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Embryo survival, uterine fluids and tubal SEM in progesterone-asynchronized rabbits

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Abstract — Survival of embryos exposed to several concentrations of uterine proteins and changes in tubal morphology in rabbits given low preovulatory doses of progesterone (P_4) that had previously not affected ovulation or fertilization, but caused severe embryo mortality, were studied. In experiment 1, 332 morulae were cultured for 24 h in a control medium containing < 0.5 to > 3.0 mg·mL^{-1} of Day 3 uterine fluid proteins. There was no difference in blastocyst development nor implantation to Day 12 following transfer of the blastocysts to recipients, except fewer implants developed in the BSA control. In experiment 2 the oviducts and uteri of control and P_4-treated does were examined by SEM for 8 days following ovulation. Secretory cells in the oviducts and to a lesser extent in the uteri were stimulated by P_4 treatment for 3 to 4 days after ovulation. Morphology of ciliated cells was unaffected. The subtle changes did not fully account for P_4-induced embryo mortality in vivo.

rabbit / embryo mortality / uterine proteins / SEM

Résumé — Altérations de la survie embryonnaire, de la morphologie et de la fonction de l'oviducte et de l'utérus par traitement de lapines à la progestérone pendant la période préovulatoire. Chez la lapine, l'administration de faibles doses de progestérone qui n'interfèrent pas avec l'ovulation et la fécondation produit une mortalité embryonnaire massive en induisant une asynchronie entre l'environnement utérin et les embryons. Les conséquences d'un tel traitement sur les protéines utérines et la morphologie des trompes en microscopie électronique sont analysées dans cette étude. Dans une première expérience, 332 morulae ont été cultivées pendant 24 h dans un milieu supplémenté avec deux conservateurs de fluide utérin obtenu à j 3 après l'ovulation (< 0.5 à 30 mg·mL^{-1}). Aucune différence dans la formation de blastocytes ni dans le taux d'implantation observé à j 12 après transfert n'a pu être mis en évidence. Dans la seconde expérience, les oviductes et les utérus de lapines traitées ou non à la progestérone ont été examinées en microscopie électronique pendant les 8 jours après l'ovulation. Les cellules sécrétoires de l'oviducte et également de l'utérus ont grandi plus rapidement chez les lapines traitées à la progestérone, mais aucun effet de traitement n'a été observé pour les cellules ciliées. Il est improbable que de telles modifications soient responsables de la mortalité embryonnaire massive observée après traitement à la progestérone.

lapine / embryon / survie / protéines utérines / microscopie électronique

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1. INTRODUCTION

Administration of a low dose of progesterone that does not block ovulation, sperm capacitation, fertilization, or initial development of the rabbit embryo results in almost complete embryonic mortality by Day 12 of gestation [1]. This preovulatory administration of progesterone alters uterine protein secretory patterns [23, 32], and exposure of embryos to asynchronous uterine fluid secreted particularly at Days 3 to 4 of pregnancy, can cause embryonic death [23]. The importance of the progesterone-induced asynchrony on Days 3 to 4 after ovulation is supported by the observed detrimental effect on Day 3 and 4 embryos temporarily exposed to Day 3 and 4 progesterone-primed rabbits, but not by exposure of these embryos to Day 3 and 4 controls [1, 29].

Protein content of uterine fluids of pregnant rabbits increases during preimplantation, with a major decrease in albumin and an increase in uteroglobin on Days 3 and 4 postovulation [2, 23, 32]. McCarthy et al. [23] reported that exposure of Day 3 morulae to 1-day asynchronous uterine fluid proteins was detrimental compared to controls. Low concentrations of amino acids [20] in culture media are essential for rabbit blastocyst formation, and Maurer and Beier [22] reported that 0.1 to 0.8 $mg \cdot mL^{-1}$ of uterine protein fractions obtained 5 and 6 days postovulation were equally effective in promoting morulae to develop into blastocysts. Concentrations of 1.0 to 3.2 $mg \cdot mL^{-1}$ of individual fractions were essentially ineffective. In contrast 2.0 $mg \cdot mL^{-1}$ of unfractionated uterine protein (from Day 6 pregnant does was superior to 0.5 $mg \cdot mL^{-1}$ in promoting development of 8- to 16-cell embryos into blastocysts). Collectively, these studies indicate that synchrony between protein mixtures and embryo age early in development is important, but protein concentration and other factors, in addition to asynchrony, may be responsible for the observed detrimental effect of the premature presence of progesterone on embryo development.

Transport of the embryo to the uterus, as this affects fertility, has been extensively reviewed [12, 14]. Hormonal imbalances, including elevated progesterone concentrations, have been reported to increase the rate of transport of the embryo through the oviduct to the uterus [5, 11]. However the low dose used by McCarthy et al. [23], and used here was found to slightly delay entrance of the embryos into the uterus. Any alteration in transport could increase the asynchrony between the developing embryo and the uterine environment.

Another possible factor affecting embryo survival could be changes in the tubal surface epithelium as it interacts with the developing embryo. Scanning electron microscopy has been used to detect changes in the surface epithelium of the rabbit female reproductive tract under various hormonal conditions [3, 8, 12, 24, 28, 30, 33]. Although, some SEM observations have been made in the rabbit under control and progesterone-modified conditions [15, 24], no systematic investigation has been reported under conditions allowing fertilization, but associated with embryonic mortality.

The objectives of the present studies were to investigate the effects of several concentrations of Day 3 uterine fluids that previously were associated with impaired embryo development in vitro, and to utilize scanning electron microscopy (SEM) to determine if differences in the morphology of the surface epithelium of oviducts and uteri existed that could provide a partial explanation for the embryo mortality previously observed as a result of the preovulatory progesterone treatment. Animals were treated with progesterone in a protocol identical to earlier studies causing embryonic death so that the new information could be compared with the previous experiments.

2. MATERIALS AND METHODS

2.1. Animals

Mature Dutch Belted rabbits, raised in our colony in separate cages at 21 °C under

a 12/12 h light to dark cycle, were used. They were fed pelleted feed, and given fresh water ad libitum. The rabbits weighed approximately 2 kg.

2.2. Experiment 1

This experiment was designed to study the effect of < 0.5 to ≥ 3.0 mg·mL^{-1} of proteins found in uterine fluids, collected from pregnant rabbits 3 days after ovulation, on embryos collected 3 days after ovulation and insemination of the donors. The range of concentrations exceeded the differences previously reported [23] for uterine fluids collected from control and P_4-treated does on Day 3 that were associated with embryo toxicity of Day 3 morulae cultured in fluids from Day 3 P_4-treated does. The does were inseminated with semen collected from fertile males, and ovulated with an intravenous injection of LH [34] at the time of insemination.

Embryos from 52 donor rabbits were flushed with BSMII without BSA [16], and examined microscopically to eliminate any unfertilized oocytes. Embryos at the morulae stage were cultured in nine media consisting of BSMII with and without BSA and several concentrations of uterine fluids. Ten embryos in 1 mL of medium were cultured for 24 h with 5% CO_2 and 95% air in a humidified incubator at 37 °C.

The uterine fluid was collected into a centrifuge tube from 3-day pseudopregnant does by flushing BSMII consecutively through the uterine horns of a series of does. The uterine fluids were centrifuged and filtered to remove any debris, refrigerated at 5 °C and used in the culture medium within 24 h. Protein content was measured by the procedure of Lowry et al. [21].

Subsequently the incubated embryos were transferred to 25 recipient does ovulated with LH 4 days previously. Five embryos from each of two treatments were transferred to opposite uteri of each recipient and recorded so as to identify the treatment associated with implants 8 days later.

Laparotomies were performed on Day 12 of gestation. The number of implantations in each uterus was counted, and recorded by treatment.

2.3. Experiment 2

This experiment was designed to examine by SEM the reproductive organs of females treated with doses of progesterone used previously to prevent development of rabbit embryos [1, 23]. Thirteen rabbits received preovulatory progesterone injections, while 16 served as controls. Progesterone was dissolved in sesame oil and treated females were given subcutaneous injections of 0.5 mg on Day –2, 1.0 mg on Day –1 and 1.0 mg on Day 0. Day 0 was the day LH was given to induce ovulation [34]. The control rabbits received subcutaneous injections of the vehicle, sesame oil, following the same schedule.

On Days 1 through 8 the rabbits were euthanized and the reproductive tracts of the rabbits were removed. Immediately thereafter 4 mm sections from the ampulla of the oviducts, 2 × 4 mm pieces of the uterus, and whole cervices from each animal were fixed in 4% glutaraldehyde in phosphate-buffered saline (PBS). After at least 24 h of fixation the tissue samples were rinsed in PBS and prepared for drying by trimming the samples of uterus and cervix and by cutting the sections of oviduct longitudinally. All the samples were dehydrated through a series of 25%, 50%, 75%, 90% and 100% acetone. The dehydrated specimens and 100% acetone were placed in a Sorvall CO_2 critical point drying apparatus. Following critical point drying the samples were mounted on aluminum stubs with double stick tape. They were coated with a 60:40 mixture of gold and palladium in a vacuum coater. Specimens were examined at various magnifications using an Hitachi HHS-2R scanning electron microscope operated at 20 kV.

Many pieces of tissue were examined and photographed at various magnifications. Photographs of control tissue and tissue from the progesterone-treated group were used to quantify the proportion of ciliated cells. Also cell diameter at the different stages was measured in two directions at right angles to each other and the average diameter calculated.

3. RESULTS

3.1. Experiment 1

A total of 332 morulae were cultured in BSMII (no BSA) with varying concentrations of uterine fluid. Also, two control media, consisting of BSMII without protein and with 15 mg·mL^{-1} of purified, 4 × crystallized BSA were included (Tab. I). Blastocysts from 7 of the 8 treatments were transferred to recipients and the percentages of blastocysts that were implanted on Day 12 were obtained (Tab. I). Only seven blastocysts were transferred in the medium with 1.0–1.4 mg·mL^{-1} of uterine proteins, and none implanted. The failure to implant was not likely related to this concentration of uterine proteins, as the implantation rate was otherwise unaffected by protein concentration ($P > 0.05$).

3.2. Experiment 2

Random samples of the ampullary portion of the oviducts of 23 control and progesterone-treated rabbits and 59 samples of uterine epithelium from control and treated does, as well as corresponding cervical samples were examined by SEM. Considerable variation was noted among samples in the proportion of ciliated cells in each group. The results are tabulated in Tab. II. There was no difference in the proportion of ciliated cells within either location in control or progesterone-treated animals over the 8-day period ($P > 0.05$). However, there were about twice as many ciliated cells in proportion to nonciliated cells in the oviducts as there were in the uterus ($P < 0.05$).

The secretory cells of the oviduct appeared to reflect an increased secretory activity while the embryos were traversing it during Days 1 to 3. On Days 2 and 3 the secretory cells protruded more. The protrusion of the secretory cells was especially prominent by Day 3 in the P_4-treated animals and microvilli were less prominent than in the controls (Figs. 1A and 1B). Subsequently, these cells tended to flatten and the microvilli were less apparent. Also the diameter of 220 secretory cells was measured by averaging two diameters taken at right angles to each other. The diameter of

Table I. Experiment 1. Effect of Day 3 uterine fluid proteins on development of Day 3 embryos cultured for 24 h at 37 °C, and then transferred to Day 4 recipients.

Uterine proteins or BSA (mg·mL^{-1})[a]	Number of morulae cultured	Blastocysts formed (%)	Number of blastocysts transferred	Implants (%)
0	63	37[b]	23	44[b]
< 0.5	77	75[b]	39	54[b]
0.5–0.9	23	0[c]	contaminated	– –
1.0–1.4	15	47[b]	7	0[c]
1.5–1.9	32	69[b]	12	33[b]
2.0–2.5	30	73[b]	21	48[b]
≥ 3.0	42	62[b]	15	80[b]
15.0 BSA	50	66[b]	33	21[c]

[a] Complex medium BSMII without BSA was the basic medium to which proteins were added.
[b,c] Percentages within columns with different superscripts differ.

Table II. Experiment 2. The proportion of ciliated cells in the oviductal ampulla and the uterus of control and progesterone-treated rabbits.

Days after ovulation	Ciliated cells, mean ± S.E.[a]			
	Oviductal ampulla		Uterus	
	Control	P_4-treated	Control	P_4-treated
1	50	– –	29	48
2	50	38 ± 1.5	30 ± 3.1	51 ± 28
3	47 ± 9	58 ± 0.5	< 1 ± 0.3	16 ± 4.8
4	76	63	11 ± 6.6	8
5	63	64 ± 1.5	23 ± 6.0	29 ± 10
6	59 ± 1.7	54	88 ± 4	28 ± 5
7	– –	– –	22 ± 4	25 ± 4
8	39	73 ± 1.0	Implant site	31 ± 15
Mean	56[b] ± 5.3	58[b] ± 4.9	25[b] ± 11.2	30[b] ± 5.1

[a] The number of micrographs examined per subclass varied from 1 to 4. With 1 subclass there was no S.E.
[b] Treatment means within tissue location did not differ ($P > 0.05$).

the secretory cells in P_4-treated does enlarged more rapidly than in the controls, possibly associated with cell fusion [30], with their average diameter exceeding controls by 18% up to Day 4 ($P < 0.05$). After Day 4 the control and treated groups did not differ significantly ($P > 0.05$) in size. No difference in the appearance of the ciliated cells was detectable between the two groups initially or over the 8-day period.

The ciliated cells in the uterus were less numerous than in the oviducts. The nonciliated cells of the uterine endometrium exhibited an increase in cellular activity during the first few days postovulation. A few small blebs of secreted material can be seen in Figure 1C. There were no striking differences between treated and control groups (Figs. 1C, D, E and F), and the appearance of cells with microvilli suggests they were nearing the end of their secretory stage. This was unexpected as the proteins in uterine fluids increased at this time [23].

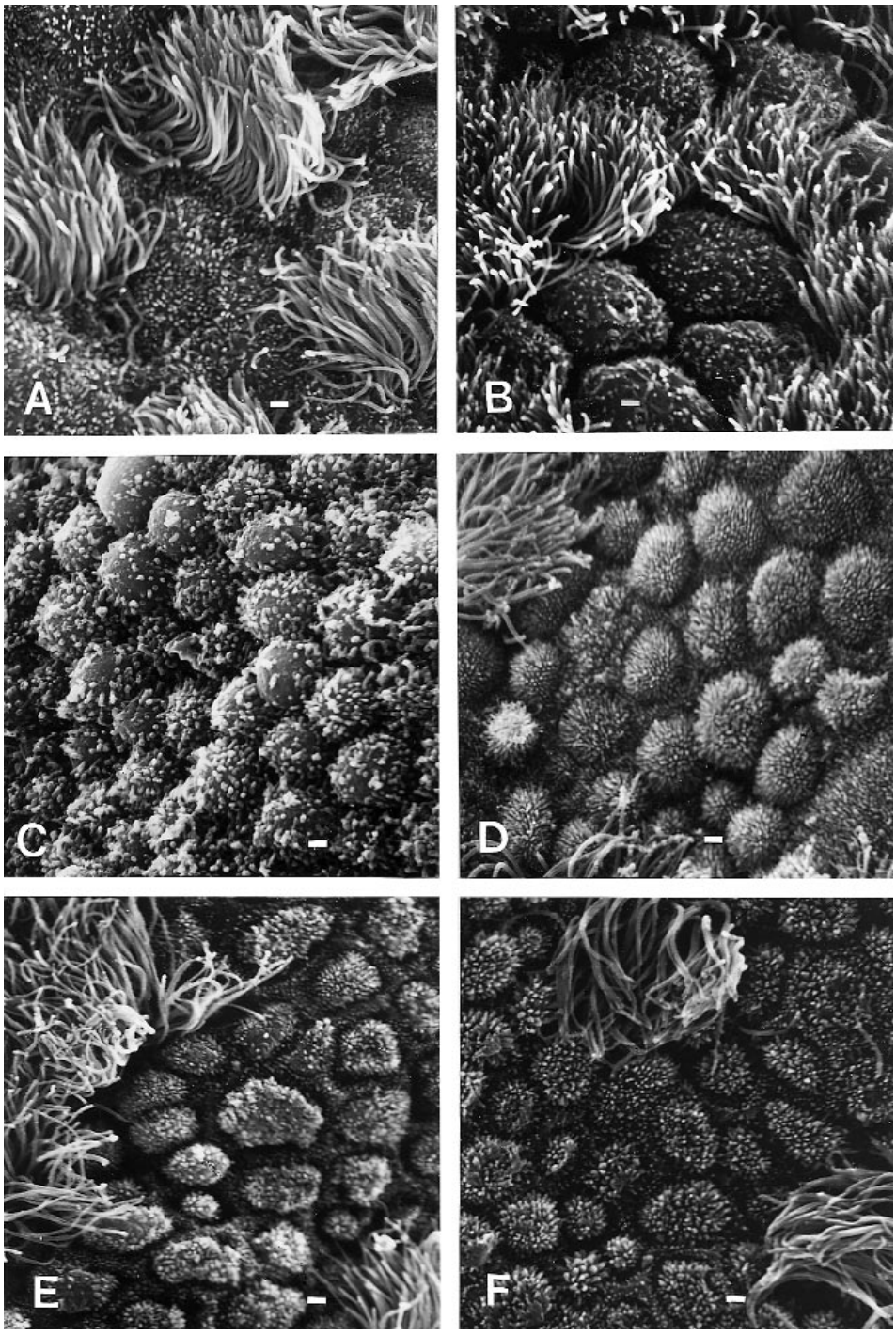
The cervical epithelium was mostly ciliated, as reported by others [7, 15, 26]. Appearance was similar in samples taken at all stages and in control versus treated rabbits.

Embryos collected from the rabbits at different stages were selected at random for study by SEM. By Day 4, and thereafter, the embryos were enlarging, particularly in the control group. When maximal and minimal diameters were measured in 10 embryos from each of the two groups on Day 4, nine of the embryos from the control group had larger average diameters. However, there was great variation, partly due to shrinkage during preparation for SEM. Because of this shrinkage, artefacts of the embryo volumes were not quantified. There was no difference in the appearance of the zona pellucida or remnants of the mucin coat covering the surface of the blastocysts. This covering precluded examining the blastomeres by SEM in the early stages.

4. DISCUSSION

4.1. General

In the studies with low doses of P_4 which did not block ovulation or fertilization, Allen and Foote [1] reported that blastocyst development in vivo was inhibited by Day 4 and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implantation severely reduced by Day 12, regardless of whether or not the embryo was removed from treated does and transferred to untreated synchronized recipients. This stage is a critical one in the initial allocation of cells to the inner cell mass [9]. Additional studies with the same preovulatory doses of P_4 [23, 29] provided further evidence that the treatment resulted in asynchrony between the embryo and uterus or uterine proteins of recipients. However, Maurer and Beier [22] and Schacht and Foote [29] reported that morulae exposed in vitro to uterine proteins from or in vivo to uteri of does 5 days postovulation, had little effect on embryo survival. Uterine protein secretion increases during early stages of pregnancy, but the potential effect of protein concentration on early embryo survival was not tested.

4.2. Experiment 1: Embryos cultured in uterine proteins

Uterine fluid proteins increased during the first 5 days of pregnancy in control and P_4-treated rabbits to a maximum of 3.39 mg of protein per doe [23]. Based upon these data and the protein concentrations reported by Kulangara [18] the in vitro study conducted here was designed to cover an extensive range of protein concentrations. There was no advantage of including more than 0.5 $mg \cdot mL^{-1}$ of uterine protein in the culture media, nor were the higher concentrations toxic (Tab. I). Furthermore, Liu et al. [20] demonstrated that 100% blastocyst formation from zygotes cultured in vitro was possible when a simple protein-free medium included an amino acid supplement totaling 0.36 $mg \cdot mL^{-1}$ of culture medium. These blastocysts developed into normal young [19]. Also, the 15 $mg \cdot mL^{-1}$ of BSA included in BSMII (Tab. I) was less effective in promoting blastocyst development than < 0.5 $mg \cdot mL^{-1}$ of uterine proteins. Thus, the substantial increase in protein in uterine fluids [2, 23, 32], as blastocysts start to grow in the uterus, is not a prerequisite, per se, for development of blastocysts capable of implantation, but may be associated with uterine preparation for ensuing pregnancy. Furthermore, the developing embryos appear to tolerate a wide range of concentrations of amino acids and proteins (Tab. I; [20, 22]), as increasing concentrations were not inhibitory. The higher concentrations of proteins tested included individual proteins at concentrations similar to those found in P_4-treated does at this same stage, Day 3 postovulation [23]. Thus, the toxic effects of one day asynchrony reported previously [1, 23, 29] do not appear to be caused primarily by changes in protein concentration. In fact, if uterine fluid volume is only a few microliters [18], concentration of proteins in the uterus of pregnant does may be much higher than tested here.

4.3. Experiment 2: SEM studies

The blastocysts examined by SEM were mostly contained within the zona pellucida, and surrounding mucin coat. No difference in this surface was seen between embryos collected from control and P_4-treated does. An occasional break in the zona pellucida, as they shrank during preparation for SEM, did not reveal anything unusual. Blastomere surfaces were similar to those reported by Koyama et al. [17]. The size of the shrunken embryos was not quantified, but

Figure 1. All figures are × 3500. The bar = 1 μm. Day 3 control oviductal cells (**A**) are compared with the more prominent secretory oviductal cells of Day 3 P_4-treated rabbits (**B**). Areas selected here have more secretory cells than the average. Uterine views from Day 3 control (**C**) and P_4-treated rabbits (**D**), and Day 4 control (**E**) and P_4-treated (**F**) rabbits. The nonciliated cells did not reveal the expected increase in secretory activity in P_4-treated animals (**C** and **F**). Areas shown have a higher proportion of secretory cells than the average.

the ones examined by light microscopy from P_4-treated does tended to be smaller by Day 4 [1, 29]. In a similar earlier study [29] embryos collected from P_4-treated does were only 32% the size (estimated volume) of control embryos. This is consistent with the subsequent embryonic death reported for embryos exposed to uterine secretions of P_4-asynchronized rabbits [23].

Examination of sections from the oviduct revealed considerable variation among locations within oviducts in the proportion of ciliated to secretory cells, as reported by others [3, 8, 33]. There was a slight preponderance of ciliated to secretory cells, with no significant change observed from Day 2 to Day 8 (Tab. II). Kanagawa et al. [15] reported about 50% ciliated cells in the oviduct. Likewise, there was no qualitative difference in the appearance of the cilia over time or treatment. This is consistent with the fact that estrogen is the hormone primarily influencing growth of cilia and estrogen therapy in ovariectomized rabbits reinitiates ciliary growth rapidly [3, 28]. There was no observed difference in ciliated cells detectable by SEM during the time that the embryos were in the oviducts.

The rate of transport of the embryos into the uterus was not timed precisely, but the tendency was to recover fewer embryos from the uterus of P_4-treated does, suggesting retarded transport. This was consistent with previous studies [1, 23]. The acceleration of embryo transport [5, 6] is associated with higher doses of P_4. Movement of embryos involves both ciliary and muscular action of the oviduct in a complex fashion not fully understood [12, 13, 31], as well as rate of passage of embryos through the ampullary-isthmic junction [27].

The secretory cells of the oviduct were protruding by Day 2 with a moderate number of microvilli. This pattern continued on Day 3 (Figs. 1A and 1B), with protruding cells, particularly in the P_4-treated group. Secretory granules have been observed discharged into the lumen on Days 2 and 3 [4], and some were observed in both control and treated groups. Progesterone increases secretion of mucin by these cells [10], but the thickness of the mucin coat was not affected by P_4 [29]. Thereafter the secretory cells tended to be flatter with fewer microvilli. This indicates that the secretory activity of the oviduct is greatest during the period of embryo transport through the oviduct.

The uterus contained only about half as many ciliated cells in proportion to secretory cells when compared to the oviduct (Tab. II). The only prominent treatment difference observed by SEM over the 8-day period was the increase in microvilli during the first 5 days, particularly in the P_4-treated does (Figs. 1C–1F). The proportion of ciliated cells tended to decrease with time, as reported by others [8, 30].

Blood P_4 is elevated by P_4 administration [23], and P_4 stimulates secretion by the secretory cells of the uterus, as indicated by transmission electron microscopy [4, 8], and by secretion of uterine proteins [2, 15, 22, 23]. The SEM studies revealed an increase in secretory activity the first few days after ovulation, consistent with the secretory patterns reported previously for uterine proteins [2, 15, 22, 23], but differences between P_4-treated and control animals was small. However, the fact that the oviductal secretory cells appeared to be more active in the P_4-treated does could indicate that some of the early increase in uterine proteins [2, 22, 23] may result from possible drainage of oviductal fluids into the uterus at the time of embryo passage. By Day 7 and 8 more variability among locations in the uteri was encountered, associated with the preparation of embryos for implantation. This was particularly noticed in the controls, with more advanced blastocysts. Also, it was more difficult to collect embryos from the controls probably because these embryos were beginning to implant. Areas appearing to be implantation sites were avoided in the SEM studies.

The cervix was not studied in detail as this organ was not relevant to the study of

early embryo development. However, the lack of any substantial differences within this study is consistent with studies demonstrating that estrogens and not progesterone primarily affect cervical morphology and secretion [7].

5. CONCLUSIONS

The preimplantation development and implantation of embryos obviously is a complex process. Interference with development may reflect both immediate and latent effects of an improper environment. The ovulation and fertilization rates were not affected and appear not to have a latent effect, as embryos produced in or temporarily exposed to the uteri of P_4-treated does implanted at similarly depressed rates compared with controls [1, 23, 29]. The toxic effect was associated primarily with some asynchrony during Days 3 and 4 postovulation, as embryos up to Day 2 were not affected by P_4-treatment of does [29], and after Day 4 various experimentally imposed asynchronies had less effect on development [22, 23, 29]. The proportions and concentrations of proteins in uterine fluids change considerably during Days 3 and 4 [2, 23]. However, the previous studies and data presented here on blastocyst formation do not fully account for the implantation failure of embryos exposed to the low dose P_4-treated rabbits, or varied concentrations of uterine proteins. The thickness of the mucin coat has been reported to affect implantation [25], but the preovulatory P_4 treatment of does did not affect the thickness of the mucin coat [29]. The low dose P_4 treatment tended to slightly delay embryo transport into the uterus in all studies, and this could increase slightly the asynchrony between embryo development and the uterine environment. However, 1 day asynchrony between Day 4 blastocysts and Day 3 recipients had no effect on development [29].

Giles and Foote [9] reported that major allocation of embryonic cells to the inner cell mass and trophectoderm occurs in the rabbit during this critical period. This partitioning of progenitor cells of fetal and placental tissues in P_4-treated rabbits has not been studied, and future research may determine this to be a useful model for examining embryo versus placental defects on implantation failure.

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