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Research Article

The Link Between Oxidative Stress Response and Tumor Necrosis Factor-Alpha (TNF-alpha) in Hepatic Tissue of Rats With Induced Thyroid Dysfunction

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ABSTRACT

Thyroid hormones are essential for normal organ growth, development and function. They regulate the basal metabolic rate of different types of cells, including hepatocytes. Oxidative stress plays an essential role in the pathogenesis of thyroid disorders and disturbed tissue functions. Tumor necrosis factor-alpha (TNF-α) is a cytokine with numerous immunological and metabolic activities. Several studies relate this cytokine to thyroid dysfunction and chronic liver diseases. This study aims to determine the relationship between the oxidative stress and the hepatic inflammatory cytokines TNF-α in rats with disturbed thyroid functions. Also, to investigate how this can affect normal hepatic tissues. Twenty-seven rats were divided into three groups: euthyroid group, hyperthyroid group; hyperthyroidism was induced by daily intraperitoneal injection of L-thyroxine and hypothyroid group; hypothyroidism was induced by administering propylthiouracil using oral gastric tube. At the end of the experiments, rats were fasted for 12 hours then blood samples were collected to measure the level of free T_3, free T_4, TSH, serum malondialdehyde (MDA), and total antioxidant (TAC). Then animals were scarified by cervical decapitation, slices of livers were submitted to histopathological examination, the rest of liver tissues were extracted and homogenized for measurements of TNF-α. The data showed that serum MDA was significantly elevated while TAC levels were significantly decreased in both hyperthyroid and hypothyroid groups compared to their corresponding values in euthyroid group. TNF-α level was also significantly increased in hyperthyroid and hypothyroid groups compared to its level in euthyroid group. Histopathological examination of the liver revealed prominent lymphocytic infiltration with some aggregate formation in portal tract in both hypothyroid and hyperthyroid groups. The lymphocytic infiltrate was more prominent in hypothyroid rats compared to the hyperthyroid rats. This study suggests that the oxidative stress and elevated level of TNF-α play an essential role in hepatic cell injury associated with thyroid dysfunction. A complex relationship exists between the thyroid gland and the liver in health and disease.

INTRODUCTION

Thyroid hormones are essential for growth, development and function of all tissues of the body by regulating BMR of different types of cells including hepatocytes. The liver metabolizes thyroid hormones and regulates their systemic effects. Therefore thyroid dysfunction may disturb liver functions and liver diseases modulate thyroid hormones metabolism (Malik & Hodgson, 2002).

It is proved that a state of hypermetabolism occurs as a result of thyroid hormone administration to experimental animals. This hypermetabolic state is accompanied by oxidative stress in several target tissues (Venditti *et al*, 2006). This oxidative stress is due to increased formation of free radicals in mitochondria (Aslan et al, 2011).

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Hypothyroidism is associated with lower metabolic rate. So, it is expected to lower free radical production. However, many studies found that hypothyroidism is associated with oxidative stress (Torun et al, 2009), and elevated lipid peroxidation (Konukoglu et al, 2002).

Free radicals can oxidize various cellular substances including DNA, proteins and lipids leading to alterations in normal cell and tissue functions. Normally this oxidation process is minimized by a fairly complex system of antioxidant defenses. However, when free radical generation exceeds the antioxidant capacity of cells, oxidative stress develops (Videla, 2000). In hyperthyroidism measurable changes occur in the anti oxidant capacity of the cell, leading to the development of oxidative stress (Kumari, et al, 2011).

Moreover, the experimental hyperthyroidism was found to be associated with increased hepatic levels of oxidized lipids and proteins. This causes an enhanced rate of free radical production in the liver (Venditti et al, 2004). Also, hyperthyroidism enhances the prooxidant activity of the liver by increasing the activity of nitric oxide synthase enzyme. This effect is exerted at hepatocytes and Kupffer cells levels (Ferna´ndez et al, 1997).

TNF-α is a multifunctional cytokine 17-kDa protein consisting of 157 amino acids that is a homotrimer in solution. In humans, the gene is mapped to chromosome 6. Its bioactivity is mainly regulated by soluble TNF-α–binding receptors. TNF-α is mainly produced by activated macrophages, T lymphocytes, and natural killer (NK) cells. TNF- α is an important mediator of inflammatory and immune functions therefore; it is linked to the pathogenesis of many inflammatory and autoimmune diseases such as Graves' disease (Gu et al, 2010).

Liver injury caused by thyrotoxicosis is relatively common. The mechanism of injury appears to be due to relative hypoxia in the perivenular regions, due to an increase in hepatic oxygen demand. There is also an evidence that hypothyroidism may directly affect the liver structure or function (Malik & Hodgson 2002). Many previous studies reflected a strong relationship between thyroid and liver in health and disease.

In this investigation we studied the relationship between oxidative stress and hepatic inflammatory cytokines TNF-α in rats with disturbed thyroid functions and investigated how this can affect the histopathology of hepatic tissues.

MATERIALS AND METHODS

Twenty seven male Wister adult albino rats of local strain weighing 150:200 grams were used in this study. Animals were fed with standard laboratory chow and water "ad libitum" and housed in animal house at faculty of Medicine Menoufiya University under artificial light/dark cycle of 12hours. The animals were acclimatized to these conditions for 10 days before the experiment. The animals were housed in groups of 9 each in identical wire-bottomed cages and were divided into 3 groups: Euthyroid (control) group: This group was not subjected to any procedure, except receiving daily intraperitoneal injections of 0.9% saline solution. Hyperthyroid group: the induction of hyperthyroidism in this group was done by daily intraperitoneal injection of L-thyroxine (T4) (0.1µg/g) for 4 weeks (Kobori et al, 1997). Hypothyroid group: The hypothyroidism was induced by administering propylthiouracil (PTU) by oral gastric tube at a dose of 1mg/kg for 30 days (Silva et al, 1984).

Blood sampling and assay:

After 12 hours of fasting, blood samples were collected from the retro-orbital venous plexus of rats, using fine heparinized capillary tubes that were introduced into the medical epicanthi of rat's eye (Schermer, 1968). 4 ml of blood were collected then delivered to serum separator tubes and allowed to clot for 30 minute at room temperature. Then the serum was separated by centrifugation at 3000 rpm for 10 minute. Sera were divided into aliquots and kept in tightly closed aliquots at – 20 °C until analysis of the following, thyroid function tests (TSH, Free T3 and Free T4). Quantitative measurement of TSH in serum was done by using the immulite 1000 analyzer using a solid phase two site chemiluminescent immunometric assay capable of measuring TSH at low concentration (Nicoloff and Spencer, 1990). Quantitative measurement of non-protein bound thyroxine (free T3 and free T4) was done by using immulite 1000 using direct one step hormone analogue immunoassay (Ekins,1990). Colometeric estimation of malondialdehyde (MDA) was done by using the protocol described in Deniz et al (1997) by using thiobarbituric acid reactive substance for measuring the peroxidation of fatty acids as oxidative stress marker. The assay of total antioxidant capacity was done according to Koracevic (2001), by allowing the reaction of antioxidants in the sample with a defined amount of exogenously provided hydrogen peroxide (H_2O_2). The antioxidants in the sample eliminate a certain amount of the provided (H_2O_2). The residual H_2O_2 as determined colorimetrically.

Tissue preparation:

The animals were scarified by cervical decapitation under ether anaesthesia. The livers were rapidly excised and placed into petri dishes containing ice-cold

isolation medium (IM) consisting of 125 mM KCl, 15 mM Tris with pH equals 7.4. Livers were freed from connective tissue. After weighing the tissues, 20% homogenates were prepared with a Potter–Elvehjem homogenizer set at a standard velocity of 500 rpm for 2 min in the isolation medium.

Tissue extract and assay:

Samples of 10% (w/v) homogenates were obtained by diluting the 20% homogenates with equal volumes of 0.2% Lubrol in 15 mM Tris with pH 8·5. Several dilutions of samples were done up to a tissue concentration of 0.002% in 15 mM Tris with pH 8.5. Tissue extracts were kept in aliquots in -20˚C until the time of assay of rat-TNF- α. Its measurement was done according to the protocol described in Brouckaert et al (1993) by using enzyme linked immunosorbent assay for quantitative measurement of rat TNF- α tissue lysate.

Histopathological examination of the liver:

The dissected livers from different groups were sent to the Pathology Department, Faculty of Medicine Menoufiya University, where they were submitted to routine tissue processing, including fixation in 10% neutral buffered formalin then dehydration in ascending grades of ethanol followed by immersion in xylene and finally impregnation in paraffin. 4 micron thick sections were cut from paraffin embedded blocks to be stained by haematoxylin and eosin stains for histopathological examination.

Statistical analysis

The data were tabulated and analyzed by SPSS (statistical package for the social science software) using statistical package version 11 on IBM compatible computer. Quantitative data were expressed as mean ± standard deviation of mean (X± SD). The data from control and test groups were compared using an independent Student's t-test. Probability value of less than 0.05 was considered as statistically significant (* P < 0.05). "n" indicates the number of tested rats.

RESULTS

Induction of thyroid dysfunction:

Table 1 shows that administration of L-thyroxine to rats significantly increase the level of free T3 (8.45±0.95), free T4 (4.67±0.75) and significantly decreased the level of TSH (0.055±0.017) compared with euthyroid group (3.19±0.52, 1.44±0.32 and 0.81±0.62 respectively). Administration of PTU significantly decreased the level of free T3 (0.98±0.10), free T4 (0.63±0.18) and significantly increased the level of TSH (4.99±0.21) compared with euthyroid group.

Table 1:

Hormonal laboratory findings in euthyroid, hyperthyroid and hypothyroid groups.

Hormonal levels	Euthyroid Group	Hyperthyroid Group	Hypothyroid Group
Free T3 (pg/dl)	3.19±0.52	8.45±0.95	0.98±0.10
P value		*<0.001*	*<0.001*
Free T4 (ng/dl)	1.44±0.32	4.67±0.75	0.63±0.18
P value		*<0.001*	*<0.001*
TSH (uIU/ml)	0.81±0.62	0.055±0.017	4.99±0.21
P value		*<0.01*	*<0.001*

Results are expressed as mean ±SD (n=9).; P value: compared to euthyroid group.

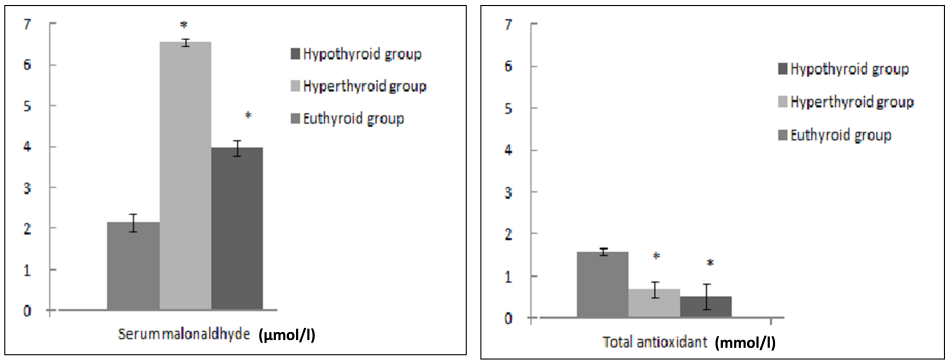

Figure 1:

The effect of disturbed thyroid functions on the level of serum malondialdehyde (µmol/l) and total antioxidant status (mmol/l). Results are expressed as mean ±SD (n=9); *Significant when compared to euthyroid group.

Oxidative stress in thyroid dysfunction:

Figure 1 shows that serum MDA was significantly increased in both hyperthyroid and hypothyroid groups (6.55±1.13 and 3.97±0.74) respectively compared with euthyroid group (2.15±0.65). TAC levels were significantly decreased in both hyperthyroid and hypothyroid groups (0.68±0.57 and 0.53±0.39) when compared to their corresponding value in euthyroid group (1.58±0.28).

Table 2:
The effect of disturbed thyroid functions on the level of TNF- α.

	Euthyroid group	Hyperthyroid group	Hypothyroid group
TNF- (pg/m	47.20±18.71	748.88±84.20	1288.03±327.5′
P valu		*<0.001*	*<0.001*

Results are expressed as mean ±SD (n=9).
P value: compared to euthyroid group.

Serum level of TNF-α in thyroid dysfunction:

Table 2 shows that TNF-α was significantly increased in both hyperthyroid and hypothyroid groups (748.88±84.20 and 1288.03±327.57) respectively when compared with euthyroid group (47.20±18.71).

Pathological changes in the liver:

Plate 1 shows the pathological changes in the liver. As seen in the upper half of the figure hyperthyroidism produced chronic inflammatory infiltrate in portal tract that spilled over adjacent hepatocytes. In the lower half of the figure prominent lympocytic infiltration with sometimes aggregate formation was seen in portal tract and spread within sinusoids in between cords of hepatocytes of hypothyroid rats and the the adjacent hepatocytes appeared normal without any evidence of degeneration, fatty changes or necrosis.

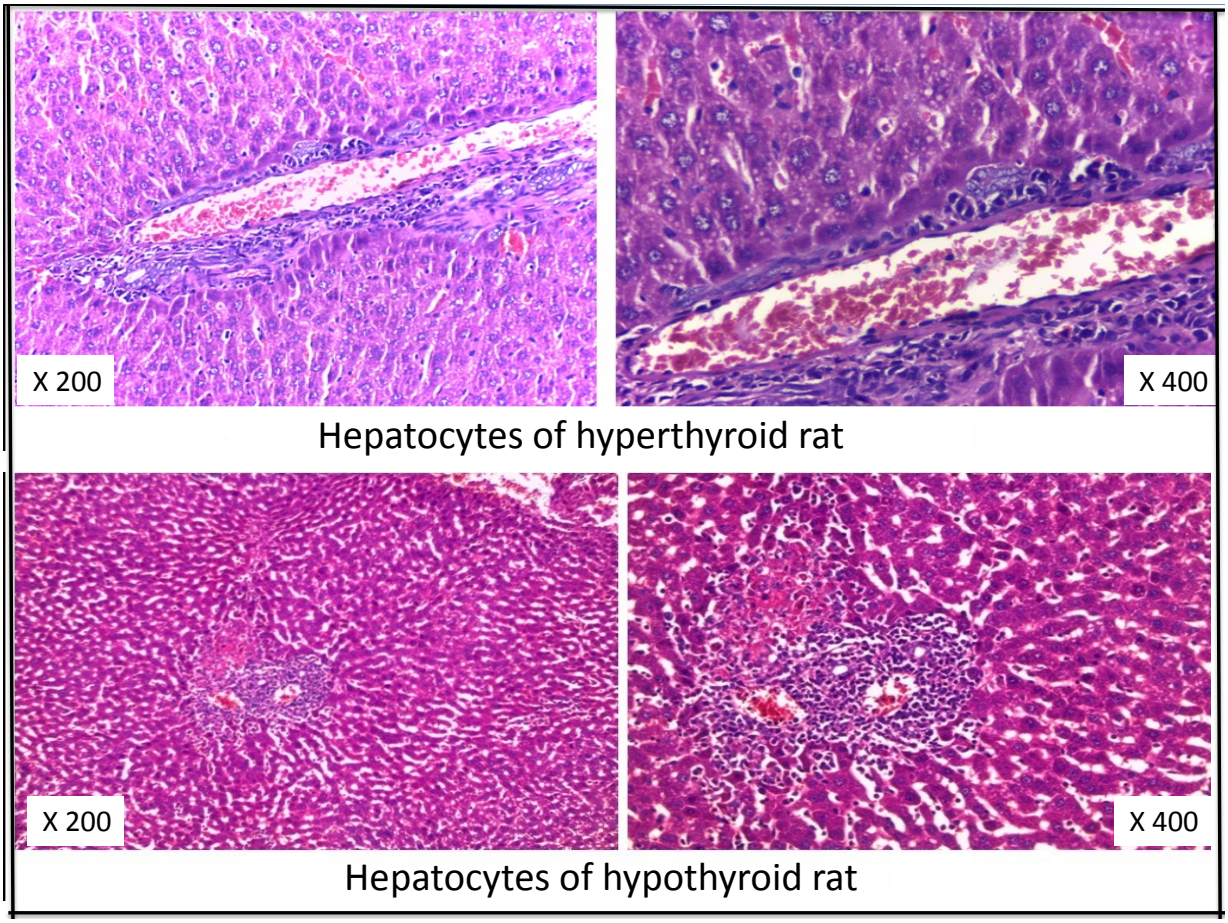

Plate 1:
Histopathological changes in the liver of hyperthyroid and hypothyroid groups
Haematoxylin and eosin stained sections with magnification power X 200 and X 400

DISCUSSION

The liver has an essential role in thyroid hormone metabolism and thyroid hormones are important to normal hepatic functions. Associations between thyroid and liver diseases of an autoimmune nature were found, such as that between primary biliary cirrhosis and hypothyroidism. Also, thyroid diseases are frequently associated with liver injuries or biochemical test abnormalities.

The study showed that the induced hyperthyroid and hypothyroid states produced oxidative stress as indicated by significant increase in the level of MDA and significant decrease in the TAC levels. Also, TNF-α was found to be significantly increased in both groups. Hepatic tissue cells showed pathological changes and lymphocytic infiltration. Rats were confirmed to be hyperthyroid by the elevation of free T_3, free T_4 and decrease in TSH level. While hypothyroid rats were identified by decreased level of free T_3, free T_4 and increase in TSH level.

Oxidative stress can be defined as an imbalance between the oxidant and antioxidant system, with deviation towards the oxidant system. Total antioxidant capacity (TAC) considers the cumulative effect of all antioxidants present in blood and body fluids (Suresh *et al*, 2009). Thyroid hormones are suggested to be linked to oxidative stress because their levels were found to be elevated in different stress conditions.

Thyroid hormones regulate oxidative metabolism and thus play an important role in free radical production (Erdamar et al 2008). They regulate the synthesis and degradation of enzymes, such as superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), and glutathione reductase, and non-enzymatic antioxidants, such as vitamin E & C, glutathione, uric acid, ferritin, transferrin, and ceruloplasmin. Undoubtedly, the changes in these enzymes and non-enzymatic substances affect the redox balance in the body. One of the major effects of thyroid hormones is to increase mitochondrial respiration, which results in upregulation of ROS generation, leading to oxidative damage to membrane lipids (Wang et al, 2011)

The significant increase in the level of MDA in hyperthyroid rats can be explained by the fact that hyperthyroidism is a hypermetabolic state associated with increase in the mitochondrial respiration by increasing the activity of mitochondrial respiratory chain. This may be responsible for the generation of reactive oxygen species. These reactive oxygen species can be initiators of free radical chain reactions that can lead to oxidative damage to membrane lipids and other cellular structures. This oxidative stress was demonstrated in many tissues of hyperthyroid rats specially the liver (Venditti et al, 2004) and it is responsible for increasing the lipid peroxidation in the heart and liver of hyperthyroid rats (Kumari et al 2011). Also, T_3 administration was found to increase the activity of nitric oxide synthase enzyme in the liver. This can contribute to the increase in the prooxidant activity in the liver of hyperthyroid rats (Ferna´ndez et al, 1997).

The induction of hyperthyroid state by T3 administration in animals cause elevation in O_2 consumption by the liver with progressive accumulation of products of hepatic lipid peroxidation and protein oxidation, enhancing the oxidative stress in the liver (Tapia et al, 2003). Moreover, Ferna´ndez et al (1997) showed that in experimental hyperthyroid rats, there was increased activity of NADH-cytochrome P450 reductase and NADPH oxidases in rat liver microsomes. These effects were accompanied by increased levels of lipid peroxidation, suggesting disruption of polyunsaturated fatty acids by free radical oxidation. On the other hand, Venditti et al (2004) attributed the oxidative stress condition to the decrease in the antioxidant capacity in these rats. This reduction in the antioxidant capacity causes more increase in the oxidative stress. Moreover, Aslan et al (2011) observed significant increase in the total oxidant status and significant decrease in the total antioxidant capacity in hyperthyroid patients compared to their controls which indicates oxidative stress; as a result, the oxidative–antioxidative balance is shifted to the oxidative side. Mogulkoc et al (2006) observed that MDA levels in the hyperthyroid group were significantly higher in the cerebral cortex, liver and ventriculer tissue of heart compared to their control group. So, the liver is affected by oxidative stress in hyperthyroidism.

From the previous studies, it can be notice that the data about the effect of hypothyroidism on oxidative stress are conflicting and controversial. In hypothyroidism, the basal metabolic rate is reduced and therefore a reduction in free radical production is expected because of the metabolic suppression caused by low thyroid hormone levels.

In the present study the hypothyroid state was found to be associated with increase in the oxidative stress which was noticed by the significant increase in the level of MDA and the significant decrease in the level of TAC. The mechanism of increased oxidative stress in hypothyroidism is attributed to insufficient antioxidant defense system because thyroid hormones are important in the synthesis and degradation of antioxidant enzymes.

Also, a chronic state of hypothyroidism is characterized by impairment in the redox potential leading to free radical chain reactions and metabolic suppression of antioxidant capacity. The oxidative stress may be due to increased free radical production which was not compensated by insufficient antioxidants (Babu et al, 2011). This hypothyroid induced oxidative stress occurs in the liver, heart and skeletal muscles with consequent lipid peroxidative response (Nanda et al, 2008).

The data in the present study are similar to that obtained by Konukoglu et al (2002) who found that the level of antioxidant decreased in hypothyroid patients. and Venditi et al (2004) who found that the antioxidant capacity in hypothyroid rats was low compared to the euthyroid rats. On the other hand, Coria et al (2009) demonstrated no significant change in the parameters of oxidative stress between hypothyroid and euthyroid women.

Also, Venditi et al (2006) showed that the levels of antioxidants are not affected equally in different tissues of hypothyroid rats; some increased, others decreased or unchanged. Torun et al (2009) found that the level of TAC was insignificantly lower in hypothyroid patients than in normal controls. The response of the antioxidant systems to both hypothyroidism and hyperthyroidism is unclear (Ferna´ndez et al, 1997).

The changes in the levels and activities of antioxidant enzymes in various tissues were found to be imbalanced and often opposite. Furthermore, there is disagreement on the effect of hyperthyroidism on the level of some antioxidants in the liver as glutathione peroxidase, which has been reported to both decrease and increase. This can be explained by the complexity of the intracellular network of various antioxidants that makes it difficult to understand the overall protective efficacy of the cellular defense system (Venditti et al, 2004).

In the present study TNF-α was found to be significantly increased in both hyperthyroid and hypothyroid groups when compared to the euthyroid group. The cytokines are group of polypeptide mediators. They are not only an integral part of normal homeostasis, but also have various biological effects in many pathophysiological processes. TNF-α and interleukin-1 may attract polymorph nuclear leucocytes (PMNs) to the site of inflammation. Which is a direct stimulus for the respiratory burst, and the generation of reactive oxygen-derived free radicals and their metabolites (Teoh and Farrell, 2003). The increase in respiratory burst activity of Kupffer cells could increases the expression of redox-sensitive genes, including those coding for cytokines. This oxidative stress would markedly increase the production of TNF-α by Kupffer cells and its release into the circulation (Ferna´ndez et al, 2002).

Kupffer cell hyperplasia is a major finding after in-vivo T_3 administration, an effect that may involve the expansion of Kupffer cell precursors or the differentiation of pre-existing local Kupffer cell precursors into mature liver macrophages, or both (Tapia et al, 2003).

In hyperthyroidism the elevation of TNF- α may be induced by an increase in its production by Kupffer cells of the liver. Ferna´ndez et al (2002) found that hyperthyroidism elicited 80-fold increase in the serum levels of TNF-α after 22 hours from T_3 administration to rats. They also proved that by pretreatment with Kupffer cell inactivator gadolinium chloride ($GdCl_3$) which virtually abolished this effect and markedly reduced the T_3-induced liver oxidative stress and the TNF-α level. In Hypothyroidism high TSH level may be responsible for the secretion of inflammatory cytokines.

Ashok et al (2007) observed an elevation in the levels of death legends', TNF-α, Fas ligand and their cognate receptors, TNF-α -receptor-1 and Fas, and 8-fold increase in caspase-8 activation lead to apoptosis in hyperthyroid rat liver. Chopra et al (1991) observed an elevation in the level of TNF-α in both hyperthyroid and hypothyroid patients. However, there was no significant correlation between serum levels of TNF-α and serum T_4 or T_3.

Also, Díez et al (2002) confirmed the activation of the TNF-α system in patients with thyroid dysfunction. They found high plasma concentrations of TNF-α and soluble receptor for TNF-α in patient with hypothyroidism and hyperthyroidism.

The sequential steps of the TNF-α induced death receptor pathway have been well characterized as follow: Binding of TNF-α to TNF-α receptor 1 (TNFR1) results in receptor trimerization and the recruitment of a series of intracellular proteins that ultimately bind caspase-8, leading to its activation. Activated caspase-8 initiates a proteolytic cascade that results in release of lysosomal cathepsin B, cleavage of the proapoptotic BCl-2 family member, initiation of the mitochondrial death pathway with release of cytochrome c, and activation of downstream effector caspases that ultimately induce apoptosis (Yongjun et al, 2006).

In the present study histological examination of the liver in hyperthyroidism and hypothyroidism showed chronic inflammatory infiltration in portal tract that spilled over the adjacent hepatocytes this was in agreement with the study of Malik and Hodgson (2002) which showed mild lobular inflammatory infiltrate consisting of polymorphic neutrophils, eosinophils and

lymphocytes, associated with nuclear changes and Kupffer cell hyperplasia. According to other studies progressive liver necrosis is manifested histologically by centrizonal necrosis. Perivenular fibrosis was also reported in a small proportion of patients suffering from thyrotoxicosis due to the effect of hypoxia. In hypothyroid state, the presence of portal mononuclear inflammatory infiltrate was also described by others (Mostaghni et al, 2008). The latter authors induced hypothyroism in sheep and found other changes such as fatty changes and bile duct proliferation, which was not seen in our trial. An association exists between Hashimoto's thyroiditis and hypothyroidism with autoimmune liver diseases such as chronic active hepatitis (Tran et al, 1993).

From the present study, it can be concluded that there is a significant relationship between oxidative stress and elevation of TNF-α in hepatic tissues in rats with disturbed thyroid functions. A complex relationship exists between the thyroid gland and the liver in both health and disease. A multisystem approach to treat patients with diseases affecting either organ is vital. Our study revealed thyroid-liver associations which can cause diagnostic confusions. Neglecting these associations results in over or under diagnosis of associated liver or thyroid diseases and thereby causes errors in patient's care. It is advisable to measure free T_3, free T_4 and TSH in patients with liver disease and to assess liver functions in patients with hyper or hypothyroidism.

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