THE HIDDEN PHENOMENON OF OXIDATIVE STRESS DURING TREATMENT OF SUBCLINICAL-MILD HYPOTHYROIDISM: A PROTECTIVE NUTRACEUTICAL INTERVENTION

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Introduction

It is known that thyroid hormones are associated with the redox-balance homeostatic regulation. Indeed, thyroid dysfunctions increase lipoperoxides which is an autocatalytic mechanism leading to oxidative damage of cellular membranes (1). Such damage may bring about cell death and to the production of toxic and reactive aldehyde metabolites, where malondialdehyde (MDA) is the most important. Subclinical hypothyroidism (SH), defined as an elevated serum thyroid stimulating hormone (TSH) level associated with serum thyroid hormone concentrations within the reference range, has been reported to affect 4-10% of Western populations (2, 3). Moreover, although depression of metabolism due to hypothyroidism has been described to decrease oxidant production and thus theoretically protecting tissues against oxidative damage, clinical evidences point out, on the contrary, that patients with hypothyroidism have an increased risk of developing atherosclerosis (4) and SH is also considered either experimentally and clinically a definite risk factor for overall cardiovascular aging and disease (5, 6). Accordingly, there are several data associating an hypothyroid status to enhanced oxidative stress (7-9). However, this issue is far from being completely settled in that, for instance, when hypothyroid rats were treated with 3,3,,5-triiodothyronine (T3), a significant increase in the level of oxidative stress parameters in mitochondrial fraction (MF) was recorded in adult rat brain (10). However, hydrogen peroxide content of MF as well as post-mitochondrial fractions of cerebral cortex was elevated by induced-hypothyroidism and reverted to normal level by subsequent treatment of T3. Indeed, when applying thyroid hormone supplementation (THS), it should be considered that T3 exerts calorigenic action by accelerating mitochondrial O_2 consumption through the triggering of the transcription of respiratory genes, which enhances reactive oxygen species (ROS) production. Existing data suggest that THS calorigenesis is reached by either a short-term nongenomic signaling mechanism mediated by 3,5- diiodothyronine and T3 leading to the allosteric stimulation of cytochrome-c oxidase and also a long-term pathway upregulating nuclear and mitochondrial gene transcription via T3 signaling. This respiratory factor accounts for up to 25% of the net increase in total O_2.
consumption and, as a matter of fact, increased ROS is also a feature of hyperthyroidism (11). Thus, it is not surprising the finding of a very recent study suggesting that oxidative stress in subjects with primary hypothyroidism under therapy with L-T4 might be the cause of the side effects following L-T4 therapy in relation to oxidative stress (12). This might one of the reasons behind the discomfort and loss of working activity often experienced in these patients not considering also further potential ROS-mediated subclinical phenomena to be manifested on the long run. When considering a possible antioxidant intervention, we relied on a certified fermented papaya-based nutraceutical (FPP, Immun-Age®, ORI, Gifu, Japan, made under ISO 9001 and ISO 14001 from a patented biofermentation process of non-OGM carica papaya) which has been shown to possess effective redox-modulating properties either in in vitro, experimental and in clinical setting, as summarized in a recent review (13). Thus, the aim of the present study was to test such redox-balance modulator, FPP, in association with treatment of SH or mild hypothyroidism (MH) in terms of: clinical symptom score, oxidative stress, lipid profile and gene expression involved in thyroid regulation.

**Material and Methods**

**Patients.** 60 generally healthy females, aged 18-55, not on birth pill control or soy supplement. Exclusion criteria were: main chronic diseases, relevant medications, major dislipidemia disorders, heavy physical activity and psychiatric disorders. All subjects presented with SH or MH and had been previously found to have normal level of zinc, selenium and copper.

Patients were put on a 2-week wash-out period and then divided into two groups (30 each) matched as for age, routine biochemical status, dietary profile and thyroid status assessment. Both groups received similar medical treatment for their hypothyroidism. One group was given: FPP 3gr 1 sachet twice-a-day for 3 months while the other group was given a flavored sugar sachet as placebo. A matched group of normal thyroid function subjects was our healthy control (HC). Moreover, the
web-based version of the National Institutes of Health Diet History Questionnaire was employed to assess diet history over the past month and along the study period and were given written and verbal instructions by a registered dietitian.

Biochemical tests.

After a 2-week wash-out period, besides routine biochemistry, unbound free T3 (FT3), free thyroxine (FT4) and TSH levels were measured in the serum using Microparticle Enzyme Immuno-Assay (Abbott Laboratories, Abbott Park, IL, USA). The reference range for FT3 was 2.05-3.65 pg/ml; for FT4, 0.71-1.85 ng/dl and for TSH, 0.47-5.01 mIU/l. Plasma oxidized glutathione, superoxide dismutase, lipid hydro-peroxide, glutathione peroxidase and malondyaldehyde were assessed by spectrophotometric analysis.

RNA isolation and thyroid receptors gene expression analysis

Total mRNA was isolated from mononuclear cells with the RNeasy Mini Kit (Qiagen GmbH). The mRNA was then treated with DNase I (Gibco BRL) before RT with Moloney murine leukaemia virus RT. Quantitative evaluation of TH receptors (TRα-1, TRβ-1) was carried out using real-time quantitative RT-PCR (qRT-PCR) with an ABI PRISM 7000 sequence detection apparatus (Applied Biosystems, Foster City, CA, USA). Primers were designed with Primer Express software (Applied Biosystems). The 25-μl reaction mixture contained 12.5 μl of SYBR Green PCR Master Mix, 10 ng of cDNA template and one pair of the primers 5′-GCT GCA GGC TGT GCT GCT A-3′ (forward) and 5′-CGA TCA TGC GGA GGT CAG T-3′ (reverse) for TRα-1, 5′-GTG TCT CAA GTG CCC AGA CCT T-3′ (forward) and 5′-CAC AGA GCT CGT CCT TGT CTA AGT AA-3′ (reverse) for TRβ-1. Relative quantification of TRα-1, TRβ-mRNA expression was analyzed by the comparative threshold cycle method. The relative quantization value of the target, normalized to an endogenous control BMG (housekeeping) gene and relative to a calibrator is expressed as 2ΔCt.
Statistical analysis

All measurements were performed in duplicate and were repeated at least three times. SPSS 15.0 software was used for analysis of the data. Comparisons of data among groups were analyzed by Kruskal-Wallis test. Further comparisons between either two groups were analyzed by Nemenyi test. The values were considered to be statistically significant at p<0.05.

Results

No reduced intake of main dietary trace minerals and vitamins appeared from the dietary questionnaire data while lipid profile and thyroid hormone parameters remained unchanged and unaffected by FPP supplementation (data not shown). As compared to HC a significant increase of all oxidative markers was observed in MH subjects (p<0.05) but not in SH. Treatment with T4 brought about a further increase (p<0.05 vs baseline) in MH and a significant increase also in SH subjects. While placebo was ineffective, FPP-supplemented individuals showed a significant normalization of redox markers in all tested subjects (p<0.01). As compared to baseline symptom score, treatment with FPP showed a non-significant trend improvement (n.s). However, at the entry only 6 (20%) subjects in the placebo group and 8 (27%) in the FPP-treated group reported symptoms affecting their quality of life. Although the limited number didn’t allow a deeper analysis, interestingly all 3 subjects in the FPP-treated group reporting a long-standing gastrointestinal discomfort often requiring to lower the dosage of the T3 therapy when cyclically being prescribed this in association to their established T4, reported a complete recovery. THS case a significant down-regulation of TRα-1 mRNA (p<0.01 vs baseline) but not of TRβ-1 genes and this pattern was unaffected by FPP.
Discussion

It is known that either genomic and nongenomic molecular mechanisms are involved in the functionality of thyroid hormone. The genomic mechanisms mainly act in the interaction between T3 with nuclear thyroid hormone receptor (TR) proteins, such as TRβ1, and the development of intranuclear complexes of either coactivators or corepressors. These modulate the transcription by binding to the promoter regions of thyroid hormone-responsive genes, (14). It has been experimentally shown that following thyroid hormone-enhanced ROS generation in the liver, it occurs a damage to polyunsaturated fatty acids, proteins and DNA (15) and worsening hepatic injury caused by other injurious factors by amplifying ROS generation and macrophage hyperplasia- and hypertrophy-induced Kupffer cell activity (16). Indeed, in humans, hyperthyroidism is characterized by significant changes in redox balance, including increased levels of conjugated dienes, H$_2$O$_2$ and lipid hydroperoxides (11) with reduced levels of thiols. However, literature data and our present one confirm that under normal THS either in SH and MH this pro-oxidant state increases the oxidative stress generation when the reduction in the antioxidant potential is not adequately compensated while an expected TR and redox gene upregulation takes place.

It appears that not only FPP can counteract the thyroid hormone-induced oxidative stress but it does not impair the physiological primary hormone-related receptors. This may be speculatively advocated for by a possible enhancement of the inner mitochondrial efficiency due to better antioxidant mechanisms and avoiding the weakening antioxidant defense as shown by Zhang et al (17). It remains mandatory to implement a careful tailor-made THS because even limited increases of TSH may allow considerable health benefits (18). However, given the failure of vitamin C to counteract the associated redox-inbalance during THS (19), these findings prove that FPP intervention might be an advisable integrative treatment to be associated to long-standing THS.
Redox-gene and other transcription factor regulation studies are under way, given the increased complexity of these metabolic pathways (20).

References

1) Catalá A. A synopsis of the process of lipid peroxidation since the discovery of the essential fatty acids. Biochem Biophys Res Commun. 2010; 399:318-323.


18) Taylor PN, Razvi S, Pearce SH, Dayan C. A Review of the Clinical Consequences of Variation in Thyroid Function Within the Reference Range. J Clin Endocrinol Metab. 2013 Jul 3. [Epub ahead of print]


**1A**

**REDOX BALANCE PARAMETERS**

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<thead>
<tr>
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<th>Before T4</th>
<th>T4 + placebo</th>
<th>T4 + FPP</th>
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<tr>
<td>MDA (μmol/L)</td>
<td>0.26±0.19</td>
<td>0.73±0.21*</td>
<td>0.33±0.26**</td>
</tr>
<tr>
<td>L-HPX (μmol/L)</td>
<td>2.2±0.5</td>
<td>6.2±0.6*</td>
<td>3.1±0.8**</td>
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<tr>
<td>GPX (U/L)</td>
<td>634.2±91.6</td>
<td>688.5±102.2*</td>
<td>648.7±94.3**</td>
</tr>
<tr>
<td>SOD (U/L)</td>
<td>24.4±4.1</td>
<td>23.6±2.2</td>
<td>30.8±2.6**</td>
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* p<0.01 vs. baseline at wash out time; ** p<0.05 vs. placebo group

**1B**

**THYROID RECEPTOR TRα-1 GENE EXPRESSION**

![Graph showing gene expression over time](image)

Fig. 1A
Redox Balance Parameters
MDA: malondialdehyde; L-HPX: hydroperoxides; GPX: glutathione peroxidase; SOD: superoxide dismutase
*p<0.01 vs. baseline at wash out time; **p<0.05 vs. placebo group

Fig 1B
Thyroid Receptors gene expression: effect of THS and supplementation
White bars: placebo-treated; grey bars: FPP-treated
*p<0.05 vs. baseline at wash out time;

254x190mm (96 x 96 DPI)
Legend

Fig. 1A

**Redox Balance Parameters**

MDA: malondialdehyde; L-HPX: hydroperoxides; GPX: glutathione peroxidase; SOD: superoxide dismutase

*p<0.01 vs. baseline at wash out time; **p<0.05 vs. placebo group

Fig 1B

**Thyroid Receptors gene expression: effect of THS and supplementation**

White bars: placebo-treated; grey bars: FPP-treated

*p<0.05 vs. baseline at wash out time;