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Different chemical fractions of fetal fluids account for their attractiveness at parturition and their repulsiveness during late-gestation in the ewe

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ABSTRACT

We have investigated whether the chemical components of fetal fluids (FFs), which elicit repulsion in late gestating ewes, are also those responsible for the attractiveness of fetal fluids at parturition. An aqueous fraction of FFs (A1), obtained after extraction with hexane, was tested for repulsion in late-pregnant ewes and for attraction at parturition. We also investigated if the repulsive and attractive characteristics of this A1 fraction were maintained after an additional extraction with dichloromethane (DCM, CH_2Cl_2) that produced two more fractions (aqueous/high polarity: A2 and dichloromethane/medium polarity: DCM). Thus, late-pregnant ewes were tested for repulsion of aqueous extracts of FFs (A1, A2 and DCM fractions) in a two-choice test of food preference, whereas parturient ewes were tested for attraction toward these same fractions in a two-choice test of licking warm spongy cloths. The A1 fraction was repulsive to late-pregnant ewes and attractive to parturient females. In contrast, neither the A2 nor the DCM fractions were repulsive to late-pregnant ewes, whereas both fractions were attractive to parturient ones. The discordance between the repulsive and attractive properties of the A2 and DCM fractions suggests that the attractiveness of FFs for parturient ewes and its repulsiveness for females outside the peripartum period depend on mixtures of substances that are at least partially different. Some compounds with high and medium polarity in the A2 and DCM fractions would act synergistically to generate the repulsiveness of FFs, whereas both high and medium polarity compounds can evoke attraction independently of each other.

1. Introduction

Birth fluids and placenta are potent chemosensory cues in many mammalian species and play an important role in the behavior of the mother and her young during the postpartum period [1–8]. Parturient ewes and goats lick their neonate very actively during the first postpartum hours [4]; in sheep this has been shown to depend on the attraction of the mother to birth fluids [9]. Indeed, the fetal fluids (FFs) and afterbirth materials are strongly repulsive to ewes through their whole reproductive cycle, but become highly attractive at parturition [10,11]. The compounds responsible for this phenomenon of repulsion–attraction have not been identified so far. In an attempt to characterize the chemical nature of the substances involved in the repulsiveness of amniotic fluid (AF) in non-pregnant ewes, Lévy [12] determined that after an extraction of this fluid with hexane, repulsive substances were contained only in the aqueous polar fraction and not in the low polar one. In addition, after a second extraction of the aqueous fraction with dichloromethane, none of the five fractions obtained showed a significant repulsive activity (see Fig. 1, [12]).

However, to our knowledge, whether the aqueous fraction obtained by hexane extraction or the fractions obtained after extraction by dichloromethane are attractive to parturient ewes has never been investigated. Therefore, it is not clear whether the chemosensory cues responsible for the attractiveness of AF to parturient ewes are the same as those responsible for repulsiveness observed in non-parturient ones. A comparison of the reactions of non-parturient and parturient ewes to these fractions should provide some indication. To assess this question, we studied 1) whether the aqueous fraction (A1) of fetal fluids (FFs) obtained after extraction with hexane was not only repulsive to late-pregnant ewes but also attractive to parturient ewes, and 2) whether this was also the case after performing an additional extraction on the A1 fraction with dichloromethane obtaining two more fractions (aqueous: A2 and dichloromethane:

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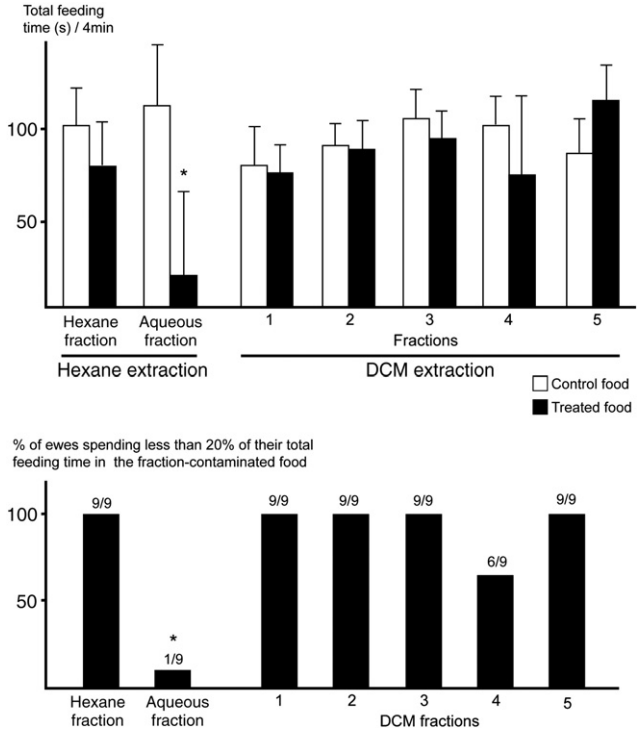

Fig. 1. Consumption of various fractions of amniotic fluids in a two-choice test of food preference between two troughs contaminated or not contaminated with the amniotic fluid extracts (dark and empty bars respectively). Fractions were obtained first from an extraction by hexane (left-hand part of the figure), and then another extraction procedure was carried out on the first aqueous fraction with dichloromethane (DCM), dividing it into five more fractions (right-hand part of the figure).
Adapted from Lévy, unpublished Master Thesis, 1981, with permission of the author.

DCM). Differing from Lévy's initial study [12], we used a mixture of allantoic and amniotic fluids, to emulate the natural conditions that occur when parturient ewes lick their neonates, which is when they become attracted to a mix of these two fetal fluids.

2. Materials and methods

2.1. Experimental design

Different fractions of FFs were tested in double choice tests using different ewes for repulsion and attraction tests. During late pregnancy, we assessed the repulsion of the ewes to the experimental fractions with a two-choice test between food mixed with the fraction to be tested or with the control substance compound, according to the procedure described by Lévy [10]. On the other hand, at parturition, attraction was measured by a two-choice test between experimental and control fractions placed on warm cloths, since it is not required to increase the ewes' interest with food at that time.

2.2. Animals

We used multiparous ideal ewes from the facilities of the National Agricultural Research Institute (INIA) "La Estanzuela", Colonia, Uruguay. Reproduction was synchronized at ovulation with vaginal sponges (Sintex®) and an injection of pregnant mare stimulating hormone (250 UI, im, Novormon®) at sponge withdrawal, followed by controlled mating with rams. Parturitions were induced by the administration of 15.0 mg of dexamethasone (2.0 mg/mL, im, Disperdex, Dispert, Montevideo), on day 144 of gestation [13]. Deliveries occurred within a 24 h period, starting in average 36 h after the dexamethasone injection. It has already been demonstrated that the endocrine events of parturition induced by dexamethasone are similar to those of spontaneous parturition and that this procedure does not interfere with the olfactory preference of ewes [14], the establishment of maternal behavior, or the length of the sensitive period [13,15].

During most of the gestation period, ewes were maintained outside on green pasture with access to alfalfa and maize. One week before the injection of dexamethasone, the ewes were habituated daily to stay indoors in individual pens of 2.0×2.0 m for 3 h. During this period, two identical troughs of maize were placed on a tray, 10 cm apart, in front of the ewes, to habituate the ewes to the food and troughs in the individual pens. Females remained permanently in individual pens from the time when the dexamethasone injection was applied until the tests were performed during late gestation and parturition. Different ewes were tested in both periods.

This study was approved by the Ethical Committee of the Facultad de Ciencias of the Universidad de la República, Uruguay.

2.3. Collection of FFs and solvent partition of the various fractions

FFs were collected in a first wave of parturitions ($n = 10$) by puncturing the amniotic and allantoidal vesicles when they became accessible at the beginning of the parturition process. A pool of FFs (about half of each fluid) was filtered, aliquoted and stored at −20 °C until subjected to purification procedures or testing. We verified in a preliminary study that this pool of FFs was repulsive to non-parturient ewes and attractive to parturient ones (results not shown; also, below, see Validation of two-choice test procedure with warm cloths).

Before solvent extraction, the FFs were thawed, filtered through gauze and centrifuged (5000 rpm; 20 min; 4 °C). The supernatant was then fractionated by liquid–liquid partitioning, first with hexane and then with dichloromethane. While hexane removes lipophilic compounds, such as fats and hydrocarbons, dichloromethane extracts medium-polarity compounds such as steroids. Solvent extractions were performed sequentially in a 6 L separation funnel. First, 5.5 L of FFs was treated with three successive extractions by 0.5 L of hexane each time, obtaining 4.0 L of an aqueous fraction (A1). This fraction was divided in two halves. One half (2.0 L) was mixed with carboxymethyl cellulose (CMC) to mimic the viscosity of FFs, obtaining the A1 fraction used in the behavioral tests. The other half was further treated with three successive extractions by 0.5 L of dichloromethane each time, yielding a second aqueous fraction that was added with CMC to obtain fraction A2. The remaining dichloromethane fraction was evaporated under vacuum and the solid residue was re-suspended in a solution of CMC in water to obtain the DCM fraction.

As a prerequisite, we verified that the CMC and the residues of the solvents used for extraction did not influence the outcome of the tests. In addition, we took into account the fact that the aqueous fractions A1 and A2 had been saturated with organic solvents during the extracting process. Therefore the water to be used for the control fractions in the behavioral tests was also saturated in the same way. First water was saturated with hexane; the hexane was then extracted and half of this water was mixed with CMC, in the same proportion as the experimental fractions, to produce a neutral fraction of CMC that served as the control for A1. The remaining half of water was saturated with DCM, which was then extracted and the water mixed with CMC; this fraction was used as control for A2 and DCM. The aqueous/CMC substance in itself was neither repulsive nor attractive to late-pregnant and parturient ewes in the preliminary observations in which the validation of attraction for raw FFs was carried out ($n = 10$; results not shown).

2.4. Behavioral tests

2.4.1. Late-pregnancy: food preference tests

Late-pregnancy tests were performed on day 145 of gestation, 12 to 24 h before parturition. Food and water were removed from the pen 2 h before the tests, to standardize the physiological state of