REVIEW

The role of thyroid hormone in testicular development and function

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Abstract

Thyroid hormone is a critical regulator of growth, development, and metabolism in virtually all tissues, and altered thyroid status affects many organs and systems. Although for many years testis has been regarded as a thyroid hormone unresponsive organ, it is now evident that thyroid hormone plays an important role in testicular development and function. A considerable amount of data show that thyroid hormone influences steroidogenesis as well as spermatogenesis. The involvement of tri-iodothyronine (T_3) in the control of Sertoli cell proliferation and functional maturation is widely accepted, as well as its role in postnatal Leydig cell differentiation and steroidogenesis. The presence of thyroid hormone receptors in testicular cells throughout development and in adulthood implies that T_3 may act directly on these cells to bring about its effects. Several recent studies have employed different methodologies and techniques in an attempt to understand the mechanisms underlying thyroid hormone effects on testicular cells. The current review aims at presenting an updated picture of the recent advances made regarding the role of thyroid hormones in male gonadal function.

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Introduction

In mammals, altered thyroid status is known to adversely affect many organs and tissues. Nevertheless, for many years, the impact of thyroid disorders on male reproduction remained controversial. This was partly due to the demonstration that the adult testis of experimental animals was metabolically unresponsive to thyroid hormones (Barker & Klitgaard 1952), and to the low number of thyroid hormone-binding sites found in the adult organ (Oppenheimer *et al.* 1974). These early reports led to the widespread view that the testis was unaffected by iodothyronines. Additionally, clinical data correlating male sexual function with thyroid disorders are limited, probably because thyroid diseases are more common in females than in males. However, in the past two decades, several experimental and clinical studies have demonstrated that thyroid hormone plays an important role in testicular development and function. It is now established that tri-iodothyronine (T_3) regulates the maturation and growth of testis, controlling Sertoli cell and Leydig cell proliferation and differentiation during testicular development in rats and other mammal species (Holsberger & Cooke 2005, Mendis-Handagama & Siril Ariyaratne 2005). Furthermore, changes in thyroid hormone levels during early testis development have been shown to affect testicular maturation and reproduction later in life (Jannini *et al.* 1995).

An extensive body of data shows that thyroid hormone inhibits Sertoli cell proliferation and stimulates their functional maturation in prepubertal rat testis. The efficiency of spermatogenesis, reflected by the daily sperm production in adulthood, correlates with the total number of functional Sertoli cells that is established during prepubertal life (Orth *et al.* 1988). These data, in conjunction with the findings that thyroid hormone receptors (TRs) are present in human and rat testes from birth to adult life (Buzzard *et al.* 2000, Jannini *et al.* 2000), further confirm that thyroid hormone plays a key role in testicular development. Interestingly, the presence of iodothyronine deiodinases, enzymes that modulate the concentration, and thus the actions of thyroid hormones in different tissues were also identified in the rodent testis from fetal to adult life (Bates *et al.* 1999, Wagner *et al.* 2003, Wajner *et al.* 2007). Although the mechanism(s) whereby T_3 regulates Sertoli cell proliferation remains unclear, recent studies have suggested that the suppressive effects of T_3 on Sertoli cell proliferation might be mediated by increased levels of expression of cyclin-dependent kinase inhibitors (CDKIs) and/or connexin43 (Cx43; Holsberger *et al.* 2003, Gilleron *et al.* 2006).

Insights into the role of thyroid hormone in the adult testis have also been gained from studies with rats subjected to prolonged thyroid hormone deficiency (Sakai *et al.* 2004). These animals presented marked morphological and

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functional testicular alterations. Moreover, clinical literature indicates that most patients with thyroid hormone disorders experience some kind of sexual dysfunction, which improves or normalizes when patients become euthyroid (Jannini *et al.* 1995, Krassas & Pontikides 2004, Carani *et al.* 2005). Hence, although thyroid hormone was not historically viewed as a major regulator of the male gonad, it is now clear that it has critical effects on the testis especially during development. The aim of the current review is to present an updated picture of the recent advances of our knowledge regarding the role of the thyroid hormones on male gonadal function.

Overview of testis structural organization

The testes are mainly comprised of tightly coiled seminiferous tubules, which are supported by loose interstitial connective tissue where the steroidogenic Leydig cells are located (Griffin & Wilson 2002). Each tubule consists of a basement membrane, elastic fibers, and peritubular myoid cells. Within the basement membrane, the seminiferous tubules are lined by a columnar epithelium composed of germ cells and the somatic Sertoli cells. Adjacent Sertoli cells are connected by tight specialized junctions to form a diffusion barrier, the so-called blood–testis barrier, which divides the seminiferous tubule into two functional compartments, basal, and adluminal (Fig. 1). The basal compartment consists of Sertoli cells, spermatogonia and preleptotene/leptotene spermatocytes (Cheng & Mruk 2002). In the adluminal compartment, primary spermatocytes divide and differentiate into germ cells in more advanced stages of spermatogenesis. Functionally, the blood–testis barrier creates a controlled microenvironment providing the nutrients, appropriate mitogens, differentiation factors as well as an immunological protected ambient required for the full development of germ cells (Yan *et al.* 2008).

Although gonadotropins play an essential role in modulating spermatogenesis and androgen synthesis, the full hormonal requirements for the entire germ cell maturation process and general maintenance of a well-functioning testis remain unclear. In addition to gonadotropins and testosterone, a number of other factors play a critical role in modulating spermatogenesis including genes, several paracrine/autocrine factors, and other hormones, such as growth hormone (GH) and thyroid hormones (Jegou & Sharpe 1993, Sharpe 1994).

Role of thyroid hormone in testicular development

The main pathway for the production of the thyroid hormone bioactive form, T_3, is via outer ring deiodination of thyroxin (T_4) by iodothyronine deiodinases type 1 and 2 (D1 and D2) in peripheral tissues. Although the actions of thyroid hormones on target tissues are predominantly mediated by specific nuclear receptors, these hormones also have well-known non-genomic actions (Davis *et al.* 2008).

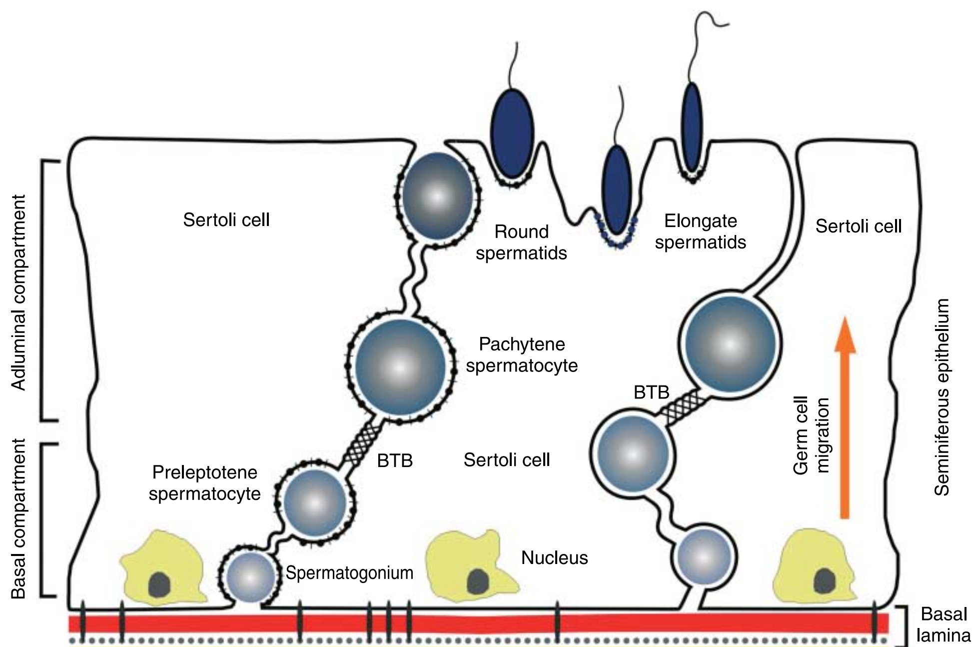

Figure 1 Schematic illustrating the morphological structure of adult Sertoli cells and their interactions with the different germ cells within the seminiferous epithelium. The relative locations of tight junctions between adjacent Sertoli cells, which create the blood–testis barrier (BTB) and divide the seminiferous tubule into basal and adluminal compartments, are indicated.

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The role of thyroid hormone in testicular development and function has received much attention since the report that functional TRs were present in high quantities in neonatal Sertoli cells (Palmero *et al*. 1988, Jannini *et al*. 1990, Francavilla *et al*. 1991). These findings changed the classical view of the testis as a thyroid hormone unresponsive organ, indicating that thyroid hormone could have direct effects on testis.

The role of thyroid hormone in Sertoli cell proliferation and functional maturation

In the mammalian testis, Sertoli cells represent the main structural component of the seminiferous epithelium playing a key role in the initiation and maintenance of spermatogenesis (Sharpe 1994). These are the first cell type known to differentiate within the fetal gonad, by expressing the *SRY* gene, an event that acts as the organizing center of the male gonad enabling the formation of the primitive seminiferous cords (Mackay 2000, Brennan & Capel 2004). After birth, the immature Sertoli cells continue to proliferate until the beginning of puberty when they stop dividing and start differentiating into their non-proliferative adult form. It is well established that the number of Sertoli cells present at puberty is closely correlated with both adult testicular size and sperm output (Orth *et al*. 1988). At this point in time, the establishment of an adequate number of Sertoli cells is crucial for future male fertility. The number of Sertoli cells present in the adult testis depends on both the duration of the proliferative phase and the rate of division during that phase. In rats, Sertoli cell proliferation starts during fetal life and is complete on approximately day 16 *post partum* (Orth 1982, Wang *et al*. 1989). Follicle-stimulating hormone (FSH) signaling is a critical factor in determining the rate of Sertoli cell division (Meachem *et al*. 1996, Kumar *et al*. 1997, Dierich *et al*. 1998, Griswold 1998), but other factors also have an effect on the final number of Sertoli cells (Griswold *et al*. 1977, Kirby *et al*. 1992). Several studies performed in rats have demonstrated that thyroid hormone determines the duration of Sertoli cell division and may be involved in the maturational changes that decrease and eliminate mitogenic responses to FSH (Holsberger & Cooke 2005).

Although hypothyroidism had no effect on testicular development during fetal life (Francavilla *et al*. 1991, Hamouli-Said *et al*. 2007), when induced in newborn rats, it was associated, at puberty, with impaired testicular development including testicular growth, germ cell maturation, and seminiferous tubule formation (Palmero *et al*. 1989, Francavilla *et al*. 1991). However, as the animals made hypothyroid were allowed to recover back to the euthyroid state, a significant increase in testis size and daily sperm production (80 and 140% respectively, compared with control animals) was observed in adulthood (Cooke & Meisami 1991, Cooke *et al*. 1991). Subsequently, the mechanism underlying these unpredictable testicular changes was established. It has been shown that transient neonatal/prepubertal hypothyroidism extends the length of Sertoli cell proliferation by delaying their maturation, resulting in an increased number of Sertoli cells in the adult testis (Francavilla *et al*. 1991, Van Haaster *et al*. 1992, Hess *et al*. 1993, Joyce *et al*. 1993, De Franca *et al*. 1995). The adult number of Sertoli cells in rats that had been subjected to transient neonatal hypothyroidism was shown to increase 157% compared with control animals (Hess *et al*. 1993). Conversely, transient juvenile hyperthyroidism resulted in an early cessation of Sertoli cell proliferation and had a concomitant stimulatory effect on their maturation, resulting in premature canalization of seminiferous tubules, decreased testis size, and sperm production (van Haaster *et al*. 1993, Cooke *et al*. 1994, Palmero *et al*. 1995*b*).

The above data together with the reported high levels of expression of functional T_3 receptors in proliferating Sertoli cells (Buzzard *et al*. 2000, Jannini *et al*. 2000) indicate that Sertoli cells are a major testicular target for thyroid hormone. It appears that thyroid hormone acts directly on Sertoli cells to inhibit proliferation while stimulating differentiation, not only in rodents (Cooke & Meisami 1991, Joyce *et al*. 1993, Kirby *et al*. 1993) but also in many other vertebrate species (Jannini *et al*. 1995, Kirby *et al*. 1996, Majdic *et al*. 1998, Matta *et al*. 2002, Jansen *et al*. 2007). Although several factors are presumed to play a role in proliferation and maturation of Sertoli cells (Sharpe *et al*. 2003, Mackay & Smith 2007), T_3 is likely to represent a major hormonal signal involved in the establishment of the adult Sertoli cell population.

Thyroid hormone and the mechanisms involved in Sertoli cell proliferation

The mechanism(s) by which thyroid hormone suppress proliferation and induce differentiation in Sertoli cells is still unknown. Recent studies indicate that T_3 might be able to control Sertoli cell proliferation by acting through specific CDKIs (Holsberger *et al*. 2005*b*), a family of proteins that directly interact with the cell cycle (Sherr & Roberts 1995), and/or by a mechanism involving Cx43, a constitutive protein of gap junctions (Gilleron *et al*. 2006).

In vivo and *in vitro* experiments demonstrated that thyroid hormone induces the expression of two CDKIs, p27Kip1 and p21Cip1, in neonatal Sertoli cells, whereas hypothyroidism decreases p27Kip1 in these cells (Buzzard *et al*. 2003, Holsberger *et al*. 2003). Indeed, the expression of p27Kip1, a critical regulator of proliferation in many cell types (Coats *et al*. 1996, Lu *et al*. 2002, Tokumoto *et al*. 2002), has been shown to be inversely correlated with Sertoli cell proliferation (Beumer *et al*. 1999). Accordingly, adult p27Kip1 knockout (p27KO), p21Cip1 KO (p21KO), and p27/p21 double-KO (DBKO) mice presented enlarged testes, increased Sertoli cell numbers, and increased daily sperm production compared with wild-type animal (Holsberger *et al*. 2005*b*). Although loss of p27 and/or p21 results in increased Sertoli cell proliferation, the magnitude of their roles in establishing the final number of adult Sertoli cells and daily sperm production has not yet been established. Nevertheless, these data suggest that the suppressive effects of T_3 on Sertoli cell proliferation might be, at least in part, mediated by suppression of the cell cycle.

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As puberty approaches, Sertoli cells form a complex network of specific intercellular junctions with each other and with adjacent germ cells (Cheng & Mruk 2002, Yan *et al.* 2008). Among these junctional complexes, the connexin-based gap junctions are unique because they form cell membrane channels, which allow intercellular communication that, in turn, plays a critical role in the control of cell proliferation and differentiation (Loewenstein & Rose 1992, Risley *et al.* 1992, Decrouy *et al.* 2004). In testicular cells, Cx43 is the most abundant gap junction protein (Risley *et al.* 1992, Tan *et al.* 1996, Batias *et al.* 2000) and recent studies demonstrated that the inhibitory effect of T_3 on Sertoli cell proliferation is associated with increased levels of this protein in postnatal testis (Gilleron *et al.* 2006). This observation was further verified when specific blockers of gap junctions coupling, such as oleamide and glycyrrhetinic acid, reverse the inhibitory effect of T_3 (Gilleron *et al.* 2006). These results are in agreement with what has been observed in the recently developed Sertoli cell-specific Cx43 knockout (SC-Cx43 KO) mouse. Two laboratories have independently demonstrated that, in these animals, loss of Cx43 in Sertoli cells is associated with continued Sertoli cell proliferation and delayed maturation in adulthood (Brehm *et al.* 2007, Sridharan *et al.* 2007*b*). In addition, seminiferous tubules of SC-Cx43 KO mice contained only Sertoli cells and actively proliferating early spermatogonia, indicating that loss of Cx43 prevents initiation of spermatogenesis and leads to a significant reduction of germ cells and infertility (Sridharan *et al.* 2007*a*).

Thyroid hormone and markers of Sertoli cell maturation

The maturation of Sertoli cells is a complex multistep process involving a cascade of changes that lead to a radical switch in their morphology and function (Sharpe *et al.* 2003, Brehm & Steger 2005). This process is characterized by either suppression or upregulation of specific proteins associated with the Sertoli cell differentiation (Sharpe *et al.* 2003) and thyroid hormone seems to affect the expression of a number of these markers.

Thyroid hormone has been reported as a possible negative regulator of anti-Müllerian hormone (AMH) expression, a Sertoli cell secretory protein that plays a critical role in the early stages of testicular development. AMH expression is sharply downregulated as Sertoli cells mature (Hirobe *et al.* 1992, Lee & Donahoe 1993, Brehm & Steger 2005). The hypothesis that thyroid hormone would be involved in this phenomenon was based on the fact that neonatal hypothyroidism in rats delayed the fall of *Amh* mRNA levels (Bunick *et al.* 1994), whereas T_3 administration decreased *Amh* transcripts in cultured neonatal rat Sertoli cells (Arambepola *et al.* 1998*b*). Nevertheless, recently, Mendis-Handagama & Siril Ariyaratne (2008) showed that AMH content in Sertoli cells gradually declines with age, irrespective of the thyroid hormone status in prepubertal rats, suggesting that AMH production is not regulated by T_3.

Loss of aromatase activity is also a marker of final maturation of Sertoli cells in rats. It is maximally expressed at perinatal age, and then it decreases sharply at puberty to become virtually absent in fully differentiated cells (Sharpe *et al.* 2003). Thyroid hormone was shown to decrease aromatase activity in Sertoli cells by direct inhibition of the aromatase gene transcription (Catalano *et al.* 2003). Moreover, precocious terminal differentiation concomitant with a dramatic decrease of aromatase activity was observed in T_3-treated prepubertal Sertoli cells (Ulisse *et al.* 1994, Palmero *et al.* 1995*a*, Panno *et al.* 1995, Andò *et al.* 2001). Thyroid hormone has also been shown to downregulate the expression of the neural cell adhesion molecule (NCAM) in cocultures of Sertoli cell–gonocytes isolated from neonatal rat testis (Laslett *et al.* 2000). The downregulation of NCAM, involved in Sertoli cell–gonocytes interactions in seminiferous cords, seems to mark the appropriate differentiation of Sertoli cells since its expression decreases dramatically in the first week of postnatal life and eventually disappears in parallel with Sertoli cell maturation in rats (Orth *et al.* 2000). Another feature of mature Sertoli cells is the nuclear expression of androgen receptor (AR), since it first appears in their nucleus before final maturation in humans, rats, and marmoset monkeys (Williams *et al.* 2001, Weber *et al.* 2002, Sharpe *et al.* 2003). *In vitro* studies have shown that T_3 increases androgen binding (Panno *et al.* 1995) and *AR* mRNA levels in immature rat Sertoli cells (Arambepola *et al.* 1998*a*), indicating that thyroid hormone might regulate the postnatal increase in *AR* expression in these cells. As already mentioned, T_3 upregulates the cyclin-dependent kinase inhibitors p27Kip1 and p21Cip1 (Buzzard *et al.* 2003, Holsberger *et al.* 2003) and Cx43 in Sertoli cells (Gilleron *et al.* 2006). Expression of both p27Kip1 and Cx43 coincides with maturation of Sertoli cells in mice, rats, and humans (Beumer *et al.* 1999, Cipriano *et al.* 2001, Brehm & Steger 2005). Thyroid hormone was also shown to differentially regulate the expression of the major components of the basement membrane (BM), laminin, entactin/nidogen, and type IV collagen, in rat Sertoli cell cultures. T_3 induced a significant increase in the number of cells expressing laminin and/or entactin, whereas type IV collagen expression was greatly reduced (Ulisse *et al.* 1998). These results obtained by *in vitro* studies suggest that T_3-induced remodeling of BM components might play a role in enhancing structural differentiation and/or in maintaining the Sertoli cell differentiated state, although similar effects *in vivo* have not been reported so far.

Effect of thyroid hormone on Sertoli cell metabolism

It is well known that the germ cells survival within the seminiferous tubules depends on the supply of many factors produced by Sertoli cells. Several studies have demonstrated that Sertoli cells actively metabolize glucose that is converted to lactate and used as energy substrate by germ cells (Jutte *et al.* 1981, Robinson & Fritz 1981, Mita & Hall 1982, Grootegoed *et al.* 1986*a*,*b*). The provision of adequate levels of lactate for germ cells seems to be essential for normal spermatogenesis

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(Courtens & Ploen 1999). Although thyroid hormone stimulates lactate production in immature Sertoli cells (Palmero *et al.* 1995*b*), its role in the different biochemical steps involved in this stimulatory effect has not yet been determined. The increase in lactate production is associated with increased levels of the glucose transporter-1 (GLUT1; now known as *SLC2A1*) mRNA (Ulisse *et al.* 1992). The increase in SLC2A1 might represent a cellular mechanism involved in the effect of T_3 on lactate production; however, it cannot be ascribed to a direct action of T_3 on the *SLC2A1* gene promoter since any thyroid responsive element has been identified in this region (Carosa *et al.* 2005). In addition to the effects on glucose metabolism, thyroid hormones also stimulate protein synthesis in immature Sertoli cells (Palmero *et al.* 1995*b*, 1996). Both T_4 and T_3 promote amino acid accumulation in Sertoli cells by distinct mechanisms (Menegaz *et al.* 2006). While the T_3 effect is partially blocked by cycloheximide, an inhibitor of protein biosynthesis, the potent stimulatory effect of T_4 remained unchanged, thus indicating that T_4 effects are modulated by non-genomic mechanisms.

The above-mentioned observations suggest that thyroid hormones use different signaling pathways to regulate critical biochemical steps in the Sertoli cell metabolism.

The role of thyroid hormone in Leydig cell differentiation and function

A considerable amount of data indicates that thyroid hormone plays a role in several aspects of Leydig cell development and function (Mendis-Handagama & Siril Ariyaratne 2005). Two distinct populations of Leydig cells are present in the testis of mammals. The fetal Leydig cells are responsible for the production of androgens for fetal masculinization and the primary source of testicular testosterone in the neonatal period (Kerr & Knell 1988, Mendis-Handagama *et al.* 1998). The adult Leydig cells are unrelated to their fetal counterparts and differentiate postnatally from the peritubular mesenchymal Leydig cell precursors of testicular interstitium (Ariyaratne *et al.* 2000*a*). The population of adult Leydig cells is the most abundant and the primary source of androgens in the mature mammalian testis.

Several studies have shown that altered thyroid status has marked effects on mesenchymal cell differentiation in the prepubertal and adult rat testis (Maran 2003, Mendis-Handagama & Siril Ariyaratne 2005). Initial reports showed that transient neonatal hypothyroidism increase the number of Leydig cells in adult rat testis (Hardy *et al.* 1993, Mendis-Handagama & Sharma 1994). Subsequent studies have demonstrated that neonatal hypothyroidism produces this effect by arresting Leydig cell differentiation and allowing continuous proliferation of precursor mesenchymal cells that accumulate in the interstitium, which will become available for differentiation later when euthyroidism is restored (Hardy *et al.* 1996, Mendis-Handagama *et al.* 1998, Teerds *et al.* 1998). Conversely, hyperthyroidism was shown to stimulate the differentiation of mesenchymal cells into progenitor Leydig cells and to increase the number of mesenchymal cells produced in prepubertal rat testis (Teerds *et al.* 1998, Ariyaratne *et al.* 2000*a*). Moreover, T_3 has been shown to induce Leydig cell differentiation in the testes of adult rats previously treated with ethane-dimethane sulfonate (EDS), a toxin that selectively kills Leydig cells within 48 h after administration (Ariyaratne *et al.* 2000*b*). These results indicate that thyroid hormone is crucial for triggering the onset of mesenchymal cell differentiation into a steroidogenic progenitor Leydig cell in prepubertal and adult rat testis. Indeed, the onset of the adult Leydig cell differentiation in the rat and mouse testes appears to be independent of luteinizing hormone (LH; Siril Ariyaratne *et al.* 2000, Baker *et al.* 2003). Nevertheless, LH is essential for the steps beyond the initial differentiation stage for further development and maturation of adult Leydig cells (Mendis-Handagama & Ariyaratne 2001).

The molecular mechanism(s) whereby thyroid hormone affects Leydig cell differentiation is still unclear. The AMH has been reported as a possible negative regulator of Leydig cell differentiation. This suggestion was based on the findings that AMH overexpression in male transgenic mice blocks the differentiation of Leydig cell precursors (Racine *et al.* 1998), whereas AMH-deficient mice presented Leydig cell hyperplasia (Behringer *et al.* 1994). Additionally, AMH was shown to inhibit Leydig cell regeneration following EDS treatment in adult rats (Salva *et al.* 2004). These results have brought into question whether T_3 would affect neonatal Leydig cell differentiation indirectly by induction of Sertoli cell maturation and consequently decrease in AMH levels. However, this seems to be unlikely since, as previously mentioned, AMH production by prepubertal Sertoli cells was shown to be independent of Sertoli cell maturation and not regulated by thyroid hormone (Mendis-Handagama & Ariyaratne 2008).

On the other hand, several studies have suggested a potential role of Sertoli cells paracrine factors in the regulation of Leydig cells (Verhoeven & Cailleau 1985, 1987, Papadopoulos 1991, Cheng *et al.* 1993). During testicular development, signaling molecules secreted by Sertoli cells, such as desert hedgehog (DHH) and platelet-derived growth factor (PDGF), seem to regulate Leydig cell differentiation and function (Clark *et al.* 2000, Pierucci-Alves *et al.* 2001). Moreover, several authors have shown that proteins secreted by Sertoli cells present stimulatory effects on Leydig cells (Verhoeven & Cailleau 1985, 1987, Papadopoulos 1991, Cheng *et al.* 1993). In this context, some thyroid hormone-mediated changes observed in Sertoli cells, such as the increase in insulin-like growth factor-1 (IGF-1) secretion (Palmero *et al.* 1990) and decrease in estrogen production due to downregulation of aromatase activity (Ulisse *et al.* 1994, Catalano *et al.* 2003), might indirectly affect Leydig cell differentiation. IGF-1 was shown to stimulate differentiation and mitosis of Leydig cells (Lin *et al.* 1998). Conversely, the decrease in estrogen production seems to inhibit Leydig cell differentiation in prepubertal as well as adult rat testis (Dhar & Setty 1976, Abney & Myers 1991). Therefore, it seems reasonable to speculate that thyroid hormone actions on Leydig cells might be, at least in part, mediated through Sertoli cells.

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Thyroid gland disorders were also shown to be associated with alterations in the hypothalamo-pituitary–testicular axis, which indirectly could affect Leydig cells. However, inconsistent alterations in the pattern of circulating gonadotropins and testosterone have been reported in hypothyroid males. Hypothyroidism was found to be associated with a significant decrease in plasma gonadotropins and testosterone levels in several reports (Chandrasekhar *et al*. 1986, Ruiz *et al*. 1989, Antony *et al*. 1995, Jannini *et al*. 1995, Kirby *et al*. 1997, Chiao *et al*. 1999, Maran *et al*. 2000*b*, 2001, Rao *et al*. 2003), while in others no such effects were observed (Kalland *et al*. 1978, Corrales Hernandez *et al*. 1990, Maia *et al*. 1990, Cristovao *et al*. 2002). These inconsistencies have been attributed to differences in the age, duration of treatment, and method of inducing the hypothyroid state in experimental animals (Maran *et al*. 2001, Maran 2003, Mendis-Handagama & Siril Ariyaratne 2005).

Likewise, evidence of direct actions of thyroid hormones on Leydig cell steroidogenesis has been demonstrated in different studies (Jana & Bhattacharya 1994, Manna *et al*. 1999, Maran *et al*. 2000*a*). It has been reported that T_3 directly stimulates and enhances LH-induced androgen secretion in goat Leydig cells (Jana *et al*. 1996), whereas hypothyroidism decreased testosterone and cAMP production in response to LH in rat testis (Antony *et al*. 1995). Decreased 3β-hydroxy steroid dehydrogenase (HSD) and 17β-HSD activities were also associated with decreased thyroid hormone levels (Antony *et al*. 1995). Similarly, thyroidectomy in adult rats led to decreased secretion of testosterone and decreased activity of 17β-HSD (Chiao *et al*. 1999). T_3 treatment of Leydig cells isolated from adult rats resulted in increased secretion of testosterone and estrogen under basal conditions as well as in response to LH stimulation, in a dose-dependent manner (Maran *et al*. 2000*a*). It has also been observed that chronic stimulatory effect of T_3 on Leydig cells increases the mRNA levels of the cytochrome P450 side-chain cleavage enzyme, while it decreases cytochrome P450 17α-hydroxylase and 3β-HSD (Manna *et al*. 2001*b*).

Recent studies have shown that T_3 treatment of mouse Leydig cells increases the levels of the steroidogenic acute regulatory (*Star*) mRNA and protein, as well as steroid production, and these responses were dependent on the expression of steroidogenic factor 1 (SF-1; Manna *et al*. 1999, 2001*a*,*b*). STAR protein mediates a rate-limiting step in Leydig cell steroidogenesis, the translocation of cholesterol from the outer to the inner mitochondrial membrane (Clark *et al*. 1994, Stocco & Clark 1996). Additionally, these studies showed that the inhibition of SF-1 expression by DAX-1 markedly abolished T_3-mediated STAR expression concurrently with steroid biosynthesis decrease. These findings suggest that thyroid hormone and STAR protein work in a coordinated manner to regulate steroid hormone biosynthesis in Leydig cells (Manna *et al*. 2001*b*).

The above reviewed data support the concept that thyroid hormone plays an important role on Leydig cell differentiation and function. However, a direct thyroid hormone effect on Leydig cells is still a matter of debate. The presence of TRs in Leydig cells is an issue that has not been completely resolved. Although TRs have been described in a subset of testicular interstitial cells in rats by immunocytochemistry, the specific cell type was not identified (Tagami *et al*. 1990, Buzzard *et al*. 2000). Further studies focus in this issue will be particularly important to identify the mechanisms by which thyroid hormone affects Leydig cells.

TRs and transporters in testicular cells

The first studies describing the presence of specific thyroid hormone nuclear-binding sites in Sertoli cell-enriched extracts and developing rat testis were of great significance, since these findings changed the classic view of the testis as a thyroid hormone unresponsive organ (Palmero *et al*. 1988, Jannini *et al*. 1990). Subsequently, several molecular techniques, such as RT-PCR (mRNA expression), *in situ* hybridization, western blotting, and immunohistochemistry, were used to demonstrate the presence of functional TR isoforms, TRα1 and TRβ1, in testicular cells. An ontogenic pattern of TRs expression in rat and human testis was established (Jannini *et al*. 1994, 1999, 2000). These studies showed that the active TRα1 isoform was expressed in human and rat testis at different levels throughout development, and that TRβ1 was completely absent in the testes of both species. The TRα1 expression was found to be maximal in late fetal and early neonatal life and restricted to Sertoli cells, suggesting these as the main target cells for T_3 action in testis. Nevertheless, current analysis of published data indicates that active TR isoforms, including TRβ1, are also found in interstital and germ cells, not only during neonatal development but also in the adult testis (Arambepola *et al*. 1998*a*, Buzzard *et al*. 2000, Canale *et al*. 2001, Rao *et al*. 2003). These results emphasized that, although TRs expression was maximal during the perinatal period and subsequently declined, T_3-binding capacity is not completely absent in adult testis (Buzzard *et al*. 2000, Canale *et al*. 2001).

Because TRα1 and TRβ1 isoforms are expressed mainly in the neonatal Sertoli cells, either or both TRs could potentially mediate the effects of T_3 on Sertoli cells. To address this issue, Holsberger *et al*. (2005*a*) used TRα KO and TRβ KO (TRβKO) transgenic mice, lacking TRα or TRβ isoforms respectively, to determine the relative roles of these receptors in mediating T_3 effects on Sertoli cells and testicular development. Whereas neonatal hyperthyroidism reduced Sertoli cell proliferation to minimal levels and induced their maturation similarly in both wild-type and TRβKO mice, minimal changes were observed in Sertoli cell proliferation in the TrαKO mice. More interestingly, the TrαKO mice showed testicular phenotypic changes comparable with those observed in the wild-type mice following neonatal hypothyroidism. These observations indicate that TRα1 is the specific TR isoform mediating T_3 effects in neonatal Sertoli cells.

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In order to interact with specific nuclear receptors and generate a biological response, thyroid hormones have to cross cell membranes. It was originally believed that thyroid hormones, due to their lipophilic nature, enter target cells by passive diffusion. Currently, however, there is growing evidence indicating that T_4 and T_3 cross the plasma membrane by carrier-mediated mechanisms (Hennemann *et al.* 2001, Neves *et al.* 2002, Jansen *et al.* 2005). Several membrane transporter families have been identified, however, only monocarboxylate transporter (MCT) 8, MCT 10, and organic anion-transporting polypeptides (OATPs) demonstrate a high degree of specificity towards thyroid hormone (Visser *et al.* 2008). The OATPs form a novel family of transporter proteins that have been detected in several tissues, including testis, in rodents and humans (Suzuki *et al.* 2003, Hagenbuch & Meier 2004, Hagenbuch 2007, Westholm *et al.* 2008). The OATPs are involved in transporting organic anions such as steroid conjugates, bile salts, drugs, and thyroid hormones into the cells. Some OATPs show preference for the transport of certain substances and are predominantly expressed in a particular tissue, rendering their action more specific (Fujiwara *et al.* 2001).

Specific thyroid hormone membrane transporters have also been identified in testes. In the human testis, a specific OATP molecule named OATP-F, which transports T_4 and reverse T_3 (rT_3) with high affinity, was isolated and shown to be expressed only in Leydig cells (Pizzagalli *et al.* 2002). Three novel members of the OATPs family designated gonad-specific transporters (GSTs) were identified in human and rat (GST-1 and GST-2) testis (Suzuki *et al.* 2003). The rat GST-1 and GST-2 is highly expressed in Sertoli cells, spermatogonia, and Leydig cells, and functional studies revealed both transport T_4 and T_3 in these cells. Additionally, two novel splice variants of OATPs, OATP3A1-V1 and OATP3A1-V2, recently isolated from human brain, were also found to be expressed in testicular germ cells and Sertoli cells respectively (Huber *et al.* 2007). However, the physiological relevance of these transporters in regulating thyroid hormone bioavailability to testicular cells is currently unknown.

Thyroid hormone actions on target tissues are predominantly mediated by specific nuclear receptors able to bind to regulatory regions of target genes modifying their expression (Yen *et al.* 2006). Nevertheless, thyroid hormones also have well-known non-genomic actions (Davis & Davis 1996, Shibusawa *et al.* 2003). Contrary to the genomic events, a number of thyroid hormones effects on plasma membrane, cytoplasm, and sub-cellular organelles occur rapidly and are unaffected by transcription and translation inhibitors. These non-genomic actions include the regulation of ion channels, oxidative phosphorylation and mitochondrial gene transcription, and generation of intracellular secondary messengers (Bassett *et al.* 2003, Davis *et al.* 2008). Recently, an increasing number of thyroid hormone non-genomic effects have been described in tissues such as brain (Leonard 2008), heart (Portman 2008), skeletal muscle (Irrcher *et al.* 2008), fibroblasts (Bhargava *et al.* 2007), and vascular endothelial cells (Hiroi *et al.* 2006).

In addition to classical genomic effects, non-genomic responses to thyroid hormones have also been described in testis. Electrophysiological studies demonstrated that both hormones, T_4 and T_3, produced immediate hyperpolarization of Sertoli cell membrane potential that involved $K^{(+)}$ channels (Menegaz *et al.* 2006). This study also showed a potent T_4 stimulatory effect on amino acid accumulation probably related to its effects on Sertoli cell membrane potential, since amino acid accumulation was independent of active protein synthesis. It has also been reported that *in vitro* administration of T_3 to isolated rat testis stimulates, by non-genomic mechanisms, the phosphorylation of vimentin (Zamoner *et al.* 2005), a cytoskeletal-associated protein that seems to be involved in the modifications of Sertoli cell morphology throughout development (Tanemura *et al.* 1994). The thyroid hormone-induced increase in *SLC2A1* mRNA levels in immature Sertoli cells (Ulisse *et al.* 1992) also seems to be mediated by a non-genomic mechanism. Recently, studies using transient transfections in primary Sertoli cell cultures have shown that T_3 does not directly regulate *SLC2A1* gene promoter (Carosa *et al.* 2005). This observation was further confirmed by the absence of any recognized thyroid responsive element (TRE) in the rat *SLC2A1* promoter (Carosa *et al.* 2005). Thus, it might be possible that T_3 modulates *SLC2A1* mRNA levels by interfering with *SLC2A1* mRNA stability.

Recently, it was shown that T_3 promotes a rapid up regulation of gap junction plaque number on Cx43-GFP-transfected cells (Gilleron *et al.* 2006). This effect seems to be mediated through actin cytoskeleton control, since cytochalasin D totally reversed T_3 stimulatory effect. The rapid non-genomic responses to thyroid hormones are currently viewed as a complementary pathway to genomic mechanisms, which may improve cell regulation by these hormones.

Expression of iodothyronine deiodinases in testis

The availability of the biologically active T_3 is essential for normal developmental processes in mammals and other vertebrates. As different tissues have specific temporal patterns of development, it is likely that their T_3 requirement varies widely, suggesting a need for the regulation of intracellular T_3 generation (Escobar-Morreale *et al.* 1996). Thyroid hormone metabolism by deiodinases regulates the local availability of T_3 (Bianco *et al.* 2005, St Germain *et al.* 2005, Gereben *et al.* 2008) and plays a critical role in the adaptation of the organism to environmental and internal changes such as exposure to cold, starvation, illness, and thyroid status (Kohrle 2007).

All three Deiodinases, D1, D2, and D3, are expressed in testis at different levels from weanling to adult life (Bates *et al.* 1999). D3 activity predominates in the developmental period and then declines in adult life. Although both D1 and D2 are present in testis, their relative levels of activity indicate that D2 is the predominant activating enzyme in this organ. It is noteworthy that the highest level of D2 expression, known to play a major role in the intracellular conversion of T_4 to T_3, occurs at a prepubertal age, a critical period of testicular

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development when TRs are highly expressed in testis (Buzzard *et al.* 2000, Jannini *et al.* 2000). Interestingly, D2 activity was significantly induced in the testis of neonatal hypothyroid rats, suggesting a D2 role in maintaining T_3 concentration in testis when T_4 levels are reduced in plasma (Bates *et al.* 1999). Similarly, studies performed by our group demonstrated that induction of even mild hypothyroidism in adult mice also significantly increases D2 activity in testis (Wagner *et al.* 2003). Unexpectedly, we found that D2 expression in the adult rat testes is highly concentrated in elongated spermatids (Fig. 2), whereas other germ cells and Sertoli cells were virtually negative for this enzyme (Wajner *et al.* 2007). This suggests that thyroid hormone may play a role in spermatogenesis in the adult rat testis, specifically on the spermiogenic phase. The coexpression of D2 and D3 in testis from weanling to adult life seems to indicate a need for tight control of intracellular T_3 levels in this organ.

Thyroid hormone effects on the adult testis

It is now well established that thyroid hormone deficiency during early stages of testicular development affects testis growth and physiology adversely. However, the role of thyroid hormone on the adult testis is unclear and contradictory results have been reported. Early studies showed that induction of hypothyroidism in adult male rats has little effect on testicular morphology, spermatogenesis, and serum testosterone levels (Vilchez-Martinez 1973, Weiss & Burns 1988). In contrast, chronic hypothyroidism induced in rats, from birth to adulthood, was shown to be associated with delayed maturation of the testis, impaired spermatogenesis, germ cells degeneration, and reduced seminiferous tubule diameter (Francavilla *et al.* 1991, Meisami *et al.* 1994, Simorangkir *et al.* 1997, Maran & Aruldhas 2002). The congenital hypothyroid *rdw* rat is a strain of dwarf mutant that has decreased serum T_4 levels due to a missense mutation in the thyroglobulin gene (Hishinuma *et al.* 2000, Kim *et al.* 2000). These animals constitute an interesting model to study the consequences of prolonged thyroid hormone deficiency on testes at different ages, from early neonatal life to the adult stage. Studies performed by Sakai *et al.* (2004) showed that, although it took more time, normal structures developed in the testes of adult *rdw* rats (Fig. 3). However, soon after full testicular maturation was accomplished, normal morphology began to degenerate. Many germ cells underwent apoptosis and the germinal epithelium became thin, changes rarely observed in normal rat testes (Sakai *et al.* 2004).

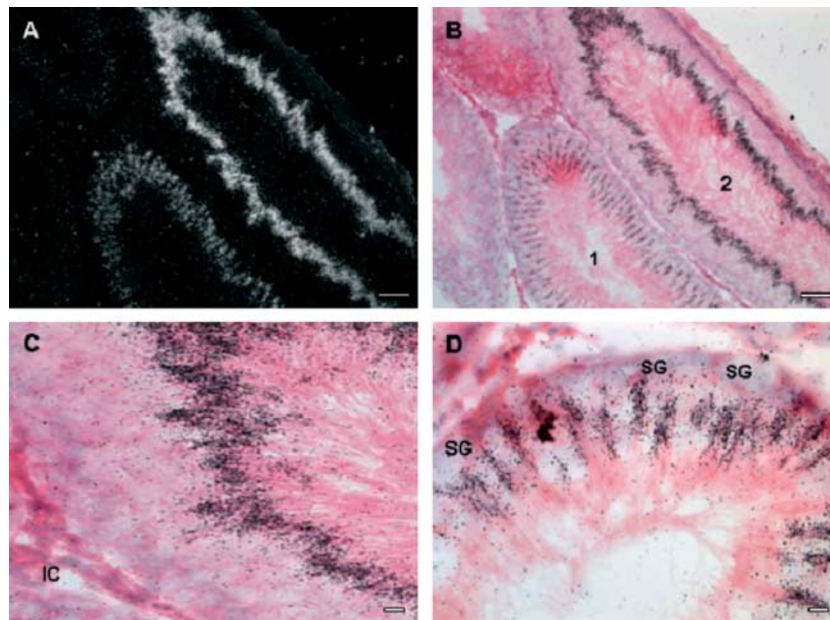

Figure 2 *In situ* hybridization autoradiograms of type 2 iodothyronine deiodinase (D2) expression in rat seminiferous epithelium. Dark (A) and bright (B) field microscopy show longitudinal sections of the seminiferous epithelium with intense labeling for D2 mRNA in spermatids. Tubule 1 is on stage III/IV of the cycle, in which spermatids are in the process of elongation and localized more internally in the tube wall. Tubule 2 is on stage VII/VIII of the cycle. (C) Higher magnification of part of tubule 2 showing interstitial cells (IC) negative for D2 mRNA and intense D2 labeling in elongated spermatids close to the lumen. A negligible background can de observed. In (D), a high magnification of a cross-section of seminiferous tubule in stage V of the cycle shows D2-positive spermatids localized in the middle of the tubule. Note that spermatogonia (SG) are negative. Scale bars, 50 μm (A and B) and 12 μm (C and D).

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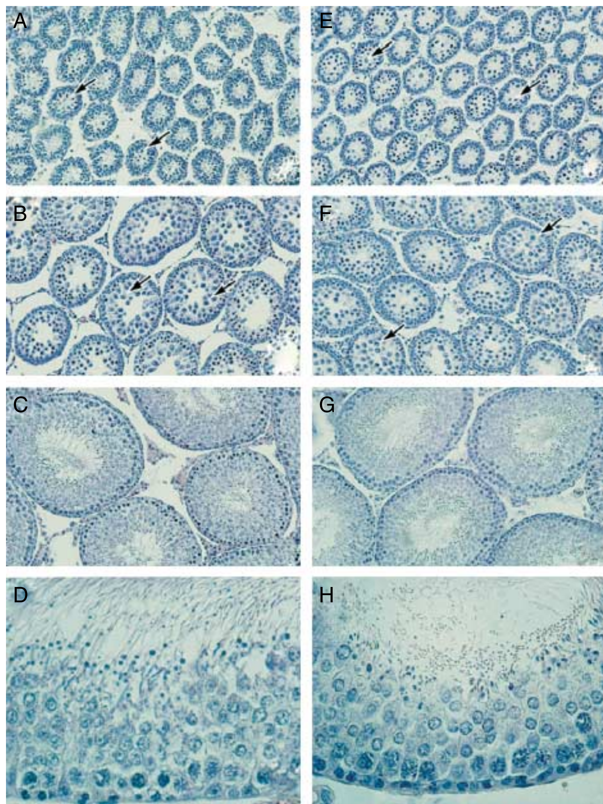

Figure 3 The control (+/?) and *rdw* rat testes stained with HE. (A) Two-week-old control (+/?) rat testis. Some spermatocytes can be seen in the center of the seminiferous tubules (arrows; magnification: ×140). (B) Four-week-old control (+/?) rat testis. Spermatocytes (arrows) become large but initial meiotic division has not yet been detected (magnification: ×140). (C) Eight-week-old control (+/?) rat testis. Seminiferous tubules fully developed (magnification: ×140). (D) Enlarged picture of (C). Even at high magnification (×560), no difference from the normal adult germinal epithelium can be detected. (E) Four-week-old *rdw* rat testis. Some spermatocytes (arrows) can be seen in the seminiferous tubules. This corresponds to the 2-week-old normal one (magnification: ×140). (F) Eight-week-old *rdw* rat testis. Spermatocytes (arrows) become large but initial meiotic division has not yet been detected. This corresponds to the four-week-old normal one (magnification: ×140). (G) Twenty-two-week-old *rdw* rat testis. Seminiferous tubules apparently seem to be fully developed. This corresponds to the 8-week-old normal one (magnification: ×140). (H) Enlarged picture of Figure g (magnification: ×560). (Permission taken from the publisher, Development, Growth and Differentiation (2004) **46**, 327–334).

The degeneration gradually proceeded and finally produced atrophic testes. The spermatocytes and spermatids were in direct contact with each other, Sertoli cells did not completely enclose the germ cells in *rdw* testes. Of note, the infertility described in the male *rdw* rat is partially reversed by T_4 treatment (Jiang *et al.* 2000, Umezu *et al.* 2004).

Similar histological changes were observed in the testis of adult rats subjected to chronic thyroid hormone deficiency due to thyroidectomy performed early in life (Oncu *et al.* 2004). In addition to the histological changes, reduced serum gonadotropins and testosterone levels were observed in both rat models. Accordingly, prolonged PTU treatment in rats, from birth to 90 days, was shown to result in a significant decrease in germ cell number and in the percentage of live sperm in the epididymis (Sahoo *et al.* 2008). Observations in the above studies indicate that prolonged thyroid hormone deficiency results in marked testicular degenerative changes, suggesting that thyroid hormone plays an important role not only in controlling normal testicular development but also in maintaining normal testicular function. However, one should keep in mind that hypothyroidism is a complex hormonal dysfunction rather than a single hormonal defect (Gomez Dumm *et al.* 1985). Hypothyroidism has been also shown to reduce the secretion of gonadotropin-releasing hormones, LH, FSH, GH, and testicular testosterone in rats, and all these changes seem to be corrected by T_4 administration. Therefore, many of the testicular changes observed in prolonged hypothyroidism could result in some degree of diminished levels of the aforementioned hormones.

Thyroid hormones and testicular antioxidant defense system

Thyroid hormones have recently been associated with the induction of oxidative stress in tissues, such as brain, heart, blood, muscle and liver (Zaiton *et al.* 1993, Huh *et al.* 1998, Shinohara *et al.* 2000, Bednarek *et al.* 2004, Das & Chainy 2004). Non-radical oxygen species, such as hydrogen peroxide, superoxide and hydroxyl radicals, which can be toxic to cells, are called reactive oxygen species (ROS; Venditti & Di Meo 2006). When ROS generation exceeds the antioxidant capacity of cells, oxidative stress develops. Cells are equipped with an enzymatic and non-enzymatic defense system to counteract ROS (Johnson & Giulivi 2005).

Interestingly, altered thyroid status has been shown to influence several oxidative stress and enzymatic antioxidant defense parameters in rat testis (Choudhury *et al.* 2003). For example, hyperthyroidism in the rat testis was associated with increased lipid peroxidation (LPx), indicative of oxidative stress, increased levels of reduced glutathione (GSH), an important component of non-enzymatic antioxidant defense, and increased levels of mitochondrial hydrogen peroxide (Sahoo *et al.* 2008). Increased activity levels of most antioxidant defense enzymes such as glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-*S*-transferase (GST), and catalase (CAT) have also been demonstrated (Zamoner *et al.* 2007). These results indicate that thyroid hormone treatment caused a high oxidative insult to the testis and are consistent with data showing that hyperthyroid tissues exhibit increased ROS production (Venditti & Di Meo 2006). Conversely, congenital and transient hypothyroidism seems to induce oxidative stress in testis by reducing the levels of testicular enzymatic and non-enzymatic defenses (Sahoo *et al.* 2008, Zamoner *et al.* 2008). The activities of superoxide

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dismutase (SOD), GR, GPx, and CAT as well as GSH content were significantly reduced in testis of transient hypothyroid rats (Sahoo *et al.* 2007).

Conclusion

Since the identification of functional thyroid receptors in Sertoli cells about two decades ago, greater insights have been gained into the role of thyroid hormone in testicular physiology. It has become clear that disturbance of the normal euthyroid state affects the morphological and functional development of the testis. The proliferation of immature Sertoli cells, an event that determines the extent of sperm production, was shown to be under the control of thyroid hormone. Furthermore, the Sertoli cell maturation process is at least in part regulated by T_3. Similarly, thyroid hormone was shown to play a critical role in the onset of Leydig cell differentiation in postnatal testis as well as in maintaining steroidogenic function with advancement of age. Thyroid hormone is also likely to contribute to normal spermatogenesis and metabolic processes in the adult testis, but these aspects are not well understood at present. The available data do not allow us to determine whether the adverse effects of prolonged hypothyroidism on testes development are mediated directly by low levels of circulating hormones, indirectly by testicular metabolic impairment, or both.

The molecular mechanisms by which thyroid hormone acts on Sertoli and Leydig cells are still unclear and further studies are necessary to establish how thyroid hormone controls Sertoli and Leydig cells proliferation, regulates testicular paracrine factors and how these impact on other events such as spermatogenesis, sperm motility, and ultimately fertility. Nevertheless, despite the gaps in our knowledge, the data reviewed here provide considerable evidence to conclude that thyroid hormone is an important hormonal regulator of testicular development and function.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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