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Original Article

Cerebral Ischemia/Reperfusion Injury in the Hyperthyroid Rat

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Abstract

Background: Hyperthyroidism as a risk factor for stroke is not conclusive. There are no definite data on the relationship between ischemic cerebrovascular injury and hyperthyroidism. This study was designed to define whether the outcomes of post-ischemic stroke injury are influenced by chronic hyperthyroidism.

Methods: Two groups of hyperthyroid (HT) and control euthyroid rats of equal numbers (n=22) were included in the study. Hyperthyroidism was induced for 4 weeks by adding L-thyroxine (300 µg/kg) to drinking water. The middle cerebral artery occlusion technique was used to induce focal cerebral ischemia. Neurological disability (neurological deficit score [NDS]) was evaluated after 24 hours, and the rats were sacrificed to obtain their brain. Triphenyl Tetrazolium Chloride (TTC) staining and Evans Blue (EB) extravasation were used to quantify cerebral infarct volume and cerebrovascular integrity disruption. Data analysis was done using SPSS, version 21.

Results: Thyroid hormones levels, T_3 (314±7 vs. 198±3 ng/dL; P=0.001) and T_4 (9.8±0.3 vs. 3.08±0.07 µg/dL; P=0.001), were significantly higher in the HT group than in the controls. Furthermore, most clinical signs seen in hyperthyroid patients were also present in the HT group. Comparison of the data on cerebral ischemia between the HT and control groups showed significant increases in the NDS (2.76±0.16 vs. 2.23±0.09; P=0.03), cerebral infarct volume (479±12 vs. 266±17 mm^3; P=0.001), and EB extravasation (50.08±2.4 vs. 32.6±1.2 µg/g; P=0.001) in the former group.

Conclusion: The intensified cerebral infarct size and cerebrovascular integrity disruption suggested that chronic hyperthyroidism aggravated post-stroke injury in the rats. More investigation is required to analyze the pathological mechanisms underlying the association between cerebrovascular disease and hyperthyroidism.

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Keywords ● Hyperthyroidism ● Stroke ● Cerebral infarction ● Blood-brain barrier

What's Known

- Association between thyroid hormones and the clinical outcome of stroke is controversial.
- Hyperthyroidism may act as a risk factor for poor recovery from ischemic stroke. Current data do not show the interference of hyperthyroidism in the intensity of stroke injuries seen in the general population or laboratory animals.

What's New

- The impression is that chronic hyperthyroidism aggravates post-stroke injuries by extending the disruption in the blood-brain-barrier integrity, vasogenic edema, and cerebral infarction. Combination of these parameters as well as other unknown factors may be the cause of poor recovery or high mortality rates after acute stroke.

Introduction

Recent clinical data have suggested that thyroid hormones may play an important role in the pathophysiology of acute ischemic stroke.[1,2] The recovery in hypothyroid patients from stroke appears to have better outcomes and lower mortality rates.[3,4] Nonetheless, the possible associations between thyroid status and the clinical outcome of acute stroke in hyperthyroid (HT) patients are

debatable. Hyperthyroidism may be associated with various types of cerebrovascular disease, a well-known risk factor for ischemic stroke, in young patients.[5,6] Subclinical hyperthyroidism may reduce the chance of recovery from ischemic stroke.[1] While the causes of deterioration in neurological disabilities in stroke patients with hyperthyroidism are diverse, thrombotic stroke has been described as a common risk factor in young patients with hyperthyroidism.[6] A few case reports in untreated thyrotoxic patients have reiterated that ischemic stroke may display a high degree of neurological disability.[7-10]

Some reports have indicated that thyroid hormones, depending on their concentration, may intensify the ischemic/reperfusion (I/R) injury of stroke in patients or in animals with experimentally induced stroke.[4,11,12] A few studies performed on laboratory animals with permanent middle cerebral artery (MCA) occlusion have indicated that the short-term treatment of stroke animals with high doses of thyroid hormones may increase the risk of I/R injury.[13] Nonetheless, data on the interaction between acute focal cerebral ischemia and chronic hyperthyroidism are trivial and inconclusive. Therefore, the present study was designed to induce transient focal cerebral ischemia, a model of stroke in the rat, and investigate the relationship between chronic hyperthyroidism and cerebral I/R injury.

The microvascular integrity of cerebral capillaries is crucial for the normal neurophysiological functions of the brain.[14,15] Clinical and animal studies have indicated that the endothelial permeability of the cerebral vascular beds, which is altered in patients with hypothyroidism, is mostly corrected by thyroid hormone therapy.[15-17] Previous studies performed on euthyroid rats have indicated that cerebral ischemia disrupts the blood-brain barrier (BBB) and that the swelling of the injured hemisphere would expand the cerebral infarct size.[18] Some reports have restated that stroke caused by a blocked artery (ischemic stroke), if followed by vasogenic edema, may intensify stroke injury after reperfusion.[19,20] Nevertheless, poor recovery after stroke in hyperthyroidism is still a mystery.

The supportive role of thyroid hormones in the protection of the brain vasculature is well defined,[21] but there is a dearth of data considering hyperthyroidism as a potential risk factor for cell death and intensified I/R injury in the wake of stroke. In this study, we tried to verify whether chronic hyperthyroidism interferes with the stroke disruption of the BBB. Hence, chronic hyperthyroidism was induced in adult rats for 4 weeks with a daily oral intake of 300 μg/kg of L-thyroxine in their drinking water. Subsequently, transient focal ischemia was induced by 60 minutes of MCA occlusion, followed by 24 hours of reperfusion. Thereafter, the study was continued to see whether chronic hyperthyroidism would change the intensity of infarction and I/R injury imposed on the brain during reperfusion.

Materials and Methods

Animals

Male adult Sprague–Dawley rats (weight=200–250 g) were obtained from the Animal House of Shiraz University of Medical Sciences. All procedures were in accordance with the Institutional Animal Ethics Committee of Shiraz University of Medical Sciences and followed the NIH Guidelines for care and use of animals (NIH Publication No. 85–23, revised in 1996). The animals were housed under a controlled temperature (22°C–24°C), humidity (40%–60%), and 12-hour light/dark cycle and had access to rat chow (Pars Dam, Iran) and water *ad libitum*.

Animal Treatment and Grouping

Two groups of HT and control normal (CN) rats were prepared. In the HT group, hyperthyroidism was induced over a 4-week period by the oral administration of L-thyroxine (Natrium Levothyroxine, Iran Hormone Company, Iran) in drinking water. During the first week of treatment, with the average daily drinking water of 30 mL/rat, the concentration of L-thyroxine was about 2.3 μg/mL. With the increased daily water consumption, the concentration of the drug was decreased and adjusted to avoid thyroid toxicity. Overall, during the 4 weeks of treatment, the daily dose of L-thyroxine never exceeded 300 μg/kg. In the CN group, the rats were treated in the same manner as the HT group except that they were given tap water for the 4-week period.

Parameters Measured during the First Period of the Experiment

In both HT and CN groups, the drinking water was measured daily during the first week and every other day afterward. Body weight and systolic blood pressure (with a tail-cuff plethysmography and a Power Lab system) were measured once a week. The levels of thyroid hormones and core body temperature were checked at the beginning and at the end of the treatment period. Blood sampling was done by slightly anesthetizing the animal with ether and collecting 1 mL of blood from the tip of the snipped tail. The samples were subsequently

centrifuged (4,000 × g), and the serum was stored in a freezer (-70°C). Plasma thyroid hormones (T_3 and T_4) were measured using an Eliza assay kit (DiaPlus, Canada). Moreover, the accomplished hyperthyroidism was verified by various clinical signs such as increased body temperature, systolic blood pressure, tachycardia, and decreased body weight.

Experimental Protocol and Groups

Four weeks after the treatments, the animals of each group were randomly selected for surgery as follows:

Ischemic CN (ICN, n=14) group: Surgery was accomplished at the neck region, and the right MCA was occluded for 60 minutes, followed by reopening and 24 hours of reperfusion.

Sham CN (SCN, n=8) group: All procedures were done like the ICN group with the exception of MCA occlusion.

Ischemic HT (IHT, n=14) group: Experiments were performed like the ICN group.

Sham HT (SHT, n=8) group: All procedures were accomplished like the SCN group.

Subsequently, the rats of each group were further divided into 2 subgroups for the evaluation of 1) cerebral infarct size and 2) BBB integrity.

The overall success rate of the induction of brain ischemia was 60% in all groups, but the mortality rate of ischemia during the 24-hour period of reperfusion was 50% in the HT group compared to 10% in the CN group. The data presented in this study were obtained from animals that survived 60 minutes of brain ischemia and 24 hours of reperfusion.

Surgical Procedures

The animals were fasted overnight prior to surgery, but they had free access to water. Anesthesia was conducted through a facemask with isoflurane (1.5%–3% isoflurane, 30% oxygen in 70% nitrous oxide). Continuous recording of blood pressure was done during surgery. MCA occlusion was done in the first 15 minutes of the reperfusion period via a cannula placed in the right femoral artery. Similarly, core temperature was continuously recorded and maintained at 37±1°C with a heating lamp or an ice bag.

Measurement of Regional Blood Flow

A laser Doppler flowmeter (AD Instrument, model: ML191, Australia) was used to measure the regional cerebral blood flow (rCBF) of the right hemisphere.[18] MCA occlusion was considered successful if there was a 75%–85% decrease in rCBF and a swift return to the pre-occluded level after reopening (figure 1).

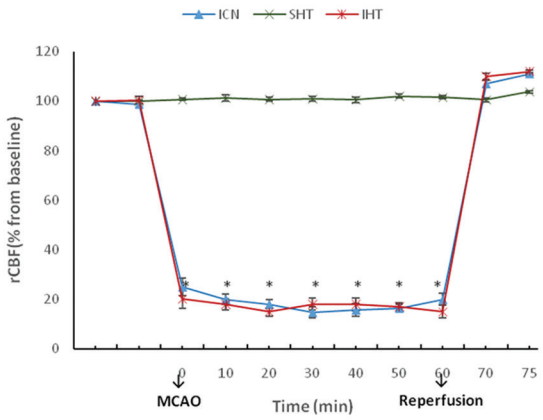

Figure 1: Regional cerebral blood flow (rCBF) in the ischemic control normal (ICN, n=4), sham hyperthyroid (SHT, n=8), and ischemic hyperthyroid (IHT, n=4) rats before and during middle cerebral artery occlusion (MCAO) and during the reperfusion period. Values are presented as means±SEMs. *Significantly different from the sham group (P=0.0001).

Induction of Focal Cerebral Ischemia

Transient focal cerebral ischemia was induced with the method of Longa modified by Vakili et al.[22,23] Right MCA occlusion was started by advancing prepared nylon thread through the exposed common carotid artery up to the cranium. Occlusion was confirmed by the observation of a sharp decline in rCBF trace. Reperfusion of the ischemic hemisphere, started after pulling out the thread, was confirmed by restored rCBF. Finally, all instruments were removed, incisions were sutured, and the animals were returned to a warm cage for recuperation during a 24-hour reperfusion period.

Evaluation of Neurological Disability

Neurological function was evaluated blindly 24 hours after surgery or ischemia with a 5-point neurological deficit score (NDS) described previously.[2,24] Then the animals were scarified and their brain was prepared for the evaluation of ischemia infarct volume or BBB disruption.

Measurements of Cerebral Infarct Volume

Cerebral infarct volume was measured according to the method of Swanson et al.[25] After deep anesthesia, the animals were beheaded and their brains were removed, cleaned, and sliced. Staining was achieved with 2% TTC (2, 3, 5-triphenyltetrazolium chloride, Sigma) as previously described.[18,26] The images of the slices were digitized by using a Cannon camera. The visible infarct zones were quantified using image analysis software. Finally, cerebral infarct volume was calculated as described previously.[18,26]

Quantitative Measurement of the Blood-Brain Barrier Permeability

The integrity of the brain vessels was quantitatively assessed using Evans Blue (EB) extravasation.[25,26] After preparation, the optical density values of the prepared samples were measured at 620 nm with a Microplate reader (BioTek model: Gen 5, Germany). The tissue contents of EB were calculated according to the standard curves of dyes described, and the results are expressed as μg/g wet tissue weight.[2,24]

Statistical Analysis

All values are presented as means±SEMs. The analyses were performed using SPSS, version 21. One-way ANOVA with the Tukey post hoc test was used to evaluate the changes that had occurred during the 4-week treatment period in body weight, daily drinking water, heart rate, blood pressure, and core temperature. The same test was used to evaluate variations in the rCBF, infarct volume, and BBB permeability. The Kruskal–Wallis test was also utilized to evaluate the NDS. Values of $P<0.05$ were considered statistically significant.

Results

Circulating Levels of Thyroid Hormones, Water Consumption, Systolic Blood Pressure, Heart Rate, Body Temperature, and Body Weight

The data presented in table 1 show that the total levels of T_3 and T_4 of the HT group were significantly higher than those in the CN group. This increase indicated that we had successfully induced experimental hyperthyroidism. While the daily water consumption of the CN rats was unchanged during the treatment, a steady and significant rise was seen in the HT group. Furthermore, the steady increase in body weight seen in the CN group was reversed in the HT animals accordingly. While at the start of treatment, systolic blood pressure, heart rate, and body temperature in both groups were almost the same, these parameters had significant and progressive increments after the 2nd week of treatment in the HT group.

The systolic, diastolic, arterial, and mean pressures as well as heart rates of both groups during ischemia are also presented in table 2. As was expected from the results of the tail-cuff pressure, prior to surgery, systolic blood pressure in the IHT group was significantly higher. Although before MCA occlusion, mean blood pressure in both ischemic groups was statistically similar, in the IHT group there was a significant increase in mean blood pressure during ischemia.

Table 1: Average tail systolic blood pressure, heart rate, daily water intake (water), body weight, core temperature, and plasma concentrations of T_3 and T_4

Parameters	CN (n=22)	HT (n=22)	P value
Systolic blood pressure (mm Hg)	114±1	151±3	0.001
Heart rate (beat/min)	372±6	551±11	0.001
Water (mL/d)	31±1	101±2	0.001
Body weight (g)	255±1	215±1	0.01
Temperature (°C)	37.2±0.08	38.5±0.08	0.01
T_3 (μg/dL)	198±3	314±7	0.001
T_4 (μg/dL)	3.08±0.07	9.8±0.3	0.001

Data are presented as means ± SEMs. CN: Control normal; HT: Hyperthyroid

Regional Cerebral Blood Flow

Alterations in rCBF (% from baseline) with time are depicted in figure 1. There was no significant variation in rCBF in the sham groups of normal or HT rats (SCN or SHT). MCA occlusion caused a 75%–85% reduction in rCBF in both ICN and IHT groups. After MCA reopening, rCBF swiftly returned to its pre-occlusion level. Comparison of rCBF traces between the sham and ischemic groups indicated that hyperthyroidism *per se* did not alter blood flow in the sham groups or during ischemia.

Neurological Disability Scales

The values of the NDS in the sham and ischemic rats are presented in figure 2. The NDS of the sham groups indicated that anesthesia and neck surgery did not impair neural activity. The significant increase in the NDS of the IHT group compared to the ICN group (2.76±0.16 vs. 2.23±0.09) denoted that ischemia neural disability was intensified in the HT rats.

Cerebral Infarct Volumes

MCA occlusion induced different magnitudes of infarctions in the right hemispheres of the ICN and IHT rats without affecting the left sides (figure 3). The quantitative comparisons of the results of infarct volumes between the 2 groups indicated that hyperthyroidism intensified the degree of infarction (266±17 vs. 479±12 mm^3; $P=0.001$).

Blood-Brain Barrier Integrity Disruption

A sample photograph of the EB extravasation of the brain of the SHT, ICN, and IHT groups is presented in figure 4. The spread bluish color on the surface of the ischemic hemispheres of both ischemic groups is a sign of BBB disruption. The absence of the blue color in the non-lesions of contralateral sides indicated that the reduced focal ischemia in the right hemispheres did

Table 2: Arterial blood pressure and heart rate before and during occlusion as well as reopening of the middle cerebral artery in the ICN and IHT rats

Parameters	Conditions	ICN (n=14)	IHT (n=14)	P_1	P_2
Systolic blood pressure (mm Hg)	Before occlusion	115±4	153±7	0.02	-
	During occlusion	120±3	164±7	0.03	0.02
	Reopening	118±5	160±6	0.03	ns
Diastolic blood pressure (mm Hg)	Before occlusion	85±3	79±2	ns	-
	During occlusion	85±2	105±2	0.01	0.001
	Reopening	83±4	103±2	0.01	0.001
Mean blood pressure (mm Hg)	Before occlusion	100±3	104±1	ns	-
	During occlusion	100±3	124±3	0.01	0.001
	Reopening	95±4	122±2	0.01	0.001
Heart rate (beats/min)	Before occlusion	379±10	507±12	0.01	-
	During occlusion	380±12	523±12	0.001	ns
	Reopening	380±7	529±17	0.001	ns

All values are presented as means±SEMs. Ns: Non-significant; ICN: Ischemic control normal; IHT: Ischemic hyperthyroid; P_1: comparisons between 2 groups of IHT and ICN; P_2: Comparison of data on the occlusion or reopening periods and data on the pre-occlusion period in the IHT group

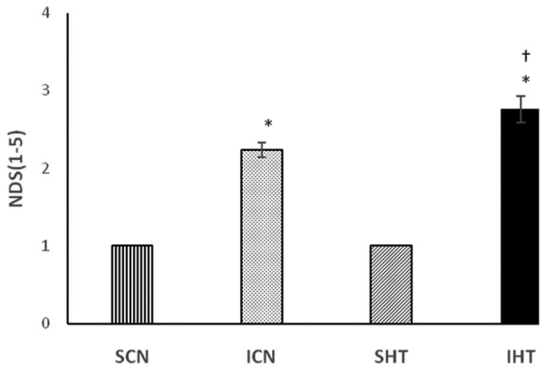

Figure 2: Neurological deficit score (NDS) 24 hours after sham surgery or middle cerebral artery occlusion (MCAO) in the sham control (SCN), ischemic control (ICN), sham hyperthyroid (SHT), and ischemic hyperthyroid (IHT). All values are presented as means±SEMs. *Significantly different from the SCN group or the SHT group (P=0.001). †Significantly different from the ICN group (P=0.03).

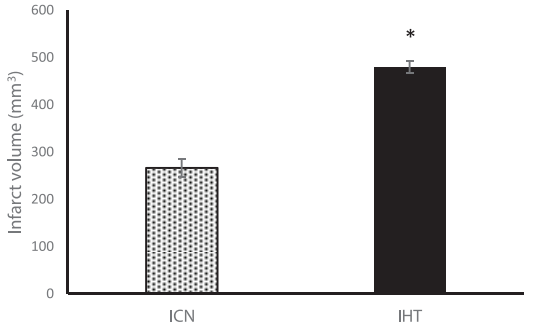

Figure 3: Infarct volumes of the right hemispheres of the ischemic control normal (ICN) and ischemic hyperthyroid (IHT) groups. All values are presented as means±SEMs. *Significantly different from the ICN group (P=0.001).

not affect the BBB of the left side of the ischemic groups. Accordingly, the quantitative measurement of EB extravasation indicated that the BBB was significantly disrupted in the lesioned hemispheres. Additionally, hyperthyroidism significantly intensified ischemia BBB disruption in the HT rats (figure 5).

Discussion

The key finding of the present study was that the brain injury occurring during ischemia or the reperfusion period was more noticeable in the HT rats. Our data also indicated that the magnitude of the cerebral infarct size and BBB disruption was potentially greater in the chronic HT rats. The relationship between thyroid hormones and cardiac diseases has long been established.[27,28] One study showed that hyperthyroidism acted as risk factor for ischemic stroke.[29] While a neuroprotective role for normal levels of T_3 in ischemic stroke has been reported,[30] the contribution of elevated thyroid hormones during stroke or recovery in patients with hyperthyroidism is still a mystery.

A few clinical studies have shed some light on the interaction between thyroid hormones and the susceptibility of the brain during ischemic stroke. Subclinical hyperthyroidism is thought to worsen functional cardiovascular disability, begetting for instance atrial fibrillation, during stroke or recovery in young patients with hyperthyroidism.[7-9] Nonetheless, the clinical interference of high levels of thyroid hormones with ischemic stroke injury, usually seen during recovery in patients with hyperthyroidism, has yet to be clarified.[1] In our study, we induced 4 weeks' chronic hyperthyroidism in the rat by

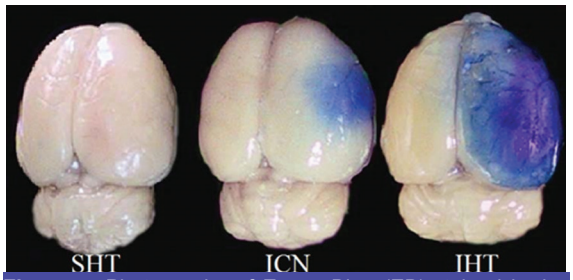

Figure 4: Photographs of Evans Blue (EB)-stained brains from the rats killed 24 hours after sham surgery or middle cerebral artery occlusion. In the sham hyperthyroid (SHT) group, the blood-brain barrier is intact and the EB extravasation is absent. The presence of the blue color indicates that the extravasation of EB has occurred in the ischemic control normal (ICN) and ischemic hyperthyroid (IHT) rats during ischemia/reperfusion injury. Marked staining is seen in the ischemic area in the IHT group.

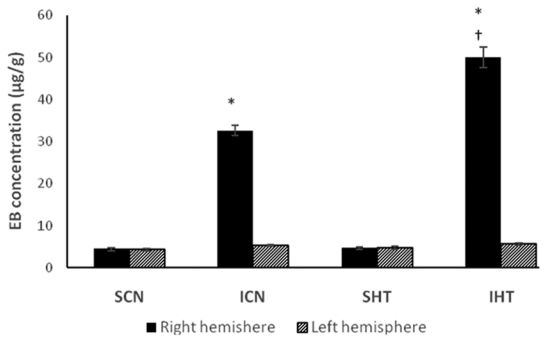

Figure 5: Evans Blue (EB) concentrations (µg/g) in the left and the right hemispheres of the sham control (SCN), ischemic control (ICN), sham hyperthyroid (SHT), and ischemic hyperthyroid (IHT) rats. All values are presented as means±SEMs. *Values are significant from sham and their own contralateral hemispheres (P=0.001). †Significantly different from the right hemispheres of the ICN group (P=0.001)

daily oral intakes of L-thyroxine solution. Chronic experimental hyperthyroidism was documented by respectively seeing a 60% increase in the level of T_3 and 226% in the level of T_4. In addition, the clinical signs of hyperthyroidism (3.5% increase in body temperature, >3 times increase in the daily water consumption, 33% increase in systolic blood pressure, 50% increase in heart rate, and 6.1% weight-loss) were well documented in the HT rats.

In the rat, the MCA supplies the lateral surface of the cortical and subcortical areas of the brain, which participate in the process of sensorimotor information.[31,32] Stroke injury in this area may challenge neural and synaptic electrophysiological information.[32] In most animal studies, a 5-point NDS is used to evaluate stroke and reperfusion injury imposed on the lateral surface of the brain.[14,18] This procedure has indicated an acceptable correlation with the intensity of the damage imposed on the brain during recovery after ischemia.[14] Comparison of the NDS between the IHT and ICN groups demonstrated that chronic hyperthyroidism potentially worsened the motor neuron damage that had occurred during ischemia or reperfusion in the right lateral hemisphere. In most cases, the NDS values helped us to estimate the intensity of ischemia brain infarction before sacrifice.

The values of cerebral infarction usually show the scale of ischemic stroke injury. Our results revealed that cerebral infarct volume was highly significant in both ischemic groups. Moreover, a comparison of the results between the 2 ischemic groups implied that hyperthyroidism significantly intensified I/R brain injury (figure 3). Clinical reports have elucidated that mild hyperthyroidism may act as a risk factor for poor recovery of ischemic stroke.[1] Experimental hyperthyroidism induced in the rat also indicated that acute thyrotoxicosis expanded ischemic cerebral infarction areas and aggravated post-stroke injury.[33] Although the levels and durations of increased thyroxin levels of our experiments were totally different from the experiments of Rastgi and colleagues,[33] their overall statement fully supported our notion that chronic hyperthyroidism would intensify I/R injury.

Thyroid hormones are well known to associate with the regulation of energy homeostasis, thermogenesis, and basal metabolic rate.[34] Reports have indicated that thyroid hormones exert their positive calorigenic actions and augmented oxygen demands by increasing the activity and the numbers of the mitochondria in tissues like skeletal muscle, heart, kidney, and liver.[35,36] Moreover, recent human studies have demonstrated that thyroid hormones, depending on their levels, may actively increase the level of the blood flow and metabolism of the adult's brain.[37,38] From these primary reports, it is possible to conclude that hyperthyroidism may have strengthened the sensitivity of the neural cells and that the reduced blood flow may have intensified I/R injury imposed on the brain of the HT rats.

Brain edema is generally classified as cytotoxic and vasogenic edema. Cytotoxic edema refers to intracellular accumulation of water due to the inability of neural cells to regulate their own volume.[39] So far data published in the literature do not present conclusive evidence about the impact of ischemic stroke injury upon cerebrovascular beds in patients with hyperthyroidism or in HT animals. The present study showed that the BBB disruption and brain injury that occurred during the ischemia

or reperfusion periods were more destructive in the HT rats. Reports have specified that in young patients, subclinical hyperthyroidism may act as a risk factor for ischemic stroke,[7-9] but the involvement of hyperthyroidism in the recovery of stroke is not clearly elucidated.[1]

Cerebral vasogenic edema refers to the influx and accumulation of fluid and solutes into the brain tissue through disrupted BBB or damaged capillary beds.[18] The excess accumulation of fluid in the intra- or extracellular space my put more pressure on the cerebral blood vessels and more reduction of blood flow further strengthens stroke injury.[40] During the first few hours of stroke recovery, vasogenic brain edema is the leading element of disability or survival.[3,41] The results of the current study showed that the BBB disruption that happened during the acute stroke or reperfusion period in the HT rats was significantly higher than that in the ICN rats. From data presented so far, it is too early to make an assumption about the interaction between high thyroid hormones and stroke inabilities occurring after recovery in patients with hyperthyroidism. Extensive investigations are needed to elucidate how hyperthyroidism, which intensified the mortality rate of focal cerebral ischemia in the chronic HT rats of this study, would increase the morbidity or mortality rates of patients with hyperthyroidism after stroke.

Conclusion

The results of this study demonstrated that hyperthyroidism *per se* did not alter cerebrovascular permeability. The increased efflux of EB extravasation in the euthyroid rats may suggest that endothelial dysfunction after stroke was mainly the cause of BBB disruption. Be that as it may, conjunction of chronic hyperthyroidism with I/R injury, happening during acute stroke, worsened the disruption of blood-brain integrity and aggravated the stroke symptoms and mortality rate. Further studies are required to characterize the pathophysiological mechanisms that exaggerate cerebrovascular injury in chronic ischemic HT rats.

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Conflict of Interest: None declared.

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