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Alterations of testicular selenium-dependent and independent glutathione peroxidase activities during experimentally L-thyroxine induced hyperthyroidism and n-propyl thiouracil induced hypothyroidism in adult rats

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ABSTRACT

Hyper- and hypothyroidism states are known to alter testicular antioxidant defence parameters in adult as well as in immature rats. Glutathione peroxidases represent a family of enzymes which metabolizes toxic hydroperoxides and are, along with superoxide dismutase and catalase, part of the enzymatic antioxidant defenses to protect cells from free-radical-mediated attacks. In mammalian testis, total GPx activity is contributed by both Selenium-dependent glutathione peroxidases (Se-D GPxs) and the Se-independent glutathione peroxidaes (Se-I GPx). Due to persistent hypothyroidism, both Se-D-GPx and Se-I-GPx were reduced in adult testicular post-mitochondrial as well as in mitochondrial fraction (MF); while, during transient hypothyroidism, both Se-D-GPx and Se-I-GPx were reduced in adult testicular post-mitochondrial fraction (PMF) with only decrease in mitochondrial Se-D-GPx activity. However, both Se-dependent and Se-independent GPx activities elevated in adult testicular PMF of L-thyroxine treated (group- T_4), T_4 with vitamin E (group- T_4+Vit.E), T_4 along with curcumin and vitamin E (group- T_4+Cur+Vit.E) with only increase in SeD-GPx in T_4 with curcumin (group- T_4+Cur) treated rats. In case of MF, Se-dependent GPx showed no alteration in response to curcumin and/or vitamin E treatment to T_4 treated rats (groups-T_4+Cur, T_4+Vit.E and T_4+Cur+Vit.E), however its level increased in T_4 treated rats (group-T_4). Se-independent GPx elevated significantly in response to T_4 (group-T_4), T_4 with curcumin (group- T_4+Cur), T_4 with vitamin E (group- T_4+Vit.E) and T_4 along with curcumin and vitamin E (group- T_4+Cur+Vit.E) treated rats respectively. Hence, hypothyroid as well as hyperthyroid states influencing testicular Se-D- and Se-I- GPx activities along with other antioxidant defence enzymes, might regulate testicular physiology and oxidative stress.

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INTRODUCTION

Thyroid hormones are known to play a significant role in growth, differentiation, maturation and metabolism in vertebrates[1]. Thyroid hormone elevates oxygen consumption and metabolic rate of nearly all tissues of vertebrates[2-4]. Relation between thyroid hormones and testis is established by demonstration of presence of T_3 receptors in Sertoli cells of testis in fetal and perinatal periods[5]. The regulatory role of thyroid hormone on functions of Leydig cells was also proposed[6] as it affects steroidogenesis by modifying the hypothalamo-hypophysial-testicular axis. Hyperthyroid as well as hypothyroid conditions also lead to reduced

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sperm number and motility influencing male fertility[7-13]. Moreover, both hyperthyroidism and hypothyroidism states are known to alter testicular antioxidant defence parameters in rats[8-15].

Glutathione peroxidases (GPXs, EC 1.11.1.9) represent a family of enzymes which metabolizes toxic hydroperoxides and are, along with superoxide dismutase (SOD, EC 1.15.1.1) and catalase (CAT, EC 1.11.1.6), part of the enzymatic antioxidant defenses that virtually any cell uses to retaliate free-radical-mediated attacks resulting from the metabolism of oxygen[16]. Selenium-dependent glutathione peroxidases (Se-D GPxs) are the foremost selenoprotein-containing gene family in mammals[17]. Among the different types of selenium-dependent hydroperoxide reducing isozymes, phospholipid hydroperoxide glutathione peroxidase (PH-GPx/GPx-4; EC 1.11.1.12) and classic cellular glutathione peroxidase (cGPX/GPx-1; EC 1.11.1.9) are mainly found in testis[13]. PHGPx is a monomeric seleno-enzyme present in different mammalian tissues in soluble and bound form[18]. Selenium-dependent glutathione peroxidases contribute to a part of the total GPx activity. Other GPx activities in mammalian systems are selenium-independent and the Se-independent GPx (Se-I GPx), component of GST alfa class (Accession: IPR003080 GST_alpha) is accountable for GPx activity in testis[13,19,20].

In our previous study, we report about reduced testicular SOD, CAT and glutathione reductase (GR) and GPx activities during transient hypothyroidism along with an elevation in SOD and CAT activities with a significant decline in GPx and GR activities following persistent hypothyroidism[10]. In another study, we report that treatment of curcumin and/or vitamin E to T4-treated rats resulted in increased SOD level in postmitochondrial fraction (PMF) and mitochondrial fraction (MF) and CAT in PMF, with decreased GPx activity in MF. However, curcumin or vitamin E was unable to alter GPx activity alone but in together they elevated the GPx in PMF of T4-treated rat testis[11]. However, in both of these studies[10,11] total GPx activity was measured. As GPx can be of Se-D and Se-I types, it is imperative to know which type of GPx is mostly effected in above conditions. The key objective of the present work is to evaluate Se-D and Se-I GPx activities in testicular PMF and MF fractions modulated during hypo- as well as hyper-thyroid rats in previously described experimental conditions[10,11].

MATERIALS AND METHODS

Animal treatment and experimental procedure

In our first experiment, as described in the earlier report[10], male pups of Wistar rats obtained from breeding were made hypothyroid from day 1 of neonatal age till day 30 or day 90 of postnatal age. Neonates were made hypothyroid by feeding the lactating mothers with 0.05% PTU (n-propyl thiouracil) through 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. Animals were divided into three groups each containing five animals.

Control	:	Control rats of 90days old.
Persistent hypothyroid	:	Rats were treated with PTU for 90 days after birth.
Transient hypothyroid	:	Rats were treated with PTU for 30 days after birth and thereafter the treatment was withdrawn for 60 days.

As described earlier[11] for the second experiment, Wistar male rats (24–25 weeks of age) weighing 300–400 g were procured from the National Institute of Nutrition, Hyderabad, India. The rats were acclimatized under laboratory conditions prior to the experiment. Twenty-five male Wistar strain rats were divided randomly into five groups, each group containing five animals. L-Thyroxine (0.0012%) was dissolved in drinkingwater, curcumin (30 mg/ml) and vitamin E (200mg/ml) dissolved in olive oil and orally administrated for 30 days according to groups as below. Group I animals (control) received orally 1 ml of placebo solution (olive oil) only for 30 days. Group II received 1ml olive oil and 0.0012% l-thyroxine (T4). Group III animals received 0.0012% T4 along with 30mg curcumin/kg body weight. Group IV animals received 0.0012% T4 along with 200mg vitamin E/kg body weight. Group V animals received 0.0012% T4 along with 200mg vitamin E and 30mg curcumin/kg body weight.

Rats were fed with freshly cooked food containing flour of Bengalgram (*Cicer arietnum*, 20%), wheat (*Triticum sativum*, 40%), rice (*Oryza sativa*, 30%), skimmed milk powder supplemented with vitamins (5%), refined vegetable oil (3%), egg (1%) and com-

mon salt (1%) in the morning and water soaked whole gram (*C. arietnum*) (approximately 5/100g body weight) in the afternoon with free access to tap water and were kept at room temperature maintained at 23±2 ?C and controlled 12:12 light and dark cycles[11]. Rats were sacrificed after 24 h of the last treatment and testes were dissected out quickly, cleaned in 0.9% (w/v) cold normal saline, pat dried and weighed. Testes were kept at -80 ?C until use. Animal care, maintenance and experiments were done 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.

Testicular mitochondrial and post-mitochondrial fractionation

The whole procedure of tissue processing was completed at 0–4^{0}C. A 20% (w/v) homogenate of testis was prepared in 50 mM phosphate buffer, pH 7.4, containing 0.25M sucrose with a Potter–Elvejhem type motor-driven homogenizer and then filtered through four layers of sterilized gauze. The filtrate was the crude homogenate and it was centrifuged at 600g for 10 min to precipitate nuclei and other cellular debris. The resulted supernatant was again centrifuged at 10,000g for 20 min to separate mitochondria. The supernatant obtained was the post-mitochondrial fraction (PMF)[9]. The mitochondrial pellet was washed thrice in 50 mM phosphate buffer, pH 7.4 (10,000g for 5 min each), and finally suspended in the same buffer to obtain mitochondrial fraction (MF)[9]. Protein content of samples was estimated using bovine serum albumin as standard[21].

Estimation of selenium-dependent and selenium independent glutathione peroxidase activities

Glutathione peroxidase (GPx) activity was assayed in PMF and MF by measuring oxidation rate of NADPH in presence of hydroperoxide, GSH, and glutathione reductase (GR)[9,22]. Total and selenium-dependent GPx activities were estimated by using cumene and tert-butyl hydroperoxides, respectively. The difference between total glutathione peroxidase and selenium-dependent glutathione peroxidase (Se-D-GPx) activities represents the selenium independent glutathione peroxidase (Se-I-GPx) activity[23,24].

Statistics

For the first experiment, all data represent means ± standard deviation and were subjected to unpaired Student's *t*-test to find out the level of significance between control and experimental rats.Minimal statistical significance was accepted at $P < 0.05$.

For the second experiment, data were subjected to one-way analysis of variance (ANOVA) followed by Duncan's new multiple range tests to find out the level of significance among mean values. A difference was considered significant at $P < 0.05$ levels.

RESULTS

Testicular Se-dependent and Se-independent glutathione peroxidase activities in persistent as well as transient hypothyroid states

Due to persistent hypothyroidism, both Se-D-GPx and Se-I-GPx were reduced by 14% and 15.5%, respectively in testicular post-mitochondrial fraction (Figure 1); whereas in mitochondrial fraction, Se-D-GPx and Se-I-GPx were reduced by 41% and 34%, respectively (Figure 2). In contrast, during transient

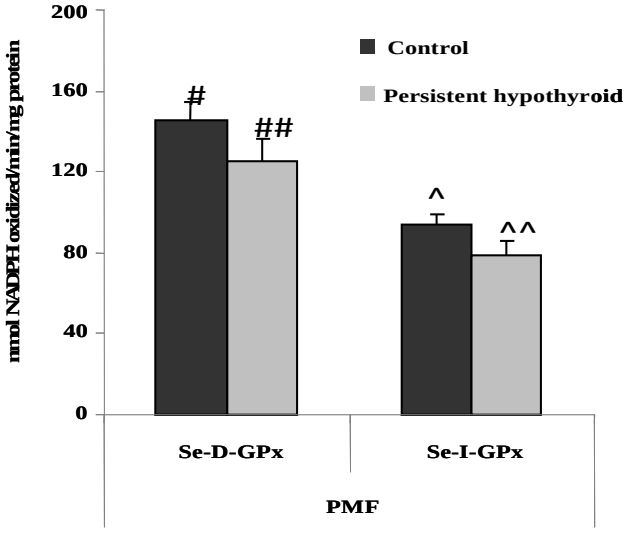

Figure 1 : Effect of persistent hypothyroidism on Se-dependent (Se-D) and Se-independent (Se-I) glutathione peroxidase activities (nmol NADPH oxidized/min/mg protein) in postmitochondrial fraction (PMF) of testes of rats. Data are expressed as mean ± S.D. of 5 observations and subjected to unpaired Student's *t*-test. Statistical significance was accepted at $P < 0.05$. Control and hypothyroid groups were found to differ significantly at $P < 0.05$ as represented by different superscripts. Control: 90-day-old control rats; persistent hypothyroid: 90-day-old rats with PTU treatment from day 1 postpartum to day 90 postpartum.

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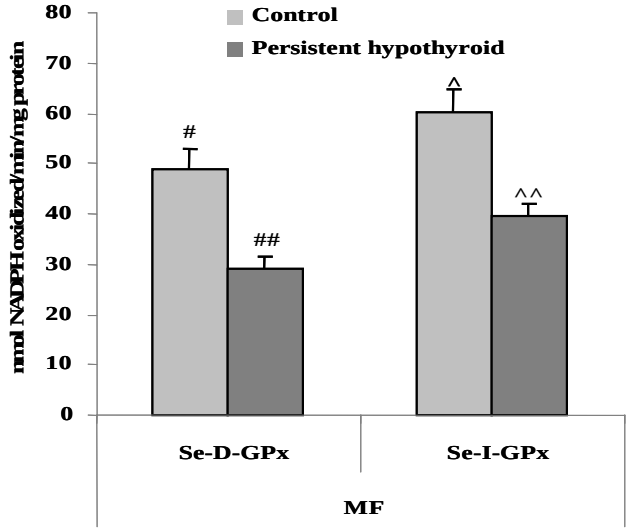

Figure 2 : Effect of persistent hypothyroidism on Se-dependent (Se-D) and Se-independent (Se-I) glutathione peroxidase activities (nmol NADPH oxidized/min/mg protein) in mitochondrial fraction (MF) of testes of rats. Data are expressed as mean ± S.D. of 5 observations and subjected to unpaired Student's *t*-test. Statistical significance was accepted at *P* <0.05. Control and hypothyroid groups were found to differ significantly at *P* < 0.05 as represented by different superscripts. Control: 90-day-old control rats; persistent hypothyroid: 90-day-old rats with PTU treatment from day 1 postpartum to day 90 postpartum.

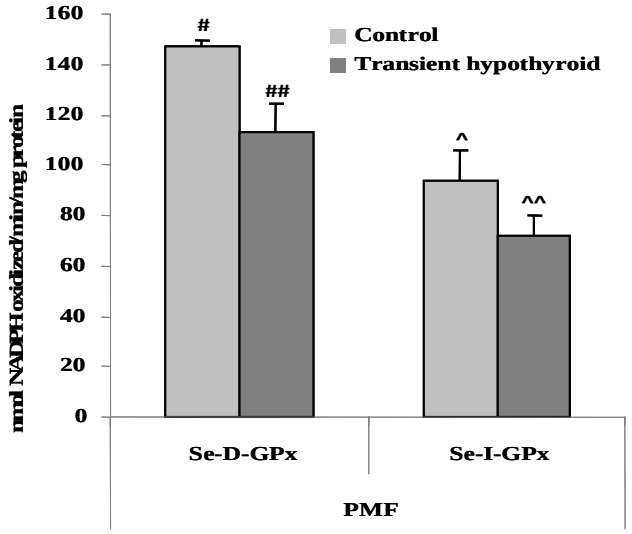

Figure 3 : Effect of persistent hypothyroidism on Se-dependent (Se-D) and Se-independent (Se-I) glutathione peroxidase activities (nmol NADPH oxidized/min/mg protein) in postmitochondrial fraction (PMF) of testes of rats. Data are expressed as mean ± S.D. of 5 observations and subjected to unpaired Student's *t*-test. Statistical significance was accepted at *P* <0.05. Control and hypothyroid groups were found to differ significantly at *P* < 0.05 as represented by different superscripts. Control: 90-day-old control rats; transient hypothyroid: 90 day old rats with PTU treatment from day 1 postpartum to day 30 postpartum and left untreated up to day 90 postpartum).

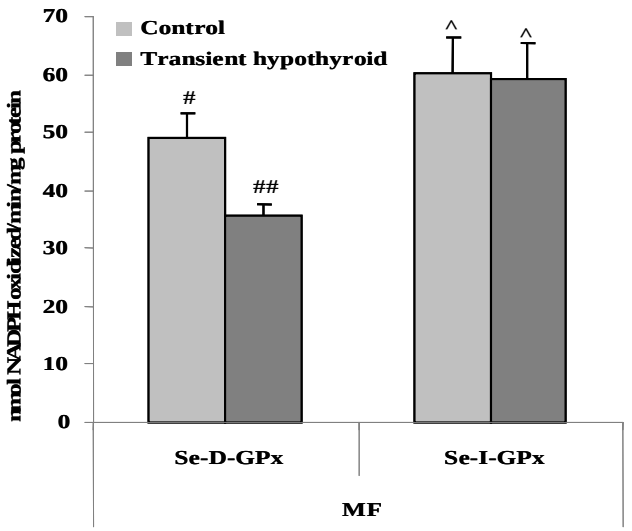

Figure 4 : Effect of persistent hypothyroidism on Se-dependent (Se-D) and Se-independent (Se-I) glutathione peroxidase activities (nmol NADPH oxidized/min/mg protein) in mitochondrial fraction (MF) of testes of rats. Data are expressed as mean ± S.D. of 5 observations and subjected to unpaired Student's *t*-test. Statistical significance was accepted at *P* <0.05. Control and hypothyroid groups were found to differ significantly at *P* < 0.05 as represented by different superscripts. Control: 90-day-old control rats; transient hypothyroid: 90 day old rats with PTU treatment from day 1 postpartum to day 30 postpartum and left untreated up to day 90 postpartum).

hypothyroidism, both Se-D-GPx and Se-I-GPx were reduced by 23%, respectively in testicular post-mitochondrial fraction (Figure 3) but in mitochondrial fraction, Se-D-GPx was reduced by 27% without any change in Se-I-GPx (Figure 4).

Testicular Se-dependent and Se-independent glutathione peroxidase activities in hyperthyroid states with or without vitamin E and/or curcumin treatments

In postmitochondrial fraction, Se-dependent and Se-independent GPx elevated respectively by 18% and 19% in T_4 (group- T_4), by 14% and with no change in T_4 with curcumin (group- T_4+Cur), by 10.35% and 21.11% in T_4 with vitamin E (group- T_4+Vit.E) and by 19% and 57% in T_4 along with curcumin and vitamin E (group- T_4+Cur+Vit.E) treated rats (Figure 5).

In case of mitochondrial fraction, Se-dependent GPx showed no alteration in response to curcumin and/or vitamin E treatment to T_4 treated rats (groups- T_4+Cur, T_4+Vit.E and T_4+Cur+Vit.E), however its level increased by 28.7% in T_4 treated rats (group- T_4). Se-independent GPx elevated significantly by

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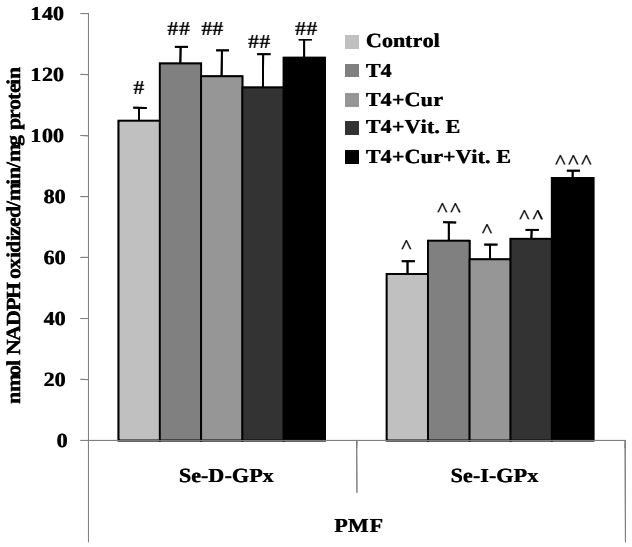

Figure 5 : Effect of thyroid hormone (T_4), curcumin, vitamin E on Se-dependent (Se-D) and Se-independent (Se-I) glutathione peroxidase activities (nmol NADPH oxidized/min/mg protein) in postmitochondrial fraction (PMF) of testes of rats. Data are expressed as mean ± S.D. of 5 observations. Different superscripts differ significantly ($p<0.05$) from each other. Group-Control (Control); Group- T_4 (T_4 treated); Group- T_4+Cur (T_4 with curcumin treated); Group- T_4+Vit.E (T_4 with vitamin E treated); Group- T_4+Cur+Vit.E (T_4 along with curcumin and vitamin E treated).

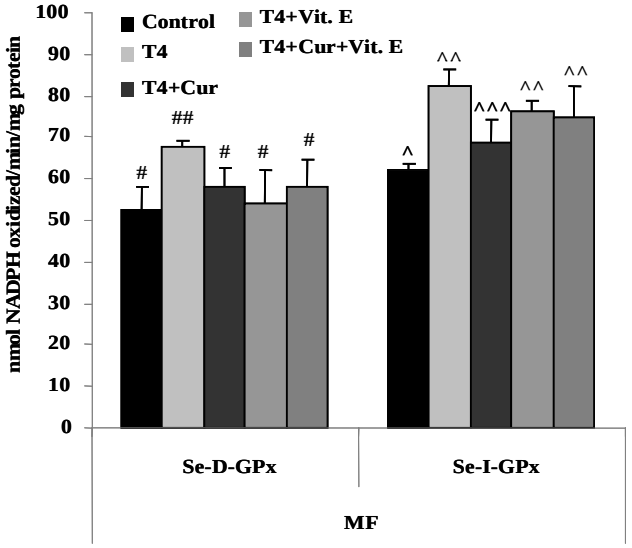

Figure 6 : Effect of thyroid hormone (T_4), curcumin, vitamin E on Se-dependent (Se-D) and Se-independent (Se-I) glutathione peroxidase activities (nmol NADPH oxidized/min/mg protein) in mitochondrial fraction (MF) of testes of rats. Data are expressed as mean ± S.D. of 5 observations. Different superscripts differ significantly ($p<0.05$) from each other. Group-Control (Control); Group- T_4 (T_4 treated); Group-T_4+Cur (T_4 with curcumin treated); Group- T_4+Vit.E (T_4 with vitamin E treated); Group- T_4+Cur+Vit.E (T_4 along with curcumin and vitamin E treated).

32%, 10%, 22% and 20% in response to T_4 (group-T_4), T_4 with curcumin (group- T_4+Cur), T_4 with vitamin E (group- T_4+Vit.E) and T_4 along with curcumin and vitamin E (group- T_4+Cur+Vit.E) treated rats respectively (Figure 6).

DISCUSSION

Aerobes protect themselves from the oxidative stress generated due to the ROS by neutralizing them by their highly evolved antioxidant defences[25]. Testis is well equipped with both small molecular weight antioxidants (like reducedglutathione, ascorbic acid, vitamin E, uric acid, ubiquinone and carotenoids) as well as antioxidant enzymes (like superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glutathione-S-transferase), that efficiently counteract ROS[12]. Superoxide dismutase (SOD; EC 1.15.1.1) constitutes the first line of coordinated enzymatic defense against ROS by dismutating O_2•- into O_2 and H_2O_2. Catalase (CAT; EC 1.11.1.6) and glutathione peroxidase (GPx; EC 1.11.1.9) are most crucial for detoxifying H_2O_2, thereby preventing the generation of hydroxyl radical by the Fenton reaction[25].

Glutathione peroxidases are believed to have a prominent role in the defense against oxidative damage to cells[26,27]. The decrease in Se-D GPx (GPx-1 and GPx-4) as well as Se- I GPx in the testis suggested that antioxidant enzymes like SOD and CAT have predominant role to combat oxidative stress than GPx in hypothyroid rats as elevated SOD and CAT levels were reported earlier[10]. The decrease in GPx activities in both in MF and PMF during persistent hypothyroidism and in PMF of transient hypothyroid rat testis reported earlier[10] was due to decline in both Se-D-GPx as well as Se-I-GPx activities. However, persistent hypothyroidism causes reduction in Se-I-GPx activities in testicular PMF of 30 day old immature rats[13]. Most probably, the Se-D-GPx activity is also decreased in PMF due to prolonged hypothyroidism in adult rats as shown by current study. In transient hypothyroidism, the declined GPx in MF (as reported earlier by Sahoo et al., 2008[10]) is due to the reduction in Se-D-GPx activity only. The significant decrease in Se-D GPx and Se- I GPx in testis suggested the prevailed oxidative stress in hypothyroid rats. The majority of the cytosolic GPx (PMF) in rat testis existed as selenium- and non-sele-

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nium-dependent GPx is present in the Leydig cells. Much lower levels are associated in sertoli and spermatogenetic cells[28]. GPx is primarily responsible for H_2O_2 removal in testicular mitochondria that doesn't contain catalase. GPx plays a crucial role in scavenging peroxyl radicals and thereby maintains functional integrity of the cell membrane, spermatogenesis, sperm morphology and motility[29]. In testis, PH-GPx or GPx-4 which is partially cytosolic and partially bound to nucli and mitochondria is localized within maturating spermatogenetic cells[28]. GPx-1 has mitochondrial and cytosolic subcellular localizations in all mammalian tissues[30]. It has also been suggested that the metabolic pathway of testosterone biosynthesis requires protection against peroxidation and will be affected by a decrease in the GPx activity[31]. The lower serum testosterone level in hypothyroid rats[10,13] also corroborates the fact. In contrast, during T_4 induced hyperthyroid conditions rat testicular Se-D and Se-I GPx remained elevated in both PMF and MF. In our earlier study, we report about changes of Se-D and Se-I-GPx activities only in PMF due to 3, 3', 5-triiodothyronine treatment in rat testis[9]. Increase in both Se-D and Se-I-GPx levels in response to hyperthyroidism (in the present study) may be an adaptive response to neutralize toxic hydrogen peroxides generated due to impairment of normooxidant status of the organ. However, vitamin E and/or curcumin treatment, caused reduction of Se-D-GPx (GPx-1 and GPx-4) in MF and Se-I-GPx in PMF up to normal level as controls. This could be the result of decreased oxidative stress due to vitamin E and/or curcumin treatment. A good number of studies have established the effectiveness of antioxidants like vitamin E and curcumin against oxidative stress[32-34]. Moreover, this fact is corroborated by our previous report that when the T_4-treated rats were fed with vitamin E and/or curcumin, the lipid peroxide and protein carbonyl contents in crude homogenates of testes decreased to normal level[11]. Hence, hypothyroid as well as hyperthyroid states influencing testicular Se-D- and Se-I- GPx activities, might regulate testicular physiology and oxidative stress.

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REFERENCES

[1] C.D.Turner, J.T.Bagnara; In: General Endocrinology. W.B.Saunders Company, Philadelphia, PA, USA, **(1976)**.
[2] J.R.Tata, L.Ernster, O.Lindberg, E.Arrhenius, S.Pedersen, R.Hedman; Biochemical.Journal, **86**, 408-428 **(1963)**.
[3] M.Moreno, A.Lanni, A.Lombardi, F.Goglia; The Journal of Physiology, **505**, 529-538 **(1997)**.
[4] P.Venditti, S.Di Meo; Cellular and Molecular Life Sciences, **63**, 414-434 **(2006)**.
[5] E.A.Jannini, M.Olivieri, S.Francavilla et al.; Endocrinology, **126**, 2521-2526 **(1990)**.
[6] P.R.Manna, J.Kero, M.Tena-Sempere et al.; Endocrinology, **142**, 319-331 **(2001)**.
[7] E.A.Jannini, S.Ulisse, M.D.Armiento; Endocrine Reviews, **16**, 443-459 **(1995)**.
[8] D.K.Sahoo, A.Roy, S.Bhanja, G.B.N.Chainy; Indian Journal of Experimental Biology, **43**, 1058-1067 **(2005)**.
[9] D.K.Sahoo, A.Roy, S.Chattopadhyay, G.B.N.Chainy; Indian Journal of Experimental Biology, **45**, 338-346 **(2007)**.
[10] D.K.Sahoo, A.Roy, S.Bhanja, G.B.N.Chainy; General and Comparative Endocrinology, **156**, 63-70 **(2008)**.
[11] D.K.Sahoo, A.Roy, G.B.N.Chainy; Chemico-Biological Interactions, **176**, 121-128 **(2008)**.
[12] D.K.Sahoo; Biochemistry: An Indian Journal, **5(6)**, 337-345 **(2011)**.
[13] D.K.Sahoo, A.Roy; International Journal of Endocrinology, 2012, Article ID 637825, 11 **(2012)**.
[14] D.K.Sahoo, A.Roy, G.B.N.Chainy; National Academy Science Letters-India, 29(3&4), 133-135 **(2006)**.
[15] S.Choudhury, G.B.N.Chainy, M.M.Mishro; Andrologia, **35**, 131-140 **(2003)**.
[16] G.Gills, K.SiegIer; Folia Microbiologica., **30(2)**, 131-152 **(1995)**.
[17] R.S.Esworthy, R.Aranda, M.G.Martín, et al.; American Journal of Physiology - Gastrointestinal and Liver Physiology, **281(3)**, G848-855 **(2001)**.
[18] F.Tramer, F.Micali, G.Sandri, A.Bertoni, A.Lenzi, L.Gandini, E.Panfili; International Journal of Andrology, **25(2)**, 72-83 **(2002)**.

[19] P.Doyen, P.Vasseur, F.Rodius; Comparative Biochemistry and Physiology, Part C, **144**, 122-129 **(2006)**.

[20] E.Institoris, H.Eid, I.Bodrogi, M.Bak; Anticancer Research, **15(4)**, 1371-1374 **(1995)**.

[21] O.H.Lowry, N.J.Rosebrough, A.L.Farr, R.J.Randall; Journal of Biological Chemistry, **193**, 265-275 **(1951)**.

[22] D.E.Paglia, W.N.Valentine; Journal of Laboratory and Clinical Medicine, **70**, 158-169 **(1967)**.

[23] F.Carmagnol, P.M.Sinet, H.Jerome; Biochimica et Biophysica Acta, **759**, 49-57 **(1983)**.

[24] S.Chattopadhyay, S.Choudhury, A.Roy et al.; General and Comparative Endocrinology, **169**, 39-47 **(2010)**.

[25] B.Halliwell, J.M.C.Gutteridge; Free Radicals in Biology and Medicine, 3rd Edition, Oxford University Press, New York, **(2001)**.

[26] F.Ursini, A.Bindoli; Chemistry and Physics of Lipids, **44**, 255-276 **(1987)**.

[27] R.Brigelius-Flohé, C.Müller, J.Menard, S.Florian, K.Schmehl, K.Wingler; Biofactors, **14(1-4)**, 101-106 **(2001)**.

[28] K.Mehrotra, R.Morgenstern, M.B.Ahlberg, A.Georgellis; Chemico-Biological Interactions, **122**, 73-87 **(1999)**.

[29] H.I.Calvin, S.I.Turner; Journal of Experimental Zoology, **219**, 389-393 **(1982)**.

[30] R.S.Esworthy, Y.S.Ho, F.F.Chu; Archives of Biochemistry and Biophysics, **340(1)**, 59-63 **(1997)**.

[31] R.Chandra, R.Aneja, C.Rewal, R.Konduri, K.Das, S.Agarwal; Indian Journal of Clinical Biochemistry, **15**, 155-160 **(2000)**.

[32] E.Brzezinska-Slebodzinska; Journal of Physiology and Pharmacology, **52**, 275-284 **(2001)**.

[33] R.J.Verma, A.Nair; Asian Journal of Andrology, **3**, 217-221 **(2001)**.

[34] M.Naito, X.Wu, H.Nomura, M.Kodama, Y.Kato, Y.Kato, T.Osawa; Journal of Atherosclerosis and Thrombosis, **9**, 243-250 **(2002)**.