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Uterine Vascular Permeability, Extracellular Fluid Volume And Blood Flow Following Deciduogenic Stimulation Of Rats

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UTERINE VASCULAR PERMEABILITY, EXTRACELLULAR FLUID VOLUME AND BLOOD FLOW FOLLOWING DECIDUOGENIC STIMULATION OF RATS

by

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ABSTRACT

The present study examined the possible importance of uterine vascular permeability (VP), extracellular fluid volume (ECFV) and blood flow (BF) in the enhanced accumulation of serum proteins in uterine tissue destined to undergo decidualization. This process is identifiable by endometrial blueing after intravenous (i.v.) injection of Evans blue dye. It was hypothesized that VP, ECFV and BF must increase to promote the enhanced intrauterine accumulation of serum proteins and that these uterine vascular and perivascular changes require optimal endometrial sensitization and prostaglandins (PGs).

ECFV was estimated by the uterine volume of distribution of i.v. injected ^{51}Cr-EDTA, VP by the rate of increase of the uterine volume of distribution of albumin after i.v. injection of ^{125}I-albumin, and BF by the microsphere technique. In one experimental manipulation, unilateral, artificial deciduogenic stimulation was given to the uteri of rats after either optimal or suboptimal sensitization for decidualization. In another manipulation, decidualization was first inhibited by intrauterine infusion of indomethacin (IM) then reinstated by simultaneous infusion of prostaglandin E_2 (PGE_2) with the IM.

When stimulation was given to an optimally sensitized uterus, both VP and ECFV were significantly higher in stimulated than nonstimulated horns during the time when the endometrial blueing reaction can be demonstrated by i.v. injection of Evans blue dye. VP became significantly increased by 4 hours after stimulation while significant increases in ECFV followed at 8 hours. Deviation from optimal sensitization and infusion with IM eliminated or significantly reduced the elevated ECFV and VP in stimulated horns.

This effect of IM was reversed by coinfusion with PGE_2. In most cases, increases in total BF were proportional to increases in uterine weight producing consistent uterine tissue BF (3-4 μl/min/mg). Excessive estrogen sensitization and coinfusion of PGE_2 and IM for 20 hours resulted in uterine weight gains which were not accompanied by proportional increases in total uterine BF. For these treatments, uterine tissue BF was significantly reduced.

The results of this study indicate that transient increases in uterine VP and ECFV occur during the time when the uterine blueing reaction can be demonstrated. The fact that uterine VP and ECFV nearly always changed in parallel suggests that these variables are either directly related or share a common control element. However, the possibility that the 2 variables play independent roles in the uterine blueing reaction cannot be excluded. Increased uterine BF does not appear to be an essential component of the blueing phenomenon. The use of contrast-enhanced magnetic resonance imaging techniques provided identification of predecidual tissue in dissected uteri and in the uteri of intact, anaesthetized rats. The elevated ECFV in predecidual tissue appears to be largely responsible for the image enhancement.

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Having finally completed my university education, I am reminded of the scarecrow in the movie "The Wizard of Oz". He reached the end of his tortuous journey along the yellow brick road only to find that he was as brainless as when he had first set out. Like the hapless strawman, I will take solace in knowing that I now possess something more important than brains; I have a degree and for that, I have a great many people to thank. Above all, Dr. Tom Kennedy has my utmost appreciation as I am convinced that I could not have accomplished this thesis and the work that comprises it without his help. Tom has been a supervisor, advisor, role model and friend throughout my PhD studies. I thank the members of my advisory committee: Drs. Steve Karlik; Bob Kline; and Brian Richardson for their numerous useful suggestions regarding the conduction and interpretation of my experiments. I especially appreciate the extensive help of Dr. Steve Karlik with MR imaging techniques and preparation of manuscripts describing that work. I would also like to thank Liz Ross for her generous and expert assistance with animal care, surgery and RIAs. I thank Gerry Barbe for his assistance in solving the numerous technical problems associated with the computers and other equipment essential to the research described in this thesis. In addition, I thank Barbara Lowery who helped me maximize the presentation quality of various abstracts, manuscripts and applications as well as Joan Barber who ensured that required chemicals were available when I needed them.

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ABBREVIATIONS

ANOVA	analysis of variance
BF	blood flow
cpm	counts per minute
dpm	disintegrations per minute
E_2	17B-estradiol: 1,3,5(10)-estratriene-3,17B-diol
ECFV	extracellular fluid volume
EDTA	ethylenediaminetetraacetic acid
Gd-DTPA	Gadolinium diethylenetriaminepentaacetic acid
GnRH-a	gonadotropin-releasing hormone agonist
h	hour
IM	indomethacin; 1-(p-chlorobenzoyl)-5-methoxy-2-methylindole-3-acetic acid
i.p.	intraperitoneal(ly)
i.v.	intravenous(ly)
min	minute
msec	milliseconds
MRI	magnetic resonance imaging
NaCl	sodium chloride
P_4	progesterone: 4-pregnene-3,20-dione
PBS	phosphate-buffered saline
PG	prostaglandin
PGE_2	prostaglandin E_2
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PGI_2	prostacyclin
RF	radio frequency
RIA	radioimmunoassay
s.c.	subcutaneous(ly)
sec	seconds
SEM	standard error of the mean
T_1	longitudinal relaxation time
T_2	horizantal relaxation time
TGF	transforming growth factor
VP	vascular permeability

CHAPTER 1: HISTORICAL REVIEW

1.1 INTRODUCTION

The mammalian uterus is designed to provide an isolated environment in which developing offspring may be supplied with nutrients and factors essential for optimal growth and development while being protected from other potentially damaging agents. During the non-pregnant state and during early pregnancy, the uterus, ovary and brain function in a co-ordinated fashion to ensure that the uterine endometrium is prepared to receive an embryo for implantation at the appropriate time after ovulation, mating and fertilization. The following literature review will describe the current state of knowledge regarding early events of embryo implantation. Particular attention will be given to uterine vascular changes occurring during the peri-implantation period. Although some of the information will be applicable to mammals in general, this review will focus on select groups including rodents and primates where embryo implantation is invasive.

Numbering System for Days of Pregnancy: In the literature, 2 different numbering systems have been used for recounting events of pregnancy. To avoid confusion, a single numbering system is used throughout this thesis. For the animals used in this study, day 1 of pregnancy represents the day when sperm are first detected in vaginal smears and day 1 of pseudopregnancy represents the day of cervical stimulation. In addition, the findings of other laboratories and the timing of events in pregnancy for other mammalian species will also be described using the same numbering system.

1.2 IMPLANTATION

In mammals, implantation is a complex series of events which begins when the embryo makes contact with the uterine wall and extends to the formation of a definitive placenta (Weitlauf, 1988). Successful completion of this process requires precise synchrony of embryo development and uterine function; the embryo must be at the blastocyst stage and the uterus must have completed a hormone-dependent series of changes producing a short-lived "sensitized" or receptive endometrium. Typically, the endocrine changes which sensitize the uterus also retain the embryo within the oviduct, foster its development and allow it to be released to the sensitized uterus at the appropriate time.

Following ovulation from the ovarian follicle, the oocyte is transported to the ampullary region of the oviduct where fertilization and early embryo development occurs. During a period of several days (depending on the species), a series of cleavages increases the number of embryonic blastomeres without increasing the overall size of the embryo. Subsequently, compaction occurs where each blastomere forms extensive intercellular junctions with all surrounding blastomeres. At this stage of development, the embryo is released from the oviduct into the uterus where its development progresses to the blastocyst stage. In the rat, the compacted embryo (morula) moves to the uterus on the afternoon of day 4 of pregnancy, the blastocyst stage of development is achieved on the morning of day 5 and implantation begins later that day (Jacobs et al. 1990).

Although the basic goal of ovoimplantation is to bring the embryonic blood

vessels into functional communication with the maternal circulation (Renfree, 1982), different mammalian species utilize different strategies to achieve this goal. Attachment of the embryo to the endometrial wall occurs in two stages; *apposition* and *adhesion*. During apposition, there is an increase in the intimacy of contact between the embryo and the wall of the endometrium. In mammals such as the rabbit, the increased intimacy occurs as a consequence of the expansion of the blastocyst (Enders and Schlafke, 1971). In other mammals including the rat, the increasing intimacy of contact occurs more as a result of the uterus "closing down" around the embryo (Schlafke et al. 1985). The closing of the uterine lumen appears to be a function of the maternal endocrine status since this phenomenon occurs without the presence of embryos (Weitlauf, 1988).

During adhesion, the embryo becomes fixed within the uterus. This stage of attachment is achieved by various mechanisms which appear related to the degree of invasiveness of the implantation process and except for species with superficial placentation, all mammalian embryos penetrate the endometrial epithelium to establish the link with the maternal circulation (Weitlauf, 1988). Highly invasive embryos such as those of the ferret (Enders and Schlafke, 1972) have adopted an *intrusive* approach to traversing the epithelial layer. For those embryos, a proportion of the trophoblast cells enlarge forming syncytial plaques. These plaques extend protoplasmic projections which simply intrude between the adjacent luminal epithelial cells (Enders and Schlafke, 1972). Rabbits utilize *fusion penetration* where trophoblastic knobs extend to the basal lamina of the uterine epithelium forming junctional complexes between trophoblast and luminal epithelial cells. The knobs give rise to functional syncytia with subsequent penetration

of basal lamina and maternal blood vessels (Enders and Schlafke, 1971). *Displacement penetration* is exhibited by the embryos of rats and mice where a progressive increase in the intimacy of contact between trophoblast cells and endometrial epithelium is followed by the death and detachment of these luminal epithelial cells. The basal lamina is subsequently breached by underlying decidual cells of maternal origin (Schlafke et al. 1985). Programmed death of the luminal epithelial cells appears to be an essential component of this type of implantation since mouse blastocysts are unable to fully implant if epithelial delamination is prevented by actinomycin D (Finn and Bredl, 1973). During their metamorphosis, the decidualizing endometrial stromal cells undergo significant differentiation and proliferation, ultimately giving rise to the maternal component of the placenta. Because of the profound structural and functional changes associated with decidualization and because of its occurrence in humans, this process has received considerable attention in the literature.

1.3 DECIDUALIZATION

In higher primates, rodents and insectivores, the uterus undergoes "decidualization" during embryo implantation (Renfree, 1982). This process is associated with a substantial increase in uterine weight resulting from the enlargement and proliferation of endometrial subepithelial stromal cells and from increases in endometrial tissue water (De Feo, 1967; Psychoyos, 1973). Transformation of the fibroblastic stromal cells into decidual cells is preceded by local vascular changes which

produce an endometrium possessing many of the hallmarks of pathological edema (Lundkvist and Ljungkvist, 1977). A detailed description of these vascular changes will be presented in the subsequent section of this chapter (section 1.4).

Differentiating stromal cells are identified in the antimesometrial endometrium as early as Day 6 of pregnancy in the rat and by Day 8, various types of decidual cells may be identified (De Feo, 1967). At this stage, antimesometrial decidual cells are large and contain abundant stores of glycogen which are ultimately replaced by lipid several days later. The cells are multinucleated and each nucleus contains several eccentrically located nucleoli (Krehbiel, 1937). Polyploidy occurs as a result of endomitosis and each decidual cell may contain 32 or more sets of chromosomes (De Feo, 1967). Meanwhile, mesometrial decidual cells are uninucleate and also contain glycogen during Day 8 of pregnancy in the rat. These cells persist during pregnancy comprising the decidua basalis (Krehbiel, 1937).

Although the exact function of the decidua is not known at this time, over the years a variety of plausible functions have been proposed. The manner in which the decidua is shed during parturition led early investigators to suggest that the decidua might function to provide a cleavage zone for separation of placental membranes at the time of delivery. In addition, the expanding decidual tissue around implanting blastocysts indicated that the decidua might provide a mechanism for spacing embryos in polytocous (more than one offspring per pregnancy) animals while the abundant stores of glycogen and lipid were believed to provide nourishment for the developing embryos (De Feo, 1967). More recently, the decidua has been shown to produce numerous hormones and

growth factors suggesting that this tissue plays other roles in the establishment and maintenance of pregnancy. Decidual cells of the rat (Basuray et al. 1983; Jayatilak et al. 1985) and the human (Riddick and Kusmik, 1976; Kubota et al. 1981) have been shown to produce prolactin or prolactin-related proteins. Although the role of these decidua-derived factors is unclear in the human, they appear to play an important role in luteal function in the rat (Basuray et al. 1983). The production and release of prostaglandin E_2 (PGE_2) by decidual cells may impart an immunoprotective effect on the conceptus since decidua-derived PGE_2 is able to inactivate T cells (Parhar et al. 1988, 1989) and natural killer cells (Parhar et al. 1989; Sodras et al. 1990). Finally, the decidua may serve to control trophoblast growth and invasion (Graham and Lala, 1991, 1992; Graham et al. 1993) by producing a variety of growth factors including transforming growth factor (TGF)-α (Lysiak et al. 1993) and TGF-βs (Clark et al. 1990; Graham et al. 1992).

In certain rodents, decidualization can be induced artificially if appropriate stimulation is given to a uterus which has been "sensitized" by the endocrine milieu of pseudopregnancy (Finn and Keen, 1963) or by ovariectomy and suitable hormone replacement therapy (DeFeo, 1963a; Yochim and DeFeo, 1963; Milligan, 1987; Kennedy and Ross, 1993). The phenomenon of artificially-induced decidualization was first demonstrated by Loeb (1907) who stabbed the uteri of guinea pigs during pseudopregnancy. Since that time many artificial stimuli have been shown to be effective in various rodents. These forms of stimulation have included: splitting open the uterine lumen (Frank, 1911); scratching the endometrium (Corner and Warren, 1919; Long and

Evans, 1922; De Feo, 1954, 1963; Herlyn, 1964); transluminal insertion of threads (Krehbiel, 1937; Fainstat, 1963), electrical stimulation of the myometrium (Krehbiel, 1937; Ansbro and Schwartz, 1965); and systemic injection of pyrathiazine (Kraicer and Shelesnyak, 1959; Psychoyos, 1960; Kraicer and Shelesnyak, 1963). In addition, many substances have been shown to represent suitable deciduogenic stimuli when injected into the uterine lumen. Sesame oil (Finn and Keen, 1962; Lundvist et al. 1977; Kennedy, 1979; Milligan and Mirembe, 1985); air (Orsini, 1963) and phosphate-buffered saline (PBS; Kennedy and Lukash, 1982; Milligan, 1987) have all been shown to be effective when delivered in this manner. However, it should be noted that these deciduogenic stimuli vary in effectiveness from species to species. For example, when instilled into the uterine lumen, PBS is an effective deciduogenic stimulus in rats (Kennedy and Lukash, 1982; Mitchell et al. 1983a; Milligan, 1987) but not in mice (Milligan, 1987).

Although all of these types of stimulation are capable of eliciting a decidual response, the endometrial presensitization necessary to permit this response varies with the type of stimulus (De Feo, 1967). In general, less traumatic forms of stimulation have rigid requirements of presensitization while more traumatic stimuli, such as scratching the endometrium, elicit a decidual response after suboptimal presensitization (the concept of endometrial sensitization for decidualization will be addressed in section 1.5 of this chapter). Injection of sesame oil into the uterine lumen represents a relatively nontraumatic form of deciduogenic stimulation and studies with mice indicate that morphological changes (Finn, 1965) and hormonal requirements (Finn and Hinchliffe, 1964; 1965) are similar to those of normal blastocyst implantation. While electron

microscopy of mouse uteri indicated that both implanting embryos and instillation of sesame oil produce breakdown and death of the luminal epithelial cells, greater numbers of the dead cells were identified following oil treatment than during a similar time after initiation of embryo implantation (Lundvist et al. 1977). However, this difference may also be due to an acceleration of the autolytic changes induced by the oil treatment (Hinchliffe and El-Shershaby, 1975).

Despite subtle differences between the endometrial decidualization elicited by natural embryo implantation and that elicited by artificial means, artificially-induced decidualization offers two important advantages: 1) While natural ovoimplantation can be initiated anywhere along the uterus and at anytime within a window of several hours of receptivity, the site and time of artificial stimulation are controlled by the experimenter, thus allowing accurate localization and timing of the ensuing events of decidualization; 2) The artificial model allows the conditions of endometrial sensitization and the form of deciduogenic stimulation to be manipulated thereby providing a means of identifying factors important to decidualization and other related events.

The use of these artificial decidualization models has provided much information regarding decidualization and embryo implantation. Furthermore, similarities between implantation in rodents and humans may ultimately allow information obtained with the rodent models to be extended to humans.

1.4 UTERINE VASCULAR CHANGES IN OVOIMPLANTATION AND ARTIFICIALLY-INDUCED DECIDUALIZATION

One of the first macroscopically identifiable signs of imminent embryo implantation is a positive "blueing reaction" after i.v. injection of Evans Blue or Pontamine Sky blue dyes. The dyes bind rapidly and strongly to serum proteins and accumulate in endometrial tissue producing blue spots in the vicinities of implanting embryos (Psychoyos, 1973). The phenomenon can be produced in most animals studied including rats (Psychoyos, 1960; Lundkvist and Ljungkvist, 1977; Rogers et al. 1983), guinea-pigs (Deanesly, 1967), hamsters (Evans and Kennedy, 1978), rabbits (Hoffman et al. 1978), sheep (Boshier, 1970) and pigs (Keys et al. 1986). Furthermore, because decidualization is always preceded by the ability to demonstrate an endometrial blueing reaction, the vascular changes which give rise to this phenomenon may be essential to decidual metamorphosis (Psychoyos, 1973).

Traditionally, the blueing reaction has been attributed to increased vascular permeability (VP) of the local endometrial blood vessels (Psychoyos, 1973) and the magnitude of this supposed VP response has been quantitated by determining concentrations of radioactivity in uterine tissue after i.v. injection of albumin labeled with radio-active iodine (Psychoyos, 1960; Kennedy, 1979; Milligan and Lytton, 1983). In fact, this conclusion that the enhanced accumulation of serum proteins in predecidual tissue results from increased VP may well be incomplete since several other variables are

known to influence the movement of macromolecules across blood vessel walls. While the permeability of a blood vessel wall (permeability coefficient) determines if a given solute can cross the wall, the rate and extent of solute transport are increased by fluid filtration (bulk flow or convective transport). Although the mechanism by which macromolecules are transported across blood vessel walls is unclear, these molecules appear to respond to altered Starling forces much as smaller solutes do. Thus when vessel permeability is sufficient, an inbalance of hydrostatic and/or oncotic pressures between plasma and interstitial compartments will result in fluid filtration and a concommitant convective transport of macromolecules as well as smaller solutes (Michel, 1989). It is thus quite possible that increases in local blood flow (BF) could contribute to the enhanced intrauterine accumulation of serum proteins through increasing plasma hydrostatic pressure. In addition, local increases in extracellular fluid volume (ECFV) could contribute to the phenomenon by creating a larger compartment in which serum proteins can accumulate following extravasation (McRae and Heap, 1988). For these reasons, each of these variables should be considered separately to determine their contributions to the changing uterine environment which characterizes embryo implantation and endometrial decidualization.

Although increased BF has been implicated in the extravasation of plasma protein during inflammation (Williams, 1977; Williams and Peck, 1977), it is not certain whether increased local BF plays a similar role in the uterine blueing reaction. Quantitative estimates of uterine BF during early embryo implantation have yielded unclear results. Assessment of BF to the uteri of pregnant rats by both the radioactive microsphere

method (McRae and Heap, 1988) and the rubidium fractionation method (Mitchell and Goldman, 1991) indicated slight but significant increases in tissue BF (BF on a per weight basis) to implantation sites as compared to adjacent uterine tissue (McRae and Heap, 1988). These increases were noted at a time when the blueing reaction can be demonstrated and results of various anatomical and histological studies have provided qualitative support for the measured increases in uterine BF during early decidualization. Examination of resin vascular casts from uteri of pregnant rats indicated that blood vessel diameters are greater in the vicinities of implanting blastocysts during Day 6 of pregnancy (Rogers et al. 1982) but that densities of these capillary beds do not increase until at least Day 7 (Takemori et al. 1984). This suggests that vasodilation, rather than neoangeogenesis, causes these increases in local BF. Recently, the uterine microcirculation of the rat was examined during early embryo implantation by *in vivo* microscopy (Tawia and Rogers, 1992). The results of that study indicated that embryos were surrounded by an avascular region of endometrium and that although capillaries adjacent to this region were large in diameter, the movement of blood was sluggish with frequent reversals and stoppages. These observations demonstrate the complexity of uterine perfusion during embryo implantation and suggest that BF to implantation sites could increase, decrease or remain unchanged. The avascular region of endometrium and the sluggish movement of blood would imply that uterine BF is reduced in the regions of implanting embryos while the large diameters of surrounding capillaries implies that local BF increases.

Uterine BF has also been reported to increase during artificially-induced

decidualization in rats (Garris et al. 1983) and mice (Edwards and Milligan, 1987) at times when the blueing reaction can be demonstrated. However, in both cases, these increases in BF were directly proportional to the increase in uterine weight which accompanies decidualization. Therefore, uterine tissue BF was unchanged during the initial 24 hours after deciduogenic stimulation (Garris et al. 1983; Edwards and Milligan, 1987). The finding that tissue BF increases during embryo implantation (McRae and Heap, 1988; Mitchell and Goldman, 1991) but not after deciduogenic stimulation might indicate that the embryo secretes a vasodilatory factor not liberated during deciduogenic stimulation. However, this possibilty should be considered critically because of the complexity of uterine perfusion (Tawia and Rogers, 1992) and because of the relatively small increases in uterine BF observed during implantation (McRae and Heap, 1988; Mitchell and Goldman, 1991).

Although the uterine BF changes associated with decidualization and implantation may be slight, various studies have shown that manipulations which moderately alter uterine BF detrimentally affect embryo implantation and decidualization. Surgical impairment of uterine BF (Franklin and Brent, 1964; Antebi et al. 1991) and systemic treatment with serotonin (Mitchell and Hammer, 1983; Mitchell et al. 1983) significantly reduce implantation, the decidual response and fetal development. Since these treatments have the greatest effect on the decidual response when surgical impairment (Franklin and Brent, 1964) or serotonin treatment (Mitchell and Hammer, 1983; Mitchell et al. 1983) precedes implantation or deciduogenic stimulation, controlled uterine perfusion may be crucial for the establishment of endometrial sensitization. In mice, it is possible that

serotonin reduces predecidual sensitization by decreasing luteal progesterone production (Marley, 1969); however, in rats, serotonin treatment does not reduce serum progesterone concentrations (Mitchell and Hammer, 1983), nor does exogenous progesterone reverse serotonin-induced termination of pregnancy (Marley, 1969; 1974). The fact that ethanol increases uterine BF (Mitchell and Goldman, 1991) but abolishes the decidual response when given prior to deciduogenic stimulation (Mitchell and Van Kainen, 1992) provides further evidence for the importance of controlled uterine BF during the predecidual period. The fact that similar treatment with ethanol increases the decidual response when given after deciduogenic stimulation (Mitchell and Van Kainen, 1992) indicates that elevated uterine BF may be beneficial to decidualization, following initiation of the process.

Extracellular fluid volume has been assessed in numerous tissues by the distribution of ^{51}Cr-EDTA after i.v. injection. Because this tracer passes out of blood vessels easily but enters cells with great difficulty, it rapidly equilibrates between plasma and interstitial compartments after i.v. injection (Larsson et al. 1980). With this approach, increases in uterine ECFV have been reported during early decidualization in rodents. Uterine ECFV was found to be higher in sites of embryo implantation than in surrounding uterine tissue of rats (McRae and Heap, 1988) and higher in uterine horns which received deciduogenic stimulation than in contralateral nonstimulated horns of mice (Milligan and Edwards, 1990). Although plasma makes up approximately 25% of the ECFV, previous studies have indicated that increases in uterine blood volume are negligible (Milligan and Edwards, 1990) or nonexistent (Bitton et al. 1965; Milligan and

Mirembe, 1984) during the same early stages of decidualization. In rats, no increase in uterine blood volume on a per weight basis has been demonstrated (Bitton et al. 1965; Milligan and Mirembe, 1984), while in mice the increased blood volume exactly matched the total increase in ECFV, consistently making up about 25% of that value (Milligan and Edwards, 1990). Therefore, most of the increased uterine ECFV during decidualization occurs in the interstitial compartment. This notion is supported by the results of histological studies after deciduogenic stimulation (Lundvist et al. 1977) and during embryo implantation (Lundkvist and Ljungkvist, 1977). In both cases, alterations to the endometrial extracellular matrix consistent with pathological edema were noted. Furthermore, metalloproteinases and plasminogen activators, capable of modifying the extracellular matrix, are secreted by cells of the uterus (Salamonsen et al. 1991) and embryos (Strickland et al. 1976; Brenner et al. 1989; Lala and Graham, 1990). Together, these observations suggest that the endometrial extracellular matrix may be modified in a manner which reduces tissue hydrostatic pressure, thereby providing a larger compartment in which extravasated vascular components can accumulate.

Despite the accumulating evidence which suggests that several uterine vascular changes may give rise to the phenomenon of the endometrial blueing reaction, there is little doubt that increased uterine VP plays a significant role. Numerous studies have provided solid qualitative evidence that endometrial VP does in fact increase during embryo implantation and decidualization. Ultrastructural examination of rat endometrial blood vessels during embryo implantation has indicated the existence of numerous endothelial fenestrations and interendothelial gaps (Abrahamsohn et al. 1983). Endothelia

with these characteristics were identified in postcapillary venules of mesometrial and antimesometrial endometrium at implantation sites but not at interimplantation sites. Similar types of endothelia have been identified in the endometrium of nonpregnant animals following the administration of estradiol to induce uterine edema (Ham et al. 1970; Martin et al. 1973). Although the existence of numerous endothelial gaps and fenestrations is indicative of a highly permeable vascular bed, these characteristics only confer enhanced permeability to water and small molecules (Michel, 1988). Endothelium of this type may or may not exhibit increased permeability to macromolecules. However, histological studies using labeled tracer-proteins have confirmed that endometrial blood vessels are in fact more permeable to macromolecules at sites and times where the blueing reaction may be demonstrated. Fluorescence microscopy indicated that appreciable amounts of fluorescein isothiocyanate-tagged albumin (Rogers et al. 1983; Parr and Parr, 1986) and IgG (Parr and Parr, 1986) gained access to the endometrial interstitium around implanting blastocysts after i.v. injection. In contrast, the avascular primary decidual zone adjacent to the blastocyst and endometrial tissue outside implantation sites were both found to be devoid of the tracers (Rogers et al. 1983; Parr and Parr, 1986).

The results of the studies described in this section suggest that the endometrium exhibits significant alterations in both structure and function soon after the initiation of embryo implantation and after deciduogenic stimulation. These alterations appear to be accompanied by changes in uterine VP and ECFV, while uterine BF may or may not be altered. The existence of such a complex array of uterine vascular and perivascular

changes suggests that several chemical mediators may play important roles in these early events of implantation. This type of multiple mediator system is similar to the "two mediator hypothesis" proposed for inflammation (Williams, 1977; Williams and Peck, 1977). In that system, a vasodilatory mediator such as PGE_2 or PGI_2 would augment the effects of a second mediator such as bradykinin or histamine which would increase VP. Both types of mediators would be required to produce maximal extravasation and accumulation of serum proteins (Williams, 1977; Williams and Peck, 1977).

At this time, it is not clear which chemical mediators actually produce the uterine vascular changes preceding endometrial decidualization. However, numerous vasoactive mediators capable of effecting these vascular changes have been examined and implicated in the processes of implantation and decidualization. Histamine (Shelesnyak, 1952; Shelesnyak, 1954), prostaglandins (Tobert, 1976; Kennedy, 1979; Phillips and Poyser, 1981), leukotrienes (Tawfik and Dey, 1988), estrogen (Dickmann et al. 1976; Wise and Heap, 1983) and platelet-activating factor (Acker et al. 1988) have been demonstrated in sites of ovoimplantation and artificial deciduogenic stimulation. This list of mediators continues to increase with reports that various growth factors including epidermal growth factor (EGF) (Paria et al. 1992) and leukemia inhibiting factor (LIF) (Stewart et al. 1992) play important roles in ovoimplantation and decidualization. The large number of mediators potentially involved in decidualization and the diversity of their vascular effects demonstrates the need to use techniques which selectively address individual uterine vascular changes.

1.5 ENDOMETRIAL SENSITIZATION IN OVOIMPLANTATION AND DECIDUALIZATION

A blastocyst will implant in the uterus during only a limited time of pregnancy and at this time the uterus is said to be "sensitized". Results of experiments involving both the asynchronous transfer of donor embryos to recipient uteri and the transplantation of embryos to various ectopic sites indicate that the uterus determines if and when implantation will be initiated. The blastocyst can wait several days for the uterus to become sensitized as demonstrated by successful implantation following transfer of blastocysts to the uterus up to 2 days early (McLaren and Michie, 1956). In contrast, implantation rates are significantly reduced when embryo development trails uterine sensitization by even 1 day of pregnancy (Dickmann and Noyes, 1960). The innate invasive capacity of the blastocyst is demonstrated by observations that trophoblast invasion and embryonic growth begin nearly immediately upon transplantation of a blastocyst to spleen (Kirby, 1963b), kidney (Kirby, 1960) or even to the testis of a male of another species (Kirby, 1963a).

As indicated in section 1.3, many different types of artificial stimuli can induce decidualization in various rodents. However, as during normal blastocyst implantation, decidualization can be induced only during a limited time of pregnancy, pseudopregnancy (Finn and Keen, 1963) or following treatment with an appropriate hormone regimen (Kennedy, 1980a;b). Although all of these artificial stimuli are effective during a limited window of time, the actual length of the "sensitive period" varies with the type of

stimulation. Stimuli of long duration and greater associated trauma appear to induce decidualization over a broader period of time than stimuli of short duration and low trauma (DeFeo, 1963b). For example, instillation of sesame oil into the uterine lumen induces limited decidualization when stimulation is given 1 day either side of peak uterine sensitization (Kennedy, 1980a).

Although several natural and experimental models have provided a good deal of information regarding endocrine factors which give rise to the transient period of sensitivity, relatively little has been learned about the underlying nature of endometrial sensitization. Numerous mammalian species exhibit delays of embryo implantation (diapauses) induced by seasonal cues or lactation (Psychoyos, 1973). These naturally occurring phenomena involving the maintenance of the blastocyst(s) in a state of quiescence within the uterine lumen can be mimmicked by ovariectomizing pregnant rodents 2 days prior to embryo implantation and supplying exogenous progesterone (P_4) on a daily basis (Cochrane and Meyer, 1957). The daily injections of P_4 allow this experimental delay of implantation to be maintained for several days, during which time, embryo implantation can be initiated by treatment with estradiol-17β (E_2). Both E_2 and P_4 are essential for this re-initiation of implantation indicating that synergistic effects of these hormones are integral components of the endometrial sensitization which renders the uterus receptive to the implanting blastocyst (Psychoyos, 1962). Similarly, for relatively non-traumatic stimuli, successful artificial induction of decidualization in ovariectomized rats requires treatment with P_4 and a limited dose of E_2 (Kennedy, 1980b).

Despite several decades of study, relatively few overt signs of endometrial sensitization have been identified and in many cases, these observable changes during sensitization appear to be coincidental rather than causal. In many species including humans (Noyes et al. 1950), sensitization is associated with endometrial edema formation which disperses cells of the stroma and occludes the uterine lumen (Fainstat, 1963). Although the resulting occlusion of the uterine lumen may facilitate the apposition phase of implantation in species whose embryos fail to expand, the edema phase occurs in the absence of embryos as well as in animals such as the rabbit, whose embryos do undergo preimplantation expansion (Psychoyos, 1973). At the ultrastructural level, several observations have been made with respect to the luminal epithelial cells of the rat uterus during the time of peak endometrial sensitization. The nuclei become localized close to the epithelial basal membranes (Allen, 1931; Psychoyos, 1973) while large cytoplasmic lipid drops, present at the beginning of the sensitive period, are replaced by small vacuoles (Alden, 1947). In addition, various biochemical changes have been correlated with endometrial sensitization including increases in endometrial RNA synthesis and changes in the activities of a variety of enzymes (Psychoyos, 1973).

1.6 PROSTAGLANDINS (PGs) IN OVOIMPLANTATION AND DECIDUALIZATION

Among the many chemical factors implicated in ovoimplantation and decidualization, most evidence suggests that prostaglandins (PGs) have an obligatory role. Much of the information regarding the importance of PGs in embryo implantation has

been acquired by the ability of indomethacin (IM), an inhibitor of PG synthesis (Vane, 1971), to interfere with implantation and its associated events. Treatment with IM abolishes or significantly delays implantation in mice (Lau et al. 1973; Saksena et al. 1976; Holmes and Gordashko, 1980; Lundkvist and Nilsson, 1980), rats (Gavin et al. 1974; Kennedy, 1977; Phillips and Poyser, 1981; Garg and Chaudhury, 1983), rabbits (Hoffman et al. 1978; El-Banna, 1980; Cao et al. 1985) and hamsters (Evans and Kennedy, 1978). When artificial stimulation is given to sensitized uteri, IM treatment reduces the extent of decidualization in mice (Rankin et al. 1979; Buxton and Murdoch, 1982) and rats (Tobert, 1976; Sananes et al. 1976; 1981; Kennedy and Lukash, 1982; Kennedy, 1985). The fact that the blueing reaction is impaired by IM pretreatment in rats (Kennedy, 1977; Phillips and Poyser, 1981), mice (Lundkvist and Nilsson, 1980), hamsters (Evans and Kennedy, 1978) and rabbits (Hoffman et al. 1978) indicates that PGs play an important role during the predecidual period. However, the decidual response also is greatly reduced even when IM administration follows the time when the blueing reaction can be demonstrated (Tobert, 1976; Kennedy, 1985). Therefore, PGs appear to be crucial for both the uterine vascular changes which precede decidualization and also to decidual metamorphosis itself.

The relative abundance of various PGs in implantation sites and decidual tissue has provided further evidence for PG mediation of events associated with embryo implantation. Concentrations of PGs of the E, F and I series are greater in implantation sites than surrounding uterine tissue (Kennedy, 1977; Kennedy and Zamecnik, 1978; Sharma, 1979; Pakrasi and Dey, 1982) during early pregnancy and greater in stimulated

than nonstimulated uterine horns (Kennedy, 1979; 1980a; b; Rankin et al. 1979; Jonsson et al. 1979) after deciduogenic stimulation. Furthermore, embryos and uterine tissue components have been shown to be sources of PGs. Embryos of rabbits (Dey et al. 1980; Harper et al. 1983), pigs (Watson and Patek, 1979), cows (Shemesh et al. 1979; Lewis et al. 1982), sheep (Holmes et al. 1990) and rats (Niimura and Ishida, 1987) produce various PGs while uterine PGs and/or PG synthase have been identified in rats (Anteby et al. 1975; Fenwick et al. 1977; Pakrasi et al. 1985; Parr et al. 1988); rabbits (Hoffman et al. 1984; Peplow and Hurst, 1989); hamsters (Evans and Kennedy, 1978); mice (Rankin et al. 1979; Milligan and Lytton, 1983) and humans (Smith and Kelly, 1988; Alecozay et al. 1991). In fact, uterine epithelial (Moulton, 1984; Jacobs et al. 1990) and stromal cells (Yee and Kennedy, 1991) have both been identified as sources of PGs. Originally, the embryo was a popular choice as the most important source of PGs because such a model would explain the localized nature of the uterine vascular responses during early implantation. The blastocyst would release PGs which would then diffuse to the nearby endometrium where localized vascular responses would be elicited. However, the ability to induce decidualization by artificial means (Loeb, 1907) suggests that the embryo is not an obligatory source of PGs. Furthermore, in rabbits, the uterus seems to be the more important source of PG synthesis since blastocysts from IM-treated donor rabbits implant readily when transferred to untreated recipients while blastocysts from untreated donors fail to implant in IM-treated recipients (Snabes and Harper, 1984). The reported ability of cultured uterine epithelial cells to preferentially secrete $PGF_{2\alpha}$ basally (Jacobs et al. 1990) could also explain the localized nature of the uterine vascular

responses if the initiation of vectorial secretion was dependent on a local signal at the apical surface of the luminal epithelial cells.

In addition to being present in elevated concentrations, PGs have been proven capable of mediating a variety of events associtated with ovoimplantation and decidualization. Although limited information is available regarding the direct role of PGs in embryo implantation, systemic treatment with $PGF_{2\alpha}$ has been shown to induce implantation during experimental diapauses in mice (Saksena et al. 1976) and rats (Oettel et al. 1979). When infused into the uterine lumen of rats sensitized for decidualization, PGE_2 and $PGF_{2\alpha}$ both reverse IM inhibition of artificially-induced decidualization and the enhanced uterine accumulation of labeled proteins which precedes it (Kennedy and Lukash, 1982; Kennedy, 1985). In similar studies, neither PGI_2 nor a stable analogue of PGI_2 could produce the same responses during IM inhibition (Kennedy et al. 1980). Therefore, for the rat at least, PGE_2 and $PGF_{2\alpha}$ appear to be the most important PGs for implantation-associated events. Two further lines of evidence indicate that PGE_2 is the active PG in these events and that $PGF_{2\alpha}$ may exert its effects through conversion to PGE_2. When stable analogues of PGE_2 or $PGF_{2\alpha}$ are infused into the uterine lumina of IM-treated rats, only the PGE_2 analogue produces subsequent decidualization (Kennedy and Doktorcik, 1988). In addition, while high affinity PGE_2 binding sites have been identified in uteri of sensitized rats (Kennedy et al. 1983), no such binding sites could be identified for $PGF_{2\alpha}$ (Martel et al. 1985).

In addition to PGs, numerous other chemical mediators have been implicated in the processes of implantation and decidualization as mentioned in section 1.4. However,

in many cases, the actions of these compounds reinforce the importance of PGs in implantation and decidualization since these compounds either alter PG synthesis or are themselves affected by PGs. Estrogen, which is crucial to implantation and decidualization, modulates PG synthesis by the uterus *in vivo* (Ham et al. 1975) by a mechanism which may involve conversion of the estrogens to catechol estrogens (Kelly and Abel, 1980) while histamine also appears to be capable of altering the metabolism of PGs (Peplow and Hurst, 1989). Although their importance is unclear, LTs (Pakrasi and Dey, 1985) and PAF (Ryan et al. 1990) have also been implicated in implantation and decidualization. It has been proposed that the actions of both of these compounds relate to PG synthesis; PAF by stimulating PG synthesis (Smith and Kelly, 1988; Alecozay et al. 1991) and LTs through a shift in arachidonic acid metabolism from the cyclooxygenase to the lipoxygenase pathway (Pakrasi et al. 1985).

Thus, the ability of IM to inhibit events associated with embryo implantation, the abilities of certain PGs to override that inhibition and the existence of PG synthesis and maximum concentrations of high affinity PG binding sites within the sensitized uterus all suggest that PGs are involved in implantation-associated events. However, PGs produce many diverse effects in many different tissues and, at this time, it is difficult to ascertain the true role(s) of PGs in embryo implantation.

1.7 IDENTIFICATION OF VASCULAR CHANGES BY CONTRAST-ENHANCED MAGNETIC RESONANCE (MR) IMAGING

Magnetic Resonance (MR) imaging techniques have allowed noninvasive examination of many pathological and physiological processes in humans as well as in a variety of animals. The development of numerous contrast agents has made it possible to assess selected vascular changes in desired tissues. Gd-DTPA is a contrast agent which enhances T_1-weighted MR images and which is eliminated via the kidneys within several hours of i.v. injection (Weinmann et al. 1984; Brasch et al. 1984). Because of its small size (560d), Gd-DTPA rapidly equilibrates amongst extracellular fluids of all tissues except brain (Strich et al. 1985). The agent enters the brain only when the blood-brain-barrier is compromised (Karlik et al. 1990).

In the past, there has been a relative inability to enhance T_1-weighted uterine images with contrast agents and as a result, T_2-weighted imaging techniques have been used most commonly for diagnostic procedures involving this organ (Hricak and Stevens, 1992). Increases in endometrial VP and ECFV have been described during ovoimplantation in rodents (McRae and Heap, 1988) and during the normal human menstrual cycle (Noyes et al. 1950). This suggests that uterine images might be enhanced at these times using T_1-weighted MR imaging with Gd-containing contrast agents as has been used to demonstrate similar vascular changes in other tissues. The ability to identify the uterine vascular changes by Gd-enhanced T_1-weighted imaging would be beneficial in both clinical and experimental settings. From a clinical standpoint, the identification of stromal edema during the normal menstrual cycle by MR

imaging could replace the invasive endometrial biopsy traditionally used in the diagnosis of luteal phase deffects (Hecht et al. 1990; Yeko et al. 1992; Johannisson et al. 1987; Li et al. 1988). In experimental settings, the behaviors of various uterine vascular components could be examined with minimal surgical intervention using T_1-weighted MR imaging enhanced by Gd-DTPA chelated to a variety of molecules.

Gd-DTPA enhanced MR imaging has been useful, not only in demonstrating changes in ECFV but also in VP. The permeability of the blood-brain-barrier has been assessed by measuring the flux or blood-to-tissue transport of this marker (Brasch et al. 1991). This method assesses VP using the changes in image enhancement by Gd-DTPA accumulation and accounts for plasma volume as well as plasma and tissue concentrations of Gd-DTPA after i.v. injection of the agent. Recent studies involving the chelation of Gd-DTPA to various macromolecules including albumin (Lauffer and Brady, 1985; Spanoghe et al. 1992; Lauffer and Brady, 1985) and polylysine polymers of various sizes (Spanoghe et al. 1992) has allowed various processes to be assessed by MR imaging.

In the past, tissue-specific vascular changes have often been monitored with techniques that use radioactive tracers and that require animals to be killed in order to obtain the results. Such an approach makes it impossible to examine vascular variables in individual animals over time. During the past decade, magnetic resonance (MR) imaging techniques have been used increasingly to examine vascular changes associated with a variety of physiological and pathological processes. Often it is altered VP (Karnik et al. 1990) and tissue water content (Weinmann et al. 1984; Brasch et al. 1984) which have allowed these processes to be examined by MR imaging.

A blood-uterine lumen barrier has been described for the uterus (McRae, 1988a) and although the most restrictive component of this barrier is thought to be the epithelium lining the uterine lumen (McRae, 1988), studies with electron microscopy have demonstrated that the endothelium of endometrial blood vessels may also be restrictive depending on the endocrine state of the animal (Abrahamsohn et al. 1983). In addition, changes in endometrial VP and ECFV during embryo implantation (McRae and Heap, 1988b) suggest that MR imaging techniques might ultimately be used to detect these events and to provide information currently inaccessable with conventional tools used in the field of reproductive biology.

1.8 SUMMARY

Embryo implantation and artificially-induced decidualization are complex processes involving significant structural and biochemical modifications of the uterine endometrium. Soon after the initiation of embryo implantation or after deciduogenic stimulation, a series of endometrial vascular changes make it possible to identify sites of imminent decidualization by macroscopic blueing after i.v. injection of Evans blue dye. Evidence reviewed in preceding sections of this chapter suggests that increases in uterine VP, BF and ECFV could all contribute to the endometrial blueing which follows the dye injection. In addition, ideal endometrial sensitization for decidualization and certain PGs

appear necessary to promote the combination of uterine vascular changes which gives rise to endometrial blueing. Finally, the types of vascular changes implicated in the endometrial bluing reaction suggest that MR imaging techniques might be used to identify sites of imminent decidualization.

CHAPTER 2: RATIONALE AND OBJECTIVES

In the rat, decidualization is always preceded by the ability to produce an endometrial blueing reaction after i.v. injection of Evans blue dye (Psychoyos, 1973). This relationship suggests that events associated with the blueing reaction may be important for successful progression to later events of embryo implantation. The present study is based on the hypothesis that increasing uterine VP, ECFV and BF all contribute to the phenomenon known as the endometrial blueing reaction. Each of these variables would be under independent control such that the co-ordinated induction of the necessary responses would require both optimum endometrial sensitization and endometrial stimulation by a blastocyst (or suitable artificial stimulus) at the appropriate time. The hypothesis was tested using a model involving unilateral artificial induction of decidualization in ovariectomized rats (Kennedy and Ross, 1993). With this model, E_2 and P_4 are administered exogenously to provide different extents of uterine sensitization. In addition, different substances can be infused into the uterine lumen to determine their abilities to induce decidualization. Therefore, the objectives of the present study are:

1) To determine if uterine VP, ECFV and/or BF are altered when unilateral artificial uterine stimulation is given to rats after optimal hormonal sensitization for decidualization.

These experiments will determine which of the variables may be involved in the enhanced accumulation of serum proteins which comprises the uterine blueing reaction.

2) To determine if uterine VP, ECFV and BF behave differently when the uterine stimulation is given after various forms of inadequate sensitization.

Because these forms of inadequate sensitization reduce the extents of i.v.-injected tracer proteins accumulated in stimulated horns, this experimental manipulation should permit the identification of uterine vascular changes which facilitate maximal protein accumulation.

3) To assess uterine VP, ECFV and BF after animals have been given optimal hormonal sensitization and subsequent intrauterine infusion of PBS or IM with and without PGE_2

PGs are considered to be obligatory mediators of decidualization and, for the rat, PGE_2 is considered to be the most likely PG involved in this process. Treatment with IM has been shown to reduce uterine accumulations of i.v.-injected tracer proteins in predecidual tissue while simultaneous infusion of PGE_2 with IM produces accumulations consistent with imminent decidualization (Kennedy, 1986). Therefore, the treatments with IM and PGE_2 should determine which uterine vascular changes (if any) are mediated by PGE_2.

4) To determine if MR imaging techniques can be used to identify uterine vascular changes associated with early decidualization.

Similar approaches have already provided successful identification of vascular changes associated with pathological and physiological events affecting numerous other tissues. MR imaging techniques offer the potential to examine various vascular and perivascular changes with minimal surgical intervention.

CHAPTER 3: GENERAL MATERIALS AND METHODS

3.1 MATERIALS

The chemicals and materials used in this study are listed by manufacturer (alphabetically).

Alza Corporation, Palo Alto, CA, USA

Alzet osmotic minipumps (model 2001)

Amersham Canada, Oakville, ON, Canada

^{125}NaI(SA=15.2mCi/μg I, ^{51}Cr-EDTA(SA=1μg/μg Cr)

BDH Inc., Toronto, ON, Canada

diethyl ether (ACS 288), Norit A charcoal (B26997)

Becton Dickinson and Co., Parsippany, NJ, USA

polyethylene tubing, (i.d. 0.580 mm, o.d. 0.985 mm)

Berlex

Magnevist; Gd-DTPA

Cayman Chemical Company, Ann Arbor, MI, USA

PGE_2; prostaglandin E_2 (14010)

Dupont Canada Inc., Markham, ON, Canada

^{57}Co-microspheres (15.5 $\pm$ 0.1 μm diameter; SA=72 DPM/microsphere), ^{3}H-PGE_2 (SA=154 Ci/mmol)

Fisher Scientific Co., Fair Lawn, NJ, USA

scintiverse I (SO-X-1B), sesame oil, Evans blue dye

Gibco Laboratories, Life Technologies Inc., Chagrin Falls, OH. USA

penicillin-streptomycin (600-5075AE)

M.T.C. Pharmaceuticals, Cambridge, ON, Canada

somnotol; sodium pentobarbital

Pharmacia Fine Chemicals HB, Uppsala, Sweden

dextran T70 (17-0280-01)

Sabex, Boucherville, PQ, Canada

diazepam

Sigma Chemical Co., St. Louis, MO, USA

E_2; estradiol-17B; 1,3,5(10)-estratrien-3,17β-diol (E-8875), bovine serum albumin (A-6918), EDTA; ethylene diaminetetraacetic acid (ED455); indomethacin; IM; 1-(p-chlorobenzoyl)-5-methoxy-2-methyindole-3-acetic acid

3.2 ANIMALS

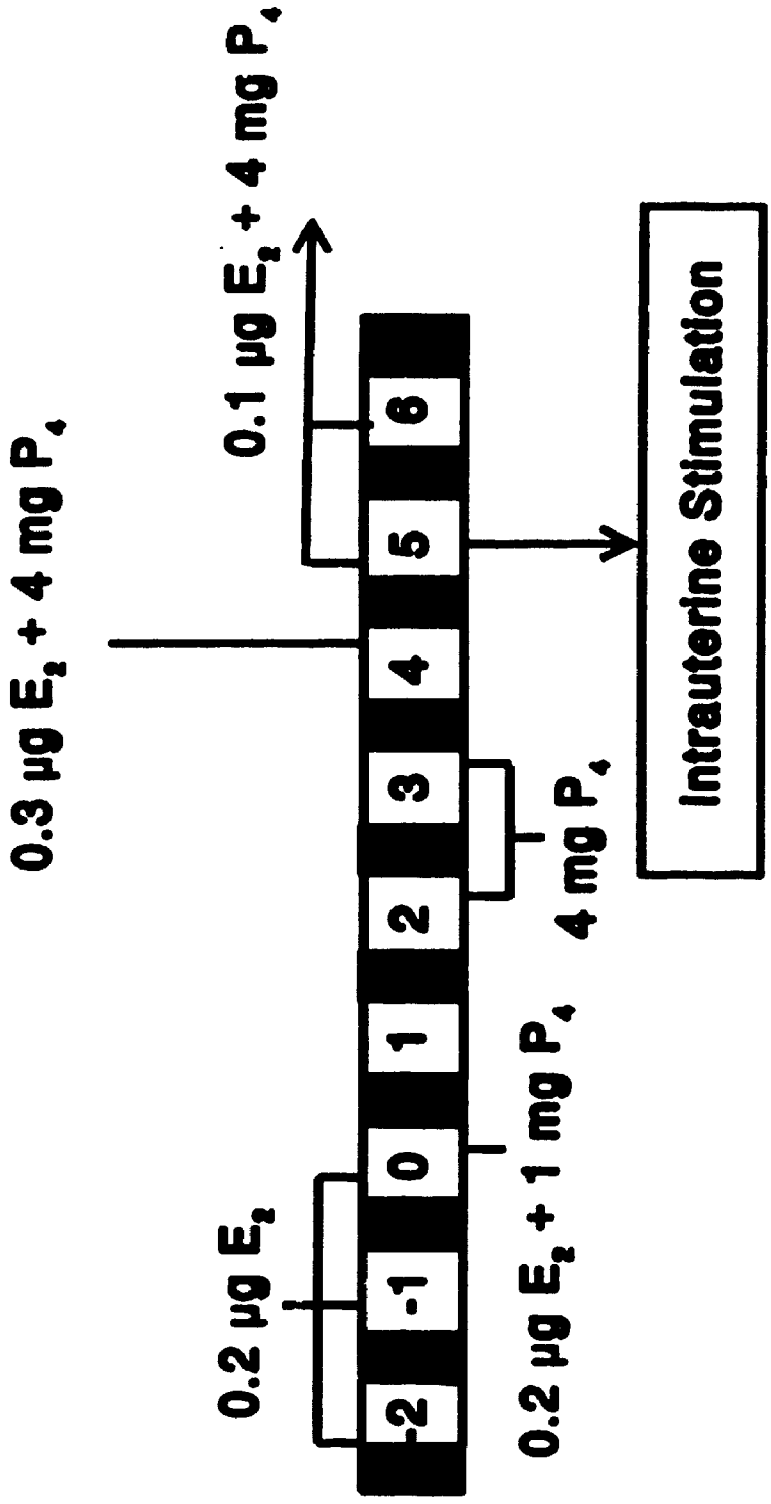
Female, Harlan Sprague-Dawley rats (Indianapolis, IN) were housed under temperature- and light-controlled conditions (14L:10D with 1200 h as midpoint of the light phase) and given free access to food (commercial rat ration) and water.

3.3 PREPARATION OF ANIMALS FOR ARTIFICIALLY-INDUCED DECIDUALIZATION

At 200-225 grams body weight, animals were anesthetized with ether and ovaries were exposed via bilateral incisions made just caudal to the rib cage and ventral to the spine. Ovaries and oviducts were removed with a small piece of uterus, the remainder of each uterine horn was returned to the abdominal cavity and incisions were closed with wound clips. The animals were given at least 4 days to recover from the surgery, at which time, a regimen of E_2 and/or P_4 was initiated to sensitize the uterus for decidualization. These hormones were administered s.c. in sesame oil according to a protocol published previously (Kennedy and Ross, 1993). The regimen (*FIGURE 1*) has been shown to create a hormonal state analogous to pseudopregnancy.

On equivalent to Day 5 of pseudopregnancy, at approximately noon (range $\pm$ 1.5 hours), animals were anesthetized with ether and decidualization was induced in one uterine horn. Briefly, a uterine horn was exposed by reopening one of the ovariectomy scars and 100 μl of sesame oil was injected into the uterine lumen via a 22-gauge needle inserted through the myometrium at the utero-tubal end. In order to prolong the interaction of the oil with the luminal walls, surgical silk was used to ligate the horn just below the site of needle entry. The hormone regimen and intraluminal oil injection have been shown to produce an increase in uterine weight (Kennedy and Ross, 1993; Psychoyos, 1961), accumulations of labeled proteins (Kennedy and Lukash, 1982; Psychoyos, 1961) and histological changes (Lundvist et al. 1977) similar to those which

FIGURE 3.1. Schematic representation of the treatment protocol for induction of unilateral decidualization in ovariectomized rats. Black areas represent periods of darkness and numbers within light areas represent equivalent day of pseudopregnancy.


0.3 µg E_2 + 4 mg P_4
0.1 µg E_2 + 4 mg P_4
0.2 µg E_2
0.2 µg E_2 + 1 mg P_4
4 mg P_4
-2
-1
0
1
2
3
4
5
6
Intrauterine Stimulation

accompany the decidualization of normal ovoimplantation.

3.4 ESTIMATION OF UTERINE EXTRACELLULAR FLUID VOLUME (ECFV)

ECFV was estimated by the uterine volume of distribution of ^{51}Cr-EDTA 15 minutes after its injection into a tail vein (McRae and Heap, 1988). EDTA rapidly equilibrates between plasma and interstitial fluids since it passes in and out of the vascular system freely, but crosses cell membranes with great difficulty (Larsson et al. 1980). As a result, the concentration of EDTA in plasma is equal to the concentration of EDTA throughout the ECF. The animals were nephrectomized immediately prior to ECFV estimation to prevent elimination of EDTA by the kidneys and the associated fall in plasma EDTA concentration.

At the times of ECFV determinations, animals were anesthetized with diazepam (3 mg/kg, i.v.) and sodium pentobarbital (20 mg/kg, i.v.) and the kidneys exposed via a midventral incision. The renal pedicles were ligated, kidneys removed and 5 μCi ^{51}Cr-EDTA in 0.5 ml 0.9% NaCl was injected into a lateral tail vein. Fifteen minutes later, animals were decapitated, trunk blood was collected and allowed to clot. Uteri were removed, horns were separated, extraneous connective tissue was removed and individual horns were weighed. Radioactivity was measured in each uterine horn as well as in a 50 μl aliquot of serum using a Nuclear Chicago gamma counter. The uterine volume of distribution of EDTA (ECFV) was calculated in each uterine horn as:

$$\frac{\text{uterine horn radioactivity (cpm/mg)}}{\text{serum radioactivity (cpm/}\mu\text{l)}} = \mu\text{l/mg}$$

3.5 ESTIMATION OF UTERINE TISSUE WATER

At desired times, animals were killed by decaptation, uteri were removed and individual horns were separated. Adherent connective tissue was removed, horns were weighed individually and heated in a dry oven at 60°C for 48 hours or until no further reduction in weight occurred (McRae and Heap, 1988). This treatment allows the evaporation of tissue water with minimal destruction of composite protein and lipid. The uterine tissue water was calculated as the percentage difference between wet weight and dry weight.

3.6 ESTIMATION OF UTERINE VASCULAR PERMEABILITY (VP)

Uterine VP was estimated by the rate at which the uterine volume of distribution of ^{125}I-albumin approached the total uterine ECFV after i.v. injection of the tracer (Setchell and Sharpe, 1981). Bovine serum albumin (BSA) was radioiodinated by the chloramine-T method as described previously for growth hormone (Greenwood et al. 1963). The ^{125}I-albumin was purified on a Sephadex G75 column yielding a product with specific activity of approximately 50 μCi/μg. At the time of each VP determination, rats were anesthetized with ether and 2 μCi ^{125}I-albumin in 0.5 ml 0.9% sodium chloride (NaCl) was injected into a tail vein. At various times from 5 to 120 minutes later, animals were killed by decapitation, trunk blood was collected and allowed to clot. Uteri were removed, separated, connective tissue was removed and individual uterine horns

were weighed. Concentrations of radioactivity were determined in individual horns and in a sample of serum using a Nuclear Chicago gamma counter. The volume of distribution of labeled albumin was determined by dividing the tissue radioactivity (cpm/mg) by the serum radioactivity (cpm/μl). Uterine VP was determined from plots of log[1-(albumin volume/ECFV)] against time after tracer injection (Setchell and Sharpe, 1981). The slopes of the lines of log[1-(albumin volume/ECFV)] versus time and the extrapolated $t_{1/2ECFV}$ (time in minutes for the uterine albumin volume of distribution to reach half the uterine ECFV) were used as indices of uterine VP. Greater negative slopes and shorter $t_{1/2ECFV}$ both represent higher VP. Treatments were considered to have significantly affected VP when ANOVA showed significant ($p<0.05$) interactions between the effects of time and treatment, a finding which indicates that the slopes of the lines differ (Steel and Torrie, 1960).

3.7 ESTIMATION OF UTERINE BLOOD FLOW (BF)

Uterine BF was determined by the radioactively-labelled microsphere technique (Rudolph and Heymann, 1967). At the time of BF determination, animals were anesthetized with diazepam and sodium pentabarbital as described above and the right carotid and right femoral arteries were cannulated with polyethylene tubing (i.d.=0.58mm, o.d.=0.985mm). The carotid cannula was attached to a pressure transducer and recording apparatus and the femoral cannula was attached to an Harvard withdrawal pump. The carotid cannula was advanced to the left ventricle as indicated

by the pressure tracing. The pump was set to withdraw at 200 μl/min, switched on and 10 μCi (in 100 μl 20% dextran, 0.01% tween 80 in 0.9% saline) of ^{57}Co-labeled microspheres (diameter = 15.5±0.1 μm) were injected as a bolus into the carotid cannula. After 2 minutes, the animal was killed by injection of concentrated KCl into the carotid cannula, the femoral cannula was removed and the remaining blood within the cannula was drawn into the syringe. Uterine horns, kidneys and pieces of liver, duodenum and shoulder muscles were removed and weighed. Radioactivities of withdrawn blood, tissue samples, cannulae, syringes and microsphere storage tubes were measured with the gamma counter. The number of spheres injected (approximately 800,000) was determined by subracting the residual radioactivity in cannulae, syringes and microsphere storage tubes from the original radioactivity contained in the storage tubes. Total organ BF was calculated as:

$$Q_{ref} \times (N_{org}/N_{ref}) = \mu l/min$$

where Q_{ref} = rate of withdrawal of the reference sample, N_{org} = number of microspheres (based on radioactivity) present in the organ and N_{ref} = number of microspheres in the reference sample. Tissue BFs were calculated by dividing total BF by tissue weights and cardiac output (CO) was calculated as:

$$Q_{ref} \times (N_{ref}/N_{inj}) = \mu l/min$$

where N_{inj} = the number of microspheres injected into the carotid cannula. Intraventricular mixing of microspheres was considered adequate when tissue BF for left and right kidneys differed by less than 15%. BF estimates associated with inadequate mixing of the spheres were not considered (< 10% of the cases). In all cases, the

procedure resulted in more than 400 microspheres being trapped in individual kidneys and uterine horns. This represents the recommended value to achieve sufficient precision of blood flow measurements (Buckberg et al. 1971).

The potential for this technique to identify uterine BF differences was assessed and reported in APPENDIX 2.

3.8 STATISTICAL TREATMENT

Data were analyzed using Bartlett's test for homogeneity of variance and ANOVA. When variance was not homogeneous, data were logarithmically transformed to achieve homogeneity and tested again using the Bartlett's test. Variance was partitioned within and between animals when appropriate, and when significant interactions were present, between-animal comparisons were made using Duncan's New Multiple Range Test. Separate between-animal comparisons were made for stimulated (or infused) and nonstimulated (or noninfused) uterine horns. Differences were considered significant at $p < 0.05$.

CHAPTER 4: UTERINE VASCULAR CHANGES AFTER UNILATERAL DECIDUOGENIC STIMULATION OF RATS SENSITIZED FOR THE DECIDUAL CELL REACTION

4.1 INTRODUCTION

Ovoimplantation and imminent decidualization are first macroscopically identifiable in the uterus by a localized increase in radioactivity or blueing after intravenous (i.v.) injection of radio-iodinated albumin (Psychoyos, 1961; Kennedy, 1979; Milligan and Mireinbe, 1985) or Evans blue dye which binds to and travels with endogenous plasma proteins (Psychoyos, 1973). The intrauterine accumulation of these labeled proteins has been attributed to increased endometrial VP (Psychoyos, 1973; Kennedy, 1979) and accordingly, endometrial VP has typically been quantitated by the uterine volume of distribution of radio-labeled albumin at a single time after i.v. injection of the tracer (Psychoyos, 1961; Kennedy, 1979; Milligan and Mirembe, 1985). This approach to assessing endometrial VP is problematic because changes in other uterine variables may also contribute to the enhanced accumulation of i.v.-injected tracer proteins. Uterine ECFV has been shown to increase in sites of ovoimplantation as compared to surrounding uterine tissue (McRae and Heap, 1988) and in uterine horns given deciduogenic stimulation (Milligan and Edwards, 1990). Any increase in the size of this space, which comprises plasma and interstitial compartments, could contribute significantly to increases in volumes of distribution of ^{125}I-albumin by creating a larger

compartment in which the tracer can accumulate. In addition, an increase in local BF has also been demonstrated in sites of embryo implantation in rats (Mitchell and Hammer, 1983; McRae and Heap, 1988) and this uterine vascular change could contribute to the extravasation of tracers by increasing plasma hydrostatic pressure. In fact, increased BF has already been shown to have this role in the extravasation of plasma proteins which occurs during inflammation (Williams, 1977; Williams and Peck, 1977). The potential for changing uterine ECFV and BF to influence uterine accumulations of tracer proteins indicates the need to examine uterine VP with approaches which assess this variable more selectively.

Setchell and Sharpe (1981) assessed VP in the testis by measuring the rate at which the volume of distribution of ^{125}I-albumin approaches the testicular ECFV after i.v. injection of the tracer. This method of assessing VP takes into account changes in ECFV, endothelial pore size and surface area (Setchell and Sharpe, 1981). A modified version of the method has already been used to examine uterine VP during ovoimplantation in rats (McRae and Heap, 1988) at approximately 12 and 24 hours after initiation of ovoimplantation. The times are approximate because the exact timing of the initiation of implantation is uncertain and varies among animals even within a colony.

In the experiments described in this chapter, the method of Setchell and Sharpe (1981) has been used to estimate uterine VP at various times from 2 to 32 hours after unilateral deciduogenic stimulation of rats. In addition, uterine ECFV and BF have been assessed at similar times to provide a more complete description of changes in uterine variables which could potentially contribute to the enhanced accumulation of i.v.-injected

tracer proteins preceding decidualization. Since these experiments examine uterine responses to deciduogenic stimulation, the exact time and site of stimulation are known. Therefore, changes in uterine VP, ECFV and BF were examined at precise times after stimulation, including times before sites of imminent decidualization could be visualized by injection of Evans blue dye.

4.2 METHODS

4.2.1 Animals

Rats were ovariectomized, sensitized for decidualization and given unilateral deciduogenic stimulation as described in sections 3.2, 3.3 and illustrated in *FIGURE 3.1*.

4.2.2 Estimation of Extracellular Fluid Volume (ECFV) and Tissue Water

At 2, 4, 8, 16 and 32 hours after unilateral deciduogenic stimulation, uterine ECFV and tissue water were estimated in stimulated and nonstimulated uterine horns as described in sections 3.4 and 3.5.

4.2.3 Estimation of Uterine Vascular Permeability (VP)

At 2, 4, 8, 16 and 32 hours after unilateral deciduogenic stimulation, uterine VP was assessed in stimulated and nonstimulated uterine horns as described in section 3.6.

4.2.4 Estimation of Uterine Blood Flow (BF)

At 3, 9 and 27 hours after unilateral deciduogenic stimulation, uterine BF was estimated in stimulated and nonstimulated uterine horns as described in section 3.7.

4.3 RESULTS

4.3.1 Uterine Extracellular Fluid Volume (ECFV) and Tissue Water

ECFV was relatively constant in nonstimulated horns for all times beyond 2 hours after deciduogenic stimulation (*FIGURE 4.1*). Among nonstimulated horns, ECFV was significantly higher at 2 hours than at other times after stimulation ($p<0.05$); however, the value was not significantly different from that of stimulated horns at 2 hours ($p>0.05$). ECFV in stimulated horns became significantly increased by 8 hours post-stimulation and reached a peak of 0.63 ± 0.06 μl/mg at 16 hours ($p<0.05$; *FIGURE 4.1*). ECFV for stimulated horns fell significantly (0.56 ± 0.04 μl/mg) between 16 and 32 hours after deciduogenic stimulation.

Estimates of uterine tissue water revealed a similar trend to that of uterine ECFV. Nonstimulated horns had similar tissue water contents at all times after deciduogenic stimulation while those of oil-injected horns increased from $81.1\pm0.4\%$ at 2 hours to a maximum of $85.6\pm0.8\%$ ($p<0.05$) at 16 hours after deciduogenic stimulation (*FIGURE 4.2*). In contrast to ECFV estimates, the tissue water contents in oil-injected horns fell only slightly ($84.8\pm0.4\%$) between 16 and 32 hours post-stimulation.

FIGURE 4.1. Uterine ECFV of nonstimulated and stimulated horns at various times from 2 to 32 hours after unilateral deciduogenic stimulation by instillation of sesame oil into the uterine lumen. ECFV was determined by the uterine volumes of distribution of i.v.-injected ^{51}Cr-EDTA and each bar represents the mean $\pm$ SEM for 7 uterine horns. Bars not sharing the same superscript indicate statistically significant differences in ECFV among stimulated horns ($p<0.05$).

Summary of Analysis of Variance			
Source	df	Mean Square	F Ratio
Between Animals			
A = Time after Stimulation	4	0.012	3.94*
Error	30	0.00312	
Within Animals			
B = Uterine stimulation	1	0.040	24.91***
Interaction	4	0.014	8.63***
Error	30	0.00160	

* $p<0.05$

*** $p<0.001$

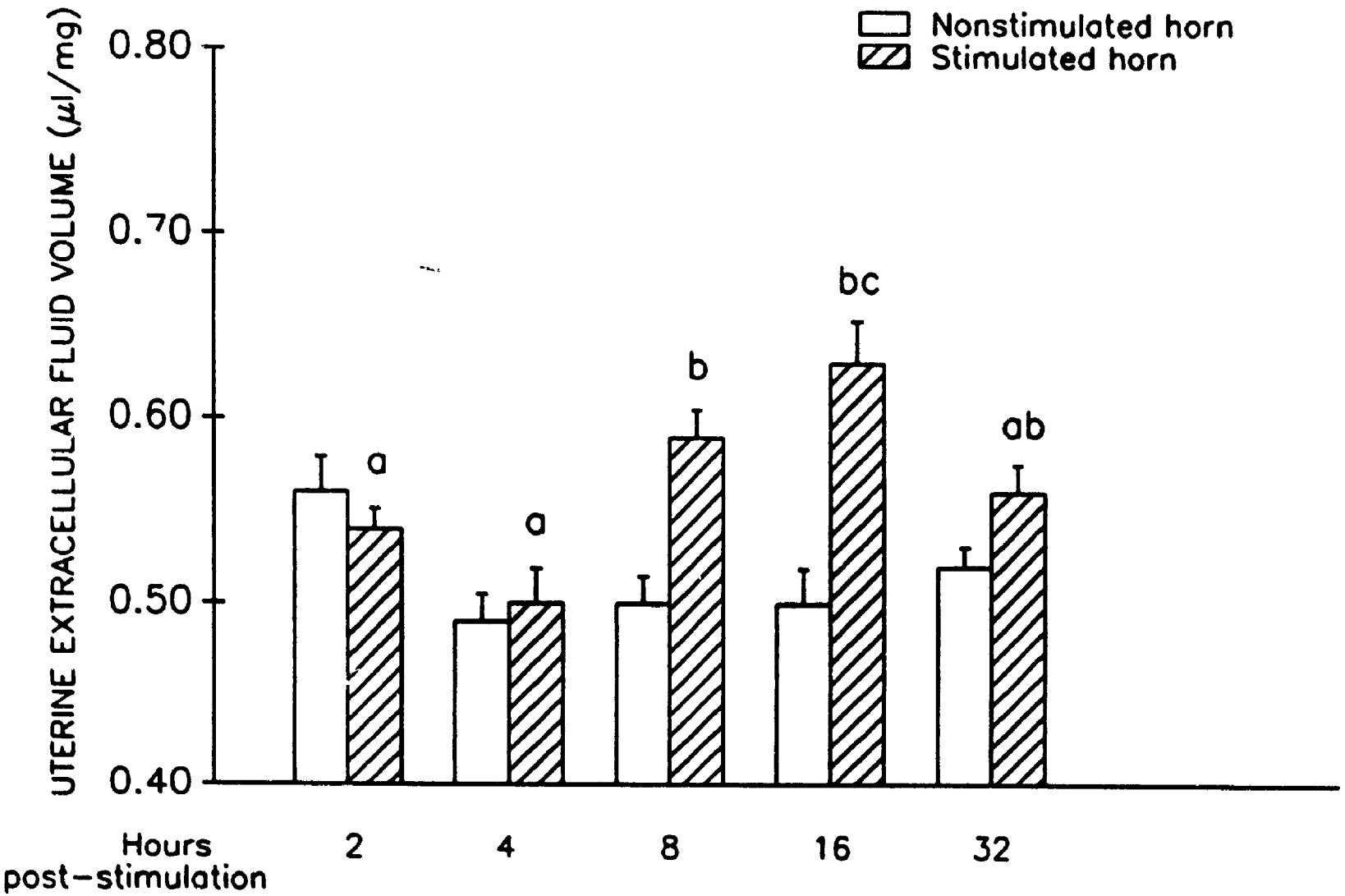
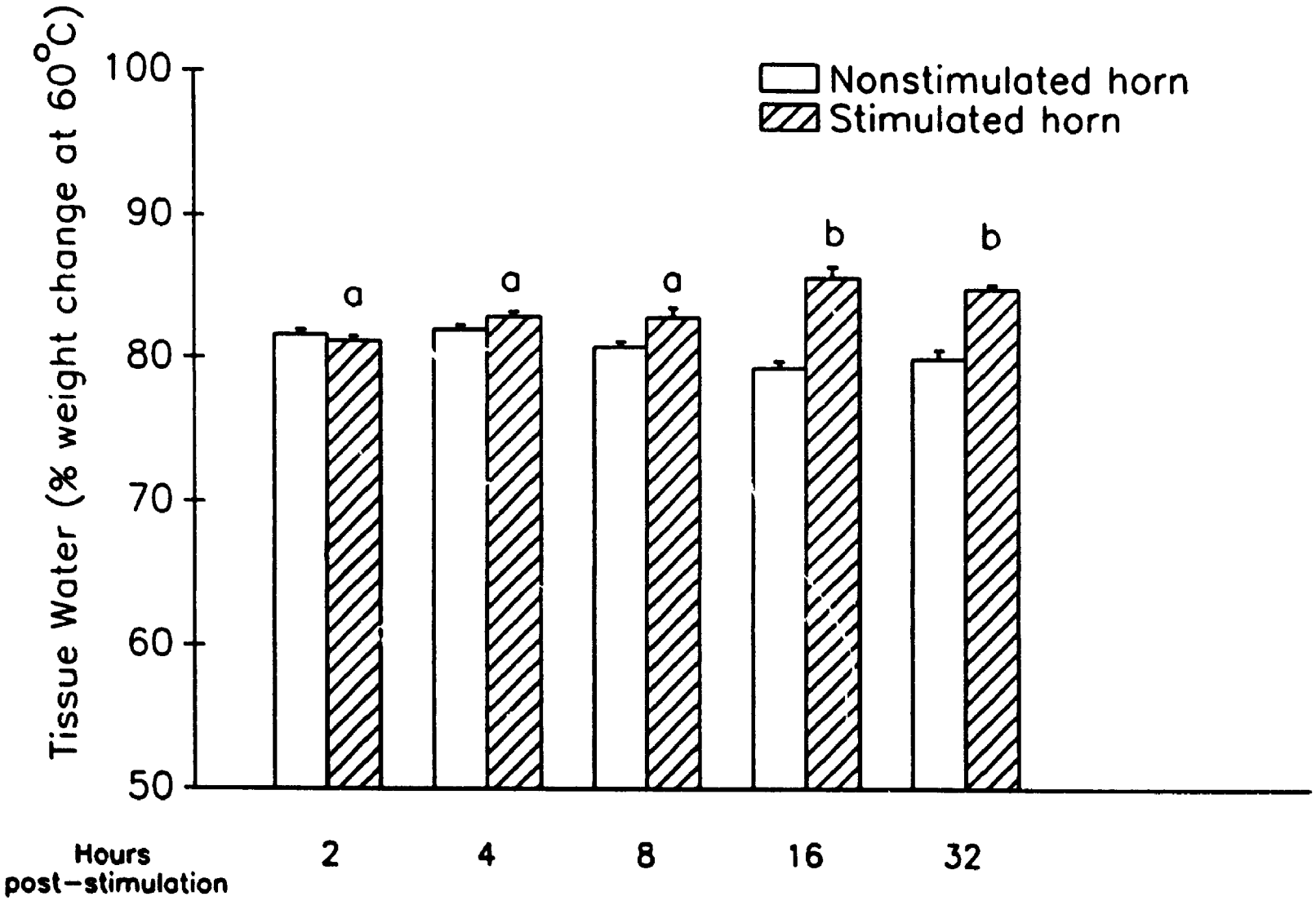

Nonstimulated horn
Stimulated horn
UTERINE EXTRACELLULAR FLUID VOLUME (µl/mg)
0.80
0.70
0.60
0.50
0.40
a
a
b
bc
ab
Hours post-stimulation
2
4
8
16
32

FIGURE 4.2. Uterine tissue water contents of stimulated and nonstimulated horns at various times from 2 to 32 hours after unilateral deciduogenic stimulation by instillation of sesame oil into the uterine lumen. Uterine tissue water was determined by the % difference between wet and dry weight and each bar represents the mean $\pm$ SEM for 7 uterine horns. Asterisks indicate statistically significant differences among stimulated horns ($p < 0.05$).

Summary of Analysis of Variance			
Source	df	Mean Square	F ratio
Between Animals			
A = Time after Stimulation	4	4.09	1.28^{NS}
Error	30	3.209	
Within Animals			
B = Uterine stimulation	1	168.9	107.5^{***}
Interaction	4	36.3	23.1^{***}
Error	30	1.572	

* $p < 0.05$.

*** $p < 0.001$.


Tissue Water (% weight change at 60°C)
100
90
80
70
60
50
Nonstimulated horn
Stimulated horn
a
a
a
b
b
Hours post-stimulation
2
4
8
16
32

4.3.2 Uterine Vascular Permeability (VP)

For nonstimulated horns, analysis (by ANOVA) of the plots log[1-(albumin volume/ECFV)] versus time after tracer injection revealed no significant interaction between these variables and time after deciduogenic stimulation ($p > 0.05$). The finding indicates that the slopes of the plots for nonstimulated horns (*FIGURE 4.3* panels *a,b,c,d* and *e*) are not different and implies that uterine VP did not change from 2 to 32 hours after stimulation of contralateral uterine horns. When plots of log[1-(alubmin volume/ECFV) versus time after tracer injection were compared in stimulated and nonstimulated horns by similar analyses, the inferred VP was significantly higher in stimulated than nonstimulated horns at 4, 8, 16 (all $p < 0.001$) and 32 ($p < 0.05$; *FIGURE 4.3*) hours after unilateral deciduogenic stimulation. Uterine VP was not different in stimulated and nonstimulated horns at 2 hours after stimulation ($p > 0.05$; *FIGURE 4.3a*). For stimulated horns, comparisons of the plots indicated that the inferred VP at 8 hours after stimulation was significantly greater than at 2 and 32 h ($p < 0.05$ by ANOVA) but not different from that estimated at 4 and 16 h ($p > 0.05$ by ANOVA).

4.3.3 Uterine Blood Flow (BF)

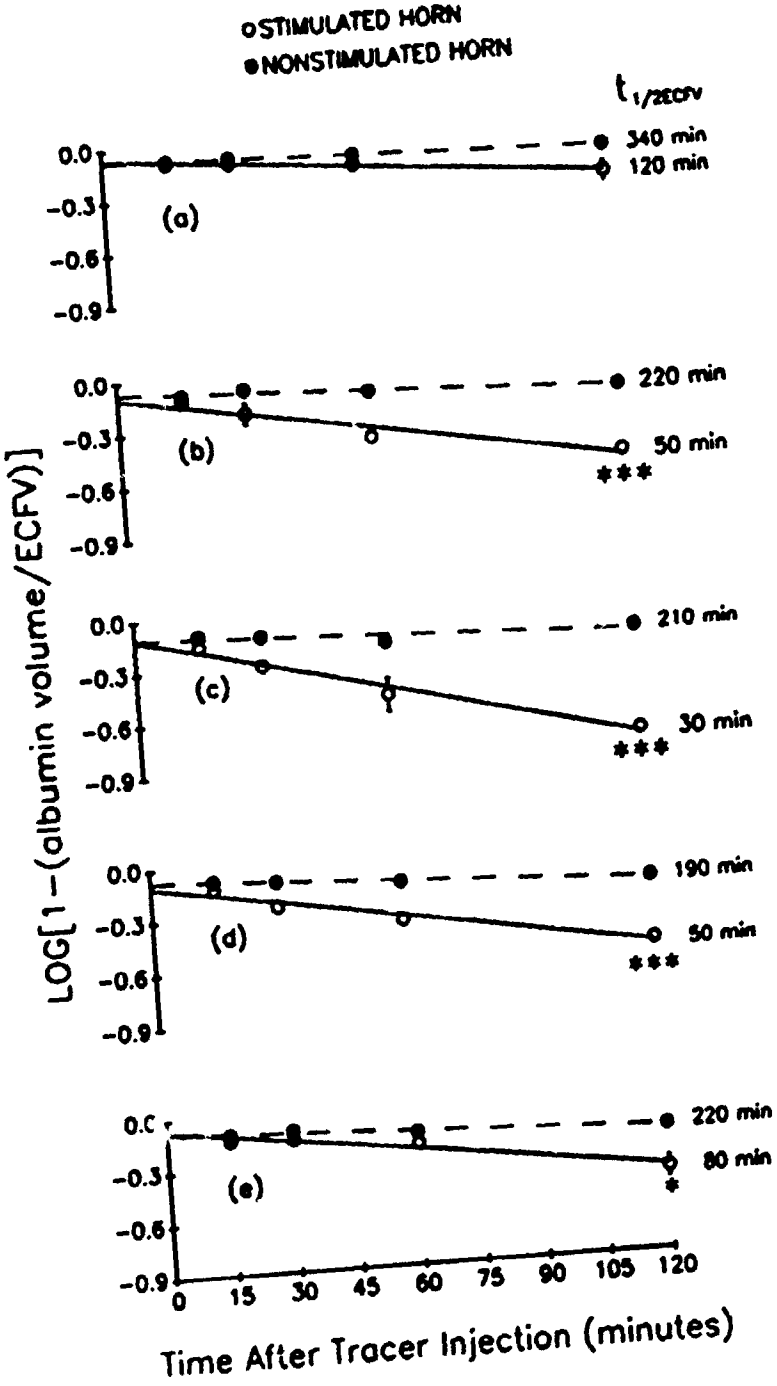
Total uterine BF (μl/min) to stimulated horns increased between 9 and 27 hours after deciduogenic stimulation (*FIGURE 4.4a*); however, the increases in BF matched the increases in uterine horn weight associated with decidualization (*FIGURE 4.4b*). Therefore, tissue BF (BF per unit tissue weight) did not change significantly in either

FIGURE 4.3. Plots of log[1-(albumin volume/ECFV)] versus time after i.v. injection of ^{125}I-albumin for nonstimulated and stimulated horns at 2 (a), 4 (b), 8 (c), 16 (d) and 32 (e) hours after unilateral deciduogenic stimulation by instillation of sesame oil into the uterine lumen. Each point represents the mean $\pm$ SEM for 5 uterine horns. Asterisks indicate statistically significant differences in VP between stimulated and nonstimulated horns (* = $p<0.05$, *** = $p<0.001$).

Summary of Analysis of Variance			
Source	df	Mean Square	F Ratio
Between Animals			
A = Time After Stimulation	4	0.147	24.4***
B = Time After Tracer Injection	3	0.585	97.4***
A x B	12	0.017	2.80*
Error	80	0.00601	
Within Animals			
C = Uterine stimulation	1	1.363	311***
A x C	4	0.076	17.4***
B x C	3	0.202	46.2***
A x B x C	12	0.020	2.74*
Error	80	0.00438	

* $p<0.05$.

*** $p<0.001$.


oSTIMULATED HORN
●NONSTIMULATED HORN
$t_{1/2ECFV}$
340 min
120 min
220 min
50 min
210 min
30 min
190 min
50 min
220 min
80 min
(a)
(b)
(c)
(d)
(e)
0.0
-0.3
-0.6
-0.9
***
*
LOG[1−(albumin volume/ECFV)]
0
15
30
45
60
75
90
105
120
Time After Tracer Injection (minutes)

horn from 3 to 27 hours after unilateral deciduogenic stimulation (3 to 4.5 μl/min/mg for both horns; $p>0.05$; *FIGURE 4.4c*). A surprising finding was that in many cases, tissue BF was slightly higher in nonstimulated horns than in oil-injected horns; however, this difference was not significant ($p>0.05$). Cardiac output (122 ± 7 ml/min) and renal tissue BF (3.35 ± 0.17 μl/mg/min; similar for both kidneys) did not change significantly at any time after deciduogenic stimulation ($n=27$).

4.4 DISCUSSION

Predecidual increases in endometrial VP have been described in rodents for several decades (Psychoyos, 1960; Psychoyos, 1961; Kennedy, 1979; Milligan and Mirembe, 1985; Parr and Parr, 1986); however, previous methods of quantitating these VP changes have not accounted for the increase in uterine ECFV which also precedes decidualization. In rats, uterine ECFV has been shown to increase in sites of blastocyst implantation (McRae and Heap, 1988) and results presented in this chapter indicate that similar increases occur in uterine horns given deciduogenic stimulation (*FIGURE 4.1*). Since uterine blood volume does not change under similar circumstances (Bitton et al. 1965; Milligan and Mirembe, 1985; Milligan and Edwards, 1990), the uterine interstitial compartment appears to be the major site of the expanded uterine ECFV.

In the present study, uterine VP was estimated by a method based on the rate of equilibration of ^{125}I-albumin between plasma and interstitial fluid after i.v. injection. The method also accounted for the predecidual increases in uterine ECFV. Thus, although

FIGURE 4.4. Total uterine BF (a), uterine weight (b) and uterine tissue BF (c) for nonstimulated and stimulated horns at various times from 3 to 27 hours after unilateral deciduogenic stimulation. Uterine BF was determined by the microsphere technique and bars represent mean $\pm$ SEM for 9 animals. Asterisks indicate statistically significant differences among stimulated horns ($p<0.05$).

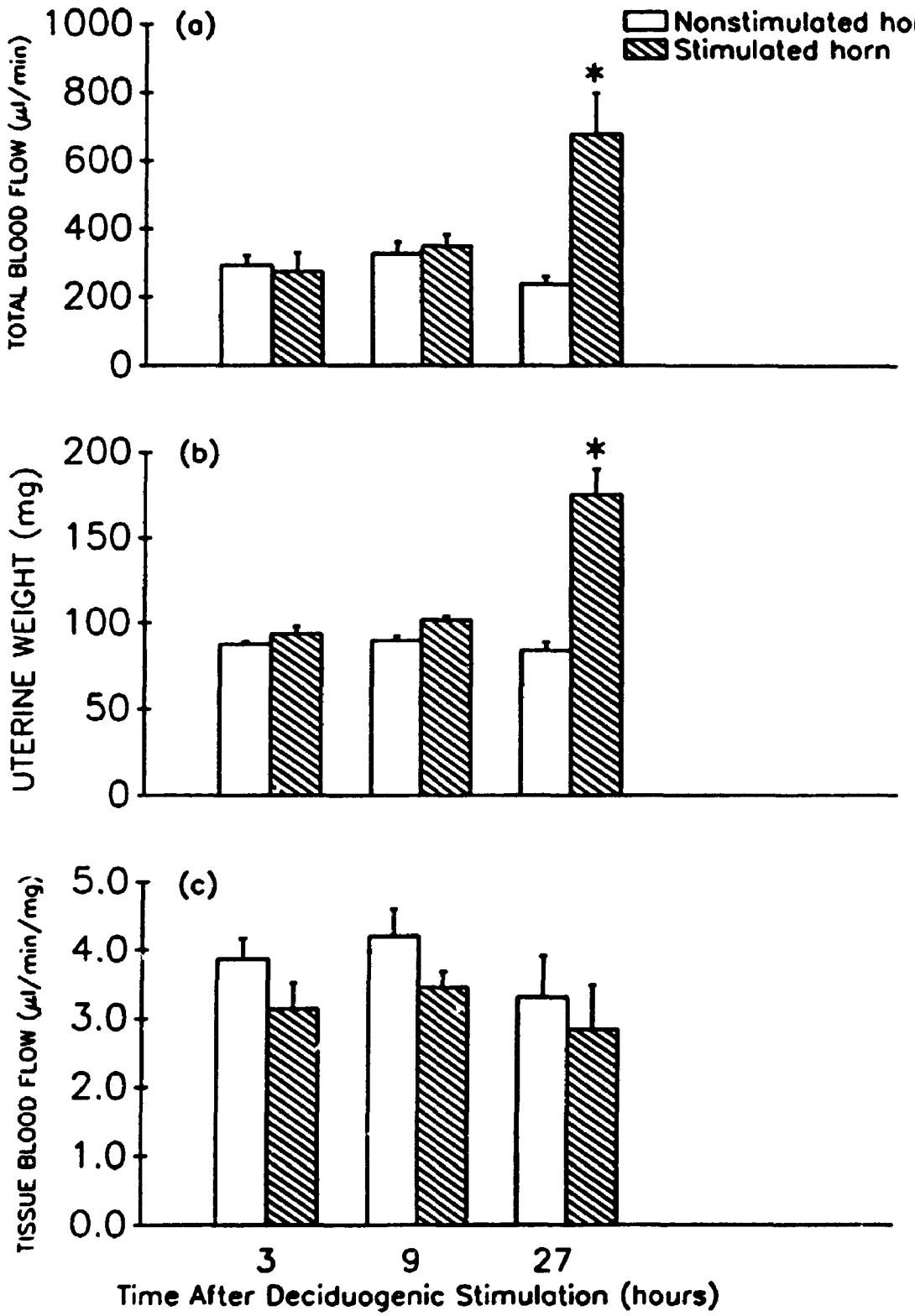
Summary of Analysis of Variance							
	Total BF			Uterine Weight[a]		Tissue BF	
Source	df	Mean Square	F Ratio[b]	Mean Square	F Ratio[b]	Mean Square	F Ratio
Between							
A=Time	2	96636	2.99^{NS}	0.262	11.31^{***}	0.456	0.254^{NS}
Error	15	32315		0.0231		1.796	
Within							
B=Ut.stim	1	197432	10.36^{*}	0.837	73.87^{***}	0.108	0.206^{NS}
Interaction	2	196162	10.29^{*}	0.400	35.32^{***}	1.74	
Error	15	19064		0.0113		0.523	

[a] Denotes data which were logarithmically transformed to achieve homogeneity

[b] Superscripts denote levels of significance: $p<0.05^{*}$; $p<0.001^{***}$; not significant^{NS}.

* $p<0.05$.

*** $p<0.001$


(a)
Nonstimulated horn
Stimulated horn
TOTAL BLOOD FLOW (µl/min)
1000
800
600
400
200
0
*
(b)
UTERINE WEIGHT (mg)
200
150
100
50
0
*
(c)
TISSUE BLOOD FLOW (µl/min/mg)
5.0
4.0
3.0
2.0
1.0
0.0
3
9
27
Time After Deciduogenic Stimulation (hours)

the trends in uterine VP are similar to those obtained using uterine ^{125}I-albumin volumes of distribution at a single time after i.v. injection of the tracer (Kennedy, 1979), the results of the present study validate conclusions drawn from the previous studies.

An exclusion volume for albumin has been described (Kwong and Egan, 1985) in various tissues where components of the extracellular matrix prevent labeled albumin from actually permeating the interstitial fluid. To prevent the uterine albumin exclusion volume from distorting VP estimates, McRae and Heap (1988) chose to assess uterine VP by the rate at which the volume of distribution of albumin approached steady state. By contrast, in the present study, uterine volumes of distribution of albumin were assessed prior to the time when the exclusion volume is achieved. As indicated by the linear relationship between time and log[1-(albumin volume/ECFV)], this approach proved to be successful except possibly for uterine VP estimates obtained 8 h after deciduogenic stimulation. During some of those estimates, log[1-(albumin volume/ECFV)] did not always continue to increase in a linear fashion from 60 to 120 minutes after i.v. injection of the tracer. This may have resulted in a slight underestimation of uterine VP at 8 hours after deciduogenic stimulation.

Significant increases in uterine VP (*FIGURE 4.3*) and ECFV (*FIGURE 4.1*) were noted in stimulated horns at similar times after application of the deciduogenic stimulus. However, the observed peak ECFV at 16 hours after stimulation is several hours later than that of peak VP at 8 hours. The relationship between uterine ECFV and VP is not surprising since the increased VP permits proteins from the blood to enter the uterine interstitium (Parr and Parr, 1986) where they can become osmotically active, promoting

a concommitant movement of water as a consequence of increased tissue osmotic pressure and bulk flow. Such a model implies that the increase in ECFV is dependent on a previous increase in VP. However, the results of these experiments do not exclude the possibility that increased uterine ECFV itself augments the intrauterine accumulation of i.v. injected tracer proteins by creating a larger compartment in which tracers can accumulate. This inference is supported by histological studies which have indicated predecidual modifications to the endometrial interstitium consistent with pathological edema formation (Fainstat, 1963; Lundkvist, 1978) and by the ability of embryos and endometrial cells to secrete plasminogen activators (Strickland et al. 1976) and metalloproteinases (Brenner et al. 1989; Lala and Graham, 1990) capable of modifying extracellular matrices. These observations indicate an expanding interstitial compartment producing measurable increases in tissue ECFV. Because quantitative estimates of endometrial VP have traditionally involved measuring the volume of distribution of ^{125}I-albumin at a single time after its i.v. injection, it is possible that the expanded uterine interstitium biased these estimates by providing an enlarged compartment in which the tracers could accumulate.

The similar trends in uterine ECFV (*FIGURE 4.1*) and tissue water (*FIGURE 4.2*) reflect the direct relationship between these variables. Since the estimate of uterine tissue water encompasses both the intracellular and extracellular compartments, any increase in ECFV should also result in a concommitant increase in tissue water. With the exception of 32 hours post-stimulation, this relationship was consistent for all times after deciduogenic stimulation. Between 16 and 32 hours, there was a significant fall in

stimulated horn ECFV which was not accompanied by a similar fall in tissue water. Such a finding might suggest that a portion of the diminished ECFV results from a shift of extracellular fluid to intracellular fluid. This conclusion is not unreasonable since cells of the endometrial stroma undergo numerous changes (structural and functional) during decidual metamorphosis (De Feo, 1967). These changes could certainly be accompanied by an increase in the size of the intracellular fluid compartment.

In the present study, total uterine blood flow (BF) increased following deciduogenic stimulation; however, at all times, the increase in uterine weight matched the increases in BF resulting in no significant changes in uterine tissue BF (BF on a per weight basis). A previous study with pseudopregnant rats using an electromagnetic flow probe (Garris et al. 1983) reported an increase in uterine tissue BF during early decidualization; however, the difference was not apparent until 48 hours after deciduogenic stimulation. Although this is a time when endometrial neoangiogenesis has been reported on the basis of vascular casts (Takemori et al. 1984), it is too late after stimulation to implicate these BF increases in the extravasation of and accumulation of proteins which give rise to the Evans blue reaction. When the uterine weights and BFs at 24 hours from the previous study (Garris et al. 1983) are considered, the tissue BFs for decidualizing and control horns are similar to those which we have reported (*FIGURE 4.4*). In addition, the absence of a change in uterine tissue BF during early decidualization for the rat is consistent with findings for the mouse (Edwards and Milligan, 1987) where increases in uterine BF also matched the increases in uterine weight. It is doubtful that increases in total uterine BF contribute to the extravasation

of tracer proteins since significant uterine weight gains and the concommitant BF increases are not observed until after the enhanced accumulation of tracer proteins is observed.

A slight but significant increase in uterine tissue BF has been reported for the uterine tissue of implantation sites in rats as compared with uterine tissue outside the sites (Mitchell and Hammer, 1983; Mitchell et al. 1983; McRae and Heap, 1988). This contrasts with our finding of no change after a deciduogenic stimulus. Perhaps the blastocyst provides a vasodilatory mediator not present during artificial deciduogenic stimulation. If a blastocyst-derived mediator does increase uterine BF, the increase does not appear to be essential for the events associated with the uterine blueing reaction since this phenomenon occurs after deciduogenic stimulation in the absence of significant increases in uterine tissue BF. Alternatively, our use of the microsphere technique may not have been sufficiently precise to have revealed small changes in endometrial BF which may have occurred.

The endometrial microcirculation has recently been examined during implantation, by in vivo microscopy (Tawia and Rogers, 1992). In that study, embryos were reported to be surrounded by an avascular region of endometrium with adjacent capillaries being large in diameter and displaying sluggish blood flows with frequent reversals and stoppages. This description demonstrates the complexity of the endometrial circulation during implantation and makes it difficult to predict what net BF changes, if any, might be obtained using the microsphere technique. An increased BF might be observed as a result of the vasodilation or a decrease might be seen as a result of the reduced capillary

density and the sluggish movement through the vessels.

Although available evidence indicates that most of the elevated uterine BF is associated with the uterine weight gain during decidualization, we feel that the absence of a change in uterine tissue BF soon after deciduogenic stimulation does not negate the importance of BF in augmenting the uterine vascular changes associated with the Evans blue reaction. A minimal BF during early pregnancy has been shown to be important in rats since surgical impairment of uterine BF prior to pregnancy reduced the number of fetuses (Franklin and Brent, 1964; Antebi et al. 1991) and fetal weights (Antebi et al. 1991). We propose that BF to both uterine horns is sufficiently high prior to decidualization to form a favourable condition to augment the effects of increased VP and ECFV as occurs during early decidualization. This possiblility is supported by the fact that, throughout these experiments, uterine tissue BF to stimulated and nonstimulated horns was similar to that of the kidney; a tissue which preferentially receives a high tissue BF.

4.5 SIGNIFICANCE

The experiments described in this chapter have examined uterine VP, ECFV and BF at several times after unilateral deciduogenic stimulation. The times of assessment encompass the period when the endometrial blueing reaction can be demonstrated by i.v. injection of Evans blue dye; extending from soon after deciduogenic stimulation to beyond the first day. The results indicate that uterine VP and ECFV increase during the

time when the blueing reaction can be demonstrated, thus implicating these two variables in the blueing reaction. Uterine tissue BF did not increase during the experimental period and although total uterine BF increased to stimulated horns, significant increases occurred after the time of maximal accumulation of serum proteins in predecidual tissue. These findings indicate that increased uterine BF is not essential to the enhanced extravasation of serum proteins which gives rise to the endometrial blueing reaction.

CHAPTER 5: MAGNETIC RESONANCE (MR) IMAGING DEMONSTRATES UTERINE VASCULAR CHANGES DURING EARLY DECIDUALIZATION

5.1 INTRODUCTION

Sites of blastocyst implantation and imminent decidualization can be identified by the presence of blue staining on uteri removed after i.v. injection of Evans blue dye (Psychoyos, 1973). This transient phenomenon can be demonstrated during the first day of embryo implantation or within 1 day of deciduogenic stimulation (Psychoyos, 1973). Although originally attributed to local increases in endometrial VP (Psychoyos, 1960), the occurrence of the endometrial blueing reaction also coincides with local increases in uterine ECFV (Milligan and Edwards, 1990; McRae and Heap, 1988) and BF (Mitchell and Hammer, 1983; McRae and Heap, 1988; Mitchell and Goldman, 1991), both of which could contribute to the extravasation and accumulation of i.v. injected tracers. In fact, results presented in chapter 4 of this thesis indicate that uterine VP (***FIGURE 4.3***) and ECFV (***FIGURE 4.2***) are elevated in predecidual tissue and that uterine tissue BF is high (***FIGURE 4.4***) during the time when the blueing reaction can be demonstrated.

Various types of circumscribed vascular changes have permitted physiological and pathological condititions to be identified in numerous tissues by magnetic resonance (MR) techniques. This noninvasive approach offers the potential to examine these processes in intact animals. Furthermore, in some cases, it has been possible to quantitate various vascular changes by the MR image intensities of the involved tissues after i.v. injection

of contrast agents.

Gadolinium-diethylene triaminepentaacetic acid (Gd-DTPA) is a contrast agent which enhances T_1-weighted MR images and which is eliminated by the kidneys within several hours of i.v. injection (Weinmann et al. 1984; Brasch et al. 1984). This agent is sufficiently small (560 d) to pass out of blood vessels and permeate the extracellular fluids (ECF) of most tissues (Strich et al. 1985), although it is restricted from brain unless the blood-brain-barrier is compromised by a pathological process (Karlik et al. 1990). Because of these characteristics, Gd-DTPA-enhanced MR imaging has been used to demonstrate many physiological and pathological processes that result in locally increased VP, ECFV or both. This chapter, as well as appendices 3 and 4, describe attempts to use this technology to identify uterine vascular changes which precede endometrial decidualization.

Studies involving electron microscopy have suggested that the endothelial layer of endometrial blood vessels can present a formidable barrier to the passage of blood-borne molecules in certain endocrine states (Martin et al. 1973) and in uterine tissue outside the implantation sites of pregnant rats (Abrahamsohn et al. 1983). Therefore, the uterine volume of distribution of ^{153}Gd-DTPA has also been measured in both decidualizing and non-decidualizing uterine tissue to determine if VP is a limiting factor in MR image enhancement of uterine tissue as it is for brain (Karlik et al. 1990).

5.2 METHODS

5.2.1 Animals

Rats were ovariectomized, sensitized for decidualization and given deciduogenic stimulation as described in sections 3.2, 3.3 and illustrated in *FIGURE 3.1*.

5.2.2 Magnetic Resonance (MR) Imaging

At 10 hours after unilateral deciduogenic stimulation (by intraluminal injection of sesame oil), animals were anesthetized with ether and 0.3 ml of a 1:1 mixture of Gd-DTPA (Magnevist) and 0.5% Evans blue dye was injected i.v. via a tail vein. This time after stimulation was chosen because it is a time when endometrial VP and ECFV have been shown to be significantly increased (Chapter 4, *FIGURE 4.2* and *FIGURE 4.4*). Evans blue dye was included in the mixture to confirm that the bluein$_{\beta}$ reaction could be demonstrated. At various times from 0 (prior to injection of the mixture) to 320 minutes after i.v. injection of the Gd-DTPA and Evans blue dye, animals were killed by decapitation. Uteri were removed, trimmed free of adherent tissue and individual uterine horns were separated. Four uterine horns (stimulated and nonstimulated horns from 2 animals) were then placed, longitudinally, in a 4-well carrier and scanned in a Bruker 1.9T-MSL 30 system using a 3.5 cm loop-gap resonator. T_1 scans were coronal, non-contiguous 5 mm slices at a separation of 1 mm with TR 600 msec and TE 30 msec. Mean image intensities were calculated for each uterine horn using computer-assisted image analysis (ImagePro II; Cold Springs, MA).

5.2.3 Uterine Volume of Distribution of i.v.-Injected ^{153}Gd-DTPA

At 10 hours after unilateral deciduogenic stimulation, animals were anesthetized and 25 μCi of ^{153}Gd-DTPA (SA = 2.61×10^{-4} μCi/mmole; prepared by a Radiopharmacist at University Hospital, London) was injected i.v. via a tail vein. Animals were killed by decaptitation 5, 20, 80 or 320 minutes later, uteri removed and a sample of trunk blood was collected. Stimulated and nonstimulated uterine horns were weighed and radioactivity in individual uterine horns as well as a 100 μl sample of serum was measured using a Nuclear Chicago gamma counter. Uterine volumes of distribution of ^{153}Gd-DTPA (μl/mg) were calculated by dividing the concentration of radioactivity in uterine horns (cpm/mg) by the concentration of radioactivity in serum (cpm/μl).

5.2.4 Magnetic Resonance (MR) Imaging after Deciduogenic Stimulation

At 3, 9 or 27 hours after unilateral deciduogenic stimulation, animals were injected with 0.8 ml of the 1:1 mixture of Gd-DTPA and Evans blue. Animals were killed 15 minutes later and uteri were imaged as described above. Mean image intensities of stimulated and control horns were calculated by computer-assisted image analysis. As a control, uteri from several animals were imaged 27 hours after deciduogenic stimulation, 15 minutes after injection of Evans blue dye only. This was done to determine possible image enhancement due directly to events associated with decidualization. The time of 15 minutes after Gd-DTPA and Evans blue injection was chosen on the basis of results or section 5.2.2.

5.3 RESULTS

5.3.1 Magnetic Resonance (MR) Imaging after i.v. Injection of Gd-DTPA

Intravenous injection of the Gd-DTPA and Evans blue dye mixture resulted in macroscopic blueing which was confined to stimulated horns. The blueing was apparent by 5 minutes and increased in intensity until 320 minutes after injection of the mixture. An example of this macroscopic blueing is depicted in *PLATE 5.1a* where a uterus is shown 80 minutes after injection of the Gd-DTPA and Evans blue. MR imaging of the uteri revealed patterns of enhancement which were similar to those of macroscopic blueing. However; image intensification of stimulated horns was short-lived compared to macroscopic blueing. An example of the image intensification of stimulated horns is shown in *PLATE 5.1b* where a uterus has been imaged 20 minutes after i.v. injection of Gd-DTPA and Evans blue. Subsequent image analysis indicated that mean image intensities of stimulated horns were significantly greater than those of nonstimulated horns by 5 minutes after injection of the mixture ($p<0.05$; *FIGURE 5.1*). Although peak image intensity of stimulated horns appeared to occur at 20 minutes after tracer injection, the values for 5, 20 and 80 minutes were not statistically different ($p>0.05$). Mean intensities of stimulated horns returned to values not significantly different ($p>0.05$) from those of nonstimulated horns by 320 minutes after injection of the mixture. Mean intensity of nonstimulated horns did not change significantly ($p>0.05$) at any time after injection of Gd-DTPA and Evans blue dye (*FIGURE 5.1*).

(a)

(b)

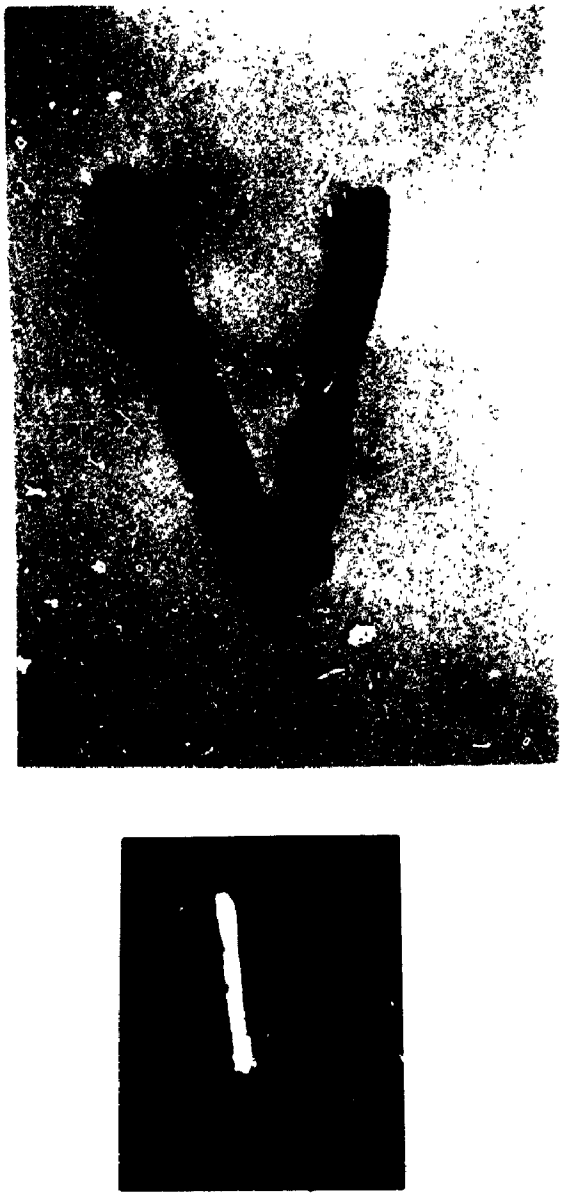


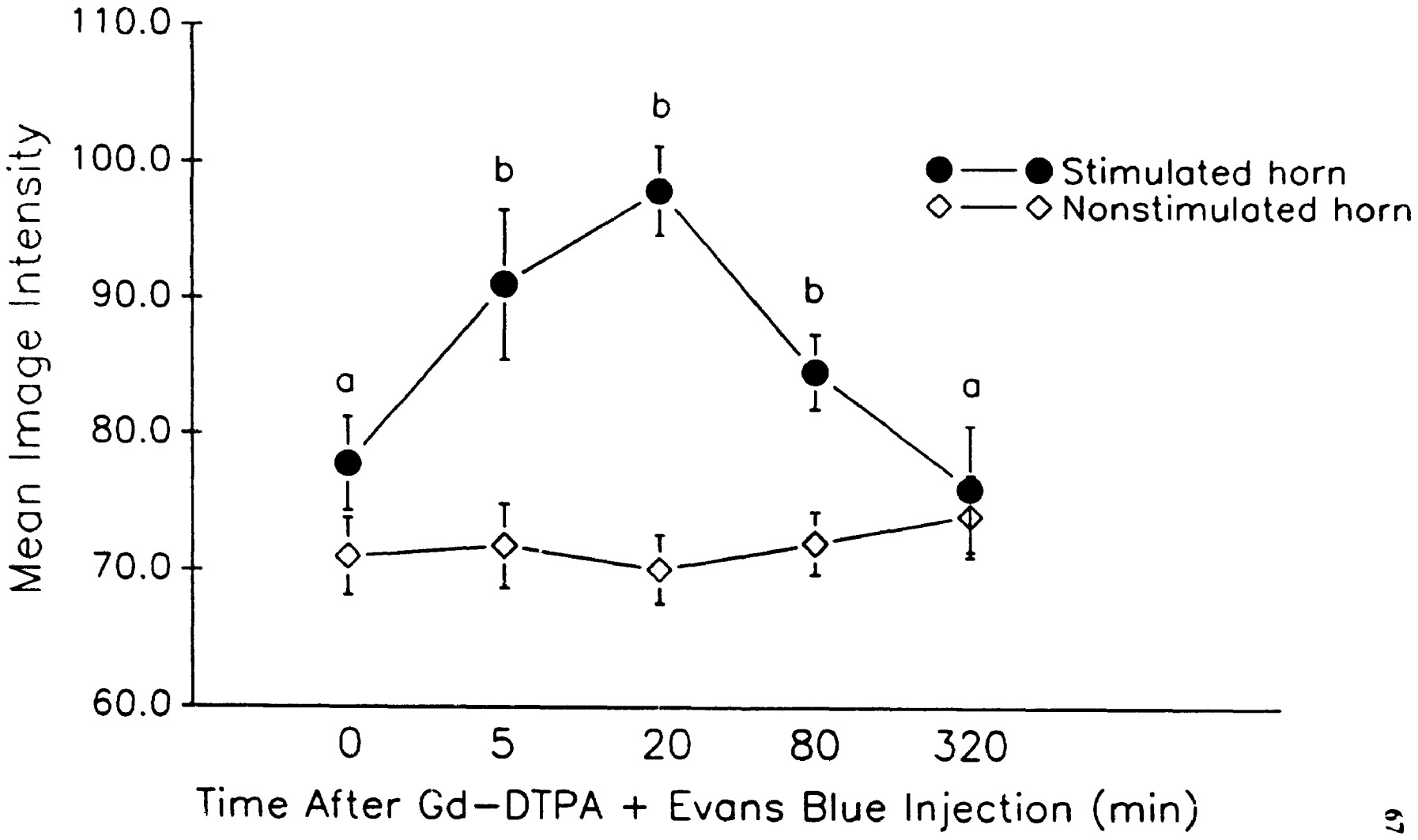
PLATE 5.1. Comparison of patterns of macroscopic blueing and Gd-DTPA MR image enhancement for uterus removed from rats after unilateral deciduogenic stimulation. a) Photograph of uterus removed from a rat 80 minutes after i.v. injection of Gd-DTPA and Evans blue dye, 10 hours after unilateral deciduogenic stimulation (**Magnification: approximately 1x**). b) MR image of a uterus removed from a similarly treated animal, 20 minutes after injection of the mixture (**Magnification: approximately 0.7x**).

FIGURE 5.1. Mean image intensities of stimulated and nonstimulated uterine horns removed 10 hours after unilateral deciduogenic stimulation at various times after i.v. injection of a 1:1 mixture of Gd-DTPA and Evans blue dye. Data are expressed in computer specific units of intensity per pixle and each point represents the mean $\pm$ SEM for 6 uterine horns. Asterisks indicate statistically significant increases in image enhancement among stimulated horns ($p < 0.05$).

Summary of Analysis of Variance			
Source	df	Mean Square	F ratio
Between Animals			
A = Time after Tracer Injection	4	290.7	2.44^{NS}
Error	35	118.93	
Within Animals			
B = Uterine Stimulation	1	4076	85.6^{***}
Interaction	4	364.3	7.66^{***}
Error	35	47.59	

^{NS} $p > 0.05$

*** $p < 0.001$


110.0
100.0
90.0
80.0
70.0
60.0
Mean Image Intensity
a
b
b
b
a
Stimulated horn
Nonstimulated horn
0
5
20
80
320
Time After Gd–DTPA + Evans Blue Injection (min)

5.3.2 Uterine Volume of Distribution of ^{153}Gd-DTPA after i.v. Injection

The concentration of radioactivity in serum was highest 5 minutes after injection of the ^{153}Gd-DTPA (the earliest time of sampling), falling to near background levels at 320 minutes. (*FIGURE 5.2*). Radioactivities of stimulated and nonstimulated horns varied in a manner which parallelled that of serum. At 5 and 20 minutes after tracer injection, concentrations of radioactivity were significantly greater in stimulated than nonstimulated horns ($p < 0.05$; *FIGURE 5.2*). When the concentrations of radioactivity in the uterine horns (cpm/mg) were divided by those of serum (cpm/μl) for each animal, the volumes of distribution of ^{153}Gd-DTPA were found to be 0.65 ± 0.04 μl/mg for stimulated and 0.53 ± 0.01 μl/mg for nonstimulated horns 5 minutes after injection of the radioactive tracer. Since serum radioactivity fell at a greater rate than uterine horn radioactivity; these values increased until 320 minutes after tracer injection.

5.3.3 MR Imaging after Unilateral Deciduogenic Stimulation

When uteri were removed 15 minutes after Gd-DTPA and Evans blue injection, macroscopic blueing, confined to stimulated horns, was apparent at 9 and 27 hours but not at 3 hours after deciduogenic stimulation with sesame oil. Image analysis produced mean image intensities which were significantly greater for stimulated than nonstimulated horns at both 9 and 27 hours after injection of the tracers ($p < 0.05$; *FIGURE 5.3*). Mean intensities of nonstimulated horns did not change significantly at any time after deciduogenic stimulation. Image analysis of uteri 27 hours after deciduogenic stimulation and 15 minutes after i.v. injection of Evans blue only yielded mean image intensities

FIGURE 5.2. Concentrations of radioactivity in serum, stimulated and nonstimulated uterine horns taken from animals at various times after i.v. injection of ^{153}Gd-DTPA. Each point represents the mean $\pm$ SEM for 5 animals. Asterisks indicate statistically significant differences between stimulated and nonstimulated horns after data were logarithmically transformed ($p < 0.05$).

Summary of Analysis of Variance			
Source	df	Mean Square	F ratio
Between Animals			
A = Time after Tracer Injection	3	47.6	1187***
Error	16	0.00401	
Within Animals			
B = Uterine Stimulation	1	0.163	8.38*
Interaction	3	0.078	4.01*
Error	16	0.00194	

* $p < 0.05$

*** $p < 0.001$

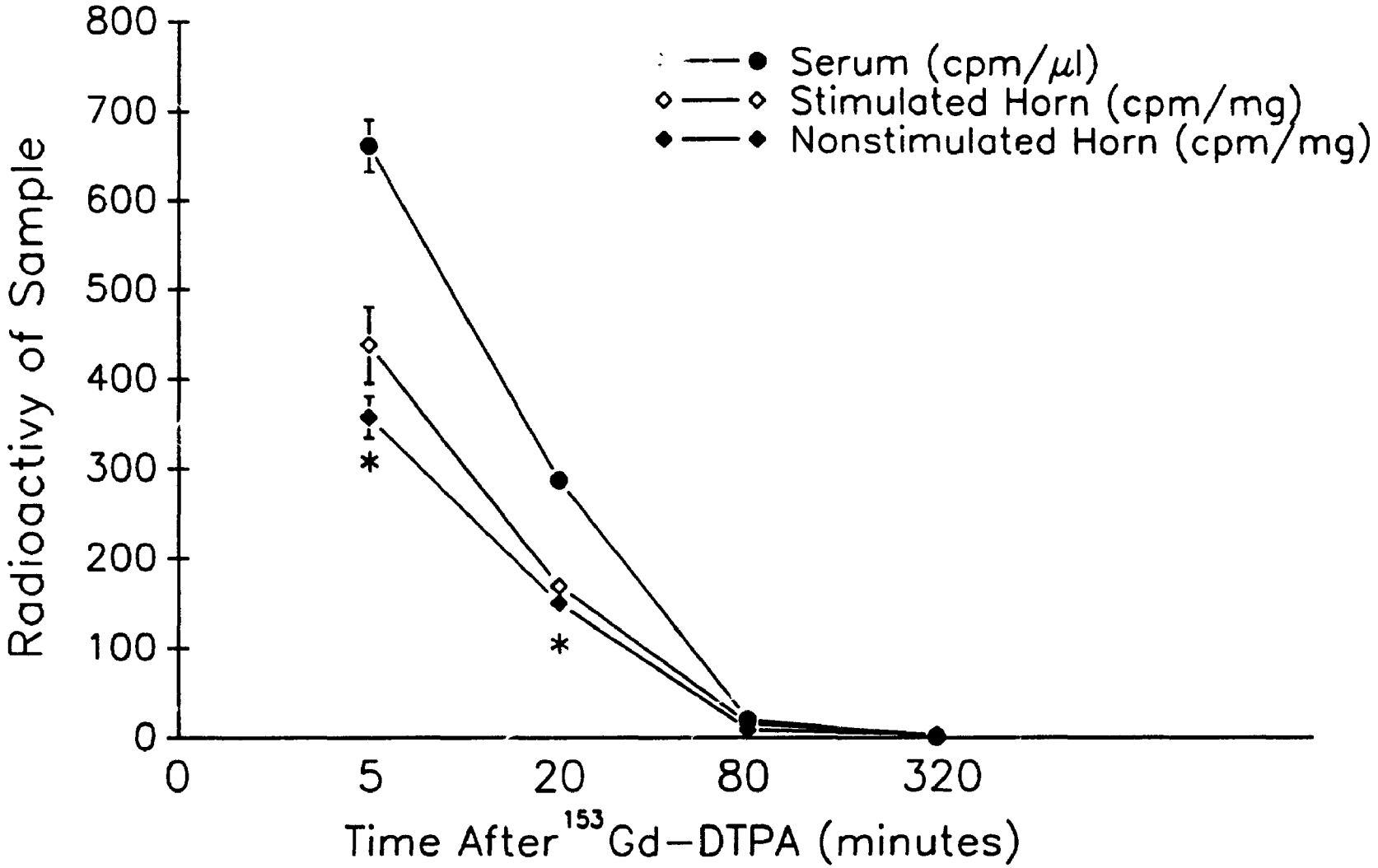
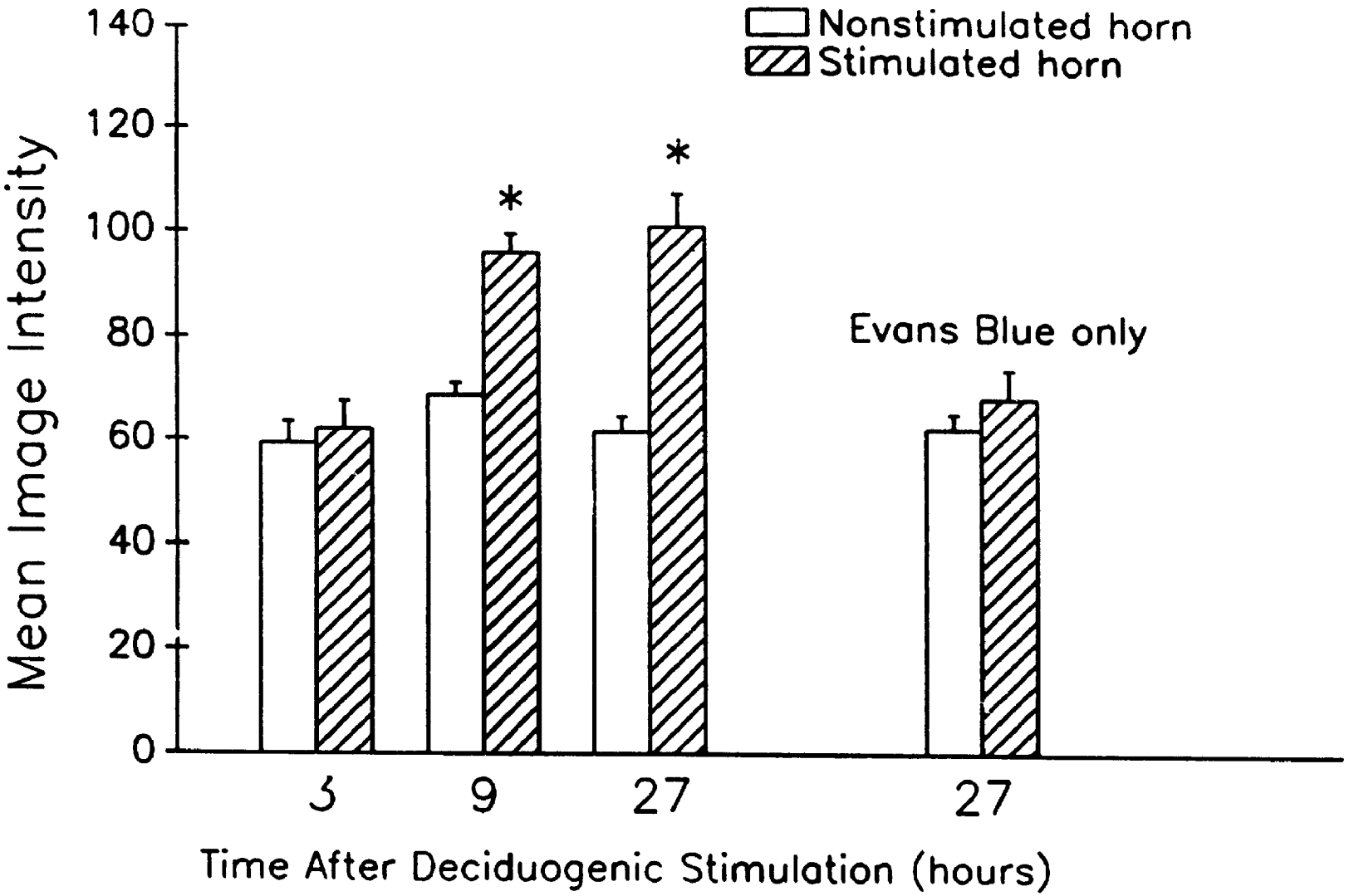

800
700
600
500
400
300
200
100
0
Radioactivy of Sample
Serum (cpm/μl)
Stimulated Horn (cpm/mg)
Nonstimulated Horn (cpm/mg)
*
*
0
5
20
80
320
Time After 153Gd–DTPA (minutes)

FIGURE 5.3. Mean image intensities of stimulated and nonstimulated uterine horns removed 15 minutes after i.v. injection of Gd-DTPA and Evans blue at various times after unilateral deciduogenic stimulation. Data are expressed in computer specific units of intensity per pixle and each bar represents the mean $\pm$ SEM for 7-9 uterine horns. Asterisks indicate statistically significant increases in image intensity among stimulated horns ($p < 0.05$).

Summary of Analysis of Variance			
Source	df	Mean Square	F ratio
Between Animals			
A = Time after Stimulation	2	1892	9.00*
Error	15	210.3	
Within Animals			
B = Uterine Stimulation	1	4692	50.2***
Interaction	2	916	9.80*
Error	15	93.5	

* $p < 0.05$

*** $p < 0.001$


Nonstimulated horn
Stimulated horn
Mean Image Intensity
140
120
100
80
60
40
20
0
*
*
Evans Blue only
3
9
27
27
Time After Deciduogenic Stimulation (hours)

which were not significanly different for stimulated and control horns ($p > 0.05$).

5.4 DISCUSSION

The results of this study clearly demonstrate that Gd-DTPA enhanced MR imaging provides a useful tool for demonstrating uterine vascular changes which precede decidualization. Patterns of image enhancement in the predecidual endometrial tissue after i.v. injection of Gd-DTPA exactly match those of macroscopic blueing which follows i.v. injection of Evans blue dye. Although increased uterine ECFV may contribute to the blueing reaction (McRae and Heap, 1988; Milligan and Edwards, 1990), the predominant determining factor is reputed to be increased VP of the endometrial vessels allowing extravasation of protein-dye complexes which are otherwise confined to the vascular system of non-implantation and non-decidualizing endometrial tissue (Finn, 1966; Lundkvist and Ljungkvist, 1977; Weitlauf, 1988).

The results of image analysis following i.v. Gd-DTPA injection (*FIGURE 5.1*) suggested that increased VP produced the image enhancement of stimulated uterine horns. Mean image intensity did not change for nonstimulated horns throughout the 320 minutes following tracer injection suggesting that Gd-DTPA did not pass from blood to the uterine interstitium in appreciable amounts. In contrast, mean intensities of stimulated horns increased soon after tracer injection when serum concentrations of Gd-DTPA were high and decreased when the agent was eliminated by the kidneys (Weinmann et al. 1984). This bidirectional movement of Gd-DTPA is identical to that which is observed

in brain when the blood-brain-barrier is compromised by pathological processes induced experimentally or by disease (Karlik et al. 1990). However, the notion that increased endometrial VP produced the image enhancement of predecidual tissue was rejected when ^{153}Gd-DTPA was shown to permeate the extracellular fluid of both stimulated and control uterine horns. The ^{153}Gd-DTPA volumes of distribution in stimulated and nonstimulated horns 5 minutes after i.v. injection of the tracer are similar to previously reported estimates of ECFV assessed by the volumes of distribution of ^{51}Cr-EDTA for stimulated and nonstimulated uterine horns (Chapter 4, *FIGURE 4.2*) as well as for implantation and non-implantation sites, respectively (McRae and Heap, 1988). Therefore, in contrast to the macroscopic blueing which follows i.v. injection of Evans blue dye, increased uterine ECFV appears largely responsible for the image enhancement of predecidual uterine tissue after injection of Gd-DTPA during the present study. Although the contrast agent is sufficiently small to escape the endometrial blood vessels and permeate the ECF of both stimulated and nonstimulated horns (*FIGURE 5.2*), significant image enhancement was only demonstrated for stimulated horns. However, the tissue remodelling which precedes decidualization (Lundkvist and Ljungkvist, 1977; Lundvist et al. 1977) could also contribute to the enhancement of stimulated horns since the constitution of the local macromolecular environment is an important component of Gd-DTPA enhancement (Goldstein et al. 1984) and since the endometrial tissue remodelling would certainly alter the local macromolecular environment.

The trends in Gd-DTPA MR image enhancement during decidualization (*FIGURE 5.3*) provide additional evidence that increased ECFV is a significant factor in the image

enhancement of decidualizing tissue. The further increase in mean image intensity between 9 and 27 hours after deciduogenic stimulation is consistent with the increase in ECFV which occurs during the same time (Chapter 4, *FIGURE 4.2*). In contrast estimates of endometrial VP (Chapter 4, *FIGURE 4.4*) indicate a fall in VP during this time. This suggests that increasing VP has little direct impact on the image intensification produced by Gd-DTPA injection. Therefore, while Gd-DTPA enhanced MR imaging appears to offer a useful method of quantitating changes in uterine ECFV, the approach must be modified in order to permit measurement of uterine VP.

5.5 SIGNIFICANCE

The results of this chapter show that decidualizing tissue in uteri from rats can be identified using Gd-DTPA enhanced MR imaging. Furthermore, this technology offers the potential to demonstrate the process of ovoimplantation in living animals. In fact, the results of preliminary experiments using Gd-DTPA enhanced MR imaging with pregnant rats indicate that this technology can provide identification of sites of recent embryo implantation in dissected uteri (Appendix 3) and in the uteri of intact rats (Appendix 4).

Limitations: During these studies, several attempts were made to use contrast-enhanced MR imaging for assessing uterine VP during artificially-induced decidualization. The experiments were conducted in an effort to design a method of assessing uterine VP

similar to that based on the rate of accumulation of ^{125}I-albumin. It was hypothesized that the use of intact, anesthetized rats would allow changes in image enhancement of uterine horns to be examined over time. Such a method should be superior to that based on the rate of accumulation of ^{125}I-albumin because the use of contrast-enhanced MR imaging would allow the changes in uterine image intensity over time to be examined in individual animals. In contrast, to determine the rate of change of uterine ^{125}I-albumin volumes of distribution, several animals must be killed at different times after tracer injection. Therefore, the use of several animals for individual VP determinations involves an element of variability which could be eliminated by the use contrast-enhanced MR imaging.

When intact rats were imaged after unilateral deciduogenic stimulation, it was possible to identify individual uterine horns on MR images of anesthetized rats by encasing them in rubber cuffs as described for pregnant animals in appendix 4. However, after i.v. injection of Gd-DTPA, maximum image enhancement of uterine horns was invariably achieved during the time required to obtain the first image (approximately 8 minutes). This result made it impossible to determine uterine VP by the rate of change of image intensity as has been accomplished for blood-brain-barrier permeability (Kenney et al. 1992). Apparently, uterine blood vessels of both decidualizing and non-decidualizing uterine tissue do not present an adequate barrier to the passage of Gd-DTPA.

The second attempt to assess uterine VP by contrast-enhanced MR imaging involved the use of Gd-DTPA-albumin. Recently, there has been an interest in

developing "blood-pool" contrast agents. Many of these agents involve the chelation of Gd-DTPA to large molecules such as albumin (Lauffer and Brady, 1985; Spanoghe et al. 1992), IgG (Lauffer and Brady, 1985) and polylysine polymers of different sizes (Spanoghe et al. 1992). The ability to synthesize Gd-DTPA-albumin should provide a contrast agent which would accumulate in uterine tissue after i.v. injection at a rate similar to that of ^{125}I-albumin. Therefore, it was hoped that rate of change of uterine image enhancement would be sufficiently slow to produce incremental changes in image intensity over the period of time necessary to acquire several consecutive images. Despite the successful production of Gd-DTPA-albumin chelates by 2 different methods (Lauffer and Brady, 1985; Spanoghe et al. 1992), the use of these agents produced no apparent enhancement of either uterine horn. Similarly, no enhancement of implantation sites could be demonstrated when pregnant animals were prepared as described in Appendix 4 and injected with Gd-DTPA-albumin. It appears that the relaxivity of Gd-DTPA-albumin (Appendix 5) is too low to enhance T_1-weighted images of uterine tissue. Thus, the failure of Gd-DTPA-albumin to represent a viable tracer for estimating uterine VP can be attributed to its poor specific activity. Administration of 100 μg of albumin labeled with Gd-DTPA fails to produce MR image enhancement of either stimulated or nonstimulated uterine horns at any time after injection of the contrast agent. In contrast, administration of 0.04 μg of albumin labeled with ^{125}I (as described in section 3.7) can produce in excess of twice the concentration of radioactivity in stimulated versus nonstimulated horns within 15 minutes of injection.

In summary, although the use of Gd-DTPA enhanced MR imaging has provided

a useful method for identifying sites of embryo implantation and uterine vascular changes which precede decidual metamorphosis, currently, this approach is limited in its ability to provide accurate quantitation of these vascular changes. However, the ongoing development of new contrast agents with higher specific activities and more selective tissue distributions suggests that contrast-enhanced MR imaging strategies may provide a viable approach to assessing uterine VP in the near future.

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CHAPTER 6: UTERINE VASCULAR CHANGES AFTER UNILATERAL ARTIFICIAL UTERINE STIMULATION OF RATS DIFFERENTIALLY SENISITIZED FOR THE DECIDUAL CELL REACTION

6.1 INTRODUCTION

Successful embryo implantation requires that the endometrium be sensitized by a complex series of changes to the endocrine milieu (Weitlauf, 1988). Although a blastocyst can wait several days for the uterus to become sensitized as demonstrated by successful implantation following transfer of blastocysts to the uterus up to 2 days early (McLaren and Michie, 1956), implantation rates are significantly reduced when embryo development trails uterine sensitization by even 1 day of pregnancy (Dickmann and Noyes, 1960). In fact, optimal endometrial sensitization has very specific hormonal requirements and lasts for only a limited time of several hours (Finn, 1977). Deviation from optimal sensitization interferes not only with blastocyst implantation (Dickmann and Noyes, 1960), but also with artificially-induced decidualization (Yochim and De Feo, 1963; Kennedy, 1980a; b) and the accumulation of i.v. injected labeled tracer proteins in predecidual endometrial tissue (Kennedy, 1980a; b; Milligan and Mirembe, 1985). Although numerous factors probably contribute to the transient nature of optimal endometrial sensitivity, the dosage of estrogen and the period of exposure to estrogen are crucial aspects of endometrial sensitization (Yochim and De Feo, 1963; Kennedy, 1980b).

Increased uterine VP (Psychoyos, 1961; Lundkvist and Ljungkvist, 1977; Parr and Parr, 1986; McRae and Heap, 1988), ECFV (Lundkvist and Ljungkvist, 1977; McRae and Heap, 1988) and BF (McRae and Heap, 1988) have all been implicated in the intrauterine accumulation of labeled proteins which precedes decidualization. In addition, results presented in chapter 4 of this thesis indicate that uterine VP and ECFV are significantly increased in stimulated uterine horns following application of a deciduogenic stimulus and that although uterine BF is not significantly increased, both uterine horns exhibit high tissue BFs. Since each of these vascular variables may influence the predecidual accumulations of labeled proteins, optimal sensitization may ensure maximal protein accumulations after uterine stimulation by altering uterine VP, ECFV and/or BF. Therefore, the experiments presented in this chapter address uterine VP, ECFV and BF in stimulated and nonstimulated uterine horns after unilateral instillation of sesame oil to rats given optimal sensitization for decidualization, or to rats given one of various forms of sub-optimal sensitization known to reduce the extent of uterine protein accumulation in stimulated horns.

6.2 METHODS

6.2.1 Animals

Rats were ovariectomized, sensitized for decidualization and given unilateral uterine stimulation as described in sections 3.2 and 3.3.

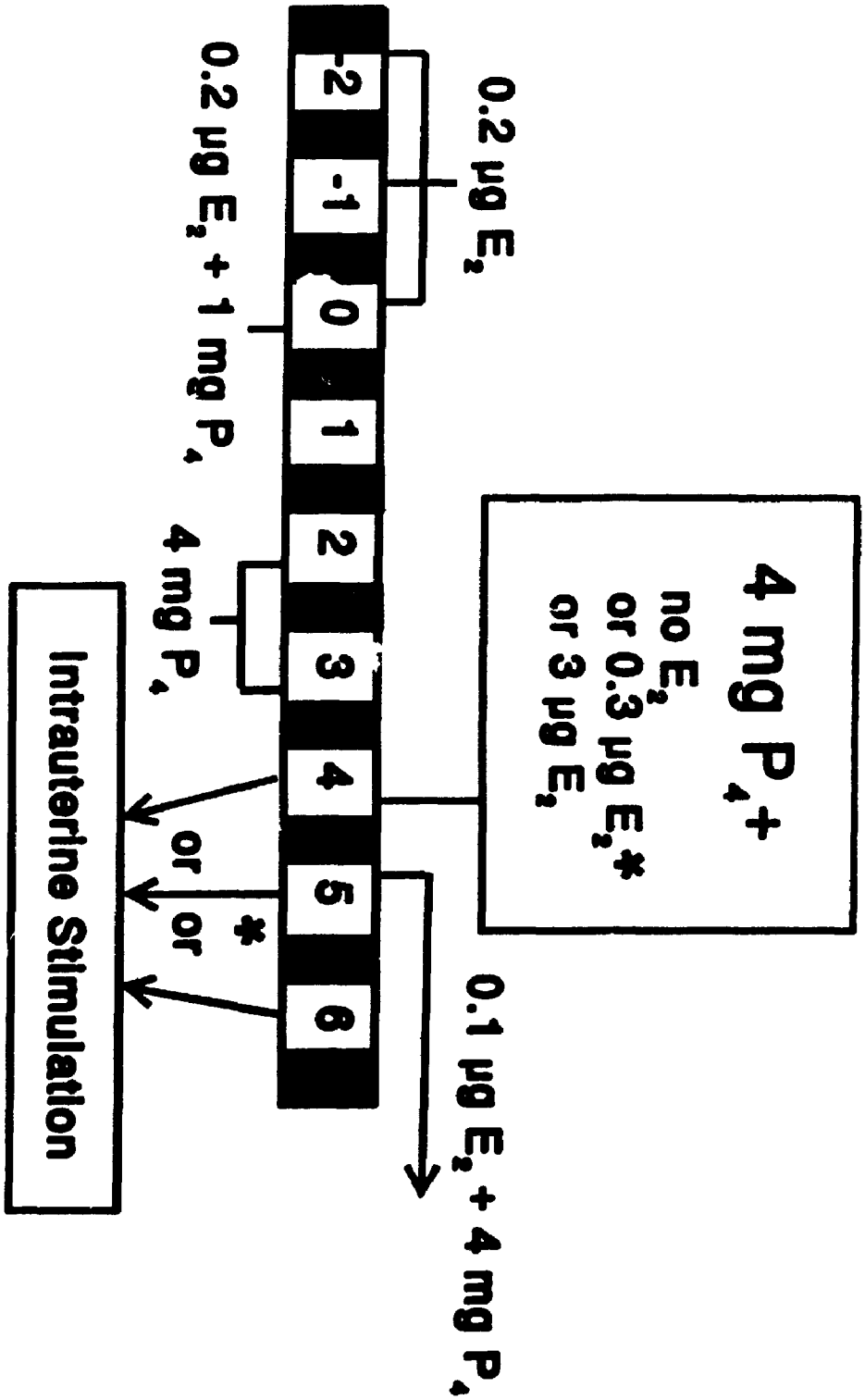
6.2.2 Differential Sensitization for Decidualization

In order to assess the effect of differential temporal sensitization on uterine ECFV, VP and BF, these variables were estimated when the uterine intraluminal injection of sesame oil was given at noon $\pm$ 1.5 hours on the equivalent of Day 4 (one day prior to optimal temporal sensitization), Day 5 (optimal temporal sensitization) or Day 6 (one day after optimal temporal sensitization). The effect of differential estrogen sensitization was assessed by estimating the vascular variables after giving the uterine intraluminal injection of sesame oil at noon $\pm$ 1.5 hours on equivalent to Day 5 of pseudopregnancy to rats receiving 4 mg of progesterone during the previous evening with either 0.3 μg estradiol (optimal estrogen treatment), 0 μg estradiol (insufficient estrogen treatment) or 3.0 μg estradiol (excessive estrogen treatment). Similar deviations from optimal sensitization have been shown to reduce the extent of intrauterine protein accumulation following deciduogenic stimulation (Kennedy, 1980a; Kennedy, 1980b). The protocols for differential temporal and hormonal sensitization are displayed in *FIGURE 6.1*.

6.2.3 Estimation of Uterine Extracellular Fluid Volume

Uterine ECFV was estimated in stimulated and nonstimulated uterine horns as described in section 3.4. In all treatment groups, ECFV was estimated at 20 hours after deciduogenic stimulation. This time was chosen because deciduogenic stimulation produced the greatest uterine ECFV at around this time during the experiments presented in chapter 4 (*FIGURE 4.1*).

FIGURE 6.1. Schematic representation of treatment protocols for obtaining rats which are optimally and differentially sensitized for the decidual cell reaction. Differential temporal sensitization was produced by giving the deciduogenic stimulus at around noon on equivalent to Day 4, 5* or 6 of pseudopregnancy. Differential sensitization with estrogen was produced by giving 3.0, 0.3* or 0 μg of estradiol during the evening of equivalent to Day 4 of pseudopregnancy. Black areas represent periods of darkness and numbers within white areas indicate equivalent day of pseudopregnancy. Asterisks indicate treatments for optimal sensitization.


-2
-1
0
1
2
3
4
5
6
0.2 µg E2
0.2 µg E2 + 1 mg P4
4 mg P4
4 mg P4 +
no E2
or 0.3 µg E2*
or 3 µg E2
0.1 µg E2 + 4 mg P4
Intrauterine Stimulation
or
or
*

6.2.4 Estimation of Uterine Vascular Permeability (VP)

At 10 hours after unilateral uterine stimulation, uterine VP was estimated in stimulated and nonstimulated uterine horns as described in section 3.6.

6.2.5 Estimation of Uterine Blood Flow (BF)

At 9 hours after unilateral uterine stimulation, uterine BF was estimated in stimulated and nonstimulated uterine horns as described in section 3.7.

6.3 RESULTS

6.3.1 Uterine Extracellular Fluid Volume (ECFV)

As shown in *FIGURE 6.2*, deciduogenic stimulation significantly increased uterine ECFV only after optimal temporal sensitization and optimal sensitization with estrogen (0.72 ± 0.03 μl/mg in stimulated horns; $p<0.05$). ECFV was significantly lower in both stimulated ($0.56\pm.01$ μl/mg) and nonstimulated (0.54 ± 0.01 μl/mg) horns when stimulatation was given on equivalent to Day 6 pseudopregnancy ($p<0.05$). No other statistically significant effects were observed (*FIGURE 6.2*).

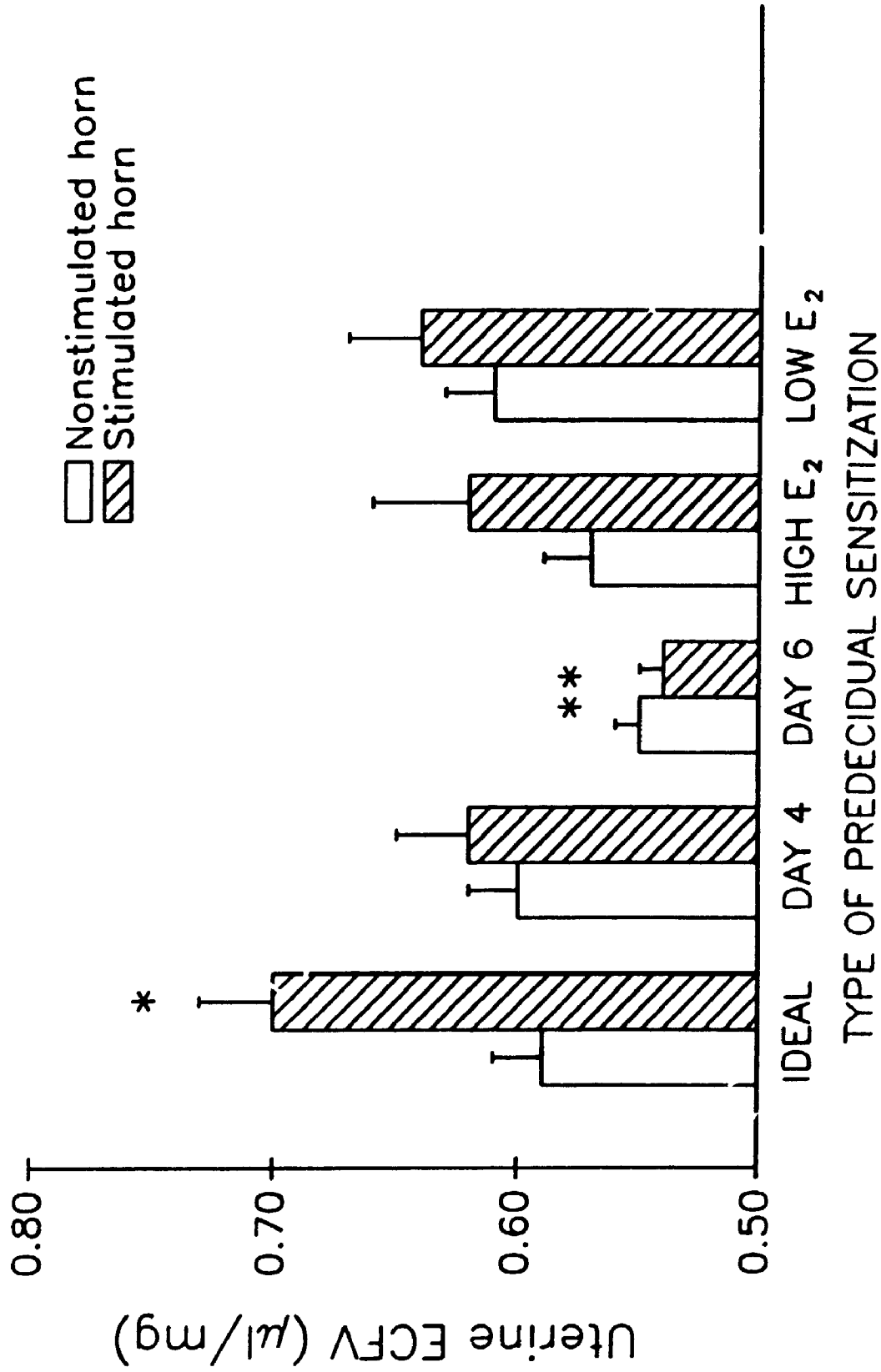
6.3.2 Uterine Vascular Permeability (VP)

For nonstimulated horns, analysis (by ANOVA) of the plots log[1-(albumin volume/ECFV)] versus time after tracer injection revealed significant interaction between

FIGURE 6.2. Uterine ECFV of nonstimulated and stimulated horns 20 hours after unilateral deciduogenic stimulation to rats given differential temporal or hormonal sensitization. Differential temporal sensitization involved uterine stimulation on equivalent to Day 4, 5 (ideal) or 6 of pseudopregnancy. Differential estrogen sensitization involved uterine stimulation on equivalent to Day 5 of pseudopregnancy after treatment with no estrogen (low estrogen), 0.3 μg estrogen (Ideal) or 3.0 μg estrogen (high estrogen) during the evening prior to stimulation. Bars represent mean $\pm$ SEM for 7 animals. * indicates a significant effect of deciduogenic stimulation ($p < 0.05$). ** indicates a significant reduction in ECFV of both uterine horns ($p < 0.05$).

Summary of Analysis of Variance			
Source	df	Mean Square	F Ratio
Between Animals			
A = Type of Sensitization	4	2.58	3.78*
Error	30	0.00683	
Within Animals			
B = Uterine Stimulation	1	0.0030	8.69*
Interaction	4	0.0034	2.75*
Error	30	0.00341	

* $P < 0.05$


Nonstimulated horn
Stimulated horn
Uterine ECFV (μl/mg)
0.80
0.70
0.60
0.50
*
**
IDEAL
DAY 4
DAY 6 HIGH E_2
LOW E_2
TYPE OF PREDECIDUAL SENSITIZATION

these variables and type of predecidual sensitization ($p<0.05$). The finding indicates that the plots for nonstimulated horns (*FIGURE 6.3 a*) are not all parallel and implies that uterine VP is not the same among all nonstimulated horns. Pairwise comparisons indicated that uterine VP was significantly reduced in nonstimulated horns when unilateral deciduogenic stimulation was given on equivalent to Day 6 of pseudopregnancy (*FIGURE 6.3*). Similar analysis of the slopes of the lines log[1-(albumin volume/ECFV)] indicated that uterine VP of stimulated horns was significantly reduced when stimulation was given on Day 4 ($p<0.05$) or Day 6 ($p<0.001$; *FIGURE 6.3*) and when insufficient sensitization with estrogen was given ($p<0.001$; *FIGURE 6.4*). Sensitization with high doses of estrogen was not associated with reduced uterine VP of stimulated horns when compared to groups receiving optimal estrogen sensitization ($p>0.05$; *FIGURE 6.4*).

6.3.3 Uterine Blood Flow

Total uterine BF was similar for stimulated and nonstimulated horns for all treatment groups ($p>0.05$; data not shown). With the exception of animals receiving sensitization with high doses of estrogen, uterine weights were also similar for stimulated and nonstimulated horns. Uterine tissue BF changed significantly only for the group receiving excessive estrogen treatment (1.58 ± 0.40 μl/mg/min for stimulated horns, 2.03 ± 0.36 μl/mg/min for nonstimulated horns; $p<0.05$) where both horns had slight weight increases which were not accompanied by similar increases in total uterine BF (*FIGURE 6.5*). In all other treatment groups, uterine tissue BF was similar for both

FIGURE 6.3. Plots of log[1-(albumin volume/ECFV)] versus time for assessing uterine VP of nonstimulated (panel *a*) and stimulated (panel *b*) uterine horns 10 hours after unilateral deciduogenic stimulation to rats given differential temporal sensitization. Each point represents the mean $\pm$ SEM for 4 uterine horns. Asterisks indicate significant significant differences in VP as determined by ANOVA (*** = $p<0.001$; * = $p<0.05$).

Summary of Analysis of Variance			
Source	df	Mean Square	F Ratio
Between Animals			
A = Type of Sensitization	4	0.0017	5.26*
B = Time After Tracer Injection	3	0.0284	89.67***
A x B	12	0.0007	2.10*
Error	60	0.00032	
Within Animals			
C = Uterine Stimulation	1	0.0089	67.49***
A x C	4	0.0012	9.35***
B x C	3	0.0010	7.61***
A x B x C	12	0.0004	3.24*
Error	60	0.00013	

* $p<0.05$

* $p<0.001$

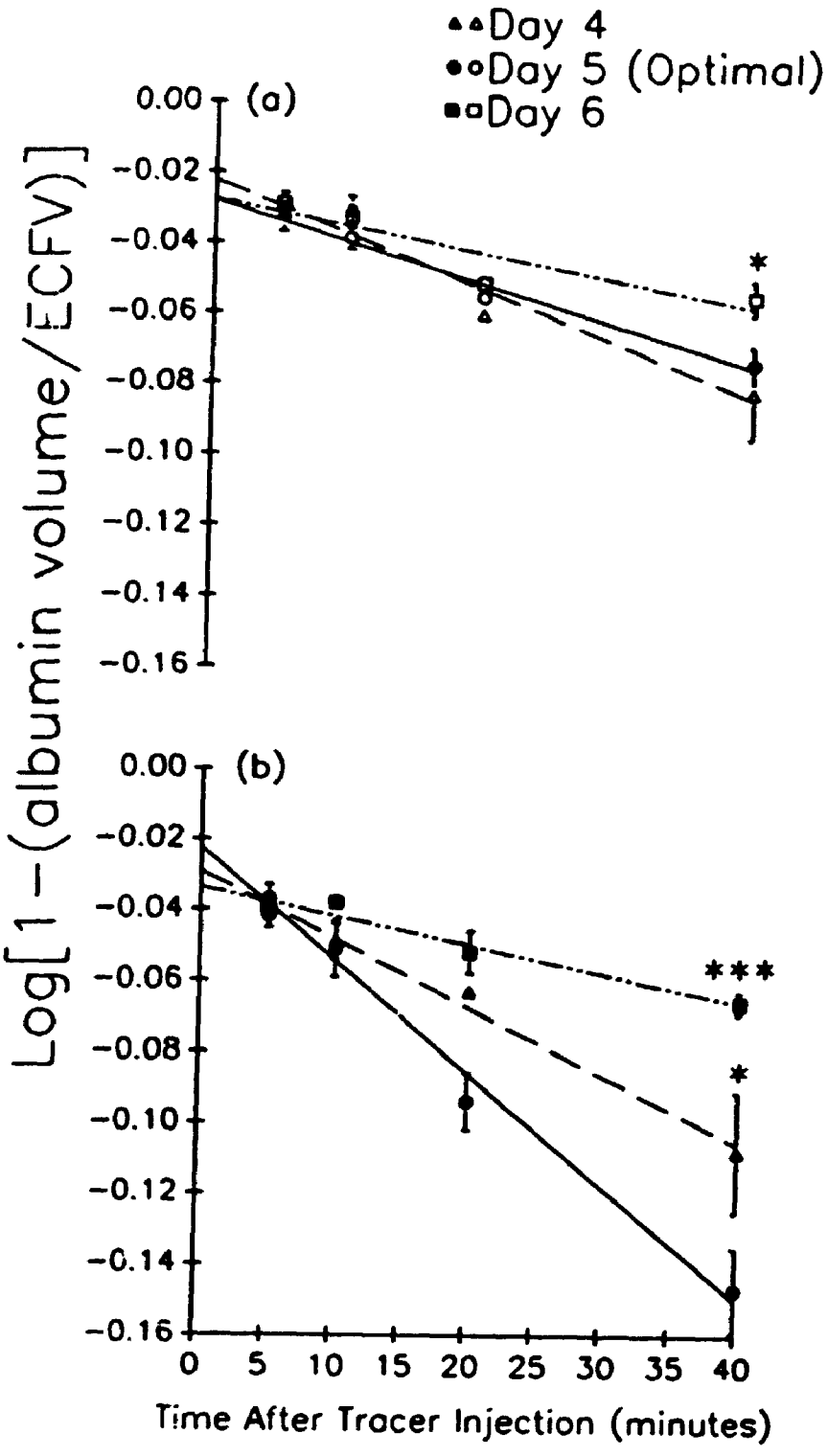

Day 4
Day 5 (Optimal)
Day 6
(a)
(b)
0.00
-0.02
-0.04
-0.06
-0.08
-0.10
-0.12
-0.14
-0.16
*
***
0
5
10
15
20
25
30
35
40
Log[1-(albumin volume/ECFV)]
Time After Tracer Injection (minutes)

FIGURE 6.4. Plots of log[1-(albumin volume/ECFV)] for assessing uterine VP of nonstimulated (panel *a*) and stimulated (panel *b*) horns 10 hours after unilateral uterine stimulation of rats on equivalent to Day 5 of pseudopregnancy following differential sensitization with estrogen. Each point represents the mean $\pm$ SEM for 4 uterine horns. Asterisks indicate significant differences as determined by ANOVA (*** = $p<0.001$). The summary of analysis of variance displayed in the legend for ***FIGURE 6.3*** comprises all forms of differential sensitization.

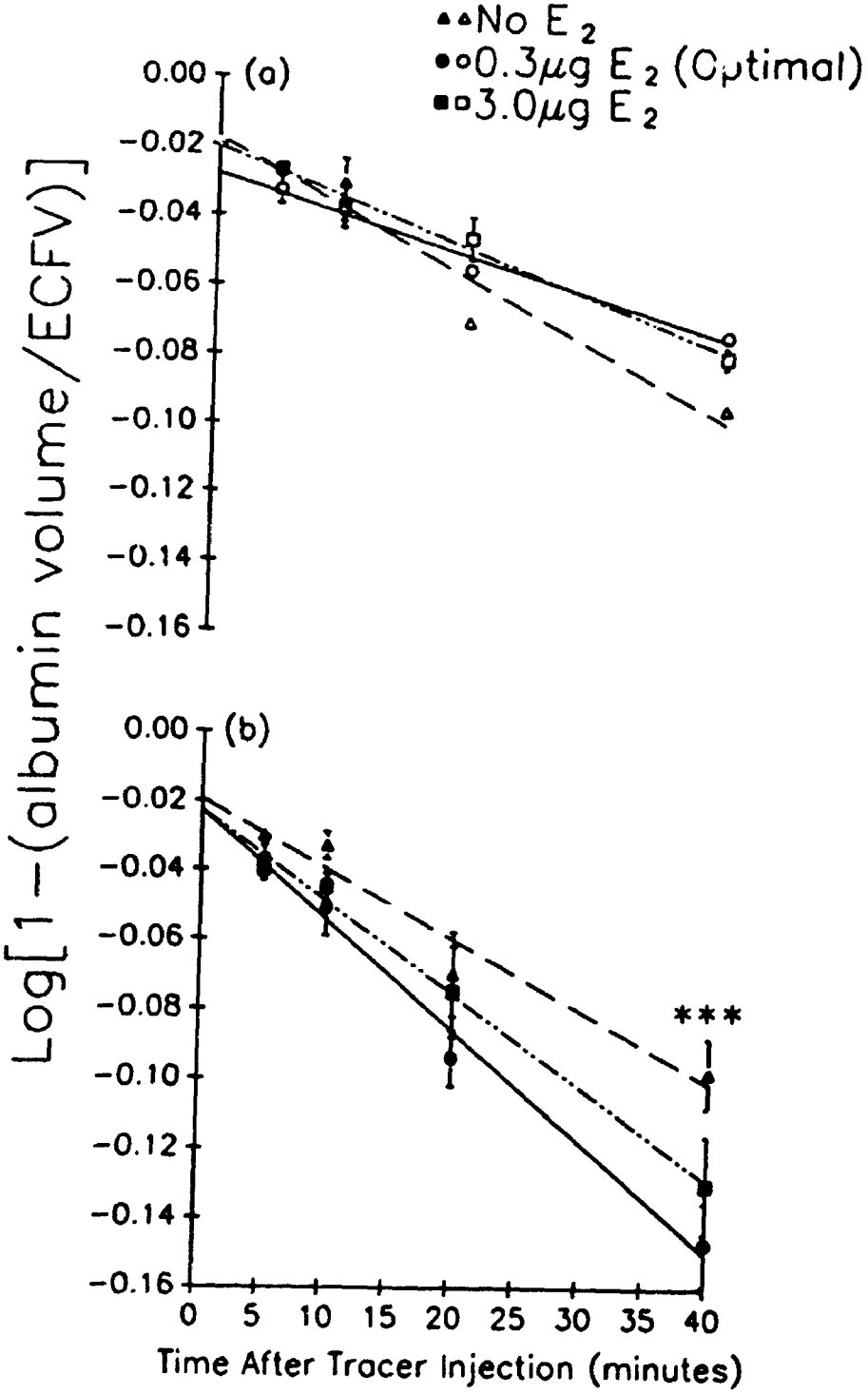
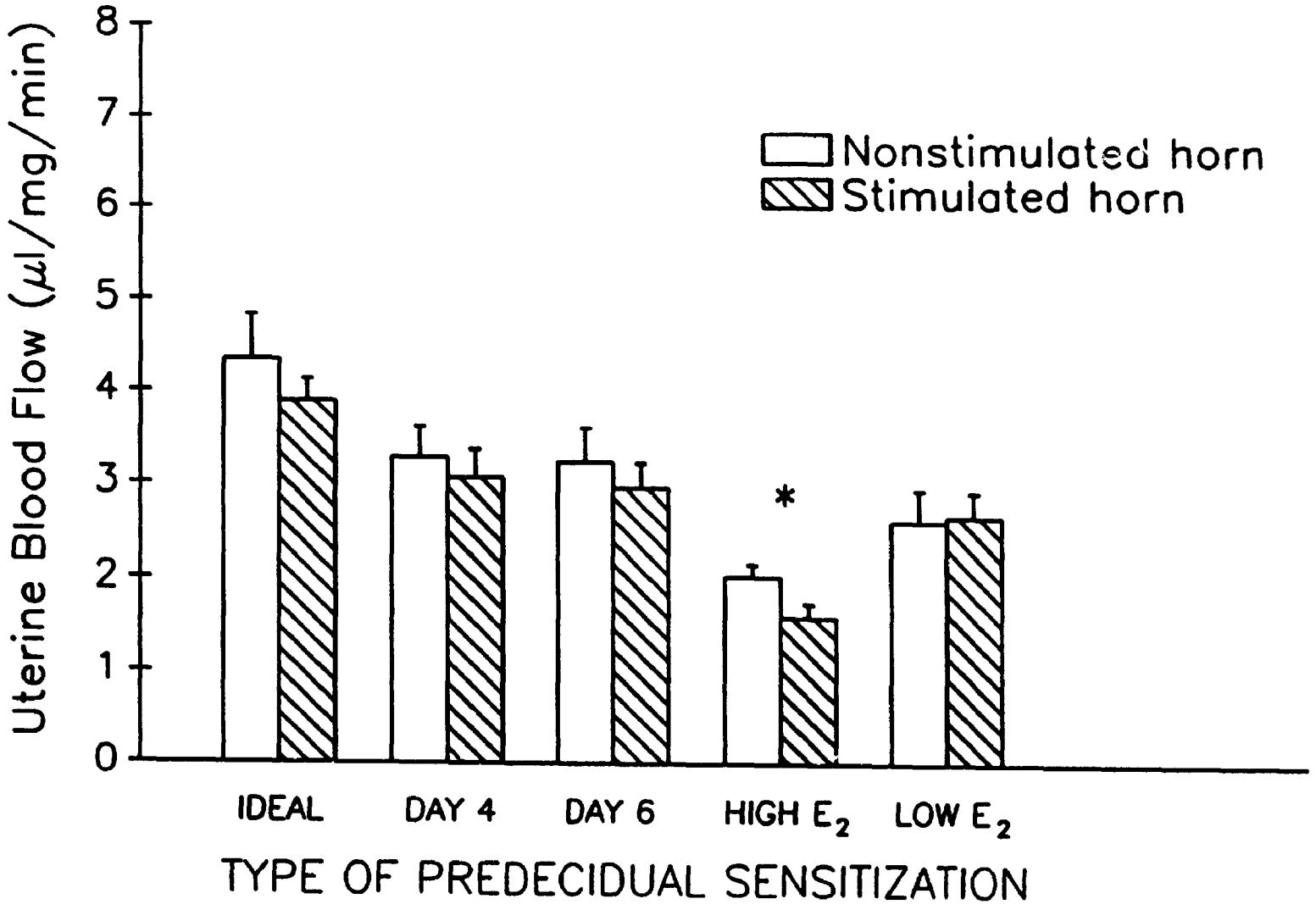

▲△No E 2
●○0.3μg E 2 (Optimal)
■□3.0μg E 2
(a)
(b)
0.00
-0.02
-0.04
-0.06
-0.08
-0.10
-0.12
-0.14
-0.16
***
0 5 10 15 20 25 30 35 40
Log[1-(albumin volume/ECFV)]
Time After Tracer Injection (minutes)

FIGURE 6.5. Uterine BF for nonstimulated and stimulated uterine horns 9 hours after unilateral deciduogenic stimulation to rats given differential temporal or hormonal sensitization. Bars represent mean $\pm$ SEM for 6 animals. * indicates a significant effect of differential sensitization.

Summary of Analysis of Variance			
Source	df	Mean Square	F Ratio
Between Animals			
A = Type of Sensitization	4	9.055	9.55^{*}
Error	30	0.948	
Within Animals			
B = Uterine horn Tissue BF	1	1.21	1.83^{NS}
Interaction	4	0.145	0.22^{NS}
Error	30	0.661	

* $p<0.05$

^{NS} = not significant


Uterine Blood Flow (μl/mg/min)
8
7
6
5
4
3
2
1
0
Nonstimulated horn
Stimulated horn
*
IDEAL
DAY 4
DAY 6
HIGH E_2
LOW E_2
TYPE OF PREDECIDUAL SENSITIZATION

stimulated and nonstimulated horns and did not differ significantly from tissue BFs associated with optimal sensitization (*FIGURE 6.5*).

6.4 DISCUSSION

The results presented in this chapter demonstrate that sub-optimal predecidual sensitization can significantly affect uterine VP, ECFV and tissue BF. In addition, the fact that optimal temporal sensitization and sensitization with estrogen produced the greatest increases in uterine VP and ECFV in stimulated uterine horns indicates that these uterine vascular responses have rigid requirements of endometrial sensitization, similar to those essential to embryo implantation and decidualization (Dickmann and Noyes, 1960; DeFeo, 1963 a, b; McLaren, 1971; Kennedy, 1980a; b).

With one exception, uterine stimulation did not significantly affect any of the uterine vascular variables after sub-optimal sensitization. Surprisingly, uterine VP was significantly higher in stimulated versus nonstimulated horns following sensitization with high doses of E_2. This result is inconsistent with that of a previous study which showed that accumulations of ^{125}I-albumin in stimulated horns were more detrimentally affected by sensitization with high doses of E_2 than by sensitization without E_2 (Kennedy, 1980b). The difference may be due to the use of immature rats or to the use of a slightly different protocol for differential sensitization (Kennedy, 1980b). However, the difference may also reflect the use of a more precise method of assessing uterine VP during the present study since the method also revealed a significant reduction in uterine VP of both

stimulated and nonstimulated uterine horns when stimulation was given on the equivalent of Day 6 of pseudopregnancy (*FIGURE 6.3*).

Although the high dose of E_2 used in the present study did not eliminate the significant increase in uterine VP in stimulated horns, it did eliminate the significant increase in uterine ECFV (*FIGURE 6.2*). This finding may be related to altered hydrostatic pressures in uterine plasma or interstitial tissue compartments suggested by the reduced uterine tissue BF (*FIGURE 6.5*). Alternatively, the differential responses of uterine VP and ECFV may provide some support for the notion that these variables are under independent control. One would predict that a significant increase in uterine VP would be followed by a significant increase in uterine ECFV since the higher permeability would allow proteins from blood to enter the uterine interstitium (Parr and Parr, 1986). The elevated interstitial protein concentration would then favour the movement of water into the interstitial space through both the increase in tissue osmotic pressure and bulk flow. In fact, this predicted relationship between uterine VP and ECFV was realized throughout the experiments described in chapter 4. Furthermore, all forms of differential sensitization (with the exception of sensitization with high doses of E_2 described above) produced similar uterine VP and ECFV responses. This includes the significant reduction in both uterine VP and ECFV for nonstimulated horns when uterine stimulation was not given until the equivalent of Day 6 of pseudopregnancy (*FIGURE 6.2* and *6.3*). Although uterine stimulation after sensitization with high doses of E_2 represents the only potential evidence that uterine ECFV and VP might be under independent control, such a theory is supported by reports that metalloproteinases and

plasminogen activators are secreted by cells of the uterus (Salamonsen et al. 1991) and by embryos (Strickland et al. 1976; Brenner et al. 1989; Lala and Graham, 1990). In addition, the possibility that these enzymes modify the uterine extracellular matrix is supported by histological studies involving embryo implantation (Lundkvist and Ljungkvist, 1977) and artificially-induced decidualization (Lundvist et al. 1977) which have described alterations to the uterine extracellular matrix consistent with pathological edema. If enzymatic alterations of the uterine extracellular matrix contribute to the enhanced accumulation of serum proteins in predecidual tissue, this process has even more rigid requirements of endometrial sensitization than the VP response since sensitization with high doses of E_2 abolished significant increases in uterine ECFV in stimulated horns (*FIGURE 6.2*).

Results presented in chapter 4 indicated that uterine BF does not increase significantly in uterine tissue destined to undergo decidualization (*FIGURE 4.4*) but that both uterine horns receive a high tissue BF during the 27 hours which follow unilateral deciduogenic stimulation. On this basis, it was speculated that endometrial sensitization might give rise to a high basal BF which would augment the effects of increased VP and ECFV following deciduogenic stimulation. Estimation of uterine BF after different forms of sub-optimal sensitization indicated only one instance where uterine tissue BF was significantly affected. When animals were sensitized with high doses of estrogen, uterine tissue BF for both stimulated and nonstimulated uterine horns was significantly reduced when uterine weights increased without similar increases in total uterine BF (*FIGURE 6.5*). With all other treatments known to significantly reduce the extent of accumulation

of i.v. injected tracer proteins in uterine tissue, uterine tissue BF was not significantly affected in either stimulated or nonstimulated horns (*FIGURE 6.5*). The fact that optimal sensitization is not essential for the high basal uterine BFs during early decidualization does not diminish the importance of BF in the decidual response since other work indicates that decidual responses are significantly reduced when uterine BF is reduced by previous exposure to serotonin (Mitchell et al. 1983) or increased by previous exposure to ethanol (Mitchell and Goldman, 1991; Mitchell and Van Kainen, 1992).

6.5 SIGNIFICANCE

Results presented in this chapter further implicate local increases in uterine VP and ECFV in the enhanced extravasation and accumulation of serum proteins in predecidual uterine tissue. Optimal temporal sensitization and sensitization with estrogen both were shown to be essential for maximal increases in uterine VP and ECFV which follow deciduogenic stimulation. The method by which uterine VP was assessed made it possible to identify a spectrum of VP responses associated with the different forms of sensitization. The fact that uterine VP but not ECFV was significantly increased in stimulated horns when uterine stimulation was given after sensitization with high doses of E_2 provides some support for the notion that these uterine vascular variables may be under independent control. Optimal sensitization does not appear to alter uterine BF in a manner which would implicate this variable in the enhanced accumulation of serum proteins in predecidual tissue.

CHAPTER 7: UTERINE VASCULAR CHANGES AFTER INFUSION OF INDOMETHACIN AND PROSTAGLANDIN E_2 INTO UTERI OF RATS SENSITIZED FOR DECIDUALIZATION

7.1 INTRODUCTION

Ovoimplantation and imminent decidualization may be macroscopically identified in the uterus by a localized increase in radioactivity or "blueing" after i.v. injection of radio-iodinated albumin (Psychoyos, 1961; Kennedy, 1979; Milligan and Mirembe, 1985) or Evans blue dye which binds to and travels with endogenous plasma proteins (Psychoyos, 1973). The enhanced intrauterine accumulation of these labeled proteins was originally attributed to increased permeability (VP) of the local endometrial blood vessels, allowing extravasation of the tracers. However, recent work indicates that uterine blood flow (BF) (McRae and Heap, 1988) and extracellular fluid volume (ECFV) (McRae and Heap, 1988) (chapter 4 of this thesis) also increase in predecidual tissue. Thus, it is possible that these vascular responses may also contribute to the extravasation of i.v.-injected tracers by increasing plasma hydrostatic pressure and decreasing tissue hydrostatic pressure respectively. The existence of independent uterine vascular changes suggests that more than one chemical factor may be involved and provides a potential explanation for the numerous chemical factors linked to the processes of implantation and decidualization (Kennedy et al. 1989). A "two mediator hypothesis" has already been proposed for the extravasation of proteins which occurs during inflammation (Williams,

1977; Williams and Peck, 1977). According to that hypothesis, a prostaglandin (PG) produces vasodilation in the inflamed tissue thereby augmenting an increase in VP which is mediated by a second compound such as bradykinin or histamine (Williams, 1977; Williams and Peck, 1977).

Among the chemical factors implicated in ovoimplantation, most evidence suggests that PGs have an obligatory role. Indomethacin (IM), an inhibitor of PG synthesis (Vane, 1971), abolishes or significantly delays implantation in mice, rats, hamsters and rabbits (Kennedy et al. 1989). In addition, IM also inhibits decidualization and numerous associated events (Kennedy et al. 1989). When ovariectomized rats are sensitized for decidualization, phosphate-buffered saline (PBS) is a suitable deciduogenic stimulus when infused into the uterine lumen via osmotic mini-pumps (Kennedy and Lukash, 1982; Kennedy, 1986). Treatment with IM prior to pump insertion significantly reduces both the decidual response (Kennedy, 1986) and the uterine accumulation of i.v. injected tracer proteins which precedes decidualization (Kennedy and Lukash, 1982). Simultaneous infusion of PGE_2 or $PGF_{2\alpha}$ with IM returns both these responses to levels similar to those associated with infusion of PBS (Kennedy, 1986).

In this chapter uterine BF, ECFV and VP are assessed when PBS, IM or IM+PGE_2 were infused into one uterine horn of rats sensitized for decidualization. Because all three variables may contribute to the accumulation of labeled proteins in the uterus, the aim was to determine which of these variables (if any) are regulated by PGs. PGE_2 was used in this study because while both PGE_2 and $PGF_{2\alpha}$ can override the inhibitory effect of IM on the enhanced uterine accumulation of tracer proteins, high

affinity binding sites for PGE (Kennedy et al. 1983) but not for $PGF_{2\alpha}$ (Martel et al. 1985) have been identified in rat endometrium at the time when uterine protein accumulations are maximal (Kennedy et al. 1983).

7.2 METHODS

7.2.1 Animals

Rats were ovariectomized and sensitized for decidualization as described in sections 3.2, 3.3 and illustrated in Fig. 3.1.

7.2.2 Preparation of Osmotic Pumps

Alzet osmotic minipumps were incubated overnight at room temperature in 0.9% NaCl. The following morning, the pumps were loaded with sodium phosphate buffer (0.05 M, 0.154 M NaCl, pH 7.4; PBS), 0.05 mM (pumping rate 0.018 μg/h) indomethacin (IM; Sigma, St Louis, MO) in PBS (IM-PBS) or 1 mg/ml (pumping rate 1 μg/h) prostaglandin E_2 (PGE_2) in IM-PBS. Note: Because of its poor solubility in water, the IM was first dissolved in a small volume of absolute ethanol. Final solutions were made such that PBS, IM-PBS and PGE_2-IM-PBS all contained 1% ethanol when loaded into pumps. All solutions were made on the days of the experiments.

7.2.3 Pump Insertion

On equivalent to Day 5 of pseudopregnancy, at 0800 h, animals were randomly

assigned to 1 of 3 groups. The first group was injected s.c. with 0.2 ml sesame oil and later received intrauterine infusion of PBS. The second and third groups were given s.c. injections of 2 mg IM in 0.2 ml sesame oil and subsequently received intrauterine infusions of IM and IM+PGE_2, respectively. The s.c. injections of IM serve to inhibit the production of uterine PGs during subsequent insertion of the pumps into the uteri. At approximately 1200 h (range $\pm$ 1.5 h), animals were anesthetized with ether and pumps containing previously assigned infusates were implanted with flow regulators inserted into the uterine lumen at the utero-tubal end (Kennedy and Lukash, 1982). After 10 or 20 h of infusion, uterine ECFV, VP and BF were determined in infused and noninfused uterine horns.

7.2.4 Estimation of Uterine Prostaglandin E_2 Concentrations

After 10 or 20 h of infusion of PBS, IM or PGE_2+IM, animals were killed by decapitation and uteri were removed. In order to terminate uterine PG synthesis, the uteri were quickly transferred to a chilled (on ice) solution of 20 mg IM/100 ml 0.9% NaCl. After 2 minutes, uteri were taken out of the IM-saline solution and extraneous connective tissue was removed. The infused and non-infused horns were then separated, frozen on dry ice, weighed, placed in 1.0 ml of chilled ethanol and stored at -20°C for future radioimmunoassay (RIA). Uterine concentrations of PGE_2 were determined as described previously (Kennedy, 1979; Kennedy, 1980b). Prior to RIA, the horns were homogenized in ethanol using Ten-Broeck tissue grinders. The homogenates were centrifuged and aliquots of supernatants were assayed in triplicate as described previously

(Behrman, 1971) using PGE_2 as standard. The antiserum is specific for PGE_2 and has been characterized previously (Olson et al. 1984) (Appendix 6). For 4 assays, the intra- and interassay coefficients of variation were 3.9 % and 4.8 %, respectively at 500 pg.

7.2.5 Estimation of Extracellular Fluid Volume (ECFV)

After 10 or 20 hours of infusion, uterine ECFV was estimated in infused and noninfused uterine horns as described in sections 3.4.

7.2.6 Estimation of Uterine Vascular Permeability (VP)

After 10 or 20 hours of infusion, uterine VP was estimated in infused and noninfused uterine horns as described in section 3.6.

7.2.7 Estimation of Uterine Blood Flow (BF)

After 10 or 20 hours of infusion, uterine BF was estimated in infused and noninfused uterine horns as described in section 3.7.

7.3 RESULTS

7.3.1 Uterine Prostaglandin E_2 Concentrations

Uterine PGE_2 concentrations were significantly affected by infusion and by the

	Uterine Horn PGE Concentration (pg/mg)			
	10 Hours of Infusion		20 Hours of Infusion	
Infusate	Infused	Noninfused	Infused	Noninfused
PBS	7.8 ± 1.3*	4.7 ± 0.7	6.7 ± 2.1*	4.3 ± 0.7
IM	0.5 ± 0.3	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
IM + PGE_2	31.4 ± 5.7***	1.1 ± 0.5	23.6 ± 3.5***	0.6 ± 0.3

TABLE 7.1. Concentrations of PGE_2 in infused and noninfused uterine horns after 10 and 20 hours of unilateral infusion of PBS, IM or IM+PGE_2. Data are expressed as mean±SEM for 7 uterine horns. * and *** indicate significant differences between infused and noninfused horns at $p<0.05$ and $p<0.001$ respectively. For statistical purposes, PGE_2 concentrations below the limit of detection of the assay were considered 0.

type of infusate (*TABLE 7.1*; $p < 0.05$, ANOVA). However, the duration of infusion had no significant effect on these concentrations ($p > 0.05$, ANOVA). Infusion of PBS produced uterine PGE_2 concentrations which were significantly higher in infused than noninfused horns for both 10 and 20 hours of infusion (*TABLE 7.1*, $p < 0.05$). The combined administration of IM by s.c. injection and infusion into the uterine lumen reduced PGE_2 concentrations of both infused and noninfused horns to levels below the minimum detectable dose of the assay in 60 to 80% of the samples (Note: For statistical purposes, values below the level of detection were taken as 0). Simultaneous infusion of PGE_2 (1 μg/h) with the IM for both 10 and 20 hours increased uterine PGE_2 concentrations in infused horns to levels several fold greater than those associated with infusion of PBS ($p < 0.05$). In contrast, PGE_2 concentrations in contralateral, noninfused horns remained near or below the minimum detectable concentration after 10 or 20 h of infusion (*TABLE 7.1*).

7.3.2 Uterine Extracellular Fluid Volume

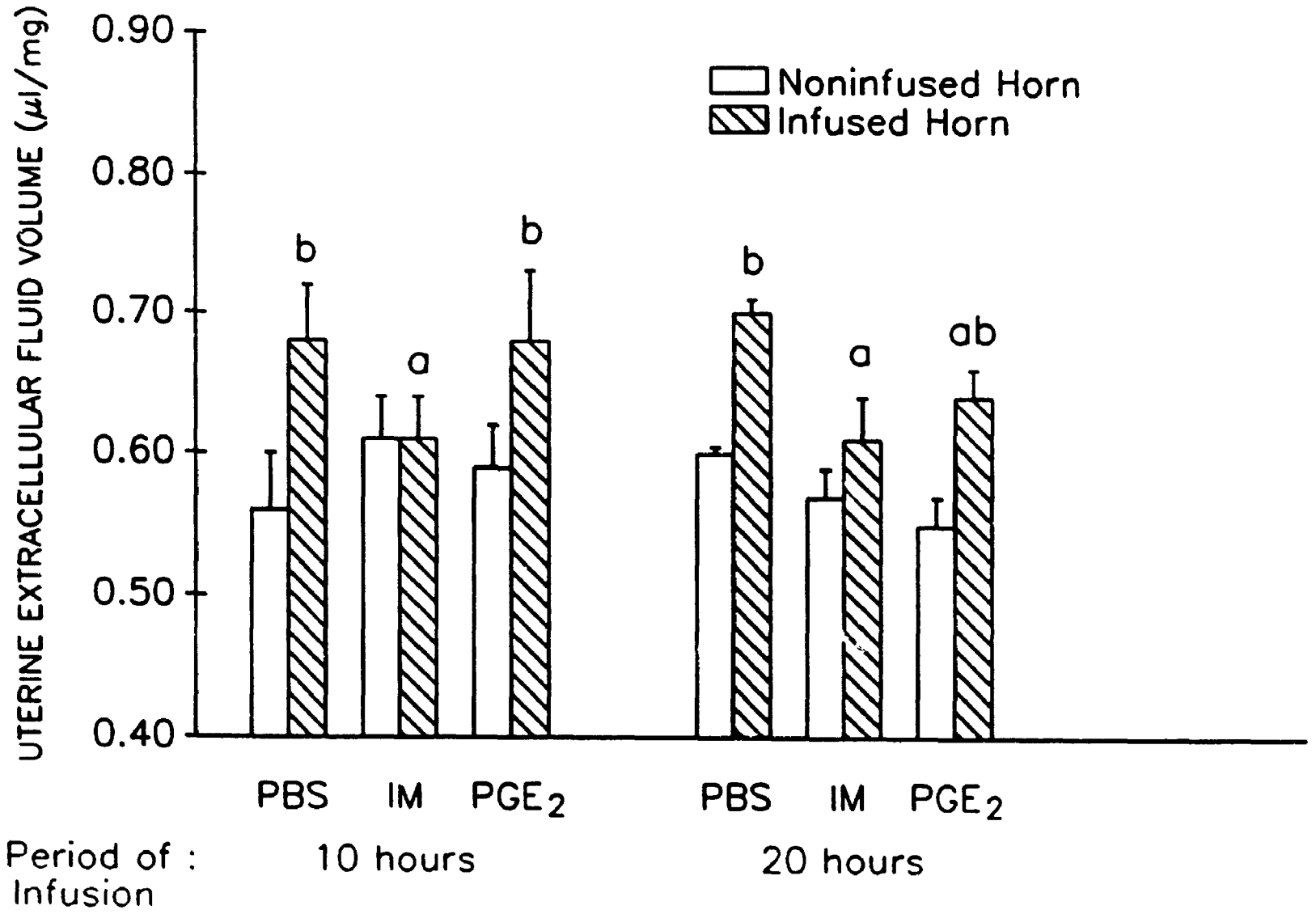
Infusion of PBS for 10 or 20 hours produced uterine ECFVs which were significantly higher in infused (0.68 and 0.70 μl/mg) than noninfused (0.56 and 0.60 μl/mg) horns (*FIGURE 7.1*, $p < 0.05$). When IM was injected s.c. and infused into the uterine lumen, ECFVs of infused horns were not different from those of noninfused horns for both periods of infusion ($p > 0.05$). Simultaneous infusion of PGE_2 with the IM returned ECFV of infused horns to levels not different ($p > 0.05$) from that associated with infusion of PBS (*FIGURE 7.1*).

FIGURE 7.1. Uterine ECFV of infused and noninfused uterine horns after 10 and 20 hours of unilateral infusion of PBS, IM or IM+PGE_2 to rats sensitized for decidualization. Each bar represents the mean $\pm$ SEM for 6 uterine horns and bars not sharing the same superscript represent significantly different ECFVs among infused horns ($p<0.05$).

Summary of Analysis of Variance			
Source	df	Mean Square	F Ratio
Between Animals			
A = Period of Infusion	1	0.00370	0.320
B = Infusate	2	0.01089	0.045
A x B	2	0.00856	0.821
Error	30	0.01042	
Within Animals			
C = Infusion vs. no infusion	1	0.0889	41.30***
A x C	1	0.00151	0.701*
B x C	2	0.00103	4.78
A x B x C	2	0.00324	1.50
Error	30	0.00215	

* $p<0.05$

* $p<0.001$


UTERINE EXTRACELLULAR FLUID VOLUME (μl/mg)
0.90
0.80
0.70
0.60
0.50
0.40
Noninfused Horn
Infused Horn
b
a
b
b
a
ab
PBS
IM
PGE2
PBS
IM
PGE2
Period of : Infusion
10 hours
20 hours

7.3.3 Uterine Vascular Permeability

For noninfused uterine horns, the slopes of the lines of log[1-(albumin volume/ECFV) against time were not different among treatment groups or time of killing, as indicated by the absence of significant ($p<0.05$) interactions on ANOVA. Thus VP in noninfused horns did not differ (*FIGURE 7.2a* and *FIGURE 7.3a*). Infusion of PBS for 10 or 20 h resulted in lines whose slopes were significantly ($p<0.05$) different from those of the contralateral noninfused horns, thus indicating that VP was increased (*FIGURE 7.2b* and *FIGURE 7.3b*). For IM infused horns, VP was significantly lower than PBS infused horns at both 10 (*FIGURE 7.2b*) and 20 (*FIGURE 7.3b*) h and not significantly different from that of noninfused horns (*FIGURE 7.2a* and *FIGURE 7.3a*). By contrast, VP of uterine horns infused with IM+PGE_2 (*FIGURE 7.2b* and *FIGURE 7.3b*) was greater than for noninfused horns and not different from that of PBS infused horns after both 10 and 20 hours of infusion.

7.3.4 Uterine Blood Flow

Uterine BF data are reported both as BF to whole uterine horns (total BF) and as tissue BF corrected for uterine weight (*FIGURE 7.4*). For noninfused horns, analysis of variance indicated that total uterine BF, uterine weight and tissue BF were not affected by any of the treatments after either period of infusion ($p>0.05$). For infused horns, total BF, uterine weight and tissue BF were not significantly affected by any of the infusates after only 10 hours of infusion ($p>0.05$). Infusion of PBS for 20 hours resulted in total BF which was significantly greater to infused than to noninfused horns

FIGURE 7.2. Uterine VP indices for noninfused (panel a) and infused (panel b) uterine horns after 10 hours of unilateral infusion of PBS, IM or IM+PGE_2 to rats sensitized for decidualization. Greater negative slopes of the lines defined by log[1-(albumin volume/ECFV)] indicate greater VP. Each point represents log[1-(albumin volume/ECFV)] for 4 uterine horns. Asterisks indicate significantly reduced VP among infused horns ($p<0.05$).

Summary of Analysis of Variance			
Source	df	Mean Square	F Ratio
Between Animals			
A = Infusate	2	0.00159	6.11*
B = Time After Tracer Injection	3	0.0144	55.38***
A x B	6	0.00065	2.56*
Error	36	0.00026	
Within Animals			
C = Uterine Infusion	1	0.0146	95.90***
A x C	2	0.00190	12.38*
B x C	3	0.00327	21.49***
A x B x C	6	0.00067	4.40***
Error	36	0.00015	

* $p<0.05$

* $p<0.001$

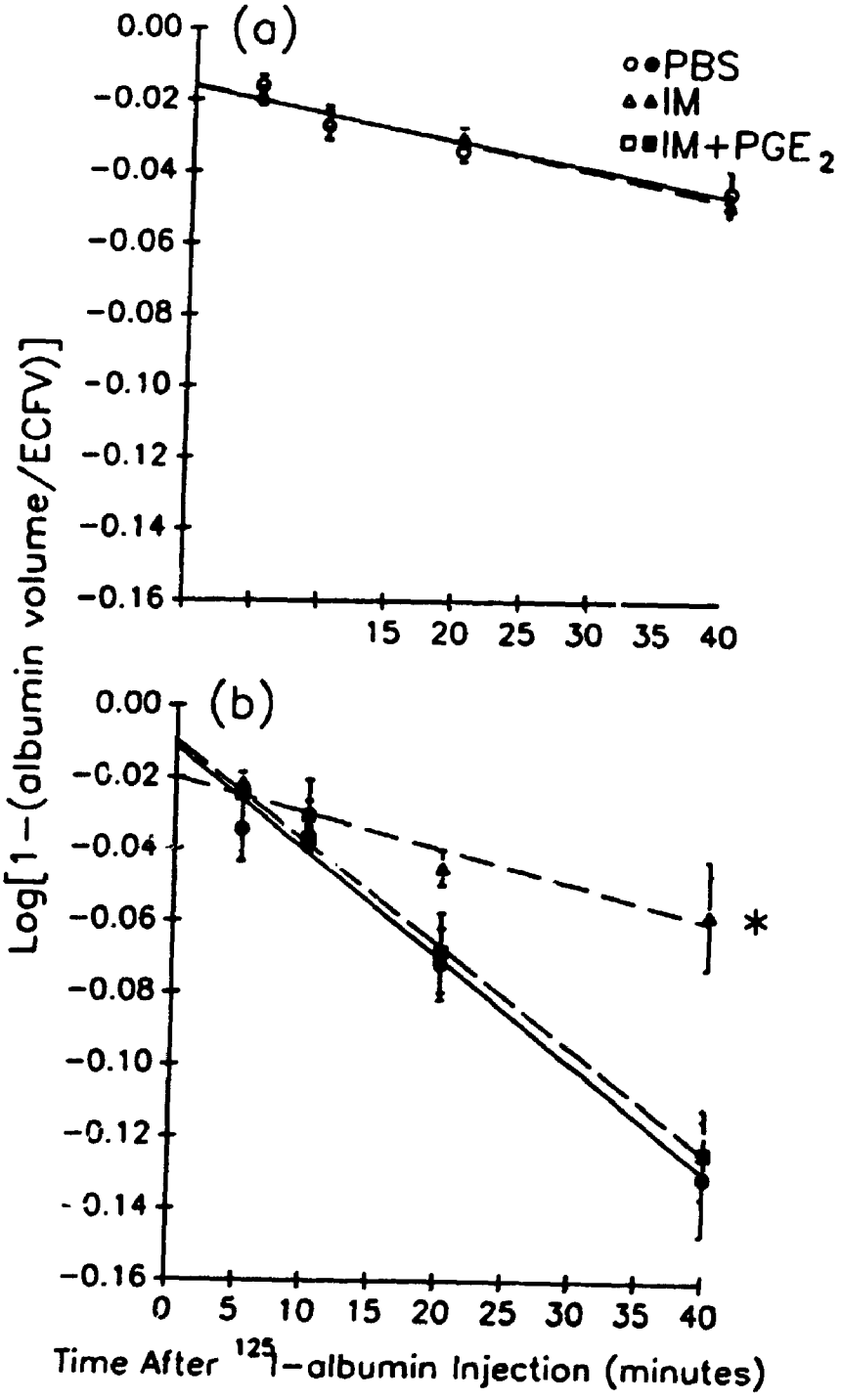

(a)
(b)
PBS
IM
IM+PGE2
0.00
-0.02
-0.04
-0.06
-0.08
-0.10
-0.12
-0.14
-0.16
15 20 25 30 35 40
0 5 10 15 20 25 30 35 40
*
Log[1-(albumin volume/ECFV)]
Time After 125I-albumin Injection (minutes)

FIGURE 7.3. Uterine VP indices for noninfused (panel a) and infused (panel b) uterine horns after 20 hours of unilateral infusion of PBS, IM or IM+PGE_2 to rats sensitized for decidualization. Greater negative slopes of the lines defined by log[1-(albumin volume/ECFV)] indicate greater VP. Each point represents log[1-(albumin volume/ECFV)] for 4 uterine horns. Asterisks indicate significantly reduced VP among infused horns ($p<0.05$).

Summary of Analysis of Variance			
Source	df	Mean Square	F Ratio
Between Animals			
A = Infusate	2	0.00471	11.40***
B = Time After Tracer Injection	3	0.0098	23.53***
A x B	6	0.00128	3.07*
Error	36	0.00042	
Within Animals			
C = Uterine Infusion	1	0.0316	119.67***
A x C	2	0.00186	7.08*
B x C	3	0.00239	8.87***
A x B x C	6	0.00094	3.55***
Error	36	0.00026	

* $p<0.05$

* $p<0.001$

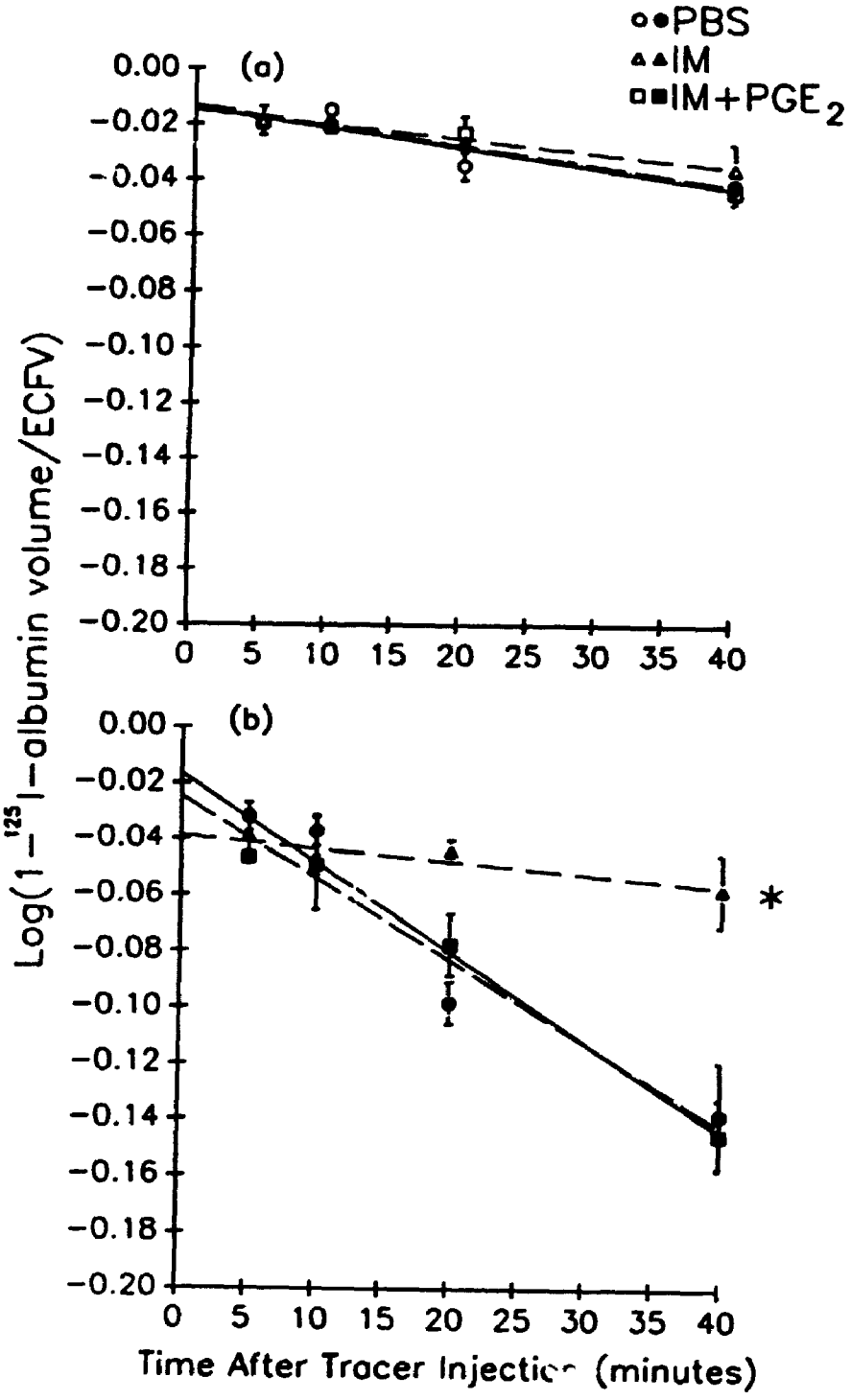

PBS
IM
IM+PGE2
(a)
(b)
0.00
-0.02
-0.04
-0.06
-0.08
-0.10
-0.12
-0.14
-0.16
-0.18
-0.20
0
5
10
15
20
25
30
35
40
*
Log(1−125I−albumin volume/ECFV)
Time After Tracer Injection (minutes)

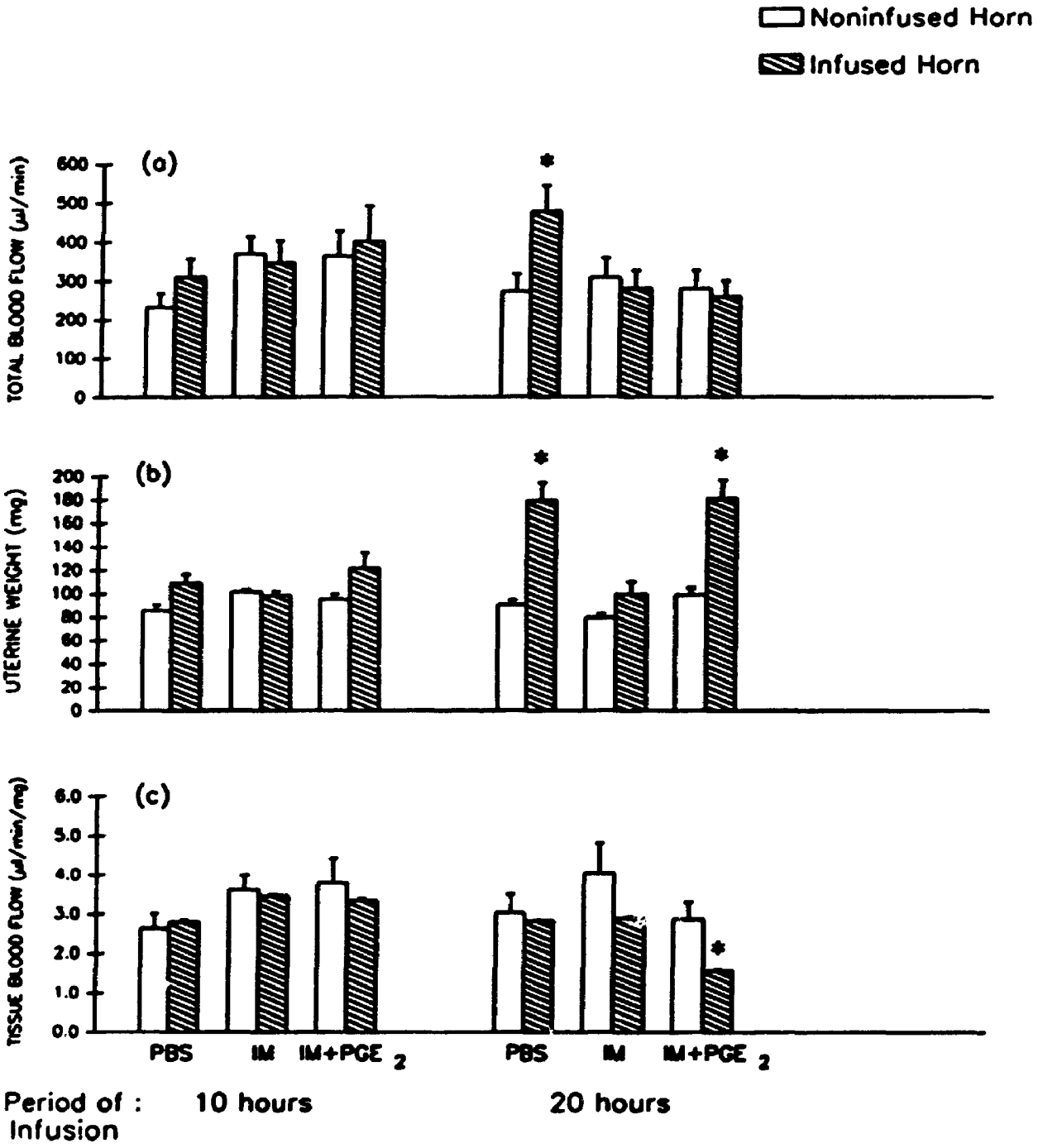
FIGURE 7.4. Total uterine BF (a), uterine weight (b) and tissue BF (c) for infused and noninfused uterine horns after 10 or 20 hours of infusion of PBS, IM or IM+PGE_2 to rats sensitized for decidualization. Bars represent mean $\pm$ SEM for 6 or 7 animals. Bars not sharing the same superscript represent significant differences among infused horns ($p<0.05$).

Summary of Analysis of Variance

	Total BF		Uterine Weight[a]		Tissue BF	
Source	df	Mean Square	F Ratio[b]	Mean Square	F Ratio[b]	Mean Square	F Ratio[b]
Between An.							
A=Time	1	15067	0.400^{NS}	5107.4	4.35^{*}	3.49	1.09^{NS}
B=Infusate	2	4082.5	0.108^{NS}	4192.2	3.57^{*}	3.85	1.20^{NS}
A x B	2	106569	2.82^{NS}	2722.9	2.32^{NS}	4.91	1.53^{NS}
Error	36	37470		1173.5		3.21	
Within An.							
C=Infusion	1	37423	4.68^{*}	29983	65.05^{***}	5.52	9.67^{*}
A x C	1	2575	0.32^{NS}	10230	22.20^{*}	2.82	4.93^{*}
B x C	2	57731	7.21^{*}	4396.1	9.54^{***}	1.38	2.41^{NS}
A x B x C	2	14199	1.77^{NS}	555.2	1.20^{NS}	0.197	0.345^{NS}
Error	36	7863.7		460.9		0.57	

[a] Denotes data which were logarithmically transformed to achieve homogeneity

[b] Superscripts denote levels of significance: $p<0.05^{*}$; $p<0.001^{***}$; not significant^{NS}.


Noninfused Horn
Infused Horn
(a)
TOTAL BLOOD FLOW (µl/min)
600
500
400
300
200
100
0
*
(b)
UTERINE WEIGHT (mg)
200
180
160
140
120
100
80
60
40
20
0
*
*
(c)
TISSUE BLOOD FLOW (µl/min/mg)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
*
PBS
IM
IM+PGE 2
PBS
IM
IM+PGE 2
Period of : 10 hours
Infusion
20 hours

and significantly greater than total BF to horns infused with IM or IM+PGE_2 (*FIGURE 7.4a*, $p<0.05$). This increase was accompanied by a proportionate increase in uterine weight (*FIGURE 7.4b*, $p<0.05$) resulting in unchanged tissue BF (*FIGURE 7.4c*, $p>0.05$). Infusion of IM+PGE_2 for 20 hours also produced a significant increase in uterine horn weight (*FIGURE 7.4b*, $p<0.05$). Since this was not accompanied by a proportionate increase in total BF to infused horns (*FIGURE 7.4a*), a significant reduction in tissue BF was observed (*FIGURE 4c*, $p<0.05$). Neither cardiac output (137 ± 9 ml/min) nor renal tissue BF (3.19 ± 0.21 μl/min/mg) changed significantly as a result of any of the infusates ($n=40$; $p>0.05$).

7.4 DISCUSSION

The results of this study demonstrate that uterine PGs are essential for the increases in uterine ECFV and VP which precede decidualization. Furthermore, the fact that uterine ECFV and VP changed in parallel throughout this study provides further support for the notion that these two vascular variables are either directly related or share a common control element. In contrast, our results suggest that PG-dependent increases in uterine BF are not essential for subsequent endometrial decidualization.

Phosphate-buffered saline (PBS) has been shown to be a suitable deciduogenic stimulus when infused into the uterine lumen of rats sensitized for the decidual cell reaction (Kennedy and Lukash, 1982; Kennedy, 1986). This treatment proved to be

effective in the present study since uterine ECFV and VP both increased in a manner consistent with imminent decidualization (Chapter 4 of this thesis). Combined administration of IM by s.c. injection and intraluminal infusion prevented the increases in uterine ECFV and VP (*FIGURE 7.1* and *FIGURE 7.3*). Since similar treatment also reduced uterine PGE_2 concentrations to very low levels (*TABLE 7.1*) as has been demonstrated previously (Kennedy, 1985), the data suggest that synthesis of uterine PGs is essential to produce these uterine vascular responses. The fact that coinfusion of PGE_2 and IM reinstated the increases in uterine ECFV and VP indicates that the presence of this PG is sufficient to permit these uterine vascular changes. Such a finding is not surprising since other studies have shown that PGE_2 alone can override the inhibitory effects of IM on decidualization (Kennedy and Lukash, 1982; Kennedy, 1985; Kennedy, 1986) and the enhanced protein accumulations which precede it (Kennedy and Lukash, 1982). The mechanism by which PGE_2 effects the increases in VP and ECFV is not clear at this time. These uterine PGE_2 concentrations following delivery of the various infusates to the uterine lumen confirm that the osmotic pumps functioned as expected and that adequate concentrations of IM and PGE_2 reached the uterine lumen. Infusion of IM effectively eliminated uterine PGE_2 while simultaneous infusion of PGE_2 with the IM produced uterine PGE_2 concentrations several fold greater than those associated with PBS infusion.

In the present study, total uterine BF increased to horns infused with PBS; however, uterine weight gains matched the BF increase producing no significant change in uterine tissue BF (*FIGURE 7.3*). This finding supports those reported previously

(Garris et al. 1983; Edwards and Milligan, 1987) as well as in chapter 4 of this thesis (*FIGURE 4.5*) demonstrating that tissue BF does not increase during similar stages of decidualization in rats and mice. Neither total uterine BF nor uterine tissue BF were affected by IM treatment during these experiments indicating that the high basal uterine BFs during the predecidual period are not dependent on prostanoid production. Coinfusion of PGE_2 with IM produced significant increases in the weights of infused horns as has been demonstrated previously (Kennedy, 1986). Surprisingly, the decidual weight gain was not accompanied by any increase in total BF. As a result, there was a significant reduction in tissue BF for horns infused with IM+PGE_2. This finding suggests that, unlike its effect on local BF during inflammation (Williams, 1977; Williams and Peck, 1977), PGE_2 does not effect an increase in local BF during early decidualization. However; it seems likely that PGE_2 contributes either directly or indirectly to the tissue growth which occurs during this stage of decidualization (Kennedy, 1986).

Since total BF did not increase in proportion to uterine weight when IM and PGE_2 were infused, a prostanoid other than PGE_2 probably modulates uterine BF during decidualization. PGI_2 represents a likely candidate because of its vasodilatory actions on a variety of vascular beds (Williams, 1977; Williams and Peck, 1977) and because the hydrolysis product of PGI_2 is elevated at implantation sites (Kennedy and Zamecnik, 1978) and in the uterus following artificial deciduogenic stimulation (Jonsson et al. 1979). In addition, it has been suggested that PGI_2 may act on uterine blood vessels during late ovine pregnancy to attenuate local vasoconstriction in response to angiotensin

II (Magness et al. 1992). Since in that system PGI_2 served to maintain rather than to increase BF, PGI_2 might play a similar role during decidualization, that of maintaining constant relative BF during rapid tissue growth. However, it seems clear that the early events of decidualization are not limited by BF because even in the absence of an increase in total BF, ECFV, VP and uterine weight all increased with infusion of IM+PGE_2. A minimum BF has been shown to be important during early pregnancy in rats since surgical impairment of uterine BF prior to pregnancy reduces the number of fetuses (Franklin and Brent, 1964; Antebi et al. 1991) and the fetal weights (Antebi et al. 1991). In addition, the importance of uterine BF in sensitization was demonstrated when rats responded to deciduogenic stimulation with reduced uterine weight gains when uterine BF was reduced by previous exposure to serotonin (Mitchell et al. 1983) or enhanced by exposure to ethanol (Mitchell and Goldman, 1991).

In summary, our results indicate that PGs are essential to the increases in uterine ECFV and VP which immediately precede decidualization and that PGE_2 is capable of producing these vascular changes. In addition, a PG other than PGE_2 appears to increase total BF in a manner which maintains constant relative BF during the uterine growth which accompanies decidualization.

7.5 SIGNIFICANCE

Results presented in this chapter again indicate that increased uterine VP and ECFV are associated with the enhanced extravasation and accumulation of serum proteins

during early decidualization. Uterine prostaglandins are essential to the increases in uterine VP and ECFV and PGE_2 alone is sufficient to override IM inhibition of these uterine vascular responses. In addition, a PG other than PGE_2 appears to increase absolute uterine BF to match increases in uterine weight during early decidualization.

CHAPTER 8: GENERAL DISCUSSION

In the preceding chapters, uterine BF, ECFV and VP have been examined nder conditions which allow the endometrial blueing reaction to be demonstrated by i.v. injection of Evans blue dye. In addition, these vascular variables have also been assessed using experimental manipulations known to modify the extent of endometrial decidualization and the enhanced intrauterine accumulation of plasma proteins which precedes it. The experimental manipulations were intended to determine if optimal uterine sensitization and unimpeded uterine PG synthesis promote increased uterine VP, ECFV and/or BF after deciduogenic stimulation and to determine if suboptimal sensitization or inhibition of uterine PG synthesis affect these uterine vascular responses.

8.1 UTERINE BLOOD FLOW (BF)

During the present study, uterine BF was examined using the radioactive microsphere method (Rudolph and Heymann, 1967). Total uterine BF to stimulated horns increased significantly between 9 and 27 hours after unilateral deciduogenic stimulation of rats sensitized for decidualization (*FIGURE 4.4a*). However, the increase was directly proportional to the increase in uterine weight during the same period after deciduogenic stimulation (*FIGURE 4.4b*). As a result, uterine tissue BF did not change significantly in either stimulated or nonstimulated uterine horns at any time after stimulation (*FIGURE 4.4c*). It is unlikely that the increased total uterine BF contributes

to the enhanced extravasation and intrauterine accumulation of plasma proteins in predecidual tissue since significant increases in total uterine BF (*FIGURE 4.4a*) occur several hours after peak intrauterine accumulations of i.v.-injected tracer proteins in stimulated uterine horns (*FIGURE A1.1*).

Although uterine tissue BF was not found to increase after deciduogenic stimulation, the high tissue BF in nonstimulated, as well as stimulated horns (*FIGURE 4.4c*), suggested that this high basal flow might create a favourable condition which would enhance the extravasation of serum proteins should VP increase as a result of deciduogenic stimulation. In fact, a minimal BF during early pregnancy has been shown to be important in rats since surgical impairment of uterine BF prior to pregnancy reduced the number of fetuses (Franklin and Brent, 1964; Antebi et al. 1991) and fetal weights (Antebi et al. 1991). Furthermore, decidual responses have been shown to be detrimentally affected when uterine BF is reduced by previous exposure to serotonin (Mitchell et al. 1983) or increased by previous exposure to ethanol (Mitchell and Goldman, 1991; Mitchell and Van Kainen, 1992). However, the results of chapter 6 indicate that total uterine BF to stimulated and nonstimulated horns is not affected by various forms of sub-optimal sensitization and that uterine tissue BF is significantly affected only treatment with high doses of E_2 increases uterine weight without increasing total uterine BF (*FIGURE 6.4*). These findings suggest that, although the high uterine tissue BF may augment the extravasation of serum proteins which follows deciduogenic stimulation, optimal sensitization for decidualization is not essential to ensure the high basal BF.

The failure of IM to affect uterine tissue BF during early decidualization provides further evidence that high uterine BF is not crucial to the events which comprise the uterine blueing reaction. It has been proposed that vasodilation of endometrial blood vessels might be an important role of PGE_2 during decidualization in rats (Kennedy and Armstrong, 1981). This hypothesis is based on the fact that PGE_2 is a crucial mediator of decidualization (Kennedy et al. 1989) and on the fact that PGE_2 can enhance the accumulation of labeled serum proteins durin$_{\epsilon}$ inflammation through increasing local tissue BF (Williams, 1977; Williams and Peck, 1977). In chapter 7, IM treatment was shown to reduce uterine PGE_2 concentrations to levels undetectable by RIA (*TABLE 7.1*). Although intrauterine infusion of IM reduced total uterine BF to infused horns (as compared with PBS infusion; *FIGURE 7.4a*), this effect was noted after 20 hours of infusion and not after 10 hours of infusion. In addition, uterine tissue BF to infused and noninfused horns was not significantly affected by this IM treatment (*FIGURE 7.4c*), although it has been shown to inhibit decidualization (Kennedy, 1986). Thus, the high basal uterine BFs during the p‹edecidual period (chapter 4) are not dependent on prostanoid production. Interestingly, coinfusion of PGE_2 with IM produced significant increases in the weights of infused horns which were not accompanied by increases in total BF. As a result, there was a significant reduction in tissue BF for horns infused with IM+PGE_2. It appears that a prostanoid other than PGE_2 probably modulates uterine BF in a manner which allows total BF to match the uterine weight gain associated with decidualization. PGI_2 represents a likely canditate because of its vasodilatory actions on a variety of vascular beds (Williams, 1977; Williams and Peck, 1977) and because the

hydrolysis product of PGI_2 is elevated at implantation sites (Kennedy and Zamecnik, 1978) and in the uterus following artificial deciduogenic stimulation (Jonsson et al. 1979). In addition, it has been suggested that PGI_2 may act on uterine blood vessels during late ovine pregnancy to attenuate local vasoconstriction in response to angiotensin II (Magness et al. 1992). Since in that system PGI_2 served to maintain rather than to increase BF, PGI_2 might play a similar role during decidualization, that of maintaining constant relative BF during rapid tissue growth. Thus, although the results presented in this thesis support the notion that PGE_2 is an important mediator of decidualization, vasodilation does not appear to be an important component of that mediation. Furthermore, other PGs which may increase uterine BF during decidualization do so at a time which is too late to suggest that PG-mediated vasodilation contributes to the extravasation of serum proteins preceding decidualization.

Therefore, while maintenance of a minimum uterine BF may be essential for certain events of early pregnancy, the results of this study indicate that this uterine vascular variable does not change in a manner which would suggest that it is an important determining factor in the uterine blueing reaction.

8.2 UTERINE EXTRACELLULAR FLUID VOLUME (ECFV)

When assessed by the uterine volume of distribution of i.v.-injected ^{51}Cr-EDTA, uterine ECFV was shown to increase only under conditions which ensure subsequent decidualization. Significant increases in ECFV occurred only after optimal sensitization

(*FIGURE 6.2*) for decidualization and only when elevated concentrations of uterine PGs were present (*FIGURE 7.1*). In addition, the increases in ECFV occurred at times when the blueing reaction can be demonstrated by i.v. injection of Evans blue dye.

The fact that increases in uterine ECFV coincide with the ability to demonstrate the uterine blueing reaction provides support for the notion that the increased uterine ECFV contribute to the phenomenon by creating a larger compartment in which protein-dye complexes accumulate following extravasation. However, the fact that increases in uterine ECFV nearly always coincided with increases in uterine VP indicates that these vascular responses are intimately related. This relationship is predictable since an increase in uterine VP allows extravasation of serum proteins which in turn would promote net movement of plasma water to the interstial compartment through both the altered osmotic gradient and through bulk flow. The fact that sensitization with high doses of E_2 prevented the increases in uterine ECFV in stimulated horns (*FIGURE 6.2*) without significantly reducing uterine VP (*FIGURE 6.4*) at least raises the possibility that uterine ECFV and VP are under separate control. The notion that uterine ECFV increases (at least partially) by a mechanism independent of increased VP is supported by histological studies which have indicated predecidual modifications to the endometrial interstitium consistent with pathological edema formation (Fainstat, 1963; Lundkvist, 1978) and by the ability of embryos and endometrial cells to secrete plasminogen activators (Strickland et al. 1976) and metalloproteinases (Brenner et al. 1989; Lala and Graham, 1990), both of which are capable of modifying extracellular matrices. If enzymatic digestion of the extracellular matix is an important component of the enhanced

accumulation of proteins in the predecidual endometrium, this process has rigid requirements, similar to those which give rise to increased uterine VP; very specific uterine sensitization and uterine PGs are essential. In fact, the requirements of uterine sensitization for increases in uterine ECFV are more rigid than for increases in uterine VP since significant increases in uterine ECFV followed treatment with high doses of E_2 (*FIGURE 6.4*) while significant increases in uterine VP did not (*FIGURE 6.2*).

Although plasma volume is an important component of the uterine ECFV, previous studies have indicated that increases in uterine blood volume are negligible (Milligan and Edwards, 1990) or nonexistent (Bitton et al. 1965; Milligan and Mirembe, 1984) during early stages of decidualization. In rats, no increase in uterine blood volume on a per weight basis has been demonstrated (Bitton et al. 1965; Milligan and Mirembe, 1984), while in mice the increased blood volume exactly matched the total increase in ECFV, consistently making up about 25% of that value (Milligan and Edwards, 1990). Thus, much of the increased uterine ECFV reported during the present study almost certainy occurs in the interstitial compartment.

Lymph flow is another factor which might be expected to influence the uterine ECFV during early decidualization. This variable was not assessed during the present study and, in fact, other studies have indicated that the rat endometrium contains very few lymphatic vessels (Head and Seelig, 1984; Lauweryns and Cornillie, 1984). Such a finding suggests that it would be extremely difficult to identify changes in uterine lymph flow with the experimental manipulations used in the present study. However, one might predict that the limited numbers of lymphatic vessels contribute to the

expanding uterine ECFV through a limited capacity to remove excess interstitial fluid which appears as a result of increased local VP and tissue remodelling.

Therefore, the results of this study indicate that increased uterine ECFV may be an important component of the uterine blueing reaction, although the tendency for increased uterine ECFV to coincide with increases in uterine VP suggests that these two vascular variables are directly related. However, the fact that uterine VP but not ECFV increased when uterine stimulation was given to rats sensitized with high doses of E_2 at least raises the possibity that uterine ECFV and VP are under independent control.

8.3 UTERINE VASCULAR PERMEABILITY (VP)

Throughout this study, uterine VP was increased during the time when the uterine blueing reaction can be demonstrated by i.v. injection of Evans blue dye. Uterine VP increased significantly only to stimulated (or infused) uterine horns, only when uterine PGs were available (*FIGURES 7.2* and *7.3*) and only after specific hormonal sensitization had been provided (*FIGURE 6.3* and *6.4*). With one exception, these conditions were consistent with those necessary to promote subsequent endometrial decidualization. Surprisingly, uterine VP was significantly increased in stimulated uterine horns after sensitization with high doses of E_2 (*FIGURE 6.4*), a condition which does not result in subsequent decidualization.

The increases in uterine VP after unilateral deciduogenic stimulation of rats are

not surprising since numerous studies have provided solid qualitative evidence that endometrial VP increases during embryo implantation and in uterine tissue destined to undergo decidualization. Ultrastructural studies have indicated numerous endothelial fenestrations and interendothelial gaps along postcapillary venules of the endometrium at implantation sites (Abrahamsohn et al. 1983). Although the endothelial gaps and fenestrations are indicative of a highly permeable vascular bed, these characteristics only confer enhanced permeability to water and small molecules (Michel, 1988). Evidence that predecidual tissue also exhibits greater permeability to certain macromolecules than nondecidualizing endometrial tissue has been provided by studies involving fluorescence microscopy. In those studies, it was shown that appreciable amounts of fluorescein isothiocyanate-tagged albumin (Rogers et al. 1983; Parr and Parr, 1986) and IgG (Parr and Parr, 1986) gained access to the endometrial interstitium around implanting blastocysts after i.v. injection. In contrast, the avascular primary decidual zone adjacent to the blastocyst and endometrial tissue outside implantation sites were both found to be devoid of the tracers (Rogers et al. 1983; Parr and Parr, 1986).

Until now, most quantitative studies of uterine VP have been based on the volume of distribution of ^{125}I-albumin at a single time after i.v. injection. However, this type of estimate can be distorted by changes in uterine ECFV which are also associated with various conditions including the treatments used in this thesis. During the experiments described here, VP was estimated by the rate at which the uterine volume of distribution approaches the total uterine ECFV (Setchell and Sharpe, 1981). In nearly all cases, trends in uterine VP were similar to those reported when uterine VP was assessed by the

uterine volume of distribution of ^{125}I-albumin at a single time after tracer injection. The similar results obtained by these two methods suggests that increased VP is, in fact, a major factor in determining the uterine volume of distribution of ^{125}I-albumin. However, the tendency for uterine ECFV and VP to change in parallel when assessed following nearly all treatments used in this study may have masked the ability of altered ECFV to affect uterine ^{125}I-albumin volumes of distribution. This possibility is supported by the fact that treatment with high doses of E_2 resulted in a significant increase in uterine VP but not ECFV of stimulated horns (*FIGURES 6.2*, *6.3* and *6.4*). In fact this measurable increase in VP after treatment E_2 is somewhat consistent with previous histological studies which indicated that endothelium in the endometrium of nonpregnant rats possess numerous fenestrations and intercellular gaps after administration of estradiol (Ham et al. 1970; Martin et al. 1973). However, in the present study, the increased uterine VP in animals treated with high doses of E_2 was confined to stimulated horns.

Therefore, the results of this study indicate that significant increases in uterine VP precede endometrial decidualization, confirming the results of previous qualitative studies. The temporal relationship between increased uterine VP and the ability to demonstrate the uterine blueing reaction reinforces the notion that increased uterine VP is an important component of this phenomenon.

8.4 THE USE OF MAGNETIC RESONANCE IMAGING TECHNIQUES TO ASSESS UTERINE VASCULAR CHANGES DURING DECIDUALIZATION

During this study, Gd-DTPA enhanced MR imaging allowed sites of imminent decidualization to be demonstrated in uterine horns given deciduogenic stimulation (*PLATE 5.1*) and in implantation sites of pregnant rats (*PLATE A3.1* and *A4.1*). This demonstration is exciting because MR imaging techniques offer the opportunity to demonstrate these vascular changes in intact animals. In fact this hope has now been realized using pregnant animals (*PLATE A4.1*). Uterine volumes of distribution of ^{153}Gd-DTPA (*FIGURE 5.3*) indicated that this contrast agent permeates the ECF of both decidualizing and nondecidualizing uterine tissue after i.v. injection. Therefore, the increased uterine ECFV appears directly responsible for the enhancement of decidualizing uterine tissue.

These results indicate that Gd-DTPA-enhanced MR imaging can provide a useful method for identifying and quantitating changes in uterine ECFV during decidualization. However, the original aim had been to use this technology to provide a novel, improved method of assessing uterine VP since it was thought that the ability to measure the rate of accumulation of i.v.-injected tracers in the uteri of individual animals would greatly reduce variability associated with the method based on ^{125}I-albumin accumulation. Unfortunately, efforts to assess uterine VP by contrast-enhanced MR imaging met with failure. Although, Gd-DTPA produced good enhancement of decidualizing uterine tissue, maximum image intensity was achieved during the time to produce 1 image, making it

impossible to assess the rate of change of image intensity accurately. Gd-DTPA was succussfully combined with albumin; however, the relatively low relaxivity of this compound made it impossible for i.v.-injected Gd-DTPA-albumin to produce significant enhancement of decidualizing tissue. It is hoped that the ongoing development of blood-pool contrast agents will ultimately provide an agent which is sufficiently small to pass from the blood to the uterine interstitium, yet large enough to do so at a rate which will allow the process to be assessed over the time necessary to acquire several consecutive images.

8.5 LIMITATIONS OF THE STUDY

Although the work presented in this thesis has provided an extensive description of uterine vascular responses to deciduogenic stimulation, the approaches used to assess the various vascular changes possess inherent limitations. Throughout these experiments, uterine vascular variables were assessed in whole uterine horns while histological studies have indicated that the most significant predecidual vascular changes are confined to the endometrium (Fainstat, 1963; Finn and McLaren, 1967; Lundkvist and Ljungkvist, 1977). Since the endometrium covers less than half the cross-sectional area of the rat uterus, the increases in uterine VP and ECFV reported in various chapters of this thesis have undoubtedly underestimated the increases in endometrial VP and ECFV, respectively. In addition, the inability to separate myometrial and endometrial regions may have contributed to the failure to demonstrate significant predecidual changes in

uterine tissue BF. Finally, many of the changes in uterine ECFV described in the thesis have been attributed to increases in interstitial fluid volume. Although the results of several published works were used to support the possibility that the interstitium is the most significant site of increased uterine ECFV, it should be noted that plasma volume was not assessed during the experiments described in this thesis.

8.6 SUMMARY and CONCLUSIONS

1) Uterine VP and ECFV increase in stimulated horns after unilateral deciduogenic stimulation.

2) The increased VP and ECFV in stimulated horns both require the availability of uterine PGs and hormonal sensitization which is quite specific.

3) Increases in uterine VP and ECFV occur during a transient period after stimulation which coincides with the time that the uterine blueing reaction can be demonstrated by i.v. injection of Evans blue dye. Such a temporal relationship suggests that increased VP and ECFV may each contribute to the events which allow the blueing reaction to be demonstrated.

4) Uterine BF does not change significantly during the time when the uterine blueing reaction can be demonstrated. In addition, the high basal uterine tissue BF does not require the availability of uterine PGs, nor does it require specific sensitization for decidualization.

5) The increased uterine ECFV makes it possible to identify sites of imminent decidualization using Gd-DTPA-enhanced MR imaging.

In conclusion, endometrial decidualization is preceded by increases in uterine VP and ECFV. Evidence was presented to suggest that these vascular responses may contribute to the uterine blueing reaction which can be demonstrated in predecidual tissue by i.v. injection of Evans blue dye. The increased uterine VP and ECFV occur at similar times after uterine stimulation, require the availability of uterine PGs and require similar hormonal presensitization. The similar trends for uterine VP and ECFV supports the notion that these variables are either directly related or share a common control element. However, evidence was presented to suggest that they may also have individual control elements. Although uterine tissue BF is high during the predecidual period, the results of these experiments suggest that changes in uterine BF do not play a significant role in the extravasation and accumulation of labeled serum proteins which precede endometrial decidualization.

8.7 OVERALL SIGNIFICANCE

Uterine VP, ECFV and BF have been examined during numerous previous studies and all of these variables are known to change during embryo implantation and artificially-induced decidualization. However, the experiments presented in this thesis have provided perhaps the most comprehensive examination of these variables to date with respect to their involvement in the endometrial blueing reaction. This phenomenon, which can be demonstrated by i.v. injection of Evans blue dye during the first day of implantation or artificially-induced decidualization, is believed to involve vascular and perivascular events essential for the progression to later stages of pregnancy. While the trends in uterine VP during the present study were similar to those described in previous studies, the use of a more valid method of VP assessment during the present study provides important confirmation of conclusions drawn from the results of those previous studies. The examination of uterine ECFV and BF soon after deciduogenic stimulation and the the examination of effects of differential endometrial sensitization and indomethacin treatment on these variable have provided important new information regarding the control and potential importance of altered uterine BF and ECFV during implantation and decidualization. Finally, the work with Gd-DTPA enhanced MR imaging has introduced a novel approach to examining uterine vascular responses to implantation; one which offers the potential to identify a variety of uterine vascular changes while using minimal surigical intervention.

APPENDIX 1: UTERINE WEIGHT GAINS AND PREDECIDUAL ACCUMULATIONS OF i.v.-INJECTED ^{125}I-ALBUMIN AFTER UNILATERAL DECIDUOGENIC STIMULATION

A1.1 INTRODUCTION

The work described in this thesis involves a study of the uterine vascular changes which occur during artificially-induced decidualization in rats. Therefore, the two experiments presented below were intended to verify that unilateral decidualization and the preceding accumulation of i.v.-injected ^{125}I-albumin occur when rats are ovariectomized, treated with the steroid regimen and given deciduogenic stimulation with sesame as described in sections 3.2, 3.3 and illustrated in *FIGURE 3.1.*

A1.2 METHODS

Rats were ovariectomized, treated with the steroid regimen and given unilateral deciduogenic stimulation with sesame oil as described in sections 3.2, 3.3 and illustrated in *FIGURE 3.1.*

^{125}I-Albumin Volumes of Distribution After Deciduogenic Stimulation: Albumin was iodinated, diluted and injected i.v. via lateral tail veins as described in section 3.6. After 15 minutes, animals were processed and uterine albumin volumes of distribution were

determined in stimulated and nonstimulated horns as described in section 3.6.

Assessment of Decidual Responses: On equivalent to Day 10 of pseudopregnancy, animals were killed by decapitation. Uteri were removed, trimmed free of adherent connective tissue and stimulated and nonstimulated horns were weighed separately.

A1.3 RESULTS

^{125}I-Albumin Uterine Volumes of Distribution: Uterine volumes of distribution of ^{125}I-albumin were significantly greater in stimulated than nonstimulated uterine horns from 4 hours to 16 hours after stimulation (*FIGURE A1*; $p<0.05$). The maximum response occurred at 8 hours after stimulation ($p<0.05$).

Decidual Response: When the steroid regimen was continued until the equivalent of Day 10 of pseudopregnancy, weights of stimulated uterine horns were approximately 10-fold greater than those of nonstimulated horns (*FIGURE A2*; $p<0.001$).

A1.4. CONCLUSIONS

The transient increase in the volume of distribution of ^{125}I-albumin (*FIGURE A1*) and the large increase in weight (*FIGURE A2*) of stimulated horns are the expected uterine responses to artificially-induced decidualization and ovoimplantation. Thus, the

animal preparation is appropriate for the study of events associated with artificially-induced decidualization.

FIGURE A1.1. Uterine volumes of distribution of ^{125}I-albumin in stimulated and nonstimulated horns, 15 minutes after i.v. injection into a tail vein. Rats had been ovariectomized, treated with the hormone protocol and given an injection of sesame oil into the lumen of one uterine horn as illustrated in *FIGURE 3.1.* Bars not sharing the same superscript indicate statistically significant differences among stimulated horns ($p < 0.05$).

Summary of Analysis of Variance			
Source	df	Mean Square	F Ratio
Between Animals			
A = Time after stimulation	4	0.00582	13.03***
Error	20	0.00045	
Within Animals			
B = Uterine Stimulation	1	0.037	158.77***
Interaction	4	0.00419	17.97***
Error	20	0.00023	

*** indicates $p < 0.001$.

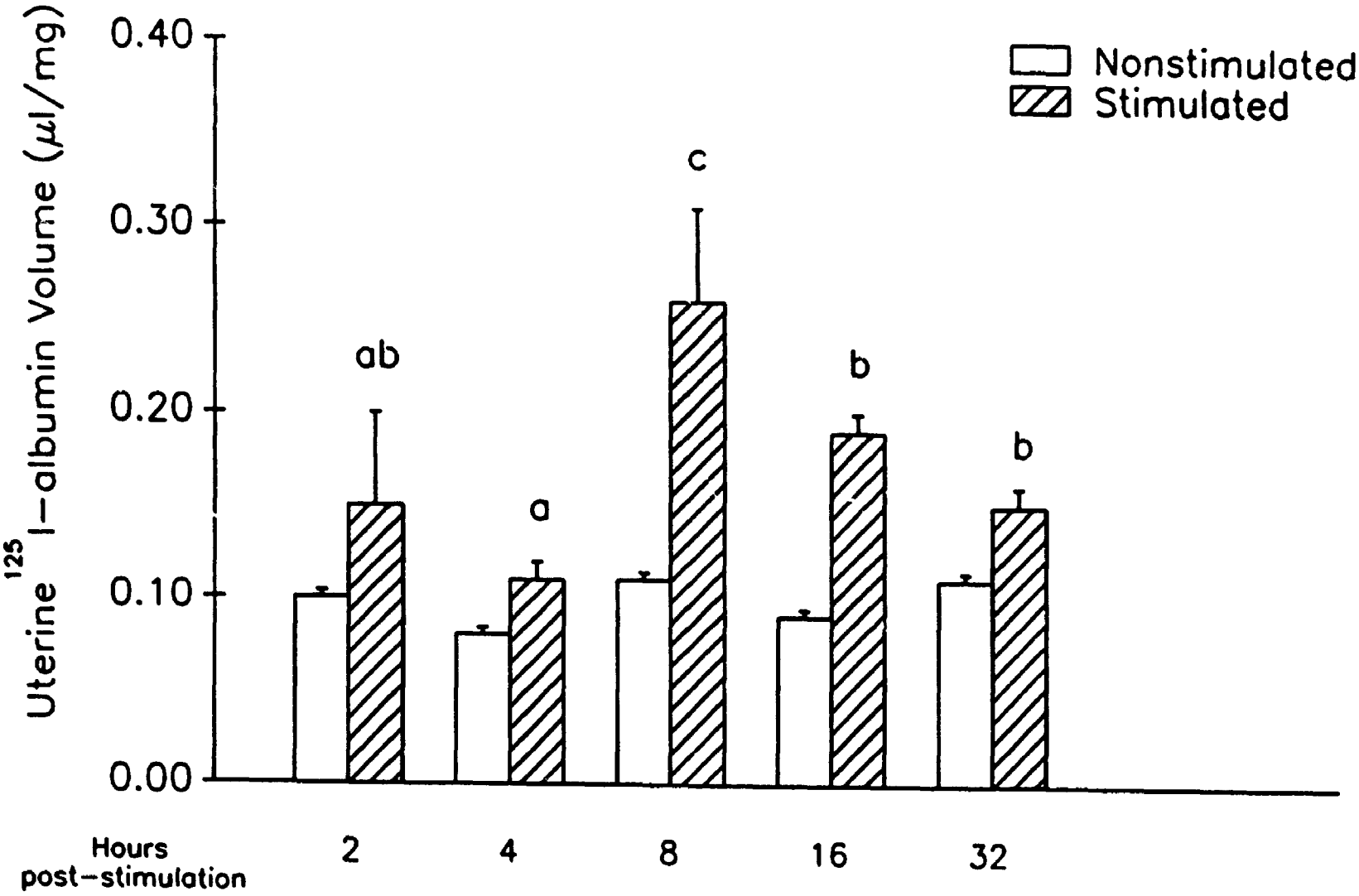
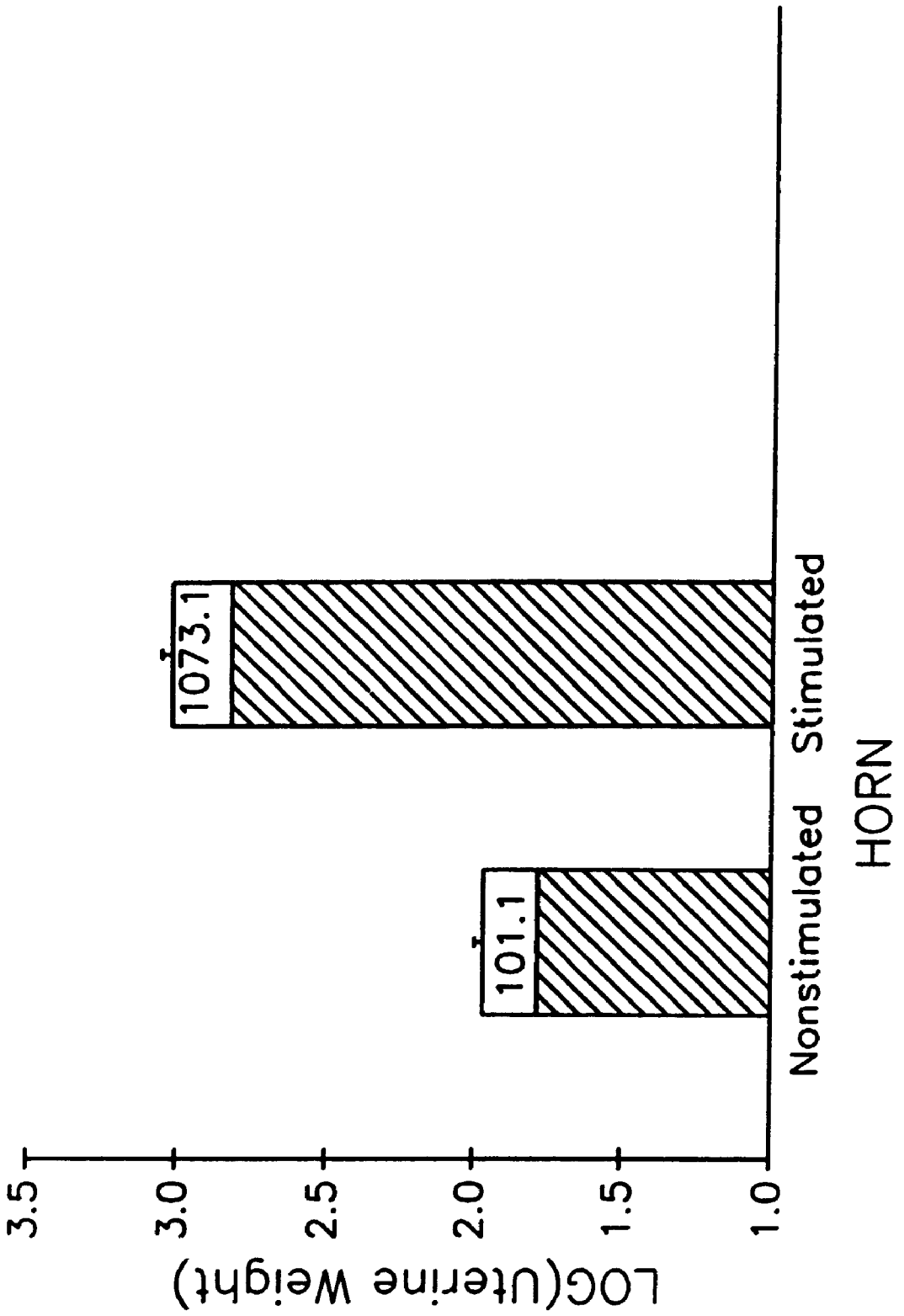

Uterine ^{125}I-albumin Volume (µl/mg)
0.40
0.30
0.20
0.10
0.00
Nonstimulated
Stimulated
ab
a
c
b
b
Hours post-stimulation
2
4
8
16
32

FIGURE A1.2. Weights of stimulated and nonstimulated uterine horns on the equivalent of Day 10 of pseudopregnancy following treatment with the protocol illustrated in *FIGURE 3.1.* Bars represent mean $\pm$ SEM of log-transformed data. Arithmetic means are indicated within each bar.


LOG(Uterine Weight)
3.5
3.0
2.5
2.0
1.5
1.0
101.1
1073.1
Nonstimulated
Stimulated
HORN

APPENDIX 2: UTERINE TISSUE BLOOD FLOW IN OVARIECTOMIZED AND IN PREGNANT RATS

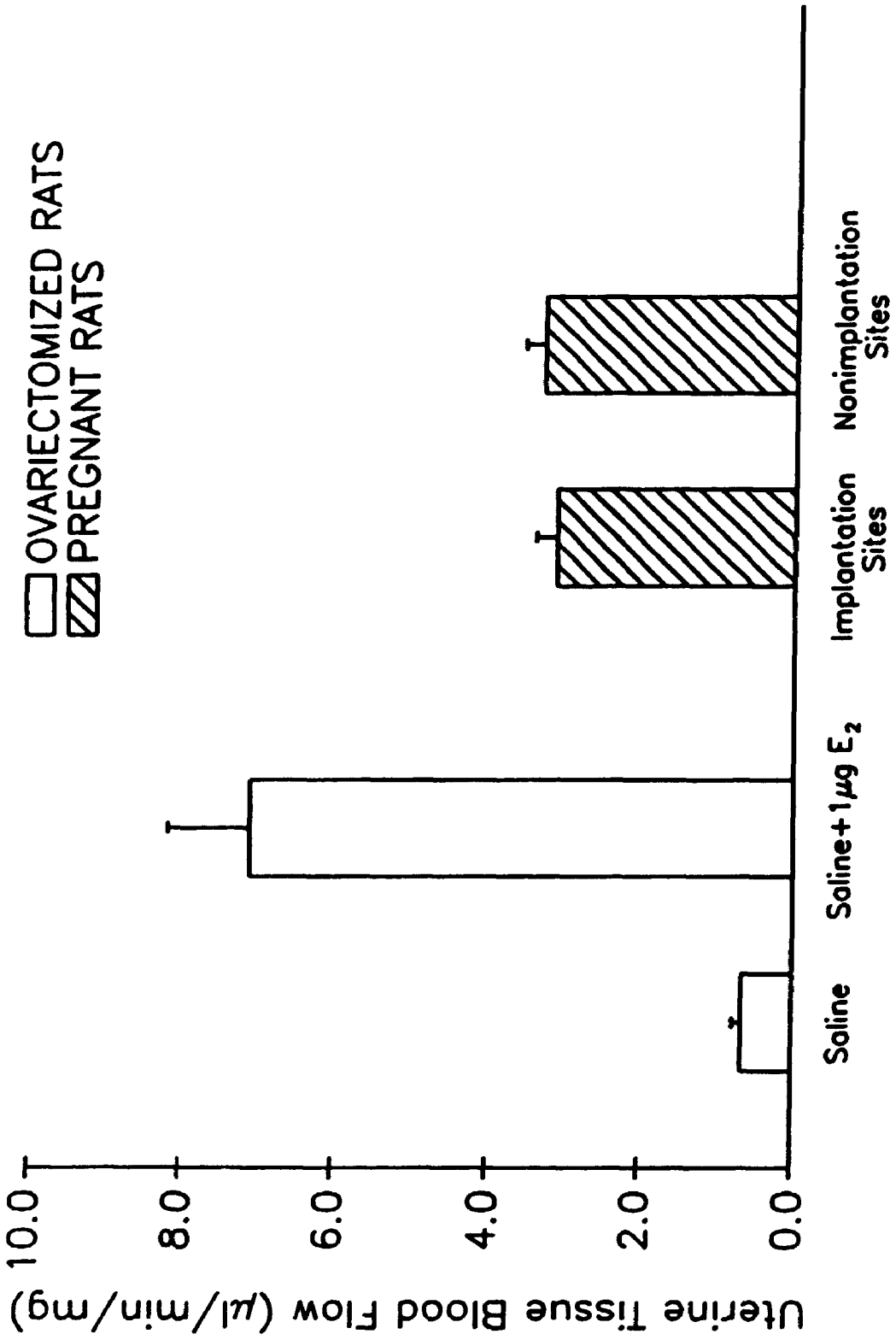
In order to verify that the microsphere method could identify differences in uterine tissue BF when performed as described in section 3.7, uterine tissue BF was estimated in ovariectomized rats 20 minutes after i.v. injection of 0.9% saline or 0.1 μg E_2 in 0.9% saline. In addition, uterine tissue BF was estimated in implantation sites (Evans blue dye sites) and nonimplantation sites of Day 6 pregnant rats. The values for uterine tissue BF were then compared to those published previously (Phaily and Senior, 1978; McRae and Heap, 1988b), respectively.

Results: In ovariectomized rats, i.v. injection of E_2 produced a ten-fold increase in uterine tissue BF (7.1 $\pm$ 1.1 μl/min/mg) compared to saline-injected animals (0.7 $\pm$ 0.1 μl/min/mg; *FIGURE A2.1*). In pregnant animals, uterine tissue BF was similar for implantation sites and uterine tissue between implantation sites (3.1 $\pm$ 0.3 and 3.3 $\pm$ 0.3 μl/min/min, respectively; *FIGURE A2.1*).

Conclusions: These values for uterine tissue BF are similar to those reported previously for ovariectomized (Phaily and Senior, 1978) and pregnant (McRae and Heap, 1988b) rats when assessed by the radioactive microsphere method. The uterine tissue BF for implantation sites and nonimplantation sites were not statistically significant, a finding which is not consitstent with that reported previously (McRae and Heap, 1988b).

However, the values presented in *FIGURE A2.1* are based on only 5 animals. The large difference in uterine tissue BF for saline-treated and E_2-treated rats (*FIGURE A2.1*) indicates that the radioactive microsphere method is capable of identifying changes in uterine BF when performed as described in section 3.7.

FIGURE A2.1. Uterine tissue BF (μl/mg/min) for ovariectomized rats and Day 6 pregnant rats as assessed by the radioactive microsphere method described in section 3.7.


OVARIECTOMIZED RATS
PREGNANT RATS
Uterine Tissue Blood Flow (μl/min/mg)
10.0
8.0
6.0
4.0
2.0
0.0
Saline
Saline+1μg E2
Implantation Sites
Nonimplantation Sites

APPENDIX 3: Gd-DTPA ENHANCED MAGNETIC RESONANCE IMAGING OF UTERI REMOVED FROM DAY 6 PREGNANT RATS

A3.1. INTRODUCTION

Results presented in Chapter 5 indicate that Gd-DTPA enhanced MR imaging can identify uterine vascular changes associated with artificial deciduogenic stimulation of rats. The following experiment was conducted to determine if a similar approach can identify sites of embryo implantation in uteri removed from rats within 24 hours of the initiation of implantation.

A3.2 METHODS

A3.2.1 Preparation of Pregnant Animals: Animals were made pregnant as described previously (Hamilton and Armstrong, 1991) and illustrated in *FIGURE A3.1*. Briefly, at 200-225 g body weight, intact rats were injected with 50 μg gonadotropin releasing hormone agonist (GnRH-a) and 4 days later (proestrus), animals were placed with males of proven fertility. The following day was taken as Day 1 of pregnancy when sperm was identified in vaginal smears.

A3.2.2 Magnetic Resonance Imaging: On the morning of Day 6 of pregnancy, 0.8 ml of a 1:1 mixture of Gd-DTPA and Evans blue was injected into a tail vein as described in section 5.2.2. Animals were killed by decapitation 15 minutes later and uteri were

removed and imaged as described in section 5.2.2.

A3.3 RESULTS

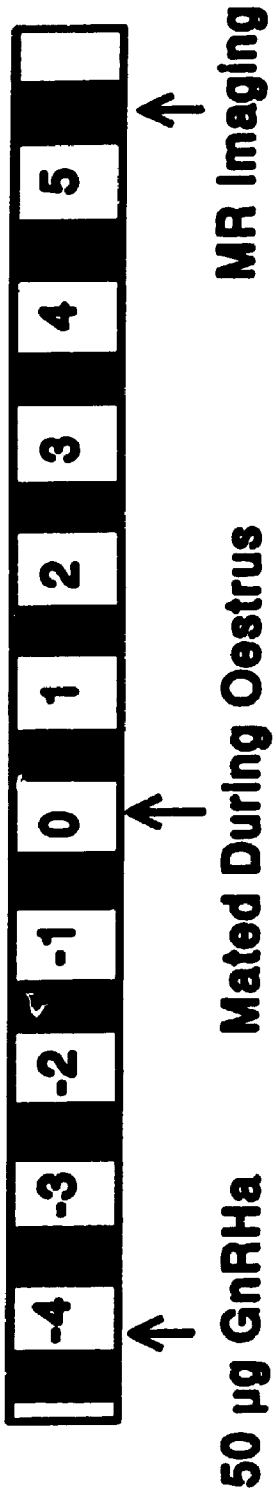
A3.3.1 MR Imaging of Uteri from Day 6 Pregnant Animals

Intravenous injection of Evans blue dye and Gd-DTPA produced punctate areas of macroscopic blueing (*PLATE A3.1a*) and similar patterns of MR image enhancement at corresponding sites of the uterus (*PLATE A3.1b*).

A3.4. DISCUSSION

The patterns of Gd-DTPA enhancement of uterine images from Day 6 pregnant animals further support the notion that uterine vascular changes are similar in the decidualizing tissue of both artificially-stimulated uteri and sites of ovoimplantation. This is not surprising since these decidualizing tissues are similar in histological appearance (Lundkvist and Ljungkvist, 1977; Lundvist et al. 1977) and in response to a variety of i.v.-injected tracers including Evans blue dye (Psychoyos, 1973), ^{125}I-albumin (Kennedy, 1979; McRae and Heap, 1988) and ^{51}Cr-EDTA (McRae and Heap, 1988), and section 4.3.1 of this thesis.

FIGURE A3.1. Schematic representation of protocol for obtaining pregnant rats. Black spares indicate periods of darkness and numbers within light squares indicate day of pseudopregnancy.


-4
-3
-2
-1
0
1
2
3
4
5
50 µg GnRHa
Mated During Oestrus
MR Imaging

(a)

(b)

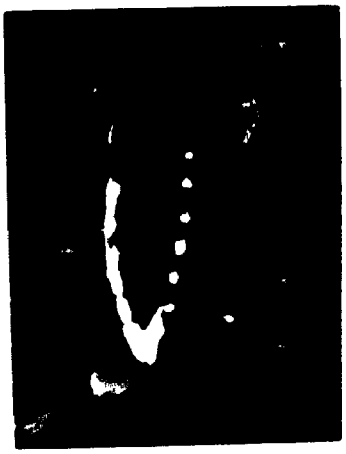


PLATE A3.1. Comparison of patterns of macroscopic blueing and Gd-DTPA MR image enhancement for uteri removed from day 6 pregnant rats. a) Photograph of uterus removed from a rat 80 minutes after i.v. injection of Gd-DTPA and Evans blue dye. **(Magnification: approximately 1x)** b) MR image of a uterus removed from a similarly treated animal, 20 minutes after injection of the mixture **(Magnification: approximately 0.7x).**

APPENDIX 4: IDENTIFICATION OF SITES OF EMBRYO IMPLANTATION IN ANESTHETIZED RATS BY Gd-DTPA ENHANCED MAGNETIC RESONANCE IMAGING

A4.1 INTRODUCTION

In rodents, it is possible to demonstrate sites of blastocyst implantation early in the process by the presence of stained areas on uteri removed after intravenous injection of dyes such as Evans blue (Psychoyos, 1973; McRae and Heap, 1988) (Appendix 3, *PLATE A3.1a*). The dyes bind to serum proteins and accumulate in the interstitial tissue around implanting embryos through a combination of local vascular changes. The endometrial "blueing reaction" makes it possible to identify sites of blastocyst implantation in dissected uteri many hours before proliferation and differentiation of endometrial stromal cells make these sites visible as uterine swellings (De Feo, 1967). Results presented in Chapter 5 and Appendix 3 indicated that sites of implantation and imminent decidualization can be identified on magnetic resonance (MR) images of uteri removed from rats after intravenous injection of Gd-DTPA. This contrast agent equilibrates amongst extracellular fluids, enhances T_1-weighted MRIs in a concentration-dependent manner and is eliminated via the kidneys within several hours of injection(Weinmann et al. 1984; Brasch et al. 1984). Furthermore, Gd-DTPA enhanced MRI is a safe, non-invasive procedure which has been used in humans and various animal models to demonstrate a variety of physiological and pathological processes

involving changes in ECFV, VP or both. Therefore, this chapter describes attempts to identify sites of early embryo implantation in intact animals by Gd-DTPA enhanced MRI.

A4.2 METHODS

Female rats were made pregnant as described in Appendix 2 (*FIGURE A3.1*). On the evening of Day 5 of pregnancy, approximately 12 hours after initiation of embryo implantation, animals were anesthetized with diazepam and sodium pentabarbital and prepared for imaging. The small size and the random, meandering arrangement of the rat uterine horns make it difficult to identify them on MR images. Therefore, rubber cuffs were placed around individual uterine horns to arrange the two horns in a uniform plane and in close proximity to each other. Uterine horns were exposed via midventral incisions and cuffs made by slitting pieces of rubber tubing (approximately 25 mm long, 4 mm internal diameter, 5 mm external diameter) were placed around individual horns. Mesometrial adipose tissue with the associated blood vessels was gently pulled through the slits and the ends of the 2 tubes were tied together loosely. The uteri then were returned to the abdominal cavity and the incision was sutured. Right femoral veins were catheterized with polyethylene tubing (0.8 mm diameter) for delivery of 1 ml Gd-DTPA diluted 1:10 in 0.9% saline. The surgical procedures did not interfere with the vascular changes associated with the endometrial blueing reaction since this response was still producible by injection of Evans blue dye into the catheter. Animals were imaged

in a 8 cm wrist coil using a 1.5 T GE Signa system. After uteri were localized with a brief T_1-weighted coronal scan, the volume of abdomen containing the uterine horns was imaged before and after Gd-DTPA injection using T_1-weighted, 3D "spoiled gradient recalled acquisition in a steady state" (SPGR; TR=50 msec, TE=11 msec) sequences. This approach allows subsequent reformatting and image analysis to be performed on slices of tissue taken from any region within the volume imaged (Harms et al. 1992). Uterine images were generated by Interactive Vascular Imaging (IVI), typically used to reconstruct MRI angiograms.

A4.3 RESULTS

Prior to injection of the contrast agent (Gd-DTPA), uteri were localized with brief scans using the transverse plane. Since the uteri and their surrounding rubber cuffs were easily identified on the MRIs (black rings of rubber cuff with grey tissue within), these transverse views were used to define the minimum volume of abdomen containing the uterine horns. It was this volume of tissue which was imaged with the SPGR sequence. Longitudinal views of both uterine horns were obtained by examining images of the volume of tissue in many different planes. Uterine tissue throughout these unenhanced images was homogeneous in appearance and implantation sites could not be identified. SPGR images obtained immediately after Gd-DTPA injection (scanning time approximately 8 minutes) displayed enhancement of a variety of tissue types as is characteristic for this contrast agent. Uterine tissue exhibited excellent contrast

enhancement which allowed easy identification of both uterine horns on these images. Furthermore, analysis of the SPGR signal information by Interactive Vascular Imaging (IVI) generated an abdominal image containing most of both uterine horns (*PLATE A4.1*). This type of image generation identifies tissues exhibiting strong enhancement and is generally used for reconstruction of MRI angiograms (Harms et al. 1992), hence the strong enhancement of the vasculature on the image. For all four pregnant animals imaged, the uteri on the images displayed punctate patterns of enhancement resembling peas in a pod (*PLATE A4.1*). It can be seen that the enhancement of the implantation sites is greater than that of the blood vessels and similar to that of the bladder where the Gd-DTPA has been concentrated by the kidneys for elimination from the body.

A4.4 DISCUSSION

The results of this study clearly show that Gd-DTPA enhanced MRI can allow identification of sites of embryo implantation in intact animals during the first day of the process. The punctate patterns of enhancement on the uterus are similar to those shown in Appendix 3 (*PLATE A3.1b*) for images of uteri removed from rats after injection of Gd-DTPA during the same time of pregnancy. In addition, the patterns match those of macroscopic blueing after i.v. injection of Evans blue dye (Psychoyos, 1973; McRae and Heap, 1988) (*PLATE A3.1a*, Appendix 3). Since increased uterine ECFV appears largely responsible for the enhancement of implantation sites in dissected uteri (Chapter 5), the similar appearance of implantation sites in intact animals suggests that local

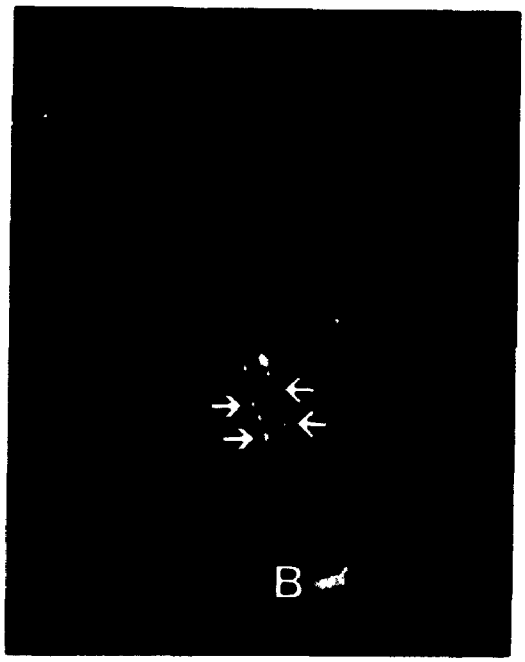


PLATE A4.1. T_1-weighted Interactive Vascular Imaging (IVI) of a rat abdomen approximately 10 h after the onset of embryo implantation. Arrows indicate punctate areas of enhancent at implantation sites along the 2 uterine horns. The bladder is labeled (B) at the bottom of the figure (**Magnification: approximately 0.5x**).

increases in ECFV also play a major role in the image enhancement of these sites in intact rats. High uterine VP and BF undoubtly contribute to the excellent enhancement of uterine images since both these vascular variables are elevated during embryo implantation in the rat (McRae and Heap, 1988) and since both are known to contribute to image enhancement of a variety of other tissues (Kenney et al. 1992).

The ability to examine early implantation sites in intact animals has important potential applications for both clinical and experimental settings. In experimental settings, the ability to examine the endometrial vascular changes in progress within intact animals will provide access to information regarding embryo implantation previously unavailable by conventional approaches. Although the vascular changes which comprise the endometrial blueing reaction are known to be essential for progression to later events of pregnancy (Psychoyos, 1973), relatively little is known about the mechanism by which this occurs.

In rodents and primates, embryo implantation is similar, both in invasiveness and in many of the associated uterine responses (De Feo, 1967). These similarities suggest that contrast-enhanced MRI may permit the identification of early sites of embryo implantation in humans. Such an approach might be applied to fertility programs to determine whether failed pregnancies are due to failures of embryo implantation or to spontaneous abortion of implanted embryos. This information is important since cases of spontaneous abortion have been estimated to exceed 30% even in couples having normal fertility (Wilcox et al. 1988). Thus, Gd-DTPA enhanced MRI could be used to examine cases of idiopathic infertility where poor fertilization and obstructed egg

transport do not appear to be causes. Alternatively, it might be used to examine the fates of embryos transferred back to the reproductive tract after in vitro fertilization.

Studies involving histological examination of endometrial biopsies (Noyes et al. 1950) suggest that some endometrial vascular changes associated with embryo implantation in rodents actually occur during nonpregnant human menstrual cycles as a result of normal luteal function. Because these changes occur at a time after ovulation when implantation would ensue if fertilization had occurred (Noyes et al. 1950), Gd-DTPA enhanced MRI also offers a potential replacement for the invasive endometrial biopsy currently used to diagnose luteal phase defects associated with female infertility (Hecht et al. 1990; Yeko et al. 1992; Johannisson et al. 1987).

In summary, Gd-DTPA enhanced MRI provides a useful tool for identifying uterine vascular changes associated with embryo implantation. It is hoped that this approach will have important applications in both experimental and clinical settings.

APPENDIX 5: T_1 RELAXIVITIES OF Gd-DTPA, ALBUMIN AND Gd-DTPA-ALBUMIN

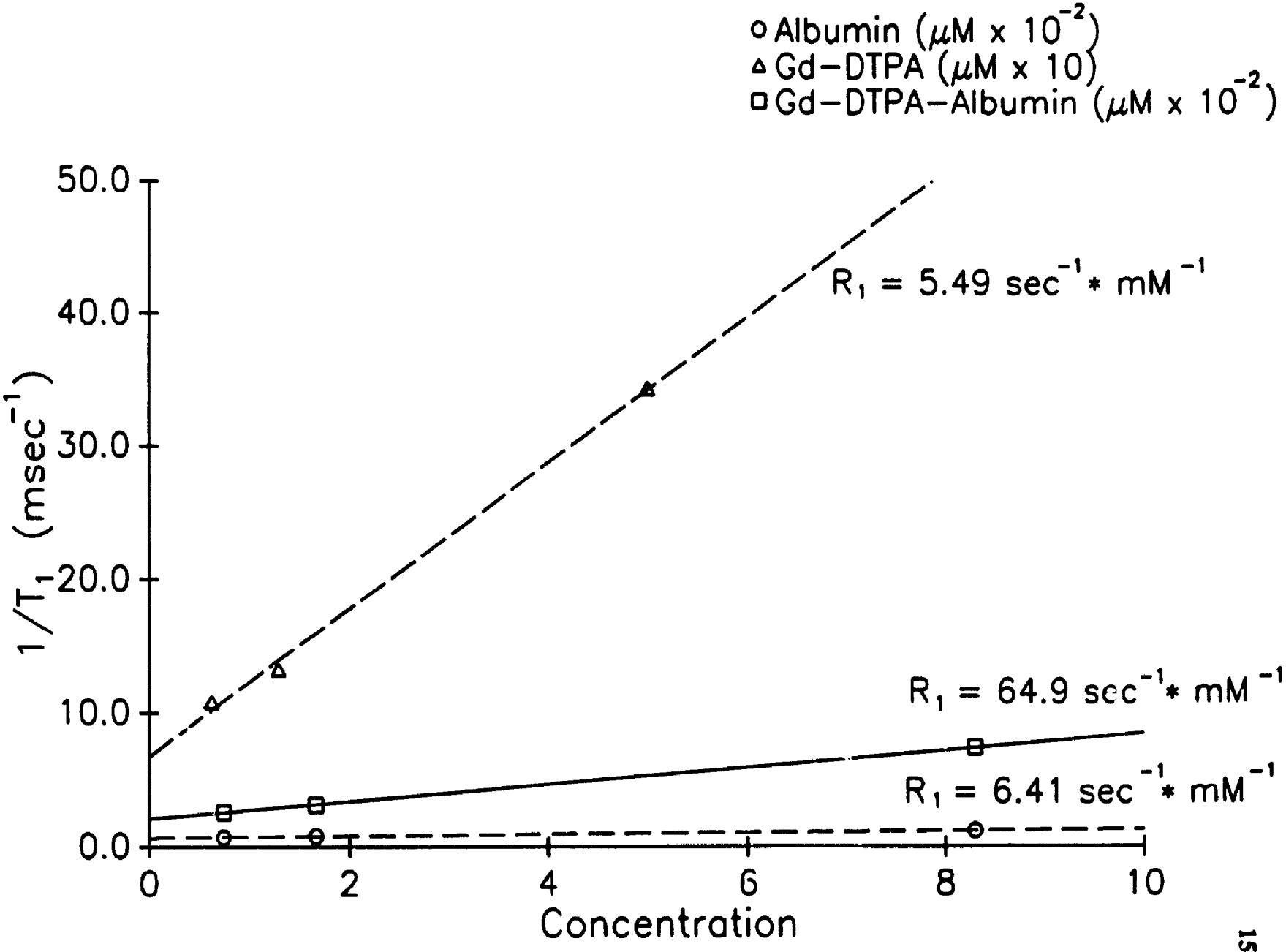
In order to assess the capacity of the Gd-DTPA-ablumin to enhance T_1-weighted images, the relaxivity was calculated. This value relates the concentration of a contrast agent to the T_1 relaxation time and is calculated from the slope of the line plotting concentration of the compound against $1/T_1$. Solutions of Gd-DTPA, albumin and Gd-DTPA albumin were serially diluted in 0.9% saline and 2 ml alliquots were placed in 25 mm tubes. T_1 relaxation times were measured on a Praxis II NMR (Praxis Corporation, Texas) at 37°C and 10.7 MHz, by saturation recovery sequences.

Results: Combination of Gd-DTPA with albumin produced a compound with a relaxivity significantly greater than Gd-DTPA (*FIGURE A5.1*) and much greater than albumin alone. The relaxivity of Gd-DTPA suggests that approximately 2 Gd-DTPAs were attached per albumin molecule (this number is inferred because the relaxivities of individual compounds are additive such that $1/T_1$ of the solution = [alb] x $1/T_{1albumin}$ + [Gd-DTPA] x $1/T_{1Gd\text{-}DTPA}$).

Conclusion: Although the higher relaxivity of Gd-DTPA-albumin compared to Gd-DTPA appears promising, the high molecular weight of albumin means that Gd-DTPA-albumin would have to be injected at a concentration in excess of 5 mg/ml to achieve the molar concentration of Gd-DTPA used for the experiment reported in Appendix 5. Since the

uterine VP assessment which uses ^{125}I-albumin requires only 40μg (2 mCi of 50 μCi/μg albumin), this concentration of Gd-DTPA-albumin is more than 100-fold greater than that injected for the deterimination of uterine ^{125}I-albumin volumes of distribution.

FIGURE A5.1. T_1 relaxivities of albumin, Gd-DTPA albumin and Gd-DTPA as determined by plotting compound concentration against $1/T_1$.


Albumin (μM x 10^{-2})
Gd–DTPA (μM x 10)
Gd–DTPA–Albumin (μM x 10^{-2})
R_1 = 5.49 sec^{-1} * mM^{-1}
R_1 = 64.9 sec^{-1} * mM^{-1}
R_1 = 6.41 sec^{-1} * mM^{-1}
$1/T_1$ ($msec^{-1}$)
50.0
40.0
30.0
20.0
10.0
0.0
0
2
4
6
8
10
Concentration

APPENDIX 6: CHARACTERIZATION OF PGE_2 RADIOIMMUNOASSAY

Extracts from homogenates of infused and noninfused uterine horns were assayed for PGE_2 as described previously (Olson et al. 1984). Briefly, 150 μl aliquots of uterine homogenates were dried under nitrogen gas, resuspended in gelatin-PBS and assayed in triplicate. By extrapolation, the minimum detectable dose (registering at 2 standard deviations above the reagent blank) was less than or equal to 5 pg/tube and concentrations used for the standard curves ranged from 17.8 to 1000 pg PGE_2/tube. Intra- and inter-assay coefficients of variation were 3.9% and 4.8%, respectively using 500 pg of standard. Cross-reactivities of the antibody at 50% displacement of [^{3}H]PGE_2 are displayed in *TABLE A6.1*.

TABLE A6.1: Cross-Reactivity Characteristics of PGE_2 Antibody

Compound	*% Cross-reaction*
PGE_2	100
PGE_1	1.6
PGA_2	0.46
PGB_2	0.001
$PGF_{1\alpha}$	0.7
$PGF_{2\alpha}$	2.0
6-keto PGE_1	2.7
6-keto $PGF_{1\alpha}$	2.2
15-keto PGE_1	0.46
15-keto PGE_2	1.1
15-keto $PGF_{2\alpha}$	0.04
13,14-dihydro-15-keto PGA_1	0.02
13,14-dihydro-15-keto PGE_1	0.04
13,14-dihydro-15-keto $PGF_{2\alpha}$	0.04

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