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Blood Flow in the Uterine Loop Artery and Loop Vein Is Bidirectional in the Mouse: Implications for Transport of Steroids Between Fetuses

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VOM SAAL, F. S. AND M. G. DHAR. *Blood flow in the uterine loop artery and loop vein is bidirectional in the mouse: Implications for transport of steroids between fetuses.* PHYSIOL BEHAV **52**(1) 163–171, 1992.—In rodents, steroids are able to pass between fetuses. Although not previously examined in mice, we have provided evidence that blood flow in the uterine loop artery in rats is bidirectional and that steroids are transported between fetuses by diffusion via the uterine lumen, not via the uterine blood vessels of the mother. The direction of blood flow in the uterine loop artery and vein feeding each uterine horn in house mice was examined on day 17 of pregnancy. Dye was injected into the heart to determine the direction of blood flow in the uterine artery while injection of dye into individual placentae was used to determine the direction of blood flow in the uterine vein. Blood entered the loop artery from both the dorsal and caudal ends and was thus bidirectional. Venus blood flow from placentae was in a rostral direction from placentae in the rostral portion of a uterine horn and in a caudal direction from placentae in the caudal portion of a uterine horn. Comparison of anogenital distance and body weight at birth using a variety of classification schemes, based on different assumptions about the mechanism and direction of transport of steroids between fetuses, showed that the only scheme which accounted for variation in anogenital distance at birth in female mice was that which was based on the number of directly adjacent male siblings in utero. Taken together with recent findings in rats reported elsewhere, we conclude that blood flow in the uterine blood vessels is bidirectional, and steroids are transported between fetuses by diffusing through the amniotic fluid and across the fetal membranes of adjacent fetuses. We found that the common method of using the ratio of anogenital distance/body weight can lead to erroneous conclusions concerning intrauterine position effects on anogenital distance. Analysis of covariance tests whether variation in body weight contributes to differences in anogenital distance due to intrauterine position, and this method of analysis should be used in future studies.

Intrauterine position Uterine blood flow Sexual differentiation Anogenital distance Body weight

IN every litter-bearing species so far examined [mice (19,20), rats (4), gerbils (1,2), and pigs (15,16)], animals of the same sex which were situated between two male fetuses (2M animals) during intrauterine development have been found to differ in a wide variety of postnatal traits when compared to animals of the same sex which were not positioned between male fetuses (0M fetuses). Correlative studies have provided evidence that the basis for the differences between 2M and 0M animals is that fetuses communicate hormonally with each other when there is more than one fetus within the uterus. For example, we have shown (19,21–23) that during the last few days of prenatal development in mice, males have higher serum concentrations of testosterone than females, and 2M fetuses have elevated serum concentrations of testosterone relative to 0M fetuses of the same sex.

The elevated blood concentrations of testosterone in 2M animals had been predicted based on the observation in rats (4) and mice (20,23) that 2M females had a longer space between the anus and genital papilla at birth (a bioassay for prenatal testosterone exposure) relative to 0M females. These findings also appeared to justify the use of the male as the reference for classifying the intrauterine position of other fetuses. Female fetuses were thus not assumed to have any impact on the development of adjacent fetuses. In contrast to this prediction, we observed that in mice, female fetuses have higher serum concentrations of estradiol than male fetuses, and 0M fetuses have elevated serum concentrations of estradiol relative to 2M fetuses of the same sex. Serum estradiol levels during fetal life are correlated with postnatal reproductive traits in male mice (19,22). It thus appears that there is transport of both testosterone and

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estradiol [and possibly other steroids (13)] between adjacent fetuses. Taken together, these findings suggest that differences in adult characteristics due to prior intrauterine position are mediated by the passage of both testosterone and estradiol from one fetus to another within a uterine horn.

The uterus of the mouse is composed of two independent horns, each with its own cervix, and is thus referred to as a duplex uterus. The origin of the blood vessels feeding the rostral portion of the left and right uterine horns is different due to the proximity of the kidney and ovary on the left side (Fig. 1). In rodents, fertilized ova entering one uterine horn do not pass into the contralateral horn. Since each uterine horn is physiologically independent, hormonal communication between fetuses should thus only occur within a uterine horn.

In most studies concerning the intrauterine position phenomenon, the classification of the intrauterine position of a fetus has been based on the sex of the adjacent fetus. This classification is based on the assumption that gonadal steroids are transported between fetuses via diffusion from the fetus, through the amniotic fluid across the fetal membranes surrounding an adjacent fetus. Studies that we have conducted using intracardial infusions of radiolabelled microspheres (6) and intraamniotic implants of ^{3}H-testosterone (7) in pregnant rats have provided evidence supporting this hypothesis.

An alternative to the adjacent male hypothesis is referred to as the caudal male hypothesis, which was initially proposed by Meisel and Ward (11), and subsequently tested by others (9,14). Specifically, testosterone was proposed to pass from the blood stream of a male fetus across the placenta into the maternal uterine vein draining the placenta. Transport of testosterone from the uterine vein into the uterine loop artery was hypothesized to occur via a putative counter current exchange system. This type of exchange system has been proposed to be involved in the transport of prostaglandins between the uterus and ovary, but it likely depends on a specific anatomical relationship between veins and arteries, which is not seen in the uterine vessels in rats (5). Based on postnatal morphological and behavioral findings (not a study of intrauterine steroid transport or uterine blood flow), Meisel and Ward (11) proposed that in rats, blood flowed in a rostral direction from the cervix toward the ovary in the uterine loop artery. Once the testosterone entered the maternal uterine loop artery (via transport from the uterine vein), the testosterone would flow into placentae and then into the blood stream of female fetuses located anywhere within the uterine horn on the ovarian side of a male fetus. Female fetuses would thus be masculinized by any male located within a uterine horn on the caudal (cervical) side of the female fetuses. However, the fact that the intrauterine position phenomenon occurs in a variety of species (rodents and pigs) with marked differences in uterine vasculature [see (6)] argues against the hypothesis that transport of steroids between fetuses is via the maternal uterine blood vessels.

The studies reported here were conducted to examine the direction of blood flow in the uterine loop artery and vein in pregnant mice. We also conducted analyses of anogenital distance and body weight of fetuses at the time of cesarean delivery. We used a variety of classification schemes to determine which scheme(s) would show significant differences and thus provide indirect information about the possible mechanism of intrauterine transport of steroids in mice. The relationship between the position of fetuses within each uterine horn relative to the ovary or cervix and body weight of females at birth was also examined in relation to the direction of blood flow at different points within the uterine loop artery. This analysis was conducted, because it is well documented that position within the uterus (relative to the ovary and cervix) influences fetal body weight, which has been proposed to be due to differences in blood flow to placentae in the middle vs. ends of a uterine horn (10).

GENERAL METHOD

Animals and Housing

CF-1 house mice (*Mus domesticus*) were housed in 18 × 29 × 13 cm polypropylene cages and maintained at 25 ± 2°C on a 12:12 light:dark cycle, with lights on at 1200 h. Mouse breeder chow (Purina 5008) and water were available ad lib.

Mating Procedure

Adult female mice were time mated by being placed daily with a stud male beginning at 0800 h and examined for vaginal plugs 4 h later (day 0 of pregnancy). Inseminated females were housed three per cage and not disturbed until day 10 of pregnancy, at which time the cages were changed. Pregnant females were housed individually 1 day prior to delivery of the litter by cesarean section or their use in the blood flow experiment.

EXPERIMENTAL PROCEDURES AND RESULTS

EXPERIMENT 1: DIRECTION OF UTERINE BLOOD FLOW IN PREGNANT HOUSE MICE

Based on findings concerning the pattern of body weights of fetuses located in the middle (which are lightest) vs. either end (which are heaviest) within a uterine horn in house mice, McLaren and Michie (10) proposed that blood flow in the uterine artery was bidirectional. They suggested that blood should enter the loop uterine artery from both the rostral end and the caudal end. We directly tested the hypothesis that blood flow in the uterine loop artery was bidirectional by injecting carbon dye into the heart and observing the movement of dye in the uterine loop artery in pregnant house mice. We also examined the direction of blood flow in the uterine loop vein by injecting dye into individual placentae.

Method

Pregnant females were individually housed on the morning of day 16 and injected with dye at 1300 h on day 17 of pregnancy (the mean time of onset of parturition is 1430 h on day 19). Six females were anesthetized with Metofane, and a mid-ventral incision was made which exposed the uterine horns and chest. The two uterine horns were carefully laid out on moist towels so that movement of dye within the uterine vessels could be visualized. The heart was then exposed by cutting open the chest, and 0.25 cc of india ink (carbon dye) was injected into the left ventricle. The dye was observed moving into the uterine loop artery feeding one of the uterine horns; half of the observations were of the left uterine horn and half of the right uterine horn.

In four other females, each of the two uterine horns was exposed as described above, and 0.1 cc dye was injected into placentae in each of the two uterine horns using a 30-ga needle. In four uterine horns the dye was injected into placentae beginning with the placenta located at the rostral end of the uterine horn and then moving caudally until flow from a placenta occurred in a caudal direction. In four other uterine horns the dye was injected beginning with the placenta located at the caudal end of the uterine horn. The direction of movement of the dye as it passed into the uterine loop vein was observed.

Results

The structure of the uterine loop artery and vein was the same for both the right and left uterine horns in all of the mice

examined in this experiment and was similar to what is depicted in Fig. 1 for the right uterine horn. In about 50% of CF-1 mice the origin of the left uterine artery and vein is from the renal artery and vein, respectively (as shown in Fig. 1), although this was not observed in any of the females used in this experiment. In addition, there were individual differences in the degree to which the loop artery and loop vein were, in fact, continuous loops. For example, in two animals the vessels coming from the rostral and caudal ends of the horn were connected at one point by a small shunt and thus did not form a continuous loop.

Arterial blood flow. Intracardial injection of carbon dye resulted in passage of dye into the uterine loop artery from both the rostral and caudal ends in both the left and right uterine horns in all animals. For example, one uterine horn contained three fetuses, and the dye entered the rostral end of the loop artery and passed down to just below the second fetus. The dye entering this uterine loop artery from the caudal end flowed through the vessel to a point just above the third (most caudal) fetus, where it merged with the dye that had entered the vessel from its rostral end. With four fetuses in a uterine horn, the dye entering from the rostral and caudal ends of the uterine loop artery met exactly in the middle of the loop. In a uterine horn with five fetuses, the dye entered the rostral end of the loop artery and passed into the arteries feeding the placentae of the two fetuses nearest the ovary. Dye entered the arteries feeding the remaining 3 fetuses from the caudal end of the loop uterine artery. In three uterine horns containing seven fetuses, the dye passed from the rostral end into the first four fetuses below the ovary in two cases, while in the third case the dye passed into the first five fetuses below the ovary from the rostral direction; the remainder of the placentae were perfused from the caudal direction.

Venous blood flow. Injection of dye into placentae beginning with the placentae located at the caudal end of a uterine horn showed that for the most caudal placenta in a uterine horn, blood flowed out of the placenta in a caudal direction within the uterine loop vein; this was observed with four to eight fetuses within a uterine horn. However, in the uterine horn with four fetuses, blood flowed from the most caudal placenta in both a rostral and caudal direction after entering the loop uterine vein. With five fetuses per horn, blood flow from the fourth placenta (starting from the ovary) was in both a rostral and caudal direction in the uterine vein. With six fetuses per horn, blood flow from the fifth placenta was in a rostral and caudal direction in the uterine vein. With eight fetuses per horn, blood flow from the sixth placenta was in a rostral and caudal direction (dye moved out of the seventh and eighth placentae and passed in a caudal direction in the uterine vein).

Injection of dye into placentae beginning at the rostral end showed that for uterine horns containing seven or eight fetuses, flow within the uterine loop vein was only in a rostral direction toward the postcava from the first five placentae (beginning at the ovarian end), while flow from the placentae feeding the sixth fetus within the uterine horn was in both a rostral and caudal direction (dye leaving the remaining placentae moved caudally). In a uterine horn with five fetuses, flow from the first two placentae was only in a rostral direction in the uterine loop vein, while flow from the third placenta was bidirectional in the uterine loop vein. However, in another uterine horn with five fetuses, blood flow in the uterine loop vein was only in a rostral direction for the first three placentae, while flow from the fourth placenta was bidirectional.

Taken together, these findings show that in pregnant house mice, blood flow in both the uterine loop artery and uterine loop vein is bidirectional, but the exact positions within the vessels at which blood flows in a rostral or caudal direction differs slightly for each uterine horn.

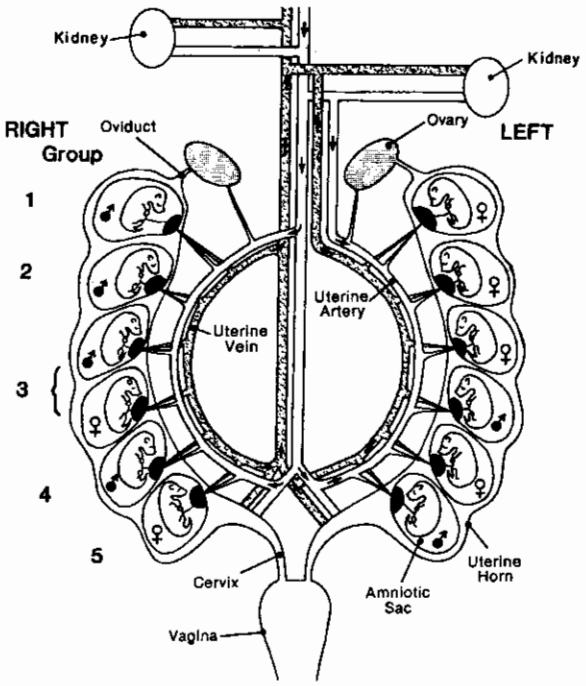

FIG. 1. A schematic diagram depicting the uterine horns and loop vessels in a pregnant mouse near the end of pregnancy. To determine the structure of the uterine vessels, 80 pregnant females were examined. For the right uterine horn, the rostral end of the uterine loop artery and vein began as branches off of the descending aorta and postcava, respectively, for 78/80 (98%) of the females; for the remaining two females, a single vessel branched off of the descending aorta and postcava and then bifurcated, with one branch leading to the uterine artery and uterine vein, respectively, and the other branch leading to the renal artery and renal vein, respectively. For the left uterine loop artery and vein, in 39/80 (49%) of pregnant CF-1 mice, the rostral origin of the uterine loop artery and vein was from the renal artery and vein rather than the descending aorta and postcava, respectively; this is similar to what is observed for the left uterine horn in the majority of Sprague–Dawley rats (7). In 34 cases (43%), the left uterine loop artery and vein began below (and independent from) the renal artery and vein, respectively. In seven cases (8%), the left uterine loop artery and vein bifurcated near the descending aorta and postcava, with one branch entering the renal artery and vein, and the other entering the descending aorta and postcava, respectively. The caudal end of the uterine loop artery and vein begin as branches off of the internal iliac artery and vein, respectively, on both the right and left side. The arrows within the blood vessels show the direction of movement of dye observed in Experiment 1. Group 1–5 refers to the classification of animals in Experiment 3.

EXPERIMENT 2: ANOGENITAL DISTANCE AND BODY WEIGHT AT BIRTH OF FEMALE MICE: INTRAUTERINE POSITION CLASSIFICATION SCHEMES

The previous experiment showed that in mice, as in rats (6), blood flow in the uterine loop vessels is bidirectional, confirming the prediction of McLaren and Michie (10). We thus propose that in mice, the best predictor of anogenital distance at birth in females should be the number of adjacent males in utero. However, we observed that movement of ^{3}H-testosterone between fetuses in pregnant rats is greater toward the cervix than toward the ovary [possibly due to the movement of fluid within the uterine lumen from the fallopian tubes toward the cervix (7)], and this could also occur in mice. We thus predicted that

for female fetuses located next to one male (1M females), 1M female fetuses located on the cervical side of the adjacent male should have a longer anogenital distance than 1M females located on the ovarian side of the adjacent male.

In this experiment we compared the anogenital distance and body weight of female mice at the time of cesarean delivery using a number of different classification schemes. The analyses were conducted on the same data set using six different classification schemes:

1. number of adjacent males;
2. number of adjacent males as well as number of adjacent females;
3. and 4. number of adjacent males located on the cervical vs. ovarian side;
5. number of males located toward the cervix without regard to proximity;
6. number of males located toward the ovary without regard to proximity.

Mating and Measurement Procedures

To obtain mouse fetuses from known intrauterine positions, adult CF-1 female mice were time mated as described in the previous experiment. Fifty-two pregnant females were housed individually on day 18 and were killed by cervical dislocation beginning at 9000 h on day 19 of pregnancy. The pups were removed sequentially from each uterine horn, and the female pups were weighed. Sex was determined by examining anogenital distance, which is about two times longer in males than in females, and the position of each animal within the uterine horns was recorded. Anogenital distance of all females was then measured using a dissecting microscope fitted with a micrometer disc accurate to 0.05 mm by an investigator who was unaware of the intrauterine position of the animal being measured; anogenital distance does not vary as a function of intrauterine position in male mice (unpublished observation).

Data Analysis

The data were analyzed both by analysis of variance (ANOVA) and analysis of covariance (ANCOVA) using a number of different strategies. First, anogenital distance and body weight of females were analyzed independently by ANOVA. Analysis of variance was also conducted on the ratio of anogenital distance/body weight for each female. The strategy of correcting anogenital distance for body weight by creating a ratio explicitly assumes that these variables are related. However, when there is a difference between the groups in body weight, this could lead to the finding of a significant ratio of anogenital distance/body weight based only on group differences in body weight. Analysis of covariance was conducted for anogenital distance with body weight as the covariate. The F value in ANCOVA associated with anogenital distance is based on differences between the groups in anogenital distance after the anogenital distance scores are adjusted to a common body weight.

The Statistical Analysis System (SAS, GLM procedure) was used to analyze all data. Planned comparisons of group means were made using the LS means test, with $p < 0.05$ set as the probability level for rejecting the null hypothesis.

2-A: The Effect of Being Adjacent to Two, One, or No Males

The results shown in Fig. 2 for the comparison (by ANOVA) of females classified based on the number of adjacent males [2M (between 2 males), 1M (next to one male) and 0M (not next to a male)] confirm previous reports that the number of males

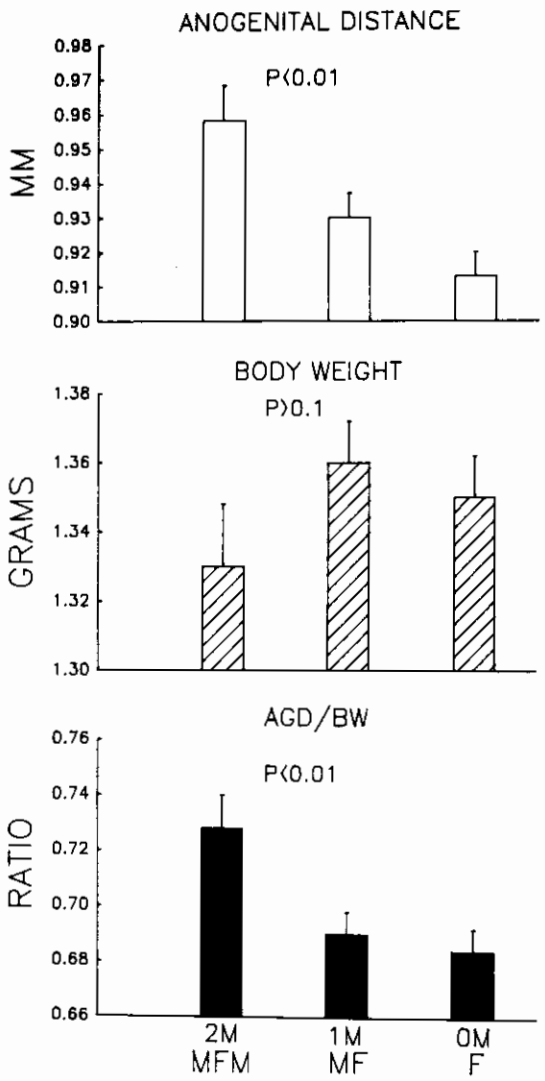

FIG. 2. Mean (+SEM) anogenital distance, body weight, and ratio of anogenital distance/body weight for females at cesarean delivery that developed between two male fetuses (2M), next to one male fetus (1M), or not next to any male fetuses (0M). This classification scheme only takes into account the number of males directly adjacent to the female being measured.

adjacent to a female fetus influences anogenital distance (20,23). Body weight did not vary significantly ($p > 0.1$) between the three groups of females. Analysis of variance on the ratio of anogenital distance/body weight showed significant differences ($p < 0.01$) between the groups. However, analysis of anogenital distance and body weight for all females showed no correlation (Pearson's $r = 0.008$, $p = 0.9$); similar nonsignificant r values were obtained when data for 0M, 1M, and 2M females were analyzed separately. Analysis of covariance also showed that body weight accounted for virtually none of the variance in anogenital distance in newborn female mice ($p = 0.9$), which is in marked contrast to the finding for newborn female rats (7). Thus, when adjusted for body weight by analysis of covariance, the mean anogenital distance for each of the three groups was virtually identical to the unadjusted means (based on ANOVA), and the groups still differed significantly ($p < 0.01$) from each other.

There were 257 females classified as either 0M females (n = 100; 39%), 1M females (n = 111; 43%), or 2M females (n = 46; 18%); this proportion of 0M, 1M, and 2M females is very close to what is predicted based on the assumption that a female being 0M, 1M, or 2M is entirely due to chance (18). The proportion of fetuses that were males in these 52 litters was 46%, although in a previous analysis of 1074 CF-1 mouse litters, the proportion of males was 52.5% (2). Overall litter size was 11.5 ± 0.13 pups, and the total number of fetuses in the same or the opposite uterine horn did not differ significantly for 0M, 1M, and 2M females. The number of males and females (and thus the sex ratio) in the same uterine horn differed significantly for 0M females (1.63 ± 0.12 males; 4.43 ± 0.15 females), 1M females (2.81 ± 0.12 males; 3.32 ± 0.13 females), and 2M females (3.76 ± 0.21 males; 2.30 ± 0.18 females; $p < 0.001$ for ANOVAs on number of females and number of males).

When the data from all females were analyzed without regard to adjacent males, there was a small, but significant, correlation (Pearson's $r = 0.20$, $n = 257$, $p < 0.01$) between the number of males present in the same uterine horn and anogenital distance of females at birth. Since 0M, 1M, and 2M females have significantly different numbers of males within the same uterine horn, ANCOVA was conducted to determine whether there was a significant effect of adjacent males on anogenital distance (i.e., 0M vs. 1M vs. 2M) when the number of males in the same uterine horn was used as the covariate. The number of males in the same horn accounted for a significant ($p < 0.01$) portion of the variance in anogenital distance at birth, but 0M and 2M females still differed significantly ($p < 0.05$) in anogenital distance when anogenital distance was adjusted for number of males in the same uterine horn, and the adjusted means were very similar to the data presented in Fig. 2.

The number of males and females in the opposite uterine horn did not differ ($p > 0.1$) for 0M, 1M, and 2M females. When the data for all females were analyzed without regard to intrauterine position, there was no correlation ($p = 0.7$) between the number of males in the opposite uterine horn and anogenital distance; ANCOVA on anogenital distance in 0M, 1M, and 2M females using number of males in the opposite uterine horn showed no significant effect of number of males in the opposite horn, and 0M, 1M, and 2M differences were still observed ($p < 0.01$). ANCOVA was also conducted with the number of females in the same or opposite uterine horn used as the covariate, but in neither case was a significant ($p > 0.1$) component of the variance in anogenital distance at birth observed due to the number of other females, and significant differences ($p < 0.01$) in anogenital distance between 0M, 1M, and 2M females described above were still observed.

2-B: The Effect of Being Positioned Between Two Males, a Male and a Female, or Two Females

In the above classification scheme, only the number of adjacent males was used as the basis for inclusion in a group. Thus, 1M females could be located at the end of a uterine horn next to a male or between a male and a female fetus. Also, 0M females might be a single fetus in a uterine horn (a rare event), at the end of a uterine horn next to a female, or between two female fetuses. Here we compared females located between two males, between a male and a female, and between two females in utero. Using this classification scheme, females differ in serum estradiol as well as testosterone (23), while using the scheme in 2-A, females differ in serum testosterone but not estradiol (21).

When the MFM (n = 46), MFF (n = 77), and FFF (n = 50) scheme was used to analyze anogenital distance and body weight at birth, the anogenital distance data for the three groups of females were virtually identical to the data presented in Fig. 2; using this classification scheme or the one in 2-A, the females in the 2M group would be the same. Specifically, the mean (±SEM) anogenital distances (in mm) were: MFM = 0.96 ± 0.01, MFF = 0.93 ± 0.01, FFF = 0.92 ± 0.01 ($p < 0.01$). Body weight did differ significantly ($p < 0.05$); post hoc analysis showed that this was due to the MFF females (1.36 ± 0.01 g) being significantly ($p < 0.01$) heavier than the FFF females (1.31 ± 0.02 g), while MFM females (1.33 ± 0.02 g) did not differ significantly from either of the other groups. Again, ANCOVA showed that virtually none of the variance in anogenital distance was due to body weight, and anogenital distance still differed significantly between the three groups of females.

2-C: The Effect of Adjacent Males Located on the Cervical or Ovarian Side of a 1M Female

To examine the possibility of greater transport of testosterone in the cervical than in the rostral direction from a male to a female fetus [based on findings in rats (7)], the anogenital distance and body weight of only 1M females (females situated next to one male) were examined as a function of whether the male was located on the cervical or ovarian side of the female. Females situated at the ends of the uterine horns were included in this analysis.

The results showed no difference in anogenital distance between 1M females based on whether the adjacent male was located on the cervical side (0.93 ± 0.01 mm; n = 56) or on the ovarian side (0.93 ± 0.01 mm; n = 55) of the female. There was also no difference between these two groups of 1M females in body weight. The ratio of anogenital distance/body weight, as well as the analysis of covariance for anogenital distance, showed no significant differences.

2-D: The Effect of a Male Located on the Cervical or Ovarian Side of a Female When Separated by an Intervening Fetus

To examine the possibility of interfetal transport of testosterone in the cervical direction from a male to a female fetus, even when the male was separated by another fetus from the female being measured [based on studies in rats (7)], we compared the anogenital distance of 1M females with two males positioned on her ovarian side (ovary—MMFFF; 0.93 ± 0.01 mm; n = 21) and 1M females with two males positioned on her cervical side (cervix—FFFMM; 0.95 ± 0.01 mm; n = 20). Anogenital distance for these two groups of 1M females did not differ significantly ($p > 0.1$). Using the adjacent male scheme, these females would be classified as 1M females and would be subsets of the groups used in the comparison of the 1M females located on the ovarian side of an adjacent male or on the cervical side of an adjacent male (in which we did not consider the sex of the fetus on the other side of the adjacent fetuses).

Anogenital distance was also compared in two subgroups of 0M females which were catagorized based on the presence of a male on the ovarian side (ovary—MFFFF; 0.93 ± 0.02 mm; n = 23) or on the cervical side (cervix—FFFFM; 0.92 ± 0.01 mm; n = 20; $p > 0.1$) of the adjacent females. The presence of a male which was separated from the female being measured by an intervening female (regardless of whether the male was on the ovarian or cervical side) thus did not appear to have any effect on anogenital distance in 0M females. Taken together, these two findings suggest that only adjacent males influence anogenital distance of females.

2-E: The Effect of Cervical-Side Males Without Regard to Proximity

We compared five groups of females based on the number of males located between a female and the cervix, without regard

to the total number of fetuses in the uterine horn or proximity of the males to the female being measured. Based on the scheme proposed by Meisel and Ward (11) in their study with rats, there were five separate groups: no males (0-CV female; $n = 19$), one male (1-CV female; $n = 22$), two males (2-CV female; $n = 16$), three males (3-CV female; $n = 5$), and four males (4-CV female; $n = 6$).

There was no effect of cervical males on anogenital distance based on ANOVA ($p = 0.7$) or on ANCOVA ($p = 0.7$), with body weight used as the covariate; body weight also did not account for a significant component of the variance in anogenital distance ($p = 0.9$). Specifically, the mean (±SEM) anogenital distance for 0-CV, 1-CV, 2-CV, 3-CV, and 4-CV females was: 0.92 ± 0.01, 0.94 ± 0.01, 0.93 ± 0.01, 0.93 ± 0.01, and 0.95 ± 0.02 mm, respectively. Body weight did not differ in females as a function of the number of cervical males ($p = 0.8$), and the ratio of anogenital distance/body weight was also not different ($p = 0.9$).

2-F: The Effect of Ovarian-Side Males Without Regard to Proximity

We also compared females based on the number of males located on the ovarian side, without regard to proximity of the males to the female being measured. Again, five groups were examined: no males (0-OV female; $n = 23$), one male (1-OV female; $n = 19$), two males (2-OV female; $n = 13$), three males (3-OV female; $n = 9$), and four males (4-OV female; $n = 4$).

The results showed no significant ($p = 0.2$) relationship between the number of ovarian males and anogenital distance at birth in female mice using ANOVA (Fig. 3). However, the 0-OV females did have the shortest mean anogenital distance while 2-OV and 4-OV females had the longest anogenital distances. Body weight did vary significantly as a function of the number of ovarian males ($p < 0.01$): 4-OV females were significantly lighter than females in any other group, and 0-OV females were also significantly ($p < 0.01$) heavier than 1-OV females. Due to the significant differences in body weight, when the ratio of anogenital distance/body weight was analyzed, the groups differed significantly ($p < 0.01$): both 0-OV females and 4-OV females differed significantly from all other groups ($p < 0.05$).

As was true for the other classification schemes, ANCOVA showed that body weight accounted for virtually none of the variance in anogenital distance ($p = 0.9$). The group means adjusted for body weight were virtually identical to the unadjusted means. Similar to the ANOVA, the ANCOVA showed that the groups did not differ significantly from each other ($p = 0.1$), although the post hoc comparison of the adjusted means using the LS means test showed that anogenital distance was longer ($p < 0.05$) in 2-OV than in 0-OV females, while 4-OV females tended ($p = 0.06$) to have a longer anogenital distance than 0-OV females.

One of the problems with the ovarian (and cervical) male analysis is that the likelihood of a female having no males vs. four males located between her and the ovary within a uterine horn is correlated with the position of the female within the uterine horn. In this, as in other studies with CF-1 mice (18), the mean number of fetuses per uterine horn was about six, and the mean (±SEM) position within the uterus for 0-OV females (with the first fetus being located next to the ovary) was 2.0 ± 0.1, while the mean position occupied by 4-OV females was 4.8 ± 0.2 fetuses from the ovary (position of 0-OV vs. 4-OV females: $p < 0.01$). Absolute position within a uterine horn influences body weight in mice (10).

A second issue concerns overlap between the various intrauterine position classification schemes. Figure 4 shows the likelihood of being a 0M, 1M, or 2M female (using the adjacent male scheme described in 2-A) when female mice delivered from the 52 litters used in this study were classified based on the number of ovarian males: there were no 2M females (adjacent scheme) in the 0-OV group, while only 1/14 4-OV females was a 0M female (adjacent scheme). Females classified as 1-OV, 2-OV, or 3-OV all had similar proportions of 0M, 1M, and 2M females (adjacent scheme). Thus, any differences between females in the 0-OV or 4-OV groups and the females in any of the other groups using the ovarian-male scheme could be due to the fact that the proportion of 0M, 1M, and 2M females (using the adjacent scheme) differed significantly, $\chi^2(8) = 39$, $p < 0.001$.

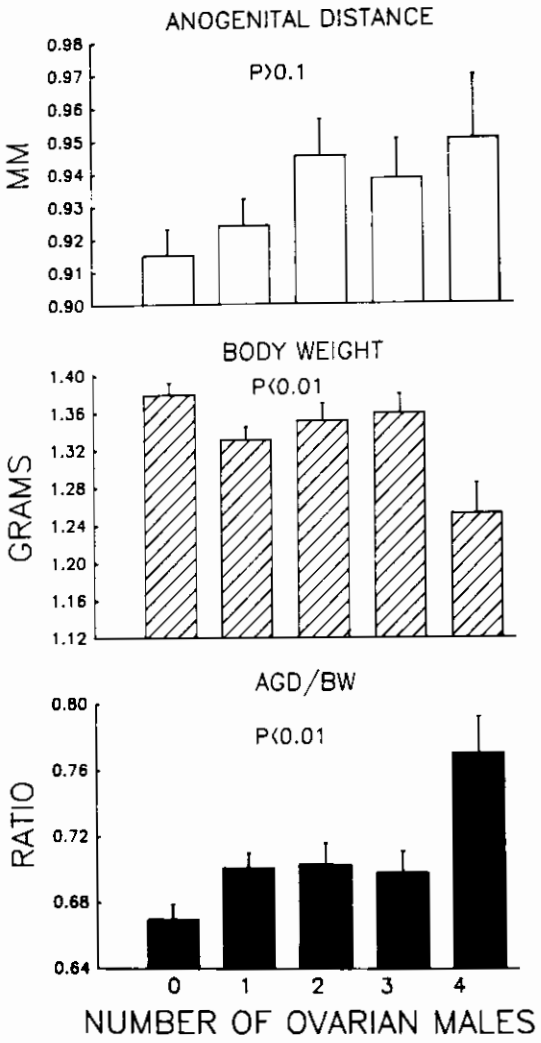

FIG. 3. Mean (+SEM) anogenital distance, body weight, and ratio of anogenital distance/body weight for females at cesarean delivery as a function of the number of male fetuses located at any point within a uterine horn between the female being measured and the ovary.

EXPERIMENT 3: VARIANCE IN ANOGENITAL DISTANCE AND BODY WEIGHT OF FEMALES AT BIRTH DUE TO INTRAUTERINE POSITION: COMPARISON WITH VARIANCE DUE TO LITTER (MATERNAL) EFFECTS

In developmental studies involving litter-bearing animals, animals within litters are typically assigned to different treatment conditions to control for similarities between animals within a

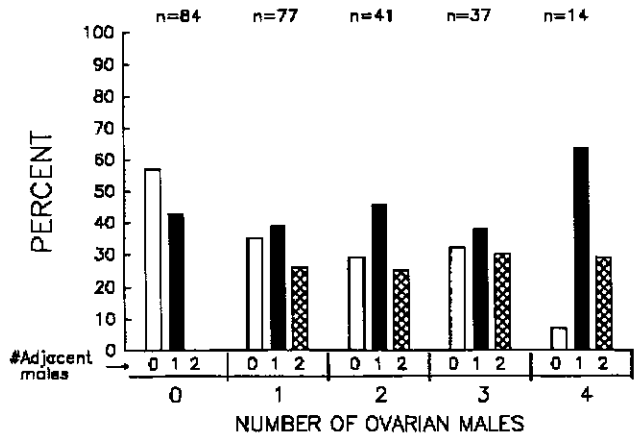

FIG. 4. The frequency of 2M, 1M, and 0M females (using the directly adjacent male scheme) in each of the groups compared in Fig. 3 using the ovarian male scheme. In the ovarian male scheme, females are classified based on the number of male fetuses located at any point within a uterine horn between the female being measured and the ovary. The proportion of 2M, 1M, and 0M females in the different ovarian male groups differed significantly ($p < 0.001$).

litter. Similarities among siblings may be due to genetic effects as well as nongenetic effects, which can also be transmitted to offspring from the mother (for instance, fetal growth differs due to differences in maternal nutrition or drug use). This procedure is based on the assumption that between-litter variation is typically greater than within-litter variation. When all animals in all litters are treated in the same manner and measured independently, litter can be analyzed as a main effect to see if the variance between and within litters is significantly different.

We examined whether females from the different intrauterine positions (relative to siblings of the same or opposite sex) within a litter differed from each other to a greater or lesser degree than did females from the same intrauterine position but produced in separate litters. To address this question, the variance in both anogenital distance and body weight of females at birth associated with litter and intrauterine position (0M, 1M, and 2M adjacent male scheme) were compared using the Nested Procedure in SAS.

Results

The percent of the overall variance in anogenital distance at birth associated with litter (16.9%) was virtually identical to the percent of the variance accounted for by the adjacent male (0M, 1M, 2M) intrauterine position classification scheme (17.8%). In contrast, for body weight of females at birth, the adjacent male intrauterine position scheme accounted for virtually none of the variance (2.3%), while litter accounted for most (65.3%) of the variance.

Using implants of ^{3}H-testosterone into the amniotic sac of individual rat fetuses and monitoring its passage into other fetuses within the uterine horn, we found evidence that in uterine horns with only a few fetuses, very little passage of ^{3}H-testosterone occurred between fetuses (7). This finding suggests that the amount of steroid which passes between fetuses and thus differences due to intrauterine position might be greatest in a crowded uterine horn. We also conducted the same analysis described above after excluding data from females which developed in uterine horns with five or less fetuses. The results showed that intrauterine position still only accounted for a small (6.4%) portion of the variance while litter accounted for most (68.2%) of the variance in body weight at birth. In contrast, intrauterine position accounted for significantly ($p < 0.01$) more (28.0%) of the variance in anogenital distance at birth than did litter (10.1%).

EXPERIMENT 4: EFFECT OF PROXIMITY TO THE OVARY OR CERVIX ON BODY WEIGHT: COMPARISON WITH VARIANCE DUE TO LITTER (MATERNAL) EFFECTS

McLaren and Michie (10) reported that mouse fetuses located in the middle of a crowded uterine horn were significantly smaller than fetuses located next to the ovary or cervix. Their findings added support for a hemodynamic model predicting that the blood pressure in a placenta should be inversely related to the number of placentae fed by the uterine loop artery; this is referred to as a local (within horn) effect. They correctly assumed (based on our findings in Experiment 1) that blood flow in the uterine loop artery was from both ends toward the middle and predicted that blood pressure in the placenta of the middle fetus in a uterine horn would be low relative to placentae of fetuses at the ends of the horn. It has also been suggested (10) that the number of fetuses in the opposite horn can influence fetal body weight; this effect would presumably be mediated via the systemic circulation of the mother. In this experiment anogenital distance was not examined, since the results of Experiment 2-F showed that being at the ends or middle of a uterine horn is correlated with the likelihood of being classified as a 0M, 1M, or 2M female (using the adjacent male scheme), which influences anogenital distance but not body weight.

Data Analysis

We classified newborn mice based on the scheme shown in Fig. 1, with female mice located at the ovarian end of the uterine horn classified as group 1 and female mice located next to the cervix as group 5, as described for studies in rats by Even et al. (6). The data for body weight of females were analyzed by ANOVA using group and litter as the main effects. The variance associated with litter then was compared to the variance associated with group (placement within the uterus) using the Nested Procedure in SAS.

The data for body weight of females in the five different groups were also analyzed using ANCOVA. The objective was to examine the hypothesis that fetal weight is dependent on local effects due to the number of fetuses within the uterine horn by using the number of animals (male and female) within the same uterine horn as the covariate. The possibility of effects of fetuses present in the opposite uterine horn, which would be mediated via the systemic circulation of the mother, on body weight was also examined by using the number of animals (males and females) in the opposite uterine horn as the covariate. Only the body weight of females was measured in this study, so data for body weight of males are not included.

Results

The body weight of females in groups 1–5 tended to differ ($p = 0.07$; Table 1). Based on findings by McLaren and Michie (10), a planned comparison of females in group 3 (in the middle of the uterine horn) with females in group 1 and group 5 showed a significant difference between group 1 and group 3 ($p < 0.05$) but not between group 5 and group 3 ($p > 0.1$). Females from different litters ($n = 52$) also differed significantly ($p < 0.001$) from each other in body weight at birth. The Nested ANOVA revealed that litter accounted for a larger percent of the total variance in body weight of females at birth (63.8%) than did the group that the female was in relative to the ovary or cervix (9.3%).

The number of fetuses (male and female) in the same uterine horn accounted for a significant ($p < 0.001$) component of the

TABLE 1
FEMALE MICE AT CESAREAN DELIVERY

	Body Weight
Group 1	1.376 ± 0.019
Group 2	1.358 ± 0.019
Group 3	1.329 ± 0.014
Group 4	1.336 ± 0.018
Group 5	1.346 ± 0.019

Values are mean ± SEM. Group 1 consisted of females next to the ovary, while group 5 consisted of females located next to the cervix, as shown in Fig. 1. Only group 1 and group 3 differed significantly ($p < 0.05$).

variance in body weight at birth of females, although a significant ($p < 0.05$) interaction between group and number of fetuses in the same horn showed that the covariance relationship between these variables differed for females in different positions within the uterus. The number of fetuses (male and female) in the opposite horn also accounted for a significant ($p < 0.01$) component of the variance in body weight of females, and the interaction term was not significant. These findings thus provide support for the hypothesis of McLaren and Michie (10) that fetuses in the middle of the uterus are smaller than fetuses at the ovarian end of the uterus. There also is a relationship between the number of fetuses in both the same and the opposite uterine horn, body weight of females at birth, and, subsequently, characteristics in adulthood (8,10).

These same analyses were conducted after eliminating data for animals which developed in uterine horns with fewer than six fetuses, since the effect of position within a uterine horn relative to the ovary or cervix on body weight is greatest when the uterine horn is crowded (10). However, virtually the same results described above (when all data were analyzed) were found.

GENERAL DISCUSSION

The results of Experiment 1 demonstrate that blood flow in the uterine loop artery and vein is bidirectional. Blood flows into the uterine loop artery from both the rostral and caudal ends. Blood flows out of placentae located near the ovary into the uterine vein only in a rostral direction, while blood flows out of placentae located next to the cervix in a caudal direction. These findings support the model of uterine blood flow proposed by McLaren and Michie (10) and refute the model proposed by Meisel and Ward (11) that blood flow in the uterine loop artery is only toward the ovary from the cervix.

We have also conducted experiments to examine indirectly the direction of blood flow in the uterus during pregnancy in rats (6). Radiolabelled microspheres were injected into the heart of pregnant rats. Microspheres incorporate into tissues as a function of the rate of blood flow to the tissue. We found that the ovarian and cervical ends of the uterine horns had higher rates of blood flow than did the middle region of the uterine horns, which is consistent with our direct observation of a bidirectional flow of blood in the uterine loop artery in pregnant mice.

In rats, we have examined the mechanism by which testosterone is transported between fetuses by implanting a silastic capsule containing ^{3}H-testosterone into the amniotic sac of fetuses located in different positions within the uterine horn; amniotic fluid of other fetuses was examined for the presence of ^{3}H-testosterone 12 h later. The results showed that there was a 3-fold greater movement of ^{3}H-testosterone into fetuses located toward the cervix than the ovary relative to the implanted fetus, regardless of where in the uterus the implanted fetus was located (7). This finding ruled out passage via the uterine vasculature as the mechanism by which steroids are transported between fetuses in rats, since at the cervical end of the uterine horn the greater transport toward the cervix was opposite to the direction of blood flow in the uterine artery (6). Instead, passage of steroids via diffusion across the fetal membranes appears to mediate the intrauterine position phenomenon. Fluid movement within the uterine lumen possibly produces a greater rate of transport toward the cervix than the ovary (7).

While we expect that experiments with mice should lead to similar findings concerning the transport of ^{3}H-testosterone between fetuses, the much smaller size of mouse fetuses and maternal blood vessels make the studies conducted with rats on blood flow and steroid transport (6,7) much more difficult to accomplish. In the present study we used anogenital distance at birth as a bioassay for indirectly testing models concerning the mechanism by which steroids pass between fetuses in mice. The perineal tissue separating the anus and genital papilla elongates into the scrotum in males under the influence of circulating testosterone, which is reduced to 5α-dihydrotestosterone within this tissue. Anogenital distance at birth in females directly reflects the levels of testosterone to which they were exposed during the last 4 days of pregnancy in mice.

We classified females based on the number of adjacent males [as proposed by Clemens et al. (4)] and the number of males located between the female and the cervix [as proposed by Meisel and Ward (11)]. In addition, since we found a greater transport of ^{3}H-testosterone toward the cervix than the ovary within the uterus in rats (7), we had predicted that the presence of males on the ovarian side of a female (either directly adjacent to the female in Experiment 2-C or not directly adjacent to the female in Experiments 2-D and 2-F) could have a greater effect on anogenital distance than the presence of males on the cervical side of a female in mice. However, this prediction was not confirmed. The results were clear in that only when females were classified based on the number of adjacent males was a significant difference in anogenital distance at birth observed.

When females are classified as having different numbers of either ovarian or cervical males (without regard to direct proximity of the males to the female), the proportion of females within these groups which are 0M, 1M, or 2M (based on the adjacent male scheme) differs significantly (Fig. 4). Thus, differences in morphology or behavior attributed to the number of ovarian or cervical males without regard to proximity of the males to the female could, instead, be due to differences based on the number of males directly adjacent to the female.

The various analyses which we conducted show that the appropriate method of analyzing morphological data, such as anogenital distance at birth, is to use analysis of covariance, with body weight used as the covariate. Correcting anogenital distance for body weight by using a ratio creates the possibility of attributing significant differences in the ratio to differences in anogenital distance when only body weight is actually significantly different. An interesting finding from Experiment 2-F was that significant differences were observed based on the number of ovarian males if the ratio of anogenital distance/body weight was used as the dependent measure. However, the significant difference between the groups in body weight created the significant difference in the ratio of anogenital distance/body weight. This finding clearly demonstrates that only reporting the ratio of these two variables can lead to erroneous conclusions con-

cerning the potential masculinizing effect of males within the uterus on anogenital distance of females.

In the Meisel and Ward study, females with different numbers of cervical males actually differed significantly in body weight but not anogenital distance. In our studies with rats (unpublished observation) and in Experiment 2-E here in mice, the scheme of Meisel and Ward did not account for a significant component of the variance in anogenital distance of females at birth. The basis for the difference in anogenital distance/body weight due to the number of cervical males in the Meisel and Ward (11) study, as well as our similar finding in Experiment 2-F, is thus not due to effects of the number of ovarian or cervical male fetuses on anogenital distance. Instead, McLaren and Michie (10) reported, and we confirmed in Experiment 4, that fetuses at either end of the uterine horns are heavier than fetuses located in the middle of the horn. We showed in Experiment 2-F that a consequence of classifying females as having different numbers of ovarian males is that the more males which are located between the ovary and a female, the farther away from the ovary the female is located, thus possibly accounting for differences in body weight between the groups. While our findings here are in agreement with those of McLaren and Michie (10) with regard to position of fetuses within the uterus and body weight at birth, findings in rats are not in agreement [reviewed in (6)].

Using analysis of covariance (and Pearson's correlation analysis), we found that body weight accounted for virtually none of the variance in anogenital distance of females at birth in mice. In contrast, in similar studies with rats, body weight did account for a significant component of the variance in anogenital distance of females at birth (unpublished observation). An additional finding from the present study (Experiment 4) is that most (64%) of the variance in body weight of females at birth is due to developing in different litters (referred to as maternal or litter effects). A much small portion of the variance (9%) in body weight at birth is accounted for by position of a female fetus relative to the ovary or cervix. The findings of McLaren and Michie (10) suggest that in very crowded uterine horns, variance due to position within the uterine horn is much greater than what we found here in relatively uncrowded uterine horns.

Taken together, our findings reveal that in mice, only females situated between two adjacent male fetuses (2M females) have significantly masculinized anogenital spaces at birth. In addition, Experiment 3 showed that the adjacent male scheme accounted for a significantly greater amount of the variance in anogenital distance at birth than the variance due to maternal (litter) effects. These findings thus lead to the recommendation that in studies of the effect of intrauterine position on mice or other rodents, the adjacent male scheme should be used to classify animals. These experiments provide indirect support for the hypothesis, which has been directly confirmed in rats (7), that the intrauterine position phenomenon occurs due to diffusion of steroids through the amniotic fluid across the fetal membranes separating adjacent fetuses.

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