Development and viability of rabbit embryos after exposure to an electromagnetic field of 1.5 Gauss at 60 Hz during *in vitro* culture and after transfer of exposed embryos to recipient does

by

Kaitkanoke Kamolpatana

A Thesis Submitted to the

Graduate Faculty in Partial Fulfillment of the

Requirements for the Degree of

MASTER OF SCIENCE

Department: Veterinary Physiology and Pharmacology
Major: Physiology

Iowa State University
Ames, Iowa

1995

TABLE OF CONTENTS

INTRODUCTION

An electromagnetic field is an association of an electric field and a magnetic field (Novini, 1993) generated by the transmission, distribution, and/or use of electric power (Halliday and Resnick, 1966). Hence, for more than a century, our lives have become increasingly linked to electrical uses including food processing and cooking, entertainment, temperature control, transportation, lighting, and medical diagnosis and treatment, all of which makes exposure to electromagnetic fields unavoidable. There are reports that isolated cells (Goodman et al., 1993), plants, and animals ranging from unicellular organisms to man are sensitive to electromagnetic fields (Presman, 1970). Recently, electromagnetic fields have received considerable media attention because of public concern that routine exposure to electromagnetic fields may adversely affect human health.

Electric and Magnetic Fields

Electric fields

An electric field is normally generated by electric charges which are an electron, a negative electric charge surrounding a nucleus, and a proton, a positive electric charge inside a nucleus. The electric field, E, is defined as $E = F/q$, where F is the force and q is the charge (Halliday and Resnick, 1966). This field, E, is defined in terms of both the magnitude and direction of the force exerted on a static unit charge.

The electric field around a charged rod can be described not only by a vector electric field strength E but also by a scalar quantity called an electric potential. If there is a different potential at any point of a conductor, the electric field will act on the electrons and give them a resultant movement of electrons (Halliday and Resnick, 1966). The movement of the electrons is called an electrical current. Electrical currents can be classified as either a direct current (DC) or an alternating current (AC). A direct current flows in only one direction and at a constant rate and generates a static electric field. In an alternating current, both the rate and direction of current flow changes in magnitude and direction periodically over time, and generates an alternating electric field. The frequency of change for this alternating current is referred to as cycles per second or hertz (Hz) (Halliday and Resnick 1966).

A DC electric field can be generated either from natural or man-made sources. Examples of naturally generated DC electric fields are those found in the electrically charged sky in fair weather or during storms (Halliday and Resnick, 1966) or those generated by the physiological activity of a neuron (Ruckebush et al. 1991). The electric fields generated by an electrically charged sky has a variety of frequencies which arise primarily from atmospheric conditions and the sun. Thus the magnitude of the natural electric field is very dependent upon the weather. In fair weather, the earth has a static vertical electric field between 90 to 120 Volt/meter; and during lightning and/or thunderstorms, DC electric fields of 5 to 20 kVolt/meter can occur from the sky to the ground (Deno and Carpenter, 1994). A neuronal electric field is generated whenever a suitable stimulus is applied to a nerve axon, then the plasma membrane becomes excited and an electrical event occurs within the plasma membrane at the

point of stimulation (Ruckebush et al. 1991). A battery is a common man-made source of a DC electric field. However, most man-made electric fields are AC electric fields and have frequencies of either 50 or 60 Hz, and are generated by electrical appliances such as in the work place or household appliances.

Magnetic fields

A magnetic field is generated from an electric current flow or a movement of electric charges. The magnetic field, B, is also defined by the magnitude and direction of the force exerted on a moving charge. The formula, $B = F/qv$, where B is the magnetic flux density, F is the force exerted on the moving electric charge, q is a unit charge, and v is the vector describing the magnitude and direction of the relative motions of the field and the charge (Halliday and Resnick, 1966).

As for electric fields, DC magnetic fields can be generated either from natural or man-made sources. A naturally generated magnetic field is the geomagnetic field which exists between the poles of the Earth (Presman, 1970). This geomagnetic field averages about 0.5 Gauss (G). However, it varies at some particular sites such as the equator and the poles. Measured vertically, the geomagnetic field is greatest at the magnetic poles, about 0.6 to 0.7 G and has a value of zero at the magnetic equator. In the horizontal direction, the geomagnetic field reaches a maximum of about 0.23 G at the equator and is zero at the magnetic poles (Deno and Carpenter, 1994). Magnetite is another example of natural source of a DC magnetic field. A battery is a common man-made source of a DC magnetic field.

An AC magnetic field is also generated either from natural or man-made sources. Natural AC magnetic fields are superimposed on the geomagnetic field and are in the range between 0.4 μG to 4 μG at 60 Hz (Deno and Carpenter, 1994). These fields are generated from ultraviolet and visible light from the sun (Zervins, 1973) or from solar storms (Deno and Carpenter, 1994). The intensity of natural AC magnetic field is small compared to man-made magnetic fields. Most man-made magnetic fields are AC magnetic fields at the frequencies of either 50 or 60 Hz, and are generated by electrical appliances (Novini, 1993 and World Health Organization, 1989).

In practice, an electrical appliance that is connected to a 50 or 60 Hz AC circuit, but not turned on, generates only an electric field. This same appliance, if plugged into the electrical socket and then turned on, generates both electric and magnetic fields (Novini, 1993). Electric fields are blocked by most matter, including rocks, trees, animals, and humans. Unlike electric fields, magnetic fields are much more difficult to block by most matter, including steel, concrete, and lead (Novini, 1993). The strength of electric fields and intensity of magnetic fields both decrease with distance from the source (World Health Organization, 1989).

Electromagnetic fields generated from alternating electric current are commonly referred to as Very Low Frequency and Extremely Low Frequency electromagnetic fields (Novini, 1993). In general, the term Very Low Frequency is used to refer to electromagnetic fields which are generated from alternating electrical currents in the frequency range of 2 kHz to 400 kHz, such as those generated from navigation radio signals used for aircraft and marine (Novini, 1993). An Extremely Low Frequency is used to refer to electromagnetic fields which are generated by alternating electrical

currents in the frequency range of 5 to 2,000 Hz (Novini, 1993). Most humans are exposed to electromagnetic fields of Extremely Low Frequency that occur in the range of 50 to 300 Hz (Goodman et al., 1993). Some examples of exposures to Extremely Low Frequency electromagnetic fields occurring in the work place are electromagnetic fields generated from factory equipment (Novini, 1993), and by electric devices, such as computers and video display terminals, and fax and copy machines (Goodman et al., 1993 and Novini, 1993). Extremely Low Frequency electromagnetic fields are also generated from household appliances, such as electric fans, electric blankets (World Health Organization, 1989), microwave ovens (Zervins, 1973), electric stoves and ovens, clothes washers and dryers, electric shavers, hair dryers and curlers, clock radios (Novini, 1993), cellular phones (Goodman et al., 1993), and television receivers (Presman, 1970). Representative examples of magnetic field intensities caused by home appliances at various distances from the sources are presented in Table 1.

Units of Measurement Used to Quantify Electric and Magnetic Fields

The Volt/meter or Newton/coulomb is used to define the strength of an electric field. The Newton-second/coulomb-meter, or Weber/meter^2, or Tesla, or Gauss (G) are all units of measurement which have been used to define the intensity of a magnetic field.

In the United States, the Gauss is the unit of magnetic field intensity which is widely used. In countries where the metric system is official, the Tesla is the unit of magnetic field used. For conversion, the following formulas are used:

One Tesla = 10,000 Gauss; One microTesla = 10 milliGauss.

Table 1. Magnetic field intensity at 60 Hz measured at 0.03, 0.3, and 1 meter from various electric appliances

Type of Appliance	Range for magnetic field intensity in milliGauss					
	0.03 meter		0.3 meter		1 meter	
	Low	High	Low	High	Low	High
Clothes washer	8	500	0.4	20.0	0.1	1.5
Electric range	60	2,000	3.5	40.0	0.1	1.0
Electric shaver	150	15,000	0.8	90.0	1.0	3.0
Fluorescent lamp	400	4,000	5.0	20.0	2.0	2.5
Hair dryer	60	20,000	0.1	70.0	0.1	3.0
Microwave oven	750	2,000	40.0	80.0	2.5	6.0
Television	25	500	0.4	20.0	0.1	1.5
Vacuum cleaner	2,000	8,000	20.0	200.0	1.3	20.0

Adapted from World Health Organization, 1989.

Medical Uses

Electromagnetic fields are widely used in human medicine either for diagnosis or treatment of functional abnormalities or diseases.

Therapeutic uses

There are enough evidences to suggest that exposures to electromagnetic fields stimulated osteogenesis and wound healing in the long bones of patients which were previously refractory to neosteogenesis (Brighton et al., 1979; Bassett et al., 1982 and Nordstrom et al., 1983). Since 1979, the Food and Drug Administration has approved the exposure to electromagnetic fields ranging from 50 to 500 G at 50 or 60 Hz (McCleary et al., 1991) or ranging from 1,000 to 10,000 G at 3 Hz (World Health Organization, 1989) as a treatment of non-union fractures. Electromagnetic fields of 3 to 25 mG at 60 Hz are also used to enhance wound healing and tissue regeneration from wounds that do not heal well (World Health Organization, 1989).

Recently, Sandyk (1993) reported that mobility symptoms of Parkinson's disease could be attenuated by treatment with an electromagnetic field of 0.075 mG at 2 to 7 Hz for 6 minutes. The mechanisms by which this low intensity electromagnetic field ameliorated Parkinsonian symptoms are unknown. However, the electromagnetic field inhibited melatonin secretion from the pineal gland of Parkinson's disease patients, and was associated with reduced blood glucose levels after exposure to an electromagnetic field for 6 minutes (Sandyk, 1993).

Diagnostic uses

Computerized Axial Tomography (Carney and Anderson, 1981) and Nuclear Magnetic Resonance Imaging (Witcofski et al., 1981) are diagnostic methods which expose patients to AC and DC magnetic fields. The intensity of Nuclear Magnetic Resonance Imaging ranges from 20,000 to 200,000 G at 60 Hz (World Health Organization, 1989). These procedures are widely used for diagnosis of morphological and/or functional abnormalities in humans.

LITERATURE REVIEW

Effects of Electromagnetic Fields on Humans

Effects on the circulatory system

Graham et al. (1994) performed an experiment to determine the effects of exposure to electromagnetic fields of 0.1 G, 0.2 G, and 0.3 G at 60 Hz on the human circulatory system. Humans exposed to electromagnetic fields of 0.2 G at 60 Hz for two 3-hour periods, separated by a 30 minute break had a significantly reduced heart rate. The result of Electrocardiogram measurements in the study of Graham et al. (1994) showed a latency from the R wave to the end of the T wave, and the amplitude of the T wave was greater than before the exposure to the electromagnetic field. However, exposures to a lower (0.1 G) or to a higher (0.3 G) intensity of electromagnetic fields had no influence on heart rates or cardiac waves.

Epidemiological studies

Association with the development of cancer

In an epidemiological survey, Wertheimer and Leeper (1979), found an increased incidence of cancer in children, but not in adults, associated with exposure to electromagnetic fields ranging from 0.002 to 0.007 G at 60 Hz generated by high-voltage power lines. The fact that an increased risk of cancer was detected for children reared near these power lines, but not in adults, suggested that there may be critical pre- or post-natal periods during which exposure to electromagnetic fields may be more

damaging. Feychting and Ahlbom (1993) also found an association between exposure to electromagnetic fields (intensities of $\geq$ 0.002 G at 50 Hz) generated by high-voltage (220 to 400 kV) power lines and leukemia in children. Their results suggested that the risk of developing leukemia increased with the intensity of the electromagnetic field within the environment. However, Sahl et al. (1993) found no association between exposure of workers in the electrical utility industry to electromagnetic fields ranging from 0.007 to 0.3 G at 60 Hz and death of these workers from brain cancer, leukemia, or lymphoma.

Effects on fertility

Epidemiological studies have also suggested a potential relationship between exposures to elevated electromagnetic fields and reduced fertility in men (Nordstrom et al., 1983) and increased abortion rate in women (Wertheimer and Leeper, 1989). Nordstrom et al. (1983) found that switchyard workers exposed to electromagnetic fields generated from high-voltage (400 kV) power lines had decreased pregnancy rates and increased frequency of congenital malformations in their offspring. Lindbohm et al. (1992) also reported that female employees using video display terminals which generated electromagnetic fields of $>$0.009 G at 60 Hz had a higher incidence of spontaneous abortion, when compared to employees using video display terminals which generated electromagnetic fields of $<$0.004 G at 60 Hz.

Other studies have also associated exposure of gametes and/or embryos to electromagnetic fields with fetal growth retardation and increased risk of abortion (Wertheimer and Leeper, 1986 and Windham et al., 1990) or with increased frequency

of congenital defects (Nordstrom et al., 1983). In studies performed by Wertheimer and Leeper (1989), pregnant women who were exposed to electromagnetic fields ranging from 0.004 to 0.015 G at 60 Hz generated from electric blankets or heated waterbeds had an increased rate of abortion during the first trimester of pregnancy and when they had a full-term gestation, there was an increased number of infants born with low birth-weight (Wertheimer and Leeper, 1986).

Effects of Electromagnetic Fields on Vertebrates

Several studies have shown that exposure to electromagnetic fields affects the endocrine processes, and embryonic development, and induces teratogenic development in laboratory animals.

Endocrinological effects

Kato et al. (1994) reported that nocturnal concentrations of melatonin were reduced in male rats exposed to an electromagnetic field of 0.01 G at 50 Hz for 6 weeks, and that the plasma melatonin concentrations for these rats returned to normal levels within one week after cessation of exposure. These authors (Kato et al., 1994) concluded that the effects of electromagnetic field exposure on the pineal glands of rats were short-lived and did not cause any persistent, substantial metabolic changes with respect to melatonin level. In contrast, Lee et al. (1995) reported that they did not detect any effect on serum level of melatonin after exposure of female lambs to an electromagnetic field of 0.037 G at 60 Hz.

Teratogenic developmental effects

Recently, Chiang et al. (1995) reported that the teratogenic effects of cytosine arabinoside were increased in mice which were given cytosine arabinoside and simultaneously exposed to electromagnetic fields of 0.4 G at 15.6 Hz. The study of Chiang et al. (1995) was performed in pregnant mice which were injected intraperitoneally with 10 mg/kg of body weight of cytosine arabinoside on day 9 of gestation and exposed to electromagnetic fields of 0.4 G for 4 hours daily on days 6 to 17 of gestation. On day 18 of gestation, mice were euthanized and the feti were examined and then dissected. Feti showed malformations of cleft palate and/or cleft lip. The incidence of abnormalities in the mice exposed to both cytosine arabinoside and the electromagnetic field was higher ($P < 0.01$) than in the mice which only received cytosine arabinoside or were only exposed to the electromagnetic field. Chiang et al. (1995) concluded that the embryo-toxic effects of exposure to electromagnetic fields may be concurrently enhanced by the administration of a teratogenic agent. Baum et al. (1995) also reported that female rats given a single oral dose of 5 mg of 7,12-dimethylbenz[α]anthracene and were continuously exposed to an electromagnetic field of 1 G at 50 Hz for a 3-month period had a significantly ($P < 0.05$) increased growth rate and size of mammary tumors when compared to female rats which received only 5 mg of 7,12-dimethylbenz[α]anthracene or which were only exposed to the electromagnetic field.

Embryonic mortality and developmental effects

Avian studies

Delgado et al. (1982) reported an increased incidence of developmental abnormalities in chick embryos exposed for 48 hours to an electromagnetic field of 0.012 G at 100 Hz during incubation. Developmental abnormalities found in chick embryos included lack of development of brain vesicles, neural tube, foregut, heart vessels, and somites, and also a reduced amount of glycoaminoglycans, which are essential elements in cellular activities and migration. Ubeda et al. (1983) subsequently reported a "window effect" on chick embryos for pulsed electromagnetic fields which were pulse rise and fall times of 100 μsec and pulse duration of 500 μsec. Ubeda et al. (1983) in their study found that only intensities of 0.01 to 0.139 G at 100 Hz during an exposure time of 48 hours induced developmental abnormalities, including a thick and disorganized neural tube, abnormalities of foregut and heart development. Ubeda et al. also reported in 1994 that exposure of chick embryos for 48 hours to pulsed electromagnetic fields with pulse rise and fall times of 2.1 μsec, a pulse duration of 500 μsec, and an intensity of 0.01 G at 100 Hz caused encephalocele, anophalocele, microphthalmia, and malformed beaks. Zhang et al. (1993) provided evidence that exposures of chick embryos to electromagnetic fields of 2 G at 60 Hz may be responsible for increased incidence of developmental abnormalities, such as open abdominal cavity, missing eyeballs, wing deformities, liver cirrhosis, and other detrimental effects, including retardation of post-hatching development. Furthermore, Moses and Martin (1992) reported that avian embryos exposed for 78 to 88 hours to an electromagnetic field of 0.04 G at 60 Hz had significantly reduced activities of enzymes

involved in cell proliferation and differentiation, such as 5'nucleotidase, alkaline phosphatase, and acetylcholinesterase.

Mammalian studies

Although relatively few studies have been performed in mammals, there is evidence to suggest that electromagnetic fields ranging from 0.23 to 0.66 G may decreased lymphocyte counts and cause abnormalities of size and shape of the skeleton in rats (Stuchly et al., 1988). In mice, continuous exposure to an electromagnetic field of 0.15 G at 20 kHz from days 0 to 14 of gestation was associated with increased incidence of developmental abnormalities such as umbilical hernia, hydrocephaly, enlarged digits, and malformations of the ears and eyes (Tribukait et al., 1987). Mezhevikina et al. (1990) reported that exposure of eight-cell mouse embryos for short periods to an electromagnetic field generated by an electric field at a frequency of 915 MHz, which is an ultra-high-frequency radiation, was found to induce morphological changes in the exposed embryos, but the exposure did not affect the capability of these embryos to develop to the blastocyst stage during *in vitro* culture. Unfortunately, Mezhevikina et al. (1990) did not identify in their report the levels of the magnetic field that was generated and applied to the mouse embryos.

Frolen et al. (1993) reported that continuous exposure from conception to birth of CBA/S-strain pregnant mice to an electromagnetic field of 0.15 G at 20 kHz, induced detrimental effects on embryos and feti. The reported effects included increased rates of placental resorption and increased number of dead feti at term for exposures initiated on the first, second, and fifth days after conception and continued to birth. However,

no effects were observed when exposure to the electromagnetic field was initiated on the seventh day after conception and continued to birth. Because the female mice were exposed to the electromagnetic fields after mating, in the study of Frolen et al. (1993), it was not possible to determine conclusively whether the effects of exposure to the electromagnetic fields were predominately on the embryos or were secondary effects due to electromagnetic field exposures of the mothers. Notwithstanding, their results suggest that there were electromagnetic field effects which were either exerted directly on the embryos, or on the mother prior to or around the time of implantation, but not afterwards because increased rates of placental resorption and increased number of dead feti were found only in the pregnant mice exposed during the first five days after conception, the preimplantation period, but this phenomenon was not observed in the pregnant mice exposed on the seventh day after conception, during the postimplantation period. Unfortunately, Frolen et al. (1993) in their study did not assess for an effect of exposure to the electromagnetic fields on the number of offspring born or on their survival to weaning.

In the rabbit, Hansson (1981) reported that continuous exposure of pregnant does to an electromagnetic field at 50 Hz throughout the period from conception to birth was associated with reduced postnatal growth and neurological impairment of the offspring. Unfortunately, Hansson (1981) did not identify the intensity of the magnetic field used in the study.

Effects of Electromagnetic Fields on Cultured Cells

Researchers have attempted to study the effects of an electromagnetic field on cultured cells with the expectation that molecular and cell biological technologies applied to the cellular model might identify a cellular basis for some of the biological effects of exposing humans or animals to electromagnetic fields. Recently Lindstrom et al. (1995) reported that the exposure of human leukemic T-cells to an electromagnetic field of 1.5 G at 50 Hz for 2 minutes increased the concentration of intracellular calcium in the exposed cells. Carson et al. (1990) had reported that the exposure for 23 minutes of HL-60 cells to an electromagnetic field generated from magnetic resonance imaging increased the concentration of intracellular calcium in the exposed cells. Unfortunately Carson et al. (1990) did not identify G or Hz for the electromagnetic field used in their study. Although, the reports of Lindstrom et al. (1995) and Carson et al. (1990) provided evidence for cellular effects of electromagnetic field exposure, no attempt was made to determine how electromagnetic fields can increase the concentration of intracellular calcium in the cultured cells. However, they propose that an effect of electromagnetic fields to increase the concentration of intracellular calcium may relate to one or more mechanisms by which an electromagnetic field might affect cells including an increase in plasma membrane permeability, especially opening of calcium channels, or an inhibition of plasma-membrane calcium-efflux mechanisms, or an increase in rates of calcium endocytosis (Lindstrom et al., 1995 and Carson et al., 1990). Alternatively, electromagnetic fields might affect the mobilization of intracellular calcium stores or inhibit calcium sequestration into the endoplasmic reticulum and/or

mitochondria (Carson et al., 1990). However, Blackman et al. (1985) reported that exposures of culture of chicken brain tissues to electromagnetic fields of 0.235 G and 0.760 G at 30 Hz caused the efflux of calcium ions from the brain cells. Others, Garcia-Sancho et al. (1994) found that exposures to electromagnetic fields of 1 to 2 G at 14.5 to 15.5 Hz for 60 minutes increased potassium uptake in Ehrlich ascites tumor cells and U397 human leukemic cells, apparently by stimulation of the sodium, potassium-pump, but these levels of electromagnetic field exposure did not affect intracellular pH, or the concentrations of intracellular calcium and sodium ions.

The effects of electromagnetic field on cellular proliferation would be expected to influence DNA and RNA synthesis, as well as transcription and translation. Goodman et al. (1989) reported that exposure to an electromagnetic field of 15 G for 30 minutes increased the rates of RNA transcription and translation in HL-60 cells. Furthermore, an exposure of Simian virus-40-transformed human fibroblast cells to an electromagnetic field of 0.08 G at 60 Hz for 20 minutes increased the levels of virally derived mRNA and levels of a large T-antigen, a virus-encoded protein which is responsible for transformation of nonpermissive and semipermissive mammalian host cells (Gold et al., 1994). Gold et al. (1994) provided evidence that the viral DNA (Simian virus-40), which can be integrated into the DNA of host cells, is affected by electromagnetic fields such that increased transcription and translation of DNA from cellular genes of host cells.

STATEMENT OF THE RESEARCH PROBLEM

A vexing aspect of the research performed to determine the effects of electromagnetic fields on avian embryos or cultured cells is the lack of consistency between experimental results in laboratories. Lindstrom et al. (1995) and Carson et al. (1990) found that only higher intensity levels of electromagnetic fields (1.5 G at 50 Hz) increased the concentration of intracellular calcium in cultured cells. However, other researchers (Blackman et al., 1985) reported that lower intensities ranging from 0.235 to 0.760 G of electromagnetic fields at 30 Hz decreased the concentration of intracellular calcium. Moreover, well-planned studies performed in various laboratories (Maffeo et al., 1984 and Maffeo et al., 1988) found no effect for exposures to electromagnetic fields ranging from 0.01 to 0.12 G at 100 Hz or 1 kHz on avian embryos, despite the fact that these authors used the same avian model and comparable intervals and intensities of electromagnetic field exposures as those used by Delgado et al. (1982) which, reportedly, had affected the avian embryos. Furthermore, even when there appears to be relative agreement that exposures to an electromagnetic field could influence cell growth or embryonic differentiation, analyses of the authors' conclusion (Delgado et al., 1982; Zhang et al., 1993; Ubeda et al., 1983; Ubeda et al., 1994; Moses and Martin, 1992 and Berman, 1990) provides no evidence for a consensus as to when the losses occurred, which developmental defects were induced, or which characteristic of the electromagnetic field, including frequency, pulse shape, and/or intensity, was responsible for the detrimental effects claimed to have occurred due to the electromagnetic field exposure.

RATIONALE FOR USING THE RABBIT EMBRYO AS A CELLULAR MODEL

Animal Model

It would be inappropriate to use the human embryo to evaluate the potentially detrimental effects of the exposure to electromagnetic fields. Therefore, I propose to use rabbit embryos as the animal model. Rabbits are among several species which have been used as a non-rodent model for toxicity testing. I selected the rabbit as the species of choice for this study because rabbits are successfully superovulated (Harper, 1961 and Foote et al., 1963), and rabbit embryos can be readily flushed from the reproductive tracts of donors. Furthermore the female rabbit has a uterus with independent uterine horns (Hafez, 1970 and Kozma et al., 1974) and cervices (Hartman, 1974). This anatomical feature allows for the transfer of control and treated embryos to separate horns of the same recipient and these embryos and feti are maintained as separate groups up to the time of parturition.

The Preimplantation Embryo as a Cellular Model

The preimplantation stage mammalian embryo provides a cellular model in which a single cell, or a group of blastomeres in a multicellular embryo contains all of the DNA needed to direct the development of an entire organism. I believe that the mammalian embryo could be developed as an ideal experimental model to study the effects of exposure to electromagnetic fields because whole embryos or isolated blastomeres from

early stages of embryonic development are totipotent. They can be maintained outside the maternal environment in an incubator or in intermediate recipients for definite periods, and perhaps more importantly, they can be exposed to electromagnetic fields of defined intensity and duration whether as individual embryos or as a group.

EXPLANATION OF THESIS FORMAT

To facilitate the description of my studies and the presentation of my results, data analysis, and discussion, I have presented my Thesis as follows: The first part is composed of 5 Experiments (Experiments 1 to 5), which I considered to be preliminary studies addressed to find a suitable medium to culture rabbit embryos *in vitro* and to determine the most suitable stage of embryonic development at which embryos could be successfully cultured to the blastocyst stage and could develop to term after transfer to a recipient doe. In addition, these preliminary experiments were designed to provide me with technical know-how and experience.

The second part is composed of 4 Experiments (Experiments 6 to 9), which were performed to test the effects of exposure to an electromagnetic field of 1.5 G at 60 Hz on the development and viability of rabbit embryos during the *in vitro* culture for 24 and 48 hours and after transfer to synchronized recipient does. For Experiment 6, rabbit morulae were exposed to an electromagnetic field of 1.5 G at 60 Hz for 24 hours during *in vitro* culture, for Experiment 7, control and treated embryos resulting from Experiment 6 were transferred to synchronized recipients does. For Experiment 8, rabbit morulae were exposed to an electromagnetic field of 1.5 G at 60 Hz for 48 hours during *in vitro* culture, and for Experiment 9 control and treated embryos resulting from Experiment 8 were transferred to synchronized recipients does.

MATERIALS AND METHODS

Preliminary Studies

Experiment 1. Effect of synthetic culture media on the *in vitro* development and viability of 1-cell and 2-cell rabbit embryos

For the purpose of finding a suitable medium to culture 1-cell and 2-cell embryos to the morula or blastocyst stages, which are the optimal transferable stages for transfer of embryos to synchronized recipient does, I selected 4 culture media for evaluation of their capability to support the *in vitro* development and viability of 1-cell and 2-cell rabbit embryos. These 4 synthetic culture media were selected based on published reports in the literature (Carney and Foote, 1991) and on prior studies performed in our laboratory.

The 4 synthetic culture media to be tested were:

HL-1 = HL-1 Medium (Hycor Biomedical Inc., Irvine, California) supplemented with 2.5 mM glutamine (Fisher Scientific Inc., Fair Lawn, New Jersey).

DMEM/F-12 = 1:1 (v/v) of Dubecco's Modified Eagle's Medium and Ham's F-12 (Hycor Biomedical Inc., Irvine, California) supplemented with 2.5 mM glutamine.

WBSA = Whitten's Medium (Pollard, 1987) supplemented with 1.5 % crystallized bovine serum albumin (Cat No. A.7638, Sigma Chemical Inc., St. Louis, Missouri).

RD Medium = 1:1 (v/v) of RPMI-1640 (Celox Inc., Hopkins, Minnesota) and Dulbecco's Modified Eagle's Medium (Celox Inc., Hopkins, Minnesota) containing 4500 mg/L glucose (Carney and Foote, 1991).

The HL-1, DMEM/F-12 (Dulbecco's Modified Eagle's Medium and Ham's F-12), and RD (RPMI-1640) culture media were obtained from commercial sources. WBSA medium was formulated in our laboratory, as described by Pollard (1987).

Hypotheses

1. Development of 1-cell and 2-cell rabbit embryos during *in vitro* culture for 96 hours is comparable among the 4 synthetic culture media.
2. There is no difference in the viability of rabbit embryos cultured *in vitro* for 96 hours in each of the culture media.

Experimental design

One-cell and 2-cell embryos recovered from 7 does were allocated into 4 groups, and each group was randomly assigned for *in vitro* culture for 96 hours in one of the 4 synthetic culture media.

Procedures

Animals A total of 7 New Zealand White does, 20 weeks of age or older and weighing a minimum of 3 kg, were randomly selected from 15 does and used as embryo donors to compare the effects of these 4 synthetic culture media on the *in vitro* development and viability of 1-cell and 2-cell rabbit embryos. Each doe was caged individually, fed commercial rabbit feed, and maintained during the experimental period in a room with controlled temperature (20 to 22 °C), and light (14 hours' light/10 hours' dark). Mature New Zealand White bucks of known fertility were used for mating. Bucks were keep in a separate room, and caged individually and maintained, as described for the does.

Superovulatory treatment Each doe was induced to superovulate by subcutaneous injections of 0.3 mg of porcine follicle stimulating hormone (FSH-P, Schering-Plough Animal Health Inc., Kenilworth, New Jersey) administered twice a day for 3 days, for a total dose of 1.8 mg of FSH-P. To induce a superovulatory response, a single intramuscular injection of 25 IU per kg of body weight of human chorionic gonadotropin (hCG, Solvay Animal Health Inc., Princeton, New Jersey) was given to each doe 6 hours after the last dose of FSH-P. Each doe was then placed in a cage with a fertile buck, and was left overnight in the male's cage for mating following the procedure recommended by Kennelly and Foote (1965). Mating was confirmed for each doe by the presence of spermatozoa in the vaginal smears obtained in the morning after the doe was paired with the male.

Embryo recovery and culture To recover embryos, each superovulated and mated doe was anesthetized 19 to 22 hours after mating by an intramuscular injection of 5 mg per kg of body weight of Acepromazine Maleate (CEVA laboratories Inc., Overland Park, Kansas), followed 10 minutes later an intramuscular injection of 40 mg per kg of body weight of Ketamine Hydrochloride (Fort Dodge Laboratories Inc., Fort Dodge, Iowa). Once the doe was in a surgical plane of anesthesia, the abdominal cavity was opened and the reproductive tract was exposed. To minimize bleeding during recovery of the reproductive tracts, and blood contamination of the embryos, each doe was euthanized by inhalation of CO_2 at low pressure (2-3 psi) until death ($\leq$ 3 minutes). Immediately after death, the respective ovary, oviduct, and a short segment of the proximal part of uterine horn from each side was removed, and placed in a collecting bowl containing HEPES-buffered saline at a pH of 7.4. It was expected that at the time of euthanasia the oocytes released in response to superovulatory treatment were fertilized and embryos at the late 1-cell and early 2-cell stages were located in the ampullary region of the oviducts. To recover embryos, each oviduct was flushed from the uterotubal junction with 3 ml of HEPES-buffered saline into a flushing bowl containing a bicarbonate-buffered saline solution at a pH of 7.4, supplemented with 0.3% (w/v) of gelatin type B (Sigma Chemical Inc., St. Louis, Missouri). The fluids, embryos, and cell debris flushed from each doe were maintained in separate dishes. To confirm the passage of fluid through the entire length of the excised oviduct, the oviduct was reflushed using a 2 ml air chase. After flushing and recovery, the oocytes and/or embryos recovered were examined for stage and morphology with an inverted microscope at 100X. Oocytes that had spermatozoa in the perivitelline space or

embryos that had extruded the second polar body or embryos that had two visible pronuclei were considered to be fertilized and were classified as 1-cell embryos. Embryos that had two blastomeres of comparable size were classified as 2-cell embryos.

Immediately after evaluation, all embryos were transferred to a culture dish, and randomly allocated to one of the 4 groups and each group of embryos was washed three times in a bicarbonate-buffered saline solution and then transferred to a culture well containing 500 μl of a 240 μM solution of eosin B in bicarbonate-buffered saline. Embryo viability was determined by exposure of embryos to 240 μM solution of eosin B for 10 minutes (Dooley et al., 1984 and Dooley et al., 1989). Embryos that had at least one unstained blastomere (Dooley et al., 1984 and Dooley et al., 1989) were classified as viable, and only viable embryos were used in this Experiment. After staining, embryos were washed two times in bicarbonate-buffered saline solution to remove the dye. After washing, viable 1-cell and 2-cell embryos were randomly allocated to *in vitro* culture in 1 of 4 media groups and each embryo of the group assigned to a particular medium was cultured individually within a well of a 24 well-plate (Becton Dickinson Inc., Lincoln Park, New Jersey) containing 500 μL of the culture media. All of the embryos were cultured for 96 hours at 37 °C within an incubator, supplemented to contain 5% CO_2 in a humidified air atmosphere. Embryos were removed from the incubator and assessed for stage of development and morphological characteristics at 24, 48, 72, and 96 hours of culture. At the end of the 96-hour period of culture, embryos were again evaluated for viability using the eosin B assay as

described above. Cleavage indices assigned for developmental stages of rabbit embryos were determined during *in vitro* culture are shown in Table 2.

Endpoints

1. Stage of embryonic development at 0, 24, 48, 72, and 96 hours of culture.
2. Maximal stage of development reached for each embryo within the 96-hour period of culture.
3. Embryonic viability before culture and at the end of the 96-hour period of *in vitro* culture.

Statistical analysis Analysis of variance (Steel and Torrie, 1980) was used to evaluate the effects of embryo donor, treatment, time, and the interaction between treatments and time, and to compare cleavage indices (Dooley, 1988) of embryos. The analysis of variance was performed using SAS (Version 6) (SAS Institute Inc.). Allocation of embryos to treatment was performed using a randomized block design, and the statistical analysis of the data was performed using a split-plot model. Tukey's procedure (Steel and Torrie, 1980) was used to test the differences between means of maximal, peak stage of embryonic development. Chi-square analyses of ratios (Steel and Torrie, 1980) were used to compare developmental stages and viability of embryos. Statistical significance was established at $P \leq 0.05$.

Table 2. Cleavage indices for developmental stages of rabbit embryos identifiable during *in vitro* culture

Cleavage index	Developmental stage of rabbit embryo
1	Fertilized, 1-cell embryo
2	2-cell embryo
3	3-cell embryo
4	4-cell embryo
5	5- to 7-cell embryo
6	8-cell embryo
7	9- to 16-cell embryo
8	17- to 32-cell embryo
9	Early morula
10	Late morula
11	Early blastocyst
12	Blastocyst
13	Expanding blastocyst
14	Hatching blastocyst
15	Hatched blastocyst

Adapted from Dooley (1988).

Morula = Multicellular embryo containing > 16 blastomeres, without a blastocoel. At this stage the extent of cellular associations formed between blastomeres results in greater membrane continuity between cells such that identification and counting of individual blastomeres is no longer possible during morphological assessment. The classification of early or late morulae are as follows:

Early morula: Outline of individual cell is still evident.
Late morula: Compacted mass; no individual blastomeres evident in cell mass.

Early blastocyst = Embryo which has a clearly defined blastocoel less than 40 % of the cell mass.

Blastocyst = Embryo which has a clearly defined blastocoel ranging between 40 % to 60 % of the cell mass.

Expanding blastocyst = Embryo which has a clearly defined blastocoel comprising more than 60 % of the cell mass.

Hatching blastocyst = Embryos which had developed to the blastocyst stage and had some blastomeres passed through the zona pellucida.

Experiment 2. *In vitro* culture of rabbit embryos in defined medium (HL-1) within the Shielded Culture Cell or within the Electromagnetic Field Exposure System (ISU-EFG)

In Experiment 1, the highest proportion of 1-cell and 2-cell rabbit embryos to reach the blastocyst stage during *in vitro* culture was obtained using HL-1 medium. Experiment 2 was performed to determine whether this medium would produce a sufficient number of embryos at the optimal transferable stages of morula and blastocyst when cultured *in vitro* for 96 hours within a Shielded Culture Cell or an Electromagnetic Field Exposure System (ISU-EFG).

Hypotheses

1. Development of 1-cell and 2-cell rabbit embryos during *in vitro* culture for 96 hours within the Shielded Culture Cell is not different from the development of embryos cultured for 96 hours within the Electromagnetic Field Exposure System (ISU-EFG).
2. There is no difference in the viability of rabbit embryos cultured *in vitro* for 96 hours within the Shielded Culture Cell or the Electromagnetic Field Exposure System (ISU-EFG).

Experimental design

One-cell and 2-cell embryos recovered from 5 does were allocated to 2 groups, and each group was randomly assigned for *in vitro* culture for 96 hours in HL-1 medium

within the Shielded Culture Cell or for culture in HL-1 medium within the Electromagnetic Field Exposure System (ISU-EFG).

Procedures

A total of 5 New Zealand White does, 20 weeks of age or older and weighing a minimum of 3 kg, were randomly selected from 10 does and used as embryo donors. Each doe was caged individually, maintained and cared for as described for Experiment 1. Superovulation was induced by treatment with FSH-P, followed by an injection of hCG at the dose and schedule described for Experiment 1. After the injection of hCG, each doe was left overnight for mating, in the cage of a male of known fertility. Embryos at the 1-cell and 2-cell stages were then surgically recovered from anesthetized does 19 to 22 hours after mating, as described for Experiment 1.

Embryos were recovered, handled, and processed for assessment of viability as described for Experiment 1. Embryos that had at least one unstained blastomere were considered viable and only viable embryos were used in this Experiment. Viable 1-cell and 2-cell embryos were then randomly allocated to two groups, and cultured *in vitro* within a:

Shielded Chamber: Embryos were cultured within the shielded Culture Cell (Lamont et al., 1994) positioned on Shelf Q of the incubator. Embryos were exposed to a background electromagnetic field of $\leq$ 0.005 G at 60 Hz.

Unshielded Chamber: Embryos were cultured within the Electromagnetic Field Exposure Chamber (ISU-EFG) (Lamont et al., 1994) which

was also positioned on Shelf Q of the incubator. No current was applied to the coils, hence embryos were exposed during the period of culture to background fields estimated to range between 0.02 and 0.06 G at 60 Hz.

For culture, each embryo was placed in a well of a Multi-well plate (LUX 5250, SAS plate, Lab-Tek Division, Miles Laboratories Inc., Naperville, Illinois). Each culture well contained 50 μl of HL-1 culture medium supplemented with 2.5 mM glutamine and overlaid with 40 μl of light paraffin oil (Fisher Scientific Inc., Fair Lawn, New Jersey). Embryos were cultured for 96 hours at 37 °C within the assigned chambers (Shielded or Unshielded, see above) which were placed in an incubator in a humidified atmosphere supplemented to contain 5 % CO_2 in air. Embryos were evaluated for stage of development at 0, 24, 48, 72, and 96 hours of culture. At the end of the 96-hour culture period, embryos were evaluated for viability, as described for Experiment 1.

Endpoints

1. Stage of embryonic development at 0, 24, 48, 72, and 96 hours of exposure.
2. Maximal stage of development reached within the 96-hour period of culture.
3. Viability before culture and at the end of 96-hour period of *in vitro* culture.

Statistical analysis Analysis of variance (Steel and Torrie, 1980) was used to evaluate the effects of embryo donor, treatment, time, and the interaction between treatments and time and to compare cleavage indices (Dooley, 1988) of embryos. The analysis of variance was performed using SAS (Version 6). Allocation of embryos to treatment was performed using a randomized block design, and the statistical analysis of the data was performed using a split-plot model. Chi-square analyses of ratios (Steel and Torrie, 1980) were used to compare developmental stages and viability of embryos. Statistical significance was established at $P \leq 0.05$.

Experiment 3. Viability after transfer of rabbit embryos cultured *in vitro* in HL-1 medium within the Shielded Culture Cell or within the Electromagnetic Field Exposure System (ISU-EFG) to 92-hour synchronized recipient does

In Experiment 2, 1-cell and 2-cell rabbit embryos were cultured *in vitro* for 96 hours in HL-1 medium and this resulted in a suitable number of embryos that reached the transferable stages of morula and blastocyst. In Experiment 3, embryo transfers were performed to confirm that the morula and blastocyst stage embryos obtained after culture within the Shielded or the Unshielded chambers of Experiment 2 were capable of producing live offspring after transfer to synchronized recipient does.

Hypothesis

Embryos cultured in HL-1 medium for 96 hours within the Shielded Culture Cell or within the Electromagnetic Field Exposure System (ISU-EFG) will develop to term and produce offspring when transferred to synchronized recipient does.

Experimental design

Embryos from each donor were cultured in HL-1 medium for 96 hours *in vitro*, within the Shielded Culture Cell or within the Electromagnetic Field Exposure System (ISU-EFG) and at the end of the culture period, embryos from the same donor that developed to the morula or blastocyst stages were randomly assigned for transfer to each of 2 synchronized recipient does, such that not less than 2 and no more than 5 embryos were transferred to each uterine horn. For this purpose, embryos recovered from each of 5 donors that met these requirements were transferred to 10 synchronized recipient does.

Procedures

Refer to the description provided as part of Experiment 2 for procedures for embryo recovery and culture. Only viable morulae and blastocysts were selected for transfer to synchronized recipient does.

A total of 10 New Zealand White does, 20 weeks of age or older and weighing a minimum of 3 kg, were randomly selected from 20 does and used as recipient does. Each doe was caged individually, maintained and cared for as described for Experiment 1. Embryos were transferred surgically, using aseptic techniques, into the uterine horns

of each of 10 synchronized recipient does, according to procedures described by Adams (1982) and Pollard (1987). Embryos cultured within the Shielded or Unshielded chambers from a given donor were transferred to each of 2 recipient does. To synchronize a recipient doe, each doe was induced to ovulate 90 to 92 hours prior to embryo transfer by an intramuscular injection of 25 IU per kg of body weight of hCG.

Embryo transfer Each recipient doe was fasted at least 6 to 8 hours before surgery. Prior to embryo transfer, each recipient doe was anesthetized with an intramuscular injection of 5 mg per kg of body weight of Acepromazine Maleate, followed 10 minutes later by an intramuscular injection of 40 mg per kg of body weight of Ketamine Hydrochloride. After the injection of Ketamine Hydrochloride, the does were maintained in a surgical plane of anesthesia with Methoxyflurane inhalation (Pitman-Moore Inc., Washington Crossing, New Jersey) until surgery was completed. Each recipient doe was positioned in dorsal recumbency, and the ventral area of the abdominal wall was shaved, disinfected, and draped. The uterine horns were exposed and exteriorized through a 4 to 6 cm midventral abdominal incision along the linea alba. Each uterine horn was randomly allocated between embryos from the Shielded Culture Cell or Electromagnetic Field Exposure System (ISU-EFG), such that if the embryos cultured within the Shielded Culture Cell were to be placed in the right uterine horn of a given doe, the embryos cultured within the ISU-EFG were transferred to the left horn of the same doe. In view of the fact that a minimum of 2 implantations are required for fetal survival and normal parturition (Adams, 1982), and the litter size of a doe ranges from 8 to 10 bunnies (Hafez, 1970), I transferred not less than 2 and no more than 5

embryos from each treatment group to the lumen of each uterine horn. To transfer embryos, each horn was punctured in the proximal portion of the uterine horn using a No. 22 suture needle, and embryos from each treatment group were transferred to the lumen of the assigned horn using a 5 μl Wiretrol pipette (Drummond Scientific Inc., Broomall, Pennsylvania). After transfer of the embryos, the exposed tissues were moistened periodically with a 0.9% solution of warm saline, supplemented to contain 10 % glycerol (Fisher Scientific Inc., Fair lawn, New Jersey) and 50 μg/ml of Gentamicin sulfate (Sigma Chemical Inc., St. Louis, Missouri) (Dooley, 1988). The abdominal muscular incision was closed by suture with 2-0 surgical chromic catgut. Surgical wire was used to suture the skin incision, and then each recipient doe was given an intramuscularly injection of 100 mg of long-acting Oxytetracycline (Pfizer Animal Health Inc., New York). The skin sutures were removed 7 days after surgery. Does were monitored daily after surgery and throughout the duration of pregnancy. At parturition, the number and normality of bunnies born were recorded. Bunnies born dead were submitted for gross and microscopic pathological examinations. Bunnies born alive were maintained with their foster mother and weaned on day 35 after birth.

Endpoints

1. Number of bunnies born alive or dead.
2. All bunnies born alive were examined for gross morphology.
3. Dead bunnies were necropsied and assessed for gross and microscopic abnormalities.

Experiment 4. Viability after transfer of rabbit embryos recovered from donor does 92 hours after mating and then transferred to 92-hour synchronized recipient does

In Experiment 3, none of the 1-cell and 2-cell rabbit embryos that were cultured *in vitro* for 96 hours in HL-1 medium produced live offspring after transfer to synchronized recipient does. The objective of Experiment 4 was to assess whether:

1. The system developed for the recovery and culture of 1-cell or 2-cell rabbit embryos to the late morula or blastocyst stages impaired the post-transfer viability of embryos.
2. Rabbit embryos recovered at the stages of morula and blastocyst from donors 92 hours after mating resulted in the birth of live offspring after transfer to synchronized recipients does.
3. The surgical techniques and anesthetic protocols used for embryo transfer did not interfere with embryo implantation.
4. HCG by itself and the time of administration of hCG to recipient does induced proper synchronization of recipient does.

Hypothesis

Morula or blastocyst embryos recovered from 92-hour post-mating donor does will develop to term after transfer to 92-hour synchronized recipient does.

Experimental design

Morulae or blastocysts recovered from each donor doe 92 hours after mating were randomly assigned for transfer to each of 2 synchronized recipient does. As for Experiment 3, not less than 2 and no more than 5 embryos recovered from each of 5 donors were allocated for transfer to 5 pairs of recipients.

Procedures

A total of 5 New Zealand White does, 20 weeks of age or older and weighing a minimum of 3 kg, were randomly selected from 10 does and used as embryo donors. Each doe was caged individually, maintained and cared for as described for Experiment 1. Superovulation was induced by treatment with FSH-P, followed by an injection of hCG at the dose and schedule described for Experiment 1. After the injection of hCG, each doe was left overnight for mating in the cage of a male of known fertility. Embryos at the morula and blastocyst stages were then surgically recovered from anesthetized does 90 to 92 hours after mating. Once the doe was in a surgical plane of anesthesia, the abdominal cavity was opened and the reproductive tract was exposed. To minimize bleeding, donor does were euthanized by inhalation of CO_2 at low pressure (2 to 3 psi) until death ($\leq$ 3 minutes). Immediately after death, the respective ovary, oviduct, and uterine horn from each side was removed and placed in a collecting bowl containing HEPES-buffered saline at a pH of 7.4. Because at the interval of 90 to 92 hours after mating, morulae or blastocysts are located near the junction of the uterine horn of rabbits, then to recover embryos, each uterine horn was cut into two segments. The first segment was composed of the oviduct and the proximal part of uterine horn,

and the second segment was the distal 2/3 of uterine horn. Each segment was then flushed with 3 ml of HEPES buffered saline and with a 2 ml air chase, and the fluids, embryos, and cell debris were collected in a bowl containing a bicarbonate-buffered saline solution, supplemented with 0.3% (w/v) of gelatin type B. The flushings from each uterine horn were maintained in separate dishes. After flushing and recovery, the embryos were examined with an inverted microscope at 100X. The embryos were then handled and processed as described for Experiment 1. Embryos were allocated at random to 1 of 2 groups and each group of embryos was then transferred to a culture well containing 500 μl of a 240 μM solution of eosin B in bicarbonate-buffered saline to determine embryonic viability. Embryonic viability was determined immediately prior to transfer and only viable morulae and blastocysts were transferred to recipient does.

Embryos were surgically transferred to the uterine horns of 10, 92-hour synchronized recipient does, as described for Experiment 3, such that embryos from a given donor were randomly transferred to each of 2 recipient does, and that at least 2 and a maximum of 5 embryos were transferred to each horn of the recipient doe. Similarly, the surgical, post-surgical and post-transfer care was as described for Experiment 3. At parturition, the number and normality of the bunnies born were recorded. Bunnies born dead were submitted for gross and microscopic pathological examinations. Bunnies born alive were maintained with their foster mother and weaned on day 35 after birth.

Endpoints

1. Number of bunnies born alive or dead.
2. All bunnies born alive were examined for gross morphology.
3. Dead bunnies were necropsied and assessed for gross and microscopic abnormalities.

Experiment 5. Viability after transfer of rabbit embryos recovered from donor does 68 hours after mating and then transferred to 92-hour synchronized recipient does

In Experiment 3, none of the 1-cell and 2-cell rabbit embryos that had been cultured *in vitro* for 96 hours in HL-1 medium produced live offspring after transfer to synchronized recipient does. However, in Experiment 4 the pregnancy rate of rabbit embryos recovered from donors 92 hours after mating at the stages of morula and blastocyst resulted in a 60 % pregnancy rate after transfer to synchronized recipient does. Inspection of the data for Experiments 3 and 4 suggested the highest rates of post-transfer survival of embryos were obtained when embryos were transferred at the blastocyst stage. However, in Experiment 2 most of the embryos recovered after *in vitro* culture were morulae. Hence, the objective of Experiment 5 was to assess whether:

1. Rabbit morulae recovered from donors 68 hours after mating, could result in the birth of live offspring when transferred to 92-hour synchronized recipients.

2. The post-transfer survival rates were comparable to those obtained using "older" 92-hour, embryos.

Hypothesis

Early morulae recovered from donor does 68 hours post-mating will develop to term after transfer to 92-hour synchronized recipient does.

Experimental design

Morulae recovered from each donor doe, 68 hours after mating, were randomly transferred to each of 2 synchronized recipient does, and as for the previous Experiments, not less than 2 and no more than 5 morulae were transferred to each uterine horn. For this purpose, embryos from 5 donors were transferred to 10 synchronized recipient does.

Procedures

A total of 5 New Zealand White does, 20 weeks of age or older and weighing a minimum of 3 kg, were randomly selected from 10 does and used as embryo donors. Each doe was caged individually, maintained and cared for as described for Experiment 1. Superovulation was induced by treatment with FSH-P, followed by an injection of hCG at the dose and schedule described for Experiment 1. After the injection of hCG, each doe was left overnight for mating in the cage of a male of known fertility.

Embryos at the morula stage were then surgically recovered from anesthetized does 68 to 70 hours after mating, handled and processed, as described for Experiment 4.

Embryos were allocated at random to 1 of 2 groups and each group of embryos was then transferred to a culture well containing 500 μl of a 240 μM solution of eosin B in bicarbonate-buffered saline to determine embryonic viability. Embryo viability was determined prior to transfer and only viable morulae were transferred to recipient does.

Embryos were surgically transferred to the uterine horns of 10, 92-hour synchronized recipient does, as described for Experiment 3. Similarly, the surgical, post-surgical and post-transfer care was as described for Experiment 3. At parturition, the number and normality of the bunnies born were recorded. Bunnies born dead were submitted for gross and microscopic pathological examinations. Bunnies born alive were maintained with their foster mother and weaned on day 35 after birth.

Endpoints

1. Number of bunnies born alive or dead.
2. All bunnies born alive were examined for gross morphology.
3. Dead bunnies were necropsied and assessed for gross and microscopic abnormalities.

Continuous Exposure of Rabbit Morulae to an Electromagnetic Field of 1.5 G at 60 Hz During *In Vitro* Culture for 24 and 48 Hours and the Survivability of Exposed Embryos after Transfer to Synchronized Recipient Does

Experiment 6. Continuous exposure for 24 hours of rabbit morulae to an electromagnetic field of 1.5 G at 60 Hz during *in vitro* culture

Because of the success in obtaining offspring born after transfer of morula and blastocyst stage embryos recovered from donors at 92 hours after mating in Experiment 4, I decided to initiate my studies on the effects of continuous exposure of rabbit morulae during *in vitro* culture for 24 hours to specific and controlled intensities of electromagnetic fields. To reduce potential variation among recipients due to variation in responsiveness to hCG treatment, I vasectomized a Dutch-belted rabbit (see Appendix A), and all recipient does were exposed immediately after the injection of hCG and allowed to copulate with the vasectomized male. The objective of the study was to assess whether continuous exposure of rabbit morulae to an electromagnetic field of 1.5 G at 60 Hz for 24 hours during *in vitro* culture affects the *in vitro* development and viability of embryos.

Hypotheses

Exposure of rabbit embryos at the morula stage to an electromagnetic field of 1.5 G at 60 Hz during *in vitro* culture for 24 hours will not affect:

1. Embryonic development.

2. Embryonic viability.

Experimental design

Viable embryos at the morula stage recovered from 10 donor does 68 to 70 hours after mating were randomly allocated to control and treated groups such that each group had 5 embryos from the same donor. Control embryos were continuously exposed for 24 hours to an electromagnetic field of $\leq$ 0.005 G and treated embryos were exposed for 24 hours to an electromagnetic field of 1.5 G at 60 Hz. Embryos were evaluated for stage of development at 0 and 24 hours of culture.

Procedures

A total of 10 New Zealand White does, 20 weeks of age or older and weighing a minimum of 3 kg of body weight, were randomly selected from 20 does and used as embryo donors. Each doe was caged individually and cared for as described for Experiment 1. Superovulation was induced by treatment with FSH-P, followed by an injection of hCG at the dose and schedule described for Experiment 1. After injection of hCG, each doe was left overnight for mating in the cage of a male of known fertility. Embryos at the stage of morula were then surgically recovered from anesthetized does 68 to 70 hours after mating, handled and processed, as described for Experiments 4 and 5. Morulae were allocated at random to 1 of 2 groups and each group of embryos was then transferred to a culture dishes, washed 3 times in bicarbonate-buffered saline solution and transferred to culture wells containing 500 µl of a 240 µM solution of

eosin B in bicarbonate-buffered saline to determine embryonic viability. Embryo viability was determined prior to transfer and only viable morulae were used in this Experiment.

Viable morulae were then randomly allocated to a control or a treated group of n = 5 embryos per group. Morulae were placed in individual culture wells containing 50 µl of HL-1 culture medium and overlaid with 40 µl of light paraffin oil, as described for Experiment 2. Control embryos were cultured within the Shielded Culture Chamber (Lamont et al., 1994) and while in this chamber, the maximal exposure during the 24 hours of culture to electromagnetic fields was ≤ 0.005 G at 60 Hz. Treated embryos were cultured within the Electromagnetic Field Exposure Chamber (ISU-EFG) (Lamont et al., 1994) and this device was programmed to continuously generate a 1.5 G magnetic field at 60 Hz during the 24-hour culture period. Morulae were cultured for 24 hours at 37 °C within their respective chambers which had been placed in a CO_2 incubator containing a humidified atmosphere supplemented to contain 5 % CO_2 in air. Morulae were evaluated for stage of development at 0 and 24 hours of culture. At the end of the 24-hour period of culture, control and treated morulae and blastocysts were removed from the respective culture chambers and exposed to eosin B to assess viability. Embryos which had at least one unstained blastomere were considered viable.

Endpoints

1. Stage of development of control and treated embryos at 0 and after 24 hours of culture.
2. Viability of control and treated embryos before culture and again at the end of the 24-hour period of culture.

Statistical analysis Analysis of variance (Steel and Torrie, 1980) was used to evaluate the effects of embryo donor, treatment, time, and interaction between treatments and time, and to compare cleavage indices (Dooley, 1988) of control and treated embryos. The analysis of variance was performed using SAS (Version 6). Allocation of embryos to treatment was performed using a randomized block design, and the statistical analysis of the data was performed using a split-plot model. Chi-square analyses of ratios (Steel and Torrie, 1980) were used to compare developmental stages and viability of cultured embryos. Statistical significance was established at $P \leq 0.05$.

Experiment 7. Transfer of rabbit embryos to synchronized recipients after continuous exposure for 24 hours to an electromagnetic field of 1.5 G at 60 Hz during *in vitro* culture

The objective of this Experiment was to assess whether continuous exposure for 24 hours to an electromagnetic field has delayed effects on the *in vivo* development of these blastocysts after transfer to synchronized recipient does.

Hypothesis

The proportion of rabbit morulae that developed to blastocysts after continuous exposure for 24 hours to an electromagnetic field of 1.5 G at 60 Hz during *in vitro* culture which develop to term after transfer to recipient does is comparable to that of control embryos.

Experimental design

Blastocysts that had developed from control and treated morulae and were viable when exposed to eosin B in Experiment 6, were surgically transferred to synchronized recipient does as described for Experiment 3. For each doe, uterine horns were assigned by random allocation to receive embryos from the control or treated groups from Experiment 6.

Procedures

To synchronize a recipient doe, each doe was induced to ovulate 90 to 92 hours prior to the time of embryo transfer by an intramuscular injection of 25 IU per kg of body weight of hCG, and immediately after injection of hCG, each doe was placed with a vasectomized rabbit (Adams, 1982) and left overnight in his cage. For each doe, the uterine horn to receive embryos from the control or treated groups from Experiment 6 was assigned by random allocation, such that, for each doe, control or treated blastocysts would be transferred alternately to the left or right horn, and not less than 2 and no more than 5 blastocysts from each treatment group were transferred to the lumen of the uterine horn. Similarly, the surgical, post-surgical and post-transfer care was as described for Experiment 3. The skin sutures were removed 7 days after surgery. Does were monitored daily throughout the duration of pregnancy.

Intrauterine fetal marking An exploratory laparotomy (Pollard, 1987 and Pollard and Pineda, 1988) was performed on each recipient doe on day 24 of gestation to determine the number and the source (control or treated embryos) of the feti that

had developed during gestation and to evaluate and record the presence of resorption sites. For this purpose, each doe was fasted at least 6 to 8 hours before surgery and does were anesthetized with an intramuscular injection of 5 mg per kg of body weight of Acepromazine Maleate, followed 10 minutes later with an intramuscular injection of 40 mg per kg of body weight of Ketamine Hydrochloride. The recipient doe was positioned in lateral recumbency on the right side, and the left flank of the rabbit was then shaved, disinfected, and draped. Immediately before the skin was opened, 2 to 3 ml of a solution of 2 % Lidocaine Hydrochloride (Astra Pharmaceutical Products Inc., Worcestor, Massachusetts) was injected subcutaneously at the surgical site located midway between the last rib and the pelvic bone. An incision of approximately 4 to 6 cm, which was perpendicular to the ventral abdominal wall, was made in the left flank to open the abdominal cavity. The left uterine horn was then exteriorized through the incision. The number of developing feti and resorption sites were determined for each uterine horn. Each fetus present in the left uterine horn, whether control or treated, was gently held by grasping the uterine horn and palpated to locate the fetal head or tail. To mark each fetus, a Tuberculin syringe with No. 26 needle containing 0.5 ml of India ink (Pelikan Inc., Hannover 1, Germany R.F.A.) was inserted through the uterine wall and into the fetal skin. Each fetus was then marked in the rump or the back regions with a subcutaneous drop of India ink to identify the source of feti and their respective treatment group at the time of birth. The left horn was selected because this horn is easier to exteriorize in rabbits, minimizing stress to the feti and mother. After marking each of the feti in the left horn, the muscle layers of the incision were closed by suture with 2-0 surgical chromic catgut. Surgical wire suture was used

to close the skin incision, and the recipient doe was given 100 mg of long-acting Oxytetracycline in an intramuscular injection. The skin sutures were removed 7 days after surgery. At parturition, the number and normality of the bunnies born alive or dead, source and treatment of the bunnies were recorded. Bunnies born dead were submitted for gross and microscopic pathological examinations. Bunnies born alive were maintained with their foster mothers and weaned on day 35 after birth.

Endpoints

1. Number of fetal resorption sites and number of feti in the uterine horns of does from the control and treated groups at laparotomy on day 24 of gestation.
2. Number of bunnies born alive or dead from the control and treated groups.
3. All control and treated bunnies born alive were examined for gross morphology.
4. All control and treated bunnies born dead were necropsied and assessed for gross and microscopic abnormalities.

Statistical analysis Chi-square analyses of ratios (Steel and Torrie, 1980) were used to compare number of fetal resorption sites and number of feti formed at laparotomy on day 24 of gestation, and number of bunnies born alive or dead in control and treated groups. Statistical significance was established at $P \leq 0.05$.

Experiment 8. Continuous exposure for 48 hours of rabbit morulae to an electromagnetic field of 1.5 G at 60 Hz during *in vitro* culture

The objective of the study was to assess whether continuous exposure of rabbit morulae to an electromagnetic field of 1.5 G at 60 Hz for 48 hours during *in vitro* culture affects the *in vitro* development and viability of embryos.

Hypotheses

Exposure of rabbit embryos at the morula stage to an electromagnetic field of 1.5 G at 60 Hz during *in vitro* culture for 48 hours will not affect:

1. Embryonic development.
2. Embryonic viability.

Experimental design

Viable embryos at the morula stage recovered from 10 donors does at 68 to 70 hours after mating were randomly allocated to control and treated groups such that each group had 5 embryos from the same donor. Control morulae were continuously exposed for 48 hours to an electromagnetic field of $\leq$ 0.005 G and treated embryos were exposed for 48 hours to an electromagnetic field of 1.5 G at 60 Hz. Embryos were evaluated for stage of development at 0, 24, and 48 hours of culture.

Procedures

A total of 10 New Zealand White does, 20 weeks of age or older and weighing a minimum of 3 kg of body weight, were randomly selected from 20 does and used as embryo donors. Each doe was caged individually, fed commercial rabbit feed, and maintained as described for Experiment 6. Superovulation was induced by treatment with FSH-P, followed by an injection of hCG as described for Experiment 1. After injection of hCG, each doe was left overnight for mating in the cage of a male of known fertility.

Embryos at the stage of morula were then surgically recovered from anesthetized does at 68 to 70 hours after mating, handled, processed, maintained, and randomly allocated to control and treated groups, as described for Experiment 6.

In this Experiment, as for Experiment 6, viable morulae were randomly allocated to a control or a treated group and placed in individual culture wells containing 50 µl of HL-1 culture medium. Each well containing medium was overlaid with 40 µl of light paraffin oil. Control embryos were placed within the Shielded Culture Chamber (Lamont et al., 1994), and while in this chamber, the maximal exposure during the 48 hours of culture to electromagnetic fields was $\leq$ 0.005 G at 60 Hz. Treated embryos were cultured within the Electromagnetic Field Exposure Chamber (ISU-EFG) (Lamont et al., 1994) and this device was programmed to continuously generate a 1.5 G magnetic field at 60 Hz during the 48-hour culture period. Embryos were cultured for 48 hours at 37 $^{\circ}$C in their respective chambers as described for Experiment 6. Embryos were evaluated for stage of development at 0, 24, and 48 hours of *in vitro* culture. At the end of the 48-hour period of culture, control and treated embryos were removed from the respective

culture chambers and exposed to eosin B assay to assess viability. Embryos which had at least one unstained blastomere were considered viable.

Endpoints

1. Stage of development of control and treated embryos at 0, 24, and 48 hours culture.
2. Maximal development and cleavage indices of control and treated embryos during the 48-hour period of culture.
3. Viability of control and treated embryos before culture and again at the end of the 48-hour period of culture.

Statistical analysis Analysis of variance (Steel and Torrie, 1980) was used to evaluate the effects of embryo donor, treatment, time, and interaction between treatments and time, and to compare cleavage indices (Dooley, 1988) of control and treated embryos. The analysis of variance was performed using SAS (Version 6). Allocation of embryos to treatment was performed using a randomized block design, and the statistical analysis of the data was performed using a split-plot model. Chi-square analyses of ratios (Steel and Torrie, 1980) were used to compare developmental stages and viability of control and treated embryos. Statistical significance was established at $P \leq 0.05$.

Experiment 9. Transfer of rabbit embryos to synchronized recipients after continuous exposure for 48 hours to an electromagnetic field of 1.5 G at 60 Hz during *in vitro* culture

The objective of this Experiment was to assess whether continuous exposure for 48 hours to an electromagnetic field has delayed effects on the *in vivo* development of these blastocysts after transfer to synchronized recipient does.

Hypothesis

The proportion of rabbit morulae that developed to blastocysts after continuous exposure for 48 hours to an electromagnetic field of 1.5 G at 60 Hz during *in vitro* culture which develop to term after transfer to recipient does is comparable to that of control embryos.

Experimental design

Blastocysts that had developed from control and treated morulae and were viable when exposed to eosin B in Experiment 8, were surgically transferred to synchronized recipient does as described for Experiment 7. For each doe, uterine horns were assigned by random allocation to receive embryos from the control or treated groups from Experiment 8.

Procedures

Blastocysts that had developed from control and treated morulae and were viable when exposed to eosin B in Experiment 8, were surgically transferred to synchronized recipient does as described for Experiment 7. For each doe, the uterine horn to receive embryos from the control or treated groups from Experiment 8 were assigned by random allocation, such that for each doe, control or treated blastocysts would be transferred alternately to the left or right horn.

An exploratory laparotomy was then performed on recipient does on day 24 of gestation to determine the number and the source (control or treated embryos) of the feti that had developed during gestation and to evaluate and record the presence of resorption sites, as described for Experiment 7. At parturition, the number and normality of the bunnies born alive or dead, source and treatment of the bunnies were recorded. Bunnies born dead were submitted for gross and microscopic pathological examinations. Bunnies born alive were maintained with their foster mothers and weaned on day 35 after birth.

Endpoints

1. Number of fetal resorption sites and number of feti in the uterine horns of does from control and treated groups at laparotomy on day 24 of gestation.
2. Number of bunnies born alive or dead from the control and treated groups.

3. All control and treated bunnies born alive were examined for gross morphology.
4. All control and treated bunnies born dead were necropsied and assessed for gross and microscopic abnormalities.

Statistical analysis Chi-square analyses of ratios (Steel and Torrie, 1980) was used to compare number of fetal resorption sites and number of feti formed at laparotomy on day 24 of gestation and the number of bunnies born alive or dead in the control and treated groups. Statistical significance was established at $P \leq 0.05$.

RESULTS

Preliminary Studies

Experiment 1. Effect of synthetic culture media on the *in vitro* development and viability of 1-cell and 2-cell rabbit embryos

A total of 204, 1-cell and 2-cell embryos (from data presented in Table 3) recovered from 7 donors were used to compare the effect of synthetic culture media on the development of rabbit embryos during *in vitro* culture for 96 hours. The development of rabbit embryos (Figure 1) in each of the 4 synthetic culture media is shown in Table 3 and the Chi-square analyses used to determine the effects of the synthetic culture media on the development of the 1-cell and 2-cell rabbit embryos to morulae or blastocysts are presented in Tables 4 and 5. The *in vitro* development of 1-cell and 2-cell stage rabbit embryos during a 96-hour period of culture was significantly ($P < 0.0005$, Table 5) affected by the medium used for embryo culture.

The number of morulae (Tables 3 and 4) resulting from the *in vitro* culture of 1-cell and 2-cell embryos for 96 hours in RD medium (28/52) was higher than for the DMEM/F-12 medium (13/53, $P < 0.005$) and for WBSA medium (10/51, $P < 0.001$). RD medium produced more morulae ($P < 0.05$) than for the HL-1 medium (15/48). This was interpreted to be the consequence of a delayed cleavage of embryos to the blastocyst stage in the RD medium. This interpretation is supported by the greater number of embryos which had developed into morulae in HL-1 medium and which

Table 3. Development of rabbit embryos during *in vitro* culture for 96 hours in 4 synthetic culture media (Experiment 1)

		Stage of development					
		Did not cleave		Cleaved at least twice		Morula	Blastocyst
Treatment	Number of embryos	No.	(%)	No.	(%)	No. (%)	No. (%)
HL-1	48	1	(2)	6	(13)	15 (31)	26 (54)
DMEM/F-12	53	1	(2)	38	(71)	13 (25)	1 (2)
WBSA	51	3	(5)	37	(73)	10 (20)	1 (2)
RD	52	0	(0)	17	(32)	28 (54)	7 (14)

Did not cleave = Number of embryos in which no further development was obtained within the 96-hour period of *in vitro* culture.

Cleaved at least twice = Number of embryos which completed at least 2 cell cycles within the 96-hour period of *in vitro* culture but did not reach either the morula or blastocyst stages.

Morula = Multicellular embryo containing > 16 blastomeres, without a blastocoel. At this stage the extent of cellular associations formed between blastomeres results in greater membrane continuity between cells such that identification and counting of individual blastomeres is no longer possible during morphological assessment.

Blastocyst = Number of embryos which developed to the blastocyst stage (formed a clearly defined blastocoel) within the 96-hour period of *in vitro* culture.

HL-1 = HL-1 Medium supplemented with 2.5 mM glutamine.

DMEM/F-12 = 1:1 (v/v) of Dubecco's Modified Eagle's Medium and Ham's F-12 supplemented with 2.5 mM glutamine.

WBSA = Whitten's Medium (Pollard, 1987); supplemented with 1.5% crystallized bovine serum albumin.

RD Medium = 1:1 (v/v) of RPMI-1640 and Dulbecco's Modified Eagle's Medium, containing 4500 mg/L glucose (Carney and Foote, 1991).

Figure 1. Rabbit embryos at different stages of development. Pictures taken during *in vitro* culture (220 X).

A = Fertilized 1-cell embryo with pronuclei
B = 2-cell embryo
C = 3-cell embryo
D = 4-cell embryo
E = 5- to 7-cell embryo
F = 8-cell embryo
G = 17- to 32-cell embryo
H = Early morula
I = Late morula
J = Early blastocyst
K = Blastocyst
L = Hatching blastocyst

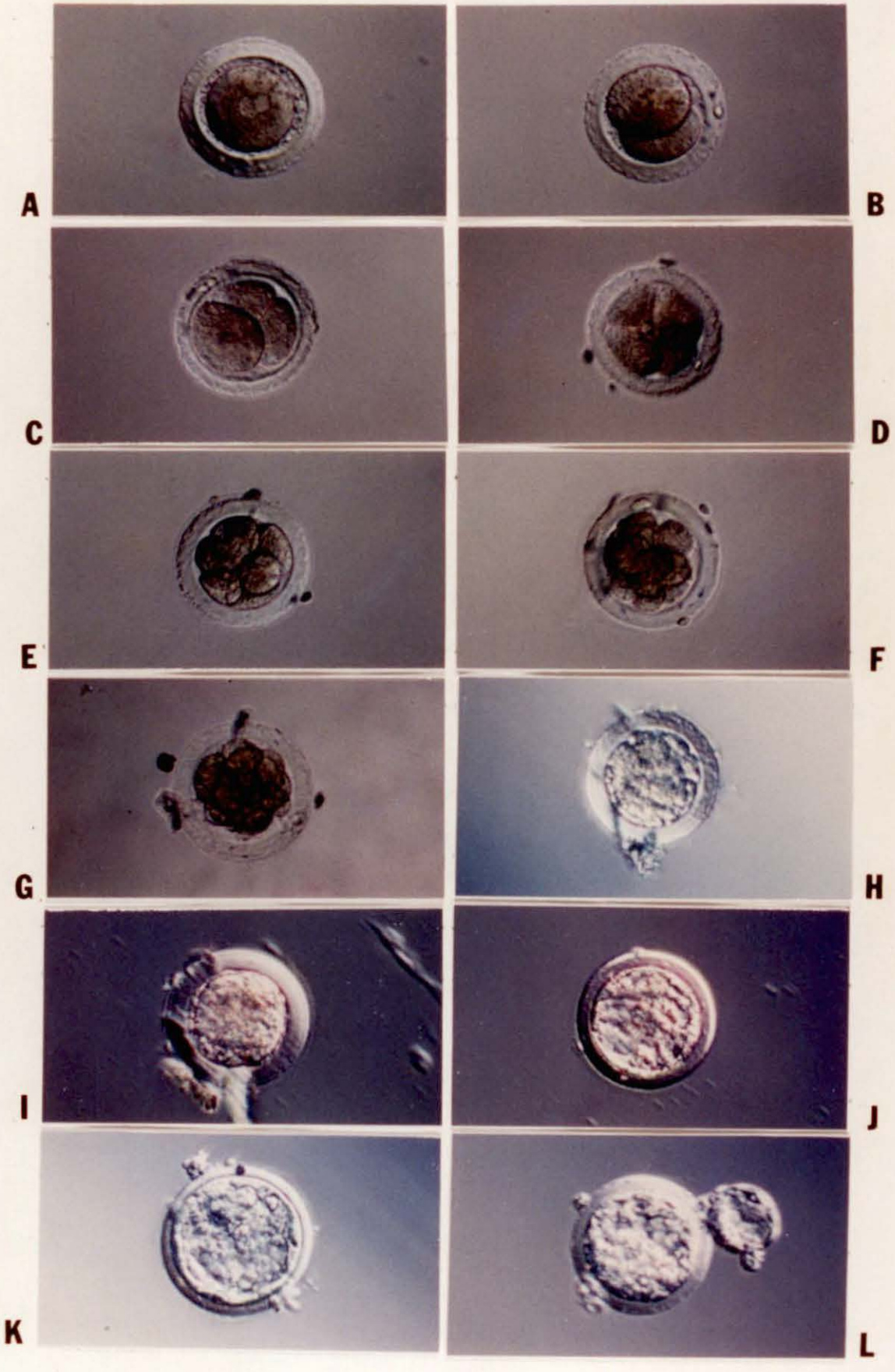

A
B
C
D
E
F
G
H
I
J
K
L

Table 4. Chi-square analyses to determine the effects of synthetic culture media on the development of 1-cell and 2-cell rabbit embryos to morula stage during *in vitro* culture for 96 hours, emphasizing the ratio formed by the number of morulae in the numerator and the total number of embryos in the denominator (Experiment 1)

Treatment	Ratio	X^2	df	P
Four culture media*	15/48 vs 13/53 vs 10/51 vs 28/52	16.27	3	< 0.001
HL-1 and DMEM/F-12	15/48 vs 13/53	0.57	1	> 0.5
HL-1 and WBSA	15/48 vs 10/51	1.78	1	> 0.25
HL-1 and RD	15/48 vs 28/52	5.20	1	< 0.05
DMEM/F-12 and WBSA	13/53 vs 10/51	0.37	1	> 0.5
DMEM/F-12 and RD	13/53 vs 28/52	9.48	1	< 0.005
WBSA and RD	10/51 vs 28/52	12.96	1	< 0.001

*The 4 culture media were as follows:

HL-1 = HL-1 Medium supplemented with 2.5 mM glutamine.

DMEM/F-12 = 1:1 (v/v) of Dubecco's Modified Eagle's Medium and Ham's F-12 supplemented with 2.5 mM glutamine.

WBSA = Whitten's Medium (Pollard, 1987); supplemented with 1.5% crystallized bovine serum albumin.

RD Medium = 1:1 (v/v) of RPMI-1640 and Dulbecco's Modified Eagle's Medium, containing 4500 mg/L glucose (Carney and Foote, 1991).

df = degrees of freedom.

P = Probability level.

Table 5. Chi-square analyses to determine the effects of synthetic culture media on the development of 1-cell and 2-cell rabbit embryos to blastocyst stage during *in vitro* culture for 96 hours, emphasizing the ratio formed by the number of blastocysts in the numerator and the total number of embryos in the denominator (Experiment 1)

Treatment	Ratio	X^2	df	P
Four culture media*	26/48 vs 1/53 vs 1/51 vs 7/52	63.74	3	< 0.0005
HL-1 and DMEM/F-12	26/48 vs 1/53	35.15	1	< 0.0005
HL-1 and WBSA	26/48 vs 1/51	33.98	1	< 0.0005
HL-1 and RD	26/48 vs 7/52	18.71	1	< 0.0005
DMEM/F-12 and WBSA	1/53 vs 1/51	0.01	1	> 0.5
DMEM/F-12 and RD	1/53 vs 7/52	5.00	1	< 0.05
WBSA and RD	1/51 vs 7/52	4.75	1	< 0.05

*Four culture media are as follows:

HL-1 = HL-1 Medium supplemented with 2.5 mM glutamine.

DMEM/F-12 = 1:1 (v/v) of Dubecco's Modified Eagle's Medium and Ham's F-12 supplemented with 2.5 mM glutamine.

WBSA = Whitten's Medium (Pollard, 1987); supplemented with 1.5% crystallized bovine serum albumin.

RD Medium = 1:1 (v/v) of RPMI-1640 and Dulbecco's Modified Eagle's Medium, containing 4500 mg/L glucose (Carney and Foote, 1991).

df = degrees of freedom.

P = Probability level.

completed their development to the blastocyst stage during the period of *in vitro* culture. The number of morulae that resulted from the culture of 1-cell and 2-cell rabbit embryos in DMEM/F-12 medium (13/53), WBSA medium (10/51, $P > 0.5$, Table 4) or HL-1 medium (15/48) were not different ($P > 0.25$, Table 4).

The number of blastocysts resulting from the *in vitro* culture of 1- cell and 2-cell embryos for 96 hours in HL-1 medium (26/48) was significantly higher ($P < 0.0005$, Table 5) than for the RD medium (7/52), DMEM/F-12 medium (1/53) or for WBSA medium (1/51). Excluding the embryos cultured in the HL-1 medium, RD medium produced more blastocysts than either DMEM/F-12 or WBSA and the number of blastocysts that resulted from the culture of 1-cell and 2-cell rabbit embryos in DMEM/F12 (1/53) and WBSA medium (1/51) were not different ($P > 0.5$, Table 5).

Overall, the combined number of embryos reaching the transferable stages of morula and blastocyst (from data presented in Table 3) from the culture of 1-cell and 2-cell embryos for 96 hours in HL-1 medium (41/48) was significantly higher ($P < 0.0005$, Chi-square analyses not shown in Tables) than for the number of morulae and blastocysts from the culture of 1-cell and 2-cell embryos for 96 Hours in DMEM/F-12 (14/53) and WBSA (11/51) media. Culture of 1-cell and 2-cell embryos in HL-1 medium also resulted in more morulae and blastocysts (41/48, $P < 0.05$, Chi-square analyses not shown in Tables) than embryos (35/52) cultured in RD medium. The combined number of 1-cell and 2-cell embryos to reach the stages of morulae and blastocysts when cultured for 96 hours in DMEM/F-12 (14/53) and in WBSA (11/51) were not different ($P > 0.5$, Chi-square analyses not shown in tables).

The differential rate of development of embryos cultured in the 4 synthetic media is reflected in their cleavage and peak indices. Analysis of variance (Table 6) of cleavage indices revealed a significant effect for treatment ($P < 0.005$), time ($P < 0.0001$) and the interaction treatment by time was also significant ($P < 0.0001$). The source of embryos, the doe, was not a factor ($P > 0.1$) affecting the cleavage rate of embryos, suggesting that the differential cleavage indices among culture media were due to the medium used and not to the source of embryos. I did not statistically analyze cleavage indices as affected by time for each medium used in the Experiment; but I concentrated in the analysis of the peak index, applying Tukey's test (Steel and Torrie, 1980) to detect difference among means. The peak index was higher ($P < 0.05$) for embryos cultured in HL-1 medium (10.5 ± 2.6, Table 7) than for embryos cultured in DMEM/F-12 (7.6 ± 1.9), WBSA (7.2 ± 2.1), and RD (8.7 ± 1.8) media.

All 1-cell and 2-cell embryos were viable before culture. The viability of embryos at the end of the 96-hour period of culture is presented in Table 8, and the Chi-square analyses for embryonic viability is shown in Table 9. At the end of 96-hour period of culture, 94 % (45/48), 79 % (42/53), 90 % (46/51), and 98 % (51/52) of the embryos were viable in HL-1, DMEM/F-12, WBSA, and RD media, respectively.

The viability of rabbit embryos during the 96-hour period of *in vitro* culture was affected by the composition of the culture medium. Most of the variation was due to the lower survival rate for embryos cultured in DMEM/F-12 medium. The number of dead embryos was lower for embryos cultured in RD medium (1/52, $P < 0.005$,

Table 6. Analysis of variance of cleavage and peak indices of rabbit embryos during *in vitro* culture in synthetic culture media (Experiment 1)

Source	df	SS	MS	F	P
Doe	6	348.43	58.07	3.36	> 0.1
Treatment	3	592.77	197.59	11.44	< 0.005
Treatment × Doe *(Error a)	18	310.80	17.26	-	-
Time	4	5494.88	1373.72	317.32	< 0.0001
Treatment ×Time	12	578.40	48.20	11.13	< 0.0001
*(Error b)	976	4225.29	4.32	-	-

Allocation of embryos to treatment was performed in a randomized block design and statistical analysis of the data was performed using a split-plot model.

Treatment = Effect due to the 4 synthetic culture media.

*The general error term was partitioned, to obtain the **Error a** term to test for the effects of treatment and doe, and the **Error b** term to test the effects of time and the interaction, treatment by time.

Table 7. Cleavage and peak indices of rabbit embryos at the time of recovery and during culture in synthetic culture media for 96 hours (Experiment 1)

Treatment	Number of embryos	Cleavage index					Peak index*
		0 h	24 h	48 h	72 h	96 h	
HL-1	48	1.5 (0.5)	5.3 (0.8)	7.6 (1.6)	8.9 (2.3)	10.3 (3.2)	10.5[a] (2.6)
DMEM/F-12	53	1.3 (0.5)	5.2 (1.2)	6.5 (2.0)	7.1 (2.4)	5.3 (4.2)	7.6[b] (1.9)
WBSA	51	1.2 (0.4)	4.7 (1.4)	6.4 (2.1)	6.4 (2.9)	5.1 (4.1)	7.2[b] (2.1)
RD	52	1.2 (0.4)	5.1 (0.9)	7.3 (1.6)	8.1 (2.4)	8.2 (3.0)	8.7[c] (1.8)

Data are presented as Mean (± SD).

h = Hour of culture.

HL-1 = HL-1 Medium supplemented with 2.5 mM glutamine.

DMEM/F-12 = 1:1 (v/v) of Dubecco's Modified Eagle's Medium and Ham's F-12 supplemented with 2.5 mM glutamine.

WBSA = Whitten's Medium (Pollard, 1987); supplemented with 1.5% crystallized bovine serum albumin.

RD Medium = 1:1 (v/v) of RPMI-1640 and Dulbecco's Modified Eagle's Medium, containing 4500 mg/L glucose (Carney and Foote, 1991).

*Peak index = The maximal cleavage index that was recorded for the embryo during the 96-hour period of culture.

For peak index, means which have a common superscript letter are not different ($P > 0.05$).

Table 8. Viability of rabbit embryos at the end of 96-hour period of culture in 4 synthetic culture media (Experiment 1)

Treatment	Number of embryos	Viable				Nonviable	
		Unstained		Partially stained		Completely stained	
		No.	(%)	No.	(%)	No.	(%)
HL-1	48	0	(0)	45	(94)	3	(6)
DMEM/F-12	53	0	(0)	42	(79)	11	(21)
WBSA	51	2	(4)	44	(86)	5	(10)
RD	52	1	(2)	50	(96)	1	(2)

Unstained = Number of embryos for which all of the blastomeres were viable at the end of the 96-hour period of culture.

Partially stained = Number of viable embryos in which one or more blastomeres had died during the 96-hour period of culture.

Completely stained = Number of embryos which had died during the 96-hour period of culture.

HL-1 = HL-1 Medium supplemented with 2.5 mM glutamine.

DMEM/F-12 = 1:1 (v/v) of Dubecco's Modified Eagle's Medium and Ham's F-12 supplemented with 2.5 mM glutamine.

WBSA = Whitten's Medium (Pollard, 1987); supplemented with 1.5% crystallized bovine serum albumin.

RD Medium = 1:1 (v/v) of RPMI-1640 and Dulbecco's Modified Eagle's Medium, containing 4500 mg/L glucose (Carney and Foote, 1991).

Table 9. Chi-square analyses to determine the effects of synthetic culture media on embryonic death of rabbit embryos during *in vitro* culture for 96 hours, emphasizing the ratio formed by the number of dead embryos in the numerator and the total number of embryos in the denominator (Experiment 1)

Treatment	Ratio	X^2	df	P
Culture media*	3/48 vs 11/53 vs 5/51 vs 1/52	11.53	3	< 0.01
HL-1 and DMEM/F-12	3/48 vs 11/53	4.44	1	< 0.05
HL-1 and WBSA	3/48 vs 5/51	0.42	1	> 0.5
HL-1 and RD	3/48 vs 1/52	1.21	1	> 0.25
DMEM/F-12 and WBSA	11/53 vs 5/51	2.39	1	> 0.1
DMEM/F-12 and RD	11/53 vs 1/52	9.20	1	< 0.005
WBSA and RD	5/51 vs 1/52	2.91	1	< 0.1

*The 4 culture media were as follows:

HL-1 = HL-1 Medium supplemented with 2.5 mM glutamine.

DMEM/F-12 = 1:1 (v/v) of Dubecco's Modified Eagle's Medium and Ham's F-12 supplemented with 2.5 mM glutamine.

WBSA = Whitten's Medium (Pollard, 1987); supplemented with 1.5% crystallized bovine serum albumin.

RD Medium = 1:1 (v/v) of RPMI-1640 and Dulbecco's Modified Eagle's Medium, containing 4500 mg/L glucose (Carney and Foote, 1991).

df = degrees of freedom.

P = Probability level.

Table 9) and in HL-1 medium (3/48, $P < 0.05$) than for embryos cultured in DMEM/F-12 medium (11/53) and tended ($P < 0.1$) to be less than for embryos cultured RD medium (1/52), as compared to WBSA medium (5/51).

Experiment 2. *In vitro* culture of rabbit embryos in defined medium (HL-1) within the Shielded Culture Cell or within the Electromagnetic Field Exposure System (ISU-EFG)

A total of 99, 1-cell and 2-cell embryos recovered from 5 donors were used to compare the *in vitro* development of embryos for 96 hours in HL-1 medium within the Shielded chamber or the Electromagnetic Field Exposure system, called the Unshielded chamber (Table 10) for short. A total of 50 embryos were cultured within the Shielded chamber and 49 embryos were cultured within the Unshielded chamber. The maximal development of rabbit embryos during *in vitro* culture for 96 hours in HL-1 medium within the Shielded chamber or Unshielded (ISU-EFG) chamber is shown in Table 10.

The *in vitro* development of 1-cell and 2-cell stage rabbit embryos during a 96-hour period of culture was not affected ($P > 0.1$) by the chamber used to hold the embryos during *in vitro* culture. Chi-square analyses of ratios (Table 11) for the proportion of rabbit embryos to reach the blastocyst stage during culture revealed that there were comparable numbers ($P > 0.25$, Table 11) of embryos to develop to the blastocyst stage in the Shielded (13/50) and Unshielded (9/49) chambers. However, Chi-square analysis of ratios for the proportion of rabbit embryos to reach the morula stage during

Table 10. Development of rabbit embryos during *in vitro* culture for 96 hours in HL-1 medium within the Shielded chamber (Shielded Culture Cell) or within the Unshielded (ISU-EFG) chamber (Experiment 2)

Treatment	Number of embryos	Stage of development					
		Did not cleave		Cleaved at least twice		Morula		Blastocyst	
		No.	(%)	No.	(%)	No.	(%)	No.	(%)
Shielded chamber	50	0	(0)	19	(38)	18	(36)	13	(26)
Unshielded (ISU-EFG) chamber	49	1	(2)	11	(23)	28	(57)	9	(18)

Did not cleave = Number of embryos in which no further development was obtained within the 96-hour period of *in vitro* culture.

Cleaved at least twice = Number of embryos which completed at least 2 cell cycles within the 96-hour period of *in vitro* culture but did not reach either the morula or blastocyst stages.

Morula = Multicellular embryo containing > 16 blastomeres, without a blastocoel. At this stage the extent of cellular associations formed between blastomeres results in greater membrane continuity between cells such that identification and counting of individual blastomeres is no longer possible during morphological assessment.

Blastocyst = Number of embryos which developed to the blastocyst stage (formed a clearly defined blastocoel) within the 96-hour period of *in vitro* culture.

Embryos were cultured in HL-1 = HL-1 medium supplemented with 2.5 mM glutamine.

Both the Shielded Culture Cell and the Unshielded (ISU-EFG) chamber were placed on the same shelf (Q) of the incubator. The background electromagnetic field in the Shielded chamber was ≤ 0.005 G, and the background electromagnetic field in the Unshielded chamber was estimated to range from 0.02 to 0.06 G.

Table 11. Chi-square analyses to compare the development of rabbit embryos during *in vitro* culture for 96 hours in HL-1 medium within the Shielded chamber (Shielded Culture Cell) or within the Unshielded (ISU-EFG) chamber, emphasizing the ratio formed by the number of morulae and/or blastocysts in the numerator and the total number of embryos in the denominator (Experiment 2)

Development	Ratio	X^2	df	P
Morula	Shielded, 18/50 vs Unshielded, 28/49	4.45	1	< 0.05
Blastocyst	Shielded, 13/50 vs Unshielded, 9/49	0.83	1	> 0.25
Morula and blastocyst	Shielded, 31/50 vs Unshielded, 37/49	2.10	1	> 0.25

df = degrees of freedom.

P = Probability level.

culture indicated that there were fewer ($P < 0.05$) morulae in the Shielded chamber (18/50) in which the embryos were protected from exposure to electromagnetic fields ≥ 0.005 G at 60 Hz during the 96-hour period of culture, as compared to the Unshielded (28/49) chamber in which the embryos were exposed to electromagnetic fields ranging from 0.02 to 0.06 G at 60 Hz.

Analysis of variance of cleavage indices indicated a significant ($P < 0.0001$, Table 12) effect of time, but the effects of doe, treatment, and treatment by time interaction were not significant ($P > 0.5$, Table 12). This finding further corroborates that the development during culture of 1-cell and 2-cell rabbit embryos for 96 hours in

Table 12. Analysis of variance of cleavage indices of rabbit embryos during *in vitro* culture in HL-1 medium within the Shielded chamber (Shielded Culture Cell) or within the Unshielded (ISU-EFG) chamber (Experiment 2)

Source	df	SS	MS	F	P
Doe	4	22.21	5.55	0.57	> 0.5
Treatment	1	0.07	0.07	0.007	> 0.5
Doe × Treatment *(Error a)	4	38.50	9.62	-	-
Time	5	4628.01	925.60	370.88	< 0.0001
Treatment × Time	5	4.45	0.89	0.04	> 0.5
*(Error b)	574	1432.53	2.49	-	-

Allocation of embryos to treatment was performed in a randomized block design and statistical analysis of the data was performed using a split-plot model.

Treatment = Effects due to culture of embryos in the Shielded Culture Cell or the ISU-EFG.

*The general error term was partitioned, to obtain the **Error a** term to test for the effects of treatment and doe, and the **Error b** term to test the effects of time and the interaction, treatment by time.

Table 13. Cleavage and peak indices of rabbit embryos at the time of recovery and during *in vitro* culture in HL-1 medium within the Shielded chamber (Shielded Culture Cell) or within the Unshielded (ISU-EFG) chamber for 96 hours (Experiment 2)

Treatment	Number of embryos	Cleavage index					Peak index*
		0 h	24 h	48 h	72 h	96 h	
Shielded chamber	50	1.3 (0.5)	6.1 (1.0)	8.0 (1.2)	8.3 (2.4)	8.9 (2.8)	8.9 (2.8)
Unshielded (ISU-EFG) chamber	49	1.3 (0.5)	6.6 (0.9)	7.9 (1.4)	8.5 (1.9)	9.1 (2.3)	9.1 (2.3)

Data are presented as mean (± SD).

h = Hour of culture.

Shielded chamber = Embryos were exposed to a background electromagnetic field of ≤ 0.005 G at 60 Hz.

Unshielded chamber = Embryos were exposed to background electromagnetic fields estimated to range from 0.02 to 0.06 G at 60 Hz.

HL-1 = HL-1 medium supplemented with 2.5 mM glutamine.

*Peak index = The maximal cleavage index that was recorded for the embryo during the 96-hour period of culture.

Table 14. Viability of rabbit embryos at the end of 96-hour period of *in vitro* culture in HL-1 medium within the Shielded chamber (Shielded Culture Cell) or within the Unshielded (ISU-EFG) chamber (Experiment 2)

Treatment	Number of embryos	Viable				Nonviable	
		Unstained		Partially stained		Completely stained	
		No.	(%)	No.	(%)	No.	(%)
Shielded chamber	50	0	(0)	48	(96)	2	(4)
Unshielded (ISU-EFG) chamber	49	2	(4)	46	(94)	1	(2)

Unstained = Number of embryos for which all of the blastomeres were viable at the end of the 96-hour period of culture.

Partially stained = Number of viable embryos in which one or more blastomeres had died during the 96-hour period of culture.

Completely stained = Number of embryos which had died during the 96-hour period of culture.

Shielded chamber = Embryos were exposed to an electromagnetic field of ≤ 0.005 G at 60 Hz.

Unshielded chamber = Embryos were exposed to background electromagnetic fields estimated to range from 0.02 to 0.06 G at 60 Hz.

Table 15. Chi-square analysis to determine the viability of rabbit embryos during **in vitro** culture for 96 hours in HL-1 medium within the Shielded chamber (Shielded Culture Cell) or within the Unshielded (ISU-EFG) chamber, emphasizing the ratio formed by the number of viable embryos in the numerator and the total number of embryos in the denominator (Experiment 2)

Viability	Ratio	X^2	df	P
Viable embryo	Shielded, 48/50 vs Unshielded, 48/49	0.32	1	> 0.5

df = degree of freedom.

P = Probability level.

HL-1 medium was not affected by the chamber, but as expected, by time of culture. Peak cleavage index was not affected by treatment ($P > 0.5$, Table 12, Table 13), therefore Tukey's test was not applied.

The viability of embryos at the end of the 96-hour period of culture (Table 14) was not affected ($P > 0.5$, Table 15) by the chambers used to culture embryos.

Experiment 3. Viability after transfer of rabbit embryos cultured *in vitro* in HL-1 medium within the Shielded Culture Cell or within the Electromagnetic Field Exposure System (ISU-EFG) to 92-hour synchronized recipient does

A total of 82 embryos, 59 morulae and 23 blastocysts, obtained after culture *in vitro* for 96 hours in HL-1 medium within the Shielded or Unshielded chambers from Experiment 2 were transferred to 10 synchronized recipient does. The numbers of embryos transferred are shown in Table 16.

None of the 1-cell and 2-cell rabbit embryos that were cultured *in vitro* for 96 hours in HL-1 medium and had reached the stage of morulae or blastocysts, produced live offspring after transfer to synchronized recipient does.

Experiment 4. Viability after transfer of rabbit embryos recovered from donor does 92 hours after mating and then transferred to 92-hour synchronized recipient does

The number of embryos recovered at the stages of morula or blastocyst from donor does 92 hours after mating and then transferred to recipient does, and the number of bunnies born from transfer of these embryos are shown in Table 17.

A total of 10 morulae and 59 blastocysts were recovered from 5 donor does and prepared for transfer. The transfer of 4 to 10 embryos (2 to 5 per horn) resulted in 6

Table 16. Viability after transfer of rabbit embryos cultured *in vitro* for 96 hours in HL-1 medium to 92-hour synchronized recipient does (Experiment 3)

Donor	Recipient	Number of embryos (stage)[a] transferred	Number of offspring born
1	1	7 (M), 1 (B)	0
	2	9 (M), 1 (B)	0
2	3	5 (B)	0
	4	9 (M)	0
3	5	9 (M), 1 (B)	0
	6	10 (M)	0
4	7	1 (M), 7 (B)	0
	8	2 (M), 5 (B)	0
5	9	6 (M), 3 (B)	0
	10	6 (M)	0
Total		59 (M), 23 (B)	0

[a]Stage: (M), morula stage; (B), blastocyst stage.

Table 17. Viability after transfer of rabbit embryos recovered from donor does 92 hours after mating to 92-hour synchronized recipient does (Experiment 4)

Donor	Recipient	Number of embryos (stage)[a] transferred	Number of offspring born
1	1	2 (M), 4 (B)	2
	2	1 (M), 3 (B)	0
2	3	9 (B)	9
	4	10 (B)	0
3	5	4 (M), 2 (B)	0
	6	2 (M), 3 (B)	3
4	7	8 (B)	5
	8	10 (B)	2
5	9	1 (M), 5 (B)	1
	10	5 (B)	0
Total		10 (M), 59 (B)	22

[a]Stage: (M), morula stage; (B), blastocyst stage.

pregnant does (60 % pregnancy rate, Table 17) and in the birth of 22 bunnies from the 69 embryos transferred, a 32 % embryo survival rate (22/69).

Experiment 5. Viability after transfer of rabbit embryos recovered from donor does 68 hours after mating and then transferred to 92-hour synchronized recipient does

The number of morulae recovered from donor does 68 hours after mating and transferred to 92-hour synchronized recipient does, and the number of offspring born are shown in Table 18.

A total of 92 morulae were recovered from 5 donors and prepared for transfer. The transfer of 7 to 10 morulae (3, 4, or 5 per horn) resulted in 3 pregnant does (30 % pregnancy rate, Table 18), and in the birth of 5 bunnies from 92 morulae transferred, a 5 % embryo survival rate (5/92).

Table 18. Viability after transfer of rabbit embryos recovered from donor does 68 hours after mating to 92-hour synchronized recipient does (Experiment 5)

Donor	Recipient	Number of embryos (stage)[a] transferred	Number of offspring born
1	1	10 (M)	1
	2	9 (M)	0
2	3	8 (M)	0
	4	7 (M)	3
3	5	10 (M)	0
	6	8 (M)	0
4	7	10 (M)	0
	8	10 (M)	0
5	9	10 (M)	1
	10	10 (M)	0
Total		92 (M)	5

[a]Stage: (M), morula stage.

Continuous Exposure of Rabbit Morulae to an Electromagnetic Field of 1.5 G at 60 Hz During *In Vitro* Culture for 24 and 48 Hours and the Survivability of Exposed Embryos after Transfer to Synchronized Recipient Does

Experiment 6. Continuous exposure for 24 hours of rabbit morulae to an electromagnetic field of 1.5 G at 60 Hz during *in vitro* culture

A total of 97 morulae (Figure 2) recovered from 10 donors were used in this Experiment (Table 19). Forty-eight morulae were allocated to the control group and exposed to an electromagnetic field of $\leq$ 0.005 G at 60 Hz during *in vitro* culture within the Shielded Culture Cell for 24 hours, and 49 morulae were allocated to the treated group and exposed to an electromagnetic field of 1.5 G at 60 Hz during *in vitro* culture for 24 hours. The maximal development for both control and treated embryos is shown in Table 19. The *in vitro* development of rabbit morulae during a 24-hour period of culture was not affected ($P > 0.25$, Table 20) by the intensity of the AC electromagnetic field present during *in vitro* culture. Chi-square analysis of ratios for the proportion of rabbit morulae that reached the blastocyst stage during culture revealed that there were comparable numbers ($P > 0.25$) of embryos that developed to the blastocyst stage in the control (47/48) and treated (48/49) groups.

Figure 2. Rabbit morula recovered from a donor doe 68 hours after mating and before exposure to an electromagnetic field of 1.5 G at 60 Hz during *in vitro* culture (350 X).

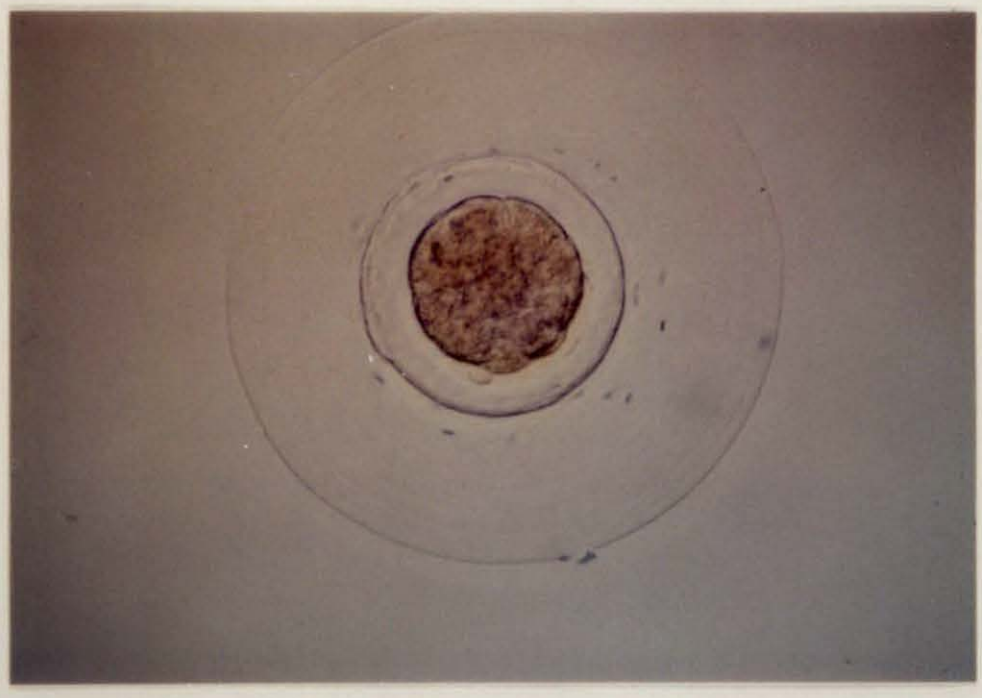


Table 19. Development of rabbit morulae continuously exposed to an electromagnetic field of 1.5 G at 60 Hz during *in vitro* culture for 24 hours (Experiment 6)

Treatment	Number of embryos	Stage of development					
		Morula		Blastocyst		Hatching blastocyst	
		No.	(%)	No.	(%)	No.	(%)
Control	48	1	(2)	31	(65)	16	(33)
Treated	49	1	(2)	40	(82)	8	(16)

Control embryos were exposed during culture to an electromagnetic field of ≤ 0.005 G at 60 Hz.

Treated embryos were continuously exposed during culture to an electromagnetic field of 1.5 G at 60 Hz.

Table 20. Chi-square analysis to determine the effects of continuous exposure to an electromagnetic field of 1.5 G at 60 Hz on the development of rabbit morulae during *in vitro* culture for 24 hours, emphasizing the ratio formed by the number of blastocysts in the numerator and the total number of embryos in the denominator (Experiment 6)

Development	Ratio	X^2	df	P
Blastocyst and hatching blastocyst	Control, 47/48 vs Treated, 48/49	0.002	1	> 0.25

df = degree of freedom.

P = Probability level.

Table 21. Analysis of variance of cleavage indices of rabbit embryos at the end of 24-hour exposure period of an electromagnetic field of 1.5 G at 60 Hz during *in vitro* culture (Experiment 6)

Source	df	SS	MS	F	P
Doe	9	16.71	1.86	7.05	< 0.005
Treatment	1	0.64	0.65	2.30	> 0.25
Treatment × Doe *(Error a)	9	2.30	0.26	-	-
Time	1	460.83	460.83	747.31	< 0.0001
Treatment × Time	1	2.51	2.51	4.08	> 0.25
*(Error b)	172	106.06	0.62	-	-

Allocation of embryos to treatment was performed in a randomized block design and statistical analysis of the data was performed using a split-plot model.

Treatment = Control ($\leq$ 0.005 G) and treated (1.5 G) groups of embryos were exposed to 60 Hz electromagnetic fields during *in vitro* culture.

*The general error term was partitioned, to obtain the **Error a** term to test for the effects of treatment and doe, and the **Error b** term to test the effects of time and the interaction, treatment by time.

Analysis of variance of cleavage indices (Table 21) revealed a significant ($P < 0.005$) effect of doe and, as expected, a significant ($P < 0.0001$) effect of time, but the effects of treatment and the interaction treatment by time were not significant ($P > 0.25$, Table 21), corroborating that the development of embryos during culture was not affected by the exposure of morulae to the electromagnetic fields. The lack of significance ($P > 0.25$) for an interaction of treatment by time, indicates that the effect

Table 22. Cleavage index of rabbits embryos at the time of recovery and after continuous exposure to an electromagnetic field of 1.5 G at 60 Hz during *in vitro* culture for 24 hours (Experiment 6)

Treatment	Number of embryos	Cleavage index after culture for 24 hours
Control	48	12.8 ± 1.6
Treated	49	12.5 ± 1.0

Data are presented as mean ± SD.

Control embryos were exposed during culture to an electromagnetic field of ≤ 0.005 G at 60 Hz.

Treated embryos were exposed during culture to an electromagnetic field of 1.5 G at 60 Hz.

of time was independent of treatment. Peak index equaled to the cleavage index at the end of culture period because embryos were cultured only 24 hours, and the cleavage index was not affected by treatment ($P > 0.25$, Tables 21 and 22), therefore Tukey's test was not applied.

The viability of rabbit embryos after 24 hours of *in vitro* culture and exposure to an electromagnetic field of 1.5 G at 60 Hz is shown in Table 23. The viability of rabbit morulae during a 24-hour period of culture was not affected by the intensity of the AC electromagnetic field (1.5 G at 60 Hz) during *in vitro* culture. Chi-square analysis of

Table 23. Viability of rabbit embryos at the end of a 24-hour period of *in vitro* culture and exposure to an electromagnetic field of 1.5 G at 60 Hz (Experiment 6)

		Viability of embryos after exposure for 24 hours					
		Viable				Nonviable	
		Unstained		Partially stained		Completely stained	
Treatment	Number of embryos	No.	(%)	No.	(%)	No.	(%)
Control	48	28	(58)	20	(42)	0	(0)
Treated	49	38	(78)	11	(22)	0	(0)

Unstained = Number of embryos for which all of the blastomeres were viable at the end of the 24-hour period of culture and exposure to an electromagnetic field.

Partially stained = Number of viable embryos in which one or more blastomeres had died during the 24-hour period of culture and exposure to an electromagnetic field.

Completely stained = Number of embryos during which had died during a 24-hour period of culture and exposure to an electromagnetic field.

Control embryos were exposed during culture to an electromagnetic field of ≤ 0.005 G at 60 Hz.

Treated embryos were continuously exposed during culture to an electromagnetic field of 1.5 G at 60 Hz.

ratios (Table 24) for the proportion of viable embryos at the end of the period of culture indicated that the number of viable embryos in the control (48/48) and treated (49/49) were comparable ($P > 0.25$). However, though the comparison was not planned, the number of embryos that had dead blastomeres (partially stained column of Table 23)

Table 24. Chi-square analysis to determine the differences in ratio of viable embryos from total number of embryos at the end of a 24-hour period of *in vitro* culture and exposure to an electromagnetic field of 1.5 G at 60 Hz, emphasizing the ratio formed by the number of viable embryos with dead blastomeres in the numerator and the total number of embryos in the denominator (Experiment 6)

Viability	Ratio	X^2	df	P
Viable embryos with dead blastomeres	Control, 20/48 vs Treated, 11/49	4.11	1	< 0.05

df = degree of freedom.

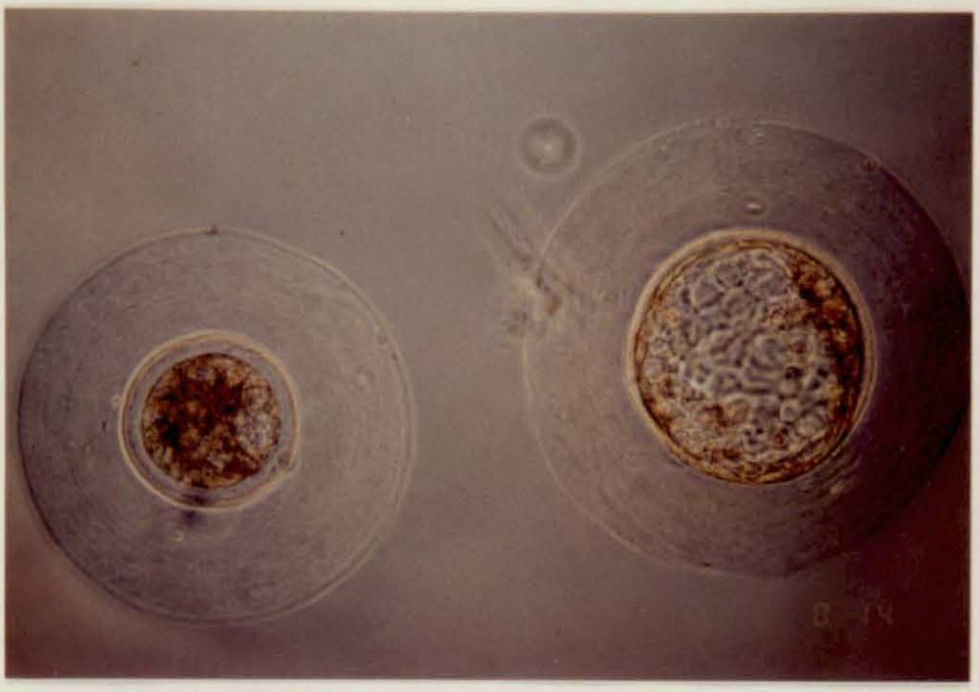
P = Probability level.

was larger ($P < 0.05$, Table 24) in embryos from the control group (20/48) than in embryos of the treated group (11/49). Figure 3 displays viable rabbit blastocysts at the end of the 24 hours of culture, before and after exposure to a 240 μM of solution of eosin B.

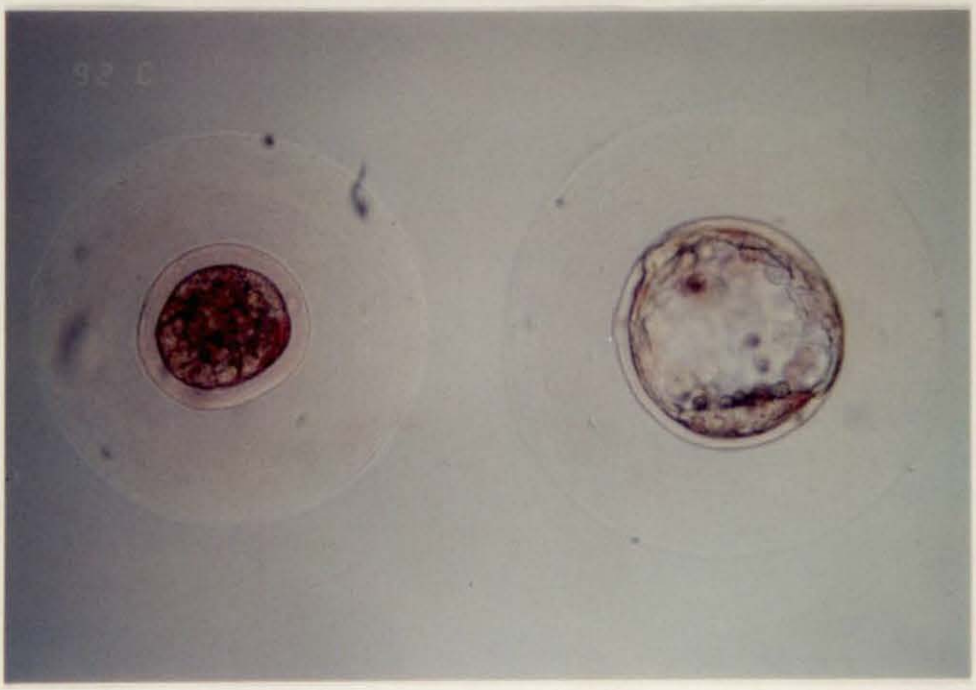
Figure 3. Composite picture of rabbit blastocysts at the end of exposure for 24 hours to an electromagnetic field of 1.5 G at 60 Hz. Early blastocyst (left) and expanding blastocyst (right) embryos (300 X).

A = Before exposure to a 240 μM solution of eosin B.

B = After exposure to a 240 μM solution of eosin B. The blastocysts display partial staining due to the presence of dead blastomeres. However, these blastocysts were classified as viable.



A



B

Experiment 7. Transfer of rabbit embryos to synchronized recipients after continuous exposure for 24 hours to an electromagnetic field of 1.5 G at 60 Hz during *in vitro* culture

A total of 96 blastocysts obtained from embryos in the control and treated groups during culture of morulae in Experiment 6 were transferred to 10 synchronized recipient does. The number of embryos transferred, number of resorption sites, and number of feti at the time of laparotomy on day 24 of gestation, and number of offspring born are shown in Table 25. A total of 47 viable, control blastocysts and a total of 49 viable, treated blastocysts were transferred to either the right or left uterine horn of 10 recipient does. Six of these does (60 %) became pregnant, however only 2 of these does delivered a total of 6 live offspring. One of the does (No. 8, Table 25) that became pregnant and delivered 2 bunnies, one alive and the other dead, had 6 resorption sites (4 sites in the left horn, corresponding to control embryos and 2 sites in the right horn, corresponding to treated embryos) when laparotomized at day 24 of gestation. Two other does (Nos. 9 and 10, Table 25) did not produce offspring and had resorption sites in the right and/or left uterine horns corresponding to losses attributable to control embryos (doe No. 9) and both control and treated embryos (doe No. 10). The number of implanted embryos that died and were detected at the time of laparotomy on day 24 of gestation were not different ($P > 0.25$, Table 26) between control (7/47) and treated (5/49) groups. The ratios for the number of offspring that developed to term and were born after transfer of control embryos (2/47 embryos, Table 25) or treated

Table 25. Viability after transfer of rabbit blastocysts derived from morulae continuously exposed for 24 hours to an electromagnetic field of 1.5 G at 60 Hz during *in vitro* culture (Experiment 7)

Recipient number	Treatment	Number of blastocysts transferred	Laparotomy on day 24 of gestation: Uterine horn	Resorption sites	Number of feti	Number of offspring born
1	Treated	5	Left	0	3	3(A)
	Control	5	Right	0	2	2(A)
2	Control	5	Left	0	0	0
	Treated	5	Right	0	0	0
3	Control	5	Left	0	1	0
	Treated	5	Right	0	0	0
4	Treated	5	Left	0	1	0
	Control	5	Right	0	0	0
5	Treated	5	Left	0	0	0
	Control	5	Right	0	0	0
6	Treated	4	Left	0	0	0
	Control	5	Right	0	0	0
7	Control	3	Left	0	0	0
	Treated	5	Right	0	0	0
8	Control	4	Left	4	0	0
	Treated	5	Right	2	2	1(A),1(D)
9	Treated	5	Left	0	0	0
	Control	5	Right	2	0	0
10	Control	5	Left	1	0	0
	Treated	5	Right	3	0	0
Total	Control	47		7	3	2(A)
	Treated	49		5	6	4(A),1(D)

Control embryos were exposed during culture to an electromagnetic field of ≤ 0.005 G at 60 Hz. **Treated** embryos were exposed during culture to an electromagnetic field of 1.5 G at 60 Hz.

A = Bunny born alive, D = Bunny born dead.

Table 26. Chi-square analyses to determine differences in viability after transfer of rabbit blastocysts which were derived from morulae continuously exposed for 24 hours to an electromagnetic field of 1.5 G at 60 Hz during *in vitro* culture, emphasizing the ratio formed by the number of resorption sites and number of offspring at birth in the numerator and the total number of embryos in the denominator (Experiment 7)

Viability	Ratio	X^2	df	P
Presence of resorption sites	Control, 7/47 vs Treated, 5/49	0.48	1	> 0.25
Produce offspring at birth	Control, 2/47 vs Treated, 5/49	1.25	1	> 0.25

df = degrees of freedom.

P = Probability level.

embryos (5/49 embryos) were not different (P > 0.25, Table 25). The bunnies born alive from the control and treated groups were normal. No developmental abnormalities were found at necropsy in the bunny from treated group which was born dead. The cause of death was not determined.

Experiment 8. Continuous exposure for 48 hours of rabbit morulae to an electromagnetic field of 1.5 G at 60 Hz during *in vitro* culture

A total of 98 rabbit morulae recovered from 10 donors were used in this Experiment (Table 27). A total of 50 morulae were assigned to the control group, and exposed to an electromagnetic field of $\leq$ 0.005 G at 60 Hz during *in vitro* culture in the Shielded Culture Cell for 48 hours. A total of 48 morulae were assigned to the treated group and exposed to an electromagnetic field of 1.5 G at 60 Hz during *in vitro* culture for 48 hours. Exposure of rabbit morulae for 48 hours during culture to an electromagnetic field of 1.5 G at 60 Hz did not affect ($P > 0.25$, Table 28) embryonic development to the blastocyst and hatching blastocyst stages. In fact, the number of blastocysts and hatching blastocysts were comparable ($P > 0.25$, Table 28) for the embryos in the control (50/50) (from data in Table 27) and treated (47/48) groups.

The analysis of variance of cleavage indices for rabbit morulae before (0 hour), at 24 hours, and after 48 hours of *in vitro* culture revealed a significant ($P < 0.0001$, Table 29) effect of doe and time, but the effect of treatment and the interaction treatment by time were not significant ($P > 0.25$, Table 29). The cleavage indices and the peak index are displayed in Table 30. There was no effect of treatment ($P > 0.25$) but there was an effect of time ($P < 0.0001$). However, because the interaction treatment by time was also not significant ($P > 0.25$), one can conclude that the development of rabbit morulae during the 48-hour period of culture, as expressed by the cleavage indices and peak index was not affected ($P > 0.25$) by the intensity of the

Table 27. Development of rabbit morulae continuously exposed to an electromagnetic field of 1.5 G at 60 Hz during *in vitro* culture for 48 hours (Experiment 8)

Treatment	Number of embryos	Stage of development					
		Morula		Blastocyst		Hatching blastocyst	
		No.	(%)	No.	(%)	No.	(%)
Control	50	0	(0)	9	(18)	41	(82)
Treated	48	1	(2)	13	(27)	34	(71)

Control embryos were exposed during culture to an electromagnetic field of ≤ 0.005 G at 60 Hz.

Treated embryos were continuously exposed during culture to an electromagnetic field of 1.5 G at 60 Hz.

Table 28. Chi-square analysis to determine the effects of continuous exposure for 48 hours to an electromagnetic field of 1.5 G at 60 Hz on the development of rabbit embryos during *in vitro* culture, emphasizing the ratio formed by the number of blastocysts in the numerator and the total number of embryos in the denominator (Experiment 8)

Development	Ratio	X^2	df	P
Blastocysts and hatching blastocysts	Control, 50/50 vs Treated, 47/48	1.05	1	> 0.25

df = degree of freedom.

P = Probability level.

Table 29. Analysis of variance of cleavage indices of rabbit embryos at the time of recovery, at 24 hours, and at the end of 48-hour period of exposure to an electromagnetic field of 1.5 G at 60 Hz during *in vitro* culture (Experiment 8)

Source	df	SS	MS	F	P
Doe	9	39.60	4.40	10.14	< 0.0001
Treatment	1	0.03	0.03	0.06	> 0.25
Treatment × Doe *(Error a)	9	3.90	0.43	-	-
Time	2	872.19	436.09	828.66	< 0.0001
Treatment × Time	2	1.15	0.05	1.09	> 0.25
*(Error b)	270	142.09	0.53	-	-

Allocation of embryos to treatment was performed in a randomized block design and statistical analysis of the data was performed using a split-plot model.

Treatment = Control (≤ 0.005 G) and treated (1.5 G) groups of embryos exposed to 60 Hz electromagnetic fields during *in vitro* culture.

*The general error term was partitioned, to obtain the **Error a** term to test for the effects of treatment and doe, and the **Error b** term to test the effects of time and the interaction, treatment by time.

electromagnetic field present during *in vitro* culture. Peak cleavage index was not affected by treatment (P > 0.25, Table 29), therefore Tukey's test was not applied.

All rabbit morulae were viable at the time of recovery. The viability of rabbit embryos after 48 hours of *in vitro* culture and exposure to an electromagnetic field of 1.5 G at 60 Hz is presented in Table 31. The viability of blastocysts derived from the

Table 30. Cleavage and peak indices of rabbits embryos at the time of recovery and after continuous exposure to an electromagnetic field of 1.5 G at 60 Hz during *in vitro* culture for 48 hours (Experiment 8)

Treatment	Number of embryos	Cleavage index: Before culture 0 h	Cleavage index: Time of exposure 24 h	Cleavage index: Time of exposure 48 h	Peak index*
Control	50	9.6 (0.5)	12.6 (0.9)	13.8 (0.5)	13.8 (0.5)
Treated	48	9.7 (0.5)	12.3 (1.3)	13.6 (0.9)	13.6 (0.9)

Data are presented as mean (± SD).

Control embryos were exposed during culture to an electromagnetic field of ≤ 0.005 G at 60 Hz.

Treated embryos were continuously exposed during culture to an electromagnetic field of 1.5 G at 60 Hz.

*Peak index was the maximal cleavage index that was recorded for the embryo during the 48-hour period of culture and exposure to an electromagnetic field.

rabbit morulae during the 48-hour period of culture (Table 31) was not affected by the intensity of the AC electromagnetic field present during *in vitro* culture. Chi-square analysis of ratios (Table 32) for the proportion of viable embryos at the end of culture period were comparable for the control (50/50) and treated (48/48) groups. Contrary to Experiment 6, the number of viable embryos that had dead blastomeres (partially

Table 31. Viability of rabbit embryos at the end of a 48-hour period of *in vitro* culture and exposure to an electromagnetic field of 1.5 G at 60 Hz (Experiment 8)

Treatment	Number of embryos	Viability of embryos after exposure for 48 hours					
		Viable				Nonviable	
		Unstained		Partially stained		Completely stained	
		No.	(%)	No.	(%)	No.	(%)
Control	50	8	(16)	42	(84)	0	(0)
Treated	48	12	(25)	36	(75)	0	(0)

Unstained = Number of embryos for which all of the blastomeres were viable at the end of the 48-hour period of culture and exposure to an electromagnetic field.

Partially stained = Number of viable embryos in which one or more blastomeres had died during the 48-hour period of culture and exposure to an electromagnetic field.

Completely stained = Number of embryos which had died during a 48-hour period of culture and exposure to an electromagnetic field.

Control embryos were exposed during culture to an electromagnetic field of ≤ 0.005 G at 60 Hz.

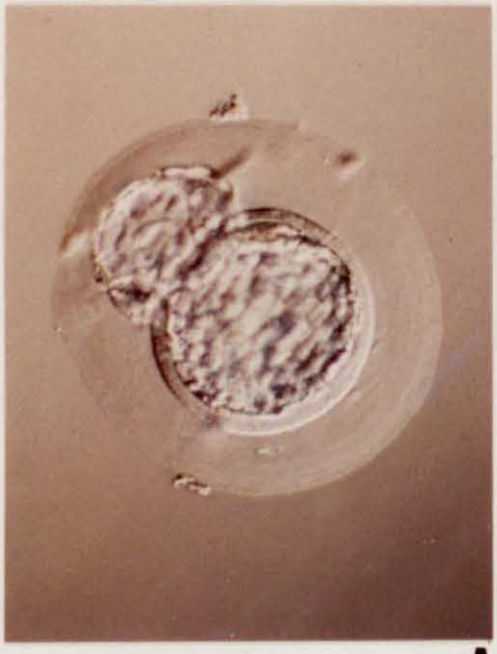
Treated embryos were exposed during culture to an electromagnetic field of 1.5 G at 60 Hz.

stained column of Table 31) was not different ($P > 0.25$) for embryos in the control (42/50) and in the treated (36/48) groups. This comparison was also done after the fact. Rabbit blastocysts at the end of the 48 hours of culture, before and after exposure to a 240 μM of solution of eosin B are shown in Figure 4.

Figure 4. Hatching rabbit blastocysts at the end of exposure for 48 hours to an electromagnetic field of 1.5 G at 60 Hz.

A = Before exposure to a 240 μM solution of eosin B (280 X).

B = After exposure to a 240 μM solution of eosin B. The blastocyst had partial staining indicating that some blastomeres had died during culture. Thus, the blastocyst was classified as viable (250 X).



A

B

Table 32. Chi-square analysis to determine the differences in ratio of viable embryos from total number of embryos at the end of a 48-hour period of *in vitro* culture and exposure to an electromagnetic field of 1.5 G at 60 Hz, emphasizing the ratio formed by the number of viable embryos with dead blastomeres in the numerator and the total number of embryos in the denominator (Experiment 8)

Viability	Ratio	X^2	df	P
Viable embryos with dead blastomeres	Control, 42/50 vs Treated, 36/48	1.22	1	> 0.25

df = degree of freedom.

P = Probability level.

Experiment 9. Transfer of rabbit embryos to synchronized recipients after continuous exposure for 48 hours to an electromagnetic field of 1.5 G at 60 Hz during *in vitro* culture

A total of 95 blastocysts obtained from embryos in the control and treated groups during culture of morulae in Experiment 8 were transferred to 10 synchronized recipient does. The number of embryos transferred, number of resorption sites and number of feti at the time of laparotomy on day 24 of gestation, and number of offspring born are shown in Table 33. A total of 50 viable, control blastocysts and a total of 45 viable, treated blastocysts were transferred to either the right or left uterine horns of 10

Table 33. Viability after transfer of rabbit blastocysts derived from morulae continuously exposed for 48 hours to an electromagnetic field of 1.5 G at 60 Hz during *in vitro* culture (Experiment 9)

Recipient number	Treatment	Number of blastocysts transferred	Laparotomy on day 24 of gestation: Uterine horn	Resorption sites	Number of feti	Number of offspring born
1	Control	5	Left	0	0	0
	Treated	4	Right	0	0	0
2	Treated	5	Left	0	3	2(A),1(D)
	Control	5	Right	1	0	0
3	Treated	4	Left	0	0	0
	Control	5	Right	0	0	0
4	Control	5	Left	0	0	0
	Treated	5	Right	0	0	0
5	Control	5	Left	0	0	0
	Treated	4	Right	0	0	0
6	Control	5	Left	0	0	0
	Treated	5	Right	0	0	0
7	Treated	4	Left	2	1	0
	Control	5	Right	0	0	0
8	Treated	4	Left	2	0	0
	Control	5	Right	0	0	0
9	Control	5	Left	0	0	0
	Treated	5	Right	0	0	0
10	Treated	5	Left	0	4	3(A),1(D)
	Control	5	Right	0	2	2(A)
Total	Control	50		1	2	2(A)
	Treated	45		4	8	5(A),2(D)

Control embryos were exposed during culture to an electromagnetic field of ≤ 0.005 G at 60 Hz. **Treated** embryos were exposed during culture to an electromagnetic field of 1.5 G at 60 Hz.

A = Bunny born alive, D = Bunny born dead.

Table 34. Chi-square analyses to determine differences in viability after transfer of rabbit blastocysts which were derived from morulae continuously exposed for 48 hours to an electromagnetic field of 1.5 G at 60 Hz during *in vitro* culture, emphasizing the ratio formed by the number of resorption sites and number of offspring at birth in the numerator and the total number of embryos in the denominator (Experiment 9)

Viability	Ratio	X^2	df	P
Presence of resorption sites	Control, 1/50 vs Treated, 4/45	2.25	1	> 0.1
Produce offspring at birth	Control, 2/50 vs Treated, 7/45	3.69	1	< 0.1

df = degrees of freedom.

P = Probability level.

recipient does. Only 4 of these does (40 %) became pregnant and 2 of these does delivered a total of 7 live offspring. The number of implanted embryos that died and were detected at the time of laparotomy on day 24 of gestation were not different ($P > 0.1$, Table 34) between control (1/50) and treated (4/45) groups. The ratios for the survival of treated embryos producing offspring at birth tended to be higher ($P < 0.1$, Table 34) than that of control embryos. The bunnies born alive from the control and treated groups were normal. No developmental abnormalities were found at necropsy in the bunnies from treated group which were born dead. No determination was made for the cause of death of these bunnies.

DISCUSSION

Overall, the results of this study demonstrated that there were no harmful effects of exposure to an electromagnetic field of 1.5 G at 60 Hz on the *in vitro* development of rabbit morulae during a 24-hour or 48-hour period of exposure or on the *in vivo* development of embryos after transfer to synchronized recipient does. For the purpose of discussing the results of the research performed for this Thesis, I will follow the sequence presented as: Preliminary studies composed of Experiments 1 through 5, and studies regarding to determine the effects of continuous exposure of rabbit morulae to an electromagnetic field of 1.5 G at 60 Hz during *in vitro* culture and after transfer to synchronized recipient does, composed of Experiments 6 through 9.

Preliminary Studies

Experiment 1. Effect of synthetic culture media on the *in vitro* development and viability of 1-cell and 2-cell rabbit embryos

To expose 1-cell and 2-cell rabbit embryos to an electromagnetic field during culture and provide these embryos with the necessary environment for them to reach the transferable stages of morula or blastocyst, it was necessary to select a suitable medium to produce a high yield of morulae and blastocysts.

The HL-1 culture medium was identified as the most suitable culture medium for

in vitro culture of 1-cell and 2-cell rabbit embryos. Fifty-four percent of embryos cultured in HL-1 medium reached the blastocyst stage, and 31 % of the embryos reached the morula stage at the end of 96-hour period of *in vitro* culture (Table 3). Therefore, a total of 85 % of 1-cell and 2-cell embryos cultured in HL-1 medium reached the transferable stages of morula and blastocyst. Ninety-four percent of embryos cultured in HL-1 medium were viable at the end of the 96-hour period of *in vitro* culture. The second best medium was the RD medium, and 54 % of embryos cultured in RD medium reached the morula stage. However, most of the morula embryos cultured in RD medium were not able to develop to blastocysts.

It seems that one of the most important components of HL-1 medium, as compared to the other 3 media used in the studies reported in this Thesis, was the mixtures of growth factors that allowed for the increased development of rabbit embryos during *in vitro* culture as compared to DMEM/F-12, WBSA, and RD media. Pollard (1987) reported that 1-cell and 2-cell embryos cultured in HL-1 medium (apparently without supplemental glutamine. I could not obtain this information from the manufacture of the medium.) resulted in 70 % (14/20) embryos reaching the morula, 20 % (4/20) embryos reaching the blastocyst, and 10 % (2/20) embryos reaching the hatching or hatched blastocyst stages during *in vitro* culture for 120 hours. These results obtained in my studies appear to be comparable to those obtained by Pollard (1987).

In conclusion, culture medium HL-1 allows for the *in vitro* development of rabbit 1-cell and 2-cell embryos to the blastocyst stage and was selected as the most suitable culture medium for use in my electromagnetic field studies.

Experiment 2. *In vitro* culture of rabbit embryos in defined medium (HL-1) within the Shielded Culture Cell or within the Electromagnetic Field Exposure System (ISU-EFG)

Experiment 2 was performed to confirm whether the HL-1 medium would provide all of the nutrients needed to promote development of 1-cell and 2-cell embryos to the optimal transferable stages of morula and blastocyst during culture *in vitro* for 96 hours within the Shielded Culture Cell (Shielded chamber) or the Electromagnetic Field Exposure System, ISU-EFG, (Unshielded chamber). The results of Experiment 2 indicated that my hypothesis was correct in the sense that I could obtain sufficient numbers of morulae and blastocysts to study the effects of exposure of embryos to electromagnetic fields of defined intensity, as I had planned.

The *in vitro* development of 1-cell and 2-cell rabbit embryos to reach the blastocyst stage and the embryonic viability during a 96-hour period culture was not affected by the exposure to electromagnetic fields ranging 0.02 to 0.06 G at 60 Hz. On the contrary, the results showed that significantly fewer (36 %, $P < 0.05$) embryos cultured within the Shielded chamber reached the morula stage than for the embryos (57 %) cultured within the Unshielded chamber and thus, exposed to the electromagnetic fields. Twenty-six percent of embryos cultured within the Shielded chamber and 18 % of embryos cultured within the Unshielded chambers reached the blastocyst stage. The difference was not significant ($P > 0.25$). Thus, a total of 62 % of the embryos cultured within the Shielded chamber, and 75 % of the embryos cultured within the Unshielded chamber reached the transferable stages of morula and

blastocyst, and this difference was not significant ($P > 0.25$). Ninety-six percent of embryos cultured within the Shielded chamber, and 98 % of the embryos cultured within the Unshielded chamber were viable at the end of 96-hour period of *in vitro* culture. This difference was also not significant ($P > 0.5$). These results provided credible evidence to conclude that the HL-1 was a suitable medium to culture rabbit embryos while being exposed to electromagnetic fields of moderate intensity. Furthermore, the culture of embryos within the Shielded and Unshielded chambers, **per se**, did not interfere with embryonic development. Thus, I could expect the obtainment of sufficient numbers of morulae and blastocysts after exposure during culture for transfer to synchronized recipient does planned for Experiment 3.

Experiment 3. Viability after transfer of rabbit embryos cultured *in vitro* in HL-1 medium within the Shielded Culture Cell or within the Electromagnetic Field Exposure System (ISU-EFG) to 92-hour synchronized recipient does

Experiment 3 was performed to confirm whether the viable morulae and blastocysts obtained after culture of 1-cell and 2-cell rabbit embryos for 96 hours in HL-1 medium within the Shielded or Unshielded chambers in Experiment 2 were capable of producing live offspring after transfer to 92-hour synchronized does. I endeavored to transfer at least 2 embryos per uterine horn because a minimum of 2 implantations are required in the rabbit for fetal survival and normal parturition (Adams, 1982). In addition, the litter size ranges from 8 to 10 bunnies (Hafez, 1970).

None of the 82, 1-cell and 2-cell rabbit embryos that were cultured *in vitro* in HL-1 medium and had reached the stage of morula or blastocysts, produced live offspring after transfer to synchronized recipient does. I could not identify the cause of failure to produce live offspring after transfer of these embryos. Maurer et al. (1970) failed to induce pregnancy after transfer of 15 morulae and 15 blastocysts developed from 2-cell to 4-cell rabbit embryos cultured for 97 hours in media containing rabbit serum or bovine serum to 3, 63-hour synchronized recipient does. The failure to induce pregnancy in Experiment 3 may reflect unforeseen problems due to donor or to embryo quality, duration of *in vitro* culture, asynchrony with the recipient does, or simply due to inadequacies in the embryo transfer technique I used.

Experiment 4. Viability after transfer of rabbit embryos recovered from donor does 92 hours after mating and then transferred to 92-hour synchronized recipient does

Because none of the 1-cell and 2-cell rabbit embryos that were cultured *in vitro* for 96 hours in HL-1 medium produced live offspring after transfer to synchronized recipient does in Experiment 3, I performed Experiment 4 to assess whether: 1) the system I had developed for the recovery and culture of 1-cell and 2-cell rabbit embryos to the late morula or blastocyst stages impaired the viability of embryos and altered their survivability after transfer and 2) that rabbit embryos recovered from donors 92 hours after mating, at the stages of morula and blastocyst instead of embryos recovered at the 1-cell and 2-cell stages and cultured to morulae and

blastocysts *in vitro* resulted in the birth of live offspring after transfer to synchronized recipients.

Transfer of 10 morulae and 59 blastocysts recovered from 5 donors, 92 hours after mating to 10, 92-hour synchronized recipient does resulted in the pregnancy of 6 of 10 recipients and in the birth of 22 offspring born from the 69 embryos transferred, a 32 % rate of survival (22/69). From the result of Experiment 4, I could not experimentally establish the causes for the failure in obtaining pregnancy and birth of offspring in Experiment 3. However, I can, for Experiment 3, at least eliminate the exposure of embryos to the eosin B solution or to inadequacies in the embryo transfer technique as potential causes of failure to produce live offspring after transfer because for both Experiments, the evaluation of embryonic viability and transfer techniques were identical.

Inspection of the data in Table 17 suggest that though there was considerable variation, all of the embryo donors were capable of producing embryos that could implant after transfer and produce live offspring at term. However, for the five embryo donors, the post-transfer survival of embryos ranged from 1/6 embryos (16%) for donor No. 5 to 9/9 embryos (100%) for donor No. 2. Within individual recipients, the embryo survival rate ranged from 0 % (0/4 embryos for recipient doe No. 2, 0/10 embryos for recipient doe No. 4, and 0/5 embryos for recipient doe No. 10) to 100 % (9/9 embryos for recipient doe No. 3). Only donor No. 4 provided embryos which established a pregnancy for both of the recipient does which received embryos from this donor. Furthermore, for 3 of 6 recipients that became pregnant (recipient does No. 3, 6, 7), 60 % or more of the embryos transferred survived to term. This extreme variation in

embryo survival rates among recipients suggests differences among recipients in uterine receptivity. Notwithstanding, the obtainment of a 60 % of rate of pregnancy is comparable to that obtained by Kendle and Telford (1970). These authors reported that a total of 41 New Zealand White rabbit embryos recovered from 5 donors 96 hours after mating, and transfer of these embryos to 5, 96-hour synchronized recipient does resulted in 60 % of pregnancy rate and 66 % of feti developing on day 16 of gestation.

Experiment 5. Viability after transfer of rabbit embryos recovered from donor does 68 hours after mating and then transferred to 92-hour synchronized recipient does

To do the experiments that I had planned to test for the effects of exposure of embryos to defined intensities of electromagnetic fields (Experiments 6 through 9 of this Thesis), it was necessary to provide for a system of culture for embryos at the earliest possible stage of development, so that the length of exposure during culture could also be the longest. To this point, though I succeeded in identifying a medium (HL-1, Experiment 1) that allowed me to culture and maintain viability of rabbit embryos for 96 hours from the 1-cell and 2-cell stages to the morula and blastocyst stages (Experiments 1 and 2), I could not obtain live offspring from the transfer of viable morulae and blastocysts derived from embryos cultured for 96 hours (Experiment 3).

The results of Experiment 4 clearly demonstrated that there was nothing wrong with my technical abilities to induce ovulation, synchronize recipients, and transfer the embryos because I successfully transferred embryos and obtained live offspring.

However, it must be noticed that I succeeded in obtaining live offspring in Experiment 4 when the embryos I transferred developed *in vivo* in the doe and were recovered from the doe 92 hours after mating at the more advanced stages of development of late morulae and blastocysts. In order to further extend the potential period of exposure of embryos to electromagnetic fields without losing the capability of these embryos to develop to term when transferred, I designed and did Experiment 5.

Experiment 5 was designed to determine whether I could obtain pregnancy and birth of live offspring by transferring morulae recovered from donors 68 hours after mating. Thus, to attempt to determine the degree of success, or failure from the transfer of embryos at an early stage, which had developed *in vivo* within the donor doe, as those successfully used in Experiment 4.

Morulae recovered from one donor were transferred to each of 2 recipient does. The transfer of 92 morulae recovered from 5 donors, 68 hours after mating, transferred to 10, 92-hour synchronized recipient does, resulted in the pregnancy of 3 of 10 recipient does, and in the birth of 5 offspring. The post-transfer survival of embryos ranged from 1/10 embryos (10%) for donor No. 1 and 5 to 3/7 embryos (42%) for donor No. 2. Within individual recipients, the embryo survival rate varied from 0 % (0/9 embryos for recipient No. 2; 0/8 embryos for recipient No. 3 and 6; 0/10 embryos for recipient doe No. 5, 7, 8, and 10) to 42 % (3/7 embryos for recipient doe No. 4). The overall embryo survival rate was only 5 % (5/92). Thus, for Experiment 4, the transfer of 10 morulae and 59 blastocysts resulted in the 32 % embryonic survival rate. This suggests that embryos at the stage of blastocyst are more likely to survive to term when transferred to a 92-hour synchronized recipient does. Pollard (1987) reported

that a total of 357, 1-cell rabbit embryos from 10 donors placed in pHema chambers and cultured *in vivo* in peritoneal cavity of male or female mice for 72 hours resulted in 95 morulae and 93 blastocysts at the end of the *in vivo* culture period. Transfer of these morulae and blastocysts to 10, 82-hour synchronized recipient does resulted in 60 % pregnancy rate and 12 % embryo survival rate (Pollard, 1987 and Pollard and Pineda, 1988). The results of Experiment 4 appear to be comparable to those obtained by Pollard (1987) and Pollard and Pineda (1988). The extreme variation in embryo survival rates among recipients suggested differences among recipient does in uterine receptivity that may somehow affect embryonic survival.

Summary and Conclusions for Preliminary Studies

For the purpose of adjusting and fine tuning the next series of Experiments designed to determine the immediate effects of a 1.5 G at 60 Hz electromagnetic field exposure on the *in vitro* development and viability of embryos, and also to determine the delayed effects of exposed embryos during the preimplantation period on the *in vivo* development after transfer to recipient does, I summarized the data from Experiments 1 through 5, as follows:

1. The HL-1 medium was selected as the most suitable culture medium for *in vitro* culture and exposure to an electromagnetic field of rabbit embryos.
2. Rabbit morulae was the stage of embryonic development selected as the most suitable stage for studying embryonic development during *in vitro* culture in HL-1

medium, while the embryos were exposed to an electromagnetic field of 1.5 G at 60 Hz.

3. Instead of culturing embryos for 96 hours, I selected to culture and expose rabbit morulae to an electromagnetic field of 1.5 G at 60 Hz for 24 or 48 hours, then to transfer the exposed embryos to synchronized recipient does.
4. To reduce a potential variation among recipient does in responsiveness to hCG treatment, I vasectomized a Dutch-belted rabbit (Adams, 1982. See details in Appendix A), to ensure that all recipient does used in Experiments 7 and 9 were exposed to this vasectomized male immediately after injection of hCG and allowed to copulate with the vasectomized buck, to ensure a more fined-tuned postovulatory synchronization.

Summary and Conclusions for the Studies on the Effects of Continuous Exposure of Rabbit Morulae to an Electromagnetic Field of 1.5 G at 60 Hz During *In Vitro* Culture for 24 and 48 Hours and the Survivability of Exposed Embryos after Transfer to Synchronized Recipient Does

Experiment 6. Continuous exposure for 24 hours of rabbit morulae to an electromagnetic field of 1.5 G at 60 Hz during *in vitro* culture

Ninety-eight percent of rabbit morulae exposed to an electromagnetic field of $\leq$ 0.005 G at 60 Hz (control group) developed to blastocysts and hatching blastocysts, and 98 % of rabbit morulae exposed to an electromagnetic field of 1.5 G at 60 Hz

(treated group) developed to blastocysts and hatching blastocysts during the 24-hour period. Obviously, the differences were not significant ($P > 0.25$). The trend ($P < 0.1$) for a higher proportion of embryos from the control group to develop to hatching blastocyst stage than for the treated group could be interpreted, perhaps, to a retarding effect of the exposure to the electromagnetic fields. However after the 24-hour period of *in vitro* culture and exposure to this electromagnetic field, all of the control and treated embryos were viable (Table 23). On the other hand, the proportions of viable embryos with dead blastomeres was larger ($P < 0.05$, Table 24) for embryos in the control group (20/48) than for embryos in the treated group (11/49). In conclusion, the results of this Experiment suggest that exposure to an electromagnetic field of 1.5 G at 60 Hz during *in vitro* culture for 24 hours has no harmful effect on the development and viability of blastocysts derived from exposed rabbit morulae.

Experiment 7. Transfer of rabbit embryos to synchronized recipients after continuous exposure for 24 hours to an electromagnetic field of 1.5 G at 60 Hz during *in vitro* culture

The number of transferred embryos that had died after implantation, detected at the time of laparotomy on day 24 of gestation, was not different ($P > 0.25$, Table 26) between control (7/47) and treated (5/49) groups. Furthermore, no abnormalities of development were found in alive or dead bunnies from either of the treatment groups. However, because of the small number of positive outcomes in the ratios formed by the number of bunnies born from the total number of embryos transferred, the conclusion

that exposure of rabbit embryos during culture for 24 hours to 1.5 G at 60 Hz does not affect survivability to term after transfer to synchronized recipients, though valid, must be interpreted with caution.

Experiment 8. Continuous exposure for 48 hours of rabbit morulae to an electromagnetic field of 1.5 G at 60 Hz during *in vitro* culture

During the 48-hour period of *in vitro* culture and exposure to electromagnetic fields, 100 % of control embryos and 98 % of treated embryos (Table 27) reached the stages of blastocyst or hatching blastocyst. This difference was not significant ($P > 0.25$). The cleavage index between treatment groups (Table 30) was not significantly different ($P > 0.25$, see Table 29). All rabbit morulae selected for study were viable before culture and after exposure to a 1.5 G at 60 Hz electromagnetic field (Table 31). Cell death was evident in these embryos, but the proportion of control and treated embryos that contained dead cells was not significantly different ($P > 0.25$) between treatment groups. In conclusion, exposure of rabbit morulae for 48 hours to an electromagnetic field of 1.5 G at 60 Hz during *in vitro* culture had no harmful effect on the development and viability of rabbit morulae.

Experiment 9. Transfer of rabbit embryos to synchronized recipients after continuous exposure for 48 hours to an electromagnetic field of 1.5 G at 60 Hz during *in vitro* culture

In Experiment 9, embryos that had been exposed for 48 hours were transferred to synchronized recipients, and doubled the amount of exposure to electromagnetic fields, as compared to the embryos transferred in Experiment 7. As for Experiment 7, only embryos which were viable at the end of the culture period were transferred to synchronized recipients to determine their post-transfer rate of implantation, as detected by laparotomy on day 24 of gestation, and by their survival to term.

A total of 50 viable, control blastocysts and a total of 45 viable, treated blastocysts were transferred to 10 recipient does. For each recipient, embryos were transferred either to the right or to the left uterine horn. Though the number of implanted embryos detected at laparotomy on day 24 of gestation that died were not different ($P > 0.1$, Table 34) between control (1/50) and treated (4/45) groups, the ratio for survival of treated embryos (7/45) producing live offspring at birth tended to be higher ($P < 0.1$, Table 34) than that of control embryos (2/50). However, because of the small number of positive outcomes in the ratios formed by the number of offspring born from the total number of embryos transferred, the tempting conclusion that exposure of rabbit embryos during culture for 48 hours to 1.5 G at 60 Hz may tend to improve survivability to term after transfer to synchronized recipients must be interpreted with caution. Furthermore, no abnormalities of development were found in alive or dead bunnies from either of the treatment groups.

Dooley et al. (1994) reported that there was no harmful effect on the development or viability of rat embryos exposed to an electromagnetic field of 1.5 G at 60 Hz during a 72-hour period of culture and continuous exposure to the electromagnetic field. However, in the study of Dooley et al. (1994), none of embryos was transferred to recipient rats.

General Conclusions

For the embryo transfer Experiments 7 and 9 of this Thesis, the survival rate for the 24-hour or 48-hour of exposure to electromagnetic fields, and the percentage of feti that developed to term from transfer of the exposed embryos to recipient does were 7 % (7/96) and 9 % (9/95), respectively. Adams (1982), Staples (1971) and Hafez (1993) reported that transfer of early rabbit blastocysts had higher survival rates compared to the transfer of late, hatching blastocysts. Thus, it is possible that one could expect a higher rate of survival to term after transfer of late morulae or early blastocysts to synchronized recipient does. In addition, though synthetic medium HL-1 was adequate to culture rabbit embryos and resulted in development of 1-cell and 2-cell embryos to the transferable stages of morula and blastocyst, it appears that this medium either did not provide all the nutrients that were need for such a prolonged period of culture or that culture itself was too stressful for a successful pregnancy after transfer of those embryos to recipient does (Experiments 2 and 3). Medium HL-1 was adequate for short-term culture of embryos at the stage of early morula (Experiments 6 and 8) since pregnancy resulted from the transfer of these embryos (Experiments 7

and 9). However, it seems that additional growth factors need to be considered for an improved formulation of HL-1 medium for the culture of rabbit embryos. It must be noticed that for the first 24 to 36 hours of development, the rate of cell division is high, such that 3 to 4 cell cycles may be completed, even during *in vitro* culture. Then, it is quite possible that rapidly cleaving rabbit blastomeres are missing, due to incompleteness of factors in the HL-1 medium, certain chemical signals at critical points in the development process of cleavage-divisions and growth. Support for this assumption is provided by previous studies from our laboratory (Pollard and Pineda, 1988). These studies provided strong evidence that rabbit embryos recovered as 1-cell and 2-cell embryos developed to blastocysts in the peritoneal cavity of mice, while contained in Hydrogel pHema chambers. These blastocysts resulted in live offspring when transferred to synchronized recipient does (Pollard, 1987 and Pollard and Pineda, 1988). Hence, it seems reasonable to anticipate that additional growth factors must be added to the HL-1 medium to ensure normal development of rabbit embryos from the 1-cell and 2-cell stages to late morula or blastocyst stages outside the maternal environment.

The totipotency of embryos, or of their blastomeres when isolated from embryos at early stages of development, provides a unique cellular model to study the acute and latent effects of exposure to electromagnetic fields. After fertilization, all of the blastomeres of embryos undergo mitosis. Thus, the preimplantation stage mammalian embryo is an ideal model system to study the effects of magnetic fields on actively dividing cells.

The electromagnetic field exposure system developed in collaboration with Drs. Lamont and Weber in the College of Engineering, which I used in my studies, provides for the precise control of the intensity of the AC electromagnetic field to which embryos are exposed.

In the studies reported in this Thesis (Tables 21 to 24, Experiment 6, and Tables 29 to 32, Experiment 8) continuous exposure of rabbit embryos to an electromagnetic field of 1.5 G during *in vitro* culture for 24 or 48 hours did not affect the cleavage and/or viability of blastomeres. The proportion of control embryos to develop to the blastocyst stage and their rate of development during culture in a $\leq$ 0.005 G magnetic field was not different from the proportion of treated embryos to develop to the blastocyst stage during exposure to an uniaxial, 1.5 G electromagnetic field which was 300-fold greater than that received by the control embryos. This strongly indicates that acute exposure of rabbit embryos to a 1.5 G electromagnetic field during *in vitro* culture did not harm the embryos. Transfer of these embryos resulted in the birth of live offspring suggesting that for the bunnies born there were not delayed effects of exposure of embryos to a 1.5 G, AC magnetic field. The effects of exposure of embryos at earlier stages and other intensities, including intermittent fields, remains to be determined.

Ubeda et al. (1983) had reported a "window effect" indicating that only intensities between 0.01 to 0.139 G of electromagnetic fields at 100 Hz during *in vitro* culture for 48 hours induced developmental abnormalities on chick embryos. Yet, others (Zhang et al. 1993) provided evidence that the electromagnetic field intensity of 2 G at 60 Hz induced deformities in chick embryos during incubation for 21 days but not at the intensities of 0.5 G or 1 G. However, Khalil and Qassem (1991) observed that

there was no effect of exposure for 24 to 48 hours to an electromagnetic field of 10.5 G at 50 Hz on human-lymphocytes when these cells were exposed during culture. However, when cells were exposed for a 72-hour period there was a significant suppression of mitotic activity and a higher incidence of chromosomal aberrations.

Though the results of my studies clearly demonstrated that there were no harmful effects of exposure during culture to an electromagnetic field of 1.5 G at 60 Hz on the *in vitro* development of rabbit morulae or on the *in vivo* development of exposed embryos after transfer to synchronized recipient does, it is obvious that more and possibly finer tuned studies are needed to further define harmful effects of exposure to electromagnetic fields on embryos and offspring resulting from the transfer of these embryos.

REFERENCES

Adams, C. E. 1982. Egg transfer in the rabbit. Pages 29-48 in C. E. Adams, ed. Mammalian egg transfer. CRC Press Inc., Boca Raton, Florida.

Bassett, C. A. L., Mitchell, S. N., and Gaston, S. R. 1982. Pulsing electromagnetic field treatment in ununited fractures and failed arthrodeses. J. Am. Med. Assoc. 247:623-628.

Baum, A., Mevissen, M., Kamino, K., Mohr, U., and Loscher, W. 1995. A histopathological study on alterations in DMBA-induced mammary carcinogenesis in rats with 50 Hz, 100 μT magnetic field exposure. Carcinogenesis 16:119-125.

Berman, E. 1990. The developmental effects of pulsed magnetic fields on animal embryos. Reprod. Toxicol. 4:45-49.

Blackman, C. F., Benane, S. G., Rabinowitz, J. R., House, D. E., and Joines, W. T. 1985. A role for the magnetic field in the radiation-induced efflux of calcium ions from brain tissue *in vitro*. Bioelectromagnetics 6:327-337.

Brighton, C. T., Friedenberg, Z. B., and Black, J. 1979. Evaluation of the use of constant direct current in the treatment of nonunion. Pages 519-545 in C. T. Brighton, J. Black, and S. Pollack, eds. Electrical properties of bone and cartilage: Experimental effects and clinical applications. Grune and Stratton, New York.

Carney, A. L., and Anderson, E. M. 1981. Diagnosis and treatment of brain ischemia. Advance in neurology: Diagnosis and treatment of brain ischemia (Vol. 30). Raven Press, New York 401 pp.

Carney, E. W., and Foote, R. H. 1991. Improved development of rabbit one-cell embryos to the hatching blastocyst stage by culture in a defined, protein-free culture medium. J. Reprod. Fert. 91:113-123.

Carson, J. J. L., Prato, F. S., Drost, D. J., Diesbourg, L. D., and Dixon, S. J. 1990. Time-varying magnetic fields increase cytosolic free Ca^{2+} in HL-60 cells. Am. J. Physiol. 259:C687-C692.

Chiang, H., Wu, R. Y., Shao, B. J., Fu, Y. D., Yao, G. D., and Lu, D. J. 1995. Pulsed magnetic field from video display terminals enhances teratogenic effects of Cytosine Arabinoside in mice. Bioelectromagnetics 16:70-74.

Delgado, J. M. R., Leal, J., Monteagudo, J. L., and Gracia, M. G. 1982. Embryological changes induced by weak, extremely low frequency electromagnetic fields. J. Anat. 134:533-551.

Deno, D. W., and Carpenter, D. O. 1994. Sources and characteristics of electric and magnetic fields in the environment. Pages 3-49 in D. O. Carpenter, and S. Ayrapetyan, eds. Biological effects of electric and magnetic fields: Sources and mechanisms. Academic Press, New York.

Dooley, M. P. 1988. The use of eosin B to assess the viability and developmental potential of rat embryos. Ph.D. Dissertation, Iowa State University, Ames, Iowa.

Dooley, M. P., Pineda, M. H., and Martin, P. A. 1984. A dye-exclusion assay using eosin B to estimate the viability of rat embryos. 10th Internat. Congr. Anim. Reprod. & AI. Univ. Illinois, Urbana/Champaign. Brief Communications, Vol. II. No. 225.

Dooley, M. P., Pineda, M. H., and Martin, P. A. 1989. Single or intermittent exposure of rat embryos to eosin B does not affect *in vitro* development, survival after transfer, birth of live offspring, and development to weaning. Biol. of Reprod. 40 (Supp 1):160.

Dooley, M. P., Pineda, M. H., Lamont, J. W., Weber, R. J., and Moye, D. J. 1994. Continuous exposure of rat embryos to a 1.5 G electromagnetic field (EMF) does not affect *in vitro* development and viability. The FASEB Journal 8(4):A398.

Feychting, M., and Ahlbom, A. 1993. Magnetic fields and cancer in children residing near Swedish high-voltage power lines. Am. J. Epidemiol. 138:467-481.

Foote, R. H., Hafs, H. D., Staples, R. E., Gregoire, A. T., and Bratton, R. W. 1963. Ovulation rates and litter sizes in sexually receptive and nonreceptive artificially inseminated rabbits given varying dosages of luteinizing hormone. J. Reprod. Fertil. 5:59-66.

Frolen, H., Svedenstal, B. M., and Paulsson, L. E. 1993. Effects of pulsed magnetic fields on the developing mouse embryo. Bioelectromagnetics 14:197-204.

Garcia-Sancho, J., Montero, M., Alvarez, J., Fonteriz, R. I., and Sanchez, A. 1994. Effects of extremely-low-frequency electromagnetic fields on ion transport in several mammalian cells. Bioelectromagnetics 15:579-588.

Gold, S., Goodman, R., and Shirley-Henderson, A. 1994. Exposure of Simian virus-40-transformed human cells to magnetic fields results in increased levels of T-antigen mRNA and protein. Bioelectromagnetics 15:329-336.

Goodman, R., Chizmadzhev, Y., and Shirley-Henderson, A. 1993. Electromagnetic fields and cells. Journal of Cellular Biochemistry 51:436-441.

Goodman, R., Wei, L.-X., Xu, J.-C., and Henderson, A. 1989. Exposure of human cells to low-frequency electromagnetic fields results in quantitative changes in transcripts. Biochimica et Biophysica Acta 1009:216-220.

Graham, C., Cook, M. R., Cohen, H. D., and Gerkovich, M. M. 1994. Dose response study of human exposure to 60 Hz electric and magnetic fields. Bioelectromagnetics 15:447-463.

Hafez, E. S. E. 1970. Rabbits. Pages 94-104 and 278 in E. S. E. Hafez, ed. Reproduction and breeding techniques for laboratory animals. Lea & Febiger, Philadelphia, Pennsylvania.

Hafez, E. S. E. 1993. Assisted reproductive technology: Ovulation manipulation, **in vitro** fertilization/embryo transfer (IVF/ET). Pages 473-475 in E. S. E. Hafez, ed. Reproduction in farm animals. Lea & Febiger, Philadelphia, Pennsylvania.

Halliday, D., and Resnick, R. 1966. Physics. John Wiley & Sons Inc., New York. pp. 647-740.

Hansson, H. A. 1981. Lamellar bodies in Purkinje nerve cells experimentally induced by electric field. Brain Research 216:187-191.

Harper, M. J. K. 1961. The time of ovulation in the rabbit following the injection of luteinizing hormone. J. Endocrin. 22:147-152.

Hartman H. A. 1974. The fetus in experimental teratology. Page 93 in S. H. Weisbroth, R. E. Flatt, and A. L. Kraus, eds. The biology of the laboratory rabbit. Academic Press, New York and London.

Hernandez, F. I. 1992. Effect of xylazine on the retrograde flow of spermatozoa into the bladder and postvasectomy clearance of spermatozoa from electroejaculates of rams. M. S. Thesis, Iowa State University, Ames, Iowa.

Kato, M., Honma, K., Shigemitsu, T., and Shiga, Y. 1994. Recovery of nocturnal melatonin concentration takes place within one week following cessation of 50 Hz circularly polarized magnetic field exposure for six weeks. Bioelectromagnetics 15:489-492.

Kendle, K. E., and Telford, J. M. 1970. Investigation into the mechanism of the antifertility action of minimal doses of megestrol acetate in the rabbit. Br. J. Pharmacol. 40:759-774.

Kennelly, J. J., and Foote, R. H. 1965. Superovulatory response of pre-and post-pubertal rabbits to commercially available gonadotrophins. J. Reprod. Fertil. 9:177-188.

Khalil, A. M., and Qassem, W. 1991. Cytogenetic effects of pulsing electromagnetic field on human lymphocytes *in vitro*: chromosome aberrations, sister-chromatid exchanges and cell kinetics. Mutation Research 247:141-146.

Kozma, C., Macklin, W. Cummins, L. M., and Maurer, R. 1974. Anatomy, physiology, and biochemistry of the rabbit. Pages 58-60 in S. H. Weisbroth, R. E. Flatt, and A. L. Kraus, eds. The biology of the laboratory rabbit. Academic Press, New York.

Lamont, J. W., Weber, R. J., Dooley, M. P., Pineda, M. H., and Moye, D. J. 1994. Shielded culture chamber and controlled uniaxial magnetic field generator for very low frequency (VLF) magnetic field exposure of cells during *in vitro* culture. The FASEB Journal 8(4):A398.

Lee, J. M., Stormshak, F., Thompson, J. M., Hess, D. L., and Foster, D. L. 1995. Melatonin and puberty in female lambs exposed to EMF. Bioelectromagnetics 16:119-123.

Lindbohm, M. L., Hietanen, M., Kyyronen, P., Sallmen, M., Nandelstadh, P., Taskinen, H., Pekkarinen, M., Ylikoski, M., and Hemminki, K. 1992. Magnetic fields of video display terminals and spontaneous abortion. Am. J. Epidemiol. 136:1041-1051.

Lindstrom, E., Lindstrom, P., Berglund, A., Lundgren, E., and Mild, K. H. 1995. Intracellular calcium oscillations in a T-cell line after exposure to extremely-low-frequency magnetic fields with variable frequencies and flux densities. Bioelectromagnetics 16:41-47.

Maffeo, S., Miller, M. W., and Carstensen, E. L. 1984. Lack of effect of weak low frequency electromagnetic fields on chick embryogenesis. J. Anat. 139:613-618.

Maffeo, S., Brayman, A. A., Miller, M. W., Carstensen, E. L., Ciaravino, V., and Cox, C. 1988. Weak low frequency electromagnetic fields and chick embryogenesis: Failure to reproduce positive findings. J. Anat. 157:101-104.

Maurer, R. R., Onuma, H., and Foote, R. H. 1970. Viability of cultured and transferred rabbit embryos. J. Reprod. Fertil. 21:417-422.

McCleary, V. L., Akers, T. K., and Aasen, G. H. 1991. Low magnetic field effects on embryonic bone growth. Biomed. Sci. Instrum. 27:205-217.

Mezhevikina, L. M., Koltun, S. V., Goryushkin, G. Y., and Tigranyan, R. E. 1990. Effect of electromagnetic u.h.f. radiation on the morphofunctional state of early mouse embryos. Biophysics 35:843-847.

Moses, G. C., and Martin, A. H. 1992. Effects of extremely low-frequency electromagnetic fields on three plasma membrane-associated enzymes in early chicken embryos. Biochemistry International 28:659-664.

Nordstrom, S., Birke, E., and Gustavsson, L. 1983. Reproductive hazards among workers at high voltage substations. Bioelectromagnetics 4:91-101.

Novini, A. 1993. Fundamental issues on electromagnetic fields (EMF). Acupuncture and Electro-Therapeutics Res., Int. J. 18:23-31.

Pollard, J. W. 1987. Controlled *in vivo* culture of mammalian embryos and isolated blastomeres. M. S. Thesis, Iowa State University, Ames, Iowa.

Pollard, J. W., and Pineda, M. H. 1988. Culture of rabbit embryos and isolated blastomeres in hydrogel chambers implanted in the peritoneal cavity of intermediate mouse recipients. J. *In Vitro* Fertilization and Embryo Transfer. 5:207-215.

Presman, A. S. 1970. Electromagnetic fields and life. Plenum Press, New York. pp. 26-33, 97-98, 107-148, and 161-169.

Ruckebusch, Y., Phaneuf, L., and Dunlop, R. 1991. Physiology of small and large animals. B. C. Decker, Inc., Philadelphia. pp. 299-303.

Sahl, J. D., Kelsh, M. A., and Greenland, S. 1993. Cohort and nested case-control studies of hematopoietic cancers and brain cancer among electric utility workers. Epidemiology 4:104-114.

Sandyk, R. 1993. Weak magnetic fields antagonize the effects of melatonin on blood glucose levels in Parkinson's disease. Intern. J. Neuroscience 68:85-91.

SAS Institute Inc. 1989. SAS language and procedures: Usage, version 6 (1st ed.). Cary, North Carolina.

Staples, R. E. 1971. Blastocyst transplantation in the rabbit. Page 290 in J. C. Daniel, ed. Methods in mammalian embryology. W. H. Freeman Inc., San Francisco, California.

Steel, R. G. D., and Torrie, J. H. 1980. Principles and procedures of statistics: A biometrical approach (2nd ed.). McGraw-Hill Inc., New York. pp. 185-186, 195-233 and 469-494.

Stuchly, M. A., Ruddick, J., Villeneuve, D., Robinson, K., Reed, B., Lecuyer, D. W., Tan, K., and Wong, J. 1988. Teratological assessment of exposure to time-varying magnetic field. Teratology 38:461-466.

Tribukait, B., Cekan, E., and Paulsson, L. E. 1987. Effects of pulsed magnetic fields on embryonic development in mice. Pages 129-134 in B. Knave, and P. G. Wideback, eds. Work with display units 86. Elsevier Science Publishers, Amsterdam.

Ubeda, A., Leal, J., Trillo, M. A., Jimenez, M. A., and Delgado, J. M. R. 1983. Pulse shape of magnetic fields influences chick embryogenesis. J. Anat. 137:513-536.

Ubeda, A., Trillo, M. A., Chacon, L., Blanco, M. J., and Leal, J. 1994. Chick embryo development can be irreversibly altered by early exposure to weak extremely-low-frequency magnetic fields. Bioelectromagnetics 15:385-398.

Wertheimer, N., and Leeper, E. 1979. Electric wiring configurations and childhood cancer. Am. J. Epidemiol. 109:273-284.

Wertheimer, N., and Leeper, E. 1986. Possible effects of electric blankets and heated waterbeds on fetal development. Bioelectromagnetics 7:13-22.

Wertheimer, N., and Leeper, E. 1989. Fetal loss associated with two seasonal sources of electromagnetic field exposure. Am. J. Epidemiol. 129:220-224.

Windham, G. C., Fenster, L., Swan, S. H., and Neutra, R. R. 1990. Use of video display terminals during pregnancy and the risk of spontaneous abortion, low birthweight, or intrauterine growth retardation. Am. J. Ind. Med. 18:675-688.

Witcofski, R. L., Karstaedt, N., and Partain, C. L. 1981. NMR imaging. The Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, North Carolina. 193 pp.

World Health Organization. 1989. Magnetic fields health and safety guide. Page 10 in Health and safety guide, No. 27. Geneva.

Zervins, A. 1973. Chick embryo development in a 26-kHz electromagnetic field. American Industrial Hygiene Association Journal Pages 120-127, March issue.

Zhang, Q., Tabrah, F. L., and Whittow, G. C. 1993. Effects of 60-Hz sinusoidal electromagnetic field on avian embryonic growth and oxygen consumption. Electro- and Magnetobiology 12:27-37.

ACKNOWLEDGMENTS

I would like to thank my Major Professor, Dr. Mauricio H. Pineda, who gave me an opportunity to study in the United States, and always supported and advised me during my graduate studies.

I wish to recognize and thank the members of my committee; Dr. Michael P. Dooley, Dr. Lawrence E. Evans, Dr. Steven M. Hopkins and Dr. Paul A. Martin for their support and advice throughout these studies. In particular, I would like to recognize Dr. Michael P. Dooley who advised me and offered a variety of challenges over the past few years.

I would like to thank Dr. Theerapol Sirinarumitr who has always helped and advised me during my studies and cheered me up during the research.

I would like to recognize Dr. D. F. Cox, Dr. Siriwan Prapong, and Dr. Chalermpol Lekcharoensuk who advised me in the procedures used for statistical analysis of my data.

During the course of my studies I have also benefited from the technical support provided by Ms. Kim Adams and support provided by the Laboratory Animal Resources division of the College of Veterinary Medicine. Especially, I would like to thank Dr. Kathleen Mullin who helped me to necropsy the dead bunnies. I also would like to thank Biomedical Communications, especially Jim Fosse who helped by taking pictures during my experimental studies, otherwise this Thesis would not be complete.

Lastly, and above all I wish to recognize and dedicate this thesis to my parents, sisters, brother, the Thai Government Scholarship, and the Faculty of Veterinary Medicine of Kasetsart University, Thailand, who supported and gave me an opportunity

to study in the United States, and finally I would like to recognize the contribution of all the rabbits I used as experimental animals in my research studies.

APPENDIX A VASECTOMY IN RABBITS

To reduce potential for variation among recipient does due to responsiveness to hCG treatment, I vasectomized a Dutch-belted rabbit (Adams, 1982), and ensured that all recipient does used in Experiments 7 and 9 were mated to this vasectomized male immediately after injection of hCG.

A fertile Dutch-belted male rabbit, 20 weeks of age, and weighing about 5 pounds was vasectomized using the technic described by Hernandez (1992). This rabbit was caged individually, fed commercial rabbit feed, and maintained during the experimental period in a room separate from the female rabbits, with controlled temperature (20 to 22 °C), and light (14 hours' light/10 hours' dark). To perform the vasectomy, the rabbit was fasted for 6 to 8 hours before surgery. Anesthesia was induced by using 5 mg per kg of body weight of Acepromazine Maleate, followed 15 minutes later with 40 mg per kg of body weight of Ketamine Hydrochloride. After injection of Ketamine Hydrochloride, the rabbit was maintained in a surgical plane of anesthesia with Methoxyflurothane inhalation until surgery was completed. The rabbit was positioned in dorsal recumbency, and the scrotal sac and surrounding areas were clipped and disinfected. A 1-cm incision was made at the cranial midline of the upper part of the scrotal sacs. Soft tissues were bluntly dissected using arterial forceps until the tunica sac was exposed. The left and right vas deferens were isolated from the blood vessels and nerves of the spermatid cords, and then each vas was fixed with two arterial forceps. Each vas deferens was ligated with 3-0 surgical silk by using a double-ligation technique. Initially the caudal end of each vas deferens was ligated and tied firmly, but

the cranial end of the vas deferens was ligated loosely, and then the vas deferens was severed between the ligatures. The loosely ligated, cranial end of the vas deferens was then held by the ligature and positioned with artery forceps so that the opening of the lumen could be visualized. The vasa deferentia were cannulated with the blunt end of a 1.0 inch long, 24-gauge needle and flushed with 5 μg solution of Gentamicin diluted with 2 ml of 0.9% warm normal saline and a 0.01% trypan blue solution (Hernandez, 1992). The cranial end of each vas deferens was tied firmly. The subcutaneous tissues were closed with 3-0 surgical chromic catgut, and the skin incision was closed with 3-0 surgical silk. The rabbit received an intramuscular injection of 0.5 ml of long-acting Oxytetracycline. The vasectomized rabbit was monitored daily, and examined for the blue coloration of urine to confirm that the vasa deferentia had been flushed and that the flushing fluid had passed into the urinary bladder. The skin sutures were removed 7 days after surgery. To confirm that the vasectomized rabbit was infertile and still maintained libido, one month after surgery the vasectomized rabbit was paired with a superovulated doe. There were no spermatozoa present in vaginal lavages and only oocytes were recovered from the superovulated doe, 19 to 22 hours after pairing with vasectomized rabbit.

I selected a Dutch-belted rabbit instead of New Zealand White rabbit because Dutch-belted rabbits have colored fur of white and black or brown. Had this vasectomized rabbit cause a pregnancy, the bunnies born will show a difference in the color from New Zealand White bunnies. The blue color (from trypan blue solution) of the urine after surgery and the fact that only oocytes were recovered from a superovulated doe

paired and left overnight with the vasectomized rabbit confirmed that the vasa deferentia were successfully flushed and cleared of spermatozoa.

APPENDIX B EFFECT OF PROTEIN SUPPLEMENTATION OF HL-1 MEDIUM ON DEVELOPMENT AND VIABILITY OF RABBIT MORULAE DURING *IN VITRO* CULTURE FOR 96 HOURS

None of the 1-cell and 2-cell rabbit embryos that were cultured *in vitro* for 96 hours in HL-1 medium produced live offspring after transfer to synchronized recipient does (see Experiments 2 and 3). The objective of this Experiment in Appendix B was to assess whether: Supplementation of HL-1 medium with bovine serum albumin (BSA) would improved the rate of development and viability of rabbit morulae during *in vitro* culture for 96 hours.

Two types of bovine serum albumin obtained from 2 commercial sources were used as supplements to HL-1 medium;

a) Crystallized bovine serum albumin fraction V (BSA-i) (Intergen, Inc., Purchase, New York).

b) Bovine serum albumin (BSA-s) (Sigma Chemical Inc., St. Louis, Missouri).

The synthetic culture medium and medium supplemented with proteins to be tested were as follows:

HL-1 = HL-1 Medium supplemented with 2.5 mM glutamine.

HL-1/BSA-i = HL-1 Medium supplemented with 2.5 mM glutamine and 0.3 % crystallized bovine serum albumin fraction V.

HL-1/BSA-s = HL-1 Medium (Hycor Biomedical Inc.) supplemented with 2.5 mM glutamine and 0.3 % bovine serum albumin.

Unused morulae recovered from 8 donor does of Experiments 6 and 8 were used for this study. Each doe was caged individually, fed commercial rabbit feed, and maintained during the experimental period in a room with controlled temperature (20 to 22 °C), and light (14 hours' light/10 hours' dark). Superovulation was induced by treatment with FSH-P, followed by an injection of hCG, as described for Experiments 6 and 8. After the injection of hCG, each doe was left overnight for mating in the cage of a male of known fertility.

Embryos at the morula stage were then surgically recovered from anesthetized does 68 to 70 hours after mating. To recover embryos, the oviducts and proximal and distal portions of the uterine horns of each donor were flushed, as described for Experiments 1 and 3, using a HEPES-buffered saline solution, handled, and processed, as described for Experiments 6 and 8. Morulae were allocated at random to 3 groups and each group of embryos was then transferred to a culture well containing 500 µl of a 240 µM solution of eosin B in bicarbonate-buffered saline to determine embryonic viability. Only viable morulae were used in this Experiment. Embryos from each treatment group were cultured individually in wells of a 24 well-plate containing 500 µL of the culture medium assigned for that group. All embryos were cultured at 37 °C within an incubator supplemented to contain 5 % CO_2 in a humidified air atmosphere. Embryos were removed from the incubator and assessed for stage of development and morphological characteristics at 0, 24, 48, 72, and 96 hours of culture. At the end of the 96-hour period of culture embryos were again assessed for viability using the eosin B assay. For the purpose of this study, embryos that had at least one unstained blastomere were considered to be viable (Dooley, 1988 and Dooley et al., 1989).

The Experimental endpoints were:

1. Stage of embryonic development at 0, 24, 48, 72, and 96 hours of culture.
2. Maximal stage of development reached during the 96-hour period of culture.
3. Viability before culture and at the end of 96-hour period of *in vitro* culture.

Analysis of variance (Steel and Torrie, 1980) was used to evaluate the effects of embryo donor, treatment, time and the interaction between treatment and time, and to compare cleavage indices (Dooley, 1988) of embryos. The analysis of variance was performed using SAS (Version 6). Allocation of embryos to treatment was performed using a randomized block design, and the statistical analysis of the data was performed using a split-plot model. Chi-square analyses of ratios (Steel and Torrie, 1980) were used to compare developmental stages and viability of embryos. Statistical significance was established at $P \leq 0.05$.

A total of 98 morulae embryos recovered from 8 donors were used to compare the effect of proteins on the embryonic development and viability during *in vitro* culture for 96 hours. Thirty-five morulae were cultured in HL-1 medium, and twenty nine and thirty four morulae were cultured in HL-1/BSA-i and in HL-1/BSA-s, respectively. The maximal development of rabbit embryos in HL-1 medium supplemented with proteins is shown in Table B-1. Chi-square analysis of ratios for the proportion of rabbit morulae that reached the blastocyst stage during culture in HL-1 medium supplemented with proteins is shown in Table B-2. Analyses of variance, cleavage and peak indices of embryos are shown in Tables B-3 and B-4, respectively. The viability of embryos at the end of 96-hour period of *in vitro* culture is shown in Table B-5.

Table B-1. Development of rabbit embryos during *in vitro* culture for 96 hours in HL-1 medium supplemented with proteins

Treatment	Number of embryos	Stage of development: Did not cleave No.	(%)	Blastocyst No.	(%)	Hatching blastocyst No.	(%)
HL-1	35	1	(3)	32	(91)	2	(6)
HL-1/BSA-i	29	1	(4)	26	(88)	2	(8)
HL-1/BSA-s	34	0	(0)	31	(91)	3	(9)

HL-1 = HL-1 Medium supplemented with 2.5 mM glutamine.

HL-1/BSA-i = HL-1 Medium supplemented with 2.5 mM glutamine and 0.3 % crystallized bovine serum albumin fraction V.

HL-1/ BSA-s = HL-1 Medium supplemented with 2.5 mM glutamine and 0.3 % bovine serum albumin.

Ninety-seven percent of the morulae (34/35, from data presented in Table B-1) cultured in HL-1, 96 % of the morulae (28/29) cultured in HL-1/BSA-i, and 100 % of the morulae (34/34) cultured in HL-1/BSA-s reached the blastocyst stage by the end of 96 hours of culture. These differences were not significant ($P > 0.1$, Table B-2). All morulae were viable before culture, and all of the embryos regardless of stage of development were viable at the end of 96-hour period of *in vitro* culture in these 3 synthetic culture media. None of these embryos were transferred to recipient does.

Table B-2. Chi-square analysis to determine the effects of protein supplementation of HL-1 medium on development of rabbit morulae during *in vitro* culture for 96 hours

Development	Ratio	X^2	df	P
Blastocyst and hatching blastocyst	34/35 vs 28/29 vs 34/34	1.11	2	> 0.1

df = degrees of freedom.

P = Probability level.

Table B-3. Analysis of variance of cleavage indices of rabbit embryos during *in vitro* culture in HL-1 medium supplemented with proteins

Source	df	SS	MS	F	P
Doe	7	34.19	4.88	3.85	< 0.05
Treatment	2	4.38	2.19	1.73	> 0.05
Treatment × Doe *(Error a)	14	17.75	1.26	-	-
Time	4	1534.21	383.55	711.87	< 0.0001
Treatment × Time	8	10.19	1.27	2.36	> 0.05
*(Error b)	454	244.61	0.53	-	-

Allocation of embryos to treatment was performed in a randomized block design and statistical analysis of the data was performed using a split-plot model.

Treatment = Effects due to the protein supplementation of culture medium HL-1.

*The general error term was partitioned, to obtain the **Error a** term to test for the effects of treatment and doe, and the **Error b** term to test the effects of time and the interaction, treatment by time.

Table B-4. Cleavage indices and peak index of rabbit embryos at the time of recovery and after culture in HL-1 medium supplemented with proteins

Treatment	Number of embryos	Cleavage index					Peak index*
		0 h	24 h	48 h	72 h	96 h	
HL-1	35	9.3 (0.5)	12.1 (1.3)	13.7 (0.9)	13.9 (0.7)	13.9 (0.7)	13.9 (0.7)
HL-1/BSA-i	29	9.3 (0.5)	12.5 (1.3)	13.8 (0.7)	13.8 (0.6)	13.9 (0.6)	13.9 (0.6)
HL-1/BSA-s	34	9.3 (0.5)	13.0 (1.2)	13.8 (0.8)	14.0 (0.5)	14.1 (0.3)	14.1 (0.3)

Data are presented as Mean (± SD).

*Peak index = The maximal cleavage index that was recorded for the embryo at some point during the 96-hour period of culture.

h = Hour of culture.

HL-1 = HL-1 Medium supplemented with 2.5 mM glutamine.

HL-1/BSA-i = HL-1 Medium supplemented with 2.5 mM glutamine and 0.3 % crystallized bovine serum albumin fraction V.

HL-1/ BSA-s = HL-1 Medium supplemented with 2.5 mM glutamine and 0.3 % bovine serum albumin.

Table B-5. Viability of rabbit embryos at the end of 96-hour period of culture in HL-1 medium supplemented with proteins

Treatment	Number of embryos	Viable				Nonviable	
		Unstained		Partially stained		Completely stained	
		No.	(%)	No.	(%)	No.	(%)
HL-1	35	0	(0)	35	(100)	0	(0)
HL-1/BSA-i	29	0	(0)	29	(100)	0	(0)
HL-1/ BSA-s	34	0	(0)	34	(100)	0	(0)

HL-1 = HL-1 Medium supplemented with 2.5 mM glutamine.

HL-1/BSA-i = HL-1 Medium supplemented with 2.5 mM glutamine and 0.3 % crystallized bovine serum albumin fraction V.

HL-1/ BSA-s = HL-1 Medium supplemented with 2.5 mM glutamine and 0.3 % bovine serum albumin.

Analysis of variance of cleavage indices (Table B-3) revealed a significant ($P < 0.05$) effect of doe, but the effect of treatment was not significant ($P > 0.05$). As expected, there was a significant effect of time ($P < 0.0001$) on embryonic development, however the interaction, treatment by time, was not significant ($P > 0.05$). For data presented in Table B-4, the cleavage index among media and peak indices were affected significantly by time of culture ($P < 0.0001$) as expected, but not

by treatment ($P > 0.05$) and the interaction of treatment by time was also not significant ($P > 0.05$), therefore Tukey's test was not applied.

The viability of embryos (Table B-5) at the end of the 96-hour period of culture was obviously not affected by the protein supplementation of HL-1 medium.

Protein supplementation of medium HL-1 did not affect the *in vitro* development, cleavage indices, and peak index or viability of rabbit morulae during culture for 96 hours. HL-1 medium or HL-1 medium supplemented with proteins appeared to be suitable culture media for rabbit morulae. All embryos cultured in HL-1 medium or HL-1 medium supplemented with proteins were viable at the of the 96-hour period of *in vitro* culture. In my studies, I did not transfer embryos cultured in these HL-1 media supplemented with proteins. Hence, it remains to be determined whether the survivability after transfer to recipient does is affected by culture in these media. Pollard (1987) reported that 5 % of the 1-cell and 2-cell rabbit embryos cultured in HL-1 medium (apparently without glutamine supplement) and 10 % of the 1-cell and 2-cell embryos cultured in HL-1 medium supplemented with 15 mg/ml of bovine serum albumin (BSA) (without glutamine supplement) reached the blastocyst or hatching blastocyst stages during *in vitro* culture for 96 hours. In conclusion, the addition of a protein supplement does not improve the suitability of HL-1 medium for the *in vitro* culture of rabbit morulae.