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Abstract

Our aim was to study the effect of iron supplementation on the following aspects of erythrocyte metabolism in experimental hyperthyroidism: glutathione (GSH) levels, glutathione peroxidase (GSH-Px), and superoxide dismutase (SOD) activities. Hyperthyroidism induced by L-thyroxine administrations significantly raised erythrocyte GSH, GSH-Px and SOD levels of the rats ($P < 0.001$). Likewise, we observed that iron supplementation induced significant rises in erythrocyte GSH, GSH-Px and SOD levels ($P < 0.001$) as compared with the control group. The erythrocyte GSH, GSH-Px and SOD levels of hyperthyroidism-induced iron-supplemented animals were significantly higher when compared with either the iron-supplemented group ($P < 0.001$) or the only L-thyroxine-administered hyperthyroid group ($P < 0.001$, $P < 0.05$, $P < 0.01$, respectively). The results of this study show that L-thyroxine administration and/or iron supplementation increases GSH, GSH-Px and SOD levels of erythrocytes.

KEYWORDS: iron, hyperthyroidism, glutathione, superoxide dismutase, glutathione peroxidase

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The Effect of Iron Supplementation on GSH Levels, GSH-Px, and SOD Activities of Erythrocytes in L-Thyroxine Administration

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Our aim was to study the effect of iron supplementation on the following aspects of erythrocyte metabolism in experimental hyperthyroidism: glutathione (GSH) levels, glutathione peroxidase (GSH-Px), and superoxide dismutase (SOD) activities. Hyperthyroidism induced by L-thyroxine administrations significantly raised erythrocyte GSH, GSH-Px and SOD levels of the rats ($P < 0.001$). Likewise, we observed that iron supplementation induced significant rises in erythrocyte GSH, GSH-Px and SOD levels ($P < 0.001$) as compared with the control group. The erythrocyte GSH, GSH-Px and SOD levels of hyperthyroidism-induced iron-supplemented animals were significantly higher when compared with either the iron-supplemented group ($P < 0.001$) or the only L-thyroxine-administered hyperthyroid group ($P < 0.001$, $P < 0.05$, $P < 0.01$, respectively). The results of this study show that L-thyroxine administration and/or iron supplementation increases GSH, GSH-Px and SOD levels of erythrocytes.

Key words: iron, hyperthyroidism, glutathione, superoxide dismutase, glutathione peroxidase

The human body is under constant assault by reactive oxygen molecules, formed as a natural consequence of normal biochemical activity. Within aerobically living cells molecular oxygen is reduced in several ways to produce reactive oxygen species such as superoxide radicals ($O_2^{\cdot-}$), hydroxyl radicals (OH^{\cdot}) and oxygen centered radicals of organic compounds (ROO^{\cdot} and RO^{\cdot}). Under normal conditions, these radicals are effectively scavenged by enzymes and small molecules (1, 2).

Free radical-mediated oxidant stress is involved in

several diseases. The delicate balance maintained between oxidants and antioxidants may be tilted towards the oxidative side under pathological conditions. That free radical-mediated oxidant stress is involved in the genesis of hyperthyroidism-induced tissue damage has been demonstrated in clinical and experimental studies (3, 4).

It has been postulated that accelerated mitochondrial respiratory electron transport brought about by a hypermetabolic state results in increased generation of superoxide at the site of ubiquinones (5, 6). This species and other active oxygen radicals generated by a cascade of free radical reactions can cause rapid spread and amplification of oxidative injury in biologic systems (7, 8).

Iron, the most common redox-active transition element present in biological systems, is known to be an active participant in the oxidant/antioxidant balance (9, 10).

This study was performed to determine the impact of L-thyroxine administration and/or iron supplementation on the antioxidant status of erythrocytes. To this end, erythrocyte SOD, GSH-Px and GSH levels were determined.

Materials and Methods

Animal treatment. Adult male Wistar albino rats weighing 200-250 g were used. The rats were permitted *ad libitum* access to standard lab chow and tap water for 10 days prior to experimental procedures.

The animals were divided into 4 weight-matched groups. Group 1: Control group ($n = 9$); Group 2: L-thyroxine-administered group ($n = 7$); Group 3: Iron-supplemented group ($n = 8$) and Group 4: L-thyroxine-administered and iron-supplemented group ($n = 9$).

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The rats in the control group received standard lab chow and tap water. The rats in groups 2 and 4 were rendered hyperthyroid by the administration of L-thyroxine (0.4 mg/100 g food) for 24 days. The rats in groups 3 and 4 received oral iron (Ferro III hydroxide polymaltose) orally at a dose of 200 mg/kg body weight. At the end of the administration period, the rats were killed under ether anesthesia (50 mg/kg).

Preparation of erythrocyte lysate. Heparinised blood samples were obtained by heart puncture. After centrifugation at 2,500 *g* for 5 min, the plasma was removed and erythrocytes were washed three times in 5 ml of sterile 9 g/L NaCl solution, hemolyzed by diluting fourfold with water and stored at -80°C until use.

Analytical methods. T_4 and TSH (TKC4, IKNT1 Diagnostic Products Corp., Los Angeles, CA, USA) analyses were performed by radio immuno assay.

Assay of SOD activity. Erythrocyte SOD activity was determined using Sun's method (11). Zero point one ml of erythrocytes was lysed with 0.9 ml of ice-cold water (4°C). Hemoglobin (and Mn SOD) was removed by adding 0.3 ml of chloroform and 0.5 ml of ethanol and vigorously vortex mixing for 1 min. The mixture was centrifuged at 18,000 *g* for 60 min. The supernatant was diluted by a factor of 100, and 0.5 ml of the diluted solution was used. This assay for SOD activity involves inhibition of nitroblue tetrazolium (NBT) reduction with xanthine-xanthine oxidase used as a superoxide generator. One unit of SOD is defined as the amount of protein that inhibits the rate of NBT reduction by 50 %.

Assay of GSH-Px activity. GSH-Px activity was determined by a modification of the method used by Paglia and Valentine (12). GSSG produced by the action of GSH-Px and peroxide was reduced by glutathione reductase (GSH-RD) and NADPH, the decrease in concentration of NADPH being recorded at 340 nm. After centrifugation to remove cell debris, the hemolysate was diluted fivefold with Drabkin's reagent (1.0 g of sodium bicarbonate, 0.05 g of potassium cyanide and 0.20 g of potassium ferricyanide diluted to 1 L with deionized water) (12). Twenty microliters of hemolysate was transferred to a 1 ml quartz cuvette containing 980 μl of the reaction mixture (Tris buffer, 50 mM/L, pH 7.6, containing per liter, 1 mM of Na_2EDTA , 2 mM of GSH, 0.2 mM of NADPH, 4 mM of sodium azide and 1,000 U of GSH-RD). The mixture was incubated for 5 min at 37°C . The reaction was initiated by adding 10 μl of H_2O_2

(8.8 mM/L). The decrease in NADPH absorbance was followed at 340 nm for 3 min. The assay kinetics were calculated using a molar absorption rate for NADPH of $6.22 \times 10^{-3} \text{M}^{-1} \text{cm}^{-1}$ at 340 nm. Enzyme activity was expressed in terms of U/g of Hb. The hemoglobin concentration was determined in the hemolysate by the cyanmethemoglobin method (13).

Assay of glutathione. Erythrocyte glutathione values were determined using the method used by Beutler *et al.* (14), using metaphosphoric acid for protein precipitation and 5,5'-dithiobis-(2-nitrobenzoic acid) for colour development.

Statistical analysis. The data are presented as the mean \pm SD. Statistical significances between the groups were determined by Student's *t*-test and $P < 0.05$ was considered significant.

Results

Table 1 shows T_4 , TSH and plasma free iron levels of erythrocytes in the experimental groups.

Significantly high T_4 ($P < 0.001$) and undetectable TSH values confirmed the establishment of the hyperthyroid state in the L-thyroxine-administered group. Plasma free iron levels of the L-thyroxine-administered group were significantly lower ($P < 0.001$) as compared with the control group.

The T_4 level of iron-supplemented group was almost the same level as that of control group, but the TSH level was lower than the control level ($P < 0.01$). Plasma free iron values, though nonsignificant ($P > 0.05$), were higher than those of the control rats.

In the hyperthyroidism-induced iron-supplemented group, the T_4 level was significantly higher ($P < 0.001$) than the control group, but the TSH level was detectable. The TSH levels of the L-thyroxine-administered and iron-supplemented group were significantly lower ($P < 0.01$) as compared with the control group. Plasma free iron levels were significantly higher than hyperthyroid rats ($P < 0.001$).

Table 2 shows SOD, GSH-Px and GSH levels of erythrocytes in the experimental groups and a statistical comparison of the experimental groups.

In the L-thyroxine-administered group, GSH, GSH-Px and SOD levels of erythrocytes were significantly higher ($P < 0.001$) than the control group.

In the iron-supplemented group, GSH, GSH-Px and SOD levels of the erythrocytes were significantly in-

Table 1 Value of the determined parameters of plasma in the experimental groups (mean \pm SD)

	Control group (n = 9)	L-thyroxine- administered group (n = 7)	Iron- supplemented group (n = 9)	L-thyroxine-administered and iron-supplemented group (n = 8)
T ₄ (ng/dl)	7.79 \pm 2.85	27.4 \pm 15.05	8.87 \pm 3.72	24.56 \pm 8.48
TSH (μ IU/ml)	1.50 \pm 0.60	Undetectable	0.88 \pm 0.43	0.87 \pm 0.50
Fe ⁺² (μ g/100ml)	240.22 \pm 50.56	138.29 \pm 33.83	273.44 \pm 40.69	220.37 \pm 57.51

Table 2 Value of the antioxidant status parameters of erythrocytes in the experimental groups (mean \pm SD) and statistical comparisons

	Control group (n = 9)	L-thyroxine- administered group (n = 7)	Iron- supplemented group (n = 9)	L-thyroxine-administered and iron-supplemented group (n = 8)
SOD (mg/L)	164.90 \pm 22.08	246.11 \pm 13.20 *** a	214.61 \pm 40.72 *** b	267.20 \pm 24.00 *** a/ *** b/ ** c
GSH-Px (U/g Hb)	148.91 \pm 29.70	225.29 \pm 40.54 *** a	190.15 \pm 24.95 *** b	256.56 \pm 30.75 *** a/ *** b/ * c
GSH (mg/g Hb)	1.97 \pm 0.17	5.37 \pm 0.44 *** a	2.81 \pm 0.48 *** b	7.74 \pm 0.46 *** a/ *** b/ *** c

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. SOD: Superoxide dismutase; GSH-Px: Glutathione peroxidase.

a: Control group vs. L-thyroxine-administered group

b: Control group vs. Iron-supplemented group

a/: Control group vs. L-thyroxine-administered and iron-supplemented group

b/: Iron supplemented group vs. L-thyroxine-administered and iron-supplemented group

c: L-thyroxine-administered group vs. L-thyroxine-administered and iron-supplemented group

creased ($P < 0.001$) as compared with the control group.

Erythrocyte GSH, GSH-Px and SOD levels of the hyperthyroidism-induced iron-supplemented group were significantly higher ($P < 0.001$) than the control group.

When compared with iron-supplemented rats, GSH, GSH-Px and SOD levels of the hyperthyroidism-induced iron-supplemented group were significantly higher ($P < 0.001$).

Statistical by significant difference of GSH, GSH-Px and SOD levels of the hyperthyroidism-induced iron-supplemented group ($P < 0.001$, $P < 0.05$ and $P < 0.01$, respectively) was observed when compared with the

group given L-thyroxine alone.

Discussion

From experimental studies and epidemiological data, it can be inferred that hyperthyroidism is associated with a general increase in tissue oxidative stress. Great controversy exists as to whether hyperthyroidism is associated with an increase or decrease in the activities of antioxidant enzymes (15, 16).

In this study we aimed to investigate the response of antioxidant components in the erythrocytes to L-thyroxine

administration, iron supplementation and hyperthyroidism-induced iron supplementation. We chose to examine erythrocytes, because they are the cells that continuously produce oxygen radicals and H_2O_2 concomitant to spontaneous oxidation of Hb to met Hb.

Our findings of significantly increased T_4 values in L-thyroxine-administered group confirmed the establishment of a hyperthyroid state.

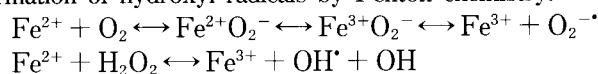
In these L-thyroxine-administered rats, SOD and GSH-Px activities and GSH values were found to be significantly increased in comparison to the control group. Activities of oxygen radical scavenging enzymes are thought to increase as an adaptive response to sustained oxidative stress, such as that in hyperthyroidism. The rate of metabolic processes is markedly influenced by a change in thyroid function. In thyroid functional diseases, disorders in tissue oxygenation and activation of free radical processes are followed by the stimulation of the antioxidant system in the blood (17, 18). During hyperthyroidism, antioxidants accumulate as increasing energy requirements are met to a greater extent by fatty acids. Recently Shull *et al.* (15) reported induction of SOD and GSH-Px with different forms of oxidative stress. There appears to be a mutually supportive interaction among the antioxidant enzymes which provides a defence against oxyradical toxicity. It is possible that an increase in oxyradicals during hyperthyroidism could increase GSH-Px activity which in turn would protect SOD inactivation by H_2O_2 and hence an increase in SOD activity. H_2O_2 is known to inactivate SOD (19). Increases in SOD activity would protect GSH-Px against inactivation by $O_2^{\cdot-}$. $O_2^{\cdot-}$ is known to inactivate GSH-Px (20). The net effect would be increases in SOD and GSH-Px activities, as confirmed by the findings of this study.

The high levels of GSH that we observed in the erythrocytes of L-thyroxine-administered rats are open to various interpretations. It is known that the erythrocyte membrane is impermeable to GSH. We measured the intraerythrocytic GSH concentration. On the basis of the suggestion by Morini *et al.* (3) that thyroid hormones alter the membrane fluidity; we might well suppose a change in GSH concentration due to altered transport in the hyperthyroid state. Furthermore, increased levels of GSH in the erythrocytes of hyperthyroid rats also may be due to the direct inductive effect of the hyperthyroid state on the components of the antioxidant system, also reflected by our findings of increased oxygen radical-scavenging enzymes.

Iron, bound to transferrin, is transported in the ferric state in a complex that is especially difficult to reduce. Importantly, the superoxide radical is capable of reducing ferritin-bound iron to a ferrous state, whereupon it is released. It is this iron, liberated by the inevitable production of superoxide, that is now free to catalyze Haber-Weiss chemistry, now commonly referred to as superoxide-driven Fenton chemistry. The liberated iron can also cause the reductive lysis of the oxygen-oxygen bond in a preexisting lipid molecule, giving rise to a lipid alkoxyl radical that may then serve as an initiator. Iron, as the most common redox-active transition element present in biological systems, is an active participant in the precarious balance between oxidants and antioxidants. Disturbance of the balance between the production of reactive oxygen species such as superoxide, H_2O_2 , hypochlorous acid, hydroxyl, alkoxyl and peroxy radicals and antioxidant defences produces oxidative stress, which amplifies tissue damage by releasing prooxidative forms of reactive iron that can drive Fenton chemistry and lipid peroxidation (16, 21, 22).

Superoxide radicals, a one-electron reduction product of oxygen, can co-react with H_2O_2 to produce a species with much greater oxidizing potential than either of the reactants. This new species is presumed to be the hydroxyl radical generated by the Haber-Weiss reaction.

Iron ions are themselves free radicals and ferrous ions can take part in electron transfer reactions with molecular oxygens. The intermediates $Fe^{2+}O_2$ and $Fe^{3+}O_2^-$ are known as perferryl ions, in which iron is said to have an oxidation state of five (23-26). Generation of superoxide, by any source, in the presence of iron ions can lead to the formation of hydroxyl radicals by Fenton chemistry.



The permissive impact of iron on oxidative processes was also observed in the significantly high SOD, GSH-Px and GSH levels in iron-supplemented rats *vs.* control rats. Furthermore, the TSH level of the L-thyroxine-administered iron-supplemented group was detectable. This led us to think that iron ions might have affected TSH metabolism.

To conclude: in our study increases in the activities of SOD, GSH-Px and GSH levels of L-thyroxine-administered iron-supplemented rats were found to be significantly higher than those of both L-thyroxine-administered and iron-supplemented rats.

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