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EFFECTS OF MATERNAL SUBCLINICAL HYPOTHYROIDISM ON NEURODEVELOPMENT OF OFFSPRING- AN ANIMAL-BASED STUDY

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ABSTRACT

Background and objective:

Maternal subclinical hypothyroidism (SCH) during pregnancy is associated with adverse maternal and neonatal outcomes, including cognitive and neuropsychiatric effects. This study aimed to assess the impact of maternal SCH on young rats, examining behavioral and gross brain structure changes, and evaluating reversibility after levothyroxine treatment.

Methods:

This experimental animal study was conducted at the Aga Khan University for a duration of 10 months. The study was approved by the Ethical committee for Animal Care and Use. Fourteen female Sprague Dawley rats were grouped into Treated (induced with SCH, treated with levothyroxine), Untreated (induced with SCH, no treatment), and Control (administered saline). Pups' body weight was monitored, and histological procedures were conducted at postnatal days 7, 14, and 21. Behavioral tests (elevated plus maze, forced swim, and tail suspension) assessed anxiety and depression. The data was analysed using SPSS (version 25.0). The mean, median, and standard deviation were calculated for each quantitative parameter. A one-way ANOVA was performed to identify any variation in mean thyroid levels between groups, and an independent sample t-test was utilized to confirm mean differences in anxiety levels.

Results:

On postnatal day 7, untreated pups showed lower body weight compared to treated and control groups. This trend continued on days 14 and 21. By day 21, an elevated plus maze (EPM) test indicated anxiety-like behaviour in untreated pups, while they also exhibited more signs of depression in tests like the forced swim test (FST) and tail suspension test (TST). Brain structure, including the prefrontal cortex, remained intact in offspring affected by maternal thyroid dysfunction, with no significant changes observed in brain morphology across the groups.

Conclusion:

Despite unchanged brain structure, untreated rat pups exhibited significant behavioral differences indicative of depression. This underscores the importance of understanding the behavioral impacts of maternal SCH even in the absence of gross anatomical alterations.

Keywords: Subclinical hypothyroidism, Behavioral tests, Depression, Brain structure

INTRODUCTION

Subclinical hypothyroidism (SCH) is characterized by absent or mild symptoms of hypothyroidism, high thyroid stimulating hormone (TSH) levels and normal T3/ T4 levels. It can cause significant complications during pregnancy.[1] Maternal thyroxin is particularly critical early in pregnancy because the fetal thyroid gland cannot synthesize iodothyronines until after 10 weeks of gestation (Figure 1).[2] From this time onward, in cases where maternal and fetal thyroid insufficiency occurs, the fetus has profound neurologic impairment, mental retardation, preterm birth, low birth weight, and fetal death.[3] The importance of maternal thyroid hormones (THs) for fetal central nervous system

development is well established and maternal and neonatal outcomes are yet to be explored. It has been reported that SCH is associated with multiple adverse maternal outcomes, and neurological impairments such as decrease in pre-pulse inhibition in Startle Reflex test.[4,5] Studies have revealed that prenatal thyroid hormone deficiency increases the risk of bipolar disorder with psychotic features.[6] MRI-based studies have shown that children born to mothers with thyroid dysfunction have structural defects in the brain.[7]

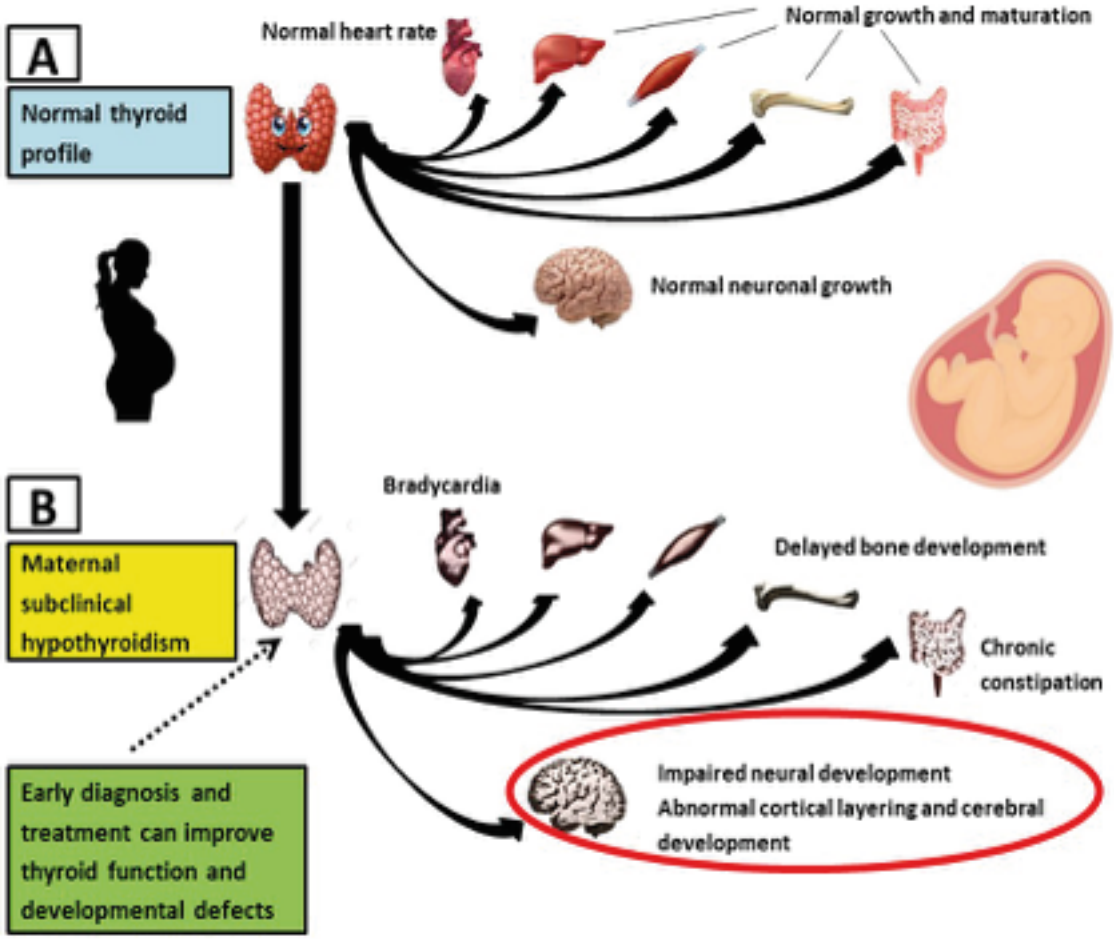

Figure 1: Role of maternal thyroid hormone on the development of fetus. A is showing physiological condition in which thyroid hormones make sure that the correct pattern of cell proliferation, differentiation, and maturation is followed in each developing organ or tissue by turning on and off the expression of many genes. B is showing effects of maternal subclinical hypothyroidism on organ development of the fetus resulting in cardiac and gastric system abnormalities, delayed bone growth and impaired neural development.

Animal models have been found to be very useful in research on hypothyroidism and SCH.[8] Studies on rat brains show that thyroid hormone dysfunction results in reduced excitatory potential in barrel cortical neurons and tactile discrimination abilities.[5] Maternal hypothyroidism adversely affected the morphology of Pyramidal and Purkinje cells by enhancing apoptosis, which increases further in second generation pups.[9] Additionally, maternal care influences development of brain regions includes prefrontal cortex, hippocampus and ventral striatum[10]. maternal hypothyroidism and hyperthyroidism have been reported to affect structural development of cerebellum and cerebrum.[11]

Adequate treatment of hypothyroidism during gestation minimizes risks and generally, makes it possible for pregnancies to be carried to term without complications (Figure 2).[12] Levothyroxine has been used to treat hypothyroidism in adults and pregnant women between 8 and 20 weeks of gestation

and results have shown that there was no significant improvement in cognition of 5-year-old children.[13,14] Animal studies have shown that levothyroxine treatment for hypothyroidism improves symptoms of anxiety.[15] Despite these research studies the relationship of SCH and fetal brain development remains unexplored. This study demonstrates developmental changes in the fore brain due to maternal thyroid insufficiency.

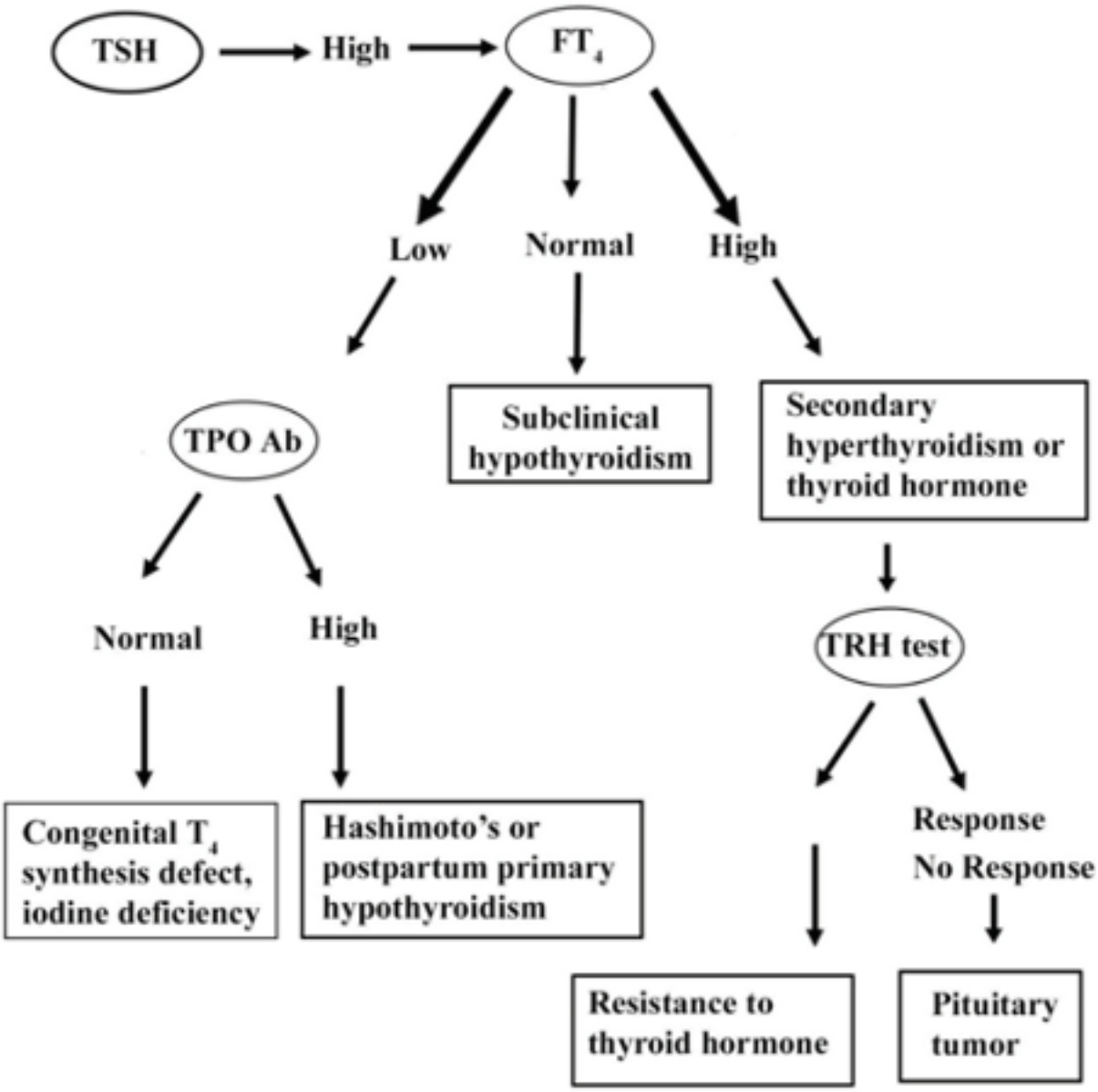

Figure 2: The flowchart demonstrates diagnostic strategy for thyroid dysfunction with increased serum TSH. The procedure includes further estimation of T4 and Thyroid peroxidase antibody (TPO Ab) levels. If the values are below the free thyroxine (FT4) reference interval further characterization is done by testing TPO Ab levels. In case of high FT4 levels testing for Thyrotropin-releasing hormone (TRH) stimulation is helpful for identifying disorders that may be brought on by pituitary gland malfunction., while normal FT4 levels indicate SCH presence.

METHODS

This experimental animal study was conducted at the Animal house facility of Aga Khan University for a duration of 10 months from January 2024 to October 2024. It was approved by the Ethical committee for Animal Care and Use (75-ECACU-BBS-18). Sprague-Dawley female rats aging from eight to 10 weeks and weighing about 235.5±25.2g were used. Rats were kept under observation in the Animal House of Aga Khan University, for two weeks for acclimatization. Male and female rats were housed separately in standard ventilated cages, for 12 hours of daily light/dark periods each, at normal atmospheric temperature (23 ± 2 □C). Food and water were provided ad libitum. Periodic measurements for body weight were recorded. Two female and one male rat were housed for one or two days to impregnate the female rats. Pregnancy was confirmed by the presence of sperm in vaginal smear, and it was recorded as the first day of gestation.

Experimental groups

On gestation day (GD = 6), the rat dams were divided into three groups:

1. Treated group (n = 4) was administered Propylthiouracil (PTU) (4-5μg/kg/day and treated for SCH with thyroxine (10 μg/kg/day).
2. Untreated group (n = 4) was administered Propylthiouracil (PTU) (4-5μg/kg/day) and left untreated.
3. Control group, (n = 4) was provided with a corresponding volume of water as placebo).

Water consumption and the PTU dose were adjusted every other day according to the last drinking water and body weight (during and after gestation) to maintain a SCH model.

Biochemical analysis

Serum samples of female rats were taken at GD=10, GD=17 and at post pregnancy day 10 to estimate TSH (ELISA kit Cat. No. E0180Ra), total T3 (ELISA kit Cat. No. PRS-30642Ra) and total T4 concentrations (ELISA kit Cat. No. PRS-30622Ra) in each group to confirm successful establishment and maintenance of SCH model.

Histological analysis

Animals were anesthetized and brain samples from nine pups per group were collected at P7, P14 and P21 and preserved in paraformaldehyde (PFA) for a minimum of 24 hours. Brains sections were dehydrated and cleared using alcohol and xylene, before preserving them in cassettes using molten wax. At the Nissl staining, 5 μm sections were placed on charged slides, deparaffinized with xylene and rehydrated before incubation in Cresyl violet.

Behavioral analysis

At postnatal day (PD) 21, the nine pups per group were tested in behavioural tasks such as elevated plus maze (EPM), tail suspension test (TST) and forced swim test (FST) to assess for symptoms of anxiety and depression.[16-19] For EPM test the pups were placed in the centre of plus maze and their movement was monitored for 5 minutes. The parameters scored were number of entries and the time spent into each area (entries were considered when four paws were placed on any arm or on the central area). Time spent in each arm was calculated in percentage [(secs in each arm / 300s) x 100].

For FST, previously described protocol was used. [16, 17] Pups were placed in a Plexiglas cylinder of 25 cm diameter and 65 cm height containing 30 cm of tap water (maintained at 25°C) for 15 min pre-test on Day 1- and 5-min test on Day 2. Cylinder was cleaned and refilled after each animal. During the test, the parameters recorded were 1) duration of immobility (lack of motion of the whole body, except minimum motion for floating), swimming (actively moving around in the cylinder), 2) climbing (vigorous movements with the forepaws in and out of the water, usually directed against the wall of the cylinder) and 3) latency to immobility (delay between start of test and first bout of immobility i.e. immobile for at least 1 sec).

For TST, the pups were placed in the experimental room at least 60 min before beginning of experiment, for acclimatization. An adhesive tape was wrapped in a constant position three quarters of the distance from the base of the tail (to avoid injury, suspend the animals by passing the hook through the adhesive tape as close as possible to the tape); ensure animal hangs with its tail in a straight line. Pups were observed continuously for five minutes and the time spent immobile was noted down.

Statistics

The data was analysed using SPSS (version 25.0). Mean, median, and standard deviation were computed for all quantitative parameters. One-way ANOVA was done to identify any difference in mean thyroid levels among groups. An independent sample t-test was also used to check mean differences in anxiety levels.

RESULTS

TSH levels were lower in untreated pregnant females From GD 10 to GD 17, higher serum TSH and normal T3 and T4 levels were observed in the PTU group when compared to the control group. After delivery, TSH levels remained significantly higher in the untreated group (p value=0.04), whereas the treated group showed normal thyroid profile following treatment with exogenous thyroxin (Table1).

Table 1: Thyroid profile of SCH mothers at GD 10, 17 and PD 10 and 17.

Days	TSH 0.45– 4.5 IU/L			P value	T3 1.59-1.63 (ug/dl)			P value	T4 9.8-11.5 ug/100ml			P value
	C	T	UT		C	T	UT		C	T	UT	
Baseline	1.28±0.38	1.50±0.63	1.62±0.39	0.65	0.72±0.24	1.02±0.17	0.93±0.13	0.17	5.37±1.12	6.07±0.23	5.93±0.90	0.58
GD 10	1.87±0.45	3.59±1.79	4.44±0.52	0.04*	1.99±0.63	3.18±1.20	3.46±1.19	0.24	5.62±0.93	6.93±1.61	5.33±1.49	0.08
GD 17	2.00±0.21	4.14±1.88	4.45±0.82	0.05*	2.67±1.51	3.76±0.51	3.7±0.46	0.24	5.87±0.99	5.70±0.66	6.05±1.29	0.96
PD 10	1.72±0.25	4.00±0.05	6.6±0.91	0.06	1.79±0.28	1.44±0.49	2.3±0.55	0.28	7.10±0.9	5.8±0.59	5.1±0.95	0.51

Data is shown as average. with 4 animals in each group. Groups: C=control, T= treated, UT=untreated. *The mean difference is significant at the level of 0.05.

Body weight of the pups

Figure 3 shows average body weights of the pups of all groups from PD7 to PD40. There was a significant difference between the weights of pups from untreated groups when compared to controls. At PD 7, 14 and 21 pups from both treated and untreated groups showed significantly lower body weight.

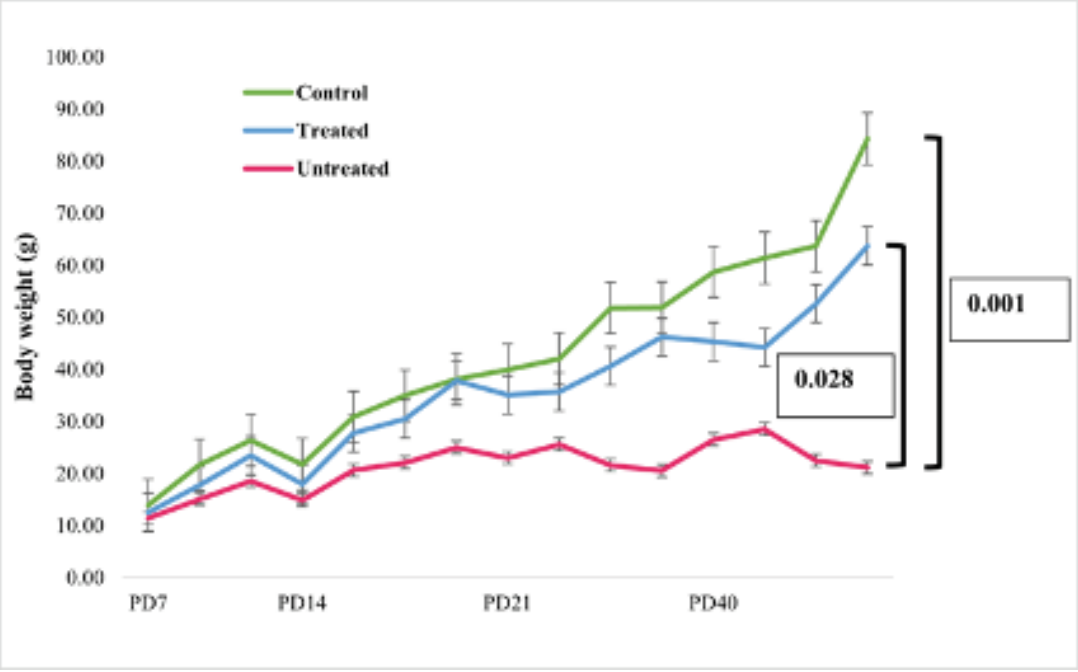

Figure 03: The average Body weight of the pups of all groups from PD7 to PD40. Body weight was measured daily

Anxiety observed in pups

Elevated Plus Maze Test

Elevated plus maze test was conducted to assess the changes in motor function and anxiety in all groups. Number of closed arm entries was used to measure locomotor activity. In general, treated group was found to be most active as shown by the trend of arm entries (Figure 4C). Pups from treated group more frequently entered sheltered arms than open arms.

The time spent in each arm was calculated and compared among groups. Overall, animals spent more time in closed arm (Figure 4E). However, time spent in closed arm by control group was found to be increased when compared to treated and untreated groups (Figure 4F). In addition, results from independent sample T-test revealed that pups of untreated group spent significantly less (p value=0.031) time in open arm compared to controls, showing a state of anxiety.

Forced Swim TestPups were tested for their active and passive behaviour through FST. Healthy pups were found to be more active compared to treated and untreated groups as shown by the duration of swimming and climbing (Figure 4G, H and I). Likewise, behaviour of pups from treated dams was like the healthy ones, showing that treatment with thyroxin was effective. The treatment also improved swimming and climbing behaviour. On the other hand, one way ANOVA showed that pups from untreated mothers had least active behaviour in immobility time and decreased duration of swimming (p value<0.05).

Tail Suspension Test

Results, using One-way ANOVA, showed that there was minor but significant difference in the duration of immobility in treated group compared to controls. Whereas pups from untreated mothers remained immobile for even longer period than any group (Figure 4J).

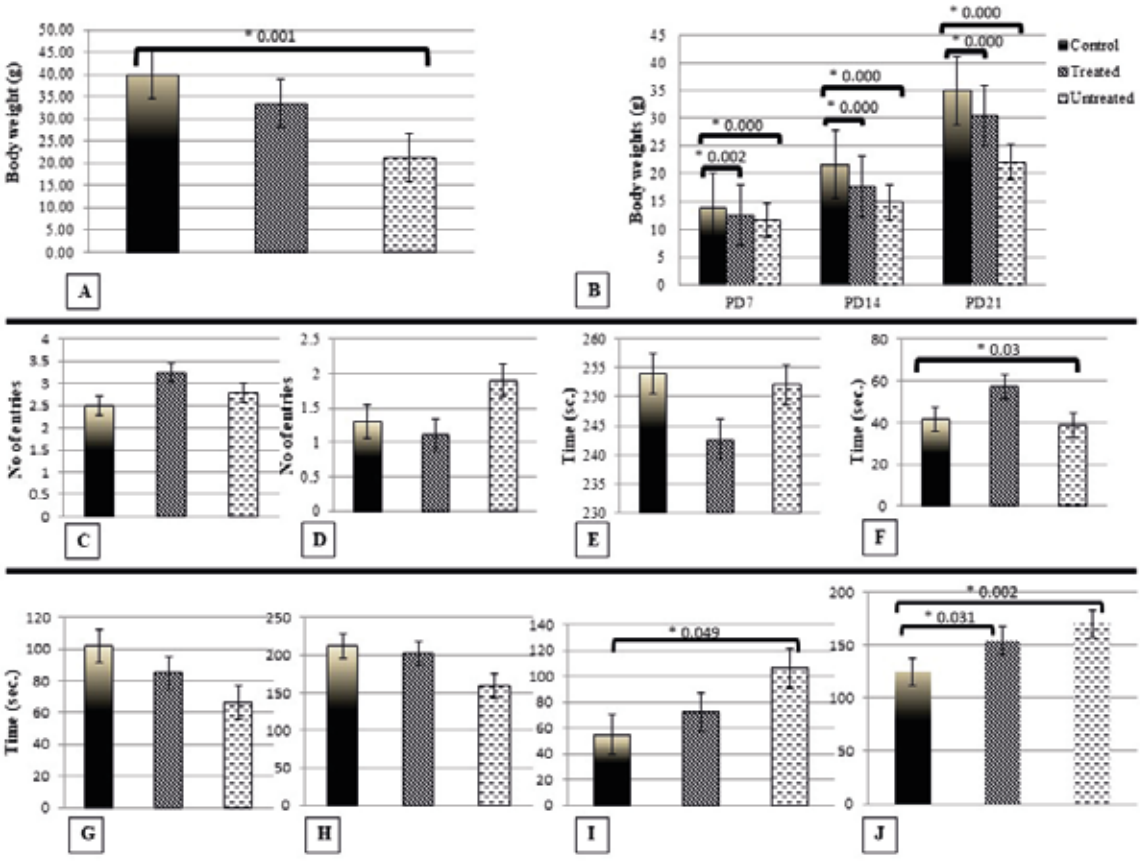

Figure 04: (A) Mean body weight of the pups (measured in 40 days). (B) Mean body weight of the pups at PD 7, 14 and 21. (C) Closed arm entries. (D) Open arm entries. (E) Time spent in closed arm. (F) Time spent in open arm. (G) Duration of swimming (FST). (H) Latency to immobility (FST). (I) Immobility measured in forced swim test. (J) Immobility measured in tail suspension test. (*The mean difference is significant at the level of 0.05).

Histological Analysis

On postnatal days 7, 14 and 21 brain samples from all pups were obtained, fixed using paraformaldehyde, sectioned, stained, and analysed (Figure 5). No significant structural changes were observed in the brains of pups belonging control, treated and untreated groups. The effect of maternal hypothyroidism on pups did not change the structure of brain.

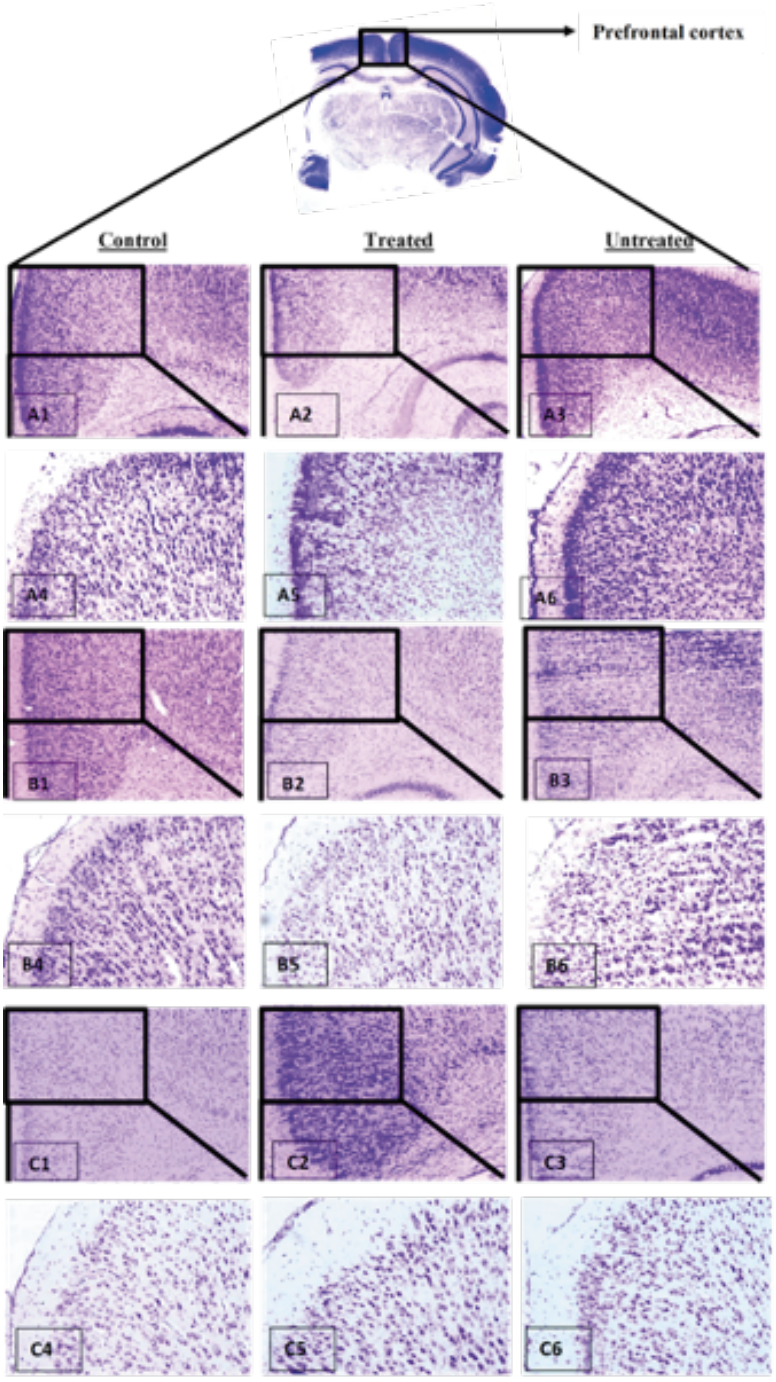

Figure 5: Nissl staining of 7-, 14-, and 21-days old brain tissues. Prefrontal cortex has been magnified at 10x on PD7 (A1 control, A2 Treated, A3 untreated), PD 14 (B1 control, B2 Treated, B3 untreated), PD21 (C1 control, C2 Treated, C3 untreated) and 20x on PD7 (A4 control, A5 Treated, A6 untreated), PD 14 (B4 control, B5 Treated, B6 untreated), PD21 (C4 control, C5 Treated, C6 untreated).

DISCUSSION

Thyroid hormones are essential for normal fetal brain development.[20, 21] The importance of maternal THs for fetal brain development and the outcome of SCH during pregnancy is well established. Maternal SCH has been known to cause reduced verbal and motor ability and impaired perceptual performance and cognitive development in offspring.[22] To determine specific cognitive abilities and underlying changes in brain structure, this study developed a model for SCH in Sprague Dawley rats and found significant changes in body weight and neurocognitive behaviour of SCH-affected offspring compared to healthy ones. There was no apparent change found in the gross structure of the brain.

Thyroid profile

Subclinical hypothyroidism was produced in rat dams following the method described by Royland et.al.[23] At GD6, well before the onset of neural tube formation, SCH dams were administered with PTU to develop maternal thyroid hormone deficiency. By determining the thyroid profile, it was assured that the maternal SCH model was established. Treatment for SCH was started around mid-gestation when dendritic development and synaptogenesis are started. Half of the SCH group was injected with levothyroxine from GD16 to PD40 (at which dendritic development, synaptogenesis, and synaptic pruning are completed) and half of the group was left untreated.

Body Weight of pups

At the end of the 1st postnatal week (PD 7), pups from the untreated group showed a marked decline in body weight presenting that maternal SCH, if not treated, can cause metabolic disturbances in offspring. These results are in accordance with previously published study, which indicated a decrease in body weight of pups born with SCH mothers.[24] By contrast treatment with levothyroxine has improved weight gain in offspring after two weeks of birth. A significant difference between the body weights of pups became more profound with age (at PD14 and 21). This means that body weight of control and treated pups increased gradually while in untreated pups no significant weight gain was observed.

Behaviour of pups

Previously published studies have shown that THs play an essential role in many central processes during brain development, including proliferation and migration of neurons, outgrowth and guidance of dendrites, formation of synapses and myelin sheath.[2,25] Maternal THs are responsible for regulating tactile and auditory information and processing in neonatal rats. [5] Similarly, the performance of human children in neuropsychological tests can be affected by asymptomatic or mild maternal hypothyroidism.[26] This can lead to neurocognitive dysfunction such as lower IQ and reduced locomotor abilities during early childhood. [27,28]

Elevated plus-maze test

Elevated plus-maze is primarily used to test anxiety as an effect of anxiolytic and anxiogenic compounds. It is also used as a common research tool to study neurobiological anxiety caused by trauma related stress disorder and brain injury.[29] Locomotor activity can be measured by counting the number of closed arm entries since there shouldn't be any anxiety entering the sheltered arms. Anxiety can be measured by observing the time spent in and counting entries to the open arm. Animals with anxiety tend to spend less time in the open arms and enter the closed arms more frequently as compared to open arms. In the present study at the end of the third postnatal week (at PD 21), these identifiers were investigated through an elevated plus-maze test which showed anxiety-like behaviour characterized by a significant reduction in the duration spent in open arm showing the involvement of maternal SCH in the pathogenesis of cognitive impairment. These results are in contrast with a study that shows that hypothyroid animals stayed for a prolonged time in the open arm.[30] However, our results are following a study conducted on both male and female Wister rats. [15] It shows that PTU-induced hypothyroidism can affect social performance and behaviour in both genders. It may be due to SCH related deficiency of dopamine precursor causing reduced dopaminergic activity and the reduction in the levels of some enzymes (such as tyrosine hydroxylase and deiodinase) and growth factors (brain-derived neurotropic factor) which are regulated by THs and are responsible for important functions in hippocampus, cortex and brainstem.[31] These are the formation and maintenance of neurons and synaptic plasticity. Other emotional disorders such as depression have also been correlated with SCH. [32]

Forced swim test and tail suspension tests

In some studies, forced swim test is used to measure susceptibility to negative moods such as hopelessness i.e., rodents' response to the fear of drowning. [33, 34] In our study, FST and TST were conducted to measure depression as described earlier.[35-37] The most depressed behaviour was found in pups from untreated dams evident from significantly increased immobility time in FST and TST as compared to healthy ones. Exact cause of depression is unclear, however reduced stimulation of postsynaptic β-adrenergic receptors in cerebral cortex and cerebellum may be responsible to cause depression like behavior and lower impulse rate of neurons and serotonin neurotransmission. [38-40]

Histological analysis

At Postnatal day 7 volume of the cortex, distribution of neurons, and glial cells were observed under different magnifications and found to be similar among groups. A previously published study has shown the presence of apoptotic bodies in the cerebral cortex in the brain of one-week-old pups of Wister rat dams.[36,41] Our investigations did not find any major change in the structure and volume of the cortex and neuronal and glial cells distribution in the brain samples from 7 days old pups. This could be a result of the timing of maternal SCH which, in our study, was induced after the neocortical development has already begun.

On Postnatal day 14 increased cortex volume was due to the formation and proliferation of neuroblasts under the influence of internal transcription factors and external growth factors. Results show that there was no apparent change found in cortical layer thickness between groups which might be because of the same transcription and growth factor levels in the body. These findings are in contrast with a study conducted on mice strain (C57 BL/6J) showing a decrease in total thickness of cortical layers on PD14. [42]

On Postnatal day 21, also no abnormal morphological variation was found in the neuronal and glial cell distribution and cortical layer thickness in 21 days old pups' brain tissues. This finding is in contrast with a previously published study, which showed changes in the cytoarchitecture of the cortex of 40 days old pups, affected by maternal thyroid dysfunction.[24]

Interestingly, the gross structure of the brain was found to be normal in offspring affected by maternal thyroid dysfunction. This may be caused by several factors, some of which are Unbalanced neurotransmission: The control of neurotransmitters in the developing fetal brain is greatly influenced by maternal thyroid hormones. Even minor changes in thyroid hormone levels during pregnancy can have an impact on the neurotransmitter balance, which is essential for healthy brain development and behavior. Subclinical hypothyroidism during pregnancy may result in epigenetic alterations in the embryonic brain that affect gene expression patterns and may contribute to the development of aberrant behaviors. Increased inflammation in the maternal and fetal brains may be linked to maternal subclinical hypothyroidism. Neuroinflammation can impair brain development and may be a factor in the child's aberrant behavior. Subclinical hypothyroidism can result in hormonal abnormalities.

The study was limited due to a varying litter size. A more comprehensive investigation could have included analysis of fetal thyroid levels and specific biomarkers, correlating them with the measured parameters. Nevertheless, the present study found the adverse outcomes of SCH during pregnancy. Based on results of the present study, it is recommended that SCH should be diagnosed and treated in a timely fashion. The treatment should be started preferably in the early stages of the first trimester. Continuous monitoring should be done for thyroid profile as the dysfunction adversely affects the cognitive abilities in new-borns. The present study leaves many promising avenues open for future research. Several biomarkers need to be investigated for the determination of their role in the pathogenesis of SCH and resulting cognitive impairments in new-borns. In the future gene expression in different regions of the prefrontal cortex may provide meaningful insights into the development of brain and its effect on behavioural disorders.

CONCLUSION

Subclinical hypothyroidism affects newborns' growth, development, and cognitive abilities, evidenced by reduced pup body weight. Offspring of hypothyroid rat dams show no gross changes in brain cortex structure compared to healthy dams. Cortical development remains stable during postnatal periods (PD7, PD14, and PD21), but minor yet significant behavioral differences suggest maternal hypothyroidism's role in cognitive impairment. Treatment can enhance physiological, behavioral, and hormonal aspects.

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The authors have approved the final version of the article, and agree to be accountable for all aspects of the work.