Inhibition of the basal and oestradiol-stimulated mitotic activity of primary spermatogonia by melatonin in the testis of the frog, *Rana esculenta*, *in vivo* and *in vitro*

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Melatonin has a direct inhibitory effect on the basal and oestradiol-stimulated mitotic activity of primary spermatogonia in the testis of the frog, *Rana esculenta*. In this study oestradiol was used to induce spermatogonial proliferation to verify the anti-proliferative effect of melatonin. The colchicine metaphase arrest technique was used. The results obtained from *in vivo* experiments confirm that oestradiol increases the mitotic index of primary spermatogonia and, for the first time, indicate that melatonin has an inhibitory role on the proliferation of primary spermatogonia in the frog testis. Similar results were obtained from testes of melatonin-injected frogs that were exposed to oestradiol *in vitro*; in fact spermatogonia were unresponsive to hormonal stimulation. In addition, in short-term cultured testes, melatonin (at physiological

concentration) interferes with the effects of oestradiol on spermatogonial proliferation, supporting the hypothesis that melatonin exerts the inhibitory effect directly via its local action on the frog gonads. Morphological observation after *in vivo* or *in vitro* melatonin treatments indicates that Leydig cells display degenerative features, whereas in adjacent germinal tubules, Sertoli cells show heterochromatic nuclei. These results indicate that melatonin may act on Leydig cells and confirm that there is a paracrine interaction between interstitial and germinal compartments. The results of the present study indicate, for the first time, that melatonin may be directly involved in the inhibitory control of spermatogonial proliferation in the testis of the frog, *R. esculenta*.

Introduction

Primary spermatogonia in the frog *Rana esculenta* are very dynamic cells exhibiting a marked seasonal variation in the mitotic index ratio. It has been shown that, in the frog testis, the multiplication of primary spermatogonia is temperature dependent (Rastogi *et al.*, 1985). In addition to high temperature, gonadotrophins and, to a certain extent, testosterone regulate the multiplication of primary spermatogonia (Rastogi *et al.*, 1990). It is well known that GnRH and its long acting agonist, buserelin, directly stimulate androgen production and multiplication of primary spermatogonia in the frog testis (Minucci *et al.*, 1986) and that oestradiol increases the mitotic index of primary spermatogonia via intratesticular mechanisms (Minucci *et al.*, 1997). However, to date, there is no information concerning the effects of melatonin on the proliferation of primary spermatogonia. Melatonin, the primary hormone of the pineal gland, plays a key role in a variety of important physiological responses, including reproduction (Morgan *et al.*, 1994).

In particular, melatonin provokes marked reproductive alterations in response to changes in daylength in seasonally breeding mammals (for reviews, see Reiter, 1991; Bartness *et al.*, 1993). Several lines of evidence indicate that melatonin is involved in basic mechanisms that control cell proliferation and differentiation (Hill and Blask, 1988; Cos *et al.*, 1991). Melatonin has a direct inhibitory effect on the proliferation of many types of cell (Persengiev and Kyurkchiev, 1993; Ying *et al.*, 1993; Chen *et al.*, 1995; Cos *et al.*, 1996; Gilad *et al.*, 1996; Petranka *et al.*, 1999; Shiu *et al.*, 1999), including the oestrogen-responsive MCF-7 human breast cancer cells (Liburdy *et al.*, 1993; Crespo *et al.*, 1994). In addition, Zhao *et al.* (2002) demonstrated that melatonin also inhibited the incorporation of [³H]-thymidine in the rat uterine anti-mesometrial stromal cells, showing an anti-proliferative effect on the uterus.

Considering that some preliminary investigations indicate a role for melatonin in inhibiting the multiplication of primary spermatogonia, in the present study the stathokinetic method was used to determine the mitotic activity by using a metaphase arresting agent (colchicine) to examine whether there are any effects of the administration of melatonin alone or in combination

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with oestradiol on the mitotic activity of primary spermatogonia in the testis of the frog, *R. esculenta*.

Materials and Methods

Animals

Adult male frogs, *R. esculenta*, were captured during March (10 h light:14 h dark) and October (12 h light:12 h dark) from the surroundings of Naples by a local dealer. The frogs were maintained in plastic tanks (23 cm × 16 cm × 11 cm) with water and food (meal worms) available *ad libitum*. Soon after capture or at the end of the experimental treatments, the frogs were killed by decapitation after anaesthesia with 1 g tricaine methane sulphonate (MS222) l⁻¹ (Sigma, St Louis, MO).

In vivo experiments

The frogs were divided into two groups: (i) vehicle treated (amphibian Krebs' Ringer bicarbonate buffer, pH 7.4; KRB, 100 µl) and (ii) melatonin (1 µg dissolved in 100 µl KRB) treated. The injections were administered into the dorsal lymph sac during the scotophase. At 24 h after the start of the experiment the animals of each group were further divided for oestradiol treatment (2 µg oestradiol dissolved in 100 µl KRB) as reported in Expts A and B (see below). The frogs of each experimental group were killed at 24 h after the last oestradiol injection. All the frogs were injected with colchicine dissolved in KRB (100 µg per 100 µl) 24 h before they were killed for the evaluation of the spermatogonial mitotic index.

Experiment A. The frogs ($n = 40$) of both vehicle- and melatonin-treated groups were further divided as follows: frogs ($n = 5$) that immediately received a single injection of oestradiol and frogs that also received a second injection of oestradiol 24 h later. The remaining frogs ($n = 20$) were used as untreated controls and melatonin-injected groups at 24 and 48 h.

Experiment B. The frogs ($n = 30$) of both vehicle- and melatonin-treated groups were further injected with a single dose of oestradiol immediately at 24 h and on day 7 after melatonin treatment. Five additional frogs were used as untreated initial controls (0 h).

In vivo and in vitro experiments

The frogs ($n = 20$) were divided into two groups: (i) vehicle treatment and (ii) melatonin treatment as in the *in vivo* experiments. At 24 h after the start of the experiment the frogs of each group were killed and each testis was cut in half and half of the testis was placed in a tube containing KRB alone, whereas the remaining half of the testis was placed in a tube containing KRB plus oestradiol (10^{-6} mol l⁻¹) for different time points (6, 12

or 24 h). For the evaluation of the spermatogonial mitotic index, colchicine (50 µg) was added to the tubes of each group at 6 h before fixation. All the tubes were placed at room temperature (20°C) in a shaking water bath and the incubation medium was replaced after 12 h.

In vitro experiments

Experiment A. The frogs ($n = 10$) were decapitated and each testis was cut in half. Half testes of five animals were incubated in KRB alone or in KRB plus oestradiol (10^{-6} mol l⁻¹) for 6 or 12 h. Half testes of the remaining five animals were used for incubations in KRB plus melatonin (10^{-6} mol l⁻¹) alone or in KRB plus melatonin (10^{-6} mol l⁻¹) plus oestradiol (10^{-6} mol l⁻¹), for the same times. All the tubes containing 50 µg colchicine were placed at room temperature in a shaking water bath.

Experiment B. Five animals were killed and each testis was cut in half. Half of the testis of each animal was incubated for 6 h in a tube containing KRB plus oestradiol (10^{-6} mol l⁻¹), KRB plus melatonin (10^{-6} mol l⁻¹) plus oestradiol (10^{-6} mol l⁻¹), KRB plus melatonin (10^{-9} mol l⁻¹) plus oestradiol (10^{-6} mol l⁻¹) or with KRB plus melatonin (10^{-12} mol l⁻¹) plus oestradiol (10^{-6} mol l⁻¹). All the tubes containing 50 µg colchicine were placed at room temperature in a shaking water bath. Half testes of five additional animals were incubated in a tube containing only KRB in the presence of colchicine and were used as 6 h incubation controls.

Histology and ultrastructure

The testes were fixed by immersion in Bouin's fluid, dehydrated in ethanol, cleared in xylene and embedded in paraffin wax. Tissue sections (5 µm) were prepared and stained with haematoxylin–eosin for histological studies.

For electron microscopy, small pieces (approximately 1 mm³) of testis were fixed for 2 h at 4°C in Karnovsky's fluid (0.1 mol l⁻¹) at pH 7.4 and post-fixed in 1% (w/v) osmium tetroxide in the same buffer at 4°C. Samples were then dehydrated in a graded series of ethanol and embedded in TAAB 812 (epoxy resin-araldite 812; TAAB Lab, Berkshire). Ultrathin sections were counterstained with 4% (w/v) uranyl acetate followed by 1% (w/v) lead citrate and observed under a Philips 301 transmission electron microscope.

Statistical analysis

Each value is the mean of five experimental animals. At least five sections, about 60 µm apart from each testis, were used for quantitative analysis. The mitotic index of the primary spermatogonia was expressed as the number of metaphases × total primary spermatogonia counted multiplied by 100. All the data are expressed as mean with standard deviations. The groups were compared by

Fig. 1. *In vivo* effect of oestradiol, oestradiol administered in combination with melatonin and melatonin alone on the mitotic index of primary spermatogonia (ISPG) after 24 (□) or 48 (■) h of treatment in testes of frogs (*Rana esculenta*) captured during March. Values are $\pm$ SD, a versus b $P < 0.01$, b versus c $P < 0.01$.

one-way ANOVA and Duncan's test (at $P < 0.05$ and $P < 0.01$) for multi-group comparisons.

Results

In vivo experiments

Experiment A. Testes obtained from frogs captured in March that were injected with oestradiol showed a significant increase in the mitotic index of primary spermatogonia at both 24 and 48 h in comparison with the untreated control testes (Fig. 1; $P < 0.01$). No significant differences were found in the testes of frogs injected with melatonin followed by oestradiol at 24 and 48 h before they were killed compared with testes of the untreated control testes (Fig. 1, $P < 0.01$). The mitotic index of the primary spermatogonia evaluated in the testes of frogs exposed to melatonin alone was higher compared with the mitotic index of the untreated control testes at both 24 and 48 h (Fig. 1; $P < 0.01$). Morphological observations indicate that testes of the untreated control frogs showed the characteristic features for March. Testes are characterized by the presence of primary and secondary spermatogonia, and several cysts containing primary and secondary spermatocytes. Few spermatids and residual spermatozoa were found in the centre of the tubules. It is interesting to note that primary spermatogonia (the largest type of germ cell present in the adult testis) consisting of voluminous eosinophilic cytoplasm and usually an irregularly-shaped nucleus are distributed singly and located adjacent to the basement membrane of the seminiferous tubules, each associated with one to several Sertoli cells. Primary spermatogonia divide mitotically to give rise to either two daughter primary spermatogonia or through repeated divisions, form a germinal cyst composed of secondary spermatogonia.

The testis of a frog treated with oestradiol at 24 h showing several primary spermatogonia in metaphases in the germinal compartments is shown (Fig. 2).

Fig. 2. Light micrograph showing primary spermatogonia (arrows) and secondary spermatogonia (asterisk) in metaphase in the testis of the frog (*Rana esculenta*) injected with oestradiol at 24 h after treatment. SPC: spermatocytes; SPT: spermatid; SPZ: spermatozoa. Scale bar represents 23 μ m.

Fig. 3. Electron micrographs showing (a) a primary spermatogonia (ISPG) in metaphase and (b) a Sertoli cell (S) surrounding a nest of secondary spermatogonia (IISPG) in metaphase in the germinal compartment of the testis of the frog (*Rana esculenta*) at 24 h after oestradiol injection. Scale bars represent (a) 2 μ m and (b) 7 μ m.

The electron micrographs of a primary spermatogonia and of a nest containing secondary spermatogonia that are surrounded by a Sertoli cell in metaphase in the testis of a frog treated with oestradiol at 24 h is shown (Fig. 3). In addition, in the testes of frogs treated with oestradiol both at 24 and 48 h, germinal tubules surrounded by normal Leydig cells were of typical morphology and architecture with respect to Sertoli cells and germ cells under both the light (Fig. 4) and electron microscope. In contrast, in the interstitial compartment of frogs injected with melatonin alone or with melatonin plus oestradiol, at any time, many Leydig cells displayed heterochromatic nuclei, cytoplasmic fragmentation and loss of cellular adhesion. In the adjacent germinal compartment, several Sertoli cells showed heterochromatic nuclei (Fig. 5). The electron micrograph of a primary spermatogonia enveloped by a shrunken Sertoli cell observed in the

Fig. 4. Electron micrograph showing two Sertoli cells (S) adjacent to a primary spermatogonium (ISPG) in the testis of the frog (*Rana esculenta*) at 48 h after treatment with oestradiol. m: mitochondria. Scale bar represents 3 μ m.

Fig. 5. Light micrograph showing many degenerate Leydig cells (asterisks) in interstitial tissue (Int) and several heterochromatic nuclei of Sertoli cells (arrows) adjacent to primary spermatogonia (ISPG) in the testis of the frog (*Rana esculenta*) at 24 h after melatonin injection. SPC: spermatocytes; SPT: spermatids. Scale bar represents 25 μ m.

testis of a frog injected with melatonin after 48 h is shown (Fig. 6).

Experiment B. Testes of frogs captured during October that were injected with oestradiol showed a significantly higher mitotic index of primary spermatogonia either at

Fig. 6. Electron micrograph showing a primary spermatogonium (ISPG) enveloped by a shrunken Sertoli cell (arrows) in the testis of the frog (*Rana esculenta*) at 48 h after melatonin injection. m: Mitochondria; N: nucleus; n: nucleolus; S: Sertoli cell. Scale bar represents 2.5 μ m.

Fig. 7. *In vivo* effect of oestradiol and oestradiol administered in combination with melatonin on the mitotic index of the primary spermatogonia (ISPG) in the frog *Rana esculenta*. Frogs were injected with oestradiol plus colchicine or melatonin and then with a single dose of oestradiol plus colchicine immediately, at 24 h and on day 7 from the melatonin treatment. The animals were killed at 24 h after the last injection (24 h, 48 h and on day 8). Values are $\pm$ SD, a versus b $P < 0.01$. ■: 0 h; ▨: 48 h; □: 72 h; ▩: 8 days.

48 h, 72 h or day 8 after treatment compared with the untreated control testes at 0 h ($P < 0.01$; Fig. 7). The increase in the mitotic index of primary spermatogonia is less pronounced at day 8 after the oestradiol injection.

No significant differences were found in the mitotic index of primary spermatogonia of frogs injected with melatonin and then with oestradiol at 48 and 72 h (Fig. 7). On the contrary, the mitotic index of primary spermatogonia observed in the testes of frogs treated with oestradiol on day 8 after the melatonin injection sharply

Fig. 8. *In vitro* effect of oestradiol (10^{-6} mol l $^{-1}$) on the mitotic index of the primary spermatogonia (ISPG) after 6 (▨), 12 (■) or 24 (□) h of incubation in testis of frogs captured in May that were injected with melatonin (1 µg). Vertical bars indicate values $\pm$ SD, a versus b and b' $P < 0.01$, b versus c $P < 0.05$, b' and b versus d $P < 0.01$.

increased compared with those of the oestradiol-injected frogs at both 48 and 72 h (Fig. 7, $P < 0.01$).

In vivo and in vitro experiments

Testes of frogs captured in May that were incubated in medium containing oestradiol showed a significant increase in the mitotic index of primary spermatogonia by both 6 as well as 12 and 24 h of incubation as compared with untreated control testes (Fig. 8; $P < 0.01$).

No significant differences were found in the mitotic index of primary spermatogonia observed in the testes of melatonin-treated animals incubated in medium containing oestradiol after 6 h as compared with that of the control testes at the same time (Fig. 8), whereas a small but significant decrease was observed at both 12 and 24 h of incubation as compared with that of the respective untreated control testes (Fig. 8; $P < 0.05$). A significant decrease in the mitotic index of primary spermatogonia was observed in the testes of frogs that received melatonin alone as compared with that of the untreated control group at any time of incubation (Fig. 7; $P < 0.01$).

In vitro experiments

Experiment A. Testes of frogs captured in October that were exposed to oestradiol for 6 and 12 h showed a significant increase in the mitotic index of primary spermatogonia (Fig. 9; $P < 0.01$). No differences were found in the mitotic index of primary spermatogonia of melatonin plus oestradiol-treated testis at both times of incubations as compared with that of the untreated control testes (Fig. 9).

The mitotic index of primary spermatogonia observed in the testes incubated in melatonin alone both at 6 or at 12 h of incubation was markedly lower in comparison with the values observed in the untreated control testes (Fig. 9; $P < 0.01$).

Fig. 9. *In vitro* effect of oestradiol (10^{-6} mol l $^{-1}$), oestradiol given in combination with melatonin (10^{-6} mol l $^{-1}$) and melatonin alone on the mitotic index of the primary spermatogonia (ISPG) after 6 (□) or 12 (■) h of incubation in testis of frogs captured in October. Values are $\pm$ SD, a versus b $P < 0.01$, b versus c $P < 0.01$.

Fig. 10. *In vitro* effect of oestradiol (10^{-6} mol l $^{-1}$) and oestradiol given in combination with melatonin (M) (10^{-6} mol l $^{-1}$, 10^{-9} mol l $^{-1}$, 10^{-12} mol l $^{-1}$) on the mitotic index of the primary spermatogonia (ISPG) after 6 h of incubation in testis of frogs captured in October. Vertical bars indicate values $\pm$ SD, a versus b $P < 0.01$, a versus c $P < 0.01$.

Experiment B. Testes of frogs captured in October that were incubated for 6 h in medium containing oestradiol alone showed a significant increase in the mitotic index of primary spermatogonia in comparison with the untreated control testes (Fig. 10; $P < 0.01$), whereas the mitotic index of primary spermatogonia observed in the testes incubated in medium containing oestradiol and melatonin at any doses used (10^{-6} mol l $^{-1}$, 10^{-9} mol l $^{-1}$ and 10^{-12} mol l $^{-1}$) after 6 h of incubation was markedly lower as compared with that of the untreated control testes (Fig. 10).

Discussion

The gametogenic activity of the frog testis shows profound seasonal modifications that are closely correlated with the seasonal average number of primary spermatogonia. In addition, spermatogonial multiplication and differentiation into committed elements (Rastogi *et al.*, 1985) appear to be required for active spermatogenesis in frogs.

Rastogi *et al.* (1978) have described the influence of temperature on spermatogenesis in frogs and, in

particular, on the proliferative activity of primary spermatogonia. In addition, the proportion of cells in mitosis within the primary spermatogonia cell crop of the frog testis exhibits both seasonal and a daily rhythmic variation (Minucci *et al.*, 1987). Several lines of evidence indicate that temperature represents the constraint which controls the responsiveness of primary spermatogonia to hormone factors (Rastogi *et al.*, 1990) and that the mitotic activity of primary spermatogonia is modulated, synergistically, by gonadotrophins and testosterone (Rastogi *et al.*, 1981, 1985; Minucci *et al.*, 1992). In addition, the presence of spermatids seems to exert a negative intratesticular regulation of primary spermatogonia multiplication (Minucci *et al.*, 1992).

In vertebrates, mitotic proliferation of germ cells seems to be dependent on several factors, including the stem cell factor and c-kit system (Sorrentino *et al.*, 1988; Loveland and Schlatt, 1997; Munsie *et al.*, 1997), GnRH-like substances (Minucci *et al.*, 1986; Di Matteo *et al.*, 1988) and oestradiol concentrations (Li *et al.*, 1997; Minucci *et al.*, 1997; Cobellis *et al.*, 1999). In particular, there is accumulating evidence that oestrogens have a proliferative effect on rat and sea star gonocytes (March and Walker, 1995; Li *et al.*, 1997; Walker *et al.*, 1998). In addition, it has been demonstrated that oestradiol treatment increases mitotic activity of primary spermatogonia in the testis of the frog *Rana esculenta* (Minucci *et al.*, 1997; Cobellis *et al.*, 1999; this study) and these findings are in accordance with the strong mitogenic activity of spermatogonia detected during the annual reproductive cycle (Rastogi *et al.*, 1985) in the period of the year characterized by the oestradiol peak (Varriale *et al.*, 1986; Fasano *et al.*, 1989).

Several studies have demonstrated the anti-proliferative effects of melatonin using MCF7 cells as a model to study the anti-oestrogenic effect of this hormone (Hill and Blask, 1988; Wilson *et al.*, 1992; Molis *et al.*, 1994, 1995; Lissoni *et al.*, 1995). Oestradiol was used in the present study to induce the spermatogonial proliferation to verify whether melatonin has an anti-oestrogenic role in the frog testis too. The results obtained from the *in vivo* experiments confirm that administration of oestradiol increases the mitotic index of primary spermatogonia within both 24 and 48 h of treatment. For the first time, these results indicate an inhibitory role for melatonin on proliferation of oestradiol-induced primary spermatogonia in the frog testis. This inhibitory effect ends on day 7 after melatonin injection, indicating that this inhibition is reversible. In addition, melatonin treatment also inhibited the mitotic index of primary spermatogonia in the testis of animals that were not injected with oestradiol.

It is well known that exposure to oestradiol induces an increase in the multiplication of primary spermatogonia *in vitro* too (Minucci *et al.*, 1997). Moreover, testes from melatonin-injected animals, exposed to oestrogen *in vitro*, were unresponsive to hormonal stimulation.

These observations indicate that melatonin counteracts the increase in primary spermatogonia mitotic index induced *in vitro* by oestradiol.

The inhibitory effect exerted by melatonin on spermatogonial proliferation could be explained either through a mechanism involving the hypothalamic–pituitary–gonadal axis or directly via the local action of indoleamine on the frog gonads. This last hypothesis is strongly supported by observations from experiments *in vitro*: in short-term cultured testes, melatonin at physiological concentration (10^{-12} mol l⁻¹) during the night interferes with the effects of oestradiol on proliferation of primary spermatogonia. Although these results clearly indicate that melatonin prevents an oestrogen-induced proliferation of primary spermatogonia, more data are needed to verify the mechanism of action of melatonin in the gonads. In fact, consideration should be given to the fact that melatonin interferes with the activation of the oestrogen receptor and destabilizes the binding of the oestradiol–oestrogen receptor complex to the oestrogen responsive element (Rato *et al.*, 1999). Moreover, melatonin binding sites in the gonads of several vertebrate species have been demonstrated (Vera *et al.*, 1997; Shiu *et al.*, 2000; Clemens *et al.*, 2001) and melatonin receptors coupled through a pertussis toxin-sensitive G-protein are present in adult rat Leydig cells (Valenti *et al.*, 1997). In addition, the presence of a transcript of melatonin family receptors has been described in adult rat testis (De Rienzo *et al.*, 2001).

After *in vivo* or *in vitro* melatonin treatments, many Leydig cells display degenerative features, whereas in the germinal compartment adjacent to them, several Sertoli cells show heterochromatic nuclei. This observation indicates that melatonin could act on Leydig cells and supports a paracrine interaction between interstitial and germinal compartments previously described after the administration of multiple ethane dimethane sulphate injections, an alkylating agent which causes the degeneration of Leydig cells in several vertebrate testes (Minucci *et al.*, 2000).

The possible action of melatonin on Leydig cells is supported in a study by Valenti *et al.* (1995) in which an acute inhibitory role of melatonin was confirmed on steroidogenesis of rat Leydig cells via reduced cAMP production. In addition, prolonged exposure to melatonin reduces the number and affinity of melatonin receptors on Leydig cell membranes and causes hypersensitization to LH challenge resulting in higher cAMP and testosterone secretion (Valenti *et al.*, 2001). However, it has been demonstrated that in the *Rana esculenta* melatonin acts on the testis by modulating the GnRH-induced androgen release (d'Istria *et al.*, 2001).

In conclusion, the results of the present study show, for the first time, that melatonin interacts with the cellular proliferation of primary spermatogonia in the testis of *Rana esculenta*, indicating that it has a role in the regulation of frog spermatogenesis and, in particular,

indicates that melatonin may have a direct inhibitory effect on the basal and oestradiol-stimulated mitotic activity of primary spermatogonia either *in vivo* or *in vitro*. Moreover, the results from the present study do not exclude an intratesticular regulative role of melatonin in the modulation of steroidogenesis activity that supports the progression of spermatogenesis.

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