THE DETERMINATION OF THE TIME OF OVULATION, FIRST CLEAVAGE, SUBSEQUENT CLEAVAGE DIVISIONS AND IMPLANTATION IN FEMALE MICE TREATED WITH GONADOTROPHINS

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ABSTRACT OF THESIS

THE DETERMINATION OF THE TIME OF OVULATION, FIRST CLEAVAGE, SUBSEQUENT CLEAVAGE DIVISIONS AND IMPLANTATION IN FEMALE MICE TREATED WITH GONADOTROPHINS.

The time of ovulation, cleavage stages to blastocyst and implantation have been timed in female mice treated with gonadotrophins. Ovulation was induced by an injection of pregnant mares' serum (PMS) followed, after a 45 hr interval, by an injection of human chorionic gonadotrophin (HCG). Ovulation began as evidenced by the presence of ova in the oviduct, at 12 hr after HCG injection and was complete at 14 hr after HCG injection. First cleavage was observed at 37 hr post HCG and was not complete until 48 hr post HCG, however some 80% had cleaved by 38 hr. The 4-cell stage was recovered at 48 hr, 8-cell at 59 hr, 16-cell at 68 hr and 32-cell at 70 hr post HCG.

The time of implantation was determined using intravenous dye injections and a "violent" vs "gentle" flushing technique. The uterine horns were examined for the presence of blue rings which indicated sites of blastocyst attachment. Comparison of the results of the two techniques indicated that implantation occurs at approximately 94 hr post HCG.

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Date

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INTRODUCTION

Many scientific studies in reproductive physiology, teratology and related sciences could be greatly facilitated by the accessibility of large numbers of fertilized ova at a given cell stage. The most stringent study conducted to date on the time of ovulation and subsequent cleavage of mouse ova has been conducted by Edwards and Gates (13). Ovulation in the female mouse was found to occur 12±1 hr after HCG injection. First cleavage was observed at approximately 37 hr after HCG injection.

The exact time intervals after HCG injection at which subsequent cell stages up to and including blastocyst can be recovered was uncertain prior to the present study.

The determination of the exact time of blastocyst implantation in mice is considered by Edwards and Fowler (12), to be important in human infertility studies. Blastocyst implantation has been determined by Finn and McLaren (17) to occur at approximately 96 hr after HCG injection.

It was the purpose of this study to determine the time of ovulation in female mice and was extended to include first cleavage and subsequent cleavages up to and including implantation.

LITERATURE REVIEW

OVULATION INDUCTION IN THE MOUSE

The female mouse copulates only during estrus when ovulation has occurred or is ready to occur (45). Estrus usually begins around midnight and mating is most common around 2:00 A.M., however, mating can occur at variable times in the morning. Therefore, the timing of ovulation in spontaneous ovulators, such as the mouse, is difficult to ascertain. Much of the difficulty in determining the time of ovulation can be eliminated by the injection of exogenous gonadotrophins. The induction of estrus and ovulation in the mouse by treatment with gonadotrophins has become an established technique after many years of investigation.

Smith and Engle (49) were among the first to induce ovulation in the mouse by the use of gonadotrophins. The capacity of human chorionic gonadotrophin (HCG) to induce ovulation was established by a successful recovery of ova from the oviducts of mice injected with HCG (7). In an attempt to devise a rapid test for the presence of pregnancy gonadotrophins in the urine of pregnant women based on the induction of ovulation in the mouse, it was found that a one International Unit (IU) injection of HCG caused ovulation

in 100% of diestrus mice (7). Various time intervals between the injection of pregnant mares' serum (PMS) and HCG have been used. Runner and Gates (46) obtained a follicular response 48 hr after the injection of serum gonadotrophin. Ovulation was observed 13 hr after the injection of HCG. Female mice injected with PMS, followed 41 hr later, by an injection of HCG were found to ovulate approximately 12±1 hr after the injection of HCG (47, 13, 21). Cumulus encased ova have been found in the oviduct as early as 11½ hr post HCG (13). Ovulation was induced in diestrus mice by Lamond (11) with both HCG and PMS. Spears (51) noted that more constant results are produced when female mice are injected with 5 IU of PMS at 1600 hr, followed by an injection of 5 IU of HCG 45 hr later. Pincus (38) concluded that gonadotrophins affect the meiotic or reduction division of the egg, which results in the extrusion of the first polar body. This action is initiated indirectly by inducing the separation of the cumulus from the main mass of the membrana granulosa. The extrusion of the first polar body is initiated by gonadotrophins in all species except the dog.

OVULATION INDUCTION IN THE RAT

The ovulation-producing capacity of HCG in the female rat was first described by Cole (9), who observed the presence of ova in the Fallopian tubes of female rats at about 72 hr after a single injection of HCG. Ovulation was observed in 90% of a group of 25 rats sacrificed 24 hr after the end of a three-injection sequence (8). Rowlands (43) injected HCG at the height of the follicular response to serum gonadotrophin (48 to 72 hr), and observed ovulation 14 to 16 hr later. Austin (2) confirmed this method of inducing ovulation in the female rat.

OVULATION INDUCTION IN THE RABBIT

Bellerby (5) observed ovulation in the rabbit at 12 hr after an intravenous injection of an acid extract of the hypophysis. He noted that this corresponded to the time of ovulation after copulation. Chorionic gonadotrophin has also been used successfully to induce ovulation in the female rabbit (19). The interval between injection and ovulation of the rabbit could not be reduced by increasing the dose of chorionic gonadotrophin; it resulted only in the rupture of a greater number of follicles (29). The capacity of gonadotrophins to induce ovulation in

the adult rabbit was fully explored by Parkes (37). The reaction was carried out in two stages to duplicate the two phases of the ovarian cycle of this mammal in which ovulation occurs spontaneously. A horse pituitary gonadotrophin was injected subcutaneously daily each day for 5 days to secure the growth of an abnormally large number of follicles. Ovulation was then induced by means of a single intravenous injection of chorionic gonadotrophin. Parkes (37) also observed that horse pituitary extract caused ovulation much more readily than did serum gonadotrophin. Pincus (39) induced ovulation in the immature rabbit using gonadotrophins after previous gonadotrophic treatment designed to stimulate follicular growth. Pincus favored the use of FSH from sheep pituitary glands to stimulate follicular growth.

OVULATION INDUCTION IN THE CAT, FERRET, BAT, FOX, AND HAMSTER

Intravenous injections of chorionic gonadotrophin in an amount that induced ovulation in the rabbit was found by Snyder and Wislocki (50) to be ineffective in the cat. McPhail (34) induced ovulation in the unmated estrus ferret by a single intravenous injection of ox pituitary extract. The interval between injection and ovulation was found to be 30 to 40 hr. Smith (48)

was successful in inducing ovulation in the bat. He found that as the follicle approaches maturity, a smaller dose of chorionic gonadotrophin is required to induce ovulation. The use of 30 IU of PMS on the expected day of ovulation led to the recovery of an average of 70 eggs in the cycling Syrian hamster (24). Chorionic gonadotrophin has been used with little success in the adult fox (30) and was successful in only 3 of 32 vixens.

OVULATION INDUCTION IN DOMESTIC ANIMALS

In sheep, Robinson (42) observed that a single injection of 500, 1000, or 2000 IU of serum gonadotrophin on the 12th day of the diestrus cycle induced superovulation about 4 days later. The ovulatory response in cattle injected with various gonadotrophins at different stages in the ovarian cycle has been compared by a number of workers, but no agreement has been reached. Rowson (44) reported a higher rate of ovulation in cows injected consecutively with serum and chorionic gonadotrophin when a large corpus luteum was present. However, Golley and Malpress (22) found no difference in the response of cows treated with serum gonadotrophin in the follicular and luteal phases. Superovulation has been used in various mammals to produce ova in

quantity for scientific studies. An average of 28.13 ova were obtained from 61 cows or calves treated with pig FSH and LH by Avery et al (4). Similiar results have been produced by the use of PMS and HCG (25). Avian, but not mammalian, gonadotrophins produced extensive follicular development with yellow yolk deposition in the immature ovary of 110 day old chickens (33). Lorenz (33) also induced ovulation in the chicken by using PMS injections followed by HCG injections. Ovulation was observed 6 - 7 hr after HCG injection.

OVULATION INDUCTION IN HUMANS

Edwards and Fowler (12) have successfully induced ovulation in a woman. The patient was given 300 IU of human menopausal gonadotrophin between the third and the ninth day of her menstrual cycle to stimulate growth of the follicles. A single injection of 5,000 IU of HCG between the ninth and eleventh day of the cycle induced ovulation. Ovulation occurred 36 hr after HCG injection.

OVULATION TIMES

Many investigations concerning the time of ovulation in the hormone-treated female mammal have been completed. It is necessary at this point to

reiterate the ovulation times reported by these workers. Runner and Palm (47) reported ovulation at 11½ hr after treating immature mice with HCG. Gates and Beatty (21) induced ovulation at 12±1 hr after treatment with HCG. This is in agreement with the work of Edwards and Gates (13). The time of ovulation in female mice induced to ovulate with gonadotrophins is not affected by mating time (36). In the rat, ovulation occurs naturally 10 to 12 hr after a neurogenic stimulus to tha anterior pituitary causes the release of luteinizing hormone (14). Rowlands (43) reported ovulation in the diestrus female rat 12 hr after an injection of HCG. In the rabbit, ovulation occurs 10 hr post HCG (53, 15, 40, 35)

CLEAVAGE STAGES

Initially, the salient feature of embryonic development is a special form of cell divisions known as "Cleavage" during which the protoplasmic mass is progressively divided until it consists of a large number of cells. Cleavage involves no gain of total protoplasmic mass (3). First cleavage in the female mouse occurs at approximately 37 hr after HCG injection (32, 23, 11, 13). Few investigations into the cleavage stages of mouse ova from first cleavage to blastocyst

have been completed, consequently, knowledge in this area is limited. A preliminary investigation to determine the post HCG ovulation and cleavage times of mouse ova to the blastocyst stage has been completed by Spears and Walker (52). Austin (2) reported that the rabbit embryo reaches the 16-cell stage in just under 2 days following ovulation, and reaches the blastocyst stage in 4 days. In general, the embryos of domestic animals and rodents cleave at a slower rate than do those of the rabbit (2). The blastocyst stage is reached in the ewe, goat, and sow, in about 6 days, and in the cow in about 8 or 9 days (11).

VIABILITY OF OVA

The viability and developmental capacity of eggs from immature mice treated with gonadotrophins has been studied and these ova have been found to be comparable to ova from spontaneous ovulators (52, 20). Some differences have been noted in the time of ovulation between mice which were placed with males immediately after injection of HCG and those placed with males a few hrs after the injection of HCG (13). However, this difference is slight and was not taken into consideration.

IMPLANTATION

Implantation of ova in rodents involves a complex series of morphological and biochemical interactions between the blastocyst and the uterus with subsequent attachment of the blastocyst to the uterine wall. In farm animals, blastocyst implantation is a relatively slow and gradual process, the precise timing of which is still in dispute (6). In the ewe, variable times have been given for implantation including day 11 and 17-20 days postocoitus (26). In the roe deer, the "free vesicle" (unattached blastocyst) stage lasts about 5 months (28). In contrast, this stage lasts about a year in the carnivore (10). The transition from the free-floating blastocyst to the definitely implanted blastocyst occurs at: 6-8 days in man; 7 days in rabbits; and 13 days in cats (1).

The site of implantation in the uterine horn of rodents has been described by Psychoyos (41), as an area containing blood vessels which have become more permeable. This increased permeability can be revealed by the injection of a large molecular weight dye, such as Evans Blue, which produces a colored band across the uterus where a blastocyst is present (17). The colored band is considered evidence of a blastocyst that is surrounded by "primary envasive cells"

which have entered the uterine epitheluim (16). The early stages of implantation in mice have been studied and implantation has been found to occur at approximately 94 hr after HCG injection (17).

MATERIALS AND METHODS

ANIMALS

Swiss Webster albino mice, originally obtained from S and S Research Animals, La Grange, Ky. and maintained by Morehead State University, were utilized in this investigation. All animals were 6-8 weeks of age at the time of use. Water and food (Purina Laboratory Chow) ad lib were given for the duration of the experiment.

OVULATION INDUCTION

Female mice, 6-8 weeks old, were injected intraperitoneally with 5 IU of PMS ("Equinex", Ayerst Laboratories) at 1600 hr followed 45 hr later by a 5 IU intraperitoneal injection of HCG ("Pregnal" Organon, USA). Immediately after the injection of HCG, 2 females were caged with a male. The presence of a vaginal plug was used as evidence that mating had occurred.

OVA RECOVERY

The animals were sacrificed by cervical separation. The oviducts were excised after external laparotomy and placed in 3 ml of 0.87% saline. The ova were recovered by inserting a 30 ga needle attached to a

2-ml syringe into the fimbrial end of the oviduct and flushing with 0.87% saline. This technique was used by Spears and Walker (52). The flushed material was examined under a 20X stereomicroscope for the presence of ova. Observed ova were counted and classified according to their appearance.

DETERMINATION OF THE TIME OF OVULATION

Females were sacrificed at 9, 10, 11, 12, and 14 hr after HCG injection and their oviducts excised and flushed with 0.87% saline. Ova, if present, were counted and classified according to their appearance.

TIME OF FIRST CLEAVAGE TO 64-CELL STAGE

Females were autopsied at the following hr intervals after injection of HCG: 32, 36, 37, 38, 42, 48, 49, 50, 51, 52, 58, 59, 60, 61, 62, 63, 64, 66, 68, 70, 72, 74, and 76 hr. The oviducts and uterine horns were flushed to recover the fertilized ova. The flushed material containing the ova was examined and the ova were classified according to the cell stage attained. Ova that had progressed beyond 72 hr post HCG were not classified as 64-cell stage because the morphology of these ova did not permit actual cell number to be ascertained. The blastocyst stage was easily recognized by the presence of the blastocoel.

IMPLANTATION

Females were selected at 90, 92, 93, 93½, 94, 94½, 95, 95½, 96, 96½, 97, 98, 99, and 100 hr after HCG injection. The tail of each mouse used was cleaned with alcohol to provide a better view of the tail vein. Evans blue, a high molecular weight dye, was injected intravenously into the tail vein using a 27 ga needle. During a time interval of 10 min after injection, the circulatory system carried the dye throughout the animal. At the permeable areas of the implantation sites in the uterine horns, the dye formed blue rings. The females were then sacrificed by cervical separation. The oviducts and uterine horns were excised and placed on a paper saturated with 0.87% saline. The oviducts and uterine horns were flushed "gently" with 0.87% saline. The flushed material was then examined for blastocysts under a 20X stereomicroscope. Blastocysts observed after this process were considered not to be implanted. The oviducts and uterine horns were then distended and flushed "violently". This technique was employed in an attempt to dislodge the fragilly implanted blastocysts, if they were present. The flushed material was examined for the presence of blastocysts. The wall of the uterine horns was then examined for

the presence of a blue ring. The colored rings were also used as evidence of implantation.

An attempt was made to correlate the number of blue rings found in the uterine wall with the number of blastocysts present in the material "violently" flushed from the uterine horns. The dye technique was similiar to that used by Finn and McLaren (17).

RESULTS

OVULATION

Twenty-four females injected with 5 IU PMS and 5 IU HCG were sacrificed between 10 and 14 hr after HCG injection and the number of ova counted. Eight of these females sacrificed at 10 hr had not ovulated. Two out of nine females sacrificed at 12 hr had ovulated, but only three eggs were counted. At 14 hr after HCG, 7 females had ovulated and 107 ova were recovered. The duration of ovulation was determined by two criteria: the presence of ova in the oviduct indicated that ovulation had begun, and the presence of 10 or more ova per mouse indicated that ovulation was advanced. At this stage of development, abnormalities manifested later by abortive cleavage are not often evident, hence, the ova appeared to be morphologically normal. The total number of ova recovered and the number of female mice sacrificed from the onset to completion of ovulation are given in Table 1.

Table 1. CLEAVAGE STAGE OF OVA FROM FEMALE MICE TREATED WITH GONADOTROPHINS

Hr. after HCG injection when females were sacrificed	No. of females sacrificed	No. of ova recovered	Condition of ova Cell stages							
			1	2	4	8	16	32	64	Blast.
10	8	0	0							
11	7	0	0							
12	9	3	3							
13	8	76	76							
14	7	107	107							
16	6	116	116							
32	7	126	126							
36	6	111	111							
37	7	105	102	3						
38	7	138	24	114						
42	6	135	10	125						
48	8	148	4	119	25					
49	7	116		97	17					
50	7	122		78	44					

Table 1. (CONTINUED)

Hr after HCG injection when females were sacrificed	No. of females sacrificed	No. of ova recovered	Condition of ova Cell stages							
			1	2	4	8	16	32	64	Blast
51	6	109		64	45					
52	9	162		100	62					
58	6	120		8	112					
59	7	113		6	105	2				
60	8	147			140	7				
61	8	140			56	84				
62	7	128			4	124				
63	7	118			7	111				
64	7	112				105	7			
65	8	105				60	45			
66	7	132				90	42			
67	9	141				47	94			
68	9	163				7	156			
69	6	108					108			

Table 1. (CONTINUED)

Hr after HCG injection when females were sacrificed	No. of females autopsied	No. of ova recovered	Condition of ova Cell stages							
			1	2	4	8	16	32	64	Blast.
70	7	131					111	20		
71	7	129					52	77		
72	6	108						54*	54*	
73	7	120							120	
74	7	109							104	5
75	7	111							13	98
76	6	95								95
77	7	97								97
78	7	106								106

* At this point it was impossible to tell any morphological difference, therefore data beyond this point were not included in the statistical analysis.

FIRST CLEAVAGE TO 64-CELL STAGE

A total of 23 ova in an uncleaved state were recovered from 13 females at 32 hr and 36 hr after injection of HCG. Seven females were sacrificed at 37 hr post HCG and 105 ova were recovered, of these, 3 had advanced to the 2-cell stage. The appearance of the 2-cell ova at 37 hr was in agreement with the work of Edwards and Gates (13). It can then be concluded that the first cleavage stage begins approximately at 37 hr after injection of HCG and is not complete until about 48 hr after HCG, however, some 80% have cleaved by 38 hr. At 48 hr after HCG injection, 148 ova were recovered and 25 ova were observed to be in the 4-cell stage. The 8-cell cleavage stage was first observed at 59 hr after HCG injection and reached its zenith at 62 hr. Those ova recovered at 62 hr, were predominantly 8-cell stage (96%). One hundred and twelve ova were recovered at 64 hr after HCG injection and 6% had reached the 16-cell stage. The incidence of 16-cell ova increased from 6% to 95% at 68 hr after HCG injection. The 64-cell stage could not be determined because of the similiarity in morphology to the late 32-cell stage. Fifteen per cent of the ova recovered at 70 hr after HCG injection had reached the 32-cell

stage. The results of this phase of experimentation are summarized in Table 1.

IMPLANTATION

Ova were recovered using the "gentle" vs "violent" technique previously described. Implantation was initially observed at 94 hr after HCG injection. This was also verified by observation of blue rings in the uterine wall at 94 hr post HCG. Data concerning implantation can be found in Table 2.

STATISTICAL ANALYSIS

The cleavage rate of ova was determined by the regression method. The data were subjected to both linear and curvilinear regression analysis but the determined F value indicated the data to best fit the curvilinear regression. The average correlation coefficant determined was .9, which indicated the data to be significant. The curvilinear regression can be found in Figure 1.

DISCUSSION

The present study provides broad-spectrum knowledge concerning the fate of ova from hormone-treated female mice. The time of ovulation, first cleavage, subsequent cleavages, up to and including implantation,

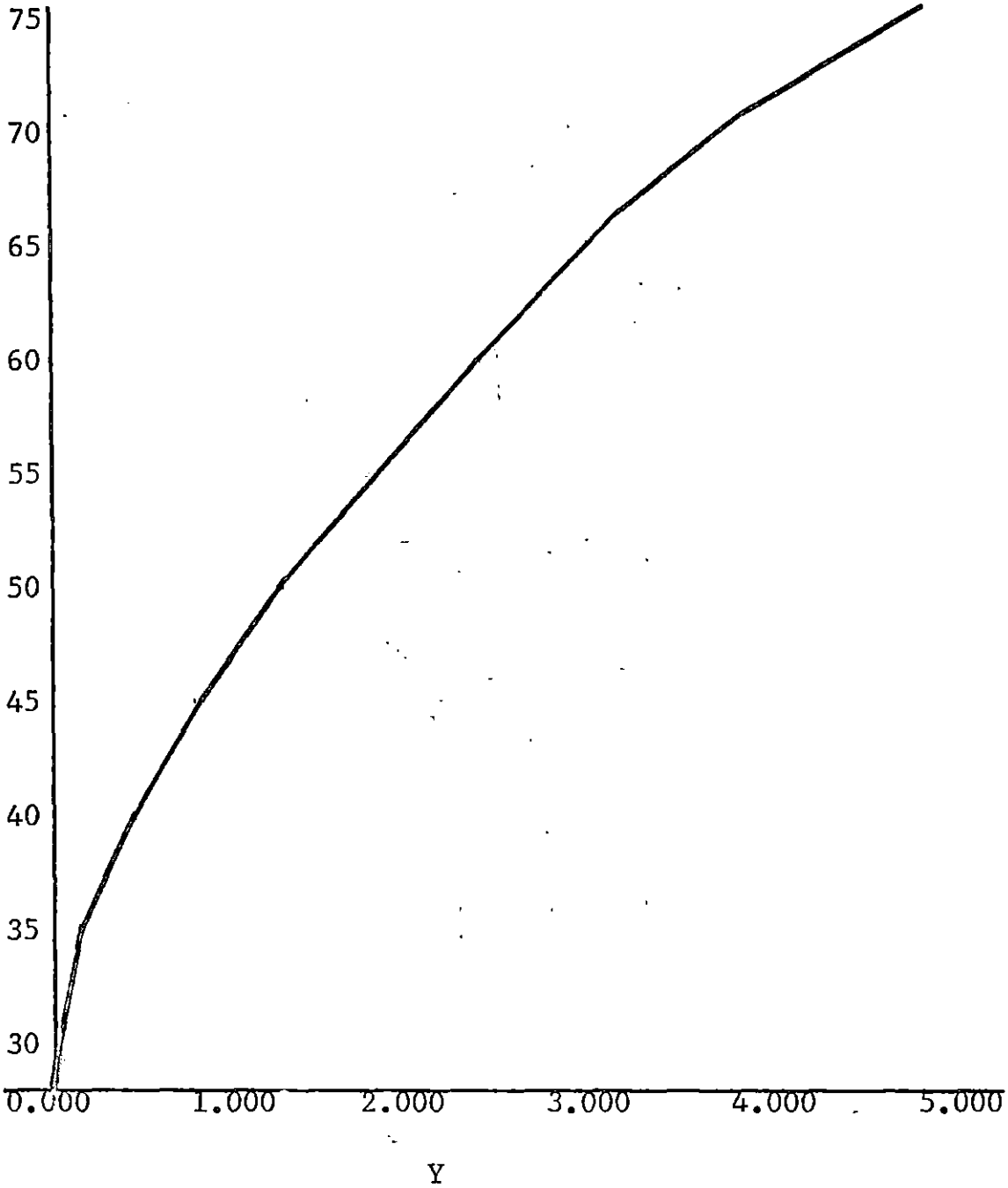

Figure 1. Curvilinear regression of data
X - Hr after HCG injection
Y - Cell stage converted to $\log_2$

can now be predicted.

Knowledge of the exact time of ovulation, cleavage stages and implantation is of obvious importance in many experiments. Cytology and cytogenic studies should be greatly facilitated by the access to large numbers of ova in a needed cell stage. Various treatments of particular cell stages, e.g. radiation damage, freezing damage, and human infertility studies (12) could be simplified.

The results indicate that 6-8 week old mice, induced to ovulate with gonadotrophins, produced an average of 11 ova per mouse. This average is somewhat high in comparison with data compiled by Spears and Walker (52). The use of 5 IU doses of gonadotrophins was superior to the doses used by Edwards and Gates (13) as evidenced by the relatively constant number of ova ovulated. Approximately 75% of the females induced to ovulate became pregnant. Similiar results have been reported by Edwards and Gates (13). The data indicates that superovulation was effected by treatment with gonadotrophins. This is in agreement with the data reported by Cole (9). There was no evidence of loss and fragmentation of ova before and after fertilization as reported by Austin (2).

Ovulation, as judged by the appearance of ova in the oviduct, began at 12 hr and was complete by 14 hr after the injection of HCG. Mating had little effect in advancing the time of ovulation in treated females. The time taken for the ova to move from the ruptured follicle to the ampulla is not great and the error involved in neglecting this interval is probably small. The results give some indication of the accuracy to be obtained in predicting the time of ovulation in hormone-treated females.

The first cleavage division observed at 37 hr after HCG injection verified the data reported by Edwards and Gates (13). The data concerning the development of the 4, 8, 16, 32-cell, and blastocyst stages can best be appreciated if the data is displayed as in Table 1.

The data collected present a consistent picture of the chronology of the pre-implantation stages of development in the mouse. The Evans Blue reaction, an index of locally increased capillary permeability in the implantation areas was positive in the majority of pregnant females at 94 hr after HCG injection. In the present study, this was an indication that the uterus was reacting locally to the presence of the blastocyst. The time of the appearance of the Evans

Blue reaction is consistant with that reported by Finn and McLaren (17). The "gentle" vs "violent" technique used in flushing the uterine horns also indicated implantation to occur at 94 hr after HCG injection. This data can be found in Table 2.

Edema was observed in females positive for Evans Blue dye test. Blastocyst elongation reported by Finn and Hinchliffe (16), was observed in females at 94 hr post HCG. Fragmentation and abnormalities of ova were in abundance after the expected time of implantation. Apparently these ova did not implant and were undergoing abortive processes. The relative ordering of the phenomena studied is shown in Table 3.

Selective measures were utilized in the recording of data. Ova that appeared morphologically normal were recovered in determining ovulation and subsequent cleavage divisions up to blastocyst stage. The frequency of abnormality observed was not significant and was not taken into consideration. A schematic representation of the results obtained in this study is shown in Figure 2.

Table 2. DETERMINATION OF IMPLANTATION BY EVANS BLUE DYE AND FLUSHING TECHNIQUES

Hr after HCG injection	No. of mice	Ova rec. "Gentle" flush	Ova rec. "Violent" flush	No. of blue rings obs. in uterine lumen
90	7	121	0	0
92	6	102	0	0
93	8	132	0	0
93½	6	96	0	0
94	7	41	77	73
94½	7	50	72	67
95	7	28	81	79
95½	6	33	61	67
96	8	64	63	65
96½	8	43	70	65
97	7	47	69	71
98	6	38	54	51
99	8	29	76	76
100	7	46	67	61

Table 3. MORPHOLOGICAL CHANGES IN UTERINE HORNS AND OVA FROM 90 TO 100 HR POST HCG.

Hr after HCG injection	Evans Blue reaction	Edema uterine horn	Elongated ova	Fragmented ova
90	–	–	–	–
92	–	–	–	–
93	–	–	–	–
93½	–	+	–	–
94	+	+	+	–
94½	+	+	+	–
95	+	+	+	+
95½	+	+	+	+
96	+	+	+	+
96½	+	+	+	+
97	+	+	+	+
98	+	+	+	+
99	+	+	+	+
100	+	+	+	+

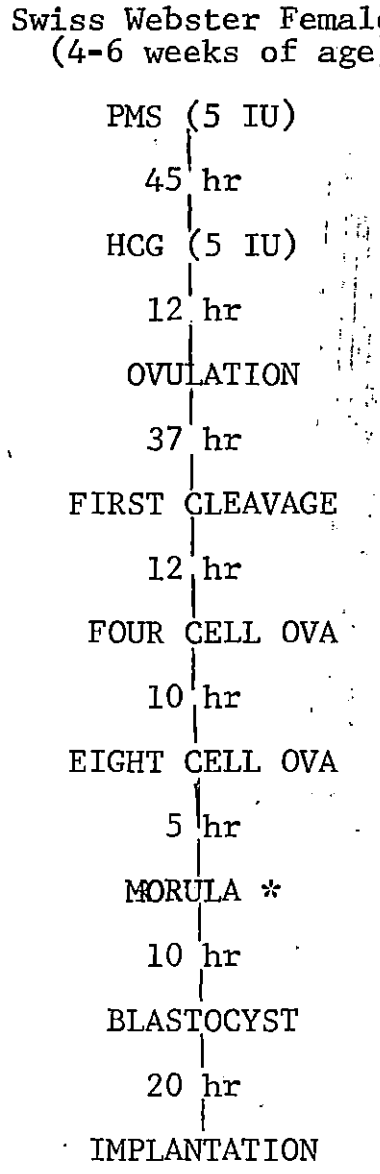

Figure 2. SCHEMATIC REPRESENTATION OF THE RESULTS OBTAINED IN THIS STUDY.

* 16 and 32-cell stages combined.

SUMMARY

The stages of ovulation, subsequent cleavage stages to blastocyst and implantation have been timed in female mice treated with gonadotrophins. Ovulation began as judged by presence of ova in the oviduct at 12 hr after human chorionic gonadotrophin (HCG) and was complete at 14 hr post HCG. First cleavage was observed at 37 hr post HCG and is not complete until 48 hr after HCG, however 80% have cleaved by 38 hr. The 4-cell stage was recovered at 48 hr, 8-cell at 59 hr, 16-cell at 68 hr and 32-cell at 70 hr after HCG injection.

Implantation was initially observed at 94 hr post HCG. In determining implantation, ova were recovered using the "gentle" vs "violent" technique. The results obtained utilizing this method were verified by injections of Evans Blue Dye to indicate the more permeable areas of implantation sites in the uterine wall. The data was subjected to the curvilinear regression analysis and was found to be significant.

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