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ORIGINAL ARTICLE

Oxidative DNA damage: the thyroid hormone-mediated effects of insulin on liver tissue

Nilgün Altan · Aylin Sepici-Dinçel · Duygu Şahin · Nilgün Kocamanoğlu · Funda Kosova · Atilla Engin

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Abstract Thyroid hormone affects glucose homeostasis with its actions between the skeletal muscle and liver and the altered oxidative and non-oxidative glucose metabolism. In our study three chemicals are considered biomarkers associated with oxidative stress for protein modifications were measured; 8-hydroxy-2-deoxyyguanosine (8-OHdG), a major lesion that can be generated by reactive oxygen species for DNA damage, protein carbonyl content (PCO), products of protein oxidation and advanced oxidation protein products (AOPPs) a dithyrosine containing cross-linked protein products. The purpose of the recent study was to determine the effects of insulin and T_4 or their combination in diabetic, thyroidectomized, or diabetic-thyroidectomized rats and possible relations with oxidative DNA and protein damages. For this purpose, rats were assigned to eight groups: Group 1; control, Group 2; diabetes, Group 3; diabetes + insulin, Group 4; surgically thyroidectomized control, Group 5; thyroidectomized + diabetes, Group 6; thyroidectomized + diabetes + insulin, Group 7; thyroidectomized + diabetes + insulin + thyroid hormone, levothyroxin sodium, 2.5 μg/kg and Group 8; thyroidectomized + diabetes + insulin + thyroid hormone, levothyroxin sodium, 5.0 μg/kg for 5 weeks. After the genomic DNA of liver tissues was extracted, the ratio of 8-OHdG to deoxyguanosine and liver tissue protein oxidation markers was determined. The main findings of our recent study were the increased 8-OHdG levels during the diabetes, hypothyroidism, and hypothyroidism with diabetes, which can be regulated in different percentages with the treatment of 2.5 and 5.0 μg/kg doses of thyroid hormone and the altered protein carbonyl and AOPP levels of liver tissue. Consequently, it was observed that the DNA and protein damage induced by oxidative stress in diabetes could be regulated by dose-dependent thyroid hormone-mediated effects to insulin treatment.

Keywords Diabetes mellitus · Thyroid hormone · Deoxyguanosine (dG), 8-hydroxydeoxyguanosine (8-OHdG) · Protein oxidation

All the authors in the affiliations were mentioned during the study.

N. Altan (✉) · A. Sepici-Dinçel · D. Şahin
Department of Medical Biochemistry, Faculty of Medicine, Gazi University, Ankara, Turkey
e-mail: naltan@gazi.edu.tr

N. Kocamanoğlu
Ministry of Health, Ankara, Turkey

F. Kosova
Celal Bayar University, Manisa, Turkey

A. Engin
Department of Surgery, Faculty of Medicine, Gazi University, Ankara, Turkey

Introduction

In the presence of diabetes, persistent hyperglycaemia causes increased production of free radicals via both auto-oxidation of glucose and enzymatic protein glycation that may lead to disruption of cellular function and oxidative damage to membranes [1, 2]. Recent investigations were shown that reactive oxygen species (ROS) could be important factors in the cellular homeostasis maintenance and physiological signal transduction. Besides, they claimed to participate in the regulation of the cellular replication, differentiation, senescence, and apoptosis [3].

Oxidative stress may also play an important role in the pathogenesis of early and long-term complications of human diabetes. The most important oxygen-free radical causing damage to basic biomolecules (proteins, membrane lipids, and DNA) is the hydroxyl radical ($HO^{\bullet}$). The interaction of $HO^{\bullet}$ with the nucleobases of the DNA strand, such as guanine, leads to the formation of C8 hydroxyguanine (8OHGua) or its nucleoside form deoxyguanosine (8-hydroxy-2-deoxyguanosine). In nuclear and mitochondrial DNA, 8-hydroxy-2-deoxyguanosine (8-OHdG) or 8-oxo-7,8-dihydro-2-deoxyguanosine (8-oxodG) is one of the predominant forms of free radical induced oxidative lesions and has, therefore, been widely used as a biomarker for oxidative stress [4]. In addition, protein carbonyl content (PCO) is used as a marker of oxidative modification of proteins that is the general and well-used biomarker of severe protein oxidation [5] and considered to be an important player in a variety of diseases [6]. Another marker of protein oxidation, advanced oxidation protein products (AOPPs) has begun to attract the attention of investigators [7], which was described by Witko-Sarsat et al. [8], for the first time as a dithyrosine containing cross-linked protein products, and are also considered as reliable markers to estimate the degree of oxidant-mediated protein damage.

Induction of diabetes by streptozotocin (stz) has been shown to produce a hypothyroid state in experimental animals. In diabetic patients, it is also difficult to control the blood glucose levels since the function of thyroid tissue is on hypothyroid state. Thyroid hormones are known to regulate glucose metabolism, and hypothyroidism is often associated with hyperglycemia [9]. In our previous study, same groups of animals serum samples were used, so we concluded from the manuscript with the idea that during diabetic hypothyroidism, insulin is not sufficient to balance the metabolic pathways, so mediated effects of insulin in leptin regulation via thyroid hormones are an increased possibility [10]. Thyroid hormone affects glucose homeostasis with its actions on a variety of organs including increased hepatic glucose output, increased unsuccessful cycling of glucose degradation products between the skeletal muscle and liver and the altered oxidative and non-oxidative glucose metabolism [11]. Variations of the levels of thyroid hormones can be one of the main physiological modulators of in vivo cellular oxidative stress that may effect the liver, heart, and skeletal muscles tissues, and also thyroid hormones are believed to be an antiperoxidative in nature [12].

The metabolic interactions were put together in our present study by liver tissue oxidative damage markers to DNA and protein that aimed mainly to describe the relationship between diabetes, hypothyroidism, insulin and thyroid hormones treatments via free radical changes. The aim of the recent study was to determine the effects of insulin and T_4 or their combination in diabetic, thyroidectomized, or diabetic-thyroidectomized rats and possible relations with oxidative DNA and protein damages.

Materials and methods

The study protocol was reviewed and approved by the Animal Care Committee and Surgical Research Center of Gazi University Faculty of Medicine (GUDAM). Guiding principles for experimental procedures found in the Declaration of Helsinki of the World Medical Association regarding animal experimentation were followed in the study. In this study, male Sprague–Dawley rats, weighting 200–250 g were maintained with 12 h of light and dark cycle, controlled humidity, temperature and free access to standard diet, and tap water for 7 days before experiment. Details about the body weights and induction of experimental diabetes mellitus were given in our first manuscript [10]. In brief, rats were assigned to eight groups:

Group 1; control ($n = 6$),
Group 2; diabetes ($n = 6$),
Group 3; diabetes + insulin ($n = 5$) (after diabetes induction, rats were treated with insulin for 5 weeks (7–10 U/kg/d, Insulatard-HM® Penfill®, Novo Nordisk, 100 IU/ml NPH, subcutaneously (s.c.) single injection per day),
Group 4; surgically thyroidectomized control ($n = 6$),
Group 5; thyroidectomized + diabetes ($n = 5$) (diabetes was done 3 weeks after the surgical operation at hypothyroid state),
Group 6; thyroidectomized + diabetes + insulin ($n = 6$),
Group 7; thyroidectomized + diabetes + insulin + thyroid hormone ($n = 4$) (after diabetes induction, rats were treated both with insulin and thyroid hormone, levothyroxin sodium, 2.5 μg/kg, Tefor® (Organon) for 5 weeks,
Group 8; thyroidectomized + diabetes + insulin + thyroid hormone, levothyroxin sodium, 5 μg/kg ($n = 5$). We used two different doses, one was the least and the other was the highest, so as to decide the critical dose.

Biochemical analysis

Determination of DNA damage

For the measurement of oxidative DNA damage (lesions/10^6 DNA nucleosides), after the genomic DNA of liver tissues was extracted by Qiagen DNA extraction kit, it was denatured by heating at 95°C for 3 min and then cooled on ice. 100 μl 2 mM DFAM in 20 mM acetate buffer (pH = 5) were added to the denaturated DNA. DNA

content was analyzed spectrophotometrically at 260 nm, and then hydrolyzed to nucleotides by incubation with 4 µl of 3.3 mg/ml suspension of nuclease P1. The Tris–HCl buffer (pH = 8.5) was added to the mixture and hydrolyzed to the corresponding nucleosides by incubation with calf intestine alkaline phosphatase for 1 h at 37°C. After adding acetate buffer and 50 mM EDTA/10 mM DFAM solution, the mixture was filtered through a 0.22-µm Millipore filter unit (UltraFree, Bedford, MA), and then centrifuged at 10,000×*g* for 20 min at 4°C. Reverse-phase HPLC-EC was performed as described by Floyd et al. [13]. The DNA hydrolysate was injected onto a Waters C18 reverse-phase column (5 µm, 0.46 × 25 cm; Waters Assoc, Milford, MA, USA) at a flow rate of 1 ml/min. The mobile phase was 50 mmol/l phosphate buffer (pH = 5.5) with 5% methanol [14, 15]. The eluant was monitored at 290 nm for the ultraviolet detection of dG and at 0.6 V for the electrochemical detection of 8-OHdG. System was calibrated with authentic dG and 8-OHdG standards (Sigma Chemical, St Louis, MO, USA). dG had a retention time of 10–12 min, and 8-OHdG had a retention time of 8.7–13.8 min. Standards were run after every fifth sample for verification, and the data were expressed as the ratio of 8-OHdG to 10^6 dG.

Determination of protein carbonyl group levels (PCO)

Protein carbonyl levels were measured by a spectrophotometric method of Levine et al. [16]. Liver samples of 100–200 mg were weighed and diluted with 20% w/v in 50 mM HEPES, pH = 7.2, homogenized, and centrifuged at 12,000×*g* for 10 min at 4°C, and then determination was performed in the supernatant fraction. Supernatants were submitted to 2,4-dinitrophenyl hydrazine (DNPH), followed by deproteinization with trichloroacetic acid, then pellets were washed in ethanol/ethyl acetate and solubilized in guanidine-HCl. The carbonyl contents were calculated by obtaining the spectra at 355–390 nm. Results were calculated as nmol carbonyl/mg protein.

Determination of advanced oxidation protein products (AOPPs)

The AOPP levels were measured spectrophotometrically and calibrated with chloramine-T solutions, in the presence of potassium iodide, absorb at 340 nm. AOPP levels were expressed in µmol chloramine-T equivalents per mg protein. 100–200 mg of liver sample was weighed and diluted 20% w/v in 20 mM ice-cold Tris–HCl, pH 7.4, homogenized, and centrifuged at 5,000×*g* for 10 min, then the analyte determination was performed in the supernatant fraction [8].

Statistical analysis

Data analysis was carried out using the SPSS 11.5 statistical package (SPSS, Chicago, IL, USA). The Kruskal–Wallis (non-parametric) test was applied to evaluate differences among all the groups, while differences between pairs of groups were evaluated by means of the Mann–Whitney test. The results were expressed as mean ± standard deviation values. Each of the results was evaluated for statistical significance at $P < 0.05$.

Results

After stz administration, rats demonstrated polyphagia, polydipsia, polyuria, and stable hyperglycemia for 5 weeks as determined by measuring blood glucose levels for every 3 days. Blood glucose concentrations, free T_3 (FT_3), free T_4 (FT_4), total T_3 (T_3) and T4 (T_4) concentrations, and HbA1c levels were given in the previous manuscript [10]. In brief, the body weight (g) measurements revealed a significant difference in all the groups compared to group 2 (159.0 ± 32.2), and also group 2 and 4 (285.0 ± 34.7) revealed a significant difference compared to group 1 (235.0 ± 35.3) and 5 (235.0 ± 22.7) at the time of sacrifice ($P < 0.05$). Blood glucose (mg/dL) determination showed a significant hyperglycemia in group 2 (424.0 ± 22.8) and 5 (487.4 ± 35.7) compared to group 1(109.5 ± 4.6) ($P < 0.05$). Serum FT_3 (pg/mL), FT_4 (pmol/L), T_3 (ng/mL), and T_4 (nmol/L) levels were significantly decreased ($P < 0.05$) in group 2 (1.39 ± 0.35, 7.54 ± 3.17, 0.28 ± 0.07, and 19.47 ± 7.23, respectively) after 5 weeks of diabetes, and serum FT3 levels were normalized in group 3 (4.33 ± 0.77) depending on insulin treatment compared to control, group 1 (3.87 ± 0.63). In group 5, FT_4 (4.20 ± 2.31) and T_4 (6.54 ± 0.01) levels were significantly decreased after the induction of diabetes ($P < 0.05$) compared to group 4 (9.14 ± 2.06, 15.54 ± 4.15, respectively). In group 7 and 8, all the levels of thyroid hormones were significantly increased after the treatment ($P < 0.05$). All values represent mean ± SD.

Increased 8-OHdG/10^6dG liver tissue levels were found in Group 2 (18.45 ± 9.32), Group 3 (62.50 ± 11.14), Group 4 (10.01 ± 5.77), Group 5 (82.14 ± 6.27), Group 6 (50.69 ± 7.87), Group 7 (25.19 ± 4.88), Group 8 (78.92 ± 2.86) when compared to control; Group 1 (2.80 ± 1.45) ($P < 0.05$). Similarly, 8-OHdG/10^6dG levels were significantly increased in Group 3, 5, 6, and 8 compared to Group 2 ($P < 0.05$). On the other hand, decreased 8-OHdG/10^6dG levels were found in Group 3, 4, 6, and 8 when compared to the Group 5 ($P < 0.05$) Fig. 1).

Liver AOPP (µmol/mg protein) levels in Group 2 (2.57 ± 0.26) and Group 6 (2.27 ± 0.58) were increased

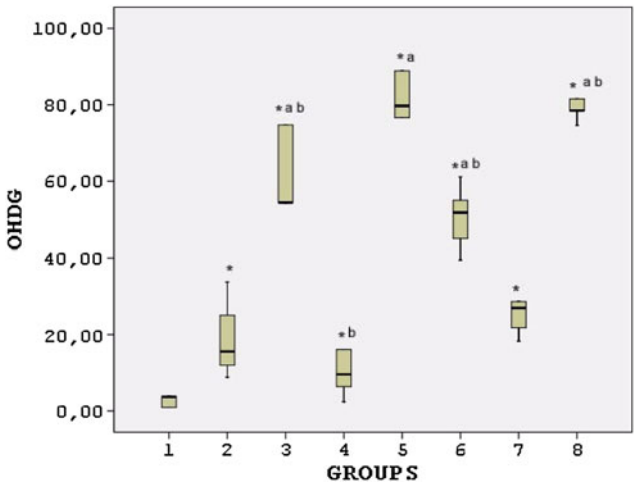

Fig. 1 Alterations of 8-hydroxy-2′-deoxyguanosine ratio to deoxyguanosine (8-OHdG/10^6 dG) levels in liver tissue of all the groups. All the values are expressed as mean ± SD values. *1*; control ($n = 6$), *2*; diabetes ($n = 6$), *3*; diabetes + insulin ($n = 5$) (after diabetes induction, rats were treated with insulin for 5 weeks), *4*; surgically thyroidectomized control ($n = 6$), *5*; thyroidectomized + diabetes ($n = 5$), *6*; thyroidectomized + diabetes + insulin ($n = 6$), *7*; thyroidectomized + diabetes + insulin + thyroid hormone, 2.5 μg/kg, Tefor® ($n = 4$), *8*; thyroidectomized + diabetes + insulin + thyroid hormone, 5 μg/kg, ($n = 5$). * $P < 0.05$ compared to control group 1. [a] $P < 0.05$ compared to diabetes group 2. [b] $P < 0.05$ compared to hypothyroidism + diabetes group 5

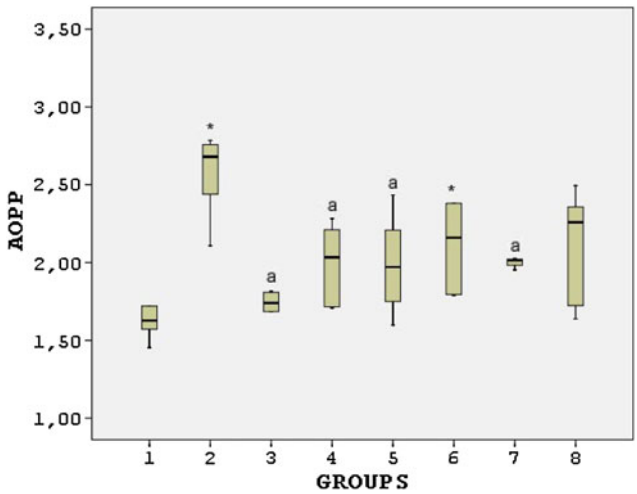

Fig. 2 Changes in the liver tissue of advanced oxidation protein product levels (AOPP, μmol/mg protein) in all the groups. All the values are expressed as mean ± SD values. *1*; control ($n = 6$), *2*; diabetes ($n = 6$), *3*; diabetes + insulin ($n = 5$) (after diabetes induction, rats were treated with insulin for 5 weeks), *4*; surgically thyroidectomized control ($n = 6$), *5*; thyroidectomized + diabetes ($n = 5$), *6*; thyroidectomized + diabetes + insulin ($n = 6$), *7*; thyroidectomized + diabetes + insulin + thyroid hormone, 2.5 μg/kg, Tefor® ($n = 4$), *8*; thyroidectomized + diabetes + insulin + thyroid hormone, 5 μg/kg, ($n = 5$). * $P < 0.05$ compared to control group 1. [a] $P < 0.05$ compared to diabetes group 2. [b] $P < 0.05$ compared to hypothyroidism + diabetes group 5

significantly with respect to control;Group 1 (1.72 ± 0.32) ($P < 0.05$). Decreased AOPP levels were found in Group 3 (1.61 ± 0.34), Group 4 (1.99 ± 0.25), Group 5 (1.99 ± 0.33), and Group 7 (2.00 ± 0.03) with respect to Group 2 ($P < 0.05$). No statistically significant difference was found in all the groups when compared to Group 5 Fig. 2).

Liver tissue PCO (nmol/mg protein) levels were significantly higher in Group 4 (2.78 ± 0.95), Group 5 (2.28 ± 0.82), and Group 7 (3.07 ± 0.17) when compared to control; Group 1 (0.61 ± 0.56) ($P < 0.05$). There was no significant difference observed between Group 2 (1.19 ± 1.44) and other groups. The decreased PCO levels were found in Group 3 (0.82 ± 0.46) and Group 6 (1.01 ± 0.67) when compared to Group 5 ($P < 0.05$) (Fig. 3). All the values represent mean ± SD.

Discussion

Our present study mainly investigates the effects of diabetes and thyroidectomy and replacements with insulin and/or T4 on three chemicals that are considered biomarkers associated with oxidative stress; DNA damage and protein oxidation. The main findings were the increased 8-OHdG levels of all the groups compared to control that could differ when compared to diabetes and thyroidectomy with diabetes groups. 8-OHdG is a major lesion that can be generated by ROS and like protein oxidation products it is mentioned as a marker of oxidative stress [17, 18]. In their study, Erkoc et al. [19] investigated the structural and electronic properties of guanine and guanosine theoretically by performing semiempirical and ab initio molecular orbital theory calculations and concluded that guanine is a highly polar molecule, therefore, it may interact with its surrounding, especially with other polar molecules in the cell more strongly; this makes the guanine molecule as a potential source of damage in the cells. Plasma 8-OHdG level can generally increase with age, smoking, radiotherapy, and also increased levels were observed with type 1 and 2 diabetes [20, 21]. In our study, we expressed our results as a ratio of 8-OHdG/dG in liver tissues to be an indicator of DNA damage, and our results were mostly in agreement with other studies [22, 23]. However, one of the striking levels was the increased DNA damage measurements observed in diabetes treated with insulin group compared to diabetes group, while the biochemical results such as glucose and thyroid hormones of the group were reasonable and insulin treatment can be considered sufficient. First, insulin thought to be an oxidative agent although it can regulate glucose metabolism, it is not able to protect the tissue from DNA damage during diabetes.

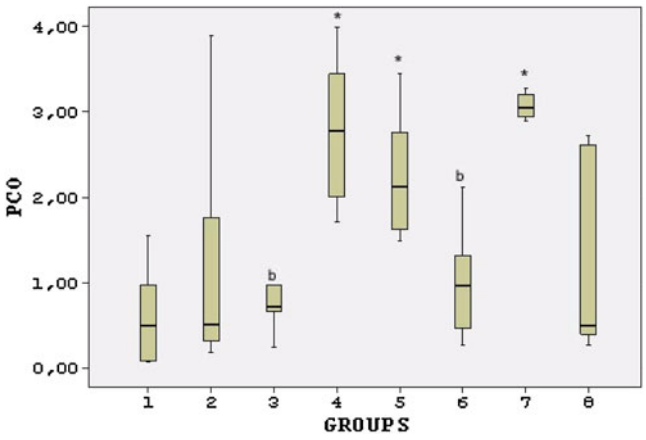

Fig. 3 Changes in the liver tissue of protein carbonyl group levels (PCO, nmol/mg protein) in all the groups. All the values are expressed as mean ± SD values. *1*; control ($n = 6$), *2*; diabetes ($n = 6$), *3*; diabetes + insulin ($n = 5$) (after diabetes induction, rats were treated with insulin for 5 weeks), *4*; surgically thyroidectomized control ($n = 6$), *5*; thyroidectomized + diabetes ($n = 5$), *6*; thyroidectomized + diabetes + insulin ($n = 6$), *7*; thyroidectomized + diabetes + insulin + thyroid hormone, 2.5 μg/kg, Tefor® ($n = 4$), *8*; thyroidectomized + diabetes + insulin + thyroid hormone, 5 μg/kg, ($n = 5$). * $P < 0.05$ compared to control group 1. [a] $P < 0.05$ compared to diabetes group 2. [b] $P < 0.05$ compared to hypothyroidism + diabetes group 5

Afterward, we also observed the increased levels of damage in thyroidectomized with diabetes group compared to diabetes group so that we can speculate on thyroid hormones that they have to be in the normal expected values during steady state metabolism. There is a synergism between thyroid hormones and insulin that affects known metabolic pathways without exact mechanism [24]. There are contradictory results in different studies whether the hypothyroidism or the hyperthyroidism cause the DNA damage. In their study, Andican et al. [25] observed increased damage in hyperthyroid rat liver tissue and other study suggested the decreased levels in rat heart tissue during hypothyroidism [26].

In addition to that in our previous study, we demonstrated that thyroid hormones were needed for insulin effect to reverse diabetes induced alterations in heart tissue [27], in a different study with experimental diabetic rats, decreased levels of thyroid hormones were maintained by insulin treatment [28], and different treatment protocols also reduced the 8-OHdG levels in various tissues [29, 30]. In our study, DNA damage was not able to be controlled with insulin treatment, but in surgically thyroidectomized rats with diabetes treated with both with insulin and appropriate thyroid hormone doses were succeeded. Thus, we suggest that the effects of insulin can occur differently in various tissue damages depending on dose and duration of treatment. Without relevant thyroid hormone levels, it is not possible to control the DNA damage, and also hypothyroidism with diabetes and high dose treatments are not able to assure the homeostasis. The detected DNA damage was worst in those groups. Pamplona et al. also proposed that thyroid hormones were the physiological regulators of both the tissue oxidative stress and the protein degradation. Besides, they were commited on the relationship between thyroid hormone levels and DNA damage and suggested that DNA was bewaring to oxidative stress [31].

Results of different studies showed that radical-mediated oxidation of proteins leads to fragmentation of the polypeptide chain, oxidation of thiol groups, nitration of aromatic amino acid residues, oxidation of amino acid side chains, AOPP formation, conversion of some amino acid residues to carbonyl derivatives, and generation of protein–protein cross linkages [32, 33]. Oxidative damage often induces loss of specific protein functions involved in the regulation of cell structure, signaling, and various enzymatic processes of the cell. The increased AOPP levels which is known as cross-linked protein products including dithyrosine [7] during diabetes and hypothyroidism with diabetes could be related with the damage to biological membranes and endothelium as a part of diabetic complications [34]. In our study, we observed significant increase in diabetes group. Surgical hypothyroidism with thyroid hormones was at low levels by itself and with diabetes was not able to form oxidative protein damage as diabetes did as observed in our study. Treatments applied with insulin and low-dose thyroid hormones with insulin were able to prevent the formation of AOPPs. On the other hand, the generation of carbonyl derivatives is orders of magnitude greater than other kinds of protein oxidation [35]. The carbonyl content is the general and well-used biomarker of severe protein oxidation. The usage of PCOs as a marker provides advantages as their early formation and their relative stability compared to the other oxidation products. The decreased PCO levels in insulin treated; diabetes

(group 2) and hypothyroid with diabetes (group 6) groups compared to hypothyroid with diabetes suggested that the therapeutic effects of insulin via thyroid hormones in which induction of diabetes by stz have been shown to produce a hypothyroid state in experimental animals. However, the higher levels of PCO were also observed in surgically hypothyroid, hypothyroid with diabetes group, and low-dose thyroid hormone-treated group when compared to control group. In their study, Resch et al. [36] showed both hyperthyroidism, and hypothyroidism was associated with enhanced oxidative stress involving enzymatic and non-enzymatic antioxidants, and Cakatay [37] suggested that the increase in plasma PCO, AOPP levels in the diabetic patients with poor glycaemic control might contribute to the development of diabetic complications.

In the absence of thyroid hormones, insulin may not work as sufficient as expected to control and balance the effects of oxidative stress to protein, lipids, and DNA, as a result insulin and thyroid hormones must work together.

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