

Hematological parameters and osmotic fragility of red blood cells in experimentally induced hyperthyroidism in rats

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ABSTRACT

Background: Although hyperthyroidism is associated with anemia in men, the exact mechanism is not very well known. In the hyperthyroid state, the activity of this pump in red blood cells is decreased, so changes in the maintenance of the cell volume and the fluidity of the membrane of RBCs occur and changes in both the osmotic resistance, as well as changes in the blood profile in the hyperthyroid state are expected.

Objectives: This study was designed to investigate the possible impact of hyperthyroidism on the hematological parameters and osmotic fragility of the red blood cells (RBC) in male rats.

Materials and Methods: Forty-eight male wistar rats (body weight, 221 ± 4 g) were divided into 4 groups (10-13 each). Levo-thyroxine treated groups (I and III given 12mg/L levo-thyroxine in drinking water for 30 and 60 days respectively), while the control groups (groups II and IV) received only tap water. Blood samples were collected to measure hormone levels and osmotic fragility. The osmotic fragility was tested by incubation of RBCs at 37°C for 30 min in different concentrations of NaCl (0 - 0.9 g/100 ml). The extent of hemolysis was measured by colorimetry of the supernatant. Percent hemolysis was calculated on the basis of the 100% hemolysis in the first tube (zero NaCl).

Results: Results of the study show that although hemoglobin (16.4 ± 0.3 g/dL), haematocrit ($52.2 \pm 1.4\%$), mean corpuscular hemoglobin (18.2 ± 0.3 pg) and mean corpuscular volume (57.4 ± 0.8 fL) in group III differed significantly ($P < 0.05$) compared to the control group (15 ± 0.4 , 46.7 ± 1.2 , 17.2 ± 0.3 , 53.6 ± 0.5 respectively), the osmotic fragility showed no significant difference.

Conclusions: The results of this study indicate that not only does experimentally induced hyperthyroidism not induce anemia in rats, but it apparently enhances the erythropoiesis without alteration of the osmotic fragility of the RBC. Exploring the mechanism may further help to explain the altered osmotic fragility observed in humans.

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Background

Thyroid hormones are essential for the normal development, differentiation, metabolic balance, and physio-

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logical function of virtually all tissues and thyroid function disorders are among the most common endocrine diseases (1). Anemia can have several reasons, such as, abnormality of the formation (2) and reduction on the half lifetime of the cell (3-5). Patients with hyperthyroidism have anemia (6, 7). On the other hand, cholesterol, one of the main compositions of the cells membrane (8) with an essential role in the fluidity of the membrane of the cells (9), increases in the membrane of red blood cells

(RBCs) of hyperthyroid patients (10, 11). Also the number and the activity of the $\text{Na}^{(+)}\text{-K}^{(+)}\text{-ATP-ase}$ pump in the membranes of the cells including RBC membrane (12, 13), which is affected by of thyroid hormone concentration, plays an important role in maintaining the cell volume and RBCs (14). In the hyperthyroid state, the activity of this pump in red blood cells is decreased (12, 13). Yucel *et al.* (15) studied the relationship between osmotic fragility of red blood cells and lipid peroxidation in hyperthyroid rat without testing the blood profile and reported an increased osmotic fragility. Hence, as a result of the above mentioned alterations, changes in the maintenance of the cell volume and the fluidity of the membrane of RBCs occur and changes in both the osmotic resistance, as well as changes in the blood profile in the hyperthyroid state are expected.

Objectives

In this study, blood quantities and erythrocyte osmotic resistance of the RBC from hyperthyroid rats have been compared with those of control rats.

Materials and Methods

Forty-eight male Wistar rats (weight 221 ± 4 g), purchased from the Pasteur Institute Karaj Iran, were divided into 4 groups of 30 day hyperthyroid, 30 day control, 60 day hyperthyroid and 60 day controls (No. = 10-13). Animals were kept under the standard conditions of 12 hour light and 12 hour dark cycles, at a temperature of $22 \pm 3^\circ\text{C}$ in polystyrene cages (4 in each cage) with free access to water and standard rat diet, (Pars Co, Tehran, Iran). Hyperthyroidism was induced by adding 12 mg/L of L-thyroxine (gift from Iran-Hormone, Tehran, Iran) into the drinking water (16) for desired duration. At the end of 30 or 60 days of thyroxine administration, the animals

were anesthetized with Ketamine (50 mg.kg body wt-1) and xylazine (2.61 mg.kg body wt-1), the abdomen was opened and a 5ml blood sample was obtained through the abdominal aorta. Two ml of the obtained sample was collected on EDTA to measure blood parameters (Coulter Counter TH90, France) and osmotic fragility and the remaining was centrifuged ($3000 \times g$ for 5 min); plasma was separated and kept at -20°C for biochemical and hormonal measurements. To assess the applicability of the counter for blood samples from rats, five similar samples were analyzed by both the counter and a routine laboratory method and the values proved to be comparable.

To assess the osmotic fragility of the RBC, a routine method with slight modification was used. In brief, RBCs were washed three times with normal saline (0.9 g/100mL), the cells were resuspended in normal saline in the same volume of original blood sample and 25 μl of the prepared cell suspension was added in triplicate into different concentrations of NaCl solution (zero to 0.9 g/100 mL) at increments of 0.025 g/100 mL, and incubated at 37°C for 30 min (17). After the incubation period, the tubes were centrifuged for 10 min at $3000 \times g$, and supernatant was used to determine the optic density as the indicators of the hemolysis at 540 nm, using an ELISA reader (Tecan, Austria). Percent hemolysis of the tubes was calculated on the basis of 100% hemolysis in the first tube (zero NaCl).

The plasma cholesterol concentration was determined using kits obtained from the Zistchimi Company (Iran). Intra-assay coefficient of variation and the sensitivity for cholesterol measurement were 1.9% and 3mg/100dl respectively.

Thyroid hormones were measured by the ELISA method (Monobind, USA) and the intra-assay coefficients of variation for total Triiodothyronine (tT3), free triiodothyronine (fT3), total thyroxine (tT4) and free thyroxine

Table 1. The first weight, second weight and the weight difference (Mean \pm SE) in 30 and 60 day old hyperthyroid and control rats

Variables	Hyperthyroid group I (30 days) (No.=12)	Control group II (30 days) (No.=10)	Hyperthyroid group III (60 days) (No.=12)	Control group VI (60 days)
Before the experiment (g)	241.9 \pm 3.3	251.1 \pm 5.7	206.5 \pm 4.7	202.0 \pm 3.4 (n = 13)
After the experiment (g)	271.8 \pm 5.7	288.5 \pm 7.5	241.1 \pm 8.1 ^a	266.8 \pm 6.7
Weight differences (g)	29.8 \pm 4.1	37.4 \pm 6.4	34.6 \pm 6.6 ^b	68.6 \pm 5.5

Student t test was used to compare each test group with its control group

^a $P < 0.05$, ^b $P < 0.001$

Table 2. Results of the blood cell variables and serum cholesterol concentration (mean \pm SE) in 30 and 60 day old hyperthyroid and control rats

Quantity	Hyperthyroid group I (30 days)	Control group II (30 days)	Hyperthyroid group III (60 days)	Control group VI (60 days)
RBC (million/mm ³)	8.4 \pm 0.2 (No. = 9)	8.0 \pm 0.2 (No. = 9)	9.2 \pm 0.3 (No. = 9)	8.7 \pm 0.2 (No. = 11)
Hb (g/dL)	15.5 \pm 0.5 (No. = 9)	14.7 \pm 0.3 (No. = 9)	16.4 \pm 0.3 ^a (No. = 8)	15.0 \pm 0.4 (No. = 11)
Hematocrit (%)	47.1 \pm 1.1 (No. = 9)	44.9 \pm 1 (No. = 9)	52.2 \pm 1.4 ^a (No. = 8)	46.7 \pm 1.2 (No. = 11)
MCH (pg)	17.8 \pm 0.3 (No. = 9)	18.4 \pm 0.2 (No. = 9)	18.2 \pm 0.3 ^b (No. = 9)	17.2 \pm 0.3 (No. = 11)
MCV (fl)	55.9 \pm 1.0 (No. = 9)	56.4 \pm 0.3 (No. = 9)	57.4 \pm 0.8 ^b (No. = 8)	53.6 \pm 0.5 (No. = 11)
Cholesterol (mg/dL)	58.9 \pm 5.4 (No. = 10)	51.2 \pm 4.7 (No. = 6)	48.1 \pm 2.7 ^b (No. = 10)	56.7 \pm 2.9 (No. = 12)

Student t test was used to compare each test group with its control group

^a $P < 0.01$

^b $P < 0.05$

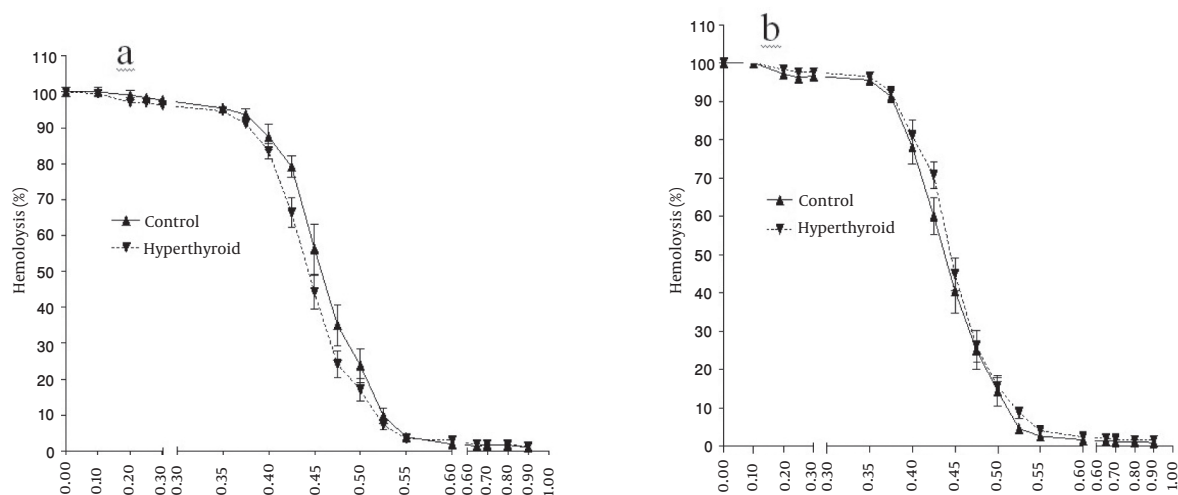


Figure 1. Osmotic fragility of RBC in 30 (a) and 60 day hyperthyroid animals compared to their control groups (0.9 g/100 mL)

(tT3) measurements were 6, 6.3, 4 and 6.9 percent respectively. The sensitivity of the assays for tT3, fT3, tT4 and fT4 were 0.04 ng/ml, 0.05 pg/ml, 0.4 ug dl and 0.05 ng/dl respectively. Data are expressed as Mean \pm SE. Two way ANOVA, followed by Tukey's HSD test, was used to compare the fragility of the RBCs of the groups. Student t-test was used to compare other findings, P-values below 0.05 being considered significant.

Results

Mean weights of the animals at the beginning of the experiments for the 30 and 60 day hyperthyroid group and their control groups were not significantly different. However body weights of the 60 day group, but not the 30 day hyperthyroid one were significantly ($p < 0.05$) lower, compared to its control group at the end of the experiment period (Table 1). No significant difference was observed in the RBC counts of the 30 and 60 day hyperthyroid rats and their control groups (Table 2). The Hb level of the 30 day hyperthyroid rats was not significantly different from its control group, whereas in the 60 day hyperthyroid group it was significantly ($P < 0.01$) higher, compared to the control group (Table 2).

Mean Corpuscular Hemoglobin (MCH), mean corpuscular volume (MCV) and hematocrit (HCT) levels in the 30 day hyperthyroid rats were not significantly different from their control groups, but in the 60 day hyperthyroid animals, these values were significantly ($p < 0.05$) increased, compared to their control group (Table 2). Figures 1a and 1b show the osmotic hemolysis of the RBCs in different groups, with no significant differences observed between the RBCs osmotic fragility of the 30 and 60 day hyperthyroid rats and their control groups. Total plasma thyroxine (tT4) concentration in the 30 and 60 day hyperthyroid animals were significantly ($p < 0.0001$) higher compared with their control groups (1.55 ± 0.1 and $2.28 \pm 0.1 \mu\text{g/dL}$ respectively) (Table 3). Total Triiodothyronine (tT3) of the 30 day and 60 day hyperthyroid rats also

were significantly ($p < 0.0001$) higher compared to the their respective control groups (Table 3).

Free Thyroxine concentration in the 30 and 60 day hyperthyroid groups were significantly higher compared with their corresponding control groups (Table 3). Free Triiodothyronine concentrations of 30 and 60 days hyperthyroid groups were 5.3 ± 0.4 and $7.1 \pm 0.7 \text{ pg/mL}$ respectively, significantly ($p < 0.0001$) higher compared to their control groups (Table 3). Plasma cholesterol concentrations of the 30 day hyperthyroid animals were not significantly different to those of the controls, whereas in the 60 day hyperthyroid animals these levels were significantly ($p < 0.05$) decreased compared to the control group (Table 2).

Discussion

The results of this study indicate that hyperthyroidism induced for 2 months in rats not only did not change the fragility of the RBC, but it could not cause anemia implying that the effect of hyperthyroidism in rats differs from that in humans. Many studies report anemia and the abnormalities of blood profiles in patients with hyperthyroidism (6, 8, 10, 18), suggesting the direct and indirect effects of thyroid hormones. The existence of microcytic anemia in both the hyperthyroid or hypothyroid patients has been reported (10, 18). In hyperthyroid patients, decreased Hb and the MCV has also been reported, parameters which after being treated have returned to normal values (6, 19). In a very recent report, Gianoukakis *et al.* (20) reported that the anemia in patients with Grave's disease is associated with inflammatory markers, results, which obviously, from the hematological point of view, do not match those reported in humans; this is not surprising because Ozkan *et al.* have shown that in hyper and hypothyroid rats, total plasma homocysteine, serum folic acid and serum vitamin B12 levels were not significantly different from control animals; they concluded that the hyper- and hypothyroid rat models cannot rep-

resen thyper- and hypothyroidism status in humans (21). Nevertheless some reports have shown that thyroid hormones can alter erythropoietic activities in rodents (22).

The results of this study also indicate that the osmotic resistance of the RBCs from 30 and 60 day hyperthyroid rats, as compared to their control groups showed no significant difference. To our knowledge although no study has examined the osmotic resistance of RBC in hyperthyroid rats, in a recent report we have shown that the osmoresistance of RBCs increased in untreated newly diagnosed hyperthyroid patients with anemia, compared to their well matched age and sex matched controls (23). In another study, Keshelava, et al. reported a significant correlation between the level of methemoglobin, osmoresistance of erythrocytes and the degree of the thyrotoxicosis (24). In 1982, Tkachev reported that erythrocyte osmotic resistance was enhanced in patients with the mild and/or moderate stages of the disease and was reduced in severe thyrotoxicosis (25). Although measurements of tT_4 , tT_3 , fT_4 and fT_3 in the current study confirm hyperthyroidism, nevertheless different degrees of hyperthyroidism were not explored. It is possible that the level of thyroid hormones or the duration of hyperthyroidism may affect the outcome. In the Zahedi, *et al.* study, hypothyroid animals had significantly lower osmotic resistance compared with controls (26). The lower weights of the 60 day hyperthyroid animals too confirm the induction of hyperthyroidism.

From the results of this study, although it is not possible to draw a conclusion as to why osmoresistance of RBCs in experimentally induced hyperthyroid rats is different from what we and others have found in hyperthyroid patients, the differences in structure and the physiology of the RBCs (22) may explain the differences; this effect may explain the differences between these findings and the report by Yücel *et al.* (15), who reported that in rats too, osmotic fragility of RBC increased in hyperthyroid rats at 60 days, when the thyroid hormones concentrations were much lower compared to those found in this study. It has been shown that in vitro conditions the deformability of erythrocytes was improved in a dose dependent manner by thyroxine. Mechanical hemolysis was found to be lower if thyroxine was included in erythrocyte suspensions at concentrations close to the physiological levels (10^{-9} M) (29); The direct effect of thyroxine on osmotic resistance of RBCs in rats has not been studied but since the study was performed on washed RBCs the effect of high thyroxine level present in the plasma of the hyperthyroid animals can be ruled out. Of course the long lasting effects of the hormone need more studies. The reason for lower hormone concentration may be the route of hormone administration. In the Yucel, *et al.* study (15) the hormone was added to the diet, while in current study it was added into the drinking water.

Studies have shown that the activity and the number of $Na^{(+)}-K^{(+)}-ATP$ -ase are decreased in the RBC membranes of hypothyroid rats (12, 13), leading obviously to increase in the RBC volume (27) and decrease in the os-

motric resistance of the cell, unless some other structural changes compensate for the alteration. In this study, plasma cholesterol levels in the 60 day but not in the 30 day hyperthyroid rats were significantly decreased, possibly reflecting the increased cholesterol content of the membrane (28). Changes in the fluidity and deformability affect the mechanical resistance of the cells, which was not tested in this study. Decrease of plasma cholesterol in hyperthyroid rats (29) and its increase in hypothyroid rats have been reported before (10, 26). The membrane cholesterol content influences the membrane fluidity and the cholesterol/ phospholipids molar ratio is the most important factor in membrane fluidity (31). The high amount of the cholesterol in the membrane causes stiffness of the membrane and reduces its permeability (29, 30); it has been shown that the amount of cholesterol in plasma is under the effect of thyroid hormones and any changes in it are due to the transfer of cholesterol from the plasma to the membrane of the RBCs and vice versa (10, 11).

In the present study Hb, hematocrite MCH, and MCV were significantly increased in 60 day hyperthyroid rats, compared to their controls, a profile which is again different from results found in hyperthyroid men. In hyperthyroid patients, decrease in the MCV has been reported (19, 30). Cohen, *et al.* attributed the decrease of MCV and Hb in hyperthyroid patients to the increase in the activity of monoester lipase enzyme, also present in the RBC membrane and to the increased lipolytic potency of RBCs (19). Regarding the increase of MCV, the decrease in the fragility resistance of the RBCs is expected. Therefore the lack changes seen in this study could be due to other causes, which need to be elucidated. It is interesting to notice that despite induction of the hyperthyroidism at 30 day, some blood parameters remained normal; although from the findings of the study it is not possible to explain this observation, but one can speculate that the effects on these parameters need a longer follow-up period; again, of course this speculation also needs to be explored by future experiments. In conclusion, the results of this study indicate that not only does experimentally induced hyperthyroidism in rats not induce anemia but it apparently enhances the erythropoiesis without alteration of the osmotic fragility of the RBC. Exploring the mechanism may help to explain the altered osmotic fragility observed in humans.

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Conflict of interest

None declared.

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