Maternal Marginal Iodine Deficiency Affects the Expression of Relative Proteins during Brain Development in Rat Offspring

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\textbf{Running title}: effects of Marginal Iodine Deficiency on offspring

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Abstract

Marginal iodine deficiency is a major health problem in pregnant women, but its impact on nerve and intelligence development in offspring has been rarely reported. Our study aimed to investigate the effects of maternal marginal iodine deficiency on nerve and cognitive development in offspring and the related mechanisms. Marginal iodine deficient rats were given 3 µg of iodine per day, while normal control rats were given 4 µg of iodine daily. Western blot was used to detect the amounts of BDNF (Brain-derived neurotrophic factor) and EGR1 (Early Growth Response Protein 1) in the hippocampus of each group. Immunohistochemistry was used to measure c-jun and c-fos expression in the hippocampal CA1 region. Finally, the water maze method was used to measure spatial performance. Free T4 (FT4) levels in marginal iodine deficient rats decreased by about 30%. Seven days after birth, EGR1 and BDNF protein levels significantly decreased in the hippocampus of marginal iodine deficiency rats compared with the normal control group. In addition, c-jun and c-fos expression in the hippocampus of 40-day-old rats was decreased in marginal iodine deficient rats, as compared to control. The spatial learning and memory ability of 40-day-old marginal iodine deficient rats had a downward trend compared with the normal control group. FT4 significantly decreased after pregnancy in rats with marginal iodine deficiency, affecting the expression of related proteins in the brain of offspring.

Key words: pregnancy, marginal iodine deficiency, offspring, brain development, protein
Introduction

It is well known that iodine is a trace element essential for the synthesis of triiodothyronine (T3) and thyroxine (T4) which play a crucial role in the process of early growth and development of most organs, especially the brain (Sethi & Kapil 2004) through genomic and nongenomic actions in neurons (Bernal 2005; Davis et al. 2008). During gestation, the mother is the only source of T4 before midgestation and the major source afterwards for the developing brain since the fetal thyroid gland is not functional and is unable to produce TH until the second trimester (Pop 2003). So maternal inadequate intake of iodine leads to insufficient production of thyroid hormones which is potentially damaging for neurodevelopment of the fetus throughout pregnancy (Morreale et al. 2004) and results in permanent and profound effects on neurological functions that contribute to cognitive and neurological impairments (Bernal J 2003; Tremont G 2003). Hippocampus is a brain region involved in cognitive skills such as learning and memory (Gerges & Alkadhi 2004) and the CA1 area in hippocampus is the most important region for spatial learning (Huang et al. 1995) where memory is encoded, consolidated and stored (Vara et al. 2003). The maturation and function of hippocampus are dependent upon thyroid hormone (Gerges & Alkadhi 2004). Congenital hypothyroidism can reduce the expression of many proteins in the hippocampus of neonatal rats (Li et al. 2010; Dong 2005) and affect its function.

In 1996, China implemented the Universal Salt Iodization (USI) regulation.
Since, the iodine nutrition condition of Chinese people has been at an appropriate or excessive level. Marginal iodine deficiency is a major health problem for pregnant women. According to the evaluation criteria set for pregnant women by the World Health Organization (WHO) in 2007, marginal iodine deficiency is defined as a urinary iodine concentration between 100 µg/L and 150 µg/L (WHO/UNICEF/ICC 2007). From our survey in Shenyang, almost half of the pregnant women were in a state of marginal iodine deficiency (Shi et al. 2009), similar to survey results from other areas (Zhang et al. 2005; Li et al. 2004). It has been shown that the cognitive performance of offspring is severely affected by moderate to severe iodine deficiency in pregnant women. However, studies investigating the impacts of marginal iodine deficiency in pregnant women on nerve and cognitive performance of their offspring have been rarely reported. According to the characteristics of marginal iodine deficiency obtained from epidemiological surveys, we established an animal model of marginal iodine deficiency in Wistar rats that were relatively sensitive to iodine. With this established rat model, we investigated the influences and mechanisms of marginal iodine deficiency on nerve and cognitive performance in offspring.

Subjects and Methods

Experimental animals and grouping
For the first part, 80 female Specific-Pathogen-Free (SPF) Wistar rats that had been weaned for 1 month and weighed 100-110g were purchased from Beijing Vital River Experimental Animal Technology LLC. After adaptive feeding for 1 week, the rats were assigned to one of the five treatment groups and counterbalanced for weight (mean weight, 107 g). The groups were divided as follows: (1) low iodine group (L group, n = 20); (2) marginal iodine deficiency group 1 (2 ug of iodine, n = 10); (3) marginal iodine deficiency group 2 (3 ug of iodine, n = 20); (4) normal control group (N group, n = 20); and (5) normal control group 1 (N1 group, n = 10). Rats were fed for 3 months and then mated with normal male rats with a female-to-male ratio of 2:1. And after pregnancy the above five group were named LP, 2ugP, 3ugP, NP, N1P group respectively.

Based on the findings of first part, for the second part, after adaptive feeding for 1 week, 90 female SPF Wistar rats were divided into three groups (n = 30 for each group) and counterbalanced for weight as follows: (1) low iodine group (L group); (2) marginal iodine deficiency group (M group); and (3) normal control group (N group). Rats were raised and mated according to the methods used in the first experiment and the offspring were fed the same diet as their mothers.

**Feeding**

The L and N1 groups drank deionized water, while the marginal iodine deficiency and N groups drank deionized water with dissolved potassium iodate of different concentrations. The low-iodine chow was made by mixing low iodine foods (corns,
soybeans and millets) supplemented with inorganic salts and trace elements necessary for animal survival. The iodine content in the chow was 60 µg/kg, as measured by the Shenyang Academy of Agricultural Sciences. The N1 group was fed a normal rat diet (Institute of Endocrinology, China Medical University, Shenyang, China), but the food composition and the proportions were the same as the low-iodine diet. The average iodine content in the normal diet was 200 µg/kg. According to the daily food-intake (20 mg) and daily water-intake (30 ml) for rats, the daily iodine-intakes were 1.2 µg/d (L group), 2 µg/d (2 ug group), 3 µg/d (3 ug group), 4 µg/d (N group) and 4 µg/d (N1 group), respectively.

Collection and measurement of urinary iodine

Chemicals were purchased from John Chem Co.Ltd Linyi China for the measurement of urinary iodine excretion using the ammonium persulfate -arsenic cerium catalytic spectrophotometry (WHO/UNICEF/ICC 2007). Urine was collected with metabolic cages every 3 days after 2 months of feeding.

Observation of fertilization and procreation conditions in pregnant rats

In the next morning after mating, the vaginal smears of the female rats were examined. A positive record of pessus and vaginal smears were defined as embryonic day 0 (E0). The pregnancy reaction, fertilization condition and stillbirth condition were observed on a daily basis. Also, the total number and death number were observed and recorded. The following calculations were used for each group: Pregnancy rate = number of
pregnant rats / number of female rats × 100%; Mortality of pregnant rats = death number of pregnant rats / total number of pregnant rats × 100%; and newborn rat mortality = total death number of newborn rats / total number of newborn rats.

**Hormone determination**

We used a solid phase chemiluminescence enzyme immunoassay to measure thyroid-stimulating hormone (TSH), total thyroxine (TT4), free thyroxine (FT4), total triiodothyronine (TT3) free, and triiodothyronine (FT3) (Diagnostic Products Corporation, Los Angeles, CA, USA). The functional sensitivity of the TSH assay was 0.02 mIU/l. The intra-assay coefficients of variation (CV) of serum for TSH, TT4, FT4, TT3 and FT3 were 1.57-4.12%, 1.26-3.20%, 2.24-6.33%, 2.42-5.63% and 0.57-4.31%, respectively. The inter-assay CV values were 1.26-5.76%, 3.58-6.67%, 4.53-8.23%, 3.50-5.19% and 5.23-8.16%, respectively.

**Measurement of hormone and iodine content in the thyroid**

Thyroid tissues were precisely weighed, sheared into pieces, added in cold homogenate and prepared into tissue homogenate, which was then centrifuged at 1000 rpm for 15 min. Supernatant was removed and diluted into 3 ml/mg for measurement. The hormone was measured using solid phase chemiluminescence enzyme immunoassay and iodine content in the thyroid was measured with the ammonium persulfate-arsenic cerium catalytic spectrophotometry (WHO/UNICEF/ICC 2007).

**Immunohistochemistry (IHC)**
Rat brains were fixed in 4% paraformaldehyde and embedded in paraffin. Coronal sections (5 µm) were collected on Super Frost glass slides (Hehyglass, Haimen, China). All sections were deparaffinized, rehydrated, and then treated for endogenous peroxidase with 3% H2O2 for 10 min. After washing with phosphate buffered saline (PBS), all sections were incubated with a rabbit anti-c-fos or anti-c-jun antibody (Santa Cruz Biotechnology, USA, 1:100 dilution) at 37°C for 30 min. Sections were then washed three times in PBS followed by additional incubation with biotinylated secondary antibodies (Maixin, Fuzhou, China) for 30 min at 37°C. Then, the sections were incubated for 10 min in avidin–biotin–peroxidase (Maixin) and washed three times in PBS. Sections were then reacted with a solution of 3,3′-diaminobenzidine (DAB, Sigma, USA). Finally, the sections were counterstained with Mayer’s hematoxylin, rinsed, and mounted in neutral gum (China National Medicines, Shanghai, China).

**Image analysis**

The Microscopic Image Analysis System (MetaMorph/DP10/Bx41/ UIC/OLYMPUS, US/JP) was used to analyze the c-fos and c-jun immunoreaction products in the bilateral dorsal hippocampal CA1 region (400× magnification).

**Western blotting**

On post-natal day 7 (PND7), pups in each group were deeply anesthetized and euthanized by ether. The brain was removed and hippocampus dissected on ice rapidly,
frozen in liquid nitrogen, and stored at -70 until use.

Hippocampi dissected from pups at post-natal day 7 (PND7) were homogenized in ice-cold solubilizing buffer containing protease inhibitors. Homogenate was centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant was collected for protein concentration measurement. Samples (50 ug for Egr1 and 60 ug for BDNF) were separated by SDS-PAGE, and then transferred onto PDVF membranes (Millipore, USA) by electrophoresis. Blots were blocked for 2 hours at room temperature with 5% BSA, and then incubated overnight at 4°C with antibodies to Egr1 (1: Signal, Danvers, USA) and BDNF (1:1000 Millipore, Billerica, USA). Blots were then incubated with horseradish-peroxidase-conjugated secondary antibodies (Zhongshan Goldenbridge, Beijing, China). Finally, the blots were visualized with an enhanced chemiluminescence Western blot detection system (Alphaview1.3, USA).

Morris water maze

The Morris Water maze experiments were conducted 40-day-old rats. On the first day, the rats were put into a water maze for 120 seconds to swim freely and adapt to the environment of the water maze. On the second day, a platform was set randomly in the middle of one quadrant. The rats were trained with two procedures per day – one in the morning and one in the afternoon. In each procedure, four separate training times were performed. In the first procedure, one quadrant was chosen. The rats were placed in the water at the center of the pool wall and the procedure was repeated in the other three quadrants. A computer was used to monitor and record the path, time
(Escape Latency, EL), swimming length, swimming speed, and the strategy used to locate the transparent platform. The procedure was immediately stopped if a rat found the platform within 120 seconds. Rats were allowed to stay on the platform for 10 sec for memory strengthening. If the rats did not find the platform within 120 seconds, then the escape latency was recorded as 120 seconds. At this point, the researchers subsequently guided the rats to the platform and allowed them stay on the platform for 10 sec before removing the rats from the platform. After resting for 30-60 sec, the rats proceeded to the next round of training. The interval of every training session was 10 sec. The test environment and position stayed the same during training. The rats were trained eight times per day.

Statistical analysis

Data were analyzed using SPSS 17.0 software (Chicago, IL, USA) and expressed as mean ±SD. A two-sample t-test was used to compare between two groups, and ANOVA was used for multi-group comparisons. A p < 0.05 was considered statistically significant.

Results

Body weight, thyroid weight to body weight ratio, and urinary iodine level

After being fed for 3 months, the average weight of the 2 ug iodine group was 270.60±20.72 g (p < 0.05 vs. N group), which did not meet the standards (Mun et al.
2006; Glinoer et al. 1995) for marginal iodine deficiency, and thus the following experiments were discontinued for the 2 ug group. The average body weight of the L group was significantly decreased compared with the other three groups (p<0.05), but the average body weight between the 3 ug iodine, N and N1 groups were similar (p>0.05). The thyroid weight to body weight ratio increased significantly in the L group (p<0.05) than the 3ug ,N and N2 groups, while there was no difference among the 3ug, N and N2 groups . (p>0.05). The urinary iodine value in the L group (63.50±13.16 ug/L) and the 3 ug group (107.00±24.40 ug/L) was significantly lower than the other two groups (p<0.01 and p<0.05, respectively), while the urinary iodine value in the N group (202.75±31.60 ug/L) and the N1 group (210.30±23.19 ug/L) did not change (p>0.05).

The thyroid function of rats before pregnancy and on E17 (Table1)

All indexes of thyroid function between the N and N1 groups were similar before pregnancy (p>0.05; Table 1). Serum TSH levels in the L group were significantly higher than the other groups (p<0.01), and no obvious difference was observed among the other groups (p>0.05). After pregnancy (E17: 17 days after their pregnancy), the TSH levels had a downward trend in all the groups. In addition, on E17, FT4 had a downward trend in all the groups, and FT4 in the L group was significantly lower than the 3 ug and N groups (p<0.05 and p<0.01, respectively) after pregnancy for 17 days. The FT4 of 3 ug group decreased by 30%, while FT4 in the N group only decreased by 4%. TT4 in the L group was significantly lower when
compared to the N group (p<0.01), while TT4 in the 3 ug group was decreased as compared to the N group, but not statistically significant. FT3 was not significantly different among all groups, but the 3 ug group had slightly higher FT3 levels than the N group and the increased trend continued after pregnancy. TT3 decreased after pregnancy in all the groups. TT3/TT4 and FT3/FT4 levels in the L group were significantly lower than the low iodine pregnancy group (LP) (p<0.05), 3ug and N groups. No obvious differences were observed among the other groups. TT3/TT4 and FT3/FT4 levels in the 3 ug and the marginal iodine deficiency pregnancy (3ugP) groups were slightly higher than the N and normal iodine pregnancy (NP) groups, but the differences were not statistically significant (p>0.05).

**T3/T4 and iodine content in thyroid (Table 2)**

In the L group, the levels of T3 and T4 were too low to be measured. In addition, the iodine content in the thyroid of the L group was significantly lower than that in the other groups (p<0.05), and there was no obvious difference among other groups (p>0.05). T3/T4 levels in the 3ug group were slightly higher than the N and N1 groups, but were not statistically significant (p>0.05). At E17, the iodine content in the thyroids of the 3ugP and NP groups was lower than in the non-pregnancy groups. There was no significant difference between the above two groups (p>0.05).

**State of pregnancy, delivery and thyroid function of rat offspring on P7 (Table 3)**

Fertilization rate, mortality of pregnant rats and mortality of newborn rats in the low
iodine group (1.2ug iodine intake daily) were higher than those in the 3 ug and N groups (p<0.05), and the number of newborn rats in the low iodine group was less than the other two groups (p<0.05). No difference was observed between the 3 ug and N groups. At P7, serum TSH levels in the offspring of the low iodine group were significantly higher than that of the normal control group (p < 0.05). Serum TT4 and FT4 levels of the low iodine group were also significantly lower than the N group. A significant difference was not observed between the 3 ug and N groups (p > 0.05).

*Index analysis of Morris water maze behaviors of 40-day-old rats (Table 4)*

On the first day of adaptive training, the time to find the platform was long and a difference in the average escape latency among the three groups was not obvious. On the second day, rats in all groups adapted to the pool environment and most of the rats actively searched for the platform. The average escape latency of all groups continuously decreased, but the rate differed among groups, indicating that rats in each group had partial memory loss between training sessions. ANOVA indicated that a difference was not observed in escape latency between the N and M groups, while the escape latency of the N and M groups was significantly shorter than L group. The results of the third day were similar to the second day. On the fourth day, the escape latency in the N group was still significantly shorter than the L group. In addition, the escape latency of the M group was longer than N group, but was not statistically significant. The results of the fifth day were similar to the fourth day.
The expression of c-fos and c-jun proteins (Fig. 1)

As observed from the results of IHC, the expression levels of c-fos and c-jun in the M group were significantly lower than that in the N group (p < 0.05). However, when compared with the low iodine group, c-fos and c-jun expression in the M group was higher, but not statistically significant.

Hippocampus Egr1 and BDNF protein expression in PND7 rats (Fig. 2)

Egr1 and BDNF protein expression was measured by Western blot. The results showed that the L and M groups had significantly lower Egr1 expression than the N group (p < 0.05). Similarly, the BDNF protein expression in the L and M groups were significantly lower than the N group (p<0.05).

Discussion

The main focus of previous studies regarding iodine deficiency was to establish severe iodine deficiency models and investigate the impact of iodine deficiency on offspring development. Our study established a marginal iodine deficiency model in rats based on the following principles: in the normal physiological status, there were no symptoms of iodine deficiency, but during pregnancy the requirement for iodine increased, resulting in iodine deficiency. This study also investigated the changes in iodine metabolism and thyroid function, as well as the influences of maternal marginal iodine deficiency on offspring neural intelligence development and the
related molecular mechanisms.

The marginal iodine deficiency in our study was very mild, and therefore before pregnancy, the thyroid was auto-regulated and had no functional changes. However, after pregnancy, the iodine requirement increased significantly, which resulted in over stimulation of the thyroid and changes in thyroid function. We found that to synthesize 100 g of thyroid hormone, which is physiologically required by a Wistar rat, 0.68 µg of iodine was needed. The average body weight of our experimental rats was less than 300 g, so a daily iodine-intake of 2 µg only met the physiological requirements (Escobar-Morreale et al. 1996). According to a literature report (Heninger & Albright 1975), when the daily iodine-intake of a Wistar rat was 3 µg, the thyroxine concentration reached most of the tissues. Furthermore, if the daily iodine-intake of a Wistar rat was 4 µg, all the physiological requirements were satisfied. According to the ANI93 standard of animal diets, the iodine content of the diet used in our study was 200 µg/kg. Assuming the daily food-intake was 20 g, the daily iodine intake requirement was 4 µg.

In our study, we chose daily iodine-intakes of 2 µg and 3 µg as the marginal iodine deficiency. We evaluated the model according to the results of epidemiology surve. The epidemiological characteristics of people in marginal iodine deficient areas are prevalent before and after pregnancy: Before pregnancy, the auto-regulatory mechanism of the thyroid is active and serum TSH does not increase. Serum FT4 has a downward trend and T3 has an upward trend. Additionally, the urinary iodine is lower than the normal, but has no influences on normal growth. After pregnancy,
obvious symptoms of thyroid over-stimulation appear, and TSH increases within the normal range, the iodine content of the thyroid decreases, the T3/T4 value further increases, and the number of newborn rats and their survival rate stays the same (Glinoer 1997; Mun et al. 2006; Glinoer et al. 1995; Pedraza et al. 2006; Lavado-Autric et al. 2003; Morreale de et al. 2003).

In our study, growth, thyroid function, iodine content in the thyroid and urinary iodine showed no difference between the N group (iodine-intake was 4µg/d) and the N1 group (normal diet), indicating that low iodine foods with water containing iodine have no deficiency of nutritive elements and can be used as an alternative for a normal diet. After 3 months of feeding, the body weight of rats in the 2 ug group (iodine-intake was 2 µg/d) was significantly lower than the N group, suggesting that the iodine-intake affected the growth of rats, and thus did not meet our standard for marginal iodine deficiency. Before pregnancy, the urinary iodine of rats in the 3 ug group (iodine-intake was 3 µg/d) was significantly lower than the normal control groups (N group and N1 group), indicating a lower iodine excretion rate and iodine intake. However, the iodine content in the thyroid, hormone content in the thyroid, thyroid function, pregnancy status and the procreation conditions were not different between these two groups, indicating that the rats in the 3 ug group were not iodine deficient but only marginally deficient.

After pregnancy, the iodine content in the thyroid of the 3 ug iodine and N groups decreased, but the rate of decrease was similar. Before and after pregnancy, the T3/T4 value of the 3 ug iodine group was slightly higher than the N group and the rate
of increase after pregnancy was slightly higher than the N group. After pregnancy, the FT4 levels of the 3 ug group decreased by about 30%, while the value in the N group only decreased by 4%, which was similar to the result of the epidemiological survey (Pedraza et al. 2006). In the survey, the decrease of FT4 did not exceed 15% after pregnancy in subjects with sufficient iodine. In our study, the 3 ug group met the requirements for marginal iodine deficiency.

The hippocampus is the part of the brain that is related to cognitive ability, including learning and memory ability (Gerges & Alkadhi 2004). The CA1 area in the hippocampus is important for spatial learning. The memories are coded and incorporated, and then stored plastically by synapse. The hippocampus is also a sensitive area regulated by the thyroid hormone. The abnormal expression of related proteins in the hippocampus affects brain intelligence. In this study, although there was no difference in the water maze test between the M and N groups, there was increased escape latency in the M group, indicating impairment in learning and memory abilities.

BDNF expression can be directly regulated by thyroid hormone (Fujinoto et al. 2004). Some studies have shown that thyroid hormone deficiency results in a significant decrease of BDNF content in the brain of newborn rats (Lasley 2011). However, if the rats are supplied with thyroid hormone, the BDNF content will increase rapidly (Camboni et al. 2003). BDNF receptor binding with TrkB activates intracellular signal transduction. MARK plays an important role in the differentiation and migration of nerve cells, the reconstruction of synaptic plasticity and the
consolidation of learning and memory. Furthermore, MARK induces hippocampus granular cells to produce continuous LTP (Kobayashi et al. 2006; Koibuchi et al. 1999), which is currently recognized as the neural basis for learning and memory. The influences of BDNF on learning and memory are mainly due to the regulatory effects of BDNF on LTP (Jia et al. 2010; Messaoudi et al. 2002). In our study, BDNF expression in the PND7 newborn rats in the M group was significantly lower than the N group, which suggests that the relative thyroid hormone deficiency in female rats with marginal iodine deficiency has a negative impact on BDNF and can further influence the brain development of offspring.

Egr1 belongs to the early growth reactive protein family, and is also referred to as krox24 or Zif268. The expression of Egr1 is mainly regulated by thyroid hormone through TRE. Egr1 is related to the connectivity of regulatory nerves and the adaptability of synapses. Thyroid hormone deficiency can result in the decrease of Egr1 content in the cortex and hippocampus of the central nervous system (Kobayashi 2009). Synaptic activation can induce the generation of Egr1 and regulate the synaptic plasticity related to learning and memory, which is critical for the maintenance of late LTP. The formation of long-term memory depends on the hippocampus and the consolidation of early constructed memory. During the process of LTP induction by the hippocampus dentate gyrus, the transcription of EGR1 is quick and robust. Studies have shown that the expression of EGR1 is a critical genetic mechanism for the maintenance of late LTP and the stability of long-term memory (Ranieri 2012). In our study, the Egr1 expression of PND7 newborn rats in the low iodine group and the
marginal iodine deficiency group were significantly lower than the normal control group, indicating that marginal iodine deficiency in female pregnant rats has a great influence on Egr1 expression of offspring, which in turn affects learning and memory ability.

Immediate early genes (IEGs) can couple the short-term function of the extracellular signal and the long-term change in cell function, suggesting that IEGs are tightly related to learning and memory regulation (Herdegen 1997; Guzowski 2002). *c-fos* and *c-jun* are the most important characteristic genes in the IEG families. Additionally, if cells have been stimulated by a series of physicochemical and biological factors, *c-fos* and *c-jun* can be rapidly expressed and encoded to generate the Fos and Jun proteins, which regulate neuronal plasticity. Neuronal plasticity is considered to play an important role in memory formation, especially long-term memory formation (French et al. 2001). In our study, the expression of *c-fos* and *c-jun* in the hippocampus CA1 area decreased significantly in the marginal iodine deficiency group. Immunoreaction products were mainly distributed in the cytomembrane and cytoplasm. In the nucleus, the expression of *c-fos* and *c-jun* was significantly lower than the N group, but higher than the low iodine group, suggesting that the changes in *c-fos* and *c-jun* expression and their distribution during marginal iodine deficient conditions play a role in the learning and memory abilities of newborn rats.

Expression of C-fos and c-jun protein in the M group was significantly lower than that of normal control group, but higher than that of low iodine group. The above
result indicates that in MWM the learning and memory capacity of the M group was on a downward trend compared to the normal control group, while it was still different from the low-iodine group which has significantly decreased learning and memory capacity.

Acknowledgments

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Figure legends:

**Figure 1.** Immunohistochemistry pictures of c-fos and c-jun in newborn rat hippocampi in each group (P40, ×400). A) represents c-fos and B) represents c-jun. C and D) show the IHC IOD values of c-fos and c-jun, respectively, in newborn rat hippocampi in each group on P40. *compared with N group, P<0.05.

**Figure 2.** Western blot of post-natal day 7 rat hippocampi of Egr1 and BDNF protein. A) is Egr1 and B) is BDNF. C and D) show Western blot grey values for EGR1 and BDNF, respectively. *compared with normal control group (N), p < 0.05.
Table 1. The levels of various hormones reflecting the thyroid function of rats before pregnancy and on E17 (mean ± SE)

<table>
<thead>
<tr>
<th>Group n</th>
<th>TT4</th>
<th>FT4</th>
<th>TT3</th>
<th>FT3</th>
<th>TSH</th>
<th>FT3/FT4</th>
<th>TT3/TT4</th>
</tr>
</thead>
<tbody>
<tr>
<td>L 7</td>
<td>2.41±0.70*</td>
<td>18.82±4.45*</td>
<td>89.34±5.94</td>
<td>5.40±0.33</td>
<td>0.92±0.29*</td>
<td>0.32±0.063</td>
<td>0.04±0.012</td>
</tr>
<tr>
<td>LP 6</td>
<td>1.56±0.55*</td>
<td>9.28±0.12*</td>
<td>72.35±5.96</td>
<td>4.71±0.87</td>
<td>0.23±0.03*</td>
<td>0.53±0.08</td>
<td>0.075±0.011</td>
</tr>
<tr>
<td>3ug 7</td>
<td>4.35±0.60</td>
<td>31.03±4.17</td>
<td>83.40±10.48</td>
<td>5.67±0.35</td>
<td>0.039±0.04</td>
<td>0.215±0.043</td>
<td>0.0210±0.003</td>
</tr>
<tr>
<td>3ugP 7</td>
<td>2.50±0.25</td>
<td>22.46±1.51</td>
<td>64.36±5.24</td>
<td>5.02±0.55</td>
<td>0.016±0.001</td>
<td>0.228±0.016</td>
<td>0.0284±0.004</td>
</tr>
<tr>
<td>N 7</td>
<td>4.59±0.33</td>
<td>27.28±2.77</td>
<td>90.82±8.91</td>
<td>5.30±0.35</td>
<td>0.032±0.005</td>
<td>0.206±0.023</td>
<td>0.0203±0.002</td>
</tr>
<tr>
<td>NP 7</td>
<td>3.05±0.53</td>
<td>27.12±3.56</td>
<td>59.17±5.88</td>
<td>4.43±0.22</td>
<td>0.017±0.03</td>
<td>0.213±0.024</td>
<td>0.0271±0.007</td>
</tr>
<tr>
<td>N1 7</td>
<td>4.61±0.27</td>
<td>23.07±1.86</td>
<td>91.00±7.67</td>
<td>5.41±0.43</td>
<td>0.033±0.003</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The P labeling represents the results for the groups in their 17 days pregnancy period. TSH (Thyroid Stimulating Hormone): mIU/L; TT4 (Total Thyroxine): µg/dL; TT3 (Total Triiodothyronine): ng/dl; FT3 (Free Triiodothyronine), FT4 (Free Thyroxine): pmol/l, SE(standard error of the mean) *Compared with N group, p<0.05.
### Table 2. T3/T4 and iodine content in thyroid (mean ± SE)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>T3/T4</th>
<th>Iodine content in thyroid (µg/100mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L</td>
<td>7</td>
<td>34.97±9.15</td>
<td>178.21±10.10</td>
</tr>
<tr>
<td>N</td>
<td>7</td>
<td>32.63±6.49</td>
<td>180.86±13.24</td>
</tr>
<tr>
<td>N1</td>
<td>7</td>
<td>32.94±5.45</td>
<td>178.00±15.07</td>
</tr>
<tr>
<td>3ugP</td>
<td>7</td>
<td>169.49±18.09</td>
<td>169.49±18.09</td>
</tr>
<tr>
<td>NP</td>
<td>7</td>
<td>169.13±12.81</td>
<td>169.13±12.81</td>
</tr>
</tbody>
</table>

T3: triiodothyronine, T4: thyroxine, SE (standard error of the mean)

*compared with N group, P<0.05.

3ugP and NP groups measured on rats from M group and N group 17 days after their pregnancy.
Table 3. Thyroid function of PND7 rats (mean ± SE)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>TT4 (µg/dL)</th>
<th>FT4 (pmol/l)</th>
<th>TSH (mIU/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L</td>
<td>12</td>
<td>1.12±0.10*</td>
<td>6.62±0.45*</td>
<td>0.138±0.02*</td>
</tr>
<tr>
<td>M</td>
<td>12</td>
<td>2.45±0.38</td>
<td>10.22±0.58</td>
<td>0.077±0.048</td>
</tr>
<tr>
<td>N</td>
<td>12</td>
<td>2.44±0.07</td>
<td>10.47±0.60</td>
<td>0.089±0.004</td>
</tr>
</tbody>
</table>

*compared with N group, p<0.05.
Table 4. Escape latency (seconds) in the Morris water maze experiment (mean±SE)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Second day</th>
<th>Third day</th>
<th>Fourth day</th>
<th>Fifth day</th>
</tr>
</thead>
<tbody>
<tr>
<td>L</td>
<td>8</td>
<td>86.688±8.02*#</td>
<td>64.566±6.41*#</td>
<td>27.22±5.06*</td>
<td>28.725±4.65*</td>
</tr>
<tr>
<td>M</td>
<td>8</td>
<td>59.038±7.86</td>
<td>37.656±7.31</td>
<td>20.806±5.13</td>
<td>19.819±3.41</td>
</tr>
<tr>
<td>N</td>
<td>8</td>
<td>56.728±7.19</td>
<td>37.434±6.81</td>
<td>12.656±2.03</td>
<td>10.970±1.44</td>
</tr>
</tbody>
</table>

*compared with N group, P<0.05, #compared with M group, P<0.05.
Figure 1. Immunohistochemistry pictures of c-fos and c-jun in newborn rat hippocampi in each group (P40, ×400). A) represents c-fos and B) represents c-jun. C and D) show the IHC IOD values of c-fos and c-jun, respectively, in newborn rat hippocampi in each group on P40. *compared with N group, P<0.05.
Figure 2. Western blot of post-natal day 7 rat hippocampi of Egr1 and BDNF protein. A) is Egr1 and B) is BDNF. C and D) show Western blot grey values for EGR1 and BDNF, respectively. *compared with normal control group (N), p < 0.05.