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# NOTE

# Effect of Morning and Evening Injections of Melatonin on the Testis of Toad (*Bufo melanostictus*)

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## Abstract

Melatonin injection either in the morning or in the evening decreases spermatogenesis as well as the Leydig Cell nuclear area in toad, whereas if administered twice daily (morning+evening) it shows no such effect.

Melatonin, an indoleamine synthesised and secreted by the pineal gland (Ralph, 1976) is reported to have both pro-and antigonadal effects on mammals, depending upon the mode of its administration (Hoffmann, 1974; Turek *et al.*, 1975; Reiter *et al.*, 1974, 1977; Banks and Reiter, 1976). Conversely, a limited number of studies on some lower vertebrates indicate that exogenous melatonin elicits only antigonadal effects on these animals (Levey, 1973; De Vlaming *et al.*, 1974; Sundararaj and Keshavnath, 1976; Packard and Packard, 1977; Biswas *et al.*, 1978a). Recently, Tamarkin *et al.* (1976) and Reiter *et al.* (1976) have shown that the effects of melatonin treatment on hamsters can vary according to the clock time of the injection. Moreover, the additional injection in the morning prevents the antigonadal effects of evening injection of melatonin on hamsters (Chen *et al.*, 1980). As there is a lack of information on the time dependent action of melatonin in lower vertebrates, this preliminary experiment was designed to examine whether the testicular response to malatonin depends on the time of injection to toad—a common lower vertebrate species.

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## Materials and Methods

Adult male toads (*Bufo melanostictus*) weighing 45 50 g were collected during their breeding season (May). Toads were maintained in natural photoperiodic conditions (0500–1900 h) and fed with ant-pupa every alternate day for the entire period of the experiments. After allowing them to acclimatize in the laboratory for a few days, the animals were randomly divided into 4 groups and subjected to different treatment schedules as follows:

Group 1: Control toads injected with vehicle both morning and evening.
Group 2: Toads treated with vehicle in the morning and melatonin in the evening.
Group 3: Toads treated with melatonin in the morning and vehicle in the evening.
Group 4: Toads treated with melatonin both morning and evening.

Morning and evening injections were given between 0600–0700 h and 1800–1900 h respectively. Calculated amounts of melatonin (Sigma Chemical Co. U.S.A.) was dissolved in 10% ethanol (400 $\mu$g/ml). Each of the treated toads was injected subcutaneously with 0.1 ml of this solution containing 40 $\mu$g melatonin (lower doses were found to be ineffective). The treatment was continued for 7 days and on the 8th day all the animals were sacrificed. Their testes were immedi-

ately removed and fixed in Bouin's fluid. Paraffin sections (5 $\mu$m) of testis were stained with haematoxylin and eosin for histological evaluation of spermatogenesis and Leydig Cell nuclear area (LCNA) as described by Biswas *et al.* (1978a; 1978b).

For quantitative study of spermatogenesis the process was divided into the following 5 stages:

Stage 0 : Primary spermatogonia in resting phase.
Stage I : Small cell nests of secondary spermatogonia containing not more than ten cells.
Stage II : Large cell nests of secondary spermatogonia containing more than ten cells.
Stage III: Primary spermatocytes.
Stage IV: Secondary spermatocytes.

The number of germ cell nests at different stages were counted in 30 seminiferous tubules, randomly selected from each testis. The mean counts of germ cell nests per tubule at each stage were used as the index of spermatogenic activity. 50 round Leydig cell nuclei were drawn from each testis by camera lucida on mm$^2$ graph paper at a magnification of camera iucida $\times$ 800 for measuring the Leydig cell nuclear area (LCNA).

## Results

The results are shown in Table 1. Injection of melatonin in toads either in the evening (group 2) or morning (group 3) resulted in a significant fall in the counts of germ cell nests representing the primary (stage 0) and secondary (stage I and II) spermatogonia and primary (stage III) and secondary (stage IV) spermatocytes in comparison with the vehicle treated controls (Group 1). The LCNA was also significantly decreased in each of these melatonin treated groups (2 and 3). On the other hand, when melatonin was injected twice daily (morning+evening) to the animals of group 4, neither the nest counts of the spermatogenic stages nor the LCNA varied significantly from the controls.

## Discussion

Several investigators have shown that melatonin exerts antigonadal effects both in mammals (Wurtman *et al.*, 1968; Rust and Meyer, 1969; Reiter, 1973; Turek *et al.*, 1975) and in lower vertebrates (Levey, 1973; De Vlaming *et al.*, 1974; Sundarraj and Keshavnath, 1976; Packard and Packard, 1977; Biswas *et al.*, 1978a). The present data shows that melatonin treatment in toad (groups 2 and 3) decreases spermatogenesis and LCNA, further decumenting the antigonadal action of this pineal hormone. It has also been established that in order to exert antigonadal actions in hamsters, melatonin must be administered late in the light phase whereas the morning injections of melatonin produced no such effect (Tamarkin *et al.*, 1976; Chen *et al.*, 1980). However, the present experiment shows that melatonin can produce antigonadal effects on toad, irrespective of whether it is injected in the morning or in the evening.

Table 1. Effect of morning and evening injections of melatonin on spermatogenesis and Leydig Cell nuclear area (LCNA) in toad.

| Group and Treatment | No. of toads | Stages of spermatogenesis (Nest/tubule) | | | | | LCNA (mm$^2$) (Camera lucida $\times$800) |
|---|---|---|---|---|---|---|---|
| | | 0 | I | II | III | IV | |
| 1. Control (vehicle) | 10 | 1.98 $\pm$0.22 | 1.65 $\pm$0.10 | 0.86 $\pm$0.05 | 1.42 $\pm$0.08 | 0.43 $\pm$0.03 | 16.97 $\pm$0.42 |
| 2. Melatonin (evening) | 10 | 0.96 $\pm$0.11* | 0.74 $\pm$0.08* | 0.37 $\pm$0.06* | 0.72 $\pm$0.05* | 0.20 $\pm$0.03* | 10.40 $\pm$0.30* |
| 3. Melatonin (morning) | 10 | 1.00 $\pm$0.11* | 0.77 $\pm$0.06* | 0.32 $\pm$0.04* | 0.58 $\pm$0.06* | 0.25 $\pm$0.04** | 10.52 $\pm$0.22* |
| 4. Melatonin (morning+evening) | 9 | 1.81 $\pm$0.18 | 1.40 $\pm$0.09 | 0.74 $\pm$0.06 | 1.24 $\pm$0.07 | 0.36 $\pm$0.06 | 16.87 $\pm$0.36 |

Values are mean $\pm$ S.E.
* $P<0.001$, ** $P<0.01$ vs. group 1 (Student's 't' test).

Therefore it appears that in this lower vertebrate, the suppressing actions of melatonin injection on the testis is not dependent on the time of administration. Moreover, the animals in group 4, receiving melatonin both in the morning and evening showed no changes in the mean counts of germ cell nests per tubule as an index of spermatogonesis and the LCNA when compared to control animals, indicating that the antigonadal effects of its single injection (either morning or evening) are possibly counteracted by the additional injection at the opposite time (evening or morning). This can also be regarded as a progonadal effect of melatonin as described previously (Reiter *et al.*, 1977; Chen *et al.*, 1980).

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