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Transport of steroids between fetuses via amniotic fluid in relation to the intrauterine position phenomenon in rats

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Summary. In litter-bearing mammals, the course of development of male and female fetuses is affected by the presence of other fetuses of the same or opposite sex located nearby within the uterus. The transport of testosterone between rat fetuses was examined by implanting a Silastic capsule containing [^{3}H]testosterone into the amniotic sac of a fetus at either the ovarian or cervical end of a uterine horn on days 19 and 20 of pregnancy. The amount of testosterone that was recovered from the amniotic fluid of other fetuses 12 h later was determined. The amniotic fluid surrounding the adjacent fetus on the cervical side of the implanted fetus contained three times as much [^{3}H]testosterone as did the adjacent fetus on the ovarian side, regardless of where in the uterus the implant was made. The movement of dye injected into the uterine lumen was towards the cervix. Intraluminal fluid movement may thus mediate the greater transport of [^{3}H]testosterone towards the cervix than towards the ovary.

Our findings support the hypothesis that transport of testosterone between fetuses occurs across the fetal membranes via diffusion, such that any fetus (male or female) located between male fetuses receives the greatest supplement of testosterone.

Keywords: intrauterine position; intrauterine transport; sexual differentiation; rat

Introduction

Fetuses of litter-bearing mammals are influenced by the sex of the fetuses located on either side of them within a uterine horn. This has been reported in mice (vom Saal & Bronson, 1978), rats (Clemens *et al.*, 1978), gerbils (Clark & Galef, 1988) and pigs (Rohde-Parfet *et al.*, 1990). These and numerous other studies (reviewed in vom Saal, 1989) have revealed that individual differences in morphological, physiological and behavioural traits in rodents, and possibly in all litter-bearing mammals, are related to the random intrauterine position that males and females occupy during prenatal development.

It has been reported that, in mice, females located within a uterine horn between male fetuses (2M females) have higher amniotic fluid and blood concentrations of testosterone, and lower concentrations of oestradiol, than female fetuses located between other females (0M females; vom Saal & Bronson, 1980; vom Saal *et al.*, 1990); the same effect of intrauterine position on concentrations of testosterone (as well as oestradiol) in blood were also observed in comparisons of male mouse fetuses from different intrauterine positions (vom Saal *et al.*, 1983; vom Saal, 1989). In male and female gerbil fetuses, the same differences were found in blood testosterone concentrations owing to intrauterine position as were observed in mice (Clark *et al.*, 1991). These findings suggest that differences in adult characteristics due to prior intrauterine position were mediated by the passage of testosterone and oestradiol from one fetus to another within a uterine horn, but the mechanism by which this transport occurred remained unknown.

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The different hypotheses concerning the possible mechanisms by which steroids might be transported between fetuses have led to different methods of classifying fetuses with regard to the sex of other fetuses within a uterine horn. In previous studies with rats and mice, we have used the method of classifying fetuses based on the sex of only the fetuses that were directly adjacent to the fetus being classified (Fig. 1). In contrast, Meisel & Ward (1981) and others (Richmond & Sachs, 1984; Houtsmuller & Slob, 1990) have used a scheme based on males located on the cervical side of the female being measured, without regard to whether there were other female fetuses situated between the male fetuses and the female being measured.

In the first experiment a Silastic capsule containing [^{3}H]testosterone was implanted into the amniotic sac of one rat fetus and monitored the presence of ^{3}H in the amniotic fluid surrounding other fetuses within the uterine horn. The objective was to determine whether passage of [^{3}H]testosterone between fetuses would occur (1) in both directions (towards both the ovary and cervix) and (2) only between adjacent fetuses. In the second experiment we monitored the movement of dye within the uterine lumen of pregnant rats to determine whether fluid movement within the uterine lumen was in the same or opposite direction to the movement of [^{3}H]testosterone between fetuses.

Methods and Results

Animals and housing

A breeding stock of Sprague–Dawley rats was purchased from Harlan Laboratories (Indianapolis, IN) and used to found a closed, randomly bred colony of rats. Animals were maintained in 45 × 25 × 20 cm^3 polyethylene cages on Aspen bedding. Purina lab chow (5008) and water were continuously available. Animal rooms were maintained at 25 ± 2°C on a 12 h light:12 h dark cycle, with lights on at 10:00 h.

Experiment 1: transport of [^{3}H]testosterone between rat fetuses

Silastic capsules for delivery of [^{3}H]testosterone. 1,2,6,7-[^{3}H]testosterone (New England Nuclear, Boston, MA), 1 μCi was dissolved per 1·0 μl sesame oil. A 5-mm length of Silastic capsule (0·020 in i.d., 0·037 in o.d.; Dow 602-135) holds 1·0 μl oil. Capsules were cut into 7 mm lengths. One end of the capsule was sealed with Silastic Type A adhesive, the [^{3}H]testosterone was injected into the capsule with a 30 g needle, and the other end of the capsule was sealed such that the length between the sealed ends measured 5 mm. The capsules were preincubated for 24 h in physiological saline containing bovine serum albumin prior to being implanted into the amniotic sac of a fetus.

Mating and surgical procedures. Female rats (3–4 months old) were paired with stud males and examined daily at 11:00 h for spermatozoa via vaginal lavage. The day that spermatozoa were found was considered to be day 1 of pregnancy. At 08:00 h on day 20 or 21 of pregnancy, females were anaesthetized with an i.p. injection of sodium pentobarbital. The initial dose was 35 mg kg^{-1}; supplemental doses were given as needed. The fur on each flank was shaved, and an incision was made which would reveal either the top or bottom part of a uterine horn. Silastic implants were placed into the amniotic sac of a fetus located in one of two positions within a uterine horn: the second fetus below the ovary ($n = 5$) or the second fetus above the cervix ($n = 8$). Each pregnant female had a capsule placed into the amniotic sac of a single fetus located in each of the two uterine horns. Each group was matched in terms of implants being placed in both the right and left uterine horns, since we have observed differences in placental blood flow between the right and left uterine horns (M. D. Even, M. H. Laughlin, G. F. Krause & F. S. vom Saal, unpublished). The objective of placing the capsule at each end of the uterus was to control for direction of blood flow in the uterine loop artery. Blood flows in the uterine loop artery in a caudal direction at the point where the fetus was implanted at the ovarian end of the uterus and in a rostral direction where the

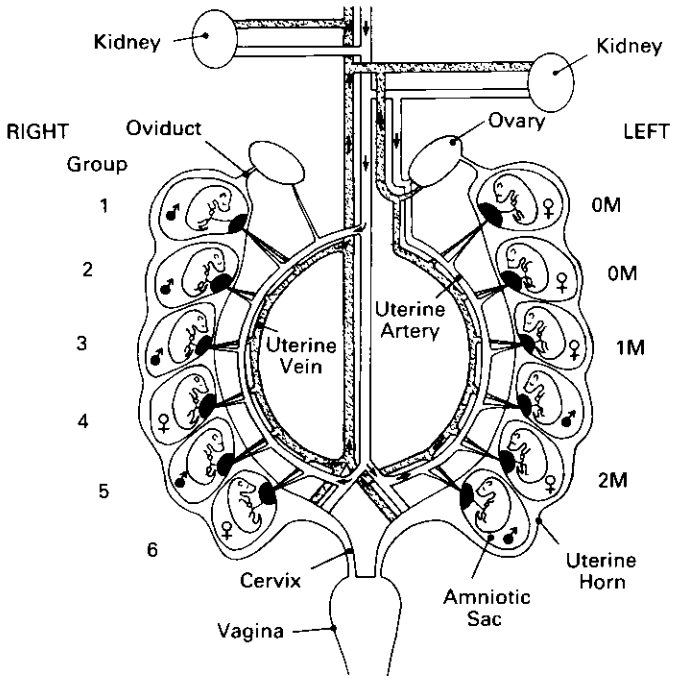

Fig. 1. Schematic diagram of the uterine horns and uterine loop artery and vein feeding each horn in a pregnant rat near the end of pregnancy. All fetuses in a horn were numbered sequentially, beginning with the fetus next to the ovary. The mean number of fetuses per uterine horn was 6·4. 2M = female between 2 males; 1M = female between a male and a female; and 0M = female between 2 females.

fetus was implanted at the cervical end of the uterus in both rats (M. D. Even, M. H. Laughlin, G. F. Krause & F. S. vom Saal, unpublished) and mice (vom Saal & Dhar, 1992).

A Silastic capsule was placed into an 18 g needle. The needle was inserted into the amniotic compartment through the uterine wall. This is possible because the uterus becomes very thin towards the end of pregnancy, and fetuses can be clearly observed through the uterus. Care was taken not to touch any of the uterine blood vessels, which are also clearly visible. The capsule was pushed out of the needle into the amniotic sac by a small rod, and the needle was withdrawn. Since the amniotic fluid is highly viscous, fluid loss was minimal.

Twelve hours after implantation of testosterone, the pregnant females were killed by CO_2 asphyxiation followed by cervical dislocation. A mid-ventral incision was made, and the uterine horns were exposed. The uterine tissue was cut away, and the fetuses were carefully removed sequentially with the fetal membranes intact. The fetuses contained within their intact fetal membranes were placed on filter paper (Whatman No. 2). The fetal membranes were then cut, and the amniotic fluid was absorbed into the filter paper. Excess amniotic fluid was wiped from the fetus onto the filter paper. The Silastic capsule was recovered from the amniotic sac of the relevant fetus. The filter paper with amniotic fluid from each fetus was then cut into small strips and placed into test tubes.

Extraction and counting of [^{3}H]testosterone. [^{3}H]testosterone was extracted twice from the amniotic fluid by addition of 5 ml diethyl ether to the test tubes containing the filter paper. The tubes were tightly covered and placed into a shaking water bath for 4 h. The ether was decanted, dried under nitrogen, and ^{3}H was counted in a scintillation counter (Beckman 5801; 55% efficiency) after addition of 5 ml Ultima Gold (Packard).

Statistical analysis. It cannot be assumed that [^{3}H]testosterone was not metabolized, and since no attempt was made to identify metabolites, the disintegrations per minute (d.p.m.) will be referred to as reflecting the presence of ^{3}H, not [^{3}H]testosterone. The d.p.m. for ^{3}H in amniotic fluid of nonimplanted fetuses within an individual uterine horn was expressed as a percentage of the d.p.m. for ^{3}H recovered from the implanted fetus, which was assigned a value of 100%. The percentage ^{3}H recovered from the amniotic fluid of fetuses located near the implanted fetus was analysed.

Data were analysed by analysis of variance using the Statistical Analysis System (SAS), General Linear Model. Comparisons of differences between group means were made using the LS mean test in SAS if the overall ANOVA showed a significant main effect or interaction. The criterion for rejecting the null hypothesis was $P < 0{\cdot}05$. Data are presented as means ± SEM.

Results. A preliminary study showed that the wound created in the amniotic sac was closed within 4 h of implantation of a Silastic capsule. All fetuses appeared healthy when the amniotic fluid was collected 12 h after implantation of the capsule (if no haemorrhaging of blood vessels occurs at capsule implantation, survival is 100%).

For fetuses at the ovarian end of a uterine horn with implantations, the percentage of ^{3}H recovered from amniotic fluid of the adjacent fetus on the cervical side of the treated fetus was over three times greater than the ^{3}H recovered from the adjacent fetus on the ovarian side (Fig. 2a). The fetus located in the fourth position away from the ovary, and thus separated by one fetus from the

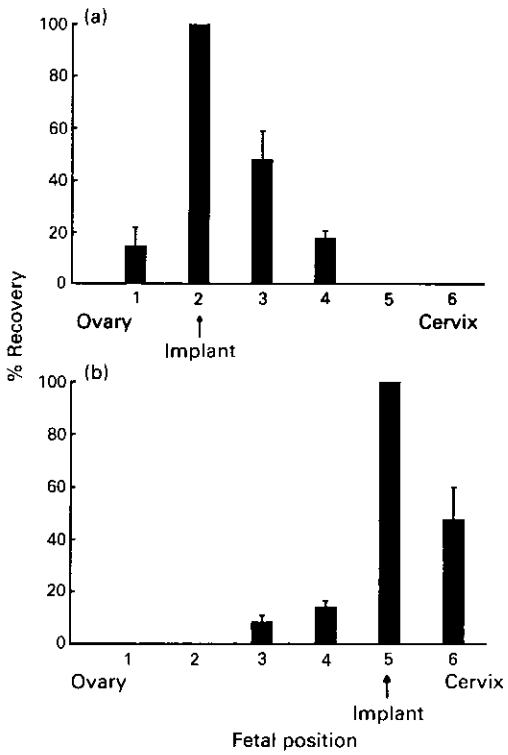

Fig. 2. Recovery of ^{3}H from amniotic fluid expressed as the percentage of ^{3}H recovered from the amniotic fluid of the fetus implanted with a capsule containing [^{3}H]testosterone, which was assigned a value of 100%. (a) Fetuses in position 2 (see Fig. 1) ($n = 5$) were implanted with a Silastic capsule containing [^{3}H]testosterone. (b) Fetuses in position 5 ($n = 8$) were implanted with a Silastic capsule containing [^{3}H]testosterone.

treated fetus, had 18 ± 2·7% of the ^3H recovered from the treated fetus; this was similar to the percent ^3H recovered from the adjacent fetus on the ovarian side of the treated fetus and significantly less than the percentage ^3H recovered from the adjacent fetus on the cervical side of the treated fetus. The means ± SEM of the number of fetuses within the uterine horn for this group was 6·4 ± 0·5 (litter size in Sprague–Dawley rats bred in our laboratory is typically around 13 pups).

Very similar findings were obtained for fetuses that had been implanted with testosterone at the cervical end of a uterine horn (Fig. 2b). The fetus on the cervical side adjacent to the treated fetus again had over three times more ^3H in the amniotic fluid relative to ^3H recovered from amniotic fluid of the fetus located adjacent to the treated fetus on the ovarian side. The fetus located in the fourth position above the cervix, and thus separated by one fetus from the treated fetus, had only 8·4 ± 2·3% of the ^3H which was recovered from the treated fetus; this value differed significantly from the 14·1 ± 2·4% recovered from the fetus which was located on the ovarian side adjacent to the treated fetus. The mean (±SEM) number of fetuses per horn for this group was 6·3 ± 0·7.

On the basis of the specific activity of the [^3H]testosterone, the mass of [^3H]testosterone which was recovered from the amniotic fluid of the implanted fetuses was calculated. The mass of [^3H]testosterone ranged from 2–8 pg per total volume of amniotic fluid collected.

Experiment 2: Transport of dye in the uterine lumen of pregnant rats

The results of the previous study showed that a threefold greater amount of ^3H was recovered from the amniotic fluid of a fetus located on the cervical side adjacent to the fetus that had been implanted with testosterone relative to that recovered from an adjacent fetus on the ovarian side. This occurred at both the ovarian and cervical end of the uterine horns. One hypothesis to explain this observation is that fluid movement within the uterine lumen is from the ovary towards the cervix, which would serve to provide drainage from the Fallopian tubes and uterus through the cervix and vagina. The direction of luminal flow would thus be different from the direction of flow of lymph within the lymphatic ducts of the uterus, since the flow of lymph within the lymphatic ducts is bi-directional (Head & Lande, 1983). To determine the direction of fluid movement within the uterine lumen of pregnant rats, we injected dye into the uterine lumen and observed the direction of movement of the dye.

Surgical procedures. Female rats were mated with males as described above. On day 19 of pregnancy, two females were anaesthetized with Nembutal (as described above), and the two uterine horns were exposed via a midventral incision. A 1% solution of pontamine blue dye was injected into each uterine lumen between the third and fourth fetus (the fetus next to the ovary was counted as the first fetus). Both of these pregnant females had six or more fetuses within each uterine horn. Care was taken to inject the dye into the lumen of the uterus and not into the amniotic sac surrounding the fetuses on either side of the point of injection.

Results. There was spreading of dye in both the rostral and caudal directions at the time of injection, such that the fetuses on either side of the point of injection were immediately surrounded by dye. However, we observed that after this initial bi-directional spreading, the dye only moved towards the cervix from the point of injection, and all fetuses on the cervical side of the injection were surrounded by dye, while there was no dye around the first two fetuses below the ovary. By 3 min after the injection, movement of dye had occurred down the entire uterus toward the cervix. The movement of dye within the uterine lumen was thus from the ovarian end towards the cervical end of each uterine horn.

Discussion

The intrauterine passage of [^3H]testosterone between fetuses occurred in both directions, although the amount of [^3H]testosterone that passed into the amniotic fluid of adjacent fetuses located

towards the cervix was over three times greater than into adjacent fetuses located towards the ovary, regardless of where in the uterine horn the fetuses were located. This finding was consistent for fetuses located in either the left or right uterine horn.

These findings support the hypothesis that testosterone diffuses from the blood in male fetuses into the amniotic fluid. It then diffuses across the fetal membranes into the uterine lumen. The amount of testosterone that diffuses across the fetal membranes into the amniotic fluid of a contiguous fetus on the cervical side of a male is greater than to a contiguous fetus on the ovarian side of a male, possibly owing to the slow movement of fluid within the uterine lumen towards the cervix. The likely explanation for the slow movement of luminal fluid that was observed on day 19 of pregnancy is that the fetuses and surrounding fetal membranes containing amniotic fluid occupy most of the uterine lumen, thus greatly restricting the flow of fluid in the uterine lumen. Finally, testosterone diffuses from the amniotic fluid into the circulation of the fetuses that are adjacent to a male, resulting in an increase in the concentration of testosterone in the blood of these fetuses (vom Saal, 1989).

Each fetus has its own placenta and is contained within both a chorionic membrane and an amniotic membrane (together these are referred to as the fetal membranes). The amniotic fluid (contained within the amniotic sac) which bathes each fetus is continuously swallowed and thus circulates through the fetal gut; steroids can thus easily pass between the fetal blood and amniotic fluid. Amniotic fluid and blood are presumed to be in equilibrium. However, because of the different concentrations of steroid-binding proteins, the exact concentration of total (protein-bound plus free) steroid in blood and amniotic fluid would likely differ (Belisle & Tulchinsky, 1980).

In three rats in which the uterine horns contained only three fetuses, the middle fetus was implanted with a Silastic capsule containing [^{3}H]testosterone and there was no difference in the amount of ^{3}H recovered from the amniotic fluid of the adjacent fetus on the ovarian side and on the cervical side (the range of values for ^{3}H recovered from amniotic fluid from fetuses on either side of the implanted fetus was only 7–11% of ^{3}H recovered from the implanted fetus) (M. D. Even, M. G. Dhar & F. S. vom Saal, unpublished). This finding, while based on a small sample and thus only preliminary, suggests that in uterine horns containing few fetuses, considerably less transport of steroid occurs between adjacent fetuses. This observation is consistent with the observation that in uterine horns containing few fetuses, the fetuses are evenly spaced, and the fetal membranes of adjacent fetuses are separated from each other; when six fetuses are located within a uterine horn, the membranes surrounding a fetus are in contact with the membranes surrounding adjacent fetuses on either side by day 19.

The findings from these experiments conflict with the hypothesis of Meisel & Ward (1981), which was based on postnatal morphological and behavioural findings from female rats using different intrauterine position classification schemes, rather than on a study of intrauterine steroid transport or uterine blood flow. Meisel & Ward (1981) proposed that movement of testosterone between fetuses was in a rostral direction and that transport of testosterone between fetuses was via the maternal uterine vasculature. Central to the hypothesis of Meisel & Ward (1981) was the assumption that blood flow in the maternal uterine artery was from the cervical end towards the ovarian end of the loop which the uterine artery forms as it branches from the descending aorta and internal iliac (this describes the right uterine horn; Fig. 1). The prediction was that testosterone passed across the placenta of a male fetus into the uterine vein draining the placenta and then into the maternal loop uterine artery. Transport of testosterone from the uterine vein to the uterine artery was suggested to occur via a putative countercurrent exchange system. This type of exchange system probably depends on a specific anatomical relationship between veins and arteries, which is not seen in the uterine loop vessels of rats (Del Campo & Ginther, 1972). Once testosterone entered the maternal uterine loop artery (via transport from the uterine vein), testosterone was predicted to flow into placentae and thus the bloodstream of female fetuses located anywhere within the uterine horn on the ovarian side of a male fetus.

In two other studies we have provided evidence that blood flow in the uterine loop artery is bi-directional in both rats and mice rather than in a rostral direction as proposed by Meisel & Ward

(1981). Specifically, uterine blood flow in pregnant rats was examined by infusing radiolabelled microspheres into the right ventricle on different days of pregnancy. Microspheres incorporate into a tissue as a function of the rate of blood flow to the tissue. Blood flow to the ovarian and cervical ends of each uterine horn was significantly greater than to the middle of the uterine horn (M. D. Even, M. H. Laughlin, G. F. Krause & F. S. vom Saal, unpublished). This finding is consistent with the direct observation in pregnant house mice that dye injected into the heart enters the loop artery feeding each uterine horn from both the upper branch and the lower branch (vom Saal & Dhar, 1992).

One interesting observation in these studies with both rats and mice is that the architecture of the left uterine loop artery and vein (specifically, the point of origin of the rostral branch of the left uterine loop artery and vein) differed among females; however, the architecture of the vessels feeding the right uterine horn did not vary among females. This difference is probably due to the close proximity of the kidney and renal vessels to the left ovary and rostral tip of the left uterine horn relative to the right side of the animal (Fig. 1). This finding suggests that the uterine horn in which a fetus developed should be considered in future studies, particularly if fetal body weight is of interest; fetal body weight may be related to placental blood flow (McLaren & Michie, 1960; M. D. Even, M. H. Laughlin, G. F. Krause & F. S. vom Saal, unpublished).

In summary, Meisel & Ward (1981) proposed that movement of steroids between fetuses is (1) in a rostral direction and does not depend on fetal contiguity, and (2) mediated by maternal uterine blood flow which moves in a rostral direction within the maternal uterine loop artery. Results of the experiments presented here and elsewhere (M. D. Even, M. H. Laughlin, G. F. Krause & F. S. vom Saal, unpublished; vom Saal & Dhar, 1992) do not support these predictions. The greater movement of [^{3}H]testosterone implanted within the amniotic sac towards the cervix, regardless of whether the capsule was placed at either the ovarian or cervical end of the uterus, rules out passage via the uterine vasculature, since blood flows in an opposite direction at the ovarian and cervical ends of the uterine loop artery. Our results provide support for the hypothesis that the mechanism by which steroids pass between fetuses (and thus the basis for the intrauterine position phenomenon) is by diffusion across the fetal membranes via the amniotic fluid of adjacent fetuses.

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