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Postnatal testis development, Sertoli cell proliferation and number of different spermatogonial types in C57BL/6J mice made transiently hypo- and hyperthyroidic during the neonatal period

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Abstract

The role of thyroid hormones in testis structure and function has been fairly well studied in laboratory rodents. However, there are no comprehensive data in the literature for mice regarding the effects of transiently induced neonatal hypo- and hyperthyroidism on testis and spermatogonial cell development from birth to adulthood. Our goals were to evaluate the effects of propylthiouracil (PTU) and triidothyronine (T3) on Sertoli cell proliferation/differentiation and to correlate these events with the evolution of the spermatogenic process, tubular lumen formation, blood vessel volume density, and size and number of different spermatogonial types. Although Sertoli cell maturation was accelerated or delayed, respectively, in T3- and PTU-treated mice, the pace of the germ cell maturation was only slightly altered before puberty and the period of Sertoli cell proliferation was apparently not affected by the treatments. However, compared with controls, the total number of Sertoli cells per testis from 10 days of age to adulthood was significantly increased and decreased in PTU- and T3-treated mice, respectively. In comparison to all other spermatogonia, type A_2 was the largest cell in all ages and groups investigated. The PTU-treated mice had a significantly increased total number of undifferentiated spermatogonia as well as volume and percentage of vessels/capillaries, probably due to the higher number of Sertoli cells, particularly at 10 days of age. Taken together, our results suggest that neonatal hypothyroidism may be a valuable tool for studying spermatogonial biology as well as a means for providing more spermatogonial stem cells that could potentially be used for spermatogonial transplantation, thereby optimizing the efficiency of this technique when young mice are used as donors.

Key words C57BL/6J mice; propylthiouracil (PTU); Sertoli cell; spermatogonia; triidothyronine (T3); testis development.

Introduction

Sertoli cells are the first somatic element to differentiate in the testis and their precursors express the sex-determining gene *Sry* in the short arm of the Y chromosome (Karl & Capel, 1998; Capel, 2000; DiNapoli & Capel, 2008). Therefore, Sertoli cells play a central role in testis differentiation, which occurs around 11.5 days post-coitus in mice (Capel, 2000). Sertoli cells proliferate more actively before birth and, in rodents, proliferation extends to 2 (mice) to 3 weeks (rats) after birth (Steinberger & Steinberger, 1971; Orth, 1982; Vergouwen et al. 1991; Joyce et al. 1993). Thereafter, the number of Sertoli cells per testis is considered stable throughout the life of the animal (França et al. 2005).

During early development, the primordial germ cells colonize the bipotential gonads and become gonocytes (Ross & Capel, 2005). In the testis of newborn mice, these gonocytes directly give rise to tyrosine kinase receptor (c-kit)-positive differentiating spermatogonia as well as Neurogenin (Ngn3)-positive undifferentiated spermatogonia (Yoshida et al. 2006). In laboratory rodents, stem cell functions reside in a small subset of spermatogonia designated undifferentiated spermatogonia (A_{und}), which are classified as A_{single} (isolated single cells), A_{paired} (chains of 2 cells) and $A_{aligned}$ (chains of 4, 8, 16 or occasionally 32 cells) (De Rooij & Russell, 2000). $A_{aligned}$ chains transform into differentiated type A_1 spermatogonia, followed by several mitotic divisions that give rise to A_2, A_3, A_4, intermediate (In) and type B (B) spermatogonia as well as primary pre-leptotene

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spermatocytes, which initiates the first meiotic prophase (De Rooij & Russell, 2000; De Rooij & Grootegoed, 1998). The spermatogonial cells are located in the basal compartment of the seminiferous epithelium, in direct contact with Sertoli cells and the basement membrane. Particularly for the A_{single} spermatogonia, this surrounding microenvironment is denominated the spermatogonial stem cell niche and spermatogonial stem cell self-renewal is maintained by the glial cell line-derived neurotrophic factor (GDNF), produced by Sertoli cells (Hofmann, 2008).

The different spermatogonial types can be characterized *in toto* by different methodologies, such as whole-mount (De Rooij & Russell, 2000), and by light and transmission electron microscopy based on the presence and distribution of heterochromatin (Chiarini-Garcia & Russell, 2001, 2002). It has been suggested that undifferentiated spermatogonia – probably including A_{single} or stem cells – are preferentially located in particular regions of the seminiferous epithelium adjacent to the intertubular compartment (Chiarini-Garcia et al. 2001; Ogawa et al. 2005), more specifically close to the blood vessels (Shetty & Meistrich, 2007; Yoshida et al. 2007).

The period of Sertoli cell mitotic activity is extended by approximately 2 weeks in laboratory rodents made transiently hypothyroidic by reversible goitrogen 6-propyl-2-thiouracil (PTU) treatment during the neonatal period, leading to a significant increase in testis size, number of Sertoli cells per testis and sperm production in adult animals (Cooke et al. 1991, 2005; Van Haaster et al. 1992; Joyce et al. 1993; Holsberger & Cooke, 2005; Holsberger et al. 2005a,b). In contrast, hyperthyroidism induced by triidothyronine (T3) accelerates Sertoli cell differentiation/maturation, resulting in a lower number of Sertoli cells per testis and lower sperm production (Van Haaster et al. 1993; Holsberger & Cooke, 2005). According to Hess et al. (1993), the larger Sertoli cell population stemming from the hypothyroidism is followed by an increased number of germ cells in the adult testis but this study did not investigate the number of the different spermatogonial types. Thus, the main objective of the present study was to evaluate the effects of neonatal hypo- and hyperthyroidism on testis development, Sertoli cell proliferation/differentiation and the correlation of these events with the evolution of spermatogenesis and sperm production. Moreover, the number and volume of undifferentiated (A_{single}, A_{paired} and $A_{aligned}$) and differentiated (type $A_{1–4}$, intermediate and type B spermatogonia) spermatogonia were evaluated for the first time in mice, along with volume density and volume of the testis vascular components from birth to adulthood.

Materials and methods

Animals and treatments

The C57BL/6J mice were bred in our colony and housed at 22 °C with a 12L : 12D photoperiod and given standard rodent food *ad libitum*. A total of 128 mice were used (control, $n = 53$; PTU-treated mice, $n = 47$; T3-treated mice, $n = 28$). Hypothyroidism was induced as described by Joyce et al. (1993) and the pups received PTU through the mother's milk from birth to 20 days of age. To make hyperthyroid pups, T3 (Sigma) was dissolved in 0.025 N sodium hydroxide and diluted in physiological saline. T3 was administered by daily subcutaneous injections of 100 $\mu g\ kg^{-1}$ body weight from day 1 to day 15 (Van Haaster et al. 1993). Testes were removed from mice at 1, 5, 10, 15, 20, 28 and 100 days of age (day of birth = 1). At least four animals were used for each age and treatment. All procedures and protocols followed approved guidelines for the ethical treatment of animals (Ethics Committee in Animal Experimentation from the Federal University of Minas Gerais - CETEA/UFMG).

Tissue preparation

The mice were killed at 1 and 5 days of age by decapitation and the testes were fixed by immersion in 5% (v/v) glutaraldehyde in 0.05 M sodium cacodylate buffer. Testes from mice of 10–100 days of age were perfused-fixed following the procedure described by Sprando (1990). After being separated from the epididymis and weighed, the testes were prepared for high-resolution light microscopy evaluations (Chiarini-Garcia & Russell, 2001) and stereological analysis. Briefly, the testes were cut into 0.5-mm slabs by perpendicular sectioning. The slabs were then cut into approximately 2-mm (length × width) portions and embedded such that cross-sections of seminiferous tubules would be obtained on sectioning. After an overnight fixation of diced slabs, the tissues were washed in three changes of buffer, post-fixed in an osmium-ferrocyanide mixture, dehydrated, embedded in Araldite resin, sectioned at approximately 1 μm thickness and stained with toluidine blue. From the knowledge of the body weight and testicular weight, the gonadosomatic index (testes mass divided by body weight) was estimated for all mice investigated.

Evolution of spermatogenesis and lumen formation

The specific basis for identifying the gonocytes and spermatogonia (undifferentiated and differentiated) followed those described by Vergouwen et al. (1991) and Chiarini-Garcia & Russell (2001), respectively. The most advanced germ cell type present in the seminiferous cords/tubules was investigated through a careful evaluation of all histological sections obtained from the mice killed from 5 to 28 days of age. This evaluation was not performed in newborn and adult mice, as these animals, respectively, presented only gonocytes (see below) or full spermatogenesis. The presence of lumen was evaluated from the analysis of 100 seminiferous cord/tubule cross-sections for each mouse – also killed from 5 to 28 days of age.

Testis stereology

The diameter of the seminiferous cords/tubules was measured at 400 × magnification using an ocular micrometer calibrated with a stage micrometer. Between 10 and 20 tubule profiles that were either round or nearly round were chosen randomly and measured for each animal. The volume densities of testicular tissue components were determined by light microscopy using a 441-intersection grid placed in the ocular of the light microscope. A total of 30 (1–15 days of age), 40 (20 days) and

80 (28 and 100 days) randomly chosen fields (13 230, 17 640 and 35 280 points, respectively) were scored for each animal at 1000 × magnification. Artifacts were rarely seen and were not considered in the total number of points used to obtain volume densities. The testicular components were classified as one of the following: gonocytes, undifferentiated spermatogonia (A_{und}), differentiated spermatogonia [(A_1, A_2, A_3 and A_4), In and B] and Sertoli cells. The vessels (capillary, artery, vein and lymphatic vessel) were also evaluated. The volume of each component of the testis was determined as the product of the volume density and testis volume. For subsequent stereological calculations, the specific gravity of testis tissue was considered to be 1.0 (França & Godinho, 2003). To obtain a more precise measure of testis volume, the testis capsule was excluded from the testis weight. The Sertoli cell nuclear volume was obtained from the knowledge of the mean nuclear diameter and 40 evident nuclei were measured for each animal. Nuclear volume was expressed in μm^3 using the formula $4/3\Pi r^3$, where R = nuclear diameter/2.

Cell counts and cell numbers

Individual volumes of the gonocytes and different types of spermatogonia (undifferentiated and differentiated) were obtained from the nucleus volume as well as the proportion between nucleus and cytoplasm. As these germ cell nuclei are usually round or ovoid, their nucleus volume was obtained from the knowledge of the mean nuclear diameter. For each cell type, 30–40 nuclei had their diameter measured for each animal. Germ cell nuclear volume was expressed in μm^3 and obtained as mentioned above. To estimate individual germ cell volume, a 441-point square lattice was placed over the sectioned material at 1000 × magnification. A total of 20 cells of each germ cell type were evaluated for each animal. The number of germ cells per testis was estimated from the individual germ cell volume and the volume occupied by the particular germ cell in the testis parenchyma. Because, from 10 days of age, the germ cell associations that compose each of the XII stages of the epithelium cycle could already be characterized, the total number of undifferentiated spermatogonia was divided by 8.7 days, which is the duration of one cycle of the seminiferous epithelium in C57BL/6J mice, according to unpublished data from our laboratory. This divisor was utilized for all undifferentiated spermatogonia of all groups investigated from 10 to 100 days of age and took into account that these spermatogonia are present in all stages of the seminiferous epithelium cycle (De Rooij, 1998). For the other cell types (A_1 to B), the duration of a particular stage in which these cells are located was used as a divisor. The total number of Sertoli cells per testis was determined as follows: total number of Sertoli cells per testis = total volume of Sertoli cell nucleus in the testicular parenchyma (μL)/Sertoli cell nuclear volume (μm^3).

In order to estimate the meiotic index (number of newly-formed round spermatids per primary pachytene spermatocyte), Sertoli cell efficiency (number of spermatids per Sertoli cell) and spermatogenic efficiency (daily sperm production per testis) in 100-day-old mice, pachytene spermatocytes, round spermatids and Sertoli cell nucleoli present in stage VII (Russell et al. 1990) were counted in 10 randomly chosen round or nearly round seminiferous tubule cross-sections for each animal. These counts were corrected for section thickness and for nucleus or nucleolus diameter, based on the procedure described by Abercrombie (1946) and modified by Amann & Almquist (1962). For this purpose, the diameters of 10 nuclei (from pachytene spermatocytes and spermatids) or nucleoli (from Sertoli cells) were measured per animal. The cell ratios were obtained from the corrected counts obtained in stage VII. Spermatogenic efficiency was obtained from the following formula: Daily Sperm Production (DSP) = [total number of Sertoli cells per testis × ratio of round spermatids to Sertoli cells in stage VII × stage VII relative frequency (%)]/stage VII duration (in days) (França, 1992).

Statistical analysis

All data are presented as mean ± SEM and were analyzed using ANOVA. Analysis was performed using the STATISTICA 3.11 software

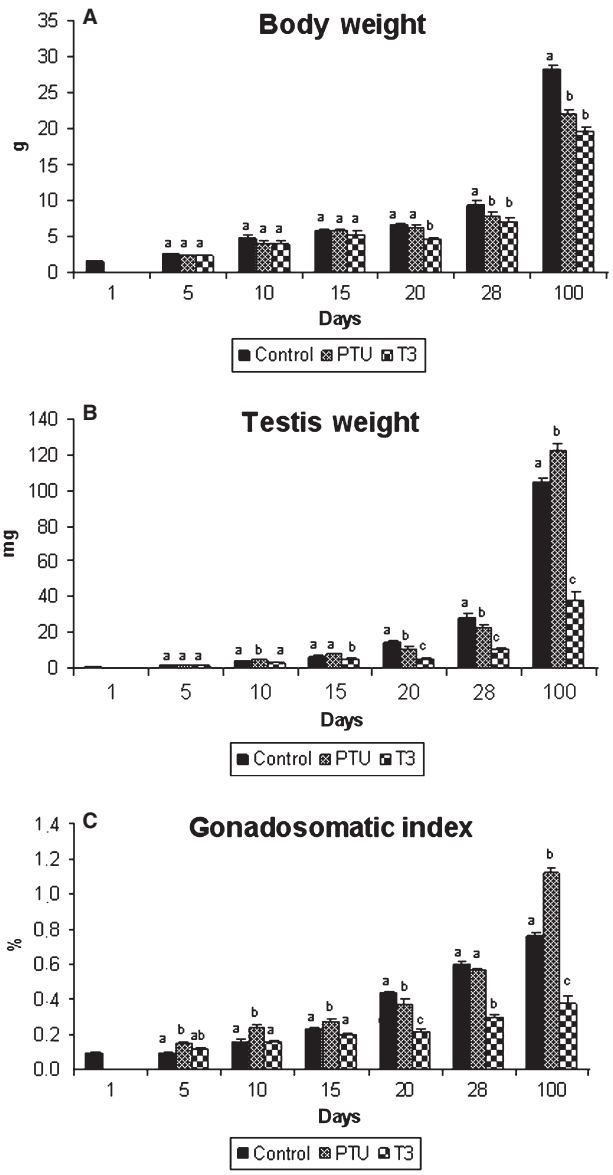

Fig. 1 Body weight (A), testis weight (B) and gonadosomatic index (C) in controls, propylthiouracil PTU- and T3-treated animals killed at different ages after birth (mean + SEM). Different letters for the same age denote significant differences ($P < 0.05$).

program for Windows (StatSoft, Inc., Tulsa, OK, USA). The significance level was set at $P < 0.05$.

Results

Biometric data

Body weight, testicular weight and gonadosomatic index for the control, PTU- and T3-treated mice are illustrated in Fig. 1. In comparison to the other two groups, body weight was significantly decreased in T3-treated mice at 20 days of age, whereas the body weight of the controls was greater at 28 and 100 days of age ($P < 0.05$) than that of mice subjected to either treatment. Compared with the controls, the testicular weight of PTU-treated animals was greater ($\sim$ 30%; $P < 0.05$) at 10 and 100 days, whereas the mice treated with T3 always had lower testis weight ($P < 0.05$) from 15 days of age to adulthood. The gonadosomatic index was higher ($P < 0.05$) in PTU-treated mice between 5 and 15 days of age as well as in adult animals. Conversely, following the same trend observed for the testis weight, the gonadosomatic index was always lower ($P < 0.05$) in T3-treated mice evaluated from 20 to 100 days of age.

Evolution of spermatogenesis, diameter of seminiferous cords/tubules and lumen formation

In all groups evaluated, gonocytes, differentiated A_3 spermatogonia, pre-leptotene spermatocytes and pachytene spermatocytes were, respectively, the most advanced germ cell types in the seminiferous epithelium at 1, 5, 10 and 15 days of age (Figs 2 and 3). Although few different germ

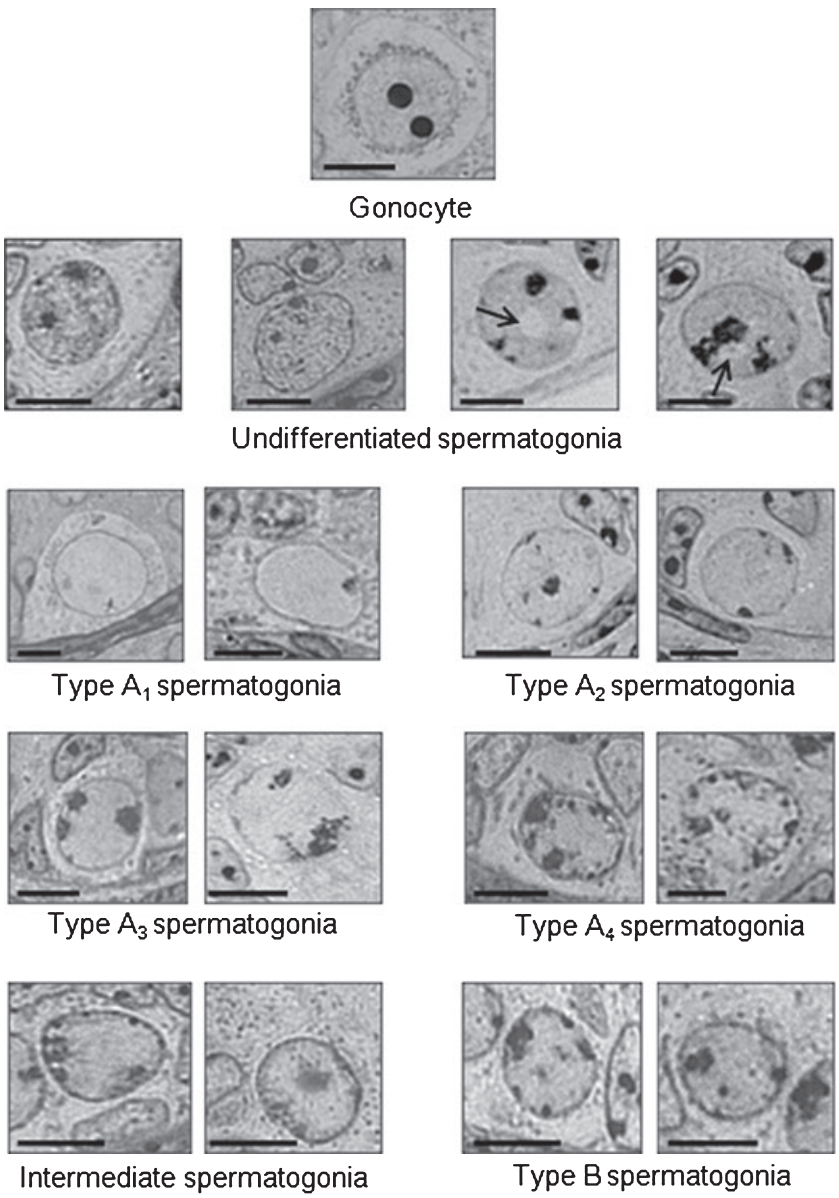

Fig. 2 The gonocyte and spermatogonia morphology in young animals followed criteria similar to those described by Vergouwen et al. (1991) and Chiarini-Garcia & Russell (2001). Briefly, gonocytes have the largest volume with one to three nucleoli. The undifferentiated spermatogonial population shows a nucleus with mottled chromatin and nuclear vacuoles are often present in the plane of the section (arrows). Differentiated type A_1 spermatogonia have a nucleus clearly demarcated from the surrounding nucleoplasm; differentiated type A_2 spermatogonia have small flakes (< 10%) of heterochromatin rimming the nuclear envelope; the differentiated type A_3 spermatogonia nucleus is slightly increased compared with A_2 and the nucleoli are generally reticulated and not well defined but extend deeply into the nucleoplasm; and differentiated type A_4 spermatogonia have 30–70% of the nuclear envelope encrusted with heterochromatin. Only small differences are noted when these cells are compared with A_3. Intermediate spermatogonia present shallow heterochromatin lining 70–100% of the nucleus, making the nuclear/cytoplasmic boundary well delineated. Type B spermatogonia (B) have small, densely stained spots of heterochromatin attached to the nuclear envelope. Bar = 7 μm.

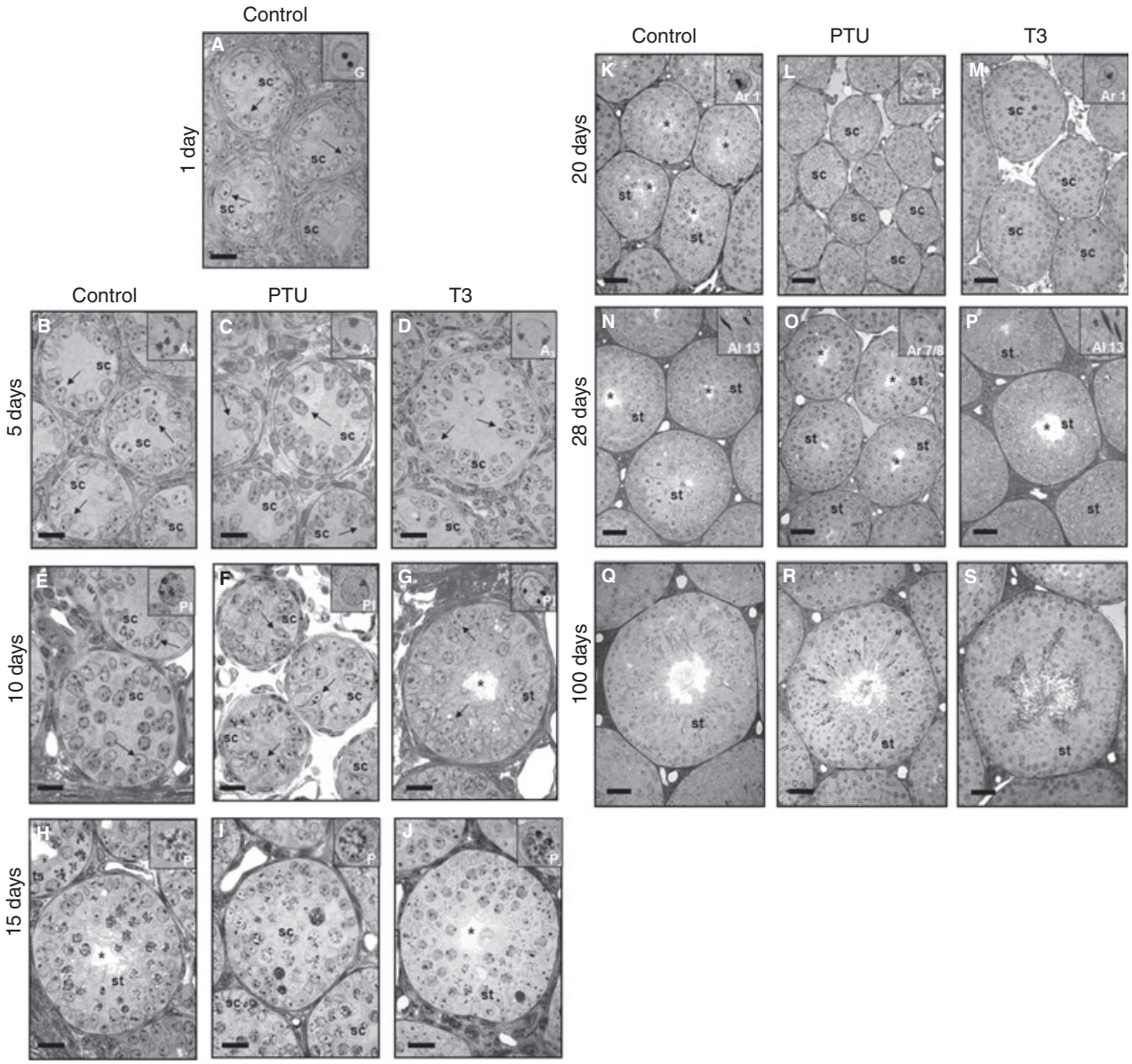

Fig. 3 Seminiferous cord/tubule sections of controls (A, B, E, H, K, N and Q), propylthiouracil PTU-(C, F, I, L, O and R) and T3-treated mice (D, G, J, M, P and S) killed between 1 and 100 days of age. Bar = 20 μm (A–J) and 40 μm (K–S). sc, seminiferous cords; st, seminiferous tubules; asterisk, lumen; arrows, Sertoli cells. The insert indicates the most advanced cell type: G, gonocyte; A_3, type A_3 spermatogonia; Pl, pre-leptotene spermatocyte; P, pachytene spermatocyte; Ar 1, step 1 round spermatid; Al 7/8, step 7/8 elongated spermatid; Al 13, step 13 elongated spermatid.

cell types were present at 10 days, it was already possible to observe the organization of these cells in the seminiferous epithelium, resembling the different cell associations, denominated stages. Except for the hypothyroidic mice, in which germ cells remained in the pachytene stage at 20 days of age, the most advanced germ cells in both control and T3-treated mice were newly-formed round spermatids. At 28 days, these cells were usually step 9/10 spermatids (one animal presented step 13 spermatids), step 7/8 spermatids and step 13 spermatids, respectively, in controls, PTU- and T3-treated mice (Fig. 3). At 100 days of age, all mice investigated exhibited full, apparently normal, spermatogenesis.

In comparison to the controls and T3-treated mice, the diameter of the seminiferous cords/tubules was always lower ($P < 0.05$) in PTU-treated animals from 10 to 28 days (Figs 3 and 4). Conversely, the data obtained for these parameters were higher ($P < 0.05$) in T3-treated animals

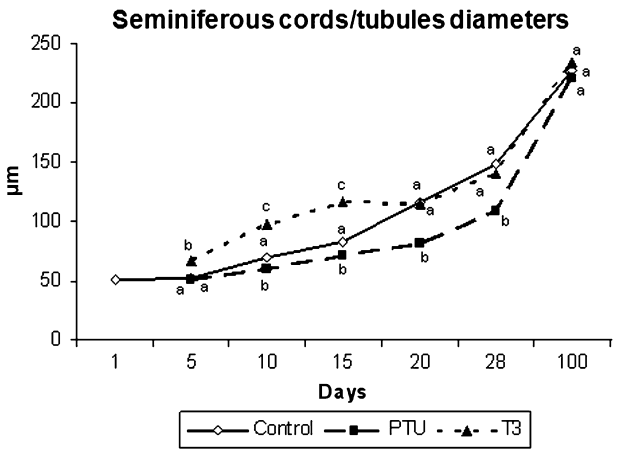

Fig. 4 Diameter of seminiferous cords/tubules in controls, propylthiouracil PTU- and T3-treated animals killed at different ages after birth (mean ± SEM). Different letters for the same age denote significant differences ($P < 0.05$).

from 5 to 15 days of age. However, there was no significant difference in tubule diameter in the three experimental groups investigated at 100 days of age.

In all groups investigated, no evidence of lumen formation was observed until 5 days of age (Figs 3 and 5). At 10 days, only T3-treated mice exhibited seminiferous tubule lumens, which were present in varying degrees in approximately 70% of the cords/tubules evaluated. The PTU-treated animals remained with no apparent lumens until 20 days, whereas about 15 and 60% of the tubules in the controls had a lumen at 15 and 20 days, respectively. Surprisingly, a prominent reduction in tubules with a lumen was observed in hyperthyroidic mice from 10 to 20 days and this trend was reversed at 28 days, which is the age that precedes the full establishment of spermatogenesis, occurring at approximately 5 weeks of age in mice, when all tubules have a patent lumen (Vergouwen et al. 1993; França L.R., unpublished data). Also, at 28 days, lumen formation was first observed in PTU-treated animals although still at a lower percentage (Figs 3 and 5). At 100 days of age, all seminiferous tubules exhibited a patent lumen in the three groups investigated.

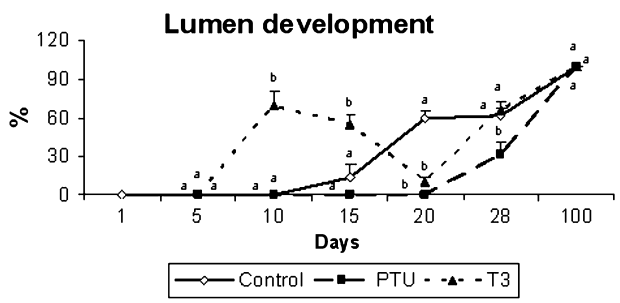

Fig. 5 Development of lumen in the seminiferous cords/tubules in controls, propylthiouracil PTU- and T3-treated animals killed at different ages after birth (mean + SEM). Different letters for the same age denote significant differences ($P < 0.05$).

Spermatogonial cell size

Figure 6 shows that the individual volume of undifferentiated spermatogonia (A_{und}) was different ($P < 0.05$) at 5 days of age in the three groups investigated, whereas the volumes for the other germ cells (types A_1–A_3 differentiated spermatogonia) at this age were significantly lower in T3-treated mice. At 10 days of age, pre-leptotene spermatocytes were present in all groups and the volumes of all different spermatogonial types could be evaluated. Therefore, it was noted that the spermatogonial volume increased 25% from undifferentiated to A_2 spermatogonia ($\sim$ 400 to $\sim$ 500 μm^3) and then decreased gradually by $\sim$ 30% until type B spermatogonia ($\sim$ 500 to $\sim$ 330 μm^3). A later decline of a similar magnitude occurred from type B spermatogonia to primary spermatocytes in pre-leptotene ($\sim$ 330 to $\sim$ 230 μm^3). The same tendency was observed for the other ages investigated (Fig. 6). Moreover, a generally similar trend was found for the values related to nuclear and cytoplasm volumes. Regarding the different experimental groups investigated at different ages, germ cell volumes exhibited the same previously described trend and, amongst all of the spermatogonial types investigated, A_2 spermatogonia were always the largest cells. Particularly at 10 and 15 days of age, practically no differences were found in cell volumes among the groups. However, from 20 to 100 days, cell volumes were generally greater in the control group and lower in T3-treated mice, whereas in PTU-treated mice these volumes were variable and no clear trend was observed. It should be emphasized that, for all spermatogonial types, the nuclear volume was proportionately higher and occupied approximately three-quarters of the total cell volume (data not shown). For pre-leptotene spermatocytes, however, this proportion was smaller at around two-thirds of the cell volume (data not shown).

Total number of spermatogonia per testis

The data for the total number of all spermatogonial types investigated are displayed in Fig. 7. At 5 days, except for the A_{und} and A_3 spermatogonia, the number of the other germ cell types present at this age (A_1 and A_2) was higher ($P < 0.05$) in hypothyroidic mice when compared with the controls. In comparison to the mice investigated at 5 days of age, the total number of gonocytes per testis obtained at birth was approximately twice the value observed for undifferentiated spermatogonia (0.5×10^5 vs. 0.24×10^5) and similar to the value found for the total number of type A spermatogonia (0.5×10^5 vs. 0.6×10^5). Therefore, some gonocytes had differentiated into spermatogonia at 5 days, whereas others probably suffered apoptosis.

In comparison to the other two groups, the hypothyroidic mice had an overall greater ($P < 0.05$) number of germ cells at 10 days of age. It is important to note that the total number of type A spermatogonia, including undifferentiated

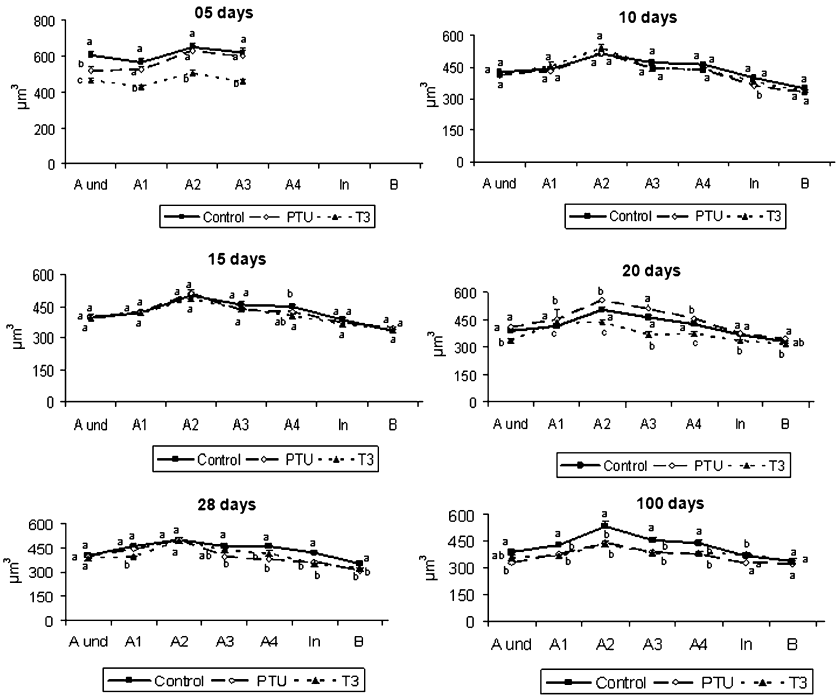

Fig. 6 Individual germ cell volume in control, propylthiouracil PTU- and T3-treated mice killed between 5 and 100 days of age. Different letters for the same age denote significant differences ($P < 0.05$) between groups. Undifferentiated type A (A_{und}), differentiated type A ($A_{1–4}$), intermediate (In) and type B (B) spermatogonia (mean ± SEM). Different letters for the same age germ cell type indicate significant differences ($P < 0.05$).

spermatogonia, at this age was significantly higher in PTU-treated mice (Figs. 7 and 8). At the other ages investigated, the PTU-treated animals generally exhibited a similar number of germ cells when compared with the controls. In contrast, T3-treated mice usually had a lower ($P < 0.05$) number of these cells. Figure 8 summarizes the total number of undifferentiated and differentiated spermatogonia as well as the total number of spermatogonia in the three groups investigated from 5 to 100 days of age. As expected and according to what was previously described for the pool of undifferentiated and differentiated spermatogonia, a similar trend was found.

Sertoli cell stereology

Data on the nuclear volume of Sertoli cells are displayed in Fig. 9A. In comparison with the two other experimental groups, hyperthyroidic mice exhibited a greater ($P < 0.05$)

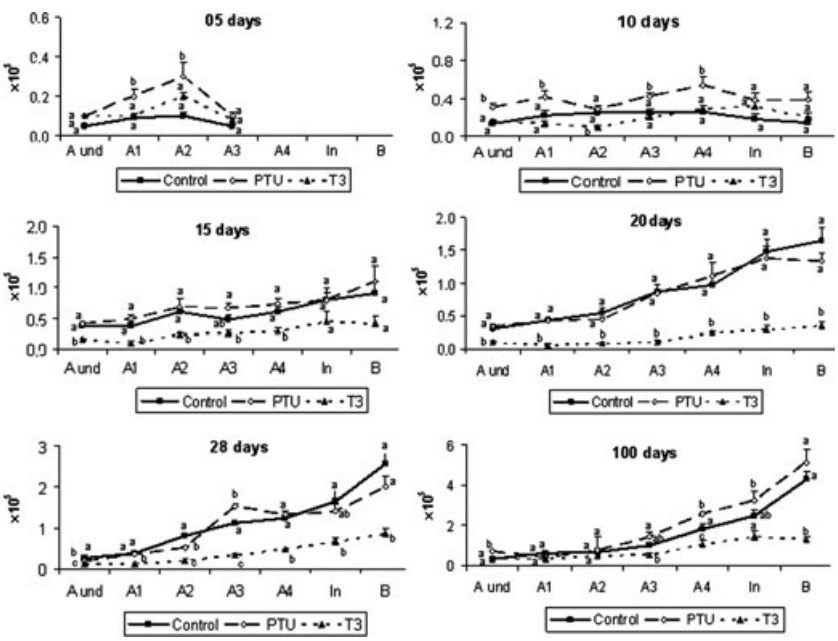

Fig. 7 Total number of germ cells per testis in control, propylthiouracil PTU- and T3-treated mice killed between 5 and 100 days of age. Different letters for the same age denote significant differences ($P < 0.05$) between groups. Undifferentiated type A (A_{und}), differentiated type A ($A_{1–4}$), intermediate (In) and type B (B) spermatogonia (mean + SEM). Different letters for the same age germ cell type indicate significant differences ($P < 0.05$).

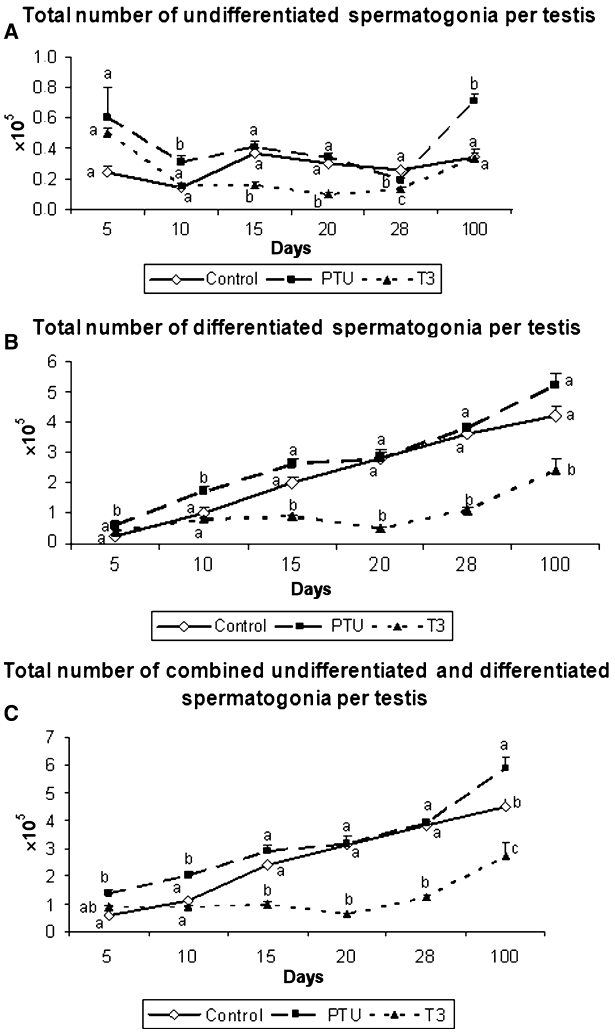

Fig. 8 Total number of undifferentiated (A) and differentiated (B) spermatogonia, and total number of combined undifferentiated and differentiated spermatogonia (C) per testis in control, propylthiouracil PTU- and T3-treated mice killed between 5 and 100 days of age. Different letters for the same age indicate significant differences ($P < 0.05$) between groups (mean + SEM).

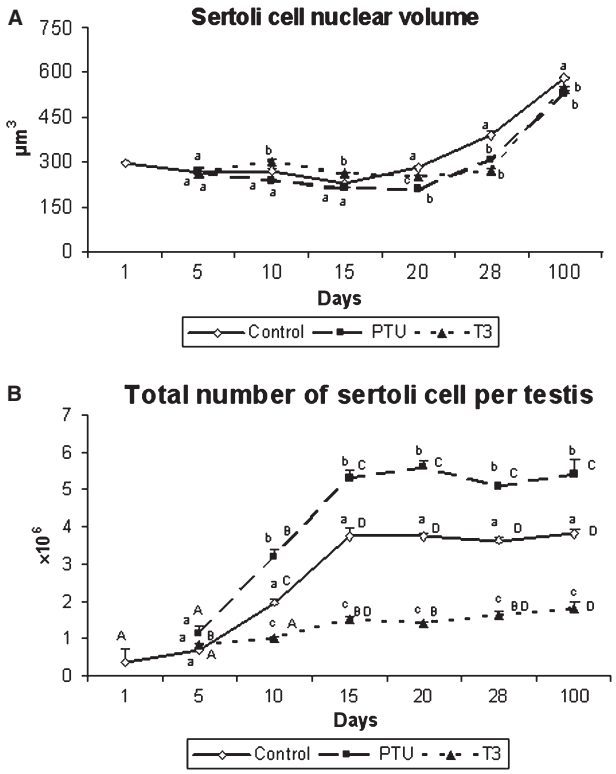

Fig. 9 Sertoli cell nuclear volume (A) and total number of Sertoli cells per testis (B) in controls, propylthiouracil PTU- and T3-treated mice killed at different ages. Different lowercase letters for the same age indicate significant differences ($P < 0.05$) between treatments and different uppercase letters indicate significant differences between the ages investigated ($P < 0.05$) (mean + SEM).

nuclear volume at 10 and 15 days of age. However, a significant reduction was observed for this parameter in T3-treated mice at 20 days, whereas the value found for PTU- and T3-treated mice was lower ($P < 0.05$) from 28 to 100 days when compared with the controls.

At 5 days of age, the total number of Sertoli cells per testis demonstrated no significant differences in the three groups (Fig. 9B). However, the total number of these cells from 10 to 100 days of age always remained higher ($P < 0.05$) in PTU-treated mice and lower ($P < 0.05$) in those that received T3 in comparison to the controls. From 15 to 100 days of age, there was a strong trend toward stabilization in the number of Sertoli cells per testis in all three groups.

Sertoli cell and spermatogenic efficiencies

These evaluations were only made for adult mice. Both the meiotic index and Sertoli cell efficiency were similar ($P > 0.05$) in the three experimental groups. These indices demonstrated that approximately 10 spermatids were supported per Sertoli cell in mice at 100 days of age and that 25–30% of germ cell loss (apoptosis) occurred during meiosis. However, in comparison to the control mice ($4.2 \pm 0.5 \times 10^6$), the total daily sperm production per testis was significantly higher ($5.6 \pm 0.5 \times 10^6$) and lower ($2.4 \pm 0.2 \times 10^6$) in PTU- and T3-treated mice, respectively.

Blood and lymphatic vessel stereology

The results related to the volume and volume density of the different testis vascular components are displayed in Table 1. For most ages investigated (particularly at 10–15 days), hypothyroidic mice had higher ($P < 0.05$) values for the percentage and absolute volume of the vascular components investigated, especially capillaries.

Table 1 Volume (μL) and volume density (% in parenthesis) of the different vascular components at different ages in C57BL/6J mice (mean ± SEM).

Age	Control	PTU	T3
5 days			
Capillary	0.02 ± 0.003^{a} (1.2 ± 0.2^{a})	0.03 ± 0.006^{b} (1.5 ± 0.2^{a})	0.01 ± 0.002^{a} (0.8 ± 0.2^{a})
Lymphatic vessel	0.006 ± 0.001^{a} (0.5 ± 0.06^{a})	0.005 ± 0.001^{a} (0.3 ± 0.03^{a})	0.004 ± 0^{a} (0.3 ± 0.1^{b})
Total vessels	0.02 ± 0.003^{a} (1.8 ± 0.2^{a})	0.03 ± 0.01^{a} (1.6 ± 0.4^{a})	0.02 ± 0.003^{a} (1.3 ± 0.2^{a})
10 days			
Capillary	0.04 ± 0.01^{a} (1.2 ± 0.3^{a})	0.17 ± 0.04^{b} (3.2 ± 0.4^{b})	0.05 ± 0.007^{a} (1.6 ± 0.2^{a})
Lymphatic vessel	0.02 ± 0.004^{a} (0.6 ± 0.1^{a})	0.05 ± 0.008^{b} (1 ± 0.1^{a})	0.02 ± 0.003^{a} (0.5 ± 0.1^{a})
Total vessels	0.07 ± 0.02^{a} (2.2 ± 0.5^{a})	0.25 ± 0.04^{b} (4.8 ± 0.7^{b})	0.07 ± 0.007^{a} (2.3 ± 0.2^{a})
15 days			
Capillary	0.1 ± 0.02^{a} (1.6 ± 0.3^{a})	0.2 ± 0.03^{b} (2.6 ± 0.3^{b})	0.1 ± 0.01^{a} (2.1 ± 0.1^{ab})
Lymphatic vessel	0.05 ± 0.01^{a} (0.8 ± 0.2^{a})	0.1 ± 0.02^{b} (1.4 ± 0.2^{b})	0.01 ± 0.01^{a} (0.2 ± 0.1^{a})
Total vessels	0.2 ± 0.05^{a} (2.5 ± 0.6^{ab})	0.3 ± 0.06^{b} (4.2 ± 0.5^{a})	0.1 ± 0.01^{a} (2.7 ± 0.1^{b})
20 days			
Capillary	0.2 ± 0.04^{a} (1.5 ± 0.3^{a})	0.3 ± 0.04^{a} (2.6 ± 0.3^{b})	0.07 ± 0.02^{b} (1.5 ± 0.4^{ab})
Lymphatic vessel	0.03 ± 0.02^{a} (0.2 ± 0.1^{a})	0.1 ± 0.02^{a} (1 ± 0.2^{b})	0.01 ± 0.005^{b} (0.3 ± 0.1^{a})
Total vessels	0.3 ± 0.05^{a} (2 ± 0.3^{a})	0.4 ± 0.05^{a} (3.7 ± 0.3^{b})	0.1 ± 0.02^{b} (1.9 ± 0.4^{a})
28 days			
Capillary	0.4 ± 0.02^{a} (1.3 ± 0.06^{a})	0.3 ± 0.06^{a} (1.7 ± 0.3^{a})	0.2 ± 0.03^{b} (1.5 ± 0.2^{a})
Lymphatic vessel	0.1 ± 0.01^{a} (0.3 ± 0.05^{a})	0.15 ± 0.04^{a} (0.8 ± 0.2^{a})	0.07 ± 0.01^{a} (0.7 ± 0.08^{a})
Total vessels	0.4 ± 0.1^{a} (1.8 ± 0.1^{b})	0.5 ± 0.1^{a} (2.8 ± 0.4^{a})	0.3 ± 0.04^{a} (2.5 ± 0.2^{ab})
100 days			
Capillary	1.1 ± 0.2^{a} (1.2 ± 0.2^{a})	1.5 ± 0.2^{a} (1.4 ± 0.2^{a})	0.4 ± 0.1^{b} (1 ± 0.3^{a})
Lymphatic vessel	0.3 ± 0.1^{a} (0.3 ± 0.1^{a})	0.4 ± 0.1^{a} (0.4 ± 0.07^{a})	0.08 ± 0.02^{a} (0.2 ± 0.07^{a})
Total vessels	1.5 ± 0.3^{a} (1.6 ± 0.3^{ab})	2.6 ± 0.2^{b} (2.3 ± 0.2^{a})	0.5 ± 0.1^{c} (1.2 ± 0.3^{b})

Different letters in same line indicate statistical difference ($P < 0.05$).
PTU, propylthiouracil; T3, triidothryronine.

Discussion

This is the first study to investigate the effects of transient neonatal hypo- and hyperthyroidism, induced, respectively, by PTU and T3, on Sertoli cell proliferation/differentiation and the correlation of these important events with the evolution of spermatogenesis, spermatogonial cell size, total number of spermatogonia per testis and total volume and volume density of vascular components in the testis. Based on the most advanced germ cell type found at 28 days of age, spermatogenesis was only slightly accelerated and delayed in T3- and PTU-treated mice, respectively, and unpublished data from our laboratory demonstrate that full spermatogenesis is present around 5 weeks of age in control, T3- and PTU-treated mice.

Unlike rats (Van Haaster et al. 1992; Hess et al. 1993; França et al. 1995) and other mouse strains (Swiss mice; Joyce et al. 1993), our results suggest that the period of Sertoli cell proliferation was apparently not affected by the treatments, at least under the conditions of the present study, in which mice were killed at 5-day intervals from birth to 20 days of age. However, the total number of Sertoli cells per testis was significantly increased in hypothyroidic mice in comparison to the controls beginning at 10 days of age, whereas the opposite occurred in the T3-treated mice. As the mice used in the present study were C57BL/6J and those investigated by Joyce et al. (1993) were Swiss-Webster, the unexpected finding that the treatments did not change the Sertoli cell proliferation period could be explained by strain specificity, at least in part. Although we have not evaluated the Sertoli cell proliferation rate, based on the Sertoli cell numbers found we assume that this mitotic index was increased and decreased, respectively, due to the hypo- and hyperthyroidic conditions of the mice. In accordance with the literature (Ross et al. 2001; Russell et al. 2001), it is worth mentioning that, in the present study, Sertoli cell apoptosis was not observed during this cell proliferation period. In particular, mature Sertoli cells only suffer apoptosis when they lack Bclw (Ross et al. 2001; Russell et al. 2001).

The total number of undifferentiated spermatogonia was increased in PTU-treated mice, particularly at 10 days of age, probably due to the higher number of Sertoli cells, and this finding was associated with significantly more vessels. Although functional experiments should be performed on this particular aspect, our data suggest that this particular age may represent a critical window in which potentially more undifferentiated cells and consequently more spermatogonial niches would be available.

We did not measure thyroid hormone levels in the present investigation. However, several critical parameters that were evaluated, such as gonadosomatic index, tubular

lumen formation and the total number of Sertoli cells per testis, strongly suggest that the treatments and doses employed were effective in inducing the hyper- and hypothyroidism conditions. Nevertheless, it should be emphasized that, as observed by Joyce et al. (1993), the magnitude of the increase in testicular weight induced by PTU in our study is much less pronounced than that observed in rats (Hess et al. 1993; França et al. 1995; Cooke et al. 2005).

The diameter of seminiferous cords/tubules is an excellent parameter for evaluating the evolution of spermatogenesis during post-natal testis development and the degree of Sertoli cell maturation and fluid secretion that result in lumen formation. Therefore, there was a parallelism between the results obtained for the lumen of the seminiferous tubules and those found for the nuclear volume of the Sertoli cells.

Although Sertoli cell maturation was accelerated in T3-treated mice, an unexpected result was the drastic decrease in the percentage of tubules with a lumen, observed in T3-treated mice killed at 20 days of age. Apparently, this treatment altered the functional activity of Sertoli cells, resulting in a smaller nuclear volume for this cell in this time period. As Sertoli cell fluid secretion is mainly regulated by androgens (De Gendt et al. 2005), the plasma levels of this male steroid could be altered before puberty in T3-treated mice. A direct effect of T3 on other testicular somatic elements such as Leydig and peritubular myoid cells could not be excluded. The results found for the lumen of seminiferous tubules and Sertoli cell efficiency in adult mice strongly suggest that Sertoli cell function was apparently normal in the treated mice, at least at 100 days of age. The changes found regarding spermatogenic efficiency at the same age were merely a reflection of the total number of Sertoli cells per testis, which resulted in smaller and larger testis size in T3- and PTU-treated mice, respectively. Although we did not evaluate any sperm parameter in the present study, in adult rats made transiently hypothyroidic during the neonatal period, sperm concentration and motility in the epididymis cauda as well as litter size have been found to be unaffected by PTU treatment (Cooke & Meisami, 1991).

To our knowledge, there are no longitudinal published data regarding the size and number of different spermatogonial types (A_{und} to B) in mice or any other mammalian species. Although the different types of undifferentiated and differentiated type A spermatogonia are not discriminated, the literature for mammals indicates that spermatogonial nuclear volume decreases gradually from type A to type B spermatogonia (França, 1991; Neves, 2001). In fish investigated at our laboratory, such as tilapia (Schulz et al. 2005) and zebrafish (Leal et al. 2009), this trend is very clear and dramatic decreases are observed for cell and nuclear sizes from undifferentiated or initial type A spermatogonia to the last generation of type B spermatogonia, which involves approximately 10 spermatogonial generations (Schulz et al. 2005; Leal et al. 2009). Our results demonstrating that undifferentiated type A spermatogonia have lesser nuclear and cell volumes may be useful for the identification of undifferentiated spermatogonia, employing approaches or methods such as *in-vitro* studies and/or confocal, scanning microscopy and image analysis programs, which could be helpful in the investigation of spermatogonial biology. Although we have no explanation for this finding, in comparison to the other type A spermatogonia evaluated in the present study, A_2 spermatogonia were consistently larger in all groups and ages investigated.

Based on the finding that the total number of undifferentiated spermatogonia at 10 days of age was higher in hypothyroidic animals and on the knowledge that undifferentiated spermatogonia are preferentially located adjacent to the interstitium (Chiarini-Garcia et al. 2001) – specifically blood vessels (Yoshida et al. 2007) – we investigated the volume and volume density of the different vascular components in the present study. These unique findings demonstrate that the testis of hypothyroidic mice had a greater volume and volume density of the vascular components evaluated, particularly capillaries at 10–15 days of age. These results suggest that capillaries and blood vessels may play an important role in the spermatogonial environment (Shetty & Meistrich, 2007; Yoshida et al. 2007). Therefore, hypothyroidism may represent an interesting approach for investigating important aspects of spermatogonial biology, including aspects involving the influence of Sertoli cells on blood vessel growth and function (Golat & Cameron, 2008).

The investigations performed in the present study provide important morphological and stereological data related to testis function, particularly spermatogonial cell development and Sertoli cell proliferation/differentiation, in mice made transiently hypo- and hyperthyroidic during the neonatal period and evaluated from birth to adulthood. Interestingly, type A_2 spermatogonia were always the largest spermatogonial cells in all groups and ages investigated. Our results suggest that neonatal hypothyroidism may be a useful tool for studying the biology of spermatogonial cells and a potential tool for providing more undifferentiated spermatogonial cells that could potentially be used for spermatogonial transplantation, thereby optimizing the efficiency of this technique when young mice are used as donors.

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