

Amniotic Fluid Ingestion Before Vaginal/Cervical Stimulation Produces a Dose-Dependent Enhancement of Analgesia and Blocks Pseudopregnancy

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THOMPSON, A. C., P. ABBOTT, J. C. DOERR, E. J. FERGUSON AND M. B. KRISTAL. *Amniotic fluid ingestion before vaginal/cervical stimulation produces a dose-dependent enhancement of analgesia and blocks pseudopregnancy.* *PHYSIOL BEHAV* 50(1) 11–15, 1991.—A substance in amniotic fluid (AF) and placenta has been shown to enhance analgesia produced by morphine, late pregnancy, footshock, and vaginal/cervical stimulation (VS). When morphine-induced analgesia was assessed previously, the degree of enhancement by ingestion of AF or placenta was found to be a function of the amount of analgesia being generated. We have extended these results to include the analgesia produced by VS. Analgesia induced by 75, 125, 175, or 225 g of vaginal/cervical pressure was measured in rats pretreated with 0.25 ml (by orogastric infusion) of either AF or saline. AF infusion enhanced the analgesia produced by 125 g VS, but did not affect the analgesia produced by 75, 175, or 225 g VS. Unexpectedly, we also found that infusion of AF shortly before the application of VS prevents VS-induced pseudopregnancy (PsP). Whereas the incidence of PsP following 75, 125, or 175 g VS was less than 19% and not statistically different for AF and saline pretreatments, the incidence of PsP after 225 g VS was 44% in saline-pretreated rats, but only 10% in AF-pretreated rats. Protection from the induction of pseudopregnancy, which could be caused by mechanical stimulation of the cervical area during delivery, may be an additional benefit of parturitional ingestion of placenta and amniotic fluid (placentophagia).

Analgesia POEF Amniotic fluid VSIA Opioids Pseudopregnancy Parturition TFL
Vaginal/cervical stimulation Placentophagia Rats

INGESTION of amniotic fluid (AF) and placenta is a characteristic of parturition in most placental mammals (10). We have presented evidence over the past few years to suggest that a major benefit of ingesting AF and placenta is to increase the analgesic action of endogenous opioids (i.e., to enhance pregnancy-induced analgesia) during delivery (14). We have named the substance in AF and placenta that enhances opioid-mediated analgesia POEF, for Placental Opioid-Enhancing Factor.

We have previously shown that POEF enhances vaginal/cervical-stimulation-induced analgesia, VSIA (5,16); vaginal/cervical stimulation, leading to stimulation of hypogastric afferents, is a primary feature of mammalian parturition (8, 9, 18). In these earlier experiments, ingestion of 0.25 ml AF or 3 placentas was found to enhance the analgesia produced by 75 g of vaginal/cervical stimulation (VS). More recently, however, we found that the effect of ingesting AF or placenta on morphine-induced analgesia or pregnancy-induced analgesia depends on the amount of AF or placenta ingested (11) as well as on the level of analgesia upon which the AF or placenta can act

(12,14). Since the amount of VSIA is a function of the amount of VS pressure applied (3), we hypothesized that, as with morphine-induced analgesia, POEF enhancement of VSIA would also depend on the base-level of analgesia being produced, and would therefore vary with the amount of pressure applied to the vaginal cervix.

EXPERIMENT 1

The effect of AF (0.25 ml) on several levels of VSIA was assessed using a radiant-heat tail-flick latency (TFL) test. Different levels of analgesia were produced by applying different amounts of mechanical pressure to the vaginal cervix. Vaginal/cervical pressures ranged from 75 to 225 g and corresponded to those used by Crowley et al. (3) that produced the smallest detectable VSIA in ovariectomized rats. Furthermore, the radiant-heat TFL assay has a rather low response ceiling; unless low levels of analgesia are used, enhancement becomes difficult to detect [e.g., (11,15)].

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Because VS produces more analgesia during estrus than at other stages of the estrous cycle (3), we only tested rats that were in diestrus. Any rat that entered pseudopregnancy (PsP) during the 3-week period after testing was replaced and the data dropped from the analysis. The relatively low pressure and short duration of VS applied in this experiment contrasted greatly with more standard procedures for mechanical induction of PsP (4,7), and the incidence of PsP, based on pilot studies, was expected to be low. The pilot data on analgesia and PsP were gathered on three occasions using 150 g VS pressure after saline orogastric infusions, in the same design used for Experiment 1 (described below). Pilot data showed that a) the incidence of PsP was found to be relatively low (3/16), and b) the level of analgesia shown by rats that subsequently became pseudopregnant was greater than the maximum level detectable in our assay (8-s ceiling), whereas the levels shown by those that did not become pseudopregnant were below the ceiling (mean TFL during VS for PsP rats = 8.00 ± 0.00 s, representing at least a 115% increase from baseline TFL; for non-PsP rats = 5.45 ± 0.51 s, representing a 46.64% increase from baseline TFL). Since the actual level of analgesia shown by rats that would become pseudopregnant would likely be unknown (beyond the 8-s ceiling), since the level of analgesia in response to VS shown by rats that would become pseudopregnant was different from that of the rats that would not become pseudopregnant, and since the incidence of PsP was likely to be very low, we felt justified in excluding PsP rats from the analysis of the effect of AF ingestion on VSIA in Experiment 1.

METHOD

Subjects

Eighty-four female Long-Evans (hooded) rats, 2–3.5 months old, were used. Rats were individually housed in hanging wire-mesh cages ($24.5 \times 18 \times 18$ cm), in a controlled environment with a 14 h on/10 h off light cycle (lights on 0500 EST). Food (Agway Prolab Rat/Mouse/Hamster Formula 3000) and water were available ad lib, except on the test day, when food and water were removed for 3 h before and during the 10-min testing period. This brief period of food and water deprivation before testing was used to assure that the stomach was empty before orogastric intubation.

Estrous cycle was monitored by vaginal smears for at least 3 weeks before testing and up to 3 weeks after testing. Rats were tested only in diestrus and only after they had shown a normal estrous cycle and weighed a minimum of 230 g.

Apparatus

Fluids were infused into the stomach through an orogastric tube consisting of a length of polyethylene tubing (PE 160) attached to a blunted 18-ga hypodermic needle, fitted to a 1/4-ml glass tuberculin syringe.

VSIA was induced by probing the vaginal cervix with a glass rod that protruded from the barrel of a 1-ml glass tuberculin syringe (3). The rod was spring-loaded and calibrated to deliver either 75, 125, 175, or 225 g of pressure. Pressure was applied for a period of 10–18 s: for 10 s before the TFL measure was taken, and up to 8 s during the TFL measurement procedure.

Pain threshold was assessed by radiant-heat TFL assay. The tail-flick algesiometer was the one described previously (15). TFL is defined as the interval between the onset of the radiant heat and the movement of the rat's tail out of the radiant-heat field. Each rat's baseline pain threshold was determined immediately before VS was applied by calculating the mean TFL

TABLE 1
NUMBER OF RATS TESTED FOR VAGINAL/CERVICAL STIMULATION-INDUCED ANALGESIA AT DIFFERENT LEVELS OF VAGINAL PRESSURE IN ORDER TO OBTAIN $n=9$ /GROUP

Infused Fluid (pretreatment)	Vaginal/Cervical Stimulation (in grams)			
	75	125	175	225
Saline (Sal)	9 (0%)	10 (10%)	10 (10%)	16 (44%)*
Amniotic fluid (AF)	9 (0%)	9 (0%)	11 (18%)	10 (10%)†

The percentage of each group showing pseudopregnancy (PsP) after testing is given in parentheses.

*Significantly more rats tested, therefore more PsP, than in other Saline groups ($p<0.05$).

†Significantly fewer rats tested, therefore less PsP, than in Sal + 225 g Group ($p<0.05$).

measure from the last 3 of 4 TFL trials. TFL trials were separated by 30 s. The pain threshold during VS was determined from a single TFL trial conducted 30 s after the last baseline TFL trial. The entire TFL test (Baseline + VS) lasted about 4 min, during which time the rat was restrained in an opaque, polyvinyl chloride tube (4.2×21 cm). If on any trial the rat failed to respond within 8 s, the TFL trial was terminated to prevent tissue damage.

AF was collected surgically from donor rats on Day 21 of pregnancy. The procedures for collection, storage, and presentation of AF have been described previously (13). Saline control fluid (0.9%) was stored and presented just as AF. All fluids were administered at body temperature (37°C).

Procedure

Pretreatment. All rats were habituated to the orogastric intubation procedure (intubation without fluid infusion) and to the restraint used during TFL testing by exposure to each procedure once each day for 5 consecutive days.

Design and statistical analysis. A $2 \times 4 \times 2$ factorial design [Fluid (0.25 ml AF, Sal) \times VS Dose (75, 125, 175, 225 g) \times Test (Baseline, VS)], with repeated measures on the Test variable, was used. The infusion of fluid preceded TFL testing by 10 min. Rats were randomly assigned to a Fluid \times VS Dose group on the test day. An n of 9/group was used. Any rat that became PsP after testing was replaced. PsP was defined as 8 consecutive days of diestrus during the 3-week posttesting observation period.

Two-way ANOVA was used to compare the VSIA of AF- and Sal-pretreated rats at each "dose" of VS. Significant 2-way interactions were analyzed with simple-effects probes. One-way ANOVA and t -tests were used to compare the incidence of PsP between VS Dose groups and between Fluid groups, respectively.

RESULTS AND DISCUSSION

The total number of rats tested to obtain an n of 9 in each Fluid \times VS Dose group is given in Table 1. A statistically significant increase in the incidence of PsP was found as VS dose increased in Sal-pretreated rats, $F(3,41) = 3.48$, $p = 0.024$, but not in AF-pretreated rats, $F(3,35) = 1.07$, $p > 0.05$.

Among Sal-pretreated rats, the largest number of replacements, and therefore the highest incidence of PsP, was found in the group receiving 225 g VS (Newman-Keuls, $p < 0.05$: 225 g > 75 g). The same effect was not observed among AF-pretreated rats; the number of replacements in all groups was low. A direct

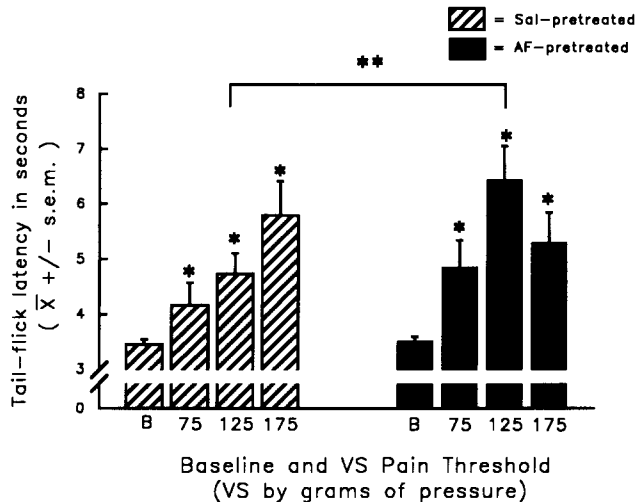


FIG. 1. Mean TFLs (\pm S.E.M.) in saline-pretreated (Sal) and amniotic-fluid-pretreated (AF) rats at Baseline (B) and during vaginal/cervical stimulation (VS), using radiant-heat TFL assay. Baselines for different levels of VS were not significantly different and were pooled within pretreatments for graphic purposes. *Significantly increased from Baseline ($p<0.03$); **significantly different ($p<0.001$).

comparison of the incidence of PsP among AF-pretreated and Sal-pretreated rats that received 225 g VS (10% vs. 44%, respectively) showed that the incidence of PsP was significantly lower in AF-pretreated rats, $t(24) = -2.08$, $p = 0.048$. Because of the significantly higher incidence of PsP in the Sal + 225 g group, we dropped the 225-g VS Dose groups from further analysis. As significantly more rats were tested in the Sal + 225 g group than in the other groups in order to obtain an n of 9, it was likely that the final sample of 9 rats in this group differed (e.g., in the level of sensitivity to 225 g VS) from those in the other groups of 9; therefore, any statistical comparison was compromised.

Figure 1 depicts the pain threshold data obtained from rats tested at 75, 125, and 175 g VS.

The statistical analyses showed that AF significantly enhanced VSIA produced by 125 g VS, but did not affect VSIA produced by 75 or 175 g VS. The 2-way ANOVA comparing pain thresholds of the AF- and Sal-pretreated groups at Baseline and during 125 g VS revealed a statistically significant Fluid \times Time interaction, $F(1,16) = 5.04$, $p < 0.04$. Subsequent probes of this interaction showed that a) TFLs at Baseline did not differ significantly between AF- and Sal-pretreated groups, $F(1,32) < 1.00$; b) TFL increased significantly during VS in both AF- and Sal-pretreated groups [AF-pretreated: $F(1,16) = 31.75$, $p < 0.001$; Sal-pretreated: $F(1,16) = 6.06$, $p < 0.03$]; and c) TFLs during VS differed significantly, in that AF pretreatment produced higher pain thresholds (more analgesia) than did Sal pretreatment, $F(1,32) = 11.31$, $p < 0.001$. The analyses of the 75-g and 175-g VS Dose groups revealed only a significant main effect of Time [75 g VS: $F(1,16) = 9.07$, $p < 0.01$; 175 g VS: $F(1,16) = 27.39$, $p < 0.001$], indicating that both levels of VS produced detectable VSIA, and neither was affected by AF pretreatment [Fluid \times Time for 75 g: $F(1,16) < 1.00$; for 175 g: $F(1,16) < 1.00$].

These results support the hypothesis that the effect of 0.25 ml AF on VSIA depends on the base level of analgesia produced by the VS; in this case, 0.25 ml AF enhanced VSIA produced by 125 g of pressure, but not that produced by 75 or 175 g. In this respect, the effect of AF on VSIA is not different from the

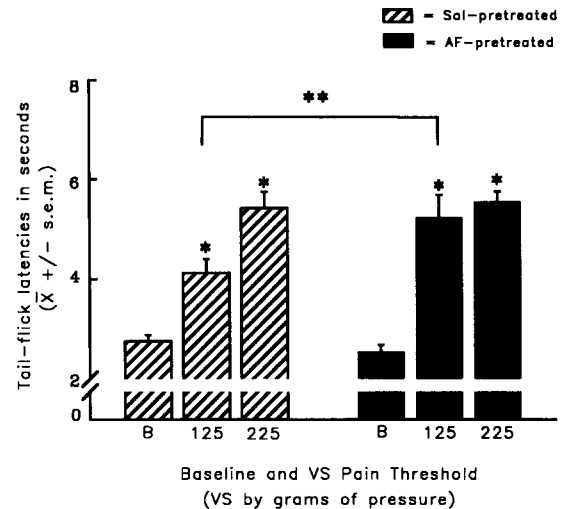


FIG. 2. Mean TFLs (\pm S.E.M.) in saline-pretreated (Sal) and amniotic-fluid-pretreated (AF) rats at Baseline (B) and during vaginal/cervical stimulation (VS), using hot-water tail-dip TFL assay. Baselines for different levels of VS were not significantly different and were pooled within pretreatments for graphic purposes. *Significantly increased from Baseline ($p < 0.001$); **significantly different ($p < 0.05$).

effect of AF on morphine-induced analgesia (12).

The low, irregular incidence of PsP among the groups receiving 125 or 175 g VS may have been due to differences in hormonal profiles among the subjects. Higher levels of estrogen, for example, would produce both increased VSIA and an increase in the likelihood of PsP after VS (2,3). Although all the rats were tested in diestrus, as determined by vaginal smear, it is possible that this technique alone is not sensitive enough to produce groups of rats that were truly hormonally homogeneous and therefore uniformly sensitive to the stimulus (1, 17, 20).

We were unable to determine the effect of 0.25 ml AF on VSIA produced by 225 g of pressure because the comparison with the Sal + 225 g control group was compromised by the concurrent increase in VS-induced PsP. The radiant-heat TFL assay could not detect a wide enough range of responses to assess VSIA in rats that subsequently became pseudopregnant, since the typical TFL during VS in those rats was greater than the 8-s latency ceiling. Although it seemed unlikely that AF ingestion would enhance 225 g VS, in view of the finding that it did not enhance 175 g VS, it was possible that AF ingestion might actually reduce the analgesia produced by 225 g VS. An inhibitory effect of AF on higher levels of analgesia, possibly due to a mixed agonist/antagonist effect, has been suggested by us in previous reports (14,15). To test the effect of AF ingestion on analgesia produced by 225 g VS, a hot-water tail-dip assay, which can detect a wider range of responses, was employed to assess VSIA in Experiment 2.

The second experiment also provided a test of the reliability of the significant difference between AF- and Sal-pretreated groups in the incidence of PsP following 225 g VS.

EXPERIMENT 2

In Experiment 1, we were unable to assess the analgesia induced by 225 g VS using the radiant-heat tail-flick assay; therefore, we repeated Experiment 1 (using only the 125-g and 225-g doses of VS) with a different pain threshold assay, one that could detect a wider range of responses. The hot-water tail-dip

TABLE 2
TAIL-FLICK LATENCIES (MEAN \pm S.E.M. IN s) AND CONSEQUENT
PSEUDOPREGNANCY OF SALINE- AND AF-PRETREATED RATS RECEIVING 225 g VS

Infusion	PsP	n	Baseline TFL	VS TFL	% Change
Saline (Sal)	Yes	4	2.49 \pm 0.18	5.52 \pm 0.79	120.92 \pm 21.91
	No	5	2.76 \pm 0.16	5.32 \pm 0.11	96.23 \pm 15.36
Amniotic fluid (AF)	Yes	1	2.09 \pm 0.00	4.80 \pm 0.00	129.67 \pm 0.00
	No	8	2.67 \pm 0.16	5.63 \pm 0.21	116.38 \pm 14.10

assay was the best choice, since it required only one change in procedure: Instead of placing the restrained rat's tail over a radiant heat source as in Experiment 1, we placed the distal 2–3 cm of the tail into a hot water bath ($55.5^\circ \pm 0.5^\circ\text{C}$). The hot-water tail-dip assay could detect up to a 28-s difference in pain threshold because, although Baseline pain threshold remains low (2 to 3 s), prolonged exposure (up to 30 s per measure) in rats experiencing analgesia does not produce tissue damage at that water temperature (6).

METHOD

Subjects

Forty-two female Long-Evans rats, 2 to 3.5 months old, were used. Rats were maintained as in Experiment 1.

Apparatus

Orogastric infusions and VS were performed as in Experiment 1.

Pain threshold was determined by hot-water tail-dip assay, using a temperature-regulated hot-water bath (Precision, Model 181, GCA Corp.) maintained at 55.5°C ($\pm 0.5^\circ$). The procedures used to determine Baseline and VS pain thresholds were the same as those used in Experiment 1, except that Baseline pain threshold was calculated as the mean of the last 2 of 3 trials [as in (14)] rather than the last 3 of 4, as used in the radiant-heat TFL assay. Although the cut-off criterion for the hot-water tail-dip assay was set at 30 s, no measure exceeded 10 s in this experiment.

Procedure

Pretreatment. All rats were habituated to orogastric intubations and to the restraint used during the pain threshold test as described in Experiment 1.

Design and statistical analysis. A $2 \times 2 \times 2$ factorial design [Fluid (0.25 ml AF, Sal) \times VS Dose (125 g, 225 g) \times Test (Baseline, VS)], with repeated measures on the Test variable, was used. The testing procedure was identical to that used in Experiment 1. A sufficient number of rats was tested to obtain a group size of 9 in each of the 4 groups. Seven rats were dropped, 6 of which were replaced, because their Baseline pain thresholds fell above the expected range for the hot-water tail-dip assay (2 to 3.2 s). Statistical analyses of pain threshold data and PsP frequencies were the same as in Experiment 1.

RESULTS AND DISCUSSION

Pseudopregnancy

The incidence of PsP in each Fluid \times VS Dose group was similar to that found in Experiment 1 (AF + 125 g VS=0/9;

Sal + 125 g=0/8; AF + 225 g=1/9; Sal + 225 g=4/9). The number of rats that entered PsP among Sal-pretreated rats increased significantly when the VS increased to 225 g, $t(8) = -2.53$, $p=0.035$, whereas the number of rats that entered PsP among AF-pretreated rats remained low across VS Dose groups, $t(8) = -1.00$, $p=0.35$.

Analgesia

Figure 2 depicts the pain threshold data obtained from rats tested at 125 and 225 g VS.

As in Experiment 1, AF pretreatment enhanced the analgesia produced by 125 g VS [Fluid \times Time interaction: $F(1,15) = 9.69$, $p=0.007$; AF vs. Sal during VS: $F(1,28) = 7.57$, $p<0.05$; AF vs. Sal at Baseline: $F(1,28) < 1.00$]. The 2-way ANOVA comparing pain threshold in AF- and Sal-pretreated rats in the 225-g VS Dose group revealed only a significant effect of Time [Time: $F(1,16) = 222.1$, $p<0.0001$; Fluid \times Time: $F(1,16) < 1.00$], indicating that AF ingestion does not affect analgesia produced by 225 g VS.

A comparison of VSIA levels also revealed that, at 225 g VS, pseudopregnancy induction was not associated with significantly different levels of VSIA. A median test that was performed on the percent change from baseline VSIA (see Table 2) for the saline-pretreated rats (AF pretreatment at 225 g VS produced only 1 PsP) revealed no differences between the groups ($p=0.32$). The median test and percent change from baseline VSIA were both used in order to provide maximum conservatism (most likely to reveal significant differences); yet no differences were found. This finding suggests a dissociation between the two effects of 225 g vaginal/cervical stimulation: pseudopregnancy induction and analgesia.

GENERAL DISCUSSION

The enhancing effect of AF on VSIA was found to depend on the amount of analgesia produced by the VS, such that infusion of 0.25 ml AF enhanced the VSIA produced by 125 g of pressure but not that produced by 75, 175, or 225 g of pressure. This finding is consistent with previous results using morphine (12) and pregnancy (14) as the source of analgesia, and suggests that the enhancing effect of a given volume of AF is not linear and depends on the dose of the analgesic. Alternatively, since the proportion of opioid-mediated analgesia in VSIA changes with the amount of VS pressure, the inability of POEF to enhance the high dose of VS (225 g) may also reflect a greater nonopioid component to VSIA (i.e., a shift in the mechanism or neuroanatomical substrate for the analgesic response) (9).

The specific combination of AF and VSIA that produced enhancement in Experiment 1, 0.25 ml AF and 125 g VS pressure, differed from that in an earlier experiment (5) in which we used 0.25 ml AF to enhance VSIA produced by 75 g VS pres-

sure. The discrepancy between the two studies in the level of VS enhanced by 0.25 ml AF probably stems from differences in the procedure used to assess enhancement. In the Doerr and Kristal (5) paper, VSIA was assessed both before and after AF ingestion; during each period, 4 TFL trials were used to determine baseline without VS and 3 trials to determine baseline with VS (total VS-TFL trials=6). In the present study, VSIA was assessed only after AF ingestion, and involved 4 TFL trials to determine baseline without VS and 1 trial to determine baseline with VS (total VS-TFL trials=1). Therefore, the rats in the earlier study received a significant amount of VS "priming" that the rats in the present study did not receive. The amount of analgesia produced by VS depends on a number of factors including the number of repetitions of stimulation (3,16); therefore, the dependent variables in these two studies, although they are both ostensibly the amount of VSIA produced by 75 g VS pressure, are not comparable.

Although analgesia produced by mechanical stimulation of the cervix occurs during delivery of the fetus (8,9) and can be enhanced by ingestion of either AF or placenta (11,14), it remains unclear how the events of delivery are synchronized with the availability of varying amounts of AF and placenta to yield an optimum amount of analgesia.

The blockage by AF pretreatment of VS-induced pseudopreg-

nancy (using 225 g VS) was not associated with a difference in VSIA, since the amount of analgesia produced by 225 g VS in Experiment 2 was the same in rats pretreated with AF and with Sal. Therefore, the effect of AF ingestion on VS-induced PsP may be independent of the effect of POEF in AF on analgesia.

We have already demonstrated that ingestion of amniotic fluid during delivery enhances pregnancy-mediated analgesia (14). The inhibition of VS-induced PsP by the single infusion of AF suggests an additional advantage to the parturient female of ingestion of AF: Prevention of pseudopregnancy resulting from vaginal/cervical stimulation occurring during delivery, which might otherwise interfere with postpartum estrus. Sachs (19) showed that placentophagia did not affect postpartum estrus, but only ingestion of placenta was tested, not ingestion of amniotic fluid.

Additional research will be required to determine whether the effect of amniotic fluid ingestion on the induction of pseudopregnancy is due to POEF, and therefore to modification of opioids, or to some other component of amniotic fluid, e.g., steroids or gonadotropins.

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