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Erythrocyte Osmotic Fragility and Oxidative Stress in Experimental Hypothyroidism

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The present study was planned to explain the relation between erythrocyte osmotic fragility and oxidative stress and antioxidant statue in primary hypothyroid–induced experimental rats. Twenty-four Spraque Dawley type female rats were divided into two, as control (n = 12) and experimental (n = 12), groups weighing between 160 and 200 g. The experimental group animals have received tap water methimazole added standard fodder to block the iodine pumps for 30 d (75 mg/100 g). Control group animals were fed tap water and only standard fodder for the same period. At the end of 30 d blood samples were drawn from the abdominal aorta of the rats under ether anesthesia. T_3, T_4, and TSH levels were measured and the animals that had relatively lower T_3, T_4, and higher TSH levels were accepted as hypothyroid group. Hormone levels of the control group were at euthyroid conditions. Osmotic fragility, as a lipid peroxidation indicator malondialdehyde (MDA), antioxidant defense system indicators superoxide dismutase (SOD) and glutathione (GSH) levels were measured in the blood samples. Osmotic fragility test results: There was no statistically significant difference found between maximum osmotic hemolysis limit values of both group. Minimum osmotic hemolysis limit value of hypothyroid group was found to be higher than that of control group values ($p < 0.02$). The standard hemolysis and hemolytic increment curve of the hypothyroid group drawn according to osmotic fragility test results was found to be shifted to the right when compared to control group's curve. This situation and hemolytic increment value, which shows maximum hemolysis ratio, is the proof of increased osmotic fragility of the erythrocytes in hypothyroidism. There is no statistically significant difference found between hypothyroid and control groups in the lipid peroxidation indicator MDA and antioxidant indicators SOD and GSH levels. As a result of our study it may be concluded that hypothyroidism may lead to an increase in osmotic fragility of erythrocytes. But the increase in erythrocyte osmotic fragility does not originate from lipid peroxidation.

Key Words: Hypothyroid; erythrocyte osmotic fragility; oxidative stress.

Introduction

Hematological changes, especially anemia, are common and well characterized in hypothyroidism. Red blood cells are usually normocytic, normochromic, or, occasionally, macrocytic. Other red blood cell shape abnormalities have also been described *(1)*.

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, higher osmotic fragility limits in various diseases were described. 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 (*2*). Oxidative stress is described as impairment of equilibrium between prooxidant and antioxidant systems. In physiological conditions there is equilibrium between oxidants that are generated during normal aerobic metabolism and their detoxification *(3)*. Whenever additional aerobic oxidants are generated, prooxidant systems, lipids, carbohydrates, proteins, and nucleic acids undergo oxidative damage, and the equilibrium is broken. Lipid peroxidations are the automatical chain reactions that produce radicals in cell membranes *(4)*. Lipid peroxidation can be easily determined in plasma and accepted as an indicator of the oxidative stress. The most widely measured lipid peroxidation product is malondialdehyde (MDA) *(5)*. By products of lipid peroxidation have been shown to 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 (*6*).

Owing to the higher concentration of polyunsaturated fatty acids in their membrane and intracellular oxygen and hemoglobin content, erythrocytes are sensitive to oxidative stress *(7,8)*. On the other hand, defense mechanisms of ery-

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throcytes are also well developed and they contain a higher concentration of 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 *(9)*. The positive relationship among antioxidant status dependent, lipid peroxidation, and osmotic fragility in various diseases was determined *(8,10)*.

Thyroid hormones maintain the oxidant/antioxidant equilibrium to protect the cell *(11)*. As is well known, in oversecretion of thyroid hormones, metabolic reactions are accelerated, oxygen consumption increases, and, owing to oxidative reactions, free radical production is increased as well. Thyroid hormones, while leading to increase in free radicals they also activate the antioxidant enzymes. As energy needs increases in hyperthyroidism, it is observed that oxidants accumulate in the cell *(12)*. So thyroid hormones constitute a risk of oxidant stress for cells. On the other hand in hypothyroism there are contrary results related to oxidative stress and the antioxidant statue *(13–15)*. The hypometabolic state induced by euthyroidism is associated with a decrease in free radical production and in lipid peroxidation products. Thyroid hormones modulate the free radical induced oxidative damage of lipids. Similar results also has been seen in hypothyroidism *(16,17)*. Hypothyroidism offers some protection against lipid peroxidation *(9)*. There is a very limited study and data on oxidative stress products, oxygen derivated free radicals and membrane damage due to effect of free radicals in hypothyroidism. It has been determined that no study on antioxidant statue and osmotic fragilityt in thyroid patients especially in hypothyroidism.

The purpose of the present study is thus to determine the structural integrity of red blood cells in experimental hypothyroidism by assessing the osmotic fragility of erythrocytes in primary hypothyroid and its relation between lipid peroxidation by determining MDA concentrations SOD enzyme activity and GSH level as antioxidants. By blocking the iodine pump in experimental animals primary hypothyroidism was induced and if possible alterations are primarily either in osmotic fragility or antioxidant statue was investigated in early phase of the hypothyroidism.

Results

The statistically significant decrease in T_3, T_4, but the significant increase in TSH ($p < 0.001$) of experimental group is the evidence of induced primary hypothyroidism (Table 1).

There was no statistially significant difference detected in plasma and erythrocyte MDA levels, SOD activity, and GSH levels between hypothyroid and control group animals (Table 1). When the osmotic fragility results were examined, osmotic fragility levels were found to be maximum at 0.32% NaCl and minimum at 0.60% NaCl in control group, whereas erythrocyte osmotic fragility levels were determined maximum at 0.36% NaCl, and minimum at 0.56% NaCl in hypothyroid group (Fig. 1). When statistical evaluations were compared, there was no statistically difference detected in maximum osmotic fragility limits between both groups but minimum osmotic fragility values of the hypothyroid group were found to be statistically higher than those of the control group ($p < 0.02$). So osmotic fragility curve of hypothyroid group was found to be shifted to right when compared to osmotic fragility curve of controls (Fig. 1). The maximum hemolysis level was found to be 0.40% NaCl in the control group and 0.48% NaCl in the hypothyroid group. When the increment curves were evaluated, hemolysis in the hypothyroid group was found to be higher than that of controls ($p < 0.05$). A shift to the right was determined in the increment curve of the hypothyroid group (Fig. 2).

Table 1
Laboratory Data in Control and Hypothyroid States of Experimental Animals

Parameter	Control ($n = 12$)	Hypothyroid ($n = 12$)
T_3, ng/100 mL	69.9 ± 14.8	26.9 ± 4.47***
T_4, ng/100 mL	5.4 ± 0.64	1.01 ± 0.12***
TSH, μIU/mL	1.08 ± 0.13	2.46 ± 0.68***
Plasma MDA level, nmol/mL	5.59 ± 0.86	6.14 ± 0.95
Erythrocyte MDA level, nmol/g Hb	356.25 ± 16.50	329.12 ± 22.28
SOD, U/mL	2173.0 ± 316.1	1992.8 ± 272.8
GSH, μmol/L	25.33 ± 2.91	23.33 ± 3.46
Min ORL, %	0.60 ± 0.02	0.56 ± 0.02**
Max ORL, %	0.32 ± 0.02	0.36 ± 0.04
HI, %	0.40 ± 0.03	0.48 ± 0.04*

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

Data are the means ± SD.

*$p < 0.05$, **$p < 0.02$, ***$p < 0.001$.

Discussion

Lowered levels of T_3, T_4, and elevated TSH show that hypothyroidism has been established in rats, after administration of methimazole for 30 d. In our study, there was no change detected in plasma and erythrocyte MDA levels of experimental group animals compared to the control group. In hypothyroidism the metabolic incidents are reduced as a result of decreased oxygen utilization and energy production *(1,13)*. Therefore, the oxidative damage observed in hyperthyroidism may not be observed in hypothyroidism. There is much evidence in various pathological circumstances that show oxidative activities which end with damage in various tissues. Reactions of tissues against free radicals may be different from one another. As a matter of fact

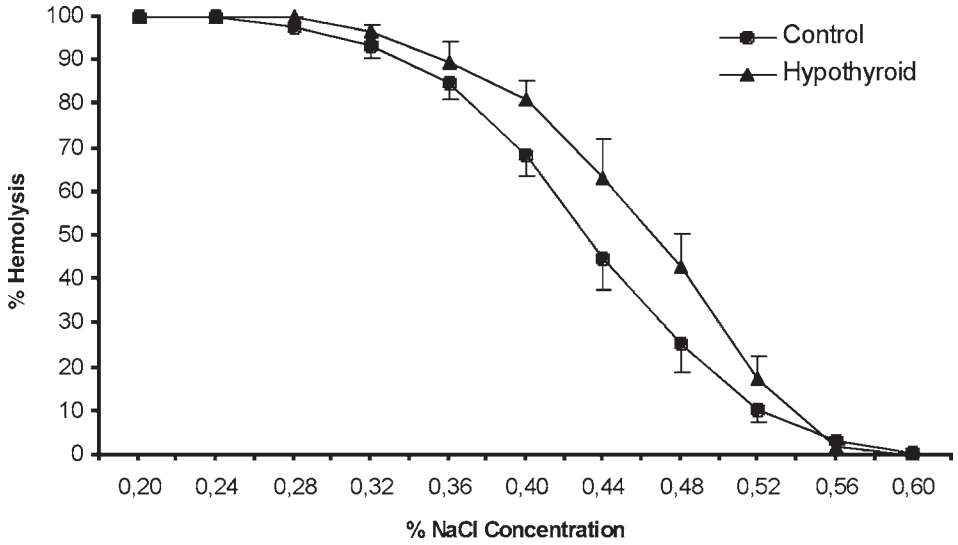

Fig. 1. Osmotic fragility curves of control and hypothyroid rats (mean ± SD; n = 12). The degree of hemolysis was calculated by comparing with 0.20% NaCl solution, which represented 100% lyses.

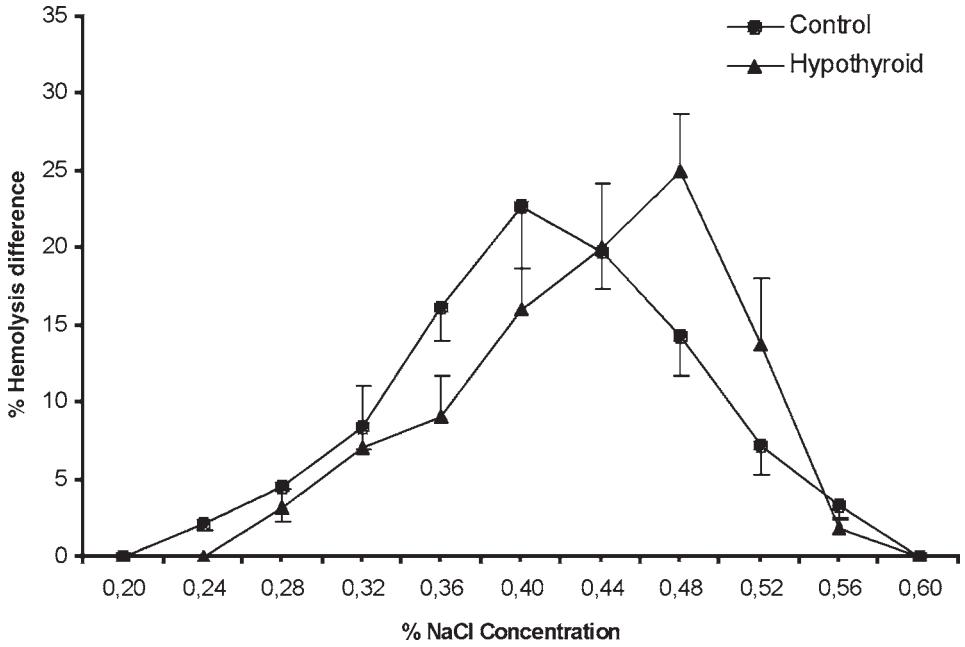

Fig. 2. Hemolytic increment curves of control and hypothyroid rats (mean ± SD; n = 12).

some researchers proposed that there was no difference in heart, liver, and skeletal muscle tissues of rats that developed hypothyroidism when compared to controls *(13,18)*. On the other hand, Krishnamurthy et al. *(14)* reported that there was a decrease in lipid peroxidation in hypothyroid patients. Pereira et al. *(19)* have shown decreased lipid peroxidation in lymphoid organs and muscle tissues of rats with induced hypothyroidism. There are also studies which report increased lipid peroxidation in hypothyroidism *(16,20)*. So, as it has been seen above, contrary results have been reported related to oxidative stress in hypothyroidism. According to results of our study there was no significant difference found between hypothyroid group and control group in either plasma or erythrocyte MDA levels. So these findings suggest that in our experimental conditions no oxidative damage occurred.

Cells contain various different cleavage enzymes responsible for scavenging free radicals. According to our results there was no change detected in SOD activity of hypothyroid animals when compared to controls. Various researchers have showed the role of thyroid hormones on antioxidant enzyme activity. Decreased MnSOD activity in experimental hypothyroidism and increased MnSOD activity in experimental hyperthyroidism have been demonstrated *(18–20)*.

In addition to enzyme defense systems, a nonenzymatic system reduced GSH with its thiol group content prevents

the cell from oxidative stress. According to our results GSH levels of experimental group have showed no change when compared to the control group. In initial studies related to effects of hypothyroidism on oxidative stress, certain decreases were determined in GSH levels. Ghosh et al. *(21)* detected a decrease in γ glutamyl cysteine synthetase activity, which plays a role in velocity limitation for GSH biosynthesis. Rahaman et al. *(22)* showed that SOD activity increased and GSH level decreased in the 4th week of postnatal development in progressive hypothyroidism in the brain of rats. So our results, which detect no significant difference in hypothyroidism when compared to controls related to oxidative stress in hypothyroidism, are similar to the results of Venditti, Mano, and colleagues *(13,18)*.

When osmotic fragility values were examined, according to our results, there was no significant difference determined in maximum osmotic resistance limits between hypothyroid and control groups but minimum osmotic resistance of hypothyroid group was found to be higher than control group values ($p < 0.02$) (Table 1). This finding may be the proof of the presence of a few resistive cells in the erythrocyte population that possibly produced by the erythropoietic stimulation in hypothyroid animals. On the other hand, when standard hemolysis curves were compared, it was observed that the curve of the hypothyroid group was seen to be shifted to the right (Fig. 1). Therefore, this shift to the right in the graph of the hypothyroid group, according to general concept, is an indicator of an increase in osmotic fragility. Other data related to increased osmotic fragility are the results of hemolytic increment curve. According to our results, the maximum hemolysis peak of the hemolytic increment curve that determine the net hemolysis amount (%) in different NaCl solutions, was 0.40% NaCl in the control group but 0.48% in the hypothyroid group. Shifted hemolytic increment curve to the right in the hypothyroid group also shows that increased osmotic fragility in this group. Therefore, hypothyroid group erythrocytes has been hemolyzed earlier than that of the control group erythrocytes in our experiment conditions (Fig. 2).

The data regarding the relationship of osmotic fragility and hypothyroidism in literature is limited. However, different studies of the hypothyroid condition report that decreased water release through kidneys is dependent on decreased ADH concentration *(23,24)*. In the hypothyroid condition due to lower plasma osmotic pressure, increased water infusion into the erythrocyte may end with the hemolysis of erythrocytes. Such a situation may end with the structural alterations that may affect the erythrocyte deformability. Owing to insufficiency of some erythrocyte metabolic enzymes in hypothyroidism, erythrocyte metabolism may slow down, and these alterations may explain the reasons of early hemolysis of erythrocytes.

As a result of present study it may be said that primary hypothyroidism may lead to changes in osmotic fragility of erythrocytes. As it has been noted in text, osmotic equilibrium shows alterations in hypothyroidism. Hypothyroid-dependent disorders in cell metabolism affect the osmotic equilibrium. Therefore, in hypothyroid conditions erythrocytes are under the hemolysis risk *(1)*. Osmotic fragility tests may not be sufficient enough when only maximum and minimum hemolysis limits are evaluated. Evaluations related to osmotic fragility have to be done after drawing whole hemolysis and increment curves. The changes in osmotic fragility are not originated from the lipid peroxidation but the osmotic gradient between plasma and intracellular fluids due to the hypothyroidism. Other possible reasons such as hemorheological parameters have to be studied and the free radical determinations should be performed after extended periods of experimental primary hypothyroidism just to examine if anything arises due to possible increased complications.

Materials and Methods

In this study 24 Spraque Dawley type female rats were used after dividing them into two groups as control ($n = 12$) and experimental ($n = 12$) that weighed between 160 and 200 g. While control group animals were being fed with tap water and standard fodder, experimental group animals received tap water plus methimazole-added fodder (75mg/100g), in order to induce hypothyroidism, for 30 d *(25)*. After the experimental period, blood samples were drawn from the abdominal aorta of the rats under ether anesthesia to measure T_3, T_4, TSH, erythrocyte osmotic fragility, as a lipid peroxidation indicator malondialdehyde (MDA) levels, and as 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 ^{125}I-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 a gamma counter (Searle, Nuclear Chicago Division, model 1185). Osmotic fragility measurements were performed according to the Suess et al. *(26)* method. According to this method 1% NaCl stock solution was prepared with phosphate buffer at a pH of 7.2. For measurement, 12 different NaCl solutions were prepared and placed in standard centrifuge tubes. Their NaCl content was between 0.20% and 0.72%. And 20 μL of freshly drawn heparinized whole blood was added to each tube. Each tube was capped with parafilm, inverted a few times. The suspensions were allowed to stand at room tem-

perature 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 which has 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.

MDA measurement: Thiobarbituric acid (TBA) test was applied for MDA level measurement that is the final product of lipid peroxidation. Measurements were done according to Slater–Sawyer method *(27)*. Absorbances were read at 532 nm. 1,1,3,3-Tetra-ethoxypropane was used as the standard. Results were calculated as nmolMDA/mL in plasma and nmol MDA/gHb in erythrocyte.

GSH measurement: The measurement was done using the Bioxytech GSH-400 kit *(28)*. Erythrocyte lysates were processed with metaphosphoric acid (MPA) for protein denaturation. After centrifugation, chromogenic reagent was added to the supernatant. After thioether production produced colored thione, which is produced with GSH, absorbance was detected at 400 nm in spectrophotometer (Shimadzu UV-VIS, model UV-160A).GSH levels were calculated as molar concentrations (µmol/L).

SOD activity measurement: Measurements were done using the Bioxytech SOD-525 kit *(29)*. 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 of both groups were done using the Mann–Whitney *U* test. Significance is accepted as $p < 0.05$ and values were indicated as mean and standard deviation.

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