In Vitro Fertilization: A Potential Means for Toxicity Testing
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Uses and potential uses of in vitro fertilization are: (1) a research tool for investigating biochemistry of fertilization, (2) an assay for fertilizing ability, (3) a potentially useful clinical approach for certain cases of infertility, and (4) a potentially useful means for improving animal breeding. In vitro fertilization methodology is sufficiently advanced for gametes of several mammalian species, especially mouse, rabbit, and rat, for use in evaluating effects imposed by toxic agents of environmental or genetic origin. Alteration of the normal events of fertilization and/or embryonic development following transfer of in vitro fertilized embryos into surrogate dams can serve as end point(s) in applying this means for toxicity testing. In vitro fertilization of mouse and rat ova has been explored as an alternative to in vivo fertilization in male contraceptive development studies. Original observations on toxicity of abnormal O₂ concentrations for rabbit fertilization in vitro are reported here. Ova were fertilized under 0, 20, 48, and 95% O₂, but toxicity from the extreme conditions, 0 and 95% was apparent from comparison of proportions of fertilized ova reaching the 4-cell stage during the 24-hr incubation. Toxicity was further evidenced by inability of embryos fertilized under 0 and 95% O₂ tensions, in contrast to those fertilized under 20% O₂, to sustain normal gestation following their transfer into recipient does. Recent success in the fertilization of cow ova in vitro in this laboratory provides encouragement to develop a useful means for testing normalcy of gametes in this species. Such studies might lead to useful screening procedures for avoidance of human infertility resulting from hazardous environmental conditions.

Introduction

The fertilization process begins with sperm penetration through the cellular investments (cumulus oophorus, and corona radiata), followed by penetration through the zona pellucida into the perivitelline space of the ovum. The fertilizing sperm cell then penetrates rapidly into the vitellus, where the sperm head containing nuclear material undergoes enlargement; the ovum undergoes activation which includes breakdown of cortical granules and extrusion of the second polar body (completion of meiosis). The remaining female chromatin condenses into the female pronucleus and the sperm head further enlarges to form the male pronucleus. The pronuclei grow and come together centrally and ultimately the membranes of the pronuclei breakdown; the chromosomes become mixed as mitosis begins and the fertilization process ends with cleavage of the zygote to form a two-cell stage embryo. This process requires 10 to 36 hr in various mammals. Initial reports providing adequate documentation for accomplishment of in vitro fertilization were carried out with sperm recovered from the uterus of mated rabbit does and consisted of cytological proof (1) and the birth of young following transfer of resulting embryos into recipient does (2). The necessity for exposure of sperm to the female reproductive tract prior to achievement of the capacity to fertilize (sperm capacitation) was recognized only a few years earlier (3-5).

The purpose of this paper is to review briefly in vitro fertilization, with emphasis on its uses and potential uses and to illustrate with original data an application of this approach to study toxicity of abnormal oxygen concentrations.

Basic Considerations

The work cited above paved the way for development of physical and chemical conditions compatible with fertilization of recently ovulated rabbit ova by capacitated sperm recovered from uteri of mated does (6). A simple defined medium

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was found capable of supporting the early stages of the fertilization process. Outstanding features of this medium were the high bicarbonate content adequate to maintain a pH of 7.8 under an atmosphere containing 5% CO₂, glucose as an energy substrate, and crystalline bovine serum albumin which was added in a concentration comparable to protein contributed by inclusion of serum in previous work. The advantageous effect of the bicarbonate-containing medium at pH 7.8 was realized from observations that when measurable volumes of uterine fluid were recovered with sperm suspensions in previous experiments optimal in vitro fertilization results were obtained and the pH of the fertilization medium in these cases was near 7.8 (7). Furthermore the same pH was detected for estrus rabbit oviductal fluid (8). The facility to combine ova and sperm cells from the same sources after varying treatments enabled comparison of resulting proportions of ova fertilized in vitro (9). This approach has been used in many subsequent experiments to learn more about the fertilization process and also to learn more about sperm capacitation (10). In the rabbit, ova can be recovered from three sites for in vitro fertilization, the oviducts just after ovulation (6, 11), the ovarian follicles just prior to ovulation (12, 13), or from the ovarian surface just after ovulation but prior to pickup by the oviduct (13-16).

Capacitation represents a change in the sperm cell which is manifested by increased metabolic activity (17-19) and involves alteration of surface antigens which otherwise prevent sperm penetration of ova (16, 20, 21). Alteration of sperm surface

![Image of rabbit sperm undergoing the acrosome reaction](image-url)

**Figure 1.** Rabbit sperm undergoing the acrosome reaction in the interstices between corona cells (CC) near the zona pellucida 1.5 hr after in vitro insemination (25). In sperm 1 (S1), the plasma membrane (PM) is swollen. In sperm 2 (S2) the plasma membrane and outer acrosomal membrane are undergoing vesiculation (V). In sperm 3 (S3), the acrosome reaction has been completed, exposing the inner acrosomal membrane (IAM).
coating antigenic components of seminal plasma by high ionic strength treatment enabled ejaculated rabbit sperm to fertilize ova in vitro (16). In addition to a possible ionic effect of oviductal fluid, the physiological mechanism may involve amylaselike enzyme activity within the female reproductive tract (22).

The sperm cell normally becomes conditioned within the female reproductive tract, i.e., capacitated, to undergo a morphological change, the acrosome reaction, upon contact with the ovum or its cellular investments (23). The acrosome reaction involves vesiculation which results from fusion of the plasma membrane with the outer acrosomal membrane ultimately leading to erosion with the inner acrosomal membrane exposed (24). Thin sections of three sperm heads in successive stages of the acrosome reaction, as seen in the electron microscope, are shown in Figure 1 (25). These events enable sperm enzymes responsible for entry into the ovum to become effective. Among the enzymes identified to play a role in sperm penetration hyaluronidase digests the matrix of the cumulus cell mass, the corona penetrating enzyme enables the sperm to reach the zona pellucida where the acrosomal proteinase, acrosin, enables digestion of the zona pellucida (26).

Following penetration through the zona pellucida, the fertilizing sperm cell rapidly contacts the vitellus and is assisted by microvilli of the ovum in penetration of the ooplasm. In most mammals, the rabbit being an exception, only one sperm cell penetrates through the zona pellucida. In all mammalian species normal fertilization involves penetration of only one sperm cell into the vitellus. Polyspermy denotes the situation in which more than one sperm cell penetrates the ovum. The abnormal condition of polyploidy may result from extra male pronuclei after polyspermy or from more than one female pronucleus, e.g., as follows suppression of meiosis and second polar body extrusion. In the latter case, triploidy results and when experimentally caused by appropriately timed colchicine treatment development of triploid embryos progresses to implantation but not to completion of gestation following transfer of experimental embryos (27). A variety of parthenogenetic and/or degenerative types of activation are recognized in the

![Figure 2](image_url)

**Figure 2.** Normal-appearing four-cell stage rabbit embryos approximately 24 hr after in vitro insemination with rabbit sperm capacitated under optimal in vivo conditions.
mouse and other mammals, at least theoretically, but the potential for complete gestational development of such “embryos” is nil (28, 29).

Although 60 to 80% of the recipients of normal-appearing four-cell stage in vitro fertilized rabbit embryos, as shown in Figure 2, become pregnant, the proportions of recipients that deliver offspring are much lower, especially following in vitro incubation of the ova (13). Embryonic wastage, pre- and post-implantation, is great. Delivery of around 20% of the total number of in vitro fertilized rabbit embryos transferred was reported from this laboratory (13). Successful embryo transfer results have also followed fertilization and early embryonic development in vitro for the mouse (30-36) and rat (37).

### Uses and Potential Uses of in Vitro Fertilization

Uses and potential uses of in vitro fertilization come under four broad headings: (1) Research, e.g., to examine discrete events of the fertilization process; (2) assessment of fertilizing ability of egg and/or sperm after various treatments of gamete donor(s) or gamete(s); (3) to overcome infertility caused by blocked oviducts in women or oligospermia in men and stallions, or—coupled with embryo transfer—by any pathological conditions of oviduct and/or uterus in animals; (4) in animal production—coupled with embryo storage and transfer—to combine selected eggs with selected sperm for production of individual(s) with predicted characteristics at an appropriate time. The applicability of in vitro fertilization methodology has greatly facilitated progress in our understanding of fertilization and gamete physiology from investigational efforts during the last decade. Methodology is adequately described for several species to enable collection of meaningful data on ability of gametes to undergo fertilization, and with appropriate controls this approach can already be applied to discern effects of various forms of toxicity. Clinical applications are still best considered as potential uses of in vitro fertilization since success has not followed the best documented efforts to overcome carefully selected cases of human infertility by this approach. Nevertheless the clinical applications especially in the veterinary medical field, e.g. to overcome certain types of infertility, to better understand infertility, and to facilitate production of desirable animals, represent very attractive goals.

That in vitro fertilization methodology can be applied in testing for toxicity affecting the male reproductive tract has been demonstrated by Tsunoda and Chang (38) in work related to male contraceptive development. α-chlorohydrin was administered to male rats and mice, and the fertilizing ability of epididymal sperm from the treated males was tested for fertilizing ability in vitro and in vivo by intrauterine insemination. Fertilizing ability was reported as percentage of ova fertilized. In the mouse, the effective dose for inhibition of fertilization in vivo was higher than that required to produce the same effect in vitro, and infertility caused by the drug in the rat was found to be of shorter duration by in vivo testing than by in vitro testing. It was concluded that the in vivo approach for testing fertilizing ability of sperm from α-chlorohydrin-treated males was less laborious and preferable since it was more physiological and was more sensitive, at least for the rat. Given a ready source of oocytes for study in an animal with a long gestation period, as in nonhuman primates or the cow, if similar results could follow in vitro fertilization as those attainable through in vivo testing the desired approach would undoubtedly be that of in vitro fertilization.

A clue to genetic toxicity might follow observations of abnormal fertilization as reported following in vivo studies in mice (39). In a recent study tertiary trisomic male mice, carrying the small translocation chromosome from the T(1;13)70H reciprocal mouse translocation as the extra chromosome, were oligospermic, and this condition led to delayed fertilization. It was concluded that there was no relationship between sperm morphology and karyotype. An earlier study involving sperm distribution in the female reproductive tract following matings with sterile t♂18/t♂32 male mice revealed normal sperm numbers in the uterus but few or no sperm at the site of fertilization in the oviducts (40).

Abnormal sperm morphology could not account for these findings. In Friesian bulls, abnormal development of acrosomes resulting in so-called “knobbed” sperm was implicated in sterility resulting from an autosomal sex-limited recessive gene influenced by local environmental conditions within the testes (41). A greater proportion of abnormal embryos might result from insemination in vitro than in vivo under conditions involving genetic aberrations and following cytogenetic evaluation insights to mechanisms of genetic toxicity, i.e., infertility, might be forthcoming.

### Experimental Observations on Fertilization of Rabbit Ova under Various Oxygen Tensions

In general, internal fertilization of mammalian species is well protected from environmental toxicity in the normal sense as it might concern other
organ systems in which a more direct effect is seen, e.g., respiratory. However, adverse influences might also alter the environment in which fertilization or events before or after fertilization take place. Experiments reported here illustrate effects that might be observed as a result of extreme alterations in the normal milieu for fertilization and very early embryonic development.

Materials and Methods

Ova were recovered from the ovarian surfaces of superovulated Dutch Belted and New Zealand White donors as previously described (14), and sperm were recovered from uterine horns of mated does (capacitors). Each capacitor was mated by two to four bucks 18 hr earlier. The medium was a simple defined medium routinely adopted for rabbit in vitro fertilization in this laboratory (16); in these experiments paraffin oil was used to completely fill the space above the 4.0 ml gamete-containing solution for incubation in small (30 mm diameter, 12 mm deep) tissue culture dishes.

In each experiment, desired combinations of O₂ and N₂ along with 5% CO₂ were mixed by flowmeters and used for equilibration of the medium and paraffin oil and for maintaining the same atmosphere above the incubation dish throughout the 24-hr incubation interval. Rapid equilibration of the gas phase was achieved following in vitro insemination by use of a small chamber (anaerobic culture apparatus, small size, Arthur H. Thomas Co., Philadelphia, PA).

Approximately 24 hr after in vitro insemination, ova were examined for evidence of fertilization, i.e., normal-appearing cleavage stages with chromatin demonstrable within each blastomere of representative ova (12). Proportions of ova that were fertilized under various conditions were compared statistically by chi-square analysis. Embryo transfers were carried out as previously described (13) to assess the potential for in vivo development.

Results

Results of 15 experiments are summarized in Table 1. The percentages of ova fertilized at various oxygen tensions in vitro did not vary significantly, suggesting that once the gametes are prepared for fertilization, the process of gamete union can precede even under adverse conditions. However, upon comparison of proportions of fertilized ova developing to at least the four-cell stage within the incubation interval highly significant differences were apparent. A significantly greater proportion of ova fertilized and incubated under an atmosphere of 20% oxygen developed to at least the four-cell stage than was the case under atmospheres of 0% oxygen (p < 0.05) or 95% oxygen (p < 0.05). Development of ova under 48% oxygen was similar to that seen under 20% oxygen in this work.

The effect of hostile conditions resulting from inappropriate levels of oxygen tension were also reflected in results of embryo transfers. Two experiments were done (using ova and sperm from the same sources) in which the viability of embryos resulting from fertilization under 20% oxygen was compared with that of embryos developing under 0% and 95% oxygen tensions, respectively. In the first, seven embryos (two in eight-cell and five in four-cell stages) that were fertilized under 20% oxygen were transferred to the left oviduct and seven embryos (four-cell stage) that were fertilized under 0% oxygen were transferred to the right oviduct of the same hormonally synchronous recipient doe; four embryos from the 20% oxygen group implanted and continued their development into normal offspring, while one of the embryos from the oxygen-deficient group implanted but did not survive to term. In the second embryo transfer experiment, five embryos (four-cell stage) that were fertilized under 20% oxygen were transferred into the right oviduct, and six embryos (two-cell stage) that were fertilized under 95% oxygen were transferred into the left oviduct of the same recipient; one of the embryos from the 20% oxygen group was resorbed after implantation, but two others developed into normal offspring while none of the embryos from the 95% oxygen group implanted.

Discussion of Experimental Observations and Concluding Remarks

In previously reported experiments fertilization of 61.8% (34/55) of tubal ova followed sperm penetration with measured oxygen tensions of 2.49 to 3.27 μl O₂/ml, or approximately 9 to 12% O₂ (9). In recent work, equilibration of medium, covering oil,
and atmosphere with 5% CO₂, 8% O₂, and balance N₂ has been adopted, since this oxygen tension is comparable to that reported in the oviduct (42, 43). Fertilization results over a wide range of oxygen tensions were similar and only under extreme conditions did toxic effects become recognizable (Table 1). This also appears to be the case for in vitro culture of mouse embryos (44). Whitten (45) found improvement of in vitro mouse embryo development under conditions with oxygen levels more comparable to those in vivo, i.e., 5% O₂. Baeckeland (46) used Whitten’s conditions and observed development of mouse embryos from the two-cell stage to blastocysts, but these conditions did not support similar development of rat embryos. Haidri, Miller and Gwatkin (47) reported best in vitro maturation of mouse oocytes under 5–10% O₂, while in later work a well-defined optimum oxygen concentration was reported to be 5% (48).

Although results following experimentally induced oxygen deprivation and oxygen toxicity do not correspond to any recognized environmental condition, aside from death in the case of 0% O₂ tension (43), these experiments serve to illustrate the use of in vitro fertilization for toxicity testing.

Although drastic conditions were required to interfere with reproduction in the original experiments reported here, less drastic conditions might over a period of time result in pathological conditions of the gonads and hence, defective gametes. For the future, insight regarding abnormal fertilization resulting from environmental (or genetic) insult might follow in vitro fertilization studies in the cow and other grazing animals. The exposure of domestic animals to environmental pollutants near major urban areas might be reflected in performance of their gametes. Some progress has already been made in development of procedures for in vitro fertilization in the pig (49) and in the cow (50, 51). In this laboratory cow ova recovered near the time of ovulation have been fertilized by in vitro capacitated sperm and cleavage observed to the four-cell stage. Future detection of gonadal toxicity might become apparent in an ancillary way through in vitro fertilization primarily designed for evaluating the breeding performance of individual bulls or cows. Although many of the thoughts presented here are highly speculative today, in vitro fertilization can realistically be considered a potential means for toxicity testing.

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