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Increased erythrocyte osmotic fragility in hypothyroidism

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Hypothyroidism a quite common thyroid disorder is often associated with anemia. Osmotic fragility is altered with an alteration of the thyroid profile. Hypothyroidism induced reduction of membrane cholesterol and compromised the activity of Na^+K^+ATPase and increased peroxidation of membrane lipids appear to be responsible for damaging the red blood corpuscle (RBC) membranes which alter the permeability of themembrane and thus ionic environment within the cell. The objective of this study was to evaluate the relation between the association of hypothyroidism and RBC membrane lipid peroxidation in the development of increased RBC osmotic fragility. A complete clinical history and biochemical investigations, including thyroid function tests namely, thyroid –stimulating hormone (TSH) and free T4 (fT4), RBC membrane osmotic fragility test by gradient concentration of NaCl, and RBC membrane peroxidation using thiobarbituric acid reactive substances (TBARS) tests were performed among thirty two hypothyroidism patients and healthy controls. Serum TSH & TBARS in the RBC membrane were significantly higher (P <0.001) than healthy controls which also showed a significant positive correlation (r =0.8, P =0.01) when compared without grouping. Osmotic fragility of RBC was found significantly increased among hypothyroid patients (P <0.001) at NaCl conc. of 0.3%, 0.4%, 0.5% & (P <0.05) at NaCl conc. of 0.6%. In conclusion, the results indicated that RBC osmotic fragility increases significantly in hypothyroidism possibility due to increased membrane lipid peroxidation as observed in the study.

Keywords: Dysferlin, Hypothyroidism, Osmotic fragility, Synaptotagmin, Thiobarbituric acid reactive substances, Thyroid–stimulating hormone

Alterations in thyroid function are quite common clinical conditions in day to day practice. Being one of the major metabolic regulators such disturbance results in many physiological alterations. Hypothyroidism with low thyroid hormones and corresponding high TSH levels is responsible for negative resting energy expenditure and thus hypometabolism which is reflected by altered lipid metabolism, obesity, decreased gluconeogenesis and many others[1]. Anemia is a common clinical association with thyroid dysfunctions and though normocytic anemia is most predominant, macrocytic and microcytic types are also dealt with[2]. Increased hemolysis and thus reduced survival period might be one of the major reasons for anemia in hypothyroidism[3] which is augmented by the altered lipid composition of the RBC membrane[4] and Na^+K^+-ATPase activity[5]. RBC membrane peroxidation and oxidative stress were reported to be significantly higher among hypothyroid patients[6-8] which further may contribute to altered membrane stability and thus increased hemolysis.

Metabolic processes control ATP levels, ROS generation and redox state within cells and thus also control ion handling by pumps and passive transport pathways[9,10]. Deranged lipid metabolism alters lipid composition and thus membrane fluidity of various cell membranes including RBC in hypothyroidism[5,11,12]. Hypothyroidism-induced oxidative stress and lipid peroxidation[13,14] may further contributes to membrane instability. Decreased Na^+K^+ATPase activity also alters net ion transport across the cell membrane with altered intra cellular ionic environment[15-16].

All these factors might play together to bring upon the increased hemolysis in hypothyroidism. However as earlier studies have ended with contradictory

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Abbreviations: ABCA1, ATP-binding cassette subfamily A member 1; ALG-2, apoptosis-linked gene-2; fT4, free thyroxine; GSH, reduced glutathione; H_2O_2, Hydrogen peroxide; HEPES, 2-hydroxyethyl-1- piperazineethanesulfonic acid ; MDA, malondialdehyde; Na^+K^+ATPase, Sodium Potassium ATPase; NO, Nitric oxide; PMSF, phenylmethylsulfonyl fluoride; TBARS, thioburbituric acid reactive substances; TSH, Thyroid–stimulating hormone; EDTA, Ethylene diamine tetraacetic acid

results, hence in the present study, we evaluated the association of RBC osmotic fragility with hypothyroidism among the treatment naïve hypothyroidism patients.

Materials and Methods

Reagents

Thyrotropin (TSH) Test System and free Thyroxine (fT4) from Monobind Inc (Lake Forest, USA), Thiobarbituric acid (TBA) from Loba Chemie, Ammonium molybdate, HEPES, EDTA, PMSF and Tris-HCl from SRL Chemical, India; All other chemicals used were of analytical grade and were purchased from the Merck India Ltd. and SRL India.

Selection of participants

For this hospital– based cross– sectional study participants were selected randomly among the patients attended the Department of Biochemistry, College of Medicine & JNM Hospital, WBUHS, Kalyani, Nadia for thyroid profile estimation referred from various OPDs of the same institute. 32 adults between 20 to 48 years of freshly detected treatment naïve patients suffering from hypothyroidism irrespective of sex were selected as cases. However, pregnant mothers, smokers, patients with a history of alcoholism, any concomitant chronic disease or patients under treatment for the same that can interfere with study results were excluded. The results obtained were compared with randomly selected 32 adult healthy volunteers, served as controls.

Hypothyroidism was characterized by TSH level (>4.2 mIU/mL) with or without fT4 level (<0.8 ng/dL)[17,18]. This study was approved by the Institutional Ethics Committee as per ICMR guideline (Ref. No. F-24/Pr/COMJNMH/IEC/16/1209 Dated 18th August 2016).

Blood sample preparation

After obtaining the written informed consent, 7 mL of fasting (12 hrs) venous blood sample was collected from each participant and divided into 2 mL for thyroid profile estimation which was centrifuged and sera separated and kept at -20^0 C until analysis. 5 mL with EDTA (1.5 mg/mL blood) was used for the osmotic fragility test and lipid peroxidation (TBARS) estimation from the membrane ghost.

Assay

Biochemical analysis of serum TSH and fT4 were done by Immuno-enzymometric assay and Enzyme Immunoassay using 96 well microplate assays using kits from Monobind Inc as mentioned earlier. Absorbance was measured by TECAN'S Magellan universal reader (Sunrise) at 450 nm.

For RBC osmotic fragility assay, cells were washed with 0.9% NaCl thrice. For each wash, 1.5 mL of the blood sample was mixed with 8.5 mL of 0.9% NaCl and centrifuged for 5 min at 1000 rpm. After careful discarding supernatant of final centrifugation cells were suspended in 1.5 mL of 0.9% NaCl and 50 µl of cell suspension was added in triplicate to the tubes containing 1 mL of different NaCl concentration (0-0.9%). Tubes were incubated for 30 min at 37°C followed by centrifugation at 3000 rpm for 10 min. The absorbance of the supernatant was measured at 540 nm using UV-VIS Spectrophotometer 117 (Spectronics). Each concentration of NaCl was made in triplicate and the average of absorbance was accepted as a final reading. The percentage of hemolysis was calculated considering absorbance for the tube containing no NaCl as 100%[19]. The percent hemolysis was calculated using following formula[20]

$$\text{Percent hemolysis} = \frac{\text{Absorbance of the test}}{\text{Absorbance of the standard (No NaCl)}} \times 100$$

Preparation of erythrocyte membranes (Ghosts)

Cytoplasm free erythrocyte membranes were prepared as per the following method[21,22]. The blood concentrates were subjected to centrifugation at 6500 × g for 10 min at 4°C. Precipitates obtained were resuspended in 20 mM Tris-Cl (pH 7.4), 130 mM KCl and 0.6 mg/ mL phenylmethylsulfonyl fluoride (PMSF). This suspension was centrifuged again at 6500 × g for 10 min at 4°C. Hemolysis obtained by freezing cell pellets in liquid nitrogen followed by thawing at room temperature. The lysed cells were then re-suspended with 5 mM 4-2-hydroxyethyl-1-piperazineethanesulfonic acid (HEPES; pH 7.4), 1 mM ethylene diamine tetra acetic acid (EDTA) and 0.6 mg/mL PMSF and centrifuged at 9000 × g for 10 min at 4°C. This washing step was repeated four times, and the resulting pellets were used for TBARS assay to measure malondialdehyde (MDA), suspended in (40 mL) 10 mM HEPES (pH 7.4), 130 mM KCl, 0.5 mM $MgCl_2$, and 0.05 mM $CaCl_2$. The solutions were spun at 9000 × g for 10 min, resuspended in a small volume of this last buffer, and

stored at –20°C until use.

The ghost membrane sample was thawed at room temperature. 1 mL sample was mixed with 2 mL 10 % trichloro acetic acid and 4 mL 0.67 % thiobarbituric acid. The mixture was heated in a water bath at 100°C for 15 min, cooled and centrifuged; absorbance was recorded at 535 nm. The extent of lipid peroxidation was calculated using a molar extinction coefficient of 1.566105 M21 cm 21 for MDA[23].

Statistical analysis

All results are expressed as the mean ± standard error (SE) using statistical software SPSS version 20. The significance of the difference of means was determined by the student's t– test. Pearson's correlation analysis was performed between TSH and TBARS without grouping to verify the relation between them. A value of P <0.05 was used to evaluate statistical significance. For a graphical representation of analysis results, GraphPad Prism (Version 8) was used.

Results

Results obtained from the study showed that TSH level and TBARS were significantly increased among the hypothyroidism group when compared to matched controls (P <0.001) (Table 1).

When the percentage of hemolysis was assessed between the control and cases it was found that at low concentrations of NaCl (0.01% & 0.02%) there was no significant change as hemolysis in both the groups. The same has been noted at higher concentrations of NaCl (0.07%, 0.08% & 0.09%) where there was minimal or no hemolysis in both groups. However, significant difference in the hemolysis was noted at NaCl concentrations of 0.03%, 0.04% & 0.05% (P <0.001) and also at 0.06% (P <0.05) when compared between hypothyroidism cases and control group (Fig. 1).

Pearson's correlation was performed between TSH concentration and TBARS level considering both groups (Control & Cases) together and it showed that there was a significant positive correlation (r =0.8, P =0.01) between the TSH and TBARS (Fig. 2).

Table 1 — Thyroid profile & TBARS in control and patients with hypothyroidism

Parameters	Control (n=32)	Hypothyroid (n=32)	P value
TSH (μIU/mL)	1.98±0.20	14.64±1.53	<0.001
fT4 (ng/dL)	1.19±0.05	1.14±0.77	0.69
TBARS (nM/mL)	3.69±0.097	5.64±0.091	<0.001

All values are expressed as mean ± SE

Discussion

Studies have been conducted earlier on RBC osmotic fragility and thyroid function with contradictory results. However, results obtained from our study showed that there was increased RBC osmotic fragility and increased lipid peroxidation of the RBC membrane among hypothyroid patients which is in agreement with earlier studies[24-26].

Thyroid hormones namely, triiodothyronine (T3) and tetraiodothyronine (T4) are synthesized in the thyroid gland under the regulation of thyroid–stimulating hormone (TSH) from the pituitary[27]. Post synthesis, the hormone is released in blood to serve as a major regulator of metabolism and thus growth and development.

When lipid metabolism is concerned in hypothyroid state the situation is worsened with the rise of serum TSH level[28]. Altered lipid metabolism results in dyslipidemia. Hypercholesterolemia and

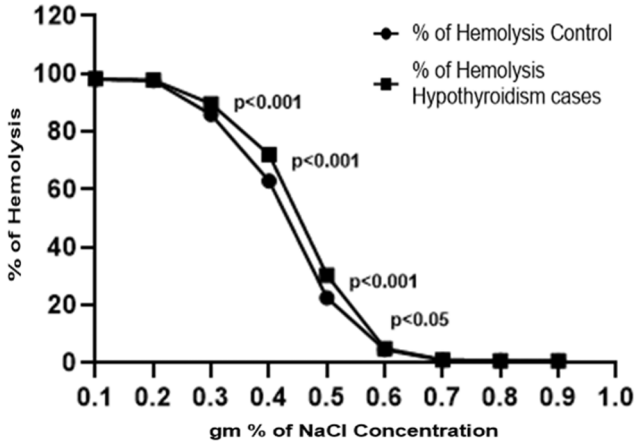

Fig 1 — Comparison of hemolysis % between control and hypothyroid patients

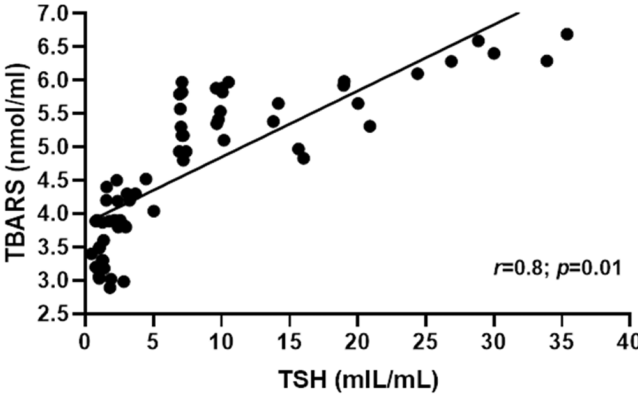

Fig 2 — Correlation between serum TSH with RBC membrane TBARS (r =0.8, P =0.01)

increased serum LDL-C are the most prominent features associated with both clinically overt and subclinical hypothyroidism[29-32]. Such hypercholesterolemia is due to TSH dependent stimulation of hepatic HMG Co-A reductase enzyme, the major and regulatory enzyme for cholesterol synthesis[33]. However, when cell membrane lipid content is concerned, the scenario is quite different from plasma. In hypothyroidism with 60% plasma cholesterol increase, there is a 22% reduction in cholesterol in the RBC membrane. This is further accompanied by a 30% reduction in RBC membrane phospholipid content[11]. Recent studies have suggested that elevated TSH level also facilitates the transfer of intracellular cholesterol to extracellular apolipoprotein through up-regulation of ATP-binding cassette subfamily A members (ABCA1) expression[34]. This can explain, at least partially the serum hypercholesterolemia with decreased cholesterol levels in cell membranes among hypothyroidism patients. Reduction in membrane cholesterol increases cellular stiffness which may be the underlying cause of increased RBC osmotic fragility[35].

In hypothyroidism membrane cholesterol decrease reduces the membrane fluidity which is reflected by decreased activity of membrane–bound Na^+K^+ATPase[4] and alteration of membrane transport of various other ions and metabolites. Increased oxidative stress in hypothyroidism[25,36] inhibits Na^+K^+ATPase by glutathionylation of β1 subunit of the enzyme and also by alteration of its conformation[37]. Inhibition of Na^+K^+ATPase induces cell lysis which has been already established *in vitro* cell culture model[38]. Suboptimal activity and failure of the Na^+K^+ ATPase can result in swelling of the cell by alteration of intracellular ion concentration which further may contribute to increased RBC lysis[39].

Though theoretically membrane cholesterol content does not affect cellular drug uptake[40] but it has been shown that reduction of cholesterol content in the erythrocyte membrane further reduces Ca^{2+} transport across the membrane and reduces intracellular Ca^{2+} content. Ca^{2+} influx through the Ca^{2+} channel is reduced significantly if the membrane cholesterol content is decreased[41]. Various calcium sensors like synaptotagmin (Syt) VII, dysferlin and apoptosis-linked gene-2 (ALG-2) are associated with membrane repair mechanism which suggests that Ca^{2+} may regulate several steps of the membrane repair process. Therefore, the reduction of intracellular Ca^{2+} may decrease the ability of a cell to repair its membrane injury[42].

Increased oxidative stress is an important finding among hypothyroid patients both in subclinical and clinically overt cases[25,36]. In the hypothyroid state, there is a significant reduction in total antioxidant capacity[43] and decreased total reduced glutathione (GSH) content within RBC[44]. These together could play an important role in the development of oxidative stress and ultimately put the cell under risk of hemolysis.

Membrane lipid peroxidation has always been a major concern in thyroid dysfunction which is the reflection of oxidative stress. Reduction in catalase activity has been reported in hypothyroidism[45] which leads to the accumulation of H_2O_2. Excess H_2O_2 could react with NO to generate peroxynitrite and various other hydroxyl free radicals. These radicals could further react with and result in damage of cellular structures which is known as lipid peroxidation[46,47]. In our study we have found there is a significant increase of RBC membrane lipid peroxidation with increased serum TSH level which is in agreement with earlier studies[8,25,43]. Increased membrane lipid peroxidation alters lipid bilayer physiology and physiological properties and functions of cell membranes that result in the modification of proteins and even nucleic acids[48,49]. Such changes result in altered lipid-lipid interactions, ion transport across the cell membrane, membrane fluidity and membrane permeability[46]. It has been also hypothesized that when the membrane is deficient in cholesterol, phospholipid peroxidation decreases membrane thickness[50].

Conclusion

This study suggested that the osmotic fragility of erythrocytes increases significantly in hypothyroidism. Augmentation of hemolysis might be the result of the interplay between reduced cholesterol content of cell membrane along with reduced membrane– bound Na^+K^+ATPase activity and Ca^{2+} transport across the cell membrane. Increased membrane lipid peroxidation has an additive effect on thyroid dysfunction by altering lipid-lipid interactions, ion transport across the cell membrane, membrane fluidity and membrane permeability.

Conflict of Interest

All authors declare no conflict of interest.

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