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The use of eosin B to assess the viability and developmental potential of rat embryos

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The use of eosin B to assess the viability and developmental potential of rat embryos

by

Michael Patrick Dooley

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ABSTRACT

It is estimated that up to 40 % of mammalian embryos die prior to implantation. The causes of early embryonic death are poorly understood, despite widespread recognition of this phenomenon and research efforts to determine the reasons for this significant wastage of embryos. The determination of factors that cause embryonic losses is hampered by the lack of reliable methods to evaluate the viability of the embryo prior to implantation. The major objective of this dissertation was to develop an assay, based on the exclusion of eosin B, for the assessment of the viability of preimplantation embryos. The rat embryo was used as the experimental model for these studies.

Heating of rat embryos to 55°C blocked in vitro development and killed the embryos. Dead embryos were completely stained when exposed to a 120 μM, 600 μM, or 1200 μM solution of eosin B. The interval from dye exposure to complete staining of dead embryos ranged from 1 to 14 minutes and was influenced by the concentration of dye and the stage of embryonic development. A single exposure of 4- to 8-cell embryos to 120 μM or 600 μM eosin B prior to culture or multiple exposures to 120 μM eosin B during culture did not influence the in vitro development of embryos. However, continuous culture in medium which contained 120 μM eosin B reduced the number of embryos that cleaved and continuous exposure to 600 μM eosin B was toxic to the embryo. A single exposure of morulae and blastocysts to the 600 μM concentration of eosin B allowed for the discrimination of viable and nonviable embryos, but did not affect the viability of embryos

after transfer to synchronized recipients. Live offspring were born when embryos, which were unstained when exposed to eosin B, were transferred into the uterine horns of pseudopregnant or naturally mated recipients. Thus, the embryos which were shown to be alive by not staining with eosin B competed successfully for implantation sites with the "self" embryos of the naturally mated rats and offsprings were born.

The results of these studies conclusively indicate that the eosin B dye-exclusion assay is a reliable method to evaluate the viability of rat embryos. The staining response of embryos to eosin B can be used alone, or in combination with the development of the embryo, for the discrimination of embryostatic and embryolethal effectors.

INTRODUCTION

Preimplantation Period of Embryonic Development

In mammals, the preimplantation period of embryonic development extends from the time of fertilization of the oocyte to the initiation of the decidual response of the endometrium and attachment of the embryo. The preimplantation period is relatively short in nondiapausing mammals, and in general comprises 3 % to 27 % of the length of gestation. During the preimplantation period, the embryo, also called conceptus, undergoes a series of morphological and biochemical changes. These include the formation of blastomeres from cellular divisions of the fertilized oocyte, differentiation of the blastomeres into trophoblast and inner cell mass, and blastulation. It is customary to consider oocytes with spermatozoa attached to the zona pellucida or within the perivitelline space, and oocytes that have extruded the second polar body as fertilized oocytes or 1-cell embryos. The fertilized oocyte is sometimes called a zygote, even though the term may not be applicable to mammals because at this stage the maternal and paternal chromosomes are not surrounded by a nuclear envelope, but are dispersed in the cytoplasm (Longo 1973). Mammals have an Ascaris type of fertilization and the nucleus becomes surrounded by a nuclear envelope at the 2-cell stage, after the first cleavage division. The first cleavage originates 2, apparently identical, embryonic cells called blastomeres. From this point, the stage of embryonic development up to blastulation can be designated by the number of blastomeres present at the

time of observation. Thus, I will refer throughout the text to 1-, 2-, 4-, 8-, etc., cell embryos. Additionally, embryos that have cleaved beyond the 16 to 32 cell stages, but had not yet developed a clearly defined cavity or blastocoel, are called morulae, and those multicellular embryos that have developed a blastocoel, are called blastocysts.

Hatching of the blastocyst from the zona pellucida, growth and elongation of the hatched blastocyst, and initiation of the decidual response of the uterus, all of which are necessary for implantation, also occur within the preimplantation period.

Surrounded by the zona pellucida, the blastomeres of the preimplantation embryo do not contact the oviductal or uterine mucosa until hatching. Thus, before implantation, the embryo depends on its own reserves and the secretions of the oviducts and uterus for cellular maintenance. It is only after hatching that direct connections between the trophoblastic tissue and the uterine mucosa begin to develop. The embryo depends on the contractile and ciliary activities of the oviduct for transport from the site of fertilization, in the ampullar region of the oviduct, to the site(s) of implantation within the uterine lumen. The fact that embryos are "free-living" within the tubular genitalia during the preimplantation period facilitates retrieval by either surgical or nonsurgical methods.

It is estimated that up to 40% of embryonic losses occur within this preimplantation period of development (Cartwright and Gerrits 1980). Present technology does not allow for the identification of the status of the preimplantation embryo unless the embryo is removed from the maternal

environment. The inability to detect in vivo whether fertilization has occurred or that the subsequent development of the preimplantation embryo(s) is proceeding normally within the maternal host, precludes the diagnosis of the causes of embryonic death and the correction of the losses for the livestock industry.

Aside from the fact that it has been difficult or impossible to use non-invasive methods to identify the occurrence of fertilization, the normal development of preimplantation embryos, or post-fertilization embryonic losses, this preimplantation period has provided numerous opportunities for mankind to intervene for either intellectual or economic benefit. Embryos obtained from the oviduct and/or uterus of mammals have been maintained in vitro for hours to several days, stored at low temperatures for days to weeks, and preserved for indefinite periods after freezing, or transferred to a different female host for completion of preimplantation development (Hunter 1980). Exposure of embryos to any or all of the above manipulations does not prevent them from implanting after transfer to an appropriate recipient and gestation to term is now an expected consequence.

Assessment of Preimplantation Embryonic Viability

Viability refers to the ability of an organism or some other form of "biological unit" to live and "implies the presence of a vital force in that unit" (Malinin and Perry 1967). Embryonic viability is frequently defined as the ability of the embryo to implant and develop into a live

offspring. That is, the capability of an embryo to exist and develop into an independent unit. However, this definition is not suitable for all cases, particularly when preimplantation embryos from many species are removed from the maternal environment, cultured in vitro or in vivo, or stored for subsequent study. Thus, embryonic viability has also been defined as the capability of the preimplantation embryo to cleave, develop a blastocoel, and/or hatch from the zona pellucida within a defined period of in vitro or in vivo culture. Unfortunately, embryo culture, particularly in vitro culture, cannot be widely applied since embryos from many species do not develop in vitro or can only be cultured from the more advanced stages. Furthermore, the extended period (hours to days) necessary to detect whether an embryo placed in culture is capable of developing is not compatible with the needs and routine methodology presently used for commercial embryo transfer. Thus, criteria to diagnose the viability of the preimplantation embryo, without regard to the surrounding environment, need to be established.

Dye tests have been routinely applied to many cell types to detect membrane damage, changes in membrane permeability, and have also been used to estimate embryo viability. Unfortunately, many of these tests are cumbersome and expensive to use, produce unreliable results, or damage the embryos.

Dye-exclusion tests, utilizing dyes such as eosin B and Y, erythrosin B, and trypan blue have been used as indicators of viability for a number of cell types (Hanks and Wallace 1958, Holmberg 1961, Phillips 1973), including spermatozoa (Dott and Foster 1972, Dooley 1979), but they have

not been developed for the assessment of the embryonic viability.

The major goal of the studies that form the body of this dissertation was to develop a nonlethal method for the repeated examination of the viability of mammalian embryos, based on the capability of live rat embryos to exclude eosin B. The rat embryo, because of availability and reduced cost, was used as the experimental model for the studies reported in this thesis. The ultimate test of viability for embryos is their ability to develop into normal offsprings after transfer to appropriately synchronized recipients. However, this approach for testing the viability of embryos is not only impractical, expensive, and time-consuming, particularly for farm animal species which have a prolonged gestation, but also does not allow for the day to day evaluation of viability and therefore, is not suitable for the study of factors that are associated with embryonic mortality.

The ideal viability assay, either in single or repeated applications should not interfere with the subsequent development to term of the embryo. This is particularly important for livestock species which produce, even after superovulatory treatments, a limited number of embryos. This is also true for the zoo animal and endangered wildlife, where the number and availability of animals is limited. A method which is reliable and also harmless for single or repeated assessment of oocytes during maturation and fertilization, would facilitate the manipulation of gametes for technological advance and livestock improvement.

Background and Review of Literature

Those basic criteria used to assess the viability of preimplantation embryos (see for review: Whittingham 1978) and selected methods (see for review: Knight and Farrant 1978, Malinin and Perry 1967, Shannon 1978) which have been proposed for or have the potential to be used for the evaluation of the preimplantation stage embryos are presented below.

Morphological evaluation

This is the simplest and most direct technique to identify and evaluate embryos. Low levels of magnification are required to visualize and classify preimplantation mammalian embryos by their gross morphological characteristics. Morphologic evaluation, as performed with the dissection microscope, is common to commercial embryo transfer units. The stereomicroscope provides a level of magnification which is sufficient to obtain an overall impression of the conformation of the embryo and to determine, on most occasions, the number of blastomeres present in an embryo. Compound microscopes, however, particularly those in which the objective is situated below the vessel which contains the embryo (inverted microscopes), provide greater magnification and resolution than stereomicroscopes. Compound microscopes are useful for a more refined examination of the embryo as a whole, as well as for evaluation of the individual blastomeres of morulae and blastocysts, or to estimate the degree of association between blastomeres. Furthermore, the compound microscope offers the additional advantages of brightfield, phase-contrast, differential interference contrast, fluorescent, and other forms of image

modulation and/or enhancement, which are particularly suitable for the examination of embryos. In practice, both stereoscopic and compound microscopes are routinely used to evaluate the morphological integrity of embryos, because these systems are complementary. The relatively large visual and depth of field of the stereoscopic microscope allows for the simultaneous visualization, isolation, and manipulation of large numbers of embryos. These advantages of the stereoscopic microscope are inherent limitations of the compound microscope. In addition, the image of the compound microscope is inverted, making manipulation difficult. However, the increased magnification of the compound microscope facilitates the evaluation of a single embryo, the assessment of the degree of cellular association between blastomeres, and the identification of spermatozoa or spermatozoal remnants within the zona pellucida, or within the cytoplasm.

As an alternative to these noninvasive methods of examination of the embryo, as described above, embryos can be fixed, stained and embedded before evaluation with the light microscope, or processed for evaluation using scanning or transmission electron microscopes. Unfortunately, the discrimination provided by differential staining, or the greater resolution afforded by the electron microscope for the assessment of embryonic status, cannot be obtained unless the embryos have been killed and fixed. Hence, the assessment is postmortem and, therefore, is useful only for determining the structural integrity, degree of cellular association, or extent of degeneration of the embryo prior to or at the time of death.

There are obvious changes in size and the shape of embryos during the preimplantation period, although, morphometric techniques (Linares and King

1980) are seldom applied to evaluate preimplantation embryos. Such forms of assessment have not been widely used, probably because the embryo does not reveal dramatic changes in volume prior to blastulation or that there is an inherent plasticity to embryonic form during development.

In commercial embryo transfer units, the morphological assessment of the embryo is commonly restricted to that obtained with a stereomicroscope (Greve et al. 1979). This routine assessment of embryonic quality is based upon both the morphological appearance of the embryo and on the temporal association presumed to exist between the embryo and the maternal host environment. It should be noted that in such applications, evaluation of the morphological integrity of the embryo is rarely limited to the evaluation of an individual embryo. After superovulation, groups of embryos are evaluated in terms of their stage of development and morphological appearance, and then compared to the other embryos recovered from that same donor. As such, embryos are frequently referred to as normal for those embryos that are at the expected stage of embryonic development, or retarded when they are not (Hasler et al. 1987, Seidel 1981). In such instances, the morphological assessment of the embryo encompasses the "brief developmental history" of that embryo within the maternal environment. This form of embryonic assessment has proved to be particularly useful, when applied in commercial embryo transfer units, because the degree of asynchrony between the donor embryo and the recipient environment can have a profound effect on embryonic survival (Dickmann and Noyes 1960, Donaldson 1985, Hancock and Hovell 1961, Hasler et al. 1987, Hunter 1980, Noyes and Dickmann 1960, Noyes et al. 1961). It is

unfortunate however that, in most cases, the precise temporal relationship between the host environment and the stage of embryonic development cannot be ascertained. This frequently results in variable and inconsistent assessments of embryonic viability or quality, which may not reflect any inherent differences in developmental potential of the individual embryos (Greve et al. 1979, Hasler et al. 1987).

Evaluation of the morphological status of an individual embryo requires an understanding of the morphological and functional changes that the embryo undergoes during the preimplantation period. Morphologically, cleavage stage embryos can be classified by the number and appearance of individual blastomeres. Membrane disintegration, rupture, variations in the distribution of cytoplasmic components, and asynchronous cleavage between blastomeres are readily identified within individual embryos (O'Neill et al. 1985). As development proceeds, the degree of cellular association between blastomeres increases and compaction of the embryonic mass often occurs before the formation of the blastocoel is initiated (McLaren 1980). The segregation of cellular elements into trophoblastic tissues and inner cell mass can be identified and the appearance of the blastocoel can be monitored (Tarkowski and Wroblewska 1967). Since changes in the number of blastomeres and/or cellular associations can be readily observed, the morphological assessment of the embryo has proven to be extremely useful to the embryologist. Unfortunately, as embryonic development proceeds to the morula stage, the embryo becomes more difficult to grade (Donaldson 1985, Greve et al. 1979).

Once embryos are manipulated, however, there is no longer a clear

source of reference for normalcy, nor is it clear to what extent the morphology of manipulated embryos, or embryos derived from single blastomeres, are influenced by external and internal factors (Tarkowski 1971, Tarkowski and Wroblewska 1967). The morphological evaluation of embryos stressed by freezing and thawing or following culture for extended periods was less reliable than for freshly collected embryos (Tervit and Elsden 1981). Furthermore, it has been recognized that there can be marked differences in the survival of embryos that are classified as normal, based on morphological criteria (Linder and Wright 1983, Shea 1981, Shea et al. 1983) and even those embryos rated as poor or abnormal have been observed to produce live offspring (Donaldson 1985, Seidel 1981, Shea 1981).

In summary, the morphological assessment of embryos is limited by: 1) an evaluation which provides only a static view of a rapidly changing and continuous process, 2) the fact that the appearance of the embryo often reflects the temporal and physical aspects of the prior relationship with the host environment, and 3) classification schemes which are either too simplistic or too unwieldy. Despite these limitations, morphological assessment remains to date as the most widely accepted and applied method of evaluation of the preimplantation embryo.

In vitro development

Complete in vitro development of embryos from the 1-cell to the blastocyst stages implies that an embryo can be recovered shortly after fertilization, placed in appropriate culturing media and observed, over time, to cleave and assume apparently normal spatial and cellular associations, including formation of a blastocoel and then proceed to

"hatch" from the confines of the zona pellucida, all of this outside of the animal body. To date, despite numerous attempts, only embryos of a few species develop in vitro (for review, see: Biggers 1971, Brackett 1981, Brinster 1969, Foote 1987, Gwatkin 1966, Wright and Bondioli 1981). Embryos from certain strains of mice (Biggers 1971, Whitten and Biggers 1968) and rabbits (Pollard 1987) can be cultured in vitro from the 1-cell to the blastocyst stage. However, embryos from other strains of mice (Biggers 1971, Whitten and Biggers 1968) or from other mammalian species (Whittingham and Bavister 1974), including livestock species (Wright and Bondioli 1981), exhibit limited development or display an in vitro block at the 2- and/or 4-cell or later stages (Eyestone and First 1986, Thibault 1966).

In general, the in vitro developmental responses that have been observed for mammalian embryos are influenced by the species (Whittingham and Bavister 1974, Wright and Bondioli 1981), stage of embryonic development at the time of collection (Whitten and Biggers 1968), and the composition of the medium (Biggers 1971, Gwatkin 1966, Wright et al. 1976a, Wright et al. 1976b, Wright 1977, Wright and Bondioli 1981). Successful embryo culture in chemically defined media, as reported in the late 1960s and 1970s, was obtained with media containing serum supplements or bovine serum albumin (BSA). The commercial preparations of BSA frequently used for embryo culture media are relatively impure (Kane and Headon 1980), often containing unknown, low molecular weight proteins, fatty acids, and steroids (Wright and Bondioli 1981), and may vary considerably in their growth-promoting effects (Kane 1983). In fact, a recent study (Kane 1985)

reported that the growth-promoting influence of BSA may be due to a low molecular weight contaminant that can be extracted from BSA. Despite the confusion that may have developed from the inclusion of BSA or serum in many media considered to be "chemically defined", there is now evidence that mouse embryos can be cultured in a medium which is truly chemically defined and devoid of protein (Boulton and Whittingham 1987, Caro and Trounson 1984, Mahadevan et al. 1986). Similarly, 1-cell rabbit embryos can develop to the blastocyst stage in a low-protein medium (Pollard 1987). Under most circumstances, however, mouse and rabbit embryos are cultured in media containing a protein supplement, (Brinster 1965a,b; Kane and Headon 1980). Frequently these media are supplemented with purified biological products or processed biological fluids (Maurer 1978, Menino et al. 1985) for optimal development in vitro, even though, in some circumstances, exogenous proteins may have a detrimental effect on preimplantation development in vitro and the post-transfer viability of the embryo (Caro and Trounson 1984). In general, systematic studies have not been performed to identify essential and nonessential media components for the in vitro culture of preimplantation stage embryos from most mammals. Studies of this nature have only been performed for mouse embryos. The mouse embryo can metabolize a variety of substrates (Biggers 1971, Brinster 1965a) but the concentration of substrates in a given medium interacts, either positively or negatively on the survival of the embryo. In addition, certain physicochemical characteristics of the medium, such as ionic composition, pH, and osmotic pressure can influence the metabolism and the in vitro developmental response of embryos (Bavister 1981, Biggers 1971,

Brinster 1965b).

Attempts to circumvent the block preventing the in vitro development of embryos from the 1-cell to the blastocyst stage include organ culture using oviductal tissues, embryo culture on Hela cell monolayers, or co-culture of embryos with cumulus cells (see for review: Biggers et al. 1971, Brinster 1969, 1970). Biggers et al. (1962) demonstrated that 1-cell mouse embryos could be cultured within a segment of the oviducts in a chemically defined medium. Subsequently, Whittingham and Biggers (1967) obtained normal fetuses from embryos which had been initially cultured from the 1-cell to 2-cell stage in chemically defined medium and then transferred to the oviducts of synchronized mice, for their subsequent in vivo culture to the blastocyst stage. Recently, studies have focused on the co-culture of livestock embryos with oviductal or trophoblastic cells (Camous et al. 1984, Eyestone et al. 1987, Rexroad and Powell 1988). The co-culture of bovine (Camous et al. 1984) and ovine (Heyman et al. 1987) embryos with trophoblastic vesicles promoted the in vitro development of 1-cell to 8-cell stage embryos to morulae.

Despite the inherent limitations of in vitro culture, as described above, the capability of in vitro culture systems to allow for embryonic development has promoted the study of embryonic cell physiology. In addition, these systems have facilitated the development of manipulative technology to elucidate essential events which occur during the preimplantation period. The combination of in vitro culture and morphological evaluation has provided the means to monitor developmental changes within single embryos.

In vivo development

As noted earlier, preimplantation embryos exist in a "free-living" state within the tubular genitalia for a relatively short portion of their embryonic life. During this period, removal of these embryos from the host environment, even for short periods, often seriously limits their potential to produce live offspring after transfer to a suitable recipient (Farrell and Bavister 1984). As an alternative to in vitro culture, for the short-term maintenance of preimplantation mammalian embryos, and in an attempt to identify satisfactory host environments that will permit embryonic development to continue, embryos have been transferred into various body cavities and organs of individuals from the same (homo-transfer) or different (hetero-transfer) species. Species commonly used to serve as intermediate recipients in hetero-transfer include mice, rabbits, and sheep.

Restricted development of embryos has been obtained when placed in ectopic sites, such as the anterior chamber of the eye (Grobstein 1949, Runner 1947), beneath the capsule of the kidney (Kirby 1962), spleen (Kirby 1963a), testis (Kirby 1963b), or in the peritoneal cavity (Briones and Beatty 1954, Fawcett et al. 1947). Most of these studies were performed primarily to study factors which influenced implantation events, as opposed to the development of methods for short-term embryo culture (Kirby 1971). Interestingly, embryonic development within an ectopic site has also been shown to be influenced by the stage of embryonic development and the environment from which the embryo had been recovered (Kirby 1962). Furthermore, studies performed using ectopic sites within intermediate

recipients have often revealed that the embryos transferred to the intermediate host were either never recovered or only the extra-embryonic tissues from these transplanted embryos survived (Kirby 1971). A greater degree of success has been obtained by the in vivo, homo- or hetero-culture of embryos, within the nonligated (Anderson and Foote 1975, Farrell and Bavister 1984) or ligated (Boland 1984, Brinster and TenBroeck 1969, Briones and Beatty 1954, Eyestone et al. 1985) oviduct and uterus. Although the recovery rate of these embryos from the reproductive tracts of the intermediate recipients is low, the method has provided a means to assess embryonic viability or to obtain live offspring after transfer of such embryos to the appropriate, terminal recipient (see for review: Boland 1984).

For certain species, such as the bovine, hamster, and ovine, the use of the intermediate recipient appears to be the only reliable method to maintain embryonic growth and differentiation from the 1-cell to the blastocyst stage, outside of the natural maternal environment. To date, the most serious limitations to the use of intermediate recipients for the in vivo culture of embryos are the low recovery rates (Brinster and TenBroeck 1969, Briones and Beatty 1954, Eyestone et al. 1985), failure of the system to maintain adequate separation of embryos in discrete groups within the intermediate host (Boland 1984, Eyestone et al. 1985), and the added cost of the intermediate recipient and attendant surgical procedures. In addition, the developmental response of embryos may be species (Briones and Beatty 1954) or stage specific (Brinster and TenBroeck 1969). Recently, an artificial chamber made of polymerized 2-Hydroxyethyl-

methacrylate (pHema) has been shown to have potential for the short-term culture of mammalian embryos (Pollard 1987, Pollard and Pineda 1988). A significant advantage to the pHema chamber, as compared to the other experimental approaches that have been used (Farrell and Bavister 1984, Willadsen 1980, Willadsen and Fehilly 1983), is the suitability of the chamber to culture an embryo, or groups of embryos, in vivo for extended periods in the same intermediate recipient and be assured that virtually all of the embryos which were placed within the chamber and incubated in the intermediate recipient will be recovered.

Metabolic studies

The developing embryo, as with other biological systems, requires certain nutrients to survive and releases by-products resulting from cellular metabolism. Furthermore, as growth and differentiation proceeds, changes in metabolism and metabolic rate may occur. While it is extremely difficult, if not impossible, to monitor embryonic metabolism in vivo, the uptake of certain substrates, the metabolism of precursors, and the release of by-products can be determined by micro-methods to study metabolic processes of embryos, immediately after recovery from the maternal environment or during in vitro culture. In most cases, however, the low sensitivity of these micro-methods require groups of embryos and can not, or are not, applied to the evaluation of single embryos (Biggers et al. 1971). Furthermore, metabolic tests do not differentiate between irreversible and reversible effects on cell survival (Freshney 1983).

Precursor uptake Cellular metabolism requires that nutrients are obtained from the surrounding environment. The preimplantation embryo

depends on aerobic respiration to a large extent (Suzuki 1973). The quantitative estimation of oxygen consumption by a single embryo or groups of embryos can be monitored manometrically (Fridhandler 1971), spectrophotometrically (Hultborn 1974, Magnusson et al. 1983) or using electrochemical techniques. While embryos can often be maintained using a variety of nutrient sources, as long as the appropriate metabolic pathways exist within the embryo (Brinster 1965a,b), it is possible to monitor the embryonic uptake of selected nutrients through the use of radio-labelled compounds (Fridhandler 1971). Micro-methods have now been developed, which can detect and quantitate glucose utilization by single embryos at the blastocyst stage (Renard et al. 1980), or the uptake of pyruvate and glucose from the incubation medium (Leese and Barton 1984). Alternatively, isotopically labelled compounds such as glucose, pyruvate, lactate, and histidine can be provided to the embryo and their uptake and conversion can be monitored extracellularly, by trapping CO_2 and estimating the amount of lactate, pyruvate, and labelled glucose (Brinster 1971, Rieger 1984), histidine (Dey and Johnson 1980) that was metabolized, or by the amount of interconversion into labelled pools of metabolites (Fridhandler 1968). Nuclear and cytoplasmic division, as well as cellular maintenance, require substrate for replication and repair of the nuclear genome and the multiplication of cellular organelles. Uptake of amino acids and nucleic acid precursors or the incorporation of compounds as cellular constituents can be monitored by following their removal from the micro-environment or by fixing the cells and then demonstrating that the radio-labelled compound had been incorporated into one or more of the cellular organelles through

autoradiographic analysis (Chan et al. 1982, Macieira-Coelho 1973, Skalko 1971), or can be quantitated by extraction of the RNA and DNA (Fridhandler 1971).

Metabolic by-product As described previously, CO_2 is released from metabolically active cells and the amount of CO_2 released can be quantitated (Brinster 1971, Rieger 1984). In experiments utilizing isotopically labelled carbon, only a fraction of the total CO_2 generated by the embryo is actually labelled. This value can be determined, however, and then used to estimate the metabolic rate of the embryo (Fridhandler et al. 1957) or to relate metabolite utilization with oxygen consumption (Brinster 1971). Alternatively, the total amount of CO_2 produced and released into a liquid or gas phase can be determined indirectly under aerobic conditions (Fridhandler 1971). In this approach, the CO_2 production from all sources is estimated, including the bicarbonate and metabolic substrates which were present within the embryo at the time of collection. Unfortunately this approach does not take into account that CO_2 can be incorporated or reincorporated into the embryo (Pike et al. 1975). These findings, the fact that there may be significant substrate reserves within the embryo at the time of collection (Ozias and Stern 1973), and that these reserves may vary with the stage of development, are likely the reasons why this approach has not been vigorously applied or studied. Even though it is possible to use micro-techniques, which take advantage of the amplification associated with enzymatic cycling, to assay the metabolites of the tricarboxylic acid cycle, glycolytic intermediates, and endogenous nucleotides in single embryos (Wales 1978), this methodology

is cumbersome and the embryo is destroyed in the measuring process.

To date, most attempts to monitor metabolic processes have focused on consumption or uptake of nutrients from the environment or their production and release from the cellular mass (Fridhandler 1971, Holden et al. 1973, Spielmann et al. 1984). Studies which focus on metabolic interconversions or on the net uptake and release of byproducts, the net of all anabolic and catabolic processes occurring within the embryo, are needed (Kaye 1986). Optimal cellular activity and the maintenance of viability are likely to be provided by those environments that maintain cellular homeostasis. It has been established that mammalian embryos tend to lose their totipotential capacity, as the period of in vitro culture increases (Davis and Day 1978, Farrell and Bavister 1984, Seidel 1981). This alteration may simply be a reflection of the embryonic response to an imbalanced environment.

Isotope release

The assay based on the release of ^{51}Cr has been used in a wide variety of cell lines (Holden et al. 1973, Zawydiwski and Duncan 1978) and chromium release has been used for evaluation of the embryotoxic effects on mice (Croy et al. 1985). Radioactive chromium, supplied as $Na_2{}^{51}CrO_4$ is taken up by living cells in the reduced form $^{51}Cr^{+3}$ and then oxidized intracellularly to $^{51}Cr^{+2}$ to which the cell membrane is impermeable (Freshney 1983). After washing away the excess extracellular chromium ions, the release of $^{51}Cr^{+2}$ into the extracellular environment can be monitored. While there will be a continual leakage of ^{51}Cr into the extracellular compartment from these labelled cells, regardless of their viability (Holden et al. 1973), the loss of cell viability is associated with the

release of relatively large amounts of chromium into the surrounding medium. The amount of ^{51}Cr lost per unit of time can be determined and used to obtain a quantitative estimate of the degree of toxicity of various agents or to monitor whether cellular integrity is maintained over time. The chromium release assay has been particularly useful to evaluate the cytotoxicity of immune cells in vitro, because the assay can be performed using large numbers of cells.

Dye assays

Dyes have long been used for the postmortem identification of cellular elements. A particular advantage of dyes and related substances is that the presence and relative concentration of these substances can be determined visually. An additional advantage of dye-based assays is that the visual, subjective impression of the observer regarding the staining and the degree of staining can be confirmed using conventional colorimetric techniques. Hence, dye assays can be developed to become an extremely useful adjunct to the continued or repeated morphological evaluation of cellular integrity. Despite this potential usage, the application of dye-assays to evaluate viability of embryonic cells has been limited to relatively few studies.

Dye metabolism The optical properties of dye molecules are influenced by the physicochemical characteristics of their environment. Oxidation reduction indicators, such as methylene blue (also used as a nucleic acid stain) and various salts of tetrazolium (tetrazoleum dyes) have been used to detect cellular activity and to obtain quantitative estimates of cellular metabolism (Umbreit et al. 1972). Methylene blue

reduction by hydrogen results in the shift from a blue solution to a colorless liquid, due to electron transfer to the dye molecule. Due to the activation of dehydrogenases, the resultant transfer of hydrogen to the dye molecule can be monitored and used as an index of cellular metabolism (Umbreit et al. 1972). Methylene blue reduction has been used to monitor the activity of spermatozoa (Bishop et al. 1954), but has not been used for the evaluation of viability of preimplantation embryos. Tetrazolium and derivatives of this compound have also been used to test for metabolic activity in vitro or for the release of dehydrogenases into the medium by embryos during culture (Renard et al. 1977). Tetrazolium is a colorless substance which serves as an electron acceptor. As a result of metabolic processes, tetrazolium is reduced enzymatically to form water insoluble colored "formazans" which cannot be re-oxidized (Alley et al. 1982). This association can be monitored visually as a shift from a colorless to a colored solution or quantitated spectrophotometrically (Renard et al. 1977, Umbreit et al. 1972). Although tetrazolium has not been used to directly assess the metabolic activity of preimplantation mammalian embryos, it has been used to identify the release of different enzymes from bovine embryos during in vitro culture (Renard et al. 1977). Tetrazolium reduction is inhibited when oxygen is present (Umbreit et al. 1972).

Vital staining Vital staining refers to the staining which occurs when the cell or tissue being stained by the indicator dye is alive. Staining results from the active uptake of the substance by the cell, entrapment within cellular milieu, and increased concentration of dye product intra-cellularly. Staining may also be the result of a color shift

due to metabolic alteration or association of the dye molecule with cellular elements. The staining response may be generalized, in which case the entire cell or cellular mass is stained, or may be localized to one or more organelles within the cell. Vital stains include acridine orange, neutral red, and trypan blue (see for review: Malinin 1966). Trypan blue also acts as a dye-exclusion agent in cell lines as discussed below. Acridine orange was shown to stain the nuclei of bovine embryos, however, the dye was cytotoxic to the embryo (Schilling et al. 1979). Neutral red was used as an indicator of viability of sheep embryos (Kardymowicz 1972), based on the ability of embryos to retain neutral red for 2 hours. Neutral red was used with trypan blue for the evaluation of frozen-thawed, mouse embryos (Thadani et al. 1982). Toxicity due to dye exposure was not evident in these studies. More recently, a series of bisbenzimide dyes referred to as Hoechst dyes (American Hoechst Company, Sommerville, NJ) have been developed and several of these have been tested using mammalian cells (Durand and Olive 1982, Lalande et al. 1980). These dyes tend to associate with DNA or DNA-protein complexes and are visualized by fluorescent microscopy, after washing dye-exposed cells to remove unbound dye. Hoechst dyes (33342; 33258) have primarily been used for detection of pronuclei or nuclei in mammalian embryos. To date, the major limitation to the application of dyes to estimate embryonic viability has been the toxic effects that have been associated with dye exposure to the fluorescent dyes, fluorescence activation, and quenching and the cost of equipment essential to visualize the staining response (Critser and First 1986, Tsunoda et al. 1988). Rhodamine dyes stain cellular organelles (Gurr 1971)

and rhodamine 123 staining is specific for mitochondria (Johnson et al. 1980). Rhodamine 123 has an obvious potential for assessment of the integrity of mitochondria of embryos during freezing and thawing and indirectly to estimate viability but has not been used for this purpose. Rhodamine 123 and ethidium bromide has been used to estimate viability of human spermatozoa (Evenson et al. 1982).

One variation to the vital staining technique involves the use of fluorescein diacetate. Fluorescein belongs to the xanthene family of dyes and is generally excluded by living cells. The addition of the acetate moieties to the fluorescein molecule facilitates passage of the dye molecule across the cell membrane. Once fluorescein diacetate (FDA) has entered the cytoplasm, it is modified by the action of intracellular esterases, resulting in the release and entrapment of fluorescein in the cytoplasmic compartment (Persidsky and Baillie 1977, Rotman and Papermaster 1966). After washing to remove excess fluorescein and fluorescein diacetate, live cells fluoresce when observed under fluorescence microscopy. Fluorescein diacetate has been used to estimate embryonic viability in the cow (Church and Raines 1980, Hoppe and Bavister 1984, Renard et al. 1982, Schilling et al. 1979), hamster (Hoppe and Bavister 1984, Hutz et al. 1985), mouse (Jackowski 1977, Mohr and Trounson 1980), rabbit (Schilling et al. 1979), and squirrel monkey (Chan et al. 1982). While the fluorescein diacetate response appears to be useful for the evaluation embryonic viability, its application has been limited by the fact that dye exposure and fluorescence can be toxic to the cell. Furthermore, morphological and staining evaluations cannot be performed

simultaneously and the repeated examination of the embryos is not possible. Added to this disadvantage is the cost of the equipment needed for fluorescent evaluation. It is interesting to note that the exposure times that have been applied to mammalian embryos ranged from 1 (Mohr and Trounson 1980) to 5 (Schilling et al. 1979) to 10 (Church and Raines 1980) minutes. While these brief exposures have been sufficient to discern qualitative differences in staining within and between embryos, they are not long enough to establish staining equilibrium (Mohr and Trounson 1980, Rotman and Papermaster 1966). Therefore, it is not clear whether the relatively brief periods of exposure that have been recommended for the evaluation of mammalian embryos exposed to fluorescein diacetate and other vital dyes were selected to simply shorten and streamline the assay, or that they reflect conscious efforts to avoid or at least minimize the potential deleterious effects of such treatments. One aspect of cellular physiology that has not been adequately addressed during the evaluation of vital staining techniques is the fact that there is a great deal of communication between blastomeres of the developing embryo (Goodall and Johnson 1982). Injection of a substance into one blastomere often is followed by the rapid dissemination of the substance into the other cells of the embryo (Lo and Gilula 1979). It should also be noted that the degree of intercellular communication may vary between embryonic stages or between embryos which appear to be at a given stage of embryonic development. Hence, the observation that multicellular embryos may exhibit varying degrees of fluorescence within the cellular mass may simply reflect inter-embryonic differences in cellular communication that do not directly

relate to cellular integrity (Lo and Gilula 1979). Alternatively, the fact that several embryos may reveal uniform fluorescent intensities would not necessarily mean that all cells of a given embryo are responding similarly in regard to dye uptake and esterase activity or that there are not marked differences between blastomeres of embryos that evince a similar degree of fluorescence after dye exposure and illumination. Vital staining therefore may reflect a composite cellular response that includes one or more of the following: 1) passive or facilitated diffusion, 2) active transport, 3) intracellular localization and/or binding, 4) cytoplasmic and/or nuclear alteration, and 5) entrapment of dye within the cellular space.

Nonvital staining As opposed to vital staining, where the apparent concentration and localization of dye molecules within the cell is interpreted to reflect an ongoing vital cellular processes, nonvital staining refers to techniques in which the staining is assumed to occur only when the cell is dead. The discussion of nonvital staining will be confined only to those dyes and staining protocols in which staining can be detected even after removal of the cells from the staining mixture. These are ethidium bromide, diamino-phenyl-indol (DAPI), trypan blue and Evan's blue. Ethidium bromide is known to intercalate with DNA of nonviable cells, which stain and exhibit a red fluorescence after incubation, washing and illumination (Freshney 1983). DAPI is also a fluorescent dye and the binding of DAPI to the nucleus of dead cells has been used for the identification of nonviable embryonic cells in the cow (Linder et al. 1982, Renard et al. 1982). However, Renard et al. (1980) reported that some morphologically normal embryos stained by DAPI, developed in vitro and live

offspring were obtained from some of these embryos. Trypan blue is the most widely used dye substance for the assessment of cellular viability. In most systems trypan blue is presumed to be excluded by viable cells (e.g., dye-exclusion--see below) and will stain only those cells that have died. The degree of association between trypan blue and cellular organelles has not been clearly defined. Furthermore, in some cell lines it has been shown that the dye is actively sequestered within those cells which are considered to be viable (Sawicki et al. 1967). Trypan blue in combination with vital stains has been used to assess embryonic cells of the hamster (Hutz et al. 1985), mouse (Thadani et al. 1982) and monkey (Chan et al. 1982). In these studies, there was a negative association between the staining of the cells of the hamster or monkey embryo with trypan blue and FDA (Chan et al. 1982, Hutz et al. 1985) or of mouse embryos and phenol red (Thadani et al. 1982). Although the teratogenic activity of trypan blue for mammals is widely recognized (see for review: Shepard 1983), trypan blue is generally regarded to be nontoxic to differentiated cells in tissue culture. An additional drawback to the use of azo dyes, such as trypan blue, has been their greater affinity for protein in solution, as compared to the xanthene dyes (Dooley 1979, Phillips 1973). Evan's blue was reported to stain nonviable bovine embryos (Brock and Rowson 1950), while viable blastomeres were apparently not penetrated by the dye. Numerous other nonvital dyes or staining combinations have been used on a limited basis to identify live and dead cells, but they have not been applied for the evaluation of the preimplantation embryos of mammals.

Dye-exclusion assays The basic precept of dye-exclusion is that viable cells are impermeable to dye in the surrounding medium (Kaltenbach et al. 1958), whereas, dead cells are permeable to dye and the dye is not trapped within the cell. The cells which are viable do not stain and appear, when examined using brightfield illumination, lighter (white) than the surrounding background of the dye. Nonviable cells do not exclude dye and stain when the concentration of dye within these cells is equal to or exceeds that of the surrounding environment. Hence, direct examination of cells when mixed with dye gives the impression that there is exclusion of dye by those cells which are viable and the dead cells are stained. The cellular processes which are responsible for dye-exclusion and the appearance of staining are largely unknown. Nonviable cells which take up dye do not respire, glycolyse, or extend cellular processes when replanted in a tissue culture system (Phillips 1973). Dye-exclusion may be a passive response attributable to the presence of an intact cell membrane; however, it is also possible that viable cells do not prevent entry of the dye into the cell, but actively transport the dye out of the cell after it has entered, or alter the dye molecules that are present within the cell, such that their presence cannot be detected visually. As defined here, dye-exclusion does not require any particular association of dye with the intercellular constituents, nor does the assay presuppose any permanent binding of the dye molecules to cellular elements. In practice, dye-exclusion by cells or cellular aggregates suspended in a fluid containing the indicator dye, can be determined after a short exposure and removal from this staining solution, or after extended exposure. On occasion, the

cells are removed from the dye solution and dried immediately after removal, or dried in the dye solution, to ensure that the dye molecules will remain within the cell. This is done because, by definition, there should not be any permanent association of the dye molecule with the components of the cell and the staining of cells which fail to exclude the dye would be transient. Thus, for cells removed from the dye solution, the stain would diffuse out from these cells within minutes to a period of several hours and staining would no longer be detected. As previously mentioned, DAPI and trypan blue are frequently considered to be dyes which are normally excluded by viable cells. However, these dyes either do not appear to act uniformly in all cell lines, or do not dissociate rapidly from cellular organelles. Dyes which appear to be readily excluded by viable cells, include nigrosin, and several dyes of the xanthene series, such as eosin B, eosin Y, erythrosin B, fluorescein, procion B, and rose bengal.

Embryonic and/or maternal factors

At present, there is a tremendous research effort to identify those essential and/or beneficial factors for embryonic development in vivo and in vitro, including factors released by the developing embryo or maternal factors which are induced by the presence of a viable embryo. The identification of essential factors indicative of the presence of a viable embryo or embryos in a female would permit the development of assays to monitor the status of pregnancy prior to or shortly after implantation or to assess the viability of embryos while cultured in vitro or in an intermediate host. Such assays are needed to supplement existing methods

such as rectal palpation, nonreturn to estrus, ultrasound or radiological examination, and immunological diagnosis of pregnancy, which are usually not effective until after implantation has occurred and embryonic growth has advanced. It has been reported for mice (O'Neill 1985a) and humans (O'Neill et al. 1985) that fertilization and/or the presence of an embryo within the oviducts or uterus, stimulates a generalized and quantifiable thrombocytopenia. The occurrence and persistence of this generalized thrombocytopenia has been promoted (O'Neill 1985a) as a method to monitor in vivo embryonic survival during the early preimplantation period of pregnancy. This approach is promising in that reportedly none of the human recipients which failed to display a reduction in blood platelet count subsequently established a pregnancy (O'Neill et al. 1985). In addition to the apparent physiological response to embryonic activity described above, there appears to be an association between the amount of platelet activating factor(s) produced by viable mouse (O'Neill 1985b) and human embryos (O'Neill et al. 1985) during in vitro culture.

Others, have identified high molecular weight glycoproteins and low molecular weight factors released from mouse embryos. To date, a high molecular weight glycoprotein released by the mouse blastocyst (Fishel and Surani 1980), early pregnancy factor (Gidley-Baird et al. 1980), and ovum factor (Cavanagh et al. 1980) have been isolated and the identification of these or related factors may eventually provide a successful tool to monitor in vivo the presence or absence of viable embryos.

STATEMENT OF THE RESEARCH PROBLEM

Several approaches are now used to assess the viability of mammalian embryos during the preimplantation period of development. In practice, the morphological appearance of the embryo remains as the primary and most widely used tool used for the evaluation of embryonic viability. Clearly, the application of any technique for embryonic assessment is limited by the effectiveness and reliability of the technique, by the expense, in terms of equipment, and by the amount of time required to perform the task. None of the dye assays, which have been developed to date, enjoys widespread application toward the evaluation of viability of mammalian embryos, probably because these approaches are either too expensive and time consuming or they simply do not provide a reliable estimate of the status of the embryos that are being evaluated. Rapid and inexpensive techniques that permit or enhance the discrimination between viable and nonviable cells and/or embryos, which can be performed simultaneously or in conjunction with the morphological evaluation of the embryo, are badly needed. Such techniques must not compromise the embryo and should complement the morphological assessment. Ideally, the technique would provide supplemental information regarding the developmental competence or status of the embryo that cannot be obtained by direct microscopic observation.

RESEARCH OBJECTIVE

The overall objective of the studies reported in this thesis was to develop a non-toxic assay based on the exclusion of eosin B for the repeated evaluation of the viability and biological potency of preimplantation embryos. Eosin B, a member of the xanthene dye series, has a formula weight of 624.098 and has been assigned the color index of 45400 by the Biological Stain Commission (Lillie 1969). Eosin B, also referred to as Eosine I Bluish or Acid Red 91 (Merck Index 1983), is a water and alcohol soluble dye, which provides an orange to red-tinged solution, over the pH range of 6.5 to 8.0 and reflects the longer (red) wavelengths, when illuminated with visible light. Eosin B absorbs light over a broad range, 450 to 575 nm (Gurr 1971), with an absorption maximum of 516 to 519 nm (Lillie 1969). The xanthene dyes are weakly fluorescent compounds with green fluorescence, absorbing light at 450 to 500 nm and fluorescing at 510 to 520 nm. Traditionally, eosin B and a chemically and structurally related form, eosin Y, have been used for differential staining of histological preparations (Junqueira et al. 1977, Lillie 1969). Furthermore, the capacity of live cells to exclude this dye has long been known and more recently, eosin B has been developed and validated as a tool to estimate the viability of feline spermatozoa (Dooley 1979, Dooley and Pineda 1986, Dooley et al. 1980).

The rat embryo was selected as the experimental model for these studies because of cost and availability. In addition, we had established a rat colony in the laboratory and mastered the ancillary techniques that would guarantee a regular supply of embryos for the studies. Specific objectives of the research reported in this thesis were:

1) To define the conditions essential to the evaluation of the staining response of embryos exposed to eosin B.
2) To determine the relationship between the staining response and the in vitro developmental capacity of preimplantation embryos.
3) To determine whether a single or multiple exposures to eosin B influenced the cleavage of embryos.
4) To determine the influence of continuous exposure to eosin B on the in vitro development and the staining response of embryos.
5) To determine whether the development of embryos transferred to synchronized recipient rats was influenced by exposure to eosin B and examination prior to transfer.

The results from Experiments 1 to 4 of these studies have been published (Dooley et al. 1984) and the eosin B assay has been successfully used to estimate embryonic viability of preimplantation mouse (Alcivar 1987, Alcivar et al. 1986) and bovine (Dooley et al. 1987) embryos.

MATERIALS AND METHODS

This section of the dissertation has been organized into two parts. The first part includes a description of the animals, procedures and techniques, and general aspects of the experimentation that are common to most or all of the experiments. Subsequently, in the second part, a description is provided for the specific aspects of each experiment, including the objectives, experimental design and procedural details, and the statistical analyses performed to evaluate the data collected.

Animals

Strains utilized

The rat (Rattus norvegicus) was used as the source of preimplantation embryos for the studies reported in this dissertation (see: Robinson 1965 for a review of the taxonomy and the origin of the strains used for these studies). The majority of the experiments were performed using the S-D line of SASCO (SASCO INC., Omaha, NE) Albino Laboratory Rats, derived from the Sprague-Dawley strain of albino rats. The experiments that involved embryo transfer were designed to take advantage of phenotypic markers, coat and eye pigmentation, that could be obtained by using white spotting (piebald or hooded spotting) rats. For this purpose, breeding stock (male) displaying a variety of coat color and pigment patterns were obtained from the Department of Food and Nutrition, Iowa State University (Zucker Rat Colony) and "Irish" spotted (Robinson 1965) male and female rats. These

"Irish spotted" rats were offspring derived from the pairing of hooded rats by the Department of Psychology, Iowa State University. The parental stock from which these offspring were derived had been purchased from Blue Spruce Farms, Inc., Altamont, NY.

Selection criteria

Only apparently healthy and sexually mature rats were used. The animals used as embryo donors were naturally mated with fertile males. Pseudopregnant recipients were obtained after pairing with nonfertile, vasectomized males (Alcivar 1987). All of these rats had a minimal body weight of 200 g and were $\geq$ 100 days of age at the time of assignment to an experiment. The mean body weight and age for the embryo donors and/or recipients and ranges are provided for each experiment.

Care and maintenance

Female and male rats were maintained under controlled temperature (18 to 22°C) and light (14 h light:10 h dark). Female rats were caged individually or in groups, as required by the experimental protocol. Breeding males of proven fertility were maintained in individual cages within the colony. The dark phase of the light cycle extended from midnight to 10:00 AM. The relative humidity of the animal room could not be controlled. The relative humidity was usually within 40 % to 60 %, but was observed to fluctuate seasonally over the range of 25 % to 90 %.

Animals were provided tap water ad libitum and fed a pelleted rodent diet. Dietary source varied during the period in which these studies were performed (Teklad, Winfield, IA; Simonson Mill, Inc., Quimby, IA). However, for all studies, the control and treated animals assigned to a

given experiment were provided pellets of the same composition and from the same commercial source. Routine animal care was provided by Laboratory Animal Resources, College of Veterinary Medicine, Iowa State University. Rats that became unthrifty and obviously diseased were eliminated from the colony (see below for mycoplasma detection and treatment), as soon as they were detected.

Mycoplasma detection and routine medication

A decline in the reproductive performance of animals within the colony was noted during 1985 and by the Spring of 1986, the low reproductive performance of rats in our colony required an aggressive program to diagnose the causes and to monitor and treat the colony. Based upon the clinical signs prevalent in certain animals and the microbiological and pathological examination of body fluids and tissues, it was concluded that the reproductive inefficiency of these rats was likely due to or exacerbated by mycoplasmosis (Mycoplasma pneumoniae, related varieties or species) and possibly Sendai or related viruses. This presumptive diagnosis was later confirmed by 1) culture of biological fluids from affected animals (Dr. R. Ross, Veterinary Medical Research Institute, College of Veterinary Medicine, Iowa State University), 2) indirect immunofluorescent test (Gardella et al. 1983) for antibodies to Mycoplasma pulmonis in rats, and 3) culture of the fluids obtained after lavage of lung tissues and reproductive tracts of apparently healthy donor animals (Dr. F. C. Minion, Veterinary Medical Research Institute, College of Veterinary Medicine, Iowa State University). The presence of antibodies to M. pulmonis was subsequently confirmed by ELISA (Cassell and Brown 1983)

and fluorescent antibody tests. Once the presence of mycoplasma within the colony had been confirmed, Dr. Minion recommended that the entire colony be eliminated and the colony reestablished with noninfected animals. This approach could not be followed because: 1) the cost to replace and reestablish the animal colony, 2) certified sources of mycoplasma-free animals were not readily available and mycoplasma infections are frequently detected in animals from colonies thought to be noninfected, 3) isolation facilities to maintain a mycoplasma-free colony were not available, and 4) the studies reported in this thesis were partially completed at the time the mycoplasma infection was first diagnosed and it was not possible to determine when mycoplasma exposure of the experimental rats had occurred. Therefore the degree of influence, if any, that the mycoplasma infection had on the results of these experiments could not be determined. However, once the diagnosis of mycoplasmosis was confirmed, to correct or minimize the effects caused by mycoplasmosis, the following steps were followed: 1) all rats that evidenced any clinical symptoms of murine respiratory mycoplasmosis were immediately eliminated from the colony, 2) the colony was closed and no further introduction of animals from commercial sources was permitted, and 3) rats were given tetracycline (395 to 500 mg/L; TechAmerica Group, Inc., Elwood, KS) in their drinking water for a minimum of 7 days every month. Tetracycline treatment was repeated at approximately 4 week intervals for the remainder of the study. Once assigned to an experiment, preventive medication with oral tetracycline was discontinued for the embryo donors, which were euthanized for embryo collection. Those experimental rats in which survival surgery was

performed (embryo recipients) received additional treatment of oxytetracycline (Liquamycin, LA-200, Pfizer Agricultural Division, New York, NY) at the time of surgery in the form of two doses (0.1 ml; 4 mg/rat) administered subcutaneously (Experiment 7) or two doses (0.2 ml) of LA-200 which was diluted 1:1 with 0.9 % physiological saline solution (Experiments 8, 9, 10).

Vaginal Smear, Mating, and Assessment of Mating

Vaginal smear

The relationship between the exfoliative cytology of the vaginal epithelium, estrous cyclicity, changes in the reproductive organs, and sexual receptivity have been described (Allen 1931, Bertalanffy and Lau 1963, Hebel and Stromberg 1986, Long and Evans 1922, Mandl 1951, Young et al. 1941). Samples for vaginal cytology were initially obtained by introducing into the posterior vagina a cotton-tipped swab, which had been moistened with 0.9 % physiological saline solution (PSS). The vaginal cells adsorbed to the cotton swab were then smeared onto a glass slide by rolling the swab over the surface of the slide. The slide was subsequently air-dried, prior to fixation and staining. Fixation and staining was performed following guidelines described in commercially available kits (Diff-Quik, Dade Diagnostic, Inc., Aguada, PR; or Leukostat, Fisher Scientific, Fair Lawn, NJ). This swab technique was subsequently replaced by the vaginal lavage technique (Bertalanffy and Lau 1963) to decrease vaginal inflammation and/or bleeding induced by the swabbing, to reduce the

likelihood of inducing cornification due to the smearing technique (Astwood 1939), and to facilitate the detection of spermatozoa in the vaginal secretions. Vaginal lavage was performed by the successive infusion into and withdrawal of 60 to 120 μl of fluid from the rat's vagina. A variety of solutions were used over the course of the studies that form the body of this thesis, for their suitability in terms of cytological appearance, integrity of the vaginal cells, and evidence of not causing vaginal inflammation associated with repetitive examinations. These included: deionized water, PSS, PSS containing 5 to 20 mg/ml oxytetracycline, and PSS containing 0.002, 0.004, and 0.02 % nitrofurazone (Nitrofurazone Solution 0.2 %, TechAmerica Group, Inc., Elwood, KS). The 0.004 % solution of nitrofurazone in PSS appeared to be optimal for the routine vaginal examination and repeated lavage over time in the same rat. The vaginal secretions and exfoliated cells obtained by lavage were assessed directly, as a wet mount and/or air-dried at 70°C. The air-dried smears were fixed, stained, and rinsed, as previously described. The fixed and stained preparations were stored to serve as a permanent record of the vaginal cytology of individual rats.

Mating and assessment of mating

Only those females whose vaginal cytology was indicative of proestrus or estrus were paired with male rats. Because a marked increase in the proportion of abnormal embryos is associated with post-ovulatory mating in the rat (see for review: Austin 1970), the proestrus or estrous rats selected for mating were placed in the male's cage during an 8 hour period preceding the onset of the dark cycle or shortly after the onset of the

dark cycle. This approach minimized the occurrence of post-ovulatory matings between rats and decreased the likelihood that fertilization of aged oocytes would occur, had a post-ovulatory mating taken place (Blandau and Jordan 1941). The female rat was left with the male overnight and mating was confirmed during the light phase of the following morning by vaginal lavage, for the recovery of spermatozoa and/or spermatozoal remnants. On some occasions, estrous rats were observed to exhibit proceptive behavior when placed in the male's cage and/or mating was monitored directly. Additionally, a vaginal copulatory plug was identified on the day after mating for some rats. However, when intact males were used, the most reliable test for the occurrence of mating was the presence of spermatozoa in the vaginal smear. The mated females were caged individually or in groups of no more than 4 mated rats. Rats were randomly paired with a given male, such that no male was used more than 3 times in any given week and in most cases the male was not used more than once per week. Based on prior studies on the rat (Hebel and Stromberg 1986, Shalgi and Kraicer 1978) and preliminary studies in our laboratory, fertilization was predicted to have occurred 12 hours after the onset of the dark cycle (2 hours after the subsequent light period). Thus, the day in which a sperm positive vaginal smear was obtained was designated as day 0 and, for each donor, the stage of embryonic development was predicted on the basis of the time elapsed from the estimated time fertilization to the time of euthanasia and embryo recovery. In this regimen, one could reliably expect to recover 1-cell embryos during the light phase of day 0 and into the dark phase of day 1; 2-cell embryos during the end of the dark phase of day 1

and the light phase of day 1; 4-cell embryos during the light phase of day 2, the dark phase of day 3, and into the light phase of day 3; 8-cell embryos during the light phase of day 3; morulae during the dark phase of day 4 and into the light phase of day 4; blastocysts during the light phase of day 4 and the dark phase of day 5.

Induction of pseudopregnancy

To induce pseudopregnancy, rats to be used as embryo recipients were paired with vasectomized males and observed for evidence of mating, as described above for mating with intact males. Mating of the recipient female with the vasectomized male was confirmed on the basis of one or more of the following criteria: 1) proceptive behavior and receptivity to the male, including the presentation of the lordotic posture when mounted, 2) presence of a vaginal plug on the day following pairing, 3) appearance of flocculence of the vaginal fluids collected during lavage, consistent with that observed in the post-pairing vaginal smear obtained for intact males, except for the absence of spermatozoa, and 4) a marked staining of the vaginal exudate and the cytological profile characteristic of the post-pairing vaginal smear. Rats mated with vasectomized males were assumed to be pseudopregnant and synchronous with females which were mated to intact males during the same period. There were no systematic attempts to monitor the vaginal cytology in pregnant or pseudopregnant rats.

Media for Flushing and Recovery of Embryos

Rats to serve as embryo donors were euthanized at predetermined intervals after mating, according to the previously described regimen, to collect embryos at the needed stage of development. Donors were euthanized by cervical dislocation (Cervical Dislocators, Inc. Shofield, WI). The abdominal cavity was entered using surgical scissors. The uterine horn, oviduct, and ovary were located and then lifted with tissue forceps to facilitate the removal of the supporting connective tissues, ligaments, vascular, and adipose tissues from the tubular reproductive tract and the ovary. Dissection was performed using a scalpel and dissection scissors. The reproductive tract was isolated as the left or right, ovary, oviduct, and uterine horn. Each ovary, oviduct, and uterine horn was transferred to a 60 X 15 mm culture dish (Falcon 1007, Oxnard, CA) containing 8 ml of Ham's supplemented F-10 medium (Whittaker M. A. Bioproducts, Inc., Walkersville, MD). The F-10 medium was supplemented to contain 50 μg/ml of gentamicin sulfate solution (Sigma Chemical Company, St. Louis, MO). The culture dishes containing the supplemented F-10 medium had been pre-equilibrated for a minimum of 30 minutes in an incubator at 37°C, in a humidified atmosphere containing 5 % CO_2 in air. The collection dishes containing the supplemented F-10 medium and the respective left or right reproductive tracts were placed on a countertop, covered with an opaque plastic hood, and allowed to cool to room temperature (20 to 24°C). A gas mixture containing 90 % N_2, 5 % O_2, 5 % CO_2 was bubbled into deionized water and then passed through a 0.2 μm filter, before entering the hood, to

provide humidification and control the pH of the culture media. The collection dishes were maintained under this hood except during the periods of manipulation of the tract necessary for the flushing and recovery of embryos. To recover embryos, the right or left horn and oviduct were transferred to a 60 X 15 mm culture dish containing the supplemented Ham's F-10 medium, as previously described, and also 0.3 % gelatin (Unflavored Gelatine, Knox Gelatine, Inc., Englewood Cliffs, NJ). The culture dishes containing the supplemented F-10 medium and gelatin had also been pre-equilibrated for a minimum of 30 minutes in an incubator at 37°C, in a humidified atmosphere containing 5 % CO_2 in air. Flushing was performed, during observation with a dissection microscope at 7 X or 15 X, using a 0.5 ml glass syringe (B-D Glaspak No. 5290, Becton, Dickinson and Company, Rutherford, NJ) and a 27 or 30 gauge blunted hypodermic needle. The flushing syringe was filled with supplemented Ham's F-10 medium that did not contain gelatin, to avoid plugging of the small bore needles. Embryos expected to be at the 1-, 2-, 3-, and early 4-cell stages were recovered from the oviducts. The oviduct was isolated from the uterine horn at the uterotubal junction and the blunted-end of the hypodermic needle was introduced into the infundibular end of the oviduct. The infundibular end of the oviduct was then clamped around the needle with Dumont No. 5 forceps and the oviduct was flushed, in a pulsatile fashion, with 0.5 ml of fluid. Embryos expected to be at the late 4- and 8-cell stages were recovered from both the oviduct and the oviductal end of the uterine horn. Subsequently, 0.1 to 0.2 ml of flushing fluid was infused into the uterine horn and the uterine horn was clamped with forceps, approximately 1 to 1.5 cm below the

uterotubal junction. The engorged horn and uterotubal junction was then manipulated to mechanically displace fluids and embryos trapped in the oviduct and the uterotubal junction. Embryos expected to be at the late 8-cell, morula, and blastocyst stages were recovered by first manipulating the uterotubal junction, as previously described, and subsequently by severing the uterine horn 1 to 2 cm below the uterotubal junction and then flushing the upper and lower segments of the uterine horn with 0.5 ml of supplemented Ham's F-10 medium without gelatin.

Embryo Retrieval and Handling

Embryo retrieval from the flushing dishes and all subsequent manipulations were performed with the aid of a stereomicroscope. To avoid the potential influence of variation due to manipulation, all embryos were handled similarly for each experiment and the embryos recovered from the right or left side were maintained in separate dishes or wells. Embryos were identified as to the donor and horn of origin from the time of collection until assignment to treatment. This approach was used to minimize variation due to embryonic differences attributable to the micro-environment within the reproductive horn and to the interval from time of euthanasia to isolation.

Embryos were transferred from the flushing dishes with the aid of a mouthpipet aspirator (Dade No. P4519-1, Dade Diagnostics, Inc., Miami, FL). The aspirator was fitted with a 0.2 μm sterile filter, near the site of attachment of the pipet adapter (Dade No. P-4518-1) to the disposable glass

pipet (Dade No. P4518-5X). All of the embryos that were recovered from each donor were transferred to 35 X 10 mm culture dishes (Falcon 1008, Oxnard, CA or Lux 5214, Miles Laboratories, Inc., Naperville, IL) containing culture medium modified (Appendix 1) after Yamamura and Markert (1981).

Embryo Evaluation and Assignment to Treatment

Embryos were initially examined and classified on the basis of morphological characteristics, using a dissection microscope at 50 X. Those embryos selected for study were then transferred to a Multiwell culture dish (Falcon 3047, Oxnard, CA) containing 500 μl of modified culture medium in each well. Embryos were washed 2 more times by transfer between wells and maintained in wells containing 500 μl of modified culture medium, until assignment to treatment.

The number of randomization wells used for a given embryo donor was dictated by the design of the experiment. For each donor, embryos were distributed to wells containing 500 μl of modified culture medium by stage of development and morphological characteristics. Thus, for each experiment, embryos were not assigned to treatment until after the embryos had been washed in modified culture medium at least 3 times and then grouped into approximately equal subsets, based on the number of embryos and the stage(s) of embryonic development. These embryos were then assigned to treatment using randomization procedures which will be described for each experiment.

Embryo Staging

After assignment to treatment and distribution of embryos to individual wells, a further evaluation and classification of each embryo, based upon morphological appearance with regard to the stage of development, was performed with an inverted microscope. Embryos could be examined with the inverted microscope over a range of magnification from 25 X to 250 X and a minimum of two different magnifications were used during each evaluation. However, the 100 X magnification, appeared to be the most convenient magnification for the determination of the developmental stage of rat embryos. During observation, embryos were rotated by swirling the dish to facilitate the determination of the appropriate stage of embryonic development. For the purposes of recording and subsequent statistical evaluation of the data, each embryo was assigned a numerical score (Table 1), which will be referred to as a cleavage index. The scale and descriptive aspects of embryonic development applied to the rat embryo in this study were adapted from the published guidelines of Seidel et al. (1980) for the bovine embryo and correspond to the preimplantation stages of embryos from the rat (Hebel and Stromberg 1986, Shalgi and Kraicer 1978, Suzuki 1973), as well as with stages of embryonic development reported for other species (Boyd and Hamilton 1952, Hasler et al. 1987, Linder and Wright 1983). It should be noted that the later cleavage stages (particularly stage 8 of Table 1) are not frequently detected in the rat and that some sources (Hasler et al. 1987, Seidel et al. 1980) consider bovine embryos with 16 or more cells as morulae, regardless of the apparent

Table 1. Numerical index for the classification of rat embryos

Numerical index	Stage of embryonic development	Comments
0	Indeterminate stage	Includes: unfertilized oocytes; degenerate embryos; embryos with no discernible blastomeres or the stage was unrecognizable
1	1-cell embryo	Oocytes with spermatozoa in the perivitelline space, or with 2nd polar body extruded
2;3;4	2-, 3-, or 4-cell embryos	Early cleavage stages; clearly recognizable blastomeres
5;6	5-7 or 8-cell embryos;	Intermediate cleavage stages
7;8	8-16 or > 16-cell embryo;	Late cleavage stage; individual blastomeres discernible
9	Early morula	Outline of individual blastomeres is still evident
10	Tight morula	Compacted mass; no individual blastomeres evident in cell mass
11	Early blastocyst	Small blastocoel, < 40 % of cell mass
12	Blastocyst	Blastocoel, 40 % to 70 % of cell mass
13	Expanding blastocyst	Blastocoel, > 70 % of cell mass
14	Hatching blastocyst	Zona pellucida ruptured; portion of embryonic mass extruded from zona
15	Hatched blastocyst	Blastocyst is outside of the zona pellucida

degree of cellular association and appearance. Oocytes or those embryos that had extensive morphological abnormalities and/or were fragmented were assigned a value of 0.

Conditions for Embryo Culture

After the embryos had been randomized and assigned to treatment, the dishes containing the embryos to be cultured were placed in an incubator. For all of the studies reported in this thesis, embryo culture was performed at 37.0 ± 0.2°C in a humidified atmosphere of room air supplemented to contain 5.0 ± 0.5 % CO_2. Embryos were maintained in the incubator under this culture regimen, except for those brief periods needed for the manipulation, morphological evaluation, and exposure to dye, as described for each experiment. Unless specified otherwise, embryos were cultured individually in Multiwell dishes (Falcon 3047) containing 500 μl of modified culture medium per well. The culture dishes containing this modified medium for embryo culture had also been pre-equilibrated for a minimum of 30 minutes in an incubator at 37°C in a humidified atmosphere containing 5 % CO_2 in air. As previously stated, the medium used to culture embryos was modified after Yamamura and Markert (1981) and the formulation and characteristics of this medium is described in Appendix 1.

Dye Formulation and Evaluation of Staining

Dye formulation

Dye was prepared as a stock solution to contain 1.0 mg eosin B (Cat. No. 2739; Lot No. 1060; Polysciences, Inc., Warrington, PA) per ml of modified embryo culture medium. According to the manufacturer's specifications, the powdered eosin B used for the studies reported here contained only 88 % eosin B, thus the actual concentration of eosin B in a 1.0 mg/ml solution would be 1,410.0 μM. To prepare the stock solution of eosin B, dye was weighed to ± 0.01 mg (± 1 %) and dissolved during mixing with an appropriate volume of modified culture medium. The stock dye solution was stored in sealed vials at 4°C. Based upon observations made during preliminary studies, 3 dye concentrations, based on total dye content, were selected for the embryo studies reported here: 1X = 120.9 μM or 75.4 μg/ml; 5X = 604.3 μM or 377.1 μg/ml and 10X = 1208.7 μM or 754.3 μg/ml. The different concentrations of dye used for the embryo assays were obtained by dilution of the eosin B stock solution with an appropriate volume of modified culture medium.

Evaluation of the staining response

The staining response of embryos exposed to eosin B was determined by visual evaluation with an inverted microscope under brightfield illumination. The microscope was equipped with a didymium filter (BG 20, Carl Zeiss, Inc., New York, NY) which could be inserted into the light path to enhance the distinction between stained and unstained blastomeres. Details of the staining procedures utilized are described for each

experiment. The staining response of embryos exposed to eosin B was recorded as follows: US = unstained; PS = partially stained, one or more blastomeres remained unstained, during or after exposure to eosin B, in an embryo in which staining was evident; CS = the embryo appeared to be completely stained. In addition to this basic classification, the proportion of the cytoplasmic mass that was stained was estimated. For the purposes of data presentation in tables and for statistical analysis the following numerical values were assigned to the staining responses described above: US = 1; PS = 2; CS = 3.

Embryo Transfer, Monitoring of Pregnancy, and Determination of Embryo Survival

Embryo transfer

Embryo transfer was performed under general anesthesia. Recipient rats received an intramuscular injection of 0.12 to 0.13 ml of Ketamine Hydrochloride (Vetalar, 100 mg/ml, Parke-Davis, Morris Plains, NJ) and were allowed a 10 minute period for induction of anesthesia prior to surgery. During this 10 minute period of induction, the treated and/or control embryos assigned for transfer to the recipient rat were aspirated into a 5 μl Wiretrol Pipet (Drummond Scientific Company, Broomall, PA). The end of each transfer pipet had been previously ground to form a blunted but conical tip to facilitate transfer of embryos into the recipient horn. Approximately 1 to 3 μl of modified culture medium was used as vehicle for embryo loading and deposition within the uterine horn. The presence of the

embryos in the lumen of the transfer pipet before transfer and the absence of embryos within the pipet after transfer was confirmed by examining the glass barrel with a dissection microscope at 20 X. The embryo-loaded pipets were maintained at 37°C in an incubator until embryo transfer. The embryo recipient was removed from the cage and prepared for sterile surgery by clipping the abdominal hair between the nipple lines from the vaginal orifice to the level of the 3rd (from cranial end) pair of mammary glands. A surgical plane of anesthesia was induced by placing the head of the recipient within an anesthesia cone containing a cotton plug wetted with 0.6 to 0.8 ml of methoxyflurane (Metofane, Pitman-Moore, Inc., Washington Crossing, NJ). The abdomen was scrubbed with surgical soap (Nolvasan Surgical Scrub, Fort Dodge Laboratories, Inc., Fort Dodge, IA) and then wiped with a gauze sponge moistened with 70 % ethanol. The recipient rat was placed in dorsal recumbency on a surgical board (Germfree Laboratories, Inc., Miami, FL). A surgical plane of anesthesia was maintained by repositioning the anesthesia cone, as needed. A midventral, 1.5 to 2.0 cm long incision was made through the skin of the abdomen with a scalpel and a No. 25 surgical blade. The abdominal muscles were then clasped with toothed forceps, lifted approximately 1 to 2 cm above the underlying viscera and then penetrated with the blade of the scalpel. The incision was expanded to approximately 1.0 to 1.5 cm with blunted probes. Either a blunted probe (Mall probe) or a feline spay forceps (Snook hook) was used to locate and expose the uterine horn(s). After exposure of the horn, a probe was inserted through the broad ligament of the uterus, positioned underneath the horn, and braced to hold the exposed horn above the level of

the abdomen. The exposed horn was then penetrated with the tip of a Mayo No. 1 atraumatic needle, at a point which did not appear to contain large blood vessels. The amount of force necessary to penetrate the uterine horns appeared to vary within and between rats. During penetration into the lumen of the horn, the tip of the Mayo needle was directed craniad, toward the ovarian end of the tract, and entry of the needle into the lumen did not exceed 5 mm in any rat. The penetrating needle was withdrawn, the embryo transfer pipet was inserted in a craniad direction, through the opening left by the penetrating needle, into the lumen of the exposed horn and threaded approximately 1.0 cm into the lumen of the horn. The embryos loaded in the pipet were then slowly deposited in the horn by advancing the plunger of the Wiretrol pipet. This approach ensured that the embryos were deposited within the lumen of the uterine horn, but beyond the point of penetration by the Mayo needle. The transfer pipet was slowly withdrawn and the uterine horn was then repositioned within the abdominal cavity. The exposed tissues were moistened periodically with a solution of PSS, supplemented to contain 10 % glycerol and 50 μg/ml of gentamicin sulfate. After transfer of the embryos, the incision of the abdominal muscles was sutured with 3-0 surgical silk (Arista, New York, NY), and the opposing skin surfaces were then stapled together (Clay Adams, 9 mm Autoclip Wound Clips, Becton Dickinson and Company, Parsippany, NJ). After surgery, each rat was given a subcutaneous dose of 40 mg of oxytetracycline. The skin staples were removed 17 days after surgery.

Monitoring of pregnancy

Recipient rats were individually caged and monitored daily throughout

the expected gestational period and for at least 4 days beyond the expected date of parturition. Two days before the expected date of parturition (17 days after surgery or 20 days after mating), wood chips were added to each cage as nesting material, and the rat was examined for pregnancy based on abdominal enlargement. The number of pups born, their general health, and evidence of phenotypic markers, when applicable, were recorded. Pups were weaned after at least 28 days had elapsed from the date of parturition. At weaning, the pups were examined for evidence of abnormalities and the sex and phenotypic appearance of the surviving offspring were recorded.

Determination of embryo survival

For the purposes of validation of the eosin B dye-exclusion assay, unstained embryos were considered to be alive. Therefore, any sign of fetal development to late gestation, or implantation remnants after transfer of the dye-exposed embryos to the recipient, was considered proof of embryonic viability at the time of transfer. Thus, those pups that were found dead at the time of parturition, particularly when fetal size was estimated to be $\geq$ 30 mm (crown/rump), and embryonic development was consistent with that detectable by day 18 (Hebel and Stromberg 1986), were identified and included in the data.

Statistical Analysis and Criteria for Significance

The procedures applied for the statistical evaluation of the data will be identified in the description provided for each experiment. For the purposes of interpreting the results of these studies, statistical

significance was established at $P \leq 0.05$. A trend for significance was defined as: $0.05 < P \leq 0.1$. Differences between means or ratios of embryonic responses were tested for significance when the overall statistical analysis indicated an effect of treatment. The specific tests used will be identified in the description for each experiment.

Experiment 1. Determination of the Minimal Temperature Needed to Block Development and Induce Staining of 4- to 16-Cell Rat Embryos when Exposed to Eosin B

The working hypothesis for the studies reported in this thesis was that eosin B could be used to discriminate between dead or alive embryos or blastomeres of the same embryo. Dead embryos would stain when exposed to eosin B, whereas live embryos would remain unstained.

Preliminary studies (Appendix 2), indicated that both the staining response to eosin B and the subsequent development of rat embryos could be influenced by exposure to temperatures over the range of 40°C to 70°C. Further, these preliminary studies suggested that exposing embryos to temperatures between 40°C and 55°C, for as little as 30 minutes, would block development and induce embryonic death because these embryos would stain when exposed to eosin B. Thus, it became necessary to determine the minimal temperature that would kill embryos in order to develop a technique or system of exposing embryos to ensure a readily available source of dead embryos. This source was needed to define the conditions necessary for staining and to characterize the staining responses of embryos, as well as

to provide evidence for a relationship between the staining response and the developmental potential of rat embryos.

Experimental objectives

The objectives of this experiment were to determine: 1) the lowest temperature, within the range of 40°C to 55°C, which would result in developmental arrest and loss of the capacity of rat embryos at the 4- to 16-cell stages to exclude eosin B and 2) whether there is a relationship between the developmental and staining responses of rat embryos.

Experimental design and procedure

A total of 34 albino female rats, weighing 235 to 320 g (mean ± SD = 274 ± 21) at the time of assignment, were mated and then euthanized on day 3 after mating. At the time of euthanasia these donors ranged from 152 to 275 days of age (mean ± SD = 205 ± 27). The embryos recovered from each donor were assessed for stage of development and only the 4- to 16-cell embryos were used in this experiment. To minimize the potential influence of donor on the response of embryos to heat-treatment, the embryos recovered from each donor were divided into 2 groups which were then assigned at random to a control (37°C) or to 1 of the 4 treatment temperatures selected (Appendix 2) for testing: 40°C, 45°C, 50°C, and 55°C. A minimum of 20 embryos were assigned to each control or treatment temperature. The control or treated embryos from each donor were then placed in glass culture tubes (12 mm X 55 mm) containing 1.0 ml of modified culture medium, sealed with polyethylene caps, placed in water baths, and incubated for 30 minutes at the 37°C control temperature or at the assigned treatment temperature. At the end of the 30 minute treatment period, the

tubes containing control or treated embryos were removed from the respective water baths, the polyethylene caps were replaced with gas permeable membranes (Kimble 73670, 13 mm P.M. Cap, Toledo, OH) and the control and treated embryos were cultured in the incubator, 3 to 9 embryos per tube, at 37°C for 24 hours. In this culture system, it was not possible to trace the development of individual embryos because the embryos assigned to control or treatment were maintained and handled as a group for each temperature throughout the period of treatment and culture. Therefore, the assessment of embryonic development was estimated on the basis of the developmental responses of the embryos within the corresponding group. At the end of the post-treatment culture period, the embryos were recovered from the culture tubes and assessed for stage of development. Each embryo was then placed in 35 μl of modified culture medium containing the 1X concentration of eosin B and the staining response was determined after a 15 minute exposure to dye. Exposure of the embryo to eosin B and evaluation of the staining response were performed within special chambers. These chambers were made from 1 X 3 inch culture slides (No. 63-2200, No. 63-2205, No. 63-2210, Carolina Biological Supply Co., Burlington, NC) by cementing (Torr Seal, low vapor pressure resin, Varian, Inc., Palo Alto, CA) a 22 mm outer diameter X 19 mm inner diameter X 2 mm high plastic ring to the flat surface surrounding each depression on the glass slide. A clean glass slide, placed on the plastic ring, served as a transparent cover to minimize evaporation during stain exposure and evaluation of the staining response.

Statistical analysis

For each of the 4 treatment temperatures (40°C, 45°C, 50°C or 55°C), differences in overall developmental and staining responses of treated embryos were compared with those of the corresponding control embryos by χ^2 analysis, using 2 X 3 contingency tables with 2 degrees of freedom (Steel and Torrie 1980). χ^2 analysis, using a 2 X 2 contingency table with 1 degree of freedom and correction for continuity (Yates 1934), was then used to test for differences in ratios for those specific end points in the developmental and staining responses of embryos for which the overall χ^2 analysis indicated a significant effect of treatment. The cleavage and staining indices of the treated embryos, at a given temperature, were compared with those of the corresponding control embryos, using a one-way analysis of variance (Steel and Torrie 1980).

Experiment 2. Influence of Stage of Embryonic Development on the Staining Response of Rat Embryos Exposed to 55°C

The results of Experiment 1 demonstrated that the in vitro development of 4- to 16-cell embryos was blocked by exposure to 55°C for 30 minutes. By my definition, these embryos were "killed", because all of the embryos exposed to this temperature stained completely when exposed to the 1X solution of eosin B. Once these results were obtained for the 4- to 16-cell embryos, it then became necessary to determine whether heating of embryos to 55°C, as performed in Experiment 1, would induce similar effects in rat embryos at stages of embryonic development other than those used for

Experiment 1. This would provide a source of embryos over a range of preimplantation stages to further define the staining response rat embryos to eosin B.

Experimental objective

The objective of this experiment was to determine whether the exposure of rat embryos to 55°C for 30 minutes induces loss of the capacity to exclude eosin B in 1-cell to blastocyst stages.

Experimental design and procedure

A total of 15 albino female rats, weighing 240 to 290 g (mean ± SD = 270 ± 14) at the time of assignment, were mated and then euthanized, at varying intervals after mating, to collect 1-cell to blastocyst stage embryos. At the time of euthanasia these donors ranged from 175 to 222 days of age (mean ± SD = 204 ± 17). In view of the definite results from Experiment 1, no formal control embryos were included in this experiment. The embryos recovered from each donor were exposed to the 55 (± 1°C) treatment for 30 minutes, and then cultured at 37°C for 24 hours, as described for Experiment 1. At the end of the post-treatment culture period, the embryos were recovered from the culture tubes and assessed for stage of development and the staining response to 1X eosin B, as described for Experiment 1.

Statistical analysis

Because no formal controls were provided, statistical analyses were not performed.

Experiment 3. Effect of the Concentration of Eosin B and the Stage of Embryonic Development on the Time Required to Stain Dead Embryos

The results of Experiments 1 and 2 indicated that exposing rat embryos from all of the preimplantation stages to 55°C for 30 minutes blocked development and produced dead embryos, because the embryos became stained during exposure to a 1X solution of eosin B. The 1X solution of eosin B was empirically determined to be the lowest concentration of dye in the modified culture medium that would produce detectable staining of the rat embryo.

Experimental objective

The objective of this experiment was to determine if the interval from exposure to eosin B to initial and to complete staining of embryos, from all of the preimplantation stages, is influenced by the concentration of eosin B and the stage of embryonic development.

Experimental design and procedure

A 3 X 4 factorial design was used to test for the effects of dye concentration and embryonic stage on the time required to stain dead embryos. The factors were concentration of eosin B (1X, 5X, 10X) and stage of embryonic development (Group I = 1- to 4-cell, Group II = 5- to 8-cell, Group III = 9-cell to morula, and Group IV = Blastocyst). A total of 25 albino female rats, weighing 245 to 320 g (mean ± SD = 272 ± 18) at the time of assignment, were mated and then euthanized at varying intervals after mating, to collect embryos from the 1-cell to blastocyst stages. At

the time of euthanasia these donors ranged from 152 to 276 days of age (mean ± SD = 181 ± 37). All of the embryos recovered from each donor were placed in a tube containing modified culture medium, killed by exposure to 55°C for 30 minutes, and then cultured in the incubator, as described for Experiment 1. After heat-treatment and culture, the embryos were removed from the culture tube, the stage of embryonic development was determined, and the embryos were allocated to 1 of 4 groups by stage of development. Then, the embryos from each developmental group were randomly assigned to 1 of the 3 dye concentrations. To determine the staining response to 1X, 5X or 10X eosin B, each embryo was placed in a well containing dye at the assigned concentration in 35 μl of modified culture medium and evaluated for staining at 1 minute intervals, as described for Experiment 1. The time of initial staining and the time at which the embryo was completely stained were determined for each embryo. Initial staining was defined as the time required from exposure to the first detectable staining in an embryo. The time of complete staining was defined as the time elapsed from exposure to dye to staining of > 90 % of the embryonic mass of embryos presumed to be dead. Once complete staining was detected, embryos were further evaluated at 3 minute intervals in an attempt to determine the final staining intensity. The final staining intensity of the embryo was graded as follows: A = light; faint, light-pink tint to the cytoplasm, B = moderate; pink to red hue of the cytoplasm, C = intense; deep-red coloration of the cytoplasm.

Statistical analysis

Analysis of variance (SAS Institute, Inc., Release 5.16, 1985) was used to determine the effects of stage of embryonic development, dye concentration, and the interaction stage of development x dye concentration on the time elapsed from dye exposure to initial and to complete staining. Tukey's ω-procedure was used to test for differences between means of end points for which the analysis of variance indicated a significant ($P \leq 0.05$) F-ratio.

Experiment 4. Effect of a Single Exposure to 1X Eosin B on the In Vitro Development of Rat Embryos

Experiment 3 revealed that killed rat embryos at all preimplantation stages would stain within 8 minutes after exposure to the 1X or higher concentrations of eosin B. Having characterized the staining responses of killed embryos, it then became necessary to determine whether the exposure of live embryos to eosin B would alter their subsequent developmental potential. Because the rat embryo at the 1-, 2-, or early 4-cell stages does not cleave or displays only limited development in vitro (Alcivar 1987) and embryos at the blastocyst stage do not develop further in vitro, only embryos at the late 4- to 8-cell stages were used in this and subsequent culture experiments.

Experimental objective

The objective of this experiment was to determine the effect on the in vitro development of 4- to 8-cell rat embryos of a single, 10 minute

exposure to culture medium containing the lowest effective concentration of dye, 1X eosin B.

Experimental design and procedure

A 2 x 7 factorial design with embryos nested within time was used to test for the influence of eosin B exposure on the in vitro development of 4- to 8-cell rat embryos. The factors were control or exposure to 1X eosin B and development of embryos after exposure to eosin B, as observed at 0 h, immediately after exposure, and at 12 h, 24 h, 36 h, 48 h, 60 h, and 72 h after exposure. A total of 23 albino female rats, weighing 220 to 330 g (mean ± SD = 268 ± 28) at the time of assignment, were mated with fertile males and then euthanized on day 3 after mating. At the time of euthanasia the donors ranged from 125 to 350 days of age (mean ± SD = 207 ± 77). To minimize the variation caused by donor on the response of embryos to dye exposure, the 4- to 8-cell embryos recovered from each donor were divided into 2 groups which were then assigned at random to the control or to the eosin B treatment group. A minimum of 50 embryos at the 4- to 8-cell stage were assigned to each group. Exposure of embryos for 10 minutes to 1X eosin B was performed in a shallow-well depression slide containing the 1X concentration of eosin B in 35 µl of modified culture medium. All of the embryos from the donor that were assigned to treatment were exposed to dye at the same time and in the same eosin B solution. The staining responses of the embryos were determined at the end of the 10 minute period of exposure. Each group of embryos was washed once in 30 µl of eosin B-free, modified culture medium, and each embryo was then transferred for culture to an individual well of Multiwell dishes (Falcon 3047) containing 500 µl

of eosin-free culture medium. Control embryos were handled similarly except that the medium used for each step in the exposure and washing process did not contain any eosin B. Control and treated embryos were cultured for 72 hours. The culture dishes were removed from the incubator at 12 hour intervals and the stage of development was determined for each embryo. The peak index, defined as the highest developmental stage (cleavage index, Table 1) reached by an embryo, was determined at each time of examination, within the period of treatment and culture, and again at the end of the period of culture. As an additional observation, the post-culture staining response was determined at the end of the 72 hour period of culture for each control embryo and again for each eosin B treated embryo, after exposure to 35 μl of the 1X concentration of eosin B, as described above.

Statistical analysis

The overall developmental and staining responses of the eosin B treated embryos were compared with those of the corresponding control embryos by χ^2 analysis, using a 2 x 4 contingency table with 3 degrees of freedom for development and a 2 x 3 contingency table with 2 degrees of freedom for staining (Steel and Torrie 1980). The cleavage indices of the treated embryos were compared with those of the corresponding control embryos over time by analysis of variance (SAS Institute, Inc., Release 5.16, 1985). The conservative F value (Geisser and Greenhouse 1958) was used to establish significance for the effect of time. The peak index recorded at the end of the 72 hour period of culture and the post-culture staining index of the treated embryos were compared to the peak index and

the post-culture staining index for the corresponding control embryos, using a one-way analysis of variance (Steel and Torrie 1980). Tukey's ω-procedure was used to test for differences between means of end points for which the analysis of variance indicated a significant ($P \leq 0.05$) F-ratio.

Experiment 5. Effect of a Single Exposure to 5X Eosin B on the In Vitro Development of Rat Embryos

The results of Experiment 4 indicated that a single exposure of 4- to 8-cell rat embryos to the 1X concentration of eosin B did not block their subsequent development in vitro. Because the staining intensity of dead embryos exposed to the 1X solution of eosin B was more variable and a longer exposure was required for either the initial or complete staining (refer to Experiment 3), it became then relevant to determine whether embryonic development was affected by the exposure of an embryo to a higher concentration of the dye. The 5X concentration of dye was selected for this experiment based on the results of Experiment 3, which indicated that this concentration of dye shortened the interval from exposure to staining and increased the intensity of staining of dead embryos.

Experimental objective

The objective of this experiment was to determine the effect on the in vitro development of 4- to 8-cell rat embryos of a single, 10 minute exposure of embryos to culture medium containing the 5X concentration of eosin B.

Experimental design and procedure

A 2 x 7 factorial design with embryos nested within time was used to test for the influence of eosin B exposure on the in vitro development of 4- to 8-cell rat embryos. The factors were control or exposure to 5X eosin B and development of embryos after exposure to eosin B, as observed at 0 h, immediately after exposure, and at 12 h, 24 h, 36 h, 48 h, 60 h, and 72 h after exposure. A total of 18 albino female rats, weighing 245 to 330 g (mean ± SD = 278 ± 20) at the time of assignment, were used as embryo donors. At the time of euthanasia the donors ranged from 183 to 234 days of age (mean ± SD = 203 ± 13). To minimize the variation caused by donor on the response of embryos to dye exposure, the 4- to 8-cell embryos recovered from each donor were divided into 2 groups, which were then assigned at random to the control or to the eosin B treatment group. A minimum of 50 embryos at the 4- to 8-cell stage were assigned to each group. Exposure of embryos for 10 minutes to 5X eosin B was performed in a shallow-well plastic dish (LUX 5250, SAS-Multiwell Plate, Miles Scientific, Inc.) containing the 5X concentration of eosin B in 50 μl of modified culture medium. All of the embryos from the donor that were assigned to treatment were exposed to dye at the same time and in the same eosin B solution. At the end of the 10 minute exposure, the staining responses of the embryos were determined, as they were being washed, 3 times, by serial transfer to wells of the SAS Plate containing 50 μl of eosin B-free, modified culture medium. After the last washing, each embryo was transferred for culture to an individual well of Multiwell dishes (Falcon 3047) containing 500 μl of eosin-free culture medium. Control embryos were

handled similarly except that the medium used for each step in the exposure and washing process did not contain any eosin B. Control and treated embryos were cultured for 72 hours. The culture dishes were removed from the incubator at 12 hour intervals and the stage of development was determined for each embryo. The peak index for an embryo within the 72 hour period of culture was recorded at the end of the 72 hour period of culture. As an additional observation, the post-culture staining response was determined at the end of the 72 hour period of culture for each control and again for each eosin B treated embryo. In this case, the staining response was determined for each embryo after a 10 minute exposure to 35 μl of culture medium containing the 5X concentration of eosin B, as described for Experiment 3.

Statistical analysis

The overall developmental and staining responses of the eosin B treated embryos were compared with those of the corresponding control embryos by χ^2 analysis, using a 2 x 4 contingency table, with 3 degrees of freedom for development, and a 2 x 3 contingency table with 2 degrees of freedom for staining (Steel and Torrie 1980). The cleavage indices of the treated embryos were compared with those of the corresponding control embryos over time by analysis of variance (SAS Institute, Inc., Release 5.16, 1985). The conservative F value (Geisser and Greenhouse 1958) was used to establish significance for the effect of time. The peak index, recorded at the end of the 72 hour period of culture, and the post-culture staining index of the treated embryos, were compared to the peak index and the post-culture staining index for the corresponding control embryos

using, a one-way analysis of variance (Steel and Torrie 1980). Tukey's ω-procedure was used to test for differences between means of end points for which the analysis of variance indicated a significant ($P \leq 0.05$) F-ratio.

Experiment 6. Effect of Multiple Exposures to 1X Eosin B on the In Vitro Development of Rat Embryos

The results of Experiments 4 and 5 indicated that a single exposure of 4- to 8-cell embryos to the 1X or 5X concentrations of eosin B did not block their subsequent development in vitro. Thus, having established that a single exposure to the 1X or 5X concentrations of eosin B does not kill the embryo, it then became necessary to determine whether multiple exposures of an embryo to dye, would exert a detrimental influence on embryonic viability and development.

Experimental objective

The objective of this experiment was to determine the effect on the in vitro development of 4- to 8-cell rat embryos of multiple, 10 minute exposures to culture medium containing the 1X concentration of eosin B.

Experimental design and procedure

A 2 x 7 factorial design with embryos nested within time was used to test for the influence of exposure to eosin B on the in vitro development of 4- to 8-cell rat embryos. The factors were control or exposure to 1X eosin B and development of embryos after exposure to eosin B at 0 h and again after exposure at 12 h, 24 h, 36 h, 48 h, 60 h, and 72 h. A total of 23 albino female rats, weighing 260 to 335 g (mean + SD = 297 ± 18) at the

time of assignment, were used as embryo donors. At the time of euthanasia the donors ranged from 252 to 320 days of age (mean ± SD = 280 ± 20). To minimize the variation caused by donor on the response of embryos to dye exposure, the 4- to 8-cell embryos recovered from each donor were divided into 2 groups which were then assigned at random to the control or to the eosin B treatment group. A minimum of 50 embryos at the 4- to 8-cell stage were assigned to each group. Exposure of embryos for 10 minutes to 1X eosin B was performed within individual wells of the Multiwell dishes (Falcon 3047) containing the 1X concentration of eosin B in 500 μl of modified culture medium. The staining response and the stage of development of each embryo was determined at the end of the 10 minute exposure to the dye. After each exposure to dye, each embryo was then washed once in 500 μl of eosin B-free, modified culture medium (Appendix 1), and then transferred to a different well of the culture dish that also contained 500 μl of eosin-free modified culture medium which had been incubated at 37°C, 12 hours before the expected transfer of the embryo to the assigned well. This exposure to dye and washing procedure was repeated 6 times, at the specified intervals, during a 72 hour period of culture. At each period of exposure to the dye, the treated embryos were evaluated for the stage of development and staining response. Control embryos were handled similarly except that the culture medium used for each step in the exposure and washing process did not contain eosin B. The peak index for an embryo within the 72 hour period of culture was recorded at the end of the 72 hour period of culture.

As an additional observation, the post-culture staining response was

also determined at the end of the 72 hour period of culture for each control and again for each eosin B-treated embryo. In this case, the staining response was determined for each embryo after a 10 minute exposure to 35 μl of culture medium containing the 1X concentration of eosin B, as described for Experiment 3.

Statistical analysis

The overall developmental and staining responses of the eosin B treated embryos were compared with those of the corresponding control embryos by χ^2 analysis, using a 2 x 4 contingency table with 3 degrees of freedom for development, and a 2 x 3 contingency table with 2 degrees of freedom for staining (Steel and Torrie 1980). The cleavage indices of the treated embryos were compared with those of the corresponding control embryos over time by analysis of variance (SAS Institute, Inc., Release 5.16, 1985). The conservative F value (Geisser and Greenhouse 1958) was used to establish significance for the effect of time. The peak index, recorded at the end of the 72 hour period of culture, and the post-culture staining index of the treated embryos were compared to the peak index and the post-culture staining index for the corresponding control embryos, using a one-way analysis of variance (Steel and Torrie 1980). Tukey's ω-procedure was used to test for differences between means of end points for which the analysis of variance indicated a significant ($P \leq 0.05$) F-ratio.

Experiment 7. Effect of Continuous Exposure to 1X or 5X Eosin B on the In Vitro Development of Rat Embryos

From the results of Experiments 4, 5, and 6, it was concluded that single or multiple exposures of rat embryos to culture medium which contained the 1X concentration of eosin B, or a single exposure of rat embryos to culture medium containing the 5X concentration of eosin B did not impair the subsequent in vitro development of 4- to 8-cell rat embryos. Because a brief period or periods of exposure to eosin B appeared not to cause harm to the rat embryo, insofar as determined by embryonic development in vitro, it became necessary to determine whether continuous exposure of the embryo to the dye would be equally harmless to the embryo. If eosin B was not toxic to the embryo during continuous exposure, then it would be possible to determine the development and viability of embryos while cultured in medium containing eosin B. This would provide an ideal system to evaluate the viability of embryos because there would be no need to remove the embryos from the culture dishes for exposure to the dye for a pre-determined period at each examination.

Experimental objective

The objectives of this experiment were to determine the effects on the in vitro development and staining response of 4- to 8-cell rat embryos when continuously exposed to culture medium containing the 1X or 5X concentration of eosin B.

Experimental design and procedure

A 3 x 7 factorial design with embryos nested within time was used to test for the influence of exposure to eosin B on the in vitro development of 4- to 8-cell rat embryos. The factors were control, continuous exposure to 1X or 5X eosin B, and development of embryos during exposure to eosin B as observed at 0 h, and again at 12 h, 24 h, 36 h, 48 h, 60 h, and 72 h of continuous exposure to the dye. A total of 55 albino female rats, weighing 225 to 350 g (mean ± SD = 278 ± 30) at the time of assignment, were used as embryo donors. At the time of euthanasia these rats ranged from 119 to 293 days of age (mean ± SD = 187 ± 46). To minimize the variation caused by donor on the response of embryos to dye exposure, the 4- to 8-cell embryos recovered from each donor were divided into 3 groups which were then assigned at random to the control, or to the continuous 1X or 5X eosin B treatment groups. A minimum of 50 embryos at the 4- to 8-cell stage were assigned to each group. Embryos assigned to the control group were not exposed to eosin B prior to culture. Each control embryo was transferred to a well containing 500 µl of eosin B-free, modified culture medium and examined at 12 hour intervals for stage of embryonic development during the 72 hour period of culture. Continuous exposure of embryos to the assigned 1X or 5X concentration of eosin B in 500 µl of culture medium was performed within individual wells of the Multiwell dishes (Falcon 3047). The stage of development and the staining response were determined at 12 hour intervals during the 72 hour period of culture. For this purpose, the embryos assigned to the 1X treatment group were transferred to a different well of the culture dish that contained the 1X concentration of eosin B in

500 μl of modified culture medium which had been incubated at 37°C, 12 hours before the expected transfer of the embryo to the assigned well. The peak index was recorded for each embryo at the end of the 72 hour period of culture. The embryos exposed continuously to the 5X concentration of eosin B were handled similarly, except that at 12 hour intervals these embryos were transferred to wells which contained the 1X concentration of eosin B in 500 μl of modified culture medium and were exposed to the dye for 10 minutes before determining the stage of development and the staining response. After each observation, these embryos were then transferred to a different well of the culture dish that contained the 5X concentration of eosin B in 500 μl of modified culture medium, as was described for the embryos continuously exposed to the 1X concentration of eosin B.

As an additional observation, the post-culture staining response was also determined, as described above, for each control embryo and again for each 1X or 5X eosin B-treated embryo.

Statistical analysis

The overall developmental and staining responses of the eosin B treated embryos were compared with those of the corresponding control embryos by χ^2 analysis. A 3 x 4 contingency table, with 6 degrees of freedom, was used to determine treatment effects on development and a 3 x 3 contingency table, with 4 degrees of freedom, was used to determine treatment effects on staining (Steel and Torrie 1980). The cleavage indices of the treated embryos were compared with those of the corresponding control embryos over time by analysis of variance (SAS Institute, Inc., Release 5.16, 1985). The conservative F value (Geisser

and Greenhouse 1958) was used to establish significance for the effect of time. The peak index, recorded at the end of the 72 hour period of culture, and the post-culture staining index of the treated embryos were compared to the peak index and the post-culture staining index for the corresponding control embryos, using a one-way analysis of variance (Steel and Torrie 1980). Tukey's ω-procedure was used to test for differences between means of end points for which the analysis of variance indicated a significant ($P \leq 0.05$) F-ratio.

Experiment 8. Viability of Control or of Rat Embryos Exposed Once to 5X Eosin B and then Transferred to the Left Horn of Naturally Mated Rats

Experiments 4 and 5, demonstrated that rat embryos were developmentally competent after a single exposure to the 1X or 5X concentration of eosin B and it was concluded from Experiment 6 that periodic exposure of embryos to the 1X concentration of eosin B was not harmful. However, the results of Experiment 7 indicated that continuous exposure of the embryo to the 1X concentration of eosin B would alter embryonic development and that embryos were killed by continuous exposure to the 5X concentration of eosin B.

Cleavage of embryos during culture is generally considered to be a proof of viability for those embryos that cleave, as was reviewed earlier in this dissertation. However, the cleavage and differentiation of embryos in vitro does not ensure totipotentiality nor are these developmental

responses reliable predictors of the survivability and development to term after transfer of these embryos to recipient females. Furthermore, several studies, as reviewed earlier in this dissertation, have demonstrated that the post-transfer survival of the embryo declines with the length of in vitro culture prior to transfer. Thus, it became important to validate the results of the previous in vitro studies by determining whether assessment of the viability of rat embryos by exposure to eosin B influenced the development of these embryos to term after transfer to synchronized recipients. In previous studies from our laboratory (Alcivar 1987), pregnancy was established in 54 % of pseudopregnant recipient rats and only 2.7 % of the transferred embryos survived to term when a minimum of 20 morulae and blastocysts, collected on day 5, were transferred to asynchronous females on day 4 (as was defined for this thesis, day 3 corresponds to day 4 in Alcivar's thesis). It was alluded in Alcivar's thesis, that the low percentage of embryonic survival to term of transferred embryos may have been caused by differences in the efficacy of the vasectomized males to induce the pseudopregnant state. In view of those results, and in an attempt to optimize the conditions for transfer and survival to term of the transferred embryos, I decreased the number of embryos to be transferred to $\leq$ 9 embryos, and in addition, transferred the embryos to the uterine horn of naturally mated, instead of pseudopregnant rats. To differentiate the offspring derived from the natural mating from the offspring derived from embryo transfer, female rats were mated to a male of a different phenotype, such as to ensure that embryos of a known and recognizable phenotype were transferred, either as a control or as

eosin B-exposed embryos. Embryos were only transferred to the left uterine horn to minimize the handling of the reproductive tract during the surgical procedure and because the left horn was easier to expose when a midventral abdominal incision was made.

Experimental objective

The objective of this experiment was to determine whether a single, 10 minute exposure of embryos to the 5X concentration of eosin B influenced embryonic survival to term after transfer to the left uterine horn of naturally mated rats.

Experimental design and procedure

A total of 14 albino female rats, weighing 300 to 350 g (mean ± SD = 313 ± 24) at the time of assignment, were mated for use as embryo donors. The embryo donors were mated to albino male rats of proven fertility or to male rats proven to be homozygous and dominant for coat color and eye pigmentation. The albino males were expected to produce embryos that would result in nonpigmented offspring at term (pink eyes) and weaning (pink eyes; white fur). The colored males were expected to produce embryos that would result in pigmented offspring at term (dark eyes) and weaning (dark eyes; pigmented fur in the hooded pattern). Depending on the phenotype of the male used for mating with the embryo donor, the recipients were then mated to males of the contrasting phenotype. This approach ensured the identification of the embryonic source of any offspring that were born following treatment and surgical transfer of embryos into the naturally mated recipients. Embryos were collected from the donors on day 4 after mating. At the time of euthanasia for embryo collection, these rats ranged

from 234 to 291 days of age (mean ± SD = 264 ± 18). The embryos recovered from each donor were assessed for stage of development and only morulae and blastocysts were used in this experiment. Prior to transfer and to minimize the influence of donor on the recipient and response of the embryos after transfer, for each donor, the embryos were divided into 2 groups which were then assigned at random to a control and to a eosin B exposure group. Control and treated embryos were transferred to different, asynchronous, recipient rats on day 3 of pregnancy. This approach was not always possible due to the failure to obtain 2 synchronously mated recipients on a given day. In these instances (5 of 13 transfers) all of the donor embryos were randomly assigned to either eosin B treatment or control and only 1 transfer was performed. Although no more than 9 embryos were to be transferred to any recipient, the number of embryos transferred was also dictated by the total number of embryos available for transfer on a given day. A total of 18 albino female rats, weighing 290 to 350 g (mean ± SD = 319 ± 15) at the time of assignment, had been mated with males of known fertility, as described above, to serve as synchronized embryo recipients. At the time of transfer these recipients ranged from 231 to 283 days of age (mean ± SD = 263 ± 15). Treated embryos were exposed, as a group, for 10 minutes to the 5X concentration of eosin B in 500 μl of modified culture medium in individual wells of the Multiwell dishes (Falcon 3047). The staining responses of the embryos were determined, as they were being washed, 3 times, by serial transfer to wells containing 500 μl eosin B-free, modified culture medium. Treatment and washing was performed approximately 1 hour prior to transfer. Control embryos were handled

similarly except that these embryos were not exposed to eosin B prior to transfer. The following endpoints were recorded: phenotype, staining response of the eosin B-exposed embryos, and number of embryos that were transferred to each recipient; number of recipients which produced a litter and the overall litter size, including the number of pups born and number of pups weaned which were of the same phenotype as for the donor used to supply the control or treated embryos.

Statistical analysis

Differences in the ratios of females that carried pregnancy to term after transfer of embryos exposed to eosin B and gave birth to at least 1 pup were compared with those of the corresponding control group by χ^2 analysis, using a 2 X 2 contingency table with 1 degree of freedom and correction for continuity (Yates 1934). χ^2 analysis, using a 2 X 2 contingency table with 1 degree of freedom and correction for continuity (Yates 1934), was used to test differences in ratios for the number of pups born and number of pups weaned, which were the result of surgical transfer to left uterine horn.

Experiment 9. Viability of Control or of Rat Embryos Exposed Once to 5X Eosin B and then Transferred to Both Horns of Naturally Mated Rats

The results of Experiment 8 demonstrated that rat embryos which were found to be viable because they did not stain when exposed to the 5X concentration of eosin B, developed to term after transfer to naturally

mated recipients and produced live offspring. However, there were marked differences in the survival of transferred embryos among rats and these differences were not related to the treatment of the embryos with the 5X concentration of eosin B. An after the fact inspection of the data collected in Experiment 8 raised the question whether the transfer of embryos to only the left horn may have created an artificial overcrowding in that horn for some rats. If overcrowding had occurred, this may have prevented the implantation and subsequent development of the transferred control or eosin B-treated embryos, because of competition for implantation sites with the recipients own (self) embryos. Thus, it was necessary to determine whether the transfer of control or eosin B-exposed embryos to both horns of naturally mated recipients would produce a better distribution of transferred embryos and result in the birth of a larger number of offspring.

Experimental objective

The objective of this experiment was to determine whether a single, 10 minute exposure of embryos to the 5X concentration of eosin B influenced embryonic survival to term after transfer to both uterine horns of naturally mated rats.

Experimental design and procedure

A total of 13 albino female rats, weighing 290 to 350 g (mean ± SD = 315 ± 18) at the time of assignment, were mated to albino males of known fertility to serve as embryo donors. As described for Experiment 8, the albino recipient rats were mated to a male of a different phenotype, such as to ensure the offsprings born from the self embryos for these rats could

be distinguished from the those derived from either the control or eosin B treated, transferred embryos. Embryos were collected from the donors on day 4 after mating. At the time of euthanasia for embryo collection, these rats ranged from 237 to 365 days of age (mean ± SD = 337 ± 37). The embryos recovered from each donor were assessed for stage of development and only morulae and blastocysts were used in this experiment. Prior to transfer and to minimize the influence of donor on the recipient and response of the embryos after transfer, for each donor the embryos were divided into 2 groups which were then assigned at random to a control and to a eosin B exposure group. Control and treated embryos were transferred to different, asynchronous, recipient rats on day 3 of pregnancy. As for Experiment 8, this approach was not always possible due to the failure to obtain 2 synchronously mated recipients on a given day. In these instances (5 of 9 transfers) all of the donor embryos were randomly assigned to either eosin B treatment or control and only 1 transfer was performed. The embryo recipients were albino rats mated to males of proven fertility and proven to be homozygous and dominant for coat color and eye pigmentation, which ensured that the eyes and coats of their offspring would be pigmented. A total of 9 female rats weighing 260 to 360 g (mean ± SD = 310 ± 28) at the time of assignment had been mated with males of known fertility, as described above, to serve as synchronized embryo recipients. At the time of transfer these recipients ranged from 300 to 327 days of age (mean ± SD = 310 ± 10). Although all possible transfers were performed from the available donor population, a minimum of 6 embryos or a maximum of 10 embryos were transferred to each recipient rat. Attempts were made to

transfer equal numbers of embryos, 3 or 5, respectively, to each horn of the recipient. Exposure of the embryos to the 5X concentration of eosin B and the subsequent post-treatment evaluation and handling prior to transfer was done, as described for Experiment 8. Control embryos were handled similarly except that these embryos were not exposed to eosin B prior to transfer. The following endpoints were recorded: phenotype, staining response of the eosin B-exposed embryos, and number of embryos that were transferred to the left and right horn of each recipient; number of recipients which produced a litter and the overall litter size, including the number of pups born and number of pups weaned which were of the same phenotype as for the donor used to supply the control or treated embryos.

Statistical analysis

Differences in the ratios of females that carried pregnancy to term after transfer of embryos exposed to eosin B and gave birth to at least 1 pup were compared with those of the corresponding control group by χ^2 analysis, using a 2 X 2 contingency table with 1 degree of freedom and correction for continuity (Yates 1934). χ^2 analysis, using a 2 X 2 contingency table with 1 degree of freedom and correction for continuity (Yates 1934), was used to test differences in ratios for the number of pups born and number of pups weaned, which were the result of surgical transfer of control or eosin B treated embryos. Because the embryos transferred to the left and right uterine horns of a given rat were of the same phenotype and because the number of "self" embryos present in each uterine horn could not be ascertained a priori, it was not possible to determine whether the recipient uterine horn had influenced the survival to term of the

transferred embryos.

Experiment 10. Viability of Control or of Rat Embryos Exposed Once to 5X Eosin B and then Transferred to Opposite Horns of the Same Pseudopregnant Rat

Experiments 8 and 9 clearly established that exposure of embryos to eosin B did not affect development and survivability to term, because, regardless of treatment, live offspring were born. Unfortunately, the design used for Experiments 8 and 9 did allow me to test for the influence of the number of "self" embryos present in the uterine horn(s), contributing to the overcrowding caused by the number of embryos transferred. Thus, it became necessary to perform Experiment 10, transfer of embryos into pseudopregnant rats, to eliminate a potential overcrowding effect caused by the transfer of additional embryos into a uterine horn or horns of rats carrying "self" embryos.

Experimental objective

The objective of this experiment was to determine whether a single, 10 minute exposure of embryos to the 5X concentration of eosin B influenced embryonic survival after transfer of control and treated embryos to opposing horns of the same rat.

Experimental design and procedure

A total of 27 female rats, weighing 240 to 360 g (mean ± SD = 285 ± 28) at the time of assignment, were mated with male rats of known fertility to serve as embryo donors. The embryo donors consisted of 13 albino female

rats mated to albino males and 14 pigmented female rats mated to male rats homozygous and dominant for both coat and eye pigmentation. Embryos were collected from the donors on day 4 after mating. At the time of euthanasia for embryo collection, these rats ranged from 140 to 349 days of age (mean ± SD = 234 ± 68). For each transfer, a minimum of 1 donor female rat of each phenotype (albino or pigmented) was mated. The embryos recovered from each donor were assessed for stage of development and only morulae and blastocysts were used in this experiment. Prior to transfer, the embryos recovered from each donor were allocated by phenotype to serve as either control or eosin B treated embryos. The allocation of embryos by phenotype was done by alternating the uterine horn to ensure a balanced distribution of control or eosin B treated embryos in the left and right horns, such that the uterine horn, phenotype, and treatment could be ascertained in the offspring born to each recipient. A total of 10 virgin, female rats, weighing 250 to 340 g (mean ± SD = 283 ± 34) at the time of assignment, were used as embryo recipients. At the time of transfer, these rats ranged from 147 to 230 days of age (mean ± SD = 200 ± 29). Only those virgin females which had been observed to pair with a vasectomized male rat were use as recipients. Virgin females were used as recipients, in an attempt to have the option of identifying the implantation remnants within the horns assigned to the treatment group or the corresponding control group. To induce pseudopregnancy, each recipient was mated with a vasectomized male and mating was performed such that the female would be synchronized to be in day 3 of pseudopregnancy at the time of the transfer of embryos collected on day 4 of pregnancy from the donors. Although all possible

transfers were performed from the available donor populations, a minimum of 3 control and 3 treated embryos and a maximum of 10 control and 10 treated embryos were transferred. Exposure of the embryos to the 5X concentration of eosin B was performed, as described for Experiment 8. Control embryos were handled similarly except that these embryos were not exposed to eosin B prior to transfer. The following endpoints were recorded: phenotype, staining response of the treated embryos, and number of control and treated embryos transferred to each recipient; number of recipients which produced a litter, number of pups born, and number of pups weaned which were the result of transfer of control and treated embryos.

Recipient rats were euthanized after weaning of the pups, or 23 to 40 days after the expected day of parturition for those rats that did not deliver pups, to determine the number of implantation sites within each horn and to estimate the number of embryos to implant after transfer that may have died during gestation.

Statistical analysis

To estimate the effect of horn, the differences in the ratios of the recipient horn that carried pregnancy to term after transfer of embryos exposed to eosin B and gave birth to at least 1 pup resulting from transfer were compared with those of the corresponding control group by χ^2 analysis, using a 2 X 2 contingency table with 1 degree of freedom and correction for continuity (Yates 1934). χ^2 analysis, using a 2 X 2 contingency table with 1 degree of freedom and correction for continuity (Yates 1934), was used to test differences in ratios for the number of pups born and number of pups weaned. Rao's Test (Rao 1952) was used to determine the effects of

recipient horn, treatment and the recipient horn x treatment interaction on the ratio of control or eosin B-exposed embryos to survive to term from the corresponding total number of control or treated embryos transferred.

Experiment 11. Viability of Control or of Rat Embryos Exposed Once to 5X Eosin B and then Transferred to Both Horns of Pseudopregnant Rats

The preceding experiments clearly established the viability of embryos after exposure to eosin B and after transfer to naturally mated rats (Experiments 8 and 9) or to pseudopregnant rats (Experiment 10). However, the results of Experiment 10 indicated an extreme variation regarding the survivability of control or treated embryos transferred between horns of the same rat. Because of the small number of embryos transferred and the design used for Experiment 10, analysis of the data did not clearly differentiate between the effects of treatment, donor, and recipient horn. By random occurrence, for those 6 rats that produced offspring in Experiment 10, the majority of the embryos transferred to the left horn of these rats were treated embryos (28 eosin B-exposed embryos versus 13 control embryos, Table 32) and the majority of the embryos transferred to the right horn of these rats were control embryos (33 control embryos versus 7 eosin B-exposed embryos, Table 32). In an attempt to confirm whether the effect of horn observed in Experiment 10 was real, I performed Experiment 11.

Experimental objective

The objective of this experiment was to determine whether a single, 10 minute exposure of embryos to the 5X concentration of eosin B influenced embryonic survival after transfer to both uterine horns of the same pseudopregnant rat.

Experimental design and procedure

A total of 44 female rats, weighing 265 to 360 g (mean ± SD = 304 ± 22) at the time of assignment, were mated with male rats of known fertility to serve as embryo donors. The embryo donors consisted of 4 albino female rats mated to albino males and 40 pigmented females mated to male rats homozygous and dominant for both eye and coat pigmentation. Embryos were collected from the donors on day 4 after mating. At the time of euthanasia these rats ranged from 283 to 408 days of age (mean ± SD = 369 ± 34). The embryos recovered from each donor were assessed for stage of development and only morulae and blastocysts were used in this experiment. Prior to transfer, the embryos recovered from each donor were allocated to either a control or an eosin B treated group. Embryos were allocated to treatment groups, such that control and dye-exposed embryos were alternated in successive transfers and the number of embryos transferred was balanced between horns for each recipient. Although all possible transfers were performed from the available donor population, a minimum of 10 control or treated embryos and a maximum of 20 control or treated embryos were transferred. A total of 12 female rats, weighing 230 to 350 g (mean ± SD = 262 ± 43) at the time of assignment, were used as embryo recipients. At the time of transfer these rats ranged from 100 to 316 days of age (mean ±

SD = 212 ± 95). To induce pseudopregnancy, each recipient was mated with a vasectomized male and mating was performed, such that the female would be synchronized to be in day 3 of pseudopregnancy at the time of the transfer of embryos collected on day 4 of pregnancy from the donors. Exposure of the embryos to the 5X concentration of eosin B was performed, as described for Experiment 8. Control embryos were handled similarly except that these embryos were not exposed to eosin B prior to transfer. The following endpoints were recorded: treatment, staining response of the eosin B-exposed embryos, and number of embryos transferred to each recipient; number of recipients which produced a litter, number of pups born and number of pups weaned which were the result of transfer of control or treated embryos.

Recipient rats were euthanized after weaning of the pups, or 33 days after the expected day of parturition for the 1 virgin recipient that did not deliver any pups, to determine the number of implantation sites within each horn and to estimate the number of embryos to implant after transfer that may have died during gestation.

Statistical analysis

Differences in the ratios of females that carried pregnancy to term after transfer of eosin B-exposed embryos and gave birth to at least 1 pup, were compared with those of the corresponding control embryos by χ^2 analysis, using a 2 X 2 contingency table with 1 degree of freedom and correction for continuity (Yates 1934). χ^2 analysis, using a 2 X 2 contingency table with 1 degree of freedom and correction for continuity (Yates 1934), was used to test differences in ratios for the number of pups

born and number of pups weaned, which were the result of surgical transfer of control or treated embryos.

RESULTS

Experiment 1. Determination of the Minimal Temperature Needed to Block Development and Induce Staining of 4- to 16-Cell Rat Embryos when Exposed to Eosin B

A total of 232 embryos at the 4- to 16-cell stages were recovered from 32 donors and used for Experiment 1. The number of 4- to 16-cell embryos recovered from each donor ranged from 0 to 16 (mean ± SD = 7.3 ± 6.1). Table 2 summarizes the results of the χ^2 analyses to determine the overall effects of heat-treatment and Table 3 shows the developmental and staining responses of the control and treated embryos. The unequal number of embryos for the control and treatment groups was the result of unequal numbers of embryos at randomization and/or embryo losses during recovery. The in vitro development of rat embryos exposed to 40°C for 30 minutes was not different ($P > 0.05$) from that of the paired control embryos (Table 2). The in vitro development of rat embryos, based on the cleavage response and the formation of a blastocoel during the post-treatment in vitro culture, was reduced ($P < 0.001$, Table 2) for those embryos exposed to 45°C, 50°C, or 55°C. Nine of the 27 embryos in the 45°C group cleaved at least once, but none developed to the blastocyst stage, whereas 24 of 28 of the paired control embryos cleaved at least once and 16 of 28 embryos formed a blastocoel during the culture period (Table 3). Post-treatment development was not observed in any of the embryos exposed to 50°C or 55°C.

Table 2. Summary of χ^2 analyses to determine the overall effect of treatment temperature on the developmental and staining responses of rat embryos (Experiment 1)

Treatment temperature	Developmental response			Staining response		
	χ^2	df	P	χ^2	df	P
40°C	2.04	2	> 0.05	1.37	2	> 0.05
45°C	24.96	2	< 0.001	3.75	2	> 0.05
50°C	62.0	2	< 0.001	53.82	2	< 0.001
55°C	43.14	2	< 0.001	54.0	2	< 0.001

df = degrees of freedom.

P = Probability level.

The staining responses of embryos exposed to 40°C or 45°C was not different ($P > 0.05$) from the controls, but there was a significant ($P < 0.001$, Table 2) effect of treatment on the staining responses of embryos exposed to 50°C or 55°C. Partial or complete staining was detected in all of the embryos exposed to 50°C or 55°C (Table 3) and all of the embryos in the 55°C group were completely stained when evaluated 15 minutes after exposure to the eosin B solution (Table 3). The mean staining index tended ($P < 0.1$) to be higher and the developmental index was lower ($P < 0.001$) and less variable for the embryos in the 45°C group as compared to the 37°C control group (Table 4). Figure 1 displays the variations in staining responses that were observed among rat embryos following treatment and culture.

Table 3. Developmental and staining responses of control and heat-treated rat embryos (Experiment 1)

Treatment temperature	Number of embryos	Developmental response			Staining response		
		Did not cleave	Cleaved	Formed blastocoel	US	PS	CS
Control	27	5	6	16	25	1	1
40°C	32	9	10	13	30	2	0
Control	28	4	8	16	24	4	0
45°C	27	18[a]	9	0[a]	17	10	0
Control	32	0	8	24	28	4	0
50°C	32	32[a]	0[a]	0[a]	0[a]	7	25[a]
Control	26	3	15	8	25	1	0
55°C	28	28[a]	0[a]	0[a]	0[a]	0	28[a]

Did not cleave = number of embryos in which no further development was obtained during the culture period.

Cleaved = number of embryos that cleaved during the culture period, but did not form a blastocoel.

Blastocoel = number of embryos which formed a clearly defined blastocoel during the culture period.

[a]Significantly ($P < 0.05$) different from the corresponding controls.

Table 4. Cleavage and staining indices of control and heat-treated rat embryos (Experiment 1)

Treatment temperature	Number of Donors	Number of Embryos	Post-treatment indices* Cleavage	Post-treatment indices* Staining
Control	6	27	10.1 ± 3.1	1.1 ± 0.4
40°C		32	9.1 ± 3.1	1.1 ± 0.3
Control	5	28	10.0 ± 3.0	1.1 ± 0.4
45°C		27	6.4 ± 0.8[a]	1.4 ± 0.5[+]
Control	6	32	11.1 ± 2.5	1.1 ± 0.3
50°C		32	5.9 ± 0.3[a]	2.8 ± 0.4[a]
Control	5	26	8.6 ± 2.6	1.0 ± 0.2
55°C		28	5.8 ± 0.6[a]	3.0 ± 0.0[a]

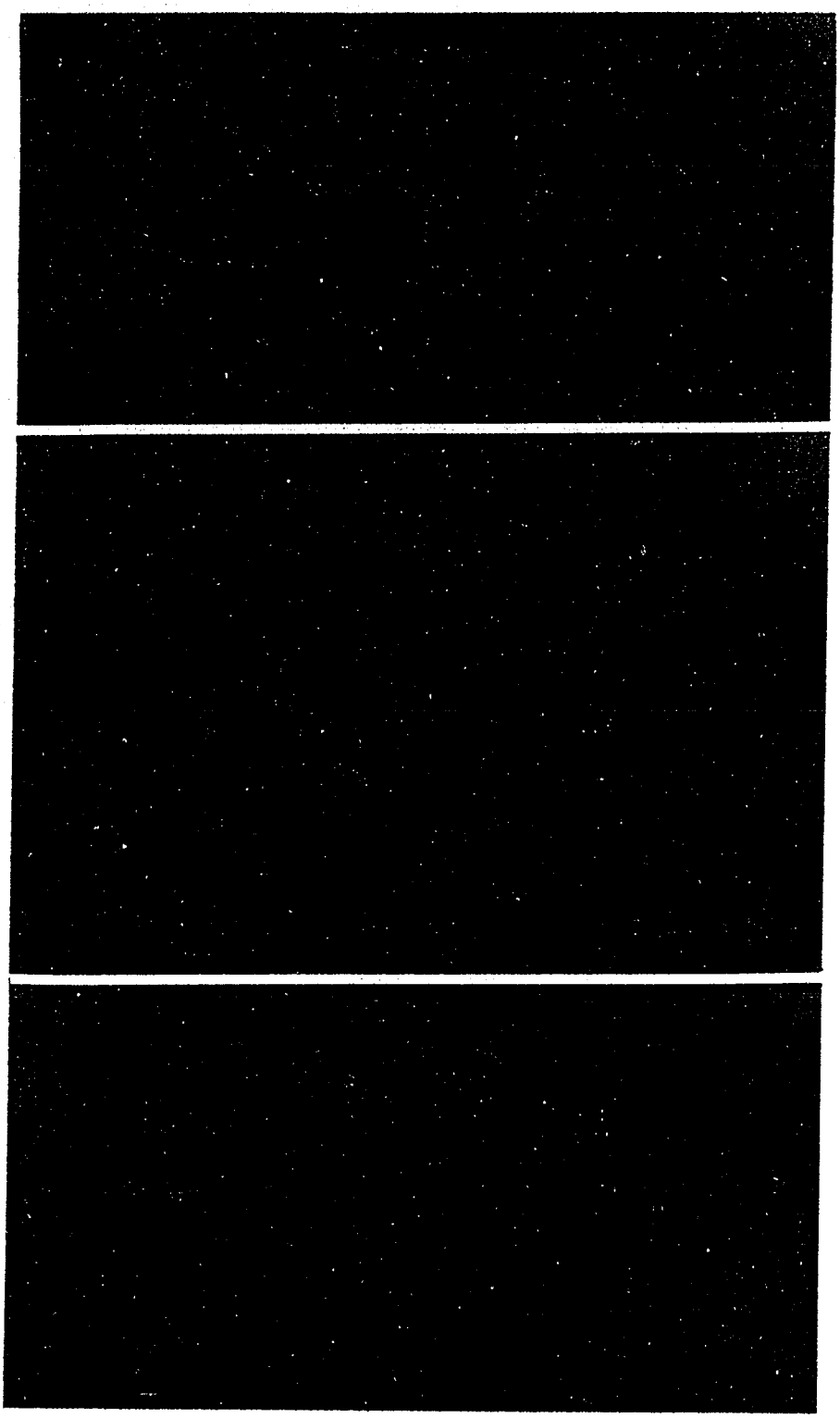
Data are presented as mean ± SD.

*Cleavage or staining index determined at the end of the post-treatment culture period.

[a]Significantly ($P < 0.001$) different from the corresponding control.

[+]There was a trend ($P < 0.1$) for an effect of treatment when compared to the corresponding control for that treatment group.

Figure 1. Heated-treated rat embryos exposed to eosin B after a 24 hour period of in vitro culture (Experiment 1).
A = Unstained and partially stained rat embryos at the 4-cell stage, which were exposed to 45°C prior to culture.
B = Completely stained rat embryos at the 5- to 8-cell stages which were exposed to 55°C prior to culture.
C = Unstained and partially stained control rat embryos classified as degenerate (upper portion of plate), early blastocyst (lower left) and expanded blastocyst (lower right)



Experiment 2. Influence of Stage of Embryonic Development on the Staining Response of Rat Embryos Exposed to 55°C

A total of 132 embryos ranging from the 1-cell to blastocyst stages were recovered from 15 donors and used for Experiment 2. The number of embryos recovered from each donor ranged from 0 to 15 (mean ± SD = 8.8 ± 5.3). As anticipated from the results of Experiment 1, exposure of embryos to 55°C resulted in embryonic death and all of the embryos, regardless of the stage of development at the time of exposure to 55°C, were completely stained by the 1X solution of eosin B (Table 5).

Table 5. Developmental and staining responses of heat-treated (55°C) rat embryos as a function of developmental stage (Experiment 2)

Stage	Number of embryos	Developmental response			Staining response		
		Did not Cleave	Cleaved	Formed blastocoel	US	PS	CS
1- to 3-cell	76	76	0	0	0	0	76
4- to 8-cell	26	26	0	0	0	0	26
Morula	4	4	0	0	0	0	4
Blastocyst	26	NA	NA	23*	0	0	26

NA = Not applicable.

*Twenty-three of the 26 embryos retained a blastocoel after treatment and culture and 3 blastocysts degenerated during culture.

Experiment 3. Effect of the Concentration of Eosin B and the Stage of Embryonic Development on the Time Required to Stain Dead Embryos

A total of 200 embryos ranging from the 1-cell to blastocyst stages were recovered from 25 donors and used for Experiment 3. The number of embryos recovered from each donor ranged from 0 to 18 (mean ± SD = 8.0 ± 6.3).

Initial staining

The interval from exposure to eosin B, to initial staining, was influenced (analysis of variance, Appendix 4) by the stage of embryonic development ($P < 0.005$), concentration of dye ($P < 0.0001$), and the interaction stage of development x concentration of dye was also significant ($P < 0.0001$). When observed at 1 minute after exposure to eosin B, staining was detected in all of the embryos exposed to the 5X and 10X concentrations of eosin B, regardless of the stage of embryonic development when killed by incubation at 55°C (Table 6). Because the "true" time of initial staining for the embryos exposed to the 5X and 10X concentrations of eosin B was obviously less than the 1 minute interval provided by the design of this experiment, the validity of applying statistical analysis to these data as a 3 x 4 factorial experiment is questionable because the variance of error would not be normally distributed among all of the groups. In view of these findings, analysis of variance was applied only to the values for initial staining obtained using the 1X concentration of eosin B (Table 6). The results of this,

Table 6. Effect of dye concentration and stage of embryonic development on the time required for initial staining of heat-treated rat embryos exposed to eosin B (Experiment 3)

Developmental group (Stage)	Mean time for initial staining (minutes) 1X eosin B	5X eosin B	10X eosin B
I (1- to 4-cell)	1.1 ± 0.3[a] (n = 17)	≤ 1.0 ± 0.0 (n = 22)	≤ 1.0 ± 0.0 (n = 14)
II (5- to 8-cell)	1.1 ± 0.5[a] (n = 14)	≤ 1.0 ± 0.0 (n = 14)	≤ 1.0 ± 0.0 (n = 12)
III (9-cell to morula)	1.2 ± 0.5[a] (n = 16)	≤ 1.0 ± 0.0 (n = 19)	≤ 1.0 ± 0.0 (n = 17)
IV (Blastocyst)	2.6 ± 2.4[b] (n = 18)	≤ 1.0 ± 0.0 (n = 18)	≤ 1.0 ± 0.0 (n = 19)

Data are presented as the mean ± SD.

Means in the column for the 1X concentration of eosin B which do not have a common superscript letter are different ($P < 0.05$).

after the fact, analysis for the 1X concentration indicated that the time of initial staining was influenced ($P < 0.005$) by the stage of development. The embryos at the early cleavage stages (Groups I to III) became stained in less ($P < 0.05$, Table 6) time than that required by embryos at the blastocyst stage. The mean time for initial staining of blastocysts by the 1X concentration of eosin B (2.6 minutes) was over 2 times longer than that required for embryos at earlier stages (Groups I to III, Table 6). The time required for all of the rat embryos from the 1-cell to the blastocyst stages to become stained by the 1X concentration of eosin B (data not shown in Tables) ranged from 1 to 8 minutes. The overall mean interval from dye exposure to initial staining of blastomeres exposed to 1X eosin B for all stages (1.6 minutes) was longer ($P < 0.05$, Table 7) than the time required for the 5X (≤ 1.0 minute) and 10X (≤ 1.0 minute) treatments.

Table 7. Influence of dye concentration on the overall mean time interval required from exposure to eosin B to initial staining of heat-treated rat embryos exposed to eosin B (Experiment 3)

Developmental group (Stages)	Mean time for initial staining (minutes) 1X eosin B	5X eosin B	10X eosin B
I to IV (1-cell to blastocyst stages)	1.6 ± 1.5[a] (n = 65)	≤ 1.0 ± 0.0[b] (n = 73)	≤ 1.0 ± 0.0[b] (n = 62)

Data are presented as the mean ± SD.

Means which do not have a common superscript letter are different ($P < 0.05$).

Complete staining

The interval required for heat-treated embryos to become completely stained by the eosin B solution was influenced (analysis of variance, Appendix 4) by the stage of embryonic development ($P < 0.0001$), concentration of dye ($P < 0.0001$), and the interaction stage of development x concentration of dye was also significant ($P < 0.0001$). Embryos at the early cleavage stages (Groups I and II) became stained in significantly ($P < 0.05$) less time than that required by the more advanced (Groups III and IV) embryonic stages (Table 8). The time required (not shown in Tables) for rat embryos to become completely stained by the eosin B ranged from 1 to 14 minutes for the 1X level, 1 to 9 minutes for the 5X level, and 1 to 5 minutes for 10X level of eosin B. The overall mean interval from dye exposure to complete staining of blastomeres exposed to 1X eosin B for all stages (4.6 minutes) was significantly ($P < 0.05$, Table 9) longer than the time required for the 5X (1.5 minutes) and 10X (1.4 minutes) eosin B concentrations. The staining intensity (Figure 2) also appeared to be influenced by the concentration of dye and was more variable for the embryos exposed to the 1X concentration of eosin B.

Table 8. Effect of dye concentration and stage of embryonic development on the time required for complete staining of heat-treated rat embryos exposed to eosin B (Experiment 3)

Developmental group (Stage)	Mean time for complete staining (minutes) 1X eosin B	5X eosin B	10X eosin B
I (1- to 4-cell)	1.5 ± 1.5[a] (n = 17)	1.0 ± 0.0[a] (n = 22)	1.0 ± 0.0[a] (n = 14)
II (5- to 8-cell)	2.5 ± 1.7[a] (n = 14)	1.7 ± 2.1[a] (n = 14)	1.1 ± 0.3[a] (n = 12)
III (9-cell to morula)	5.9 ± 2.5[b] (n = 16)	1.5 ± 0.7[a] (n = 19)	1.3 ± 0.8[a] (n = 17)
IV (Blastocyst)	8.1 ± 3.6[c] (n = 18)	2.1 ± 1.0[a] (n = 18)	1.4 ± 0.9[a] (n = 19)

Data are presented as the mean ± SD.

Means in columns or rows which do not have a common superscript letter are different ($P < 0.05$).

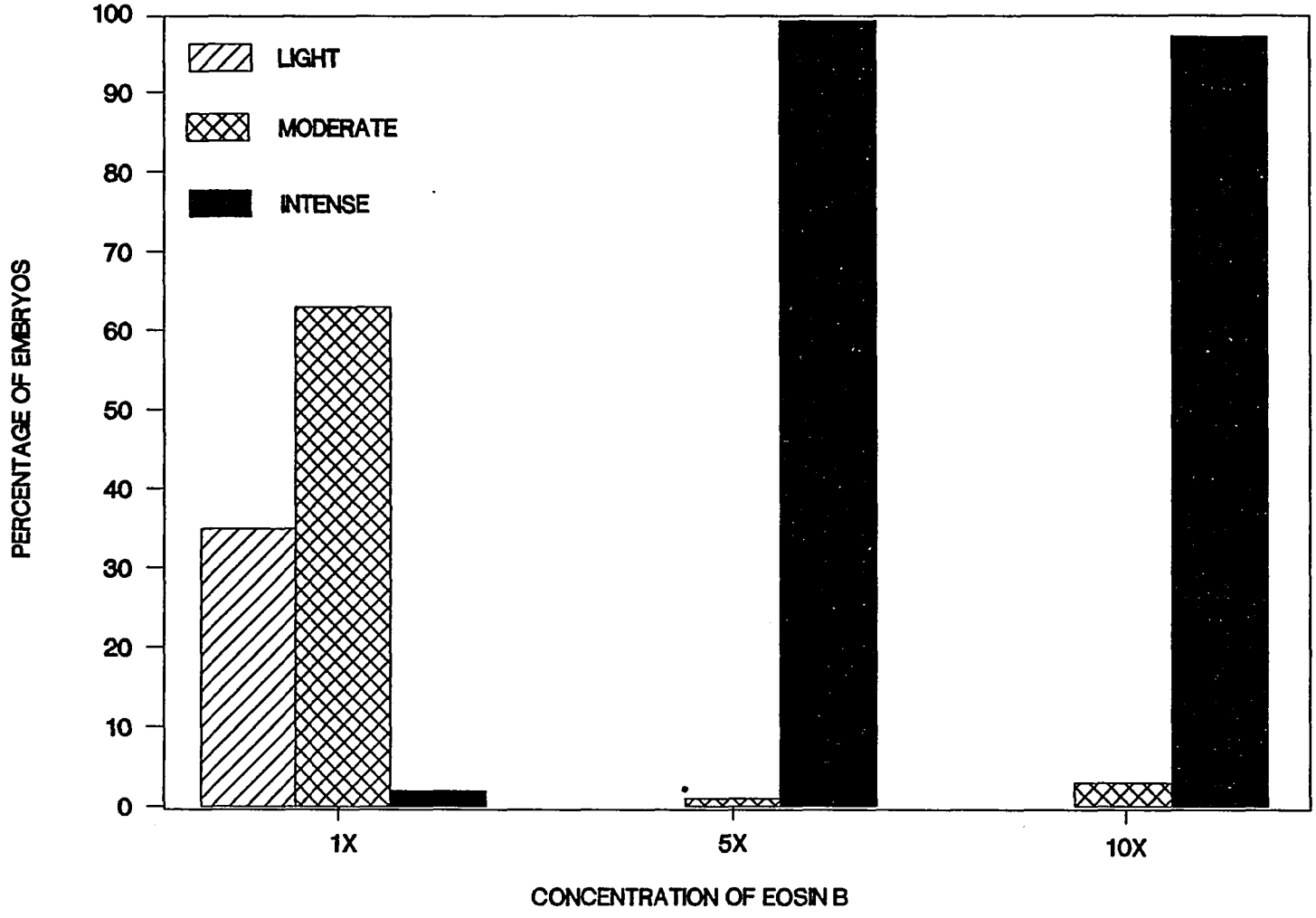
Table 9. Influence of dye concentration on the overall mean time interval required for complete staining of heat-treated rat embryos exposed to eosin B (Experiment 3)

Developmental group (Stages)	Mean time for complete staining (minutes) 1X eosin B	5X eosin B	10X eosin B
I to IV (1-cell to blastocyst stages)	4.6 ± 3.7[a] (n = 65)	1.5 ± 1.2[b] (n = 73)	1.4 ± 0.9[b] (n = 62)

Data are presented as the mean ± SD.

Means which do not have a common superscript letter are different ($P < 0.05$).

Figure 2. Percentage of heat-killed rat embryos exposed to 1X, 5X, or 10X eosin B that were classified by staining intensity as A -light, B - moderate, or C - intense (Experiment 3)


100
90
80
70
60
50
40
30
20
10
0
LIGHT
MODERATE
INTENSE
PERCENTAGE OF EMBRYOS
1X
5X
10X
CONCENTRATION OF EOSIN B

Experiment 4. Effect of a Single Exposure to 1X Eosin B on the In Vitro Development of Rat Embryos

A total of 151 embryos ranging from the 4- to 8-cell stages were recovered from 23 donors and used for Experiment 4. The number of embryos recovered from each donor ranged from 0 to 14 (mean ± SD = 6.6 ± 5.8). The number of embryos that cleaved at least once is shown in Table 10. Cleavage was detected within approximately one-half of the control and treated embryos within 12 hours of culture and most of the embryos, which cleaved at least once during the 72 hour period of in vitro culture, had cleaved within the first 36 hours of culture Table 10). The number of embryos that cleaved, cleaved and formed a blastocoel, or cleaved, formed a blastocoel, and hatched from the zona pellucida for the control and treated groups are shown in Table 11. A single exposure of rat embryos to the 1X

Table 10. Number of control and treated embryos that cleaved after a single exposure to 1X eosin B during culture (Experiment 4)

Treatment	Number of embryos	Number of embryos that cleaved at least once						
		0 h	12 h	24 h	36 h	48 h	60 h	72 h
Control	74	NA	39	50	51	51	51	51
Eosin B	77	NA	40	56	59	59	59	60

NA = Not applicable.

Table 11. Developmental response of control and eosin B treated 4- to 8-cell rat embryos during culture (Experiment 4)

Treatment	Number of embryos	Developmental response			
		Did not Cleave	Cleaved	Formed blastocoel	Hatched
Control	74	23	19	32	0
Eosin B	77	17	23	32	5

Did not cleave = number of embryos in which no further development was obtained during the culture period.

Cleaved = number of embryos that cleaved at least once during the culture period, but did not form a blastocoel.

Blastocoel = number of embryos which formed a clearly defined blastocoel during the culture period.

Hatched = number of embryos which formed a clearly defined blastocoel during the culture period and were observed to be in the process of escaping from or had escaped from the zona pellucida.

The development of the eosin B treated embryos was not different ($P > 0.05$) from the corresponding control embryos.

Table 12. Cleavage indices of control and eosin B treated 4- to 8-cell rat embryos during culture (Experiment 4)

Treatment	Mean ± (SD) cleavage index							Peak* index
	0 h	12 h	24 h	36 h	48 h	60 h	72 h	
Control	5.9	6.0	6.2	5.4	5.0	4.0	4.9	9.4
	(0.4)	(3.6)	(4.7)	(5.5)	(5.8)	(5.6)	(5.6)	(2.9)
Eosin B	5.8	6.0	6.7	6.7	5.7	6.2	5.5	10.1
	(0.5)	(3.6)	(4.6)	(5.4)	(5.8)	(6.1)	(6.2)	(2.8)

*Peak index = the maximal cleavage index that was recorded for the embryo during the 72 hour period of in vitro culture.

There was a trend ($P < 0.1$) for an effect of time on the cleavage index, but the effects of treatment and the treatment x time interaction were not significant ($P > 0.05$).

The peak indices for the control and eosin B treated groups were not different ($P > 0.05$).

concentration of eosin B did not influence ($P > 0.05$, Table 11) the subsequent in vitro embryonic development of these embryos.

There was a trend ($P < 0.1$) for an effect of time on the cleavage indices (Table 12 and Appendix 4) but the effects of treatment and the treatment x time interaction were nonsignificant ($P > 0.05$). Variation in cleavage, as reflected by the magnitude of the standard deviation, appeared to increase over time (Table 12), reflecting cleavage activity of individual embryos within the control and treated groups. In addition

(data not shown in Tables), the proportion of the embryos which were classified as degenerate appeared to increase during the period of culture. At the end of the 72 hour period of culture, 41 of 74 control embryos (55%) and 42 of 77 treated embryos (55%) were classified as morphologically degenerate.

The overall mean for the peak index of control and treated embryos during the 72 hour period of culture was not different ($P > 0.05$, Table 12). The means for the peak index of the control and treated embryos at each observation period are displayed in Figure 3.

The staining responses of embryos exposed to 1X eosin B prior to culture and for all embryos after the 72 hour period of culture are shown in Table 13. The staining responses at the end of the 72 hour period of culture of the control embryos which had not been previously exposed to eosin B and that of the treated embryos were not different ($P > 0.05$, Table 13). The staining index (data not shown in Tables) of the control embryos at the end of the 72 hour period of culture (mean $\pm$ SD = 1.3 ± 0.6) was not different ($P > 0.05$) from that of the eosin B-exposed embryos (mean $\pm$ SD = 1.3 ± 0.5). None of the 77 embryos assigned to the eosin B treatment group stained when first exposed to the eosin B solution before culture (Table 13). It appeared that a greater proportion of the embryos, which were unstained during the post-culture evaluation of the staining response, had formed a blastocoel during the 72 hour period of culture than those embryos which were either partially or completely stained. Inspection of the data from Table 13 revealed that, for the unstained embryos, 26 of 51 control (51 %) and 34 of 57 treated (60 %) embryos had formed a blastocoel during

Figure 3. Mean peak index of control 4- to 8-cell rat embryos or of embryos exposed to the 1X concentration of eosin B prior to in vitro culture (Experiment 4)

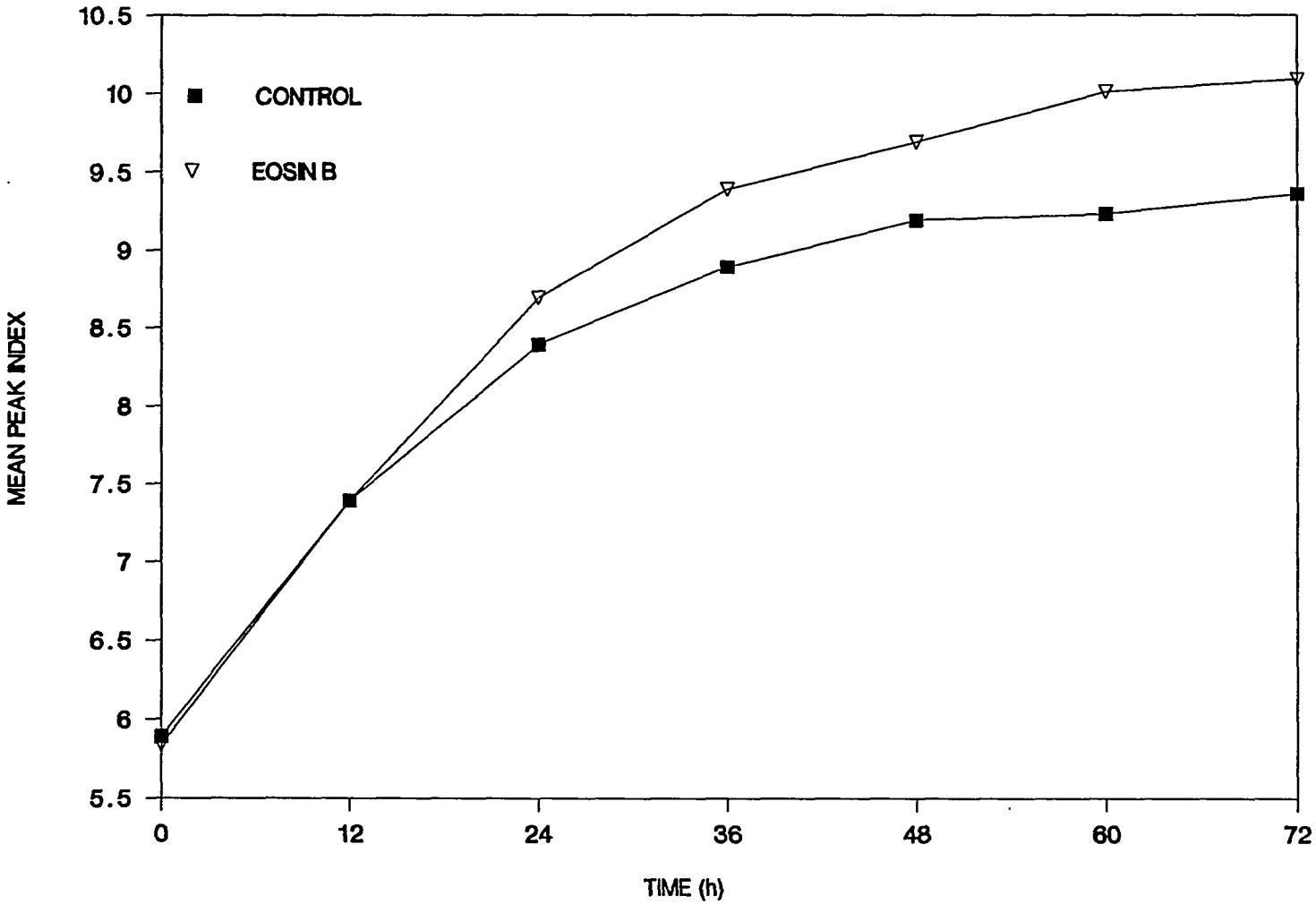

10.5
10
9.5
9
8.5
8
7.5
7
6.5
6
5.5
CONTROL
EOSIN B
MEAN PEAK INDEX
0
12
24
36
48
60
72
TIME (h)

Table 13. Staining response of 4- to 8-cell rat embryos exposed to 1X eosin B before culture and staining responses of control embryos and of treated embryos exposed again to eosin B at the end of the 72 hour period of in vitro culture (Experiment 4)

Treatment	Number of embryos	Staining response					
		Before culture			Post culture		
		US	PS	CS	US	PS	CS
Control	74*	NA	NA	NA	51	19	3
Eosin B	77	77	0	0	57	17	3

NA = Not applicable.

*One embryo in the control group was lost during transfer to the 1X eosin B solution used to evaluate of the dye-exclusion response at the end of the in vitro culture period.

At the end of the 72 hour culture period, the number of stained embryos in the eosin B-exposed group was not different ($P > 0.05$) from the corresponding control group.

in vitro culture. For those embryos which were partially stained, only 6/18 control (33 %) and 3/18 treated (17 %) embryos formed a blastocoel. None of the embryos which were completely stained at the end of the culture period had formed a blastocoel during in vitro culture. For the embryos which were partially stained at the end of culture, the proportion of the embryonic mass which was stained by the 1X concentration of eosin B ranged from 5 % to 50 %. In general, less development was observed in those partially stained embryos for which a high proportion of the embryonic mass was stained.

Experiment 5. Effect of A Single Exposure to 5X Eosin B on the In Vitro Development of Rat Embryos

A total of 165 embryos ranging from the 4- to 8-cell stages were recovered from 18 donors and used for Experiment 5. The number of embryos recovered from each donor ranged from 0 to 15 (mean ± SD = 9.2 ± 4.7). The number of embryos that cleaved at least once is shown in Table 14. Cleavage was detected within 40 % of the control and 46 % of the treated embryos within 12 hours of culture and most of the embryos which cleaved at least once during the 72 hour period of in vitro culture had cleaved within the first 36 hours of culture (Table 14). The number of embryos that cleaved, cleaved and formed a blastocoel, or cleaved, formed a blastocoel, and hatched from the zona pellucida are shown in Table 15. A single exposure of rat embryos to the 5X concentration of eosin B did not

Table 14. Number of control and treated embryos that cleaved after a single exposure to 5X eosin B during culture (Experiment 5)

Treatment	Number of embryos	Number of embryos that cleaved at least once						
		0 h	12 h	24 h	36 h	48 h	60 h	72 h
Control	81	NA	32	59	66	66	66	66
Eosin B	84	NA	39	69	69	69	69	70

NA = Not applicable.

Table 15. Developmental response of control and eosin B treated 4- to 8-cell rat embryos during culture (Experiment 5)

Treatment	Number of embryos	Developmental response			
		Did not cleave	Cleaved	Formed blastocoel	Hatched
Control	81	15	14	39	13
Eosin B	84	14	14	44	12

Did not cleave = number of embryos in which no further development was obtained during the culture period.

Cleaved = number of embryos that cleaved at least once during the culture period, but did not form a blastocoel.

Blastocoel = number of embryos which formed a clearly defined blastocoel during the culture period.

Hatched = number of embryos which formed a clearly defined blastocoel during the culture period and were observed to be in the process of escaping from or had escaped from the zona pellucida.

The development of the eosin B treated embryos was not different ($P > 0.05$) from the corresponding control embryos.

influence ($P > 0.05$; Table 15) the subsequent in vitro development of these embryos.

There was a significant ($P < 0.001$) effect of time on the cleavage indices (Table 16 and Appendix 4) but the effects of treatment and the treatment x time interaction were nonsignificant ($P > 0.05$). Variation in cleavage, as reflected by the magnitude of the standard deviation appeared to increase over time (Table 16), reflecting cleavage activity of individual embryos within the control and treated groups. In addition

Table 16. Cleavage indices of control and eosin B treated 4- to 8-cell rat embryos during culture (Experiment 5)

Treatment	Mean ± (SD) cleavage index							Peak* index
	0 h	12 h	24 h	36 h	48 h	60 h	72 h	
Control	5.5	6.1	7.4	8.4	8.3	8.3	7.5	10.9
	(0.8)	(2.0)	(3.7)	(4.8)	(5.6)	(6.1)	(6.2)	(3.4)
Eosin B	5.6	6.3	7.8	9.0	8.5	8.5	8.2	11.1
	(0.7)	(1.9)	(3.8)	(4.4)	(5.4)	(6.0)	(5.9)	(3.2)

*Peak index = the maximal cleavage index that was recorded for the embryo during the 72 hour period of in vitro culture.

There was a significant ($P < 0.001$) effect of time on the cleavage index, but the effects of treatment and the treatment x time interaction were not significant ($P > 0.05$).

The peak indices for the control and eosin B treated groups were not different ($P > 0.05$).

(data not shown in Tables), the proportion of the embryos which were classified as degenerate appeared to increase during the period of culture. At the end of the 72 hour period of culture, 30 of 81 control embryos (37 %) and 25 of 84 treated embryos (30 %) were classified as morphologically degenerate.

The overall mean for the peak index of control and treated embryos during the 72 hour period of culture was not different ($P > 0.05$, Table

16). The means for the peak index of the control and treated embryos at each observation period are displayed in Figure 4.

The staining responses of embryos exposed to 5X eosin B prior to culture and for all embryos after the 72 hour period of culture are shown in Table 17. The staining responses at the end of the 72 hour period of culture of the control embryos, which had not been previously exposed to eosin B, and and that of the treated embryos were not different ($P > 0.05$, Table 17). The staining index (data not shown in Tables) of the control embryos at the end of the 72 hour period of culture (mean ± SD = 1.4 ± 0.5) was not different ($P > 0.05$) from that of the eosin B-exposed embryos (mean ± SD = 1.3 ± 0.5). Although 2 embryos assigned to the eosin B treatment group were partially stained when exposed to the eosin B solution before culture (Table 17), the nonstaining blastomeres for these 2 embryos cleaved and formed a blastocoel within the 72 hour period of in vitro culture.

For the embryos which were partially stained at the end of culture, the proportion of the embryonic mass which was stained by the 5X concentration of eosin B ranged from 5 % to 80 %. In general, less development was observed in those embryos that had a higher proportion of the embryonic mass stained. As was observed in Experiment 4, it appeared that a greater proportion of the embryos which were unstained during the post-culture evaluation of the staining response had formed a blastocoel during the 72 hour period of culture than those embryos which were

Figure 4. Mean peak index of control 4- to 8-cell rat embryos or of embryos exposed to the 5X concentration of eosin B prior to in vitro culture (Experiment 5)

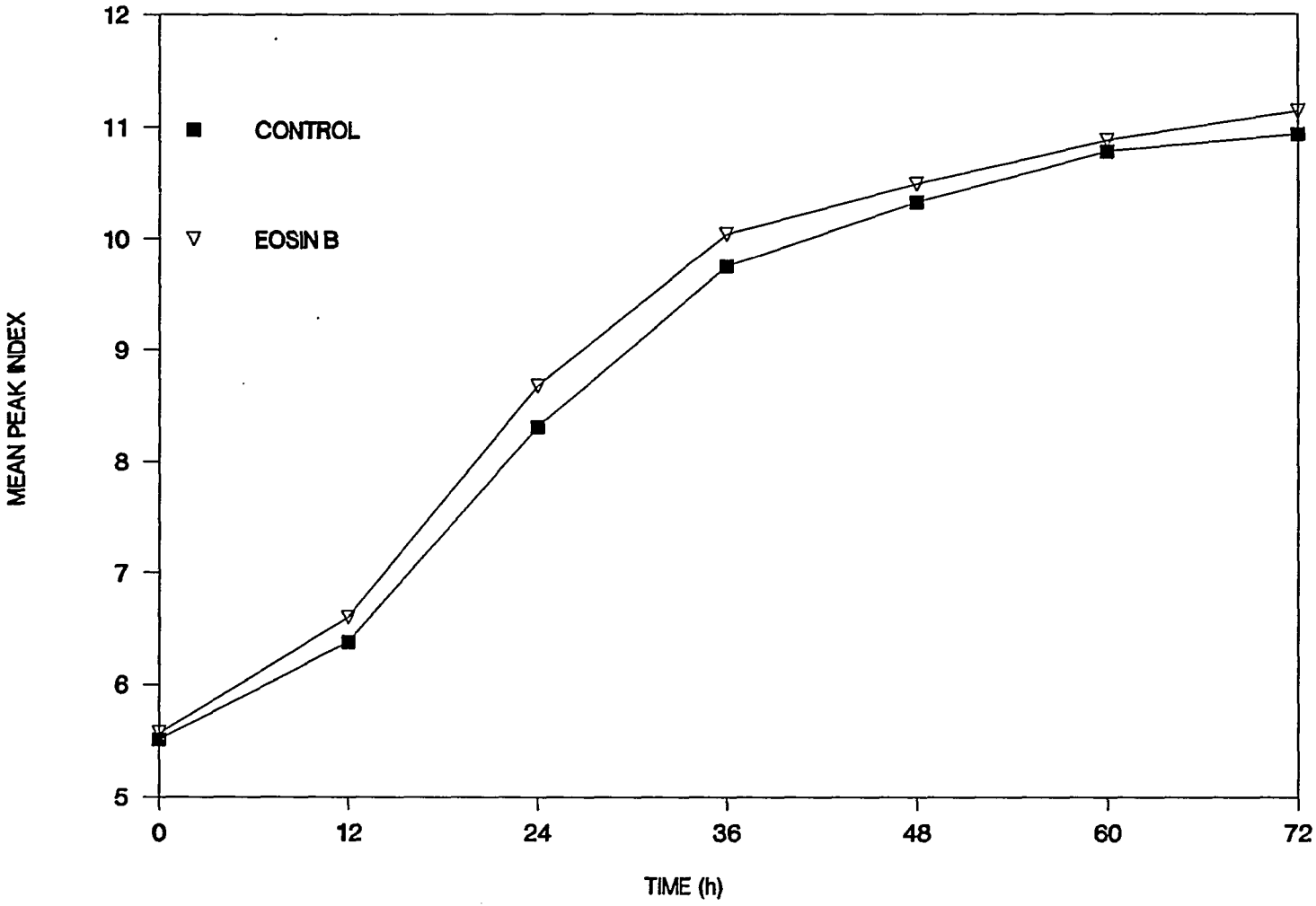

12
11
10
9
8
7
6
5
CONTROL
EOSIN B
MEAN PEAK INDEX
0
12
24
36
48
60
72
TIME (h)

Table 17. Staining response of 4- to 8-cell rat embryos exposed to 5X eosin B before culture and staining responses of control embryos and of treated embryos exposed again to 5X eosin B at the end of the 72 hour period of in vitro culture (Experiment 5)

Treatment	Number of embryos	Staining response: Before culture US	PS	CS	Post culture* US	PS	CS
Control	81	NA	NA	NA	51	28	0
Eosin B	84	82	2	0	54	29	0

NA = Not applicable.

*Two of the control and 1 of the treated embryos were lost during transfer to the 5X eosin B solution used to evaluate the dye-exclusion response at the end of the in vitro culture period.

At the end of the 72 hour culture period, the number of stained embryos in the eosin B-exposed group was not different ($P > 0.05$) from the corresponding control group.

partially stained. In this experiment, 108 embryos were classified as blastocysts within the period of in vitro culture (see Table 14) and 101 embryos retained a blastocoel at the end of the culture period. Of these 101 blastocysts, 87 were unstained and 14 were partially stained by the 5X concentration of eosin B. For the 14 blastocysts which were partially stained, 5 % to 25 % of the embryonic mass was stained. None of the embryos were completely stained by the 5X concentration of eosin B at the end of the 72 hour period of culture.

Experiment 6. Effect of Multiple Exposures to 1X Eosin B on the In Vitro Development of Rat Embryos

A total of 124 embryos ranging from the 4- to 8-cell stages were recovered from 23 donors and used for Experiment 6. The number of embryos recovered from each donor ranged from 0 to 12 (mean ± SD = 5.4 ± 4.8). The number of embryos that cleaved at least once is shown in Table 18. Cleavage was detected within approximately one-third of the control and treated embryos within 12 hours of culture and all of the embryos, which cleaved at least once during the 72 hour period of in vitro culture, had cleaved within the first 36 hours of culture (Table 18). The number of control and treated embryos that cleaved, cleaved and formed a blastocoel, or cleaved, formed a blastocoel, and hatched from the zona pellucida are shown in Table 19. Multiple exposures of rat embryos to the 1X

Table 18. Number of control embryos and embryos exposed periodically to the 1X concentration of eosin B that cleaved during culture (Experiment 6)

Treatment	Number of embryos	Number of embryos that cleaved at least once						
		0 h	12 h	24 h	36 h	48 h	60 h	72 h
Control	61	NA	20	42	45	45	45	45
Eosin B	63	NA	22	45	50	50	50	50

NA = Not applicable.

Table 19. Developmental response of control and eosin B treated 4- to 8-cell rat embryos during culture (Experiment 6)

Treatment	Number of embryos	Developmental response			
		Did not cleave	Cleaved	Formed blastocoel	Hatched
Control	61	16	17	27	1
Eosin B	63	11	16	30	6

Did not cleave = number of embryos in which no further development was obtained during the culture period.

Cleaved = number of embryos that cleaved at least once during the culture period, but did not form a blastocoel.

Blastocoel = number of embryos which formed a clearly defined blastocoel during the culture period.

Hatched = number of embryos which formed a clearly defined blastocoel during the culture period and were observed to be in the process of escaping from or had escaped from the zona pellucida.

The development of the eosin B treated embryos was not different ($P > 0.05$) from the corresponding control embryos.

concentration of eosin B at 12 hour intervals within the 72 hour period of culture did not influence ($P > 0.05$; Table 19) the in vitro development of these embryos.

There was a significant ($P < 0.025$) effect of time on the cleavage indices (Table 20 and Appendix 4), but the effects of treatment and the treatment x time interaction were nonsignificant ($P > 0.05$). Variation in cleavage, as reflected by the magnitude of the standard deviation appeared to increase over time (Table 20), reflecting cleavage activity of

Table 20. Cleavage indices of control and eosin B treated 4- to 8-cell rat embryos during culture (Experiment 6)

	Mean ± (SD) cleavage index							
Treatment	0 h	12 h	24 h	36 h	48 h	60 h	72 h	Peak* index
Control	5.7	5.3	6.6	7.0	6.2	6.8	5.0	9.6
	(0.6)	(2.9)	(3.7)	(4.9)	(5.6)	(5.7)	(5.7)	(3.2)
Eosin B	5.7	6.0	7.3	7.6	7.3	7.9	6.4	10.3
	(0.6)	(2.3)	(3.2)	(4.9)	(5.6)	(5.7)	(6.2)	(3.4)

*Peak index = the maximal cleavage index that was recorded for the embryo during the 72 hour period of in vitro culture.

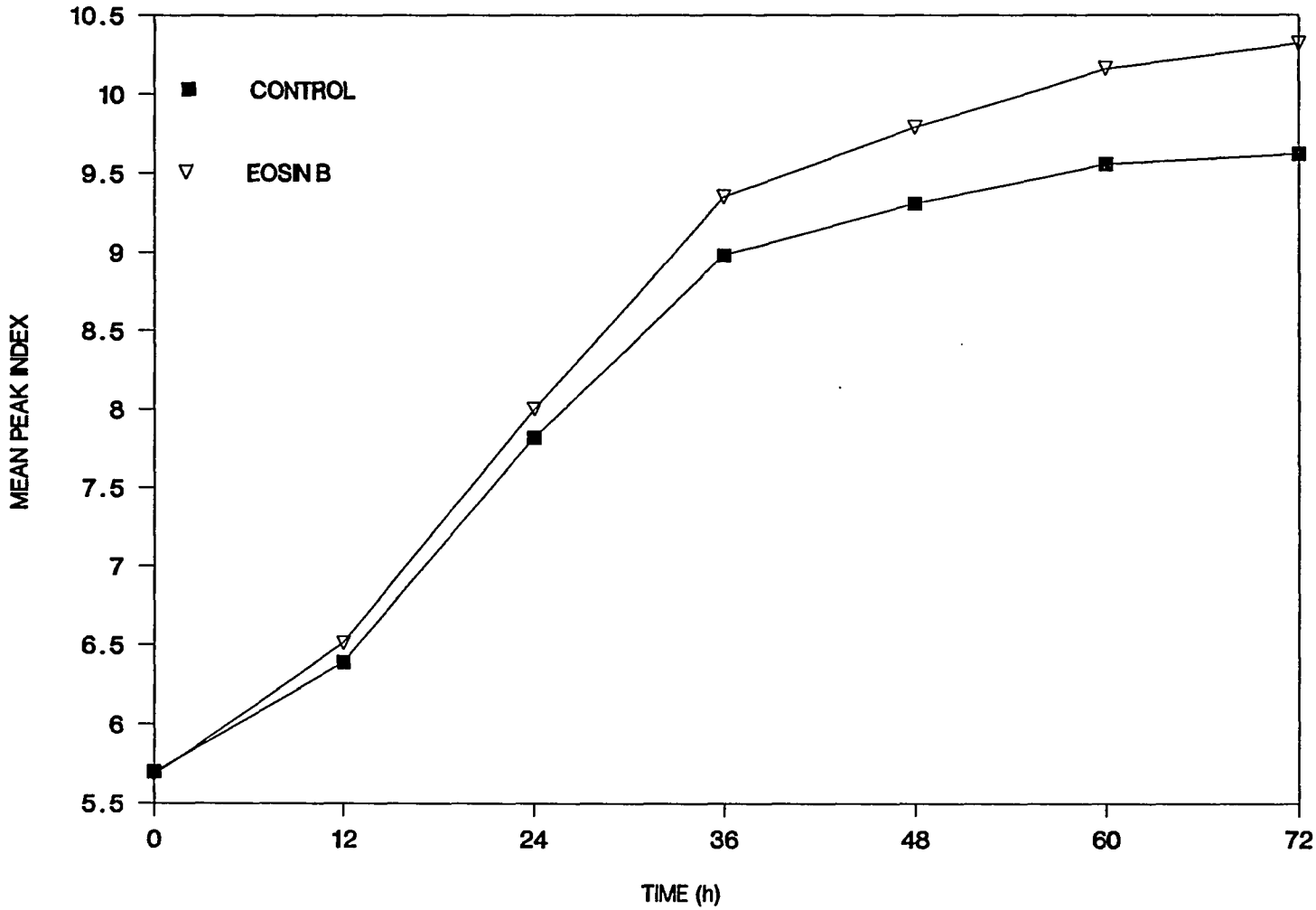
There was a significant ($P < 0.025$) effect of time on the cleavage index, but the effects of treatment and the treatment x time interaction were not significant ($P > 0.05$).

The peak indices for the control and eosin B treated group were not different ($P > 0.05$).

individual embryos within the control and treated groups. In addition (data not shown in Tables), the proportion of the embryos which were classified as degenerate appeared to increase during the period of culture. At the end of the 72 hour period of culture, 32 of 61 control embryos (52 %) and 28 of 63 treated embryos (44 %) were classified as morphologically degenerate. The overall mean for the peak index of control and treated embryos during the 72 hour period of culture was not different ($P > 0.05$; Table 20). The means for the peak index of the control and treated embryos at each observation period are displayed in Figure 5.

The staining responses of the treated embryos prior to culture and for all embryos after the 72 hour period of culture are shown in Table 21. The staining responses at the end of the 72 hour period of culture of the control embryos (Table 21) which had not been previously exposed to eosin B were significantly ($P < 0.05$) different from that of the treated embryos which had been exposed to eosin B at each observation period. The staining index (data not shown in Tables) of the control embryos at the end of the 72 hour period of culture (mean ± SD = 1.3 ± 0.5) was significantly ($P < 0.005$) different from that of the eosin B-exposed embryos (mean ± SD = 1.5 ± 0.5). Within the eosin B treatment group, the number of embryos stained by the 1X concentration of eosin B appeared to increase over time.

Figure 5. Mean peak index of control 4- to 8-cell rat embryos or of embryos exposed periodically to the 1X concentration of eosin B during in vitro culture (Experiment 6)


10.5
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8.5
8
7.5
7
6.5
6
5.5
CONTROL
EOSIN B
MEAN PEAK INDEX
0
12
24
36
48
60
72
TIME (h)

Table 21. Staining response of 4- to 8-cell treated rat embryos exposed to 1X eosin B before culture and staining responses of control embryos and of the eosin B treated embryos exposed again to eosin B at the end of the 72 hour period of culture (Experiment 6)

Treatment	Number of embryos	Staining response					
		Before culture			Post culture*		
		US	PS	CS	US	PS	CS
Control	61	NA	NA	NA	41	18	0
Eosin B	63	55	8	0	29	33	0

NA = Not applicable.

*Two of the control and 1 of the treated embryos were lost during transfer to the 1X eosin B solution used to evaluate the dye-exclusion response at the end of the in vitro culture period.

At the end of the 72 hour culture period, the number of stained embryos in the eosin B-exposed group was different ($P < 0.05$) from the corresponding control group.

Furthermore, it was observed that some of the treated embryos which did not stain, when examined during the 72 h period of culture, were partially stained when re-examined at the end of culture period. This difference was attributed to the effects of time and additional handling. Furthermore, it was easier to determine the staining response of an embryo with smaller (35 μl) volumes of dye, as was used with the "microwell" glass slides described for Experiment 1, than when larger (500 μl) volumes of the eosin B solution were used in the Multiwell culture dishes for this experiment.

During the course of this experiment, it was observed that 8 of the 63 embryos assigned to the eosin B treatment group were partially stained when first exposed to the 1X concentration of eosin B before culture (refer to Table 21). The nonstaining blastomeres from 7 of these 8 embryos cleaved during culture and 4 of these 7 embryos formed a blastocoel within the 72 hour period of in vitro culture. It was also noted that 1 embryo, which was partially stained at the beginning of culture, developed to the blastocyst stage and was unstained at the end of the culture period. Thus, the cellular elements which were stained at the start of culture had either lysed or lost their affinity for the stain over time.

For the embryos which were partially stained at the end of culture, the proportion of the embryonic mass which was stained by the 1X concentration of eosin B ranged from 5 % to 80 % for the control group and from 5 % to 90 % for the eosin B treated embryos. In general, less development was observed in those embryos that had a higher proportion of the embryonic mass which stained.

Experiment 7. Effect of Continuous Exposure to 1X or 5X Eosin B on the In Vitro Development of Rat Embryos

A total of 232 embryos ranging from the 4- to 8-cell stages were recovered from 55 donors and used for Experiment 7. The number of embryos recovered from each donor ranged from 0 to 15 (mean ± SD = 5.6 ± 5.4). The number of embryos that cleaved at least once is shown in Table 22. Cleavage was detected, within approximately one-half of the control and the 1X-eosin B treated embryos, within 12 hours of culture and all of the embryos, which cleaved at least once during the 72 hour period of in vitro culture, had cleaved within the first 48 hours of culture (Table 22). The

Table 22. Number of control embryos and embryos exposed continuously to the 1X or 5X concentrations of eosin B that cleaved during culture (Experiment 7)

Treatment	Number of embryos	Number of embryos that cleaved at least once						
		0 h	12 h	24 h	36 h	48 h	60 h	72 h
Control	80	NA	39	63	67	68	68	68
Eosin B (1X)	78	NA	38	51	64	66	66	66
Eosin B (5X)	74	NA	13	13	13	13	13	13

NA = Not applicable.

number of embryos that cleaved, cleaved and formed a blastocoel, or cleaved, formed a blastocoel, and hatched from the zona pellucida for the control and treated groups are shown in Table 23. Continuous exposure of rat embryos to eosin B over a 72 hour period of culture significantly ($P < 0.0005$; Table 23) influenced the in vitro development of these embryos. Only 13 of the 74 embryos cultured continuously in the 5X concentration of eosin B cleaved during culture, as compared to 68/80 embryos for the control group and 66/78 embryos exposed continuously to the 1X concentration of eosin B. However, only 14/78 embryos cultured in the 1X concentration of eosin B formed a blastocoel during culture as compared to 57/80 embryos in the control group. None of the embryos exposed continuously to the 1X concentration of eosin B, and which formed a blastocoel, were observed to hatch and none of the embryos cultured in the 5X concentration formed a blastocoel during culture. There was a significant ($P < 0.0001$) effect of time on the cleavage indices (Table 24 and Appendix 4) and the effects of treatment and the treatment x time interaction were also significant ($P < 0.0001$). Variation in cleavage, as reflected by the magnitude of the standard deviation, appeared to increase over time for the control and 1X continuous exposure groups (Table 24), reflecting cleavage activity of individual embryos within the control and this treatment group. For the 5X continuous exposure group, there was evidence for cleavage activity within the first 24 hours; however, the mean cleavage index then decreased to zero in the subsequent observation periods, reflecting the change in the appearance of these embryos from that

Table 23. Developmental response of control and eosin B treated 4- to 8-cell rat embryos during culture (Experiment 7)

Treatment	Number of embryos	Developmental response			
		Did not cleave	Cleaved	Formed blastocoel	Hatched
Control	80	12	11	48	9
Eosin B (1X)	78	12	52	14	0
Eosin B (5X)	74	61	13	0	0

Did not cleave = number of embryos in which no further development was obtained during the culture period.

Cleaved = number of embryos that cleaved at least once during the culture period, but did not form a blastocoel.

Blastocoel = number of embryos which formed a clearly defined blastocoel during the culture period.

Hatched = number of embryos which formed a clearly defined blastocoel during the culture period and were observed to be in the process of escaping from or had escaped from the zona pellucida.

The development of rat embryos was influenced ($P < 0.0005$) by continuous exposure to eosin B.

Table 24. Cleavage indices of control and eosin B treated 4- to 8-cell rat embryos during culture (Experiment 7)

Treatment	Mean ± (SD) cleavage index							Peak* index
	0 h	12 h	24 h	36 h	48 h	60 h	72 h	
Control	5.6	6.3	8.0	8.5	9.2	8.5	7.3	11.1[a]
	(0.7)	(2.0)	(3.4)	(4.9)	(5.0)	(5.9)	(6.0)	(3.2)
Eosin B (1X)	5.6	6.2	6.9	6.4	5.4	3.6	2.9	8.7[b]
	(0.7)	(1.3)	(2.6)	(3.9)	(4.3)	(4.6)	(4.3)	(2.3)
Eosin B (5X)	5.7	5.6	3.7	0.3	0.1	0.0	0.1	6.0[c]
	(0.5)	(1.1)	(2.5)	(1.1)	(0.6)	(0.0)	(0.7)	(0.6)

*Peak index = the maximal cleavage index that was recorded for the embryo during the 72 hour period of in vitro culture.

There was a significant ($P < 0.0001$) effect of time on the cleavage index and the effects of treatment and the treatment x time interaction were also significant ($P < 0.0001$).

There was a significant ($P < 0.0001$) effect of treatment on the peak index. Means which do not have a common superscript letter in the column for peak index are different ($P < 0.05$).

of the cleavage stages to that of morphologically degenerate embryos. As was observed in Experiments 4, 5, and 6, the proportion of the embryos which were classified as degenerate appeared to increase during the period of culture in the control and in the 1X and 5X treatment groups. At the end of the 72 hour period of culture (data not shown in Tables), 29 of 80

control embryos (36 %), 49 of 78 1X-continuously treated embryos (63 %), and 73 of 74 5X-continuously treated embryos (99 %) were classified as morphologically degenerate. The peak indices for the control and the eosin B treatment groups were significantly ($P < 0.0001$; Table 24) different. The means for the peak index of the control and treated embryos, at each observation period, are displayed in Figure 6.

The staining responses of the treated embryos prior to culture and for all embryos after the 72 hour period of culture are shown in Table 25. The staining responses at the end of the 72 hour period of culture of the control embryos (Table 25), which had not been previously exposed to eosin B, were significantly ($P < 0.0005$) different from that of the treated embryos, which had been continuously exposed to eosin B during culture. The number of embryos within the 1X and 5X continuous exposure groups, which were stained by the 1X concentration of eosin B, appeared to increase over time and there was a significant ($P < 0.0001$) effect of treatment on the staining indices at the end of the 72 hour period of culture. The staining index (data not shown in Tables) of the control embryos, at the end of the 72 hour period of culture (mean $\pm$ SD = 1.2 ± 0.4), was lower ($P < 0.05$) than that of either the 1X eosin B (mean $\pm$ SD = 1.3 ± 0.5) or the 5X eosin B (mean $\pm$ SD = 3.0 ± 0.0) continuous exposure groups, and the mean staining index for the 1X continuously exposed embryos was different ($P < 0.05$) from that of the 5X continuously exposed embryos. All of the embryos which were cultured continuously in the 5X concentration of eosin B were completely stained before the end of the 72 hour exposure period.

Figure 6. Mean peak index of control 4- to 8-cell rat embryos or of embryos exposed continuously to the 1X or 5X concentrations of eosin B during in vitro culture (Experiment 7)

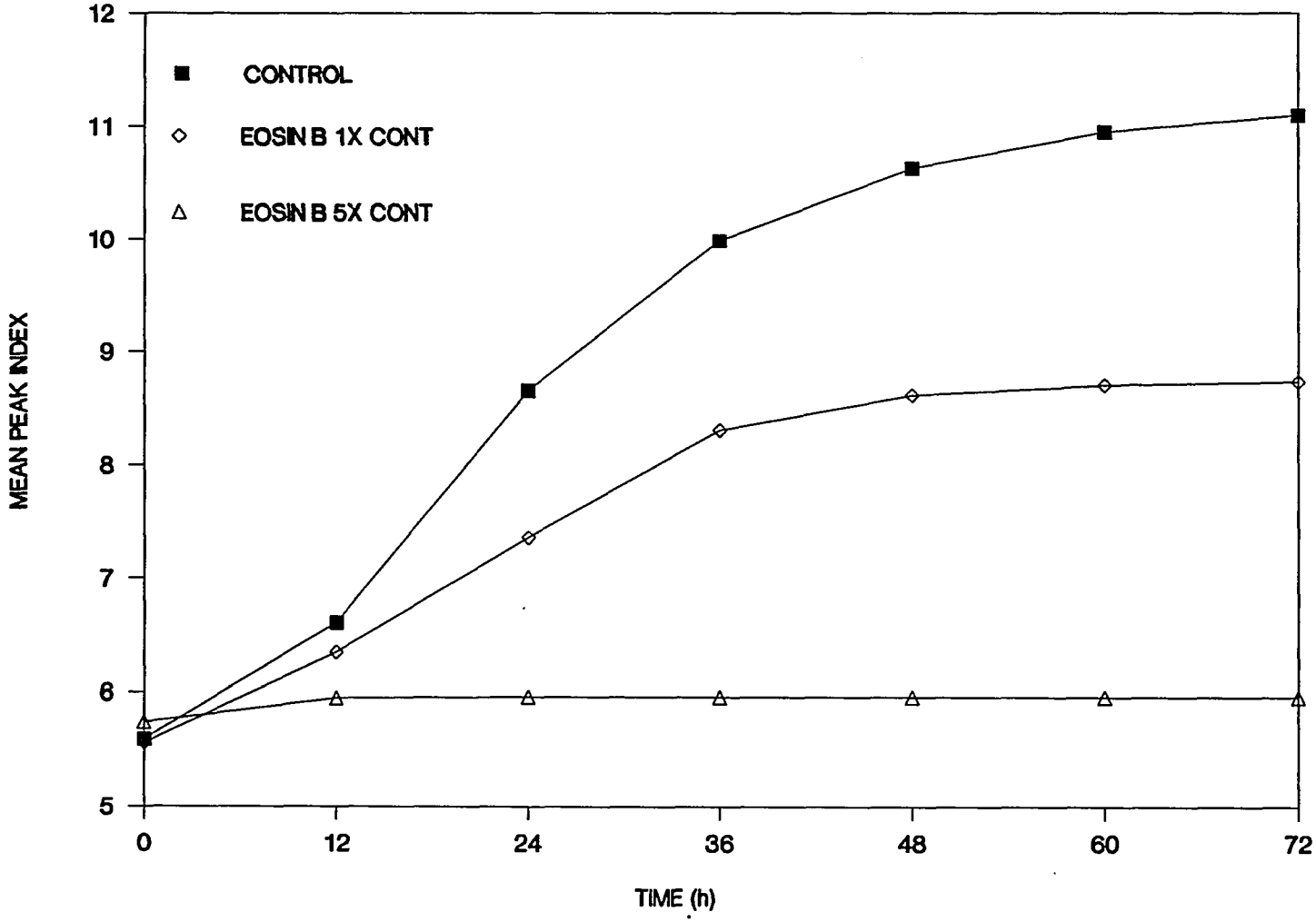
12
11
10
9
8
7
6
5
MEAN PEAK INDEX
CONTROL
EOSIN B 1X CONT
EOSIN B 5X CONT
0
12
24
36
48
60
72
TIME (h)

Table 25. Staining responses of 4- to 8-cell treated rat embryos exposed to 1X eosin B before culture and staining responses of control embryos and of the eosin B treated embryos exposed again to the 1X concentration of eosin B at the end of the 72 hour period of culture (Experiment 7)

Treatment	Number of embryos	Staining response					
		Before culture			Post culture		
		US	PS	CS	US	PS	CS
Control	80	NA	NA	NA	69	10	1
Eosin B (1X)	78	77	1	0	53	24	1
Eosin B (5X)	74	72	2	0	0	0	74

NA = Not applicable.

The staining response of rat embryos at the end of the 72 hour culture period was influenced ($P < 0.0005$) by continuous exposure to eosin B.

As shown in Table 23, only 13 of the 74 embryos exposed continuously to the 5X concentration of eosin B cleaved during in vitro culture. For these 13 embryos, 12 (92 %) were unstained and 1 was partially stained when exposed to the 1X concentration of eosin B and examined prior to culture. Cleavage occurred within the first 12 hours of culture for these 13 embryos, yet none cleaved after 24 hours of culture and continuous exposure to the 5X concentration of eosin B. At some point during the culture period, each of the embryos exposed continuously to the 5X concentration of

eosin B became completely stained. Cleavage activity was never detected in an embryo that was completely stained by the dye.

Experiment 8. Viability of Control or of Rat Embryos Exposed Once to 5X Eosin B and then Transferred to the Left Horn of Naturally Mated Rats

A total of 58 morulae and blastocysts were recovered from 14 donors and used for Experiment 8. The number of embryos recovered from each donor ranged from 0 to 9 (mean ± SD = 4.1 ± 3.5). A total of 13 embryo transfers were performed into the left horn of naturally mated rats. The expected phenotype of the donor embryos, treatment and number of embryos treated prior to transfer to a given recipient, the number of offspring born and weaned which were from embryos transferred, and the number of offspring born and weaned from "self" embryos for each recipient, are presented in Table 26. Seven of the 8 recipients that received embryos which were assigned to the control treatment produced a litter and 4 of 5 recipients that received embryos that had been exposed to the 5X concentration of eosin B before transfer produced a litter. The ratio of recipients that remained pregnant and produced a litter from the total number of recipients was not affected ($P > 0.05$) by the single exposure of the embryos to the 5X concentration of eosin B before transfer. The number of control and treated embryos transferred and their subsequent survival at term are summarized in Table 27. Overall, 6 of 58 morulae or blastocysts transferred into mated recipients survived to term (10.3 %). Of these, 17

Table 26. The expected phenotype and number of control and eosin B (5X) exposed embryos and their survival after transfer to the left horn of naturally mated rats (Experiment 8)

Recipient	Donor embryos					Self embryos	
	Phenotype	Treatment	Transferred	Born	Weaned	Born	Weaned
1	Albino	5X	8	1	1	13	12
2	Dark	Control	2	0	NA	12	12
3	Dark	5X	2	1	0	12	12
4	Dark	5X	3	0	NA	0	NA
5	Dark	Control	4	0	NA	15	12
6	Albino	5X	2	0	NA	1	0
7	Albino	Control	2	0	NA	4	4
8	Albino	Control	7	3	2	10	7
9	Dark	Control	9	0	NA	0	NA
10	Albino	5X	2	0	NA	2	2
11	Albino	Control	2	0	NA	8	8
12	Albino	Control	7	1	1	8	8
13	Albino	Control	8	0	NA	9	9

NA = Not applicable.

embryos were exposed to and were unstained by the 5X concentration of eosin B prior to transfer. From these 17 transferred embryos, 2 offspring were born at term (11.8 %) and 1 of these pups survived to weaning on day 28 (50 %). Four of the 41 control embryos transferred survived to term (9.8 %) and 3 of these pups were weaned (7.5 %). The ratio of eosin B treated embryos to survive to term and weaning from the total number of eosin B treated embryos that were transferred was not different ($P > 0.05$; Table 27) from the controls. Of the 13 mated females to which embryos were transferred, 4 produced litters that contained offspring derived from the embryos which were transferred and offspring derived from the natural mating, 7 produced litters which contained only offspring derived from the natural mating, and 2 rats did not produce a litter. In no case was there a litter obtained in which all of the offspring born could be attributed to the control or eosin B-treated embryos transferred. Individually considered, no more than 3 offspring were born as a result of embryo transfer to a given rat. On a percentage basis (Table 26), embryo survival to weaning ranged from 0 % to 29 % and averaged 7.3 % (3 of 41, Table 27), for the controls and ranged from 0 % to 13 % and averaged 6 % (1 of 17, Table 27), for the eosin B-treated group.

The overall pregnancy rate (data in Table 26) for these recipient females was 84.6 % (11/13) and the gestation interval ranged from 22 to 24 days (mean ± SD = 22.5 ± 0.7). The overall litter size ranged from 1 to 15 pups (mean ± SD = 9.1 ± 4.9). As an additional observation, 3 of 5 rats (60 %) which had been mated for use as embryo recipients, but were not used in this experiment, produced litters when allowed to go to term. The

Table 27. Effect of a single exposure of rat embryos to the 5X concentration of eosin B on embryonic survival to term (Experiment 8)

Embryo treatment	Number of recipients pregnant	Litters* to contain a nonself embryo	Number of embryos		
			Transferred	Born	Weaned
Control	7	2	41	4	3
Eosin B	4	2	17	2	1

*Number of recipients for which at least one offspring of the same phenotype as the donor embryos was born.

The ratio of recipients that remained pregnant and produced a litter from the total number of recipients was not affected ($P > 0.05$) by the single exposure of the embryos to the 5X concentration of eosin B and ratio of control embryos to survive to term and weaning were not different ($P > 0.05$) from that of the eosin B treated group.

gestation interval for these rats ranged from 23 to 24 days.

In this experiment, the interval from euthanasia of the donor to embryo transfer to the naturally mated recipients ranged from 3.5 to 9.3 hours. Overall, differences in the survival of the transferred, control or treated, embryos could not be attributed to the length of in vitro culture prior to transfer because a consistent pattern could not be detected. All of the offspring born which were the result of surgical transfer and which survived to weaning (n = 4), were males. For these same litters the offspring derived from the natural mating of the recipient resulted in a total of 43 males (50 %) and 42 females (49 %). No developmental abnormalities were evident in any of the pups at birth, or during the postnatal period.

Experiment 9. Viability of Control or of Rat Embryos Exposed Once to 5X Eosin B and then Transferred to Both Horns of Naturally Mated Rats

A total of 72 morulae and blastocysts were recovered from 13 donors and used for Experiment 9. The number of embryos recovered from each donor ranged from 0 to 11 (mean ± SD = 5.5 ± 4.2). A total of 9 embryo transfers were performed into the left and right horns of naturally mated female rats. All of these transfers involved the transfer of embryos from albino donors into recipients expected to contain "self" embryos, which would, when developed to offspring, display both coat and eye color (pigment) markers. The number of embryos transferred to each horn, the embryo

treatment prior to transfer, the number of offspring born and weaned which were from embryos transferred, and the number of offspring born and weaned from "self" embryos for each recipient, is presented in Table 28. Four of the 5 recipients that received control embryos produced a litter and 2 of these 5 recipients had offspring derived from the transfer of control embryos. All 4 recipients that received eosin B-treated embryos produced a litter and 2 of the 4 recipients had offspring derived from the transfer of

Table 28. The number of control and eosin B (5X) exposed embryos and their survival after transfer to the left and right horns of naturally mated rats (Experiment 9)

Recipient	Number of embryos transferred		Embryo treatment	Transferred embryos		Self embryos	
	Left	Right		Born	Weaned	Born	Weaned
1	3	3	Control	0	NA	0	NA
2	4	4	5X	1	1	5	5
3	5	5	Control	0	NA	6	6
4	4	3	5X	1	1	7	7
5	5	5	Control	1	1	8	8
6	5	5	5X	0	NA	3	3
7	3	4	Control	1	1	6	6
8	4	4	5X	0	NA	10	7
9	3	3	Control	0	NA	9	9

NA = Not applicable.

eosin B-treated embryos. The ratio of recipients that remained pregnant and produced a litter from the total number of recipients was not affected ($P > 0.05$) by the single exposure of the embryos to the 5X concentration of eosin B before transfer. The number of control and treated embryos transferred and their subsequent survival at term are summarized in Table 29. Overall, 4 of 72 morulae or blastocysts transferred into mated recipients survived to term and were weaned (5.6 %). Of these, 33 embryos were exposed to and were unstained by the 5X concentration of eosin B prior to transfer. From these 33 transferred embryos, 2 offspring were born at term and were weaned (6.1 %; Table 29). Two of the 39 control embryos transferred survived to term and were weaned (5.1 %; Table 29). The ratio

Table 29. Effect of a single exposure of rat embryos to the 5X concentration of eosin B on embryonic survival to term (Experiment 9)

Embryo treatment	Number of recipients pregnant	Litters* to contain a nonself embryo	Number of embryos		
			Transferred	Born	Weaned
Control	5	2	39	2	2
Eosin B	4	2	33	2	2

*Number of recipients in which at least one offspring of the same phenotype as the donor embryos was born.

The ratio of recipients that remained pregnant and produced a litter from the total number of recipients was not affected ($P > 0.05$) by the single exposure of the embryos to the 5X concentration of eosin B and ratio of control embryos to survive to term and weaning were not different ($P > 0.05$) from that of the eosin B treated group.

of eosin B-treated embryos to survive to term and weaning from the total number of eosin B treated embryos that were transferred was not different ($P > 0.05$; Table 29) from the controls. Of the 9 mated females to which embryos were transferred, 4 produced litters that contained offspring derived from the embryos which were transferred and offspring derived from the natural mating, 4 produced litters which contained only offspring derived from the natural mating, and 1 rat did not produce a litter. As was observed in Experiment 8, in no case was there a litter obtained in which all of the offspring born could be attributed to the control or eosin B-treated embryos transferred. Individually considered, no more than 1 offspring was born as a result of embryo transfer to a given rat. On a percentage basis (Table 28), embryo survival to weaning ranged from 0 % to 14 % and averaged 5.1 % (2 of 39, Table 29) for the controls and ranged from 0 % to 14 % and averaged 6.1 % (2 of 33, Table 29) for the eosin B-treated group.

The overall pregnancy rate (data in Table 28) for these recipient female rats was 89 % (8/9) and the gestation interval ranged from 22 to 23 days (mean ± SD = 22.4 ± 0.5). The overall litter size ranged from 3 to 10 pups (mean ± SD = 8.4 ± 4.7). As an additional observation, 12 of 18 rats (67 %) which had been mated for use as embryo donors or recipients, but were not used in this experiment, produced litters when allowed to go to term. The gestation interval for these rats ranged from 21 to 23 days (mean ± SD = 22.5 ± 0.7).

In this experiment, the interval from euthanasia of the donor to embryo transfer to the naturally mated recipients ranged from 3.8 to 10

hours. As was observed in Experiment 8, overall differences in the survival of the transferred, control or eosin B-treated, embryos could not be attributed to the length of in vitro culture prior to transfer. Of the offspring born which were the result of surgical transfer and which survived to weaning, 3 were males (75 %) and 1 was a female (25 %). For these same litters the offspring derived from the natural mating of the recipient resulted in a total of 21 males (41 %) and 30 females (59 %). No developmental abnormalities were evident in any of the pups at birth, or during the postnatal period.

Experiment 10. Viability of Control or of Rat Embryos Exposed Once to 5X Eosin B and then Transferred to Opposite Horns of the Same Pseudopregnant Rat

A total of 123 morulae and blastocysts were recovered from 27 donors and used for Experiment 10. The 27 donors (14 albino; 13 coat and eye color markers) were mated to intact male rats of known fertility and of the same phenotype, so that 1 donor of each phenotype was available for transfer on a given day. The number of embryos recovered from each donor ranged from 0 to 10 (mean ± SD = 4.6 ± 3.8). The number of embryos transferred into the left and right horns of each recipient, their treatment prior to transfer, the number of offspring born from the embryos transferred to each horn of the recipient, and their survival to weaning is summarized in Table 30. Six of the 10 recipients produced a litter (60 %)

Table 30. The number of control (C) and eosin B (5X) exposed embryos transferred and their survival after transfer to the left or the right horn of the same pseudopregnant rat (Experiment 10)

	Number of embryos transferred		Embryo treatment		Number of pups born:weaned			
					Left		Right	
Recipient	Left	Right	Left	Right	Born	Weaned	Born	Weaned
1	8	4	5X	C	2	2	0	NA
2	4	6	C	5X	0	NA	0	NA
3	3	10	5X	C	2	2	3	3
4	7	3	C	5X	2	0	0	NA
5	3	7	C	5X	0	NA	0	NA
6	8	8	C	5X	0	NA	0	NA
7	10	10	5X	C	5	5	1	1
8	6	4	C	5X	2	2	2	2
9	7	4	5X	C	5	5	0	NA
10	4	7	5X	C	0	NA	0	NA

NA = Not applicable.

and the overall litter size ranged from 2 to 6 pups (mean ± SD = 4.0 ± 1.7). The number of control and treated embryos transferred and their subsequent survival at term are summarized in Table 31. Overall, 24 of the 123 morulae or blastocysts transferred into these pseudopregnant recipients survived to term (19.5 %) and 22 of these 24 pups survived to weaning on day 28 (92 %). Of these, 60 embryos were exposed to and were unstained by the 5X concentration of eosin B prior to transfer. From these 60 transferred embryos, 16 offsprings were born at term and were weaned (26.7 %; Table 31). Eight of the 63 control embryos transferred survived to term (12.7 %; Table 31) and 6 pups were weaned. The ratio of eosin B treated embryos to survive to term and weaning from the total number of eosin B treated embryos that were transferred was not different ($P > 0.05$; Table 31) from the control embryos that were transferred to the opposite uterine horn. However, when compared to the controls, there was a trend ($P < 0.1$; Table 31) for more of the eosin B-treated embryos to survive to term. Individually considered, no more than 6 offspring were born as a result of embryo transfer to a given rat. On a percentage basis (Table 30), embryo survival to weaning ranged from 0 % to 33 % and averaged 9.5 % (6 of 63, Table 31) for the controls and ranged from 0 % to 71 % and averaged 26.7 % (16 of 60, Table 31) for the eosin B-treated group.

Inspection of the data presented in Table 30 revealed that 6/10 pseudopregnant recipients delivered offspring at term as a result of surgical transfer to the left uterine horn and only 3/10 recipients delivered offspring at term due to embryos which were transferred to the right uterine horn. Furthermore, most of the embryos which were exposed to

Table 31. Effect of a single exposure of rat embryos to the 5X concentration of eosin B on embryonic survival to term for pseudopregnant recipients in which control and treated embryos were transferred to opposing horns of the same pseudopregnant recipient (Experiment 10)

Treatment	Number of transfers	Number of litters*	Number of embryos		
			Transferred	Born	Weaned
Control	10	4	63	8	6
Eosin B	10	5	60	16	16

*Number of recipients in which at least one offspring was born and was of the same phenotype as expected for the embryos transferred to either the left or right horn.

The ratio of the embryo recipients to produce a litter containing offspring derived from surgical transfer of control embryos to one uterine horn and the ratio of control pups to survive to weaning were not different ($P > 0.05$) from that of the eosin B treated embryos transferred to the opposite horn.

There was a trend ($P < 0.1$) for an effect of treatment on the ratio of control pups to survive to term as compared to that of the eosin B-treated embryos transferred to the opposite horn.

eosin B and which produced offspring at term resulted from 2 litters (recipients 7 and 9) which accounted for 10 of the 16 offsprings born and both of these transfers of the eosin B-treated embryos had been to the left uterine horn of these recipients. Rao's Test of the ratios of control and eosin B-exposed embryos to survive to term (data from Table 30) indicated that there was no effect of treatment ($P > 0.05$) on embryo survival, but there was an effect of horn ($P < 0.01$), and the interaction treatment x horn was also significant ($P < 0.025$).

Overall, implantation sites were detected within the uterine horns of 7/9 rats (78 %; Table 32) post-weaning. Thus, morphological evidence indicated that at least 11 of 55 control embryos (20 %) and 25 of 57 eosin B-treated embryos (44 %) were viable at the time of transfer and had initiated the decidual reaction following transfer to these recipients. Based on the decidual responses, and the number of offspring produced at term, 5/11 of the control embryos (45 %) and 14/25 of the eosin B treated embryos (56 %), which implanted after transfer, survived to term, providing a further evidence that exposure to eosin B did not exert any harmful effects on embryonic survival. From these results, it was also apparent that many of the control and treated embryos transferred had initiated a decidual response following transfer, confirming their viability at the time of transfer. However, the number of pups born were less than the number of implantation sites. This suggests that embryo mortality had occurred and equally affected the survival of control and eosin B-treated embryos. Two of 10 recipient rats (Table 32) had no evidence of a prior implantation site and implantation sites were evident in both horns of 2

Table 32. The number of control and eosin B-exposed embryos transferred to the uterine horns of day 3 pseudopregnant rats, the number of pups born at term and the number of implantation sites detected post-weaning in the respective uterine horns (Experiment 10)

	Number of embryos transferred; treatment of embryos by horn				Number implanted or pups born			
					Left horn		Right horn	
Recipient	Left		Right		Implanted	Born	Implanted	Born
1	8	(5X)	4	(C)	2	2	0	0
2	4	(C)	6	(5X)	0	0	0	0
3	3	(5X)	10	(C)	ND	2	ND	3
4	7	(C)	3	(5X)	2	2	0	0
5	3	(C)	7	(5X)	2	0	4	0
6	8	(C)	8	(5X)	0	0	0	0
7	10	(5X)	10	(C)	7	5	3	1
8	6	(C)	4	(5X)	3	2	4	2
9	7	(5X)	4	(C)	6	5	0	0
10	4	(5X)	7	(C)	2	0	1	0

(5X) = Eosin B-exposed; (C) = Control; ND = Not determined, this rat had been re-mated and became pregnantfollowing the first postpartum weaning.

Implanted = The number of implantation remnants in the recipient uterine horn when evaluated after parturition and weaning or after the expected date of parturition for those rats that did not produce a litter.

The ratio of the recipients that had detectable implantation sites resulting from the surgical transfer of control embryos to one uterine horn was not different ($P > 0.05$) from the ratio of embryo recipients that had implantation sites resulting from the transfer of eosin B treated embryos to the opposite horn.

recipients that produced no offspring. Implantation sites were limited to 1 horn in those 2 recipients that produced a fetus at term of the same phenotype, as the embryos which had been transferred into that horn.

The gestation interval ranged from 21 to 23 days for these pseudopregnant recipient rats (mean ± SD = 22.3 ± 0.8) and the overall litter size ranged from 2 to 6 pups (mean ± SD = 4.0 ± 1.7).

In this experiment, the interval from euthanasia of the donor to transfer of the embryos into the pseudopregnant recipient ranged from 3.5 to 11.3 hours. Overall, differences in the survival of the transferred, control or treated, embryos did not appear to be influenced by the length of in vitro incubation prior to transfer, because a consistent pattern could no be detected. Of the offspring born and which survived to weaning, 13 were males (59 %) and 9 were females (41 %). Developmental abnormalities were not detected in any of the offspring derived from the control or treated embryos, including those fetuses which were born dead or offspring that died before weaning.

Experiment 11. Viability of Control or of Rat Embryos Exposed Once to 5X Eosin B and then Transferred to Both Horns of Pseudopregnant Rats

A total of 152 morulae and blastocysts were recovered from 44 donors and used for Experiment 11. The number of embryos recovered from each donor ranged from 0 to 15 (mean ± SD = 3.5 ± 4.2). The number of embryos transferred into the left and right horn of each recipient, their treatment

prior to transfer, the number of offspring born from the embryos transferred to each recipient, and their survival to weaning is summarized in Table 33. Eleven of these 12 recipients produced a litter (92 %) and the overall litter size ranged from 1 to 10 pups (mean ± SD = 4.8 ± 3.3). Five of the 6 recipients of embryos which were assigned to the control treatment produced a litter and all of the recipients that received embryos that had been exposed to the 5X concentration of eosin B before transfer produced a litter. The ratio of recipients that remained pregnant and produced a litter from the total number of recipients was not affected ($P > 0.05$) by the single exposure of the embryos to the 5X concentration of eosin B before transfer. The number of control and treated embryos transferred and their subsequent survival to term are summarized in Table 34. Overall, 53 of the 152 morulae and blastocysts transferred into these 12 pseudopregnant recipients survived to term (36.8 %) and 50 of the 53 pups born, survived to weaning on day 28 (94 %). Of these, 76 embryos were exposed to and were unstained by the 5X concentration of eosin B prior to transfer. From these 76 transferred embryos, 25 offspring were born at term (32.9 %) and 23 were weaned (92 %; Table 34). Twenty-eight of the 76 control embryos transferred survived to term (36.8 %; Table 34) and 27 pups were weaned (96 %). The ratio of eosin B treated embryos to survive to term and weaning from the total number of eosin B-treated embryos that were transferred was not different ($P > 0.05$; Table 34) from the control embryos that were transferred to different pseudopregnant recipients. Individually considered, no more than 10 offspring were born as a result of embryo transfer to a given rat. On a percentage basis (Table 33), embryo survival

Table 33. The number of control and eosin B (5X) exposed embryos and their survival when 10 or more embryos were transferred to both horns of day 3 pseudopregnant rats (Experiment 11)

Recipient	Number embryos transferred		Embryo treatment	Number of embryos	
	Left horn	Right horn		Born	Weaned
1	8	7	5X	4	3
2	7	7	Control	1	1
3	6	5	5X	2	2
4	6	5	Control	0	NA
5	6	5	5X	4	4
6	6	6	Control	5	4
7	6	5	Control	3	3
8	5	5	5X	2	2
9	9	10	5X	10	9*
10	6	7	Control	9	9
11	5	5	5X	3	3
12	8	7	Control	10	10

*Paralysis in the hind limbs became evident on day 19 for 1 pup in this litter, seizures were detected during the intervening period, and this pup was euthanized on day 24 for pathological examination. Radiological examination revealed a compression fracture or hemivertebra in the lower thoracic/upper lumbar region. Although the possibility of a congenital defect in this rat could not be totally eliminated, the primary diagnosis provided for a postnatal traumatic insult as the cause of the observed deficiency.

Table 34. Effect of a single exposure of rat embryos to the 5X concentration of eosin B on embryonic survival to term for pseudopregnant recipients in which ≥ 10 embryos were transferred to the left and right horns of the recipient (Experiment 11)

Treatment	Number of transfers	Number of litters*	Number of embryos		
			Transferred	Born	Weaned
Control	6	5	76	28	27
Eosin B	6	6	76	25	23

*Number of recipients in which at least one offspring was born and was of the same phenotype as expected for the embryo donor(s).

The ratio of the embryo recipients to produce a litter containing offspring derived from surgical transfer of control embryos to the left and right uterine horns and the ratio of control embryos to survive to term and to weaning were not different ($P > 0.05$) from that of the eosin B-treated embryos.

to weaning ranged from 0 % to 69 % and averaged 35.5 % (27 of 76, Table 34) for the controls and ranged from 18 % to 52 % and averaged 30.3 % (23 of 76, Table 34) for the eosin B-treated group.

Implantation sites were detected post-weaning in the uterine horns of all of the virgin females (n = 7, Table 35) that were used as recipients. Thus, morphological evidence from these 7 rats indicated that at least 27 of 39 control embryos (69 %) and 35 of 54 eosin B-treated embryos (65 %) were viable at the time of transfer and had initiated the decidual reaction following transfer to these virgin recipients. Based on the decidual responses and the number of offspring produced at term, 22/34 of the control embryos (65 %) and 19/35 of the eosin B treated embryos (54 %), which implanted after transfer, survived to term, providing a further indication that eosin B exposure did not exert any harmful effects on embryonic survival. Evidence for implantation was confined to a single horn in only 1 virgin recipient (No. 8), and these sites were in the right horn of this recipient. More importantly, it was observed, after inspection of the data presented in Table 35, that the total number of implantation sites in the left horns of these virgin recipients (n = 32) was comparable to that for the right horns of these same recipients (n = 30). This suggested that the horn of the recipient did not affect embryonic survival for these rats. It was also noted, that for those recipients that had previously been mated, the number of implantation remnants which could be detected was always equal to or greater than the combined number of pups these rats had produced at term for this transfer and from the prior natural mating(s).

Table 35. Number of control or eosin B (5X) exposed embryos transferred to the uterine horns of day 3 pseudopregnant rats, number of former implantation sites detected post-weaning in the uterine horns and the number of pups born at term (Experiment 11)

Recipient	Number of embryos transferred by horn		Embryo treatment	Number implanted		Number born
	Left	Right		Left horn	Right horn	
1	8	7	5X	5	4	4
2	7	7	Control	NA	NA	1
3	6	5	5X	NA	NA	2
4	6	5	Control	NA	NA	0
5	5	5	5X	NA	NA	4
6	6	6	Control	NA	NA	5
7	6	5	Control	4	1	3
8	5	5	5X	0	4	2
9	9	10	5X	7	7	10
10	6	7	Control	5	6	9
11	5	5	5X	4	4	3
12	8	7	Control	7	4	10

NA = Not applicable, recipient had been mated previously.

Overall, the pregnancy rate for these pseudopregnant recipient female rats was 92 % (11/12) and the gestation interval ranged from 21 to 23 days (mean ± SD = 22.1 ± 0.5). The overall litter size ranged from 1 to 10 pups for transfers of 10 or more embryos to the recipient females (mean ± SD = 4.8 ± 3.3).

In this experiment, the interval from euthanasia of the donor to transfer of the embryos into the pseudopregnant recipient ranged from 3.5 to 10 hours. Survival of the transferred embryos did not appear to be influenced by the length of in vitro incubation prior to transfer because 11 of 12 recipients that received ≥ 10 embryos produced offspring at term and for the 1 recipient in this group which produced no offspring, embryos had been transferred within 6 hours of collection. Of the offspring born and which survived to weaning, 21 were males (42 %) and 29 were females (58 %). Developmental abnormalities were not detected in any of the offspring derived from the control embryos and an abnormality was detected in 1 pup that was born from the eosin B-treated embryos. As was noted in the footnote of Table 33, the cause of this defect appeared to be the result of postnatal trauma to this pup.

DISCUSSION

Overall, the results of this study conclusively demonstrate that single or multiple exposures of rat embryos to micromolar concentrations of eosin B is a useful approach to estimate the viability of rat embryos. For the purpose of discussing the results of the research conducive to the development of an assay for embryonic viability, based on the exclusion of eosin B, I have grouped the experiments into 4 major categories, as follows:

1. Effects of Thermal Shock on Embryonic Development and the Staining of Rat Embryos.

2. Influence of the Concentration of Eosin B and Stage of Embryonic Development on the Staining Response of Rat Embryos.

3. Effects of Single, Multiple, or Continuous Exposure to Eosin B on the In Vitro Development of Rat Embryos.

4. Influence of Exposure to Eosin B on the Viability of Rat Embryos Transferred to Naturally Mated or Pseudopregnant Recipient Rats.

Effects of Thermal Shock on Embryonic Development and the Staining of Rat Embryos

To initiate the studies described in this dissertation, it was first necessary to obtain a source for dead embryos which, as previously defined, would stain when exposed to eosin B. The results of Experiments 1 and 2 clearly demonstrate a negative effect of temperature on the capability of the embryo to develop in vitro and to exclude eosin B. This was further confirmed by the additional observations to Experiment 1 (refer to Appendix 2) and during studies with bovine embryos (Appendix 5). As the temperature used to shock the embryo increased (Experiment 1), or the time of exposure to a given elevated temperature (≥ 45°C) was extended (Appendix 2), embryos lost the capability to develop in vitro and died.

The selection of thermal shock from the various methods tested for their potential as a source of dead embryos, was based on several criteria: 1) both temperature and time of exposure of embryos to a given temperature can be controlled and temperature variation provided a graded, repeatable response in cleavage activity and embryo staining, 2) the cellular alterations induced in the embryo by thermal shock were minimal, as compared to the other methods tested (Appendix 2), and 3) the interval between thermal insult and loss of the embryonic capacity to exclude dye was rapid, well within the 24 hour period of in vitro culture needed to assess development.

Hyperthermia induces damage and potentiates the sensitivity of cultured cells to radiation (Ben-Hur et al. 1972, Ben-Hur et al. 1974).

Dikomey (1981) demonstrated that the harmful effects of temperatures from 41°C to 46°C on the survival of cultured, non-embryonic cells increased with temperature and duration of exposure (0 to 360 minutes). Furthermore, the sensitivity to radiation insult, for those cells which survived the heat-treatment, was highest immediately after exposure to elevated temperature (Dikomey 1981). Even though exposure of 1-cell rabbit embryos to 40°C for 6 hours did not influence cleavage or the proportion of embryos to implant following transfer, significantly fewer of the embryos which had implanted after transfer, were viable on day 12 of pregnancy. The post-transfer viability of 2-cell rabbit embryos that received the same thermal treatment was not affected (Alliston et al. 1965). Heat treatment appears to induce discrete changes in embryonic cells, since the exposure of 1-cell embryos or morulae from mice to 56°C for 30 minutes reduced the intra-embryonic accumulation of fluorescein after exposure to fluorescein diacetate. However, heat-treatment did not influence membrane permeability because these same embryos excluded fluorescein when added to the culture medium (Mohr and Trounson 1980). The loss of esterase enzyme activity preceded the loss of embryo viability, as assessed by morphological appearance (Mohr and Trounson 1980). However, it was not reported (Mohr and Trounson 1980) whether attempts were made to culture or transfer the heat-treated, mouse embryos or to determine whether the embryos recovered the ability to accumulate fluorescein.

In my studies, the exposure of 4- to 16-cell rat embryos to 55°C for 30 minutes induced embryonic death, as evidenced by the fact that in vitro development was completely blocked and staining was apparent in all heat-

treated embryos when exposed to micromolar concentrations of eosin B. Exposures of rat embryos from the 1-cell to blastocyst stages to 55°C for 30 minutes (Experiment 2) had the same result on the developmental and staining responses of the rat embryo. From these results, it is concluded that exposure of rat embryos to 55°C for 30 minutes initiates irreversible physical alterations and/or induces changes in the metabolic processes of the embryo, which effectively eliminates the capacity for these embryos to develop and to exclude dye. By definition, embryos were considered dead when stained during exposure to eosin B. Thus, the original objective, to locate a source of dead embryos, was achieved. No attempt was made to define the nature of the changes in the embryonic cells induced by heat-treatment, nor to determine the rate at which these changes may have occurred. It is quite possible that heat-treatment induces ultrastructurally detectable, degenerative changes in the embryo which may be directly related to loss of embryonic viability or to the patterns of necrosis, which have been recognized for dead or dying cells (Cheville 1976). However, it would be impossible, solely on the basis of ultrastructural cellular changes, to precisely define the time at which viability is lost, because, as Cheville (1976) observed, cellular degeneration does not become cellular death until the point of irreversibility in the ongoing degenerative process is reached and that point, the exact moment of necrosis in the cell, is not precisely discernible. In addition, it is widely recognized in toxicity testing that both, the nature of the insult and duration of exposure of the cell to an agent influences the post-exposure response of the cell and that there may

be a series of events which occur in dying cells (see Malinin and Perry, 1967, for review). In some cases, e.g. anti-metabolic drugs, the effects may be reversible and/or may take several days to exert a measurable effect on cells (Freshney 1983). Such outcomes were unacceptable for the purposes of my embryological studies.

As is evident from the data presented in Tables 3, 4, and 5, the capacity to exclude the 1X concentration of eosin B was lost, for all of the rat embryos exposed to 55°C for 30 minutes, at some time within the 24 hour period following treatment. The precise time when this event occurred was not determined for each embryo. However, when embryos were examined at shorter intervals during incubation (Appendix 2), variations in the staining responses were observed, both within and between embryos. This is suggestive of a variable response of embryos to the lethal effects of heat-treatment or could also reflect undetermined postmortem differences between embryos. Exposure of bovine embryos to 55°C also blocked in vitro development and resulted in stained embryos. However, bovine embryos responded differently than the rat embryos when exposed to 45°C. Most of the bovine embryos (Appendix 5) cleaved and 11 of 18 (61 %) developed to blastocysts in vitro, whereas, none of the rat embryos exposed to 45°C formed a blastocoel. Thus, differences in embryonic responses to temperature may be species specific, and possibly, even stage specific for embryos from a given species.

Influence of the Concentration of Eosin B and Stage of Embryonic Development on the Staining Response of Rat Embryos

The results of Experiment 3 revealed that, regardless of the concentration of eosin B used, all embryos were completely stained within a 14 minute period following dye exposure. The time of exposure to the dye for initial and complete staining of embryos was influenced by the concentration of dye in the medium and by the stage of embryonic development. The time required for initial and complete staining of dead embryos was longer for the 1X concentration of eosin B, as compared to the 5X or 10X concentrations. The time required for staining was not different for the 5X and 10X concentrations. Compared to the 5X concentration, the 10X concentration of eosin B offered no advantage for the assessment of the staining response of embryos. In fact, the 10X concentration of eosin B was considered to be a "liability" because the intense color of the background made it difficult to determine the staining response for some embryos. In view of the toxicity evidenced during continuous exposure of embryos to the 5X concentration of eosin B, the 10X concentration is not recommended for the estimation of embryonic viability. The interval from exposure to the 1X concentration of eosin B to either initial or complete staining was longer for embryos at the morula or blastocyst stages. However, the stage of development did not affect the time required for staining, when the embryos were exposed to the 5X or 10X concentration of eosin B. Except for embryos recovered as hatched blastocysts, the embryos used in these studies were surrounded by the noncellular, zona pellucida.

Although, the presence of the zona pellucida could conceivably have influenced the time of staining, there was no evidence to suggest that the zona acted as a barrier to the diffusion of the dye. For all of the embryos tested which had a zona pellucida, the dye solution had diffused into the perivitelline space by the time of initial examination, even though variations in the time of initial and complete staining were observed among embryos from the same donor. In preliminary studies (Appendix 2), embryos which did not have an intact zona pellucida were observed to be quite capable of excluding dye and the loss of the dye from the perivitelline space during washing of the dye-exposed embryos surrounded by the zona pellucida appeared to be instantaneous. Furthermore, both non-hatched and hatched blastocysts were included in Experiment 3 and these embryos provided no evidence to suggest that the presence of the zona pellucida lengthened the time required for staining of embryos.

The results of Experiment 3 clearly demonstrated a concentration-dependent effect of dye on the intensity of staining and the time required for the embryo to become stained. Evaluation of the staining response of embryos while immersed in dye has been reported (Brock and Rowson 1950, Hutz et al. 1985). However, most methods for embryonic assessment using dyes require or recommend that the embryo is washed prior to evaluation of the staining response (Church and Raines 1980, Hoppe and Bavister 1984, Kardymowicz 1972, Mohr and Trounson 1980, Schilling et al. 1979, Thadani et al. 1982). Of those studies, only a few (Mohr and Trounson 1980, Thadani et al. 1982) exposed the embryo to the dye in the same medium used to

culture the embryo and then determined the staining response, after washing in this same culture medium. In fact, most reports have formulated the dye solution in buffered saline solutions, completely avoiding the use of culture media (Church and Raines 1980, Hoppe and Bavister 1984, Hutz et al. 1985, Kardymowicz 1972, Schilling et al. 1979).

In Experiment 3, the effect of dye concentration and the influence of stage of development on the staining response were determined while the embryo was immersed in culture medium containing eosin B. Therefore, the volume of the staining solution was held constant and only the concentration of dye, in this defined volume, was varied. Albumin displays a remarkable affinity for dyes (Chignell 1975, Edsall 1947). Thus, the intensity of staining may have been influenced by the bovine serum albumin included in the modified culture medium (Laurence 1952).

Both temperature and concentration of fluorescein diacetate influence the rate of fluorescein accumulation in preimplantation embryos from the mouse (Mohr and Trounson 1980) or bovine (Church and Raines 1980). Mohr and Trounson (1980) noted that the amount and rate of fluorescein accumulation varied greatly within embryos or even between embryos at the same stage of development. However, none of the studies so far reported has specifically investigated for an influence of the stage of embryonic development on the staining response (Church and Raines 1980, Hoppe and Bavister 1984, Hutz et al. 1985, Mohr and Trounson 1980, Schilling et al. 1979).

In my studies, I made no attempt to determine the "precise optimal concentration" of eosin B that will clearly stain dead embryos under all

conditions. A 2X concentration of eosin B appeared to be adequate for the assessment of bovine (Dooley et al. 1987), mouse (Alcivar et al. 1986) or rat embryos (Alcivar et al. 1986, Alcivar 1987). It seems that, at least for these three species, a 2X or 3X concentration of eosin B would be adequate for routine studies.

In the course of my studies, I observed a marked reduction in the staining intensity of rat embryos stained by eosin B a few minutes after the removal of the embryo from the dye solution. A similar response was observed for bovine embryos (Appendix 5). The decrease in the staining intensity (refer to Figures 1, 7, and 8), although variable within and among embryos, was such that staining could no longer be detected unless the embryos were re-exposed to dye. This observation suggests that simple diffusion was responsible for dye entry into the dead blastomeres and that there were non-covalent associations of the dye with the cellular components of the stained blastomeres. Xanthene dyes, such as eosin B and eosin Y, have long been used in histochemical evaluation as plasma stains, for their preferential association with cytoplasmic granules (Bailey 1913, Junqueira et al. 1977). Staining of dead embryos was likely due to electrostatic association between acidic radicals (anions) of the eosin B molecule and basic radicals (cations) of cytoplasmic proteins (Ham and Leeson 1961, Junqueira et al. 1977).

Effects of Single, Multiple, or Continuous Exposure to Eosin B on the In Vitro Development of Rat Embryos

The number of embryos to cleave and the overall development of 4- to 8-cell embryos during in vitro culture for 72 hours was not affected by a single exposure to medium containing the 1X or 5X concentration of eosin B. The most rapid development of control and eosin B-treated embryos occurred in the first 36 hours of culture. From these results, it is concluded that a single exposure of 4- to 8-cell rat embryos to the 1X or 5X concentration of eosin B does not alter the developmental potential of the embryo, as determined by its capacity to cleave and differentiate in vitro.

Cleavage and differentiation of embryos in vitro are accepted indications of embryonic viability (see for review: Bavister 1981, Brackett 1981, Seidel 1981). Cleavage of the embryo during a 24 hour period has been used to assess toxicity of dyes in the hamster (Hutz et al. 1985) and bovine (Schilling et al. 1979). Hoppe and Bavister (1984) used the development of 8-cell hamster embryos into blastocysts or the expansion of bovine blastocysts, during a 24 hour period of culture, to assess the potential toxicity of the fluorescein diacetate assay. Although cellular alterations due to insult may be observed immediately in some damaged cells, a 24 to 48 hour interval is frequently required for such changes to become evident (Evans 1963, Malinin and Perry 1967). Furthermore, it has been recognized (Seidel 1981) that despite the inadequacy of the present methods of bovine embryo culture, embryonic viability, as determined by pregnancy after transfer, does not begin to decline significantly unless

the period of culture is extended beyond 24 hours.

Multiple exposures of rat embryos to eosin B had no effect on cleavage or on the overall developmental responses of 4- to 8-cell embryos during in vitro culture for 72 hours. Again, developmental changes were observed within the first 36 hours of culture, and were similar to those observed for the embryos in Experiments 4 and 5. From these observations, it is concluded that multiple exposures of the rat embryo to micromolar concentrations of eosin B does not alter the developmental potential of the embryo. Comparable studies have not been performed for the other dye assays reported to be efficacious for the assessment of embryonic viability (Church and Raines 1980, Hoppe and Bavister 1984, Hutz et al. 1985, Mohr and Trounson 1980, Schilling et al. 1979).

It has been reported (Fukuda et al. 1987) that hatching of mouse embryos during in vitro culture is a more sensitive indicator of media quality than either cleavage or formation of the blastocoel. Thus, it is of interest to emphasize that, in Experiments 4 and 6, more eosin B-exposed rat embryos hatched from the zona pellucida during culture when compared to the corresponding control embryos.

The results of Experiment 7 clearly demonstrate that continuous exposure to eosin B, particularly to the 5X concentration, is toxic to the embryo. The number of embryos to cleave at least once during continuous exposure to the 1X concentration of eosin B was comparable to the cleavage of the control embryos. However, few of the embryos cultured continuously in the 1X concentration of eosin B were observed to form a blastocoel during culture and only a few of the embryos cultured in medium containing

the 5X concentration of eosin B cleaved. In addition, none of the embryos exposed to the 5X concentration of eosin B developed to the blastocyst stage. The cleavage indices and peak index of embryos exposed continuously to the 1X concentration of eosin B were significantly lower than that of the controls, but higher than that of embryos exposed continuously to the 5X concentration of dye. These findings confirm that there are marked and concentration-dependent differences in the cleavage and overall developmental responses of embryos exposed continuously, during culture, to eosin B. Deleterious effects of continuous exposure to the dye were evident from the gross alterations, over time, in the morphological appearance of these embryos. From these results, it is concluded that the embryo cannot survive continuous exposure to the 5X concentration of eosin B in the culturing medium and that even continuous exposure to the minimal effective, 1X concentration of eosin B, is detrimental to rat embryos. The potential detrimental influences of continuous exposure of embryos to fluorescein, fluorescein diacetate, trypan blue, neutral red or other dyes reported to provide the means to distinguish between viable and nonviable embryos, have not been reported.

Continuous culture has been used as a means to determine metabolic pathways in the developing embryo (Biggers 1971), identify protein supplements which are beneficial or detrimental to the embryo (Canfield et al. 1986, Caro and Trounson 1984, Kane 1983, 1985), and to select media or media components for embryo culture (Kane and Foote 1970). The continuous culture of embryos in medium containing viruses also has been used to determine their effects on embryonic development and then to identify those

viruses which directly affect the embryo (Gwatkin 1966, Heggie and Gaddis 1979). In my studies, embryos were observed to cleave even during continuous exposure to the 1X concentration of eosin B. Thus, the inclusion of eosin B at the minimal effective concentration in the culturing medium would provide a method for the evaluation of the effects of microorganisms and viruses on the mammalian embryo during short-term in vitro culture.

The 72 hour culture interval selected for the studies reported in this thesis compares to the in vivo period within which embryos are expected to form a blastocoel, hatch, and implant (Dickmann and Noyes 1960). Therefore, it is reasonable to assume that if eosin B induced degenerative changes, which would lead to preimplantation losses, they would have been evident by the end of this period of culture. In retrospect, the 72 hour interval of culture used for my studies might now be considered as excessive, because most of the embryos which cleaved, or cleaved and formed a blastocoel had done so within the initial 48 hours of culture.

In my studies, the primary criteria to analyze for a detrimental influence of eosin B on the in vitro development of the embryo was the morphological integrity of the embryo or detectable changes in morphology. In a review of culture of mammalian embryos, Brinster (1969) recognized that, while it is desirable to determine whether the embryonic cell lives or dies during a specified period of culture or forms a blastocyst, such obvious end points are not always available and that they often measure only severe effects. This author then concluded that these overall endpoints do not permit the demonstration of a graded response, necessary

to determine which of a series of treatments is best. Morphometric analyses could provide a quantitative measurement of the embryo for the analysis of effects on embryo development. However, as noted in the introductory section of this thesis, while morphometric analysis of embryos can be used to detect differences in diameter of blastocysts to analyze the responses of embryos to factors in culture (Kane 1985), these analyses are not generally applicable to the earlier stages of development, because the initial cleavage divisions are reductional in nature (Papaioannou and Ebert 1986, Richa and Solter 1986). In fact, Linares and King (1980) noted no difference in zona pellucida diameter between normal blastocysts and degenerate embryos and, despite the degenerative changes, the diameter of the cell mass of normal blastocysts was only slightly greater than that of degenerate blastocysts. Thus, the apparent size of the blastocoel is commonly used to classify changes in blastocysts (Hasler et al. 1987), whereas cleavage activity is recommended for use in the earlier embryonic stages.

Morphological evaluation of the embryo is particularly useful when used to evaluate embryos immediately after recovery from the reproductive tracts, because, as I have contended in this thesis, such embryos are not only being evaluated for their appearance at the time of collection, e.g., their apparent morphological integrity, but also, on their prior developmental history. In my studies, the timing of fertilization could be predicted within a few hours. Based on this predicted time of fertilization, the recovery and selection of embryos at defined intervals post-mating or post-insemination may have resulted in the selection of a

sub-population of embryos in which development was proceeding normally. I believe therefore, this approach ensured that the embryos assigned at random to control or treatment in this study had considerable uniformity. Due to the experimental designs used, the staining responses of embryos prior to (Experiments 4 and 5) or during culture (Experiment 6) were not obtained for the control embryos. Nevertheless, the staining responses of the eosin B-treated embryos prior to in vitro culture confirm that the selection of embryos based on morphologic criteria resulted in relatively homogenous populations. Nonviable embryos were not assigned to any of the eosin B-treated groups and partial staining was detected in relatively few embryos.

In Experiments 4 and 5, the post-culture staining responses and the staining indices of the control and eosin B-exposed embryos were not different. This further confirms that eosin B exposure did not induce cell death in these embryos. There were a few completely stained embryos in the control and eosin B treatment groups of Experiment 4, indicating that embryonic losses occurred during culture due to factors unrelated to the eosin B treatment.

In Experiment 6 at the end of culture, there were significantly more embryos in the eosin B-treated group which were partially stained by the 1X concentration of eosin B, as compared to the controls. This difference in the staining response of control and treated embryos could be ascribed to a toxic influence of eosin B, as evidenced by the results of Experiment 7. I now believe, however, that the effect of eosin B treatment on the post-culture staining responses was the result of an unequal distribution of

embryos. None of the embryos assigned to the eosin B treatment group was stained at the time of initial exposure in Experiment 4, and only 2 of 84 embryos were stained by the dye at the time of assignment in Experiment 5. In Experiment 6, however, 8 of 63 embryos were partially stained before culture despite the selection of embryos based on morphological appearance. Thus, it is possible that more partially stained embryos were assigned, by random occurrence, to the eosin B group in Experiment 6. The lack of an effect of eosin B on intermittently exposed embryos, as reported in Appendix 5, appears to confirm this was the case. Therefore, I conclude that multiple exposures of the embryo to eosin B do not induce cell death in the rat or bovine (Appendix 5) embryo. On the other hand, it is quite clear that continuous exposure of embryos to the 5X concentration of eosin B is lethal.

The number of embryos that stained when exposed to dye increased during culture in all experiments. Several of the control and treated embryos stained completely when exposed to eosin B at the end of culture (Experiment 4). This confirms that complete staining of the embryo, when exposed to eosin B, is not an artifact of heat-treatment. It should also be noted that less development occurred in embryos for which a high proportion of the embryonic mass was stained at the end of culture.

It is important to emphasize that cleavage was never detected in an embryo that was completely stained, no matter how long that embryo was cultured. Furthermore, for those embryos in which cleavage activity was detected, development occurred only for those embryos which had blastomeres that were unstained by the eosin B. It is clear, however, that a different

scoring system needs to be developed, which adequately reflects the proportion of the embryonic mass that is stained by eosin B. The classification system I used: unstained, partially stained, and completely stained, even though useful for the studies of this thesis, does not consider the potential of unstained blastomeres to develop over time into a normal embryo, nor does it adequately reflect the degree of embryonic heterogeneity within the subclassification referred to as partially stained. It is possible that a more refined staining index, which reflects the proportion of the embryo which is stained, can provide an accurate means to predict the developmental potential of the embryo in vitro or in vivo.

The results of Experiment 7, indicate that eosin B reduced over time the capacity of embryonic cells to cleave, and resulted in the death of blastomeres. It has been demonstrated that eosin Y can act as a photosensitizing compound that exerts a direct cytotoxic effect (Bolande and Wurz 1963). In fact Bolande and Wurz (1963) presented evidence for an effect of light and the photosensitizer (eosin Y) on the cells within a 15 to 20 minute period of illumination, and an additional indirect cytotoxic effect, which suggested that a diffusible cytotoxin was released that may have been a product of eosin Y. Bolande and Wurz (1963) concluded that the direct cytotoxic action of eosin Y was greater and more rapid, as compared to the cytotoxin that was released. In fact, the toxicity of the diffusible cytotoxin was not observed to affect the cells until after a 1 to 2 hour period of exposure had been provided.

A review by Combes and Haveland-Smith (1982) indicates that there is

no evidence to suggest that xanthene dyes are mutagenic in mammals. However, Yoshikawa et al. (1978) has demonstrated that xanthene dyes, including eosin B, do not induce nonreparable damage in the DNA of B. subtilis, unless the mixtures are irradiated by white light. Similar effects have been observed using other fluorescein derivatives, such as phloxine and phloxine B (Maus et al. 1981). Combes and Haveland-Smith (1982) proposed that xanthene dyes exert a dual activity on cells; at low concentrations, due to light and singlet O_2 production in the absence of molecular dimerization and, at high doses, due to the chemical itself. In view of the results of these in vitro studies, the widespread recognition of the cellular effects of illumination with ultraviolet and visible light (see for review: Coohill and Jacobson 1981), and the interactive effects of dye binding and photosensitization on mammalian and nonmammalian cells (see for review: Amagasa 1981), it is recommended that exposure of the eosin B solution, culture medium, and embryos to incident light be carefully controlled until the conditions for safe storage, exposure, and evaluation of the embryos can be more thoroughly determined. I consider it unlikely that continuous exposure of embryos to eosin B will ever be used for routine culture of embryos, although it may be possible to further minimize the potential toxic effects of eosin B, by the inclusion of additional reducing agents into the culture medium (Bolande and Wurz 1963). All of the embryos of this study were exposed to visible light. While it is likely that the effects of eosin B observed on the embryos were due to the indirect cytotoxic effects described previously, it is not possible to rule out an effect of the illumination, which was provided in conjunction

with embryo evaluation during culture or during the routine handling of the stock solution of eosin B.

Both parametric and nonparametric statistical procedures were applied to the results obtained for the in vitro development and staining responses of rat embryos. It has long been recognized that parametric methods are efficient and useful statistical techniques. Unfortunately, parametric methods may not be applicable to the analysis of embryonic development. While the preimplantation development of the embryo is a continuous process, the evaluation of the in vitro development of embryos is based on stages of development and these data are discrete. Such evaluations are qualitative and therefore do not allow for the arrangement of the measurement of embryonic development into an "order of size" (Tate and Clelland 1957). Biggers and Brinster (1965) applied the angular and probit transformations to the results obtained for the culture of groups of preimplantation embryos. Kane and Foote (1970) assigned numerical scores, based on developmental stage, to each embryo and then used the non-zero weighted means of embryo groups to compare treatments. Analyses of variance have been used for scores assigned to developmental stages (Canfield et al. 1986), numerical indices to compare cleavage activity among embryos (Menino and Wright 1982, Wright 1977, Wright et al. 1976a,b) and counting of the total number of cell nuclei (Kane 1983, 1985) to determine the effects of treatment on the in vitro development of the embryo. In all instances a numerical value was assigned to an embryo and used for the comparison of the effect of treatment(s) to groups embryos by analyses of variance. More recently, a coding system for mouse embryonic

development was reported (Menino et al. 1985). In that system, the coded embryo development ranged from 0 = 2 cell stage to 9 = hatched blastocyst, and over one-half of the coded range, scores from 5 to 9 referred to the various conformations of the blastocyst. These coded embryo scores were then used (Menino et al. 1985) to determine the effects of different media on embryonic development, for the correlation of embryonic development with protein levels in various media, and the calculation of regression equations for the predicted development of embryos, based on the total protein or concentration of BSA in the medium.

In my studies, all experiments were designed for analysis using the χ^2 distribution. However the cleavage indices, described in Table 1, were developed after Menino et al. (1985) to facilitate the collection, summary and analysis of data. Statistical analyses of the results of in vitro embryo culture demonstrate that the peak index, determined after culture of embryos for 72 hours, correspond with the results of statistical analyses for embryonic development by the χ^2 statistic. However, the statistical analysis using cleavage indices allowed for analyses that were not possible by the use of χ^2. Thus, cleavage indices showed that there were developmental changes over time for the control and treated embryos and the interaction, treatment x time period, indicated that the rates of development for the control and treated embryos were similar in Experiments 4, 5, and 6, but different in Experiment 7. Additional confirmation of the validity and the obvious utility of such indices was evident from the observed development and analysis for the effect of treatments of bovine embryos in Appendix 5.

I believe the cleavage indices, as shown in Table 1, offer a real advantage to biological testing of embryonic factors. This system provided a scale for the classification of the developmental responses of embryos and allowed the use of analysis of variance models to evaluate the effect of time and the interactive effects of time and treatment. This type of analysis is needed to test for "rate differences" in cleavage among embryos. This may prove to be a sensitive indicator of the effects of treatment on embryos. Others (Takahashi and Kanagawa 1986) have monitored the changes in embryonic development over time by treating each time period, in effect, as a separate experiment. This is inappropriate, because the correlation of errors are simply ignored.

Influence of Exposure to Eosin B on the Viability of Rat Embryos Transferred to Naturally Mated or Pseudopregnant Recipient Rats

The development of embryos to term after transfer to an appropriately synchronized recipient is considered to be the "ultimate" test of embryonic viability (Whittingham 1978). To ensure that eosin B exposure did not exert a delayed effect on embryonic survival, a series of experiments was performed to determine the influence of eosin B exposure on embryonic survival to term and weaning. Asynchronous transfers, in which the embryo donor was "advanced" 1 day relative to the recipient, were used for both naturally mated and pseudopregnant recipients. The results of Experiments 8, 9, 10, and 11 provide conclusive proof that embryos exposed to and

unstained by the 5X concentration of eosin B retained the capacity to develop to term and weaning. Exposure of embryos to eosin B did not reduce embryonic survival after transfer or the ongoing pregnancy in naturally mated recipients (Experiments 8 and 9). Furthermore, the eosin B-exposed embryos were capable of competing successfully with the "self" embryos of the recipient.

McLaren and Michie (1956) reported that, in the mouse, the survival rate of the "alien" embryos was highest for transfers in which the donor embryos were recovered on day 3.5 and transferred into recipients on day 2.5 of gestation. The overall embryonic survival for transfers of 5 to 18 "alien" embryos to the left horn of pregnant recipients on days 16 to 17 post-coitum was 22 %, but when larger numbers of embryos were transferred into 1 uterine horn of the recipient, the number of implantations decreased in the uninjected horn. Overall, 42 % of the mouse recipients which received an average of 9 embryos at transfer had 1 or more implanted "alien" embryos on days 16 to 17 and similar implantation rates were observed for transfers to naturally mated or pseudopregnant mice (McLaren and Michie 1956). Comparable studies have not been performed for the rat. The lower survival rates obtained for the transfer of embryos into naturally mated rats in Experiments 8 and 9 of this study may reflect species differences or may have been biased by the fewer numbers of embryos, which were transferred to these rats. Transfer of dye-exposed embryos to naturally mated recipients has not been performed for other dye-assays. However, it has been reported that exposure to fluorescein diacetate did not affect embryonic survival to day 17 of pregnancy for the

blastocyst stage embryos of the mouse (Mohr and Trounson 1980) and exposure to neutral red and trypan blue did not affect development to term following transfer of frozen-thawed mouse blastocysts to pseudopregnant recipients (Thadani et al. 1982). Others have reported that exposure of embryos to fluorescein diacetate prior to transfer did not affect the post-transfer viability of bovine blastocysts (Hoppe and Bavister 1984), 8-cell hamster embryos (Hoppe and Bavister 1984) or rabbit embryos collected 2 to 4 days after mating (Schilling et al. 1979).

In Experiment 10, live offspring were obtained from the transfer of embryos of different phenotypes, assigned to either a control or eosin B treatment, to opposing horns of the same pseudopregnant rat. The exposure of the embryo to eosin B was not detrimental to embryonic survival, and as shown in Table 31, there was a trend for more of the eosin B-treated embryos to survive to term than for the controls. Further analysis indicated that the variation in embryonic survival was due to an effect of recipient horn and not eosin B treatment. The overall pregnancy rate of 60 % was comparable to that observed for the transfer of a minimum of 20 mitochondrial DNA-typed embryos between rats of the same or different mitochondrial DNA type (Alcivar 1987) or for allotypic transfers between rats of unknown mitochondrial DNA type (Noyes et al. 1961). Higher pregnancy rates were reported for recipient rats which were euthanized on day 18 (Noyes and Dickmann 1960).

From the results of Experiment 10, it was not possible to isolate the effects of donor and recipient horn from that of the treatment provided to the embryo before transfer. This resulted because the embryos recovered

from each donor, or the combined embryos from 2 donors of the same phenotype, were assigned to either the control or treated groups and then transferred to only 1 horn of the recipient. Hence, the effects of treatment and donor were completely confounded for this experiment and, as noted in the results section for Experiment 10, there was an unequal distribution of control and treated embryos across horns for those recipients that became pregnant. The differences observed for the effect of horn in Experiment 10 could therefore reflect donor, treatment, or horn effects or a combination of these variables. Overall, a total of 24 offspring were born from the 123 morulae and blastocysts transferred (19.5 %) and this compares favorably to the survival of 80 of 640 embryos of different phenotypes to opposite horns of the same pseudopregnant recipient (12.5 %) reported by Noyes et al. (1961). Aside from the fact that both control and treated embryos were transferred to the same rat in Experiment 10, the recovery, handling, treatment, and transfer procedures used were comparable to those of Experiments 8 and 9. For Experiment 10, the percentage of embryos to survive to term in those rats which were pregnant and produced offspring at term was 31.5 % (calculated from data in Table 30) and the implantation rate in these rats was over 40 %. Therefore, the percentage of rat embryos to survive to term appeared to be twice the survival rate for embryos of Experiment 8 and was over 3 times higher than for Experiment 9, indicating that the conditions used, vasectomized male and transfer of a known complement of embryos to each horn of the pseudopregnant recipient, were superior to that provided by the use of the naturally mated recipients.

Alcivar (1987) reported that the embryonic survival rate to birth was 12.5 % when calculated only for the recipients that produced offspring at term. An effect of overcrowding was not evident from the data in Table 32, because implantation sites and/or live offspring were detected in 9 of the 12 uterine horns that received 6 to 10 embryos (75 %) and in only 4 of the 8 uterine horns that received 3 or 4 embryos (50 %). However, the implantation rate of 10.7 % for recipient horns that received 6 to 10 embryos appeared to be lower than that for uterine horns in which 3 or 4 embryos (30.8 %) were deposited. The number of recipients to become pregnant and the number of rat embryos to implant are variable and worse, have even been observed to vary markedly within the same laboratory over time (Noyes et al. 1961). McLaren and Michie (1956) had estimated that in approximately one of three transfers the entire group of mouse embryos to be transferred to a recipient is lost. Thus, two-thirds or more of the embryos transferred after selection and treatment may have been lost, due to factors that were unrelated to dye exposure. Additional factors that could have influenced the results of the studies of this thesis, but for which the effects could not be determined, include: presence and localized influences of M. pulmonis or other reproductive pathogens and/or the altered immune responsiveness of these females.

The results of Experiments 8, 9, and 10, indicate that the highest pregnancy rate that could be obtained would follow from transfer of a minimum of 5 embryos to each horn of pseudopregnant recipients. The results of Experiment 11 support this contention and, in addition, confirmed that eosin B exposure does not affect embryonic survival to term.

In Experiment 11, the implantation rates for embryos from the same donor and transferred to the left and right horns of the same virgin recipient were similar. These results compare favorably with literature reports for the rat (Dickmann and Noyes 1960, Noyes and Dickmann 1960, Noyes et al. 1961).

The blastocyst stage represents the first overt signs of differentiation in eutherian mammals (Papaioannou and Ebert 1986). Exposure of morulae and blastocysts of the rat to eosin B prior to transfer did not effect the subsequent survival of embryos. No evidence was obtained to suggest a teratological influence of dye exposure on the offspring obtained at term or at weaning. For compounds which have been shown to be teratogenic in rats, the teratogenic effects appear at concentrations below toxic levels (Cicurel and Schmid 1988). In fact, I am not aware of studies which demonstrate that any of the xanthene dyes are teratogenic in mammals. This is a clear advantage of the viability assay based on the exclusion of eosin B because exposure of post-implantation rat (Turbow 1966) or rabbit (Ferm 1956, 1971) embryos to trypan blue resulted in developmental abnormalities. It remains to be determined, however unlikely, whether genetic defects may become apparent in the offspring derived from embryos exposed to eosin B at later stages of life or in subsequent generations.

SUMMARY AND CONCLUSIONS

The evaluation of the staining response to eosin B provides a simple, rapid, and reliable method for the detection of dead blastomeres and/or embryos. The indicator dye, eosin B, is readily soluble in aqueous solutions and can be formulated within the same medium used for embryo culture. Staining was evaluated using conventional, compound or stereoscopic microscopes. This is an added advantage over the other methods, which require fluorescence illumination of the embryo for the evaluation of viability. Embryos unstained after a 10 minute exposure are viable and nonviable embryos can be identified by their staining response to eosin B, which occurs in less than 1 minute at the higher concentrations. The staining response can be determined for an embryo while immersed in a solution of eosin B or during washing. Stain is lost rapidly during washing, allowing dye exposure, staining, and stain "removal" to be accomplished within a few minutes, which is another advantage of the method I have developed.

Single or multiple exposures to the 1X concentration of eosin B did not influence the developmental potential of rat embryos during in vitro culture. Toxicity, however, was evident during continuous culture in the 1X and 5X concentrations of eosin B. Single exposure of embryos to the 5X concentration of eosin B did not influence the subsequent developmental potential of rat embryos during in vitro culture and exposure of embryos to this concentration of dye had no affect on their post-transfer survival. Live, normal offspring were born from the transfer of unstained rat embryos

which were exposed to the 5X concentration of eosin B.

We had anticipated (Dooley et al. 1984) that the exclusion of eosin B was suitable for development as a nonlethal method for the estimation of viability and evaluation of mammalian embryos. The results of this study confirm the validity of that assertion for embryos from the rat, and in part, for embryos from the mouse and cow. I anticipate that comparable results can be obtained for embryos from these species and validation assays can be extended to other laboratory and livestock species. I predict that the eosin B assay will have a significant impact on the assessment of the preimplantation embryos of domestic livestock species and it will facilitate embryo selection prior to transfer. The widespread application of this technique may eventually lead to a refined and inherently more flexible definition of morphologic normality of the preimplantation embryo.

Morphologic evaluation of cow and pig embryos is more difficult than for the rat or mouse due to the higher content of cytoplasmic granules. In the bovine, it has been observed that embryos rated on morphologic criteria as poor or degenerate, retain the capacity to develop in vitro and there are even reports that such embryos have the capacity to develop into live offspring, if transferred to appropriately synchronized recipients (Seidel 1981). Bovine embryos which, on the basis of morphological criteria were classified as abnormal, had blastomeres which retained the capacity to exclude dye and developed during culture (Dooley et al. 1987). This suggests that the capacity to exclude eosin B will be particularly useful for the identification of abnormal embryos which may have the capacity to

develop in vitro or produce live offspring after transfer. Often these embryos, because of their appearance at the time of collection, are classified as abnormal and discarded in routine studies or in commercial transfer units, which adds to the cost and decreases the margin of profits for the livestock industry.

A shift in emphasis is needed, from studies which evaluate embryonic potential based on morphological appearance of normality, to the development of a fundamental understanding of the biochemical and biophysical bases of the differential staining responses and potency of blastomeres. Furthermore, the method for the estimation embryonic viability I have developed will greatly facilitate the advancement of studies for which the manipulation of embryos is essential.

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APPENDIX 1

Formulation and Storage of Medium for the Culture of Embryos

The medium used to culture embryos was a modification of the medium developed by Yamamura and Markert (1981). The formulation, final concentration, or volume of stock solution added for each ingredient used in this modified culture medium are listed in Table 36.

The primary modifications to the original medium described by Yamamura and Markert were as follows: Additions--Polyvinyl Alcohol 0.01% (Crystalline Type II, Cold water soluble, P-8136, Sigma Chemical Company, St. Louis, MO), HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 391338, Calbiochem, La Jolla, CA), and Gentamicin; Deletions--Streptomycin Sulfate and Penicillin G, K salt.

Reagent or tissue culture grade chemicals were used at all times. The inorganic salts and glucose were obtained through Fisher Scientific Co., Inc. (Fair Lawn, NJ). The phosphorylated nucleotides (AMP, CMP, GMP, TMP, UMP, and ATP) and the Bovine Serum Albumin--Crystallized and Lyophilized, A-7638, were purchased from Sigma Chemical Co., Inc. (St. Louis, MO). The concentrates of essential and non-essential amino acids, and MEM vitamin supplements were obtained through Whittaker M. A. Bioproducts, Inc., Walkersville, MD. The phenol red solution was obtained from Gibco Laboratories, Grand Island, NY.

The modified culture medium was prepared using Type I deionized water, at 1 to 2 week intervals, in 100 ml batches for Experiments 1 to 7 and at 2

to 6 week intervals, in 250 ml batches for embryo transfer, for Experiments 8 to 11. The medium was adjusted to pH 7.40 ± 0.02 at 37°C to 39°C immediately after preparation, filter-sterilized using 0.2 μM filters (Nalgene Company No. 150-4020, Rochester, NY), and placed in sterile 125 ml glass bottles. The bottle containing this medium was gassed for 15 minutes with 90 % N_2,
5 % O_2, 5 % CO_2, sealed with a latex septum, and stored at 4°C until used. After each use, the medium was "gassed" again for 15 minutes and returned to the 4°C refrigerator for storage. Exposure of culture media to room light (fluorescent) and atmosphere was held to a minimum.

Table 36. Modified embryo culture medium*

Component	mg/100 ml	ml/100 ml
NaCl	544	
KCl	35.6	
KH_2PO_4	16.2	
$MgSO_4 \cdot 7H_2O$	29.4	
$NaHCO_3$	190	
Glucose	100	
Sodium Pyruvate	3.6	
Calcium Lactate$\cdot 5H_2O$	49.6	
Gentamicin (10 mg/ml)	--	0.5
ATP,AMP,GMP,TMP,UMP, and CMP	1.0	
NAD	0.1	
NADP	0.1	
Glutamine	14.6	
Essential Amino Acids	--	2.0
Non-essential Amino Acids	--	1.0
Vitamin Solution	--	1.0
Bovine Serum Albumin	300	
Phenol Red (5 mg/ml)	--	0.2
HEPES	358	
Polyvinyl Alcohol	10	

*pH Adjusted to 7.40 at 37°C to 39°C using 1N HCl prior to filtration.

APPENDIX 2

Preliminary Trials to Select a Method to Induce Death in Rat Embryos

To initiate the studies of this thesis, it was first imperative to find a method which would reliably produce dead embryos. Death can be defined philosophically, or even legally, in many ways, but for the purposes of my studies I had to find a method that would produce irreversible changes in the embryo such that all embryos were rendered incapable of cleaving yet, at the same time, minimally alter the gross morphological appearance of that embryo. As defined previously in the Materials and Methods section, I arbitrarily defined a dead embryo as an embryo which could not develop in vitro and was stained by exposure to micromolar concentrations of eosin B. The validation of the dye-exclusion assay I intended to develop also would require the evaluation of the staining response to be performed on embryos at various stages of preimplantation development. Thus, it was not only necessary to induce embryonic death, but also, to maintain as much of the gross morphologic features of the embryo as possible.

The following methods were considered and received at least a preliminary screening: Culture in suboptimal conditions or for extended periods, low temperature storage, repeated freezing/thawing, exposure to cyanide, and heat shock.

Culture in suboptimal conditions

Embryos were cultured for 96 to 168 hours at 20°C or 37°C in modified culture medium and exposed to an atmosphere of 5 % CO_2 or room air, or in medium that had been gassed with N_2 and was then sealed. Embryos were examined at 24 hour intervals and, in general, all were observed to degenerate and lose the ability to exclude eosin B (110 to 120 μM) as the period of culture increased. However there were marked variations in the staining response among embryos and more importantly, there were also severe morphological alterations in most of the embryos during culture. Thus, the method was deemed to be not suitable for further testing.

Low temperature storage

A total of 232 embryos ranging from 1-cell to blastocyst stages were recovered and stored at 4°C in modified culture medium for 6 to 32 days. At variable intervals, embryos were recovered from the culture dishes, exposed to the 1X concentration of eosin B, and evaluated for staining. None of the embryos cleaved while maintained at this low temperature. A total of 66 of the 232 embryos apparently disintegrated during storage, as these embryos were never recovered. The staining response was variable and increased over time. Only unstained embryos were detected when examined after 6 days of storage. Most of the embryos were stained when examined after 12, 15 or 16 days of storage, however, a few embryos were capable of excluding dye for even longer periods of storage, and 2 embryos were unstained when examined after 23 days of storage at 4°C. In general, the embryos were either unstained by the eosin B or stained completely when examined. In some cases the unstained embryos were returned to the

refrigerator and then re-examined in later periods. For these embryos, the proportion of embryos to stain also increased with the period of storage. However, as the period of storage increased, the embryos underwent severe morphologic alterations and most were degenerate at the time of complete staining. Storage of rabbit embryos for 24 hours at 10°C was reported to decrease embryonic viability (Anderson and Foote 1975). However, when the storage temperature was lowered to 4°C there was no loss of viability of rabbit embryos after a 7 day period of storage and one-third of the embryos stored for 15 days were capable of development to blastocysts during in vitro culture (Hughes and Anderson 1982). In these studies, no systematic attempts were made to culture or to transfer the rat embryos that, when exposed to eosin B, remained unstained after periods of extended storage at 4°C. I have, however, cultured rat embryos that were stored at 4°C for periods of 24 to 48 hours and observed that they retained the capacity to cleave and form a blastocoel when cultured at 37°C. Again, for the reasons stated above, the extended storage of embryos at 4°C was not suitable for my purposes.

Freezing and thawing

Suboptimal freezing has been used by others (Hutz et al. 1985, Thadani et al. 1982) as a means to damage mammalian embryos in studies intended to validate vital staining and dye-exclusion assays for the evaluation of embryonic viability. Thus, I attempted to kill rat embryos by freezing to - 20°C and subsequent thawing to room temperature. This treatment induced partial or complete staining in some embryos; however, the staining responses was variable among embryos and several freezing and thawing

cycles had to be performed to ensure complete staining. Again, a large proportion of the blastomeres were lysed during repeated freeze/thaw cycles. No attempt was made to culture these frozen-thawed embryos or to include cryoprotectants within the medium used for freezing and thawing of the embryo in an attempt to minimize structural alterations. Based on the gross appearance of these embryos after freezing and the variable staining responses, this method was also considered to be unsuitable.

Cyanide exposure

Rat embryos were placed in individual wells of culture dishes, which contained 0 to 100 mM NaCN or KCN in culture medium. All solutions tested were alkaline, in the pH range of 7 to 10. Rat embryos exposed to cyanide stained within ≤ 24 hours of culture at 37°C. At the highest concentration of cyanide tested (100 mM), exposure induced the collapse of the blastocyst within minutes and then staining by eosin B was observed at variable periods after exposure and collapse of the blastocoel. This method of killing was not adopted due to the failure of embryos to maintain the appearance of a blastocyst after cyanide exposure, the variable alkalinity of the cyanide solutions, and the inherent danger of cyanides for the operator and laboratory personnel.

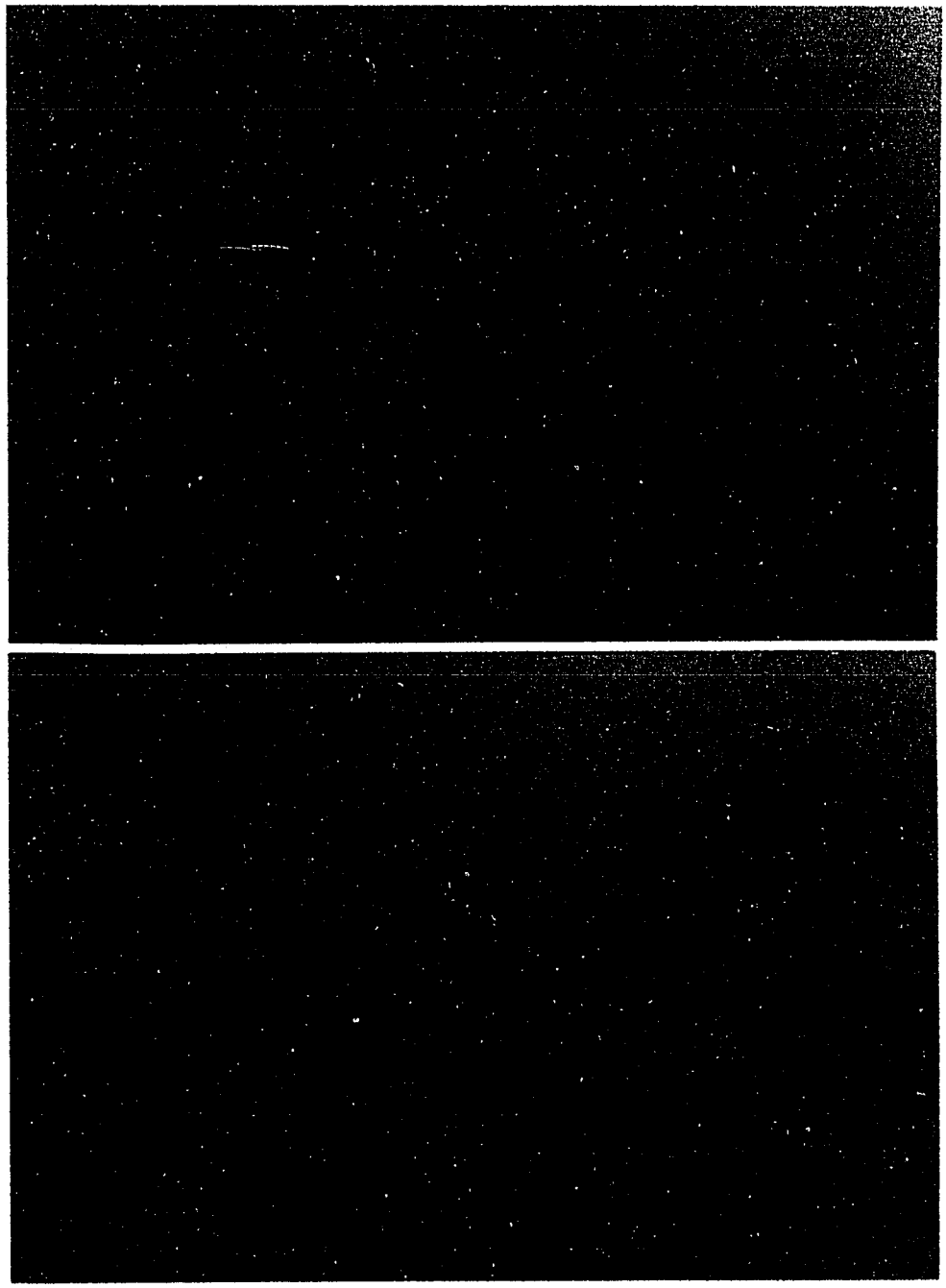
Heat shock

Embryos were heated from 1 to 60 minutes at temperatures ranging from 45°C to 70°C. A few embryos heated to 70°C for 1 to 2 minutes remained unstained when exposed to eosin B, whereas, all of the embryos heated for 3, 4, or 5 minutes at 70°C were stained when exposed to eosin B. Fragility of these embryos increased after heat-treatment, particularly at 60°C to

70°C and the zona pellucida for some embryos was destroyed by heating. In fact, most of the embryos heated at temperatures ≥ 60°C became "sticky" and could not be recovered from the culture tube after heat-treatment, or were lost during transfer to the dye solution for the evaluation of the staining response. Exposure of embryos to 45°C to 55°C resulted in staining over time and appeared to be the method of choice, since hyperthermia produced a much more consistent staining response in the embryos and, when compared to the other methods tested, the gross morphologic alterations were minimal.

Thus, heat-shock was the method selected for further evaluation as an embryo-lethal method for the validation of the eosin B assay. During the heating trials, it was observed that when embryos were heated to 50°C or 55°C for 30 minutes and exposed to eosin B immediately after heat-shock, there was a variable staining response among embryos (Figure 7). This suggested that embryonic death by heat-shock was not immediate for all embryos, but occurred over time after this brief period of heat-treatment. As is demonstrated in Experiments 1, 2, and 3, staining is evident within minutes in all embryos exposed to 50°C or 55°C.

Figure 7. Staining responses of rat embryos exposed to the 5X concentration of eosin B, immediately after heat-treatment at 50°C or 55°C for 30 minutes. A = unstained and partially stained embryos exposed to 50°C for 30 minutes. B = partially stained embryos exposed to 55°C for 30 minutes



APPENDIX 3

Effect of Intermittent Exposure to Eosin B on the In Vitro Development of Rat Embryos

A total of 310 embryos at the 4- to 8-cell stages had been recovered from the 55 donors used for Experiment 7. Of these, 232 embryos were assigned to the control or continuous exposure groups in Experiment 7, and the remaining 78 embryos from these same donors were used in this additional observation on the effect on intermittent exposure to eosin B. These 78 embryos were assigned and treated as a 1X eosin B intermittent exposure group and cultured along with the control and 1X and 5X continuous exposure groups of Experiment 7. The number of control (as reported in Experiment 7) and the intermittently exposed embryos that cleaved, cleaved and formed a blastocoel, or cleaved, formed a blastocoel, and hatched from the zona pellucida, are shown in Table 37.

A total of 69/78 embryos exposed intermittently to the 1X concentration of eosin B cleaved during culture as compared to 68/80 embryos for the control group. There was a significant ($P < 0.0001$) effect of time on the cleavage indices (Table 38 and Appendix 4), but the effects of treatment and the treatment x time interaction were not significant ($P > 0.05$). Variation in cleavage, as reflected by the magnitude of the standard deviation appeared to increase over time for the control and 1X intermittent-exposure groups (Table 38), reflecting cleavage activity of individual embryos within the control and this treatment group.

Table 37. Developmental responses of control and eosin B treated 4- to 8-cell rat embryos during culture (Appendix 3)

Treatment	Number of embryos	Developmental response			
		Did not cleave	Cleaved	Formed blastocoel	Hatched
Control	80	12	11	48	9
Eosin B	78	9	19	41	9

Did not cleave = number of embryos in which no further development was obtained during the culture period.

Cleaved = number of embryos that cleaved at least once during the culture period, but did not form a blastocoel.

Blastocoel = number of embryos which formed a clearly defined blastocoel during the culture period.

Hatched = number of embryos which formed a clearly defined blastocoel during the culture period and were observed to be in the process of hatching or had hatched from the zona pellucida.

The development of rat embryos was not influenced ($P > 0.05$) by intermittent exposure to the 1X concentration of eosin B.

Table 38. Cleavage indices of control and eosin B treated 4- to 8-cell rat embryos during culture (Appendix 3)

Treatment	Mean ± (SD) cleavage index							Peak* index
	0 h	12 h	24 h	36 h	48 h	60 h	72 h	
Control	5.6	6.3	8.0	8.5	9.2	8.5	7.3	11.1
	(0.7)	(2.0)	(3.4)	(4.9)	(5.0)	(5.9)	(6.0)	(3.2)
Eosin B	5.8	6.6	8.2	8.4	8.5	8.1	7.3	11.0
	(0.6)	(2.3)	(2.9)	(4.9)	(5.4)	(6.0)	(5.9)	(2.9)

*Peak index = the maximal cleavage index that was recorded for the embryo during the 72 hour period of in vitro culture.

There was a significant ($P < 0.0001$) effect of time on the cleavage index but the effects of treatment and the treatment x time interaction were not significant ($P > 0.05$).

The peak index of the control and intermittently exposed embryos were not different ($P > 0.05$).

At the end of the 72 hour period of culture (data not shown in Tables), 29 of 80 control embryos (36 %) and 32 of 78 1X-intermittently treated embryos (41 %) were classified as morphologically degenerate. The peak indices for the control and the intermittent eosin B treatment groups were not different ($P > 0.05$; Table 38).

The staining responses of the intermittently exposed embryos prior to culture and for all embryos after the 72 hour period of culture are shown in Table 39. The staining responses at the end of the 72 hour period of culture of the control embryos (Table 39) which had not been previously exposed to eosin B were not different ($P > 0.05$) from that of the intermittently treated embryos.

It is concluded that intermittent exposure to eosin B does not influence the subsequent in vitro development of the embryo, as compared to the control embryos which were not handled or transferred to fresh culture medium during the 72 hour period of in vitro culture. Analysis of cleavage indices and the staining responses at the end of culture confirm that multiple exposures of embryos to eosin B does not affect the developmental and staining responses of 4- to 8-cell rat embryos.

Table 39. Staining responses of 4- to 8-cell treated rat embryos exposed to 1X eosin B before culture and staining responses of control embryos and of the eosin B treated embryos exposed again to the 1X concentration of eosin B at the end of the 72 hour period of culture (Appendix 3)

Treatment	Number of embryos	Staining response					
		Before culture			Post culture		
		US	PS	CS	US	PS	CS
Control	80	NA	NA	NA	69	10	1
Eosin B	78	77	1	0	65	13	0

NA = Not applicable.

The staining response of control rat embryos at the end of the 72 hour culture period was not different from the embryos exposed intermittently to the 1X concentration of eosin B ($P > 0.05$).

APPENDIX 4

Analyses of Variance for Time of Staining

Experiment 3

The effects of treatment (trt), 1X, 5X, or 10X concentration of eosin B, developmental group (stage), and the treatment x developmental group interaction on the time of initial and complete staining were determined by ANOVA for a factorial design. Refer to Kirk (1968) for a description of the layout and computational procedures used for this design. In these studies the embryo was the experimental unit. Thus, for all experiments the model statement required that three classes, treatment, group, and embryo were specified for each data set which was then analyzed by analysis of variance (SAS Institute Inc., release 5.16, 1985). To test for an effect of eosin B concentration, embryo within treatment, designated as emb(trt) in tables was used as the error term. To test for an effect of stage of embryonic development, embryo within group, designated as emb(stage) in tables was used as the error term.

The classes and number of levels for each class for the time of initial or complete staining were: treatment, 3 levels; and developmental group, 4 levels. The data set contained a total of 200 observations. The sources of variation (source), degrees of freedom (df), sum of squares (SS), error terms used, F values, and probability levels (P) are listed in Table 40 for the time of initial staining and in Table 41 for the time of complete staining.

Table 40. Analysis of variance for time of initial staining (Experiment 3)

Source	df	SS	Error term	F	P
Treatment (TRT)	2	13.458	2	10.89	< 0.0001
Group (STAGE)	3	9.159	3	4.75	< 0.005
TRT x STAGE interaction	6	18.708	1	5.42	< 0.0001
Error:					
1. General error[+]	199	149.52			
2. Embryo(TRT)	47	29.038			
3. Embryo(STAGE)	61	39.182			

[+]The general error term was partitioned, as indicated, to test for the effects of treatment and development group.

Table 41. Analysis of variance for time of complete staining (Experiment 3)

Source	df	SS	Error term	F	P
Treatment (TRT)	2	431.453	2	40.48	< 0.0001
Group (STAGE)	3	247.749	3	14.54	< 0.0001
TRT x STAGE interaction	6	247.348	1	15.09	< 0.0001
Error:					
1. General error[+]	199	1440.0			
2. Embryo(TRT)	47	250.409			
3. Embryo(STAGE)	61	346.437			

[+]The general error term was partitioned, as indicated, to test for the effects of treatment and development group.

Analyses of Variance for Cleavage Indices

The effects of treatment, time, and the treatment x time interaction on the cleavage indices were determined by ANOVA for a split-plot factorial design. Refer to Kirk (1968) for a description of the layout and computational procedures used for this design. In these studies the embryo was the experimental unit. Thus, for Experiments 4 through 7 and for Appendix 3, the model statement required that three classes, treatment, time, and embryo were specified for each data set which was then analyzed by analysis of variance (SAS Institute Inc., release 5.16, 1985). The number of levels for embryo was variable due to differences in the number of embryos recovered from the available donors. No attempt was made to test for donor effects in any experiment. To test for an effect of treatment embryo within treatment, designated as embryo(trt) in tables, was used as the error term and the interaction time x embryo within treatment, designated as time x embryo(trt) was used as the error term for the evaluation of the effect of time.

Experiment 4

The classes and number of levels for each class were: treatment, 2 levels; and time, 7 levels. The data set contained a total of 1057 observations. The sources of variation (source), degrees of freedom (df), sum of squares (SS), error terms used, F values, and probability levels (P) are listed in Table 42.

Table 42. Analysis of variance for cleavage indices

Source	df	SS	Error term	F	P
Treatment (TRT)	1	145.405	2	1.43	> 0.05
Time[+] (TM)	6	227.158	3	4.11	< 0.001
TRT x TM interaction	6	137.988	1	0.96	> 0.05
Error:					
1. General error[++]	1043	24971.898			
2. Embryo(TRT)	21	2131.583			
3. TM x Embryo(TRT)	126	1160.019			

[+]The conservative F-test, F (1,21) = 4.11, was used to determine the effect of time, P < 0.1.

[++]The general error term was partitioned, as indicated, to test for the effects of treatment and time.

Experiment 5

The classes and number of levels for each class were: treatment, 2 levels; and time, 7 levels. The data set contained a total of 1155 observations. The sources of variation (source), degrees of freedom (df), sum of squares (SS), error terms used, F values, and probability levels (P) are listed in Table 43.

Table 43. Analysis of variance for cleavage indices

Source	df	SS	Error term	F	P
Treatment (TRT)	1	34.241	2	1.57	> 0.05
Time[+] (TM)	6	1440.990	3	26.13	< 0.0001
TRT x TM interaction	6	15.265	1	0.74	> 0.05
Error:					
1. General error[++]	1141	23511.10			
2. Embryo(TRT)	25	1073.711			
3. TM x Embryo(TRT)	150	1378.931			

[+]The conservative F-test, F (1,25) = 26.13, was used to determine the effect of time, P < 0.001.

[++]The general error term was partitioned, as indicated, to test for the effects of treatment and time.

Experiment 6

The classes and number of levels for each class were: treatment, 2 levels; and time, 7 levels. The data set contained a total of 868 observations. The sources of variation (source), degrees of freedom (df), sum of squares (SS), error terms used, F values, and probability levels (P) are listed in Table 44.

Table 44. Analysis of variance for cleavage indices

Source	df	SS	Error term	F	P
Treatment (TRT)	1	131.740	2	1.71	> 0.05
Time[+] (TM)	6	451.548	3	6.73	< 0.0001
TRT x TM interaction	6	39.496	1	0.26	> 0.05
Error:					
1. General error[++]	854	17780.163			
2. Embryo(TRT)	20	1544.058			
3. TM x Embryo(TRT)	120	1341.290			

[+]The conservative F-test, $F(1,20) = 6.73$, was used to determine the effect of time, $P < 0.025$.

[++]The general error term was partitioned, as indicated, to test for the effects of treatment and time.

Experiment 7

The classes and number of levels for each class were: treatment, 3 levels; and time, 7 levels. The data set contained a total of 1624 observations. The sources of variation (source), degrees of freedom (df), sum of squares (SS), error terms used, F values, and probability levels (P) are listed in Table 45.

Table 45. Analysis of variance for cleavage indices

Source	df	SS	Error term	F	P
Treatment (TRT)	2	7923.987	2	143.74	< 0.0001
Time[+] (TM)	6	1383.394	3	33.36	< 0.0001
TRT x TM interaction	12	3747.337	1	27.87	< 0.0001
Error:					
1. General error[++]	1605	17963.818			
2. Embryo(TRT)	39	1075.011			
3. TM x Embryo(TRT)	234	1617.086			

[+]The conservative F-test, F (1,39) = 33.36, was used to determine the effect of time, P < 0.001.

[++]The general error term was partitioned, as indicated, to test for the effects of treatment and time.

Influence of intermittent exposure to eosin B (Appendix 3)

The classes and number of levels for each class were: treatment, 2 levels; and time, 7 levels. The data set contained a total of 1106 observations. The sources of variation (source), degrees of freedom (df), sum of squares (SS), error terms used, F values, and probability levels (P) are listed in Table 46.

Table 46. Analysis of variance for cleavage indices

Source	df	SS	Error term	F	P
Treatment (TRT)	1	1.808	2	0.03	> 0.05
Time[+] (TM)	6	1310.467	3	19.74	< 0.0001
TRT x TM interaction	6	32.070	1	0.27	> 0.05
Error:					
1. General error[++]	1092	21714.749			
2. Embryo(TRT)	25	1499.604			
3. TM x Embryo(TRT)	150	1660.037			

[+]The conservative F-test, $F(1,25) = 19.74$, was used to determine the effect of time, $P < 0.001$.

[++]The general error term was partitioned, as indicated, to test for the effects of treatment and time.

APPENDIX 5

The Use of Eosin B to Assess the Viability and Developmental Potential of Bovine Embryos

The results presented below, represent a joint study which was completed with the co-operation and support of the Departments of Veterinary Physiology and Pharmacology and Veterinary Clinical Sciences, Iowa State University, Ames, Iowa 50011 and a Biomedical Research Support Grant from the National Institutes of Health (Dr. S. M. Hopkins). The following individuals, who served as co-investigators for this study: Dr. J. J. Wichtel, Dr. C. Plante, and Dr. S. M. Hopkins, are recognized for their contributions to the studies summarized below. These results were presented, in part, at the 20th Annual Meeting of the Society for the Study of Reproduction, University of Illinois, Urbana, Illinois, July 20-23, 1987, the full reference (Dooley et al. 1987) is provided in the Reference section to this thesis.

Background

As noted in the introduction to this thesis, the biological potential of livestock embryos at the time of collection, during culture, or after storage and/or manipulation has been assessed by a variety of methods. For the bovine embryo, assessment of the morphological characteristics (Linares and King 1980, Linder and Wright 1983) is the most widely used means to

predict the developmental potential of individual embryos. In commercial applications (Hasler et al. 1987), morphological evaluation is frequently performed using a stereomicroscope. Thus, the resolution available for the morphological assessment of individual embryos is limited.

In view of the fact that the exclusion of eosin B by preimplantation stage embryos had been proposed as a nonlethal method for the estimation of viability and morphologic evaluation of mammalian embryos (Dooley et al. 1984), this study was performed to determine whether: 1) Bovine embryos are killed by short-term exposure to elevated temperature, 2) periodic exposure to eosin B influences the development of the bovine embryo in vitro and 3) the eosin B dye-exclusion assay could be used to discriminate between viable and nonviable bovine embryos.

Experimental Procedures

Two experiments were performed with bovine embryos. This section provides a general description of the methods and materials which were common to both experiments. The specific details regarding the randomization and assignment of embryos to treatment and the analysis of the results obtained is provided within the experiment specified.

Superovulation

A total of 21 non-lactating dairy cows of mixed breeds were used as embryo donors. Donors were palpated rectally to monitor ovarian changes and estimate the stage of the estrous cycle. Superovulation was induced by administering 28 mg of FSH-P (Burns-Biotec, Omaha, NE) intramuscularly, in

a declining 8-dose schedule over 4 days (5 mg of FSH-P in AM and PM on day 1 declining to 2 mg of FSH-P in AM and PM on day 4) to cows estimated to be between days 9 and 13 of the estrous cycle. On day 4 of FSH-P treatment, 30 mg of prostaglandin ($PgF_{2\alpha}$; Lutalyse, Upjohn Co., Kalamazoo, MI) was administered intramuscularly, in 2 doses (15 mg) at the time of the gonadotropin injections. Each donor was then artificially inseminated at 48, 57, and 72 hours following the first of the two prostaglandin injections.

Embryo recovery

Six days after insemination embryos were collected nonsurgically (Elsden et al. 1976). Each uterine horn of the donor was flushed with 500 ml of Dulbecco's Phosphate Buffered Saline containing 2 % heat-inactivated calf serum and a 1 % antibiotic solution. The flushing fluid from each horn was recovered into a collection dish fitted with a nylon mesh filter (VCI E.T. Dish, Veterinary Concepts, Inc., Spring Valley, WI).

Embryo evaluation

The stage of embryonic development was estimated using an inverted microscope (25-160 X). A numerical index, modified slightly from that used for the rat embryo (refer to Table 1, page 46 of this thesis) was used to record the stage of preimplantation development for each embryo, as follows: 0 = degenerate, or unfertilized; 2-8 = pre-morula stages; 9 = early morula; 10 = tight morula; 11 = blastocyst (blastocoel small, but discernible); 12 = blastocyst (large blastocoel, embryonic diameter < 160 μM); 12.5 = expanding blastocyst (embryonic diameter 160 to 180 μM); 13 = expanded blastocyst (embryonic diameter > 180 μM); 14 = hatching

blastocyst; 15 = hatched blastocyst.

Morphological assessment and determination of the staining response

Embryos were provided a 10 minute exposure to 240 μM (2X) eosin B prior to the evaluation of the staining response. To expose the embryos to dye, embryos were transferred to wells containing the 2X concentration of eosin B in 500 μl of modified culture medium and returned to the incubator for 10 minutes. At the end of the 10 minute period of exposure to dye, the staining response was determined for each embryo. Each embryo was then transferred to a well containing eosin B-free culture medium to dilute the dye and to determine of staining response. Embryos were examined using a stereomicroscope (20-50 X) and the staining response of each treated embryo was classified as follows: Unstained (U), none of the blastomeres appeared to be stained by the eosin B; Partially Stained (PS), one or more of the blastomeres did not stain in an embryo where staining was evident; Completely Stained (CS), all of the blastomeres of the embryo appeared to be stained. In addition to this gross classification, the proportion of the embryonic mass that was stained assigned a staining index ranging from 0 = unstained to 10 = completely stained.

Determination of the Minimal Temperature Needed to Block Development and Induce Staining of Bovine Embryos When Exposed to Eosin B

The results of Experiments 1, 2, and 3 of this thesis indicated that short term exposure of the rat embryo to 45°C altered the subsequent

development of the embryo and exposure to 50°C or 55°C blocked development. This additional study was designed to determine whether the bovine embryo would behave like the rat embryo when exposed to the temperatures ranging from 45°C to 55°C.

Embryo assignment and treatment

For each donor, the stages of embryonic development were determined and those embryos which had developed beyond the 8-cell stage by the time of recovery (9- to 16-cell to morulae), were allocated by stage of development into 1 of 4 groups. Each group of embryos was then randomly assigned to a control (37°C) or 1 of 3 treated groups (45°C; 50°C; 55°C). A minimum of 15 embryos were assigned to each control or treatment temperature. The control or treated embryos from each donor were then placed in glass culture tubes and handled as described for Experiment 1 of this thesis. Treated embryos were exposed for 30 minutes to the assigned temperature. At the end of the 30 minute heat-treatment period, control and treated embryos were transferred to 24-well culture dishes (Falcon Multiwell-3047, Becton Dickinson Labware, Lincoln Park, NJ); each well contained 500 μl of culture medium. The stage of development and the staining response was then determined for each embryo at 0 h, immediately after exposure to the control or assigned treatment temperature, and at 24, 48, 72, and 96 h. After heat exposure, embryos were cultured individually at 37°C in a humidified atmosphere containing 5% CO_2. At each observation period within the 96 h period of culture, control and treated embryos were transferred to wells containing 240 μM (2X) eosin B in 500 μl of modified culture medium and returned to the incubator for 10 minutes. The stage of

development and the staining response was then determined for each embryo at the end of the 10 minute exposure to eosin B.

Statistical analyses

The overall developmental and staining responses of the heat-treated embryos were compared with the those of the corresponding control embryos by χ^2 analysis. A 3 x 4 contingency table, with 6 degrees of freedom, was used to determine treatment effects on development and staining (Steel and Torrie 1980). The peak index recorded at the end of the 96 hour period of culture and the post-culture staining index of the treated embryos were compared to the peak index and post-culture staining index for the corresponding control embryos using a one-way analysis of variance (Steel and Torrie 1980). Tukey's ω-procedure was used to test for differences between means of end points for which the analysis of variance indicated a significant ($P \leq 0.05$) F-ratio.

Results

A total of 94 embryos were recovered in 22 nonsurgical collections from 15 superovulated cows. Of these, 74 embryos were assigned to the control group or to 1 of 3 heat-treated groups. The number of control and treated bovine embryos and the overall developmental and staining responses of these embryos at the end of the 96 hour period of culture are shown in Table 47. The unequal number of embryos for the control and treatment groups was the result of unequal numbers of embryos at randomization and/or embryo losses during recovery. The in vitro development of bovine embryos

based on the cleavage response and the formation of a blastocoel during the post-treatment in vitro culture was significantly influenced by heat-treatment. Most of the control (86 %) and those treated embryos exposed to 45°C (61 %) formed a blastocoel during culture. None of the embryos exposed to 50°C or 55°C formed a blastocoel during culture and cleavage activity was evident in only 1 of the 16 embryos exposed to 50°C and none of the embryos exposed to 55°C cleaved.

Partial or complete staining was evident by the end of the 96 hour period of culture in all of the embryos exposed to 50°C or 55°C and all of the embryos exposed to 55°C were completely stained by the 2X concentration of eosin B. The means for the peak cleavage and staining indices for these embryos are displayed in Table 48. The peak indices for embryos exposed to 37°C or 45°C were different ($P < 0.05$) from each other and were higher ($P < 0.05$) than for the embryos exposed to 50°C or 55°C. The staining indices for embryos exposed to 37°C or 45°C were lower ($P < 0.05$) than for the embryos exposed to 50°C or 55°C.

The staining responses of control and treated bovine embryos were variable over the period of culture and both the number of the embryos which were stained and the proportion of the embryonic mass which was stained appeared to increase over time (data not shown in Tables). None of the control or treated embryos were completely stained by the 2X concentration of eosin B immediately after heat-treatment. At the end of 24 h of culture 1/16 embryos exposed to 50°C and 16/18 embryos exposed to 55°C were completely stained. For the 2 embryos in the 55°C group that were partially stained after a 24 hour period of culture, over half of the

Table 47. Developmental and staining responses of control and heat-treated bovine embryos during a 96 hour period of culture (Appendix 5)

Treatment temperature	Number of embryos	Developmental response			Staining response		
		Did not cleave	Cleaved	Formed blastocoel	US	PS	CS
Control	22	2	1	19	10	12	0
45°C	18	6	1	11	5	12	1
50°C	16	15	1	0	0	1	15
55°C	18	18	0	0	0	0	18

Did not cleave = number of embryos in which no further development was obtained during the culture period.

Cleaved = number of embryos that cleaved during the culture period, but did not form a blastocoel.

Blastocoel = number of embryos which formed a clearly defined blastocoel during the culture period.

The developmental and staining responses of bovine embryos were influenced ($P < 0.0005$) by heat-treatment prior to culture.

Table 48. Peak cleavage and staining indices of control and heat-treated bovine embryos during a 96 hour period of culture (Appendix 5)

Treatment temperature	Number of embryos	Post-treatment indices*	
		Cleavage	Staining
Control	22	14.0 ± 2.0[a]	1.3 ± 1.8[a]
45°C	18	12.0 ± 2.8[b]	2.3 ± 2.8a
50°C	16	8.6 ± 0.9[c]	9.9 ± 0.5[b]
55°C	18	9.4 ± 0.6[c]	10.0 ± 0.0[b]

Data are presented as mean ± SD.

*Peak index during culture or staining index determined at the end of the 96 hour period of culture.

There was an effect of treatment ($P < 0.0001$) on the cleavage and staining indices of heat-treated bovine embryos.

Means in the same column that do not have a common superscript letter are different ($P < 0.05$).

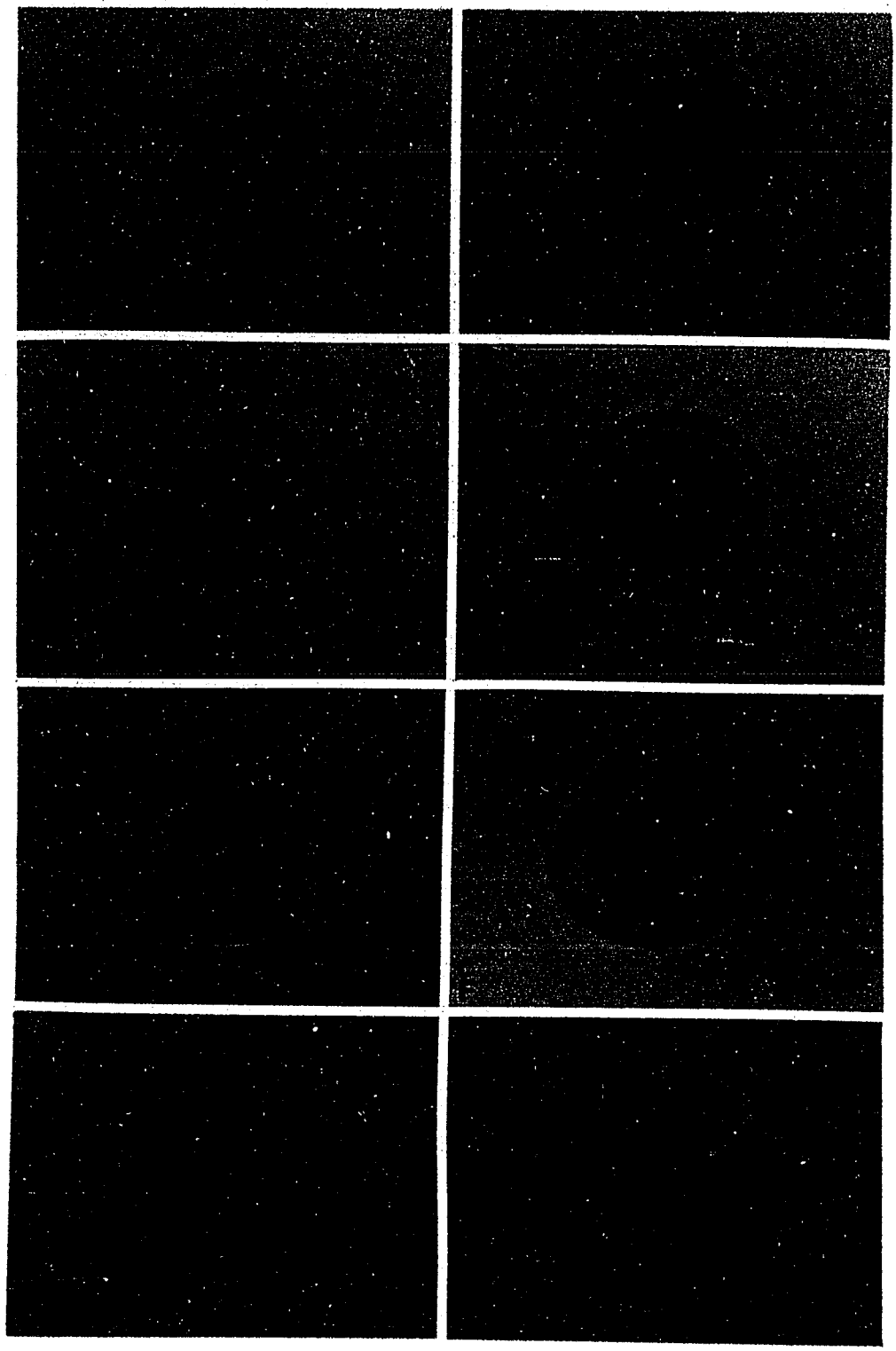
blastomeres were stained by eosin B (60 % and 80 % respectively) and both embryos were completely stained when examined at 48 hours and in subsequent evaluation periods.

The appearance of heat-treated embryos during culture but prior to the determination of the staining response, and their staining responses after exposure to 2X eosin B, are shown in Figure 8. Figure 8 (G and H) depicts differential staining responses in control embryos, which reached the blastocyst stage during in vitro culture and which were partially stained by the 2X concentration of eosin B.

Conclusions

From these results it is concluded that heat-treatment of bovine embryos to 55°C for 30 minutes blocks in vitro development and induces complete staining within 48 hours of culture. Thus, heat-treatment is a reliable method to provide a source of dead bovine embryos, which can then be used to determine the effect of stage of development and concentration of eosin B on the staining response of dead embryos. The fact that heating of bovine embryos to 45°C did not block the formation of the blastocoel during culture and that several of these embryos were observed to hatch during in vitro culture and that not all embryos exposed to 55°C were completely stained at 24 hours suggests that the bovine embryo has a greater tolerance to heat-shock, as compared to embryos of the rat.

Figure 8. Staining responses of heat-treated and control bovine embryos exposed intermittently to the 2X concentration of eosin B, during culture. A and B = bovine morula unstained by eosin B; before (A) and after (B) exposure to dye. C and D = bovine morula partially stained by eosin B; before (C) and after (D) exposure to dye. E and F = bovine morula completely stained by eosin B; before (E) and after (F) exposure to dye. Embryos G and H are control embryos which reveal partial staining of blastomeres in an early blastocyst (G) and expanding blastocyst (H) following exposure to eosin B, during the 96 hour period of culture



The Influence of Exposure to Eosin B on the In Vitro Development of Bovine Embryos

The studies performed utilizing rat embryos (Experiments 1 through 11 of this thesis) were done with embryos selected on the basis of morphological appearance at the time of collection and those rat embryos, which were developmentally retarded or appeared to be degenerate, were excluded from these experiments. For the bovine embryo, however, all of the embryos recovered were cultured regardless of morphological appearance at the time of collection. This approach was used to gain insight on the suitability of the eosin B dye-exclusion assay under conditions commonly found in bovine embryo transfer units.

Embryo assignment and treatment

For each donor, the stages of embryonic development were determined and embryos were allocated by stage of development into 1 of 2 groups which were then assigned at random to a control or eosin B-treated group. Each control or treated embryo from the donor was then transferred to an individual well of a 24-well culture dish (Falcon Multiwell-3047, Becton Dickinson Labware, Lincoln Park, NJ), each well contained 500 μl of modified culture medium. The stage of development of the control embryos and the stage of development and the staining response of the treated embryos was then determined at 0 h, immediately after exposure to the control or eosin B solution, and at 24, 48, 72, and 96 h. Embryos were cultured individually at 37°C in a humidified atmosphere containing 5% CO_2. At each observation period within the 96 h period of culture, treated

embryos were transferred to wells containing 240 μM (2X) eosin B in 500 μl of modified culture medium and returned to the incubator for 10 minutes. The stage of development and the staining response was then determined for each embryo at the end of the 10 minute exposure to the eosin B. Control embryos were handled and examined, as for the treated group, except that these embryos were transferred to wells containing eosin B-free medium.

Evaluation of the influence of eosin B exposure on development

To test for an influence of eosin B exposure on the in vitro development of the bovine embryo, the development of the control embryos which were never exposed to eosin B and those embryos exposed intermittently to eosin B during the 96 hour period of culture were compared.

Statistical analyses

The overall developmental responses of the eosin B-exposed embryos were compared with the those of the corresponding control embryos by χ^2 analysis. A 2 x 4 contingency table, with 3 degrees of freedom, was used to determine treatment effects on development and staining (Steel and Torrie 1980).

Results

A total of 241 embryos were recovered in 38 nonsurgical collections from 21 superovulated cows. Of these, 119 embryos were assigned to the control group and 122 embryos were assigned to the treated group.

The overall developmental responses of the control (n = 119) and

treated embryos (n = 122), are shown in Table 49.

The staining responses of the 122 embryos exposed to eosin B at the time of collection and the subsequent development of these embryos in vitro is summarized in Table 50. Embryos, regardless the stage of development, which were completely stained by the eosin B did not cleave in vitro. Once staining was detected within an embryo, the embryo would stain again during subsequent exposures to eosin B.

Table 49. Developmental response of control and eosin B treated bovine embryos during culture

Treatment	Number of embryos	Developmental response			
		Did not Cleave	Cleaved	Formed blastocoel	Hatched
Control	119	39	1	21	58
Eosin B	122	40	3	18	61

Did not cleave = number of embryos in which no further development was obtained during the culture period.

Cleaved = number of embryos that cleaved at least once during the culture period, but did not form a blastocoel.

Blastocoel = number of embryos which formed a clearly defined blastocoel during the culture period.

Hatched = number of embryos which formed a clearly defined blastocoel during the culture period and were observed to be in the process of hatching or had hatched from the zona pellucida.

The development of bovine embryos was not influenced ($P > 0.05$) by intermittent exposure to 2X eosin B.

Table 50. Staining responses of eosin B-treated embryos at the time of collection and their subsequent development during culture

Staining response	Number of embryos	Developmental response			
		Did not Cleave	Cleaved	Formed blastocoel	Hatched
Completely stained	17	17	0	0	0
Partially stained	33	14	NA	6	13
Unstained	72	9	3	12	48

NA = not applicable, all of the embryos which cleaved formed a blastocoel.

Did not cleave = number of embryos in which no further development was obtained during the culture period.

Cleaved = number of embryos that cleaved at least once during the culture period, but did not form a blastocoel.

Blastocoel = number of embryos which formed a clearly defined blastocoel during the culture period.

Hatched = number of embryos which formed a clearly defined blastocoel during the culture period and were observed to be in the process of hatching or had hatched from the zona pellucida.

Summary and Conclusions

These results demonstrate that multiple exposures of bovine embryos to the 2X concentration of eosin B does not affect in vitro development (refer to Table 49).

Overall, staining was detected in 41% of the bovine embryos which were collected on day 6 and assigned to the eosin B treatment group. Most of the embryos which were unstained when exposed to eosin B at the time of collection continued to develop in vitro. None of the embryos which were stained completely by the eosin B at the time of collection developed in vitro (refer to Table 50).

Based on the fact that staining was detected in such a large proportion of the bovine embryos recovered and that eosin B is not harmful to the embryo, it is concluded that the dye-exclusion response of bovine embryos can be incorporated into the routine assessment of embryos at the time of recovery or periodically during culture, in order to estimate embryonic viability. This should be of particular benefit for commercial applications.