Increased germ cell apoptosis during testicular development and maturation by experimentally induced transient and persistent hypothyroidism

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**Competing Interests:**
There are no competing interests.
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Abstract

Oxidative stress is known to be one of the major factors to induce germ cell apoptosis. In our earlier research, it was reported that neonatal persistent and transient hypothyroidism cause prevalence of oxidative stress marked by elevated lipid peroxide levels, protein carbonyl contents with decreased antioxidant enzyme levels. Alteration in germ cell population was also marked in persistent and transient hypothyroid rat testis. In the present investigation, germ cell apoptosis were assessed by TUNEL in testicular sections and was found that significant apoptosis occurred in germ cells in the experimentally induced persistent and transient hypothyroid rats by 6-n-propyl-2-thiouracil (PTU). The number of TUNEL-positive cells (bright green spots) increases dramatically in case of the PTU-treated rat testis both for transient and persistent hypothyroidism in comparison to the controls. Nuclei were stained with DAPI and emitted blue fluorescence when excited with 405nm blue-diode laser. This also establishes the fact that hypothyroidism caused by PTU is linked with high testicular germ cell death rates. Such type of altered testicular physiology by hypothyroidism is reflected in adulthood with hampered fertility as evidenced by reduced total viable germ cells and sperm counts.

Introduction

Alterations in thyroid hormone levels might cause oxidative stress in tissues (Sahoo and Chainy 2007; Chattopadhyay et al. 2007; 2010). Thyroid hormone is very much essential for the functional development of the reproductive tract (Panno et al. 1995). It plays an important role in the regulation of growth and differentiation of the somatic cells of the seminiferous epithelium (Jannini et al. 1990) that in turn influences gametogenesis (Palmero et al. 1995). As testis is very rich in polyunsaturated fatty acids and has poor antioxidant defense system (Peltola et al. 1992; Sahoo et al. 2008c), it is much more vulnerable to oxidative damage than other tissues. Hypo- and hyperthyroid states are associated with abnormal sexual function and infertility (Maqsood 1950; Chowdhury et al. 1984; Gerhard et al. 1991; Cooke and Meisami 1991; Palmero et al. 1994; Jannini et al. 1995; Sahoo et al. 2005; 2007; 2008a; 2008b; Sahoo, 2011; Sahoo and Roy 2012).

6-n-Propyl-2-thiouracil (PTU) is a reversible goitrogen that inhibits iodine uptake, T4 synthesis by the thyroid gland and peripheral deiodination of T4 to T3 (Tamasy et al. 1984; Oppenheimer and Schwartz 1997). Induction of transient hypothyroidism in rats by PTU during the neonatal period of life leads to increased testicular size in the adult (Cooke and Meisami 1991). Further, it has been reported that the testis mass under the above experimental regime is significantly reduced during the period of hypothyroidism especially on postnatal days 20 and 30 (Simorangkir et al. 1995). The reason for the above-mentioned decrease in the testis size and mass during the period of hypothyroidism is ascribed to a decrease in the number of germ cells in hypothyroid rats (Simorangkir et al. 1997). Besides, neonatal hypothyroidism has been reported to affect Sertoli cell number and androgen binding protein concentration in plasma and testicular interstitial fluid of rats (Maran et al. 1999). Oxidative stress is reported to be one of the major factors to induce germ cell apoptosis (Kasahara et al. 2002; Maneesh et al. 2005). There are few reports to indicate that the rate of apoptosis is important during the proliferative stage (Berenszteин et al. 2002).

In our earlier research, it was reported that neonatal persistent and transient hypothyroidism cause prevalence of oxidative stress marked by elevated lipid peroxide levels, protein carbonyl contents with decreased antioxidant enzyme levels. Marked changes in germ cell population, Sertoli cell counts and seminiferous tubule diameter were noticed in persistent and transient hypothyroid rat testis (Sahoo et al. 2008b). In case of transient hypothyroid rats we found some contradictory results, since we counted more germ cells in hypothyroid rats (Sahoo et al. 2008b) but we found less viable germ cells in these rats (Sahoo et al. 2006). Hence, in the present study we investigated occurrence of apoptosis in transient and persistent hypothyroidism.
Methods

Experimental design
Male pups of Wistar rats used in the present study were obtained from six months old mothers maintained in the animal house of the Department under standard conditions of controlled temperature (25°C) and light (12 hours light: 12 hours darkness). Animal care, maintenance and experiments were conducted under the supervision of the Institutional Animal Ethics Committee (IAEC) regulated by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Govt. of India.

Induction of hypothyroidism
Male pups obtained from breeding were made hypothyroid from day 1 of neonatal age till day 30 or day 90 of postnatal age. Hypothyroidism was induced in neonates by feeding the lactating mother with 0.05% PTU through the drinking water. From the day of parturition till weaning (25 day postpartum), the pups received PTU through mother’s milk (or) drinking water and then directly from drinking water containing 0.05% PTU for the remaining period of experimentation (Sahoo et al. 2008b).

For the experiment, rats were divided into three groups, each containing five animals. Group I rats served as control group. Group II rats were treated with PTU from day 1 postpartum to day 30 postpartum and left untreated up to day 90 postpartum. Group III rats were treated with PTU from day 1 postpartum to day 90 postpartum.

Serum Hormone Analysis
On the morning of day 91 postpartum (Groups I, II and III), body weight of animals was recorded; the animals were sacrificed by decapitation, trunk blood was collected for serum hormonal analyses. The serum levels of total T3, T4, TSH and testosterone were measured by using ELISA kits (Monobind, Inc., Costa Mesa, CA92627, USA and Equipar diagnostici, Italy).

Tissue Processing
Following sacrifice, testes were removed and cleaned in 0.9% (w/v) cold normal saline before fixation. Testes tissues were immediately fixed with Bouin’s fluid for histological studies as described earlier (Dutta et al., 2012). Tissues were sectioned at 5 mm and sections were used for TUNEL analysis. All these procedure was carried out at 4 °C temperature to minimize generation of false TUNEL-positive cells (Dutta et al., 2012).

Apoptotic fragmentation of DNA in histological sections of rat testes was evaluated by TUNEL (Terminal Deoxynucleotide Transferase dUTP Nick End Labeling) analysis according to the procedure of the kit (Cat# A23210; APO-BrdUTM TUNEL Assay kit, Invitrogen). Final detection of BrdU incorporation at DNA break sites was achieved through Alexa Fluor® 488 dye-labeled anti-BrdU antibody. Standard protocols for paraffin sections were followed (Grataroli et al. 2002).

Sections were observed under confocal laser scanning microscope (TCS SP5; Leica Microsystems CMS GmbH, D-68165 Mannheim, Germany) using LAS AF (Leica Application Suite Advanced Fluorescence) 1.8.1 build 1390 software under HCX PL APO CS DRY UV objective (20.0X/N.A.0.70) with confocal pinhole set at Airy 1 and 2X zoom factor for improved resolution with eight bits. For exciting alexa fluor 488 in testicular sections, argon laser (30%) with AOTF for 488 nm (at 30%) was used and the fluorescence emissions were collected between 500 to 550 nm with photomultiplier tube (PMT) detector gain set at 1130V. The fluorescent images were captured after passage through double dichroite DD 488/561 and to optimize the image quality, the offset was adjusted for a maximum range of fluorscence from 0 to 255 (50% green pixels). DAPI (4',6-diamidino-2-phenylindole) in sections were excited with 405 diode laser and fluorescence emissions were collected between 415 to 480nm with detector (PMT) gain set at 1200V. To optimize the image quality, the offset was adjusted for a maximum range of fluorescence from 0 to 255 (50% blue pixels). PI (propidium iodide) in sections were also excited with 488 line of the argon-ion laser and red fluorescence emissions were collected between 600 to 700nm with detector (PMT) gain set at 1200V. To optimize the image quality, the offset was adjusted for a maximum range of fluorescence from 0 to 255 (50% red pixels).

Results

Serum hormone profile
Induction of hypothyroidism in the treated group (Group III) was confirmed by reduction of serum T3 concentration in the present study. The effect of PTU on T3 seems to be reversed in adult rats when the treatment was withdrawn after 30 days as the levels of the T3 and TSH tends to be nearing normal levels. Nevertheless, T4 levels in these animals were still higher than controls (Table 1).

TUNEL Assay
The number of TUNEL-positive cells (bright green spots) increases dramatically in case of the PTU-treated rat testis both for transient and persistent hypothyroidism in comparison to the controls. Nuclei were stained with DAPI and emitted blue fluorescence when excited with 405nm blue-diode laser (Figure 1). Nuclei were stained with either DAPI and emitted blue fluorescence when excited with 405nm blue-diode laser (Figure 1 and 2) or with PI and emitted red fluorescence when excited with 488 nm argon-ion laser (Figure 3).

**Discussion**

It was found from the present investigation that apoptotic cells were predominantly found in the center of the tubules, and also adjacent to the wall of seminiferous tubules of rat testes during hypothyroid state, suggesting that, if any spermatogonia are present in the apoptotic cell population, they should have detached from the basal membrane of the seminiferous tubules. If this is the case for spermatogonia, the apoptotic mechanism elicited in spermatogonia might be related to a particular mode of cell death named anoikis (Frisch and Screaton 2001) and such type of cell death has been observed in adherent cells and is triggered following detachment from the extracellular matrix. In the present study, DAPI staining was used to stain nuclei and to assess gross cell morphology as DAPI is well known to visualize nuclear DNA in both living and fixed cells (Tamowski et al. 1991).

In the previous study, it has been reported that, total germ cell number increases in the testes of transient hypothyroid rats (Sahoo et al. 2008b). However, due to deprivation of thyroid hormones by PTU from birth to 30 days of age and withdrawal of PTU thereafter (transient hypothyroidism) caused not only decrease in number of viable germ cells (Sahoo et al. 2006) but also reduction in percentage of live sperms (Sahoo et al. 2008b) in adult testis. This may be due to the activation of apoptotic pathways leading to the reduction in number of viable germ cells as apoptotic cells were predominantly found in transient hypothyroid rat testes. The number of apoptotic germ cells was increased not only in transient hypothyroid rat testis but also incase of persistent hypothyroid rat testes. The trigger of apoptosis may be due to the poor rat testicular antioxidant defense status and oxidative stress under hypothyroidism. It has been reported earlier that testicular mitochondrial lipid peroxidation (LPx) and protein carbonylation are elevated with decreased glutathione peroxide (GPx) and glutathione reductase (GR) activities in persistent hypothyroidism and transient hypothyroidism is associated with reduced testicular superoxide dismutase (SOD), catalase (CAT), GR and GPx activities (Sahoo et al. 2008b). It has been further shown that particularly germ cells of transient hypothyroid state exhibit higher LPx contents and lower reduced glutathione (GSH), CAT and SOD activities (Sahoo et al. 2006). Moreover, a compromised antioxidant defense system marked by increased protein carbonylation, disturbed redox status during neonatal hypothyroidism was found to be contributed towards poor growth and development of testis by affecting spermatogenesis and steroidogenesis in rats before puberty as indicated by reduced germ cell number, complete absence of round spermatids, decreased seminiferous tubule diameter and decreased testosterone level in immature rats (Sahoo and Roy 2012). Such type of altered testicular physiology by hypothyroidism is reflected in adulthood with hampered fertility as evidenced by reduced total viable germ cells (Sahoo et al., 2006) and sperm counts (Sahoo et al. 2008b).

In the present investigation, germ cell apoptosis were evaluated by TUNEL in testicular sections and was found that significant apoptosis occurred in germ cells in the PTU treated groups. This also establishes the fact that hypothyroidism caused by PTU may have a role in testicular germ cell death. Oxidative stress is one of the contributory factors to induce germ cell apoptosis (Maneesh et al. 2005). There are few reports to indicate that the rate of apoptosis is important during the proliferative stage (Berensztein et al. 2002). In our study, neonatal hypothyroidism induced apoptosis might play a role in cell death leading to a decreased sperm count.

**Conclusions**

The prevalence of oxidative stress in the present study marked by decreased antioxidant enzymes such as SOD, CAT, GPx and GR levels in both mitochondrial as well as post-mitochondrial fractions (Sahoo et al. 2008) might be responsible for triggering germ cell apoptosis in transient hypothyroid rats and reduction in sperm number.

**Abbreviations**

LPx: Lipid Peroxidation
SOD: Superoxide dismutase
CAT: Catalase
GPx: Glutathione peroxidase
GR: Glutathione reductase
GSH: Reduced glutathione

References

24. Sahoo DK, Roy A, Chainy GBN, 2008a: Protective effects of Vitamin E and curcumin on...


**Illustrations**

**Illustration 1**

Table 1:

<table>
<thead>
<tr>
<th>Serum Hormones</th>
<th>GROUP-I</th>
<th>GROUP-II</th>
<th>GROUP-III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total T₃</td>
<td>1.02 ± 0.07ᵃ</td>
<td>1.1 ± 0.14ᵃ</td>
<td>0.54 ± 0.06ᵇ</td>
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<tr>
<td>Total T₄</td>
<td>20.00 ± 0.62ᵃ</td>
<td>33.9 ± 3.21ᵇ</td>
<td>4.63 ± 0.41ᵇ</td>
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<tr>
<td>TSH</td>
<td>0.32 ± 0.05ᵃ</td>
<td>0.26 ± 0.04ᵃ</td>
<td>17.63 ± 2.14ᵇ</td>
</tr>
<tr>
<td>Testosterone</td>
<td>0.74 ± 0.06ᵃ</td>
<td>0.42 ± 0.03ᵇ</td>
<td>0.24 ± 0.03ᵇ</td>
</tr>
</tbody>
</table>

**Table 1.** Effect of neonatal PTU treatment on serum total T₃, total T₄, testosterone levels (ng/ml) and Thyroid stimulating hormone (TSH) level in µIU/ml. Data are expressed as mean ± S.D. of 5 observations. Superscripts of different letters differ significantly (p<0.05) from each other. Group-I (90 day old control rats); Group-II (90 day old rats with PTU treatment from day 1 postpartum to day 30 postpartum and left untreated up to day 90 postpartum); Group-III (90 day old rats with PTU treatment from day 1 postpartum to day 90 postpartum).
Illustration 2

Figure 1

Groups of apoptotic germ cells in rat testicular transverse sections (with 200x magnification) with Group I (90 day old control rats); Group II (90 day old rats with PTU treatment from day 1 postpartum to day 30 postpartum and left untreated up to day 90 postpartum, transient hypothyroid rats); Group III (90 day old rats with PTU treatment from day 1 postpartum to day 90 postpartum, persistent hypothyroid rats). Note that the number of TUNEL-positive cells (bright green spots) increases dramatically in group-II and III animals. Panel 1, blue fluorescence corresponds to DAPI; panel 2, green fluorescence corresponds to Alexa Fluor® 488 (apoptotic or TUNEL-positive cells); and panel 3, confocal image recorded simultaneously in blue and green fluorescence mode (i.e., green fluorescence overlaid on the blue fluorescent image, co-localization of TUNEL positive nuclei on the DAPI stained nuclei). DAPI: 4',6-diamidino-2-phenylindole.
Illustration 3

Figure 2

Figure 2: Groups of apoptotic germ cells in rat testicular transverse sections (with 400x magnification) with Group I (90 day old control rats); Group II (90 day old rats with PTU treatment from day 1 postpartum to day 30 postpartum and left untreated up to day 90 postpartum, transient hypothyroid rats); Group III (90 day old rats with PTU treatment from day 1 postpartum to day 90 postpartum, persistent hypothyroid rats). Note that the number of TUNEL-positive cells (bright green spots) increases dramatically in group-II and III animals. Panel 1, blue fluorescence corresponds to DAPI; panel 2, green fluorescence corresponds to Alexa Fluor®488 (apoptotic or TUNEL-positive cells); and panel 3, confocal image recorded simultaneously in blue and green fluorescence mode (i.e., green fluorescence overlaid on the blue fluorescent image, co-localization of TUNEL positive nuclei on the DAPI stained nuclei). DAPI: 4',6-diamidino-2-phenylindole.
Illustration 4

Figure 3

Figure 3: Groups of apoptotic germ cells in rat testicular transverse sections (with 200x magnification) with Group I (90 day old control rats); Group II (90 day old rats with PTU treatment from day 1 postpartum to day 30 postpartum and left untreated up to day 90 postpartum, transient hypothyroid rats); Group III (90 day old rats with PTU treatment from day 1 postpartum to day 90 postpartum, persistent hypothyroid rats). Note that the number of TUNEL-positive cells (bright green spots) increases dramatically in group-II and III animals.
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