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# Thyroid hormone and anti-Mullerian hormone (AMH) on Leydig cell differentiation: studies using C57BL/6 mice and AMH over expressing mice

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**Summary** Although the thyroid hormone has stimulatory effects and anti-Mullerian hormone (AMH) has inhibitory effects on prepubertal Leydig cell (LC) differentiation, it is important to find out whether the stimulatory effect of thyroid hormone could overcome the inhibitory effect of AMH on postnatal LC differentiation. Therefore, the objective of the present study was to use the anti-Mullerian hormone overexpressing mouse ($AMH^{++}$) model to understand the simultaneous effects of AMH and thyroid hormone on postnatal LC differentiation, proliferation, maturation and function and to test whether the inhibitory effect of AMH could be overcome by the stimulatory effect of the thyroid hormone. Four age groups (7, 21, 40, 90 days) of control (C57BL/6; C) and $AMH^{++}$ were used. Mice received either saline or triiodothyronine ($T_3$) SC injections daily from birth to 21days. The four experimental groups were C, C+$T_3$, $AMH^{++}$ and AMH+$T_3$. Body and testis weights of both C+$T_3$ and AMH+$T_3$ mice were significantly reduced at days 21, 40 and 90, compared to their age-matched saline-treated mice (C and $AMH^{++}$). BrdU studies revealed the absence of LC proliferation in $AMH^{++}$ mice at day7, however, same-aged mice of C+$T_3$ and AMH+$T_3$ mice showed increased LC proliferation; the rate was highest in C+$T_3$ at day21. C+$T_3$ mice of day 21 had more LC than C mice as well as AMH+$T_3$ and $AMH^{++}$ mice. At days 40 and 90, LC number/testis in C+$T_3$ was lower than C, however, AMH+$T_3$ had higher LC numbers than $AMH^{++}$ mice. Cellular apoptosis was not seen as the cause of reduced LC numbers. Serum testosterone was not different among groups at day 21, but significantly higher levels were seen in AMH+$T_3$ compared to $AMH^{++}$ mice at days 40 and 90. Similar pattern was seen for luteinizing hormone (LH)-stimulated testicular testosterone and androstenedione production *in vitro*. Findings suggest that $T_3$-treatment for the first postnatal 21 days was able to partially counteract the inhibitory effect of AMH on prepubertal LC differentiation. Whether continuation of the $T_3$-treatment beyond 21 days would have resulted in complete removal of this inhibition, is a question that needs to be addressed.

**Key words:** AMH, Thyroid hormone, Leydig cells, Testosterone, Testis

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## Introduction

Androgens are required for the reproductive functions as well as to maintain the general health of the adult male; Leydig cells (LC) in the testis are their primary source. It is established that there are two populations of LC in mammals studied to date, namely the fetal and the adult populations (see review Mendis-Handagama and Ariyaratne, 2001). The adult population of LC differentiate postnatally from mesenchymal stem cells, located specifically, from those mesenchymal cells immediately outside the seminiferous tubules: they are referred to as the peritubular mesenchymal cells.

The fetal population of LC is present in the testis at birth (Lording and de Kretser, 1972; Mendis-Handagama et al., 1987; Kerr and Knell, 1988; Baker et al., 1999; Ariyaratne and Mendis-Handagama, 2000; O'Shaughnessy et al., 2002, 2003 ) and continue to be present in the postnatal testis in rodents studied to date (Kerr and Knell, 1988; Baker et al., 1999; Ariyaratne and Mendis-Handagama, 2000; O'Shaughnessy et al., 2002, 2003). Prepubertally differentiated adult

population of LC become the primary source of androgens in the adult male.

Differentiation of peritubular mesenchymal cells into adult LC is reported to be regulated by many factors including luteinizing hormone (LH), follicular stimulating hormone (FSH), androgens, estrogen, thyroid hormones and anti-Mullerian hormone (AMH; Mendis-Handagama and Ariyaratne, 2001; Haider, 2004). In the LC lineage in the postnatal testis, between the mesenchymal stem cell and the mature adult LC, there are three other cell types; namely, the progenitor cell, the newly formed adult LC and the immature adult LC (Mendis-Handagama and Ariyaratne, 2001). To begin the process of postnatal LC differentiation, a mesenchymal stem cell in the testis interstitium needs to be stimulated to differentiate into a progenitor cell. We (Mendis-Handagama et al., 1998; Ariyaratne et al., 2000a-d) and others (Teerds et al., 1998; Wagner et al., 2008) have shown that thyroid hormone is the triggering hormone to initiate the mesenchymal stem cell differentiation to progenitor cell to begin the process of postnatal LC differentiation. Moreover, we have also shown that luteinizing hormone (LH) is not required for this step in the process, which is the initiation of mesenchymal stem cells to differentiate into the progenitor cells (Ariyaratne et al., 2000a,b,d; Mendis-Handagama and Ariyaratne, 2001), although it is required later in the process for maturation of differentiated cells in the LC lineage. The fact that LH is not required for the initial step in the process of LC differentiation is further supported by the studies on luteinizing hormone receptor knock out mice (LURKO mice; Huhtaniemi et al., 2006) where LC differentiation occur postnatally, although their maturation is compromised in the absence of LH.

Importance of thyroid hormones for initiation of postnatal LC differentiation process came from many studies (Mendis-Handagama and Ariyaratne, 2004). Neonatal-prepubertal hypothyroidism inhibited the differentiation of the adult population of LC (Mendis-Handagama et al., 1998; Teerds et al., 1998; Ariyaratne et al., 2000b). In contrast, neonatal-prepubertal hyperthyroidism induced by administration of thyroid hormone stimulates the adult LC differentiation (Teerds et al., 1998; Ariyaratne et al., 2000b,c).

Anti-Mullerian hormone (AMH) is a member of the TGF-ß family of growth factors that expressed only in the gonads (Massague and Wotton, 2000). In the testis, AMH is produced by the Sertoli cells, and it exerts its effect on target cells through its specific receptor named as AMHR- II. The well known function of AMH during fetal life is the inhibition of the development of the Mullerian duct into the female reproductive duct system in the male fetus (Jost, 1953; Arango et al., 1999). In the postnatal testis AMH has a negative regulatory role on postnatal LC differentiation (Racine et al., 1998; Mendis-Handagama et al., 2010) and inhibition of androgen secretion from these cells (Rouiller-Fabre et al., 1998; Lee et al., 1999; Sriraman et al., 2001; Salva et al., 2004). Moreover, it is seen that transgenic mice who chronically overexpress human AMH under the control of mouse metallothionein-1 promoter ($AMH^{++}$ mice) show reduced LC number in their testes and inhibition of testicular testosterone production (Racine et al., 1998; Mendis-Handagama et al., 2010). By contrast, mice null for AMH gene (Behringer et al., 1994; Mishina et al., 1996) demonstrated LC hyperplasia and neoplasia.

Hypothetically, changes in the numbers of LC differentiating in the postnatal testis interstitium could be brought about by several mechanisms; availability of the number of mesenchymal precursor cells for their differentiation, the rate of differentiation of mesenchymal cells into LC, rate of proliferation (mitotic activity) of existing LC and apoptotic removal of LC present in the testis interstitium. Therefore, it is possible that one or more of these mechanisms are involved in producing higher numbers of LC in the adult testis.

With the previous findings on the stimulatory and inhibitory effects of thyroid hormone and AMH respectively, on postnatal LC differentiation, it is important to find out whether the stimulatory effect of thyroid hormone could overcome the inhibitory effect of AMH on postnatal LC differentiation. This is the rationale for the present study. The objective of the present study was to use the $AMH^{++}$ mouse model to understand the simultaneous effects of AMH and thyroid hormone on postnatal LC differentiation, proliferation, maturation and function and to test whether the inhibitory effect of AMH could be overcome by the stimulatory effect of the thyroid hormone.

## Material and methods

### *Animals*

Transgenic male mice over expressing AMH ($AMH^{++}$ mice) were gifted by Dr. R.R. Behringer (M.D. Anderson Cancer Center, TX). These transgenic mice were produced using C57BL\6 mice (Brinster et al., 1985; Bheringer et al., 1990).

Normal adult female C57BL\6 mice were purchased from Harlan Industries (Madison, WI). One male and a female animal were paired and housed in cages in the animal facility of The University of Tennessee College of Veterinary Medicine, with controlled temperature and lighting (14L:10D). Animals had access to mouse chow (Agway Prolab, Syracuse, NY) and water *ad libitum*.

### *Experimental design*

The experimental protocol #1519 of the current study was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Tennessee (Knoxville, TN). Each breeder cage was observed twice a day (morning and afternoon) for litters and the day of birth of pups was considered as the 1$^{st}$ day of their age. Only the male pups were used in the study. Litters were randomly selected to receive either daily subcutaneous

injections of triiodothyronine ($T_3$; Sigma, St. Louis, MO) at a dosage of 50 $\mu$g/kg body weight in 50 $\mu$l saline or 50 $\mu$l of saline (Teerds et al., 1998; Ariyaratne et al., 2000b,c) from birth to 21 days. Pups were weaned at day 21, and were sacrificed at days 21, 40 and 90. At weaning, tail clips were collected from each animal and genotyping was performed as described (Mendis-Handagama et al., 2010). This experimental design resulted in four experimental groups of mice at each age, i.e. saline treated control group (Group C), thyroid hormone treated control group (Group C+$T_3$), saline treated AMH$^{++}$ group (Group AMH$^{++}$) and thyroid hormone treated AMH over expressing group (Group AMH+$T_3$). Mice in all four treatment groups were used for stereological determinations and LH-stimulated testicular androgen secretory capacity in vitro as described below (n=6 per group).

In addition to mice in the above four experimental groups, additional litters were generated similarly and sacrificed at postnatal days 7, 14, 21, 40 and 90. Two hours before sacrifice of these mice in each age group, each mouse was given an intra-peritoneal injection of deoxybromouridine (BrdU, Sigma, St. Louis, MO) at a dose of 150 mg/kg body weight, dissolved in saline. Testes were collected from these euthanized mice and fixed in Bouin's fixative overnight, decolorized by several changes in 70% ethyl alcohol and embedded in paraplast (Fisher Sientific, Fair Lawn, NJ). These testes were used to detect the proliferating cells using an anti-BrdU antibody (BioGenex, San Ramon, CA). Cells undergoing apoptosis was detected using terminal deoxynucleotide transferase dUTP nick end labeling (TUNEL) method (Millipore Corporation, Billerica, MA) as described below (n=6 per group).

### *Fixation and processing of testicular tissue for stereology*

For determination of LC and mesenchymal cell numbers, glutaraldehyde -fixed epon-araldyte embedded tissue was used as described in detail previously (Mendis-Handagama et al., 1988; Ariyaratne and Mendis-Handagama, 2000). Briefly, animals were euthanized via inhalation of isoflurane (Abbott Laboratories, Deerpark, IL ) and one testis was removed, dissected free from the epididymis, weighed (fresh weight of the testis) and specific gravity was determined by flotation method. This testis was used for the determination of LH-stimulated testicular androgen secretory capacity *in vitro* as described below. A sample of blood was also collected at this point by cardiocentesis. The intact testis in each mouse was fixed *in situ* by whole body perfusion technique (Mendis-Handagama et al., 1988; Ariyaratne and Mendis-Handagama, 2000) using 2.5% glutaraldehyde in cacodylate buffer (pH 7.4). Fixed testis was removed, weighed (fixed testis weight) and specific gravity was determined. Then the testis was cut in to approximately 2 mm x 3mm blocks, postfixed in a mixture of osmium tetroxide and potassium ferrocyanide, dehydrated in a series of graded ethanols and embedded in epon-araldite (Mendis-Handagama et al., 1988). Separate tissue blocks were processed similarly to determine the shrinkage factor as described previously (Mendis-Handagama and Ewing, 1990), which is required to determine the final cell counts.

### *Stereology*

From epon-araldite embedded tissue blocks, 1 $\mu$m thick serial sections were cut using a LKB ultra microtome V (Pharmacia LKB, Piscataway, NJ) and glass knives. From the serial sections, two section which were four sections apart i.e. the 1$^{st}$ and the 5$^{th}$ section, were collected and mounted on a glass slide close to each other. Then, the sections were stained with a mixture of methylene blue and azure and coverslipped under permount (Fisher Scientific, Fair Lawn, NJ ). LC were not differentially counted as fetal LC and adult LC. Therefore, the term 'LC' in the rest of the text was the total of both fetal and adult types.

LC and mesenchymal cells in the testicular interstitum were identified by their characteristic morphology, as described previously (Ariyaratne and Mendis-Handagama, 2000). Both populations of LC were polyhedral cells with a pale staining cytoplasm and a relatively large nucleus, which had a distinct peripheral rim of heterochromatin. Mesesnchymal cells were fusiform cells with elongated nucleus with sparse cytoplasm. They were mainly located at the periphery of the seminiferous tubules (i.e. peritubularly), however, few were seen scattered in the testis interstitium. Myoid cells are also fusiform cells, however, they are differentially identified from the peritubular mesenchymal cells based on their intimate attachment to the periphery of the seminiferous tubules and their extreme slender appearance compared to the peritubular mesenchymal cells; myoid cells were not counted in the present study.

Volume density (volume of the cell type in a unit volume of the testis) of LC and mesenchymal cells was determined by the point counting method (Weibel, 1979) as previously described (Mendis-Handagama et al., 1988). The test system contained, a square lattice grid with 121 test points which was fitted to the eye piece of the light microscope (Olympus BH-2, Olympus, Tokyo, Japan).

The numerical density (number per unit volume of the organ) of LC and mesenchymal cells was determined using the disecter principle (Sterio, 1984) which was adapted to count cells in the testis (Mendis-Handagama and Ewing, 1990; Ariyaratne and Mendis-Handagama, 2000). The total number of LC and mesenchymal cells per testis was calculated by multiplying the numerical density of the cell type by the testis volume (Mendis-Handagama and Ewing, 1990). The average volume of an individual LC was determined by dividing the volume density of LC by its numerical density (Mendis-Handagama et al., 1988).

### *Detecting proliferating LC and mesenchymal cells*

Leydig and mesenchymal cells which were undergoing mitosis were visualized by a double immunostaining method for BrdU and 3ß-hydroxy-steroid dehydrogenase (3ß-HSD) , respectively, using specific antibodies. Anti-BrdU used was a mouse monoclonal antibody purchased from BioGenex (San Ramon, CA) and anti-3ß-HSD was a rabbit polyclonal antibody purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Control tissue slides were incubated without the antibodies. From the Bouin's fixed and paraffin embedded tissue blocks, 5 $\mu$m thick sections were cut and adhered onto Superfrost Plus (Fisher Scientific, Fair Lawn, NJ) glass slides. These sections were deparaffinized in xylene, rehydrated in a series of graded ethyl alcohols and brought to distilled water. Slides were then incubated in 2N HCL for one hour at 37°C in order to unmask DNA epitopes. After the incubation, the slides were washed twice in 0.1 M sodium borate buffer (pH8) for five minutes each. Slides were then incubated overnight with the anti-BrdU primary antibody at 4°C in a humidified chamber. The bound antibody was detected by using a supersensitive detection system purchased from BioGenex (San Ramon, CA) and following the manufacturer's instructions. The chromogen used in this detection system was fast red. Then the slides were washed twice in phosphate buffer saline (PBS pH 7.6), five minutes each, incubated in 3% $H_2O_2$ for 10 minutes, incubated with normal donkey serum for 10 minutes and incubated in 3ß-HSD primary antibody for two hours in a humidified chamber at room temperature according to a protocol and super sensitive detection system provided by Santa Cruz (Santa Cruz, CA). In this detection system bound antibodies were visualized by peroxidase labeled streptavidin and using diaminobenzidine tetrahydro-chloride (DAB) as the chromogen. This step was followed by counterstaining the sections with Mayer's hematoxylin and coverslipping under a water based mounting medium (Aquamount, Fisher Scientific, Fair Lawn, NJ). The control incubations were carried out simultaneously in the absence of the primary antibody.

The percentage of BrdU positive LC (nucleus stained red and cytoplasm stained brown because of 3ß-HSD ) and mesenchymal cells (nucleus stained red) were counted by systematic observation of the entire tissue section under Olympus BH-2 light microscope using a square lattice grid inserted into the eyepiece of the microscope. Four to five sections per animal were scored.

### *Detection of cells undergoing apoptosis*

Detection of testicular cells undergoing apoptosis was performed with the TUNEL method using an assay kit (Millipore Corporation, Billerica, MA). Briefly, tissue sections from Bouin's fixed and paraffin embedded tissues were deparaffinized, rehydrated and brought to distilled water as described above. Then tissue sections were pretreated with pepsin (25 mg/100 ml distilled water) for 30 minute at room temperature followed by washing with phosphate buffer (pH 7.4). Next, the sections were incubated with 3% $H_2O_2$ in order to quench endogenous peroxidase activity. Sections were then incubated with equilibrium buffer, TdT reaction buffer and stop buffer sequentially according to manufacturer's instructions. In this detection kit, peroxidase labeled anti-digoxigenin was the conjugate and DAB was the chromogen. Color development of the apoptotic cells was monitored under a microscope and was stopped when appropriate by washing the slides with distilled water. The control sections were processed in a similar manner except the TdT reaction buffer step was replaced with incubation in equilibrium buffer. Sections were counterstained with Mayer's hematoxylin, dehydrated in ethanol and mounted under Permount (Fisher Scientific, Fair Lawn, NJ).

### *Determination of LH-stimulated testicular testosterone secretory capacity in vitro*

Each testis removed from each mouse as described above, was used for testicular incubations containing a maximum stimulatory dose of LH (ovine LH 100 ng/ml; NIDDK Hormone Distribution Program, Torrance, CA) and 0.0004 g/ml glucose as described previously (Mendis-Handagama et al., 1990a, 1998). Briefly, each testis was weighed, decapsulated and the testicular parenchyma was teased and incubated in 2ml of Kreb-Ringer Bicarbonate buffer (PH 7.4) which was gassed for 10 minutes prior to adding glucose and LH. Incubations were carried out at 34°C in an oscillating water bath (90 oscillations per minute). After three hours, the incubation medium was collected and centrifuged at 3000g for 10 minutes and the supernatant was separated and stored at -80°C until assayed for testosterone and androstenedione.

### *Radioimmunoassay (RIA) for Testosterone and Androstenedione*

Testosterone and androstenedione concentrations in the plasma and in vitro incubation media were quantified using commercially available RIA kits (Coat-A-Count; DPC, Los Angeles, CA). The sensitivity of these assays was 0.14 nM for both hormones. The intra-assay coefficients of variation for both assays were less than 8%. The cross reactivity of the antibody used in the testosterone RIA kit was 2.8% for dehydrotestosterone, 0.5% for androstenedione, and less than 0.02% for other steroids. The cross reactivity of antibody used in the androstenedione RIA kit was 1.5% for testosterone, 0.21% for dehydrotestosterone, and 0.14% for dehydroepiandrosterone.

### *Statistical analysis*

The mean values of various parameters from different age groups were compared using General Linear Models of the Statistical Analysis Systems (SAS) program (SAS Corporation, 2003). When a significant difference was observed among groups, Duncan's New Multiple-Range Test was employed to separate the means. P values of 0.05 or lower were considered significant.

## Results

### *Body weight and testis weight*

Table 1 shows body weights and testis weights in mice of different treatments (columns) and ages (rows) of this study. Body weight increased with the advancement of age up to 90 days in all experimental groups, i.e. control, $AMH^{++}$, control+$T_3$ and AMH+$T_3$. However, body weights of control mice were not significantly different from their age-matching $AMH^{++}$ mice at any age tested. Also, body weights of control+$T_3$ and AMH+ $T_3$ mice were not significantly different. However, body weights of control+$T_3$ and AMH+ $T_3$ mice were significantly lower than their age matching control and $AMH^{++}$ mice.

Testis weight increased with the advancement of age up to day 40; there was no significant difference between day 40 and day 90 in all experimental groups. However, testis weights of $AMH^{++}$ mice were significantly reduced when compared to their age matched control mice at all ages studied. Comparison of testis weights between control+$T_3$ and their age-matching AMH+ $T_3$ showed that there was no significant difference in testis weights between these groups at any age studied. However, testis weights of control+$T_3$ and AMH+ $T_3$ mice was almost half the size that of control (C) and $AMH^{++}$ animals (Table 1), respectively.

### *Stereology*

#### Cell identification

LC and mesenchymal cells in the testis interstitium were easily identified using by morphology described by Ariyaratne and Mendis-Handagama 2000, and also described briefly under "stereology" in the Materials and Methods of this paper.

#### Leydig cells

The number of LC per testis, average volume of a LC and volume density of LC in different experimental groups at days 21, 40 and 90 are shown in Table 2. In all tested experimental groups, i.e. control, $AMH^{++}$, control+$T_3$ and AMH+$T_3$ mice, the maximum number of LC per testis was seen at day 40. However, LC number per testis in 90 day old mice in the all experimental groups was significantly higher than those at day 21.The number of LC per testis in control mice was significantly greater than their age-matching $AMH^{++}$ at all tested ages. Similar observations were seen between control+$T_3$ and AMH+ $T_3$ mice, where control+$T_3$ mice had a greater LC number per testis compared to their age matching AMH+ $T_3$ mice. At day 21, control+$T_3$ group had more LC than those in the control group. Similarly, AMH+$T_3$ group contained more LC than $AMH^{++}$ group. However, at days 40 and 90, the LC number per testis in control+$T_3$ group was lower than those in the control group, but AMH+$T_3$ group had higher numbers than $AMH^{++}$ group.

#### Average volume of a Leydig cell (size)

In control, control+$T_3$, and $AMH^{++}$ mice, the

**Table 1.** Body weights (g) and testis weights (mg).

| | Body weights (g) | | | |
|---|---|---|---|---|
| | Day 7 | Day 21 | Day 40 | Day 90 |
| Control | $5.52\pm0.3^{aA}$ | $10.23\pm0.7^{aB}$ | $22.9\pm1.9^{aC}$ | $31.8\pm1.9^{aD}$ |
| AMH | $5.62\pm0.5^{aA}$ | $10.62\pm1.7^{aB}$ | $24.17\pm1.2^{aC}$ | $29.7\pm2.4^{aD}$ |
| Control+T3 | | $8.9\pm0.4^{bA}$ | $18.85\pm1.5^{bB}$ | $23.1\pm1.9^{bC}$ |
| AMH+T3 | | $8.45\pm0.96^{bA}$ | $20.55\pm2.0^{bB}$ | $23.0\pm1.7^{bB}$ |
| | Testis weights (mg) | | | |
| | Day 7 | Day 21 | Day 40 | Day 90 |
| Control | $4.41\pm0.4^{aA}$ | $27.55\pm0.9^{aB}$ | $83.62\pm6.2^{aC}$ | $91.13\pm6.7^{aC}$ |
| AMH | $3.84\pm0.4^{bA}$ | $25.24\pm1.0^{bB}$ | $72.60\pm9.1^{bC}$ | $78.33\pm4.9^{bC}$ |
| Control+T3 | | $15.51\pm2.6^{cA}$ | $46.94\pm7.8^{cB}$ | $48.56\pm7.7^{cB}$ |
| AMH+T3 | | $14.57\pm3.9^{cA}$ | $44.70\pm5.5^{cB}$ | $48.26\pm5.7^{cB}$ |

Mean+SE. n=6, Lower-case superscripts in each column for each parameter show significant differences ($P<0.05$) among the four experimental groups at each age group. Upper-case superscripts of each row show significant differences ($P<0.05$) among the four age groups of each experimental group.

**Table 2.** Numbers of Leydig and mesenchymal cells per testis ($10^6$) and average volume of a Leydig cell ($\mu m^3$).

| | Leydig Cell Number Per Testis ($10^6$) | | | |
|---|---|---|---|---|
| | Day 7 | Day 21 | Day 40 | Day 90 |
| Control | $0.037\pm0.004^{aA}$ | $0.81\pm0.03^{aB}$ | $1.90\pm0.09^{aC}$ | $1.25\pm0.08^{aC}$ |
| AMH | $0.029\pm0.003^{bA}$ | $0.63\pm0.03^{bB}$ | $1.04\pm0.06^{Cb}$ | $0.68\pm0.08^{bB}$ |
| Control+T3 | | $0.92\pm0.02^{cA}$ | $1.43\pm0.09^{cB}$ | $1.03\pm0.11^{cA}$ |
| AMH+T3 | | $0.70\pm0.02^{dA}$ | $1.33\pm0.06^{dB}$ | $0.77\pm0.09^{dA}$ |
| | Average volume of a Leydig Cell ($\mu m^3$) | | | |
| | Day 7 | Day 21 | Day 40 | Day 90 |
| Control | $1758\pm102^{aA}$ | $1448\pm98^{aB}$ | $2431\pm112^{aC}$ | $3480\pm115^{aD}$ |
| AMH | $1648\pm130^{aA}$ | $1349\pm142^{abB}$ | $2048\pm133^{bC}$ | $2926\pm222^{bC}$ |
| Control+T3 | | $1277\pm135^{cA}$ | $2489\pm151^{aB}$ | $3291\pm137^{bC}$ |
| AMH+T3 | | $1290\pm80^{cA}$ | $1466\pm127^{cA}$ | $1288\pm126^{cA}$ |
| | Mesenchymal Cell Number Per Testis ($10^6$) | | | |
| | Day 7 | Day 21 | Day 40 | Day 90 |
| Control | $0.45\pm0.01^{aA}$ | $0.27\pm0.02^{aB}$ | $0.40\pm0.01^{aA}$ | $0.33\pm0.02^{aAB}$ |
| AMH | $0.39\pm0.02^{bA}$ | $0.40\pm0.002^{bA}$ | $0.51\pm0.04^{bB}$ | $0.49\pm0.05^{bB}$ |
| Control+T3 | | $0.14\pm0.005^{cA}$ | $0.30\pm0.01^{cB}$ | $0.21\pm0.02^{cC}$ |
| AMH+T3 | | $0.18\pm0.002^{dA}$ | $0.27\pm0.06^{dB}$ | $0.16\pm0.01^{dA}$ |

Mean+SE. n=6, Lower-case superscripts in each column for each parameter show significant differences (P<0.05) among the four experimental groups at each age group. Upper-case superscripts of each row show significant differences (P<0.05) among the four age groups of each experimental group.

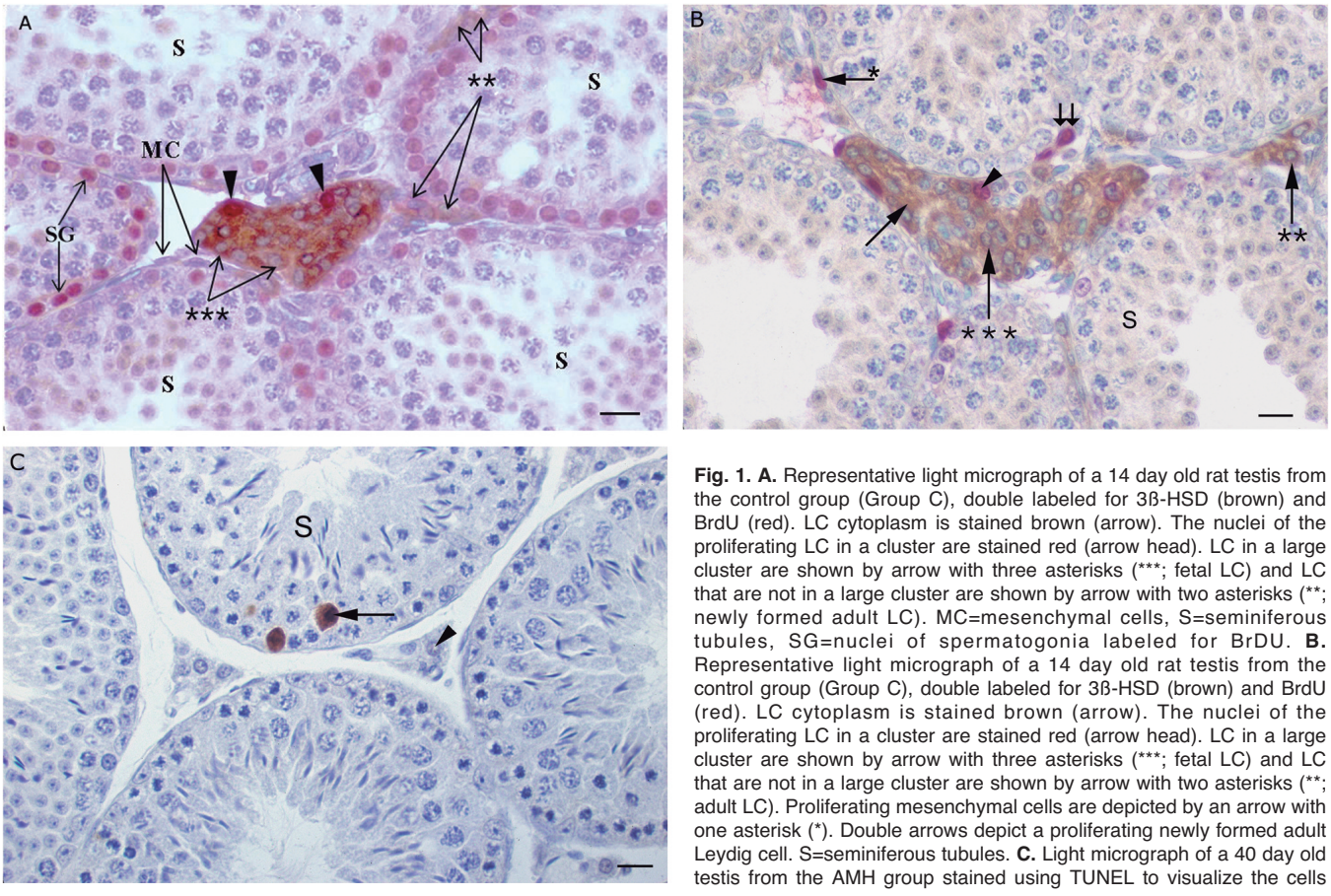


**Fig. 1. A.** Representative light micrograph of a 14 day old rat testis from the control group (Group C), double labeled for 3ß-HSD (brown) and BrdU (red). LC cytoplasm is stained brown (arrow). The nuclei of the proliferating LC in a cluster are stained red (arrow head). LC in a large cluster are shown by arrow with three asterisks (***; fetal LC) and LC that are not in a large cluster are shown by arrow with two asterisks (**; newly formed adult LC). MC=mesenchymal cells, S=seminiferous tubules, SG=nuclei of spermatogonia labeled for BrDU. **B.** Representative light micrograph of a 14 day old rat testis from the control group (Group C), double labeled for 3ß-HSD (brown) and BrdU (red). LC cytoplasm is stained brown (arrow). The nuclei of the proliferating LC in a cluster are stained red (arrow head). LC in a large cluster are shown by arrow with three asterisks (***; fetal LC) and LC that are not in a large cluster are shown by arrow with two asterisks (**; adult LC). Proliferating mesenchymal cells are depicted by an arrow with one asterisk (*). Double arrows depict a proliferating newly formed adult Leydig cell. S=seminiferous tubules. **C.** Light micrograph of a 40 day old testis from the AMH group stained using TUNEL to visualize the cells undergoing apoptosis. Germ cells with apoptotic changes in the seminiferous tubules (S) are stained brown color (arrow). LC in the interstitium (arrow head) are not stained. Bars: 20$\mu$m.

average volume of a LC showed a significant increase with age advancement from day 21 to day 90. By contrast, there was no change in the average volume of a LC in AMH+$T_3$ mice with age advancement from day 21 through 90.

Mesenchymal cell numbers

In all experimental groups, mesenchymal cell number per testis increased with age advancement up to day40. Except for the $AMH^{++}$ group, which did not show a significant difference with age advancement from day 40 to 90, all other groups showed a significant reduction compared to day 40, but still was higher than those of day 21. At days 21, 40 and 90, the highest mesenchymal cell number per testis was seen in $AMH^{++}$ mice. At day 21, the lowest number of mesenchymal cells was seen in control+$T_3$ group. However, at days 40 and 90 the lowest number of mesenchymal cells was seen in AMH+$T_3$. In all ages tested, the mesenchymal cell number in control+$T_3$ mice was less than those of controls. Similarly, the mesenchymal cell number in AMH+$T_3$ mice was less than those of $AMH^{++}$ mice.

BrdU and TUNEL Assay

Labeled cells for BrdU were observed in the germ cells located on the basal region of some seminiferous tubules (Fig. 1A) and was considered as positive controls for the staining procedure to compare with the positive stained cells in the testicular interstitium. LC and peritubular mesenchymal cells showed labeling for BrDU (red nuclei in testis interstitium of Fig. 1B ). TUNEL technique demonstrated few positively stained

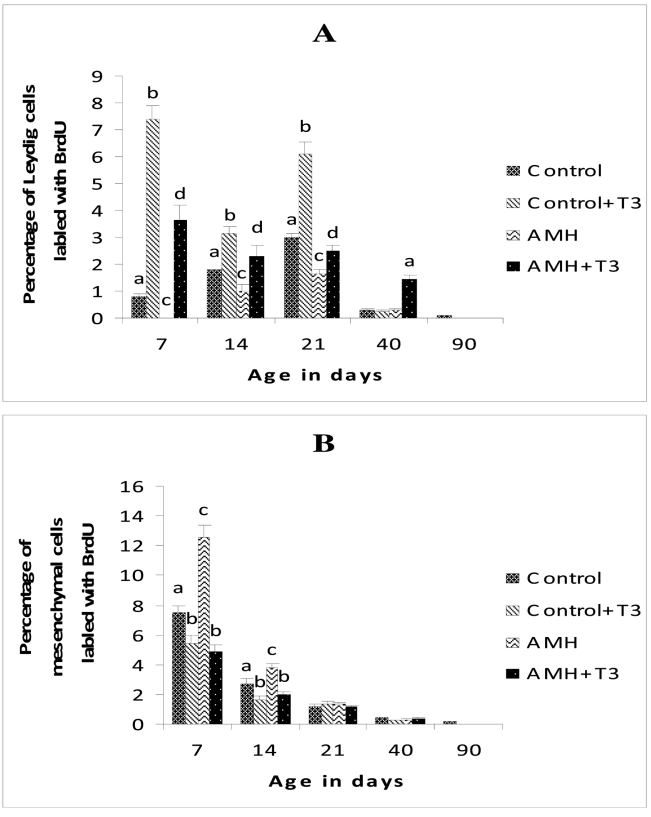


**Fig. 2.** Percentage of BrdU positive Leydig cells **(A)** and mesenchymal cells **(B)** in C, Control + T3, $AMH^{++}$ and AMH+T3 groups at different ages. The columns with different letters in each age group are significantly different (P<0.05).

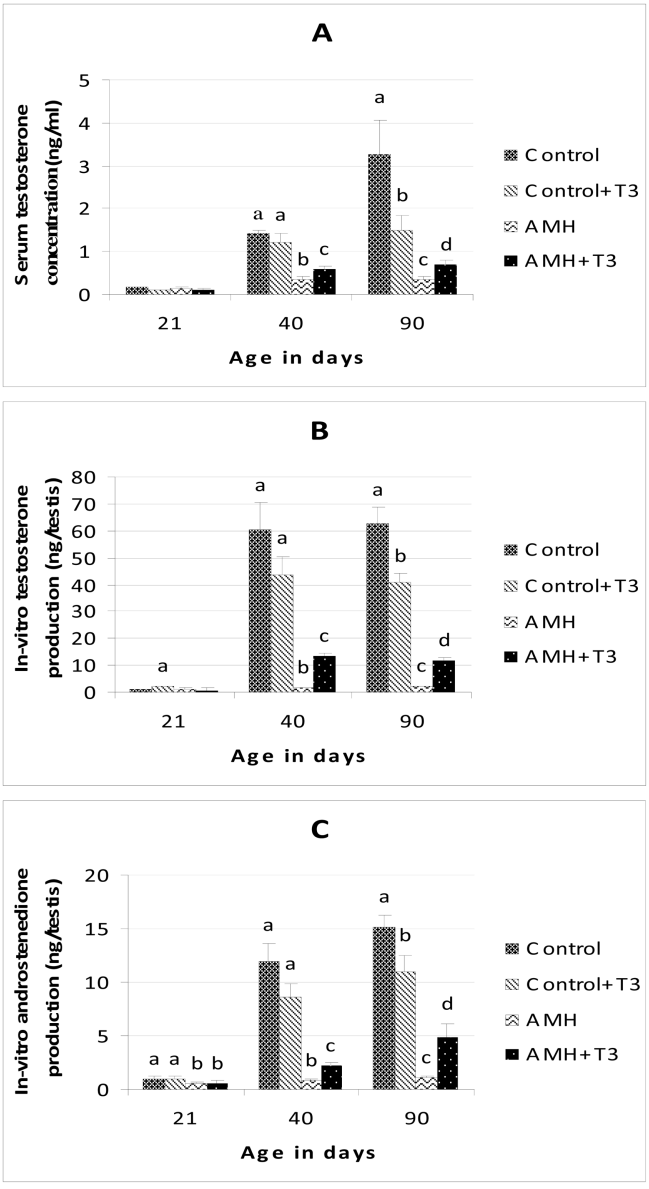


**Fig. 3. A.** Serum testosterone concentration **(B)** in vitro testicular testosterone production and **(C)** in vitro testicular androstenedione production in different experimental groups at different ages (Mean±SEM). The columns with different letters in each age group are significantly different (P<0.05).

cells in the seminiferous tubules which was considered as positive control for the procedure (Fig. 1C) and such staining was not seen in any cells in the testis interstitium of any of the experimental groups at any tested ages.

The percentages of LC positive for BrdU labeling in different treatment groups at different ages tested are shown in Fig. 2A. At 7, 14 and 21 day groups, the highest percentage of BrdU labeled LC was observed in $C+T_3$ mice, and the second highest was seen in $AMH+T_3$ mice. Control mice (C group) in these three age groups showed lower values than $C+T_3$ mice and $AMH+T_3$ mice for this parameter. In $AMH^{++}$ mice of day 7, LC labeled for BrdU were totally absent. At day 14, BrdU label was observed in mainly in the LC, which were in clusters, but few were also seen in those LC, which were not in clusters (Fig. 2A). At day 21, many of the LC were labeled. At day 40, labeled LC were lower in value compared to the younger ages. $AMH+T_3$ mice showed the highest percentage of labeling among the four experimental groups. At day 90, no labeled LC were seen.

Highest percentage of labeled mesenchymal cells was seen at day 7 (Fig. 2B) for all experimental groups, and the highest value was seen in the $AMH^{++}$ group. The labeling pattern was similar at day 14, however, the values were significantly lower than those observed at day 7. When comparing the values in $C+T_3$ and $AMH+T_3$ mice at days 7 and 14 to their age matching control mice, a decrease in percentage of BrdU labeled mesenchymal cells was observed. However, no differences were observed among the four experimental groups at day 21. Only few proliferating mesenchymal cells were seen in mice at 40 or 90 days in all experimental groups.

### *Serum testosterone levels and testicular androgens secretory capacity in vitro*

Serum testosterone levels in different experimental groups at days 21, 40 and 90 are shown in Figure 3A. At day 21, serum testosterone concentrations were not statistically different among the four experimental groups; there was high variability of the hormone levels in individual animals. At day 40, serum testosterone levels were not different between C and $C+T_3$ groups, however, a significantly higher level of was observed in $AMH+T_3$ mice than $AMH^{++}$ mice. At day 90, $C+T_3$ mice showed significantly low level of serum testosterone than control mice. In contrast, $AMH+T_3$ mice had significantly higher levels of testosterone than $AMH^{++}$ mice at this age. Similar pattern was observed for LH-stimulated testicular testosterone and androstenedione production *in vitro* (Fig. 3B,C).

## Discussion

This study revealed that the stimulatory effects of thyroid hormone could overcome the inhibitory effects of AMH on postnatal LC differentiation. No difference in body weight was observed among age matching $AMH^{++}$ and control mice at all ages studied. These findings indicated that high circulating levels of AMH do not have a significant effect on general body growth during the tested ages in these mice. However, a significant increase in body weight in five month old $AMH^{++}$ mice compared to their age matching controls was seen (Mendis-Handagama et al., 2010), which has been attributed to the deficiency of thyroid hormones in $AMH^{++}$ mice at that age. In agreement with the latter observation, the body weights of $C+T_3$ and $AMH+T_3$ mice of the present study were significantly lower than their age matching saline-treated C and $AMH^{++}$ mice at all ages tested. Also this finding compares favorably with previous studies on rats (Van Haster et al., 1993; Teerds et al, 1998; Ariyaratne et al., 2000b,c), where the same dose of $T_3$ has been used to induce hyperthyroidism. Reduced body weights in these animals could be attributed to increased tissue catabolism associated with high levels of $T_3$.

In the present study significant reductions were observed for testicular weight in both $C+T_3$ and $AMH+T_3$ compared to age matching saline-treated C and $AMH^{++}$ mice, respectively. It is not clear why $AMH^{++}$ mice of day 21 showed a significant reduction in testicular weight, however, testicular weight loss in $AMH^{++}$ mice at day 40 and 90 could be attributed at least in part to the germ cell loss in some seminiferous tubules (unpublished data), possibly resulting from reduced circulating testosterone levels in these mice as a result of low numbers of LC. This view compares favorably with our published study on five month old control and $AMH^{++}$ mice (Mendis-Handagama et al., 2010). The reduced testis weights in $C+T_3$ and $AMH+T_3$ mice compared to C and $AMH^{++}$ mice in the present study is also in consistence with previous reports which showed that neonatal-prepubertal thyroid hormone treatment of rats causes smaller testes (Van Haster et al., 1993).

It is also important to note that testis weight, LC number per testis, LH-stimulated testicular testosterone and androstenedione secretory capacity *in vitro* were observed to be highest at day 40 in the mouse; no further increase was observed at day 90. However, in the rat, which is a closely related species, day 90 values for those parameters are significantly higher (Ariyaratne and Mendis-Handagama, 2000). Therefore, it appears that mice reach puberty in days well ahead (i.e. around day 40) of the rat (i.e. around day 56). To our knowledge, this is the first study which revealed this information on testicular development and puberty in the mouse and also that such differences exist between species, even they are closely related.

The reduction in LC numbers even in control animals with the age advancement from day 40 to 90 without a corresponding reduction in the testicular androgen secretory capacity could be attributed to the LC hypertrophy at day 90 where LC volume per testis

was still maintained. This finding agrees with the concept that the LC volume per testis, and not the number is positively correlated with testicular testosterone secretory capacity (Mendis-Handagama et al., 1988).

Peritubular mesenchymal cells are the precursors for the adult type LC in the prepubertal rat testis (Ariyaratne et al., 2000a) and the adult rat testis after ethane dimethanesulphonate (EDS) treatment, which kills adult LC within 48 hours of EDS treatment (Ariyaratne et al., 2000d). The number of mesenchymal cells in the testis depends on their rates of proliferation, differentiation into LC and their removal from the interstitum by apoptosis if any. Present results show that the proliferation index (PI) of the peritubular mesenchymal cells was significantly increased and decreased in saline-treated $AMH^{++}$ mice and $AMH+T_3$ mice, respectively. These findings suggest the existence of counteracting control mechanisms exerted by $AMH^{++}$ and $T_3$ to maintain an adequate number of mesenchymal precursors at the time of prepubertal LC differentiation.

It appears that mice achieve almost the full complement of the adult LC population at day 21, where the differences in the proliferation rate in the mesenchymal cells disappear. No reports are available on proliferation of mesenchymal cells in the $AMH^{++}$ mice during the prepubertal period to compare with the present finding. However, Salva et al. (2004) observed an increase in proliferating mesenchymal cells in AMH treated animals compared to controls at day 15 after the EDS treatment. These findings agree with the present results.

To our knowledge, the present study is the first to show the inhibitory effect of thyroid hormone on testicular mesenchymal cell proliferation in mice. However, Teerds et al. (1998) did not observe an inhibitory action on mesenchymal cell proliferation in thyroid hormone treated and control rats. This difference may possibly be due to species differences since we and Teerds et al. (1998) used similar techniques to estimate proliferating mesenchymal cells. Although rat and mouse are closely related species, we have shown in the present study (i.e. testicular growth and function discussed above) and others have shown previously (Mendis-Handagama et al., 1990a,b; Kerr et al., 1979), the existence of differences in testicular responses between the rat and the mouse to the same experimental procedures; with experimentally induced cryptorchidism, LC hypertrophy, no hyperplasia and increased testicular testosterone secretion *in vitro* were observed in the rat (Kerr et al., 1979). In contrast, LC hypotrophy, hyperplasia and no difference in testicular testosterone secretion *in vitro* were observed in the mouse (Mendis-Handagama et al., 1990a,b).

Effects of AMH on its target cells are exerted via AMHR-II (Barrends et al., 1995; Josso et al., 2001; Josso and Clementi, 2003). Effects of thyroid hormones are exerted on target cells via α and ß receptors (Jannini et al., 1995). In the postnatal testis, it is shown that AMHR-II (protein) is mainly observed in LC (Mendis-Handagama et al., 2006) and they are absent in mesenchymal cells from birth to sexual maturity (Mendis-Handagama et al., 2006). Therefore, with the present data, it is not possible to predict the possible way of AMH action on mesenchymal cells. However, in the testis, paracrine interactions among different cell types through locally produced factors are well known (review Saez, 1994) . Therefore, it is possible that paracrine effects among LC, Sertoli and germ cells to regulate the mesenchymals cell acitivity. In contrast, testicular thyroid hormone receptors are found in many testicular cell types, including peritubular mesenchymal cells (Buzzard et al., 2000). Hence, the effect of thyroid hormone on LC and mesenchymal cells could be considered as a direct effect.

In consistence with our findings on reduced LC numbers in $AMH^{++}$ mice, it is reported that $AMH^{++}$ mice have 50% and 80% less LC numbers in immature and mature testis, respectively (Racine et al., 1998) and that five month old AMH mice have 75% fewer LC numbers compared to age matching controls (Mendis-Handagama et al., 2010). Furthermore, at 35 days after EDS treatment, in MIS-treatede (i.e. AMH-treated) rats, a 34% reduction of newly differentiated LC is observed (Salva et al., 2004). In contrast, disruption of AMH gene (Behringer et al., 1994) or its receptor (Mishina et al., 1996) resulted in LC hyperplasia. However, the mechanism by which these changes in LC numbers occur in these studies is not discussed in these investigations (Behringer et al., 1994; Mishina et al., 1996).

As stated earlier, we did not perform differential counts on FLC and ALC in the present study. At day7 in $AMH^{++}$ there was no LC proliferation. Lower values on LC proliferation was also seen in $AMH^{++}$ mice at day 14 and at day 21. These results suggest that inhibition of LC proliferation may be one of the mechanism which cause reduced LC number in testis of $AMH^{++}$ mice. Additionally, another mechanism that could result in fewer LC in adult $AMH^{++}$ mice is inhibition of mesenchymal cell differentiation into LC; latter process was not investigated in the present study.

The present study also showed that cell apoptosis is ruled out as a mechanism that resulted in reduced LC numbers in $AMH^{++}$ mice. In contrast, Salva et al. (2004) observed apoptotic cell nuclei in the testis interstitum of AMH-treated adult rats following 15 days after EDS treatment. These differences are difficult to explain by the results of the present investigation, however, it is important to note that the micro-environment (i.e. prepubertal mouse testis versus EDS treated adult rat testis) in which the cells differentiate in the two studies are different.

Present report is the first to show that thyroid hormone treatment results in LC differentiation in the prepubertal $AMH^{++}$ mouse. Findings revealed an increase in the proliferation (mitotic activity) of LC in $C+T_3$ animals compared to control mice with a

maximum at day 21. This observation agrees with the previous results on cell counts (Ariyaratne et al., 2000b) and of BrdU incorporated cell counts (Teerds et al., 1998) in the prepubertal rat testis. However, in contrast to our findings, Hardy et al., (1993) reported an increase in LC proliferation in hypothyroid rats until postnatal day 40. Finding of Hardy et al. (1993) were difficult to substantiate with previous reports (Mendis-Handagama et al., 1998; Teerds et al., 1998; Maran et al., 2001; Ariyaratne et al., 2000b,c) and present findings. In the present investigation, we also demonstrated the long term effects of prepubertal hyperthyroidism on the LC number and function.

In AMH+$T_3$ mice, two opposing factors, stimulatory effect of thyroid hormone and inhibitory effect of AMH are acting simultaneously on LC differentiation to determine the final LC number in the adult. Our result show that more LC are present at day 21 and nearly 30% and 15% increase in LC number at day 40 and 90, respectively in AMH+$T_3$ mice compared to the $AMH^{++}$ mice indicating the ability of thyroid hormone at this given dosage to overcome at least in part, their ihibitory effect of AMH on postnatal LC differentiation.

Androgen levels in serum and incubation media of testicular tissue of the present study provide further evidence for the effects of AMH and thyroid hormone on postnatal LC differentiation. At postnatal day 21, low levels of serum testosterone was measured in all treatment groups, despite the significant differences in LC number per testis. One contributory factor for low testosterone levels at day 21could be the maturational status of these LC, which are categorized as newly formed and immature adult LC (Mendis-Handagama et al., 1998; Mendis-Handagama and Ariyaratne, 2001). Previous studies showed that adult type LC at day17 mainly secrete 17α-reduced androgens rather than testosterone (Moger, 1977; Corpechot et al., 1981). Furthermore, in $AMH^{++}$ mice, high levels of AMH should be inhibiting the androgen production by the LC (Lyet et al., 1995) and by down regulation of androgen biosynthetic enzymes (Racine et al., 1998; Rouiller-Fabre et al., 1998; Trbovich et al., 2001, 2004). This is in addition to having reduced LC numbers in their testes.

In the presence of maximum stimulatory dose of LH, C+$T_3$ mice secreted more androgens. This observation agrees with having more LC in their testes. At day 40, in C+$T_3$ groups, the serum testosterone level as well as *in vitro* production of androgens were not different from those of C mice, despite the presence of lower number of LC in C+$T_3$ mice. Therefore, it appears that LC in 40 day old C+$T_3$ animals possess a greater capacity for androgen secretion compared to those of C. Although statistically not significantly different, a larger average size of LC were observed for C+$T_3$ mice, which agrees with their greater capacity for steroid secretion (Mendis-Handagama et al., 1988). Nevertheless, by day 90, the higher androgen secretory capacity was not evident in C+$T_3$ mice, probably due to early appearance of aging changes in their LC resulting from their early maturation, resulting from thyroid hormone exposure during early life (Ariyaratne et al., 2000c). Very low level of both testosterone and androstenedione in serum and incubation media observed in $AMH^{++}$ mice may be due to having low numbers of LC in their testes and chronic inhibition of LC steroid production due to high level of AMH in them. As expected from the cell counts, AMH+$T_3$ mice were able to secrete significantly more androgens than AMH mice, even though the increase was only a fraction of the control level. Short term thyroid hormone treatment during prepubertal period in $AMH^{++}$ mice may have produced a significant effect on LC differentiation during the treatment period. However, the long term carry-over effect of this treatment with the cessation of thyroid hormone treatment at day 21, together with the presence of high level of AMH largely inhibited, but was still able to produce a significant difference in LC differentiation.

Based on the present findings, it is possible to conclude that the negative regulation of AMH on postnatal LC differentiation on both the precursors and differentiated cells, could be overcome at least in part by the stimulatory effect of thyroid hormone on the same cell populations, given at an adequate dose.

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