T₃ Directly Stimulates Basal and Modulates LH Induced Testosterone and Oestradiol Production by Rat Leydig Cells *in vitro*

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Abstract. The effect of T₃ on basal and LH mediated synthesis and secretion of testosterone and oestradiol by puberal rat Leydig cells was studied *in vitro*. Percoll gradient purified Leydig cells (1×10^3) were cultured for 48 hours at 34 °C in a medium containing a range of 5–400 ng/mL concentration of T₃ or ovine LH after 24 hours initial culture at 37 °C. T₃ increased testosterone and oestradiol secretions in a dose dependent manner which reached the saturation point with 50 ng dose. While the minimum effective dose of T₃ (25 ng) potentiated the stimulatory effect of the minimum effective dose of LH (25 ng) on testosterone secretion, it suppressed the effect of the saturation dose of LH (100 ng). Fifty ng T₃ quelled the stimulatory effect of either dose of LH. Both doses of T₃ increased oestradiol secretion, irrespective of the dose of LH. Addition of androstenedione (500 ng/mL) to the culture medium enhanced 25 ng T₃ induced testosterone and oestradiol secretions. While androstenedione potentiated the stimulatory effect of T₃ (25 ng) on LH (25 ng) induced testosterone and oestradiol secretions, it reversed the inhibitory effect of 50 ng T₃ on LH mediated testosterone secretion which was accompanied by a decrease in oestradiol secretion. Puromycin (35 µg/mL) suppressed the stimulatory effect of T₃ on basal and LH mediated testosterone and oestradiol production. Taken together, the present results indicate a direct stimulatory effects of T₃ on basal production of testosterone and oestradiol by Leydig cells and its modulatory effect on LH mediated steroidogenic activity varies depending upon the intensity of LH stimuli.

Key words: Leydig cells, LH, Oestradiol, T₃, Testosterone

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MAMMALIAN testis, which has specific T₃ receptors (TR) is considered to be a prime target organ of thyroid hormones [1–3]. The majority of existing reports consider immature, rather than mature rat testis to be vulnerable to thyroidal influence, as TR concentration decreases significantly in adult rats [3]. Though TR- α_1 expression in the testis ceases at prepuberal age, expression of TR- α_2 persists till puberty but at a reduced rate [1, 2]. On the contra-

ry, the genomic locus of c-erb A α , the protooncogene that encodes TR- α and its variant transcribes another mRNA called Rev-erb A α (Rev) during prepuberal age of rats with maximal expression in adults [3].

Modulatory effects of thyroid hormones on testicular steroidogenesis are well known [4–6]. An increase in Leydig cell number with reduced steroidogenic activity was reported in adult rats subjected to transient neonatal hypothyroidism [7]. Recently, we have shown enhanced Leydig cell number accompanied by steroidogenic activity in puberal rats subjected to transient neonatal hypothyroidism during the first two weeks of postnatal period [8]. Early studies identified Sertoli cells as the only testicular target for T₃ in view of the presence of specific TR [3, 9]. However, recent studies have shown the presence

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of T₃ binding sites in adult rat and goat Leydig cells [10–13]. Hardy *et al.* [14] demonstrated the expression of TR- α_1 and TR- α_2 mRNA in rat Leydig cells but the functional significance of TR in Leydig cells is yet to be understood. We have shown subdued Leydig cell function in rats subjected to hypothyroidism from prepuberal age under basal and LH induced conditions [6]. Valenti *et al.* [15] reported resistance of normal and hypothyroid adult rat Leydig cells to T₃ stimulation *in vitro*. This paper is the first to report a direct dose dependent stimulatory effect of T₃ on basal testosterone and oestradiol production and a dose dependent modulatory effect on LH induced testosterone production by purified Leydig cells of puberal rats *in vitro*.

Materials and Methods

Chemicals

Collagenase (type IV), HEPES, bovine serum albumin (BSA), fetal calf serum (FCS), minimum essential medium (MEM), Percoll, sodium pyruvate, sodium lactate, trypan blue, β -nicotinamide adenine dinucleotide (β -NAD), nitro blue tetrazolium (NBT), dehydroepiandrosterone (DHEA), puromycin, androstenedione, amphotericin and L-triiodothyronine were purchased from Sigma Chemical Company, St. Louis, MO, USA. Bioactive ovine luteinizing hormone (NIDDK-LH-26; AFP-5551 B) was a gift from the National Institute of Diabetes, Digestive and Kidney Diseases, Maryland, USA. [1, 2, 6, 7-³H] Testosterone, and [2, 4, 6, 7-³H] oestradiol were purchased from Amersham International PLC, Buckinghamshire, England. Testosterone and oestradiol standards were provided by World Health Organization (WHO), Geneva, Switzerland under SMA programme. Testosterone antibody was a gift from Dr. Med. E. Nieschlag, Director, Institute of Reproductive Medicine, University of Münster, Germany. Oestradiol antibody was a gift from Dr. C. Munroe and Dr. B. Lesely, University of California, Davis, USA.

Isolation of Leydig cells

Sixty day old Wistar rats were used for Leydig cell harvest as puberal maturation of the testis is com-

plete by this age with predominance of adult type Leydig cells having the maximum number of LH receptors [16, 17].

Leydig cells were isolated by using collagenase digestion method [18] with slight modifications. Testes were decapsulated without breaking seminiferous tubules under an aseptic condition and incubated with 5 mL MEM (pH 7.4) containing collagenase (0.35 mg/mL), HEPES (1 mg/mL) and BSA (1 mg/mL) in 25 mL flasks for 15 minutes at 34°C, while gently shaking the flasks without damaging the seminiferous tubules. After this, 10 mL MEM (pH 7.4) was added, allowed to stand for 5 minutes at room temperature and the supernatants were transferred to sterilized centrifuge tubes using a Pasteur pipette. This procedure was repeated twice to obtain the maximum number of Leydig cells. Pooled Leydig cells were washed repeatedly (at least thrice) with MEM by centrifuging (1000 $\times$ g) at 4°C for 15 minutes, and the pellet was resuspended in 5 mL MEM. These crude Leydig cells were purified by Percoll density gradient method [19] and identified by histochemical localization of 3 β -hydroxysteroid dehydrogenase (3 β -HSD) [20]. The purity of Leydig cells was 80–85%. The principal contaminants were spermatogonia (12–15%), sperm (2%) and peritubular myoid cells (1–3%) which were removed by an initial 24 hours culture at 37°C (described under cell culture). The viability of Leydig cells (90–95%) was assessed using trypan blue exclusion method [20].

Cell culture studies

Purified Leydig cells (1×10^3) in MEM 300 μ L containing 4% FCS were plated in 96 well flat bottom culture plates and incubated under 5% CO₂ and 95% O₂ at 37°C for 24 hours. At the end of this initial incubation, most of the germ cell contaminants floated and were discarded along with the medium. This step helped to improve the purity of Leydig cells and to remove residual hormones secreted by the cells into the culture medium. After the initial incubation, the culture was replenished with fresh medium containing different concentrations (5, 10, 25, 50, 100, 150, 200 and 400 ng/mL) of bioactive ovine LH or T₃ at 34°C. The culture media were collected after 48 hours, centrifuged and used for the estimation of testosterone and oestradiol by radioimmunoassay (RIA). The viability of Leydig cells in each culture,

checked at the end of 48 hours incubation was 90–95%.

Modulatory effect of T₃ on LH induced testosterone and oestradiol secretion by Leydig cells

From the dose response curve of testosterone, the maximum (saturation) and minimum effective doses of LH (25 ng and 100 ng) and T₃ (25 ng and 50 ng) were selected for combination studies involving LH plus T₃. Then 1×10^3 cells were cultured for 48 hours after an initial 24 hour culture in a medium containing the following combination of T₃ and LH: 25 ng T₃+25 or 100 ng LH; 50 ng T₃+25 or 100 ng LH. Concentrations of testosterone and oestradiol in the medium were quantified by RIA.

Testosterone and oestradiol production from androstenedione

In order to check the influence of T₃ on testosterone and oestradiol production, Leydig cells were cultured for 48 hours (after the initial culture) at 34°C as described above with a medium containing 500 ng/mL androstenedione [21] and challenged with the minimum or maximum effective doses of LH/T₃ alone or in combination as described earlier. Concentrations of testosterone and oestradiol in the culture media were quantified after 48 hours.

Effect of puromycin on LH or T₃ induced changes in Leydig cell function

To check the involvement of new protein synthesis if any, in mediating T₃ induced changes in Leydig cell steroidogenic activity, a set of cultures was challenged with LH or T₃ in the presence of puromycin [22]. Then 1×10^3 Leydig cells were incubated in a fresh medium containing puromycin (35 µg/mL) along with minimum or maximum effective doses of LH or T₃ and incubated for 48 hours at 34°C after the initial culture and concentrations of testosterone and oestradiol in media were quantified.

RIA of testosterone and oestradiol

Testosterone and oestradiol concentrations in culture media were measured (for testosterone 10 µL media was taken and for oestradiol 25 µL media was

taken extracted in diethyl ether and quantified) by liquid-phase RIA [23]. The sensitivity of these assays were 14 fm/tube for testosterone and 7 fm/tube for oestradiol. Inter- and intra-assay variations were: 7% and 7.2%, respectively for testosterone; 5% and 3.2%, respectively for oestradiol.

Statistical Analysis

Data were subjected to analysis of variance (ANOVA). If the F ratio was significant, the data were subjected to multiple comparison using Student-Newman-Keul's (SNK) test to assess the individual significance [24].

Results

LH induced testosterone and oestradiol secretion by Leydig cells
Testosterone

A clear dose dependent biphasic effect of LH on testosterone secretion by purified Leydig cells was observed *in vitro*. Leydig cells stimulated with 25, 50 and 100 ng LH responded with increased secretion of testosterone in a graded manner, reaching the saturation point with 100 ng LH stimulation. On the other hand, a significant slump in testosterone secretion was evident when Leydig cells were challenged with 150, 200 or 400 ng LH, reaching the nadir level after stimulation with 200/400 ng LH (Fig. 1).

Oestradiol

LH induced oestradiol secretion presented a diametrically opposite picture to that of testosterone (Fig. 1). An initial marginal but significant increase in oestradiol secretion by cells stimulated with 50 ng LH was followed by a slump, reaching the nadir value when stimulated with 100 ng LH. This was followed by significantly increasing concentrations of oestradiol in media of Leydig cells challenged with higher doses of LH, reaching the zenith in response to 200/400 ng LH (Fig. 1).

Fig. 1. Dose response curves of testosterone and oestradiol secretions by rat Leydig cells stimulated with different doses of oLH *in vitro*. Each point represents the mean and the vertical line denotes the SEM ($n=9$). a- $p<0.05$; b- $p<0.01$; c- $p<0.001$ vs basal level. Cells were cultured for 48 hours at 34 °C after an initial culture for 24 hours at 37 °C.

T₃ induced testosterone and oestradiol secretions by Leydig cells

Testosterone

Testosterone secretion by Leydig cells stimulated with T₃ showed a dose dependent biphasic response. Testosterone concentration increased in response to stimulation with 25 ng T₃ and reached the saturation point with 50 ng T₃. This was followed by a slump in the concentration of testosterone in media of cultures challenged with 100, 150, 200 and 400 ng T₃ (except for cultures stimulated with 200 ng T₃/mL) but these testosterone levels were higher than that of Leydig cells stimulated with the minimum effective dose (25 ng) of T₃ (Fig. 2).

Oestradiol

Unlike testosterone, oestradiol concentration in culture media of Leydig cells stimulated with 5 ng T₃ showed a significant decrease, whereas stimulation with all other doses of T₃ increased oestradiol level in culture media. Nevertheless, there was a significant decrease in oestradiol secretion in culture media of cells stimulated with 150 or 200 ng T₃, when com-

pared with the peak value obtained in response to 100 ng T₃ but oestradiol secretion regained the maximum level when challenged with 400 ng T₃ (Fig. 2).

Interaction of T₃ and LH on testosterone/oestradiol secretions by Leydig cells

Testosterone

When minimum effective doses of T₃ (25 ng) and LH (25 ng) were administered together, the quantum of testosterone secreted by Leydig cells doubled from that of cognate cultures stimulated with minimum effective dose of either of these hormones. On the contrary, co-administration of the minimum effective dose of T₃ with the saturation dose of LH (100 ng) perceptibly decreased (82%) the concentration of testosterone in the medium when compared with cultures challenged with the saturation dose of LH alone. Similarly, when the saturation dose of T₃ (50 ng) was co-administered with either 25 or 100 ng LH, there was a conspicuous decrease (35% and 92%, respectively) in testosterone secretion, when compared with cognate cultures stimulated with either dose of LH alone (Fig. 3).

Fig. 2. Dose response curves of testosterone and oestradiol secretions by rat Leydig cells stimulated with different doses of T₃ *in vitro*. Each point represents the mean and the vertical line denotes the SEM (n=9). a-p<0.05; b-p<0.01; c-p<0.001 vs basal level. Cells were cultured for 48 hours at 34 °C after an initial culture for 24 hours at 37 °C.

Fig. 3. Modulatory effects of T₃ on LH mediated changes in testosterone secretion by rat Leydig cells *in vitro* in the presence and absence of androstenedione (500 ng/mL). Each point represents the mean and the vertical line denotes the SEM (n=9). A-androstenedione. a-i denote the statistical significance of the difference between values compared. Differences were regarded as significant when P<0.05. a-vs basal; b-vs androstenedione; c-vs T₃25; d-vs T₃50; e-vs LH25; f-vs LH100; g-vs LH25+T₃25; h-vs LH25+T₃50; i-vs LH100+T₃50. Cells were cultured for 48 hours at 34 °C after an initial culture for 24 hours at 37 °C.

Oestradiol

When 25/50 ng T_3 was co-administered with either 25 or 100 ng LH, it consistently increased oestradiol secretion when compared with cognate cultures stimulated with the respective dose of LH alone. The important finding being the reversal of the inhibitory effect of 100 ng LH on oestradiol secretion by T_3 (Fig. 4).

Effect of T_3 on basal and LH induced testosterone and oestradiol production from androstenedione by Leydig cells

Testosterone

LH induced increase in testosterone concentration was enhanced significantly when androstenedione (500 ng) was added to the culture medium, indicating LH mediated conversion of androstenedione into testosterone. Unlike LH, the effect of T_3 on testosterone production from androstenedione was dose dependent. While 25 ng T_3 increased the production of testosterone from androstenedione, 50 ng T_3 did not produce any appreciable change in the same when compared with cognate cultures incubated in a

medium containing androstenedione alone (Fig. 3).

The stimulatory effect of 25 ng T_3 on 25 ng LH induced testosterone production increased significantly in the presence of androstenedione in the culture medium. The inhibitory effect of 25 ng T_3 on 100 ng LH induced testosterone secretion persisted even in the presence of androstenedione. On the other hand, the inhibitory effect of 50 ng T_3 on 25 ng LH induced testosterone secretion was reversed by the addition of androstenedione to the culture medium and the concentration of testosterone in the medium was perceptibly higher than those cultures stimulated with either or both 25 ng LH and 50 ng T_3 . The inhibitory effect of 50 ng T_3 on 100 ng LH induced testosterone secretion was also reversed by the addition of androstenedione to the culture medium but the level of testosterone was significantly less than that of cognate cultures stimulated with 100 ng LH or 50 ng T_3 alone (Fig. 3).

Oestradiol

Presence of androstenedione in the culture medium increased the production of oestradiol in response to 25 ng LH stimulation and reversed the inhibiting

Fig. 4. Modulatory effects of T_3 on LH mediated changes in oestradiol secretion by rat Leydig cells *in vitro* in the presence and absence of androstenedione (500 ng/mL). Each point represents the mean and the vertical line denotes the SEM (n=9). A-androstenedione. a-h denote the statistical significance of the difference between values compared. Differences were regarded as significant when $P < 0.05$. a-vs basal; b-vs androstenedione; c-vs T_3 25; d-vs T_3 50; e-vs LH25; f-vs LH100; g-vs LH25 + T_3 25; h-vs LH25 + T_3 50. Cells were cultured for 48 hours at 34 °C after an initial culture for 24 hours at 37 °C.

effect of 100 ng LH, leading to a perceptible increase in the concentration of oestradiol in the culture medium. While the stimulatory effect of 25 ng T₃ on oestradiol production was augmented by the presence of androstenedione in the culture medium, no appreciable difference was noticed in the stimulatory effect of 50 ng T₃. Androstenedione augmented the stimulatory effect of 25 ng T₃ on 25 ng LH induced oestradiol production alone whereas, it decreased the potentiating effect of 50 ng T₃ on 25 ng LH induced oestradiol production. The stimulatory effect of 25/50 ng T₃ on 100 ng LH induced oestradiol secretion did not undergo any appreciable change due to the presence of androstenedione in the medium (Fig. 4).

Table 1. Modulatory effect of T₃ (25 or 50 ng/mL) on LH (25 or 100 ng/mL) mediated changes in testosterone and oestradiol secretions by rat Leydig cells in the presence and absence of puromycin (35 µg/mL).

Culture Status*	Testosterone ng/10 ⁶ cells	Oestradiol Pg/10 ⁶ cells
Basal	5.04±0.80	15.56±2.60
Puromycin (P)	4.78±0.30	13.42±1.06
LH25	7.82±0.50	22.66±3.40
LH25+P	2.68±0.27 ^a	8.25±0.79 ^a
LH100	65.67±8.30	0.47±0.03
LH100+P	2.01±0.42 ^a	9.36±0.89 ^a
T ₃ 25	7.61±0.30	22.68±1.10
T ₃ 25+P	3.38±0.47 ^a	6.05±1.03 ^a
T ₃ 50	28.11±4.90	31.31±4.90
T ₃ 50+P	2.61±0.39 ^a	5.49±0.95 ^a
LH25+T ₃ 25	14.40±5.50	37.64±1.74
LH25+T ₃ 25+P	4.03±0.56	6.30±0.85 ^a
LH25+T ₃ 50	4.78±1.30	97.64±3.39
LH25+T ₃ 50+P	2.80±0.62	7.36±0.85 ^a
LH100+T ₃ 25	12.56±1.80	77.83±3.31
LH100+T ₃ 25+P	4.92±0.62 ^a	5.69±0.64 ^a
LH100+T ₃ 50	4.40±0.90	105.44±4.50
LH100+T ₃ 50+P	3.08±0.60	3.19±0.58 ^a

Each value is mean±SEM (n=9).

^a Hormones alone vs Hormones+puromycin (Differences were regarded as significant when P<0.05).

* Suffix numbers indicate the concentration of the respective hormone (ng/mL).

Cells were cultured for 48 hours at 34°C in a fresh medium containing hormones and puromycin, as indicated after an initial culture for 24 hours at 37°C.

Effect of puromycin on T₃ induced changes in testosterone/oestradiol secretion

Testosterone

Presence of puromycin in the culture medium did not affect basal testosterone secretion. However, presence of this protein synthesis inhibitor blocked LH (25/100 ng) and T₃ (25/50 ng) induced testosterone secretions. Puromycin also decreased the concentration of testosterone in media of Leydig cell cultures stimulated with the minimum effective dose of T₃ plus either dose of LH. Puromycin did not produce any appreciable effect on the inhibitory effect of 50 ng T₃ on 25/100 ng LH induced testosterone secretion (Table 1).

Oestradiol

Addition of puromycin to the culture medium decreased the concentration of oestradiol secreted by Leydig cells stimulated with 25 ng LH and 25 or 50 ng T₃ whereas there was a significant increase in the concentration of oestradiol in media of cells challenged with 100 ng LH. Marked reduction in the concentration of oestradiol was also evident in culture media of Leydig cells challenged with 25 ng or 100 ng LH plus 25 or 50 ng T₃ in the presence of puromycin (Table 1).

Discussion

The present study proves the existence of a direct effect of T₃ on steroidogenic activity of adult type rat Leydig cells obtained from puberal rats. To the best of our knowledge, this is the first report to show a direct stimulatory effect on T₃ on purified rat Leydig cells. Early reports explained the effect of thyroid hormones on Leydig cell steroidogenesis as an indirect one through altered secretion of paracrine regulators from Sertoli cells [3, 25] which have specific TR [9]. This is mainly because of the inability of early researchers to show the presence of specific TR in Leydig cells [3, 9]. Valenti *et al.* [15] reported that T₃ or T₄ does not influence basal or LH mediated testosterone production by purified rat Leydig cells. Our results are inconsistent with the above study. This inconsistency may be explained by the differences in age of animals and culture con-

ditions between the two studies.

The dose response curves of testosterone/oestradiol production under LH stimulation *in vitro* demonstrates the biological potency of oLH used and the functional viability of the Leydig cells in the *in vitro* system employed. Viability of cells was also confirmed after 48 hours incubation as explained earlier. The mechanism underlying LH-induced testosterone secretion has been studied in depth and reviewed by Dufau *et al.* [26], Dufau [27], Huhtaniemi [28].

Adult type Leydig cells respond to LH stimulation in a dose dependent manner. Leydig cells showed increased steroidogenic activity in terms of testosterone production when stimulated with low and medium doses of LH and with impaired steroidogenic activity when stimulated with high doses of LH. The latter effect is due to down regulation and desensitization of LH receptors [26–29]. Down regulation of LH receptors involves sequestration of the LH receptors from plasma membrane, internalization and loss of binding (homologous down regulation) accompanied by a decrease in some other membrane receptors also (such as heterologous down regulation of lactogen receptors) [26–28]. Other evidence can be drawn from the study of Pakarinen *et al.* [30] which showed decreased LH receptor mRNA concentration in adult rat Leydig cells challenged with high dose of hCG.

In vivo [31] and *in vitro* [32] studies have shown that higher doses of LH or hCG induce up regulation of Leydig cell LH receptors and testosterone secretion in fetal Leydig cells, whereas an opposite effect was observed in adult Leydig cells. The reason behind this differential effect of LH/hCG is the lack of oestradiol receptors and aromatase activity in fetal Leydig cells [29]. Mature Leydig cells aromatize androgens into oestradiol [33, 34]. In rats, there occurs a shift in aromatization with maturation. In immature rat, the Sertoli cell is the site of aromatization, while the Leydig cell is the site of 5α -reduction of testosterone, whereas in adult rats, the Leydig cell becomes the site of aromatization of testosterone in the testis [35].

An important finding of the present study is the increased oestradiol secretion and response of Leydig cells to high doses of LH stimulation. Early studies on stimulation of Leydig cells with LH have shown increased aromatase activity and oestrogen forma-

tion [33, 34, 36, 37]. LH-induced steroidogenic lesions in the adult type Leydig cells include reduced conversion of cholesterol to pregnenolone (early lesion) and progesterone to testosterone (late lesion). The late steroidogenic lesion caused by LH/hCG was shown to be oestrogen mediated by inhibiting steroidogenic enzymes 17,20-lyase and 17α -hydroxylase [31, 33, 34]. These are oestrogen-induced steroidogenic lesions mediated by the synthesis of a specific protein (27 kD) [36, 38]. The present study clearly shows that diminished secretion of testosterone by Leydig cells challenged with high dose of LH is due to enhanced estradiol secretion.

A direct effect of T_3 on Leydig cells appeared during the first half of this decade when specific T_3 binding to Leydig cells of adult rats [11], and the expression of TR in the interstitium of rats [10] were demonstrated. The proposal for direct T_3 action on Leydig cells was strengthened by a recent demonstration of TR- α_1 and α_2 mRNA expression in immature and mature rat Leydig cells [14]. In general, the thyroid hormone acts through β form receptors. Testis is the unique organ wherein the thyroid hormones act through α form receptors [3]. The results from *in vitro* studies are consistent with early *in vivo* studies that hypothyroidism decreased and hyperthyroidism increased serum and intratesticular testosterone concentration [6, 39, 40]. The present *in vitro* study on purified adult type Leydig cells from sixty day old rats provides clear evidence for a direct effect of T_3 on Leydig cell steroidogenesis.

An important aspect of the present finding is a partial desensitization or negative modulatory effect on steroidogenesis, rather than a complete down regulation of T_3 receptors in Leydig cells challenged with high doses of T_3 . This could be evinced from the sustained elevation of testosterone secretion by Leydig cells challenged with T_3 doses higher than its saturation dose (50 ng), albeit a decrease in the quantum of testosterone secreted. This partial decrement in testosterone production by Leydig cells is attributed to the accompanying enhanced secretion of oestradiol. This could be deduced from the diametrical secretory responses of testosterone and oestradiol secretion observed in Leydig cells challenged with most of the high doses of T_3 (100, 150 and 400 ng) used. This shift in Leydig cell response to T_3 stimulation is comparable to that of LH. The difference between LH and T_3 stimulation

is that the high doses of the former depleted testosterone concentration, while the latter had only a partial effect.

The present study points out a clear stimulatory effect of T₃ on Leydig cell aromatase activity though there was an inhibitory effect of the lowest dose of T₃ employed (5 ng). To the best of our knowledge, this is the first report to show the dose dependent biphasic direct effect of T₃ on oestradiol production by Leydig cells. This is consistent with *in vivo* studies [40] when T₄ administration increased serum oestradiol titres. The quantum of oestradiol secretion by Leydig cells stimulated with T₃ suggest that Leydig cell aromatase activity is more sensitive to thyroid hormones rather than LH. T₃ inhibits the Sertoli cell aromatase activity and granulosa cell aromatase mRNA [41–43], whereas it stimulates the production of oestradiol in Leydig cells, suggesting cell specific differential role of thyroid hormones on testicular oestradiol production. Per the authors' knowledge there is no report available on the effect of thyroid hormones on Leydig cell aromatase expression, and further studies are required to confirm the effect of T₃ on Leydig cell aromatase gene expression. The exact mechanism underlying the inhibitory effect of 5 ng T₃ dose on oestradiol production is not clear at present. A recent study from this laboratory has shown the inhibition of oestradiol production by T₃ when Leydig cells are co-cultured with Sertoli cells [44]. This finding suggests that T₃ stimulates certain specific Sertoli cell paracrine factors which has an inhibitory effect on Leydig cell aromatase activity.

Results from studies on Leydig cells stimulated with LH plus T₃ attest to the stimulatory effect of T₃ on Leydig cell aromatase. T₃ favors enhanced aromatization of testosterone by Leydig cells stimulated with LH. This effect of T₃ is also associated with stimulation of testosterone production when the effect of LH is minimal i.e. when Leydig cells were stimulated with 25 ng LH. Taken together, the data on testosterone and oestradiol suggest that the inhibitory effect of T₃ on LH mediated testosterone secretion is not due to a lesion in its production; rather it is a result of enhanced rate of conversion of testosterone into oestradiol. Since oestradiol is a potent inhibitor for the synthesis of testosterone [27], the enhanced production of oestradiol might have also decreased the testosterone secretion.

Data on testosterone and oestradiol production

from androstenedione by Leydig cells stimulated with 25 ng T₃ would indicate that T₃ induced reduction in testosterone concentration is the result of enhanced aromatization of the same. Since aromatase activity seems to reach the zenith in Leydig cells challenged with 50 ng T₃, there was no added effect on the same due to the presence of exogenous androstenedione in the medium. Therefore, the difference in testosterone production in response to minimum and maximum effective doses of T₃ may be attributed to the dose dependent variation in the response of Leydig cell aromatase activity to T₃ stimulation.

Data from *in vitro* studies on testosterone and oestradiol production from androstenedione also suggest a dose dependent biphasic effect of T₃ on Leydig cell 17 β -hydroxysteroid dehydrogenase (17 β -HSD) as minimum and maximum effective doses of T₃ produced opposite results. Early studies from our laboratory [6] and others [5] showed decreased activity of Leydig cell 17 β -HSD in thyroidectomized rats. These *in vivo* studies also showed normal activity of 17 β -HSD in those hypothyroid rats supplemented with T₄, suggesting a stimulatory effect of thyroid hormones.

Studies on testosterone/oestradiol production from androstenedione by Leydig cells costimulated with LH+T₃ provide additional evidence for a modulatory effect of T₃ on LH action at the level of 17 β -HSD activity. This effect of T₃ may vary depending upon the dose of LH used as addition of androstenedione to the culture medium failed to reverse the inhibitory effect of T₃ on 100 ng LH induced testosterone production. The increased level of oestradiol in the culture medium of Leydig cells stimulated with 100 ng LH plus T₃ has probably blocked the activity of 17 β -HSD. There appears to be a dose dependent shift in the site of T₃ effects on Leydig cell testosterone production.

Data from puromycin studies indicate that stimulatory effect of T₃ on Leydig cell steroidogenic activity is mediated by new protein synthesis. At this juncture, it may be worthwhile to recall the finding of Jana and Bhattacharya [12] and Jana *et al.* [13] who showed the synthesis of a new protein during an acute stimulation of crude goat Leydig cell preparations with T₃. It is now well established that steroidogenic acute regulatory protein (StAR) is one of the major regulators of Leydig cell steroidogenesis [45]. It is not clear at present whether T₃ induced steroidogenic

activity in Leydig cell is mediated by StAR protein. This could be inferred from the reduction in T_3 mediated increase in basal and LH induced testosterone/oestradiol secretions due to puromycin. Recently Manna *et al.* (46) reported that T_3 induced a 3.6 fold increase in StAR protein mRNA expression and progesterone production by mouse Leydig tumor cell line. In light of these observations, T_3 induced steroidogenesis in rat Leydig cells may be mediated by StAR protein.

In conclusion, the present study reveals that (i) T_3 has a direct stimulatory effect on basal steroidogenic activity in puberal rat Leydig cells, and (ii) T_3 also modulates LH mediated steroidogenesis in a dose dependent manner.

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