J Endocrinol Reprod 18 (2014) 1 : 95-110 **Original Research**

Transient neonatal-onset hypothyroidism boosts estradiol synthesis in the testis of adult Wistar rat

N.S. Venkatesh, M. Kathiresh Kumar, S. Sambavi, N. Kalpana, A.M. Sashi John, J. Anbalagan, R. Ilangovan and M.M. Aruldhas

Department of Endocrinology, Dr. ALM Post Graduate Institute of Basic Medical Sciences, University of Madras, Taramani Campus, Chennai – 600 113, India.

Summary

Transient neonatal hypothyroidism is known to boost Sertoli cell (SC), Leydig cell (LC) and germ cell (GC) number at adult age. Existing reports attribute decreased steroidogenic potential of LCs to unaltered/decreased serum testosterone in such rats, despite a boost in the cell number. However, these studies have ignored the status of estradiol in such conditions. In this present study, we tested the hypothesis "transient neonatal-onset hypothyroidism may lead to a temporal shift in adult rat testicular steroidogenesis towards estradiol production". Hypothyroidism was induced in neonates by providing methimazole (MMI) in drinking water (0.05%) to lactating mothers and pups for a transient period from postnatal day 1 (PND 1) to PND 14 or from PND 1 to PND 29. After the experimental period, the pups were provided drinking water free of MMI and sacrificed on PND 91. Coeval rats without MMI exposure served as control. Radioimmunoassay revealed decreased serum titres of luteinizing hormone (LH), follicle stimulating hormone (FSH), growth hormone (GH) and prolactin (PRL); while serum testosterone remained unaltered, its level in testicular interstitial fluid (TIF) decreased. Between the two major metabolites of testosterone, 5α-dihydrotestosterone (DHT) concentration decreased in serum and TIF, whereas estradiol recorded a significant increase in both. Transient neonatal-onset hypothyroidism decreased 3β-hydroxy steroid dehydrogenase (3β-HSD) in LCs but augmented 17β-HSD activity. Radio-receptor assay revealed decreased concentration of LHR and ER in LCs of hypothyroid groups, whereas AR and PRLR increased. While aromatase activity decreased in LCs, it increased along with FSHR in SCs, when compared to control rats. The changes in LHR and FSHR levels and aromatase were consistent with the expression level of the respective genes. The present study supports the proposed hypothesis and suggests that transient neonatal-onset hypothyroidism-induced boost in estradiol in adult rat testis may be due to augmented expression and activity of aromatase, and FSHR content in SCs of these rats.

Key words: Leydig cells, Sertoli cells, LHR, FSHR, Hypothyroidism

Introduction

Mammalian testis is a target organ for thyroid hormones (Jannini et al., 1995; Aruldhas, 2002). Testis is an exocrine organ because it produces sperm and it is also an endocrine organ producing hormones (Mendis-Handagama, 2012). Histologically, the testis consists of three major cell types: Leydig cell (LCs) (steroidogenic cells), Sertoli cells (SCs) (nursery cells) and germ cells (GCs). LCs lie in cluster or groups near blood vessels in the testicular interstitum (see Saez, 1994), whereas SCs and GCs are confined to the tubular compartment. The hypothalamo-hypophyseal-testicular axis is the primary hormonal regulator of the testis (see Jannini et al., 1995; see Aruldhas, 2002).

Luteinizing hormone (LH) and follicle stimulating hormone (FSH) are required for normal growth and differentiation of LCs and SCs (Christensen & Fawcett, 1966). Testosterone is produced by LCs in response to LH stimulation. The pulsatile secretion of FSH and LH by the gonadotrophs in rat pituitary is regulated by hypothalamic gonadotrophin releasing hormone (GnRH) (Bousfield et al., 1994), gonadal steroids (Hall, 1994), gonadal peptides like activins and inhibins (Vale et al., 1994), growth hormone (GH) (Tang et al., 1993), prolactin (PRL) (Hermanns & Hafez, 1981) and leptin (Yu et al., 1997). LCs are the main source of testosterone during adult life. There are two major population of LCs in mammals, fetal and adult types (FLCs and ALCs); the ALCs differentiate post-natally

Correspondence to be addressed to: Dr. M. Michael Aruldhas, Ph.D. E-mail: aruldhasmm@gmail.com

from the mesenchymal stem cells in the testis (Mendis-Handagama and Ariyaratne, 2001).

Extensive studies performed in our laboratory and others have identified specific neonatal windows for thyroid hormone action on LCs and SCs development and differentiation (Cooke and Meisami, 1991; Maran et al., 2000; 2001; Maran & Aruldhas, 2002; Venkatesh, 2004). Protection of the testis from thyroid hormone action during this critical post-natal window results in continuous proliferation of all the three cell types at adulthood, with delayed functional differentiation of the testis. Hence, transient deficiency of thyroid hormone during neonatal-prepubertal life increases testicular size and sperm production in adult rat and mouse (Cooke et al., 1993; Maran et al., 1999a, b; Maran & Aruldhas, 2002).

Hypothyroidism is associated with altered pituitary gonadotropins, which may be the result of modified GnRH action or status of other regulators, such as gonadal steroids and peptides, which modulate the synthesis of gonadotropins (Longcope, 2000). Hypothyroidism impairs the steroidogenic response of immature and mature rat testis / LCs to LH stimulation under *in vivo* and *in vitro* conditions (Valle et al., 1985; Ando et al., 1990; Antony et al., 1995). Dihydrotestosterone (DHT) is the biologically active metabolite of testosterone and is derived from the local conversion of testosterone by the enzyme 5α reductase. Another bioactive metabolite of testosterone is estradiol, which is formed by the action of the enzyme aromatase, which is expressed in both LCs and SCs (see Akingbemi et al., 1999). Triiodothyronine (T_3) affects testosterone metabolism (Panno et al., 1996) and inhibits SC aromatase activity both *in vivo* and *in vitro* (Palmero et al., 1995). LC function in terms of testosterone secretion was shown to be decreased in adult rats exposed to transient neonatal hypothyroidism, despite increased number of LCs, and this was attributed to decreased steroidogenic potential of LCs (Cooke & Meisami, 1991). However, the probable changes in DHT and estradiol titres and their synthesis in adult rats with transient neonatal hypothyroidism remain obscure. With the above hindsight in mind, it was hypothesized that *transient neonatal-onset hypothyroidism may lead to a temporal shift in adult rat testicular steroidogenesis towards estradiol production,* and this hypothesis was appropriately put to test in this study.

Materials and Methods

Animal and treatment

The experiments in this study were approved by the Institutional Animal Ethics Committee (IAEC). Male albino Wistar rats (*Rattus norvegicus)* weighing 200–250 g were allowed to mate with proven-fertile female rats (1:2) at late proestrous phase. Successful mating was confirmed by the presence of vaginal plug or sperm in the morning vaginal smear. The day of parturition was counted as postnatal day (PND) one. Lactating rats and pups were given drinking water containing methimazole (MMI, 0.05% in drinking water) to induce hypothyroidism in the pups as MMI can pass through mother's milk (see Haynes,1991). Rats were divided into the following groups: Group I: Control male rats at PND 90; Group II: Male rats at PND 90 which had transient neonatal exposure to MMI from PND 1 to PND 14, covering the short neonatal window of thyroid hormone action on rat testis (proliferation of SCs and LCs periods of testicular differentiation from the bipotential gonad and initiation of the differentiation of ALCs) (Maran et al., 2000; 2001); Group III: Male rats at PND 90, which had transient exposure to MMI treatment from PND 1 to PND 29, covering the large neonatal window of thyroid hormone action on testis (Cooke et al., 1992) when there will be active proliferation and functional differentiation of ALC with the onset of testosterone production and degeneration of FLCs (Mendis-Handagama & Ariyaratne, 2001). On completion of MMI treatment, the rats were given normal drinking water free of MMI. Rats were sacrificed by decapitation on the morning of PND 91. Blood and testicular interstitial fluid (TIF) were collected. Testes were removed and used for the isolation of LCs and SCs.

Isolation and purification of LCs and SCs

Briefly, testes were decapsulated using fine forceps without breaking the seminiferous tubules, under aseptic conditions, and incubated in culture medium (DMEM) containing 0.1% BSA and collagenase (0.2 mg/ml) for 15 min at 34°C. After this incubation, the flasks were gently shaken and fresh DMEM (10 ml) was added, allowed to stand for 5 min at 37°C and the

supernatant was centrifuged at 1000 × g for 15 min at 4°C to obtain a crude LC suspension. This suspension was then placed on top of the discontinuous percoll density gradient (prepared by layering decreasing percentage of percoll, one on top of another - 75%, 60%, 50%, 40%, 30%, 20% and 10%) and centrifuged at 1000 × g for 20 min at 4°C. Uncontaminated LCs were observed in between 30%-45% gradients and removed carefully using a Pasteur pipette. The percentage of purity of LC was confirmed by staining the cells for 3β-HSD activity following the procedure of Aldred and Cooke (1983).

After isolating Leydig cells, the seminiferous tubules were resuspended in 5ml medium-S and incubated for 15 min at 37°C. After 15 min, 5 ml fresh DMEM was added to the flasks, shaken well and allowed to stand for 5 min. The supernatant, which contained predominantly peritubular myoid cells, was removed. Then 5 ml medium-S was again added and the flasks were incubated for another 15 min at room temperature. The supernatant, which contained predominantly mixed germ cells, was removed using a Pasteur pipette. Five ml DMEM was added to the flasks in addition to 5 ml trypsin inhibitor to arrest further proteolytic activity of trypsin, and incubated for 5 min. The SC-enriched medium was filtered through 25 µm nylon mesh to remove other contaminating cells (rat SC diameter is 60-75 µm). This step further improved the purity of SC preparation. The clusters of Sertoli cells on the top of the nylon wire mesh were taken into sterilized tubes and washed thrice, using DMEM. Finally, the pellet was resuspended in 10 ml DMEM. The purity of SC was 85-90% as assessed by Oil Red O staining (Lillie and Fullmer, 1976).

Histomorphometry of testis

Testicular cell numbers

Immediately after animal sacrifice, one testis from each rat was fixed in 7% formalin and 5.0 µm thick paraffin sections of the testis were prepared, stained with Hematoxylin and Eosin, and used for morphometric analysis (Russell et al., 1990). The numerical density (Nv) of LCs and SCs was quantified by the method of Furuya (1990) adopting the equation $Nv = Na / (D + T - 2h)$, where Na = Number of cell nuclei in an unit area of a section; D = Average diameter of the cell nucleus; T = Section thickness and h = height of the smallest section of the cell. 'h' was assumed to be 0.1 × nuclear diameter (Mori & Christensen, 1980); D was obtained by measuring long and short axes of the cell using the formula $D = \sqrt{a \times b}$, where, a = maximum length of the cell, and b = the maximum width of the cell.

Radioimmunoassay of hormones

The levels of serum LH and FSH were determined adopting standard liquid phase RIA (Banu et al., 2002) using radioactive iodine [^{125}I] (MP Biochemicals India Pvt Ltd, Mumbai). In this method, the antibody was introduced into the assay system in liquid form and the free and bound antigens were separated by using an accelerated precipitation reagent (second antibody and PEG for peptides and dextran-coated charcoal for steroids). Specific rat antigen, antibodies and reference standards were received from NIDDK (Bethesda, Maryland, USA): tracer rFSH (NIDDK-rFSH-I-8-AFP-1145B)], rFSH antiserum (NIDDK-anti-rFSH-S-AFP-C0972881), rFSH reference preparation (NIDDK-rFSH-RP2-AFP-4621B), rLH tracer (NIDDK-rLH-I-9-AFP-10250C), rLH antiserum (NIDDK-anti-rLH-S-10), and rLH reference preparation (NIDDK-rLH-RP-3-AFP-7187B). T, E_2 and DHT in both serum T and TIF were also assayed by liquid phase RIA adapting the WHO procedure (Sufi et al., 1980). The FSH intra- and inter-assay coefficient of variation was 5.7-8.9% and 12.2%, respectively. The sensitivity of the assay was 0.20 ng/ml. The LH intra- and inter-assay co-efficient of variation was 4.9-8.48% and 9.9%, respectively. Sensitivity of the assay was 5 ng/ml. The 'T' intra and inter-assay co-efficient of variation was 4-6% and 6-8%, respectively, the sensitivity of the assay was 0.3 ng/ml. The E_2 intra- and inter-assay variation was 1.5-10% and 3.2-4.9%, respectively. The sensitivity was 4 pg/ml. The DHT intra- and inter-assay variation was 3-6% and 4-8%, respectively, and the sensitivity was 0.01 ng/ml.

Assays of steroidogenic enzyme activity

The steroidogenic enzymes 17β-hydroxysteroid dehydrogenase (17β-HSD-EC 1.1.1.62), 3β-hydroxysteroid dehydrogenase (3β-HSD-EC 1.1.1.145) and aromatase (EC 1.14.14.1) enzyme activity were assayed following the method of Bergmeyer (1974) and Ulisse et al. (1994), respectively.

Real-time RT-PCR

LCs and SCs isolated from the control and experimental rats were homogenized in TRIZOL reagent (Invitrogen, USA). Total RNA was isolated from the LCs by using total RNA isolation reagent (TRIR, ABGen, Surrey, USA). A ratio of absorbance at 260 / 280 nm > 1.8 is generally considered as good quality RNA. The RNA with purity between 1.8 and 2.0 alone was used for the subsequent RT-PCR and qRT-PCR analysis. Extracted total RNA (2 µg totalRNA) was reverse-transcribed using a commercial kit (Eurogentech, Europe). RT-PCR was carried out in a MX3000p PCR system (Stratagene, Europe). Reaction was performed using Euro Gentech SyBR green PCR kit. cDNA was then amplified using the primers LHR*: sense*; -5' GTG CGC TGTC CTG GCC 3'; *anti-sense;*- 5' TTC AGG GGA ACT TAA TGA GGT CG 3'; 5α-reductase type I: *sense*;- 5' CGT CCT GCT GGC TAT GTT TG 3'; *anti-sense;*- 5' TCA CCT TTG TCT TTG GCC TTC 3'; 5α-reductase type II: *sense*;- 5'GGA CCT GAT CCT GTG GGT TA 3'; *anti-sense*;- 5'GTT GCC TTG CAT TTG TGG TGT 3'*;* Glyceraldehyde-3-phosphate dehydrogenase: *sense*;- 5' TCT ACC CAC GGC AAG TTC AAT 3'; *anti-sense*;- 5' CCG CTA ACA TCA AAT GGG GT 3'. The PCR conditions were 95°C for 10 min and 40 cycles of 95° C for 30 sec, 61° C for 30 sec and 72° C for 1 min. Denaturation analysis cycles were 95° C for 30 sec, 50°C for 50 sec and 95°C for 30 sec. Fluorescent signals were captured at 82°C, and the occurrence of primer dimers and secondary products were determined using melting curve analysis. The copy number for each sample was calculated and the data were normalized using the mRNA expression level of GAPDH. The aromatase expression was amplified by using *sense;* -5' CAC CCC TCA CAG ACT GTG GT 3'; *Antisense*; - 5' TTG ACA GTG CTG ACA GGA GC 3'. The PCR condition was intial PCR activation 95°C for 15 min; denaturation 94°C for 1.5 min; annealing 58°C for 1.5 min; extension at 72°C for 3 min; final extension at 72°C for 10 min. Number of cycles was 30.

Statistical analysis

The data were subjected to statistical analysis using one-way analysis of variance (ANOVA) and Duncan's multiple-range tests to assess the significance of individual variations between groups using the statistical analysis software SPSS 7.5, Students' version (SPSS, Chicago, USA).

Results

Effect of transient neonatal exposure to MMI on Leydig and Sertoli cells number, testis weight and TIF volume of adult rats

LCs number per testis was significantly increased in rats exposed to transient neonatal-onset hypothyroidism (Groups II and III), when compared to age-matched control group (Fig. 1). SC number also increased significantly in rats exposed to transient neonatal-onset hypothyroidism (Fig. 1a)

Transient neonatal exposure to MMI increased the absolute and relative weight of the testis of rats at PND 90, when compared to coeval control rats (Fig. 1). A significant decrease in TIF volume was observed in adult rats with transient neonatal exposure to MMI.

Effect of neonatal hypothyroidism on serum and TIF hormones of adult rats

Serum hormones

Serum LH and FSH decreased significantly in 90 day old adult rats which were subjected to transient neonatal-onset hypothyroidism, when compared to coeval control rats (Fig. 2). While serum testosterone remained normal (Fig. 3), serum DHT decreased to significant levels (Fig. 4), whereas serum estradiol increased significantly in experimental rats when compared to control rats (Fig. 5).

TIF hormones

Both testosterone and DHT titres decreased in the TIF of 90 day old experimental rats belonging to groups II and III, when compared to coeval control rats in group I (Fig. 3, 4). On the contrary, consistent with the trend in peripheral circulation, estradiol titre in the TIF also increased significantly in experimental rats belonging to groups II and III (Fig. 5).

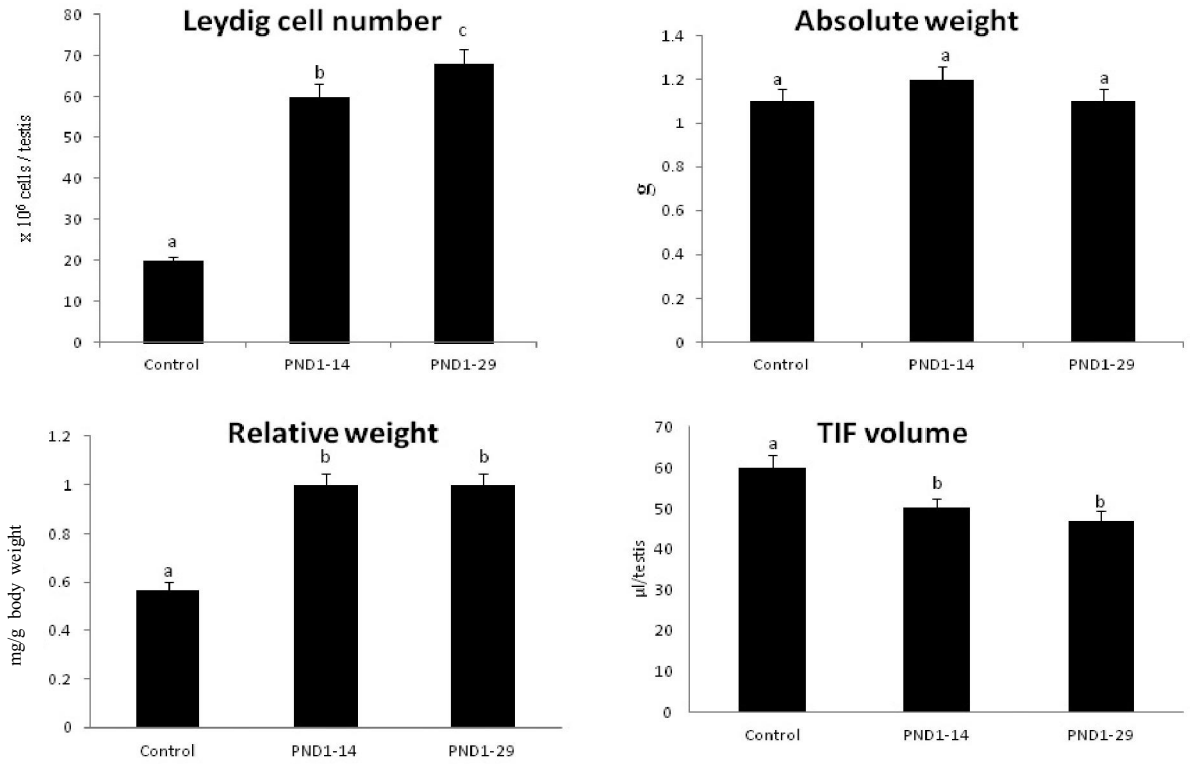

Fig. 1. Impact of transient neonatal-onset hypothyroidism on Leydig cell number, testis and TIF volume of adult rat testis. Each bar represents the mean and the vertical line denotes the SEM; n=6. Same alphabets denote statistically insignificant difference between the respective means, while different alphabets denote statistically significant difference between the means at $p<0.05$ level.

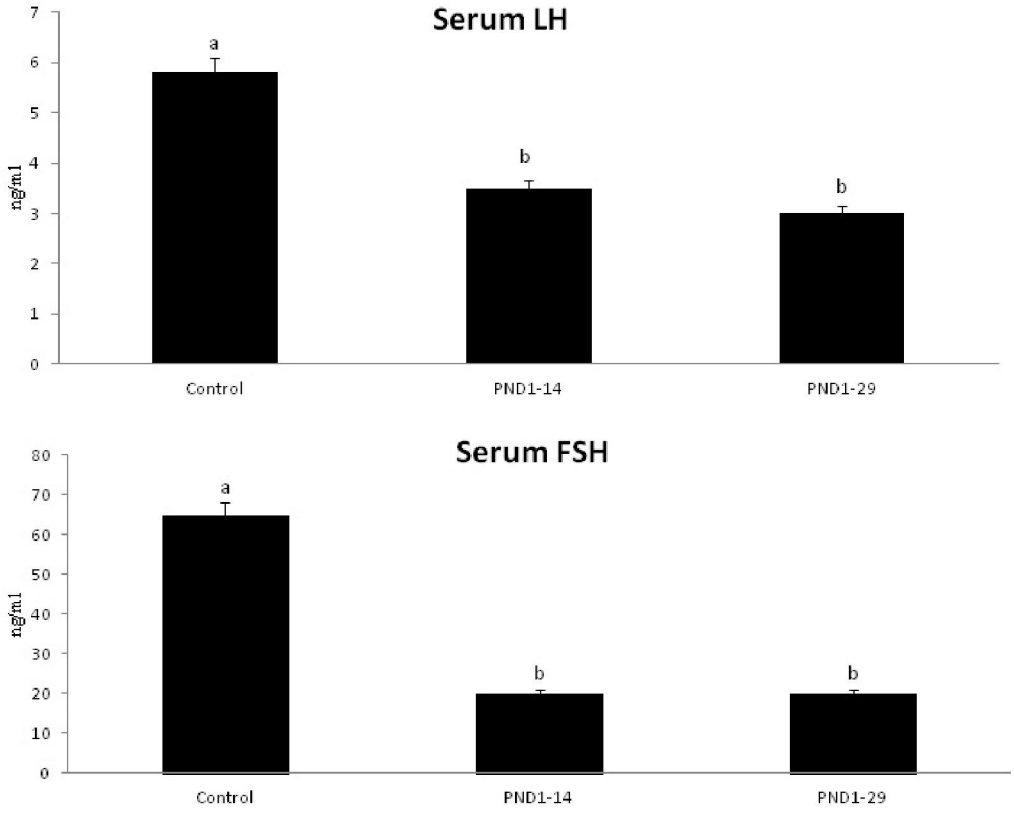

Fig. 2. Impact of transient neonatal-onset hypothyroidism on serum gonadotropins titre in adult male rats. Each bar represents the mean and the vertical line denotes the SEM; n=6. Same alphabets denote statistically insignificant difference between the respective means, while different alphabets denote statistically significant difference between the means at $p<0.05$ level.

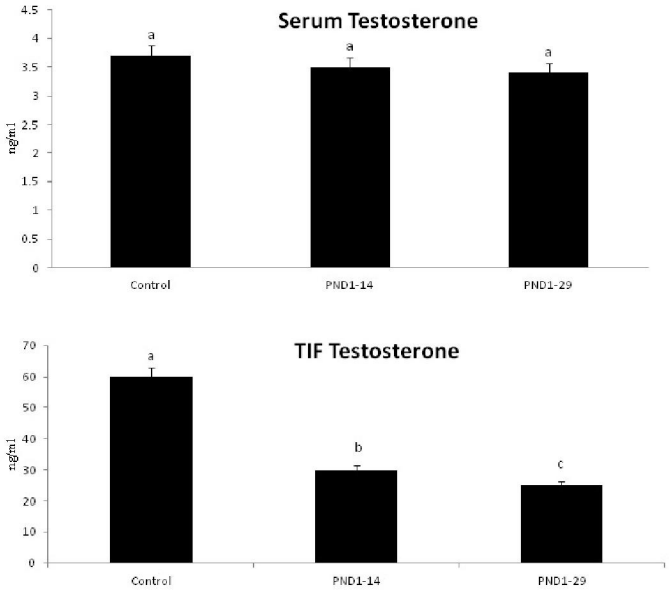

Fig. 3. Impact of transient neonatal-onset hypothyroidism on serum and TIF testosterone titres in adult male rats. Each bar represents the mean and the vertical line denotes the SEM; n=6. Same alphabets denote statistically insignificant difference between the respective means, while different alphabets denote statistically significant difference between the means at $p<0.05$ level.

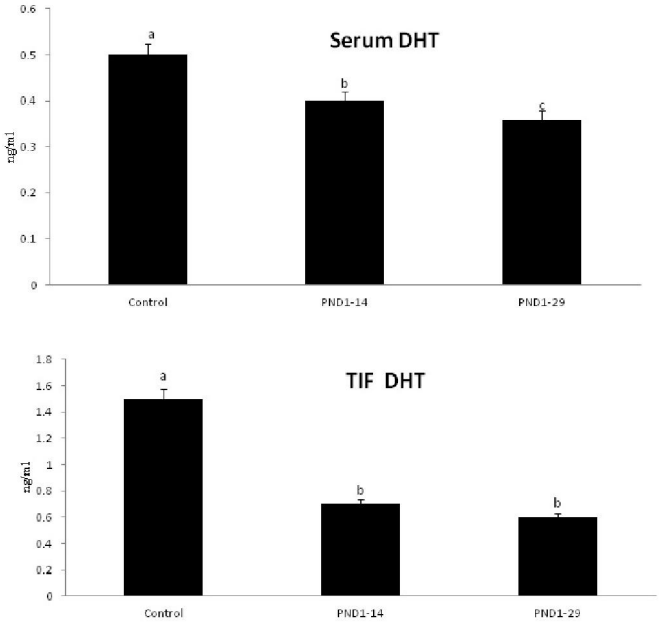

Fig. 4. Impact of transient neonatal hypothyroidism on serum and TIF DHT titres in adult male rats. Each bar represents the mean and the vertical line denotes the SEM; n=6. Same alphabets denote statistically insignificant difference between the respective means, while different alphabets denote statistically significant difference between the means at $p<0.05$ level.

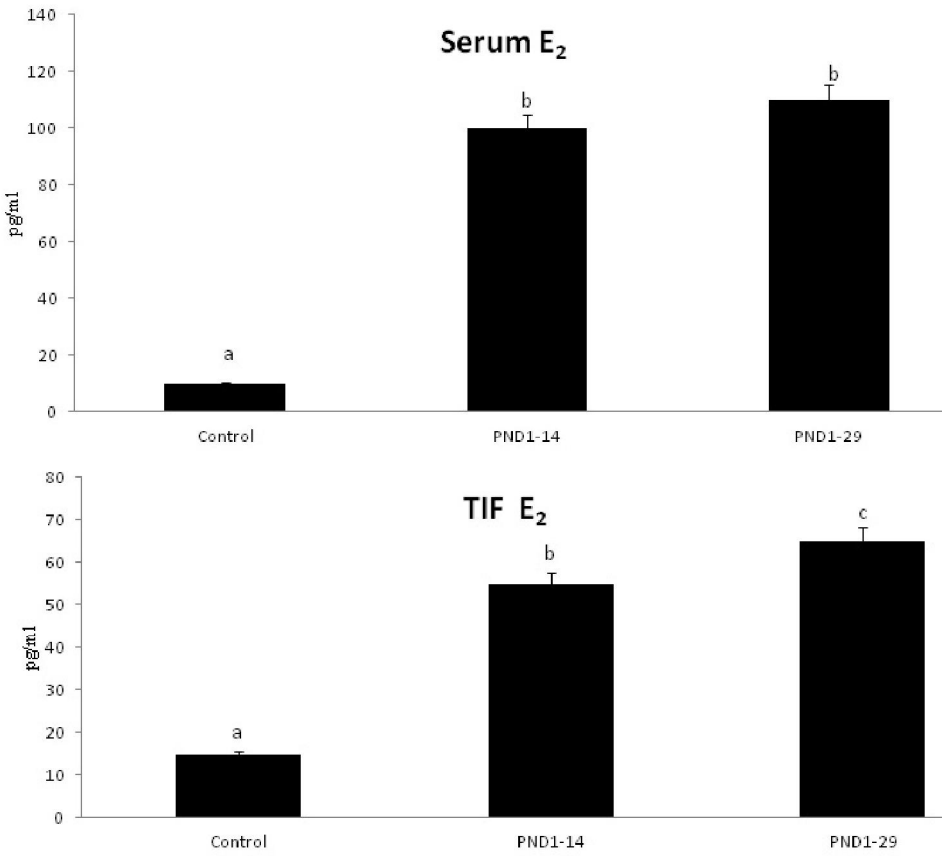

Fig. 5. Impact of transient neonatal hypothyroidism on serum and TIF E_2 titres in adult male rats. Each bar represents the mean and the vertical line denotes the SEM; n=6. Same alphabets denote statistically insignificant difference between the respective means, while different alphabets denote statistically significant difference between the means at $p<0.05$ level.

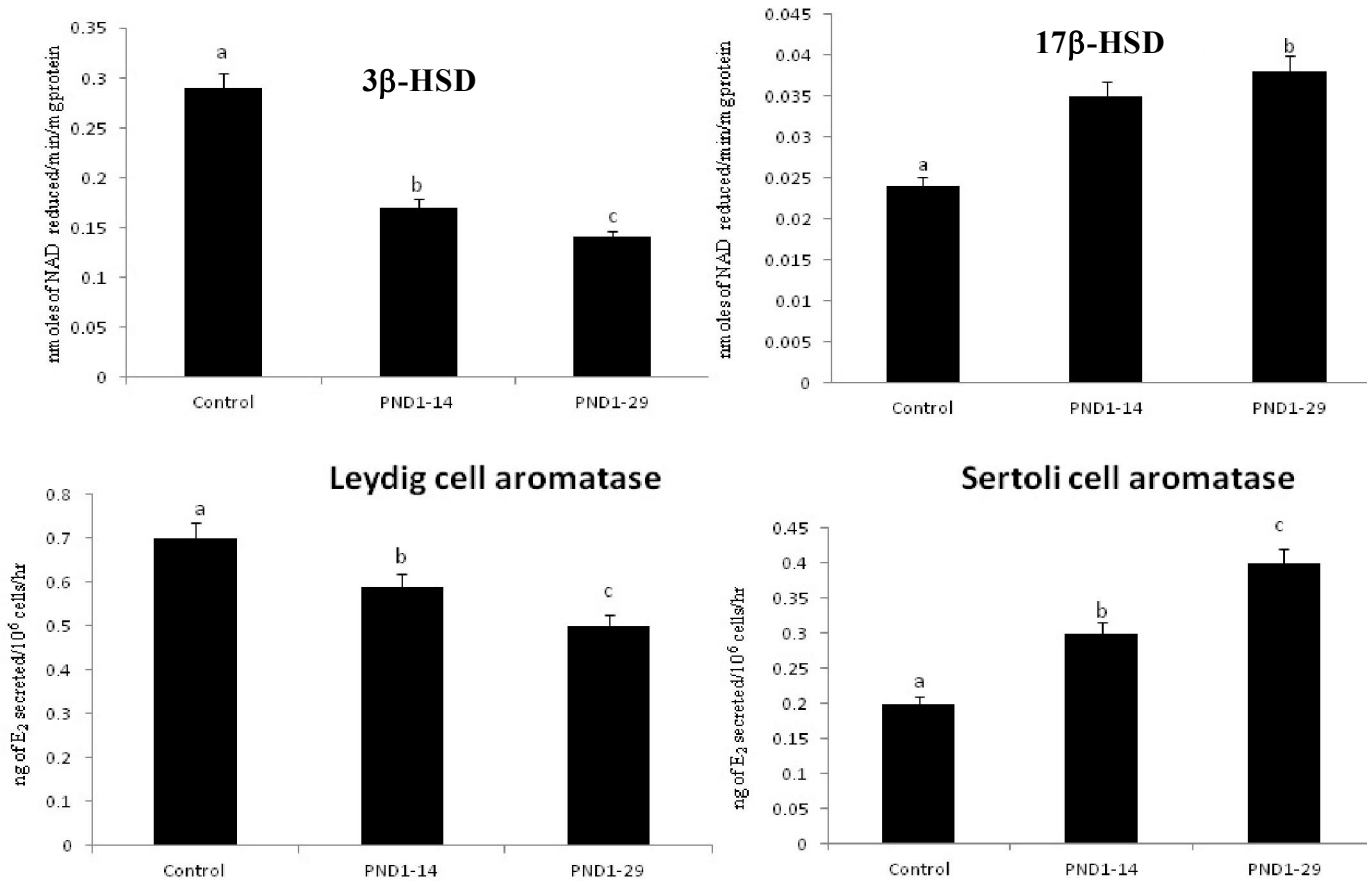

Fig. 6. Specific activity of 3β-HSD, 17β-HSD and aromatase in Leydig cells isolated from adult rats subjected to transient neonatal-onset hypothyroidism. Each bar represents the mean and the vertical line denotes the SEM; n=6. Same alphabets denote statistically insignificant difference between the respective means, while different alphabets denote statistically significant difference between the means at $p<0.05$ level.

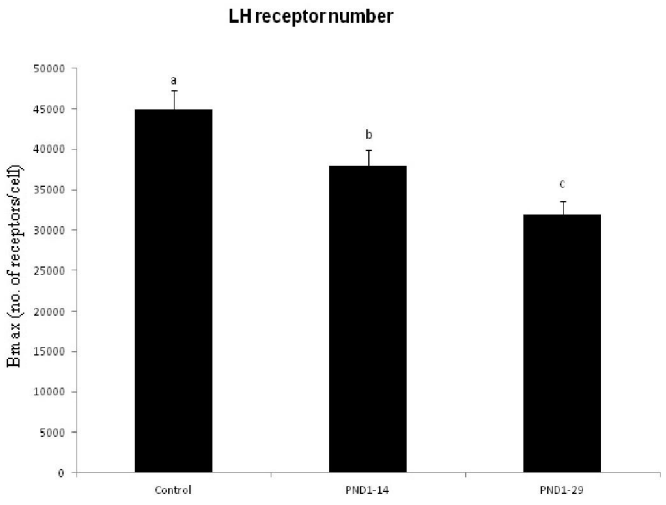

Fig. 7. LH receptor number in Leydig cells isolated from 90 day old adult rats subjected to transient neonatal-onset hypothyroidism. Each bar represents the mean and the vertical line denotes the SEM; n=6. Same alphabets denote statistically insignificant difference between the respective means, while different alphabets denote statistically significant difference between the means at $p<0.05$ level.

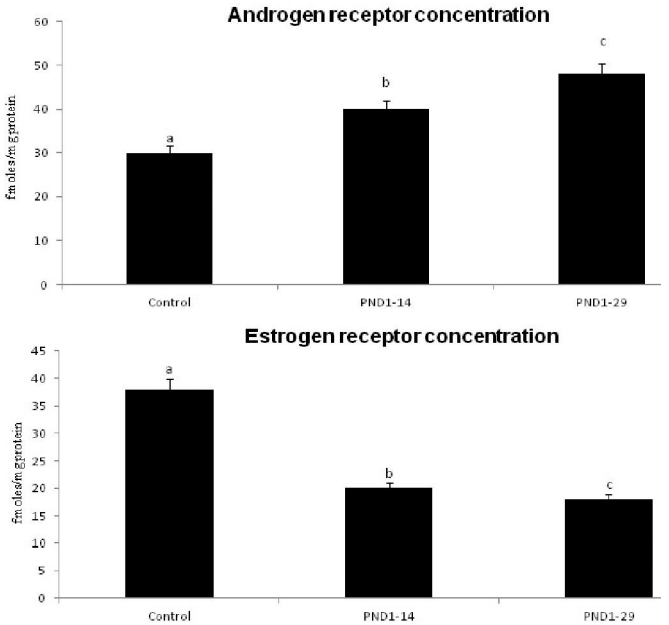

Fig. 8. Androgen and estrogen receptor concentration in Leydig cells isolated from 90 day old adult rats subjected to transient neonatal-onset hypothyroidism. Each bar represents the mean and the vertical line denotes the SEM; n=6. Same alphabets denote statistically insignificant difference between the respective means, while different alphabets denote statistically significant difference between the means at $p<0.05$ level.

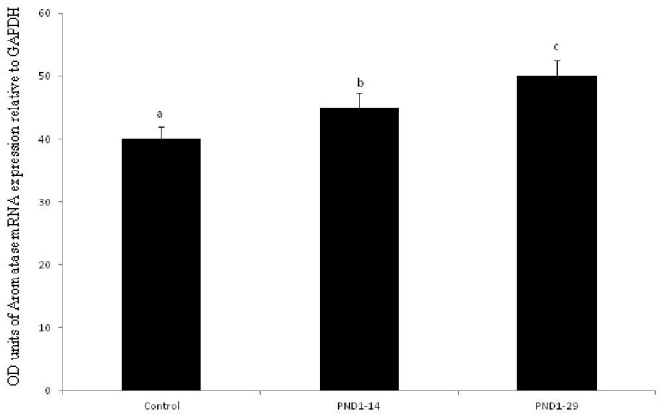

Fig. 9. Impact of transient neonatal-onset hypothyroidism on aromatase mRNA expression in the Leydig cells of adult male rats. Each bar represents the mean and the vertical line denotes the SEM; n=3. Same alphabets denote statistically insignificant difference between the respective means, while different alphabets denote statistically significant difference between those means at $p<0.05$ level.

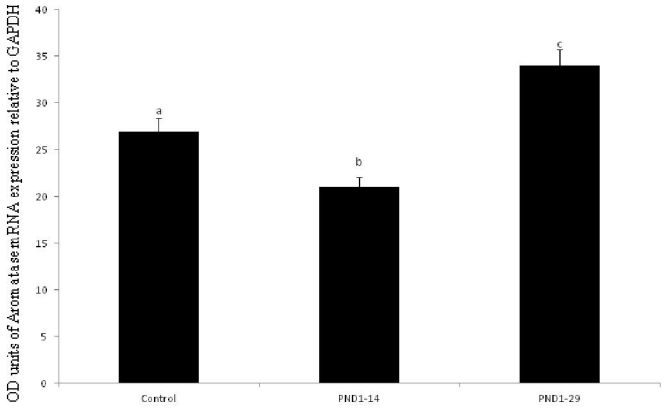

Fig. 10. Impact of transient neonatal-onset hypothyroidism on aromatase mRNA expression in the Sertoli cells of adult male rats. Each bar represents the mean and the vertical line above denotes the SEM; n=3. Same alphabets denote statistically insignificant difference between the respective means, while different alphabets denote statistically significant difference between the means at $p<0.05$ level.

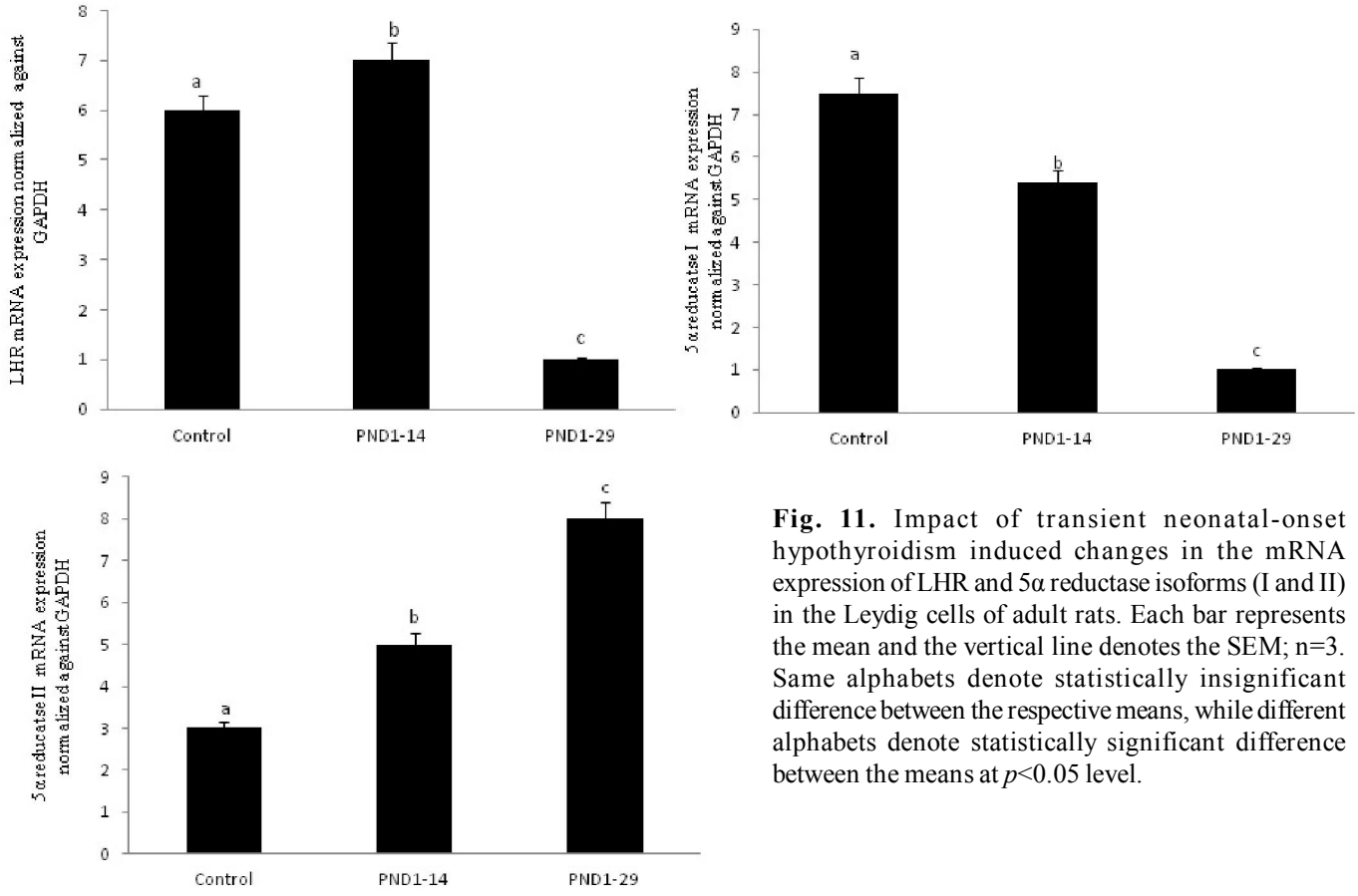

Fig. 11. Impact of transient neonatal-onset hypothyroidism induced changes in the mRNA expression of LHR and 5α reductase isoforms (I and II) in the Leydig cells of adult rats. Each bar represents the mean and the vertical line denotes the SEM; n=3. Same alphabets denote statistically insignificant difference between the respective means, while different alphabets denote statistically significant difference between the means at $p<0.05$ level.

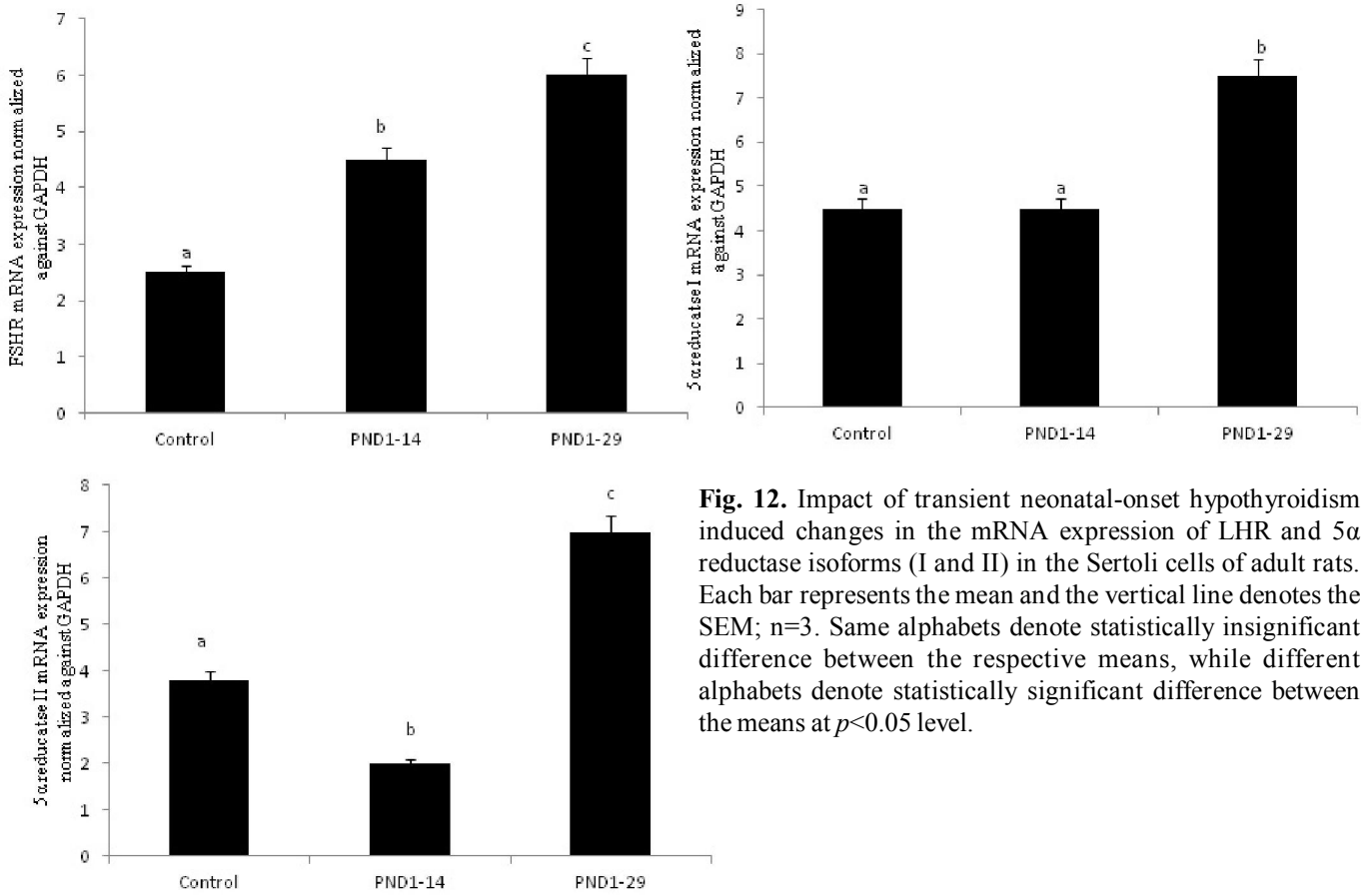

Fig. 12. Impact of transient neonatal-onset hypothyroidism induced changes in the mRNA expression of LHR and 5α reductase isoforms (I and II) in the Sertoli cells of adult rats. Each bar represents the mean and the vertical line denotes the SEM; n=3. Same alphabets denote statistically insignificant difference between the respective means, while different alphabets denote statistically significant difference between the means at $p<0.05$ level.

Effect of neonatal hypothyroidism on testicular steroidogenic enzyme activity

In order to understand the mechanism underlying the boost in estradiol in TIF and peripheral circulation, we estimated the specific activities of the steroidogenic enzymes 3β-HSD and 17β-HSD in LCs and aromatase activity in LCs and SCs. Transient neonatal-onset hypothyroidism caused significant decrease in the specific activity of 3β-HSD but increased the specific activity of 17β-HSD in the LCs of groups II and III rats, when compared with coeval control rats (Fig. 6). However, aromatase activity of LCs decreased, whereas the same increased in SCs (Fig. 6).

Effect of neonatal hypothyroidism on testicular hormone receptor

LH receptor content decreased significantly in groups II and III rats subjected to transient hypothyroidism for a short or long early postnatal life, whereas FSHR content in SCs of these rats increased significantly when compared to coeval controls (Fig. 7). However, LHR mRNA expression decreased in the LCs of group III experimental rats, when compared to coeval control rats (Fig. 11).

Transient neonatal hypothyroidism caused increase of AR concentration but decreased the concentration of ER in LCs of rats belonging to groups II and III when compared to age-matched controls (Fig. 8).

Effect of neonatal hypothyroidism on the expression of specific testicular genes

In an attempt to find if neonatal exposure to MMI interferes with testicular genes, we tested the expression of selected genes specific to LCs and SCs. Aromatase mRNA expression was increased in LCs (Fig. 9) of both experimental groups and in SCs of group III rats, whereas it decreased marginally in SCs of group II rats (Fig. 10).

Though we did not determine the activity of 5α reductase, we detected the mRNA expression of its two

isozymes, which depicted a differential response to transient neonatal-onset hypothyroidism. 5α reductase I mRNA expression significantly decreased, whereas 5α reductase II mRNA expression increased in LCs of both the groups when compared to coeval control rats (Fig. 11). In the case of SCs, expression of both isozymes of 5α reductase increased in group III rats, whereas in group II rats alone expression of 5α reductase II decreased (Fig. 12).

Lhr mRNA expression recorded a significant decrease in LCs of group III rats, whereas its expression level in group II rats was comparable to coeval control rats (Fig. 11). There was a consistent increase in *Fshr* mRNA in both groups of experimental rats when compared to control rats (Fig.12)

Discussion

Our data on testicular weight, volume and the numerical density of LCs and SCs are consistent with earlier reports on adult rats with transient neonatal exposure to antithyroid drugs such as propylthiouracil or MMI (Hardy et al., 1993; also, see Cooke, 1995). Thyroid hormone inhibits proliferation of mesenchymal cell precursors and causes acceleration of their differentiation into progenitor LCs (Ariyaratne et al., 2000 a,b,c). The presence of thyroid hormone receptor α1 and α2 mRNA in purified testicular mesenchymal LCs is known (Hardy et al., 1993). Therefore, the increase in LC and SC number in rats under neonatal exposure to MMI might be the result of decreased inhibitory effect of thyroid hormones, as these animals were in hypothyroid state during the postnatal periods encompassing differentiation and proliferation of ALCs and SCs (Aruldhas et al., 2010). Cooke (1995) attributed delayed differentiation and maturation to increased number of LCs and SCs in adult rats with transient neonatal hypothyroidism. The same explanation may be extended to explain the boost in LCs and SCs observed in the present study.

Data on serum LH and FSH and their receptors ascertained hypo-gonadotrophic state in adult rats with transient neonatal exposure to MMI. This may be attributed to the hypoandrogen (decreased testosterone and DHT titres in TIF and serum DHT) status observed in adult rats, which experienced transient neonatal-onset hypothyroidism. Nevertheless, it is surprising to note the existence of a state of hyperestrogenism in these rats, as could be inferred from elevated levels of estradiol in both serum and TIF. In addition, there was under-expression of *Lhr;* hypothyroidism-induced decreased production of LH may be responsible for the subnormal expression of *Lhr* mRNA in the LCs as an indirect consequence of hypothyroidism. This subnormal LH and LHR status might have affected functional activity of LC due to the delayed differentiation. Importantly, the present study reveals that suppression of thyroid function during the neonatal window of thyroid hormone action on the testis has an imprinting adverse effect on LCs at adulthood through inhibition of *Lhr* expression. Probably, the low level of serum LH, LHR and *Lhr* expression might have affected functional differentiation of LCs. This is reflected in the decreased activity of 3β-HSD and aromatase in LCs of the experimental rats. Growth and maturation of testis are predominantly under the control of gonadotrophins (de Kretser and Kerr, 1994; Setchell et al., 1994; Zirkin, 1995a, b), GH, and PRL (Bartlett et al., 1990). Therefore, hypogonadotrophism, accompanied by subnormal levels of GH and PRL (data not shown) due to transient hypothyroidism during lactational period could have contributed for the decreased level of TIF volume observed in these animals.

LCs secrete their product (testosterone/estradiol) in two directions, *i.e.,* towards peripheral circulation and TIF (Huhtaniemi, 1999). Peripheral circulation of testosterone may not reflect the true nature of the secretory status of LCs, as changes in sex hormone binding globulin titres and peripheral metabolism of testosterone will modify the same (see Brown, 1999). Moreover, TIF concentration of sex steroids is higher than that of peripheral circulation at all ages (Walker, 2009).

Testosterone is metabolized into biologically active metabolites estradiol and DHT by aromatase and 5α-reductase, the enzymes involved in the conversion of testosterone into DHT and estradiol, respectively (see Akingbemi et al., 1999). The rate of conversion of testosterone into estradiol insert DHT may reflect the actual status of testosterone. Therefore, decreased serum and TIF DHT levels noticed in rats exposed to transient neonatal hypothyroidism indicate the subnormal androgen

status in these animals. Nevertheless, data on the expression of *5α reductase* genes do not provide a clear picture as *5α reductase* II, which is considered to be the major isoform in the testis (Cooke, 1995; see Akingbemi et al., 1999), was increased in LCs of both the experimental groups and in SCs of group III rats with a specific down fall in SCs of group II rats. Probably, the drastic reduction in *5α reductase I* gene noticed in LCs of both the groups of experimental rats might have caused the decrease in DHT level. Taken together, the data on TIF and DHT, testosterone, LC 3β-HSD and aromatase activity and expression of type II 5α reductase support the view in vogue that transient neonatal hypothyroidism decreases the steroidogenic potential of LCs in the animals at adulthood (Cooke, 1995). Nevertheless, the increased concentration of estradiol observed in TIF and serum of the experimental rats of the present study adds a new dimension to the above view of Cooke (1995). Increased estradiol might have been contributed by augmented activity of SC aromatase. Though transient neonatal-onset hypothyroidism decreased LC aromatase activity, it enhanced aromatase activity in SCs. It is known that there is a shift in aromatase activity from LC to SC with maturation of the testis (Cooke, 1995). Since there is a delay in the maturation of SCs of rats which experience transient neonatal hypothyroidism (Cooke, 1995; Jannini et al., 1999), it is possible that aromatase activity continues in SCs, though there is subnormal activity in LCs, as observed in the present study. Therefore, the increased concentration of estradiol observed in TIF of the experimental rats of the present study might have been contributed by augmented activity of SC aromatase.

SCs of 90 day old rats subjected to transient neonatal hypothyroidism have augmented action of FSH, with a boost in FSHR number as reported here, with enhanced affinity (data not shown). It is well known that estradiol is an inhibitor of LH and FSH synthesis (Burger, 2001). FSH being the major stimulant of SC aromatase, its increased activity on SCs might have contributed to the specific increase in aromatase activity and thus, estradiol. Thus, the present study reveals that transient neonatal hypothyroidism leads to a shift in testicular steroidogenesis towards estradiol due to enhanced action of aromatase in SCs. This is achieved by increased expression of FSHR in SCs of these rats. In our study, androgen receptor concentration in LCs increased whereas estrogen receptor decreased in rats exposed to neonatal hypothyroidism. This might have also contributed to the decreased level of aromatase activity in LCs, while enhancing the same in SCs.

3β-HSD and 17β-HSD are two key enzymes involved in steroidogenesis beyond cholesterol. 3β-HSD catalyses the conversion of pregnenolone into progesterone (see Dufau, 1988) and 17β-HSD catalyses the conversion of androstenedione into testosterone (see Anderson, 1995). The present study reveals that transient neonatal hypothyroidism impairs 3β-HSD activity, whereas it enhances 17β-HSD activity in LCs of adult rats. The trend of 3β-HSD activity in LCs of experimental rats parallels the concentration of TIF testosterone. The 3β-HSD activity is stimulated by LH and androgens by binding to LHR and AR, respectively (Dufau, 1988), whereas it is negatively regulated by estradiol (Freeman, 1985; Dufau, 1988; Abney, 1999). In this study, TIF testosterone level was low, indicating subnormal stimulation of 3β-HSD enzyme activity which, along with subnormal stimulation by LH due to its bioavailability-coupled diminished concentration of LHR observed in experimental groups, may be attributed to decreased 3β-HSD activity.

In addition, increased availability of estradiol within the testis might have also contributed to the decrease in 3β-HSD activity. In the light of the findings of Hardy et al. (1993), and our data on LC 3β-HSD and TIF testosterone, it can be suggested that the steroidogenic potential of LC is decreased in adult rats subjected to transient neonatal hypothyroidism. Nevertheless, increased activity of 17β-HSD activity noticed in experimental rats of the present study points out the possibility of enhanced testosterone production as this steroidogenic enzyme is involved in the conversion of 17 ketosteroids (e.g., estrone, androstenedione and 5α-androstenedione) to their more potent 17β-hydroxysteroid forms (estradiol, testosterone and dihydrotestosterone) and *vice versa* (Nokelainen et al., 1998; O'Shaughnessy et al., 2000). Ando et al. (1990) have shown a shift from Δ^4 to Δ^5 steroidogenic pathway in mature hypothyroid rats, leading to decreased testosterone production under *in vitro* conditions. 17β-HSD is not only up-regulated by its product estradiol,

but also by PRL (Duan et al., 1997). Since 17β-HSD activity is enhanced, one may expect enhanced conversion of available androstenedione into testosterone. However, the data on TIF testosterone does not favor this idea; DHT, one of the important androgenic metabolite of testosterone, also decreased in these animals whereas estradiol, another metabolite of testosterone, increased. This suggests that the rate of conversion of testosterone into estradiol is higher than the rate of production of testosterone or its conversion into DHT in the testis of adult rats subjected to transient neonatal hypothyroidism. Probably, the overall production of testosterone was normal but more of it was converted into estradiol, resulting in subnormal levels of androgens and high concentration of estradiol in the TIF.

In general, the data on sex steroids, mainly TIF testosterone and DHT levels, in animals belonging to groups II and III suggest that neonatal-onset hypothyroidism suppresses androgen level at adulthood. This may be attributed to either decreased production as suggested by Hardy et al. (1993) and Mendis-Handagama and Sharma, (1994) or increased conversion of testosterone into estradiol as indicated in the present study. A careful look at the data on serum and TIF estradiol status, cell-specific aromatase activities in the present study clearly favor the proposed hypothesis suggesting that there is a shift in the end product of steroidogenesis from androgens to estrogens in the testis of adult rats subjected to transient neonatal hypothyroidism.

Taken together, transient neonatal hypothyroidism modifies pituitary-testicular axis at the levels of both the pituitary and testis. Boost in SC and LC number in adult rat testis is associated with a temporal shift in testicular steroidogenesis towards estradiol formation in adult rat testis. Transient neonatal-onset hypothyroidism might delay the differentiation process of LC due to low level of LH during pre-puberal period. It has cell-specific differential effect on aromatase expression and activity in LCs and SCs, due to changes in androgen and estrogen status within the testicular environment, and specific changes in LH and FSH action. Enhanced rate of estradiol formation rather than testosterone due to augmented activity of aromatase, the terminal steroidogenic enzyme, with normal or marginally elevated activity of 17β-HSD may be responsible for increased estradiol with decreased testosterone titre. The data from above studies support the hypothesis proposed and we conclude that transient neonatal-onset hypothyroidism leads to a temporal shift in adult rat testicular steroidogenesis towards estradiol formation due to a specific increase in SC aromatase, as a result of modified actions of regulators of steroidogenesis.

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