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IN VITRO CULTURE OF MOUSE SPERMATOZOA, OOCYTES, AND EARLY EMBRYOS IN THE PRESENCE OF AN INTRAUTERINE DEVICE

The Ohio State University

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IN VITRO CULTURE OF MOUSE SPERMATOZOA, OOCYTES, AND EARLY EMBRYOS IN THE PRESENCE OF AN INTRAUTERINE DEVICE

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree doctor of Philosophy in the Graduate School of The Ohio State University

By


*****

The Ohio State University

1984

Reading Committee:

H. C. Hines, Ph.D.
G. R. Wilson, Ph.D.
H. L. Barr, Ph.D.
T. L. Ludwick, Ph.D.

Approved By

H. C. Hines, Ph.D.
Department of Dairy Science
Dedicated to my wife, Anne, and my two daughters, Sarah and Hannah
ACKNOWLEDGEMENTS

This research project is the culmination of a group effort. Immeasurable advice, assistance and support from the numerous graduate and technical personnel of the Cattle Blood Typing Laboratory and The Midwest Reproductive Institute enabled this author to develop a stronger, and more comprehensive, study.

To Mary Sakey, Administrative Supervisor: Thank you for the unselfish time and dedication that it took to edit, type and print this dissertation. The superb final quality is largely a product of your extra efforts.

To Claudia L. Jenkins, Andrology Supervisor: Particular gratitude is expressed for your help in developing and providing laboratory protocols and discipline that were essential for the establishment of experimental consistancy and quality control.

To my wife, Anne, and my parents, Barbara and Charles Paschall: Their extraordinary patience and dedication provided the necessary catalyst, and impetus, to continue this project through its initial conception as well as its many years of development.
To William C. Baird, Ph.D., Co-Director, The Midwest Reproductive Institute: My sincere gratitude and personal thanks for helping to shoulder, expand and improve many of my daily work responsibilities. Without this assistance, the extra time necessary to continue my project would have been impossible to find.

To Nichols Vorys, M.D.: Your unselfish personal and intellectual guidance, in addition to your unquestioning financial support and moral leadership, has been of very special importance to me.

To Professor T.M. Ludwick, Ph.D.: A special thank you for providing the continuous, and much needed, encouragement and personal support that I required throughout my graduate education.

I would like to extend my warmest appreciation to my academic advisor, Professor H. C. Hines, Ph.D., for his constant guidance, professional leadership and his continued enthusiastic interest. I am extremely grateful for the faith that Dr. Hines placed in my ability to develop, and independently pursue, a research project of very special personal interest.

The close professional association with both of my major Professors, as well as their colleagues, has given me a clear, and awesome, understanding of words academic freedom, integrity, and responsibility.
VITA

November 19, 1949 ........... Born - Wilmington, Delaware

1971 ........................ B.A. in Economics, Tufts University
Medford, Massachusetts

1973-1974 .................... Laboratory Technician, College of
Veterinary Medicine, The Ohio State
University, Columbus, Ohio

1976 ........................ M.Sc., Department of Dairy Science,
The Ohio State University,
Columbus, Ohio

1976-1978 ..................... Research Assistant, Department of
Dairy Science, The Ohio State
University, Columbus, Ohio

1978-1979 ..................... Academic Advisor, University College
The Ohio State University,
Columbus, Ohio

1979-Present  ................ Reproductive Endocrinology and
Immunology Consultant, Infertility
and Gynecology, Inc., Columbus, Ohio

1983-Present  ................ Co-Director, The Midwest Reproductive
Institute, St. Anthony's Hospital,
Columbus, Ohio

FIELDS OF STUDY

Major Field: Reproductive Immunology and Endocrinology
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INTRODUCTION

There is a general consensus that the contraceptive effectiveness of an intrauterine device (IUD) is the result of a combination of several different phenomena. A number of these phenomena may be generic to any foreign body within the endometrial cavity while others are probably unique for a specific IUD. IUDs have demonstrated an antifertility effect in every species tested, but that the stage of the reproductive process influenced by their presence differs from model to model. It is not possible, therefore, to explain the mechanism of action in a singular manner that applies to all species. Several comprehensive reviews have discussed in detail a variety of observations that have been documented to occur in both animals and humans as a result of an IUD (Davis, 1971; Eckstein, 1971; Wallach and Kempers, 1979).

These observed differences in action are due in part not only to variations in the anatomic and physiologic features of the different reproductive systems studied but also to the marked variation in the size, configuration, placement and composition of the devices themselves. The principal contraceptive action of the IUD is still considered to reside primarily within the uterus, rather than within the tube or the ovary. The block to pregnancy induced by the IUD is therefore
thought to be exerted after the embryo has left the oviduct and before, or during, its attachment to the endometrium. Although specific modalities probably involve the creation of an ovotoxic environment and/or an inhibition of nidation, IUDs have also been proposed to: (1) stimulate uterotubular motility leading to premature expulsion of the fertilized ova or blastocyst from the reproductive tract; (2) initiate mechanical trapping of the ova, or "tube locking"; (3) reduce the life span and function of the corpus luteum (CL), thereby, depriving the embryo of progesterone essential for implantation; (4) inhibit or modify the decidual transformation of the endometrium in preparation for nidation; or (5) provoke a sterile inflammatory reaction in the endometrium in such a way as to render the uterine cavity toxic to preimplantation embryos. In addition, phagocytic elements in the cellular exudate may also alter sperm function to a point of interfering with fertilization (Eckstein and Hurst, 1978).
Animal investigations have shown a myriad of effects that IUDs can exert depending on the experimental design and the particular species in question. Clarification of the mechanisms responsible for IUD efficacy has proceeded slowly in part because of the many possible IUD effects requiring further in depth exploration. Corfman and Segal (1968) have provided an excellent review that discusses the variations of specific species with regard to experimental IUDs as well as their probable mechanisms of action.

Species Differences

A. Mouse. The placement of a length of thread (usually silk or nylon) in a segment of one horn of the bicornate uterus of the mouse prevents the establishment of nidation sites throughout the entire length of the occupied horn and renders the contralateral (control) horn comparatively infertile as well. The degree of infertility experienced in the contralateral horn depends upon the species of mouse investigated. In a study performed by Marston and Kelly (1969), conceptuses were not found in any of the IUD uterine horns and few were
present in the control horns of Swiss, CBA, and CBA x C57 mice. However, in C57 mice, normal conceptuses were developing in the control uterine horn and accounted for an implantation rate of 47 percent when compared with the number control corpus luteum (CL). The report concluded that a unilateral IUD exerted an almost complete, bilateral contraceptive effect in the first three strains of mice, but only a unilateral effect in the fourth.

The basis of the low fertility on the side not operated upon is unknown, although it is presumed to be mediated via a humoral factor transmitted from the IUD bearing horn; a phenomenon that is anatomically possible in the mouse because of an incomplete septum between the two horns at the cervical junction. This same study demonstrated that once the two horns had been completely separated from each other (following mating), CBA and CBA x C57 strains of mice were able to establish normal pregnancies in the control (contralateral) uterine horn.

B. The Rat. Foreign bodies in the bicornate uterus of the rat have a less extensive effect than they do in mice (Doyle and Margolis, 1965). Threads of silk, or nylon, will prevent blastocyst nidation in the horn in which they are inserted, but usually have no antifertility effect within the contralateral untreated horn. A foreign body must lie freely in the endometrial cavity in order for the device to be effective. If the thread lies only in the myometrium, it exerts no demonstrable antifertility effect. Surgical anastomosis between the uterine horns of the rat will allow a single suture to have a bilateral
effect, again suggesting the transfer of a humoral mediator as in the case of the mouse. There appears to be no effect on the estrous cycle and sperm transport is not believed to be altered. Removal of the IUD is associated with rapid restoration of normal fertility. These findings indicate that an IUD may induce reversible, or transient, alterations in the uterine environment that are hostile to the fertilized ova.

C. Hamster and Guinea Pig. Similar to the rat, the hamster responds to the presence of a foreign body in the uterus by the failure to manifest blastocyst nidation in the treated horn. Cycle length, ovulation, sperm transport, and fertilization are not significantly affected (Bland and Donovan, 1966).

The reproductive cycle of the guinea pig differs from that of the mouse, rat, and hamster by the occurrence of a prolonged functional luteal phase. IUDs have been found to inhibit CL function in guinea pigs much as they do in sheep and cattle. Such inhibition is not observed in other rodents, possibly due to the fact that the estrous cycle (lack of a true luteal phase in the absence of a pregnancy or pseudopregnancy) in such animals is comparatively short. In guinea pigs only the corpora lutea of the ovary on the side of the uterus bearing the foreign body are affected. It appears, therefore, that the leukolytic effect of the IUD is mediated locally rather than systemically.
D. Rabbit. Some of the earliest laboratory work on the antifertility effect of intrauterine foreign bodies was performed on the rabbit. The device is thought to affect fertility by interfering with normal nidation. Ova are released, fertilization occurs, tubal transport is normal, implantation takes place on both the treated and untreated side, and most embryos, not immediately adjacent to the device, proceed to term. However, embryos in close proximity to the IUD were usually viable for only seven days postcoitus (pc.).

Evidence also exists for a systemic effect in the rabbit, probably neurogenic in nature, by which the IUD alters the hypothalamo-hypophyseal complex with the discovery that there is prolongation of several hours in the postmating interval before ovulation occurs. Although this does not explain locally observed antifertility effects, the mechanism, which has been proposed for such delay, is a temporary prevention of the LH surge.

E. Sheep. The effect of an IUD in the ewe is unique among the mammals studied to date, since it appears to block sperm transport. When a device is placed unilaterally, no sperm were found in either oviduct after natural mating.

The device apparently does not affect ovulation or egg transport, but it does influence the size of the CL. When a CL develops on the same side as a unilaterally placed IUD, the CL is smaller than normal. The administration of human chorionic gonadotrophin (hCG) was found to overcome this inhibitory effect. If the CL develops on the ovary
contralateral to the IUD it is also normal in size. These observations indicate that IUD in sheep exert a distinctly local effect on the ipsilateral corpus luteum.

F. Swine. Studies with IUDs in the gilt have shown that ovulation and fertilization occur normally and that the estrous cycle length is unchanged, but that the device inhibits full development of CL. Although the principal effect on fertility appears to involve the survival of pre- and post-implantation embryos, like the rabbit, the effect was also observed bilaterally, as with the mouse, and was dissimilar to the more restrictive effects seen in sheep and cattle.

G. Cattle. The cow responds to the placement of an intrauterine device in a similar fashion to the ewe and sow. Ovulation and ova transport are normal, but the life span of the CL is shortened. CL inhibition occurred when the device was located on the ipsilateral side. These findings continued to support the contention that there was a local factor transmitted from the affected horn to the adjacent ovary.

H. Primates. An early report by Vorys et al. (1965) suggested that the human menstrual cycle was shortened during the first few months after the insertion of an IUD, after which normal cyclicity was resumed. No changes, from expected patterns, were found in
measurements of urinary FSH and LH. Further documentation of normal ovulatory cycles was indicated by studies utilizing endometrial biopsies, urinary progesterone levels, and visualization of CLs at laparotomy. Histochemical studies of ovaries from women with IUDs revealed no significant alterations in lactic dehydrogenase, succinic dehydrogenase, or glucose-6 phosphatase levels.

Tubal patency tests establish that the presence of an IUD does not cause mechanical obstruction of the oviduct. Normal motile sperm have been found in oviducts and uteri of women bearing IUDs at least 24 hours following coitus. Effects on normal fertilization processes are not known.

Although some evidence exists in female monkeys (macaca mulatta) that the tubal transport of ova may be accelerated, the principal contraceptive effect of IUDs in primates is focused upon the endometrium. Bacterial contamination occurs universally after insertion. Chronic infiltration of the endometrium with plasma cells and lymphocytes almost always occurs. There is tissue edema, stroma fibrosis, and increased vascularity in tissues directly adjacent to the device.

In summary, sperm transport in sheep is inhibited so that fertilization does not usually occur. In the guinea pig, cow, and ewe, function is impaired to varying degrees. This effect appears to be unilateral and local rather than bilateral. In the sow, luteal size is bilaterally decreased. In animals such as the guinea pig, cow, and pig
in which sperm transport is not affected, as it is in sheep, fertilization can be documented. IUDs do not inhibit sperm transport and fertilization in rodents and rabbits, but they do inhibit implantation within increasing effectiveness as one progresses from the rabbit, through the rat, to the mouse. Although systemic documentation of infertility, as a result of an IUD, is rare, both rabbits and sheep exert altered systemic levels of LH, consequently interfering with potentially normal ovulatory procedures. Studies in the human and rat also indicate that altered levels of serum immunoglobulins (IgG and IgM) may exert a systemically mediated contraceptive effect.

Mechanisms of Action

A. Tubal Transport. Doyle and Margolis (1966) demonstrated that in mice (Swiss-Webster, C57BL, or R3), unilaterally fitted with 5-0 braided silk and sacrificed eight to ten days pc., a time when no free ova should have been present, embryos were recovered from the control and experimental tubes as well as from the control uterine horn. Of the 23 animals without implantations on the control side, three (13 percent) had uterine ova on that side and two (9 percent) had tubal ova. Comparable data for the operated horns showed that no uterine ova could be recovered, but ten (45 percent) of the females had tubal ova.

Of 15 animals killed during the pre-implantation period, only tubal ova could be recovered from either side on days two and three pc.
while on day four ova were recovered from the uterus on the control side, but only from the tube on the operated side. Mechanical blockage of the uterotubular junction was probably not a cause of the retention of the ova since some ova were fertile. This demonstrated that spermatozoal transport had occurred, and that there was no tubal distention due to fluid accumulation which would have been expected if the junction were completely closed.

In a finding dissimilar from that of Doyle and Margolis, Marston and Kelly (1969) were not able to substantiate the observation that IUDs seriously disturbed the pattern of egg transport through the mouse fallopian tube. The authors were only able to document cases of "tube locking" in three out of their 41 experimental animals. They concluded that these infrequent cases may have been caused either by the insertion of an IUD thread into such a position that it mechanically obstructed the uterotubal junction or to the accumulation of cellular debris within the uterus.

Hawk et al. (1965) placed bilateral polyethylene spirals, or loops, into the uterine horns of 18 cows. These cows subsequently returned to estrus after a total of 42 services with no evidence of conception. Each cow was bred again, and killed a predetermined number of days post-breeding. The only evidence of pregnancy was a three day cleaving ovum recovered from one cow. Thus, this was the only possible pregnancy subsequent to 60 services, and it may be considered doubtful that a normal pregnancy would have ensued. Fertility in the control
cows was satisfactory; all 21 cows were pregnant after a total of 38 services.

According to Hawk, cleaving ova should have been recovered from bovine oviducts approximately 90 percent of the time within three days post-breeding. Nine cows were killed at this time and the oviducts flushed. A cleaving ovum of eight blastomeres was recovered from an oviduct of one cow, an uncleaved ovum was recovered from the oviducts of each of four cows, and no ova were recovered from the oviducts or from the uteri of an additional four other cows. Three cows with no devices in the uterus were also killed three days post-breeding; a cleaving ovum of eight blastomeres was recovered from the oviducts of each one.

Five experimental cows with CLs on the ovaries were also killed between 15 and 19 days post-breeding to determine whether embryos were present in the uterus. This stage of gestation is more than two weeks before implantation in the cow. No embryos were found, which suggested that contraceptive effects were occurring before this stage of gestation. Although this evidence continued to suggest that tubal transport was adversely affected, as demonstrated in mice by Doyle and Margolis (1966), opposite tubal pathology was documented. In the latter case, ova transport was enhanced, whereas in the former situation ova retention, or a "tube locking" effect, was apparent.

Using female monkeys, Mastroianni and Hongsanand (1965) were able to document inappropriate rapid transport of ova into the uterus. In eight control monkeys, 21 fresh ovulation points were noted. Eleven
ova were recovered, yielding a collection rate of 52 percent. Of the seven experimental monkeys studied, ova were recovered in one animal in which the IUD was discovered to be lying free in the vagina. In the remaining six animals there was a total of 15 ovulation points with no ova being recovered. Spermatozoa were present in the tubal washings in four of the six animals.

Ovum distribution in the fallopian tubes of the experimental animals was further studied in a series in which one fallopian tube was ligated at the uterotubal junction. Recovery of ova from the ligated side at approximately the same rate as in the non-IUD animals suggested that the coil did not impair the mechanism of ovulation or ovum pickup.

In a more detailed study by Hussein and Ledger (1969a), the paired genital tracts of New Zealand female rabbits were fixed in the presence or absence of an IUD. Staining techniques were employed to demonstrate the presence of P.A.S.-positive material and mucopolysaccharides in the uterine and tubal epithelium. The sections were studied in an attempt to detect histological differences which might account for the contraceptive action of the plastic foreign bodies.

Since the contraceptive action of IUDs in rabbits is reversible when these foreign bodies are removed early after insemination, a second group of animals was also studied in which the device was removed on day one, day three, or day four post-insemination. These groups of animals were then sacrificed on either day three or day five postbreeding.
No differences were noted in the amount of P.A.S.-positive staining material in the endometrial gland cells of the device and control uterine horns under the varying conditions of the study.

There were differences, however, between device and control fallopian tubes, particularly in the isthmic portion. Prior to ovulation, there were more positive P.A.S. staining granules in the epithelial cells on the device side than on the control side. This pattern of increased P.A.S.-positive staining granules was carried throughout the first five days following ovulation. The retention of P.A.S.-positive material was not seen when the device was removed the first day after ovulation and the animals killed three days following ovulation.

In addition to the important function of transportation provided by the cilia of nonsecretory cells within the fallopian tube itself, secretory epithelial cells may provide nutrition or protection for the fertilized ova which have progressed to the blastocyst stage by the time they reach the uterine cavity. Secretory cells are present in all portions of the tube, but are most abundant in the isthmic portion. The author continues to suggest that the inability of tubal epithelial cells to provide sufficient secretion may result in a decreased number of implantations due to increased degeneration of blastocysts reaching the uterus. A lack of mucin covering the ova was also noted to parallel the retention of granules seen histologically in the tubal epithelium.
B. Failure of implantation. Numerous studies have indicated that IUDs may create an unfavorable uterine environment which may: (1) act upon blastocysts so that they disintegrate and fail to implant or (2) result in alterations in normal uterine tone and motility so that ova may be allowed to escape into the vagina prior to the time of implantation.

In an experiment by Doyle and Margolis (1963), the reproductive tracts from nine rats, which were fitted with unilateral IUDs and sacrificed on the third and fourth days of pregnancy, were dissected free and the uterine horns and oviducts were flushed. No demonstrable differences in the number of the fertilized ova recovered, in the position of the fertilized ova within the tract, their morphology, or rate of cleavage could be found between the control and operated sides. On the fifth day of pregnancy, only one of six animals with retained IUD sutures had fertilized ova within the operated horn even though all but one of the contralateral horns contained normally developing embryos.

Additional studies by Doyle and Margolis (1964) supported original findings that alterations of implantation physiology represents the modus operandi of IUDs in rats. Although the study did not determine the exact mechanism of action it did serve to rule out several other possibilities. Estrous cycles, mating physiology, sperm transport, fertilization, cleavage, and tubal transport of fertilized ova were shown to be unaffected by the presence of an IUD.
Successful implantation is dependent upon several processes. The uterus must be sufficiently sensitized so that it may react to a normally cleaving embryo which the tube has transported and allowed to enter the uterus at the appropriate time. A failure in any one of these processes results in failure of proper implantation. Since ovulation and tubal transport are thought to be normal in rats with IUDs, the cause of implantation failures must be due to some deleterious alteration within the uterus.

As mentioned earlier, a consistent difference between the operated and control sides first became apparent on day five. A large proportion of ova were not recovered from the operated side once they reached the uterus. Although mechanical trapping, or aberrant uterine motility, may have been the cause for failure of implantation, the observation of normal sperm transport within the tubes, associated with normal fertilization rates, tend to make these two scenarios improbable.

A third possibility is that the IUD altered the intrauterine environment to one that was hostile to the ova and acted in some fashion to destroy them. This unfavorable environment was not attributed to a systemic imbalance since implantation could and did occur normally within the contralateral horn. Observations by Martson and Chang (1965) not only confirmed the work of Doyle and Margolis, but were also able to demonstrate a close association between the failure of the decidual cell reaction (DCR) and the inhibition of implantation. They found that complete inhibition of both implantation (0% vs 91%) and DCR
occurred if the IUDs were removed four or more days following mating whereas implantation (62% vs 93%) and the DCR response were only slightly affected if the devices were removed within two days of mating.

These results demonstrated a close association between the failure of the DCR response on the one hand, and the inhibition of implantation on the other. The foreign body is effective during the time of preparation for implantation, but not at an earlier time. Thus, Marston and Chang (1965) were able to conclude that some specifically timed local trigger mechanism, whether a biochemical substance or a neuroendocrine reaction necessary for the initiation of implantation, was exhausted by the presence of a foreign body. The authors also suggested, however, the possibility that the almost complete failure of implantation following removal of an IUD on day three or four, when normally developing eggs are found in the tube, may in part result from the induction of an embryotoxic condition since the extent of the DCR after the removal on day three appeared sufficient to support implantation to a certain extent.

Marston and Kelly (1969), using several strains of mice, supported the previous conclusions that the bilateral contraceptive effects of an IUD are related to the elimination of embryos from both uterine horns and to the bilateral inhibition of the decidual cell reaction. In untreated mice, all but one of the ova recovered were found in the uterus and were undergoing normal development at the morula or blastocyst stage. The recovery rates for the untreated mice amounted
to 61 and 68 percent, respectively, of the total active corpora lutea. In IUD mice, the recovery rates were low and ranged from 14 to 37 percent although the fertilized eggs recovered were apparently undergoing normal development. As a whole, the results suggested that ova from treated mice were rapidly eliminated as soon as they entered either the control or the IUD uterine horn. Even though these observations did not show how the embryos were being eliminated from the uterus, Marston and Kelly felt that they were probably being destroyed in the uterus. They did not exclude the possibility that the embryos had been prematurely expelled.

More direct proof of the IUD-induced hostility of the uterine environment to the early conceptus was provided by de Boer and Anderson (1971), using the technique of double transfer of embryos. They transferred embryos, obtained from normal rats, to females with one IUD horn and one non-IUD horn. They recovered the embryos from these hosts after a variable period of up to four hours in length, and then tested their capacity to survive and implant on retransfer to a second, pseudopregnant recipient without an IUD. Their findings demonstrated that after remaining in the IUD horn for two to four hours, only 10 to 15 percent of the embryos were recoverable and survived in untreated recipients, compared with 40 to 50 percent of the embryos kept for a similar time in the non-IUD horn before transfer. It was concluded that the IUD, in the rat, induced a uterine milieu which became more destructive the longer the pre-implantation embryo was exposed to it. The hostility continued to persist even after the IUD was removed.
Equality between the IUD and the control horns was not reestablished until 72 hours after removal of the IUD in embryo transfer.

C. Leukocytic response. The nature of the specific changes which IUDs induce in the intrauterine environment remains largely conjectural. The most common view is that the device initiates a low-grade inflammatory reaction, the products of which either directly or indirectly cause embryonic death. Work carried out in rats by Greenwald (1965), in addition to confirming previous observations that normal embryos are present in the oviduct until day four of pregnancy but are missing from the tract by day five (Doyle and Margolis, 1964), also identified a leukocytic infiltration of the endometrium in cornua containing sutures lacking embryos. The acute inflammatory response was noted in tracts of cyclic females as well as of animals killed 2 to 17 days pc. The duration of this observed process is different from the transitory post-ovulatory influx of leukocytes that is observed to occur into the uterine lumen of several animal species. The leukocytic invasion was not limited to the area adjacent to the thread but rather involved the entire uterine horn. This inflammatory response seemed to be enhanced by anti-inflammatory compounds (e.g. cortisone acetate, and phenylbutazone) which increased the production of leukocytes by bone marrow. In all instances, the contralateral uterine horn was histologically normal and pregnancy was unaffected. Polymorphonuclear leukocytes (PMNL) infiltration appeared to be essential for the inter-
ruption of pregnancy since the absence of leukocytes was always correlated with implantation. The continued presence of the thread was necessary to establish a reaction as was demonstrated by a series of sham operations. The necessity of the suture was further demonstrated by the fact that after its removal, implantation occurred at subsequent remating.

The study also indicated that the location of the thread was critical in determining whether the leukocytic response was elicited. This was shown by the presence of normally implanted and developing embryos found in some of the threaded cornua although the suture was still in situ. In all of these animals the thread had not reached the uterine lumen, but instead, at its deepest penetration, continued to be imbedded in the endometrial stroma. By deliberately inserting the thread superficially into the uterus, Greenwald showed that pregnancy continued in the threaded uterus and that the uterine epithelium was not invaded by leukocytes.

Using Holtzmann rats, Parr et al. (1967) was able to support the results found earlier by Greenwald. PMNLs, representing 95% of the total cellular infiltrate, were found along the entire length of the rat uterine horn containing a silk suture at all stages of the estrous cycle. In contrast, no PMNLs were found in the control uterine horns. This was observed as early as two days after insertion of the foreign body and remained unchanged for at least as long as 100 days. The inflammatory response was thus unusual in that the acute, or polymorphonuclear phase, persisted indefinitely.
Par, in a continuation of the same study, was also able to culture bacteria from uteri containing foreign bodies. Most of the animals contained at least $10^8$ bacteria per horn containing foreign bodies and between $10^1$ to $10^4$ bacteria per control horn. Although none of the organisms were identified, the most common one was thought to be a Vibrio and almost all were gram negative.

In mice, however, the pattern of PMNLs infiltration was different in at least one respect. Whereas the leukocytes were never observed in control horns of rats, they were found in over half of the mouse control horns examined. In the mouse horn containing the foreign body, as also observed in the rat, PMNLs occurred along the entire length. However, in contrast with the rat, the fertility of the contralateral (control) horn of the mouse was also markedly reduced. Thus, the pattern of infertility in the mouse continues to correspond to the pattern of PMNL infiltration.

Intrauterine foreign bodies placed in rabbits show an even less effective capacity to prevent pregnancy when compared with the rat or mouse. From the number and location of the embryos in rabbit uterine horns containing IUDs, Parr was able to conclude that the entire rabbit uterus, except for the segment in direct contact with the foreign body, remained fertile, as evidenced by the frequent occurrence of normal embryos immediately adjacent to a foreign body. No embryo was ever observed in the segment with the foreign body, but 42 embryos were present elsewhere in ten uterine horns. Embryos were found on the cervical side of the foreign bodies as well as on the ovarian side.
The histological examination of the rabbit horn containing a foreign body revealed relatively few leukocytes per section and furthermore, did not demonstrate markedly more leukocytes in the segment that contained the foreign body than in the remainder of the horn. Bacteriological studies on the rabbit uterus were consistently negative. No growth was ever obtained from the segment in contact with the foreign body.

Since studies using non-germ free rat uteri containing silk sutures indicated that bacterial infection regularly occurred it was thought that this infection might be responsible for all or part of the inflammation, and might also exert an anti-infertility effect. It was noted that foreign bodies in the uteri of germ free rats also caused uterine infiltration of PMNLs as well. However, in the germ free uterus, these PMNLs were present only in the tissue and the lumina immediately adjacent to the foreign body, whereas in the conventional rat uterine horn containing a foreign body, the PMNLs were distributed throughout the entire length of the horn. Even at a distance of only two millimeters from the foreign body, there were no observed PMNLs in the IUD containing uterine horns of germ-free rats. It was found that when the foreign body was in the ovarian end of the uterine horn, no embryos were able to implant or grow in the non-inflammed lower uterine segment. When the foreign body was in the cervical end, however, many proximally located blastocystes were able to survive.

One germ free animal, having five embryos above a cervically positioned foreign body and eight embryos in the control horn, was
released to a conventional environment on day eight of pregnancy. At term she delivered ten normal pups and was then mated in the postpartum estrous to a conventional male. On the tenth day of gestation, there were eight embryos in the control horn again and none in the horn with the foreign body. This horn now harbored $10^8$ bacteria of three species, and inflammation was present throughout its length. These results demonstrated that a uterus containing a silk suture could acquire a pathologic bacterial flora even when the conditions of foreign body insertion were absolutely aseptic, and that inflammation and infertility of the entire horn developed in association with this bacterial infection.

Using the assumption that the uterine physiology of conventional and germ-free rat is identical except with regard to bacteria and inflammation, Parr postulated that inflammation is largely responsible for the contraceptive action of a silk foreign body in the uterus. Although bacteria may make some contribution to the toxicity within the uterine lumen, the results obtained in germ free rats demonstrated that bacterial contamination is not essential for contraception.

Further work by Hurst et al. (1977a), using a mouse model, continued to elucidate the role of PMNLs in regard to embryo degeneration and subsequent infertility. In sham operated controls examined at 72 and 96 hours post-coitus, blastocyes were seen spaced along both uterine horns and no PMNLs were observed in the lumina of either the horns or the oviducts.
In all IUD-bearing animals, large masses of PMNLs were seen to surround the IUD and to extend from it towards both the ovarian and cervical extremities of the horn, frequently filling the lumen. In many of the animals studied, normal blastocysts were found either clustered in the isthmus of the fallopian tube, or lying in the uterine horn between the utero-tubular junction and the IUD. Many of these embryos were surrounded by PMNLs and were observed undergoing various stages of degeneration. Although fewer PMNLs were found in the lumen, epithelium, and stroma of the contralateral horn as compared with those on the IUD side, reduced rates of fertility continued to be observed. In addition, the number of embryos in the contralateral horn in relation to the corpora lutea agreed well with those in the control, continuing to suggest that ovulation rates were not significantly affected. This particular study was not able to ascertain whether PMNLs actively invade, or phagocytize, embryos or whether by directly surrounding the embryos, they form a barrier which interferes with the passage of the essential nutrients from the lumen, making autolysis of the embryos inevitable.

However, attempts to isolate and identify the factor(s) responsible for this toxicity have progressed little beyond Parr's (1969) demonstration that extracts of PMNLs are capable of killing rat morulae in vitro. In this particular study, rat morulae were incubated in vitro until they formed the blastocyst cavity during incubation. It was observed that if an extract of PMNLs was added to the culture medium the formation of the blastocyst cavity was prevented. The
concentration of PMNL extract required in the culture medium to arrest all embryonic development prior to blastulation was about equal to that which was calculated to occur in the lumen of the rat uterus containing a foreign body. It was concluded that the nature of the factors that arrested the development of the fertilized ova in vitro was that:

1. they were not associated with leukocytic lysosomes;
2. they were resistant to heating at an acidic or alkaline pH,
3. they could be separated by dialysis into three fractions, each of which was inactive by itself, but which exhibited original toxicity upon being recombined, and
4. that the toxic factors were not limited to leukocytes.

These results support the hypothesis that factors derived from degenerating PMNLs are able to exert toxic effects on fertilized ova. Sahwi et al. (1971b) investigated the effect of rabbit PMNLs extracts on the subsequent development of two cell mouse embryos in vitro. It was found that intact leukocytes, homogenized PMNLs, or lyophilized homogenates of 1-2 x 10^6 PMNLs prevented development beyond the eight-cell stage when placed in culture with in vivo fertilized two-cell mouse embryos. Moreover, the number of embryos that did not develop to the blastocyst stage in vitro was correlated with the concentration of PMNLs in the culture media just as the antifertility effect of the IUD can be correlated with the number of PMNLs in the uterine cavity in vivo.

The authors were able to draw several conclusions from their study. First, the active component was readily soluble in an aqueous medium, but not in paraffin oil. This was substantiated by the observation that the embryo-toxic activity was not transferred through the
paraffin oil covering the culture drops. Secondly, the active principle is reasonably stable after storage of whole cells at four degrees centigrade for one week since homogenates of such stored cells appeared to be just as active as those prepared from fresh PMNLs. Thirdly, the embryotoxic activity of the PMNLs in vitro does not require cellular integrity. It was previously suggested that leukocytes might phagocytize embryos, as well as sperm, in the uterine cavity. Since the embryotoxic activity of homogenized PMNLs was comparable with that of intact cells, the authors felt that it was unlikely that phagocytosis is the main mechanism for embryo destruction. Fourthly, embryo death did not appear to result from competition of the PMNLs with the embryos for nutrients in the medium. This was indicated by the observation that freeze-dried culture medium homogenate did not destroy its activity. The results of this experiment continue to lend support to the concept that some degree of leukocytic activity is responsible for the observed infertility in uterine horns containing foreign bodies.

Further studies using in vitro fertilization culture techniques continue to support the idea that fluids collected from the uteri of rodents fitted with IUDs are embryotoxic. Using mouse embryos, Breed (1965) measured the inhibitory effect of flushes from both rat and mouse uteri collected during different stages of the estrous cycle.

In culture media containing IUD experimental fluid from diestrous rats, the investigator found no reduction in either expanded blastocysts (EB) or hatching blastocysts (HB) as compared with the
controls. Similar results were obtained with supernatants collected from rats on day four pc. Only in flushes from estrous rats was there a suggestion that the IUD horn was slightly toxic to the growth of mouse morula.

In diestrous flushes from mice, again, there was no reduction in either EB or HB development versus the controlled horn. As with the rat, similar results were also noted with flushes obtained from mice on the fourth day post-coitus. The major difference however, occurred with flushes obtained from estrous mice. These supernatants demonstrated a potent embryotoxicity. Only three HB out of 79 cultured rat morula were observed and none of these blastocysts went on to hatch. The controls demonstrated 66 EB out of 104 initially cultured morula with 54 continuing on to hatching.

It appears, therefore, that mouse IUD-horn flushes are markedly more toxic to mouse ova in culture than are rat flushes. This toxicity cannot be demonstrated in flushes from rats or mice in diestrous or in day four pc uterine flushes, possibility due to a greater dilution of uterine fluids with the culture medium.

In an eloquent study, Sahwi and Moyer (1971a) were able to demonstrate a quantitative relationship between the number of PMNLs and mononuclear cells and their observed antifertility effect in rabbits. In the control animals, the mean fertility rate, as measured by a ratio of the number of implantations over the number of observed corpora lutea, was 0.86. The mean fertility rate in 33 uterine horns containing IUDs was 0.5. The mean number of leukocytes in the uterine
flushings on the IUD side was 32.6 leukocytes per cu.mm. as compared with only three leukocytes per cu.mm. on the control side.

The mean diameter of all implantations occurring in the IUD uterine horn was 15.0 mm. compared with the mean diameter of 20.5 mm. in the control horn. When implantations were present in the IUD horn, the size of the implantation site varied inversely with the number of leukocytes present. When the number of leukocytes was ten cells per cu.mm. or less, the size of the implantation sight was similar to the size found in the control horn. When the number of leukocytes per cu.mm. was 30 or greater, the size of the implantation sight ranged between one-half to one-tenth of those in the control horn.

Seventy-three implantation sites were noted in the 33 IUD uterine horns. Of those, 57 were located anteriorly to the IUD, 15 were located in a posterior position, and one was opposite the intrauterine device. The maximal antifertility effect of the IUD was exerted in the immediate vicinity of the device and the least effect was noted in the oviductal portion. In general, the fewer leukocytes present in the uterine flushings, the closer the implantations occurred to the IUD, and conversely, when larger number of leukocytes were present in the uterine flushings, the implantations occurred further away from the IUD. Additionally, as the difference in the diameter of the implantation sites between the control and IUD horns increased, a concomitant increase was also observed in the difference in number of leukocytes in the two horns.
Histologic sections of endometrium lying beneath the IUD showed a small number of PMNLs present in the superficial epithelium and stroma. Sections of endometrium taken from the proximal and distal portions of the uterus, approximately two to three centimeters from either end of the IUD showed significantly fewer PMNLs than in the areas contiguous with the IUD. The number of PMNLs present in the endometrial regions which were not contiguous with an IUD was similar to the concentration of leukocytes present in sections of endometrium taken from the control horn between the implantation sites. The number of leukocytes present in the endometrium showed no correlation to the number present in the flushings.

In general, the data suggest that an embryotoxic substance(s), either associated with or derived from the leukocytes, is present in the uterine horn containing the IUD, and that a gradient distribution of this substance(s) occurs. It appeared that the concentration of the substance(s), which was required to destroy the embryo, extended for a greater distance on a proximal side than on the distal side of the IUD. More implantations occurred in the distal than in the proximal half when the IUD was placed in the middle of the uterine horn of the rabbits. Furthermore, when implantations occurred on the IUD side (vs the control horn), the diameter of the implantation was smaller in those rabbits having larger number of leukocytes flushed from the uterine cavity. This inverse relationship suggests that a substance(s) derived from the leukocytes, when present in a concentration less than that which was lethal to the embryos, retarded the development of the
placenta and/or embryo. The diminished size of the implantations was not due to resorption as studied by histologic sessions.

Only a few studies have dealt with the effect of leukocytes on embryos obtained from primates. Hurst et al. (1977b), by flushing fluid through a needle inserted into the uterine lumen above the cervix and aspirating the fluid through a second fundal needle into a recovery syringe, was able to recover approximately 50 percent of pre-implantation embryos in monkeys examined between days 15 and 20 of the menstrual cycle. Sixteen females, from all of which embryos had been recovered in the non-IUD condition, only yielded six embryos in a total of 46 attempts. Five of these embryos (the sixth being lost in processing) were histologically prepared and representative sections were examined by light and electron microscopy. All five were considered to be abnormal or degenerating and each showed PMNLs both outside and within the embryonic tissue. In two of them, macrophages were also present. In another, judged to be an azonal morula, all blastomeres, associated with infiltrating leukocytes, were fragmenting. Two azonal embryos were blastocysts with leukocytes present in both blastocoele cavities. Another blastocyst possessed the zona pellucida and again leukocytes were found within the blastocoele and interspersed among cells of the intercell mass. In two cases, leukocytes were seen apparently passing through the zona pellucida. The fifth embryo was comprised of only five or six shrunken cells within an intact zona. Electron microscopy revealed several polymorphs and macrophages to be in direct contact with the embryonic cells. The polymorphs appeared to
be typical, mature neutrophils and the macrophages had extensive plasma membrane invaginations. Occasionally, eosinophils were also present.

By contrast, of a total of three unfertilized ova and 30 embryos so far recovered from non-IUD monkeys, none demonstrated any types of leukocytes attached to the surface, and only eight embryos were considered to be degenerating.

Pathological analysis of all IUD-flushes showed that the number of cells varied from $4.37 \times 10^5$ to $2.39 \times 10^6$, most of them being neutrophils. Flushes from non-IUD fitted uteri gave values between $1.44 \times 10^4$ and $3.92 \times 10^5$, most being epithelial cells.

The authors conjectured whether the embryos observed in the study were entirely normal before their investments had been penetration by leukocytes. It was possible that the highly cellular milieu of an IUD bearing uterine environment, perhaps derived from dead or dying PMNLs is in itself embryotoxic and that the main function of infiltrating leukocytes is the final destruction and removal of dying or damaged tissue including embryos.

Parr and Shirley (1976) were able to obtain at hysterectomy in twelve human uteri containing intrauterine contraceptive devices and ten uteri without IUDs. Samples of fluid were collected from the uterine lumen by absorbing the fluid with small pieces of lens paper. The samples of uterine fluid were then measured for the concentration of beta-galactosidase, an enzyme which is present in human neutrophilic leukocytes and whose concentration in the luminal fluid was thought to correlate with the local inflammatory response due to the intrauterine
foreign body. Samples of fluid from IUD-bearing uteri contained concentrations of beta-galactosidase in significantly greater amounts than that in the luminal fluid obtained from control uteri ($P < 0.0005$).

To determine whether a foreign body response of this magnitude could have any effect on pre-implantation embryos, the authors cultured mouse embryos, from day four to day seven of development, in culture media to which extracts of human leukocytes were added. All mouse embryos were killed when the culture media contained enough leukocyte extract to give beta-galactosidase concentrations similar to those found in human uteri containing IUDs. Although direct in vitro embryotoxic effects of uterine fluids collected from primates containing IUDs were not examined, the comparison by Parr and Shirley between the presence of leukocytic components found within the uteri of IUD fitted primates and the ability of human leukocytic extracts to inhibit mouse embryo cultures indicates that the luminal fluid of these IUD-bearing uteri contains leukocytic byproducts in sufficient concentration to be lethal for pre-implantation embryos.

D. Alterations in uterine biochemistry. A study by Hall et al. (1965), involving women fitted with a stainless steel intrauterine ring, and subsequently biopsied at fixed three month intervals, essentially revealed no significant changes in uterine histology. There was no evidence of pregnancy or abortion, and no increases in the instance of chronic inflammation were noted. Additionally, no disturbances of
endometrial function, as evidenced by normal cyclic changes and normal glycogen and alkaline phosphatase activity, were recorded.

Instances of mild neutrophilic infiltration were considered significant since these cell types are not typically found in the glands of the lumena. It was postulated that these cells may reflect some type of compositional change in the endometrial mucus. In view of the known importance of the fluid environment for nutrition and homeostasis of the implanting ovum, it was suggested that changes in the endometrial gland secretion may be involved in the prevention of pregnancy. The authors suggested that the lack of abnormal morphological changes make it appear that biochemical rather than histological methods may be more useful in the future study of the environment of the ovum.

Bonney et al. (1966) also finding no significant differences in endometrial morphology between IUD users and non-users, nevertheless, did document a thick and mucoid fluid surrounding the IUD particularly during the proliferative phase.

Lipid studies, performed by the same authors, suggested that the specific pattern of biochemical maturation, which is a prerequisite for the normal development of the postovulatory endometrium, was adversely affected. A uterine environment unfavorable to implantation results from the failure to achieve biochemical maturation of the endometrium which must be in synchrony with the changing events taking place within the ovum. Since the rate of recovery of human ova from patients using the intrauterine device was slightly greater than those not using the
device, rapid transit of tubal/uterine ova was not postulated as a probable mode of action.

In pursuing the idea that a change in endometrial fluid might create a hostile environment for the implanting ovum, Sedlis et al. (1967) measured the in vivo pH of intrauterine fluid. By inserting an electrode through the cervix until it reached the fundus of the uterus, these investigators found significantly lower post-IUD insertion values (pH 6.4) as compared with pre-IUD values (pH 6.6). This difference was maintained throughout the various stages of the menstrual cycle. The shift in pH was interpreted as an indication of changed composition of the uterine fluid, possibly due to contamination with components not normally found in utero (e.g., blood, tissue, and fluids), bacterial action, or perhaps, altered electrical potential. The pH study was thus interpreted as supporting the "changed-fluid changed-environment" theory as a mode of action exerted by the intrauterine device.

A more detailed study on the effect of intrauterine contraceptive devices on biochemical composition of human uterine fluid was carried out by Kar et al. (1968). The results of that study, although showing no changes in uterine pH, demonstrated that the most striking effect of the IUD was the marked increase of the protein level of the uterine fluid irrespective of the stage of the cycle. This increase in protein concentration was also accompanied by a concomitant rise in nonprotein nitrogen. This later phenomenon was thought to exist in the form of nitrogenous materials such as urea. Since no proportional increase in enzyme activities was observed in the fluid, it appeared that the bulk
of the excess protein in the fluid of IUD-fitted women was metabolically inactive.

It was postulated that the increased protein levels were a result of: (1) blood protein leaking into uterine fluid through alteration of vascular permeability and/or (2) the lysis of such cells as PMNLs and denuded surface epithelial elements. Leukocytic infiltration through the endometrium, into the luminal fluid, has been previously observed in both women and animals fitted with IUDs.

The authors felt that the accumulation of waste products and nonprotein materials in the uterine fluid may lead to a disturbance in the osmotic tension and consistency of the fluid, thereby rendering its milieu metabolically inappropriate for growth and survival of the pre-implantation blastocyst.

Except for studies demonstrating mild to no histologic uterine pathology, equivocal changes in pH, and increased protein content of IUD-uterine fluids, little evidence existed concerning the cyclicity of biochemical changes which occur in the human endometrium during the menstrual cycle. Determinations of total protein, alkaline, and acid phosphatase, acid-soluble nucleotides, RNA, and DNA were performed on biopsy samples obtained from pre- and post- (two month) IUD uteri during both the proliferative and secretory phases of the menstrual cycle (Joshi and Sujan-Tejuja, 1969).

These studies illustrated the cyclic changes in the metabolic activity of the human endometrium during the various phases. It is well established that the induction of active cellular growth is
associated with a sequential stimulation of synthesis of acid-soluble nucleotides, RNA, and protein. During the proliferative phase, the concentration of acid-soluble nucleotides and total protein, is increased, paralleling the changes in RNA concentration, and reflecting specifically the influence of endogenous estrogen on endometrial growth. Such estrogen-dominated growth has been found to reach a peak of activity during days 16 to 20 of the human menstrual cycle. A sharp decline in growth, as indicated by the levels of endometrial acid-soluble nucleotides, RNA, and protein during the midsecretory phase may be related to a decrease in endogenous estrogen output, to estrogen-proesterone antagonism, or to both. These studies indicated that an IUD does not interfere with these cyclic changes. A comparison of these growth-activity indicators revealed that endometrial changes during the proliferative and early secretory phases was actually stimulated by the presence of the device. Moreover, the peak concentrations of RNA and protein in the endometrium, which are normally not obtained until days 16 to 20, are reached during days 9 to 13 in the presence of the IUD, indicating an acceleration of growth. Higher concentrations of RNA and total protein in the IUD-treated endometrium were found to be maintained even during days 21 through 23 when the growth activity of the normal endometrium is significantly depressed. It was felt that these observations demonstrated that the IUD, in some manner, promotes intracellular anabolic activity of the endometrium. It was not known whether the increased protein-synthetic activity in the IUD treated endometrium was due to a hypersensitivity of the endometrium to
estrogen or to a mild, transient, local inflammation which is associated with these devices.

In later work, Hester et al. (1970) confirmed the findings of increased, but cyclic, changes in alkaline and acid phosphatase levels in women wearing IUDs. He was also able to document increased production of glycoproteins, or mucins, in these uteri as well. Muco-substances of the endometrium are thought to provide a barrier against infection, coat the ovum and sperm, aid in the implantation of the fertilized ovum, and act as a transport agent for menstrual debris. By staining these mucins, a cyclic pattern was established and a histochemical classification of the glycoprotein profile for the human endometrium was accomplished by the authors. Although these muco-substances continued to cycle normally, as did the alkaline and acid phosphatase enzymes, intrauterine devices were able to produce a more copious production of the glycoproteins throughout all phases of the cycle.

The developing hypothesis that IUDs produce endometrial and intrauterine fluid changes that discourage implantation was supported by these investigators.

E. Post-implantation embryo mortality. As previously discussed, the rabbit does not seem to be as susceptible to the antifertility effects of an IUD as compared with rodents and primates. Most
researchers have documented no significant changes in ovarian hormonal activity, ovulation, sperm and ova transportion, fertilization or early embryonic cleavage.

What Adams and Eckstein (1965) were able to demonstrate, in addition to the loss of pre-implantation embryos, was also the significant loss of post-implantation embryos or fetal wastage. This procedure was accomplished by subjecting does to a laparotomy on day 10 pc., followed by an autopsy close to term on day 27. The animals were fitted with three to four sutures attached to one horn while the other horn served as a control.

The results showed that the loss of ova, as compared with the number of CLs in the experimental horn through day 10 pc, is of the previously established order of approximately 50 percent. This compared with only 12.5 percent for the control horn. But whereas the control horn suffers a further loss (17.1 percent) between implantation (effective day 10) and day 27, the number of surviving fetuses is strikingly reduced by over 85 percent in the experimental horn. The result was that in this particular series only three of 21 ova (one in seven) represented by implantation sites on day 10 would have survived to term. It was thus clear that the effect of the devices in rabbits is not confined to the pre-implantation stage. The conspicuously high proportion of degenerating placental sites ("placental relics") present on day 27 indicates that the bulk of the embryos died during midpregnancy (days 10 through 17) possibly as a result of some interference with placental function.
These particular observations indicated that in rabbits, unlike rats, the effects of IUDs are not confined to the early stages of implantation, but persist at least through midpregnancy and possibly until later stages of gestation.

Following Adams and Eckstein's report of increased post-implantation embryonic death in rabbits, Dukelow et al. (1967) investigated the effects of an IUD on the uterine and oviductal environment at the time when embryonic death was thought to occur. While substantiating the finding of Adams and Eckstein, Dukelow was also able to document a reduction in the protein content of both uterine and oviductal fluids. It was suggested that this may be the result from the loss of activity of several enzymes, perhaps including those necessary for implantation and embryonic survival. Additional findings included an observed decrease in the carbohydrate content of uterine fluids corresponding to a similar decrease observed in eight day old blastocyst fluid. An increase in carbohydrate metabolism or a decrease in carbohydrate synthesis was suggested as a possible modus operandi.

Studies involving embryonic resorption in rabbits led to questions about whether implantations and subsequent fetal wastage occurred in primates with IUDs. Landsman (1976) found hGC-like material in 20 percent of serum samples obtained randomly during the luteal phase from women of known fertility who were using IUDs. Radioimmunoassays, and radioreceptor assays, with sensitivities of six IU. hCG/liter were utilized. No cross-reactivity with LH was stated. None of these women was noted to have developed clinical signs of pregnancy. In a similar
study, 32 out of 73 urine samples, using a hemagglutination inhibition test with a sensitivity of 30 IU. of hCG/liter, were found to contain hCG (Beling et al., 1976).

Other authors, however, have failed to substantiate these findings. For example, Sharpe et al. (1977) did not find hCG in either the blood, or the urine, of women with IUDs using a radioimmunoassay utilizing an antibody to B-hCG cross-reacting with LH in the range of 15 to 50 mIU. per ml. Any value below 25 mIU. was assumed to be LH, and most values in these patients ranged from two to 17 mIU. Current disagreement about the presence of hCG in women with IUDs appears to be partly due to the use of nonspecific assays at the limit of their sensitivity.

In reviewing the problem of the inability of assay systems to discriminate between hCG and LH, Hodgen et al. (1978) was able to document increases in urinary hCG in five out of 26 IUD users employing a highly specific radioimmunoassay system based on an antiserum reacting with the unique carboxyl-terminal peptide (residues 123 to 145) of B-hCG. In addition, physical, chemical, and biological properties of this hCG-like substance were examined by gel chromatography and a variety of immunological and biological tests to help establish its unique identity from LH.

The pattern of elution of this material from gel chromatography indicated that a symmetrical peak, analogous to hCG, had both the immunological and biological properties of hCG. Although no conclusion could be reached about whether this hCG-like gonadotrophin is of trophoblastic, hypophyseal, or other origin, equivalent peaks were not
found in urine samples from 33 women utilizing oral contraceptives, tubal ligations, or diaphragms for contraceptive protection. The duration of the rises in urinary hCG-like activity was very transitory. The magnitude of the peaks observed by Hodgen in the urinary concentrates from these five IUD users would not have permitted its detection in the peripheral circulation. Additionally, no increases in daily serum progesterone near the time of the peak in urinary hCG-like activity was observed, and the onset of the next menstrual flow did not appear to have been delayed. It was suggested that if these brief, but distinct, peaks of urinary hCG-like gonadotrophin are elicited in response to implantation, the data may support the occurrence of unsuccessful implantation during the late luteal phase of some IUD users.

An immunosuppressive early pregnancy factor (EPF), associated with mammalian reproduction, has currently attracted considerable interest (Smart et al., 1981). EPF is detected by using an in vitro rosette inhibition assay which measures the capacity of antilymphocyte serum (ALS) to inhibit rosette formation between T-cells and erythrocytes. The mechanism of the rosette inhibition test is unknown, but neither lymphocytotoxicity, nor lymphoagglutinability, is involved.

The rosette inhibition assay consistently demonstrated the suppression of maternal lymphocyte activity by EPF during early pregnancy. Studies with mice have shown a significant reduction of lymphocyte activity six hours after fertilization, coincidental with formation of the pronuclei, but before the first cell division. Titer values return to normal levels four to six days prior to parturition.
Understanding and recognizing the limitations of the technology used for hCG measurement and the lack of clinical symptoms of pregnancy in women fitted with IUDs, Smart utilized EPF as an index of fertilization. He was able to indicate a transient appearance of EPF in six of 22 cycles in IUD users. He believed the best interpretation of these results was that fertilization had more than likely occurred, but that the fertilized ovum probably failed to implant and survive in the endometrium of the uterus. This evidence suggested that the IUD was probably not an abortifacient, but rather that it appeared to act by interfering with the implantation process itself.

F. Ovarian and spermatozoal dysfunction. Depending upon the species studied, many IUD bearing animals display some degree of dysfunction involving fertilization and ova transport. Little evidence involving ovulation disturbances, or spermatozoal dysfunction, has been well documented. In addition to failing to recover ova in almost half of the cows studied (four of nine), suggesting an inappropriate alteration of ova pickup and transport, Hawk et al. (1965), was also able to detect an inhibition of normal CL function. The CL of a cow is ordinarily detectable by rectal palpation between approximately five and 18 days postestrous during a normal 21 day cycle. A detectable CL did not form during many of the estrous cycles of the experimental cows indicating inhibition of CL function by the devices in the uterus. It also appeared that with incidences of inhibited corpora
lutea, the development was influenced by the size of the device in the uterus. Corpora lutea of palpable size failed to form in cows carrying large devices more often than in those carrying small ones. The failure of CL development would in itself cause the death of embryos reaching the uterus since the level of progesterone secretion would undoubtedly be insufficient to maintain pregnancy. It was, therefore, concluded by Hawk that at least part of the contraceptive nature of an IUD in cows may have been exerted through an effect on ovarian function.

In a subsequent study by the same author (Hawk, 1966) none of the 16 ova recovered from the fallopian tubes of IUD-bearing ewes had cleaved. In control animals, out of 11 ova collected, all were cleaving normally. Furthermore, in the experimental animals, no spermatozoa were seen penetrating the zona pellucida, and the author felt that it was doubtful whether any of these ova had even been fertilized. Additionally, two ova that were transferred soon after ovulation from the oviducts of unmated experimental ewes into the oviducts of mated control ewes contained eight and nine blastomeres each two days following transfer. Each ovum also had several sperm within the zona pellucida. The oviducts, uterine horns, and cervixes of five experimental and six control ewes were flushed with saline solution between four and 24 hours after mating. Spermatozoa in the flushings were examined by phase microscopy. No sperm were found within the oviduct flushings of one control ewe, but 142,000 sperm were recovered from the oviducts of a second ewe and 2,800 to 16,000 from oviducts of the other four control ewes. In contrast, no spermatozoa were found in the
flushings of either oviduct of any experimental ewe. The toxicity to spermatozoa in the oviducts probably explains the failure of ovum fertilization. It is pertinent that the IUD was present only in one uterine horn of each experimental ewe. The author suggested that an IUD within a sheep uterus interferes with the mechanism(s) of sperm transport.

Although reports vary as to the fate of spermatozoa in the uterus and fallopian tubes of women with IUDs, Morgenstern et al. (1966), was able to recover live sperm from both anatomical regions. These consistent findings suggest that the chronic presence of an IUD does not prevent spermatozoal transport throughout the reproductive system. These results are not unexpected in light of: (1) the numerous experiments in which investigators were able to recover fertilized ova and embryos from IUD-bearing women; and (2) the earlier documentation of transient rises in both hCG and EPF.

A study undertaken by Hussein and Ledger (1969b) was designed to see if varying the time interval between ovulation and insemination would have any effect upon the number of implantations seen in the device and control horn of the rabbit. An intrauterine device was placed into one horn of 18 mature female New Zealand rabbits. After three weeks of convalescence the rabbits were given 100 units of hCG and then were artificially inseminated from two to 14 hours after this injection. The day after insemination, each rabbit was again anesthetized and the IUD was subsequently removed. The animals were killed ten days after insemination and the number of corpora lutea and implan-
IUD was then counted and recorded. The observed decline in the percentage of implantations, as the time interval between the administration of hCG and insemination was increased, was parallel for device and control horns, with failure of implantation achieved more rapidly on the device side. As expected, the difference in the number of implantations between the device and the control horns was highly significant.

Most studies involving the investigation of sperm capacitation have counted the number of fertilized ova flushed from the fallopian tube and compared this to the number of corpora lutea present in each ovary. In this particular study, the authors counted the number of implantation sites found at day ten after insemination. Utilizing a consistent finding in the laboratory that there were no differences in the number of implantations between the device and the control horns when the IUD was removed the day after insemination, the investigators suggested that the separate phenomena of gradual oocyte release by the ovary, fertilization, tubal transport, and the ability to implant are essentially the same on the device and control sides when a foreign body is removed early. Any differences noted between the device and the control sides in this current study would therefore be related to variation in the time of insemination.

Fewer implantations resulted when the time interval between the intravenous dose of hCG and insemination was increased. One possible explanation for the results was that a longer time interval was needed for sperm capacitation on the device side. Soupant and Orgebin-Christ
have shown that capacitation in a rabbit uterine horn, extended by its own fluid after ligation at the distal and proximal ends, had not occurred in ten hours. This was in contrast to the usual time interval of six hours. Following release from the ovary and transport by the fallopian tube, rabbit ova have a limited period in which fertilization by capacitated sperm can be accomplished before a mucopolysaccharide coating, acquired from tubal secretions, prevents sperm penetration. The authors suggested that if capacitation of spermatozoa in horns containing IUDs is prolonged, the subsequent successful fertilization of the ova may be impaired.

G. Immunological pathology. In one of the few reports to document that an IUD may have a systemic mechanism of action, Holub et al. (1971), observed an altered immunological response in the 35 women he analyzed for IgG and IgM between one and 52 months following the insertion of their device. The authors found that IgG and IgM levels were increased by 61.8 and 39.8 percent, respectively, over those of a control group of patients. Of interest, higher levels of immunoglobulin secretion were observed at longer than 20 months exposure to the IUD as compared with the group with less than three months of exposure. Holub suggested that the data fit the model one would expect to see if some form of chronic immune reaction were occurring.

Two probable mechanisms of action were proposed. First, a nonantigenic chronic inflammation has been demonstrated to induce the
production of autoantibodies to endogenous cellular components. Conceivably, antilymphocytic globulin (ALG), which is an IgG, could participate in the process of inflammatory cell turnover occurring within the uterus. Secondly, another possible role for IgG in the IUD inflammation process is one of acting as a chemotactic factor. Yoshinaga et al. (1970) was able to demonstrate that after an increase in vascular permeability in the skin, a protease was found which converts the exuded IgG molecule to a chemotactic factor, which in turn, causes the migration of PMNLs to the site of the inflammatory lesion.

It was concluded that if any arriving blastocyst is met at the endometrial surface by the cellular elements characteristic of the inflammatory response, its immunologic tolerance may be lost. The blastocyst, unable to implant, or to resist phagocytic attack, would disintegrate and fail to develop.

In a follow-up study, Holub (1972) examined the ability of several immunosuppressive drugs to overcome, or neutralize, the antifertility effect of IUDs in rats. Utilizing the observation that these animals respond unilaterally to a single IUD placed in one of their bicornuate horns, each animal therefore acted as its own control.

IgG levels in the rats' sera increased 52.7 percent after four weeks and 95.4 percent after eight weeks. IgG levels in uterine fluids indicated a much greater increase after four weeks than in the sera (214 percent) or control horn uterine fluid (24.8 percent). This response, although greater in magnitude, is similar to that previously reported to occur in women.
Inhibition of the antifertility effect induced by IUDs was indicated by all of the compounds studied (cytoxin, leukeran, and butylated hydroxytoluene). Although they were not able to negate the effect completely, they did allow significantly more implants to occur than in the nontreated animals. The author suggested that the mechanism of IUD function could be immunologically dependent. It was further suggested that detailed studies, with the use of immunodiffusion and fluorescent antibody labeling, would better help to determine specifically what correlation, if any, exists between IgG and IUD induced antifertility.
METHODS AND MATERIALS

A plethora of information exists concerning the general subject of mammalian, and more specifically mouse, \textit{in vitro} fertilization. Several extensive reviews and research publications of this subject include Brackett (1973, 1979, 1980), Chandley (1971), Cross and Brinster (1970), Frazer and Durury (1975), Hoppe and Pitts (1973), Miyamoto and Chang (1972), Whitten and Biggers (1968) and Whittingham (1971).

**Animals:**

All animals used throughout the experiment were mature (8-16 week, 25 to 30 gm. females or 12-24 week, 30 to 35 gm. males) Swiss albino mice purchased from a commercial breeder. They were maintained in a controlled light (day from 0600 to 2000 hours) and temperature (22°C) environment, and allowed to eat \textit{ad libitum}.

Three distinct groups of animals were utilized: (1) IUD-containing females; (2) superovulated females; and (3) males from which epididymal spermatozoa were collected, capacitated and subsequently used for further \textit{in vitro} fertilization procedures.
**IUD Surgical Procedures:**

Insertion of IUD material was best accomplished by briefly exposing the uterine horns via a midventral laparotomy. To accomplish appropriate general anesthesia, each animal was injected intraperitoneally (ip.) with 2 mg. of sodium pentobarbital (Nebutal). A pain-free response was obtained within 5 minutes of drug injection and all of the animals were conscious and active inside their cages within 2 hours of surgery. A 1 cm. incision was sufficient to allow the gentle grasping of each horn sequentially and the pulling of them individually into the incision site. A length of sterile silk (4-0 silk), threaded to a round-bodied atraumatic noncutting needle, was passed through the lumen of the horn beginning near the cervical bifurcation (proximal end) and progressing 4 to 6 mm. distally. In order to prevent the loss of the suture material during the recuperative period, both ends extending freely from the external uterine surface were knotted together and allowed to float freely within the peritoneal cavity (Eckstein and Hurst, 1978). Enough slack was allowed to exist in the loop so as to avoid tearing of the tissue during myometrial activity. To ensure complete reproductive sterility, at least one-third of the uterine lumen always remained in contact with the suture material. These procedures were performed in randomly selected animals using conventional aseptic surgical techniques. All suture and needle materials utilized were contained in pretreated, disposable, sterile packages. Any animal injured or found to be bleeding within the peritoneal cavity following IUD insertion was
immediately sacrificed. All acceptable animals were then rested at least 10 days before being used in any experimental protocol.

At time of IUD removal (the animal being sacrificed by cervical dislocation), a large, vaginal-to-sternum incision was made and the uterine horns were brought into view. Each horn was then cut twice just interior to where the IUD suture material entered and exited the tissue. Microforceps were used to remove only IUD material freely residing within the lumen. An animal found to exhibit a chronic uterine infection was removed from the experiment. The excised silk segment was then placed in culture media. One IUD was utilized per culture dish (Falcon #1003; 35 x 10 mm.) (Plates I and II).

Control IUDs consisted of identical 4-0 silk sutures that had been allowed to soak in standard culture media for at least 24 hours prior to use. All control dishes contained a three to five mm. length of this neutral silk material.

Superovulation

Superovulation was induced in randomly selected mice by the ip. injection of 5.0-7.5 I.U. of pregnant mare serum gonadotropin (PMSG) (between 1700 and 2000) followed by 5.0 I.U. of hCG 44 to 48 hours later. Thirteen hours (± 0.5 hours) following hCG, each animal was sacrificed (Baukloh et al., 1982). The oviducts were removed within two minutes of cervical dislocation and placed in 1 ml. of culture media. After placing a maximum of four intact oviducts per dish, each
PLATE I.: Silk IUD (25x) in Culture with Associated PMNLs

PLATE II.: IUD (67x) Demonstrating its Silk Weave Characteristics and Infiltrating PMNLs
cumulus mass was located using a 2.5x dissecting microscope and the specific ampullary region was pricked", or gently incised, so as to release the egg mass from the tube (Plate III). The remaining tissue was then removed and discarded. Two cumulus masses were then placed into each in vitro culture dish. This particular protocol consistently yielded between 20 and 30 ova per stimulated animal (Plates IV and V).

Retrieval and Capacitation of Epididymal Spermatozoa

One male mouse was sacrificed by cervical dislocation per experiment, and each epididymal caput was placed singly into 1 ml. of culture media. The epididymis was bisected, and by exerting gentle pressure with two pairs of microforceps, spermatozoa were forced from each epididymal half. The spermatozoa were allowed to diffuse and disperse from the epididymal halves for an additional 15 minutes after which the tissue was removed and discarded (Frazer and Duruy, 1975; Stanger and Quinn, 1982). The cells were permitted to capacitate in an environmental mixture of 5% CO₂ and 95% air for a total of 90 minutes (Baukloh et al., 1982; Brackett 1973 and 1980). During this period, a concentration, in millions/ml., and subjective motility were recorded. Only animals in which recoveries of greater than 20 million/ml./ epididymis, with motilities of greater than 50%, were utilized for subsequent fertilization.
PLATE III.: Swollen Ampullae (25x) Containing a Cumulus Mass
PLATE IV: Cumulus Mass (25x bright field) in Culture After Release From the Ampullae

PLATE V: Cumulus Mass (25x dark field). Note Individual Oocytes Visible Near the Periphery of the Mass
In Vitro Culture Techniques

Stock media, to which all additional modifications were made, consisted of a slight adaption to that of Toyoda's medium, as reviewed by Mettler et al., (1980). Specific changes consisted of using 0.55 mM. sodium pyruvate (Miyamoto and Chang, 1972) and 30 mg. BSA/ml. (Mettler et al., 1980; Frazer and Duruy, 1975; Cross and Brinster, 1970; Quinn et al., 1982a and 1982b). Unaltered components consisted of glucose (5.6 mM.), sodium lactate (32.38 mM.), sodium bicarbonate (25.06 mM.), NaCl (72.17 mM.), KCl (4.76 mM.), CaCl$_2$\cdot2$H_2$O, (1.60 mM.), MgSO$_4$\cdot7$H_2$O (1.19 mM.), and KH$_2$PO$_4$ (1.19 mM.). Streptomycin sulfate and penicillin-G potassium were also added in 50 and 75 mcg./ml. amounts, respectively.

Stock solutions, in isotonic concentrations, were freshly prepared weekly using pretreated 18 meg. glass distilled milli-Q polished water. Media pH ranged between 7.25 and 7.30 following a 20 minute period of equilibration with a filtered 5% CO$_2$ - 95% air mixture. The milliosmolarity of stock solutions consistently ranged between 278 and 288 milliosmols. New media was prepared if solutions failed to meet any of these criteria. No attempt was made to readjust media parameters. All media were filtered using a 0.22 Micron disposable nonpyrogenic, sterile Millipore filter immediately prior to use.

Twenty-four to 48 hours before each experiment, mineral (paraffin) oil (Fisher: Saybolt viscosity = 125/130) was steam autoclaved (20 minutes at 120$^\circ$C), equilibrated with a 5% CO$_2$ - 95% air mixture for 15 to 20 minutes, and stored in the culture incubator. Environmental
parameters of the incubator also consisted of a 5% CO₂ - 95% air mixture maintained at 37°C at 100% humidity.

Each culture dish was placed on a 37°C warming tray following its removal from the incubator when manipulative (e.g. ova washing, transfer, and/or observation) protocols were required. Each experimental protocol consisted of the following common elements: (1) culture dishes containing 0.5 ml. of media were completely overlayed with mineral oil; (2) all cumulus masses (two per culture dish) were allowed to equilibrate for 60 minutes prior to fertilization; (3) undiluted epididymal spermatozoa were allowed to mature/capacitate for 60 to 90 minutes; (4) fertilization was initiated by the addition 2.5 - 5.0 x 10⁵ spermatozoa/dish (0.5 to 1.0 x 10⁶ sperm/ml.); (5) fertilization was allowed to proceed for six hours before the ova were removed from the dispersed cumulus and nonfertilizing sperm cells, washed twice, and placed back into a new culture dish containing fresh media (a maximum of 40 fertilized ova per dish); and (6) all fertilized ova were evaluated 24 hours later for the presence of two equal sized blastomeres (occasionally three were noted) without evidence of fragmentation or cytoplasmic "blebbing" (Plates VI and VII). At the time of transfer from fertilization to developmental dishes (six hours post-fertilization), as many as possible degenerating, or fragmenting ova, were removed from the experimental process.
PLATE VI.: Single, 2-cell and Fragmentating Ova (100x) 24 Hours Post-Fertilization

PLATE VII.: 2-cell Ova (400x)
Experimental Design

This study consisted of six independent experimental protocols that are described as follows:

Protocol I:

Different from all other protocols, experimental and control media contained only uterine flushings from animals containing, or free from, IUDs respectively. At no time during any of the in vitro culture procedures were the IUDs placed directly into the media.

In order to obtain uterine flushings, the proximal (cervical) end of each horn was ligated closed with a small tie of 4-0 silk suture. A 25 gauge needle, fitted onto a tuberculin syringe and filled with 0.5 ml. of equilibrated media, was passed into the lumen of a single horn. Medium was gently forced into the horn until it became mildly distended. Negative pressure was then applied to the syringe plunger causing the horn to collapse as the flushed medium was returned to the syringe barrel. This process was repeated a total of three times with the same syringe and medium. The resulting 0.5 ml. of media was then placed under the oil in the usual fashion and subsequently used as the culture media. This process was carried out for each control, as well as experimental, horn.

Cumulus masses, 2 per dish, were placed into the media and allowed to equilibrate/incubate for one hour prior to fertilization. Spermatozoa were added directly to the test dishes with fertilization occurring during the following six hours. After the fertilized ova
were washed, they were transferred to fresh culture media and observed for division 24 hours later. No attempt was made to replenish the fresh media with appropriate (control or experimental) uterine fluids.

Protocol II:
In this group, and in all subsequent protocols, gametes were placed, at some time during the in vitro process, in the presence of an actual IUD instead of only being incubated with uterine flushings (Plate VIII). Specifically, Protocol II called for the appropriate experimental, or control, IUD to be present in the media throughout the oocyte incubation (1 hour), fertilization (6 hours) and subsequent developmental (24 hours) stages. Only the capacitation of the spermatozoa (90 minutes) was allowed to continue under untreated, or neutral, media conditions.

Protocol III:
In Protocol III, considered a subset of Protocol II, control and experimental IUDs were added to the in vitro culture system only at the time of fertilization. Each respective IUD remained with the oocytes throughout the 24 hour developmental period. Spermatozoa and ova were allowed to incubate for 90 minutes and one hour respectively under normal, or untreated, media conditions.
PLATE VIII.: Incubating Oocytes in the Presence of an IUD (25x)
Protocol IV:

As a subset of both Protocols II and III, Protocol IV specified that either experimental or control IUDs be in the culture system only during the 24 hour developmental time frame. All spermatozoa capacitation and ova incubation and fertilization procedures were carried out under untreated media conditions.

Protocol V:

This experimental design called for a one hour incubation of the oocytes, and their associated cumulus masses, with IUD suture material. Following this one hour treatment period, the masses were removed from IUD-contaminating medias, washed twice, and subsequently placed into fresh untreated media (without IUD). Normally capacitated spermatozoa were then added in the usual fashion. After six hours, all ova were washed according to protocol and placed into fresh developmental media for the remaining 24 hours.

Protocol VI:

This group was designed to measure whether an IUD was able to exert an effect on a spermatozoa's potential to initiate and complete normal fertilization parameters. This was accomplished by allowing epididymal spermatozoa to undergo capacitation with an experimental, or control, IUD present in the culture media. Following 90 minutes of incubation, an aliquot containing $2.5-5.0 \times 10^5$ sperm was removed and
immediately placed with dishes containing appropriately incubated ova and cumulus masses.

Control sperm, having come from the contralateral epididymis (internal male control) and capacitated in media containing a control IUD, were placed with ova treated in a similar fashion to those within the experimental group. In Protocol VI the only control contact of the IUD with the culture system was during the 90 minute capacitation period.

In Figure 1, each protocol is displayed as a bar graph that represents the various stages of the in vitro developmental processes that were affected by the presence of an IUD or intrauterine flushes.

Statistical Analysis:

Observations of all control and experimental ova for each protocol was classified as either 2-cell or non-2-cell according to their morphological appearance at the end of the 24-hour developmental stage. Since the observations were considered categorical in nature, a Chi-square analysis, comparing the number of 2-cell embryos versus the number of non 2-cell embryos, was performed for each protocol.

Specifically, a procedure utilizing the computer based Statistical Analysis System (SAS; 1982), called FUNCAT, or Functions of Categorical responses, was used to analyze the data. FUNCAT is specified like an analysis-of-variance procedure except that the response is categorical rather than continuous.
<table>
<thead>
<tr>
<th>Protocol</th>
<th>Spermatozoa Capacitation (60 - 90 minutes)</th>
<th>Oocyte Incubation (60 minutes)</th>
<th>Fertilization (6 hours)</th>
<th>Development (24 hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protocol I</td>
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<td>Protocol II</td>
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<td>Protocol V</td>
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<tr>
<td>Protocol VI</td>
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</table>

**FIGURE 1.** Stages of in vitro fertilization in which Uterine Flushings (Protocol I) or IUDs (Protocols II thru VI) were present during culture
RESULTS

Protocol I

A total of 102 control and 91 experimental ova were evaluated for successful completion of first mitotic division. Eighty-eight (86.3%) and 83 (91.2%) two-cell embryos were observed in the former and latter groups respectively (Table 1). No significant differences ($X^2=2.27$, df.=1; $P=0.13$) were demonstrated between groups of ova that had been allowed to incubate and fertilize in media containing uterine flushings obtained from animals with, or without, IUDs.

Protocol II

Groups of 848 and 889 control and experimental ova respectively were observed under conditions in which IUDs were present in the media throughout every stage of in vitro ova development. Within the control group, 587 (69.2%) ova developed into 2-cell embryos whereas only 352 (39.6%) experimental ova were able to complete first cellular division successfully (Table 1). Significant differences ($X^2=165$, df.=1; $P<0.0001$) were found to be present between the control and experimental groups of Protocol II.

64
Protocol III

When oocyte fertilization, and subsequent division, proceeded in the presence of an IUD (without a one hour IUD exposure prior to fertilization), 207 of 228 (90.7%) control and 66 of 257 (25.6%) experimental ova continued on to complete 2-cell development (Table 1). These results were different at the $P < 0.0001$ level ($X^2 = 74.7, df. = 1$).

Protocol IV

A total of 193 control and 224 experimental fertilized ova were placed in culture media containing IUDs during only the last 24 hours of development. With this protocol, 71 (36.7%) control and 95 (42.4%) experimental ova developed into 2-cell embryos (Table 1). No differences ($X^2 = 1.00, df. = 1; P = 0.31$) were found to exist between the two treatment groups.

Protocol V

When oocytes were incubated for one hour with IUDs, but were fertilized and allowed to develop under non-IUD (neutral) conditions, a significant reduction in the number of 2-cell embryos was observed ($X^2 = 12.2, df. = 1; P < 0.0005$). Sixty-nine (56.5%) of 122 control and 105 (42.8%) of 245 experimental ova were recorded as having completed first cellular division (Table 1).
Protocol VI

If an IUD was present only during sperm capacitation, while oocyte incubation, fertilization and development took place under neutral conditions, significant reductions in the number of developing 2-cell embryos were still noted (Table 1). Whereas 75.4% (123/163) of control ova were considered 2-cell embryos after 24 hours of in vitro culture, only 56.9% (234/411) of the ova were able to complete 2-cell division within the experimental group ($X^2 = 46.5$, df. = 1; $P < 0.0001$).

Figure 2 represents the data from each of the six experimental protocols expressed as a percentage of the number of 2-cell embryos observed with respect to each appropriate control. Using a basis of 100% for each control protocol, the percentage of experimental embryos for Protocols I thru VI was 106%, 57%, 28%, 115%, 76% and 75% respectively.
TABLE 1. Comparison of Numbers of Ova Developing into 2-cell Embryos in Control and Experimental Groups for each Protocol

<table>
<thead>
<tr>
<th>PROTOCOL</th>
<th>TREATMENT GROUPS</th>
<th>Control</th>
<th></th>
<th>Experimental</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Number of 2-cell Embryos</td>
<td>(%)</td>
<td>Total</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td></td>
<td>88 (86.3)</td>
<td>102</td>
<td>83 (91.2)</td>
<td>91 ns</td>
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<tr>
<td>II</td>
<td></td>
<td>587 (69.2)</td>
<td>848</td>
<td>352 (39.6)</td>
<td>889 **</td>
</tr>
<tr>
<td>III</td>
<td></td>
<td>207 (90.7)</td>
<td>228</td>
<td>66 (25.6)</td>
<td>257 **</td>
</tr>
<tr>
<td>IV</td>
<td></td>
<td>71 (36.7)</td>
<td>224</td>
<td>95 (42.4)</td>
<td>193 ns</td>
</tr>
<tr>
<td>V</td>
<td></td>
<td>69 (56.5)</td>
<td>122</td>
<td>105 (42.8)</td>
<td>245 *</td>
</tr>
<tr>
<td>VI</td>
<td></td>
<td>123 (75.4)</td>
<td>163</td>
<td>234 (56.9)</td>
<td>411 **</td>
</tr>
</tbody>
</table>

* P 0.0005
** P 0.0001
FIGURE 2. The Effect of Experimental Treatment Upon the Formation of 2-cell Embryos Expressed as a Percentage of Matched Control Development
DISCUSSION

Although specific mechanisms of action exerted by intrauterine devices (IUDs) have been demonstrated to vary from species to species, at least some degree of reduced fertility potential has been universally shown to exist in all animals studied to date. Such effects include ova "tube-locking", decreased fertilization rates, ovulatory dysfunction (shortened luteal phases and increased LH to ovulation time intervals), spermatozoa and ova phagocytosis, altered ova, spermatozoa, and/or embryo transport, inappropriate uterine environments, both histologically and biochemically, and pathological changes in systemic immunoglobulin responses.

By and large, the majority of the work has been performed under in vivo conditions. The variety of inhibitory effects was thought to be due to anatomical and physiological differences between the species examined. However, as "toxic" alterations in the uterine environment became recognized as an additional important contribution to IUD-induced infertility, a few studies employing relatively new in vitro laboratory techniques were subsequently undertaken. Many of these in vitro studies primarily documented the inhibitory activity of polymorphonuclear leukocytes (PMNL) extracts on 2-cell embryos collected after in vivo fertilization. Unfortunately, most of these extracts were obtained from either different species or different anatomical
locations (example given: peritoneal PMNLs) within a specific species. Only one study investigated the effects of fluids directly obtained from the reproductive tracts of animals bearing IUDs. This work was initiated, however, utilizing 2-cell embryos fertilized in vivo.

This current study was concerned not only with the direct effect that an IUD, or IUD-associated components, may exert on the early phases of embryonic development, but it was also interested in documenting fertilization derangements due to alterations, in ova and spermatozoal function. The first attempt to achieve this goal (Protocol I) was designed to document an effect that IUD-bearing uterine fluids might exert on in vitro ova fertilization. Although no differences were found between the control and experimental groups, certain technical and physiological difficulties were encountered that may have masked any potential adverse effect.

Obtaining sufficient, uncontaminated, fresh uterine fluids from mouse oviducts was difficult to accomplish successfully. Although several techniques were employed, the actual amount of uterine fluids retrieved was always in question. Assuming that the horns were successfully rinsed, the question of dilution, i.e. the ratio of stock culture media to experimental uterine fluid, must be addressed. In the single instance of chronically inflamed uterine horns (one out of more than two hundred animals with surgical placement of IUDs) in which large volumes of fluid (approximately 0.5 ml. per horn) containing PMNLs were collected, no 2-cell development was noted from an initial population of 114 ova fertilized. Animals possessing larger
reproductive tracts, accompanied by successful histories of in vitro culture techniques, would have provided a better model for studying this particular phenomenon.

With the single exception of Protocol IV, when only fertilized ova were incubated with an IUD, all the remaining experimental protocols (II, III, V, and VI) demonstrated significant reductions in the final number of 2-cell embryos observed. Given the present experimental conditions, IUDs were able negatively to influence spermatozoa and unfertilized oocyte function, as well as the fertilized ova's ability to undergo first mitotic division.

Although no direct comparison can be drawn between the respective Protocols, the greatest effects were noted whenever the IUD was present during the six hours of in vitro fertilization. Protocol II, when oocyte incubation, fertilization, and development were affected, demonstrated a 43 percent reduction in the number of 2-cell embryos while Protocol III, involving only the fertilization and development stages of ova/embryo growth, inhibited cellular division by 72 percent. Of interest is the observation that the influence of the IUD, in Protocol II for the extra one hour during oocyte incubation, was not able to inhibit fertilized ova division to a greater extent than when it was present only during fertilization and development. If this effect was indeed real, one possible explanation for its presence was that the IUD had lost some of its potential effect by being allowed to incubate with ova and cumulus masses for one hour before fertilization was initiated.
No inhibition of \textit{in vitro} division was noticed when the experimen-
tal treatment was introduced during the last stage of development
(Protocol IV). Even though the time of IUD exposure was the longest
for any of the Protocols, 24 hours, the ability of the fertilized ova
to proceed with cellular division was not inhibited. It was therefore
concluded that at this very early stage of development, no embryotoxic
effect could be demonstrated.

Reductions in the number of 2-cell embryos, although not as large
percentage wise as recorded for Protocols II and III, were nevertheless
significant when spermatozoa (Protocol VI) and ova (Protocol V) were
experimentally treated individually. Although not objectively quanti-
fied, the presence of an IUD in the culture media of capacitating
spermatozoa appeared to decrease motility, as well as increase
agglutinability. Whether the decrease in the sperm cell's ability to
fertilize ova was a result of quantitatively fewer motile and less
active cells being added to the culture system, or was a result of
qualitative changes in cell membrane structure, possibly affecting
capacitation or oocyte binding potential, could not be accurately
determined.

Since direct quantitative assessment of oocyte toxicity, within an
ongoing \textit{in vitro} culture environment, is even harder to evaluate than
for spermatozoa, the reasons for the observed inhibition of 2-cell
development under the conditions of Protocol V became even more specul-
ative. Two avenues of approach that may be involved are: 1) an oocyte
toxic environment that is detrimental to normal cellular physiology,
and/or 2) alteration(s) in zona pellucida biochemistry. This latter effect could cause changes in specific oocyte-spermatozoa interactions leading to actual failures of attachment and/or successful penetration.

Of note was the observation that the toxic effect exerted by the IUD during oocyte incubation was able to occur in the presence of nondispersed cumulus cells. Although the cumulus masses were not as tight, or compact, by the end of the one hour of incubation as they were when they were first collected from the fallopian tube, nevertheless each oocyte remained surrounded by numerous layers of tightly attached granulosa cells.

Two additional observations also helped to support the idea that the gametotoxic component of the IUD was soluble in nature. First, under microscopic visualization (400x) no dying, or qualitatively impaired, spermatozoa were found attached to any cells or endometrial debris derived from the uterus and subsequently transferred with the IUD into culture. At no time during any of the protocol procedures were either the gametes, or the fertilized ova and 2-cell embryos, observed to be associated with direct cell-to-cell contact as a result of the IUD.

Secondly, there appeared to be no gradient effect on whether an oocyte developed into a 2-cell embryo in relation to its position and distance from the IUD. In protocols in which significant effects were documented, the number of single-cell ova and 2-cell embryos was randomly distributed throughout the dish. It was of interest to observe normal two-cell embryos having developed in direct contact with the IUD.
along with single cell ova distributed near the periphery of the media some 1 to 2 cm. from the suture material itself.

The solubility of this IUD derived gametotoxic effect is strongly supported in spite of the observation that every IUD used in each protocol was surrounded, or coated, with cellular components. Although the predominant cell types were PMNLs, no support for the hypothesis that they were able to engage in phagocytosis of either the gametes, or of the embryos, under *in vitro* conditions, was substantiated (Plate IX).

As a part of the experimental design, each protocol consisted of multiple runs, or repeat experiments, thereby introducing an additional cause of possible statistical variation. All protocols, except for II, which comprised six separate runs, were repeated in duplicate (Appendix A). Since considerable variation was observed within control populations among the runs (the percentage of fertilized ova resulting in 2-cell embryos), the FUNCAT Chi-square analysis (SAS, 1980) was modeled to include run and run by treatment interactions. Significant run variations were documented (P < 0.0001) for all protocols. Although Protocols I, IV, V, and VI demonstrated no significant treatment by run interactions, Protocols II and III were different at the P < 0.0001 levels.

Upon further examination of individual Chi-square analyses for each run within a particular protocol, the interaction was considered to be divergent, instead of convergent, in nature. This simply indicated that even though the degree (percentage) of inhibition exerted by
PLATE IX.: IUD Associated PMNLs (1000x) Identified with Safranin and Genetian Violet Stains
the toxic effect could not be accurately predicted among runs, the
direction of the effect was always consistent. It was always signifi­
cantly directed against the experimental treatment group.

In addition to evaluating the number of cleaved 2-cell embryos, a
population of oocytes, grossly morphologically normal at the time of
postfertilization transfer, were noticed to have undergone degeneration
and fragmentation during the following 24 hours. Whether these ova had
degenerated prior to or after fertilization had occurred could not be
ascertained from this experimental design. Since this particular class
of fragmenting oocytes was unforeseen, they were considered as
non-2-cell embryos, and were therefore grouped with the morphologically
normal, nondividing, single-cell oocytes, for statistical evaluation.
Nevertheless, ex post facto Chi-square analysis, comparing the number
of degenerate ova occurring between the control and experimental treat­
ment groups for each of the protocols, was performed. No particular
significance, or trends, could be identified. The pronounced signifi­
cant effect consistently observed in comparing experimental with con­
trol groups in Protocols II, III, V, and VI was a decreased percentage
of 2-cell embryos and a concomitantly increased percentage of single
cell ova.

The role(s) of PMNLs in reproductive immunology needs further
elucidation. In addition to this particular study, where the presence
of PMNLs were linked with the inhibition of spermatozoa viability and
oocyte fertilizability, this cell type has also been associated with a
variety of gametoxic and embryotoxic events observed under both in vivo
and in vitro conditions. Recently, PMNLs in human semen have been correlated with reduction of the spermatozoa's capacity to successfully penetrate zona free hamster oocytes (Rogers, 1983). Whether the normal, or pathological, appearance of these phagocytic cells, or their products, are able to adversely affect the fertilization of species specific oocytes, the normal development of early embryos and/or the initiation of implantation under in vitro conditions, especially those being currently utilized with human in vitro fertilization and embryos transfer programs, requires further investigation.
The antifertility effects of intrauterine devices (IUDs) have been recognized for decades; however, clarification of the mechanisms of action has remained largely elusive. Speculations, in part, have included possible alteration in tubal transport, mechanical interference with implantation and local gametotoxic effects. The purpose of this study was further to elucidate the effects that an IUD, or IUD-associated substances, could exert on mouse oocytes, spermatozoa and early embryonic development utilizing in vitro fertilization techniques.

IUDs (4-0 silk), having resided within the uteri of mice for at least ten days, were placed within the in vitro culture system during various stages of gametogenic and embryogenic development. These various stages of IUD intervention were represented by Protocol I: oocyte incubation (60 minutes) and fertilization (6 hours) in culture media containing only fluids obtained from IUD-bearing uteri (no IUD material); Protocol II: oocyte incubation, fertilization and development (24 hours) in the presence of IUD suture material (the same for all subsequent protocols); Protocol III: fertilization and embryo development; Protocol IV: embryo development; Protocol V: oocyte incubation and Protocol VI:
epididymal spermatozoa capacitation. The number of 2-cell embryos vs. total ova observed for control and experimental treatment groups was: Protocol I) 88 2-cell embryos vs. 102 total ova for the control group, and 83 2-cell embryos vs. 91 total ova for the experimental group; Protocol II) 587 vs. 848 and 352 vs. 889; Protocol III) 207 vs. 228 and 66 vs. 257; Protocol IV) 71 vs. 224 and 95 vs. 193; Protocol V) 69 vs. 122 and 105 vs. 245; and Protocol VI) 123 vs. 163 and 234 vs. 411. Significant reduction in the number of embryos was noted for Protocols II (P < 0.0001), III (P < 0.0001), V (P < 0.0005), and VI (P < 0.0001). No difference between treatment groups was found for Protocols I (P=0.13) and IV (P=0.32). The percent of 2-cell embryo development, when compared with matched controls, was 106, 57, 28, 115, 76 and 75 for Protocols I, II, III, IV, V and VI, respectively.

These results suggest that in vitro capacitation of spermatozoa, oocyte incubation/maturation and oocyte fertilization are significantly inhibited when allowed to culture directly with an IUD. The development of 2-cell embryos, either in the presence of an IUD postfertilization, or with only IUD-flushes, appears to be unaffected.
APPENDIX
APPENDIX A: Numbers of Ova Developing into 2-cell Embryos in Control and Experimental Groups for each Protocol Listed by Individual Run

<table>
<thead>
<tr>
<th>PROTOCOL</th>
<th>RUN</th>
<th>TREATMENT GROUPS</th>
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<th>Treatment</th>
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<td></td>
<td></td>
<td>Number of 2-cell</td>
<td>Total (%)</td>
<td>Number of 2-cell</td>
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<td></td>
<td></td>
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<tr>
<td></td>
<td>B</td>
<td>90</td>
<td>102 (88)</td>
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BIBLIOGRAPHY


