

## Iron Supplementation in Experimental Hyperthyroidism: Effects on Oxidative Stress in Skeletal Muscle Tissue

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This study was designed to investigate the effects of iron supplementation on the parameters of oxidative stress in the skeletal muscle tissue of hyperthyroidism induced rats. Hyperthyroidism was found to cause an increase in thiobarbituric acid-reactive substances (TBARS) and copper zinc superoxide dismutase (Cu, Zn SOD) activity, but decreases in the glutathione-peroxidase (GSH Px) activity and glutathione (GSH). Iron supplementation caused an increase in TBARS and a decrease in GSH. Iron supplementation in hyperthyroid rats attenuated the hyperthyroid state, but lowered the plasma ferritin level, which is considered an indicator of thyroid hormone action. Iron supplementation caused no additional increase in the TBARS in hyperthyroid rats, ameliorated the decrease in GSH content and abolished the induction of Cu, Zn SOD.

Our findings suggested no increase, but a decrease, in the risk of oxidative stress in iron supplemented hyperthyroid rats. Whether supplementation of iron would have similar effects in humans should be further investigated in clinical studies.

**Key Words:** Hyperthyroidism, iron supplementation, skeletal muscle, lipid peroxidation, antioxidant status, ferritin

### INTRODUCTION

Thyroid hormones play a central role in differentiation, development and energy metabolism. In hyperthyroidism due to accelerated oxidative metabolism in the mitochondria, there is augmented production of reactive oxygen species (ROS).<sup>1-3</sup> It is well established that ROS induced lipid peroxidation, occurring mainly in the cell membrane, eventually leads to a loss in cellular integrity.<sup>4</sup>

Thyroid hormones have marked effects on skeletal muscle. They alter gene and isoform expressions, and thus modify the contractile and metabolic characteristics of the tissue.<sup>5-8</sup>

ROS are suggested to contribute to the muscular injury caused by thyroid hormones. Thyrotoxic myopathy and cardiomyopathy are known as major complications of hyperthyroidism.<sup>9-11</sup>

On the other hand, although uncommon, iron deficiency anemia has been reported in hyperthyroid patients, and has shown a substantial improvement on iron supplementation.<sup>12-14</sup> As ferrous iron, in the presence of a reducing agent, behaves as a potent catalyst for ROS formation and lipid peroxidation, iron supplementation in hyperthyroidism might be considered to lead to larger ROS producing and damaging potentials. According to our literature survey; no study has been performed in skeletal muscle tissue investigating the alteration in oxidative stress due to iron supplementation in hyperthyroidism. Therefore, the present study was undertaken to examine the impact of iron supplementation on the parameters of oxidative stress in skeletal muscle (M. gastrocnemius) tissue of hyperthyroid rats.<sup>15</sup>

As parameters of oxidative stress; TBARS, the indicator of lipid peroxidative damage, and Cu, Zn SOD, GSH Px and GSH, components of antioxidative defense, were measured.

### MATERIALS AND METHODS

#### Animal treatment

Adult male Wistar albino rats, obtained from

Received September 9, 2003

Accepted December 13, 2003

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Production Center of Laboratory animals of Cerrahpaşa Medical Faculty, weighing 200 - 250 g, were used. The rats were cared for in accordance with the Guide for the Care and Use of Laboratory Animals,<sup>16</sup> and permitted access to standard lab chow and tap water, ad libitum, for 10 days prior to the experimental procedures. The animals were divided into 4 weight -matched groups: control (C) (n=8), L-thyroxine (T) (n=7), Iron supplemented (I) (n=8) and L-thyroxine and iron supplemented (TI) (n=8) groups. The rats in the four experimental groups received the same standard lab chow (20 g/rat/day) and tap water. Complete consumption of food was ascertained daily. The rats in the T and TI groups were rendered hyperthyroid by the administration of L-thyroxine (Organon Inc., Istanbul, Turkey)-0.4 mg/100 g food for 24 days. Ferro III hydroxide polymaltose (Abdi İbrahim Inc., Istanbul, Turkey) was injected (250 mg/kg/day ip) into the rats in the I and TI groups. Twenty five days after the beginning of the experiments, the rats were killed by ether anesthesia (50 mg/kg), and blood samples and skeletal muscle tissues (M. gastrocnemius) obtained.

### Biochemical analyses

Heparinized blood samples were obtained by heart puncture. After centrifugation at 2500 g for 5 minutes, the plasma was removed and stored at -80°C for less than 3 weeks. T<sub>4</sub>, T<sub>3</sub> and TSH analyses were performed by radioimmunoassays (RIA) with the respective Coat-A-Count assays: TKT<sub>3</sub> 1, TCK4 and IKNT 1 (Diagnostic Products Corp. (DPC), Los Angeles, CA, USA). The plasma ferritin concentrations were measured by RIA (KFED1 DPC Los Angeles CA, USA).

### Homogenate preparation

For the determinations of TBARS and GSH; 10% (w/v) skeletal muscle tissue homogenates were prepared in 0.15 M cold KCl (pH 7). The homogenates were centrifuged at 1000 × g for 15 min to obtain the supernatant. For enzyme assays; the tissues were homogenized in 10 vols of 50 mM Tris-0.1mM EDTA (pH=7.4) at 4°C for 30 seconds (2 × 15 seconds with 15 seconds cooling intervals)

using a polytron homogenizer. Cytosolic fractions were prepared by centrifuging the homogenates for 15 min at 105000 × g in a Beckman ultracentrifuge.<sup>17</sup>

The muscle lipid peroxide concentration was measured by the reaction with thiobarbituric acid (TBA), as described by Ohkawa et al.<sup>18</sup> The reaction mixture contained: 0.2 ml 10% (w/v) tissue homogenate, 0.2 ml 8.1% sodium dodecyl sulfate (SDS), 1.5 ml 20% acetic acid solution, adjusted to pH 3.5 with NaOH, and 1.5 ml of a 0.8% aqueous solution of TBA. The mixture was made up to a final volume of 4 ml with distilled water, and then heated at 95°C for 60 minutes. After cooling with tap water, 1 ml of distilled water and 5 ml of a mixture of n-butanol and pyridine (15:1, v/v) were added, and the total mixture shaken vigorously. After centrifugation at 1500 × g for 10 minutes, the absorbance of the organic layer was measured at 532 nm. The levels of lipid peroxide were calculated using  $1.56 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$  as the molar extinction coefficient. The results were expressed as nmol of TBARS/g of protein. The tissue protein concentration was measured by the method of Lowry et al.<sup>19</sup>

The skeletal muscle Cu, Zn SOD activity was determined by the method of Sun et al.<sup>20</sup> The assay involved the inhibition of nitro blue tetrazolium (NBT) reduction with xanthine, with xanthine oxidase used as a superoxide generator. The Mn SOD in the homogenate was removed by the addition of 0.3 ml of chloroform and 0.5 ml of ethanol, by vigorous vortex-mixing for 1 minute. The mixture was centrifuged at 18000 × g for 60 minutes. The supernatant was diluted by a factor of 100, and the diluted solution used to assay the Cu, Zn SOD activity only. The results were expressed as mg/g of protein.

The skeletal muscle GSH Px activity was determined by a modification to the method of Paglia and Valentine.<sup>21</sup> The enzyme activity was measured from the oxidation of NADPH in the presence of H<sub>2</sub>O<sub>2</sub> used as substrate, which was monitored spectrophotometrically at 340 nm. The activity of the GSH Px was calculated using the molar extinction coefficient for NADPH ( $\Sigma 6.22 \cdot 10^3 \text{ M}^{-1}\text{cm}^{-1}$ ). One unit of enzyme activity was defined as 1 μmol of NADPH oxidized/minute the results were expressed as U/g of protein.

Skeletal muscle GSH content was determined in the supernatant of the tissue homogenate, according to the method of Beutler et al.,<sup>22</sup> using metaphosphoric acid to precipitate the protein and 5'5'dithiobis (2-nitro benzoic acid) for colour development. The results were expressed as mg/g of protein using  $1.36 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$  as the molar absorption coefficient.

### Statistical analysis

All values are expressed as the mean  $\pm$  standard error (SE). Analysis of variance (Oneway ANOVA) was used to compare the study groups. The Tukey test was used to determine the *p* values. A *p* < 0.05 was considered significant.

## RESULTS

In the animals fed thyroxine (T group), the induction of hyperthyroidism was established by

the elevated plasma levels of  $T_3$  (~120%) and  $T_4$  (~270%) and the decrease in the TSH to undetectable levels (Table 1).  $T_4$  administration also led to an elevation in the ferritin level (~190%). In the iron supplemented rats (I group), the plasma ferritin was increased by only (25%, while the  $T_3$  was increased by (95%. In contrast, when the hyperthyroid rats were administered iron (TI group) the  $T_4$ -induced elevations in plasma  $T_3$  were blunted without any suppression on TSH. Iron supplementation of the hyperthyroid rats also blunted the  $T_4$  induced elevation in the plasma ferritin.

The establishment of hyperthyroidism in rats (T group) caused elevation in the TBARS by 220%, increased the Cu, Zn SOD activity by (45% and decreased the GSH Px by (65% (Table 2). Hyperthyroidism also decreased the muscle GSH levels by (75%. Iron supplementation of the rats (I group) elevated the TBARS by (390% and decreased the GSH by (30%, but without affecting the Cu, Zn SOD or GSH Px activities. Iron supple-

**Table 1.** Effects of Hyperthyroidism and Iron on the Parameters of Thyroid Hormone Function

Parameters	Control(C)	L-thyroxine-administered(T)	Iron supplemented (I)	L-thyroxine administered and iron supplemented (TI)
$T_3$ (ng/100ml)	67 $\pm$ 10	146 $\pm$ 59 <sup>a</sup>	129 $\pm$ 51 <sup>a</sup>	100 $\pm$ 27 <sup>b</sup>
$T_4$ (mg/100ml)	4.31 $\pm$ 0.82	15.94 $\pm$ 4.72 <sup>a</sup>	5.31 $\pm$ 2.38	11.55 $\pm$ 1.39 <sup>a,b,c</sup>
TSH (mIU/ml)	0.38 $\pm$ 0.21	Undetectable	0.35 $\pm$ 0.32	Undetectable
Ferritin (ng/ml)	2.97 $\pm$ 0.4	8.66 $\pm$ 4.5 <sup>a</sup>	3.80 $\pm$ 0.4 <sup>a</sup>	3.9 $\pm$ 1.1 <sup>b</sup>

Data are mean  $\pm$  S.E., of 8 rats per group. Statistically significant at *p* < 0.05.

<sup>a</sup>Significantly different from control.

<sup>b</sup>Significantly different from L-thyroxine-administered.

<sup>c</sup>Significantly different from iron supplemented.

**Table 2.** Effects of Hyperthyroidism and Iron on the Antioxidant Status Parameters in Skeletal Muscle

Parameters	Control(C)	L-thyroxine-administered(T)	Iron supplemented (I)	L-thyroxine administered and iron supplemented (TI)
TBARS (nmol/g protein)	421 $\pm$ 193	1350 $\pm$ 270 <sup>a</sup>	2064 $\pm$ 469 <sup>a</sup>	1535 $\pm$ 539 <sup>a,c</sup>
Cu,ZnSOD (mg/g protein)	32.04 $\pm$ 7.7	46.23 $\pm$ 20.4 <sup>a</sup>	30.06 $\pm$ 9.6	31.32 $\pm$ 5.9 <sup>b</sup>
GSH-Px (U/g protein)	549 $\pm$ 98	204 $\pm$ 136 <sup>a</sup>	576 $\pm$ 149	161 $\pm$ 116 <sup>a,c</sup>
GSH (mg/g protein)	2.96 $\pm$ 1	0.70 $\pm$ 0.4 <sup>a</sup>	2.09 $\pm$ 0.5 <sup>a</sup>	1.69 $\pm$ 0.4 <sup>b</sup>

Data are mean  $\pm$  S.E. of 8 rats per group. Statistically significant at *p* < 0.05.

<sup>a</sup>Significantly different from control.

<sup>b</sup>Significantly different from L-thyroxine-administered.

<sup>c</sup>Significantly different from iron supplemented.

mentation of the hyperthyroid rats (TI group) decreased the muscle GSH (~45%) to levels between those found in the T and I groups and abolished the increase in the Cu, Zn SOD activity that had been induced by hyperthyroidism. The hyperthyroid rats supplemented and unsupplemented with iron displayed no significant differences with respect to either the TBARS or GSH Px activity.

## DISCUSSION

This study was performed to investigate alterations in the parameters of oxidative stress in skeletal muscle (M. gastrocnemius) tissue of hyperthyroid rats due to iron supplementation. The measured values of the thyroid status parameters confirm that hyperthyroidism was established in both of the thyroxine administered groups, (T) and (TI). However, the lower  $T_4$  and  $T_3$  values observed in the (TI) group compared to those in the (T) group suggest that iron attenuates the hyperthyroid state. Reduction in gastrointestinal absorption of orally administered levothyroxine has been attributed to ferrous sulphate intake in patients with primary hypothyroidism, and hypothyroid patients predisposed to iron deficiency anemia were reported to require an increased dosage of thyroxine.<sup>23</sup> It had been shown that three thyroxine molecules, via phenolic, carboxylate and amine groups, bind to a single ferric iron to form an insoluble ferric thyroxine complex.<sup>24</sup> Since the thyroxine and iron in this study were administered orally and intraperitoneally, respectively, the interference in the absorption was unexpected. However, after being absorbed, the thyroxine might have complexed with iron.

The plasma ferritin level, which is considered a marker of thyroid hormone action, was elevated in both the thyroxine administered groups compared to the controls. Upregulation of the ferritin expression in the liver due to  $T_3$ , and its contribution to plasma ferritin, was reported by Leedman et al.<sup>25</sup> However, the elevation was lower in the TI group. This finding supports our suggestion that iron attenuates the hyperthyroid induced state. As expected, the plasma ferritin, an indicator of available stored iron, was increased in the

iron administered rats.

Increased lipid peroxidative damage was found in the gastrocnemius muscles of the rats in all the experimental groups (T, I and TI) compared to that in the controls. Lipid peroxidative damage per se, or that due to proteolysis, may account, at least partly, for the muscle weakness found in hyperthyroidism.<sup>2,3,26,27</sup> The highest TBARS value was observed in the group administered iron alone. The hyperthyroid groups, whether iron supplemented or unsupplemented, displayed similar values. Increased lipid peroxidation has previously been demonstrated in the heart and slow oxidative muscles of hyperthyroid animals.<sup>2,3,11,27</sup> In iron supplemented rats, increased lipid peroxidation is expected. Although the plasma ferritin level was increased, and thus the administered iron sequestered, it is possible that intraperitoneally administered iron might still circulate in an unshielded state, if only in catalytic amounts. On the other hand, the elevated  $T_3$  levels in the iron supplemented rats might have also contributed to the increased lipid peroxidative damage. In rats administered both iron and  $LT_4$ , the decrease in the amount of circulating iron due to  $T_4$  binding might be responsible for the lower lipid peroxidative damage compared to the group administered iron alone, despite the weakened antioxidative defense.

Although lipid peroxidative damage in skeletal muscle has consistently been reported to be increased in hyperthyroidism, the observations of antioxidant protection are not so clear. The glutathione dependent antioxidant system, specifically, is considered important for the maintenance of the structural and functional integrity of muscular tissue. In our study, the GSH content of the gastrocnemius muscle in all the experimental groups was lower than in the control group, the lowest content being in the thyroxine administered group. As the lowest TBARS values were also observed in this group, it may be suggested that consumption of GSH, to a certain extent, restricted the increase in the lipid peroxidation. Additionally, the enzymatic conversion of  $T_4$  to  $T_3$  in the skeletal muscle tissue, which requires GSH as a co-factor, might have contributed to the decrease in the GSH.<sup>28</sup>

GSH Px, the principal scavenger of  $H_2O_2$  in

skeletal muscle tissue, was observed to be lowered in both the thyroxine administered groups (T and TI), but interestingly, not in the iron supplemented rats, which displayed the highest amount of lipid peroxidation. Thus, the decrease in the GSH Px activity in the hyperthyroid groups might not have been due to inactivation by ROS, but to either enhanced proteolysis and/or the conversion of the muscle fibers from the GSH Px rich type to GSH Px depleted type in the hyperthyroid state.<sup>2</sup>

Cu, Zn SOD is a scavenger of  $O_2^-$  and thus considered the first line of antioxidant defense. Maral et al.,<sup>29</sup> who investigated the  $O_2^-$  and  $H_2O_2$  scavenging system in erythrocytes of several species, observed that Cu, Zn SOD may be constitutively present only at low levels, but is highly inducible under oxidative stress. Accordingly, the Cu, Zn SOD activity was found to be significantly increased in the L-thyroxine administered group. Elevated Cu, Zn SOD, but lowered GSH Px, reflected the unbalanced regulation of the antioxidant enzymes in the hyperthyroid gastrocnemius muscle.<sup>2</sup> The higher stability and resistance to proteolysis of Cu, Zn SOD, relative to GSH Px, should also be considered.<sup>26</sup>

Our findings revealed that iron supplementation attenuated the hyperthyroid state. In accordance with the attenuation of the hyperthyroid state the plasma ferritin level, which is considered an indicator of thyroid hormone action, was lower in the iron supplemented than the unsupplemented hyperthyroid rats. Although both hyperthyroidism and iron supplementation individually caused an increase in the lipid peroxidative damage in skeletal muscle tissue, supplementation of iron to hyperthyroid rats caused no further damage increase. Accordingly, iron supplementation ameliorated the decrease in the GSH content and abolished the induction in Cu, Zn SOD observed in hyperthyroid rats.

Our findings suggest no increase, but a decreased, risk of oxidative stress in iron supplemented hyperthyroid rats. Whether supplementation of iron would have similar effects in humans should be further investigated in clinical studies.

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