Article ID: WMCPLS00209 ISSN 2051-0799

Increased Germ Cell Apoptosis During Testicular Development And Maturation By Experimentally Induced Transient And Persistent Hypothyroidism

Corresponding Author:
Dr. Dipak K Sahoo,
Researcher, KTRDC, University of Kentucky - United States of America

Submitting Author:
Dr. Dipak K Sahoo,
Researcher, KTRDC, University of Kentucky - United States of America

Submitted on:03-Dec-2013, 07:39:53 PM GMT

Accepted on:04-Dec-2013, 01:14:07 AM GMT

Published on:09-Dec-2013, 05:25:15 AM GMT

Article ID: WMCPLS00209

Article Type: Research articles

Article URL: http://webmedcentralplus.com/article_view/209

Subject Categories:BIOCHEMISTRY

Keywords: Hypothyroidism; Testes; Rat; Apoptosis; Oxidative stress

How to cite the article:

Sahoo DK.Increased Germ Cell Apoptosis During Testicular Development And Maturation By Experimentally Induced Transient And Persistent Hypothyroidism. WebmedCentral *plus* BIOCHEMISTRY 2013;4(12):WMCPLS00209

Source(s) of Funding:

None

Competing Interests:

None

Increased Germ Cell Apoptosis During Testicular Development And Maturation By Experimentally Induced Transient And Persistent Hypothyroidism

Author(s): Sahoo DK

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 increases dramatically in case of the PTU-treated rat testis both for transient and persistent hypothyroidism in comparison to the controls. 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. Thyroid hormone is 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 (Berensztein 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. We also marked changes in germ cell numbers, Sertoli cell numbers and seminiferous tubule diameter in persistent and transient hypothyroid rat testis (Sahoo et al. 2008b). In case of transient hypothyroid rats some contradictory results were found. The histological testicular germ cell count was more in hypothyroid rats (Sahoo et al. 2008b) whereas less viable germ cells were obtained from these rats (Sahoo et al. 2006). Hence, in the present study we investigated occurrence of apoptosis in transient and persistent hypothyroidism.

Materials And 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 (25^0C) 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).

Epididymal Sperm Count

After sacrificing the animals, epididymides were removed and cleaned in 0.9% (w/v) cold normal saline. Epididymal sperm count was done essentially following the earlier method (Sahoo et al., 2005).

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 ^{o}C temperature to minimize generation of false TUNEL-positive cells (Dutta et al., 2012).

TUNEL analysis and Confocal Microscopy

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-BrdU™ 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 fluorescence 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).

Statistics

Data were subjected to one way analysis of variance (ANOVA) followed by Duncan's new multiple range test to find out the level of significance among mean values. A difference was considered significant at p

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).

Serum Hormones	GROUP-I	GROUP-II	GROUP-III
Total T_3	1.02 ±0.07[a]	1.1 ±0.14[a]	0.54 ±0.06[b]
Total T_4	20.00 ±0.62[a]	33.9 ±3.21[b]	4.63 ±0.41[b]
TSH	0.32 ±0.05[a]	0.26 ±0.04[a]	17.63 ±2.14[b]
Testosterone	0.74 ±0.06[a]	0.42 ±0.03[b]	0.24 ±0.03[b]

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).

TUNEL Assay

The number of TUNEL-positive cells (bright green spots) increases dramatically incase of the PTU-treated rat testis both for transient and persistent hypothyroidism in comparison to the controls (Figure 1 and 2). In addition, the apoptotic cell density in the transient (group-II) and persistent (group-III) hypothyroid rat testis was higher than in the control group (group-I). Nuclei were stained with DAPI and emitted blue fluorescence when excited with 405nm blue-diode laser (Figure 1 and 2).

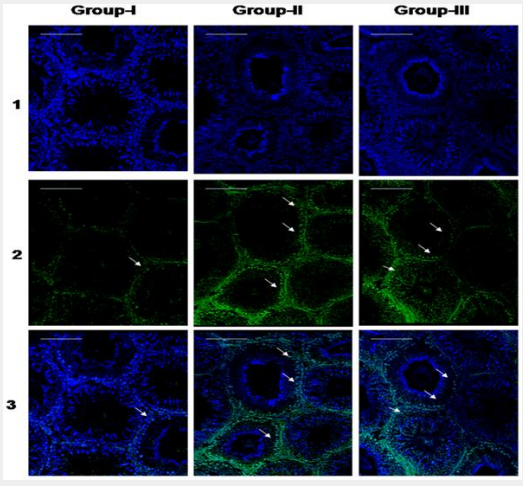

Figure 1. Groups of apoptotic germ cells in rat testicular transverse sections with Group-I (90 day old control rats); Group-II (90 day old rats with PTU treatment from day 1 postpartum to day 30 postpartum and left untreated up to day 90 postpartum, transient hypothyroid rats); Group-III (90 day old 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. Scale bar represents 80µm.

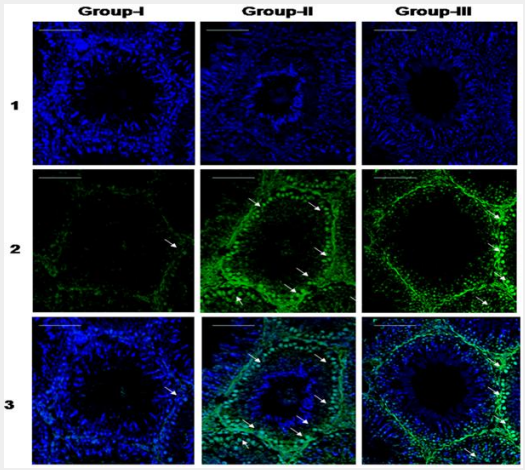

Figure 2. Groups of apoptotic germ cells in rat testicular transverse sections with Group-I (90 day old control rats); Group-II (90 day old rats with PTU treatment from day 1 postpartum to day 30 postpartum and left untreated up to day 90 postpartum, transient hypothyroid rats); Group-III (90 day old 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. Scale bar represents 50µm.

Epididymal sperm count

Epididymal sperm count reduced significantly by 78%

in group-II transient hypothyroid rats and by 68% in group-III persistent hypothyroid rats in comparison to the group-I control rats (Figure 3).

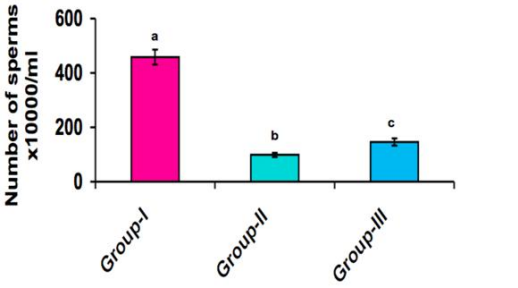

Figure 3. Effect of PTU treatment on epididymal sperm count (number/ml). Data are expressed as mean ± S.D. of five observations from five different animals. In the figure, different letters on the bar differ significantly ($p < 0.05$) from each other. Group-I (90 day old control rats); Group-II (90 day 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).

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 (Tarnowski 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 contents of reduced glutathione (GSH), CAT and SOD activities (Sahoo et al. 2006). Moreover, a compromised antioxidant defence 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 study, the observed reduction in the epididymal sperm count in transient and persistent hypothyroid rats also corroborates the earlier finding (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. 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. 2008b) might be responsible for triggering germ cell apoptosis in transient hypothyroid rats and reduction in sperm number.

Acknowledgements

We are very much thankful to Prof. G.B.N. Chainy, Head of Departments of Zoology and Biotechnology, Utkal University for his support, help, and suggestions and also for providing necessary facilities during the experiment. We are grateful to the Indian Council of Medical Research (ICMR), New Delhi, DRS-UGC and Department of Biotechnology, Govt. of India for financial assistance.

References

1. Berensztein EB, Sciara MI, Rivarola MA, Belgorosky A, 2002: Apoptosis and proliferation of human testicular somatic and germ cells during prepuberty: High rate of testicular growth in newborns mediated by decreased apoptosis. J Clin Endocrinol Metab 87, 5113-5118.

2. Chattopadhyay S, Sahoo DK, Roy A, Samanta L, Chainy GBN, 2010: Thiol redox status critically influences mitochondrial response to thyroid hormone-induced hepatic oxidative injury: A temporal analysis. Cell Biochemistry and Function 28(2), 126-134.

3. Chattopadhyay S, Sahoo DK, Subudhi U, Chainy GBN, 2007: Differential expression profiles of antioxidant enzymes and glutathione redox status in hyperthyroid rats: a temporal analysis. Comp Biochem Physiol C 146, 383-391.

4. Chowdhury AR, Gautam AK, Chatterjee BB, 1984: Thyroid-testis interrelationship during the development and sexual maturity of the rat. Arch Androl 13, 233-239.

5. Cooke PS, Meisami E, 1991: Early hypothyroidism in rat causes increased adult testis and reproductive organ size but does not change testosterone levels. Endocrinology 129, 237-243.

6. Dutta D, Park I, Mills NC, 2012: Fixation temperature affects DNA integrity in the testis as measured by the TUNEL assay. Toxicol Pathol 40(4):667-674.

7. Frisch SM, Screaton RA, 2001: Anoikis mechanisms. Curr Opin Cell Biol 13, 555–562

Gerhard I, Becker T, Eggert-Kruse W, Klinga K, Runnebaum B, 1991: Thyroid and ovarian function in infertile women. Hum Reprod 6, 338-345.

8. Grataroli R, Vindrieux D, Gougeon A, Benahmed M, 2002: Expression of tumor necrosis factor-alpha-related apoptosisinducing ligand and its receptors in rat testis during development. Biol Reprod 66, 1707–1715.

9. Jannini EA, Olivieri M, Francavilla S, Gulino A, Ziparo E, d'Armiento M, 1990: Ontogenesis of the nuclear 3,5,3'-triiodotyronine receptor in the rat testis. Endocrinol 126, 2521-2526.

10. Jannini EA, Ulisse S, d'Armiento M, 1995: Thyroid hormone and male gonadal function. Endocr Rev 16, 443-459.

11. Kasahara E, Sato EF, Miyoshi M, Konaka R, Hiramoto K, Sasaki J, Tokuda M, Nakano Y, Inoue M, 2002: Role of oxidative stress in germ cell apoptosis induced by di (2-ethyl hexyl) phthalate. Biochem J 365, 849-856.

12. Maneesh M, Jayalakshmi H, Dutta S, Chakrabarti A, Vasudevan DM, 2005: Role of oxidative stress in ethanol induced germ cell apoptosis-An experimental study in rats. Ind J Clinil Biochem 20, 62-67.

13. Maqsood M, 1950: Thyroid functions in relation to reproduction of mammals and birds. Biol Rev 27, 281-319.

14. Maran RR, Sivakumar R, Arunakaran J, Ravisankar B, Ravichandran K, Sidharthan V, Jeyaraja DA, Aruldhas MM, 1999: Duration-dependent effect of transient neonatal hypothyroidism on Sertoli and germ cell number, and plasma and testicular interstitial fluid androgen binding protein concentration. Endocr Res 25, 323-340

15. Oppenhiemer JH, Schwartz HL, 1997: Molecular basis of thyroid hormone- dependent brain development. Endocr Rev 18, 462-475.

16. Palmero S, Trucchi P, Prati M, Fugassa E, Lanni A, Goglia F, 1994: Effect of thyroid ststus on the oxidative capacity of Sertoli cells isolated from immature rat testis. Eur J Endocrinol 130, 308-312.

17. Peltola V, Huhtaniemi I, Ahotupa M, 1992: Antioxidant enzyme activity in the maturing rat testis. J Androl 13, 450-455.

18. Panno ML, Salerno M, Lanzino M, De Luca G, Maggiolini M, Straface SV, Prati M, Palmero S, Bolla E, Fugassa E, Ando S, 1995: Follow-up study on the effects of thyroid hormone administration on androgen metabolism of peripubertal rat Sertoli cells. Eur J Endocrinol 132, 236-241.

19. Sahoo DK, 2011: Effects of thyroid hormone on testicular functions and antioxidant defence status. Biochemistry: An Indian Journal 5 (6).

20. Sahoo DK, Chainy GBN, 2007: Tissue specific response of antioxidant defence systems of rat to

experimentally induced hyperthyroidism. Natl Acad Sci Lett 30 (7 & 8), 247-250.
21. Sahoo DK, Roy A, 2012: Compromised Rat Testicular Antioxidant Defence System by Hypothyroidism before Puberty. International Journal of Endocrinology, Article ID 637825, 11 pages. doi:10.1155/2012/637825.
22. Sahoo DK, Roy A, Chainy GBN, 2006: PTU-induced neonatal hypothyroidism modulates antioxidative status and population of rat testicular germ cells. Natl Acad Sci Lett 29 (3 & 4), 133-135.
23. Sahoo DK, Roy A, Chainy GBN, 2008a: Protective effects of Vitamin E and curcumin on L-thyroxine-induced rat testicular oxidative stress. Chem Biol Interact 176, 121-128
24. Sahoo DK, Roy A, Bhanja S, Chainy GBN, 2008b: Hypothyroidism impairs antioxidant defence system and testicular physiology during development and maturation. Gen Comp Endocrinol 156, 63-70.
25. Sahoo DK, Roy A, Chainy GBN, 2008c: Rat testicular mitochondrial antioxidant defence system and its modulation by aging. Acta Biologica Hungarica 59 (4), 413-424.
26. Sahoo DK, Roy A, Bhanja S, Chainy GBN, 2005: Experimental Hyperthyroidism-induced Oxidative stress and Impairment of Antioxidant Defence System in Rat Testis. Ind J Exp Biol 43, 1058-1067.
27. Sahoo DK, Roy A, Chattopadhyay S, Chainy GBN, 2007: Effect of T3 treatment on glutathione redox pool and its metabolizing enzymes in mitochondrial and post-mitochondrial fractions of adult rat testes. Ind J Exp Biol 45, 338-346.
28. Simorangkir DR, de Kretser DM, Wreford NG, 1995: Increased numbers of Sertoli and germ cells in adult rat testes induced by synergistic action of transient neonatal hypothyroidism and neonatal hemicastration. J Reprod Fertil 104, 207-213.
29. Simorangkir DR, Wreford NG, De Kretser DM, 1997: Impaired germ cell development in the testes of immature rats with neonatal hypothyroidism. J Androl 18, 186-193.
30. Tamasy V, Du JZ, Vallerga A, Meisami E, Timiras PS, 1984: Suckling ability and maternal prolactin levels in hypothyroid rats. Horm Behav 18, 457-454.
31. Tarnowski BI, Spinale FG, Nicholson JH, 1991: DAPI as a useful stain for nuclear quantitation. Biotech Histochem 66 (6), 297-302.