Effect of Ginseng on the Testis of Subclinical Hypothyroidism Model in Adult Male Albino Rat

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Introduction
Thyroid hormone plays an important role in testicular development and function. It has been established that Triiodothyronine (T3) regulates the maturation and growth of the testis, controlling Sertoli cells and Leydig cell proliferation and differentiation during testicular development in rats and other species [1]. Subclinical Hypothyroidism (SCH) could be considered as an elevated serum thyrotropic stimulating hormone (TSH) level associated with normal Thyroxine (T4) and Triiodothyronine (T3) levels with no clinical symptoms of hypothyroidism. This thyroid dysfunction occurs in 4-20% of the adult population, the possibility of SCH progression to clinical hypothyroidism is around 7% [2]. Hypothyroidism extremely affects reproductive functions including fertility, pregnancy and postnatal development in human and rat [3]. Rat SCH model was induced mostly by administration of antithyroid drug mostly Propylthiouracil (PTU) and Methimazole (MMI) [4]. PTU is a thioucaril-derived drug which can be used to treat hyperthyroidism through lowering the amount of thyroid hormone produced by the thyroid gland [5]. PTU inhibits conversion of T4 to T3 causing hypothyroidism [6]. Ginseng is considered as an aromatic herb that is commonly used in herbal medicine [7]. It contains saponins and high iodine content [8]. Ginseng saponins, which are known as ginsenosides, have been studied to be responsible for its medicinal effects, specifically anticancer, antihypertensive, anti diabetic, and antistress effects. Ginseng benefits all the endocrine system, and therefore the thyroid gland. As ginseng markedly increased epididymal sperm count, motility and hyperactivation in mice [9]. So, we studied its effect on the testis and thyroid of SCH model.

Methodology
Animals
Forty adult Westman male albino rats were used in this experiment, each weighting 150-200gm. Food and water were provided ad libitum and the rats were left for 7 days for acclimatization before use in the experiment. The animals were divided into four groups as the following:

- **Group I (Control):** included ten rats that received oral propylthiouracil in a dose of 5mg/kg once daily for three weeks by gastric tube.
- **Group II (Ginseng treated):** included ten rats that received oral ginseng (Panax ginseng) a product of Pharco Pharmaceuticals, Alexandria, Egypt, was available in the form of capsules with the trade name ‘Ginseng 100’. Each capsule contained 100mg of the dried roots of Panax ginseng. The contents of the capsule was withdrawn by a syringe and dissolved in distilled water and given to the rats in a dose of 2mg/kg once daily for three weeks by gastric tube.
- **Group III (Subclinical hypothyroid SCH group):** included ten rats that received oral propylthiouracil in a dose of 5mg/kg once daily for three weeks by gastric tube.
- **Group IV (Treated group):** included ten rats with subclinical hypothyroidism that received oral treatment of ginseng in a dose of 2mg/kg dissolved in distilled water once daily for three weeks by gastric tube after the induction of subclinical hypothyroidism.

Hormonal assay
Two weeks after subclinical hypothyroidism induction, retroorbital blood samples of all rats were collected and plasma Thyrotropic Stimulating Hormone (TSH), Plasma Thyroxine (T4) and Plasma triiodothyronine (T3) were measured. Sera were separated and stored at −20°C until the hormonal assay was performed. Serum levels of T3 and T4 were determined by chemilumines-cence immunoassay using Amerlite kits (Bunkyo-ku, Tokyo, Japan) [10]. Serum levels of TSH were assayed by radioimmunoassay [11]. Serum levels were measured at the Clinical pathology Department, Faculty of Medicine, Menoufia University.
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**Epididymal spermatozoal study**

Twenty-four hours after the last treatment, animals were euthanized under anesthesia, according to the time of the experiment. The epididymal content of each rat was obtained immediately by cutting the tail of the epididymis and squeezing it gently to obtain fresh undiluted semen in a clean Petri dish to proceed for epididymal spermatozoal examination.

**Histological study**

Thereafter, animals were killed by cervical dislocation under ether anesthesia. The testes and thyroid gland of each rat were immediately excised and prepared for histological study. The right testis and the right thyroid lobe were cut into small pieces (about 1×1mm3) and fixed in 3% phosphate-buffered glutaraldehyde and was further processed and prepared for transmission electron microscopic examination [12] using a Joel 100 CX Electron Microscope (Joel, Tokyo, Japan) at the Electron Microscopy Unit, Faculty of Science, Menoufia University. Left testis samples were fixed in Bouin’s solution while left thyroid samples were fixed in 10% formol saline and processed to prepare 5μm-thick paraffin sections for:

1. **Haematoxylin and Eosin (H&E) stain.**
2. **Immunohistochemical stains for testis and thyroid sections.**

Testicular sections were incubated with rabbit polyclonal anti-Androgen Receptors (anti-AR) antibody. Fremont, California, USA (Thermo Scientific) [13] while thyroid sections were incubated with rabbit monoclonal Anti-Thyroglobulin antibody. Abcam’s RabMAb technology, USA.

**Quantitative assessment**

The following parameters were measured:

1. **Area % of immunopositive reaction of anti-AR antibody.**
2. **Area % of immunopositive reaction of Anti-Thyroglobulin antibody.**

Five non-overlapping fixed fields, each 2mm² in area, were taken using a Leica DMLB2/11888111 microscope equipped with a Leica DFC450 camera using the Leica C PLAN 10×0.22 objectives.

**Statistical analysis**

Data from the hormonal assay and immunohistochemical study were tabulated and expressed as mean±SD. They were fed into the computer using statistical package for the social sciences (SPSS, version 20; IBM SPSS, Chicago, Illinois, USA) software package [14]. Statistical analysis was carried out using one-way analysis of variance and the post-hoc test (Scheffe) for pair wise comparison. A level of significance of p<0.05 was considered to be statistically significant.

**Results**

There was no significant difference between Group I (Control) and Group II (Ginseng treated) rats in all the outcomes; therefore, these two groups were pooled in one group (Control).

**Hormonal assay**

The biochemical profiles of the three groups are shown in (Table 1). SCH was confirmed by measuring plasma T3, T4 and TSH levels. The serum TSH concentration in the SCH group was significantly increased compared with that in the control group.

**Epididymal spermatozoal study**

Results showed a statistically significant decrease (P<0.05) in the mean sperm count, viability and motility when compared with the control group (Table 2).

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**Table 1: Serum thyroid hormone, TSH levels.**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Mean±SD</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td><strong>T3 (ng/ml)</strong></td>
<td>13.22±9.56</td>
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<tr>
<td><strong>T4 (μg/dl)</strong></td>
<td>87±0.15</td>
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<tr>
<td><strong>TSH (μlU/ml)</strong></td>
<td>3.95±0.48</td>
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*Significant difference (P<0.05) as compared with the control group.

²Significant difference (P>0.05) as compared with the SCH group.

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**Table 2: Effects of SCH on rat testes.**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Mean±SD</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td><strong>Sperm count</strong></td>
<td>72.6±1.8</td>
</tr>
<tr>
<td><strong>Sperm viability (%)</strong></td>
<td>91.37±4.34</td>
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<tr>
<td><strong>Sperm motility (%)</strong></td>
<td>84±0.65</td>
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¹Significant difference (P<0.05) as compared with the control group.

³Significant difference (P<0.05) as compared with the SCH group.

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**Figure 1: Representative H&E staining of rat testes of different experimental groups.** The testes of control rats showed normal organization of the seminiferous tubules with narrow interstitial spaces (A). Seminiferous tubules of testes of control rat exhibited normal spermatogenesis with normal arrangement of Spermatogonia (Sg) and Sertoli Cells with triangular nuclei (SC) resting on an intact basement membrane. Typical structures of Primary Spermatocytes (PS), round and elongated Spermatids (SP) were found (B). In the testes of SCH group, some seminiferous tubules showed irregular boundaries (arrows, C), and many exhibited maturation arrest with no mature spermatозoa in their lumen (D and E), sloughed germinal epithelial cells (arrow, F), multiple giant cell formation (arrows, G). In some tubules, the germinal epithelium was separated from the basement membrane (arrows, H). The interstitium showed acidophilic hyaline material and dilated congested blood vessels (arrow head, arrows, I). Many spermatocytes appeared with pyknotic nuclei and surrounded by clear halos (arrows, J). Testis sections of the treated rats showed significant improvement of histological architecture with almost normal organized seminiferous tubules with normal spermatogenesis (K and L); however, a few seminiferous tubules showed pyknotic nuclei (arrow, K). Scale bar = 200 μm (A, C, D, H, I, and K) and 100 μm (B, E-G, J, and L).
Histological study

Haematoxylin and Eosin (H&E) stain for testis sections: In the control group, testes exhibited normal histological patterns of the seminiferous tubules with normal shape and arrangement of their cellular differentiation. Spermatogonia and Sertoli cells rest on intact basement membranes. Large primary spermatocytes with characteristic large rounded nuclei, round spermatids, and elongated spermatids were observed. Spermatozoa were also visible in the lumen (Figure 1A and 1B).

In SCH group, several seminiferous tubules showed irregular outlines and the majority showed maturation arrest. Germinal epithelial cells were sloughed in the lumina of some seminiferous tubules. In addition, many seminiferous tubules exhibited multiple giant cell formation. Many of the spermatocytes appeared with pyknotic nuclei surrounded by a halo indicating apoptosis. The interstitial connective tissue showed acidophilic hyaline material and dilated congested blood vessels (Figure 1C-J).

Treatment of rats with ginseng almost completely ameliorated the histopathological changes; however, a few of the seminiferous tubules still exhibited pyknotic nuclei (Figure 1K and L).

Immunohistochemical stain for testis sections: The SCH group dramatically down-regulated the expression of Androgen receptors as compared with control group. Ginseng co-administration significantly increased the expression of Androgen receptors (Figure 2).

Electron microscopic examination of the testis: Ultrastructurally, control group showed that the seminiferous tubules were surrounded by basement membranes ensheathed by myoid cells. They were lined by spermatogenic and Sertoli cells. Spermatogonia appeared with peripheral heterochromatin in their nuclei and had a few mitochondria. Sertoli cells showed triangular indented euchromatic nuclei, prominent nucleoli; dense bodies, mitochondria were observed (Figure 3). Primary spermatocytes were distinguished by their large rounded nuclei. Spermatocytes showed thin rim of cytoplasm containing mitochondria that appeared small and peripheral (Figure 4). Spermatids appeared with large rounded euchromatic nuclei. Their cytoplasm appeared granular and showed peripherally located vesicular mitochondria (Figure 5). Interstitial Leydig cells were observed. These cells were relatively large in size with slightly elongated indented nuclei with a rim of peripheral heterochromatin. Their cytoplasm showed multiple processes (Figure 6). Transverse sections in different parts of the sperms revealed mid

and principal pieces with a central axoneme. In the mid pieces, this axoneme was surrounded by dense bundles of fibrous sheath and mitochondrial sheath. In the principal pieces, the axoneme was surrounded by a fibrous sheath only (Figure 7).

Ultrastructural examination of SCH group revealed destructed spermatogonia and Sertoli cells that showed condensed nuclei and
were rested on irregular thick basement membrane (Figure 8). Wide separations in-between the germ cells was observed (Figures 8–10). Some spermatocytes had shrunken nuclei, electron dense bodies and vacuoles that were variable in size and electron density (Figure 9). Many spermatids appeared with no nuclei and multiple cytoplasmic vacuolation (Figure 10). Leydig cell population showed irregular...
shaped heterochromatic nuclei and multiple vacuoles. Leydig cells were surrounded by a large amount of collagen fibers (Figure 11). Mid pieces of sperms showed markedly affected disarranged axoneme, fibrous and mitochondrial sheaths (Figure 12).

Treated rats showed that they regained nearly their normal general architecture. Spermatogonia, primary spermatocytes, spermatids, sperms and sertoli cells showed their normal fine structure. However, intercellular separations were still present between some cells (Figure 13).

Haematoxylin and Eosin (H&E) stain for thyroid sections: In the control group, thyroid parenchyma was composed of different sized follicles. The follicular walls were lined by a single layer of flattened to cuboidal follicular cells, with flat to oval nuclei. The follicular lumens were filled with homogenous acidophilic colloids that had peripheral small vacuoles. An apparent few number of interfollicular cells and blood capillaries were observed in between the follicles (Figure 14A and B).

In SCH group, sections from the thyroid glands showed that most of the follicles were apparently distended with vacuolated colloid whereas others showed desquamated follicular cells in their lumen. Congested blood capillaries were also observed. Some follicles appeared with no colloid in their lumens and were lined by cuboidal vacuolated cells. Other follicles were lined by multiple layers of vacuolated follicular cells. Signs of mitosis were observed in the follicular epithelial lining (Figure 14B and C).

Sections of the treated rats showed almost normal organized architecture. Spermatogonia, primary spermatocytes, spermatids, sperms and sertoli cells showed their normal fine structure. However, intercellular separations were still present between some cells (Figure 13).
glandular tissue. Few follicles were distended with vacuolated colloids (Figure 14E and F).

**Immunohistochemical stain for thyroid sections**

The SCH group dramatically down-regulated the expression of Thyroglobulin as compared with the control group. Ginseng co-administration significantly increased the expression of Thyroglobulin (Figure 15).

**Electron microscopic examination of thyroid gland:** The examination of thyroid glands of group I (control group) revealed multiple follicles, each lined by a single layer of cuboidal follicular cells with large oval euchromatic nuclei. Their cytoplasm revealed a well-developed rough Endoplasmic Reticulum (rER), mitochondria (m), a few Vacuoles (V) and dense Lysosomal granules (L). The apical border of the follicular cells exhibited a moderate number of short microvilli projected into the lumen filled with moderately dense colloid. Typical junctional complexes were between the adjacent follicular cells (Figure 16).

Electron microscopic examination of thyroid gland of the SCH group revealed evident histological alterations of the follicles. Most of the follicular cells were flattened. The nuclei of follicular and interfollicular cells became heterochromatic. They were either flattened or relatively rounded with marked irregularity in their nuclear envelope (Figure 17). Collagen fibers were seen in the interstitial spaces between the follicles (Figures 17-20). The most prominent ultrastructural feature of the follicular cells was the distorted apoptotic nuclei along with cytoplasmic rarefaction and vacuolation and apparent increase in the lysosomal content of the follicular cells. Many follicles showed a few sparse or focal loss of apical microvilli at the luminal border (Figure 18). Many cells appeared with a dark irregular nucleus, vacuolated cytoplasm and widening of the junctional complexes between the follicles; associated frequent mast cells and collagen fibers were also detected (Figure 19).

Electron microscopic examination of treated group showed restoration of normal histological structure of thyroid follicular cells however, few collagen fibers were present in the interfollicular spaces (Figure 20).

**Discussion**

The testicular function is the mirror of thyroid gland state as disturbance of the normal euthyroid state affects the structure and function of the testis. The subclinical hypothyroidism is a risky condition that may progress toward overt hypothyroidism [15], also SCH was found to be independently associated with an increased risk of all causes of mortality [16]. Both subclinical and overt hypothyroidism adversely affects the testicular function [1]. Our study is the first to describe a novel effect of propylthiouracil...
induced subclinical hypothyroidism on the function and structure of adult male albino rat testis and to evaluate the possible protective role of ginseng. The results of epididymal spermatozoa study of SCH group showed a statistically significant decrease in the mean sperm count, viability and motility when compared with the control group, also the ultrastructure examination of testicular sections of SCH group revealed disarranged axoneme of mid pieces, fibrous and mitochondrial sheaths of spermatozoa. This is in accordance with the result of other authors who stated that propylthiouracil induced overt hypothyroidism resulting in impairment of reproductive efficiency of adult male rats [17].

The testicular section of SCH group showed irregular outlines and maturation arrest in several seminiferous tubules. Other tubules showed sloughed germinal epithelial cells in the lumina and multiple giant cell formation. In other fields many of the spermatocytes appeared with pyknotic nuclei surrounded by a halo indicating apoptosis and the interstitial connective tissue showed acidophilic hyaline material with dilated congested blood vessels.

Figure 18: Electron microscopy of SCH group showing thyroid follicular cells with focal loss of apical microvilli (arrows), apoptotic Nucleus (N), numerous dense Lysosomes (L), rarified and vacuolated cytoplasm (star). Note the interfollicular connective tissue with abundant Collagen fibers (C). TEMX10000.

Figure 19: Electron microscopy of SCH group showing irregular dark Nuclei (N) of follicular and interfollicular cells with Vacuolated cytoplasm (V) and gapping of the Junctional complexes between adjacent follicular cells (J). Prominent Mast cells (M) and excess Collagen fibers (C) were present in the interfollicular spaces. TEMX8000.

The ultrathin testicular sections of SCH group revealed this damage that also included vacuolated Leydig cell populations which were surrounded by a large amount of collagen fibers. These collagen fibers explained the hyalinized appearance of the interstitial tissue that was revealed by light microscope. This observed testicular damage was explained by lipid peroxidation and excessive production of Reactive Oxygen Species (ROS) indicating the oxidative stress [18]. As the hypothyroidism markedly increased the proteins carbonylation which disturbed the redox status and compromised the antioxidant defence system [19]. Testosterone signalling through Androgen Receptor (AR) is vital for complete spermatogenesis and this is mediated by the somatic cell types of the testis. These cells included specifically the Sertoli and peritubularmyloid cells that have intimate connections with the maturing germ cells which showed also the expression of AR [20,21]. So, the decreased expression of androgen receptor indicates the impairment of spermatogenesis as well as testosterone production [17].

Regarding the thyroid sections of SCH group, some follicles were distended with vacuolated colloid whereas others showed desquamated follicular cells in their lumen. The electron microscopic examination of the ultrathin thyroid sections of SCH group also illustrated these marked follicular alterations which included distorted apoptotic nuclei along with cytoplasmic rarefaction and apparent increase in the lysosomal content of the follicular cells. This is in accordance with Gossel et al., who denoted these changes of hypothyroid follicles to the circulating antibodies [22]. Moreover, AbdAllah, 2013 stated that hypothyroidism resulted in excessive production of reactive oxygen species; oxidative stress and subsequently lipid peroxidation and cell death followed by the desquamation of the degenerated follicular cells and added that vacuolated colloid results from a defect in pinocytosis of thyroglobulin follicular cell [23].

The multiple layers of the follicular epithelium, glandular congestion and empty follicles were recorded by light microscope, together with increased mast cell in the follicular cells that was revealed by electron microscope. All the previous changes were explained by increased TSH release from the pituitary gland which leads to hypertrophy followed by hyperplasia of thyroid epithelium [24].

Figure 20: Electron microscopy of treated group showing restoration of normal histological structure of follicular cells that surrounded a lumen filled with moderately dense Colloid (CO). The cells show large euchromatic Nuclei (N) and numerous short microvilli (arrows) projecting into the lumen however, few Collagen fibers (C) were still present in the interfollicular spaces. TEMX12000.
Thyroglobulin (TGB) is produced by the thyroid gland, acting as a precursor of thyroid hormones (T3 and T4), and serving as a specific marker for thyroid follicular cell. TGB expression is diffuse in 100% of normal thyroid follicular epithelial cells [25]. The decreased thyroglobulin expression indicates the impaired function of the thyroid gland [26]. This is in agreement with our result, as thyroid sections of SCH group showed significant down regulation of the thyroglobulin expression.

However, ginseng treatment protected the testis and thyroid from most of the above changes except some testicular cells showed pyknotic nuclei and some follicles showed vacuolated colloid, this partial protection was explained by the antioxidant mechanisms to combat ROS production partially or totally through prevention of peroxidation of substrate by the endogenous antioxidant enzymes such as SOD, GSH-Px and CAT are mainly responsible for the elimination of excessive ROS [27]. This was in agreement with other authors who stated that garcinia kola and vitamin E showed slight improvement in the damaged testes with Lead II oxide when compared with Group C that received Lead II oxide only [28]. The testicular protection property of ginseng was explained by the presence of ginsenosides which are the main active compounds in ginseng [29]. Ginsenosides were considered as powerful antioxidants reducing the oxidative stress and delaying the aging [30]. Beside the antioxidant properties of the ginseng, it is considered as a thyromimetic agent as reported by Filho et al. [30]. This thyromimetic effect is implicated in the up regulation of thyroglobulin expression in the thyroid sections of ginseng treated group. So we concluded that the subclinical hypothyroidism led to reproductive deficiency that was detected biochemically, histologically and immunohistochemically. This deficiency could be reversed by ginseng usage which was considered as antioxidant and thyromimetic agent that protected the thyroid and the testis from the effects of SCH.

References