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XENOGENOUS FERTILIZATION OF BOVINE AND PORCINE OOCYTES

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XENOGENOUS FERTILIZATION OF BOVINE AND PORCINE OOCYTES

By

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ABSTRACT

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By

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Fertilization of bovine and porcine follicular oocytes and hamster oviductal oocytes in the oviduct of the pseudopregnant rabbit (xenogenous fertilization) was studied.

When oviductal recovery of hamster oocytes from the rabbit oviduct is 32 hr or earlier after insemination, no tubal ligation is necessary to avoid ovum loss.

The 2-cell stage block observed with hamster xenogenously fertilized embryos cannot be overcome with either Fetal Calf Serum (FCS) nor sheep serum heat inactivated at 56°C for 30 minutes.

Bovine and porcine follicular oocytes were capable of being fertilized xenogenously as shown by overall fertilization and recovery rates of 13.4% and 44.8%, respectively, for bovine, and 2.0% and 36.1%, respectively, for porcine oocytes. A significant decrease in recovery ($P < 0.01$) and fertilization rates ($P < 0.001$) for porcine oocytes with respect to bovine oocytes was observed.

Transport in 0.15 M NaCl solution of ovaries at 0° vs 37°C had no effect on either recovery or fertilization rates for bovine and porcine oocytes.

Several recovery times were used for bovine oocytes. Recovery times from 50 to 70 hr significantly differed ($P < 0.01$) from earlier (40-45 hr) or later (70-75 hr) times.

Fertilization was not affected by recovery time, although an increased number of 2-cell embryos was recovered at 50 hr after insemination.

In vitro culture of oocytes in medium supplemented with 5 µg/ml of follicular stimulating hormone (FSH) decreased ($P < 0.1$) the recovery rate from the rabbit oviduct (91.9% of control vs. 76.7% when treated with FSH). No effect was observed on fertilization. Cleavage to the 2-cell stage was achieved when oocytes were preincubated with FSH.

In vitro culture of bovine follicular oocytes in medium for 25 hr prior to xenogenous fertilization significantly increased the recovery from the rabbit oviduct ($P < 0.01$). No effect was observed on the fertilization rate.

To my grandparents

Rodolfo Schwarz and Hilde K. de Schwarz

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INTRODUCTION

Xenogenous fertilization is the fertilization of an oocyte of one species in the oviduct of another species using homologous sperm.

There are several ways of increasing the number of embryos available for research. Superovulation techniques (with gonadotropins) to increase ovulation rates are widely used. Follicular oocytes have been shown to be capable of maturation in culture (Pincus and Enzman, 1935; Chang, 1955a, 1955b; Edwards, 1962, 1965, 1966; Quirke, 1969; Sreenan, 1970; Cross and Brinster, 1970; Mukherjee, 1972; Foote and Thibault, 1969; Hunter et al., 1972; Jagiello et al., 1974, 1975; Shea et al., 1976) and furthermore, can be fertilized *in vitro* and reach different stages of development depending on the species (Suzuki and Mastroiani, 1968; Edwards et al., 1969; Sreenan, 1970; Bedirian et al., 1975; Edwards, 1966; Hunter et al., 1972). Xenogenous fertilization coupled with superovulation offers a means of increasing the number of embryos available for research. When superovulation techniques are used an increased number of follicles grow to the preovulatory stages. Aspiration of oocytes from these follicles and xenogenous fertilization provides the possibility of studying time development, of different stages of embryos at early times of their development.

Explanation of the occurrence of events at early times after fertilization can be more easily approached by using this technique. Early embryos obtained from the rabbit oviduct can be examined further knowing their developmental stage.

With the present status of embryo transfer and embryo freezing technology, all the transferred embryos are produced by in vivo fertilization of gonadotropin-induced ovulated oocytes. These oocytes are fertilized naturally or with frozen semen with artificial insemination techniques. These embryos must then be flushed from the donor's reproductive tract and they must be transferred to a synchronized recipient to produce a pregnancy. However, in the case of tubal or ovarian abnormalities, ovulation or fertilization are impaired. In this case, laparoscopic follicular aspiration of oocytes with further xenogenous fertilization of these oocytes can increase the percentage of embryos recovered. Follicular aspirations of ovaries obtained at slaughter or by other surgical procedures allow fertilization and the increased availability of embryos for further teratological or morfological analysis. In the present study, a review on the current literature of xenogenous fertilization, in vitro fertilization, ova culture in vitro, and embryo culture in the rabbit reproductive tract is presented. The main objective of the present work was to determine the possibility of fertilizing bovine and porcine oocytes in the pseudopregnant rabbit reproductive tract. Variables such as the effect of ligation of the oviduct, temperature of transport of the ovaries, different times of recovery after insemination from the rabbit oviduct, in vitro culture with medium supplemented with FSH and in vitro culture for 25 hr prior to xenogenous fertilization were investigated in relation to recovery and xenogenous fertilization rates.

LITERATURE REVIEW

Bovine ovum development in culture

Hamilton and Laing (1946) timed the developmental stages of bovine oocytes as shown in Table 1. They reported that the egg enters the uterus 96 hr after ovulation in the 8- to 16-cell stage. Ovulation occurred 10 to 15.5 hr after the end of estrus. Hunter et al. (1972) indicated that follicular oocytes matured *in vitro* and transferred to previously inseminated heifers are capable of fertilization and development. Oocytes that had been cultured for 24 to 26 hr were transferred. Recipients were slaughtered 21 to 23 hr after transfer and 29 of 55 (52.3%) tubal eggs were recovered. Sixteen (55.2%) of these oocytes had been penetrated by sperm. Eleven were at the pronuclear stage, six of which had the opposing pronuclei located eccentrically. The male pronucleus was larger in most cases. Three eggs were recently penetrated and were in the anaphase of the second meiotic division. The sperm head was swollen. Two 2-cell embryos were recovered. Assuming that 6 to 8 hr were required to complete oocyte maturation, cleavage was believed to have occurred 16 hr after sperm penetration.

Recently fertilized bovine oocyte would be expected to develop *in vivo* to a late morula or early blastocyst over six days (Table 1). Eight-cell ova developed *in vivo* to the late morula and blastocyst in a period of four days. However, most cultured ova are retarded in this development (Tervit et al., 1972).

Cleavage of in vivo fertilized eggs in a chemically defined medium having pyruvate as the only source of energy was achieved 48 to 72 hr after hCG or LH injection (McKenzie and Kenney, 1973). Cleavage from 4 to 8 cells occurred between 48 and 72 hr of culture. The rate of cleavage was accelerated in the donor animals when corpus luteum regression was hastened by gonadotropin injections than when manually enucleated. The rate of cleavage was similar to the in vivo cleavage rate reported by Hamilton and Laing (1947).

Whenever follicular oocytes are matured in vitro, they undergo germinal vesicle breakdown and progress to metaphase II within 19 to 31 hr (Shea et al., 1976; Edwards, 1965; Newcomb et al., 1978; Jagiello et al., 1974). Ovulation occurs approximately 28 hr after the LH peak in heifers (Christenson et al., 1974), 27 hr from onset of estrus (Hammond, 1927) or 10 to 18 hr after the end of estrus (Nalbandov and Casilda, 1942). Thus, maturation in vivo requires less than 28 hours. Undoubtedly, some oocytes commence atresia within the ovarian follicle (Edwards, 1966). This may account for the failure of some individual oocytes to mature or be fertilized in vitro.

Brackett et al. (1977, 1978) observed cleavage of the 2-cell stage within 24 hr of culture of gonadotropin-induced ovulated oocytes recovered 30 hr after onset of estrus. Further cleavage to the 4-cell stage required an additional 24 hr of culture (Table 1). Newcomb et al. (1978) showed that follicular oocytes matured in vitro are capable of being fertilized. Two bull calves were born from these follicular oocytes that, after maturation, were transferred to inseminated

Table 1. Timed stages of development of bovine oocytes *in vivo* and *in vitro*

	Hours after Ovulation (Hamilton and Laing, 1946)	Hours after Follicular Aspiration (Hunter et al., 1972)	Hours after hCG (McKenzie and Kenney, 1973)	Hours after Onset of Estrus (Wright et al., 1976)	Hours after Onset of Estrus (Brackett et al., 1977, 1978)	Hours after *in vivo* Maturation (Newcomb et al., 1978)	Hours Post-Ovulation (Thibault, 1966 Brackett et al., 1980)
2 Pronuclei	--	--	--	--	--	--	5-12
1-cell	23-52	45-49	--	--	--	--	--
2-cell	40-56	45-49	48-72	--	54	--	--
3 to 4-cell	44-66	--	--	--	78	--	--
5 to 8-cell	47-96	--	96-144	72-96	--	168	44
9 to 16-cell	71-141	--	--	--	--	--	--
Morula	144	--	--	138-174	--	--	--
Blastocyst	190	--	--	192-228	--	168	--
Expanded Blastocyst	--	--	--	207-256	--	--	--

recipients. Seven days after transfer the heifers were sacrificed and ova recovered. Of the recovered ova 23.3% cleaved at least to the 8-cell stage. Two blastocysts were transferred to another recipient and two normal bull calves were born.

In gonadotropin treated cows, pronuclear ova are found 5 to 12 hr after ovulation (Thibault, 1967; Brackett et al., 1980), and the 8-cell stage is reached at 44 hr after ovulation (Thibault, 1966; Brackett et al., 1980) (Table 1).

Porcine ovum development in culture

With the report of Leman and Dziuk (1971) indicating that porcine follicular oocytes are capable of being fertilized *in vivo* and develop to a 25 day embryo, a new area of research was opened.

Ova develop to the 4-cell stage in the oviduct. The uterine environment is required for further normal cleavage (Rundell and Vincent, 1969).

It was shown by Rundell and Vincent (1969) that ova cultured *in vitro* can develop up to the 4-cell stage. However, when ova were transferred without culture, development to 105 days of pregnancy was achieved.

Ovulation occurs 24 to 48 hr after injection of hCG (Hunter and Polge, 1966; Baker et al., 1967; Hunter 1972) or at 30 to 36 hr after the onset of estrus (Lewis, 1911; Burger, 1952; Pitkjamen, 1958). When ova were recovered from 3 to 6 hr after mating, 83% of the animals had fertilized ova. Sperm penetration was observed as early as 3 hr after mating. Mature male and female pronuclei in syngamy were seen 6 hr after mating (Hunter, 1972).

The evolution of the sperm head to an early pronucleus required 1½ to 2 hr whereas the first cleavage of pig ova was not observed until 12 to 14 hr after penetration (Hunter, 1972).

Davis and Day (1978) reported recovery of 4-cell embryos on the fourth day after the onset of estrus, and recovery of 2-cell embryos two to three days after first detection of estrus. When 1- and 2-cell embryos were cultured for 72 hr in vitro, only 16 of 29 2-cell eggs cleaved to the 4-cell stage and only 3 more than doubled their cell number. None completed two cleavage divisions (16-cell stage). However, when 4-cell embryos or later stages were cultured in vitro, blastocysts were formed. Early 4-cell embryos should reach the blastocyst stage in three days (Davis and Day, 1978). Morulae recovered on day 5 or 6 from the uterus of the sow will develop to blastocysts in vitro and undergo some expansion (Davis and Day, 1978).

One-cell fertilized ova will cleave to the 2- and 3-cell stage by 48 hr of culture. Two-cell embryos cleave to 4- to 8-cells and 4-cell cleave to 13- to 16-cells by 48 hr of culture. When 72 hr culture was used, the 1-cell ova cleaved to 3-cell, the 2-cell ova cleaved to 4- to 8-cell and the 4-cell cleaved from 4- to more than 20-cells in vitro (Davis and Day, 1978).

Cleavage of 4-cell embryos to morula was achieved by 24 hr of culture (8- to 16-cells). This appears to be near the in vivo rate of cleavage. Although some 4-cell embryos did not cleave during the first 24 hr, they eventually developed to blastocysts. This is not necessarily abnormal since Hunter (1974) observed that the 4-cell stage

lasted 20 to 24 hr in vivo. By 48 hr all embryos destined to become blastocysts had assumed the characteristics of morulae and some had developed a blastocoel. By 72 hr most embryos were blastocysts (Davis and Day, 1978). From Hunter's (1974) report, it appears that early 4-cell embryos require 72 hr to form blastocysts in vivo. This coincides with the findings of Davis and Day (1978) in vitro.

Loss of the zona pellucida is reported to occur in vivo on the sixth day after ovulation (Hunter, 1974). Loss of zona pellucida in vitro (starting from 4-cell embryos) occurs six to eight days after ovulation (Davis and Day, 1978).

In vitro fertilization of cattle ova

Several attempts to fertilize bovine oocytes in vitro have been made. Von Bregulla et al. (1974) used ovaries from slaughtered cows. The oocytes were held in TC 199 containing FCS and antibiotics. Sperm capacitation was both in vivo and in vitro. Of 1977 oocytes, they recovered 12 (0.6%) in the first polar body stage; 13 (0.7%) in the second polar body stage and 3 (0.2%) at later development stages. A total of 6 (0.3%) cells divided. Of these, one was 2-cells, two were 4-cells, two were at the 5-cell stage and one was a 9-cell embryo.

Edwards (1973) reported evidence of in vitro fertilization of bovine oocytes without giving details.

Iritani and Niwa (1977) used oocytes from ovaries of slaughtered cows. Capacitation of the sperm was in the reproductive tract of an estrous cow, estrous doe, or in a Kreb's-Ringer bicarbonate (KRB) solution. After capacitation, the sperm was washed and added to the

oocyte culture. They reported that 60% of the follicular oocytes matured to the second metaphase. No fertilization was observed when KRB was used for capacitation. However, 6 of 48 (20.7%) oocytes showed evidence of sperm penetration when the oviduct of the estrous cow was used for sperm capacitation. When the uterus of an estrous cow was used for capacitation, a total of 5 of 44 (18.5%) oocytes were penetrated. When the uterus of an estrous doe was used for capacitation, 10 of 78 (21.2%) of the oocytes were penetrated.

Brackett et al. (1978) reported in vitro fertilization for bovine at a rate of 56% when ejaculated sperm capacitated in vitro were used. These scientists also reported no difference in fertilization of follicular or oviductal oocytes. Furthermore, they reported that 71.4% of the oocytes matured to the 2-cell stage within 24 hr. Of these, 57.1% reached the 4-cell stage within 48 hr of culture (Brackett et al., 1977, 1978). The donor cows were primed with PMS and ovulation induced with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$).

In vitro fertilization of swine ova

Iritani et al. (1975) determined the rate of in vitro fertilization and cleavage rates in swine. When using spermatozoa preincubated in modified Kreb's-Ringer bicarbonate (KRB), oviduct, and uterus of an estrous sow, rates of fertilization were of 10/137 (7.3%); 8/128 (5.2%); and 54/469 (11.5%), respectively. They also reported cleavage rates of in vitro fertilized porcine eggs of: 0/128 (0%), 42/469 (9.0%), and 12/137 (8.8%) using sperm capacitated in KRB, oviduct, and uterus, respectively. Their report also demonstrated

that none of 263 eggs cultured without sperm cleaved. In these experiments, ovaries were obtained at slaughter and follicular oocytes aspirated and cultured for 26 to 30 hr to reach metaphase II. Collected semen was pretreated in one of three ways: (a) modified KRB, (b) in the oviduct, or (c) in the uterus of an estrous sow. Fertilization was determined 6 to 16 hr after addition of sperm. The criteria used for fertilization was sperm penetration through the vitellus, evidence of second meiosis and cleavage of ova. Cleaved ova were noted between 16 and 40 hr after insemination. Sperm penetration into periviteline space was observed 3 to 5 hr after insemination. Sperm penetration into the vitellus was observed from 5 to 10 hr after insemination. Second meiotic division was observed at 10 to 16 hr. Two pronuclei and syngamy were seen at 16 to 24 hr and cleavage at 16 to 30 hr.

Iritani et al. (1978) reported improved in vitro fertilization of pig oocytes. Ovarian oocytes from slaughter house and ejaculated and epididimal sperm were used. The oocytes were cultured for 24 hr and sperm were capacitated in modified KRB, oviduct, and uterus for 4.5 to 5 hr. Oocytes and sperm were cultured together for 17 to 20 hr. Again 60.6% of oocytes matured to at least metaphase after the maturation period of 24 hr. A total of 12/120 (10.0%) of the oocytes were fertilized when ejaculated sperm were used. None of 400 oocytes were fertilized when the sperm were preincubated in modified KRB, whereas 4/40 (10.0%) and 8/48 (16.7%) were fertilized when the sperm were preincubated in the oviduct and uterus of an estrous sow, respectively.

When epididimal sperm were preincubated in KRB, oviduct, or uterus of estrous sow, the fertilization rates were of 1/30 (3.3%), 9/46 (19.6%) and 14/53 (26.4%) with a total of 24 fertilized eggs from 129 cultured (18.6%).

Ovum culture in the rabbit reproductive tract

Work by Harper (1965) demonstrated that there is a decrease in the rate of ovum transport in the reproductive tract of estrus, 1 day and 2 day pseudopregnant rabbits. Rates of transport are 11.4, 8.9, and 5.7 mm/min in estrus, ovulation, and on day 2.5 of pseudopregnancy, respectively. No further change was noted between day 2.5 and 15 of pseudopregnancy.

Rabbit ova held in the oviduct by ligation develop into early blastocysts, and this development appears to be faster in the luteal phase than in estrous does, however, rabbit eggs cannot develop beyond the early blastocyst stage in the oviduct (Adams, 1973).

Averill, Adams, and Rowson (1955) reported that following transfer to the genital tract of the rabbit, fertilized sheep ova can survive for at least 5 days (120 hr) and develop to the early blastocyst stage.

Hunter et al. (1962) obtained six pregnancies and four live lambs after aerial transport of sheep embryos in the ligated oviduct of the pseudopregnant doe. A total of 17 sheep blastocysts or morulae were recovered after transport and storage in the rabbit oviducts for 101 to 128 hr. Sixteen of these were transferred.

Longer storage periods were obtained by Adams et al. (1968) when, of 20 sheep morulae that were transferred to the oviducts of follicular phase rabbits for 4-7 days, 14 developed into blastocysts. Of these, five had lost the zona pellucida. Two of the latter blastocysts, that were in the rabbit oviduct for 6 days, developed to term after re-transfer to a synchronized ewe. Of 20 cow morula cultured 3 to 4 days, all but one showed further development; however, no pregnancies resulted when some of these blastocysts were transferred to synchronized cows (Adams et al., 1968).

The maximum bovine embryo cleavage obtained in the ligated oviduct of the pseudopregnant rabbit (Sreenan et al., 1968) was of 64 blastomeres. This followed a storage of an 8-cell embryo for a period of 4 days. A total of 138/189 (73%) of the embryo transferred to the rabbit were recovered and 71/138 (51.4%) continued to cleave in the rabbit oviduct (Sreenan et al., 1968).

Sreenan and Scanlon (1968), using follicular fluid as the transfer medium and pseudopregnant does as recipients, obtained development to early blastocysts. When they used homologous serum as the transfer medium, the success was very limited (development to the 19-cell stage, maximum). The pseudopregnant ligated oviduct appears to be a very suitable environment for embryos (Sreenan and Scanlon, 1968; Averill et al., 1955; Hunter et al., 1962; Sreenan et al., 1968; Seidel et al., 1971; Lawson et al., 1972b). The follicular phase rabbit oviduct appears to sustain development of embryos as does the pseudopregnant doe (Adams et al., 1968; Lawson et al., 1972a, 1972b; Trounson et al., 1976a; Allen et al., 1976).

Cleavage of fertilized ova to the 2-, 8-, and 10-cell stages and cleavage of a 2-cell to the 8-cell stage, occurred in the pseudopregnant rabbit oviduct after two days of culture; however, no further cleavage was obtained when ova with more than two balstomeres were transferred (Seidel et al., 1971).

Neither the initial cleavage of the sheep embryos transferred to the rabbit nor the condition of the rabbit (estrus or pseudopregnant) appears to affect the development of sheep embryos. It has been shown that 93% of all sheep embryos continue to develop normally when transferred to the rabbit oviduct (Lawson et al., 1972b). It was also found that uncleaved sheep ova could develop to apparently normal blastocysts in the oviducts of estrous rabbits. This differs from the findings of Brinster and Tenbroeck (1969) who showed that mouse ova are unable to develop in the rabbit oviduct.

The rate of cleavage of sheep ova in the oviducts of estrous rabbits appears to be slightly retarded in comparison to natural in vivo conditions (Lawson et al., 1972b). Such retardation could be caused by effects of ovum manipulation (Tarkowski, 1959). However, 2- and 4-cell eggs appear to develop more rapidly in the oviducts of pseudopregnant rabbits. This could imply that the oviduct of the pseudopregnant rabbit may provide a better environment for development (Lawson et al., 1972b).

Sheep ova appear to remain viable in the rabbit oviduct for up to 5 days. Storage for more than 5 days decreased significantly the viability (Lawson et al., 1972b). These results agree with those

of Witenberger-Torres (1956) who found that sheep ova can remain in the oviduct of the ewe until day 7 without developing a retardation in their development.

When follicular phase rabbit oviducts are used to culture bovine eggs, all of the 1-cell eggs develop into morula and the majority of the 2- and 4-cell egg develop into 16-cell egg by recovery 3 days later (Lawson et al., 1972a).

The transfer of 15 ova cultured three or four days in the rabbit oviduct resulted in 11 (73%) calves (Lawson et al., 1972a).

Trounson et al. (1976a) used the rabbit follicular phase oviduct as an environment, to study development of ova subjected to different temperatures of storage in vitro. Day 5 ova were left in the rabbits' oviducts for 48 hr and day 3 ova were left in the oviduct for 96 hr. The day 3 ova were considered normal after 96 hr incubation and had reached the early blastocyst stage with little expansion of the blastocoele. Whereas blastocysts derived from day 5 ova were generally well expanded, with detectable embryonic discs after 48 hr of incubation. This suggests a retardation in development of the eggs incubated for 96 hr in the rabbit oviduct with respect to those incubated for only 48 hr (Trounson et al., 1976a).

It has also been shown that horse embryos remain viable for at least 2 days in the ligated oviduct of the estrous doe. Pregnancy in three mares of four receiving embryos transported in the oviducts of rabbits demonstrate the viability of these embryos (Allen et al., 1976).

Xenogenous fertilization

Several attempts (Sreenan, 1970; Bedirian et al., 1975; Trounson et al., 1977; Shea et al., 1976) have been made to fertilize oocytes from one species in the reproductive tract of another (xenogenous fertilization). Follicular oocytes from various species will resume meiosis up to extrusion of first polar body (Pincus and Enzmann, 1935; Chang, 1955a, 1955b; Edwards, 1962, 1965, 1966; Quirke, 1969; Sreenan, 1970; Cross and Brinster, 1970; Mukherjee, 1972; Foote and Thibault, 1969; Hunter et al., 1972; Jagiello et al., 1974; Jagiello et al., 1975; Shea et al., 1976). These matured oocytes can be fertilized *in vitro* or *in vivo* and are physiologically normal (Suzuki and Mastroianni, 1968; Edwards et al, 1969; Sreenan, 1970; Bedirian et al., 1975; Edwards, 1966; Hunter et al., 1972).

Sreenan (1970) reported successful fertilization of cattle oocytes in the oviduct of estrous ewes. He used non-gonadotropin stimulated follicular oocytes cultured *in vitro* for 27-32 hr to obtain maturation to the metaphase II stage. Of 375 bovine oocytes transferred to the oviducts of previously inseminated (whole fresh semen) estrous ewes, 15% showed evidence of fertilization. When extended semen collected 24 hr earlier was used, no fertilization occurred. Parthenogenic cleavage was ruled out in these studies, since only 1 of 28 noninseminated control oocytes fragmented to a 2-cell. This fragmented ova upon staining showed no evidence of nuclei in one blastomere and only scattered chromatin in the other blastomere.

An overall fertilization rate of 8.6% was obtained when ewes were used as recipients and inseminated with either fresh or extended

semen. Cleavage to the 8-cell stage occurred. When estrous rabbits were used as hosts, no fertilization occurred.

Bedirian et al. (1975) used oocytes from gonadotropin primed cows and transferred them to the oviduct of ovariectomized heifers that were inseminated. They showed that fertilization in vivo can occur under these conditions. However, this is not xenogenous fertilization since only a single species was used. They did, however, transfer bovine oocytes to the oviduct of prepuberal gilts. Six of nine oocytes showed evidence of fertilization.

Follicular oocytes from prepuberal heifers superovulated with PMS were another source of oocytes used for transfer. These were recovered from the uterine horn of ovarectomized cows after 16-18 hr and the results suggested that development to the pronuclear stage occurred in about 20 hr (Bedirian et al., 1975).

Trounson et al. (1977) used bovine oocytes matured in vitro or in vivo for fertilization trials. An estrous or pseudopregnant rabbit was used as the host recipient. A total of 633 oocytes were cultured for 24 hr in Fetal Calf Serum (FCS) and were transferred to does that previously had been inseminated. They found no evidence of fertilization with recovery ranging from 12 hr to 7 days. Although 24 hr culture resulted in 73% of the oocytes reaching the metaphase II stage, there was no fertilization. Control does receiving oocytes but no sperm had 8% cleaved oocytes indicating possible fragmentation. The authors suggested this might be parthenogenic activation.

When *in vivo* matured oocytes were transferred to oviducts of does which had been inseminated 2 hr previously, 3 of 50 had developed to the 2- to 4-cell stages but there was no evidence of sperm penetration.

As it has been reported for other species (hamster: Barros and Austin, 1967; rabbit: Chang, 1955b; Brackett et al., 1972; rat: Noyes, 1952; pig: Leman and Dziuk, 1971; and man: Edwards, 1973), oocytes from cows can develop normally after transplantation provided they have undergone the first meiotic division *in vivo* (Trounson et al., 1977).

Further attempts at fertilization of bovine follicular oocytes in the pig and sheep oviducts were reported by Shea et al. (1976). Oocytes were matured for 30 hr, then placed in the oviducts of immature pigs previously injected with PMS and hCG, or cycling ewes in estrus. Transfer was approximately 20 hr before ovulation. Bull semen was used for insemination. Recovery rates of 67 and 43% from the oviducts of the sheep and pig were obtained, respectively. After recovery, 88% of the oocytes from the sheep and 69% from the pig had extruded a polar body. Sperm penetration was not observed in the oocytes recovered from sheep oviducts. In the pig, 17% of the oocytes recovered appeared to be penetrated by spermatozoa and had normal appearing pronuclei.

MATERIALS AND METHODS

Animals

Four species of animals were used. The oocytes donor animals used were: cattle (Bos taurus), swine (Sus scrofa), and hamsters (Mesocricetus auratus). The host animal for the xenogenous fertilization was the rabbit (Oryctolagus cuniculus).

Hamsters. Immature hamsters (two to three weeks old) were obtained from the Health Laboratory of the State of Michigan (Lansing, Michigan). Hamsters, not exceeding six per cage, were kept in plastic cages with metal tops. Water was supplied ad libitum and the hamsters were fed a commercially prepared diet (Wayne Laboratory animal diets, Lab-Blox, Allied Mills Inc., Chicago, Illinois). The cages were washed weekly and the bedding was replaced (San-I-Cel, Paxton Processing Co., Inc., Paxton, Illinois). These animals were kept in a 14 hr light, 10 hr dark cycle. Temperature was maintained between 21° and 26°C with ambient relative humidity.

Bovine. Bovine ovaries from mixed breeds were obtained from Van Alstine Packing Co. (Okemos, Michigan). Ovary transport was in plastic bags containing 0.15 M NaCl. These bags were held in either: (a) an ice bucket at 0°C or (b) in a dewar flask (thermos) at 37°C. In other instances ovaries from surgical ovariectomies at the M.S.U. Dairy Cattle Research and Training Center were used. These ovaries were transported in the same manner as previously described. The

donor animals received no type of gonadotropin injection. Upon arrival at the laboratory, the ovaries were placed in an incubator at 37°C or held in the ice bath.

Porcine. Mature sow ovaries were obtained at Milligan's slaughter plant (Rives Junction, Michigan) or at the Michigan State University Meat Laboratory. The porcine ovaries were handled in a similar manner as the bovine ovaries.

Rabbits. Adult New Zealand female rabbits were used for xenogenous fertilization. These animals were selected because of previous work showing that the oviduct of the pseudopregnant rabbit is an excellent embryo incubation environment (Adams et al., 1961). Other factors affecting this decision were the large size of the oviduct in comparison to other laboratory animals and the fact that rabbits have a 17 to 18 day pseudopregnancy period after nonfertile ovulation.

Rabbits were isolated in individual stainless steel cages. They were maintained at a temperature of 20°C during the fall and winter and at ambient temperature during spring and summer. Water was administered ad libitum and they were fed Lab Rabbit Chow #5321 (Ralston Purina Co., St. Louis, Missouri).

Experimental Design

Xenogenous fertilization of hamsters. Because of past success with xenogenous fertilization in hamsters, this species was used to examine two procedures involved in xenogenous fertilization prior to studies with cattle and swine. The following questions were set forth to be answered.

1. What is the effect of ligation of the tubal uterine junction? Adult female hamsters were given the superovulatory regime (page 24) and one-day pseudopregnant rabbits were used as hosts for xenogenous fertilization. The ova were deposited into the ampulla following the described techniques (page 28). At the time of tubal ligation, one of the oviducts was ligated and the other was not. Oocytes were recovered (page 29) 32 hr later. This recovery time is optimal for recovery of 2-cell hamster embryos derived from xenogenous fertilization (DeMayo, personal communication).

2. What is the effect of sheep serum on cleavage rate of xenogenously fertilized hamster eggs? One problem with xenogenously fertilized hamster ova is that they will not develop beyond the 2-cell stage (DeMayo et al., 1980). This series of experiments conducted to determine if the in vitro culture with sheep serum instead of Fetal Bovine Serum can reverse the block to cleavage at the 2-cell stage.

Adult sheep at the Endocrine Research Unit were bled to provide serum. Approximately 50 ml of blood were drawn from three adult sheep. This blood was allowed to coagulate and was centrifuged at 1058 Xg for 15 min. The serum was separated from the blood clot and filtered through a 0.45 µm Millex ^{R}-HA filter (Millipore Corp., Bedford, Massachusetts).

The serum was heat inactivated at 56°C for 30 min and then cooled at 4°C. The medium was mixed keeping the same proportions as in Table 2, but replacing the GG Free Fetal Bovine Serum with the sheep serum.

Xenogenously fertilized hamster ova that were recovered from rabbit oviducts were placed in this medium. Further observations of 1-cell ova and 2-cell embryos for development was done.

Xenogenous fertilization of bovine oocytes. Eggs that were held in the 37°C incubator were transferred to the ampullae of pseudopregnant rabbits and inseminated with homologous sperm. Several hours after insemination these oocytes were recovered. Recovery and fertilization rates were calculated.

The variables studied included temperature of ovary transport, time of recovery from the rabbit oviduct, culture previous to transfer, and the addition of exogenous FSH for in vitro culture of the oocytes prior to xenogenous fertilization.

1. What is the effect of temperature of ovary transport on recovery and xenogenous fertilization rates from the oviduct of the pseudopregnant rabbit? Bovine ovaries were transported in plastic bags containing 0.5 M NaCl solution. These bags were put in ice buckets so as to maintain the temperature at 0°C.

Control ovaries were transported in a 37°C, 0.15 M NaCl solution kept in a Dewar flask (thermos). Upon arrival to the laboratory, the ovaries transported at 0°C were kept on ice and the ovaries transported at 37°C were transferred to a 37° water bath.

2. What is the effect of time of recovery from the rabbit oviduct on xenogenous fertilization and recovery rate? Experiments were conducted to determine the stages of maturation, fertilization, and recovery rates of bovine and porcine ova from the pseudopregnant

rabbit oviduct. Recovery times were calculated from the time of insemination to the time of cervical dislocation.

In some instances the oocytes were stained immediately, but in other cases a further in vitro culture was carried out.

3. What is the effect of in vitro culture after recovery from the rabbit oviduct? Morphologically normal ova or ova that seemed to be near cleavage were cultured in vitro. Ova recovered at early stages of development were nearly all tested for their ability to develop in vitro.

Ova culture was carried out for not more than 25 hr due to the need of staining ova in good conditions prior to possible degeneration.

4. What is the effect of culture of oocytes in medium containing 5 µl/ml of FSH? The cattle and swine used in these studies were not treated with gonadotropins prior to oocyte aspiration. Oocytes aspirated from the follicle were generally immature with no polar bodies and tight cumulus cells surrounding the oocyte. In lieu of gonadotropin priming, a series of experiments involving attempts at in vitro maturation of the oocytes with FSH (Burns-Biotic Laboratory, Oakland, California) were done (Eppig, 1980).

Dilutions were made from a commercial FSH-P (Burns-Biotic) as to get a final concentration of 5 µg/ml of FSH in the culture medium (Table 2). This medium with 5 µg/ml of FSH was used to aspirate the follicular oocytes and was used throughout the culture period. The time of culture (37°C) in the FSH-media ranged from 19 to 20.5 hr.

Incubations were performed in the chamber/slides as will be described (page 29). For a control treatment an equal number of oocytes were in vitro cultured with the normal culture medium (Table 2). Upon completion of the maturation period, as described (page 28), oocytes were transferred.

Xenogenous fertilization of porcine oocytes. Porcine ova were handled in a similar manner to those of cattle. Aspiration of follicular oocytes was done as described (page 25) and the oocytes maintained in a 37°C, 5% CO_2 in air incubator. Oocytes were transferred to the ampulla of the pseudopregnant rabbit using a 5 µl micropettor. Insemination was with 0.05 ml of diluted sperm of concentrations of 1.35×10^7 and 4.5×10^7 sperm/ml. Oviducts were ligated at the utero-tubal junction. At various times after insemination, oocytes were recovered. The same general criteria were used for both bovine and porcine xenogenous fertilizations.

1. What is the effect of temperature during transport? Porcine ovaries were transported in Dewar flask (thermos) that contained 37°C 0.15 M NaCl solution. The time period from slaughter to aspiration of the oocytes from the follicles, was from two to four hrs. This time is considerably greater than the transport time of bovine ovaries due to the longer distance to the slaughter plant.

In other instances porcine ovaries were transported in 0°C 0.15 M NaCl solution in plastic bags in an ice bucket. The transport was similar to that for bovine ovaries.

2. What is the effect of time of recovery from the rabbit oviduct on xenogenous fertilization? Standard procedures as described (page 29) were followed to recover the porcine oocytes from the rabbit oviduct at various times after insemination.

After recovery, oocytes were placed in a tissue culture chamber (Lab-Tek products) for further examination under a phase contrast microscope. Criteria for fertilization were as described (page 30). Differences between means were examined by chi square (Gil, personal communication).

Procedures Used

Ovulation induction in hamsters and rabbits. Hamsters were induced to ovulate with an i.p. injection of 30 IU of pregnant mare serum gonadotropin (PMSG, Biological Specialties, Middleton, Wisconsin) between 9:00 and 12:00 hr. An i.p. injection of hCG (A.P.L.® Ayerst, New York, New York) followed 52 to 64 hr later. The hamsters were killed by cervical dislocation 14 hr after hCG injection. The oviducts were dissected from the rest of the reproductive tract. Using a dissecting microscope (30X), the oviducts were flushed from the fimbrial end towards the utero-tubal junction. For this purpose, a 1 ml tuberculin syringe with 30 gauge ½" blunt needle was introduced into the oviduct through the infundibulum. A total volume of 0.3-0.5 ml of medium was flushed through the oviduct. The medium was recovered in a watch glass and the same microscope was used to locate and aspirate the oocytes. From this point, the oocytes were handled in the same way as the bovine or porcine oocytes.

Pseudopregnancies were induced in rabbits one day prior to xenogenous fertilization. An i.v. injection of 100 IU of hCG (A.P.L.® Ayerst Laboratories, Montreal) in the marginal ear vein was given in the morning of the day prior to surgery. As ovulation occurs 10 hr after hCG injection (Harper, 1963), rabbits were considered to be one day pseudopregnant at the time of oocyte transfer.

Preliminary experiments with hamsters have demonstrated that the day of pseudopregnancy does not effect the xenogenous fertility rate (DeMayo, personal communication).

Bovine and porcine follicular oocyte aspiration, medium used for ova handling. All visible follicles on the ovarian surface were aspirated regardless of size. Follicles were aspirated using a 3 ml Luer-Lok tip plastic syringe with a 25 gauge, 5/8" needle. This syringe contained 0.2-0.3 ml of 37°C medium (Table 2). This medium was used for ovum handling at all times. All equipment was kept at 37°C.

After aspiration, the contents of the syringe were transferred to a prewarmed watchglass. The aspirated contents were observed under a dissecting microscope (30X) for the presence of oocytes.

The oocytes were picked from the watchglass using a micro/pettor A (Scientific Manufacturing Industries, SMI) of 4 to 5 µl volume, and transferred to a spot plate depression chamber that contained 0.4 ml of medium. The chamber was covered with a glass slide and placed in a 37°C incubator under 5% CO_2/air.

Table 2. Medium used for ovum handling and sperm dilutions[a]

	Quantity	Source
Medium 199[b]	80%	Gibco[d] Laboratories, Grand Island, New York
GG Free Fetal Bovine Serum	20%	Gibco[d] Laboratories, Grand Island, New York
Pyruvic acid	115.2 µg/ml	Sigma Chemical Co., St. Louis, Missouri
Gentomicin reagent solution	0.1 mg/ml	Schering Corp., Kenilworth, New Jersey
Penicillin-streptomycin	100 units and 100 µg/ml	North American Biol., Miami, Florida
Hyaluronidase[f]	1 mg/ml	Sigma Chemical Co., St. Louis, Missouri
Heparin sodium[g]	1 unit/ml	Upjohn Co., Kalamazoo, Michigan

[a]Medium was filtered through a 0.45 µm millipore filter into sterile vacutainer. Medium was stored at 4°C.

[b]With 25 mM HEPES Buffer, Earle's Salt and L. Glutamine.

[c]Mycoplasma tested and virus screened, heat inactivated at 56°C for 30 minutes.

[d]Grand Island Biological Company

[e]Sodium Salt Crystalline Type II.

[f]From bovine testes, lyophilized powder type 1-5.

[g]From beef lung.

Sperm handling. Different sources of sperm were used based on availability.

1. Fresh semen. This was collected from mature bulls or boars of proven fertility using an artificial vagina and standard collection procedures. The sperm sample was then transported at 37°C to the laboratory. The sperm were washed by dilution of 1 ml of semen with 9 ml of medium and centrifuged at 1058 x g for five minutes. The pellet was resuspended and washed again. Finally, the sperm were diluted in 9 ml of 37°C medium and the motility visually estimated. The sperm suspension was held at 37°C until insemination. Whenever freshly ejaculated boar semen was collected, only the sperm-rich fraction was used.

2. Frozen semen. Bull semen ampules and straws were provided by MABC (Michigan Animal Breeders' Corporation, East Lansing, Michigan). Boar pellets were provided by International Boar Semen (United Suppliers, Eldora, Iowa). Boar semen was frozen using the technique of Pursel and Johnson (1975). Frozen semen was thawed and washed following the same procedure described above, but in same experiments nonwashed frozen semen was used.

Boar semen pellets or bull semen, frozen in straws and ampules, were held in liquid nitrogen. These were thawed in a 37°C water bath and diluted with medium (approximately 3 ml of medium). At this point they were either washed or stored at 37°C until ready for use. In preliminary experiments using hamster oocytes, the source of sperm was the cauda epididymis.

Mature male hamsters were killed by cervical dislocation and the testes removed. The cauda epididymis was minced on a watch glass and diluted with medium. A visual estimate of motility was made. The sperm suspension was then transferred to a test tube and held at 37°C until insemination.

The inseminating device consisted of a 0.25 ml glass tuberculin syringe, with a 20 gauge 1½" needle that had a 4 cm polyethylene tube attached (I.D. 0.034"; O.D., 0.050"; PE 90 Intramedic®, Clay Adams, Parsippany, New Jersey). The polyethylene tubing was inserted 1 to 2 cm into the ampulla.

Sperm concentrations were determined using a hemocytometer and motility was based on visual estimation.

Anesthesia and surgery. Rabbits were anesthetized with a sodium pentobarbital (Nembutal, Sodium, Abbot Laboratories, North Chicago, Illinois) intravenous injection at a level of 60 mg per 5 lbs of body weight. A surgical plane of anesthesia was maintained by ether inhalation.

While under anesthesia, the ventral hair was clipped and the surgical area cleaned with Betadine® surgical scrub (The Purdue Frederick Co., Norwalk, Connecticut).

The anesthetized rabbit was restrained on a surgery table tilted at a 20° angle. A 7 cm ventral incision was made to expose the reproductive tract. All instruments used were cold sterilized in a 1:750 aqueous solution of Zephiran Chloride® (benzalkonium chloride, Winthrop Laboratories, New York, New York).

Oocytes were removed from the 3-chamber culture dishes with the micropipetter and immediately transferred to the oviduct of the pseudopregnant rabbit in 4-5 µl aliquots. Twenty-two to forty-one ova were deposited 2 cm through the fimbriated end of the ampulla into each oviduct. Homologous sperm was then deposited in the oviducts as previously described in aliquots of 0.05 ml. The tubal-uterine junction was ligated with 00 chromic gut to prevent oocyte loss, and the incision closed.

Furacin soluble powder (Eaton Veterinary Laboratories, Norwich, New York) was applied on the wound before closing. Procaine Penicillin-G (300,000 IU) was injected intramuscularly to prevent infections.

Ovum recovery from the rabbit. At varying times after insemination, the oocytes were recovered from the oviducts. The rabbits were killed by cervical dislocation. A ventral incision was made and the reproductive tract removed. The tubal uterine ligation was removed and the oviducts flushed with medium from the uterine end. A 5 ml plastic syringe with a 25 gauge 5/8" blunt needle was used for this purpose. The syringe contained 2 ml of medium and 3 ml of air.

The flushing medium was collected in a watch glass at 37°C. A dissecting microscope (30X) was used to locate all oocytes. These were transferred to a tissue culture chamber/slide® (Lab-Tek Products, #4838 Miles Laboratories Inc., Naperville, Illinois). The chambers each contained 0.25 ml of 37°C medium. Fertilization was assessed as described in the next section.

In some cases further in vitro culture was done to determine if further development would occur.

Criteria of fertilization. Oocytes with two pronuclei and two polar bodies or cleaved ova were considered to be fertilized. These observations were confirmed by staining with giemsa stain (T. Asakawa, personal communication). This procedure consisted of gradual fixation using three fixatives and warm moist-air drying (Mizoguchi and Dukelow, 1981).

RESULTS

Preliminary studies were for technique development. One of these studies consisted in studying the effects of ligation of the oviduct at the tubo-uterine junction on the rates of recovery and xenogenous fertilization. Hamster oocytes were used for these studies. Table 3 shows the results for these experiments. No significant differences were observed at 32 hr recovery times from the rabbit oviduct. The criterion for fertilization was 2-cell embryos. It was concluded that it is not necessary to ligate the oviduct of the rabbit when recovery is less than 32 hr post imsemination. The need for ligation for longer periods of oviductal incubation is yet to be determined.

The effect of sheep serum vs Fetal Calf Serum (FCS) on hamster xenogenously fertilized embryos was studied. A total of 46 ova recovered from the rabbit oviduct were cultured using the standard medium with sheep serum instead of FCS. Of these, 7 were in the 2-cell stage. Ova were *in vitro* cultured for 11 hr. No further development was observed in either the 1-cell or 2-cell embryos by the end of the culture. Most of the embryos were either in early or late phase of degeneration by this time.

An experiment was undertaken to test the fertilizability and recovery rates of bovine and porcine ova xenogenously fertilized in the oviduct of the pseudopregnant rabbit. The results are shown in

Table 3. The effects of ligation at the uterotubal junction, on recovery and xenogenous fertilization of hamster embryo rates (recovery 32 hours after insemination)

	Oocytes Recovered/Transferred (%)	Oocytes[a] Fertilized/Ova Recovered (%)
Nonligated oviduct	24/41 (58.5)	4/24 (16.7)
Ligated oviduct	22/41 (53.7)	3/22 (13.6)

[a]Criterion of fertilization, 2-cell.

Table 4. The recovery rate for bovine was higher than that of swine ($P < 0.01$). Fertilization rates were also significantly different ($P < 0.001$).

Bovine ovaries were transported from the slaughter house to the laboratories at either 0° or 37°C in physiological saline. Table 5 shows the effect of the two transport temperatures on the recovery and xenogenous fertilization rates.

The number of oocytes recovered was higher when the ovaries were transported at 0°C, but this increase was not significantly different from the rate when ovaries were transported at 37°C ($\chi^2 = 1.12$ for recovery and 1.64 for fertilization). Fertilization rates were not statistically significant between the two treatments.

Table 6 shows the effect of two different temperatures of transport on recovery and fertilization rates of porcine oocytes. No significant differences between treatments were found for either

Table 4. The recovery and xenogenous fertilization rates of bovine and porcine follicular oocytes in the oviduct of the pseudopregnant rabbit

Oocytes	No. of Rabbits	Recovered/Transferred (%)	Fertilized/Recovered (%)
Bovine	10	261/582 (44.8)	35/261 (13.4)
Porcine	6	148/410 (36.1)[a]	3/148 (2.0)[b]

[a]Significantly different from bovine ($P < 0.01$).

[b]Significantly different from bovine ($P < 0.001$).

Table 5. Temperature effects on ovary transport on recovery and xenogenous fertilization rates of bovine oocytes

Temperature	No. of Rabbits	Oocytes Recovered/ Transferred (%)	Oocytes Fertilized/ Recovered (%)
0°C	4	88/228 (38.6)	10/88 (11.4)
37°C	4	88/259 (34.0)	16/88 (18.2)

Table 6. Temperature effects on ovary transport, recovery, and xenogenous fertilization rates of porcine oocytes

Temperature	No. of Rabbits	Oocytes Recovered/ Transferred (%)	Oocytes Fertilized/ Recovered (%)
0°C	3	83/241 (34.4)	1/83 (1.2)
37°C	3	65/169 (38.5)	2/65 (3.1)

recovery or fertilization rates. The recovery times for these experiments were 22.3 to 70 hr after insemination.

When bovine oocytes were xenogenously fertilized, several times of recovery were used. Table 7 shows four time intervals with their respective recovery and fertilization rates.

Intervals of 50-55 and 65-70 hr are significantly greater for recovery rate (49.8 and 39.6%, respectively) than intervals of 40-45 and 70-75 hr (11.4 and 8.5%, respectively) ($P < 0.01$). There were no significant differences between intervals for the fertilization rates ($\chi^2 = 5.595$). Ova cleavage was observed starting at times of recovery of 50 hr and beyond.

The effect of in vitro culture of bovine follicular oocytes in medium supplemented with 5 µg/ml FSH is shown in Table 8. The time of culture in the supplemented and control media was of 20 hrs. Oviductal recovery rates of 76.7 and 91.9% for the treated and control oocytes were observed, respectively. There was a tendency for oocytes treated with FSH to have lowered recovery compared with controls

Table 7. The recovery and xenogenous fertilization rates of bovine oocytes recovered at varying times after insemination

Recovery Time (Hr Post Insemination)	No. of Rabbits	Oocytes Recovered/ Transferred (%)	Oocytes Fertilized/ Recovered (%)
40-45	1	5/44 (11.4)[a]	1/5 (20.0)
50-55	3	103/207 (49.8)[b]	10/103 (9.7)
65-70	3	61/154 (39.6)[b]	13/61 (21.3)
70-75	1	7/82 (8.5)[a]	2/7 (28.6)

[a]Different superscripts indicate significant difference ($P < 0.01$).

Table 8. The effect of culture in vitro for 20 hours in medium supplemented with 5 μg/ml FSH, on recovery and xenogenous fertilization rates of bovine oocytes

	Oocytes Recovered/ Transferred (%)	Oocytes Fertilized/ Recovered (%)	Stage of Fertilization
FSH	23/30 (76.7)[a]	2/23 (8.7)	2-cell, 2 pb[b]
Control	34/37 (91.9)	1/34 (2.9)	2 pb

[a]Different from control ($P < 0.1$).

[b]2 pb = 2 polar body stage.

($P < 0.10$). There was no difference between treated and control oocyte fertility.

Further trials to obtain *in vitro* maturation of the follicular oocytes prior to sperm exposure were performed. In the rabbit oviduct a mucin coat is deposited around the oocyte which limits the fertilizable life (of rabbit oocytes) to six hours. To determine if mucin deposition was effecting xenogenous fertilization, bovine follicular oocytes were cultured *in vitro* for 25 hr prior to deposition in the rabbit oviduct. Results indicate that (Table 9) of 28 oocytes incubated *in vitro* and transferred to the rabbit oviduct, 28 (100%) were recovered. Of these 6/28 (21.4%) showed evidence of fertilization. Five of the ova were at the 2 polar body stage and showed condensed cromatin when stained. The other ovum was recovered as a 2-cell embryo. The *in vitro* culture was carried out for a period of 25 hr and the oocytes were further cultured in the oviduct for 47 hr (72 hr total). Control I oocytes were of the same age (70 hr) that had been in the rabbit oviduct for the entire culture period. Control II oocytes were not cultured *in vitro* but were in the oviduct approximately the same time as the treated oocytes (50 hr). These oocytes (Control II) were 25 hr younger than the *in vitro* cultured oocytes. Table 9 shows that there was a significant increase of the recovery rates when oocytes were cultured *in vitro* for 25 hr ($P < 0.01$). No significant effect in fertilization was noted.

Table 9. Recovery and fertilization rates of oocytes matured *in vitro* for 25 hours prior to xenogenous fertilization

	No. of Rabbits	Oocytes Recovered/ Transferred (%)	Oocytes Fertilized/ Recovered (%)
In vitro incubation for 25 hr and 47 in oviduct (72 hr total)	1	28/28[a] (100%)	6/28 (21.4%)
Control I (oocytes same age entire 70 hr in oviduct)	3	61/154[b] (39.6%)	13/61 (21.3%)
Control II (oocytes same time in oviduct 50 hr; no *in vitro* culture)	3	103/207[b] (49.8%)	10/103 (9.7%)

[a]Different superscripts indicate significant difference ($P < 0.01$).

DISCUSSION

The xenogenous fertilization technique using the pseudopregnant rabbit as host was developed using hamster oviductal oocytes that were placed in the rabbit oviduct. It was demonstrated that ligation, which is primarily performed to avoid oocyte loss, is not necessary when recovery times are 32 hr or less. This recovery time is the ideal to recover 2-cell hamster embryos (F. J. DeMayo, personal communication). Ligation does not interfere with fertilization. This confirms the findings of Adams (1973) and Sharp and Black (1975) who studied the effects of ligation on rabbit ovum fertility. The ease of xenogenous fertilization of hamster oocytes to the 2-cell stage has been reported (DeMayo et al., 1980). In the present work, similar cleavage rates were obtained. DeMayo et al. (1980) reported cleavage rates of 14%, whereas, in the present studies, a cleavage rate of 16.7% was found with the unligated oviduct and 13.6% with ligation. It still must be determined if recovery rates decrease once the oocytes enter the uterus, or if longer recovery times are used as is the case with cattle or swine follicular oocytes.

Hamster embryos fertilized xenogenously do not develop beyond the 2-cell stage in the rabbit oviduct and this could not be altered by substituting sheep serum for FCS. It appears that there is a "2-cell block" that cannot presently be overcome in either the oviduct of the pseudopregnant rabbit or *in vitro*.

Successful xenogenous fertilization of bovine oocytes had been reported in estrous ewes (Sreenan, 1970) and prepuberal gilts (Bedirian et al., 1975; Shea et al., 1976). However, Trounson et al. (1977) reported negative results when using the pseudopregnant and estrous rabbit as host for the transfer. In the present study, both bovine and porcine follicular oocytes can be fertilized in the oviduct of the one-day pseudopregnant rabbit. Parthenogenic activation has been ruled out in the present study by staining and observation of normal pronuclei, sperm tail remanants in the cytoplasm and normal nuclei after cleavage to the 2-cell stage.

A difference in the present technique and that reported by Trounson et al. (1977) is the site of insemination. Their work was done with does previously vaginally inseminated. The present work was done by oviductal insemination to the ampulla immediately after to oocyte deposition. They held oocytes matured in vivo (after gonadotropin treatment of heifers) in Phosphate Buffered Saline (PBS) at room temperature, whereas, in the present study, oocytes were held at all times after aspiration at 37°C.

Both bovine and porcine oocytes are capable of being fertilized in the reproductive tract of the pseudopregnant doe as shown in the present study. There was a significant difference between species with respect to fertilization ($P < 0.001$). The difference was not as significant ($P < 0.01$) for recovery rates. The longer transport times involved in the transport of swine ovaries could be a limiting factor, as the bovine ovaries were brought to the laboratory at times varying from 2 to 4 hrs and the porcine ovaries transport time was greater (6 to 7 hr).

No effect of temperature on recovery or xenogenous fertilization rates for both bovine nor swine ovaries was observed. Trounson et al. (1976b) reported tolerance of bovine blastocysts to cooling to 0°C, but it appears that at the 8-cell stage cooling to 0°C produces irreversible damage of the embryo (Wilmut et al., 1975). When bovine morulae are cooled at 0°C, only a small percentage of the embryos survive cooling (Trounson et al., 1976a, 1976b). In the present study, no apparent effect was observed by cooling the ovary to 0°C compared with 37°C.

Different times of recovery were used to determine stages and rates of fertilization at time intervals of 50 through 70 hr after insemination. There was a significant increase in recovery rates. The decrease of recovery after 70 hr probably reflects a degeneration and reabsorption of nonfertilized oocytes. This same loss of nonfertilized oocytes could result in an apparent increase in the fertilization rate (28.6% fertilization for 70-75 hr vs 21.3% for 65-70 hr).

Bovine follicular oocytes mature in vitro from germinal vesicle to metaphase II (GVBD) by as early as 19 hr (Jagiello et al., 1974) to as late as 31 hr (Edwards, 1965). The maturation period is from 19 to 31 hr for the follicular oocytes and 24 hr for in vitro cleavage to the 2-cell (Brackett et al., 1977, 1978). Therefore, in vitro cleavage would be expected 43-56 hr from initiation of culture. In the present studies, cleavage to the 2-cell stage was observed from 50 to 70 hr after insemination. This represents a delay from the cleavage observed in vivo by Hamilton and Laing (1946).

In the present work, pronuclear stages were observed as early as 40 hr. This can be compared with the in vivo pronuclear stage 5 to 12 hr after ovulation (Thibault, 1967; Brackett, 1980) considering maturation periods of 19-31 hr for follicular oocytes (Edwards, 1965; Jagiello et al., 1974; Shea et al., 1976; Newcomb et al., 1978).

Eppig (1980) reported cumulus cell expansion hyaluronic acid synthesis and hastened maturation of mouse follicular oocytes when they were cultured in medium containing FSH and FCS. In the present work, in vitro incubation with FSH had a deleterious effect on recovery from the rabbit oviduct. It is suggested that if maturation is hastened degeneration might occur at earlier stages. Thus, reabsorption or complete degeneration of nonfertilized oocyte devoid of protective cumulus cells could occur in the rabbit oviduct. Fertilization rate was not significantly affected by in vitro culture with FSH; however, 2-cell embryos were recovered from the oviduct after 54 hr incubation. In vivo rates of development show 8-cell stages at 44 hr after ovulation (Thibault, 1966; Brackett, 1980) or 2- to 4-cell (Hamilton and Laing, 1946). This shows that it appears to be a delay in the xenogenous fertilized embryo development when compared with in vivo fertilized embryos.

The mucin coat built up in the rabbit impairs fertilization of rabbit oocytes after six hr. An attempt to increase maturation of oocytes before transfer to the rabbit oviduct did significantly increase oocyte recovery. This appears to be due to loss of degenerated oocytes. Edwards (1966) reported the start of atresia within the ovarian follicle

but it appears that there is no readily discerned morphological difference between a degenerative oocyte and a viable one immediately after aspiration from the follicle. After 25 hr in culture, degenerative oocytes can be easily observed. Degenerated oocytes were not knowingly transferred.

Fertilization rates did not increase when oocytes were incubated *in vitro*, but a highly significant increase in recovery rates was observed. It would be advantageous to mature oocytes *in vitro* to obtain higher recovery rates from the rabbit oviduct. When oocytes were previously cultured, two of six cleaved to the 2-cell stage upon recovery (47 hr). In this case cleavage appears to be similar to that obtained *in vitro* (Brackett, 1977, 1978) and *in vivo* (Hamilton and Laing, 1946).

SUMMARY AND CONCLUSIONS

Fertilization of bovine and porcine follicular oocytes and hamster oviductal oocytes, in the oviduct of the pseudopregnant rabbit (xenogenous fertilization) was studied.

When oviductal recovery of hamster oocytes from the rabbit oviduct is 32 hr or earlier after insemination, no tubal ligation is necessary to avoid ovum loss.

The 2-cell stage block observed with hamster xenogenously fertilized embryos cannot be overcome with either Fetal Calf Serum (FCS) nor sheep serum heat inactivated at 56°C for 30 min.

Bovine and porcine follicular oocytes were capable of being fertilized xenogenously as shown by overall fertilization and recovery rates of 13.4% and 44.8%, respectively, for the bovine, and 2.0% and 36.1%, respectively, for the porcine oocytes. A significant decrease in recovery ($P < 0.01$) and fertilization rates ($P < 0.001$) for porcine oocytes with respect to bovine oocytes was observed.

Transport of ovaries at 0°C vs 37°C had no effect on either recovery or fertilization rates for both bovine and porcine oocytes.

Several recovery times were used for bovine oocytes. Recovery times from 50 to 70 hr significantly differed ($P < 0.01$) from earlier (40-45 hr) or later (70-75 hr) times.

Fertilization was not affected by recovery time, although an increased number of 2-cell embryos were recovered at 50 hr after insemination.

In vitro culture of oocytes in medium supplemented with 5 µg/ml of follicular stimulating hormone (FSH) decreased ($P < 0.1$) the recovery rate from the rabbit oviduct (76.7% vs 91.9% of control). No effect was observed on fertilization. Cleavage to the 2-cell stage was achieved when oocytes were preincubated with FSH.

In vitro culture of bovine follicular oocytes in medium for 25 hr prior to xenogenous fertilization increased significantly the recovery from the rabbit oviduct ($P < 0.01$). No effect was observed on the fertilization rate.

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APPENDIX A

PUBLICATIONS BY THE AUTHOR

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Papers: Alternative methods of fertilization for reproductive toxicology. W. R. Dukelow, P. J. Hirst, T. Asakawa, F. J. DeMayo, and P. J. Chan. Proc. 8th European Teratology Conference (in press, 1981).

Xenogenous fertilization of laboratory and domestic animals in the oviduct of the pseudopregnant rabbit. P. J. Hirst, F. J. DeMayo, and W. R. Dukelow. Theriogenology (in press, 1981).

Abstracts: Xenogenous fertilization of nonhuman primate, laboratory and domestic animals. P. J. Hirst, F. J. DeMayo and W. R. Dukelow. Proc. 31st Annual Session of American Association for Laboratory Animal Science. October 7, 1980. Indianapolis, Indiana.

APPENDIX B

VITA

APPENDIX B

VITA

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	Licenciado en Ciencias Agrárias	
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