Effects of the Administration of Thyroid Hormones in Cases of Hepatic Ischemia and Reperfusion Injury

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Objective: Hepatic ischemia and reperfusion (IR) injury is the most important cause of cellular death and hepatic dysfunction following liver transplantation and resection. Our aim in this study is to reveal the early stage effects of thyroid hormone levels on hepatic IR injury that effectively act on cellular homeostasis.

Methods: Forty-six male Wistar albino rats were divided into 6 groups as follows: euthyroid-sham (n = 8), euthyroid with IR injury (n = 8), hyperthyroid-sham (n = 7), hyperthyroid with IR injury (n = 7), hypothyroid-sham (n = 8), and hypothyroid with IR injury (n = 8). After 90 minutes of partial hepatic ischemia, 90 minutes of reperfusion was applied. Liver tissue malondialdehyde (MDA) levels, catalase (CAT), glutathion peroxidase, and superoxide dismutase (SOD) enzyme activities were measured. Hepatic tissue
was immunohistochemically analyzed.

**Results:** MDA levels of liver tissue were analyzed, and hepatic MDA levels in the hyper-IR group were found to be significantly lower ($P = 0.002$) than those of the hypo-IR and euthyroid-IR groups. Serum CAT levels did not differ between control groups, whereas CAT values in the hyper-IR group were detected to be significantly lower than in the euthyroid-IR and hypothyroid-IR groups ($P = 0.003$). However, levels of SOD and glutathione peroxidase (GPX) were not affected by the functional state of the thyroid. No statistically significant difference was seen in the results of the histopathologic evaluation and immunohistochemical staining of the liver tissue.

**Conclusion:** The administration of thyroid hormone within a short time before IR injury may have protective effects.

**Key words:** Thyroid hormones – Ischemia and reperfusion injury – Liver – Rat

Ischemia-reperfusion (IR) injury caused by disruption of cellular energy balance is a tissue injury induced by restoration of blood flow following an ischemic process. IR injury is the main cause of hepatic tissue damage and of graft dysfunction occurring during surgical procedures such as liver transplantation and resection, and it remains a problematic issue. Decrease in the oxygen supply to the tissue results in impairment of mitochondrial oxidative phosphorylation. Reinstitution of $O_2$ support after the ischemic process induces a series of cellular reactions encompassing the formation of free $O_2$ radicals, which paradoxically worsens ischemic injury.

Aerobic respiration is a process realized in mitochondrial cristae, in which oxygen is used in the cellular metabolism. The electron transport system (ETS) is the final component of aerobic respiration. As a result of redox reactions, which resemble relay races, oxygen is reduced by 4 electrons and transforms into water. During oxygen metabolism, a small amount of reactive oxygen species (ROS)—such as superoxide radical ($O_2^-$), hydrogen peroxide ($H_2O_2$), and hydroxyl radicals ($OH^*$)—are produced. In cases with ischemia, bleeding, and radioactivity, the aerobic oxidative phosphorylation balance in mitochondria is affected; electron leakage from the ETS increases, and levels of ROS are enhanced. The transient increase in ROS is balanced by various regulatory functions. However, when this increase is at a higher level and/or prolonged, it can induce injury in DNA, lipids, and proteins.

There are some defense mechanisms against the accumulation of ROS. These consist of nonenzymatic molecules (e.g., vitamins A, C, and E and flavonoids), and enzymatic ROS scavengers [e.g., superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX)]. In addition to their contributions to normal growth and development, they also control the production of heat and energy, which are components of metabolic homeostasis. The receptor–hormone complex, which is formed by the binding of thyroxine to its cellular receptor, leads to changes in the enzymes that regulate cellular function. As a result of all these processes, the widespread physiologic effects of thyroid hormones manifest. Stimulation of increased cellular respiration and production of $O_2$ in many tissues—including the liver, heart, and kidney—constitutes one of these effects. The liver is the main target organ of thyroid hormones. Deviations in the thyroid hormone balance also lead to alterations in the response to oxidative stress.

Our aim in this study is to reveal the effects of thyroid hormone levels on the regulation of cellular homeostasis during IR injury in hypothyroidism and hyperthyroidism.

**Materials and Methods**

A total of 46 male Wistar albino strain rats weighing 200–250 g were procured from the Experimental Research Center of Ondokuz Mayis University. All patients were cared for under suitable conditions (temperature: $21 \pm 1^\circ C$, humidity: 40–70%, under 12 hours of dark and 12 hours of light conditions, fed standard rat pellets, and given water ad libitum). The study protocol was approved by the Ethics Committee for Investigations in Experimental Animals of...
The Ondokuz Mayis University Faculty of Medicine (2012/65-12.18, 2012).

All surgical procedures were conducted under anesthesia after intraperitoneal injection of ketamine (50 mg/kg) and xylazine (10 mg/kg). The 46 rats were divided into 6 groups as follows:

1. hypothyroid with IR injury (n = 8)
2. hypothyroid-sham (n = 8)
3. euthyroid with IR injury (n = 8)
4. euthyroid-sham (n = 8)
5. hyperthyroid-sham (n = 7)
6. hyperthyroid with IR injury (n = 7)

Partial hepatic ischemia was induced by clamping the common vascular pedicles of the median and left lateral lobes of the liver. After 90 minutes, the ischemia vascular clamp was opened, and reperfusion was maintained for 90 minutes. A state of hypothyroidism was induced by adding 6-propyl-2-thiouracil (P3755, Sigma Aldrich, St Louis, Missouri) to the drinking water 21 days before the procedure. A state of hyperthyroidism was induced by intraperitoneal administration of 3,3',5-triiodo-L-thyronine (T2877, Sigma Aldrich) (10 μg/100 g/d) 2 days before the procedure.

Measurements of enzymatic activities of liver tissue CAT, GPX, SOD, and MDA

Liver tissue samples were prepared according to the recommendations of Celik and Suzek. The tissues were homogenized for 5 minutes in 50 mM cold KH2PO4 solution (1:5 wt/vol) using a homogenizer (Dounce homogenizer, Sigma-Aldrich). Homogenates were centrifuged at 4°C for 20 minutes (4100 rpm). To 0.5 mL of sample, 2.5 mL 20% trichloroacetic acid and 1 mL 67% thiobarbituric acid were added, and the amount of MDA was determined using the thiobarbituric acid reactivity measurement method. Measurements of the enzymatic activities of CAT, GPX, and SOD were performed in compliance with the directions for use indicated in the inserts of the kits used.

Histopathologic analysis

Tissue samples were fixated in 10% buffered formaldehyde, decalcified, and embedded in paraffin blocks. The 4- to 5-μm sections obtained were stained with the hematoxylin and eosin (H&E) method, and histopathologic changes were examined and evaluated semiquantitatively.

Histopathologic evaluation of thyroid tissue with H&E

Histological changes in the thyroid tissue were classified as euthyroidism, hypothyroidism, or hyperthyroidism based on the definitions of Serrakides et al. Histopathologic evaluation of liver injury with H&E

Hepatic injury was scored in 4 grades: grade 0, no evidence of injury; grade 1, mildly cytoplasmic vacuolization and slight injury, including focal nuclear pyknosis; grade 2, widespread nuclear pyknosis, cytoplasmic hypereosinophilia, and moderate to severe injury effacing intercellular borders; grade 3, severe necrosis with impairment of hepatocyte cords, bleeding, and neutrophilic infiltrations.

Nearby subcapsular areas were left out of the assessments because of operative manipulations and possible errors of detection.

Immunohistochemical analysis

Sections obtained from tissue blocks prepared in paraffin were treated with the streptavidin biotin peroxidase complex technique (SABC) to determine the presence of the proteins described in Table 1. Primary antibodies were diluted with phosphate-buffered saline (PBS, pH 7.4) in line with the directives of the manufacturing firm and in proportions determined in previously performed trials (Table 1). Immunohistochemical analysis staining was performed using a preprepared commercial kit for the application of the SABC technique, and all applications were performed in compliance with recommended standard procedures. The positive controls recommended by the manufacturing firms

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Table 1 Antibodies used in the study and their characteristic features

| Antibody (clone) | Type of antibody | Host of the antibody | Method for the release of the antigen | Dilution used | Duration of incubation |
|------------------|------------------|----------------------|---------------------------------------|--------------|------------------------|
| IGF-1 orb10886 Biorbyt | Polyclonal | Rabbit | Citrated | 1/300 | 1 night |
| VEGF Sc-9070 | Polyclonal | Rabbit | Citrated | 1/50–500 | 1 night |
| Ki67 Sc-30069 | Monoclonal | Rabbit | Citrated | 1/50–500 | 1 night |
for primary antibodies were used, and PBS (pH 7.4) was used as a negative control. Accordingly, cut sections were placed on slides coated with acetone 3-ethoxypropylamine (catalogue no:8.21619, 100 mL acetone, 2 mL 3-ethoxypropylamine, Merck, Darmstadt, Germany) and kept for 30 minutes at 58°C in an incubator. Sections were deparaffinized in xylol and dehydrated in a series of diluted alcohol. To eliminate the masking effect of formaldehyde on the antigenic structure of the tissue, the sections were immersed in a citrate buffer solution and boiled for 20 minutes at 600 W in a microwave oven. The tissue sections were left in 3% H2O2 in methanol for 7 minutes to eliminate endogenous peroxidase activity and then kept in a protein blocking serum for 10 minutes. Afterward, tissue sections were left for incubation with primary antibodies in the dilutions indicated in Table 1 for predetermined periods of time. Biotin-labeled secondary antibody was dropped on cut sections, and after waiting 30 minutes, the sections were incubated with streptavidin peroxidase enzyme for 30 minutes. After all procedures (except for incubation with the protein-blocking serum), the tissue sections were rinsed twice for a period of 5 minutes with PBS. Finally, tissue sections were stained with chromogen dye for 10 minutes under a microscope and counterstained with Gill’s hematoxylin. The tissue sections were covered with immune adhesives and examined under a light microscope.

Evaluation of the results of the immunohistochemical staining

Assessment of Ki67 immunohistochemical staining
Liver tissue sections immunohistochemically stained with Ki67 were examined on 10 randomly selected microscopic fields of view (360,000 μm²) under ×40 magnification, and the ratio between the number of positively stained cells and the total number of cells was calculated and expressed as percentages.8

Assessment of insulin-like growth factor-I immunohistochemical staining
Liver tissue sections immunohistochemically stained with insulin-like growth factor (IGF-I) were examined on 10 randomly selected microscopic fields of view at ×20 magnification and evaluated for the intensity and distribution of immune dyes. Positive staining on one field of view was graded as follows: no staining, 0; weakly stained, 1; moderately stained, 2; strongly stained, 3. Distribution of immunohistochemical staining was calculated based on the percentage of staining intensity of each field of view. Last, the percentage of positively stained cells on each field was multiplied by their staining intensity, and their sum was calculated. Immunohistopositivity values were expressed as percentages.9

Statistical analysis

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) for Windows (version 21.0, SPSS Inc, Chicago, Illinois). The normality assumption was analyzed using the Shapiro-Wilk test. In the comparison of data that fitted to a normal distribution, 1-way analysis of variance (ANOVA) was used. For multiple comparisons, Tukey’s honest significant difference (HSD) and Tamhane’s T2 tests were used. In the distribution of categorical data, the χ² test was used. All results were expressed as mean ± SD. A value of ≤ 0.05 was considered statistically significant.

Results

Biochemical data

MDA levels of liver tissue were analyzed, and hepatic MDA levels in the hyper-IR group were found to be significantly lower (P = 0.002) than those of the hypo-IR and euthyroid-IR groups (Fig. 1). However, MDA levels did not differ between control groups (P > 0.05).

Tissue CAT levels did not differ between control groups, whereas CAT values in the hyper-IR group were detected to be significantly lower than in the euthyroid-IR and hypothyroid-IR groups (P = 0.003; Fig. 2).

No intragroup or intergroup differences were observed regarding tissue GPX or SOD values (P > 0.05).

Data about thyroid tissue

Thyroid glands of the euthyroid rats
Histologic examination of the thyroid glands in this group revealed normal-appearing thyroid glands consisting of round or oval follicles with variable sizes, lined mostly with low cuboidal epithelium and containing a scarce number of vacuoli, as well as hypodense colloid material.5,10 The thickness of the follicular epithelium was nearly 5 μm (Fig. 3).

Thyroid glands of the hypothyroid rats
Thyroid follicles had variable sizes. They did not contain colloid material, and the follicular epithelium...
um consisted mostly of high prismatic cells with eosinophilic and vacuolar cytoplasm containing large hyperchromatic nuclei. The follicular epithelium protruded into the lumen. The thickness of the follicular epithelium was nearly 15 μm (Fig. 3).5,10,11

Thyroid glands of the hyperthyroid rats
Mostly large follicles containing abundant amounts of colloid material and lined with squamous epithelium were observed. The thickness of the follicular epithelium was nearly 5 μm (Fig. 3).5,10

Histopathologic evaluation of liver tissue
Histopathologic changes in the liver tissue were semiquantitatively evaluated based on the method described by Camargo et al7 (Fig. 4). A statistically significant difference was not found in the tissue examination (P > 0.05).

Results of immunostaining for IGF-I in the liver tissue
IGF-I immunostaining revealed homogenous or red-colored granules in the cytoplasm of hepatocytes due to the presence of 3-amino-9-ethylcarbazole.
results of immunohistochemical staining of liver tissue with Ki67

The nuclei of the hepatocytes turned red during Ki67 immunostaining due to the intranuclear presence of AEC chromogen. Pale blue cell nuclei were seen after the counterstaining of cell nuclei with hematoxylin.

Discussion

The hypothesis underlying the present study is to investigate the “thyroid state” in hepatic IR injury. In our study, pretreatment with 3,3’,5-Triiodo-L-
thyronine (T3) decreased hepatic IR injury and lipid peroxidation. The levels of CAT, which assumes defensive roles against antioxidant injury, were decreased in hyperthyroidism, although they did not change in hypothyroidism. In addition, levels of SOD and GPX did not change with thyroid state.

Given the effects of thyroid hormones on basal metabolic rate and oxygen consumption, their association with the production of ROS is already acknowledged.\textsuperscript{12,13} At the same time, they play a role in postischemic repair in various tissues. Thyroid hormone treatment decreases the levels of free oxygen radicals in the kidney\textsuperscript{14} and increases inulin clearance\textsuperscript{15} and alveolar fluid clearance in intact and hyperoxia-injured lung tissue\textsuperscript{16}; it also improves cardiac function in the myocardium after myocardial infarction.\textsuperscript{17–19} The liver is the major organ in which many metabolic reactions—such as synthesis, biotransformation, and secretion—are realized. Ischemia-induced alterations in liver func-

Fig. 5  IGF-I immunopositivity after IR injury induced in the livers of rats with altered thyroid functions. (a) Hypointense diffuse IGF-I immunopositivity. (b) Moderately intense diffuse IGF-I immunopositivity. (c) Diffuse IGF-I immunopositivity with moderate-severe intensity IGF-I. (d) Diffuse IGF-I immunopositivity with severe intensity; SABP-hematoxylin, \( \times 20 \).

Fig. 6  Ki67 immunopositivity of hepatocytes associated with IR injury induced in the livers of rats with altered thyroid functions. (a) Ki67 immunopositivity in the nuclei of hepatocytes around the portal region (arrows). (b) Ki67 immunopositivity in the nuclei of hepatocytes around the vena centralis (arrows). (c and d) Ki67 immunopositivity in the nuclei of hepatocytes (arrows); SABP-hematoxylin (a and b, \( \times 20 \); c and d, \( \times 40 \)).
tion may affect the homeostasis of the entire body. Production of free O₂ radicals induced by ischemia affects all major cellular components, including lipids, proteins, and DNA. MDA is the end-product of lipid peroxidation in oxidative stress. Therefore, levels of MDA may offer information about the metabolism of free radicals and the state of lipid peroxidation. In a recent study, hepatic tissue MDA values in the IR hyperthyroid group were found to be significantly lower when compared with the hypothyroid and euthyroid IR groups. However, no significant difference was found between the hypothyroid IR and euthyroid IR groups.

The observed decrease in tissue MDA concentration may indicate the decreased levels of lipid peroxidation after T3 administration. Similar to our study, in a study by Taki-Eldin et al, lower hepatic MDA levels were found in the hyperthyroid group, which received T3 48 hours before IR injury. T3 administered a short time before renal IR injury exerts favorable effects, and when given 6 hours prior to renal IR, it is renoprotective. It also decreases proteinuria and lipid peroxidation when given 24 hours before IR injury. However, contrary to these results, Venditti et al and Messarah et al administered thyroid hormone to the hyperthyroid rats, in which the IR model was not induced for 5 weeks and 10 days, and they reported increased MDA levels following treatment with thyroid hormone. In pathologic processes such as cellular oxidative stress, ROS are produced in excess of those eliminated by cellular defense mechanisms. Antioxidants such as SOD, CAT, and GPX, which are involved in cellular defense mechanisms, convert free oxygen radicals into less potent novel molecules, and levels of these enzymes provide information about oxidative stress. In our study, intergroup differences were not observed with respect to the levels of antioxidant enzymes such as GPX and SOD, whereas in parallel with tissue MDA levels, levels of CAT were found to be significantly lower in the hyperthyroid IR group than in the hypothyroid IR and euthyroid IR groups. However, levels of CAT did not differ between control groups. According to these results, administration of thyroid hormones seems to decrease oxidative stress. Similarly, Fernandez et al reported that the administration of a single dose of T3 exerts significant protection against hepatocellular injury induced by IR. However, some studies have reported that thyroid hormones increase the hepato-cellular injury. Application of thyroid hormone for 5 weeks increased SOD and CAT levels, whereas the use of thyroid hormone therapy for 4 weeks increased CAT and GPX levels. Dissimilar results obtained about the effect of thyroid state on antioxidative status may stem from differences in the doses of thyroid hormone, the duration of its application, or the inability to induce an IR model in all of these studies.

In our study, histopathologic examination with H&E staining revealed changes in the thyroid tissues of rats consistent with hypothyroidism and hyperthyroidism. Liver tissues were evaluated according to the presence of cytoplasmic vacuolization, nuclear pyknosis, and necrosis. No significant intergroup differences were detected. Lack of any intergroup differences in our study suggests that 90 minutes of reperfusion is not sufficiently long for anticipated tissue response. Fernandez et al demonstrated the protective effect of a single dose of T3 in rats following one hour of ischemia and with a 20-hour reperfusion period. Similarly, Taki-Eldin et al emphasized that during longer periods of reperfusion, T3 decreases neutrophilic infiltration and expression of proinflammatory cytokines, transcription factors, and adhesion molecules.

There are several limitations in this study. In the group with hyperthyroidism, it might be useful to administer thyroid hormone for various time periods. IR injury still continues to be an intraoperative problem in liver transplant patients. Starting from this fact, we wanted to see the effects of thyroid state on the acute intraoperative period, and we restricted the period of reperfusion to 90 minutes. A trial of longer reperfusion times and acute phase reactants might be more useful.

In conclusion, the administration of thyroid hormone 48 hours before IR injury attenuates lipid peroxidation and may exert protective effects. However, given the differing results cited in the literature, comparative studies related to the duration of thyroid hormone therapy are needed.

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content and writing of the paper. The experimental protocol of this study was reviewed and approved by the Animal Care and Use Committee of Ondokuz Mayis University, Samsun, Turkey (Ethical Committee No. B.30.2.0DM.020.09.00-050.04-97-18.12.2012) and carried out according to the Animals in Research: Reporting InVivo Experiments (ARRIVE) statement. Also this manuscript adheres to the Enhancing the Quality and Transparency of Health Research (EQUATOR) guidelines. Informed consent was obtained from all individual participants included in this study.

References

1. Chen XB, Xu MQ. Primary graft dysfunction after liver transplantation. Hepatobiliary Pancreat Dis Int 2014;13(2):125–137.
2. Videla LA. Oxidative stress signaling underlying liver disease and hepatoprotective mechanisms. World J Hepatol 2009;1(1):72–78.
3. Yehuda-Shnaidman E, Kalderon B, Bar-Tana J. Thyroid hormone, thyromimetics, and metabolic efficiency. Endocr Rev 2014;35(1):35–58.
4. Feng X, Jiang Y, Meltzer P, Yen PM. Thyroid hormone regulation of hepatic genes in vivo detected by complementary DNA microarray. Mol Endocrinol 2000;14(7):947–955.
5. Celik I, Suzek H. Subacute effects of methyl parathion on antioxidant defense systems and lipid peroxidation in rats. Food Chem Toxicol 2008;46(8):2796–2801.
6. Serakides R, Nunes VA, Santos RL, Cassali GD, Costa Neto PP. Histomorphometry and quantification of necrocellular organizing regions in bovine thyroid containing methylthiouracil residues. Vet Pathol 1999;36(6):574–582.
7. Camargo CA, Jr., Madden JF, Gao W, Selvan RS, Clavien PA. Interleukin-6 protects liver against warm ischemia/reperfusion injury and promotes hepatocyte proliferation in the rodent. Hepatology 1997;26(6):1513–1520.
8. Sozmen M, Tunca R, Dag Erginsoy S. Cyclin A expression is associated with apoptosis and mitosis in murine 3-methylcholanthrene-induced fibrosarcomas. Exp Toxicol Pathol 2009;61(1):41–49.
9. Sozmen M, Beytut E. An investigation of growth factors and lactoferrin in naturally occurring ovine pulmonary adenomatosis. J Comp Pathol 2012;147(4):441–451.
10. Ferreira E, Silva AE, Serakides R, Gomes AES, Cassali GD. Model of induction of thyroid dysfunctions in adult female mice. Arquivo Brasileiro Med Vet Zoot 2007;59:1245–1249.
11. Charles C, Capen RAD, Yarrington JT. Endocrine system. In: Haschek WM, Rousseaux CG, eds. Handbook of Toxicologic Pathology. 1st ed. UK: Academic Press, 1991:705–707.
liver lipid peroxidation and antioxidant status in experimental rats. Exp Toxicol Pathol 2010;62(3):301–310
26. Fernandez V, Castillo I, Tapia G, Romanque P, Uribe-Echevarría S, Uribe M et al. Thyroid hormone preconditioning: protection against ischemia-reperfusion liver injury in the rat. Hepatology 2007;45(1):170–177
27. Messarah M, Boulakoud MS, Boumendjel A, Abdennour C, El Feki A. The impact of thyroid activity variations on some oxidizing-stress parameters in rats. C R Biol 2007;330:107–112
28. Kong L, Wei Q, Fedail JS, Shi F, Nagaoka K, Watanabe G. Effects of thyroid hormones on the antioxidative status in the uterus of young adult rats. J Reprod Dev 2015;61(3):219–227
29. Fernandez V, Tapia G, Varela P, Gaete L, Vera G, Mora C et al. Causal role of oxidative stress in liver preconditioning by thyroid hormone in rats. Free Radic Biol Med 2008;44(9):1724–1731