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Triiodothyronine modulates initiation of spermatogenesis in rats depending on treatment timing and blood level of the hormone

Abbreviated title: Thyroid hormone and spermatogenesis

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ABSTRACT

Triiodothyronine (T3) stimulates spermatogenic onset but the influence of T3 on spermatogonia development is unknown. The aim of the study was to investigate the role of T3 for both processes simultaneously. Male rats were given daily injections of 100µg T3/kg body weight or vehicle from birth until postnatal day (pnd) 5 and euthanized on pnd 6 (short T3-sT3). Other rats, euthanized on pnd 16, were treated either transiently with T3 (tT3) during the initial 5 days or continuously until pnd 15 (cT3). sT3 was found to increase gonocyte differentiation, spermatogonia number, cell degeneration and proliferation. tT3 increased serum T3 level and spermatogonial development to adult values precociously, but cell degeneration or proliferation were not affected. cT3 increased serum T3 together with cell degeneration and proliferation, but cell number was not affected. In conclusion, T3 may modulate spermatogonial development quantitatively depending on treatment timing and blood level of the hormone.

Key words: Thyroid hormone, testes, maturation, gonocyte, spermatogonia, Sertoli cell.

1 INTRODUCTION

Spermatozoa differentiate from spermatogonia that arise from fetal/neonatal gonocytes at the onset of spermatogenesis. Gonocytes complete their differentiation into type A spermatogonia in the rat at postnatal day (pnd) 5, a process associated with their migration toward the basal lamina. There are both differentiating A spermatogonia that give rise to the first wave of spermatogenesis as well as spermatogonial stem cells (Clermont and Perey, 1957; de Rooij, 2001; de Rooij and Russell, 2000). Spermatogonial development finalizes on about pnd 10 with the first appearance of preleptotene spermatocytes (Clermont and Perey, 1957; Kula, 1977; Russell et al., 1987; van Haaster and de Rooij, 1993) while the differentiation of Sertoli cells finalizes on about pnd 16, together with cessation of their proliferation (Orth, 1982; Steinberger and Steinberger, 1971). The formation of junctions between Sertoli cells (St-Pierre et al., 2003; Vitale et al., 1973) is associated with the accretion of tubular lumen, reflecting fluid secretion by maturing Sertoli cells (Russell et al., 1989).

The initiation of spermatogenesis requires FSH, testosterone and estradiol action, (Almiron and Chemes, 1988; Kula et al., 2001; Russell et al., 1987; Walczak-Jedrzejowska et al., 2008). Follicle stimulating hormone (FSH) and androgens are important regulators of Sertoli cell proliferation (Atanassova et al., 2005). The body of literature shows that the mitogenic action of thyroid hormone on Sertoli and Leydig cells is limited to an early developmental window (Ariyaratne et al., 2000; Hardy et al., 1996; Kirby et al., 1992; Orth, 1982). The administration of triiodothyronine (T3) during the postnatal period shortens the Sertoli cell proliferation period and reduces testis size in adult rats (van Haaster et al., 1992). Transient neonatal hypothyroidism causes prolonged Sertoli cell proliferation, increased Sertoli cell number, testis weight, and sperm production in adulthood (Cooke and Meisami, 1991; Cooke et al., 1992). Triiodothyronine (T3) up-regulates androgen receptor mRNA in postnatal Sertoli cells (Arambepola et al., 1998), while testosterone supports differentiation and survival of immature Sertoli cells (Walczak-Jedrzejowska et al., 2007). T3 was shown to stimulate immature Sertoli cells to take up glucose (Ulisse et al., 1992) and secrete growth factors such as IGF-1 which stimulate DNA synthesis in mitotic germ cells (Palmero et al., 1990).

The action of thyroid hormones on target tissues is predominantly mediated by specific nuclear receptors, but these hormones also have well known nongenomic effects (Davis and Davis, 2002). The expression of thyroid hormone receptor (TR) in rat Sertoli cells is high on pnd 1, gradually decreases between pnds 15-20 and is minimal thereafter (Canale et al., 2001;

Holsberger and Cooke, 2005; Jannini et al., 1990). From pnd 0 to 5, expression of TR is not found in gonocytes, but all germ cells between intermediate spermatogonia and pachytene spermatocytes express TR α 1 (Buzzard et al., 2000). Thyroid hormone may be involved in the control of the premeiotic steps of the initiation of spermatogenesis, as it has been shown that at spermatogenic onset, T3 treatment increases germ cell number and decreases degeneration (Jannini et al., 1993). However, as the influence of T3 on further steps of spermatogenesis has not been studied yet, the aim of the present study was to investigate its influence on both spermatogenic onset and spermatogonial development in immature rats.

2 MATERIAL AND METHODS

2.1 Animals and treatments

Pregnant Wistar rats were obtained from the Department of Animal Facilities, Medical University of Lodz (Poland). On the day of birth (day 1), the litters were designated at random to treatment and control groups. Each group, containing 5-7 animals, was kept in a separate cage together with one lactating female. Animals were maintained at stable temperature (22°C), and diurnal light-dark cycles (12L:12D) with free access to food and water.

Experiments were performed in accordance with Polish legal requirements, under the license given by the Commission of Animal Ethics at the Medical University of Lodz, Poland.

Male pups received daily subcutaneous 0.1ml injections of 100 μ g T3/kg body weight (b.w.) (St. Louis, USA), dissolved in 0.025 N NaOH (POCH, Gliwice, Poland) in saline solution (Ariyaratne et al., 2000; van Haaster et al., 1993); this dose being established on the basis of previous studies (Holsberger et al., 2005; van Haaster et al., 1993). Two sets of experiments were performed. Experiment I (*short-term study*) concerned spermatogenic onset in young animals i.e. differentiation of gonocytes into type A spermatogonia. T3 or vehicle (control I) were administered from pnd 1 to 5 and the pups were euthanized on pnd 6 (sT3 - short T3 administration).

Experiment II (*long-term study*), concerned 16-day old rats at the completion of the premeiotic part of first spermatogenesis, displaying the appearance of pachytene spermatocytes. T3 was administered from pnd 1 to 5 followed by daily injections of vehicle up to pnd 15 (tT3 - transient T3 administration). In a parallel group, animals were continuously injected with T3 from pnd 1 to 15 (cT3 - continuous T3 administration) and were euthanized on pnd 16.. Control II (*for long term study*) received daily injections of

vehicle from birth. The rats were anaesthetised with Brieal (Eli Lilly & Co., Indianapolis, IN, USA) and Fentanyl WZF (Polfa, Poland) before autopsy and weighed. Blood samples were taken by cardiac puncture. Paired testes weight was measured at autopsy. The right testis of each rat was fixed in Bouin's fluid and, upon paraffin embedding, used for gross histology, seminiferous tubule stereometry, quantitative analysis of the seminiferous epithelium and staining for cell proliferation and degeneration.

2.2 Hormone assays

Hormone determinations were performed in experiment II. The clotted blood was centrifuged, and the serum was collected, frozen and stored at -20°C until assayed. All samples were measured in the same assay. Free triiodothyronine (fT3) concentration was determined by microparticle enzyme immunoassay (MEIA) (Abbott Diagnostic, USA) with a sensitivity of 1.0 pg/ml and a coefficient of variation (CV) of 5.2%. Rat follicle stimulating hormone (rFSH) concentration was determined by double antibody radioimmunoassay (Amersham, UK) with a sensitivity of 1.0 ng/ml, CV 4.7%. Concentrations of testosterone and estradiol were determined by competitive immunoassay (Ortho-Clinical Diagnostic Amersham, UK), with a sensitivity of 0.03 nmol/l, CV 2.3% for testosterone and 10.0 pmol/l, CV 4.1% for estradiol.

2.3 Morphometry and stereometry of seminiferous tubules

Sections 4 μm thick from equatorially transected organs were stained with haematoxylin/eosin for morphometry. The diameter and height of the seminiferous epithelium in 100 randomly-selected transverse seminiferous tubules sections for each animal were measured using LxAND image analysis software, version 3.60HM (Logitex, Lodz, Poland). The absolute volume of the seminiferous tubules and the total length of the seminiferous tubule was determined as described previously (Walczak-Jedrzejowska et al., 2008). The percentage of seminiferous tubule cross-sections containing a clear lumen (larger than 100 μm^2) and the surface area of tubular lumen were determined by scoring at least 100 subsequent tubular cross-sections.

2.4 Identification of cell proliferation and degeneration

The proliferative activity of the cells was studied by immunohistochemical labelling of proliferating cell nuclear antigen (PCNA LI) as described previously (Walczak-Jedrzejowska et al., 2008). As a negative control, sections were incubated with non-immune serum instead

of the primary antibody. The proliferating index of 500 subsequent germ cell nuclei, or 1000 subsequent Sertoli cell nuclei, was examined at 1000x magnification in the light microscope (Nikon, Eclipse E600, Japan). Germ and Sertoli cells were identified based on their location within the tubule, their size and the shape of nucleus.

Sections 4 μm thick from equatorially transected organs were stained with toluidine blue (Bio-Optica, Italy) for identification of germ cell degeneration (Jannini et al., 1993; Russell and Clermont, 1977), which was expressed as a percentage of tubular cross-sections containing at least two degenerating cells. Degenerating cells were identified as having strongly condensed, darkly stained nuclei in toluidine blue-stained sections. One hundred subsequent tubular cross-sections per each animal were scored.

2.5 Quantitative analysis of the seminiferous epithelium

Huckins and Clermont (1968) described gonocytes as cells located in the centre of the seminiferous tubules as well as on the periphery. Gonocytes have a large, light, spherical nucleus containing fine chromatin granules and two or more globular nucleoli, as well as a clearly visible cell membrane. In young rats before pnd 5, gonocytes generally appear in the centre (Gc), but some of them will have already migrated to the periphery of the tubule (Gp) and started to differentiate into type A spermatogonia. On pnd 6, the enumerations of Gc, Gp, type A spermatogonia and immature Sertoli cells were performed in 20 cross-sections of seminiferous tubules per animal.

The germ cell types present on pnd 16 were identified on the basis of previously reported morphological characteristics (Russell and Frank, 1978; Russell et al., 1987). In rats, an adult rate of premeiotic spermatogenic progression was observed from day 15 onward whereas a much higher rate of progression of spermatogenesis was observed before this age (van Haaster and de Rooij, 1993). Although pachytene spermatocytes represent the most advanced cell type from about pnd 12, they still constitute the oldest germ cell generation at 16 day of life (van Haaster and de Rooij, 1993).

Due to the paucity of spermatids, it is impossible to define the precise staging of the cycle of the seminiferous epithelium in immature rats. Hence, the abbreviated classifications described by Russell and Frank (1978) and Russell et al. (1987) were used. Association 1 refers to stages V/VI of the adult cycle, containing type B spermatogonia, and association 2 refers to

stages VII/VIII of the adult cycle, containing preleptotene spermatocytes. Huckins (1971) showed that some A spermatogonia, which can be identified among the B spermatogonia in stages V-VI, were undifferentiated type A spermatogonia. Between them, type A single spermatogonia are thought to be stem cells. Undifferentiated type A spermatogonia metamorphose without cell divisions into differentiating type A₁ spermatogonia. In stages VII-VIII, 80-90% of A spermatogonia are actually A₁ spermatogonia.

Germ and Sertoli cell enumerations were performed in 20 complete round seminiferous tubule cross-sections containing germ cell association 1 s, and in 20 cross-sections containing association 2. All counts were performed at 1000 x magnification under a light microscope (Nikon, Eclipse E600, Japan). Crude cell numbers per tubule cross-sections were corrected for section thickness and nuclear diameter of the respective cell types using Abercrombie's formula (Abercrombie, 1946):

$$T = C \times \frac{S}{S + d}$$

Where:

T - cell numbers per tubule cross-sections corrected according to Abercrombie

C - crude cell numbers per tubule cross-sections

S - section thickness

d - mean nuclear diameter of the respective cell types

Total number of each cell type per testis was expressed by multiplying the corrected cell number by the total length of the seminiferous tubules (Pineau et al., 1989).

The efficiencies of the succeeding steps of spermatogenesis (germ cell differentiation) were characterized by the ratios of the number of the following and proceeding generations of different cell types. Mature Sertoli cells have the capability to support a relatively fixed number of germ cells (Russell and Peterson, 1984). To examine the capacity of Sertoli cells to support the distinct germ cell types during testis maturation, the ratios of the number of the given germ cell type to the number of Sertoli cells in the same tubular cross-sections in each rat were evaluated.

2.6 Statistics

The distribution of the data was analysed using Shapiro-Wilk's test. The parametric data was presented as mean ±SD and the results were analyzed using either Student's *t* test (for two independent groups) or one-way ANOVA followed by the *least significant differences* (LSD)

as a *post hoc* test. On the other hand, nonparametric data was presented as median and the range [Min-Max] and a Mann-Whitney's U test (for two independent groups) or Kruskal-Wallis non-parametric test were used for analysis. Differences were considered significant at $p < 0.05$.

3 RESULTS

3.1 Hormone levels

Hormone levels were not determined in the short experiment.

In the *long term study*, the physiological blood level of fT3 at the 16th day of life was 2.01 ± 0.7 pg/ml; transient treatment increased it by 2.6 times (5.3 ± 2.7 pg/ml, $p < 0.05$), while continuous treatment increased it by 11.9 times (23.9 ± 2.7 pg/ml, $p < 0.001$). The serum level of rFSH in control II was 3.3 ± 1.4 ng/ml and was not significantly different in either experimental group (5.7 ± 4.9 ng/ml in tT3 and 6.7 ± 4.5 ng/ml in cT3). Similarly, both testosterone and estradiol serum levels were statistically unaltered in all experimental groups, which might suggest indirect T3 action. The blood level of testosterone was 0.9 ± 0.2 nmol/l in control II, 1.0 ± 0.2 nmol/l in tT3 and 1.1 ± 0.5 nmol/l in cT3. The blood level of estradiol was 249.5 ± 142.3 pmol/l in control II, 377.6 ± 133.1 pmol/l in tT3 and 245.9 ± 74.2 pmol/l in cT3.

3.2 Body and testes weights

In the *short term study*, body weight increased by 1.1 times under the influence of T3 treatment (12.8 ± 0.9 g in sT3 vs. 11.6 ± 0.6 in C I, $p < 0.05$). In the *long term study*, body weight did not change in tT3 (31.5 ± 2.9 g) and decreased by 1.2 times the control II value in cT3 (25.9 ± 1.9 vs. 29.3 ± 1.4 in control II), suggesting a higher metabolic rate (thyrotoxic effect). Relative testes weight (mg/10g b.w.) increased by 1.5 times of the control I value in sT3 (22.3 ± 5.2 mg vs. 15.0 ± 1.7 , $p < 0.01$), by 1.4 times in tT3 (44.5 ± 3.7 , $p < 0.01$) and did not change in cT3 (33.2 ± 2.5 mg) in comparison to control II (34.7 ± 6.2 mg).

3.3 Seminiferous tubule morphology

On pnd 6, the seminiferous epithelium consisted of Sertoli cells and Gc, Gp and type A spermatogonia. Irregular ovoid to elongated Sertoli cell nuclei with distinct nucleoli were oriented perpendicular and placed peripherally along a basement membrane (Fig.1A). In sT3 Sertoli cell nuclei were more irregularly located in more than one layer; Gc were fewer, and type A spermatogonia were more abundant in comparison to control I. Degenerating germ cells, when located in the centre of tubule cross-sections, resembled Gc (Fig.1B). A tubular

lumen was absent in both groups. In both control I and sT3, PCNA-positive cells were almost all Sertoli cells and mostly type A spermatogonia, while PCNA-negative were Gc and Gp (Fig.1A,B, inserts).

Fig.2A, B and C show the tubular cross-sections representing germ cell association 2, corresponding to stages VII-VIII of the adult seminiferous epithelium cycle, examined on pnd 16. In all groups, spermatogonia were present along the basement membrane, and preleptotene spermatocytes were more numerous and irregularly placed. Pachytene spermatocytes were the most mature germ cells. In tT3 and cT3, Sertoli cell nuclei were displaced from the basement membrane into the centre. The morphological appearance (lack of central nucleoli) and location of Sertoli cell nuclei indicated their morphological immaturity. The tubule lumen not present in control I and II appeared in some tubules in tT3 and was frequent in cT3. In cT3, the tubular lumen was wider than in tT3 and the Sertoli cell cytoplasm was rich in vacuoles. Seminal tubules appear to be distended, indicating regressive changes in the seminiferous epithelium. Most germ cells were PCNA-positive, while most Sertoli cells were PCNA-negative (Fig.2D, E, F).

3.4 Seminiferous tubule morphometry

sT3 Seminiferous was found to increase tubule diameter to 1.2 times the value of control I. In both older experimental groups, seminiferous tubule diameter was elevated by 1.2 times the control II value (Fig.3a). Total length of the seminiferous tubule did not change in sT3 and tT3 but was reduced by half by cT3 (Fig. 3b). The seminiferous epithelium was 1.3 times higher in younger animals treated with T3 in comparison to the control I. On the other hand reduction of this parameter was observed in both 16-day old experimental rats (Fig.3c).

The seminiferous tubules had not started the canalization process by pnd 6. The median percentage of tubules containing lumen and did not change in tT3 but increased 68-fold in cT3 (Fig.4a). The mean seminiferous tubule lumen area did not change in tT3 and increased 4-fold in cT3 (Fig.4b).

3.5 Cell degeneration and proliferation

The incidence of tubules containing degenerating germ cells was increased 2-fold in sT3 in comparison to the control I, was unchanged in tT3 and increased 1.9 times the control II value in cT3 (Fig.5a).

The PCNA LI of germ cells was increased 1.5 times in sT3 in comparison to control I with the progression of age in untreated rats, between pnds 6 and 16, (control I vs. control II). The PCNA LI of germ cells increased by 2.4 times, reaching a value of $85.1 \pm 7.4\%$ in the control II. This parameter was unchanged in tT3 but increased by 1.1 times the original (control II) value in cT3 (Fig. 5b). In *short-term study*, the total number of germ cells per testis was increased by 1.9 times the control I value in sT3. However, in *long-term study*, the increase (1.6 times the control II value) was observed only in tT3 (Fig. 5c).

The PCNA LI of Sertoli cells was decreased in sT3 to 0.84 times the control I value. In the the 16-day old animal group, the PCNA LI of Sertoli cells was significantly decreased to 3.5 ± 2.4 in tT3 and became undetectable in cT3 in comparison to control II (Fig. 5d).

3.6 Germ cell subtypes and Sertoli cell number

Table 1 and Fig. 6a. present the mean numbers of germ cell subtypes and Sertoli cells per testis ($\times 10^6$). In sT3, the number of Gc was reduced, without change in the number of Gp, while the number of type A spermatogonia increased 4-fold of the control I value.

In tT3, the number of undifferentiated type A spermatogonia decreased insignificantly with respect to control II, while the number of type A₁ spermatogonia decreased significantly. A 1.5-fold increase of type B spermatogonia number and a 1.6-fold increase of preleptotene spermatocytes in comparison to control II were also observed.

In cT3 the number of type A₁ spermatogonia was reduced, followed by a 0.6-fold decrease in both type B spermatogonia and preleptotene spermatocytes. The number of pachytene spermatocytes did not differ significantly after either treatment.

The number of Sertoli cells increased to 1.9 times the control I value in sT3. Decreases in both tT3 and cT3 by 0.6 times and 0.7 times the control values, respectively were observed with no significant difference between tT3 and cT3.

Table 2A presents the efficiencies of the succeeding steps of spermatogenesis: differentiation rates, yields. On pnd 6, sT3 stimulated differentiation of Gc into Gp by 4.6 times of control I and Gp into type A spermatogonia by 2.4 times. The yield of type A spermatogonia from Gc was 13 times greater than in the control I.

On pnd 16 (Table 2B) none of the treatments influenced the yield of undifferentiated type A spermatogonia. In tT3, the differentiation of B spermatogonia from A₁ spermatogonia increased by 2.6 times the control II value. The yield of preletotene spermatocytes from type A₁ spermatogonia increased by 2.3 times, exceeding the value described for 26-day-old rats (Clermont and Perey, 1957) and adult rats (Clermont, 1962). The yield of preleptotene spermatocytes from type B spermatogonia (result of the single mitotic division) was 1:2 in control II and was not modified by the treatments. Continuous treatment did not influence any of the examined germ cell ratios.

The capacity of Sertoli cells to support distinct germ cell types is presented in Table 3. In comparison with the control I, sT3 reduced number of Gc and increased number of type A spermatogonia per Sertoli cell. sT3 did not influence the ratio of total germ cells to Sertoli cells (Table 3 A). In tT3 germ cell – Sertoli cell ratios did not change vs. control II for undifferentiated A spermatogonia and type A₁ spermatogonia, but was 2.2 times greater ($p < 0.001$) for B spermatogonia and 2.4 times greater ($p < 0.001$) for preleptotene spermatocytes, approaching the value observed in adult rats (Berndtson and Thompson, 1990) and adult mice, as deduced from (Haywood et al., 2003). For the total number of germ cells, this ratio was also 2.4-times greater ($p < 0.001$) than in the control II. In cT3, the germ-Sertoli cell ratios did not change; they returned to the infantile, control II value (Table 3 B).

4 DISCUSSION

4.1 Main findings

Acting in a short perinatal window of time corresponding to spermatogenic onset, T3 significantly accelerated the entire spermatogonial development process. Increased germ cell differentiation, degeneration and proliferation were seen and on pnd 16, the ratios between seminiferous epithelium cells precociously attained adult values. These changes were normalised by continuous administration of the hormone.

4.2 Serum levels of hormones and testis growth

Although we did not determine blood hormones at spermatogenic onset, previous studies revealed that the serum level of FSH was either slightly increased (Jannini et al., 1993) or decreased (van Haaster et al., 1993) by T3 treatment at this point. This study revealed that FSH secretion was influenced by T3 on pnd 16, which was confirmed in a previous study on

rats (van Haaster et al., 1993), and serum levels of testosterone and estradiol were also unchanged, suggesting a direct hormonal effect of T3 on the testes.

Transient administration of T3 increased serum fT3 level by 2.6 times, despite discontinuation of the treatment 11 days earlier, while continuous treatment increased serum fT3 12-fold. T3 is prevalent within tissues, has high potency, rapid effects and rapid turnover. Panno et al. (1995) showed that in immature rats, the serum level of total T3 did not increase on the day following the 7-day intraperitoneal daily administration of T3. This discrepancy may arise from the different route of administration, here subcutaneous, and the higher dose used (100 µg/kg) instead 30 µg/kg. Indeed, it has been reported that in hypothyroid woman, erroneous oral intake of excessive T3 doses (5 mg every 8 hours) over 9 days resulted in a serum level of T3 397 times higher than normal after the treatment. T3 level was normalized 200 hours (8.3 days) after the intake of the latest dose, and clinical manifestation of toxic effects still lasted three days more (Botella de Maglia et al., 2003).

A short period of T3 treatment was seen to increase body weight, which is in accord with Bassett and Williams, (2003) who found that thyroid hormone accelerates body growth and bone age. Testes weight increased by pnd 6, which is also in accordance with an earlier study (Jannini et al., 1993; van Haaster et al., 1993). In our study, increased testes weight was noted also 11 days later, despite discontinuation of the treatment. In turn, the treatment prolonged to the end of the experiment did not affect testes weight, which again supports earlier results (van Haaster et al., 1993). The rise in testis weight during maturation is due to increase in weight of the seminiferous tubules (Bascom and Osterud, 1925). Indeed, increased seminiferous tubule diameter was observed after all treatments, however, seminiferous epithelium height decreased after prolonged treatment. A 68-fold increase in the incidence of tubular lumen was seen, which supports an earlier study (van Haaster et al., 1993) where a 4-fold increase of the lumen area might indicate tubule distention. It has been shown that T3 regulates the expression of basement membrane components in Sertoli cell extracellular matrix *in vitro* to varying degrees (Ulisse et al., 1998), which may be responsible for the varying effects of T3 on seminiferous tubule development.

4.3 Sertoli cells

T3 exerted a biphasic effect on Sertoli cell number in rats: a stimulatory influence on postnatal days, and an inhibitory influence during further seminiferous tubule development. It seems therefore that an increase of Sertoli cell number may be important for spermatogenic onset, whereas numerical stabilization of Sertoli cell compartment for spermatogonial development. Alternatively, early maturation and terminal differentiation of Sertoli cells may enhance the capacity of these cells to sustain the spermatogenic process and promote successful differentiation of the germ cells.

On pnd 6, Sertoli cell number was doubled by T3 treatment but their PCNA LI was decreased, indicating that T3 stimulated Sertoli cell proliferation earlier than within the 6 pnds window. On pnd 16, both treatments inhibited Sertoli cell PCNA LI, which is in agreement with earlier data indicating that T3 directly decreased mitogenesis in immature Sertoli cells *in vitro* together with accelerated progression of their maturation (Cooke et al., 1994). Increased Sertoli cell number on pnd 6 has been described by Jannini et al. (1993) but the biphasic effect of T3 on Sertoli cell number was not seen earlier in a single study before this.

Body weight decreased after continuous treatment exclusively, which may indicate a thyrotoxic effect. Reduced tubule length may be explained by a decrease in the number of Sertoli cells. The Sertoli cell cytoplasm became vacuolated, indicating a breakdown in Sertoli-germ cell junctions (Russell et al., 1990), which may be due to increased proteolysis in cell junctions, as has been demonstrated for cardiac myocytes in experimental thyrotoxicosis (Parmacek et al., 1986).

While transient treatment increased the spermatocyte - Sertoli cell ratio by 2.2 - 2.4 times, close to adult values in rodents (Berndtson and Thompson, 1990; Haywood et al., 2003), continuous treatment normalized it to infantile, control levels. Mature Sertoli cells have the capability to support a relatively fixed number of germ cells. Our results indicate that while short transient T3 treatment may evoke functional capability of Sertoli cells precociously, prolonged treatment and/or a high T3 level normalise it.

4.4 Germ cells

At spermatogenic onset T3 administration increased the yield of type A spermatogonia from gonocytes by 13 times, spermatogonia number by 4 times, germ cell degeneration 2 times, total germ cell number 1.9 times and their proliferation by 1.5 times. A hereto undescribed

finding of an enhancement of germ cell differentiation by T3 is complementary to an earlier observation in rats made hypothyroid since birth. In these rats spermatogenic onset was delayed in association with prolonged persistence of gonocytes (Simorangkir et al., 1997). T3 was also shown to stimulate the *in vitro* germ cell switch from somatic to germ-cell-specific gene expression which is responsible for body metamorphosis in *Xenopus laevis* (Abdallah et al., 1996).

We not only found that T3 increased germ cell number in 6-day-old rats, which is in accordance with an earlier study (Jannini et al., 1993), but we were able to specify that it increased the number of A spermatogonia rather than gonocytes. This imply that stimulation of gonocyte differentiation by T3 is inter-coordinated with increased proliferation of daughter cells. It has been previously shown that the transformation of gonocytes into spermatogonia does not involve cell divisions of gonocytes (de Rooij, 1998).

Germ cell death during spermatogenesis is necessary to remove abnormal or excessive cells and to maintain the correct cell number ratio between maturing germ cells and Sertoli-germ cell interactions (de Rooij and Grootegoed, 1998; Rodriguez et al., 1997). Increased germ cell degeneration may facilitate spermatogenic onset, but in contrast to our results, 3-day pre-treatment with T3 was shown to decrease germ cell degeneration on pnd 6 (Jannini et al., 1993). The discrepancy may arise from different duration of the treatment, here beginning from the day of birth, different route of administration, here subcutaneous rather than intraperitoneal, and the much lower daily dose of T3, 100 µg/kg b.w. instead of 1000 µg /kg as applied previously.

Transient T3 administration produced an increase in the total number of germ cells with an enhanced yield of preleptotene spermatocytes from A₁ spermatogonia, but neither germ cell proliferation nor degeneration were affected. Hence, transiently increased proliferation of A spermatogonia during first 5 pnds was responsible for an increase of germ cell number 11 days later. The progression from type A₁ spermatogonia into preleptotene spermatocytes examined here represents the entire process of spermatogonia development that, after T3 transient treatment, exceeded the values described for 26-day-old rats (Clermont and Perey, 1957) and adult rats (Clermont, 1962). TR has been localized in differentiating spermatogonia (intermediate type and type B spermatogonia) as well as in preleptotene spermatocytes (Buzzard et al., 2003). The requirement for differentiating spermatogonia to survive in the

regulation of cell density during spermatogenesis (de Rooij and Lok, 1987; Huckins, 1978) points to a potential new role of thyroid hormone in promoting germ cell differentiation and organization of the seminiferous epithelium. In analogy, thyroid hormones stimulate postnatal cellular differentiation, synaptogenesis and protect neural cells from death in the developing brain (Bernal and Nunez, 1995).

Continuous treatment and a high T3 level normalized total germ cell number and differentiation with their increased degeneration. It has been suggested that selective loss of differentiating spermatogonia in rodents is accounted for by the death of A₂, A₃, and A₄ spermatogonia (Clermont, 1962; de Rooij and Lok, 1987; Huckins, 1978; Huckins and Oakberg, 1978). It has been also described that during continual T3 administration since birth, germ cell degeneration begins to increase on pnd 12 where a simultaneous decrease in thyroid stimulating hormone secretion indicated thyrotoxicosis (van Haaster et al., 1993), which also increases germ cell apoptosis in adult rats (Faraone-Mennella et al., 2009). Increased degeneration of spermatogonia after continuous treatment could result in the absence of factors that function to block amplification of undifferentiating spermatogonia (de Rooij et al., 1985; van Keulen and de Rooij, 1975) resulting in increased proliferation of undifferentiated type A spermatogonia and stem cells between them. An unchanged secretion of endogenous FSH and estradiol, which have been shown to stimulate spermatogonia proliferation (Kula et al., 2001; Wahlgren et al., 2008; Walczak-Jedrzejowska et al., 2007; 2008), might participate in maintaining germ cell number and density on a normal level appropriate to the age.

5 CONCLUSION

T3 exerted a biphasic effect on Sertoli cell number in rats. The stimulatory influence was restricted to the first postnatal days whereas the inhibitory one appeared during further seminiferous tubule development. It seems, therefore, that an increase of Sertoli cell number is important for spermatogenic onset, whereas numerical stabilization of the Sertoli cell compartment is needed for further spermatogonial development. Alternatively, early maturation and terminal differentiation of Sertoli cells enhances their capability to support spermatogonial proliferation and differentiation, as well as ensure their own survival.

Administration of T3 in a short neonatal window increased testis growth and precociously induced quantitative completion of spermatogonial development. The stimulatory effect was

due to increased germ cell differentiation from the gonocyte onward, the proliferation of spermatogonia and degeneration of germ cells. Initiation of spermatogenesis was normalized by continuous T3 administration where an increase of germ cell degeneration and proliferation was present simultaneously. Vacuolation of Sertoli cell cytoplasm and a decrease in body weight might suggest an involvement of toxic effect of high serum level of T3. T3 may modulate the initiation of spermatogenesis in rats depending on treatment timing and T3 serum level.

Figure legends

Fig.1. Representative photomicrographs of seminiferous tubule cross-sections in 6-day-old rats (hematoxylin-eosine staining) from the control group (A) and after short T3 treatment (B). Immature Sertoli cells (white arrow), gonocytes located centrally (black arrow), gonocytes located peripherally (black arrowhead), type A spermatogonia (white arrowhead) are distinguishable. Note that after sT3, seminiferous tubule diameter is bigger and Sertoli cell nuclei are more numerous and more irregularly placed than in controls. Immunostaining for PCNA (inserts) appears with red coloured cell nuclei (PCNA - positive cells), PCNA-negative cells have blue coloured nuclei. (Bar - 10 μ m).

Fig. 2. Representative photomicrographs of seminiferous tubule cross-sections in 16-day-old rats from the control group CII (A), from rats treated transiently with T3 (tT3, B) or continuously (cT3, C) (hematoxylin/eosin staining, left panel, and immunostaining for PCNA, right panel). Seminiferous tubules represent germ cell association 2, characteristic for 16-day-old rats, and contain: Sertoli cells (white arrow), type A spermatogonia (arrowhead), preleptotene spermatocytes (black arrows) and pachytene spermatocytes (asterix, D). Note that tubule lumen (L) appears in tT3 (D), is much wider in cT3(E, F) and numerous vacuoles are present in Sertoli cell cytoplasm in cT3 (E). PCNA-positive cell nuclei are stained in red, PCNA-negative nuclei in blue. Note that in CII seldom Sertoli cells are PCNA-positive, but after tT3 or cT3 all Sertoli cell nuclei are PCNA-negative. (Bar - 10 μ m).

Fig.3. The influence of triiodothyronine after short (sT3) (6 postnatal day, pnd 6), transient (tT3) or continuous (cT3) administration (pnd 16) on seminal tubule diameter in micrometers (μ m) (a), total length of the seminiferous tubules in meters (m) (b) and the height of the seminiferous epithelium in μ m (c) (mean +S.D.). CI, control group for sT3; CII, control group for tT3 and cT3; (a) $p < 0.001$, (b) $p < 0.05$, Student's *t*-test for experimental sT3 vs. the control I group or one-way ANOVA followed by the *least significant differences* (LSD) as a *post hoc* test between tT3 and cT3 vs. the control II group. Number of animals in parentheses.

Fig.4. The influence of triiodothyronine on the 16th postnatal day after transient (tT3) and continuous (cT3) administration on the incidence of tubules containing lumen (a), and area of seminiferous tubule lumen (b). Boxplots (a) show median, the 25th and the 75th percentiles (Q1-Qu) and range (Min-Max). CII, control group; (a) $p < 0.01$, Kruskal-Wallis non-parametric test were used for experimental groups vs. CII. Charts (b) represent mean +S.D., (c) $p < 0.05$. One-way ANOVA followed by the *least significant differences* (LSD) as a *post hoc* test between tT3 and cT3 vs. the control II group. Number of animals in parentheses.

Fig.5. The influence of triiodothyronine after short (sT3) (6 postnatal day, pnd 6), transient (tT3) or continuous (cT3) administration (pnd 16) on the incidence of tubule cross-sections containing degenerating germ cells (a), immunohistochemical labelling of proliferating cell nuclear antigen (PCNA LI) of germ cells (%) (b) total number of germ cells ($\times 10^6$ per testis) (c) and PCNA LI (%) of Sertoli cells (d), (means +S.D.). CI, control group for sT3; CII, control group for tT3 and cT3; N.D., not detectable; (a) $p < 0.001$, (b) $p < 0.01$, (c) $p < 0.05$; Student's *t*-test for experimental sT3 vs. the control I group or one-way ANOVA followed by the *least significant differences* (LSD) as a *post hoc* test between tT3 and cT3 vs. the control II group. Number of animals in parentheses.

Fig.6. Graphic description of the influence of triiodothyronine after short (sT3) (6 postnatal day, pnd 6) (A), transient (tT3) and continuous (cT3) administration (pnd 16) (B) on germ cell numbers. The subsequent (from left to the right) steps (immediate or not immediate) are presented at spermatogenic onset (A), and at completion of spermatogonial development (B). Abbreviations include centrally located gonocytes (Gc), peripheral gonocytes (Gp), type A spermatogonia (SgA), undifferentiated type A spermatogonia (SgUnA), type A₁ differentiating spermatogonia (SgA1), type B spermatogonia (SgB), preleptotene spermatocytes (ScPL) and pachytene spermatocytes (ScPA). Numbers are expressed as the percentage changes vs. control groups (100%). Note the logarithmic scale. CI, control group for sT3; CII, control group for tT3 and cT3. Student's *t*-test for experimental sT3 vs. the control I group or one-way ANOVA followed by the *least significant differences* (LSD) as a *post hoc* test between tT3 and cT3 vs. the control II group were performed on absolute values, presented in Table 1; (a) $p < 0.001$, (b) $p < 0.01$, (c) $p < 0.05$ for experimental vs. the respective control group. Number of animals in parenthesis.

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Table 1.

Germ and Sertoli cell numbers (million per testis) after short, transient and continuous administration of triiodothyronine (T3) (mean \pm S.D.) *

Cell type	PND 6		PND 16		
	C I (n=7)	short T3 (n=5)	C II (n=5)	transient T3 (n=5)	countinuous T3 (n=5)
Gc	0.33 \pm 0.09	0.12 \pm 0.03 ^b	-	-	-
Gp	0.21 \pm 0.09	0.36 \pm 0.18	-	-	-
SgA	0.17 \pm 0.10	0.78 \pm 0.16 ^a	-	-	-
SgUnA	-	-	1.4 \pm 0.65	0.8 \pm 0.17	0.9 \pm 0.22
SgA ₁	-	-	1.4 \pm 0.18	0.9 \pm 0.40 ^c	0.8 \pm 0.43 ^c
SgB	-	-	10.9 \pm 1.43	16.2 \pm 4.48 ^c	6.1 \pm 1.82 ^b
ScPL	-	-	23.7 \pm 4.46	38.8 \pm 8.88 ^b	15.1 \pm 3.37 ^b
ScPA	-	-	0.5 \pm 0.40	1.6 \pm 0.29	0.1 \pm 0.30
Sertoli cells	7.01 \pm 1.56	10.70 \pm 2.40 ^c	34.5 \pm 5.89	23.1 \pm 4.27 ^c	19.2 \pm 4.63 ^b

* Abbreviations, PND, postnatal day; C, control group; *n*, number of animals; Gc, gonocytes located centrally; Gp, gonocytes located on the periphery; SgA, type A spermatogonia; SgUnA, undifferentiated type A spermatogonia; SgA₁, type A₁ spermatogonia; SgB, type B spermatogonia; ScPL, preleptotene spermatocytes; ScPA, pachytene spermatocytes.

(a) $P < 0.001$; (b) $P < 0.01$; (c) $P < 0.05$ Student's *t* test for experimental vs. control CI group .

Anova and the least significant differences (LSD) as post hoc test for experimental vs. control CII group.

Table 2.

Efficiencies of germ cell differentiation in immediate (Gc:Gp; Gp:SgA; SgUnA:SgA₁; SgB:ScPL) and not immediate (Gc:SgA SgA₁:SgB; SgA₁:ScPL) steps of spermatogenic onset (short T3, A), and entire spermatogonial development (transient and continuous T3, B) administration of triiodothyronine (T3) (mean \pm S.D.)*

A (pnd 6)

Group	<i>n</i>	Gc : Gp	Gp : SgA	Gc : SgA
Control I	7	1 : 0.7 \pm 0.2	1 : 1.0 \pm 0.8	1 : 0.5 \pm 0.4
short T3	5	1 : 3.2 \pm 2.1 ^b	1 : 2.4 \pm 1.0 ^c	1 : 6.6 \pm 2.8 ^b

B (pnd 16)

Group	<i>n</i>	SgUnA : SgA ₁	SgA ₁ : SgB	SgB : ScPL	SgA ₁ : ScPL
Control II	5	1 : 1.2 \pm 0.4	1 : 7.6 \pm 0.8	1 : 2.2 \pm 0.4	1 : 18.9 \pm 6.9
transient T3	5	1 : 1.0 \pm 0.4	1 : 19.5 \pm 8.1 ^b	1 : 2.5 \pm 0.4	1 : 44.3 \pm 8.4 ^a
continuous T3	5	1 : 0.9 \pm 0.3	1 : 8.7 \pm 3.4	1 : 2.6 \pm 0.7	1 : 16.6 \pm 3.9
12-day-old intact rat (Clermont and Perey, 1957)			1 : 6.4	1 : 1.2	1 : 7.9
26-day-old intact rat (Clermont and Perey, 1957)			1 : 12.5	1 : 2.0	1 : 25.6
Adult intact rat (Clermont, 1962)			1 : 12.2	1 : 2.1	1 : 26.3

* Abbreviations: pnd, postnatal day; *n*, number of animals; Gc, gonocytes located centrally in the tubule; Gp, gonocytes located on the periphery of the tubule; SgA, type A spermatogonia; SgUnA, undifferentiated type A spermatogonia; SgA₁, type A₁ spermatogonia; SgB, type B spermatogonia; ScPL, preleptotene spermatocytes. (a) $p < 0.001$; (b) $p < 0.01$; (c) $p < 0.05$, vs. Controls.

Table 3.

Germ cells/Sertoli cell ratios on pnd 6 (A) and pnd 16 (B) after short, transient or continuous administration of triiodothyronine (T3) (mean \pm S.D.) *

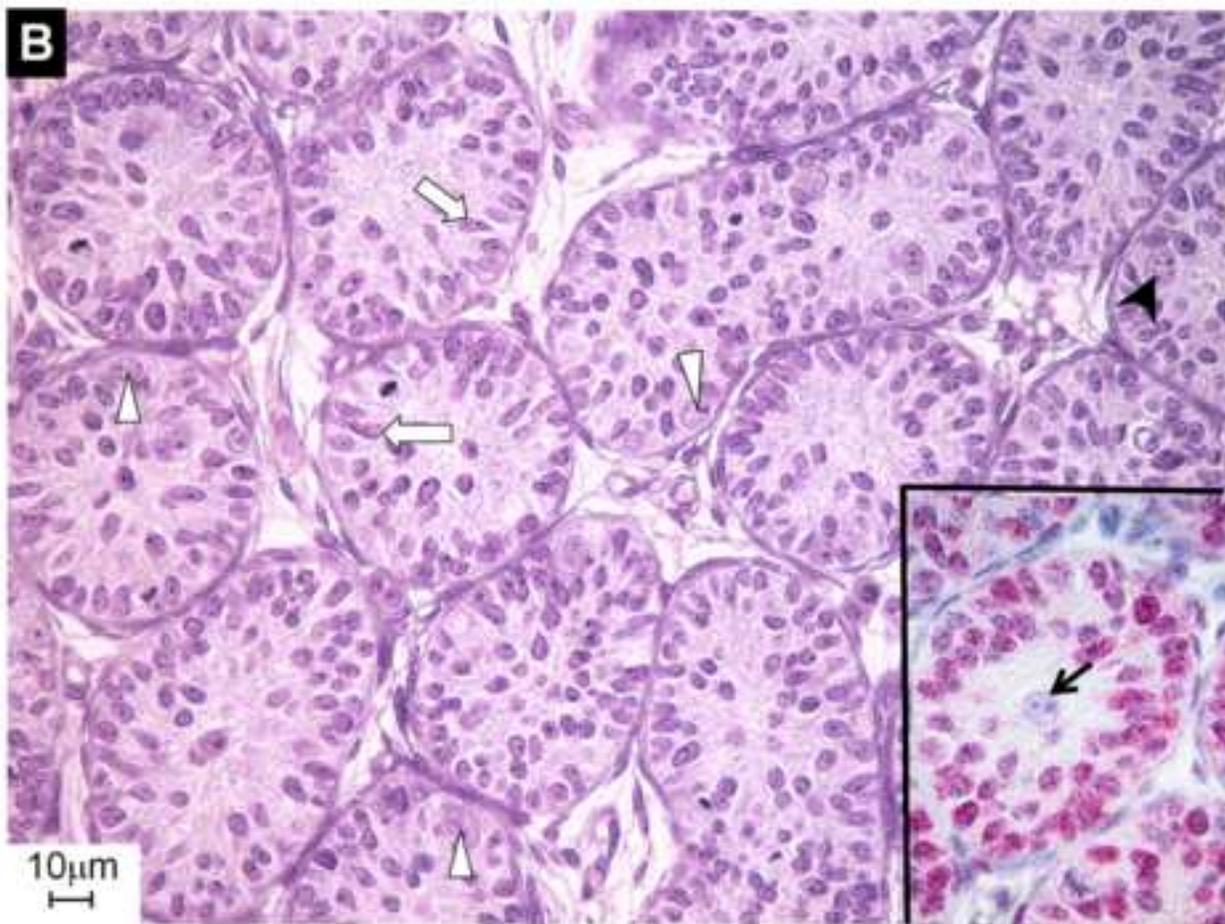
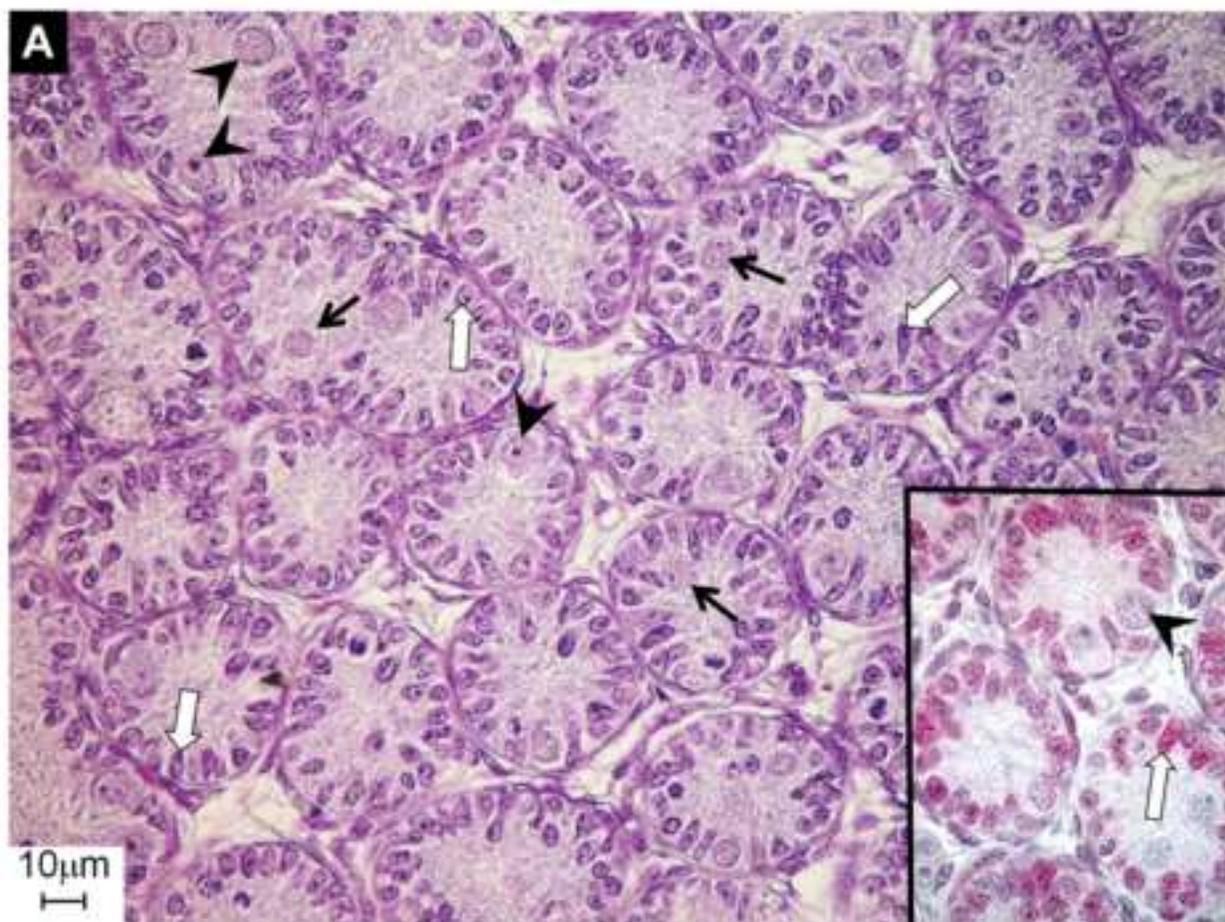
A (pnd 6)

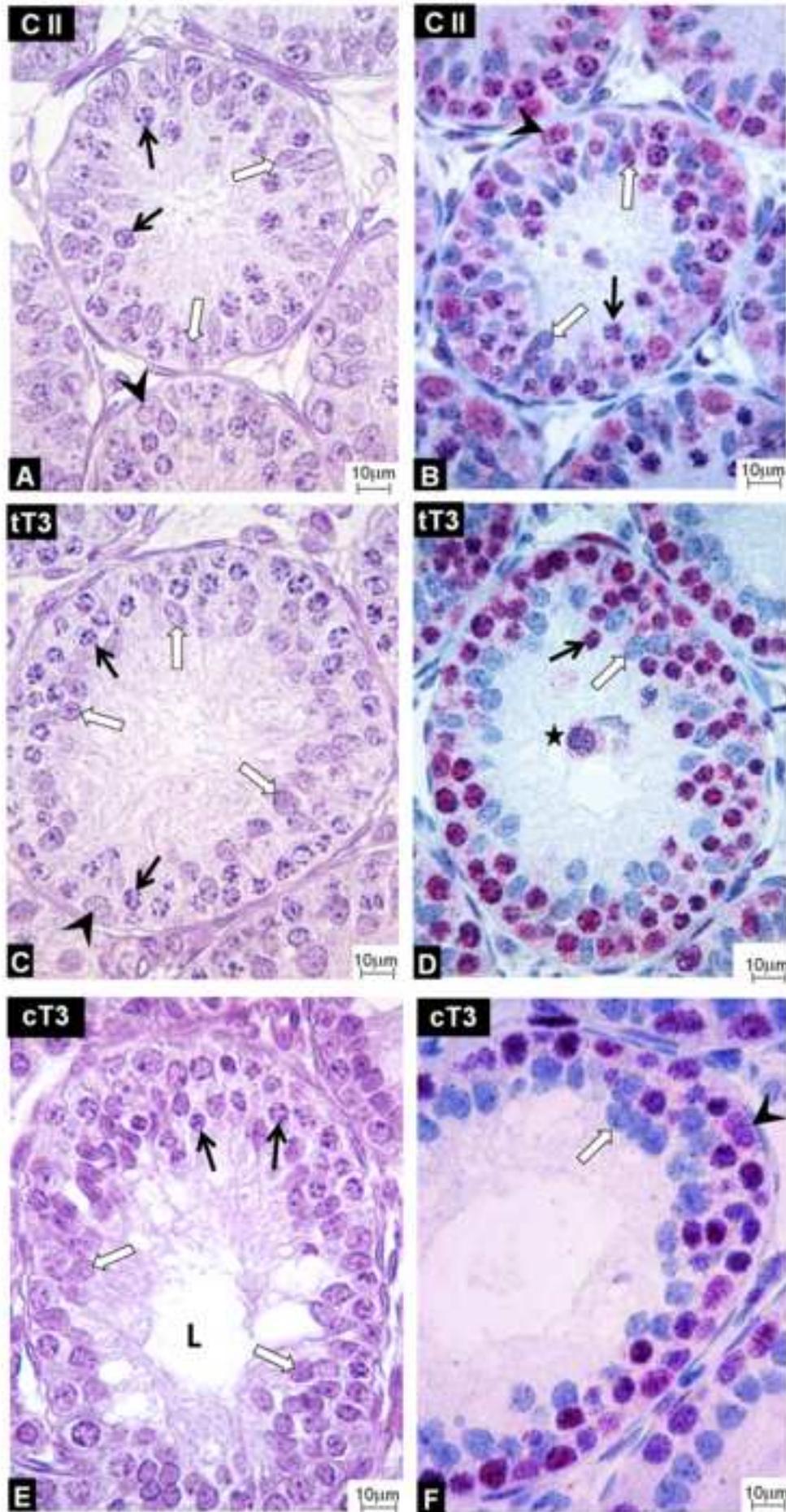
Group	<i>n</i>	Gc/S	Gp/S	SgA/S	Total/S
Control I	7	0.05 \pm 0.02	0.03 \pm 0.01	0.02 \pm 0.01	0.10 \pm 0.03
short T3	5	0.01 \pm 0.004 ^b	0.03 \pm 0.01	0.06 \pm 0.01 ^b	0.10 \pm 0.01

B (pnd 16)

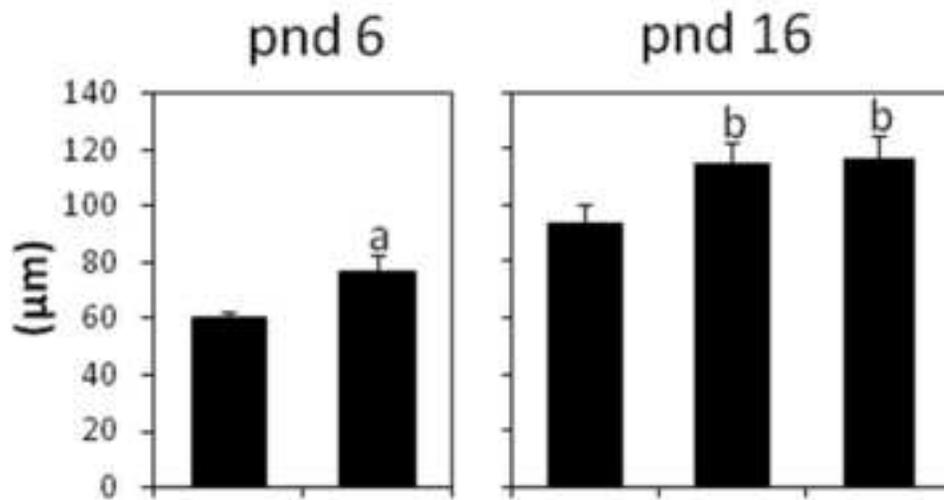
Group	<i>n</i>	SgUnA/S	SgA ₁ /S	SgB/S	ScPL/S	Total/S
Control II	5	0.04 \pm 0.01	0.04 \pm 0.01	0.31 \pm 0.07	0.67 \pm 0.11	1.08 \pm 0.16
transient T3	5	0.04 \pm 0.01	0.04 \pm 0.01	0.69 \pm 0.12 ^a	1.64 \pm 0.12 ^a	2.55 \pm 0.31 ^a
continuous T3	5	0.05 \pm 0.01	0.04 \pm 0.02	0.30 \pm 0.06	0.74 \pm 0.14	1.14 \pm 0.20
Adult intact rat (Berndtson and Thompson, 1990)					2.40 \pm 0.07	
Adult intact mice (deducted from Haywood et al., 2003)					range: 1.50 – 2.0	

* Abbreviations: pnd, postnatal day; *n*, number of animals; Gc, gonocytes located centrally in the tubule; Gp, gonocytes located on the periphery of the tubule; SgA, type A spermatogonia; SgUnA, undifferentiated A spermatogonia; SgA₁, A₁ spermatogonia; SgB, B spermatogonia; ScPL, preleptotene spermatocytes; Total, total number of germ cells; S, Sertoli cell. (a) $p < 0.001$, (b) $p < 0.01$, vs. Control.

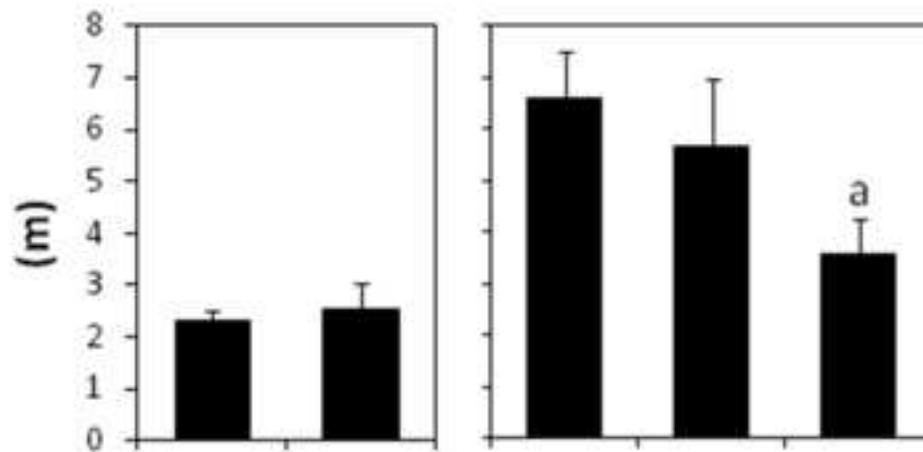




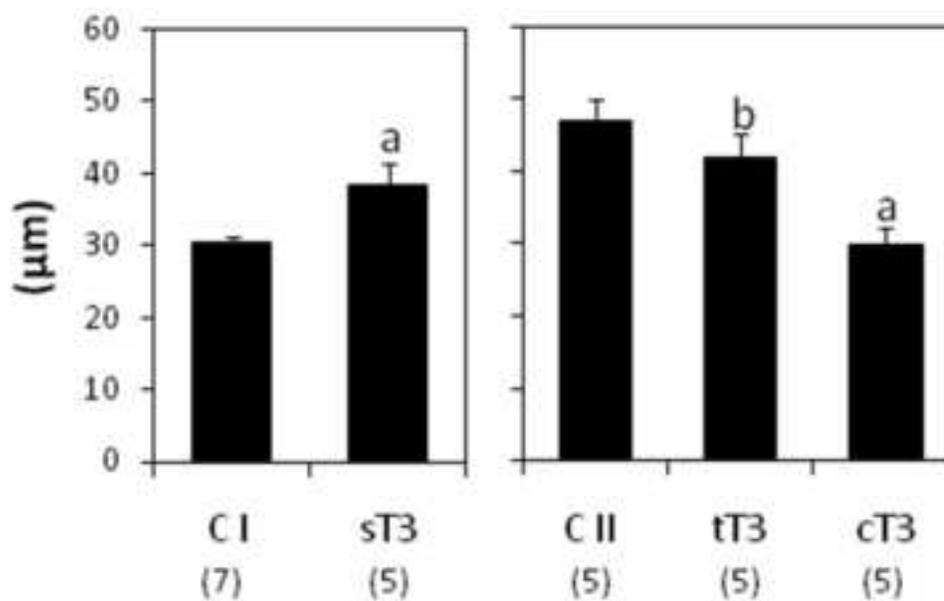
a. Seminiferous tubule diameter

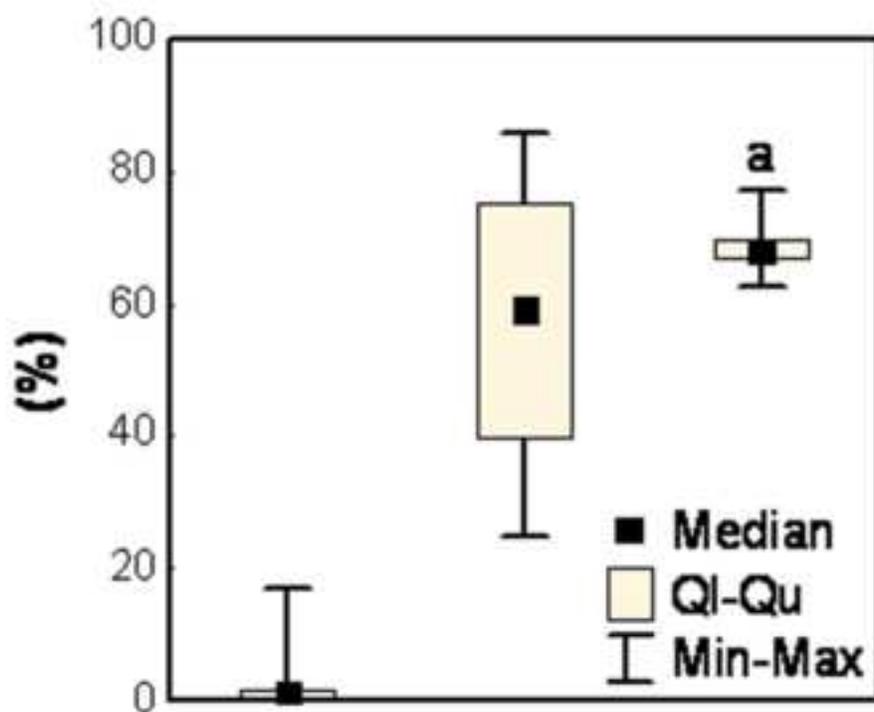


b. Seminiferous tubule length



c. Height of seminiferous epithelium



a. Tubules containing lumen**b. Tubule lumen area**