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Review

Local Actions of Melatonin in Somatic Cells of the Testis

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Abstract: Environmental darkness signal is transferred from the retina to the pineal gland triggering melatonin secretion. Melatonin influences the synthesis and release of the hypothalamic GnRH and the adenohypophyseal gonadotropin hormones and therefore, regulates testicular function in photoperiodic species. Besides the brain, direct actions of melatonin at the testicular level have also been described. Melatonin released from the pineal gland to the circulation is taken up by peripheral tissues including testes. Testicular synthesis of melatonin has also been reported. The two key somatic cell types in the testis, Leydig and Sertoli cells, express melatonergic receptors. Melatonin acts as a local modulator of the endocrine activity in Leydig cells. In Sertoli cells, melatonin influences the oxidation state and the energy metabolism, and consequently may regulate spermatogenesis. Melatonergic receptors were also described in testicular macrophages and mast cells of infertile patients. Whereas melatonin exerts anti-proliferative and anti-inflammatory effects on testicular macrophages, it provides

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protective effects against oxidative stress in testicular mast cells. These data pinpoints melatonin as a key player in the regulation of testicular physiology (i.e. steroidogenesis, spermatogenesis) mostly in seasonal breeders. More importantly, melatonin is also involved in the modulation of testicular inflammatory and oxidant/anti-oxidant states in patients with idiopathic infertility.

Keywords: melatonin; testis; androgen production; oxidative stress; inflammation; infertility; Leydig cells; Sertoli cells; mast cells; macrophages

1. Introduction

Male reproductive functions are regulated by the LH and the FSH secreted by the pituitary. Hypothalamic gonadotropin releasing and inhibitory hormones (i.e. GnRH and GnIH), via hypothalamic-pituitary portal veins, are carried directly to the anterior pituitary gland where they bind specific receptors modulating the release of LH and FSH. LH binds to Leydig cells in the testicular interstitium stimulating androgen production. FSH binds to Sertoli cells in the seminiferous tubules stimulating their secretory activity and the subsequent normal progression of spermatogenesis [1,2]. Particularly, in rodents, pituitary control of the testis also involves the hormone prolactin which binds to Leydig cells and regulates the number of LH receptors [3].

Numerous species experience seasonal changes in their reproductive activities which are dependent of the photoperiod length [4]. Thus, in seasonal breeders, the function of the hypothalamic-pituitary-testicular axis undergoes cyclic variations consisting on periods of activation (during sexual maturation and at the beginning

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of each annual breeding season), and inactivation during the hibernation period. Photoperiod influences seasonal breeders through changes in melatonin secretion by the pineal gland. Light signal is received by the photoreceptors of the retina and transferred from the eyes to the pineal gland through a circuitous connection of neurons involving retinohypothalamic fibers, the suprachiasmatic nuclei, hypothalamus-pineal fibers, and the peripheral sympathetic nervous system, leading to the regulation of melatonin synthesis and secretion [5-7]. In long-day seasonal breeders such as the hamster, the duration of daily melatonin secretion is negatively correlated with the time the seasonal animal is exposed to daylight, and therefore, light signal is interpreted as pro-gonadotropic. On the contrary, light signal results anti-gonadotropic in short-day animals such as several breeds of sheeps and goats. Short-day animals are sexually active during the shortest days of the year when melatonin levels are maximal in terms of their nocturnal duration [8]. Finally, in non seasonal breeders like humans, which are sexually active all through the year irrespective of the season, the role of melatonin on male reproduction is still poorly understood [9].

Melatonin exerts its effects via specific receptors coupled to G protein and characterized by a seven transmembrane-spanning domain. Cloning studies identified three subtypes of membrane melatonergic receptors: *MT1* receptors which are expressed in the suprachiasmatic nucleus of the hypothalamus and pars tuberalis of the pituitary, *MT2* mainly located in brain and retina, and *MT3* receptors which are not expressed in mammals but were found in fish, amphibians, birds and chickens [10]. *MT1* and *MT2* receptors were found in rodent testis including mice, rats and hamsters [11-14]. Melatonin also exerts its effects by binding to orphan

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nuclear receptors (the retinoid-related orphan nuclear hormone receptor RZR/RORα and the X-linked melatonin-related orphan receptor GPR50) and intracellular proteins such as calmoduline [10].

In males, besides its effect on the synthesis and secretion of the hypothalamic GnRH and the adenohypophyseal gonadotropin hormones, melatonin released from the pineal gland to the circulation is taken up by testis where it directly modulates testicular activity [8,15-17]. In addition, testes are also able to synthesize melatonin [18-21].

This review summarizes the current state of knowledge with regards to the local effects exerted by melatonin on the different somatic cells of the testis. Furthermore, data highlight the relevance of melatonin in testes of men with idiopathic infertility through its modulatory role on inflammation and the oxidative stress.

2. Melatonin in Leydig cells

Initial studies examined the *in vitro* effect of melatonin on Leydig cell steroidogenesis. For instance, Wu et al. [22] found that this indolamine directly inhibited hCG- or db$_c$AMP-stimulated progesterone production as well as acute regulatory (StAR) protein expression in MA-10 mouse Leydig tumor cells. However, 22R-hydroxycholesterol reversed melatonin's inhibitory effect, suggesting that melatonin does not suppress cytochrome P450 family 11 subfamily A member 1 (CYP11A) enzyme activity [22].

In accordance with this report, data from our group revealed that physiological concentrations of melatonin exert a direct inhibitory effect on hCG-stimulated

cAMP and androgen production in Leydig cells from Syrian (golden) hamsters (*Mesocricetus auratus*) [12]. Mouse and rats do not have a breeding season. Instead, reproductive activity in Syrian hamsters is restricted to spring and summer. Under artificial light conditions, male adult hamsters need to be kept under a long day (LD) photoperiod (14 h light, 10 h dark) to remain sexually active. Exposure to a short-day (SD) photoperiod (less than 12.5 h of light per day) for a period of 8-16 weeks results in a marked testicular regression as a consequence of a severe fall in LH, FSH and prolactin circulating levels and a decline in blood and gonadal concentrations of testosterone, its hormonal precursors, and its metabolites [1,23]. Besides the absence of stimulating pituitary factors, a negative regulation of steroidogenesis by signals originated within and/or outside of the testis contributes to the profound decrease detected in serum androgen concentrations during the involution phase in the Syrian hamsters. In this context, whereas melatonin reduced basal and hCG-stimulated testosterone production in Leydig cells of hamsters maintained under a LD photoperiod, it did not alter testosterone secretion from Leydig cells of regressed hamsters. However, melatonin diminished hCG-stimulated 5α-androstane-3α,17β-diol (3α-Diol) secretion in Leydig cells purified from hamsters exposed to a SD photoperiod for 16 weeks. It is important to bear in mind that although testicular and circulating levels of androgens are markedly reduced during the regression period, inactive adult hamster testes released more 5α-reduced compounds [dihydrotestosterone (DHT) and 3α-Diol] than active adult hamster testes, being 3α-Diol the main androgen produced under *in vitro* conditions [24].

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Testicular regression involves profound morphological changes in the tubular as well as in the interstitial compartments. The number of Leydig cells per testis fluctuates only very slightly during the involution phase. Nevertheless, it has been described a significant reduction in the absolute volume and surface area of nearly all of the Leydig cells organelles including mitochondria and smooth endoplasmic reticulum [1,25,26]. In spite of the morphological changes taking place in the main site of androgen biosynthetic enzymes, our findings indicate that testicular androgen biosynthetic capacity is not reduced in adult hamsters exposed to a SD photoperiod for 16 weeks. In fact, regressed testes reached an intermediate physiological state between peripubertal and active adult testes (see further details in Frungieri et al. [24]).

Subsequent studies indicated that melatonin reduced the expression of StAR protein and important steroidogenic enzymes [CYP11A, 3β-hydroxysteroid dehydrogenase (3β-HSD), and 17β-hydroxysteroid dehydrogenase type III (17β-HSD3)] in hamster Leydig cells [12]. In this context, Maitra and Ray [27] also described a significant decrease in the testicular activity of 3β-HSD and 17β-HSD in adult rats injected with melatonin. In Leydig cells of hamsters kept under a LD photoperiod, melatonin clearly inhibited mRNA expression of steroid 5α-reductase 1 (5α-R1), an enzymatic isoform that plays a crucial role for the testicular conversion of testosterone into the active and nonaromatizable testosterone metabolite DHT [12,28]. However, in melatonin-treated Leydig cells of regressed hamsters, 5α-R1 expression was significantly induced [12]. Thus, in Leydig cells of inactive hamsters, melatonin stimulates the conversion of testosterone into 5α-

reduced androgens. Supporting this assumption, 5α-reductase activity and DHT production were markedly increased in testes of hamsters exposed to a SD photoperiod for 16 weeks compared to the values detected in testes of sexually active hamsters [24]. Furthermore, the expression of 3α-hydroxysteroid dehydrogenase (3α-HSD), an enzyme that catalyzes the interconversion between DHT and 3α-Diol, was significantly reduced in the presence of melatonin in the incubation media of hCG-treated Leydig cells from inactive hamsters [12]. Consequently, in testes of inactive hamsters, melatonin modulates the production of DHT and 3α-Diol by inducing the conversion of non-5α-reduced into 5α-reduced androgens and inhibiting the production of the main androgen 3α-Diol.

Testosterone production by Leydig cells is stimulated not only by LH, which activates the cAMP pathway, but also by several endocrine and paracrine factors which act through cAMP-independent pathways [29]. For example, GnRH stimulates androgen secretion via activation of protein kinase C (PKC) and an increment in the cytosolic Ca^{2+} concentrations. Subsequent studies showed that melatonin reduced GnRH-induced testosterone secretion by suppressing the GnRH-dependent release of Ca^{2+} from intracellular stores [30].

Initial characterization of melatonin receptors in Leydig cells by 2-[125I] iodomelatonin-binding studies and by pharmacological assays using luzindole (a *MT1/MT2* receptor antagonist) were unable to discriminate among different subtypes of melatonin receptors [22,31]. Further experiments using reverse transcription polymerase chain reaction (RT-PCR) and Western blot techniques

characterized the presence of *MT1* but not *MT2* expression in hamster Leydig cells [12].

Cross-interactions between the melatonergic system and other systems located in the testis have been described. In this regard, melatonin modulates testosterone production via its interaction with the testicular corticotropin-releasing hormone (CRH) system. CRH is secreted by the hypothalamus and regulates the pituitary–adrenocortical axis [32]. However, it has also been reported the production and secretion of CRH as well as the expression of its receptor (*CRH-R1*) in mouse, rat and hamster Leydig cells [12,33-36]. CRH modulates testosterone production although controversial findings have been published in mouse and rat Leydig cells [33,34]. CRH has been proposed as a negative modulator of hCG-stimulated testicular steroidogenesis in rats. However, the basal production of testosterone and cAMP remained unchanged when rat Leydig cells were incubated in the presence of this hormone [33,34,37]. In mouse Leydig cells, it has been described that CRH increases the basal testosterone production and cAMP concentration but without affecting the maximum hCG-stimulated testosterone synthesis [36,38].

Data from our group revealed an inhibitory effect of CRH on gonadotropin-induced cAMP and androgen production in hamster Leydig cells from both, reproductively active and inactive animals [12]. Interestingly, melatonin induced CRH production in Leydig cells from hamsters kept under a LD photoperiod, but did not affect CRH levels in Leydig cells from regressed animals. Intracellular concentrations of CRH in Leydig cells from hamsters exposed to a SD photoperiod for 16 weeks duplicate those detected in Leydig cells of hamsters kept under a LD photoperiod, but are similar to those found in melatonin-treated Leydig cells from reproductively active

hamsters [12]. Thus, Leydig cells of regressed adult hamsters exposed to light deprivation and extended night melatonin secretion from pineal gland into circulation seems to be already challenged to synthesize more CRH than that produced from Leydig cells of active hamsters.

Additional experiments demonstrated that the competitive CRH receptor antagonist α-helical CRH (9–41) blocks the inhibitory effect of melatonin on hCG-stimulated cAMP and androgen production in Leydig cells from both reproductively active and inactive hamsters, suggesting that melatonin effect on steroidogenesis could take place through the local CRH system [12].

The expression of the two key enzymes involved in melatonin synthesis, arylalkylamine-*N*-acetyltransferase (AANAT) and *N*-acetylserotonin-*O*-methyltransferase (ASMT) has been reported in ram Leydig cells [21]. These enzymes catalyze the conversion of serotonin into melatonin. Such as melatonin, serotonin inhibits testosterone production in Leydig cells through the CRH system [34,39]. Furthermore, the inhibition of testosterone production via the serotonin/CRH system remains under the influence of epinephrine and norepinephrine acting through *α1/β1*-adrenergic receptors located in hamster Leydig cells [39]. Overall, interactions between the testicular melatonergic, serotoninergic, catecholaminergic, and CRH systems take part in the modulation of cAMP and testosterone production at least in Leydig cells of the Syrian hamster (Figure 1).

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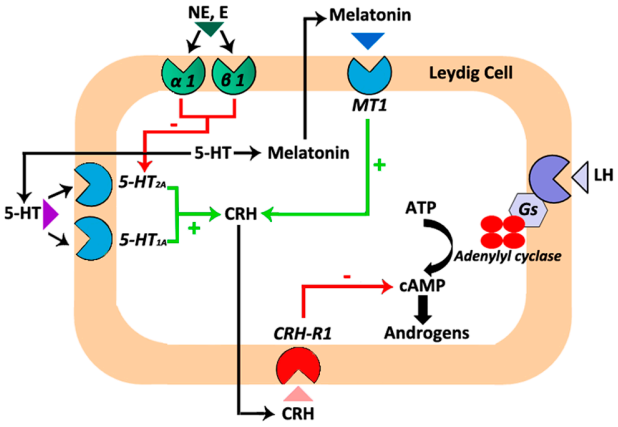

Figure 1. A summary of the interactions between the local melatonergic, serotoninergic, catecholaminergic and corticotropin-releasing hormone (CRH) systems that are involved in the regulation of androgen production in hamster Leydig cells.
Melatonin acting through specific *MT1* receptors located in hamster Leydig cells stimulates CRH production [12]. Serotonin (5-HT), the precursor of melatonin, also stimulates CRH production via *5-HT1A* and *5-HT2A* receptors [39]. Subsequently, CRH through *CRH-R1* receptors inhibits hCG-stimulated androgen production [12,39]. In addition, the inhibitory effect of the 5-HT/*5-HT2A* receptors/CRH system on androgen production is negatively modulated by epinephrine/norepinephrine and *α1/ß1*-adrenergic receptors [39].

Subsequent studies were developed to further establish the initial events of the melatonin/CRH signaling pathway. Results indicated that melatonin stimulates the activity of tyrosine phosphatases and reduces the phosphorylation levels of the transcription factors, erk and jnk, which directly or indirectly play roles in the regulation of cell cycle, apoptosis, inflammation, cell differentiation and proliferation [40]. A similar action of melatonin has been previously described in other experimental models [41-44]. It is known that erk phosphorylates and regulates the transcription of c-fos, whereas c-jun is mainly activated by the jnk pathway. In this context, melatonin also down-regulated c-fos and c-jun expression in hamster Leydig cells as well as *StAR* expression [40]. When tyrosine phosphatases activity, MAP kinases phosphorylation, early immediate genes and *StAR* expression, and testosterone production were evaluated in the presence and absence of the

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competitive CRH receptor antagonist α-helical CRH (9–41), results revealed that melatonin does not exert a direct role. On the contrary, this indolamine acts indirectly on tyrosine phosphatases, MAP kinases, transcription factors and the steroidogenic pathway via its stimulatory role on the local CRH production [40] (Figure 2).

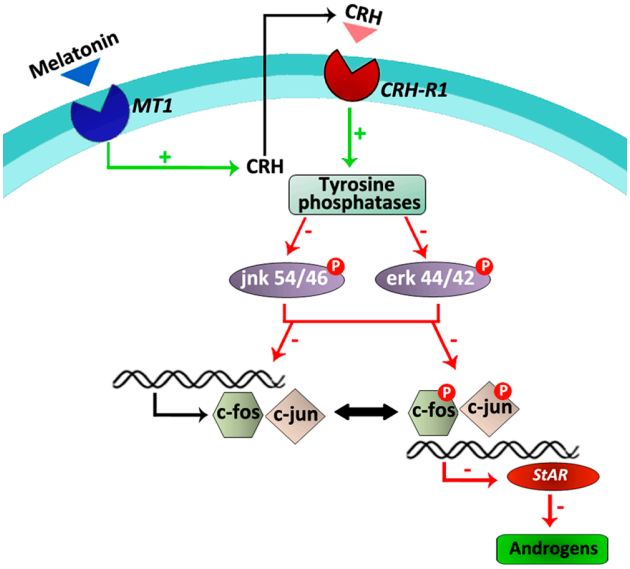

Figure 2. Schematic overview of the melatonin/CRH signaling pathway in hamster Leydig cells.
Melatonin locally stimulates CRH production in hamster Leydig cells. Thus, activation of tyrosine phosphatases via *CRH-R1* receptors, leads to reduced phosphorylation levels of erk 44/42 and jnk 54/46, down-regulation of c-jun and c-fos expression, inhibition of the transcription factors phosphorylation, decreased expression of steroidogenic acute regulatory protein (StAR) and consequently, diminished androgens production [12,40].

All components involved in melatonin/CRH-induced down-regulation of androgen production in hamster Leydig cells were also identified in testes of infertile men. For instance, testicular biopsies of patients suffering idiopathic infertility contain measurable levels of melatonin and human Leydig cells express CRH as well as *MT1* and *CRH-R1* receptors [40]. These data allow us to conjecture about the

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potential relevance of melatonin and/or CRH on human Leydig cell function in some fertility disorders.

3. Melatonin in Sertoli cells

The expression of *MT1* and *MT2* receptors has been reported in rat and bovine Sertoli cells [45,46]. In addition, unpublished data from our group reveal the expression of *MT1* and *MT2* receptors in the murine Sertoli TM4 cell line (Figure 3).

Sertoli cells are, within the seminiferous tubules, the major transducers of testosterone and FSH signals that are required to support germ cell survival and development [47]. Therefore, Sertoli cells play a key role in spermatogenesis efficiency and fertility [48].

Initial studies suggested that melatonin has adverse effects on mice and rats seminiferous tubules [49,50]. Sertoli cells provide energy substrates such as lactate required to fuel germ cell metabolism. It has been described that melatonin decreases basal lactate production, but up-regulates the insulin-stimulated lactate generation in rat Sertoli cells [46]. In this context, several biochemical mechanisms may contribute to alterations in lactate production and secretion; one of them is cellular glucose uptake, the main carbon source for lactate synthesis. In Sertoli cells, facilitated diffusion glucose transport across plasma membrane is mediated by the glucose transporters GLUT1, GLUT3 and GLUT8 [51-54]. It has been shown that melatonin increases GLUT1 protein levels and glucose consumption in rat Sertoli cells [46].

Lactate production also depends on lactate dehydrogenase expression (LDH) and activity. Melatonin decreases LDH protein levels and activity in rat Sertoli cells [46]. Not only lactate but also acetate can be used by Sertoli cells as a source and store of energy. Acetyl-CoA synthase is responsible for the esterification of acetate to acetyl-CoA in the cytosol and mitochondria, while acetyl-CoA hydrolase is responsible for the re-conversion of acetyl-CoA to acetate [55]. In addition, acetate may follow other paths. Arachidonic acid is formed from an exogenous C18 precursor (presumably derived from linoleic acid) by the addition of a C2 fragment derived from acetate. Sertoli cells are reported to play a key role in the conversion of essential fatty acids [56]. Melatonin increases acetate production and accumulation in rat Sertoli cells which may be essential for the maintenance of a high rate of lipid synthesis by developing germ cells [46].

Despite the low oxygen tension that characterizes the testicular micro-environment, the testis remains vulnerable to oxidative stress due to the abundance of highly unsaturated fatty acids and the presence of systems that generate reactive oxygen species (ROS) including the mitochondria and a variety of enzymes such as the xanthine- and NADPH- oxidases and the cytochrome P450s [57]. Oxidative stress might lead to an impaired spermatogenesis and therefore, to infertility [58]. There are many factors capable of inducing oxidative stress in the testes. For instance, we have recently described a pivotal role of PGD2 in the regulation of the oxidant/anti-oxidant status in the mouse TM4 Sertoli cell line [59]. Interestingly, in insulin-treated rat Sertoli cells, melatonin restores the intracellular redox state to its control levels [46]. Unpublished results from our group indicate that in murine TM4 Sertoli cells, melatonin decreases lipid peroxidation and activates the anti-oxidant

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system through induction of the enzymes *superoxide dismutase 1* (*Sod1*), *glutathione peroxidase (Gpx*), *peroxiredoxin 1* (*Pxr1*) and *catalase* (*Cat*) (Figure 3).

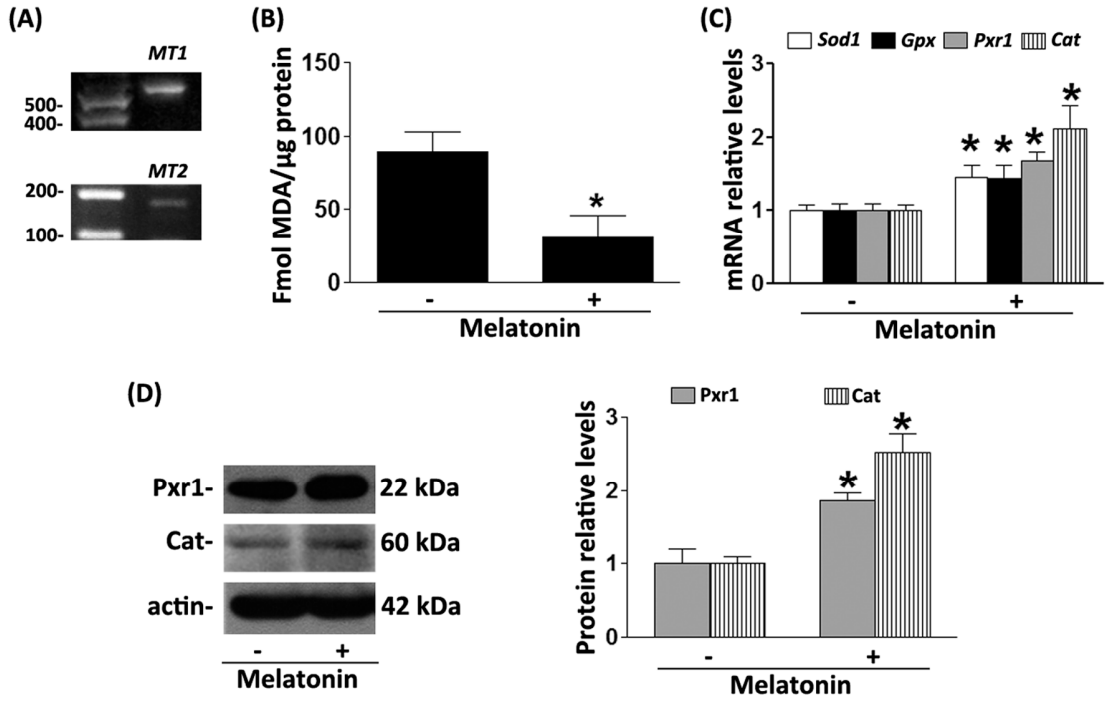

Figure 3. Melatonin regulates lipid peroxidation and the expression of anti-oxidant enzymes in mouse TM4 Sertoli cells.
(**A**) mRNA expression of *MT1* (560 bp) and *MT2* (197 bp) receptors in TM4 Sertoli cells was detected by reverse transcription polymerase chain reaction (RT-PCR) using the following oligonucleotide primers: *MT1*, 5´-GATGGAATCTGGGATATC and 5´-CCACGAACAGCCACTCTG; *MT2*, 5´-GGTGTCCTTCTGCTACCT and 5´-ATTGCCTCTGGGTTGATG. (**B**) TM4 Sertoli cells were incubated in the presence or absence of melatonin (1 µM) for 3 h. Lipid peroxidation was determined using the Thiobarbituric Acid Reactive Substances (TBARS) assay. Bar plot graph represents the mean ± SEM; n = 3. * $p < 0.05$, Student's t-test. (**C**) TM4 Sertoli cells were incubated in the presence or absence of melatonin (1 µM) for 3h. The mRNA expression of *superoxide dismutase 1* (*Sod1*), *glutathione peroxidase* (*Gpx*), *peroxiredoxin 1* (*Pxr1*) and *catalase* (*Cat*) was determined by real time-PCR using oligonucleotide primers from Rossi et al. [59]. *Gapdh* was chosen as the housekeeping gene. The mRNA anti-oxidant enzymes expression levels obtained in three independent experiments were analyzed using the mathematical model of Pfaffl. Bar plot graph represents the mean ± SEM. * $p < 0.05$, Student's t-test. (**D**) TM4 Sertoli cells were incubated in the presence or absence of melatonin (1 µM) for 5h. Protein expression of peroxiredoxin 1 (Pxr1, 22 kDa) and catalase (Cat, 60 kDa) was measured by immunoblotting. These immunoblots are representative of three experiments performed in different cell preparations that showed comparable results. Bar plot graph represents the mean ± SEM and depicts the quantification by densitometry of the bands obtained in three independent experiments. Results are expressed as fold

change relative to the control (cells incubated in basal condition) which was assigned a value of 1 and normalized to actin (42 kDa). * $p < 0.05$, Student's t-test.

On the other hand, melatonin has been shown to exert both anti- or pro-inflammatory actions depending on the physio/pathological status. As a rule, anti-inflammatory effects are the most evident under situations of high-grade of inflammation. In contrast, pro-inflammatory actions are frequently observed under basal conditions [60,61]. With regard to the above mentioned, novel results from our group indicate that melatonin induces the expression of cyclooxygenase 2 (*COX2*) and lipocalin-type prostaglandin D synthase (*L-PGDS*), key enzymes in the synthesis of prostaglandins, in murine TM4 Sertoli cells (Figure 4). Prostaglandins play a crucial role in the generation of the inflammatory response. Consequently, melatonin targeting Sertoli cells might contribute to the generation of inflammatory responses in the testis. Melatonin also affects Sertoli cell growth and proliferation. In bovine Sertoli cells, melatonin decreases the mRNA levels of P21, a potential inhibitor of G1 cyclin-dependent kinases [45]. In mouse TM4 Sertoli cells, melatonin increases proliferation (Figure 4).

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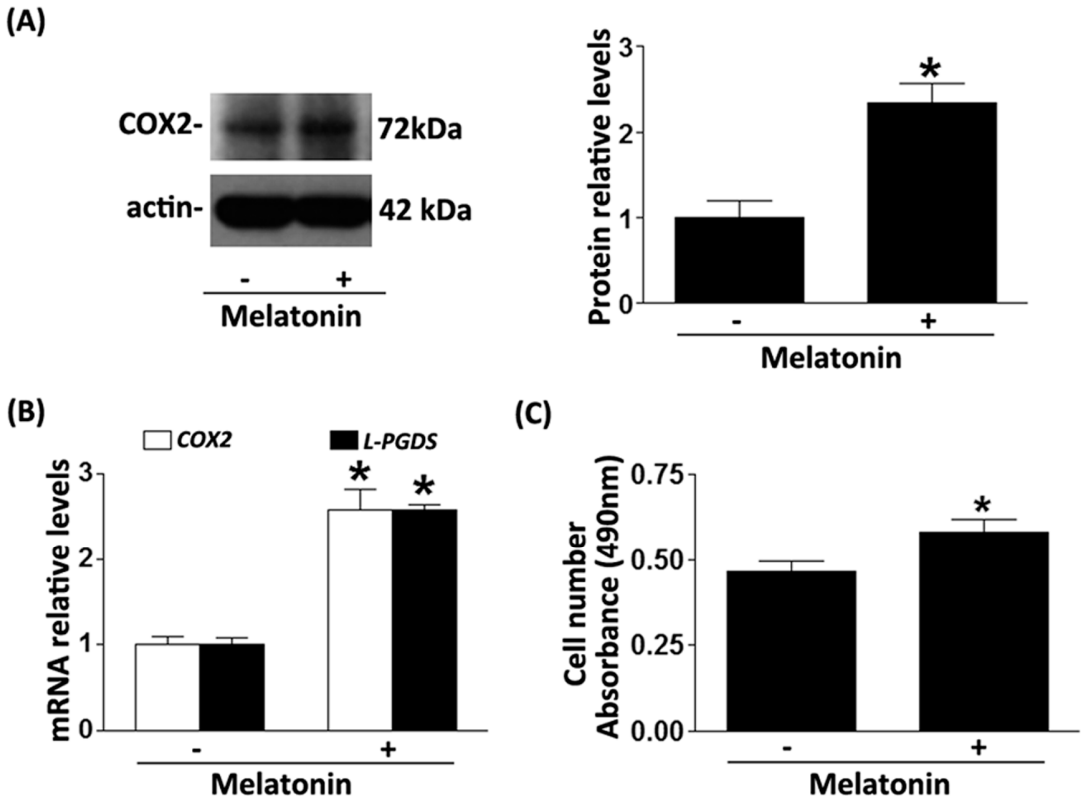

Figure 4. Melatonin regulates cell proliferation and the expression of *cyclooxigenase 2* (*COX2*) and *lipocalin-type PGD synthase* (*L-PGDS*), key enzymes in the synthesis of prostaglandins, in mouse TM4 Sertoli cells.
(**A**) Murine TM4 Sertoli cells were incubated in the presence or absence of melatonin (1 µM) for 5h. Protein expression of cycloxygenase 2 (COX2, 72 kDa) was measured by immunoblotting. These immunoblots are representative of three experiments performed in different cell preparations that showed comparable results. Bar plot graph represents the mean ± SEM and depicts the quantification by densitometry of the bands obtained in three independent experiments. Results are expressed as fold change relative to the control (cells incubated in basal condition) which was assigned a value of 1 and normalized to actin (42 kDa). * $p < 0.05$, Student's t-test. (**B**) TM4 Sertoli cells were incubated in the presence or absence of melatonin (1 µM) for 3h. The mRNA expression of *cyclooxygenase 2* (*COX2*) and *lipocalin-type PGD synthase* (*L-PGDS*) was determined by real time-PCR using oligonucleotide primers from Rossi et al. [59]. *Gapdh* was chosen as the housekeeping gene. The mRNA expression levels obtained in three independent experiments were analyzed using the mathematical model of Pfaffl. Bar plot graph represents the mean ± SEM. * $p < 0.05$, Student's t-test. (**C**) TM4 Sertoli cells increases in number after 24-h incubation in the presence of melatonin (1 µM). This figure shows data obtained from one of the three experiments that yielded comparable results. Bar plot graph represents the mean ± SEM of n = 10 replicate wells per treatment. Co, control cells incubated in the absence of melatonin.

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Moreover, melatonin promotes spermatogonial stem cells (SSCs) proliferation by stimulating glial cell line-derived neurotrophic factor (GDNF) production in Sertoli cells [62].

Melatonin also up-regulates the expression of spermatogenesis-related genes, including cyclin D1, cyclin E, Pdgfa, Dhh, ocludin, and claudin in bovine Sertoli cells and therefore, it might affect spermatogenesis and the blood-testis barrier [45].

It has been reported that in bovine Sertoli cells, melatonin significantly increases inhibin βA, inhibin βB and inhibin α mRNA expression as well as the secreted levels of inhibin B, a marker of Sertoli cell damage and spermatogenic disturbance [45]. In addition, melatonin increases the responsiveness of the Sertoli cell to FSH which may help to prevent testis damage [63].

From the aforementioned data, it is clear that melatonin exerts a plethora of functions in the Sertoli cell regulating its growth, proliferation, oxidant/anti-oxidant status, the energy metabolism and the production of prostaglandins. Because male fertility and the process of spermatogenesis are strongly dependent on Sertoli cells function, we can suppose that melatonin acts as an important modulator in the progression of germ cells to spermatozoa.

4. Melatonin in testicular immune cells

A large number of reports implicate melatonin as an immunomodulatory compound. Recently, the role of melatonin on the immune system has been described as that of a buffering agent, acting as a stimulant under basal or

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immunosuppressive conditions or as an inhibitory factor in the presence of exacerbated immune responses, such as acute inflammation [60].

Melatonin receptors are detectable in T helper cells, lymphocytes, granulocytes, mast cells and monocytes/macrophages [64-68]. On the other hand, bone marrow cells and the mast cell line RBL-2H3 are able to synthesize melatonin [68,69].

The testis is considered an immune-privileged organ. However, immune cells gain access to the testis and, some of them also undergo local proliferation. Leukocytes, including T cells, natural killer cells (NK), mast cells, eosinophils and macrophages were localized into the testis [70]. Among immune cells, mast cells and macrophages are best known for their role in inflammatory process. In the testis, mast cells and macrophages are also involved in the regulation of steroidogenesis, Sertoli cell activity, germ cell survival, and the generation of fibrosis in the wall of the seminiferous tubules [70-79].

The human testis contains melatonin as well as macrophages and mast cells expressing melatonergic receptors [80]. Data from our group described that the number of macrophages is significantly higher in testes of patients with hypospermatogenesis or Sertoli cell only (SCO) syndrome than in gonads of healthy men [76]. Moreover, testicular melatonin concentrations inversely correlate with the number of macrophages in biopsies from infertile patients. Subsequent studies established that melatonin decreases cell density and the expression of the proliferating cell nuclear antigen (PCNA) without affecting cell viability in both non-human testicular macrophages and human non-testicular macrophages [80].

In addition to its anti-proliferative role on testicular macrophages, melatonin also inhibited the expression of the pro-inflammatory cytokines TNFα and IL1β, as well

as the expression of COX2 in human non-testicular and testicular non-human macrophages [80]. Therefore, melatonin targeting testicular macrophages plays local anti-inflammatory effects. It has been already proposed that melatonin regulates the immune system by affecting cytokine production in immunocompetent cells. In this context, melatonin increases the production of IL-2, IFNγ and IL-6 in human mononuclear cells [81], the secretion of IL-1, IL-6, IL-12 and TNFα in monocytes [82], the production of IFNγ by Th1 cells [81] as well as the expression of IL-2 and IL-12 in NK cells [81,83,84].

Additionally to the anti-proliferative and anti-inflammatory effects of melatonin on testicular macrophages, Pawlak et al. [85] described that physiological concentration of this hormone significantly increased the phagocytic index in testicular macrophages of rats via a Ca^{2+}-dependent mechanism.

Although the number of testicular mast cells is significantly higher in infertile patients than in healthy men [74], no correlation was found between testicular melatonin concentrations and the number of mast cells in testes of patients suffering from hypospermatogenesis or SCO syndrome [80]. However, in the frog Rana esculenta, Izzo et al. [86] described that melatonin decreases testicular mast cell number. Mast cells secrete a plethora of potent mediators including proteases. Mast cells proteases are the most precise markers of mast cells population heterogeneity. Two types of mast cells have been recognized. Mast cells containing tryptase together with chymase, cathepsin-G like protease, and carboxypeptidase (MCTC), and mast cells which contain tryptase (MCT) but lack the other neutral proteases present in MCTC cells [87]. Therefore, the serine protease tryptase is of special interest because it is expressed in almost all

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populations of mast cells. The expression of tryptase and chymase was described in testicular mast cells of infertile patients [80]. The mast cells product tryptase decreased motility in human spermatozoa while chymase showed no such effect [88]. In peritubular cells of the human testis, tryptase induced COX2 expression, 15d-PGJ2 production and subsequently, fibrosis of the tubular wall [89-91]. Tryptase also alters the microenvironment in the human testes with regards to neurotrophin actions and the production of the extracellular matrix protein decorin [92,93]. Tryptase- and chymase-immunoreactive mast cells in testes of patients suffering from idiopathic infertility express the melatonin-synthesizing enzymes *AANAT* and *ASMT* (Figure 5).

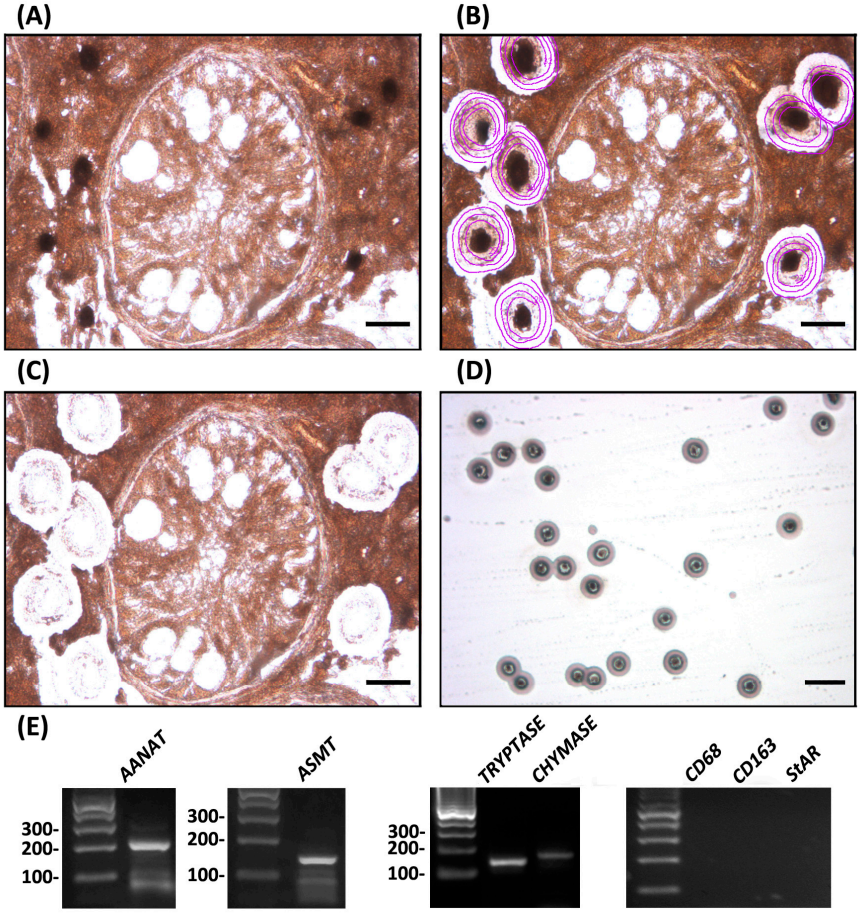

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Figure 5. Using laser capture microdissection, tryptase-immunoreactive mast cells were isolated from testicular biopsies of patients suffering from hypospermatogenesis or Sertoli cell-only syndrome, and subjected to reverse transcription polymerase chain reaction (RT-PCR) studies.
Panels depict the same specimen before laser microdissection (A), after ultraviolet laser delimitation of tryptase-immunoreactive mast cells (B) and after infrared laser microdissection of target cells (C). Bar, 25 µm. Panel D depicts tryptase-immunoreactive mast cells captured into the cap of a microfuge tube. Bar, 100 µm. A monoclonal mouse anti-human tryptase antibody (1:50, DAKO) was used.
(E) Expression of the melatonin-synthesizing enzymes *arylalkylamine-N-acetyltransferase* (*AANAT*; 234 bp) and *N-acetylserotonin-O-methyltransferase* (*ASMT;* 149 bp) was detected in the microdissected tryptase-immunoreactive mast cells by RT-PCR assays performed using the following oligonucleotide primers: *AANAT,* first set 5´-GGGACAAGGAGAGACTTA and 5´-TCAGCAGCCGCTGTTCC, heminested set 5´-CCGGCAGCAGGGCAGGGG and 5´- TCAGCAGCCGCTGTTCC; *ASMT,* 5´-GAGACGAGGGGAGGAAAAGC and 5´-GTCGTCCTTCTGCTACCT. Expression of the serine protease tryptase (142 bp) and chymase (168 bp) was used as positive controls. Expression of *CD68* and *CD163* (macrophage markers) and *StAR* (Leydig cell marker) in tryptase-immunoreactive mast cells was not found, indicating that the material employed in laser capture microdissection was not contaminated with other testicular cell populations. Expression of positive and negative controls was also determined by RT-PCR assays using oligonucleotide primers from Rossi et al. [80].
PCR products were separated on 2% agarose gels and visualized with ethidium bromide. The identity of the cDNA products was confirmed by sequencing, using a fluorescence-based dideoxy sequencing reaction and an automated sequence analysis on an ABI 373A DNA sequencer. The gene expression profile shown is representative of independent analyses performed in six different mast cell preparations (hypospermatogenesis, n = 3; Sertoli cell only syndrome, n = 3) that showed comparable results.

Furthermore, a direct correlation between testicular melatonin concentrations and the expression of these serine proteases in human testicular biopsies of patients with hypospermatogenesis and SCO syndrome was reported [80].

Under physiological conditions, the immunosuppressive testicular microenvironment protects germinal cells from being attacked by the immune system. However, in inflammatory conditions with increased density of resident macrophages and mast cells [74,76], this tolerance is disrupted and immune cells and their mediators respond to germinal cell self-antigens, inducing damage to the germinal epithelium [94]. The testicular damage induced by oxidative stress is currently considered as one of the most important causes of impaired testicular

function. Human testicular mast cells express the anti-oxidant enzymes *SOD1*, *Pxr1* and *CAT* [80]. A positive correlation between testicular melatonin concentrations and the expression of these anti-oxidant enzymes was found in biopsies of infertile men [80]. These results highlighting a potential anti-oxidant effect of melatonin on testicular mast cells, is in line with numerous reports that point out the ability of physiological concentrations of this indolamine to acts as a protector against free radical damage [95-98].

On the other hand, oxidative stress might play a pivotal role in apoptosis. In fact, it has been suggested that oxidative stress and apoptosis may be functionally linked [99]. In the testis, increasing amount of evidence suggests that oxidative stress can induce apoptosis in germ cells [100]. In agreement with this assumption, melatonin testicular concentrations showed a negative correlation with the pro-apoptotic Bax/Bcl-2 ratio in biopsies of infertile patients [80]. Consequently, the anti-oxidant role of melatonin on testicular mast cells seems to be associated with anti-apoptotic events.

In summary, testicular macrophages and mast cells are targets of melatonin. This indolamine exhibits anti-proliferative and anti-inflammatory actions on testicular macrophages, while melatonin might provide protective effects against oxidative stress and apoptosis in tryptase- and chymase-positive human testicular mast cells. Therefore, it is plausible to hypothesize about a potential biological relevance of melatonin acting as an immunomodulatory compound in the pathogenesis or maintenance of some states of infertility in humans.

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5. Concluding remarks and future perspectives

Currently, melatonin is commonly prescribed to treat sleep disorders [101]. It is considered a safe drug. However, in spite of the lack of apparent side effects, the use of melatonin in the treatment of other pathologies is still under discussion because several health benefits have only been attributed to pharmacological doses of this neurohormone.

At present, the majority of infertile men show disorders either untreatable or treatable with drugs of questionable effectiveness. Bearing in mind the research summarized in this review suggesting that melatonin therapy may improve male reproductive potential, the impact of this indolamine on male (in)fertility and/or its future as potential therapeutic targets should be further considered. In this sense, melatonin treatment reduced the severity of the testicular damage generated in animals models with hyperlipidemia, induced gonadal torsion, artificial varicocele or toxicity provoked by exogenous chemicals such as anti-cancer drugs or environmental toxicants [102-111]. In addition, serum levels of melatonin decrease with advancing age, which could be related to the therapy for diabetic and the impairment of fertility in elderly men [46]. Melatonin seems to protect human spermatozoa from apoptosis [112]. At this point, low melatonin levels have been associated with reduced sperm motility and abnormal sperm progression in infertile patients [113,114]. Moreover, melatonin levels in serum and seminal plasma of infertile patients suffering from oligoasthenozoospermia or non-obstructive azoospermia are significantly reduced compared with those quantified in fertile men [114].

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Regarding non-germ cells, a melatonergic system has been described in the two key somatic cell types of the testis: Leydig and Sertoli cells. Melatonin mainly acts as a local modulator of the endocrine activity in Leydig cells, while it regulates proliferation, oxidative stress, inflammatory process and energy metabolism in Sertoli cells. Furthermore, the existence of melatonergic receptors in testicular immune cells (mast cells and macrophages) showing a significant increase in their population number in some idiopathic pathologies, strongly suggests the importance of melatonin in the regulation of the local inflammation development that might further compromise testicular function in patients with idiopathic infertility.

Collectively, literature reports crucial roles of melatonin on testicular function. Therefore, future advances in the knowledge of the role played by melatonin and its receptors in the human testis will clarify the beneficial and/or disadvantageous effects of this indolamine for the clinical practice.

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