MATERNAL-FETAL TRANSFER OF MELATONIN IN THE NON-HUMAN PRIMATE

Steven M. Reppert, Ronald A. Chez, Allan Anderson, and David C. Klein

Section on Neuroendocrinology
Laboratory of Developmental Neurobiology
and
Pregnancy Research Branch
National Institute of Child Health and Human Development
National Institutes of Health
Bethesda, Maryland 20205

SUMMARY

Melatonin was detected in the circulation of the near-term rhesus monkey (Macaca mulatta) and baboon (Papio papio) fetus. We determined whether the source could be the mother by studying placental transfer of melatonin in the rhesus monkey. When [^{3}H]melatonin was administered i.v. to the mother it promptly appeared in the fetal circulation; the rates of disappearance of [^{3}H]melatonin in the maternal and fetal circulations were parallel. The rapid decrease in circulating [^{3}H]melatonin was associated with a rapid accumulation of [^{3}H]melatonin-metabolites in the maternal and fetal circulations. Although the pattern of appearance of metabolites was similar in both circulations, relatively less [^{3}H]melatonin-metabolites appeared in the fetal circulation.

Acute changes in total maternal plasma melatonin, experimentally produced by giving a 20 min infusion of melatonin, were rapidly reflected in the fetus. This suggests that a daily rhythm in maternal melatonin would generate a similar rhythm in the fetus.

The fetal monkey pineal was found to have the two enzymes necessary for the conversion of serotonin to melatonin. It is, however, not known whether fetal melatonin synthesis is rhythmic or the extent to which it could contribute to circulating melatonin levels at this or earlier stages of gestation.

SPECULATION

Prompt placental transfer of melatonin could result in a maternally generated daily melatonin rhythm in the fetus. This communication may introduce the developing fetus to a 24-hr chemical periodicity and coordinate certain fetal functions with the prevailing environmental lighting cycles.

INTRODUCTION

The existence of placental transfer of melatonin, the putative pineal hormone, has raised the possibility that a daily maternal rhythm in melatonin could generate a similar rhythm in the fetus. Placental transfer of melatonin has been documented in the rat and sheep (8,10), but not in a primate. In addition, it is not known whether the rate of placental transfer of melatonin is sufficient to transmit rapid changes in maternal plasma melatonin or if it would damp out such changes.

For these reasons, we have determined whether placental transfer of melatonin exists in the rhesus monkey and if rapid changes in total maternal melatonin are reflected in the fetal circulation. The presence of melatonin in fetal plasma, the appearance of [^{3}H]melatonin-metabolites in the fetus, and the enzymatic capacity of the near-term fetal pineal gland to synthesize melatonin were also studied.

MATERIALS AND METHODS

Animals were obtained from the NIH primate colony and given Purina Monkey Chow and water ad libitum, until the overnight fast prior to surgery. Lighting was automatically controlled (LD 12:12) with the lights on from 0600 hr to 1800 hr.

Plasma was collected at the time of Caesarean section (1000 hr to 1200 hr) from blood obtained from the umbilical vessels of eleven rhesus monkeys (Macaca mulatta) at term gestation and the umbilical vein of four premature baboons (Papio papio; 78% to 82% gestation). Plasma samples for each species were pooled, and duplicate samples (400 ul) from each pool were analyzed for melatonin by radioimmunoassay (RIA).

Surgical technique:

The placental transfer studies were performed with three pregnant rhesus monkeys between 150 d and 153 d of gestation (90% to 93% term). The surgical and experimental techniques have been reported in detail (5). Briefly, after ketamine hydrochloride premedication and under N_2O-O_2-halothane anesthesia, abdominal laparotomy was performed and the uterus was exposed. After the myometrium, decidua, and chorion, but not the amnion, were incised, a silastic T-tube cannula was placed in an interplacental fetal vessel, thereby allowing continuous flow through the vessel and sequential sampling of fetal blood (0.5 ml to 1.0 ml). The fetal-maternal-placental circulations were maintained, and the amniotic sac remained intact. The fetal and maternal samples were obtained between 0930 hr and 1130 hr. Maternal blood (1.0 ml) was sampled from a cannula placed in the inferior vena cava via the saphenous vein. Amniotic fluid (1.0 ml) was sampled from a catheter introduced through the anterior wall of the lower uterine segment. During the experiments, fetal and maternal blood pH, pO_2, pCO_2, and cardiovascular indices were monitored; they remained within normal limits.

At the completion of the experiments, the fetus was killed with intravenously administered barbituates, removed from the mother, and exsanguinated. Samples of cerebrospinal fluid (CSF) from the cisternal subarachnoid space and bladder urine were removed from the fetus, as was the pineal gland; these samples were frozen on solid CO_2 and stored at -20°C until analysis.

Experimental protocols:

1. [^{3}H]Melatonin[1] injection studies:

The unidirectional placental transfer of [^{3}H]melatonin from the mother to fetus was studied in all three animals. The homogeneity of [^{3}H]melatonin, (N-acetyl-5-methoxytryptamine-2-aminoethyl-2-^{3}H, specific activity = 36.4 Ci/mmol; New England Nuclear Corp., Boston, MA) was 99% as judged by one-dimensional thin layer chromatography. Immediately before injection, the [^{3}H]-melatonin stock solution (28 uM; ethanol/water, 99:1) was diluted with sterile saline (1.5:100). [^{3}H]Melatonin (1.0 ug; 150 uCi) was injected into the maternal vena cava over a 20 sec period. This amount of melatonin, based on studies in the sheep (25), was expected to elevate melatonin to normal high night values for a short period of time. Maternal and fetal blood and amniotic fluid were simultaneously withdrawn into heparinized syringes at timed intervals over a 30 min period. Amniotic fluid was stored at -20°C until analysis. Blood samples were stored at 4°C for 2 hrs; plasma was then collected by centrifugation, frozen on solid CO_2, and kept at -20°C until analysis. In separate experiments, [^{3}H]melatonin added to monkey plasma was found to be stable for 24 hr at 4°C.

2. Melatonin infusion studies:

Melatonin (Regis Chemical Co., Chicago, IL; 8 ug/20 ml normal saline) was intravanenously infused (0.4 ug/min) for 20 min into two of the mothers. The infusion was started 30 min after the [^{3}H]melatonin injection. This rate of infusion was chosen because it is approximately equal to the estimated rate of pineal secretion of melatonin at night in sheep (25). Maternal and fetal blood samples were simultaneously withdrawn into heparinized syringes at intervals over a 50 min period. The plasma was collected and frozen on solid CO_2 and stored at -20°C until analysis. Plasma samples (50 ul) were analyzed for melatonin by RIA.

Assay procedures:

1. Analysis of [^{3}H]melatonin:

To determine the amount of [^{3}H]melatonin in a sample, 100 ul was extracted with 5 volumes of water-saturated chloroform. The aqueous layer was removed and saved for analysis of [^{3}H]melatonin-metabolites[2]. The chloroform layer was washed sequentially with 100 ul of 0.1 M sodium bicarbonate, pH 10.0, and then water. One-half the volume of chloroform extract was taken to dryness in a scintillation vial by vacuum, and the radioactivity was quantified as described (21). This procedure was found to extract 94% or more of [^{3}H]-melatonin added to fetal plasma, maternal plasma, amniotic fluid, or fetal cerebrospinal fluid.

The radioactivity in pools of chloroform extracts from maternal and fetal plasma and urine, amniotic fluid, and fetal cerebrospinal fluid for each animal was authenticated as [^{3}H]melatonin by one-dimensional thin layer chromatography (chloroform: methanol: acetic acid, 90:10:1; silica gel) (Figure 1), as previously described (21). We found that 88% to 96% of the radioactivity in these pools was [^{3}H]melatonin. The reported values were not corrected for this loss.

2. Analysis of [^{3}H]melatonin-metabolites:

[^{3}H]Melatonin-metabolites were estimated by counting the water-soluble radioactivity remaining in a sample after chloroform extraction. One-half of the volume of extracted sample was dissolved in Nuclear Chicago Solubilizer (Amersham/Searle Co., Arlington Height, IL), counting fluor was added, and the sample was counted.

This appears to be a valid estimation of [^{3}H]melatonin-metabolites because the major metabolites of melatonin (6-hydroxymelatonin, the sulfate and glucuronic acid conjugates of 6-hydroxymelatonin) are not extractable into chloroform at neutral or alkaline pH (21).

3. Melatonin RIA:

The method of Rollag and Niswender (24) was used as modified (22). The extraction efficiency was 80%; the reported values have not been corrected for this loss. This technique is valid and reliable for measuring melatonin in monkey plasma (22). Maternal and fetal plasma samples were determined in the same assay run. The within-assay coefficient of variation was 12%. The limits of sensitivity varied from 0.3 pg/tube to 0.5 pg/tube.

4. Pineal enzyme analysis:

Fetal pineal glands were sonicated in 0.1 M sodium phosphate buffer, pH 6.8 (1 gland/100 ul), and the resulting homogenate was used for enzyme assays. Pineal gland protein was measured by the Lowry method (16).

The assay procedure for N-acetyltransferase (NAT) activity was a modification (18) of the method of Deguchi and Axelrod (6). The activity was analyzed by adding 20 ul of the gland homogenate to 80 ul of phosphate buffer containing 1 umol of tryptamine HCl and 50 umol of [^{14}C]acetyl CoA (SA = 1 Ci/mol). This solution was incubated at 37°C for 20 min.

Hydroxyindole-O-methyl transferase (HIOMT) activity was analyzed by adding 20 ul of the gland homogenate to 80 ul of 0.1 M sodium phosphate buffer, pH 7.9, containing 10 nmol [^{14}C]S-adenosyl methionine (SA=51.8 Ci/mol) and 100 nmol of N-acetyl serotonin. The solution was incubated at 37°C for 30 min, and the enzyme activity was determined as described (3). Fetal cerebral cortex tissue (1 mg) also was assayed for this enzyme in the same manner.

5. Statistical analysis:

Data in the text is given as either individual values or as the mean (± SE) of three values.

RESULTS

Melatonin in fetal primate plasma:

Radioimmunoassayable melatonin was identified in the pooled plasma samples from the term rhesus monkey and fetal baboon; the melatonin concentrations were 45 pg/ml and 40 pg/ml respectively.

Maternal and fetal plasma levels of melatonin in the rhesus, ten minutes prior to the [^{3}H]melatonin injection, were 100 pg/ml and 32 pg/ml respectively in animal 922, and 40 pg/ml and 16 pg/ml in animal S92.

Placental transfer of [^{3}H]melatonin (Fig. 2):

The disappearance of [^{3}H]melatonin from the maternal circulation following an injection was rapid and multiphasic. Thirty min after the injection, the

[1] [^{3}H]Melatonin refers both to the radioisotopically labelled compound that was injected and to the radioactive compound that was chloroform-extractable and co-migrated with authentic melatonin in one-dimensional thin layer chromotagraphy (see Materials and Methods).

[2] [^{3}H]Melatonin-metabolites refers to the non-chloroform-extractable radioactivity remaining in the aqueous phase (see Materials and Methods).

maternal plasma level of [^{3}H]melatonin was 18.7 ± 1.67% of the peak (one min) level.

[^{3}H]Melatonin rapidly crossed the placenta; within the first three min after the injection [^{3}H]melatonin levels in fetal plasma were 84% to 90% of that in maternal plasma in animals 606A and 922A. In animal S92, the fetal plasma [^{3}H]melatonin level at one min was 27% of the corresponding maternal plasma level; at three min, it was 63%. Thereafter, in all three animals the rates of maternal and fetal disappearance of [^{3}H]melatonin appeared parallel. Thirty min after the injection, the fetal plasma level was 66 ± 5.5% of the corresponding maternal value.

[^{3}H]Melatonin was first detected in amniotic fluid three to eight min after the maternal injection and increased over the 30 min sampling period with a value of 17.3 ± 2.67% of the maternal plasma level occurring at 30 min after the injection.

Fetal CSF obtained at delivery (90 min after the [^{3}H]melatonin injection) was found to have a similar concentration of [^{3}H]melatonin as simultaneously obtained fetal plasma; in animal 922A CSF melatonin was 1.0 nCi/ml (plasma [^{3}H]melatonin was 1.3 nCi/ml) and in animal 606A the values for both plasma and CSF were 1.1 nCi/ml.

[^{3}H]Melatonin-metabolites in the maternal and fetal circulations (Fig. 3):

[^{3}H]Melatonin-metabolites in the maternal circulation rapidly increased within the first 10 min to 15 min after the injection, and then remained relatively constant over the remainder of the sampling period. The pattern of appearance of [^{3}H]melatonin-metabolites in the fetal circulation was somewhat similar to the maternal pattern but did not exhibit a plateau within the experimental period. Further, the amounts were not similar. At 30 min after the [^{3}H]melatonin injection, the fetal [^{3}H]melatonin-metabolite plasma level was 50 ± 6.3% of the corresponding maternal plasma level.

[^{3}H]Melatonin-metabolites appeared in amniotic fluid at three min to eight min after the [^{3}H]melatonin injection and increased over the 30 min sampling period. [^{3}H]Melatonin-metabolites represented 32% to 48% of the total radioactivity in amniotic fluid at the 30 min sampling period. Analysis of maternal and fetal urine 90 min after the maternal injection (922A, S92) indicated that a higher percentage (37% and 30%) of the radioactivity in fetal urine was [^{3}H]-melatonin as compared to maternal urine, in which only 3% and 5% was [^{3}H]-melatonin.

Melatonin infusion studies (Fig. 4):

At 10 min after the start of the infusion, melatonin in the maternal circulation had increased 29- and 16-fold and that in the fetal blood 12- and 21-fold above baseline values. After the infusion was stopped, melatonin disappeared from both the maternal and fetal circulations at similar rates. Thirty min later, melatonin in both the maternal and fetal plasma was 260 pg/ml to 280 pg/ml. This represented 20% and 29% of the concentration of plasma melatonin at the termination of the infusion in the mother and 66% and 54% of that in the fetus.

Fetal pineal enzymes (Table 1):

The activities of two pineal enzymes required for melatonin synthesis, NAT and HIOMT, were detected in the pineal glands from two fetuses (922A, S92). No HIOMT activity was detected in fetal cerebral cortex.

DISCUSSION

The results of our study can be divided into two major areas; one having to do with the source, existence, and fate of melatonin in the monkey fetus, and the second bearing on the question of whether a maternal rhythm in melatonin can generate a similar rhythm in the fetus. These areas will be discussed sequentially.

Our study shows that there are two potential sources of fetal melatonin. On one hand, the detection of rapid transfer of both authentic and [^{3}H]-melatonin across the primate placenta from mother to fetus indicates that the maternal circulation could be the source of circulating fetal melatonin. This is consistent with placental transfer studies in pregnant rats and pregnant sheep (8,10). Rapid placental transfer of melatonin is probably a reflection of the lipophylic, nonionized nature of this small molecule (mol. wt. 232).

On the other hand, our data raises the possibility that the fetal pineal gland may also be a source of circulating fetal melatonin. This is in sharp contrast with the rat (9,30), which develops the capacity to synthesize melatonin one week after birth, but is similar to fetal sheep (8), which have the capacity to synthesize melatonin during the last trimester.

The relative importance of the fetal pineal at this gestation stage as a source of melatonin is difficult to assess. Although the two pineal enzymes necessary for the conversion of serotonin to melatonin are present in the fetal monkey pineal, it is not clear whether this system can be activated on a circadian basis to generate a rhythm in melatonin, as is seen in the adult (23). The generation of this rhythm in the rat not only requires the presence of melatonin synthesizing enzymes, but also requires the complete development of a neural circuit from the hypothalmus to the pineal gland (11); the presence and function of this circuit in the primate have not been proven.

Concerning the existence of melatonin in the fetal primate circulation, our data clearly shows that endogenous melatonin is present in the plasma of monkey fetuses during late third trimester pregnancy. The endogenous fetal melatonin levels that we detected in the rhesus monkey were approximately 40% of that in the maternal circulation. Assuming a dominant maternal contribution to these fetal melatonin levels, the differences in fetal and maternal levels could be a function of differences in albumin binding. In rats and humans, 70% of the melatonin in blood is bound to albumin (4). Thus, high levels of albumin or a specific melatonin binding protein in the mother, relative to the fetus, could decrease the fraction of total melatonin in the maternal circulation free to cross to the fetus. Another possibility for this difference is increased tissue uptake of melatonin in the fetus. Higher rates of metabolism of melatonin by the fetus, relative to mother, appears an unlikely explanation as will be discussed.

Concerning the fate of melatonin in the primate fetus, it appears from our results that the monkey fetus has a lower rate of metabolism of melatonin relative to mother. Several lines of evidences support this conclusion. First, the concentrations of [^{3}H]melatonin-metabolites in the fetal circulation were markedly lower (17% to 30%) than the corresponding maternal plasma levels at the time when fetal [^{3}H]melatonin plasma levels were almost equal to that in stimultaneously obtained maternal plasma. Second, our data indicates that the fetal kidney excretes a larger percentage of the total radioactivity as melatonin as compared to the mother.

Conversely, the [^{3}H]melatonin-metabolites in the fetal circulation may be simply a reflection of circulating maternal [^{3}H]melatonin-metabolites via placental transfer with little or no metabolism of [^{3}H]melatonin by the fetus. Since the major metabolites of circulating melatonin, 6-hydroxymelatonin and its conjugates (12,13,21), are relatively more polar than melatonin it would appear that on this basis [^{3}H]melatonin-metabolites may be transferred across the placenta at a slower rate. However, the extent of the contribution of maternal [^{3}H]melatonin-metabolites to circulating fetal levels is difficult to assess from these studies.

We also observed the rapid appearance of [^{3}H]melatonin and [^{3}H]melatonin-metabolites in amniotic fluid. Fetal renal clearance with susequent micturation is an unlikely explanation for this because of the remarkably rapid rate at which these compounds appeared. Other routes of entry into amniotic fluid, both fetal and maternal in origin, are probably operative.

The second major purpose of this investigation, to determine whether the rate of transfer of melatonin across the placenta was sufficient to transmit rapid changes in maternal melatonin to the fetus, is interesting because in adult mammals, including the rhesus monkey, there is a large daily rhythm in circulating melatonin with high values occurring at night (7,22). Placental transfer of melatonin could transmit this rhythm to the fetus. We attempted to simulate naturally occurring diurnal changes in maternal melatonin with an infusion of melatonin. This resulted in increases in both maternal and fetal melatonin; during this experiment the fetal:maternal plasma melatonin ratio remained constant. These observations support the conclusion that placental transport is sufficient and rapid to reflect acute maternal plasma melatonin changes in the fetal circulation.

It should be pointed out, however, that the rate and amount of melatonin infused, which was based on data provided by studies using sheep (25), raised melatonin in the mother to values which were 5- to 100-fold higher than reported peak values of melatonin in primates (1,2,7,15,20,22,26,28,29). This large variation in published circulating melatonin values may in part be a result of variation in methodology. In any case, it would appear that our peak values were higher than those which occur physiologically. Accordingly, we are reluctant to conclude from this data alone that physiological changes in maternal melatonin are reflected in the fetus. However, in our injection studies using [^{3}H]melatonin, we found that the total amount of melatonin in maternal plasma increased only 3- to 6-fold within one minute following the injection. This increase, which is similar to that seen physiologically at night, caused a rapid increase in fetal melatonin. On this basis, and in view of the results of the infusion studies, we feel it is highly probable that daily changes in maternal plasma melatonin generate a rhythm in fetal plasma melatonin.

The experiments outlined in this paper were performed on acute surgically prepared animals. Thus, the possible effect of anesthetic agents, and surgical and anesthetic stress on the results of our observations cannot be ignored. The endogenous maternal plasma melatonin concentrations found prior to the placental transfer studies but obtained after anesthetic induction are in the range of nighttime plasma melatonin levels found in the adult rhesus monkey. These somewhat elevated maternal melatonin levels could have resulted from the effect of ketamine. This compound has been found to have stimulatory effects upon the sympathetic nervous system (14). Since the rhythmic production of melatonin by the pineal is felt to be controlled by changes in the release of neurotransmitter from the sympathetic nerves that innervate the pineal, then it is conceivable that ketamine could increase melatonin production via this mechanism. Stress per se appears unlikely as a cause of these elevated levels, since it has been shown that presynaptic sympathetic nerve terminals in the pineal protect this gland from the effects of increased levels of circulating catecholamines induced by stress (19). Also, anesthetic stress does not appear to elevate circulating melatonin levels in humans (27). Finally, reduced fetal or maternal blood flow during these studies could alter the transfer of melatonin across the placenta. However, these reductions would limit transfer and result in hypoxia and metabolic acidosis; this did not appear to occur.

It is not known whether the pregnant rhesus monkey exhibits a diurnal melatonin rhythm. However, this seems likely because the diurnal rhythm in circulating melatonin in adult humans persists unaltered throughout pregnancy (17). The results of our experiments show that a diurnal rhythm in circulating maternal melatonin is probably reflected in the circulation of the fetus. In addition, this maternally generated melatonin rhythm is probably also reflected in fetal CSF. This is supported by our detection of maternally derived [^{3}H]-melatonin in fetal CSF 90 min after the maternal injection. This finding is consistent with published data in the adult monkey which shows that the daily rhythm in circulating melatonin is reflected in CSF (22). Thus, a maternally generated melatonin rhythm in the fetal circulation and CSF could provide two routes of delivery of this rhythm to the fetal CNS. Through either of these routes, the developing fetus might be introduced to 24-hr periodicity which might possibly synchronize certain fetal functions with the mother.

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32. A portion of these studies was presented at the 48th Annual Meeting of the Society for Pediatric Research, New York, April, 1978.

33. Requests for reprints should be addressed to Dr. S.M. Reppert, Bldg. 6, Rm. 128, NIH, Bethesda, Md 20205.

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Table 1

Fetal Monkey Pineal Enzyme Activity

Animal	Enzyme activity (pmol/min/mg protein) N-acetyl-transferase	Hydroxyindole-O-methyl-transferase
922A		
pineal	150.0	25.5
cerebral cortex	66.8	*
S92		
pineal	99.0	67.1

*Radioactive counts (dpms) were not significantly different from background.

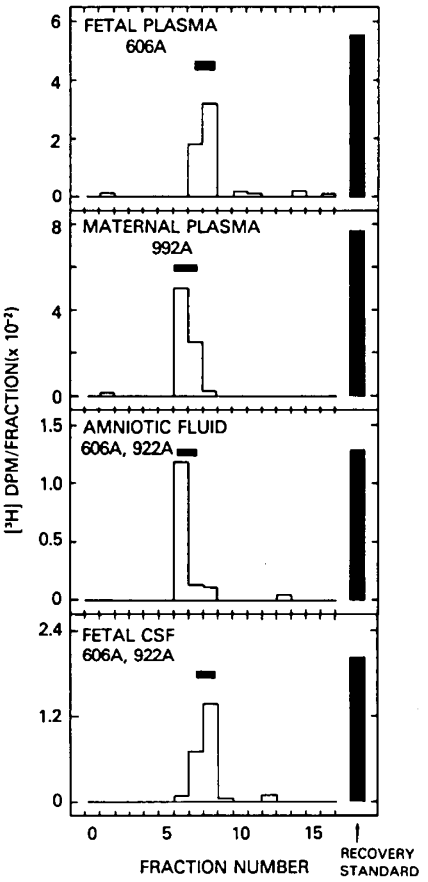

Figure 1. One-dimensional TLC of chloroform-extractable radioactivity from 4 pools. After chromatographic development sixteen 1 x 5 cm parallel sections of gel were analyzed for radioactivity. Fraction numbers represent the distance (± 0.5 cm) of each fraction from the origin. The location of authentic melatonin, dark horizontal bar, is compared to the radioactivity in the corresponding fractions. The recovery standard, dark vertical bar, represents the total radioactivity applied to the plate before chromatographic development.

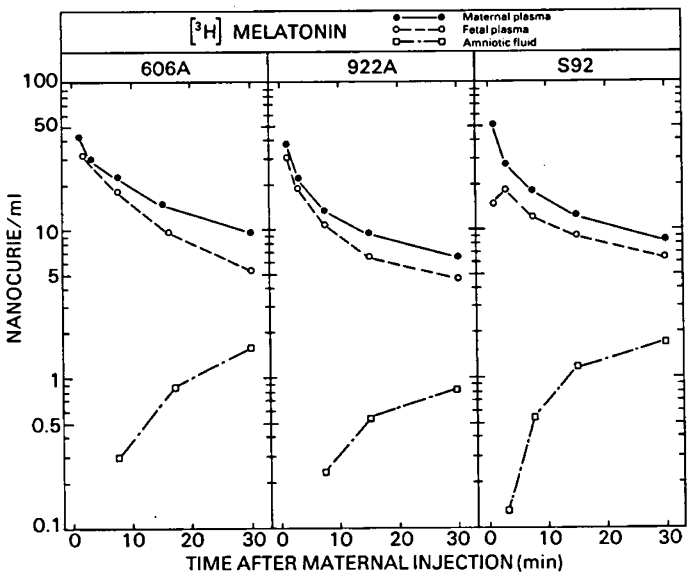

Figure 2. Maternal plasma, fetal plasma, and amniotic fluid [³H]melatonin concentrations after maternal [³H]melatonin injection.

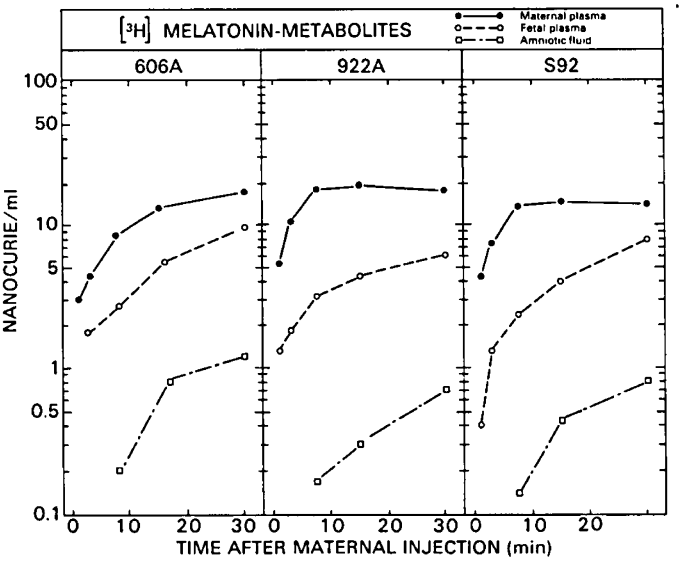

Figure 3. [³H]Melatonin-metabolite levels in fetal plasma, maternal plasma, and amniotic fluid after maternal [³H]melatonin injection.

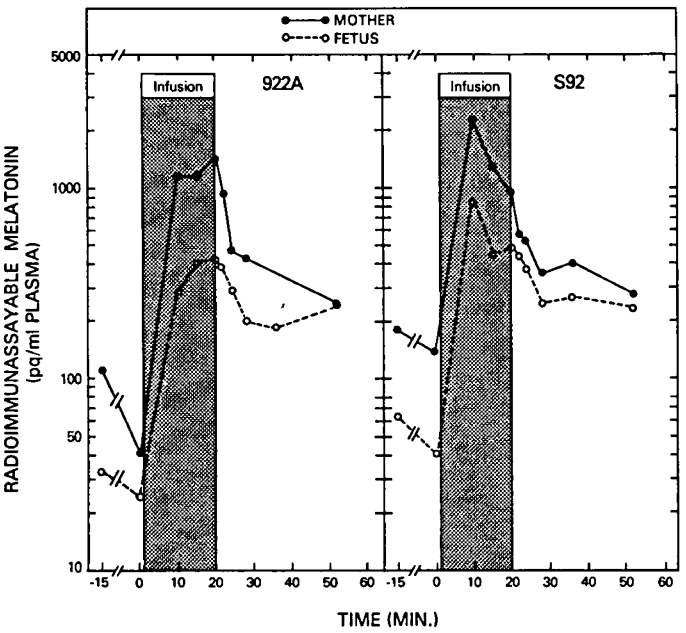

Figure 4. Melatonin in maternal and fetal plasma before, during, and after an infusion of authentic melatonin to the mother. Time -15 min is 15 min after the [³H]melatonin maternal injection. Zero time is 30 min after the [³H]melatonin injection.

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