

Advanced ovulation in gilts by the intrauterine application of a low molecular mass pronase-sensitive fraction of boar seminal plasma

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The shortening of the time interval between the onset of oestrus and ovulation in sows by the transcervical administration of seminal plasma was investigated in 23 German Landrace gilts, using the technique of single horn infusions (Mariensee model) in combination with the transcutaneous sonographic monitoring of ovaries. Preparative surgery comprised the detachment of the left uterine horn from the corpus, leaving the caudal end open to the peritoneal cavity but sealing the corpus wound. The left ovary was loosely tied to the ventral abdominal wall for better sonographic distinction. The animals were used in two to four consecutive cycles. After detection of oestrus by the teaser boar, the patent (right) horns were filled by transcervical infusion of 100 ml of a variety of test solutions. Ovulation was probed by transcutaneous sonography at intervals of 4 h thereafter. Native seminal plasma provoked ovulation in the ipsilateral ovary of the treated horn 10.7 h earlier than in the contralateral ovary. This effect was reduced to 7.3 h after charcoal treatment of seminal plasma; addition of 10 µg oestradiol restored the effect in full, while 10 µg of oestradiol in PBS shortened the time interval to only 3.3 h versus the control ovary. Little effect was seen with oestrone sulfate, none with prostaglandins in PBS or with PBS alone. The preliminary characterization of the nonsteroidal component of seminal plasma advancing ipsilateral ovulation after transcervical infusion suggests a proteinaceous nature. The activity resides in the 1–10 kDa fraction separated by ultrafiltration and is lost after treatment with pronase.

Introduction

The intervals between the onsets of oestrus and ovulation in sows vary widely (Weitze *et al.*, 1994). Maximal fertilization rates require an interval of no more than 12 h between insemination and ovulation (Waberski *et al.*, 1994). It is well known that boars exert a stimulative effect on the onset and intensity of oestrous behaviour in sows (Signoret, 1970; Hughes *et al.*, 1990). Natural mating shortens the interval between the onset of oestrus and ovulation (Signoret *et al.*, 1972). This phenomenon has been explained as a stimulatory effect of copulation on the hypothalamus–hypophyseal axis via receptors found in the vagina and the cervix with subsequent enhancement of LH concentrations in blood plasma (Ziecik *et al.*, 1981). Claus (1989) and Weiler and Claus (1991) suggested that the oestrogens present in boar semen in

amounts of up to 11.5 µg are the specific trigger for release of prostaglandins from the endometrium; prostaglandins reach the ovary via a capillary network and increase the prostaglandin concentration in the follicular fluid. In addition, the seminal oestrogens could act systemically by provoking an LH surge (Claus 1989). With the establishment of transcutaneous sonography as a non-invasive and stress-free tool for the detection of ovulation in unanaesthetized pigs (Weitze *et al.*, 1989), the effect of seminal plasma in advancing ovulation has been ascertained. Transcervical infusions of oestrogen in saline also have some effect, but this is markedly surpassed by seminal plasma containing comparable concentrations of oestrogens (Weitze *et al.*, 1990). In the present study, we investigated evidence for a local effect of seminal plasma constituents other than oestrogens. The Mariensee model (Jungblut *et al.*, 1991), which allows for a clear distinction between local and systemic effects, was used in combination with the transcutaneous sonographic monitoring of ipsi- and contralateral ovaries.

Materials and Methods

Animals and surgery

Twenty-three German landrace gilts with an average body mass of 95 kg were surgically prepared according to the Mariensee model (Jungblut *et al.*, 1991). Surgery was carried out at the Institute for Animal Husbandry and Behaviour FAL, Mariensee, under general anaesthesia using Stresnil[®] (Janssen GmbH, Neuss) and Nembotal[®] (WDT, Hannover). A schematic design of the model is given (Fig. 1). After midventral incision and propositation of the corpus uteri and the adjacent part of the cervix, a section (7 cm × 2 cm) of the parametrium at the caudal end of the left horn and the cervix was coagulated at about 150°C by 'ironing' with a custom-made 'foot' attached to a thermostated soldering rod (set to 350°C) against a Teflon 'board'. The serosa of the left horn about 5 cm above the corpus was rounded with the iron, the left horn–cervix junction was ligated with catgut and a section (about 2 cm) of the left horn between the ligation and ironed circle was excised. The ligated part of the horn left at the corpus was thoroughly cauterized at the cutting edge and in the lumen to ensure permanent sealing after the discharge of decay. All traces of blood shed during the operation were carefully removed to avoid adhesions. For easier sonographic distinction, the mesovarium of the left ovary was attached to the abdominal wall next to the incision using a synthetic yarn (Synthofil[®]; Braun, Melsungen). Animals were allowed to recover from surgery in individual pens for 14 days and were then transported to the Hannover Veterinary School. They were housed in groups of two or three with olfactory and visual contact with the boar. Oestrus was detected by contact twice a day with the teaser boar (08:00 h, 20:00 h). The gilts became quickly acquainted with the personnel and got used to the examination stand and sonographic equipment.

Transcervical infusions

Transcervical infusions of 14 different solutions into the patent horns were performed by insemination catheters immediately after tolerance to mounting by the boar. Aliquots of 100 ml were applied, and refluxes were collected for 5 min after completion of the infusion. The animals were used in two to four consecutive oestrous periods, beginning with the second oestrus after stalling: five gilts were used in four cycles, nine gilts in three cycles, and nine gilts in two cycles. Within each treatment group different gilts were used.

Assessment of the interval between the onsets of oestrus and ovulation

The beginning of oestrus was set at the middle of the time interval between the last rejection of mounting and the first tolerance. During oestrus, the ovaries were examined sonographically every 4 h (04:00, 08:00, 12:00, 16:00, 20:00, 24:00 h) to determine the time of ovulation. Transcutaneous sonography was performed using a 5 MHz sector scanner (Sonoline SX[®]; Siemens, Erlangen), as described by Weitze *et al.* (1989). Ovulation was assumed to have occurred at the middle of the time interval between the last detection of

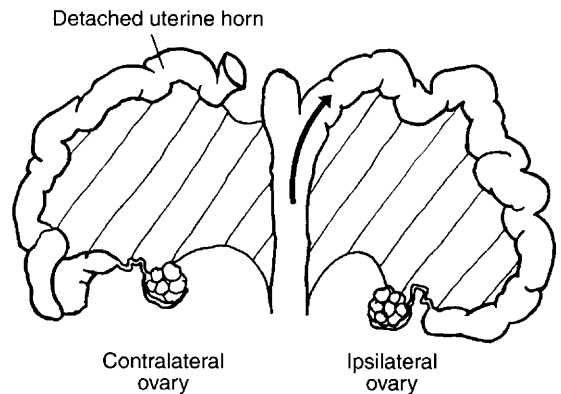


Fig. 1. Schematic design of the Mariensee model. The arrow indicates the flow of an inseminated solution.

follicles and their subsequent disappearance. Fewer follicles than were seen in the previous examination followed by complete disappearance of follicles at the next examination was taken as a sign of ongoing ovulation. In this case, the time at which a reduction in numbers of follicles was registered was taken as the time of ovulation. The criterion for distinguishing between the left and right ovaries was their position relative to the bladder and the sonograph probe. The effect of an infused solution in enhancing ovulation was calculated from the mean difference between the times of ovulation of the ipsilateral (treated horn) and the contralateral (control) ovaries in six gilts. Simultaneous ovulations at both ovaries in at least three gilts were taken as proof of the ineffectiveness of an infusate to advance ovulation by a local mechanism. In this case, it was assured that in at least two of these three gilts ovulation had been advanced after the infusion of seminal plasma, or an active component thereof, so that an animal specific cause (e.g. postsurgical adhesion) for the negative result could be eliminated.

Solutions infused

Seminal plasma. Semen samples from four fertile boars were collected over 4 months. They were centrifuged at 3000 g for 20 min and the cell-free plasma was stored frozen at -20°C . Plasma pools were prepared by thawing the frozen samples and mixing them when fully thawed.

Charcoal treatment of seminal plasma. Activated charcoal powder (5 g; Merck, Darmstadt) was added to 1 l of thawed seminal plasma; the suspension was stirred for 1 h at 4°C and then centrifuged at 3000 g for 20 min. The supernatant was again treated with charcoal for 24 h, and the suspension cleared by centrifugation and filtration through fluted filters. Aliquots of 100 ml were frozen and stored at -20°C until use.

Spiking of charcoal-treated seminal plasma with oestradiol. Immediately before use, 10 μg oestradiol (Sigma, St Louis, MO) dissolved in 0.1 ml 96% (v/v) ethanol was added to 100 ml of charcoal-treated seminal plasma.

Oestradiol (10 μg) was dissolved in 0.1 ml 96% (v/v) ethanol and added to 100 ml PBS containing 3% (w/v) BSA.

Oestrone sulfate. Since most of the oestrogens found in seminal plasma are conjugated, the effect of 7.8 µg oestrone sulfate (Sigma) in 100 ml PBS containing 3% (w/v) BSA was tested.

Prostaglandins. Prostaglandin F_{2α} (PGF_{2α}) (250 µg Dinoprost; Dinolytic[®]: Upjohn Heppenheim) or PGF₂ (250 µg Dinoprost; Minprostin[®]: Upjohn Heppenheim) was dissolved in 100 ml PBS.

Fractionation of charcoal-treated seminal plasma by gel-exclusion chromatography and ultrafiltration. The plasma was adjusted to pH 4.5 and passed through two columns (5 cm × 100 cm) of Sephadex G-50 superfine (Pharmacia-LKB, Uppsala) set in series, equilibrated with 1 mol sodium formate l⁻¹, pH 4.5. Two macromolecular peaks were obtained: peak 1 contained > 60 kDa proteins, peak 2 consisted of non-aggregated 16–20 kDa spermadhesin proteins. Both peaks were neutralized, concentrated by ultrafiltration and adjusted to the starting volume of 100 ml. For fractionation by ultrafiltration, 100 ml portions of seminal plasma were first passed through PM 10 membranes (Amicon GmbH, Witten). The filtrate was either tested as such or passed through YM 1 membranes, and the filtrates and retentates recovered. All fractions were analysed by SDS-PAGE and reconstituted to the original volume by addition of PBS before infusion.

Digestion with pronase. The 1–10 kDa fractions of the ultrafiltration experiment were digested with pronase by adding 0.1 mg enzyme to 10 ml of concentrate and incubating the mixture for 2 h at 38°C. The enzyme was recovered by passage through Centricon[®]-30 (Amicon GmbH) and the filtrate was adjusted to 100 ml by addition of PBS.

Hormone assays

Unconjugated oestrogens. Total unconjugated oestrogens were extracted from seminal plasma aliquots (5–50 µl) with ethyl acetate and determined by radioimmunoassay. The anti-serum directed against oestrone-17-CMO-BSA (G. Emons and R. Knuppen, Med. Univ. Lübeck, Germany) cross-reacted 100% with oestradiol, 19.1% with 17α-oestradiol and < 0.1% with testosterone, androstendione, progesterone and cortisol. Dried sample extracts were resuspended in 100 µl assay buffer (0.01 mol NaH₂PO₄ l⁻¹, 0.01 mol EDTA l⁻¹, containing 0.1% (w/v) lysozyme, pH 7.2). [2,4,6,7-³H]oestradiol (Amersham, Buchler) was added as tracer at 12 000 c.p.m. in 100 µl assay buffer, and the mixture was incubated with 100 µl of a 1:120 000 antiserum dilution at 4°C for 18 h. Free and bound steroids were separated by dextran-coated charcoal (Separex[®]: S. Klinger, St Albans; 2 mg per 0.5 ml assay buffer), and the bound fraction was mixed with 1 ml scintillation fluid (Ready Organic, Beckmann Instruments, Fullerton, CA) and radioactivity was measured in a β counter. The minimal detectable concentration was 2 pg; zero binding was 37%; and intra- and interassay coefficients of variation were 8.3% and 12.4%, respectively.

Conjugated oestrogens. Oestrogen sulfates and glucuronides were cleaved by enzyme hydrolysis and the steroids were

extracted with ethyl acetate and estimated by radioimmunoassay. Seminal plasma aliquots (5–50 µl) were diluted with 300 µl CH₃COONa (0.05 mol l⁻¹, pH 4.5) and incubated with β-glucuronidase/arylsulfatase (10 µl, *Helix pomatia*, 5.5 U β-glucuronidase ml⁻¹ and 2.6 U arylsulfatase ml⁻¹ (Boehringer, Mannheim) at 37°C for 15 h. After cooling, the mixture was extracted with ethyl acetate (3 ml) and the dried extract was subjected to the total oestrogen radioimmunoassay as described above. Recovery of oestradiol-3-glucuronide (R. Knuppen, Med. Univ. Lübeck) or oestrone sulfate (Sigma, Deisenhofen) was 75% and 82%, respectively.

Oestradiol. Concentrations of oestradiol were determined by radioimmunoassay as described by Behrens *et al.* (1993). The minimal detectable concentration was 2 pg ml⁻¹. Intra- and interassay coefficients of variation were 9.55 and 11.8%, respectively.

Prostaglandin. Concentrations of PGF_{2α} and PGE₂ in frozen-thawed seminal plasma were measured by fluoroimmunoassay after extraction of 500 µl aliquots with 8 ml ethyl acetate at pH 3.5. The organic phase was dried with a gentle stream of nitrogen gas, and samples were further treated as described by Lüke and Schlegel (1990). The detection limit for PGF_{2α} was 2.2 pg, and for PGE₂ 1.3 pg per microtitre well. The average intra-assay coefficient of variation for both types of prostaglandins was below 10%. The interassay coefficient of variation for PGF_{2α} was 13.4%, and for PGE₂ 13.0%.

Statistical analyses

Statistical analyses were made using SAS (SAS/STAT, 1989). The effects of treatment and each of the following variables: number of cycle, volume of reflux and duration of oestrus were tested by two-way analysis of variance. Since none of the variables showed a statistically significant influence on the time difference between ipsi- and contralateral ovulations, a one-way analysis of variance with the factor treatment neglecting the covariables was used to test for differences between the infused solutions. The pairwise comparisons between any two of the means were made using the Ryan-Einot-Gabriel-Welsch multiple range test (SAS/STAT, 1989).

In the groups showing the highest effects and the highest ranges (seminal plasma and charcoal-treated seminal plasma + 10 µg oestradiol), the dependence of the time differences between ipsilateral and contralateral ovulations on the interval between the onset of oestrus and ovulation in contralateral ovaries was shown using the linear regression coefficient.

Results

Hormone contents of native and charcoal-treated seminal plasma

From the total amount of 9.2 ng oestrogens ml⁻¹ measured in the native seminal plasma, 8.6 ng ml⁻¹ were conjugated. Charcoal treatment reduced the oestrogen content by 98.7% (Table 1). The concentration of oestradiol in charcoal-treated seminal plasma was below the detection limit. Neither PGF_{2α} nor PGE₂ were found in frozen-thawed seminal plasma.

Table 1. Concentrations of oestrogens and prostaglandins in native and charcoal-treated seminal plasma of boars

Constituents (pg ml ⁻¹)	Seminal plasma	
	Native	Charcoal-treated
Total oestrogens	9200	120
Unconjugated oestrogens	600	5
Oestradiol	135	nd
Prostaglandin F _{2α}	nd	nd
Prostaglandin E ₂	nd	nd

nd: not detectable.

Effects of infusates on the time interval between the onsets of oestrus and ovulation

In gilts infused with seminal plasma, the intervals between the onset of oestrus and ovulation were, on average, 10.7 h (8–14 h) shorter in the ipsilateral than in the contralateral (control) ovaries (Table 2). The duration of oestrus, the number of cycles and the volumes of refluxes, ranging from 0 to 52 ml, had no influence on the results. Treatment of seminal plasma with activated charcoal reduced the difference between the treated and untreated side by 3.4 h. This reduction was apparently equalized by adding 10 µg oestradiol to the charcoal-treated seminal plasma. The mixture advanced ovulation in the ipsilateral ovaries by 10 h, compared with the control. In this group, and in the seminal plasma group, the differences between ipsi- and contralateral ovulations depended ($r^2 = 0.43$, $P < 0.05$) on the interval from the onset of oestrus to ovulation in the contralateral (control) ovary (Fig. 2). Oestradiol (10 µg) in PBS resulted in ovulations occurring 3.3 h earlier in the experimental, right-side ovaries. The same figure was obtained with 7.8 µg oestrone sulfate in PBS. Treatments with prostaglandins and with fractions of the separation experiments with molecular masses < 1 and > 10 kDa were ineffective. Advanced ovulations were seen with the PM 10 filtrate of charcoal-treated seminal plasma (6.8 h on average) and its YM 1 retentate (5.3 h on average). The earlier ovulation of 6.8 h is not different from the 7.3 h observed with charcoal-treated seminal plasma. The interval of 5.3 h seen with the 1–10 kDa YM 1 retentate was significantly different from the 7.3 h value, but not from the 6.8 h of the PM 10 filtrate. Treatment of the 1–10 kDa YM 1 retentate with pronase resulted in a complete loss of the effect.

Discussion

We previously reported that the transcervical infusion of boar seminal plasma shortens the interval between the onset of oestrus and ovulation by about 14 h compared with untreated controls (Weitze *et al.*, 1990). The high variations in oestrous behaviour and time of ovulations in sow herds (Weitze *et al.*, 1994) required the study of a large number of cycles to obtain significant data. The Mariensee model used in this study requires fewer animals, since the times of ovulations at the patent sides of the uterus can be compared with those in the

control ovaries of the detached uterine horns. The model allows for the distinction between local and systemic effects. The differences observed identify the effects seen as local phenomena. It was demonstrated that a transcervical infusion of seminal plasma in one uterine horn advanced ovulation on the ipsilateral ovary for 10.7 h, on average, compared with the contralateral ovary. The effect is more pronounced in gilts that show long intervals between the onset of oestrus and ovulation on the control ovary, compared with gilts with earlier contralateral ovulations. Previous data from 303 spontaneously ovulating gilts, collected during 3 years in the Institute of the Hannover Veterinary School, revealed a mean interval between the onset of oestrus and ovulation of 39.5 h, ranging from 24 to 64 h (K. F. Weitze and D. Waberski, unpublished). Since in the present study, the mean interval from the onset of oestrus to ovulation of the control ovaries in gilts infused with seminal plasma was 45.3 h, ranging from 40 to 56 h, it can be concluded that systemic effects do not contribute to the ovulation-advancing effect. Steroid depletion of seminal plasma reduced the effect significantly, and this could be compensated for by adding oestradiol to charcoal-treated seminal plasma. The difference of 3.4 h between steroid-depleted and native seminal plasma corresponds to the effect of oestrogens in PBS, thereby indicating an additive effect of oestrogens and a non-steroidal component of seminal plasma. The ineffectiveness of some infusates also discounts the possibility of distension reflexes. The contribution of oestrogens in seminal plasma to earlier ovulations appears less pronounced than that of small proteinaceous constituents. Since oestrogens in seminal plasma occur mainly in a conjugated form and a receptor independent local effect of oestrogens in the uteri of pigs has been described (Jungblut *et al.*, 1979, 1989), oestrone sulfate was tested in the present study and was found to be as effective as oestradiol. Claus (1989) suggested that the oestrogens in seminal plasma provoke the synthesis of prostaglandins in the endometrium. They would travel from there via the parametrial/mesovarial capillary bed to the ovary, increase the prostaglandin concentrations in the follicular fluid and trigger follicle rupture. However, infusions of prostaglandins in quantities that caused a substantial rise in concentration in the uterine vein comparable with that measured by Claus (1989) after the intrauterine application of oestrogens (Okrasa *et al.*, 1985) did not advance follicle rupture in the ipsilateral ovary. The observation of Kotwica (1980) that prostaglandins infused into the anterior uterine vein on day 14 of the oestrous cycle in pigs also caused regressive changes in the contralateral ovary indicates the presence of effective pathways for prostaglandins from one uterine horn to the opposite ovary. This is a possible argument against the mechanism of seminal plasma action suggested by Claus (1989) since, in the present study, the local, unilateral effect of seminal fluid upon the time of ovulation in gilts was clearly established.

Although the concentrations of FSH and LH in the peripheral blood plasma were not measured in the present study, the different timings of ovulation between the treated and the control sides rule out the possibility of increased plasma concentrations as the ovulation elicitor. The conclusions drawn from experiments with camels in which GnRH-like factors present in seminal plasma were suspected of acting on the pituitary (Zhao *et al.*, 1990, 1992) indicate a different

Table 2. Comparison between times of ipsilateral ovulation after infusion of seminal plasma or components thereof into patent uterine horn and ovulation in control ovary

Solutions	Cycles (n)	Time interval (h) between onsets of oestrus and ovulation											
		Right ovary, patent horn					Left ovary, detached horn					Difference	
		Mean	Min.	Max.	Mean	Min.	Max.	Mean	± SD	Min.	Max.		
Seminal plasma	6	34.7	30	44	45.3	40	56	10.7 ^a	± 2.4	8	14		
Charcoal-treated seminal plasma	6	33.7	28	40	41.0	36	48	7.3 ^b	± 1.0	6	8		
10 µg oestradiol in PBS	6	36.0	32	40	39.3	36	44	3.3 ^c	± 1.6	0	4		
Charcoal-treated seminal plasma + 10 µg oestradiol	6	33.3	26	36	42.7	34	48	10.0 ^a	± 1.8	8	12		
7.8 µg oestrone sulfate in PBS	6	32.8	28	48	35.2	28	52	2.4 ^{cd}	± 1.7	0	4		
250 µg prostaglandin E ₂ in PBS	3	37.3	28	44	38.0	28	44	0.7 ^d	± 1.2	0	2		
250 µg prostaglandin E ₂ in PBS	3	36.0	32	44	36.7	32	46	0.7 ^d	± 1.2	0	2		
Charcoal-treated seminal plasma, fractions:													
G 50-fraction 1	4	39.0	32	44	39.0	32	44	0 ^d	± 0	0	0		
G 50-fraction 2	4	34.0	28	40	34.0	28	40	0 ^d	± 0	0	0		
< 10 kDa	6	36.0	32	44	42.8	38	50	6.8 ^{bc}	± 1.8	6	10		
1–10 kDa	6	30.7	26	36	36.0	30	40	5.3 ^e	± 1.6	4	8		
< 1 kDa	3	34.0	26	42	34.0	26	42	0 ^d	± 0	0	0		
1–10 kDa treated with Pronase	3	35.3	28	46	35.3	28	46	0 ^d	± 0	0	0		
PBS	3	40.0	36	44	40.0	36	44	0 ^d	± 0	0	0		

Results from 65 cycles of 23 gilts.

^{a–e}Values with different superscripts are significantly different ($P < 0.05$).

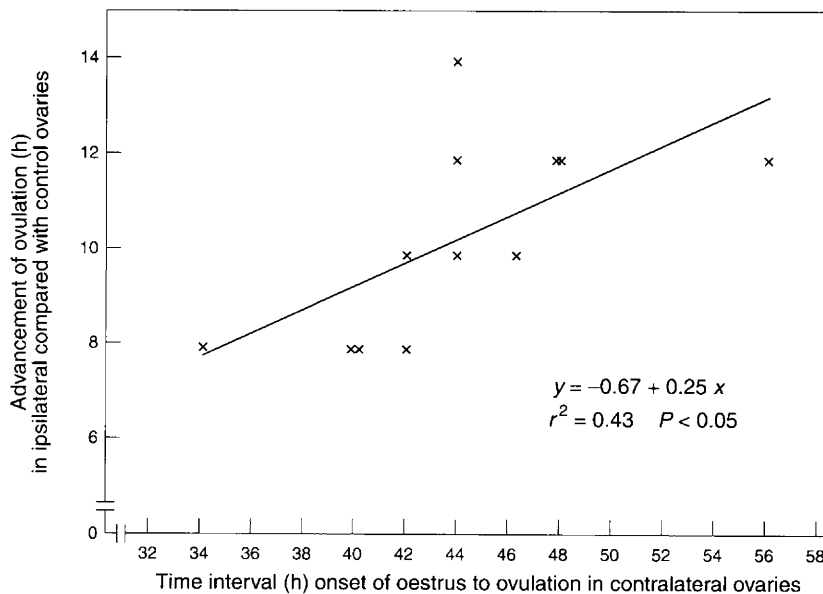


Fig. 2. Relation of the time interval between the onset of oestrus and ovulation in contralateral (control horn) ovaries and the ovulation advancing effect on ipsilateral (treatment horn) ovaries after transcervical infusion of seminal plasma or charcoal treated seminal plasma + oestradiol ($n = 12$ gilts).

mechanism of seminal plasma in this species with copulation-induced ovulation. The key role of the 1–10 kDa fraction of seminal plasma in eliciting ovulation seems to be without dispute. The isolation of the active factor(s), therefore, is of utmost importance in identifying the primary site of action and elucidating the molecular mechanisms of the subsequent events.

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