Pharmacological effects of melatonin on reproductive activity: experimental bioimplants with sustained-release polymeric systems

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Summary

A vast literature documents the role of melatonin in human reproductive function including: a) the relation between melatonin and the menstrual cycle in relation to the peak time of luteinizing hormone in the middle of the cycle [1]; b) the varying concentrations of melatonin in the control of puberty [2]; c) the fewer conceptions in some artic populations where melatonin is connected significantly to seasonal photoperiodicity during the months of the polar nights [3]. The aim of this paper is to report our findings on the pharmacological action of this molecule on reproduction in which gonadal activity is clearly connected to photoperiodicity. We used polymer bioimplants programmed for the sustained release of melatonin for experimental gynecologic protocols. These implants had beneficial results with respect to the use of progestinics because melatonin allowed ovarian activity to be induced for at least two to three consecutive cycles with one single bioimplant. We thought it indispensable to use pharmacological systems with a sustained release because different preliminary tests showed that the half-life of melatonin is limited at maximum to two to three hours and, consequently, any other tested modalities of administration would not provide any appreciable results for our study. As a model for our research we used goats to administer melatonin via targeted programs since these animals clearly respond (even against the rule of the light/dark relation) in contrast to humans in whom response is less evident. For controls, after inserting bioimplants in the animals, we tested their efficiency in vitro and subsequently in vivo, evaluating blood parameters and pharmacological effects of melatonin occurring during the treatment. The final results proved to be interesting in relation to reproductive activity in that regular and programmed births were achieved.

Key words: Melatonin; Sustained-release systems.

Biosynthesis of melatonin and its mechanism of action on the hypothalamus-hypophysis-gonad system

Melatonin is produced by pinealocytes of the epiphysis and is correlated to the chronobiologic regulation of endocrine systems, in particular to the hypothalamus-hypophysis-gonad system. The epiphysis receives information about duration and intensity of the brightness of ambient light through the ocular apparatus. In fact, light information received by the retina passes through the suprachiasmatic nuclei of the anterior hypothalamus, the posterior hypothalamus and the sympathetic hypothalamic spinal pathways, thus reaching the superior cervical ganglion from which post-ganglion fibers reach the epiphysis. Here is where all information about the duration and intensity of ambient light arrives and where the mechanisms that regulate the secretory functions and the respective biosynthesis of melatonin are activated. In order for the action mechanism of melatonin to be effective, a sufficient concentration of the active principle and abundant receptors in the target cells with different degrees of sensitivity are needed. When considering circadian cycles, the pharmacological response of melatonin is related to light intensity (light/dark) because the efficiency of melatonin rigorously depends on it. The mediation of melatonin occurs with a complex action mechanism through a specific neuroendocrine transducer capable of transforming nerve stimuli into endocrine impulses.

Genetic aspects of melatonin

In man it has been shown that on a genetic level melatonin acts pharmacologically through the receptor MTNR1A which is coupled to protein G. Such receptor is located in the pars tuberalis of the hypophysis [4] and the respective gene is positioned on chromosome 4 (4q35.1) [5]. It is composed of two exons that codify for one protein of 350 amino acids. In the rat the homologous gene (Mtnr1a) has been mapped in the proximal portion of chromosome 8. The locus of MTNR1A is probably involved in the genetic control of circadian rhythm. It has also been shown that the cyclical expression of the Period-1 gene (the so-called "clock" gene) in the pinealocytes of the rat depends on nocturnal activation of the melatonin receptor [6]. Some investigators have shown that the variability of nocturnal plasmatic concentrations of melatonin in 312 animals genetically varied and that the variability of the melatonin rate was due to a different genetic synthesis of the pineal gland [7]. Moreover,

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the genetic variability of melatonin is linked to the difference in the number of pinealocytes contained in the epiphysis [8]. A genetic polymorphism in the receptor Mel(1a) of melatonin in position 605, responsible for seasonal anovulatory activity, has been shown in sheep used as a study model [9].

Sustained-release system

In order to use melatonin in our study, we prepared capsules consisting of a controlled sustained-release polymer system using the method of Bannon-Peppas [10], allowing melatonin to pass through the pores and channels of the structural matrix made up by the polymer. The system, based on the diffusion mechanism described by Haik-Creuger [11], was adequately prepared with melatonin so that, technically, this pharmaceutical formula could be released for a fixed period of time in an effective, constant, and long-lasting manner. Consequently, the possibility of over- or under-dosage was avoided during the entire treatment period of the implanted animals, thus forcing them to undergo programmed reproductive activity. Among other things, we kept into account that the level of melatonin released could be maintained within the programmed range and that the animals could well tolerate an optimal use of the drug in question. Therefore, a biocompatible polymer system (completely non biodegradable), made up of hydroxyethyl methacrylate poles, using the method of Siegel and Langer [12] was implemented. The same system, consisting of hydrogel, was able to swell without dissolving once it was implanted subcutaneously in the treated animals. This type of implant system offered the advantage of being able to remove or interrupt the pharmacological treatment at anytime.

Materials and Methods

Preparation of biopolymer

Spheres of polymer with the addition of 2 ml of dichloromethane were placed in glass test tubes with a length of 5 cm and diameter of 1 cm. After having heated the contents of the test tubes in sand in a thermostat oven at 90°C, care was taken to mix until complete liquefaction of the polymer was obtained while keeping the mixture at its initial level by adding more dichloromethane. When a good fluidity of the preparation was obtained, a mixture formed of 20% or 30% of melatonin (Bachem) in an excipient composed of 900 µl of dichloromethane and 100 µl of methanol was added, while keeping the solution in motion to insure uniform dispersion of the polymer. Once the solvent evaporated almost to the point of solidity, the compound was sucked into a glass tube with a syringe connected to the tube, paying attention to avoid bubbles. Subsequently, the filled tube was placed in dry ice (-80°C) in a freezer and kept at -20°C. After three days, during which evaporation of the residual dichloromethane was completed, the cylinder contained in the tube was taken out and left at -20°C for one more day to complete the preparation. Finally, the polymer cylinders were recovered (standard weight 0.25 g).

Release test in vitro

A sustained-release test of the active principle was carried out by placing the polymer cylinder in a glass test tube containing 2 ml of normal saline solution in agitation in a thermostat at 38.5°C. Samples of the normal saline solution in which the polymer cylinder was immersed were taken for analysis with HPLC every 24 hours every day until the active principle was released. Each test sample consisted of 20 ml and, at the beginning of each subsequent day, the normal saline solution was renewed.

Test in vivo

Tests made in vivo began after the end of the reproductive season, which is normally in winter. Twelve Saanen goats aged one to three years and weighing 40-50 kg were used. The test was carried out on nine of these animals, while the remaining three were used as controls. Of the nine goats submitted to the treatment, seven were pluriparous and did not get pregnant during the normal reproductive season, while the other two were still too young to become pregnant at that time. All goats were initially submitted to an echographic examination to exclude any possible pregnancies occurring before the experiment. For this examination, an ULTRASCAN 45 (FOSCHI) with a 5 MHz linear type endorectal probe was used.

All the goats, excluding the controls, were under treatment in April for a total period of 80 days, the so-called "long" days. With the proper applicator, a polymer cylinder weighing 0.25 g, melatonin was implanted under the skin in the auricular pavilion of all the nine treated animals. After 30 days, a second implant identical to the previous one, was placed in the contralateral auricular pavilion of the animals. After another 30 days, both polymer cylinders were removed. After having localized the cylinders in the auricular pavilion by palpation, small incisions with a lancet were made to remove them with the help of a clamp. Before the application of the first implant, a blood sample was taken by the Vacutainer method from the jugular veins of both treated and control goats. Subsequently, blood samples were taken every 15 days for a total of four times. Each blood sample was centrifuged and the plasma subsequently stored in the freezer at -18°C. During the first days of July (80 days after the beginning of the treatment) goats pretreated with melatonin were mated. Then the animals were tested for pregnancy by means of echographic examination performed 30 days from the mating (early diagnosis) and repeated after another 30 days (late diagnosis) for confirmation. Still in vivo, we took hormonal determination with a radioimmunologic dosage (radioimmunologic assay) of melatonin in the plasma samples of goats and, thus, increased plasmatic concentrations of melatonin in the treated animals could be evaluated.

Results

Results of daily quantitative calculations of melatonin released by polymer supports

In some of our tests, the interpretation of chromatograms obtained with the HPLC equipment allowed us to calculate the daily amounts of melatonin released by the two different types of polymer supports we used. Consequently, after the release of active principle from the first preparation consisting of 20% melatonin, the polymer and the excipient were analyzed. A subsequent

analysis was repeated with another preparation in which melatonin was increased up to 30%. Analytical responses showed that on the first day the two different supports had released a high quantity of melatonin – 680 μg and 392 μg, respectively – which decreased on the second day to 408 μg and to 244 μg, respectively. The release continued to decrease on the third day down to the value of 324 μg for the preparation with 20% and of 186 μg for that with 30% melatonin. From the fourth day on and until the 12th day the release of active principle became stabilized at a value around 160 μg for the preparation with 20% and around 110 μg for that with 30% melatonin. At this point in time the release diminished, remaining however constant, and above the threshold effective for treatment for a certain period. During this period we observed that the release of the implant containing 30% melatonin was used up approximately the 40th day, while the preparation with 20% melatonin continued to release the active principle until the 51st day. The release of melatonin by this last implant was subsequently reconfirmed by a second test carried out under the same conditions for a duration of 31 days.

Results in vivo

As for the biocompatibility of the implants, no animal showed any local reaction except for goat no. 6 which developed an abscess in the area of the auricular pavilion where the second bioimplant had been inserted. The results of the manifestations connected with ovular maturation were observed approximately three weeks after the application of the second melatonin implant and the goats were fertilized by natural mating. Echographic examination (late diagnosis), performed about two months after mating, showed that seven of the nine treated goats had become pregnant while two were in a state of pseudopregnancy. In the non-treated control goats, the echographic examination turned out to be negative for all animals. Gestations proceeded regularly and the seven pregnant goats gave birth within 146-155 days from the presumed fecundation. The total number of births was 14 kids, all born without difficulty, within the normal period of gestation and in good condition. It should be noted that pregnancy in these animals is often a twin pregnancy.

Conclusions

We produced sustained-release devices consisting of non biodegradable polymer materials which, when in contact with a watery environment, did not swell. This allowed us to prepare subcutaneous implants for the release of the active principle taking advantage of only the diffusion mechanism. The polymer support did not degrade in any way during its use, and its dimensions and aspect remaining unchanged. The great advantage in using this material is that an implant inserted subcutaneously can be easily located even after a long period of time. It can thus be removed easily integrally, producing no fragments that could otherwise cause undesired effects. During the preparation of the implant, the polymer and active principle of melatonin were mixed scrupulously forming a quite homogeneous system. The polymer matrix worked as a support, like a sort of rigid scaffolding, crossed by channels and pores communicating with each other and with the surface. The molecules of the active principle of melatonin were distributed randomly in these spaces. Once the active principle was dissolved, the diffusion process took place only when the active principle passed from the polymeric matrix to the outside environment due to osmolarity. The results obtained with the melatonin implanted animals show optimal pharmacological effects for controlled, constant, and long-lasting administration in programmed periods. Considering that the animals we used have a melatonin-dependent gonadal activity linked to cyclical manifestations of variable photoperiodicity in different seasonal periods, our method provides the possibility of birth in prefixed periods and outside of the mating season with the spontaneous fecundation of these animals.

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