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ORIGINAL ARTICLE

Erythrocyte osmotic fragility and lipid peroxidation in experimental hyperthyroidism

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Abstract This study investigated the relation between erythrocyte osmotic fragility and oxidative stress and antioxidant state in primary hyperthyroidism induced experimental rats. Twenty-four Spraque–Dawley-type female rats weighing between 160 and 200 g were divided into two, as control ($n = 10$) and experimental ($n = 12$), groups. The experimental group animals have received tap water and L-Tiroksin (0.4 mg/100 g fodder) added standard fodder for 30 days to induce hyperthyroidism. Control group animals were fed tap water and standard fodder for the same period. Blood samples were drawn from the abdominal aorta of the rats under ether anesthesia. T_3, T_4, and TSH levels, osmotic fragility, malondialdehyde (MDA), superoxide dismutase, and glutathione levels were measured in the blood. There was a statistically significant deviation found in maximum and minimum osmotic hemolysis limit values of experimental group when compared to controls. The standard hemolytic increment curve of the hyperthyroid group shifted to the right when compared to control group's curve. There was a statistically significant increase found in MDA and superoxide dismutase, but statistically a significant decrease was detected in glutathione levels in hyperthyroid group when compared to controls. As a result of our study, it may be concluded that hyperthyroidism may led to an increase in osmotic fragility of erythrocytes and this situation may possibly originate from the increased lipid peroxidation in hyperthyroidism.

Keywords Osmotic fragility · Hyperthyroidism · Lipid peroxidation · Rat

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Introduction

Oxidative stress is described as impairment of equilibrium between prooxidant and antioxidant systems. Under physiological conditions, there is equilibrium between oxidants and antioxidants that are generated during normal aerobic metabolism and their detoxification [1]. Whenever additional aerobic oxidants are generated, lipids, carbohydrates, proteins, and nucleic acids undergo oxidative damage and the equilibrium is disrupted. Lipid peroxidations are the automatic chain reactions that produce radicals in cell membranes [2]. Lipid peroxidation can be easily determined in plasma and accepted as an indicator of oxidative stress. The most widely measured lipid peroxidation product is malondialdehyde (MDA) [3]. Products of lipid peroxidation may cause profound alteration in the structural organization and function of the cell membrane including decreased membrane fluidity, increased membrane permeability, inactivation of membrane bound enzymes, and loss of essential fatty acids [4]. Owing to the greater concentration of polyunsaturated fatty acids in their membrane than that in other cells and intracellular oxygen and hemoglobin content, erythrocytes are sensitive to oxidative stress. On the other hand, defense mechanisms of erythrocytes are also well developed and they contain a greater concentration of copper–zinc superoxide dismutase

CuZnSOD (enzymatic). Erythrocytes also contain, as thiol-specific antioxidant, glutathione (GSH) (nonenzymatic). Peroxidation of unsaturated chains of membrane lipids increases the erythrocyte osmotic fragility and lysis of the cell [5, 6].

Osmotic fragility, the sensitivity to change in osmotic pressure characteristic of red blood cells, has been found to be altered in various pathological conditions. In various diseases, greater osmotic fragility limits as a result of increased lipid peroxidation were described [6, 7]. The measurement of osmotic fragility of erythrocytes has been applied to the diagnosis of hemolytic diseases, studies of membrane permeability, and alterations that led to destruction of erythrocytes [6]. The osmotic fragility test of red cells is often performed to evaluate the sensitivity of erythrocytes to hypotonic saline, i.e., this test reflects the ability of RBC to take up a certain amount of water before lysing. The ability of normal red cell to withstand hypotonicity results from its biconcave shape, which allows the cell to increase its volume by about 70% before the surface membrane is stretched. Once this limit is reached, lysis occurs [8]. Therefore, measurement of osmotic fragility provides a useful indicator of whether a patient's red cells are normal or not. There is a positive relationship between lipid peroxidation, and osmotic fragility has been identified in various diseases [6, 8].

Increased thyroid hormone concentrations affect the oxidant/antioxidant equilibrium and may cause harm to the cell. As is well known, in over secretion of thyroid hormones, metabolic reactions are accelerated, oxygen consumption increases, and free radical production is increased as well. Thyroid hormones, while leading to increase in free radicals also activate the antioxidant enzymes. As energy needs increases in hyperthyroidism, oxidants accumulate in the cell. Thyroid hormones modulate the free radical induced oxidative damage of lipids [9, 10]. So elevated levels of thyroid hormones constitute a risk of oxidant stress for cells.

The purpose of this study is to determine the structural integrity of red blood cells in experimental hyperthyroidism by assessing the osmotic fragility of erythrocytes in primary hyperthyroidism and its relation between lipid peroxidation.

Results

The statistically significant increase in T_3, T_4 and the significant decrease in TSH ($P < 0.001$) of experimental group is the evidence of induced primary hyperthyroidism (Table 1). There was a statistically significant increase found in erythrocytes in the lipid peroxidation indicator MDA ($P < 0.001$), and in the antioxidant indicator SOD ($P < 0.001$), but statistically a significant decrease was detected in GSH ($P < 0.05$) levels in hyperthyroid group when compared to controls (Table 1). When the osmotic fragility results were examined, osmotic fragility levels were found to be maximum at 0.32% NaCl and minimum at 0.56% NaCl in control group, whereas erythrocyte osmotic fragility levels were determined maximum at 0.40% NaCl, and minimum at 0.64% NaCl in hyperthyroid group (Fig. 1). When statistical evaluations were compared, statistically important increases were detected in maximum and minimum osmotic fragility limits between both the groups ($P < 0.001$). The standard hemolytic increment curve of the hyperthyroid group drawn according to osmotic fragility test results was found to be shifted to the right when compared to control group's curve (Fig. 2). This finding and hemolytic increment value, which shows maximum hemolysis ratio, is the evidence of increased osmotic fragility of the erythrocytes in hyperthyroidism.

Discussion

Elevated levels of T_3, T_4, and lowered TSH show that hyperthyroidism had been established in rats, after

Table 1 Laboratory data in control and hyperthyroid states of experimental animals

Parameter	Control ($n = 10$)	Hyperthyroid ($n = 12$)
T_3 (ng/100 ml)	63.73 ± 9.74	82.49 ± 4.79***
T_4 (ng/100 ml)	3.98 ± 0.69	5.72 ± 0.48***
TSH (μIU/ml)	0.03 ± 0.01	0.01 ± 0.004***
Plasma MDA (μmol/l)	10.08 ± 1.11	10.92 ± 1.17
Erythrocyte MDA (nmol/gHb)	87.56 ± 10.71	148.69 ± 34.27***
SOD (U/g Hb)	494.95 ± 26.32	599.32 ± 80.94***
GSH (mg/100 ml)	18.57 ± 1.85	16.64 ± 1.49*
Min ORL (%)	0.56 ± 0.02	0.64 ± 0.03
Max ORL (%)	0.32 ± 0.02	0.40 ± 0.03
HI (%)	0.40 ± 0.02	0.44 ± 0.02

ORL osmotic resistance limit, *HI* hemolytic increment, *SOD* superoxide dismutase *GSH* glutathione, *MDA* malondialdehyde

Data are the means ± SD

* $P < 0.05$, *** $P < 0.001$

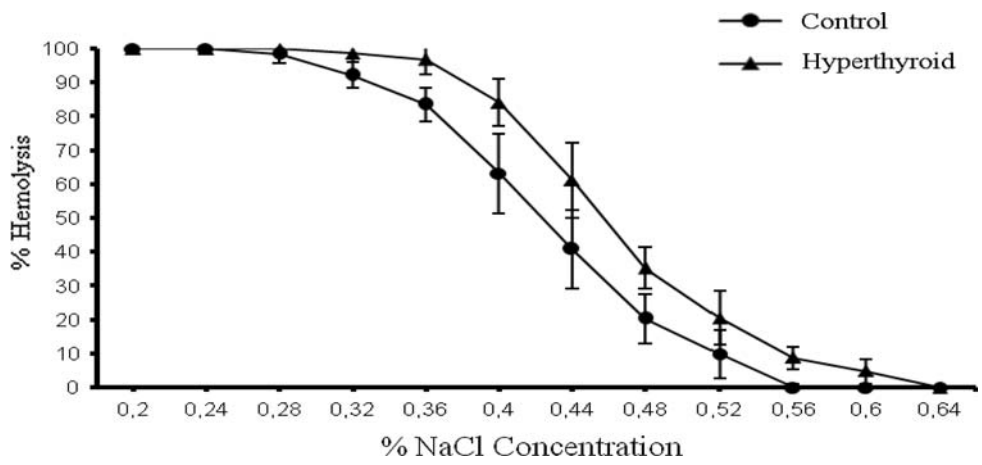

Fig. 1 Osmotic fragility curves of control and hyperthyroid rats (mean ± SD). The degree of hemolysis was calculated by comparing with 0.20% NaCl solution, which represented 100% lyses

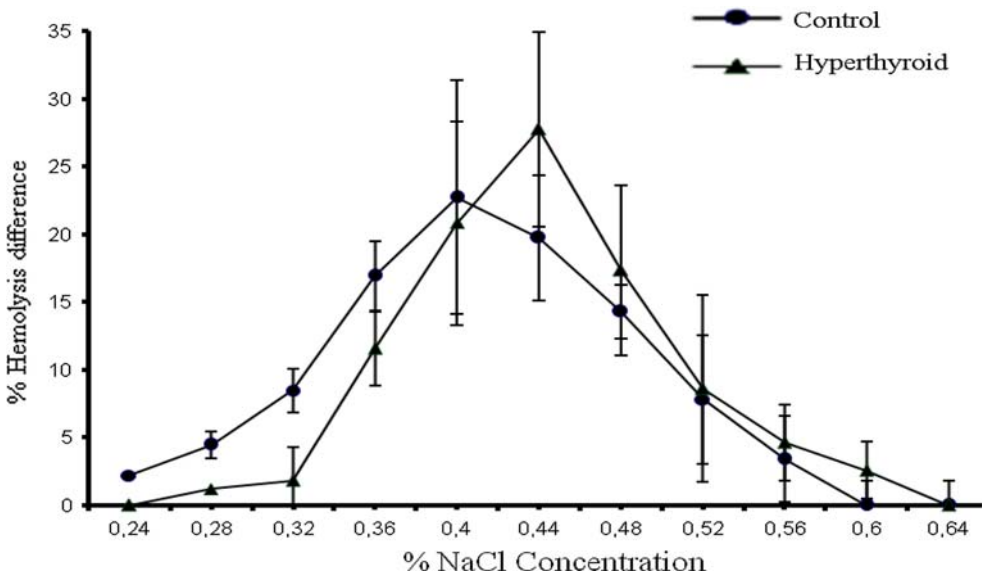

Fig. 2 Hemolytic increment curves of control and hyperthyroid rats (mean ± SD)

administration of L-Tiroksin for 30 days. Thyroid hormones have multifaceted effects on humans. Among others, they influence the basal metabolic rate; the overproduction of thyroid gland hormones results in an increased rate of metabolism and oxygen consumption by cells [9, 10]. There are also studies which report increased lipid peroxidation in hyperthyroidism. Increase in the concentration of reactive oxygen species (ROS) leads to imbalance of the oxidant/antioxidant equilibrium [11, 12]. According to the results of this study, increased erythrocyte MDA levels were detected in experimental animals as compared to controls. This result demonstrates lipid peroxidation in erythrocyte membrane.

As it is known, erythrocytes are sensitive to oxidative stress because of higher concentration of polyunsaturated fatty acid and hemoglobin content than other cell types. But they also have both enzymatic (SOD) and nonenzymatic (GSH) defense systems against oxidative damage [5]. Andryskowski and Owczarek [13] found that, SOD activity in erythrocytes of patients treated for hyperthyroidism was 11% greater than in the euthyroid population; however, this difference did not reach statistical significance. But other researchers observed the SOD activity in erythrocytes of persons with hyperthyroidism recorded decreased activity [14, 15]. But some other researchers observed an increased activity of SOD in patients with hyperthyroidism [16, 17]. In our study, SOD enzyme activity was significantly increased in hyperthyroid group. This increase reveals a primary role of SOD against oxidative damage in experimental hyperthyroidism and in our case probably lipid peroxidation is caused by increased MDA concentration.

In addition to enzyme defense systems, a nonenzymatic system (reduced GSH with its thiol group content) protects the cell from oxidative stress [18]. According to the present results GSH levels of experimental group decrease when compared to the control group values. Such results have been obtained in the study of Asayama et al. [9]. They

examined the thiol group content in plasma proteins, which are particularly sensitive to effects of free radicals. A reduction in the content of thiol groups in plasma proteins containing sulfhydryl illustrates the intensity of free radical processes [13]. A similar observation was made by Ademoglu et al. [19] who recorded a 37% reduction in the sulfhydryl content in serum of individuals with Graves–Basedow disease, with reference to the control group. The decreased erythrocyte GSH level in the hyperthyroid group may be the reason of decreased osmotic resistance and hemolysis of erythrocytes.

Osmotic fragility, the sensitivity to change in osmotic pressure characteristic of red blood cells, has been found to be altered in various pathological conditions. As a result of increased lipid peroxidation, greater osmotic fragility limits in various diseases have been described [20, 21]. The measurement of osmotic fragility of erythrocytes has been applied to the diagnosis of hemolytic diseases, studies of membrane permeability, and alterations leading to destruction of erythrocytes [6]. According to Levander and Welsu [22], RBC with decreased GSH content showed a shortened survival time and increased susceptibility to hemolysis. This study performed in nephrotic syndrome children determined an increase in lipid peroxidation and a decrease in GSH led to an increase in RBC osmotic fragility [21]. The end products of lipid peroxidation for example MDA could induce modification in structure, fluidity, and permeability of erythrocyte membrane leading to membrane damage, decreased stability, and increased sensitivity of the cells toward hypotonic saline.

Our results show that as a result of increased lipid peroxidation, erythrocyte hemolysis has been enhanced. Therefore, it could be inferred that increased lipid peroxidation and SOD and decreased GSH levels play a causal role in increasing the osmotic fragility of the erythrocytes of hyperthyroid rats.

Materials and Methods

In this study, 24 Spraque–Dawley-type female rats that weighed between 160 and 200 g were used and divided into two groups: control ($n = 10$) and experimental ($n = 12$). The experimental group has received tap water, L-Tiroksin (Tefor TM, L-Tiroksin sodium 0.1 mg, Organon-Holland) (0.4 mg/100 g fodder) added standard fodder for 30 days to induce the hyperthyroid [23]. Control group animals were fed tap water and standard fodder for the same period. After the 30-day period, blood samples were drawn from the abdominal aorta, while rats were under ether anesthesia to measure T_3, T_4, TSH, erythrocyte osmotic fragility, a lipid peroxidation indicator MDA levels, and antioxidant defense system indicators SOD, and GSH levels. Our protocol and methods were approved by the Animal Care and Use Committee of Laboratory Animal Service of the Istanbul University, Turkey.

T_3, T_4, TSH levels were measured by RIA method (Diagnostic Products Corporation). The coat-A-count procedure is a solid-phase radioimmunoassay where 125I-labeled T_3, T_4, and TSH compete for a fixed time with T_3, T_4, and TSH in the sample for antibody sites. This reaction takes place in the presence of blocking agents which serve to liberate bound triiodothyronine from carrier proteins; hence, the assay measures total T_3, T_4, and TSH, because both free and protein-bound T_3, T_4, and TSH from the sample are able to compete with radiolabeled T_3, T_4, and TSH for antibody sites. Radioactivity counting was performed in the gamma counter (Searle, Nuclear Chicago Division, model 1185).

Osmotic fragility measurements were performed according to the method of Suess et al. [24]. According to this method 1% NaCl stock solution was prepared with phosphate buffer at a pH of 7.2. For fragility measurements, 12 different NaCl solutions were prepared and placed in standard centrifuge tubes. Their NaCl contents ranged between 0.20 and 0.72%. Freshly drawn 20-µl volume of heparinized whole blood was added to each tube. Each tube was capped with parafilm and inverted a few times. The suspensions were allowed to stand at room temperature for 60 min. Then they were centrifuged for 10 min at 400 g to sediment unlysed cells and stroma, and the supernatant solution was removed. The Hb concentration of each sample was measured at 540 nm in a spectrophotometer (Shimadzu UV–VIS model no. 160A). Hemolysis percentage and hemolytic increment values were calculated and their curves were drawn. The drawn standard hemolysis curve was based on assuming the Hb concentration in 0.20% NaCl solution as 100% and calculating the Hb concentrations in other tubes as percentage of it. The hemolysis percentage ratios in each tube were determined. The NaCl solution with 90% hemolysis was taken as maximum osmotic fragility limit and the NaCl solution which has minimum hemolysis was accepted as the minimum osmotic fragility limit. According to the appropriate NaCl% and percent hemolysis difference values, the hemolytic increment curve was drawn. Hemolytic increment values were determined to find out the actual hemolysis in each tube. This calculation was performed by beginning from the maximum hemolysis limit and subtracting the hemolysis amount of each tube from the next tube value.

The thiobarbituric acid (TBA) test was applied for MDA level measurement, the final product of lipid peroxidation. Measurements were done according to the Slater–Sawyer method [25]. Absorbances were read at 532 nm. 1,1,3,3-Tetra-ethoxypropane was used as the standard. Results

were calculated as nmol MDA/ml in plasma and nmol MDA/gHb in erythrocyte.

The measurement of GSH was done using the Bioxytech GSH-400 kit [26]. Erythrocyte lysates were processed with metaphosphoric acid (MPA) for protein denaturation. After centrifugation, the chromogenic reagent was added to the supernatant. After thioether production, a colored thione absorbance was detected at 400 nm in spectrophotometer (Shimadzu UV–VIS, model UV-160A). GSH levels were calculated as molar concentrations (μmol/l).

Measurements of SOD activity were done using the Bioxytech SOD-525 kit [27]. Hemoglobin was expelled by excretion of erythrocyte lysates with ethanol/chloroform (62.5:37.5 v/v). This method is based on autoxidation rate of chromogenic reagent with SOD in alkaline conditions. SOD activity unit was taken as unit that doubled the autoxidation rate of control blank.

During the statistical evaluations, comparison of values from both the groups was done using the Mann–Whitney U test. Significance is accepted as $P < 0.05$ and values were indicated as mean and standard deviations.

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