca et al. have shown that adult male rat offspring with growth retardation in fetal life, suckling and after

the weaning period show an identical GSIS in the presence of high glucose doses, but reveal a reduced GSIS in the presence of basal and low glucose doses (31). So far, no similar study has been reported to compare the findings; however, Heywood et al. have shown that the adult male offspring of growth-retarded rats during fetal-weaning period show an increased GSIS in the presence of different glucose doses, similar k_m activity of hexokinase and decreased k_m activity of GK, as compared to the control group (32). On the other hand, Taguchi et al. have found that in the hypothyroid growth-retarded mice, insulin granules, glucose transporter 2 (GLUT-2), GK mRNA and ATP- sensitive k^+ channels in the β cells of adults are normal, although their GSIS is reduced (33). It is interesting to note that in the study by Taguchi et al, T_3 replacement led to complete recovery of glucose tolerance but only slightly improved the insulin secretion capacity. Therefore considering the findings of Taguchi et al. it is possible to presume that in this study the observed abnormalities are due to long-lasting effects of neonatal hypothyroidism. It must be pointed out that, in this study, the growth retardation was due to hypothyroidism at the time of weaning and the animals were not hypothyroid at the time of assessments. Therefore, a long-lasting effect of hypothyroidism was observed as growth retardation as well as in the reduced size of the islets. Whether changes in the carbohydrate metabolism as well as in the insulin secretion capacity are due to growth retardation or direct impact on insulin secretion mechanisms is currently under study.

In summary, neonatal hypothyroidism retards growth and decreases the size of the islets which are associated with a long-lasting effect on carbohydrate metabolism and reduced insulin secretion capacity, effects which may increase the susceptibility for development of Type 2 diabetes later in adult life. Of course this does not rule out the possible changes in the IR and insulin secretion mechanisms those remain to be investigated.

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