

Melatonin in Human Preovulatory Follicular Fluid*

AMNON BRZEZINSKI†, MACHELLE M. SEIBEL, HARRY J. LYNCH,
MEI-HUA DENG, AND RICHARD J. WURTMAN

Laboratory of Neuroendocrine Regulation, Massachusetts Institute of Technology,
Cambridge, Massachusetts 02139; and the Department of Obstetrics and Gynecology, Beth-Israel Hospital
(M.M.S.), Boston, Massachusetts 02215

ABSTRACT. Melatonin, the major hormone of the pineal gland, has antigonadotrophic activity in many mammals and may also be involved in human reproduction. Melatonin suppresses steroidogenesis by ovarian granulosa and luteal cells *in vitro*. To determine if melatonin is present in the human ovary, preovulatory follicular fluids ($n = 32$) from 15 women were assayed for melatonin by RIA after solvent extraction. The fluids were obtained by laparoscopy or sonographically controlled follicular puncture from infertile women undergoing *in vitro* fertilization and embryo transfer. All patients had received clomiphene citrate, human menopausal gonadotropin, and hCG to

stimulate follicle formation. Blood samples were obtained by venipuncture 30 min or less after follicular aspiration. All of the follicular fluids contained melatonin, in concentrations (36.5 ± 4.8 ($\pm$ SEM) pg/mL) substantially higher than those in the corresponding serum (10.0 ± 1.4 pg/mL). A positive correlation was found between follicular fluid and serum melatonin levels in each woman ($r = 0.770$; $P < 0.001$). These observations indicate that preovulatory follicles contain substantial amounts of melatonin that may affect ovarian steroidogenesis. (*J Clin Endocrinol Metab* 64: 865, 1987)

MELATONIN, the major hormone of the pineal gland, has been shown to influence reproductive function in many mammalian species (1-3). Its administration to female rats diminished ovarian weight, blocked ovulation, and suppressed the vaginal estrus cycle (1, 4). Prolonged darkness, which prolongs melatonin synthesis, suppresses gonadal function in several mammalian species (5-7). Recently, melatonin was reported to be involved in such human reproductive processes as puberty (8) and the menstrual cycle (9-11).

It is generally thought that melatonin exerts its antigonadotrophic effect mainly at the level of the brain and pituitary (12-14). However, exogenous melatonin was found to be concentrated in rat and cat ovaries (15), and specific melatonin receptors were reported (16) in hamster, rat, and human ovaries. Other reports (17-19) described direct effects of melatonin, added *in vitro*, on ovarian steroidogenesis. These observations prompted us to look for melatonin in human preovulatory follicular fluid.

Materials and Methods

Samples

Follicular fluids ($n = 32$) were recovered either by laparoscopy or sonographically controlled follicular aspiration from 15

women who were undergoing *in vitro* fertilization and embryo transfer because of tubal infertility. All women underwent induction of ovulation by sequential administration of clomiphene and human menopausal gonadotropin followed by hCG (20). Follicular fluid was obtained by aspiration using a Craft suction device and a 16-gauge needle. The aspirated follicular fluid was examined under a stereomicroscope for the presence of oocytes; the oocytes were transferred to culture medium for *in vitro* fertilization, and the remaining fluid was saved for melatonin assay. Because a relatively large volume of follicular fluids was needed to carry out the melatonin assays, only fluids from follicles more than 19 mm in diameter were saved for the study. Blood-tinged fluids were excluded. After centrifugation ($3000 \times g$), aliquots of follicular fluid were frozen and stored at -20°C until they were assayed for melatonin. Blood samples (6 mL each) were obtained by venipuncture 30 min or less after follicular aspiration. The serum was separated by centrifugation ($3000 \times g$) and stored at -20°C until assay. All follicular aspirations were performed between 0800-1100 h.

Melatonin assays

RIA. Melatonin in serum and follicular fluids was measured by RIA using CIDtech Ultraspecific melatonin antiserum (CIDtech Research, Inc., Hamilton, Ontario, Canada) (21). Samples (1 mL) were extracted with 5 mL chloroform: the organic phase then was evaporated to dryness under a stream of nitrogen. The residues were dissolved in 0.5 mL 0.01 M phosphate buffer (pH 7.5) containing 0.1% gelatin, and then washed with 1 mL petroleum ether. The buffer extracts or buffer samples containing graded concentrations of authentic melatonin were then mixed with 100 μL antiserum (diluted 1:6000) and 100 μL (3000 cpm) [^3H]melatonin (New England Nuclear Corp., Boston,

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Address requests for reprints to: Dr. A. Brzezinski, E25-604 Massachusetts Institute of Technology, Cambridge, Massachusetts 02139.

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† Fellow of the Fogarty International Center, NIH on leave from the Department of Obstetrics and Gynecology, Hadassah University Hospital, Jerusalem, Israel.

MA). After incubation for 1 h at 37 C, saturated ammonium sulfate solution was added. The mixture was then incubated overnight at 4 C, and the antibody-bound [^{3} H]melatonin precipitated by $(\text{NH}_4)_2\text{SO}_4$ was collected by centrifugation. Radioactivity was measured in a liquid scintillation counter, and melatonin concentrations were estimated by means of a logit-log plot (22). All samples were assayed together in duplicate. Recovery of 50 or 100 pg/mL melatonin added to samples of pooled serum or follicular fluid was 96–100% ($n = 10$). The intraassay coefficients of variation for serum and follicular fluid measurements were 8.1% and 10%, respectively. The corresponding interassay coefficients of variation were 17.3% and 7.3%. The sensitivity of the assay (defined as twice the SD of maximum binding) was 5 pg/mL (22 pmol/L).

Thin-layer chromatography. To confirm the identity of the assayed melatonin and to exclude cross-reactivity by interfering substances, extracts of pooled follicular fluid were subjected to thin layer chromatography (23). Authentic melatonin (1 μ g) applied to two channels of silica gel-precoated plates (Whatman Chemical Separation, Inc., Clifton, NJ) served as chromatographic reference guides. One milliliter of pooled follicular fluid was extracted, as described above, and applied to two other channels of each plate. The plates were developed by ascending chromatography using one of two organic solvent systems: ethyl-acetate (authentic melatonin; $R_f = 0.28$) or chloroform-methanol (9:1; authentic melatonin; $R_f = 0.65$). The location of authentic melatonin was determined by treating the reference channels with Erlich's reagent (dimethylaminobenzaldehyde in concentrated HCl and acetone). The other channels of each plate were then divided into 1-cm segments, with the midpoint of one segment centered at the R_f of authentic melatonin. The silica gel from each segment was eluted with acetone, which was evaporated to dryness under a nitrogen stream. The residue was then redissolved in phosphate buffer (pH 7.5) and subjected to RIA, as described above.

The immunoreactive component of chloroform extracts of follicular fluids analyzed in this manner was found to have the same R_f value as authentic melatonin. In both chromatographic solvents, at least 90% of the material detected by RIA comigrated with authentic melatonin.

Statistical analysis

All data were analyzed by correlation coefficient analysis, and differences were determined by Student's t test.

Results

Follicular fluids were aspirated from 45 preovulatory follicles of 15 women; melatonin concentrations were measured in 32 of these samples (the other 13 were contaminated with blood and, thus, were excluded from the study). The average volume of fluid per follicle was 3.2 ± 2.1 ($\pm$ SD) mL. All of the fluids contained melatonin (Fig. 1). The mean follicular fluid concentration [36.5 ± 4.8 ($\pm$ SEM) pg/mL] was substantially higher than that in the serum obtained concurrently (10.0 ± 1.4 pg/mL).

The melatonin levels in the serum and follicular fluid

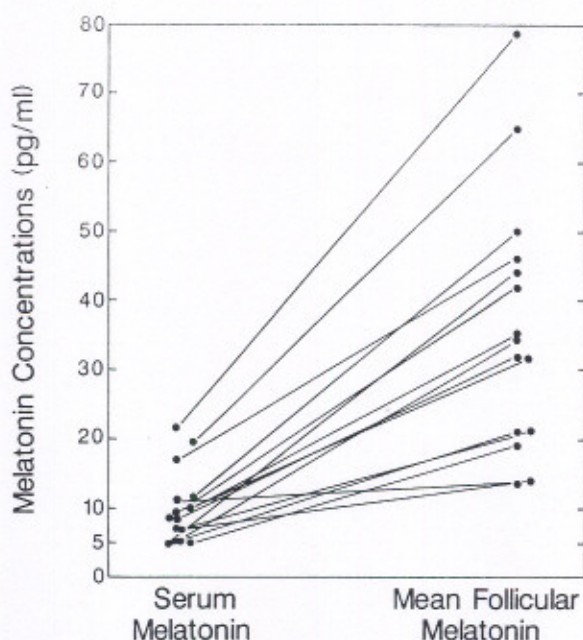


FIG. 1. Melatonin concentrations (picograms per mL) in samples of follicular fluid and serum, collected 0–30 min later, from 15 women undergoing *in vitro* oocyte retrieval. Each follicular fluid value shown represents data from 1–5 follicles (total, 32) from the 15 women. The correlation between the melatonin concentrations in each woman's serum and follicular fluid ($r = 0.770$) was statistically significant ($P = 0.001$).

of each woman were positively correlated ($r = 0.770$; $P < 0.001$). The correlation coefficient between follicular melatonin and follicular volume was only 0.120, which was not statistically significant.

Discussion

These data indicate that substantial amounts of melatonin exist in fluid from human preovulatory ovarian follicles. During the daytime, when these fluids were collected, the follicular fluid melatonin concentrations exceeded those in the corresponding serum samples. We confirmed the identity of the hormone in the follicular fluid by both an alternative highly specific RIA procedure [that of Arendt *et al.* (24); data not presented] and by thin layer chromatography. The question arises as to how the follicle retains or achieves such high melatonin levels in the presence of relatively low serum melatonin levels. There is no evidence to suggest that the human ovary can synthesize melatonin or related indoleamines. Moreover, pinealectomy reportedly decreases serum melatonin to undetectable levels in both experimental animals and humans (25, 26). Therefore, the possibility that the hormone is produced locally seems highly unlikely. Melatonin from the circulation could be retained in follicular fluid by binding to a protein or some other follicular constituent. Serum melatonin, in keeping with

its lipophilic character, is weakly bound to serum albumin (27); conceivably, a more potent binding substance may exist in follicular fluid. This possibility is indirectly supported by earlier descriptions of a high uptake of circulating [^3H]melatonin by ovaries of several mammalian species, including humans (15, 16).

What might be the physiological significance of the substantial melatonin levels in follicular fluid? Melatonin exerts an inhibitory effect on gonadal development and function in various mammalian species (1-3, 28). Its mechanism and site of action are still uncertain. Most studies suggest that melatonin exerts its main effect on the pituitary or within the central nervous system, either by suppressing pituitary responses to GnRH (14, 29) or by inhibiting pulsatile hypothalamic GnRH secretion (30). However, circulating melatonin could also have a direct effect on the gonads. It is concentrated in the ovary *in vivo* (15) and *in vitro* (16), and may modulate ovarian steroidogenesis. It causes a dose-related stimulation of progesterone synthesis by human corpus luteum (17) and granulosa cells (31) *in vitro* and increased the ovarian incorporation of [^{14}C]acetate into androstenedione. A positive correlation between melatonin and progesterone levels was recently suggested, due to apparently higher levels of melatonin during the luteal phase of the menstrual cycle (11). Other investigators (18) reported that hCG stimulation of rabbit ovarian follicles was blocked by melatonin and that rat testicular androgen synthesis was inhibited when melatonin was added to the culture medium (18). Melatonin may also influence estrogen-dependent neoplastic growth (32, 33).

In view of the above observations, melatonin could affect ovarian function by modulating steroid synthesis in follicular granulosa cells or by suppressing follicular proliferation.

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