THE ROLE FOR INSULIN-LIKE GROWTH FACTOR-I IN PREIMPLANTATION
EMBRYONIC DEVELOPMENT AND DECIDUALIZATION FOLLOWING
SUPEROVULATION IN THE RAT

by

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We accept this thesis as conforming to
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Date April 22, 1996
ABSTRACT

Superovulation causes detrimental effects, including embryonic loss and implantation failure. This study determined potential roles for insulin-like growth factor (IGF-I) in uterine environment regulation and preimplantation development, in conjunction with the detrimental effects of superovulation in the rat.

IGF-I may be beneficial to preimplantation embryonic development. IGF-I stimulated embryonic development and metabolism \textit{in vitro} and increased the rate of implantation and fetal development when the blastocysts were transferred into a receptive uterus.

However, IGF-I may be involved in embryonic loss following superovulation, by perturbing the uterine environment. Superovulation enhanced uterine IGF-I action from day 1 to 3 of pregnancy and reduced its action on days 5 and 6. Uterine luminal fluid from the uterus exposed to increased IGF-I action from day 1 to 3 was detrimental to embryonic development. This uterine luminal fluid had altered electrolyte composition that is similar to that observed following superovulation. Anti-IGF-I antibody restored superovulation-induced alterations in cations, suggesting that IGF-I may partially mediate this effect of superovulation.

Superovulation-induced alterations in IGF-I action may adversely affect decidualization, a critical step in implantation. High IGF-I levels from day 1 to 3 and low levels from day 3 to 5, inhibited decidual formation. Alterations in IGF-I action after day 5 had no effect, suggesting a role for IGF-I in uterine sensitization.

The role of IGF-I in decidualization may be complex. IGF-I cannot be substituted for growth hormone (GH) and thyroxine (T_4) during decidualization, but altered decidual formation and alkaline phosphatase (ALP) activity in the GH and T_4-dependent manner. IGF-I either stimulated or inhibited decidual formation and ALP activity, depending upon concentration and treatment period. IGF-I stimulated basal ALP activity
but inhibited prostaglandin E2-stimulated ALP activity in the endometrial stroma cells.

In conclusion, IGF-I may play an important role in the maintenance of a receptive uterine environment for embryonic development and the regulation of decidualization. Embryonic loss and failure of implantation following superovulation may be partially attributed to disturbances in uterine IGF-I action as observed in this study.
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<tr>
<td>ALP</td>
<td>alkaline phosphatase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>BW</td>
<td>body weight</td>
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<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>°C</td>
<td>degree of Celsius</td>
</tr>
<tr>
<td>Ci</td>
<td>curie (= 3.7 x 10^{10} disintegrations per second)</td>
</tr>
<tr>
<td>cm</td>
<td>centimeter</td>
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<tr>
<td>cpm</td>
<td>radioactive counts per minute</td>
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<tr>
<td>CSF</td>
<td>colony stimulating factor</td>
</tr>
<tr>
<td>DMEM:F-12</td>
<td>Dulbecco's modified Eagle's medium:Ham's F-12 nutrient mixture</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco's phosphate buffered saline</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>eFSH</td>
<td>equine follicle stimulating hormone</td>
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<tr>
<td>eLH</td>
<td>equine luteinizing hormone</td>
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<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
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<tr>
<td>ET</td>
<td>embryo transfer</td>
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<tr>
<td>FCS</td>
<td>fetal calf serum</td>
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<td>FSH</td>
<td>follicle stimulating hormone</td>
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<tr>
<td>g</td>
<td>gram</td>
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<tr>
<td>g</td>
<td>gravity</td>
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<tr>
<td>GH</td>
<td>growth hormone</td>
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<tr>
<td>h</td>
<td>hour</td>
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<tr>
<td>^3H</td>
<td>tritium, a radioactive isotope of hydrogen</td>
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<tr>
<td>HBSS</td>
<td>Hank's balanced salt solution</td>
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<tr>
<td>hCG</td>
<td>human chorionic gonadotropin</td>
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<td>hMG</td>
<td>human menopausal gonadotropin</td>
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<tr>
<td>hr-IGF-I</td>
<td>human recombinant insulin-like growth factor</td>
</tr>
<tr>
<td>^125I</td>
<td>a radioactive isotope of iodine</td>
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<tr>
<td>ICM</td>
<td>inner cell mass</td>
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<tr>
<td>IGF</td>
<td>insulin-like growth factor</td>
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<td>IGFBP</td>
<td>insulin-like growth factor binding protein</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>IU</td>
<td>international unit</td>
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<tr>
<td>IVF</td>
<td>in vitro fertilization</td>
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<tr>
<td>kDa</td>
<td>kilodalton</td>
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<tr>
<td>kb</td>
<td>kilobase</td>
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<tr>
<td>l</td>
<td>liter</td>
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<tr>
<td>LH</td>
<td>luteinizing hormone</td>
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<tr>
<td>LIF</td>
<td>leukemia inhibitory factor</td>
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<tr>
<td>M 16</td>
<td>embryo culture media</td>
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<tr>
<td>M199</td>
<td>medium 199</td>
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<tr>
<td>mCi</td>
<td>millicurie</td>
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<td>mg</td>
<td>milligram</td>
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<td>mg</td>
<td>microgram</td>
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<td>ml</td>
<td>milliliter</td>
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<td>ml</td>
<td>microliter</td>
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<td>mM</td>
<td>millimolar</td>
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<td>nM</td>
<td>nanomolar</td>
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<tr>
<td>ng</td>
<td>nanogram</td>
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<tr>
<td>PG</td>
<td>prostaglandin</td>
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<tr>
<td>pGH</td>
<td>porcine growth hormone</td>
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<tr>
<td>pH</td>
<td>$\text{-log } H^+$ concentration in a fluid</td>
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<tr>
<td>PMSG</td>
<td>pregnant mare's serum gonadotropin</td>
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<tr>
<td>PVA</td>
<td>polyvinylalchohol</td>
</tr>
<tr>
<td>RIA</td>
<td>radioimmunoassay</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of means</td>
</tr>
<tr>
<td>T4</td>
<td>thyroxine</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>TSH</td>
<td>thyroid stimulating hormone</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
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<tr>
<td>v/v</td>
<td>volume per volume</td>
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INTRODUCTION

Superovulation, in association with in vitro fertilization-embryo transfer (IVF-ET), and related techniques are now commonly used in the treatment of human infertility. With few exceptions, standard superovulatory protocols currently use pharmacological dosages of exogenous gonadotropins to obtain multiple oocytes. Fertilized embryos are then replaced into a disturbed uterine environment. Many studies aimed at improving superovulatory treatments have been focused on the ovarian functional aspects in order to obtain maximum number of fertilizable oocytes. Hence, an attempt of ovarian stimulation has typically been determined by the number and size of follicles and the level of ovarian steroid hormones that include estrogen and progesterone. Aspects of uterine function have been largely disregarded with the exception of monitoring of uterine endometrial thickness.

Recent studies have confirmed that normally functioning endometrium and a receptive uterine environment play a key role in preimplantation embryonic development and the establishment of successful pregnancy. This is compatible with high pregnancy rates observed in IVF-ET cases where the patient has not been subjected to ovarian stimulation and oocyte donation programs. In these cases, embryos are transferred to a receptive uterine environment which was not been subjected to ovarian hyperstimulation. The ability to induce development of multiple follicles and to manage the physiological status of the uterine endometrium is fundamental to the success of superovulatory treatments in a clinical setting. Further studies to improve superovulatory treatments with respect to achieving normal uterine function and a receptive uterine environment for the preimplantation embryo is clearly needed.

It is apparent that a large part of ovarian steroid hormone action is mediated by a complex local regulatory network of growth factors and cytokines in the uterus. The insulin-like growth factor (IGF) system that consists of IGF-I, IGF-I receptor, and IGF binding proteins is a part of the regulatory network. Potential roles for the uterine IGF
system in the regulation of the uterine environment and preimplantation development following superovulation are the main focus of this study. The long term goals of this study are to determine the mechanisms by which superovulatory treatments affect the uterine environment and the development of the preimplantation embryo, and the subsequent establishment of a successful pregnancy. This study was performed in an attempt to determine changes in the uterine IGF system following superovulation and their significance in preimplantation embryonic development and implantation in the rat.

UNDERLYING HYPOTHESIS

The uterine environment for preimplantation embryonic development is predominantly regulated by ovarian steroid hormones. A disturbed ovarian steroid hormone balance, especially highly elevated estrogen levels, or estrogen/progesterone ratio, following superovulation results in a variety of detrimental effects. Since the uterine IGF system is regulated by ovarian steroid hormones, this system may mediate detrimental effects such as early embryonic loss and failure of implantation by altering uterine receptivity. The disturbed IGF system may render the uterine microenvironment detrimental to preimplantation embryonic development. The disturbed IGF system may also affect uterine sensitization to a deciduogenic stimulus and endometrial stromal cell decidualization. This in turn results in failure of implantation.

OUTLINE OF THE THESIS

Chapter one reviews available information that is related to the topic of this study. The literature review is followed by chapter two that shows the beneficial effect of IGF-I on preimplantation embryonic development in vitro (Fig. 1). Chapter three examines alterations in the uterine IGF system following superovulation by using an immature rat.
superovulation model (Fig. 2). Chapter four and chapter five determine significance of the alterations in the uterine IGF system, caused by superovulation, in preimplantation embryonic development and implantation. This is followed by the study to determine the potential mechanisms by which the altered uterine IGF system increased an early embryonic loss and impaired blastocyst implantation. In particular, chapter four examines the role of IGF-I in the regulation of uterine environment for embryonic development, which includes the ionic composition of the uterine luminal fluids (Fig. 3). Chapter five focuses on decidualization, a crucial step in implantation in the rats. The role of IGF-I in the uterine sensitization process for the decidual reaction is determined (Figs. 4 and 5).
CHAPTER TWO

Embryo culture with IGF-I

- Embryo development
- Cell count
- Protein synthesis
- Embryo transfer

Figure 1 The effect of IGF-I on preimplantation embryonic development in vitro is determined. The 8-cell stage rat embryos are cultured for 36 h in the presence of IGF-I of varying concentrations. The effect of IGF-I on embryonic development is determined by the developmental stages of embryos, the number of cells in the resulting blastocysts, and the levels of protein synthesis in the blastocysts. The blastocysts are also transferred into a receptive uterus and the ability of the blastocysts to implant and develop into day 18 fetuses determined.
Figure 2 The effect of superovulatory treatment on the uterine IGF system is determined in an immature rat superovulation model. Immature female rats are injected with a single dose of 4 IU (control) pregnant mare's serum gonadotropin (PMSG) or 40 IU (superovulation) PMSG to achieve a pregnancy. Levels of IGF-I, total IGF binding protein (IGFBP), and IGF-I receptor are determined in the uterus and serum from day 1 to day 6 of pregnancy. The levels of IGF-I is determined by radioimmunoassay (RIA) and the levels of total IGFBP and IGF-I receptor determined by ligand binding assays. The serum levels of estradiol-17β and progesterone are determined by RIA from the day of the PMSG injection (day -2) to day 6 of pregnancy.
Figure 3  The potential mechanisms by which an altered uterine IGF system, caused by superovulation, increases the rate of embryonic loss are determined. A condition (concentration and infusion rate) for IGF-I infusion that achieves an increase in uterine IGF-I action after superovulation is determined. Eight-cell stage rat embryos are cultured with the uterine luminal fluids (ULF) obtained from the IGF-I infused rats, superovulated rats, and superovulated and anti-IGF-I antibody infused rats to determine if an increase in IGF-I action in the uterus mediates early embryonic, loss caused by superovulation, through an alteration of uterine environment. Electrolyte composition of each ULF is determined in an effort to identify detrimental factors in the ULF.
Figure 4  The role for IGF-I in the uterine sensitization process for the decidual reaction is determined. First, rats are ovariectomized and treated with ovarian steroid hormones for maximal uterine sensitization and are infused with IGF-I or anti-IGF-I antibody during the different time periods (day 1-3, day 3-5, and day 5-9). An artificial deciduogenic stimulus was given on day 5 and the degree of deciduoma formation were determined on day 9. Secondly, the potential mechanisms of IGF-I action in the regulation of uterine sensitization in conjunction with growth hormone (GH) and thyroid hormone (T4). Pituitary intact, hypophysectomized, and hypophysectomized-GH and T4 treated rats are ovariectomized and treated with ovarian steroid hormones. Rats are infused with IGF-I or anti-IGF-I antibody during the uterine sensitization period or from the sensitization period throughout the time of deciduoma formation. An artificial deciduogenic stimulus is given on day 5 of pseudopregnancy. The degree of deciduoma formation and levels of uterine alkaline phosphatase activity are determined on day 9 of pseudopregnancy.
The role for IGF-I in the regulation of alkaline phosphatase activity is further studied in the uterine endometrial stromal cells that is undergoing decidualization. Rats are ovariectomized and treated with ovarian steroid hormones for maximal uterine sensitization. Some rats are infused with IGF-I during the uterine sensitization period. Uterine endometrial stromal cells are obtained from the uterus after the uterine endometrium is sensitized (day 5) and are treated with varying concentrations of IGF-I. Some uterine endometrial stromal cells are treated with prostaglandin E₂ (PGE₂) in addition to IGF-I. Alkaline phosphatase activity in the endometrial stroma cells are determined after the 48 h culture period.
CHAPTER ONE

LITERATURE REVIEW

I. SUPEROVULATION

A. General

a. History

Superovulation is now commonly used in human infertility clinics, in the livestock industry and in basic reproductive biology research. The very first attempt of ovulation induction or superovulation was made in the effort of defining the role of the pituitary gland in the reproductive system, using laboratory species. The importance of the pituitary in the regulation of reproductive system became apparent during the first quarter of this century. This led to an attempt of using implants of pituitary tissue to stimulate follicular growth which resulted in multiple follicular growth and ovulation (Engle, 1927; Smith and Engle, 1927). Implants of pituitary tissue were soon replaced by pituitary extracts or pregnant mare's serum.

Biologically active elements have been separated from the pituitary extracts (follicle stimulating hormone: FSH and luteinizing hormone: LH), pregnant mare's sera (pregnant mare's serum gonadotropin: PMSG), human placentae (human chorionic gonadotropin: hCG) and urine of women in the post-menopausal period (human menopausal gonadotropin: hMG). PMSG and hMG are rich sources of FSH activity but they also contain a considerable amount of LH activity. In contrast, hCG only contains LH activity and acts through the LH receptor. In humans and laboratory animals, the standard superovulatory treatment employs gonadotropins with FSH activity to stimulate multiple follicular development in combination with LH activity to induce ovulation and oocyte maturation. PMSG has become one of the most widely used sources of FSH activity in laboratory animals, while hMG is a preferred source of FSH activity for clinical use in
humans. hCG is the standard choice as a source of LH activity in laboratory animals and humans.

b. Mechanism

The mechanisms used by exogenous gonadotropins to achieve multiple follicular development and ovulation have been previously described. Exogenous gonadotropins appear to recruit a non-growing pool of small follicles and transform them into a group of maturing follicles (Greenwald, 1973). Superovulation is also believed to involve the rescue of atretic follicles (Peters et al., 1975; Byskov, 1978; Hay and Moor, 1978; Byskov, 1979; Braw and Tsafriri, 1980; Fujimori et al., 1987). However, the term of "rescue" is ambiguous and misleading. Some investigators do not distinguish between "rescue" of follicles from atresia and "recruitment" of small follicles, as they believe that most of the small follicles will undergo atresia unless they are stimulated by exogenous gonadotropins to the ovulatory cohort of the present cycle. In contrast, several investigators use the term of "rescue" based on the evidence that a reduced ratio of atretic follicles to healthy non-atretic follicles or total number of follicles is observed following hormonal treatments. In this case, the term of "rescue of follicle from atresia" may be replaced by "reversal of atresia", while in the former case "rescue of follicle from atresia" may be replaced by "prevention of atresia" (Hirshfield, 1989).

Evidence suggests that hormonal treatments could stop or reverse the atretic process (Tsafriri and Braw, 1984). However, this is controversial, since other evidence suggests that once a follicle begins to degenerate in vivo, it will probably not return to the ovulatory pathway (Hay et al., 1979; Hirshfield, 1989). A new in vivo labeling technique with [3H]thymidine which allows us to examine the reversal of atresia in an individual follicle demonstrated that follicular atresia was an irreversible process (Hirshfield, 1989). A decreased ratio of atretic follicles to the total number of follicles following hormonal treatments may indicate the reversal of atresia by the hormonal treatments. However, a
reduction in the ratio of atretic follicles may also be the result of an increase in the number of newly developed follicles arising from a reserve of small follicles recruited by the hormonal treatments. Thus, it is unclear if "reversal of atresia" can be considered to be a mechanism through which exogenous gonadotropins achieve superovulation.

B. Pregnant Mare's Serum Gonadotropin (PMSG)

a. Gonadotropin for Superovulation

The strong FSH activity and the extraordinary long half-life of PMSG allows us to induce follicular development and superovulation with a single dose of this hormone in species other than the horse. This is a great advantage when a large number of animals are to be treated. Furthermore, the availability of commercial preparations at a low cost has led to a widespread use of this gonadotropin in basic research and in the livestock industry. Although the role of PMSG in maintaining pregnancy in the horse may be controversial, PMSG is undoubtedly an indispensable pharmacological agent in reproductive biology and in the livestock industry.

b. Origin, Secretion, and Molecular Properties

PMSG is first detected in the serum of mares on approximately day 40 of pregnancy, peaks between days 60 and 80, and declines towards base levels on day 130 of pregnancy. PMSG, also known as equine chorionic gonadotropin, is produced by chorionic cells in the uterine endometrial cup, a pale and circumscribed plaques of tissue that develop adjacent to the chorionic girdle of the embryo (Cole and Hart, 1930; Allen, 1969; Moor et al., 1975). Evidence suggests that synthesis and secretion of PMSG appears not to be regulated by endocrine and paracrine regulatory factors (Hamilton et al., 1973; Hernandez-Jauregui and Gonzales-Angulo, 1975; Nett and Pickett, 1979; Thompson et al., 1982). Instead, the size and numbers of the endometrial cups and their histological appearance are correlated with serum PMSG levels (Murphy and Martinuk, 1991; Ginther,
1992; Squires, 1993; Hoppen, 1994). The role for PMSG in the horse pregnancy is not well understood. However, this hormone is believed to induce ovulation and subsequent luteinization of accessory corpora lutea during early pregnancy through its LH activity, and thus contributes to the maintenance of pregnancy until placental steroidogenesis reaches sufficient levels (Stewart and Allen, 1981).

PMSG is a dimeric glycoprotein that is structurally similar to the pituitary glycoprotein hormones, which include FSH, LH, and thyroid stimulating hormone (TSH), and composed of an \( \alpha \)- and \( \beta \)-subunit (Murphy and Martinuk, 1991; Hoppen, 1994). The \( \alpha \)- and \( \beta \)-subunits of PMSG are composed of 96 and 149 amino acids, respectively (Stewart and Maher, 1991; Sherman et al., 1992). The gene encoding the \( \alpha \)-subunit of PMSG is identical to that encoding the \( \alpha \)-subunit of all pituitary glycoprotein hormones in the horse (Stewart et al., 1987), while the \( \beta \)-subunit of PMSG is encoded by the same gene that encodes equine LH (eLH) (Stewart and Maher, 1991; Sherman et al., 1992). Since PMSG and eLH have identical \( \alpha \)- and \( \beta \)-subunits, only differential glycosylation appears to distinguish PMSG and eLH (Smith et al., 1993; Matsui et al., 1994).

**b. Dual FSH and LH Activity**

The most unique feature of PMSG may be its strong FSH activity, in addition to its LH activity, in non-equid species (Murphy and Martinuk, 1991; Hoppen, 1994). PMSG and eLH, as well as equine FSH, bind to the FSH receptors in all non-equid species and donkey (an equid species), but not in the horse (Licht et al., 1979; Stewart and Allen, 1981; Moudgal and Papkoff, 1982; Guillou and Combranous, 1983; Combranous et al., 1984). Some possible explanations for the unique FSH activity of PMSG and eLH have been proposed. The \( \alpha \)-subunits of equine gonadotropins possess a unique transposition and non-conservative substitutions of amino acid residues, compared with those of the other species (Stewart et al., 1987). The high amount of carbohydrates, especially sialic acid, has been suggested to be responsible for the unique FSH activity of PMSG.
(Aggarwal et al., 1980a,b; Damm et al., 1990). The β-subunit of PMSG has as high as 55.3% of carbohydrate content that is 2- to 3-fold greater than that of FSH and LH. Sialic acid comprise 21.3% of the total carbohydrate weight (Papkoff, 1978). Some molecular properties of the β-subunit of PMSG, other than glycosylation, have also been discussed in relation to the dual FSH and LH activity of PMSG (Murphy and Martinuk, 1991). However, none of these molecular properties, including glycosylation, do not appear to be a single factor responsible for the dual FSH and LH activity of equine gonadotropins. Thus, the mechanism which allows the dual FSH and LH activity of PMSG and eLH is not clear.

c. Long Half-Life

PMSG has the highest carbohydrate content among the glycoprotein hormones. The heavy glycosylation of PMSG, especially by sialic acids, gives an extraordinary long half-life in the circulation in the mare and other species (Catchpole et al., 1935; Sasamoto et al., 1972; McIntosh et al., 1975; Menzer and Schams, 1979; Katagiri et al., 1991). Although the half-life of circulating FSH is generally much shorter than that of PMSG, a varying degree of sialylation is also found to be responsible for distinct half-lives of various isoforms of FSH (Chappel et al., 1983). The subunits of PMSG/eLH have structurally distinct N-linked oligosaccharides and terminal charge groups (Damm et al., 1990; Smith et al., 1993; Matsui et al., 1994). eLH has been shown to contain mono- or di-branched oligosaccharides terminating with SO₄-4-GalNAc beta 1,4GlcNAc that bind to specific receptors in liver endothelial cells (Smith et al., 1993). On the other hand, PMSG β-subunits have di- or tri-branched oligosaccharides terminating Sia alpha 2,3 or 6Gal beta 1,4GlcNAc that are not metabolized by liver (Damm et al., 1990; Smith et al., 1993). As a result, PMSG remains in the circulation for longer period (approximately 5.7-fold) than equine LH. PMSG shows remarkable heterogeneity in the structure of N-linked chains as well as the degree of glycosylation (Aggarwal et al., 1980a,b). These heterogeneity in
glycosylation has been related to the variability in biological activity and half-life of different PMSG preparations.

C. Immature Rat Superovulation Model

a. Background

Small laboratory animals, which include rats, mice and hamsters, have been extensively utilized for basic studies in reproductive biology. Early studies used rats and mice in an attempt to induce superovulation by the daily implantation of anterior pituitary tissues (Engle, 1927; Smith and Engle, 1927). Pregnant mare's serum was then introduced to induce superovulation. A single injection of crude pregnant mare's serum or partially purified PMSG was capable of inducing superovulation and producing of greater than normal litter sizes (Cole, 1936; Cole, 1937; Cole, 1940). Induction of superovulation with PMSG has become a standard choice due to the simplicity of the procedure and availability of the material. Immature rats, immediately prior to sexual maturity at 28-30 days old, were found to be more suitable than sexually mature rats (Cole, 1937; Strauss and Meyer, 1962; McCormack and Meyer, 1963; Zarrow and Quinn, 1963). Immature rats and mice produced a greater number of oocytes compared to adults (Cole, 1937; Fowler and Edwards, 1957; Biggers et al., 1971). Two possible explanations have been proposed for the better response of immature animals to PMSG. First, a greater number of developing follicles, capable of responding to PMSG, may be available in immature rats (Jones and Krohn, 1961). Second, the timing of PMSG injection in the estrous cycle may affect the results of superovulation in adults. In particular, the time of PMSG injection to obtain an optimal result may be very limited (McLaren and Michie, 1959; Edwards et al., 1963).

b. PMSG dose

The normality of a pregnancy induced with PMSG in immature rats may be a matter of concern. Numerous studies have shown that low doses (4-8 IU) of PMSG produces a
normal pregnancy in immature rats. A low dose of PMSG induces a synchronized ovulation through eliciting an endogenous LH surge (Sorrentino et al., 1972; Wilson et al., 1974; Kostyk et al., 1978). The LH surge is preceded by an increase in the levels of estradiol-17β and progesterone which are similar to those found during proestrus in the adult (Shaikh, 1971; Butcher et al., 1974; Parker et al., 1976). Hormonal profiles, timing of oocyte maturation, and quality of oocytes in immature rats treated with a low dose of PMSG appear to be compatible to those observed in the adult (Barraclough et al., 1971; Linkie and Niswender, 1972; Hillensjo et al., 1974). In addition, treatment with a low dose of PMSG does not increase embryonic loss or fetal wastage in immature rats (Nuti et al., 1975).

In contrast, treatments with greater doses (16-40 IU) of PMSG result in reduced fertility in immature rats, although an increased number of oocytes are ovulated. Reduced fertilization rates, an increase in early embryonic loss and failure of implantation were observed in immature rats following a single injection with a greater dose of PMSG (Miller and Armstrong, 1981a; Miller and Armstrong, 1982; Walton et al., 1983; Yun et al., 1987; Yun et al., 1988; Yun et al., 1989). These detrimental effects appear to become more apparent as the PMSG dose increases. Furthermore, the detrimental effects caused by a large dose of PMSG are also observed in the adult (Booth et al., 1975; Betteridge, 1977; Moor et al., 1980; Moor et al., 1985).

c. Practical Aspect

Other than the biological aspect discussed, there are some other practical advantages in using the immature rat model. Immature rats are economical, easy to handle and are readily available from a common supplier. Immature rats are especially useful in studies associated with pregnancy or pseudopregnancy. A single injection of PMSG can be used to produce timed pregnancies in large number of immature rats. This saves laborious and time-consuming examination of vaginal smears required in experiments involving adult
rats. Thus, immature rats appears to be a suitable model for basic studies in reproduction. Treatment with 4 IU PMSG could serve as a control while treatment with 40 IU PMSG achieves superovulation and has exaggerated detrimental effects.

D. Detrimental Effects of Superovulation
a. General

Remarkable progress has been made in the management of infertility during the last decade. IVF-ET procedures have become an indispensable treatment option for many types of infertility. Significant and profound advances have been achieved in both the clinical and basic science studies aimed at improving IVF-ET outcome. However, these advances have not translated into dramatically higher pregnancy rates. A number of investigators have attributed reduced fertility to abnormalities or failure in the process of fertilization, early loss or abnormal development of preimplantation embryos, implantation failure and high fetal wastage. Although no single factor can be attributed to the detrimental effects associated with IVF-ET, adverse effects of ovarian hyperstimulation caused by superovulatory treatments are undoubtedly one of the major factors (Moon et al., 1990).

The adverse effects of ovarian hyperstimulation, resulting from superovulatory treatments with exogenous gonadotropins, have been recognized since the early use of IVF-ET. Standard superovulatory protocols currently use pharmacological dosages of exogenous gonadotropins to obtain multiple oocytes. As a result, fertilized embryos are replaced into a disturbed uterine environment. Increased early embryonic loss and failure of implantation are common after superovulation and have been linked to a non-receptive endometrium. A large volume of endocrinological and histological studies suggests that superovulatory treatment prevents the uterine endometrium from becoming receptive through hyperestrogenemia or an increased ratio of estrogen/progesterone. However, the mechanisms by which imbalance in ovarian steroid hormones causes the detrimental effects on preimplantation embryonic development and the implantation process remain to be
elucidated.

b. Ovarian Steroid Hormones

Reduced fertility rates after superovulation have been attributed to hyperestrogenemia or an increase in estrogen/progesterone ratio in many species (Baranczuck and Greenwald, 1973; Morris and Van Wagenen, 1973; Booth et al., 1975; Greenwald, 1976; Fujimoto and Tanaka, 1977; Saumande, 1980; Miller and Armstrong, 1981a; Miller and Armstrong, 1982; Walton and Armstrong, 1982; Walton et al., 1983; Laufer et al., 1986; Yun et al., 1988; Safro et al., 1990). Estrogen administered after ovulation has anti-fertility effects in many species (Greenwald, 1961; Gidley-Baird et al., 1986; Forman et al., 1988). However, freshly ovulated, normal-appearing oocytes and embryos (at least during the first 36 h) after superovulation have an equal ability to fertilize and develop to the fetal stage as do the control oocytes and embryos, if they were transferred into a normal oviductal and uterine environment, respectively (Miller and Armstrong, 1982; Walton and Armstrong, 1983).

Changes in the levels of ovarian steroid hormones appears to render the oviductal and uterine environment detrimental to fertilization and embryonic development. Oviductal fluid, obtained from mice that have experienced high estrogen levels, become detrimental to early embryonic development as determined in cultures (Cline et al., 1977). The uterine microenvironment associated with elevated estrogen/progesterone ratios also detrimental to the blastocyst metabolism and RNA synthesis in mice (Safro et al., 1990). The rate of fertilization and the ability of embryos to develop to the blastocyst stage is substantially impaired in rats which have experienced high serum estrogen levels for extended periods of time prior to, and after, ovulation (Fugo and Butcher, 1966; Butcher and Pope, 1979, Miller and Armstrong, 1981a; Miller and Armstrong, 1982; Walton et al., 1983). Superovulatory treatment, with a large dose of PMSG, increases early embryonic loss and results in failure of implantation in immature rats (Miller and Armstrong, 1981a,b). The disturbed endocrine environment asynchronizes embryonic and uterine development
c. Histology

An increase in uterine wet weight of up to 2-fold has been observed after superovulatory treatment in immature rats (Miller and Armstrong, 1981a). This increase is accompanied by the presence of desquamated cellular debris in the uterine flushings. Histological examination of the debris suggests that it is epithelial hyperplasia, suggesting that there is hyperstimulation of the endometrial epithelium. It has also been shown that superovulation may cause hypertrophy of the uterine endometrial stroma (Rennels, 1951; Fang, 1988). Hyperestrogenemia interferes with the proper secretory transformation of the endometrium (Martel et al., 1987). These structural findings suggest that superovulatory doses of exogenous gonadotropins may induce abnormal changes which results in a non-receptive state in the endometrium.

II. THE INSULIN-LIKE GROWTH FACTOR (IGF) SYSTEM

A. IGF-I

a. Molecular Properties

IGF-I is a member of the insulin-like peptide family which also includes insulin and IGF-II. IGF-I is composed of 70 amino acids and has structural similarities to proinsulin, IGF-II and relaxin (Rinderknecht and Humbel, 1978). Mature IGF-I consists of B (amino-terminal domain, 29 amino acids), C (12 amino acids), A (21 amino acids), and D (8 amino acids) domains. The B and A domains exhibit approximately 40% homology to the B and A chains of insulin and approximately 60% homology to the corresponding domains of IGF-II (Foyt and Roberts, 1991). The amino acid sequence of IGF-I is highly conserved (>92%) among mammalian species. For example, human, bovine, and porcine IGF-I have identical amino acid sequence, and difference in three amino acid residues distinguish rat IGF-I from that of these species (Rinderknecht and Humbel, 1978; Shimatsu and Rotwein, 1987; Francis, 1988; Francis, 1989).

A truncated variant form of IGF-I lacking the N-terminal tripeptide which is
involved in interactions with IGFBPs has been isolated in many tissues, including human brain and porcine uterus (Sara et al., 1986; Ogasawara et al., 1989). This variant is at least 5 to 10 times more potent in its biological activity than the native peptide. It has been suggested that the N-terminal tripeptide is itself biologically active in the brain and stimulates acetylcholine release (Sara et al., 1989).

IGF-I mRNA are seen in multiple size transcripts, due to alternate splicing in the 5'-untranslated region and the distinct length of 3'-untranslated region, which is itself controlled by differential polyadenylation site usage (Lund et al., 1989; Roberts et al., 1989; Foyt and Roberts, 1991). The size of mRNA transcripts varies widely from 0.8 kb to 15 kb, although 7.0-7.5 kb appears to be the most common size of transcript in many tissues.

b. Endocrine Regulation

This growth factor was initially determined to be a mediator of the growth promoting actions of growth hormone (GH) (Scholenle et al., 1982). Accordingly, IGF-I synthesis is GH-dependent in liver and many other tissues and is present in significant quantities in plasma, due to its secretion by the liver (D'Ercole et al., 1984; Lund et al., 1986; Roberts et al., 1986; Murphy et al., 1987a,b). While GH exerts a body wide effect in IGF-I regulation, other hormones seem to exert tissue or cell-type specific effects. Of these, estrogen is the best studied and the most important regulator of IGF-I synthesis in the reproductive organs. Estrogen stimulates IGF-I synthesis in the uterus and granulosa cells but appears to have no effect, or even inhibit, hepatic IGF-I synthesis (Murphy et al., 1987c; Murphy and Friesen, 1988). GH has only a minor effect, by comparison, on IGF-I synthesis in the uterus (Murphy and Friesen, 1988). The mechanisms by which GH and estrogen increase IGF-I mRNA levels appear to be different. Protein synthesis is required in GH-induced IGF-I synthesis but not in estrogen-induced IGF-I synthesis (Murphy and Luo, 1989).
Estrogen also increases IGF-I mRNA levels in the bone. In this tissue, parathyroid hormone increases synthesis of IGF-I at mRNA and peptide levels (Ernst et al., 1989; McCarthy et al., 1989). Gonadotropins like FSH and LH stimulate gonadal IGF-I mRNA levels without an increase in circulating IGF-I levels (Hsu and Hammond, 1987; Closset et al., 1989). Dexamethasone decreases IGF-I mRNA levels in cultured brain cells and reduces the basal or GH-stimulated synthesis of IGF-I in many tissues in vivo (Adamo et al., 1988; Luo and Murphy, 1989). Thyroid hormone increases hepatic and pituitary cell IGF-I mRNA levels (Fagin et al., 1989a; Wolf et al., 1989). Although thyroid hormone alone did not affect IGF-I mRNA levels, pre- or co-treatment with thyroid hormone enhances GH-induced IGF-I synthesis in the rat liver (Wolf et al., 1989). Thyroid hormone action on IGF-I mRNA levels in a pituitary cell line could also be mediated by endogenous GH secretion (Fagin et al., 1989a).

In addition, insulin increases IGF-I mRNA levels in cultured hepatocytes (Johnson et al., 1989). Most studies examining the effect of insulin on the regulation of IGF-I has been done with respect to diabetes. In the rat diabetes model, reduced basal IGF-I levels were found in the liver and other tissues. Furthermore, GH is no longer capable of inducing IGF-I synthesis (Fagin et al., 1989b). Basal uterine IGF-I levels are not significantly reduced; however, estrogen-induced IGF-I synthesis is impaired in diabetic rats (Murphy, 1988). Insulin treatment in diabetic rats restored basal IGF-I levels and GH- and estrogen-induced IGF-I synthesis (Murphy, 1988; Fagin et al., 1989a). The regulation of IGF-I synthesis by IGFs is poorly understood. IGF-II secreting tumors decreased circulating IGF-I levels; however, the same study failed to demonstrate a decrease in hepatic IGF-I mRNA levels (Wilson et al., 1989). In rats fed an energy-restricted diet, IGF-I infusion decreased hepatic IGF-I mRNA levels. Energy-restriction or fasting per se also reduces hepatic IGF-I mRNA levels which is caused primarily by reduced GH secretion and hepatic resistance to GH resulting from down regulation in GH receptor expression (Emler and Schalch, 1987; Lowe et al., 1989; Straus and Takemoto, 1990;
c. Biological Action

Although IGFs and insulin share many structural and functional features, these ligands have significantly different biological roles. Insulin is a key regulator of systemic and cellular metabolism which includes glucose transportation, and glycogen and fat biosynthesis. In contrast, IGF-I appears to be a more potent mitogen and mediates the growth promoting actions of GH. Furthermore, IGF-I appears to function not only in the classical endocrine manner but also in the autocrine and/or paracrine mode. The ability of IGF-I to regulate DNA synthesis, cell proliferation and cellular metabolism in an autocrine and paracrine fashion has been well documented (William, 1991). IGF-I has also been implicated in the control of cell differentiation and in tissue repair and regeneration (William, 1991).

B. Insulin-Like Growth Factor Binding Proteins (IGFBPs)

a. General

IGFBPs are a group of structurally homologous proteins that bind IGF-I and II. The nomenclature of IGFBPs is somewhat confusing (Ballard et al., 1989; Drops and Hintz, 1989). Some IGFBPs have been given several names according to the source of purification and the estimated molecular weight of proteins. IGFBPs now go under the nomenclature; IGFBP-x where 'x' is the number which reflects the order in which full sequence of cDNA was published. Consequently, six IGFBPs (IGFBP-1 to IGFBP-6) have been identified and named to date. Some IGFBPs have variants with distinct molecular weights caused by posttranslational modifications that include glycosylation and phosphorylation (Rechler et al., 1989; Clemmons, 1991; Clemmons, 1993).

IGFBPs possess common structural features such as small prepeptides (signal peptides) of similar size and cysteine-rich areas in the amino and carboxyl terminal regions. The amino acid sequences in the amino and carboxyl terminal cysteine-rich areas show a
high degree of homology across IGFBPs, whereas the central areas are less conserved. Signal peptides and the cysteine-rich amino terminal are highly hydrophobic, while the remaining areas are hydrophylic (Lee et al., 1988). The cysteine-rich amino and carboxyl terminal regions appear to be responsible for ligand binding, although evidence indicates that non-cysteine amino terminal residues may also be involved in ligand binding (Huhtala et al., 1986; Lee et al., 1988; Brinkman et al., 1991). Each binding protein also has some unique properties. For example, IGFBP-1 and -2 have an RGD sequence near the carboxyl terminal. The RGD sequence is believed to mediate the binding of matrix proteins to structurally related receptors, known as the integrins (Rouslahti and Pierchbaker, 1988).

b. Regulation and Function

IGFBP-1 is present at ng/ml levels in the plasma, and is the only IGFBP where the plasma level varies from minute to minute, due to hormonal and metabolically regulations (Baxter and J.L., 1989; Hall et al., 1991; Holly, 1991; Lee et al., 1993). Insulin, GH, IGF-I, estrogens, glucose and glucocorticoids appear to suppress IGFBP-1 synthesis, while glucagon, cAMP, protein kinase C, theophylline, progesterone and fasting stimulate IGFBP-1 synthesis (Baxter and J.L., 1989; Hall et al., 1991; Holly, 1991; Lee et al., 1993). IGFBP-3 is the most abundant binding protein in the circulation existing at mg/l levels, followed by IGFBP-2 to approximately 1/10 less extent (Lee et al., 1993). IGFBP-3 forms a 140-150 kDa complex with an IGF-I or IGF-II molecule and an 88 kDa acid labile subunit in the blood. This complex is generally believed to serve as a carrier and as a reservoir for IGFs. Although the exact role of IGFBPs is unclear, the tissue-specific hormonal regulation of IGFBPs suggests that they function in both a paracrine and autocrine fashion. In general, IGFBPs inhibit IGF-I actions, primarily by regulating the amount of free IGF-I in these tissues. However, the function of these binding proteins can be diverse, depending upon the cell type and other factors. Enhancement of IGF-I actions by IGFBPs has been reported (Elgin et al., 1987). IGFBP-3 appears to have a bifunctional
role that could be based on its cell surface association (De Mellow and Baxter, 1988). Cell surface associated IGFBP-3 enhanced IGF-I action on DNA synthesis in fibroblast cultures, while free IGFBP-3 inhibited IGF-I action in the same system. Furthermore, IGFBP-3, -4, and -5 have been shown to bind to extracellular matrix proteins. Extracellular matrix-associated IGFBP-5 enhanced cell growth in response to IGF-I (Clemmons, 1993; Jones et al., 1993).

C. IGF-I Receptors

a. General

IGF-I binds to IGF-I, IGF-II, and insulin receptors with different affinities. Like their ligands, IGF-I and insulin receptors are structurally similar to each other (Bhaumick et al., 1981; Chernausek et al., 1981). In contrast, the IGF-II receptor has a very different structure which is identical to mannose 6-phosphate receptors (Morgan et al., 1987; Oshima et al., 1988). IGF-I and insulin receptors are tetrameric glycoproteins which are composed of two α-subunits and two β-subunits (Hintz et al., 1972; Le Bon et al., 1986). The α-subunit is entirely extracellular, contains a cysteine-rich domain and is responsible for ligand binding, while the β-subunit has a cytoplasmic tyrosine kinase domain. IGF-I and insulin receptors are members of the subclass II of tyrosine kinase receptor family (Yarden and Ullrich, 1988).

The IGF-I receptor demonstrates heterogeneity through distinct glycosylation. For example, a less N-glycosylated form of the α-subunit is found in the brain (McElduff et al., 1988; Ota et al., 1988). Another source of heterogeneity is formation of hybrid receptor with insulin receptor subunits. The structural similarities allow insulin and IGF-I receptors to form hybrid receptors in vitro and in vivo (Feltz et al., 1988; Lammers et al., 1989; Treadway et al., 1989; Wilden et al., 1989). A monoclonal antibody, which recognizes the C-terminal region of the insulin receptor but not the IGF-I receptor, immunoprecipitated a receptor that has two phosphorylated β-subunits of distinct molecular
weights (Garofalo and Rosen, 1989). The molecular weight of each β-subunit represents that of the phosphorylated β-subunits of insulin and IGF-I receptors, respectively. Other examples of heterogeneity of the IGF-I receptor also can be explained by introducing the hybrid receptor concept (Burant et al., 1987; Izumi et al., 1987; Kadowaki et al., 1987; Alexandrides and Smith, 1989). These hybrid receptors have been related to some pathophysiological conditions such as tumor formation and diabetes.

b. Function

IGF-I receptor recognizes IGF-II and insulin, while IGF-II and insulin receptors in turn recognize IGF-I. These cross-bindings are distinguishable by binding assay due to distinct binding affinities (Marshall et al., 1974; Rechler et al., 1980; Jonas et al., 1982; Flier et al., 1986; Lammers et al., 1989). Although the significance of these cross-binding capabilities is not fully understood, it is suggested that they may be a part of mechanisms that allow IGF-I or related peptides to exert a variety of actions in many cell types.

Despite the structural similarities and ligand cross-binding capabilities between insulin and IGF-I receptors, these two receptors appear to have distinct functional roles. A differential signaling potential of the cytoplasmic domain of the β-subunit of human insulin and IGF-I receptors has been demonstrated in a mouse fibroblast cell line (Lammers et al., 1989). Both insulin and IGF-I receptor cytoplasmic domains have a compatible signaling potential in the short-term effect, such as stimulation of glucose transportation. However, the cytoplasmic domain of IGF-I receptor appears to have a greater signaling potential in the long-term effect such as mitogenic actions (Lammers et al., 1989). Taken together, these observations demonstrate that the IGF-I receptor is more potent than insulin receptors in mediating mitogenic and growth promoting activity, while the insulin receptor has a greater potential in cellular energy metabolism (Hintz et al., 1972; Flier et al., 1986; Lammers et al., 1989; William, 1991).
D. The IGF System in the Uterus

a. IGF-I

The rat uterus is a major site of IGF-I synthesis. IGF-I synthesis in the uterus is primarily under estrogen regulation, and to a lesser extent GH regulation (Murphy et al., 1987c; Murphy and Friesen, 1988; Norstedt et al., 1989; Carlsson and Billig, 1991). Exogenous gonadotropins or estrogen injections into rats cause an increase in the levels of uterine IGF-I mRNA and peptide (Murphy et al., 1987c; Murphy and Friesen, 1988). Progesterone may also play a role in the regulation of IGF-I synthesis in the uterus (Norstedt et al., 1989; Croze et al., 1990a; Kapur et al., 1992). IGF-I mRNA has been localized to the stroma and myometrium of the rat uterus (Ghahary et al., 1990). IGF-I mRNA transcripts are abundant in the antimesometrial stromal tissue and has been further localized to the periepithelial and periglandular stromal cells (Croze et al., 1990a). IGF-I levels, both protein and mRNA, vary in the uterus during the estrous cycle and is maximal during the preimplantation period. In contrast, there is no change in serum IGF-I concentrations during the same period (Murphy et al., 1987c; Kapur et al., 1992; Katagiri et al., 1996).

Multiple size transcripts of IGF-I mRNA has been described in the uterus. Uterine IGF-I mRNA levels increase during the preimplantation period in rats, mice, pigs, sheep and cows (Letcher et al., 1989; Croze et al., 1990a; Geisert et al., 1991; Ko et al., 1991; Kapur et al., 1992). In mice, the 7.0 kb transcript decreases on Day 5 and 6, after initiation of implantation, while the levels of the other smaller transcripts remain high (Kapur et al., 1992). The decline in the levels of IGF-I mRNA transcripts at the time of implantation has also been observed in the rat uterus (Croze et al., 1990a). In pigs and sheep, the concentration of uterine luminal IGF-I and its mRNA levels in the uterine tissues is higher during early pregnancy compared with its corresponding days in the estrous cycle (Ko et al., 1991). IGF-I levels in the uterine luminal fluids and IGF-I mRNA in the uterus
increase steadily throughout the preimplantation period. This is followed by a rapid decline after the initiation of implantation in the pig (Letcher et al., 1989).

A high molecular weight variant with molecular mass of 18 kDa, but not the 7 kDa form, of IGF-I has been detected in the rat uterus (Murphy and Ghahary, 1990). A truncated IGF-I molecule has also been isolated from the pig uterus (Ogasawara et al., 1989). This variant lacks an N-terminal tripeptide which is involved in interactions with IGFBPs, indicating a greater bioactivity. The significance of these variants requires clarification.

b. IGFBPs

IGFBPs are believed to play a central role in the uterine IGF system which is involved in regulating uterine growth and function. In particular, the role of IGFBP-1, which has been associated with implantation, has drawn much attention in the rodent and primate. However, the specific role of the IGFBPs remains poorly characterized.

IGFBPs have been detected in the rat uterus. IGFBP-1 has been localized to the uterine epithelium, stroma and decidual tissue in the rat (Croze et al., 1990a; Murphy et al., 1990). IGFBP-1 levels vary during the estrous cycle, with maximal levels being observed at diestrus and nadir at proestrus (Ghahary and Murphy, 1989). This expression pattern is the inverse to the uterine IGF-I receptor profile. IGFBP-1 may act as an inhibitor and also a reservoir for IGF-I during diestrus. This would allow maximal IGF-I action during proestrus when the IGF-I receptor levels are greatest (Murphy, 1991). This system seems to assure a rapid growth of the uterine endometrium during proestrus. IGFBP-1 mRNA is abundant in the decidualized uterus, indicating a possible role for the uterine IGF system in decidualization. The role of the IGF system in decidualization will be discussed later.

IGFBP-2 and -4 have been localized to the epithelium. In contrast, IGFBP-3 has been localized to the stroma and IGFBP-5 and -6 to the myometrium and the serosa (Girvigian et al., 1994). The mRNA levels of each IGFBP varies during the estrous cycle
and early pregnancy (Girvigian et al., 1994).

c. IGF-I receptor

IGF-I receptors have been detected in the rat uterus (Ghahary and Murphy, 1989; Chandrasekhar et al., 1990). The binding affinity and capacity of uterine IGF-I receptors are similar to those found in other tissues. The majority of IGF-I binding has been localized to the smooth muscle layer by autoradiography (Ghahary and Murphy, 1989). Since IGF-I has also been localized to the myometrial and stromal layer, IGF-I may play a role in myometrial and stromal cell growth and function in the rat uterus (Ghahary et al., 1990). However, this does not exclude the presence of IGF-I receptors and their potential role in other layers of the uterus.

Administration of estrogen increases the number of IGF-I receptors in the uterus of hypophysectomized and ovariectomized adult rats and immature rats (Ghahary and Murphy, 1989; Chandrasekhar et al., 1990). In mature cycling rats, IGF-I binding capacity is greatest during proestrus and lowest during diestrus (Ghahary and Murphy, 1989). At present, it is not clear whether uterine IGF-I receptors are directly regulated by estrogen. Since the increase in IGF-I mRNA levels precedes the increase in the number of IGF-I receptors seen in response to estrogen administration, IGF-I may also mediate the estrogen effect on IGF-I receptor synthesis (Murphy, 1991).

III. GROWTH FACTORS IN PREIMPLANTATION EMBRYONIC DEVELOPMENT

A. Paracrine and Autocrine Regulation

The establishment of a successful pregnancy requires synchronized growth and differentiation of the preimplantation embryo and the uterine endometrium. It is apparent that ovarian steroid hormones coordinate the synchronized growth and differentiation of embryos and the uterine endometrium (Finn and Martin, 1967). These effects may be mediated by growth factors, derived from the oviduct and uterus in a paracrine manner.

In general, preimplantation embryos grow more slowly and less successfully in
vitro. Co-culture of embryos with somatic cells, such as oviductal and uterine cells, or even cells not derived from the genital tract, augment embryonic development (Bavister, 1988). An adequate oviductal and uterine environment may optimize preimplantation embryonic development through multiple mechanisms that include secretion of growth-promoting factors. Preimplantation embryonic development in most species requires supplementation of growth-promoting factors such as serum to the culture medium. It appears that growth factors and cytokines in serum and from co-cultured cells may be largely responsible for the growth-promoting effect on embryonic development. Many growth factors and cytokines that are found in the oviduct and uterus have been demonstrated to promote embryonic development, but some of them have been shown to inhibit or to have no effect on embryonic development (Brigstock et al., 1989; Simmen and Simmen, 1991; Adamson, 1993). The presence of functional receptors for growth factors in preimplantation embryos is compatible to these observations (Rappolee et al., 1991).

In contrast, preimplantation mouse embryos grow to the blastocyst stage in a simple defined culture media, suggesting that the developmental process of preimplantation embryos may be autonomous. Development of 2-cell mouse embryos and the number of cells per blastocyst are superior in cultures with multiple embryos to in cultures with a single embryo (Paria and Dey, 1990). This suggests that embryonic autocrine factors may be important in embryonic development, and that concentrations of these factors in culture media may not reach effective concentrations if cultured in relatively large culture medium droplet.

The presence of ligand and receptor for many growth factors supports this concept. Expression of mRNAs for a variety of growth factors and their receptors have been detected in the preimplantation embryo (Rappolee et al., 1988; Rappolee et al., 1991). Evidence suggests that these mRNAs are translated into functional proteins (Rappolee et al., 1991; Adamson, 1993; Schultz and Heyner, 1993). Taken together, these findings suggest that these growth factors regulate preimplantation development in an autocrine
Immediately after fertilization, embryonic development appears to be controlled by the expression of mRNA transcripts of maternal origin. The transition from maternal to embryonic control in early development occurs at a certain stage between the 2-cell stage and the 16-cell stage, depending upon the species (Telford et al., 1990). The transition occurs at the 2-cell stage in mice (Schultz, 1986), between the 4-cell and 8-cell stages in pig and humans (Tesarik et al., 1986; Tesarik et al., 1987a; Braude et al., 1988), between the 8-cell and 16-cell stages in cows and sheep (Camous et al., 1986; Crosby et al., 1988; Frei et al., 1989), and at the 16-cell stage or later in rabbits (Manes, 1973; Manes, 1977).

In mice, reflecting the transition of mRNA expression from maternal to embryonic origin, abundance of mRNA transcripts show four distinct temporal patterns: (1) undetectable until the blastocyst stage; (2) present as maternal transcripts which decrease in levels due to the destruction of maternal transcripts and reexpressed as embryonic transcripts; (3) transcribed only as embryonic transcripts; and (4) detectable throughout preimplantation development (Rappolee et al., 1991). The temporal expression patterns of growth factors and their receptors in preimplantation embryos may give insight into the role of these growth factors in the paracrine and autocrine regulation of their development.

B. Insulin-Like Peptides in the Preimplantation Embryonic Development

a. Gene Expression

Initial observations suggested that IGF-I mRNA transcripts were not present in the preimplantation mouse embryos prior to the blastocyst stage (Werb, 1990; Rappolee et al., 1992). However, IGF-I mRNA transcripts have recently been detected in mouse embryos (Doherty et al., 1994). The mRNA transcript levels decrease from oocytes to 8-cell stage embryos and then increase from the 8-cell to the blastocyst stages. IGF-I mRNA transcripts are also detectable at all stages of preimplantation development in bovine embryos (Schultz et al., 1992; Watson et al., 1992). However, IGF-I mRNA transcripts
have not been detected at all stages of preimplantation development in the rat (Zhang et al., 1994). The pattern of IGF-I expression in embryonic development appears to be different across the species. In contrast, information about IGF-II and insulin in preimplantation embryos is consistent across all species. The presence of IGF-II mRNA and the absence of insulin mRNA have been reported in mouse, rat and bovine embryos (Rappolee et al., 1992; Watson et al., 1992; Zhang et al., 1994).

Expression of IGF-I and insulin receptors has been detected from oocytes to blastocysts throughout preimplantation development (Zhang et al., 1994). The levels of mRNA transcripts for both receptors; however, show a temporal decline at the 4-cell and 8 cell stages (Zhang et al., 1994). Expression of IGF-I receptor mRNA becomes detectable at the 8-cell stage in the mouse (Werb, 1990; Rappolee et al., 1992; Schultz et al., 1992). Insulin receptor mRNA, which also mediates IGF-I actions, is detected in the mouse embryo at the 8-cell stage, the time when embryos enter the uterus (Werb, 1990; Rappolee et al., 1992; Schultz et al., 1992). IGF-II receptor mRNA is detectable at the 2-cell stage and at later stages in the mouse and rat (Werb, 1990; Rappolee et al., 1992; Schultz et al., 1992; Zhang et al., 1994). The stage-specific expression of these receptors has been evidenced by detection of these receptor proteins using an antibody against the receptors and gold- or radioisotope-labeled ligands (Mattson et al., 1988; Heyner et al., 1989; Harvey and Kaye, 1991a,b; Smith et al., 1993). In the cow, IGF-I, IGF-II, and insulin receptor mRNA transcripts have been detected throughout preimplantation development (Schultz et al., 1992; Watson et al., 1992). The preimplantation embryos appear to translate these mRNAs into functional receptors which allows the embryos to respond to exogenous IGF-I, IGF-II, and insulin (Harvey and Kaye, 1988; Harvey and Kaye, 1990; Zhang and Armstrong, 1990; Harvey and Kaye, 1991c; Harvey and Kaye, 1992a,b; Kaye et al., 1992; Rappolee et al., 1992; Smith et al., 1993).

Expression of IGFBPs have been detected in preimplantation mouse embryos (Hahnel and Schultz, 1994). The mRNA for IGFBP-2, -3, and -4 are detectable
throughout preimplantation development, and IGFBP-6 mRNA at the blastocyst stage. In contrast, IGFBP-5 mRNA is not detectable at any preimplantation stage. IGFBP-1 has not been examined in this study. Information for IGFBPs in preimplantation rat embryos is not available at this time.

b. Growth Promoting Effects

The effect of insulin-like peptides on the preimplantation embryonic development has been demonstrated in the mouse. IGF-I and insulin have been shown to be internalized through receptor-mediated endocytosis in the mouse embryo (Heyner et al., 1989; Smith et al., 1993). Insulin and IGF-I enhance the development of embryos, increase the number of cells in the embryo and stimulate protein synthesis in vitro (Harvey and Kaye, 1988; Harvey and Kaye, 1990; Harvey and Kaye, 1992a; Schultz et al., 1992). Although expression of embryonic IGF-II receptor mRNA is detectable as early as the 2-cell stage, there is no evidence that IGF-II plays a critical role in embryonic development prior to the compaction stage when the expression of insulin and IGF-I receptors becomes detectable (Rappolee et al., 1992). IGF-II accelerates blastocyst formation, increases the number of cells in the embryo and stimulated protein synthesis (Rappolee et al., 1992).

Insulin, in the presence of amino acids, stimulates 8-cell stage embryos to develop to the blastocyst stage in the rat (Zhang and Armstrong, 1990). Embryos cultured to the blastocyst stage in the presence of insulin are capable of developing to day 18 fetuses at a greater rate than blastocysts cultured in the absence of insulin, when transferred to the receptive uterus (Zhang and Armstrong, 1990). The presence of mRNA transcripts for IGF-I receptor, but not for IGF-I, has been detected in the preimplantation rat embryos (Zhang et al., 1994). Together, along with information regarding the IGF system in the uterus, it appears that the uterine IGF system plays a role in preimplantation embryonic development in a paracrine manner in the rat.
IV. ELECTROLYTES IN PREIMPLANTATION EMBRYONIC DEVELOPMENT

A. General

The difference in electrolyte composition between cells and their microenvironment is a universal source of energy for cell growth and function. In particular, the sodium gradient across the plasma membrane has great importance. Sodium gradients across the plasma membrane are maintained by Na\(^+\)/K\(^+\)-ATPase which transports Na\(^+\) and K\(^+\) across the plasma membrane against their electrochemical gradient, using energy obtained from the hydrolysis of ATP. The sodium gradient, in turn, provides energy to transport protons, chloride, phosphate, glucose, amino acids and other substances by co- and countertransport mechanisms across the plasma membrane (Lechene, 1988; Cohen and Lechene, 1989; Biggers et al., 1991). These co- and countertransports are required to maintain cellular homeostasis and regulate cellular metabolisms. Furthermore, the proper intracellular pH and other electrolyte composition are necessary for optimal enzyme function and actions of growth factors that play a central role in preimplantation embryonic development (Pouyssegur et al., 1985; Somero, 1985; Moolenaar et al., 1988).

The intracellular concentrations of Na\(^+\), K\(^+\), Cl\(^-\), and H\(^+\) have been determined in preimplantation mouse embryos (Powers and Tupper, 1977; Lee, 1987). These values are conflicting between the studies. For example, the intracellular concentration of Na\(^+\) of the 2-cell stage embryos is reported to be 143-151 mM and 25 mM (Lee, 1987). The extremely high Na\(^+\) concentration of the former study (Powers and Tupper, 1977) suggests that the 2-cell stage embryos of the study may be damaged to some extent. However, the same study has demonstrated that the plasma membrane permeabilities to Na\(^+\) and K\(^+\) of the 2-cell stage mouse embryo are compatible to those of other cell types (Powers and Tupper, 1977; Jain and Wagner, 1980). Furthermore, the Na\(^+\)/K\(^+\)-ATPase activity in the 2-cell stage mouse embryo is intact, suggesting that the embryos are normal (Powers and Tupper, 1977). The intracellular concentrations for electrolytes including these four ions need to be determined.
B. Electrolyte Transport in Preimplantation Embryonic Development

a. Regulation of Intracellular pH

The 2-cell stage mouse embryos appear to highly permeable to H\(^+\), being several orders of magnitude larger than that of other cell types (Baltz et al., 1990). The extremely high H\(^+\) permeability in the 2-cell stage mouse embryo is of particular interest with respect to the intracellular pH regulation. Evidence suggests that the 2-cell stage mouse embryo lacks Na\(^+\)-dependent intracellular pH regulatory systems, the Na\(^+\)/H\(^+\) antiporter and Na\(^+\)-dependent HCO\(_3^-\)/Cl\(^-\) exchanger, that are the most common systems in other cell types (Baltz et al., 1990; Baltz et al., 1991a). The 2-cell stage mouse embryo appears to have no specific mechanism for alleviating intracellular acid loads, but possess a Na\(^+\)-independent HCO\(_3^-\)/Cl\(^-\) exchange mechanism to recover from alkaline loads (Baltz et al., 1991a,b). The oviductal fluid has a high pH and K\(^+\) concentration, both of which make the cells of the embryo alkaline (Leese, 1988; Biggers et al., 1991). Therefore, the specific mechanism for acid load relief may not be necessary in this particular stage of development. The extremely high H\(^+\) permeability was diminished in the trophectoderm, but still present in the inner cell mass (ICM) (Baltz et al., 1993). Taken together, the pH and concentrations of K\(^+\), HCO\(_3^-\), and Cl\(^-\) in the oviductal and uterine environment may be critical for the intracellular pH regulation of preimplantation embryos; and therefore be critical to embryonic development.

b. Compaction

Loosely associated blastomeres in the embryos of the early cleavage stages become flattened against one another during the compaction process. The morphological change during the compaction process in the mouse embryo is believed to be caused by an extensive increase in Ca\(^+\)-dependent cell-cell adhesion that is mediated by the surface glycoprotein, uvomorulin (Hyafil et al., 1980; Hyafil et al., 1981; Peyrieras et al., 1983; Johnson et al., 1986). Calcium appears to regulate proteolytic cleavage of uvomorulin that
is identical to E-cadherin (Yoshida and Takeichi, 1982; Shirayoshi et al., 1983; Yoshida-Noro et al., 1984), and homologous with cell adhesion molecule 120/80 in the human (Damsky et al., 1983).

One of the essential features of compaction is the polarization of blastomeres, so that the blastomeres show distinct apical and basolateral membrane. Changes in the distribution pattern of uvomorulin is one of many examples of cell polarization. The protein is evenly distributed over the entire surface of the blastomere prior to compaction, while it becomes no longer detectable on apical cell surface of outer cells of compacted morulae, but remains entirely distributed on the inner cell surface (Vestweber et al., 1987).

The distribution patterns of Na\(^+\)/K\(^+\)-ATPase in both uncompacted and compacted morulae are similar to those of uvomorulin (Vestweber et al., 1987; Watson and Kidder, 1988). The polarized distribution of Na\(^+\)/K\(^+\)-ATPase is particularly important with respect to electrolyte and fluid transport. Together with the formation of tight junctions between the cells of the outer surface of the embryos, the preimplantation embryos establishes the polarity of a plasma membrane and the transport mechanisms that are essential to subsequent blastocyst formation (Wiley, 1987; Biggers et al., 1991). Although the prevention of compaction and Ca\(^+\)-dependent cell-cell adhesion do not prevent blastomeres from developing cell surface polarity (Ziomek et al., 1982), evidence suggests that uvomorulin plays a central role in the formation of tight junction and the polarization of Na\(^+\)/K\(^+\)-ATPase distribution (Johnson et al., 1986; Watson et al., 1990; Larue et al., 1994). These events were inhibited by an anti-uvomorulin (or E-cadherin) antibody in the 8-cell mouse embryos.

c. Formation of Blastocoelic Cavity

The polarized distribution of Na\(^+\)/K\(^+\)-ATPase to the basolateral plasma membrane is a commonly used marker of the basolateral domain of epithelial cells (Cereijido et al., 1989; Rodriguez-Boulan and Nelson, 1989). Expression of the protein is restricted to the
basolateral plasma membrane of the trophectoderm cells but remains evenly distributed on the surface of the ICM cells (Watson and Kidder, 1988). Indeed, the trophectoderm is believed to be the first epithelium developed during embryonic development (Wiley et al., 1990). Evidence suggests that Na⁺/K⁺-ATPase plays a central role in formation of the blastocoele; i.e. formation of blastocysts (Wiley, 1987). Blastocoele formation is inhibited or delayed by ouabain, a inhibitor of Na⁺/K⁺-ATPase, in the rabbit and mouse (DiZio and Tasca, 1977; Biggers et al., 1978; Wiley, 1984; Manejwala et al., 1989). The rate of blastocoele formation is inversely related to the concentration of extracellular K⁺ (Wiley, 1984; Wiley, 1987). Evidence suggests that the effect of varying concentrations of K⁺ is exerted through its ability to modulate Na+/K+-ATPase activity (Wiley, 1987; Dumoulin et al., 1993). Extracellular K⁺ levels affect Na⁺/K⁺-ATPase activity by changing plasma membrane potential and passive ion fluxes that are caused by the current of Na⁺ (Cohen et al., 1976; Wiley, 1984). Furthermore, substitution of either Na⁺ or Cl⁻, but not K⁺, in embryo culture media reduced the rate of blastocoele expansion in the mouse, suggesting the role of Na+/K+-ATPase in blastocoele formation (Manejwala et al., 1989).

The levels of Na⁺/K⁺-ATPase mRNA increase after compaction in the mouse and rabbit preimplantation embryos (Gardiner et al., 1990a,b). Interestingly, IGF-I has been shown to stimulate Na⁺/K⁺-ATPase synthesis (Madsen and Bern, 1993; Matsuda et al., 1993). The temporal expression patterns of mRNAs for the IGF-I and insulin receptors in the mouse and rat embryos appear to coincide with that of Na⁺/K⁺-ATPase (Rappolee et al., 1991; Zhang et al., 1994). These observations suggest that IGF-I may stimulate the formation of the blastocoele in the preimplantation embryo.

C. Preimplantation Embryonic Development In Vitro

The electrolyte composition of the embryo culture media has been well defined for laboratory animals; mostly in the mouse and hamster (Whitten, 1971; Whittingham, 1971; Bavister et al., 1983). The composition of the most commonly used culture media for
preimplantation embryos are based on Krebs-Ringer solution. All of these media are very simple and have similar electrolyte compositions. Conventional complex cell culture media, such as Ham's F-10 and tissue culture medium 199, have been chosen for embryo culture in most other species to satisfy the potentially complex but largely unknown nutritional requirements of the embryo.

Classical culture media for the preimplantation mouse embryos allow 2-cell stage mouse embryos to develop into blastocysts which are capable of implantation and subsequent fetal development (Biggers, 1987). However, with the exception of some inbred and F1 strains, the culture of 1-cell stage embryos has met with limited success due to a phenomenon termed the '2-cell block'. Modification of the culture media in compositions of energy substrates and ions, and supplementation of EDTA largely improved development of 1-cell embryos to blastocysts (Cross and Brinster, 1973; Abramczuk et al., 1977; Chatot et al., 1989; Gardner and Leese, 1990; Lawitts and Biggers, 1991).

Some ions and energy substrates, which are commonly found in the defined culture media for preimplantation development, have been shown to be detrimental to preimplantation embryonic development. High NaCl and K$^+$ concentration in culture media is detrimental on mouse embryonic development, causing cell block and inhibition of blastocyst formation (Wiley, 1984; Lawitts and Biggers, 1991; Dumoulin et al., 1993). Conversely, low levels of NaCl allow glutamine to impair embryonic development, although glutamine appears to be a preferred energy substrate during early development in the mouse (Chatot et al., 1989; Lawitts and Biggers, 1992). In contrast, glucose is detrimental to embryonic development at early stages especially prior to the morula stage (Seshagiri and Bavister, 1989a; Lawitts and Biggers, 1991). Phosphate plays a role in the toxic effects of glucose on early preimplantation embryonic development (Schini and Bavister, 1988; Seshagiri and Bavister, 1989b; Seshagiri and Bavister, 1991).

Thus, the electrolyte environment affects preimplantation embryonic development.
Although our knowledge is still limited, the electrolyte environment appears to play a central role in preimplantation embryonic development.

D. Electrolytes in the Uterus

Changes in electrolyte composition of uterine luminal fluid has been examined during delayed implantation in the rat and mouse, in which diapause of embryos at the blastocyst stage occurs as a normal part of reproduction. A decrease in Na\(^+\) levels has been found in the uterine luminal fluids of the rat and mouse (Setty et al., 1973; Van Winkel, 1977; Van Winkle et al., 1983). This has been interpreted as being part of the mechanism that regulates embryo metabolism, since embryos become metabolically less active during the delayed implantation period (McLaren, 1973; Weitlauf et al., 1979; Weitlauf and Kiessling, 1980). Low levels of Na\(^+\) have been shown to suppress embryo metabolism (Van Winkel, 1977; Van Winkel, 1981). However, low levels of Na\(^+\) in culture media may impair viability of embryos in vitro. During the delayed implantation period, the detrimental effect of the low levels of Na\(^+\) appears to be compensated against by some unknown mechanism. As progesterone is the dominant steroid hormone in the uterus at this time, progesterone may be involved in this regulatory mechanism.

V. DECIDUALIZATION

A. General

Decidualization is a conspicuous and critical part of implantation in species that have an true placenta, such as Carnivora, Rodenta and Primates. Since Loeb's original studies in 1908 (Loeb, 1908a,b), the process of decidualization has been studied in many species. Decidualization involves many cytological events and has been likened to the inflammatory reaction. Localized changes associated with decidualization are characterized by cell proliferation and differentiation, reorganization of the extracellular matrix, and infiltration of macrophages from the circulation (Aplin, 1991; Abrahamsohn and Zorn, 1993).
Decidual transformation of the stromal cells in the endometrium results in apparent increases in the size and weight of the uterus. The embryo becomes embedded in the enlarging mass of decidual tissue.

a. Deciduoma Induction Model

These histological changes are triggered by implanting embryos in naturally occurring decidualization. However, formation of the decidua can also be obtained in response to many different types of artificial stimuli (Loeb, 1907; Loeb, 1908a; De Feo, 1963a). This artificially induced decidua is usually referred to as deciduoma, to distinguish it from the naturally occurring decidua. Formation of the deciduoma and naturally occurring decidua are compatible, except for subtle differences in the timing of development and morphology (Deanesly, 1971; Lundkvist and Nilsson, 1982; Welsh and Enders, 1985). Induction of deciduoma has been used as a model to study the physiology of decidualization.

Decidualization can take place when a deciduogenic stimulus is applied during a very limited time, although this period varies with the nature of stimuli. The grossly traumatic stimuli such as cutting or crushing the uterus, can induce decidualization during day 3 to day 4 of pseudopregnancy. Less traumatic or non-traumatic stimuli such as intrauterine instillation of different chemical substances and intraperitoneal injection of pyrathiazin is only effective for a few hours during early pregnancy (De Feo, 1963a,b; Finn and Hinchliffe, 1965; Hetherington, 1968; Finn and Martin, 1972). This very limited period of sensitivity to non-traumatic stimuli seems to correspond to the period of uterine receptivity for implanting embryos (McLaren and Michie, 1956; Dickmann and Noyes, 1960; Noyes and Dickmann, 1960).
b. Function

The function of the decidua is not fully understood. The decidual tissue may play a role in controlling trophoblast invasion and serve as a source of nourishment for the embryo (Shelesnyak, 1962; Kirby, 1965). The decidua may also isolate each embryo and thus ensure the development of individual embryonic vascular systems, protecting embryos from the deleterious effects of adjacent implantation sites (De Feo, 1967). Furthermore, decidual tissue may provide a cleavage zone for placental separation at the time of delivery (Kirby, 1965). Prostaglandins (PGs) and oxytocin which appears to be important regulators in parturition is synthesized in the placenta of some species that include the human. The decidua plays a central role in steroidogenesis, as a part of the fetoplacental unit during pregnancy. Decidual tissue has been shown to produce many cytokines and growth factors in response to hormonal stimulation (Abrahamsohn and Zorn, 1993; Clark, 1993). Therefore, it is likely that decidual tissue plays a role in the endocrine and paracrine regulation of the implantation, maintenance of pregnancy and parturition.

Rodents, especially the rat, are the most commonly used animal model for studying decidualization. Therefore, the following literature review is based largely on the information obtained using this model. However, it is noteworthy that the degree of decidualization, as well as many details of this process, differ among species (De Feo, 1967).

B. Endocrine Regulation

a. Ovarian Steroid Hormones

The requirements of ovarian steroid hormones for decidualization have been well established using deciduoma induction in ovariectomized-steroid hormone primed animals (De Feo, 1967; Finn and Martin, 1972). It appears that the uterus needs to be exposed to progesterone for a minimum of 48 h, followed by a single, small dose of estrogen. The uterus becomes responsive to a deciduogenic stimulus some 24 h after exposure to estrogen
Maximal decidualization is achieved with three to four days of daily treatments of progesterone followed by a small dose of estrogen. Excessive or low doses of estrogen diminished the decidual reaction in response to deciduogenic stimuli. The process in which the uterus acquires the capability of responding to a deciduogenic stimulus is called uterine sensitization.

b. Pituitary Hormones

Evidence suggests that the pituitary gland is also involved in the regulation of decidualization (Kennedy and Doktorcik, 1988). A poor decidual response has been described in hypophysectomized rats. Treatment with GH and thyroid hormone, a substitute for TSH, restored the decidual response in ovariectomized-hypophysectomized rats (Kennedy and Doktorcik, 1988). Interestingly, the treatment with GH and thyroid hormone was able to restore the decidual response in hypophysectomized rats during the predecidualization period. Continuous treatment from the prestimulation (predecidualization) period throughout the poststimulation (decidual tissue formation) period had no further effect on decidual tissue formation. This may indicate that these hormones play a role in the uterine sensitization process rather than decidual tissue formation itself. However, the mechanisms through which pituitary hormones act in the uterus have not been defined. Pituitary hormones may restore the poor decidual reaction following hypophysectomy by improving impaired general metabolism caused by hypophysectomy, or may directly regulate uterine function in a specific manner.

C. Paracrine/Autocrine Regulation

There is a growing body of evidence to suggest that a variety of local regulators, such as cytokines and growth factors, regulate uterine growth and function (Tabibzadeh, 1991; Clark, 1993; Murphy and Ballejo, 1994; Weitlauf, 1994). It is believed that these local regulators form a complex regulatory network that contains intrinsic redundancy,
thereby assuring each step of the implantation process. These local regulators not only interact with each other but also interact with endocrine regulators such as ovarian steroid hormones. The spatio-temporal expression of these local regulators in the uterus appears to be tightly regulated mainly by ovarian steroid hormones (Murphy and Ballejo, 1994; Weitlauf, 1994). In contrast, little is known about their roles in the implantation or decidualization. However, recent evidence suggests these local factors which include such as epidermal growth factor (EGF), leukemia inhibitory factor (LIF), colony stimulating factor-1 (CSF-1) and interleukin-1β (IL-1β) may play a critical role in the process of implantation (Pollard et al., 1991; Stewart et al., 1992; Johnson and Chatterjee, 1993a,b; Simon et al., 1994a,b; Stewart, 1994a,b). This section reviews available information regarding these four cytokines and the IGF system in the uterus during the periimplantation period.

a. Epidermal growth factor (EGF)/Transforming Growth Factor (TGF)-α

EGF and its receptor have been detected in the uterus of mouse, rat and human. EGF has been further localized to the luminal and glandular epithelium (Gonzalez et al., 1984; Hoffmann et al., 1984; Mukku and Stancel, 1985a,b; Sheets et al., 1985; Chakraborty et al., 1988; DiAugustine et al., 1988; Lingham et al., 1988; Huet-Hudson et al., 1990). Estrogen appears to stimulate both ligand and receptor synthesis in the uterus of the mouse and rat (Gonzalez et al., 1984; Mukku and Stancel, 1985a,b; Lingham et al., 1988; Gargener et al., 1989), although there is little evidence to suggest that estrogen regulates the levels of the EGF receptor in the human uterus (Chengini et al., 1986; Berchuck et al., 1989). A localized increase in EGF binding at implantation sites has been shown in the mouse uterus, even before the blastocyst attachment (Brown et al., 1989). Furthermore, it has been demonstrated that EGF is capable of inducing implantation in the absence of nidatory estrogen during the experimentally-induced delayed implantation period in rats (Johnson and Chatterjee, 1993a,b). Expression of TGF-α mRNA, a cytokine that
binds to EGF receptor, increases with the progression of decidualization in the rat (Bonvissuto et al., 1992). EGF and TGF-α have also been demonstrated to mediate or even replace estrogen actions in the female genital tract in the mouse (Nelson et al., 1991; Nelson et al., 1992). EGF stimulates PG synthesis in the mouse and rat uterus which has been sensitized to decidual reaction (Paria et al., 1991; Bany and Kennedy, 1995). The EGF-mediated implantation can be reversed by a large dose of indomethacin, an inhibitor of PG synthase, and phospholipase A$_2$ (Johnson and Chatterjee, 1995). Together, along with the established roles of PGs in decidualization (Hoffman et al., 1977; Kennedy and Lukash, 1982; Kennedy, 1986a,b; Yee and Kennedy, 1988; Hamilton and Kennedy, 1994), EGF appears to initiate the decidualization and subsequent implantation through PG synthesis and/or activation of the arachidonic acid cascade in the uterine endometrial stroma.

b. Leukemia Inhibitory Factor (LIF)

The levels of LIF mRNA transcripts in the uterus increase, coinciding with the onset of blastocyst implantation, in the mouse, rabbit, and human (Bhatt et al., 1991; Charnock-Jones et al., 1994; Kojima et al., 1994; Yang et al., 1994). LIF expression is greater at implantation sites than other part of of endometrium. The time-specific expression of LIF appears to be under maternal control and precedes blastocyst implantation. Further studies using LIF-knockout mice revealed that LIF is essential for blastocyst implantation (Stewart et al., 1992). The LIF-deficient female mice are unable to become pregnant due to the failure of the blastocyst implantation. Infusion of recombinant LIF reversed the defect of implantation in LIF-deficient mice. LIF does not affect embryonic development up to blastocyst hatching and LIF-deficient embryos are capable of implanting in a normal recipient following embryo transfer. Thus, LIF undoubtedly plays an essential role in the implantation process, although the mechanism through which LIF regulates implantation has not been determined.
c. Colony Stimulating Factor (CSF)-1

CSF-1 mRNA transcripts have been localized to the uterine epithelium as early as day 3 of pregnancy in the mouse (Arceci et al., 1989). The levels of CSF-1 mRNA and protein dramatically increase during pregnancy in response to ovarian steroid hormones in the mouse and human (Muller et al., 1983; Muller et al., 1983; Arceci et al., 1989; Regenstreif and Rossant, 1989; Kauma et al., 1991; Pampfer et al., 1992). The spatio-temporal expression of this cytokine and its receptor has led to the hypothesis that this cytokine may play an important role in placentation and placental function (Pollard et al., 1987; Pollard, 1990). A role for CSF-1 in the regulation of implantation has been demonstrated in osteopetrotic (op/op) mice that lack CSF-1 synthesis due to homologous recessive mutation in the 5'-region of the CSF-1 gene (Pollard et al., 1991). These mice have severe osteopetrosis, various skeletal defects, a markedly diminished number of macrophage, are toothless and infertile. It has also been demonstrated that the injection of a small amount of CSF-1 during the preimplantation period results in a complete inhibition of implantation from certain genetic crosses (Tartakovsky et al., 1991). Thus, CSF-1 appears to be an essential local regulator of implantation. It is still not clear whether this cytokine plays a specific role in decidualization.

d. Interleukin (IL)-1β

IL-1β is found in human decidual tissue and has been localized to macrophages and trophoblast cells. In the mouse, IL-1β has been detected in the macrophage-like cells of the subepithelial stromal layer and placenta (Takacs et al., 1988; Kauma et al., 1990; De et al., 1992a,b; De et al., 1993; Simon et al., 1994a). Evidence suggests that ovarian steroid hormones may regulate this cytokine in the uterus (Polan et al., 1988; Kauma et al., 1990; De et al., 1992b; Simon et al., 1993). In the mouse, IL-1α and IL-1β mRNA levels and IL-1 activity increase from day 3 of pregnancy, peak between day 4 and day 5 when blastocyst implantation initiates, and then decrease on day 7 and day 8 (De et al., 1993).
The type I receptor is found predominantly in the uterine luminal epithelium in the human and mouse, especially at the site of implantation (Simon et al., 1994a,b).

Blastocyst implantation or, more specifically, the attachment of the blastocyst to the endometrium, can be blocked by IL-1 receptor antagonists in the mouse, indicating a role for IL-1 in mouse implantation (Simon et al., 1994a). IL-1β and -α have been shown to stimulate LIF and CSF-1 synthesis (Lubbert et al., 1991; Harty and Kauma, 1992). IL-1α also stimulates the synthesis of PGs, IL-6, and class II major histocompatibility complexes (Tabibzadeh et al., 1989; Tabibzadeh et al., 1990a,b; Jacobs et al., 1992; Bany and Kennedy, 1995). The ability of IL-1 to stimulate PG synthesis may suggest a role for this cytokine in decidualization. However, it is not known if IL-1 alone can induce implantation or decidualization. Furthermore, IL-1 has been linked to apoptosis (Van Damme et al., 1989). This may be an interesting function of this cytokine, since the implantation process contains apoptosis of the uterine endometrial epithelium (Parr et al., 1987).

e. The IGF system

Evidence suggest a potential role for the uterine IGF system in the regulation of the decidualization process. Both estrogen and GH, which regulate the uterine IGF system are involved in the regulation of decidualization in rats (Psychoyos, 1976; Kennedy and Doktorcik, 1988). The expression of IGF-I, IGFBPs, and IGF-I receptors coincides with the time of decidualization in the mouse and rat (Chandrasekhar et al., 1990; Croze et al., 1990a; Kapur et al., 1992; Sadek et al., 1994).

Temporal changes in IGF-I and IGFBP-1 mRNA expression are of particular interest. Expression of IGF-I mRNA is predominant in stromal cells on days 3 and 4, prior to decidualization, and in decidual cells. The levels of IGF-I mRNA in decidual cells decline on days 7 or 8 of pregnancy (Kapur et al., 1992). IGF-I mRNA transcripts are particularly abundant in the antimesometrial stromal tissue and is localized to the
periepithelial and periglandular stromal cells (Croze et al., 1990a). IGF-I levels increase during the preimplantation period (Katagiri et al., 1996).

The levels of IGFBP-1 and IGFBP-1 mRNA transcripts are first detected on day 5 of pregnancy. Maximal levels are observed on day 6 of pregnancy in the rat uterus (Croze et al., 1990a; Sadek et al., 1994). The majority of IGFBP-1 and its mRNA transcripts are localized to the luminal and glandular epithelium in the antimesometrial region and in the uterine luminal cavity. Interestingly, IGFBP-1 is not detected in any type of decidual cells in the rat (Sadek et al., 1994). This is in direct contrast to the expression pattern of IGFBP-1 in the human uterus where the majority of IGFBP-1 mRNA expression is localized to the decidual cells and pre-decidualized stromal cells of the late secretory phase (Seppala et al., 1994). In addition, IGF-I receptor levels increase during the sensitization period and at the time of decidualization in the rat (Chandrasekhar et al., 1990; Katagiri et al., 1996).

The spatio-temporal expression pattern of IGFBP-1, in conjunction with IGF-I mRNA expression in the adjacent periepithelial and periglandular stromal cells that surround the region of IGFBP-1 expression (Croze et al., 1990a), suggests a role for IGFBP-1 in the peri-implantation process. First, IGFBP-1 may inhibit the mitogenic action of IGF-I and IGF-II in the endometrium which allows stromal cells to differentiate into decidual cells (Croze et al., 1990a; Sadek et al., 1994). Second, it may be possible that IGFBP-1 acts as a carrier system which transports IGF-I from the adjacent stroma to the trophoblast prior to, or during, the interstitial invasion of the mesometrial decidua (Sadek et al., 1994). The expression of mRNA for IGF-I receptor, but not IGF-I, has been demonstrated in preimplantation rat embryos (Zhang et al., 1994). A paracrine role of uterine IGF-I in preimplantation embryonic development and implantation has been proposed in the mouse (Kapur et al., 1992).

Thus, the uterine IGF system appears to play a role in the decidualization process in the rat. The role for the uterine IGF system in the regulation of decidualization remains to
D. Initiation of Decidualization

a. General

The uterine sensitization for a deciduogenic stimulus appears to be regulated by maternal endocrine and paracrine regulation as discussed above. However, the exact mechanisms that initiate the decidual reaction are not fully understood. Interestingly, once the uterine endometrium has been sensitized for the decidual response, some changes occur before the trophoblast invades the endometrium. The most obvious of these are the increases in alkaline phosphatase activity levels and vascular permeability in the stroma, immediately adjacent to embryos (Finn and Hinchliffe, 1964; Kennedy, 1979; Milligan and Mirembe, 1984). However, the formation of decidual tissue and the major changes in the uterine vascular system do not occur in the absence of implanting embryos in a naturally occurring pregnancy, or unless a deciduogenic stimulus is applied in pseudopregnancy. This suggests that an embryonic factor or the implanting embryo itself may trigger decidualization.

Many factors, as well as physical stimuli by the implanting embryos, have been proposed to be the embryonic signal required for implantation. These substances appear to vary between species (Weitlauf, 1994). It seems probable that in many cases there is more than one embryonic signal. Furthermore, even if the same signal was present, the mode of action may be quite different between species (Weitlauf, 1994). Carbon dioxide, steroids, PGs, histamine, and some proteins of embryonic origin have been proposed to be the signal secreted by the embryo. Although none of these factors have been shown to play a critical role, one or more of these factors may act as the trigger which initiates decidualization in the sensitized endometrium. Many artificial deciduogenic stimuli may also trigger the same mechanism in the sensitized uterine endometrium during deciduoma formation.
b. Prostaglandins (PGs)

PGs are believed to play an important role in decidualization (Kennedy and Armstrong, 1981; Kennedy, 1983a; Kennedy, 1986b). Uterine PG levels increase during decidualization (Kennedy, 1977; Kennedy, 1979; Rankin et al., 1979; Kennedy, 1980a; Hoffmann et al., 1984; Malathy et al., 1986). Indomethacine inhibits and/or delays an increase in vascular permeability, decidual tissue formation and blastocyst implantation (Lau et al., 1973; Kennedy, 1977; Evans and Kennedy, 1978; Hoffman, 1978; Kennedy, 1979; Miller and O'Morchoe, 1982). PGs, which include PGE$_2$ and PGF$_2\alpha$, overcome the indomethacine inhibition (Kennedy, 1979; Kennedy and Lukash, 1982; Miller and O'Morchoe, 1982; Kennedy, 1986a,b). PGs also increase the alkaline phosphatase (ALP) activity which is associated with decidualization in the rat uterus (Yee and Kennedy, 1988; Yee and Kennedy, 1991).

Evidence suggests that the action of PGs is probably mediated by PGE$_2$ receptors in the uterine endometrium. PGE$_2$ receptors, but not PGF$_2\alpha$ receptors, have been detected in the rat uterus (Kennedy et al., 1983a,b; Martel et al., 1985). A single dose of PGF$_2\alpha$ is not effective in increasing vascular permeability. However, a constant infusion of PGF$_2\alpha$ appears to be as effective as PGE$_2$ (Kennedy, 1979; Kennedy and Lukash, 1982). Taken together, these observations suggest that PGF$_2\alpha$ may be converted to PGE$_2$ or that PGF$_2\alpha$ may interact with PGE$_2$ receptors to exert its effect in the rat uterus. Furthermore, evidence has linked some post receptor signaling mechanisms associated with PGE$_2$ receptors to decidualization in the rat, mouse and hamster. Uterine cAMP levels dramatically increase following the application of a deciduogenic stimulus and treatment with PGs (Leroy et al., 1974; Rankin et al., 1977; Rankin et al., 1979; Kennedy, 1983b; Johnston and Kennedy, 1984; Yee and Kennedy, 1991). The instillation of cholera toxin resulted in an increase in vascular permeability and decidual tissue formation (Rankin et al., 1977; Alleua et al., 1983; Johnston and Kennedy, 1984).
c. Histamine

Histamine may play a central role in the mechanisms that initiate decidualization in response to the "triggering" factor which is presumably of embryonic origin (De Feo, 1967; Weitlauf, 1994). First, histamine may be released by the mast cells in the uterus in response to the nidatory estrogen surge (Spaziani and Szego, 1958; Shelesnyak, 1959; Spaziani and Szego, 1959). An intraluminal histamine injection and systemic injection of histamine releaser induce decidualoma formation (Shelesnyak, 1952; Kraicer and Shelesnyak, 1958). Histamine antagonists prevent the formation of decidual tissue and reduce the number of implantation sites (Shelesnyak, 1952; Brandon and Wallis, 1977; Dey et al., 1978). Some objections have been raised based on evidence that histamine and histamine antagonists have failed to stimulate and inhibit, respectively, the decidual response in some studies (Finn and Keen, 1962a,b; Banik and Ketchel, 1964; Harper, 1965; De Feo, 1967; Humphrey and Martin, 1968). However, evidence suggests a critical role for uterine histamine release in response to the nidatory estrogen surge during the initiation of the decidual process.

E. Evaluation of Decidualization

Attempts have been made to quantitate the decidual response using many markers associated with decidualization. Markers include morphological and functional changes and various local products whose levels increase in the uterine endometrium which has been sensitized to the decidual response. However, some of these changes are noted even in the absence of implanting embryos or before any artificial stimulation to the endometrium which is sensitized to the decidual reaction. Some of these local products are unique to decidual cells, others are not unique but are present in decidual cells, while others are localized only to non-decidual cells. Interestingly, the pattern of synthesis of these local products among cell types varies among species. Furthermore, the role of these local products in decidualization are largely unknown. Nevertheless, the spatio-temporal
expression pattern of these factors in the uterus may provide a good basis for them to serve as markers for uterine sensitization and subsequent decidualization. In addition, if the uterine horn is treated locally rather than systemically, morphological and functional changes and increases in the levels of local products occur only in the treated uterine horn, particularly in species that have the duplex uterus like most of rodents. This allows investigators to use the other uterine horn as a control.

As discussed above, PGs, histamine, and other local factors play a critical role in the initiation of decidualization. Determination of increases in uterine PGs and histamine levels may serve as a marker for early decidualization. Some other factors serve as good markers for decidualization due to their appearance or an increase in the levels in the decidualizing uterus and availability of simple, yet sensitive, detection methods.

a. Decidual Tissue Mass

Since decidual tissue only forms in the presence of implanting blastocysts or an artificial stimulus, decidual tissue mass may be the most straightforward marker for decidualization. This marker can be used to determine the effect of various treatments on uterine sensitization, and the effects of different stimuli on the decidual response. Most studies have used whole uterine tissue rather than separated decidual tissue to estimate the size of decidual tissue mass. Such attempts consist of subjective gradings based on visual inspection (Astwood, 1939; Shelesnyak, 1952), measurement of the diameter of the uterine horn (Rothchild et al., 1940; Hisaw and Velardo, 1951), estimation of the length of uterine horn participating in the reaction (Shelesnyak and Kraicer, 1961), and determination of the weight of the uterus (De Feo and Rothchild, 1953; Velardo et al., 1953; Shelesnyak, 1957; De Feo, 1963a,b).

Determination of the single uterine horn weight has become a standard method. Although some studies examined the separated decidual tissue weight (Barkai et al., 1992), the majority have determined the weight of the whole uterine horn. Although weighing the
separated decidual tissue mass has some advantages, decidual tissue separation from the rest of the uterus may increase variability of the tissue weight. This artifact appears to be critical if decidual tissue mass is relatively small.

b. Vascular Permeability and Extracellular Fluid Volume

An increase in vascular permeability is one of the earliest signs of the decidual reaction (Finn and McLaren, 1967; Brandon, 1980; Kennedy, 1980b). The first detection of an increase in vascular permeability may vary, largely due to the nature of stimuli and the detection methods employed (Psychoyos, 1961; Lundkvist et al., 1977; Kennedy, 1979; Lundkvist and Nilsson, 1982). However, the increase in vascular permeability in the appropriately sensitized uterus becomes detectable as early as 15 min after an artificial stimulation (Milligan and Mirembe, 1984). Vascular permeability peaks 9 h after the stimulation, followed by a sharp decline at 12 h (Milligan and Mirembe, 1984).

Changes in uterine vascular permeability associated with the decidual response are determined by measuring the degree of leakage of molecules from the circulation that have been injected systemically. Earlier studies injected dyes such as Evans Blue and Pontamine Sky Blue (Psychoyos, 1973). Since dyes injected in the circulation are readily visible when vascular permeability increases, they have been used to identify the site of decidualization and implantation (Psychoyos, 1961; Psychoyos, 1973; Lundkvist and Nilsson, 1982). However, injection of dye may not be suitable for quantitation of changes in vascular permeability. Instead, the injection of radioisotope-labeled large molecules, in particular \(^{125}\text{I}\)-albumin, may serve as a better marker for changes in vascular permeability at the time of the decidual reaction.

Changes in the uterine vascular system associated with the decidual response can also be determined by an increase in the tissue blood space and a decrease in the extracellular space (Milligan and Edwards, 1990). The uterus has a large extracellular tissue compartment, the volume of which changes dramatically in response to the hormonal
stimulation (Spaziani, 1975). Histological changes associated with the decidual reaction are in close correlation with an increase in blood tissue space and a decrease in the extracellular space (Finn, 1977). However, since changes in the vascular permeability is more obvious and precedes these changes, the significance of these changes as a marker for the decidual reaction may be marginal.

c. Alkaline Phosphatase (ALP) Activity

The ALP activity is a widely used marker for the decidual reaction. The levels of ALP activity in the uterine endometrium, especially in the stroma, increase with decidualization in many species (Finn and Hinchliffe, 1964; Christie, 1966; Finn and McLaren, 1967; Hafez and White, 1967; Murdoch, 1970; Yee and Kennedy, 1988). The endometrial stromal cells sensitized to the decidual response appear to spontaneously decidualize in vitro (Sananes et al., 1978). This provides a convenient system to study the cellular physiology of decidualization such as intracellular signal transduction pathways for factors that may be involved in the regulation of the decidual reaction (Yee and Kennedy, 1991).

PGs stimulate uterine ALP activity as well as deciduoma tissue mass and vascular permeability (Kennedy and Armstrong, 1981; Kennedy, 1983a; Kennedy, 1986b; Yee and Kennedy, 1988). Therefore, the determination of PG-stimulated ALP activity, in addition to basal ALP activity, may allow us to examine the uterine response to PGs after different treatments for uterine sensitization. This may be of particular importance, when the study is performed in cultured endometrial stromal cells, since the other two markers; the decidual tissue mass and vascular permeability, are not available in the in vitro system.

Most reports have determined uterine ALP activity by the method of Lowry (Lowry, 1957). This classical assay is simple and sensitive enough to determine ALP activity in the rat uterine tissue.
d. Other Markers

Some of other factors have been found to be potential markers for decidualization. However, the limited availability and complexity of the assay system or detection methods compared to the aforementioned markers may prevent many of these factors from being used as markers for the decidual reaction.

IGFBP-1, also called placental protein 12, has drawn a lot of attention due to the high levels found in the decidual tissue of humans (Seppala et al., 1994). As discussed above, IGFBP-1 has also been suggested to play a role in the decidualization process in the rat (Croze et al., 1990a; Sadek et al., 1994). Interestingly, IGFBP-1 is localized to non-decidual cells in the antimesometrial region in the rat, while most IGFBP-1 expression has been localized to decidual cells in the human (Croze et al., 1990a; Sadek et al., 1994; Seppala et al., 1994).

Other potential markers for decidualization in the rat are prolactin-like proteins and $\alpha_2$-macroglobulin (Gibori et al., 1974; Bell, 1979; Jayatilak et al., 1989; Thomas and Schreiber, 1989; Croze et al., 1990b; Gu et al., 1992). Synthesis of prolactin-like proteins has been localized to the antimesometrial region of the uterus during decidualization (Jayatilak et al., 1989; Croze et al., 1990b). Prolactin-like proteins appear to stimulate $\alpha_2$-macroglobulin synthesis in the mesometrial region where the trophoblast invades the endometrium (Gu et al., 1992; Gu and Gibori, 1995). Since $\alpha_2$-macroglobulin is a potent protease inhibitor, this system may play a role in the regulation of trophoblast invasion (Gu et al., 1992). The localized expression of prolactin-like proteins and $\alpha_2$-macroglobulin suggests that the detection of mRNAs for these factors may serve as markers for the antimesometrial and mesometrial cells, respectively (Gu et al., 1992).
CHAPTER TWO

THE EFFECT OF IGF-I IN THE PREIMPLANTATION RAT EMBRYONIC DEVELOPMENT

I. INTRODUCTION

IGF-I and other related peptides such as insulin and IGF-II stimulate preimplantation embryonic development in the mouse (Harvey and Kaye, 1988; Harvey and Kaye, 1990; Harvey and Kaye, 1992a; Schultz et al., 1993; Schultz and Heyner, 1993). Insulin, in the presence of amino acids, stimulates morphological development of the 8-cell stage rat embryo to the blastocyst stage and later, if the resulting blastocysts are replaced in a receptive uterus (Zhang and Armstrong, 1990). Embryonic development is greatly impaired in diabetic rats and mice as determined by decreased cell number, especially in the ICM, and increased cell death in morulae and blastocysts, suggesting a role for maternal insulin and IGF-I during preimplantation development (Pampfer et al., 1990; Beebe and Kaye, 1991). These observations suggest that IGF-I possesses a growth promoting action in the preimplantation embryos. However, the ability of IGF-I to stimulate embryonic development may diminish after the blastocyst formation (Paria and Dey, 1990; Harvey and Kaye, 1992a).

IGF-I has been detected in the oviductal and uterine lumen (Letcher et al., 1989; Geisert et al., 1991; Ko et al., 1991; Wiseman et al., 1992; Smith et al., 1993). The levels of IGF-I in the uterus and uterine luminal fluids vary during the estrous cycle and early pregnancy period (Letcher et al., 1989; Murphy and Grahary, 1990; Geisert et al., 1991; Ko et al., 1991; Murphy, 1991; Kapur et al., 1992). The levels of uterine IGF-I increase during the preimplantation period and reaches maximal levels prior to implantation. Transcripts for the IGF-I receptor have been detected in oocytes and embryos of all
preimplantation stages in the rat (Zhang et al., 1994). However, mRNA transcripts for IGF-I have not been detected in the rat embryo throughout preimplantation development (Zhang et al., 1994).

Taken together, an emerging concept is that IGF-I, derived from the uterus, regulates preimplantation embryonic development in a paracrine manner in the rat. This chapter examines the effect of IGF-I on preimplantation embryonic development in the rat. Eight-cell stage rat embryos are cultured in the presence of IGF-I at various concentrations to determine if IGF-I stimulates embryonic development. Blastocysts are also transferred to a receptive uterus and their development examined to determine if the presence of IGF-I, during the preimplantation development period, improves the viability of blastocysts during implantation and subsequent fetal development.

II. MATERIALS AND METHODS

Preparation of IGF-I Free-Fetal Calf Serum

Fetal calf serum (FCS, GIBCO, Burlington, ON) was treated with anion exchange resin and charcoal, as previously described (Smith et al., 1988). Briefly, one liter of FCS was mixed with 56 g of AG 1-X8 (CL-) resin (Bio-Rad, Mississauga, ON) and gently shaken for 16 h. The mixture was centrifuged at 10,000 x g for 15 min and adsorbed FCS was collected. The procedure was repeated with fresh AG 1-X8 (CL-) resin. The FCS was mixed with 100 g of acid-washed charcoal and gently shaken for a further 16 h. The mixture was centrifuged at 10,000 x g for 15 min and adsorbed FCS was collected. The FCS was then passed through an AE/1 glass fiber filter (Gelman Science, Montreal, PQ), followed by two 0.2 μm filters (Gelman Science). The FCS which was considered to be IGF-I free-FCS was aliquoted and stored at -70°C.
Animal Treatments

All animals were obtained from the Animal Care Centre (the University of British Columbia, Vancouver, BC) and maintained at the animal care facility of British Columbia Children’s Hospital Research Centre (Vancouver, BC). Experiments were performed according to the guidelines of the Animal Care Committee of the University of British Columbia. Animals were kept under conditions of controlled lighting (14 h light: 10 h dark, light on at 6:00 h) and temperature (23 ± 2°C) with free access to food and water.

Immature female Sprague-Dawley rats were injected intraperitoneally (ip) with a single dose of 20 IU PMSG (Equinex, Ayerst, Montreal, PQ) prepared in 0.2 ml of 0.9% NaCl at 10:00 h at 26 to 29 days of age. Immature rats were housed overnight with a fertile male (1:2, male:female) 60 h after the PMSG injection, and examined for the presence of a vaginal plug at 8:00 h in the following morning (day 1 of pregnancy). Adult female rats (180-200 g body weight, BW) were mated with a fertile male (1:2, male:female) during the night of the proestrus or estrus stage, as determined by daily vaginal smear examinations, and examined for the presence of a vaginal plug in the following morning.

Embryo Collection

Embryos were collected at the 8-cell stage from superovulated immature rats at 2:00-3:00 h on day 4 of pregnancy by flushing the oviduct and uterine lumen with Dulbecco’s phosphate buffered saline (DPBS, GIBCO), pH 7.4, containing 1 mg/ml polyvinylalcohol (PVA, Sigma, St. Louis, USA). Eight cell embryos were pooled from at least four rats in each experiment. Blastocysts were collected from adult rats, ongoing normal pregnancy, at 11:00-12:00 h on day 5 (in vivo-blastocysts).

Embryo Culture

Eight cell embryos were washed twice in medium 199 (GIBCO) supplemented with 5% IGF-I free-FCS. This was used as the basic culture medium (M199). Fifteen to
twenty embryos were cultured in a 20 µl droplet of M199 with 0 (control culture), 0.02, 0.2, or 2.0 nM human recombinant-IGF-I (hr-IGF-I, Sigma) covered with mineral oil (Fisher Scientific, Fair Lawn, USA) for 36 h at 37°C with 5% CO₂ in humidified air. Each culture condition was repeated at least seven times.

**Developmental Stages of Embryos**

The developmental stages of cultured embryos were scored at the end of 36-h-culture under a phase-contrast microscope (200 x magnification). All cultured embryos were classified into four categories: blastocysts, morulae, uncompacted embryos and degenerated embryos. The blastocyst stage was defined by the presence of a definitive blastocoele cavity and an ICM. The morula stage was defined by the compaction of blastomeres without the blastocoele. Both of these stages were considered as growing embryos. Embryos at the morulae stage, without compaction, were considered to be non-growing embryos, but were scored as uncompacted embryos, a category distinct from degenerated embryos. Embryo degeneration was judged by the presence of multiple cell fragments with marked variation in size and shape, and a hazy blastomere outline (Miller and Armstrong, 1981a).

**Cell Count**

The number of cells in the ICM and trophectoderm of twenty blastocysts from 36-h-culture and in vivo-blastocysts were determined by the differential cell counting method (Handyside and Hunter, 1984). For differential cell staining, the zona pellucida was removed by an approximately 10 min incubation in 0.5% pronase (Sigma) in DPBS. Embryos without zona were incubated for 30 min in 10% heat-inactivated rabbit anti-rat lymphocyte antiserum (Sigma) in DPBS. Embryos were then incubated for 30 min in 5% guinea pig serum in DPBS containing 20 µg/ml bisbenzimide (Sigma) and 10 µg/ml propidium iodide (Sigma). Embryos were examined under fluorescent microscope to count
the number of nuclei in the ICM cells that stained blue, and those in trophectoderm cells which stained red. The number of cells in the ICM and trophectoderm were scored as the number of nuclei in each moiety of blastocysts. The number of cells in twelve in vivo-blastocysts were counted by the air-drying method (Tarkowsky, 1966). Cell death was judged by the presence of scattered nuclear fragments of various sizes. The dead-cell index was defined as a percentage of the number of dead cells in the total number of cells in whole blastocysts, or in the ICM and trophectoderm (Pampfer et al., 1990).

Protein Synthesis by Blastocysts

Blastocysts cultured for 36 h and in vivo-blastocysts were washed in serum free M199 medium and incubated for 3 h in the same medium. A group of ten embryos were incubated for 2 h in a 20 μl droplet containing 5 μM [4,5-3H]leucine (1 Ci/l, Amersham Canada, Oakville, ON), amino acids premixture (GIBCO) and 1 mg/ml PVA. Blastocysts were washed three times with ice-cold DPBS without radioactive leucine. Acid-insoluble material from blastocysts was subjected to scintillation counting. Protein synthesis was determined four times in each blastocyst group.

Implantation Rate and In Vivo Development

Adult female rats were mated with a vasectomized male during the night of the proestrus or estrus stage, as determined by the daily vaginal smear examination, and examined for the presence of a vaginal plug in the following morning (day 1 of pseudopregnancy). These rats were used as recipients for cultured embryos. Twelve blastocysts cultured for 36 h (six/uterine horn) were transferred to the recipient on day 5 of pseudopregnancy. Five rats/treatment group were used in these studies.

Under anesthesia, the ovary and tubal end of the uterine horn was exposed through an incision (approximately 1 cm) on the back of the rat. Six blastocysts were washed twice in DPBS containing 5% IGF-I free FCS, loaded into a transfer pipette (150-180 μm
internal diameter) and transferred with a minimum amount of medium into the uterine horn through a puncture made by a 25 G needle near the utero-tubal junction of the uterus. A further six blastocysts were transferred to the other uterine horn using the same protocol. All recipients were sacrificed on day 18, and the number of developing fetuses, resorption sites, and the weight of each fetus and placenta determined. The number of implantation sites was estimated as a total of the number of developing fetuses and resorption sites.

Statistical Analysis

The number of cells, [4,5-^3^H]leucine incorporation, and the weight of the fetus and placenta were compared by ANOVA, followed by the Tukey's test. The number of cells present in in vivo-blastocysts, determined by different counting methods, were compared by the two-tailed unpaired Student's t-test. The dead-cell indexes were compared by the Mann-Whitney Test. All other results, expressed as proportions, were compared by Chi-square test. Difference of the means was defined by a P-value of 0.01. Analysis was conducted using the computer software 'SYSTAT' (SYSTAT, Inc., Evanston, USA).

III. RESULTS

Developmental Stages of Embryos

The rate of embryonic development to the blastocyst stage increased in embryos treated with hr-IGF-I (Table 2-1). In contrast, the rate of uncompacted embryos decreased in embryos cultured in the presence of hr-IGF-I (Table 2-1). hr-IGF-I had no effect on the rate of embryo degeneration.

Cell Count

The total cell number of in vivo-blastocysts counted by the differential cell staining method was equivalent to the conventional air drying method (33.1 ± 1.0 vs. 33.4 ± 1.2,
Table 2-1. The developmental stages of 8-cell embryos following 36-h-culture with human recombinant-IGF-I at various concentrations

<table>
<thead>
<tr>
<th>IGF-I concentration (nM)</th>
<th>No. of embryos cultured</th>
<th>No. (%) of embryos at the indicated developmental stage*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>morula</td>
</tr>
<tr>
<td>0 (control)</td>
<td>207</td>
<td>62 (30.0)</td>
</tr>
<tr>
<td>0.02</td>
<td>143</td>
<td>38 (26.6)</td>
</tr>
<tr>
<td>0.2</td>
<td>131</td>
<td>38 (29.0)</td>
</tr>
<tr>
<td>2.0</td>
<td>167</td>
<td>36 (21.6)</td>
</tr>
</tbody>
</table>

a<b<c: Chi-square test (P<0.01)

*Morulae and blastocysts were considered growing embryos, while uncompacted and degenerated embryos considered non-growing embryos
mean ± SEM). The number of cells in the ICM and whole blastocysts of the 0.2 and 2.0 nM hr-IGF-I groups increased, compared to those of the control group, and was comparable to that of in vivo-blastocysts (Fig. 2-1). The presence of hr-IGF-I in the culture medium had no effect on the number of cells in the trophectoderm, at any concentration of hr-IGF-I. The dead cell index of all the culture groups was greater than that of in vivo-blastocysts, in both the ICM and trophectoderm (Fig. 2-2). The dead-cell index of the ICM in blastocysts of the 0.2 and 2.0 nM hr-IGF-I groups was less than the other two culture groups; however, the dead-cell index of the trophectoderm of all culture groups was equal.

Protein Synthesis

Protein synthesis by blastocysts of the 0.2 and 2.0 nM hr-IGF-I groups was the same as that of in vivo-blastocysts (Fig. 2-3). However, the levels of protein synthesis by blastocysts of the 0.2 nM hr-IGF-I group were not significantly different from those of the control and 0.02 hr-hr-IGF-I groups whose protein synthesis was significantly lower than that observed in the in vivo-blastocysts (Fig. 2-3).

Implantation Rate and In Vivo Development

All the transfer results were pooled by culture group. All recipients, but one in the control culture group, became pregnant. The rate of the implantation and live fetuses in the 0.2 and 2.0 nM hr-IGF-I groups was greater than those of the other two culture groups (Table 2-2). Although the implantation rate was greater in the 0.02 nM hr-IGF-I group, the rate of live fetuses was the same as the control group. There was no difference in fetal or placental weight across all the groups (Table 2-2).
The number of cells in the rat blastocysts obtained from cultures with varying concentrations of human recombinant (hr)-IGF-I. Eight-cell stage rat embryos were cultured for 36 h in the presence of 0 (control), 0.02, 0.2, or 2.0 nM hr-IGF-I. Blastocysts at the equivalent stage that had grown in vivo were obtained freshly. The number of cells in the inner cell mass (ICM) and trophectoderm of blastocysts in each culture group and blastocysts grew in vivo were determined by the differential cell counting method. Values represent the means and SEM for twenty blastocysts in each group. Letters on the top of bars indicate statistical differences of the means (a>b, P<0.01) across the different groups.
In vivo

- Control
- 0.02 nM
- 0.2 nM
- 2.0 nM

ICM
Trophectoderm
TOTAL

Number of cells in blastocysts
The dead-cell index in the rat blastocysts obtained from cultures with varying concentrations of human recombinant (hr)-IGF-I. Eight-cell stage rat embryos were cultured for 36 h in the presence of 0 (control), 0.02, 0.2, or 2.0 nM hr-IGF-I. Blastocysts at the equivalent stage that had grown in vivo were obtained freshly. Cell death was judged by the presence of the scattered nuclear fragments of various sizes. The dead-cell index was defined as a percentage of the number of dead cells in the total number of cells in whole blastocysts (TOTAL), or in the inner cell mass (ICM) and trophoderm. Values represent the means and SEM for twenty blastocysts in each group. Letters on the top of bars indicate statistical differences of the means (a<b<c, P<0.01) across the different groups.
Dead cell-index = \frac{\text{No. of fragmented nuclei}}{\text{No. of total nuclei}} \times 100 \, (%)
Figure 2-3  The levels of protein synthesis by the rat blastocysts obtained from cultures with varying concentrations of human recombinant (hr)-IGF-I. The 8-cell stage rat embryos were cultured for 36 h in the presence of 0 (control), 0.02, 0.2, or 2.0 nM hr-IGF-I. Blastocysts at the equivalent stage that had grown in vivo were obtained freshly. A group of ten blastocysts were incubated for 2 h in the presence of 5 mM [4,5-³H]leucine (1 Ci/l) and acid-insoluble material from blastocysts were subjected to scintillation counting. Values represent the means and SEM for four experiments in each group. Letters on the top of bars indicate statistical differences of the means (a>b, P<0.01) across the different groups.
In vivo
Control
0.02 nM
0.2 nM
2.0 nM

[3H]leucine incorporation
($\times 10^3$ cpm/blastoceyst)

- a
- b
- a, b
Table 2-2. The rate of implantation and development into day 18 fetuses of blastocysts obtained from the cultures with human recombinant-IGF-I at various concentrations*

<table>
<thead>
<tr>
<th>IGF-I concentration (nM)</th>
<th>Control</th>
<th>0.02</th>
<th>0.2</th>
<th>2.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. (%) of developing fetuses**</td>
<td>16 (26.7)a</td>
<td>20 (33.3)a</td>
<td>41 (68.3)b</td>
<td>44 (73.3)b</td>
</tr>
<tr>
<td>No. (%) of implantation**</td>
<td>19 (31.7)a</td>
<td>29 (48.3)b</td>
<td>48 (80.0)c</td>
<td>47 (78.3)c</td>
</tr>
<tr>
<td>Fetal weight (g)***</td>
<td>1.03 ± 0.08</td>
<td>0.97 ± 0.09</td>
<td>1.07 ± 0.11</td>
<td>1.01 ± 0.06</td>
</tr>
<tr>
<td>Placental weight (g)***</td>
<td>0.65 ± 0.19</td>
<td>0.72 ± 0.21</td>
<td>0.63 ± 0.18</td>
<td>0.66 ± 0.13</td>
</tr>
</tbody>
</table>

*A total of sixty blastocysts were transferred in each group.

**Results from five recipients of the same culture group were pooled.

***Values represent the means ± SD.

a<b<c: Chi-square test (P<0.01)
IV. DISCUSSION

The presence of hr-IGF-I in the culture medium stimulated morphological development of the rat embryo *in vitro*. IGF-I appears to stimulate blastocyst formation and compaction of morulae. hr-IGF-I at all examined concentrations (0.02-2.0 nM) increased the rate of embryonic growth into the blastocyst stage, and decreased that of uncompacted embryos (Table 2-1). The present findings are supported by previous studies. Insulin, a closely related peptide to IGF-I, enhances the growth promoting action of amino acids on preimplantation rat embryonic development (Zhang and Armstrong, 1990). IGF-I stimulates preimplantation development, by increasing compaction and blastocyst formation in the mouse embryo (Harvey and Kaye, 1992a). Insulin also stimulates blastocyst formation but has no effect on the compaction of mouse embryos (Harvey and Kaye, 1990; Gardner and Kaye, 1991). Furthermore, retardation in preimplantation embryonic development has been found associated with diabetes in the rat and mouse, suggesting a role for insulin in preimplantation development (Pampfer et al., 1990; Beebe and Kaye, 1991).

IGF-I may modulate blastocyst formation through stimulating Na\(^+\)/K\(^+\)-ATPase synthesis. IGF-I has been shown to stimulate Na\(^+\)/K\(^+\)-ATPase synthesis in many species (Madsen and Bern, 1993; Matsuda et al., 1993). As previously discussed, evidence suggests that Na\(^+\)/K\(^+\)-ATPase plays a central role in formation of the blastocoele in the rabbit and mouse (DiZio and Tasca, 1977; Biggers et al., 1978; Wiley, 1984; Vestweber et al., 1987; Manejwala et al., 1989). The levels of mRNA transcript and protein expression of Na\(^+\)/K\(^+\)-ATPase subunits increase from about the time of compaction until the expanded blastocyst stage (Gardiner et al., 1990a,b). The increase in the levels of Na\(^+\)/K\(^+\)-ATPase subunit coincides with the increase in levels of mRNA encoding IGF-I receptor in the preimplantation embryo (Rappolee et al., 1991).

It has been suggested that morphologically defined blastocysts (embryos with a
blastocoele) may not always be capable of implantation and post-implantational development. This argument is based on at least two lines of observations. First, the formation of a blastocoele cavity may simply reflect a difference in the ability of blastomeres to transport fluid (Wiley, 1984). The development of mechanisms involved in fluid transport is related to the number of cell cycles, or time after fertilization, rather than the actual number of cells in embryos or the differentiation of the ICM (Smith and McLaren, 1977; Pratt et al., 1981; Chisholm et al., 1985; Winston et al., 1991). It has been demonstrated that a substantial proportion of blastocysts with a fewer number of cells may also have a numerically deficient ICM (Pratt et al., 1981; Hardy et al., 1989). These blastocysts may be less capable or incapable of subsequent development.

Secondly, evidence suggests that morphologically normal blastocysts, especially those cultured in vitro, often contain multinucleated cells (Tesarik et al., 1987b; Winston et al., 1991). The most common method to determine the number of cells is to count the number of nuclei rather than the true cell number (Tarkowsky, 1966; Handside and Hunter, 1984). An increase in the number of nuclei does not reflect an increase in cell number in the presence of multinucleated cells. Although formation of bi- or multinucleated cells in the trophoblast appears to be a part of the normal developmental process (Barlow and Sherman, 1972; Long and Williams, 1982), failure of cytokinesis and kariokinesis may result in the formation of multinucleated cells during the earlier stages of embryonic development (Tesarik et al., 1987b; Winston et al., 1991). The formation of multinucleated cells, especially in the ICM, may be detrimental to embryonic development (Winston et al., 1991). Therefore, incorporation of other examinations, in addition to the scoring of developmental stages and counting the number of cells (nuclei), are highly desirable to determine viability of embryos following in vitro treatments.

The present study examined the viability of blastocysts from an in vitro culture using multiple criteria. hr-IGF-I, especially at concentrations of 0.2 and 2.0 nM, appears to be beneficial to the quality of blastocysts. hr-IGF-I had a mitogenic effect on the cells in
the ICM (Fig. 2-1) and decreased the dead-cell index in the ICM of the same hr-IGF-I concentration groups (Fig. 2-2). Thus, hr-IGF-I appears to increase in the number of living cells in the ICM which develop into the fetus. This may contribute to the observed increase in the viability of blastocysts which, in turn, increases the rate of implantation and subsequent fetal development (Table 2-2). Furthermore, blastocysts of the 2.0 nM hr-IGF-I group was metabolically more active than blastocysts of the control and 0.02 nM hr-IGF-I groups (Fig. 2-3). Thus, the improvement in the developmental stage of embryos by 0.2 and 2.0 nM hr-IGF-I appears to be accompanied by an improvement in viability of embryos. In contrast, hr-IGF-I at 0.02 nM improved developmental stage of embryos and increased the implantation rate to a lesser extent (Tables 2-1 and 2). Other variables, such as the number of cells, dead-cell index, protein synthesis, and the rate of developing fetuses, remained constant (Figs. 2-1, 2 and 3, and Table 2-2). These results suggest that a large portion of blastocysts of the 0.02 nM group may be less capable or incapable of implantation or postimplantation development.

The mitogenic action of hr-IGF-I and improved dead-cell index by hr-IGF-I were observed in the ICM but not in the trophectoderm (Figs. 2-1 and 2). The selective effect of IGF-I and insulin on the cells of the ICM has been shown to occur in the mouse embryo (Harvey and Kaye, 1990; Harvey and Kaye, 1992a; Smith et al., 1993), with a single exception in which insulin increased the number of cells in the ICM and trophectoderm (Smith et al., 1993). The effects of IGF-I and insulin on the isolated ICM from the trophectoderm by immunosurgery technique are compatible to those observed in the whole blastocyst (Harvey and Kaye, 1990; Harvey and Kaye, 1992a). This suggests that IGF-I and insulin act directly on the cells of the ICM rather than exerting their effects through the activation of paracrine or paracellular functions of the trophectoderm cells. However, the trophectoderm appears to play a role in transferring these peptides from their surroundings to the ICM. The cells of the trophectoderm of mouse blastocysts internalize IGF-I and insulin via receptor-mediated endocytosis, and then transfer the peptides to the ICM.
hr-IGF-I had no effect on fetal and placental weights on day 18 of pregnancy (Table 2-2). Treatment of the preimplantation rat embryo with insulin in the presence of amino acids has increased the rate of implantation and the number of live fetuses. However, insulin had no effect on fetal and placental weights determined on day 18 of pregnancy under these culture conditions (Zhang and Armstrong, 1990). It is apparent that exceptionally large or small fetuses are not beneficial for reproduction. Accordingly, evidence suggests that fetal growth is strictly regulated by unknown mechanisms that optimize the size of fetuses in a given species. The 'giant' or 'miniature' blastocyst produced by aggregation of two or multiple embryos and by bisection of embryos, respectively, result in the fetus having similar weight by the middle or end of gestational period.

The present study indicates that the effect of IGF-I on the rat embryo is mediated by the IGF-I receptor. It is believed that IGF-I can exert its biological effect through IGF-I, IGF-II, or insulin receptors. Expression of IGF-I receptor mRNA has been detected in oocytes and embryos throughout preimplantation development stages in rats (Zhang et al., 1994). Transcripts of the IGF-I receptor are readily detectable in oocytes, 1-cell and 2-cell stage embryos and in blastocysts. The transcripts are also detectable in 4-cell and 8-cell stage embryos but at highly reduced levels (Zhang et al., 1994). In contrast, levels of IGF-II receptor mRNA has been detected at consistent levels in rat oocytes and embryos of all stages. In addition, insulin receptor mRNA has been detected in a similar pattern to that of the IGF-I receptor mRNA expression in the rat (Zhang et al., 1994). The temporal decline in the levels of IGF-I and insulin receptor mRNA expression may reflect the transition of the regulation of gene expression from maternal to embryonic transcripts (Telford et al., 1990; Rappolee et al., 1991). It is not known if the levels of functional receptors for IGF-I and insulin fluctuate in a similar pattern to the levels of mRNA transcripts.

IGF-I appears to exert its effect through the IGF-I and insulin receptors in the
preimplantation mouse embryo (Harvey and Kaye, 1991c; Harvey and Kaye, 1992b). B-10 Fab fragments of IgG from anti-insulin receptor autoantiserum, a specific insulin receptor antagonist, have completely blocked the effect of IGF-I and insulin on protein synthesis in the mouse embryo (Harvey and Kaye, 1991c; Harvey and Kaye, 1992b). B-10 Fab fragments have also inhibited the growth promoting action of insulin, which include mitogenic action on the ICM and the stimulation of morphological development, but had no effect on the growth promoting actions of IGF-I (Harvey and Kaye, 1992b). These observations suggest that the growth promoting action of IGF-I in the mouse embryo may be mediated by the IGF-I and/or IGF-II receptors. Furthermore, although the IGF-II receptor is expressed in the preimplantation mouse embryo as early as the 2-cell stage, the IGF-II receptor does not appear to mediate the growth promoting actions of IGF-II (Rappolee et al., 1992). Instead, the growth promoting actions of IGF-II may be mediated by the IGF-I and insulin receptors (Rappolee et al., 1992). Therefore, the growth promoting action of IGF-I in the mouse embryo may be mediated by the IGF-I receptor. Taken together, the presence of the IGF-I receptor in the rat embryo, the growth promoting action of hr-IGF-I in the present study appears to be mediated by the IGF-I receptor.

In contrast, the action of IGF-I on metabolic stimulation like protein synthesis may be mediated by the insulin receptor. As discussed above, evidence suggests that the effect of IGF-I on protein synthesis in the preimplantation mouse embryo appears to be mediated by the insulin receptor (Harvey and Kaye, 1991c; Harvey and Kaye, 1992b). The levels of protein synthesis of the 0.2 nM hr-IGF-I group was not statistically different from those of the control group and the 2.0 nM hr-IGF-I group, where protein synthesis was greater than that of the control group, and were at intermediate levels of the two groups (Fig. 2-3). However, most of the actions of hr-IGF-I were apparent at the 0.2 nM hr-IGF-I group. The high levels of hr-IGF-I which were required to stimulate protein synthesis suggest that this action is being mediated by receptors other than the IGF-I receptor, such as the insulin and IGF-II receptors. The IGF-II receptor does not appear to mediate the action of IGF-II
on protein synthesis in the mouse (Rappolee et al., 1992). The insulin receptor may be responsible for the observed action of hr-IGF-I on protein synthesis in the preimplantation rat embryo observed in the present study.

The concept that the growth promoting action and metabolic stimulation of IGF-I are mediated through the IGF-I and insulin receptors, respectively, is consistent with previous observations. A differential signaling potential of the cytoplasmic domain of the β-subunit of human insulin and IGF-I receptors has been demonstrated using transfection techniques in the mouse NIH-3T3 fibroblast cell (Lammers et al., 1989). The cytoplasmic domain of the IGF-I receptor appears to have a greater signaling potential in the long-term effect, such as mitogenic actions (Lammers et al., 1989). The IGF-I receptor is more potent than the insulin receptor in mediating mitogenic and growth promoting activity, while the insulin receptor has a greater potential in cellular energy metabolism in many cell types (Hintz et al., 1972; Lammers et al., 1989; William, 1991).

Finally, the rat uterus is one of the major sites of IGF-I synthesis (Murphy et al., 1987a,c; Norstedt et al., 1989). It has been shown that the uterine luminal fluids contain IGF-I in various species (Letcher et al., 1989; Geisert et al., 1991; Ko et al., 1991; Smith et al., 1993). Internalization of maternally derived IGF-I via receptor-mediated endocytosis by preimplantation embryos has been demonstrated in the mouse (Smith et al., 1993). Expression of IGF-I, IGF-II, and insulin receptors, but not that of IGF-I, have been detected in the preimplantation rat embryo (Zhang et al., 1994). These, together with observed beneficial effects of hr-IGF-I on preimplantation development in the rat, suggest that IGF-I may be involved in the maternal-to-fetal signaling mechanisms that mediate synchronized development between the uterus and preimplantation embryos.
V. SUMMARY AND CONCLUSIONS

The present study demonstrated a potential role for IGF-I in the development of the preimplantation rat embryo. IGF-I appears to promote morphological development of rat embryos to the blastocyst stage. The improvement in the developmental stage of embryos by IGF-I appears to be accompanied by an improvement in viability of embryos, as determined by an increase in the number of live cells in the ICM, an increase in protein synthesis, and a greater rate of implantation and fetal development. The effects of hr-IGF-I were observed at the concentrations at which IGF-I has been shown to effect other cell types through the IGF-I receptor.

These experimental observations support the hypothesis that IGF-I may be involved in the maternal-to-fetal signaling mechanisms. This in turn may mediate synchronized development between the uterus and preimplantation embryos. Alternately, a well-regulated uterine IGF system may be required for synchronized development between the uterus and embryos. Therefore, factors that disturb the regulation of uterine IGF system may be detrimental to preimplantation embryonic development and subsequent postimplantational development in the rat.
CHAPTER THREE

THE EFFECT OF SUPEROVULATION ON THE UTERINE IGF SYSTEM

I. INTRODUCTION

The IGF system is one of a number of regulatory systems which mediate steroid hormone actions in the uterus in an autocrine/paracrine manner. IGF-I is a key member of the IGF system. Other components of this system are IGFBPs and IGF-I receptors. IGF-I synthesis in the uterus is regulated primarily by estradiol-17β rather than GH, the principal regulator of IGF-I synthesis in most other tissues (Murphy et al., 1987c; Murphy and Friesen, 1988; Norstedt et al., 1989). Other components of the IGF system are also under ovarian steroid hormone regulation in the uterus (Ghahary and Murphy, 1989; Girvigian et al., 1994). Therefore, it is likely that changes in the levels of ovarian steroid hormones following superovulation will disturb the regulation of the IGF system in the uterus. Such disturbances in the uterine IGF system may, in turn, be responsible for the detrimental effects seen with superovulation, such as early embryonic loss.

The objective of this chapter is to determine whether the uterine IGF system is changed by superovulation. The study is performed using an immature rat model, in which the reproductive physiology and the detrimental effects of superovulation have been well characterized. This chapter examines the modifications in the uterine IGF system following superovulation. In the immature superovulation model, 40 IU PMSG has been used as a superovulatory dose, while 4 IU PMSG used as a control (Miller and Armstrong, 1981a; Yun et al., 1987; Yun et al., 1989).
II. MATERIALS AND METHODS

Animal Treatments

All animals were obtained from the Animal Care Centre of the University of British Columbia. Experiments were performed according to the guidelines of the Animal Care Committee of the University of British Columbia. Animals were kept under conditions of controlled lighting (14 h light: 10 h dark, light on at 6:00 h) and temperature (23 ± 2°C) with free access to food and water. At 28 days of age, immature female Sprague-Dawley rats were injected (ip) with either 4 or 40 IU PMSG prepared in 0.2 ml of 0.9% NaCl at 9:00-10:00 h. Females were mated at 30 days of age. Day 1 of pregnancy was determined by the presence of a vaginal plug. Six females from each group were sacrificed daily at 9:00-10:00 h, from day 1 to day 6, for IGF-I and IGFBP assays. Another six rats from each group were sacrificed for IGF-I receptor assays at every time point. After decapitation, trunk blood was collected and the uterus dissected. The oviductal and uterine lumen were flushed with DPBS containing 3 mg/ml polyvinylpyrrolidone (Sigma) for embryo collection.

In separate experiments, uterine endometrial IGF-I, IGFBP, and IGF-I receptor levels were determined in rats given 4 or 40 IU PMSG as above. Rats were sacrificed on days 1, 3, 5, or 6 and the uterine horns dissected. The uterine horns were opened longitudinally and the endometrial tissue separated from the myometrium by mechanical scraping. The endometrial tissue from 4 to 6 rats were pooled and used for the IGF-I and IGFBP assays. The endometrium from another 4 to 6 rats were used for the IGF-I receptor assay. Experiments were repeated three times.

Four rats from each PMSG treatment group were sacrificed at 9:00-10:00 h daily from the day of PMSG treatments (day -2) to the day of mating (day 0) to collect the blood samples for estradiol-17β and progesterone assays. Serum ovarian steroid levels from day 1 to day 6 of pregnancy were determined by using blood samples from 4 out of 6 rats used
for the IGF-I and IGFBP assays.

**RIA for Ovarian Steroid Hormones**

Estradiol-17β and progesterone serum levels were determined by RIA (Yun et al., 1987). The cross-reactivity of the estrogen antiserum was: estrone, 2.9%; estriol, 0.5%; other major ovarian steroids, <0.2%. The cross-reactivity of the progesterone antiserum was: 5β-pregnan-3,20-dione, 35.5%; 5α-pregnan-3,20-dione, 15.7%; 3α-hydroxy-5β-pregnan-20-one, 2.0%; 20β-hydroxy-4-pregnen-3-one, 1.3%; 17-hydroxyprogesterone, 1.2%; other major ovarian steroids, <0.2%. The intra-assay coefficients of variation for both assays were <10% throughout the effective range of the standard curves. The inter-assay coefficients of variation for both assays were <15% throughout the effective range of standard curves.

**Sample Preparation for IGF-I and IGFBP Assays**

The uterus was weighed, minced and homogenized in 1 M acetic acid (1 g/5 ml) and centrifuged at 3,000 x g for 30 min at 4°C. The supernatants were saved and the pellets re-extracted. Supernatants from both centrifugation steps were combined, concentrated, and applied to a Sephadex G-75 (Sigma) column. The Sephadex G-75 column was previously calibrated with molecular weight markers (Sigma); aprotinin (6.5k), cytochrome c (12.4k), carbonic anhydrase (29k), and bovine albumin (66k). The void volume was determined by blue dextran. Fractions containing a molecular weight range between 20k and 60k were considered to contain IGFBP. Fractions with a molecular weight range below 10k were considered to contain IGF-I. In a preliminary study, 91 to 96% of IGF-I and 89 to 93% of IGFBP were collected in these fractions, respectively. An aliquot of each fraction was subjected to protein assay (Lowry et al., 1951). Fractions were lyophilized and stored at -20°C until assayed. The serum was mixed with the same volume of 1 M acetic acid and the mixture applied to a Sephadex G-75 column to separate
IGF-I from IGFBP. IGF-I and IGFBP fractions were collected, lyophilized and stored as above.

**RIA for IGF-I**

The concentration of IGF-I in the IGF-I fractions of the serum and uterine protein extract was determined by RIA using a IGF-I $^{125}$I-RIA kit (INCSTAR, Stillwater, USA). The minimum detectable concentration of the IGF-I RIA kit is $<2.0$ nM (15.4 ng/ml). Cross-reactivity to peptides; IGF-II, human GH, fibroblast growth factors, TGF, and platelet derived growth factor was determined to be $<1\%$. The intra-assay coefficients of variation were 8.4%, 10.1%, and 9.1% at the low, medium and high ends of standard curve, respectively. The inter-assay coefficients of variation were 12.5%, 10.3%, and 15.2% at the low, medium and high ends of standard curves. The lyophilized uterine protein extracts were reconstituted in 200 μl of RIA buffer.

**Ligand Binding Assay for Total IGFBP**

IGFBP levels in the serum and uterine protein extracts were determined by a ligand binding assay using the IGFBP fractions from Sephadex G-75 columns. For ligand binding assays (IGFBP and IGF-I receptor), 1 μg of hr-IGF-I was iodinated with 1 mCi Na$^{125}$I (Amersham Canada) and 2.5 mg Iodogen (Pierce, Rockford, USA). The $^{125}$I-IGF-I, with a specific activity of 220-280 μCi/mg, was purified on a Sephadex G-50 column and aliquots stored at -20°C until used. The lyophilized IGFBP fractions were reconstituted at a protein concentration of 1 mg/ml in 50 mM tris-HCl buffer, pH 7.2, containing 500 mM NaCl. One hundred μl of the reconstituted IGFBP fraction was blotted on to a nitrocellulose membrane and incubated with 2% BSA (Sigma) in the same buffer at room temperature for 3 h. The membranes were then incubated with 0.5 nM $^{125}$I-IGF-I in 100 μl of the tris-HCl buffer containing 0.5% BSA at 4°C for 12 h. The nitrocellulose filters were washed twice with 0.05% Tween-20 (Bio-Rad) in tris-HCl buffer and the
radioactivity levels determined using a γ-counter. The non-specific binding, determined by using 100 μg BSA, was always <3%. The specific binding was calculated by subtracting the non-specific binding from the total binding for each sample.

**Membrane Preparation for IGF-I Receptor Assay**

Uteri were dissected, minced and homogenized in 50 mM tris-HCl buffer (1 g/5 ml), pH 7.4, containing 250 mM sucrose and 1 mM CaCl₂. Homogenates were centrifuged at 700 x g for 10 min. Pellets were re-homogenized in fresh buffer and centrifuged. Supernatants from both centrifugations were combined and centrifuged at 40,000 x g for another 40 min. The pellets were then washed twice and resuspended in 50 mM tris-HCl, pH 7.8, containing 0.5% BSA at 2 ml/g starting tissue weight. All steps were carried out at 4°C. An aliquot of the membrane preparation was subjected to protein assay (Lowry et al., 1951).

**Ligand Binding Assay for IGF-I Receptor**

Each binding assay contained 0.1 nM ¹²⁵I-IGF-I and 50 μg membrane protein in 50 mM tris-HCl buffer, pH 7.8, containing 0.1% BSA in a volume of 100 μl. Assays were incubated for 20 h at 4°C after which 300 μl of 25% polyethylene glycol, previously chilled on ice was added to the reaction mixture. Assays were centrifuged at 3,000 x g for 15 min at 4°C and the pellets washed with 100 μl of 12.5% polyethylene glycol. The levels of radioactivity in the pellets were determined using a γ-counter. Non-specific binding, determined by adding 100-fold excess of unlabeled IGF-I, was <2%. The specific binding was calculated by subtracting the non-specific binding determined in each measurement from the total binding for each sample. Fifty percent displacement of ¹²⁵I-IGF-I binding by unlabeled IGF-I, human recombinant IGF-II (Sigma), and insulin (Sigma) was observed at peptide concentrations of approximately 2, 8, and 850 nM, respectively (Fig. 3-1).
Figure 3-1  Competition-inhibition curves for $^{125}$I-IGF-I binding to the uterine membrane preparations by human recombinant (hr)-IGF-I, hr-IGF-II, and insulin. Each competition-inhibition assay contained 0.1 nM $^{125}$I-IGF-I and 50 µg membrane protein in a volume of 100 µl with varying concentrations of each peptides. The competition-inhibition curves were determined in three separate membrane preparations for each peptides. The competition-inhibition curves in all membrane preparations were similar to each other for each peptides. The means of fifty percent displacement of $^{125}$I-IGF-I binding by hr-IGF-I, hr-IGF-II, and insulin was observed at peptide concentrations of 2, 8, and 850 nM, respectively.
Statistical Analysis

Data are expressed as the mean ± SEM. The means of IGF-I, IGFBP and IGF-I receptor levels between treatment groups were compared using the Student's t-test. Means of IGF-I, IGFBP, and IGF-I receptor levels between different days were compared by ANOVA, followed by Tukey's test. Differences were considered to be statistically significant at the 95% confidence level (P<0.05). Analysis was conducted using the computer software ‘SYSTAT’.

III. RESULTS

Serum Steroid Levels

The levels of estradiol-17β and progesterone were greater throughout the experimental period in animals undergoing superovulation compared with those of the control group (Fig. 3-2). The increase in the levels of progesterone in the superovulated rats become more apparent during the postovulatory period. A secondary peak of estradiol-17β was observed on day 2 of pregnancy in the superovulated rats, in addition to the first peak on the day of ovulation (Fig. 3-2).

Uterine and Serum IGF System

Total IGFBP and IGF-I receptor levels are presented as a percentage of the levels of the control group on day 1 (Figs. 3-3, 4, and 5). The IGFBP levels (100%) of the whole uterus, separated endometrium and serum were 61 ± 2.0, 83 ± 11, and 140 ± 10 pmol IGF-I/mg protein, respectively (Fig. 3-3, 4, and 5). The IGF-I receptor levels of the whole uterus and separated endometrium were 58 ± 2.9 and 86 ± 12 fmol IGF-I/mg membrane protein (Figs. 3-3 and 4).

IGF-I levels in the whole uterine tissue homogenates obtained from the control group increased throughout the preimplantation period. Peak levels were observed on day
Figure 3-2 Profiles of the serum levels of estradiol-17β (A) and progesterone (B) in superovulated and control rats. Immature rats were injected with a single dose of 40 IU PMSG (superovulation) or 4 IU PMSG (control). Rats were mated 60 h after the PMSG injection and sacrificed on the indicated days (day 1: day of vaginal plug). The serum levels of estradiol-17β and progesterone were determined by RIA. Values represent the means and SEM for four animals in each point.
Figure 3-3 Profiles of IGF-I (A), IGFBP (B), and IGF-I receptor (C) levels in the uterus of superovulated and control rats. Immature rats were injected with a single dose of 40 IU PMSG (superovulation) or 4 IU PMSG (control). Rats were mated 60 h after the PMSG injection and sacrificed on the indicated days (day 1: day of vaginal plug). The levels of IGF-I in the uterine extracts were determined by RIA and the levels of IGFBP and IGF-I receptor determined by ligand binding assay using $^{125}$I-IGF-I. The levels of IGFBP and IGF-I receptor are presented as a percentage of the levels of the control group observed on day 1 of pregnancy. Values represent the means and SEM for six animals in each point. Asterisks indicate statistical difference of the means between two groups (P<0.05).
Profiles of IGF-I (A), IGFBP (B), and IGF-I receptor (C) levels in the uterine endometrium of superovulated and control rats. Immature rats were injected with a single dose of 40 IU PMSG (superovulation) or 4 IU PMSG (control). Rats were mated 60 h after the PMSG injection and sacrificed on the indicated days (day 1: day of vaginal plug). Endometrial tissue, separated from the rest of the uterus, of four to six rats were pooled. The levels of IGF-I in the uterine extracts were determined by RIA and the levels of IGFBP and IGF-I receptor determined by ligand binding assay using $^{125}$I-IGF-I. The levels of IGFBP and IGF-I receptor are presented as a percentage of the levels of the control group observed on day 1 of pregnancy. Values represent the means and SEM for three pools of the endometrial tissues in each point. Asterisks indicate statistical difference of the means between two groups ($P<0.05$).
Profiles of IGF-I (A) and IGFBP (B) levels in the serum of superovulated and control rats. Immature rats were injected with a single dose of 40 IU PMSG (superovulation) or 4 IU PMSG (control). Rats were mated 60 h after the PMSG injection and sacrificed on the indicated days (day 1: vaginal plug). The levels of IGF-I were determined by RIA and the levels of IGFBP determined by ligand binding assay using $^{125}$I-IGF-I. The levels of IGFBP are presented as a percentage of the levels of the control group observed on day 1 of pregnancy. Values represent the means and SEM for six animals in each point.
(A) IGF-I (ng/ml)

- Control
- Superovulation

(B) IGFBP (%)

Day of pregnancy
5, the day on which blastocyst implantation begins (Fig. 3-3A). Total IGFBP levels in the control group remained constant from day 1 to day 3, decreased on days 4 and 5, then increased on day 6 (Fig. 3-3B). The patterns of IGF-I and IGFBP profiles following superovulation were the inverse of the control group (Fig. 3-3A, B). IGF-I levels were elevated during the first three days of pregnancy and decreased on days 5 and 6. In contrast, superovulation suppressed IGFBP levels by 20-40% during the first three days and then increased. Maximum levels in animals following superovulation were observed on day 5. The levels of IGF-I receptor in the uterus were similar in both groups until day 5 (Fig. 3-3C). On day 6, IGF-I receptor levels in the control group increased by approximately 60% from those of day 5, while the receptor levels of the superovulation group did not increase during the same period (Fig. 3-3C).

The patterns of IGF-I, IGFBP, and IGF-I receptor profiles in homogenates of the separated uterine endometrium were similar to those observed in the whole uterine tissue homogenates (Fig. 3-4). A superovulatory dose of PMSG had no effect on serum IGF-I and IGFBP levels throughout duration of this experiment (Fig. 3-5).

IV. DISCUSSION

The levels of ovarian steroid hormones observed in this study are comparable to those found in previous reports. Hyperestrogenemia during the preovulatory period and a postovulatory estradiol-17β peak following superovulatory treatment has been previously described (Miller and Armstrong, 1981a; Gidley-Baird et al., 1986). A decline in the levels of estradiol-17β between day 2 and day 3 preceded changes in the uterine IGF system, which were not observed until day 4. These observations are consistent with the hypothesis that superovulatory treatment induces hyperestrogenemia which in turn alters the uterine IGF system in the rat uterus.

Changes in the uterine IGF system following superovulation appear to be biphasic.
Changes in the first three days of pregnancy were characterized by elevated IGF-I levels and suppressed IGFBP levels, while suppressed IGF-I and enhanced IGFBP levels were found during the second half of preimplantation period (Fig. 3-3). Since IGFBPs, in general, suppress IGF-I action, these changes may indicate enhanced IGF-I action during the first half of preimplantation period and suppressed IGF-I action during the second half of preimplantation period, and at the time of implantation.

Although the daily examination of the IGF system used whole uterine tissue homogenates due to the small amount of tissue, these changes appear to be consistent with observations made using homogenates prepared from the endometrium (Fig. 3-4). Changes in the IGF system in the endometrium may have a greater impact on the environment for embryonic development. Embryos arrive in the uterus late on day 3 or early on day 4 of pregnancy. Enhanced IGF-I actions from day 1 to day 3 may render the uterine environment detrimental to preimplantation embryonic development by the time that the embryos enter the uterus. This will be examined in the following chapter.

Superovulatory treatments may also affect blastocyst implantation by interfering with uterine function. This may be caused, at least in part, by changes in the uterine IGF system following superovulation. The endocrine requirements for implantation, more particularly for decidualization, have been well characterized in the rat (Psychoyos, 1976). After the uterus has been primed with progesterone for a minimum of 48 h, a single injection with a small amount of estradiol-17β sensitizes the uterus for a decidualogenic stimulus 24 h later. Thus, the sensitization process for a decidualogenic stimulus appears to initiate as early as day 2 of pregnancy when a secondary estradiol-17β peak was observed in superovulated rats. The uterine IGF system was perturbed throughout the uterine sensitization period (Figs. 3-3 and 4).

Superovulatory treatments also changed the uterine IGF system between day 5 and day 6, i.e. around the time of implantation. Changes in the uterine IGF system following superovulation were characterized by a failure to increase receptor levels (approximately
60%) and IGFBP levels (approximately 25%) that were observed in the control group. It has been suggested that the uterine IGF system is possibly involved in the regulation of the decidual reaction (Chandrasekhar et al., 1990; Croze et al., 1990a; Yallampalli et al., 1992). Hence, disturbances in the uterine IGF system may perturb uterine preparation for implantation. This will be examined in further detail later in this thesis.

V. SUMMARY AND CONCLUSIONS

In summary, treatment with a pharmacological dose (40 IU) of PMSG creates superphysiological levels of estradiol-17β during the periovulatory period and a secondary estradiol-17β peak during the postovulatory period. Changes in the levels of estradiol-17β result in an alteration in the uterine IGF system which can be divided into two distinct phases. The first phase is observed in the first three days of pregnancy which is characterized by enhanced IGF-I action. This may be the result of both increased IGF-I levels and decreased IGFBP levels. The second phase is observed at the periimplantation period or at the time of implantation and is characterized by suppressed IGF-I action. This may be caused by a reduction in the levels of IGF-I and/or an increase in IGFBP levels. These changes may affect preimplantation embryonic development and subsequent implantation. Chapter four will examine the effect of enhanced IGF-I action on preimplantation embryonic development. Chapter five will address the significance of these changes in the uterine IGF system in the decidualization process.
CHAPTER FOUR

THE EFFECT OF IGF-I ON THE UTERINE MICROENVIRONMENT FOR PREIMPLANTATION EMBRYONIC DEVELOPMENT

I. INTRODUCTION

Increased early embryonic loss and failure of implantation after superovulation have been associated with a hostile maternal endocrine environment (Moon et al., 1990). Ovarian hyperstimulation generates elevated serum levels of estradiol-17β that may jeopardize the establishment of a successful pregnancy (Miller and Armstrong, 1981a; Gidley-Baird et al., 1986). Estrogens administered after ovulation have caused anti-fertility effects; hyperestrogenemia probably interferes with the normal secretory transformation of the endometrium which, in turn, causes a failure in implantation (Morris and Van Wagenen, 1973; Martel et al., 1987). However, the precise mechanisms through which postovulatory estrogen causes detrimental effects on preimplantation embryonic development and implantation have not been defined. A better understanding of such mechanisms is needed if we are to improve the outcome of IVF-ET in human medicine and ET in the livestock industry.

The IGF system is one of a number of regulatory systems mediating steroid hormone actions through autocrine/paracrine mechanisms in the uterus. IGF-I is a key member of the uterine IGF system. Other components of this system are IGFBPs and IGF-I receptors. Synthesis of IGF-I is primarily regulated by estrogen, rather than GH, the principal regulator of IGF-I synthesis in most other tissues (Murphy et al., 1987c; Murphy and Friesen, 1988; Norstedt et al., 1989). Other components of the IGF system are also under ovarian steroid hormone regulation in the uterus (Ghahary and Murphy, 1989; Girvigian et al., 1994). Therefore, it is likely that changes in the levels of ovarian
steroid hormones following superovulation could disturb the regulation of the IGF system in the uterus. Disturbances in the uterine IGF system may be responsible for the detrimental effects of superovulation, which include early embryonic loss.

Electrolytes in the uterine microenvironment appear to have a great impact on preimplantation embryonic development (Biggers et al., 1991). The sodium gradient across the cellular plasma membrane provides the energy to maintain cellular homeostasis and regulate cellular function and metabolism (Lechene, 1988; Cohen and Lechene, 1989). A normal intracellular pH and electrolyte composition is necessary for optimal enzyme function and actions of growth factors that play a central role in preimplantation embryonic development (Pouyssegur et al., 1985; Somero, 1985; Moolenaar et al., 1988). Electrolyte composition in the uterus and uterine luminal fluids may vary under the regulation by ovarian steroid hormones (Kao, 1967; Setty et al., 1973; Van Winkle et al., 1983). For example, in rats and mice, sodium ion content in the uterine luminal fluids decreases during delayed implantation which is experimentally induced by controlling the levels of ovarian steroid hormones (Setty et al., 1973; Van Winkle et al., 1983). Furthermore, the decreased levels of sodium ion in the uterine luminal fluids has been related to decreased levels of metabolism of the mouse embryos (Van Winkel, 1977; Van Winkel, 1981).

The previous chapter demonstrated changes in the uterine IGF system following superovulation. This chapter contains two studies. The objective of study one is to determine whether changes in the uterine IGF system, following superovulation, are responsible for an increase in early embryonic loss. It has been hypothesized that increased IGF-I action during the first three days of pregnancy may render the uterine environment hostile to preimplantation embryonic development.

Secondly, it has been hypothesized that an alteration in the electrolyte composition of the uterine luminal fluid may be one of mechanisms through which the IGF system mediates embryonic loss associated with superovulation. Thus, study two examines the effect of varying doses of PMSG, between the control and superovulatory doses, on
electrolyte composition of the uterine luminal fluids. These studies led to the determination of the effect of IGF-I on the electrolyte composition of the uterine luminal fluids.

II. MATERIALS AND METHODS

Study One

IGF-I Infusion Model

Adult female Sprague-Dawley rats (340-360 g BW) were mated overnight with males at estrus. Females were implanted with an Alzet osmotic pump (Model 1003D or 1007D, Alza Co., Palo Alto, CA) at 10:00-11:00 h on the day of vaginal plug (day 1 of pregnancy). Rats were anesthetized and a 4 to 5 cm medial incision was made in the xiphoid process and the pubic tubercle. Rats were unilaterally ovariectomized on the right and the distal end of the delivery tubule of the osmotic pump was introduced into the right ovarian artery, proximal to the uterine artery branch. The left ovary and artery were maintained intact as the control (Fig. 4-1). The incision in the abdominal wall and the skin was sutured leaving the osmotic pump in the abdominal cavity. The osmotic pump was filled with hr-IGF-I in Ringer’s solution containing 0.1% of gelatin and 20 IU heparin, prior to implantation. Ten nM hr-IGF-I was infused at 0.5 and 1.0 µl/h using the osmotic pump models 1007D and 1003D, respectively. Twenty five and 50 nM hr-IGF-I, as well as the vehicle alone, were infused at 1.0 µl/h using the 1003D model. Four rats were used in each group including the control (non-infusion) group, in which animals were unilaterally ovariectomized but not infused. Rats were sacrificed at the end of the 48 h infusion period (day 3 of pregnancy). The uterus and blood were collected for IGF-I, IGFBP and IGF-I receptor assays. The levels of IGF-I, IGFBP and IGF-I receptor in the left and right uterine horns were determined separately.
Figure 4-1 A rat uterine IGF-I infusion model. Adult rats (340-360 g BW) were mated at estrus and implanted with an alzet osmotic pump. Rats were anesthetized and a 4 to 5 cm incision was made on the median line in the middle of the xiphoid process and the pubic tubercle. Rats were unilaterally ovariectomized on the right and the distal end of the delivery tubule of the osmotic pump was introduced into the right ovarian artery proximal to the uterine artery branch. The left ovary and artery were maintained intact as control. The incision in the abdominal wall and the skin was sutured leaving the osmotic pump in the abdominal cavity.
Anti-IGF-I Antibody Preparation

Rabbit anti-IGF-I antiserum (Amersham Canada) was diluted with 100 mM phosphate buffer, pH 7.0, (1:1 v/v) and applied to a protein A column (Bio-Rad). Antibody was eluted with 1 M acetic acid and the eluate from the protein A affinity column was then applied to a Sephadex G-25 column. Antibody solution eluted in 100 mM phosphate buffer, pH 7.4, was concentrated by using a microcentrifuge filter, aliquoted and stored at -30°C. An aliquot was subjected to protein assay, in which a standard curve was prepared using rabbit IgG (Sigma).

Uterine Luminal Fluid Collection

Eight adult female rats were unilaterally ovariectomized on the right hand side, and implanted with an osmotic pump (Model 1003D) at 10:00-11:00 h on day 1 of pregnancy as described above. Four rats were infused with hr-IGF-I at a concentration of 10 nM (at an infusion rate of 1 μl/h for 48 h) from day 1 to day 3 (IGF-I group). The other four rats were infused with vehicle alone (vehicle group). As a control, four adult rats were unilaterally ovariectomized on day 1 but not infused (non-infusion group). Four immature rats were induced to superovulate by injecting with 40 IU PMSG (superovulation group). Four immature rats were induced to superovulate with 40 IU PMSG and were implanted with an osmotic pump (Model 1003D), subcutaneously on the back, at 10:00-11:00 h on day 1. Immature rats were infused with 1 mg/ml anti-IGF-I antibody for 48 h from day 1 to day 3 at an infusion rate of 1 μl/h (superovulation + IGF-I antibody group).

All uterine luminal fluids were collected at 10:00-11:00 h on day 3 of pregnancy. The uterus was dissected to separate the uterine horns. Uterine luminal fluids were collected separately from the left and right uterine horns by flushing the uterine lumen with 0.25 ml of M199 (Catalogue Number 11150, GIBCO) within 5 min of being sacrificed. In the superovulation and superovulation + IGF-I antibody groups, the uterine luminal fluid from the left and right uterine horns of the same animal were combined. Collected media were centrifuged at 3,000 x g for 10 min to remove cell debris. One hundred μl aliquots of
each supernatant was mixed with M199 (1:1 v/v) containing 10% fetal bovine serum (GIBCO) and equilibrated with 5% CO₂ in humidified air. The osmotic concentration of the collected media was determined using an osmometer. Two 20 μl-droplets were prepared from each sample. An aliquot of the uterine luminal fluids collected from the superovulation group and those from the right uterine horns of the non-infusion and IGF-I groups were dialyzed (molecular weight cutoff < 1,000) against fresh M199 for 6 h at 4°C and used for embryo culture.

Superovulation and Embryo Collection

Immature rats were injected with a single dose of 20 IU PMSG at 10:00 h at 26 to 29 days of age and mated with fertile males, 60 h after the PMSG injection. Rats were mated overnight and the presence of vaginal plugs was examined at 8:00 h on the following day (day 1 of pregnancy). Embryos at the 8-cell stage were collected at 2:00-3:00 h on day 4 of pregnancy by flushing the oviduct and uterine lumen with Dulbecco’s PBS containing 1 mg/ml PVA. Eight-cell stage embryos were pooled from at least four rats in each experiment and randomly assigned to droplets of culture media that contained the uterine luminal fluids.

Embryo Culture

Embryos were washed three times in M199 containing 5% FCS and transferred to 20 μl droplets that contained the uterine luminal fluids. Ten embryos were cultured in each droplet (a total of eighty embryos for each uterine luminal fluid group) for 48 h at 37°C in humidified air containing 5% CO₂. The developmental stages of embryos were scored and the number of cells in each blastocyst was counted by the air-drying method (Tarkowsky, 1966).
Study Two

Animal Treatments and Uterine Luminal Fluid Collection

Immature rats were injected with 4 (control), 10, 20, or 40 (superovulation) IU PMSG at 9:00-10:00 h on the day of 28-day-old and mated 60 h after the PMSG injection. Some immature rats that had been injected with 40 IU PMSG were infused with anti-IGF-I antibody from day 1 (day of vaginal plug) to day 3 of pregnancy as described above. Adult rats were treated for IGF-I infusion, superovulation, and superovulation + IGF-I antibody groups as described in study one. Control rats were unilaterally ovariectomized on day 1 but not infused (non-infusion group). Five immature or adult rats were used in each treatment group. All the rats were sacrificed at 11:00 h on day 3 and the uterine horns removed. Uterine luminal fluids were collected by flushing the uterine lumen with degassed 0.3 M mannitol solution (0.25 ml/uterine horn) and the uterine flushes were kept away from air contact to avoid changes in free CO₂ levels. The uterine flushes centrifuged at 3,000 x g for 10 min. The uterine flushes from the left and right uterine horns in the same animals, except for the infusion groups, were combined and the electrolyte composition determined. For cation assays, an aliquot of each supernatant was saved and frozen at -70°C until assayed.

Determination of Electrolyte Composition

The levels of all electrolytes were determined by at Biochemical Laboratory of Vancouver General Hospital (Vancouver, BC). Frozen supernatants were thawed, and 15 mM LaCl₃, 4 mM CsCl, and 100 mM HCl were added. The levels of Na⁺, K⁺, Ca²⁺, and Mg²⁺ were determined by atomic absorption photospectroscopy (Sanui, 1971; Sanui and Rubin, 1982). The levels of Cl⁻ were determined by the coulometric-amperometric method (Dietz and Bond, 1982). The levels of HPO₄²⁻ and HCO₃⁻ were determined, as total inorganic phosphorus and total CO₂, respectively, by using Kodak Ektachem Clinical Chemistry Slides (PHOS and ECO₂, Kodak, Eastman Kodak, Rochester, NY).
Statistical Analysis

Data are expressed as the mean ± SEM, except for Figure 4-3 where 95% confidence limit was used for error bar. The mean levels of the uterine IGF-I and IGFBP in the right and left horns of the same animals were compared by the paired Student's t-test. The mean levels of IGF-I, IGFBP, and IGF-I receptor in the same side of uterine horns across different infusion groups were compared by ANOVA, followed by Tukey's test. The developmental rates of the embryos were compared by Chi-square analysis. The mean number of cells in embryos cultured with uterine luminal fluids from the left and right uterine horns within animals were compared by the paired Student's t-test, while those obtained from the different groups were compared by ANOVA, followed by the Tukey's test. Total cation contents of uterine luminal flushes were compared by ANOVA followed by the Tukey's test. Statistical analysis was performed using the computer software 'SYSTAT'.

III. RESULTS

Study One

The IGF-I Infusion Model

Uterine IGFBP and IGF-I receptor levels are presented as a percentage of the levels in the left uterine horn of the non-infusion group. Serum IGFBP levels are presented as a percentage of the levels in the non-infusion group (Fig. 4-2). The control values for the levels of IGFBP in the uterus and serum were 79 ± 4.7 and 131 ± 26 pmol IGF-I/μg protein, respectively, while the levels of the IGF-I receptor in the uterus were 64 ± 3.8 fmol IGF-I/μg membrane protein.

Vehicle alone had no effect on the levels of IGF-I as compared to the non-infusion control group (Fig. 4-2A). IGF-I infusions increased the levels of IGF-I in the infused uterine horn but had no effect on IGF-I levels in the control horn of the same animal.
Figure 4-2  The effect of IGF-I infusions on the uterine IGF system. IGF-I was infused at the indicated conditions from day 1 to day 3 of pregnancy by an osmotic pump that was implanted in the abdominal cavity (Fig. 4-1). The levels of uterine and serum IGF-I (A) was determined by RIA and the levels of IGFBP (B) and IGF-I receptor (C) determined by ligand binding assays using $^{125}$I-IGF-I. The levels of IGF-I, IGFBP and IGF-I receptor in the left (control) uterine horn and the right (infused) uterine horn were determined separately. The levels of IGFBP and IGF-I receptor are presented as percentage of the levels of the control group that was not received an infusion. Values represent the means and SEM for four animals in each group. Letters on the top of bars indicate statistical differences of the means (a<b<c) across the treatment groups. Asterisks indicate statistical differences (*P<0.05, **P<0.01) of the means between the control and infused uterine horns within the animals.
(ng/g tissue) (ng/ml)

(A) IGF-I

Serum

Non-infusion
Vehicle-1.0 µl/h

10 nM-0.5 µl/h
10 nM-1 µl/h
25 nM-1 µl/h
50 nM-1 µl/h

(B) IGFBP

Non-infusion
Vehicle-1.0 µl/h

10 nM-0.5 µl/h
10 nM-1 µl/h
25 nM-1 µl/h
50 nM-1 µl/h

(C) IGF-I receptor

Non-infusion
Vehicle-1.0 µl/h

10 nM-0.5 µl/h
10 nM-1 µl/h
25 nM-1 µl/h
50 nM-1 µl/h
When IGF-I was infused at 1 µl/h at 10 nM or at greater concentrations, IGF-I levels in the infused horns were similar to those observed in the uterus on days 2 and 3 of pregnancy, following superovulation (Fig. 3-3A and Fig. 4-2A). The levels of IGF-I in the control horn were, however, not affected by IGF-I infusions and were not significantly different from the levels observed in the non-infusion or vehicle group (Fig. 4-2).

IGF-I infusions at 10 nM at 0.5 µl/h did not alter uterine IGFBP levels in either uterine horn (Fig. 4-2B). However, IGF-I infusions at 10 and 25 nM at 1 µl/h suppressed uterine IGFBP levels by 30% (P<0.001) in the infused uterine horn. The suppressed IGFBP levels were equivalent to approximately 60% on the scale of Fig. 3-2B and were similar to the levels of IGFBP observed on day 3 of the superovulation group (Fig. 3-2B and 4-2B). IGFBP levels in the control horn of the same animal were not significantly different from the levels observed in the non-infusion group. Interestingly, IGF-I infusion at 50 nM at 1 µl/h increased the levels of IGFBP by approximately 18% (P<0.01) in the infused horn compared with the vehicle and non-infusion controls (Fig. 4-2B). The levels of IGFBP remained at base level in the control horn. IGF-I infusions had no effect on the serum levels of IGF-I and IGFBP or uterine IGF-I receptor levels (Fig. 4-2).

Embryo Culture with Uterine Luminal Fluids

The osmotic concentration of the uterine flushes containing uterine luminal fluid was between 275-295 mOsm/kg H$_2$O. The mean concentrations of flushes between groups were not significantly different from each other (Table 4-1). Uterine luminal fluids from the non-infusion group did not affect embryonic development and the number of cells in blastocysts as compared to those of embryos that were cultured with media alone (Table 4-2). In contrast, uterine luminal fluids of the superovulation group inhibited embryonic development. The rate of blastocyst formation (P<0.001) and the number of cells in blastocysts decreased (P<0.01). In addition, the rate of embryo degeneration during the 48-hour-culture increased in the superovulation group (P<0.001), compared to those of the
Table 4-1. Osmotic concentrations of uterine luminal flushes following the IGF-I infusion and superovulation

<table>
<thead>
<tr>
<th>Uterine flushes</th>
<th>Uterine horn</th>
<th>Osmotic concentration (mOsm/kg H₂O)³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flushing medium</td>
<td></td>
<td>285b</td>
</tr>
<tr>
<td>Non-infusion</td>
<td>Left</td>
<td>288 ± 2.9</td>
</tr>
<tr>
<td></td>
<td>Right</td>
<td>287 ± 4.1</td>
</tr>
<tr>
<td>Vehicle</td>
<td>Left</td>
<td>286 ± 4.9</td>
</tr>
<tr>
<td></td>
<td>Right</td>
<td>287 ± 4.4</td>
</tr>
<tr>
<td>IGF-I</td>
<td>Left</td>
<td>286 ± 5.0</td>
</tr>
<tr>
<td></td>
<td>Right</td>
<td>285 ± 5.5</td>
</tr>
<tr>
<td>Superovulation</td>
<td>Bothc</td>
<td>284 ± 3.5</td>
</tr>
<tr>
<td>Superovulation + IGF-I antibody</td>
<td>Bothc</td>
<td>288 ± 1.7</td>
</tr>
</tbody>
</table>

*Values represent the mean and SEM for four repeated experiments.

bThe osmotic concentration of flushing medium (Medium 199, Cat. No. 11150, M199, Gibco) was adjusted 285 ± 3 mOsm/kg H₂O.

cUterine luminal flushings of both left and right horns were combined prior to determination of the osmotic concentration.
Table 4-2. The effect of uterine luminal fluids obtained from IGF-I infused or superovulated rats on 8-cell stage embryo development

<table>
<thead>
<tr>
<th>Uterine luminal fluids</th>
<th>Uterine horns</th>
<th>Number (%) of embryos after 48-h-culturea</th>
<th>Number (means ± SEM) of cells in blastocysts (n)b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>morula</td>
<td>blastocyst</td>
</tr>
<tr>
<td>Media alone</td>
<td>-</td>
<td>12 (15.0)</td>
<td>50 (62.5)</td>
</tr>
<tr>
<td>Non-infusion</td>
<td>Left</td>
<td>12 (15.0)</td>
<td>47 (58.7)</td>
</tr>
<tr>
<td></td>
<td>Right</td>
<td>10 (12.5)</td>
<td>49 (61.2)</td>
</tr>
<tr>
<td>Vehicle</td>
<td>Left</td>
<td>16 (20.0)</td>
<td>42 (52.5)</td>
</tr>
<tr>
<td></td>
<td>Right</td>
<td>14 (17.5)</td>
<td>39 (48.7)</td>
</tr>
<tr>
<td>IGF-I</td>
<td>Left</td>
<td>13 (16.3)</td>
<td>45 (56.3)</td>
</tr>
<tr>
<td></td>
<td>Right</td>
<td>13 (16.3)</td>
<td>19 (23.8)c, d</td>
</tr>
<tr>
<td>Superovulationg</td>
<td></td>
<td>15 (18.8)</td>
<td>23 (28.8)d</td>
</tr>
<tr>
<td>Superovulation + IGF-I antibodyg</td>
<td></td>
<td>14 (17.5)</td>
<td>42 (52.5)</td>
</tr>
</tbody>
</table>

aEighty embryos were cultured in each group.

bNumber of blastocysts analyzed

cChi-square, P<0.001; Values were compared to the left (Control) horn of the same animals in the infusion groups
dChi-square, P<0.001; Values were compared to the right horn of the non-infusion group.
ePaired t-test, P<0.01; Values were compared to the left (Control) horn in the same group.
fTukey's test, P<0.01; Values are different from others.
gUterine luminal fluids of the both uterine horns were combined.
non-infusion group. The anti-IGF-I antibody infusion, following superovulation, restored the rate of blastocyst formation and the number of cells in blastocysts to normal levels (Table 4-2). Vehicle infusions did not alter embryonic development and the number of cells in blastocysts.

IGF-I infusions selectively rendered uterine luminal fluids detrimental to embryonic development. Embryos cultured with the uterine luminal fluids obtained from the infused uterine horn of the IGF-I infusion group decreased the rate of blastocyst formation (P<0.001), the number of cells in the blastocysts (P<0.01), and an increased the rate of embryo degeneration (P<0.001). The developmental rate of embryos and the mean number of cells in blastocysts cultured with the uterine luminal fluids from the control horn of the IGF-I infusion group were compatible to those of the non-infusion and vehicle groups (Table 4-2). Dialysis of the uterine luminal fluids of the superovulation and IGF-I infusion groups improved embryonic development and the number of cells in blastocysts (Table 4-3). The percentage of embryos degenerating decreased (P<0.001), whereas the rate of blastocyst formation (P<0.001) and the mean number of cells in blastocysts increased.

Study Two

The levels of each electrolyte is presented as a percentage of the total amount of cations or anions (Figs. 4-3 and 4). PMSG, at the superovulatory dose (40 IU), altered both cation and anion compositions of the uterine luminal flushes on day 3 of pregnancy. In the cations, the percentage of Na\(^+\) in the superovulation group decreased by 26% and that of K\(^+\) increased 27%, as compared to those of the control (4 IU PMSG) group (Fig. 4-3). In anions, the percentage of Cl\(^-\) decreased by approximately 15% and that of HCO\(_3\)^- increased to a similar extent. Anti-IGF-I antibody infusion restored the alterations of cation composition, following superovulation, to the control levels (Fig. 4-3). In contrast, the anti-IGF-I antibody infusion had no effect on the altered anion composition. Compositions of cations and anions in the all PMSG groups, except for the superovulation group, were
Table 4-3. The effect of dialysis of uterine luminal fluid obtained from IGF-I infused or superovulated rats on 8-cell stage embryo development

<table>
<thead>
<tr>
<th>Uterine luminal fluids</th>
<th>Dialysis of uterine luminal fluids</th>
<th>Number (%) of embryos after 48-h-culture*</th>
<th>Number (mean ± SEM) of cells in blastocysts (n)†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>morula</td>
<td>blastocyst</td>
</tr>
<tr>
<td>Non-infusion</td>
<td>-</td>
<td>10 (12.5)</td>
<td>49 (61.2)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>13 (16.3)</td>
<td>47 (58.7)</td>
</tr>
<tr>
<td>IGF-I</td>
<td>-</td>
<td>13 (16.3)</td>
<td>19 (23.8)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>12 (15.0)</td>
<td>43 (53.7)‡</td>
</tr>
<tr>
<td>Superovulation</td>
<td>-</td>
<td>15 (18.8)</td>
<td>23 (28.8)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>16 (20.0)</td>
<td>38 (47.5)‡</td>
</tr>
</tbody>
</table>

* Eighty embryos were cultured in each group.
† Number of blastocysts analyzed
‡ Chi-square, P<0.001
§ t-test, P<0.05
Figure 4-3  The effect of PMSG on electrolyte composition of the uterine luminal fluid. Immature rats were injected with PMSG at the indicated doses. A group of rats that had been injected with 40 IU PMSG was infused anti-IGF-I antibody from day 1 to day 3. Uterine luminal fluids were collected by flushing the uterine lumen with degassed 0.3 M mannitol solution (0.25 ml/uterine horn) on day 3 of pregnancy. The levels of Na\(^+\), K\(^+\), Ca\(^{2+}\), and Mg\(^{2+}\) were determined by atomic absorption photospectroscopy. The levels of Cl\(^-\) were determined by the coulometric-amperometric method. The levels of HPO\(_4\)^{2-}\) and HCO\(_3\)^{-}\) were determined, as total inorganic phosphorus and total CO\(_2\), respectively, by using Kodak Ektachem Clinical Chemistry Slides. Electrolyte compositions are presented as a percentage of each component to the total anion or cation. Values represent the means and SEM for five rats in each group.
The effect of IGF-I infusion on electrolyte composition of the uterine luminal fluid. Adult rats were mated at estrus and infused with 10 nM IGF-I at 1 ml/h from day 1 to day 3 by an osmotic pump. Uterine luminal fluids were collected by flushing the uterine lumen with degassed 0.3 M mannitol solution (0.25 ml/uterine horn) on day 3 of pregnancy. The levels of Na\(^+\), K\(^+\), Ca\(^{2+}\), and Mg\(^{2+}\) were determined by atomic absorption photospectroscopy. The levels of Cl\(^-\) were determined by the coulometric-amperometric method. The levels of HPO\(_4\)\(^{2-}\) and HCO\(_3\)\(^-\) were determined, as total inorganic phosphorus and total CO\(_2\), respectively, by using Kodak Ektachem Clinical Chemistry Slides. Electrolyte composition is presented as the mean percentage of each component for five rats to the total anion or cation. The data for the superovulated rats were taken from Fig. 4-3.
similar to that of the non-infusion group (Fig. 4-3). Total cation content in the superovulation group increased by 2.1-fold compared to that of the non-infusion control (P<0.01, Table 4-4). Total cation content in the other PMSG (4, 10, and 20 IU) groups were not statistically different from that of the non-infusion group.

Unilateral ovariectomy or vehicle infusion had no effect on the electrolyte compositions and total cation content in the infused and control horns. IGF-I infusion altered the electrolyte composition of the uterine luminal fluid in the infused uterine horns but did not change the electrolyte composition of the control horns compared to that of the non-infusion group (Fig. 4-4). The alterations in electrolyte compositions of both cations and anions were compatible to those observed in superovulated immature rats. Ca\(^{2+}\), Mg\(^{2+}\), and HPO\(_4\)\(^{2-}\) levels were constant throughout these experiments. IGF-I infusion or anti-IGF-I infusion following superovulation had no effect on total cation contents in the uterine luminal flushes (Table 4-4).

IV. DISCUSSION

This study demonstrated that enhanced IGF-I actions in the uterus, resulting from superovulatory treatments, causes an increase in the rate of early embryonic loss. Superovulatory treatments elevated serum ovarian steroid levels and the postovulatory estradiol-17\(\beta\) peak (Fig. 3-1)(Miller and Armstrong, 1981a; Gidley-Baird et al., 1986). Changes in the IGF system in the uterus during the first three days of pregnancy following superovulation were characterized by increased IGF-I actions in the uterus (Chapter Three). The IGF-I infusion model achieved these changes in the uterine IGF system following superovulation, locally in the infused uterine horn, without changing the IGF system in the control uterine horn and in the circulation at control levels (Fig. 4-2). In this model, only the uterine luminal fluids from the infused uterine horn, in which the IGF system following superovulation had been mimicked, became detrimental to embryonic development (Table
Table 4-4. Total cation content in the uterine luminal flushes

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Uterine horn</th>
<th>Total cation concentration (nM)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMSG (IU)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Both$^b$</td>
<td>1.33 ± 0.30</td>
</tr>
<tr>
<td>10</td>
<td>Both</td>
<td>1.28 ± 0.22</td>
</tr>
<tr>
<td>20</td>
<td>Both</td>
<td>1.34 ± 0.26</td>
</tr>
<tr>
<td>40 (Superovulation)</td>
<td>Both</td>
<td>2.52 ± 0.29$^c$</td>
</tr>
<tr>
<td>Superovulation + IGF-I antibody$^d$</td>
<td>Both</td>
<td>2.58 ± 0.32$^c$</td>
</tr>
<tr>
<td>Infusions$^e$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-infusion</td>
<td>Left</td>
<td>1.21 ± 0.21</td>
</tr>
<tr>
<td></td>
<td>Right</td>
<td>1.22 ± 0.18</td>
</tr>
<tr>
<td>Vehicle</td>
<td>Left</td>
<td>1.19 ± 0.21</td>
</tr>
<tr>
<td></td>
<td>Right</td>
<td>1.23 ± 0.31</td>
</tr>
<tr>
<td>IGF-I</td>
<td>Left</td>
<td>1.23 ± 0.33</td>
</tr>
<tr>
<td></td>
<td>Right</td>
<td>1.31 ± 0.26</td>
</tr>
</tbody>
</table>

$^a$Values represent the means and SD for five rats.

$^b$Uterine luminal fluid from the left and right horn in the same rat were combined.

$^c$Total cation concentration is greater than that of the other groups (P<0.01).

$^d$Anti-IGF-I antibody was systemically infused from day 1 to day 3.

$^e$Infusions were performed on the right uterine horn.
Furthermore, the uterine luminal fluids of the superovulation group, which was detrimental to embryonic development, could be reversed by anti-IGF-I antibody (Table 4-2). It appears that locally enhanced IGF-I actions in the uterus, caused by superovulatory treatments, may inhibit embryonic development and increase early embryonic loss through alterations in the uterine environment.

Dialysis of the uterine luminal fluids obtained from the superovulation group and the uterine luminal fluids of the IGF infused horns restored embryonic development to normal levels (Table 4-3). One possible explanation for this result is that the osmotic concentration of these uterine luminal flushes may be unsuitable for embryonic development. However, this explanation can be excluded, since osmotic concentration of all uterine luminal flushes are within an acceptable range for embryonic development and the mean concentration of the uterine flushes of each group were not statistically different (Table 4-1). An alternative explanation is that some molecules in the uterine luminal fluids are responsible for the detrimental effect on embryonic development. These molecules are not likely to be proteins, since these molecules must be small enough (molecular weight < 1,000) to be removed by dialysis.

The detrimental nature of the uterine luminal fluids in the uterus that have been exposed high levels of IGF-I may be attributed, at least in part, to an alteration in electrolyte composition in the uterine luminal fluids observed in study two. Alterations in electrolyte composition following IGF-I infusion were observed only in the infused horns and is compatible to that observed in superovulated immature rats (Fig. 4-4). Alterations in cation composition caused by superovulation were restored by anti-IGF-I antibody infusion to control levels (Fig. 4-3). These observations suggest that exposure to high levels of IGF-I causes changes in cation composition. In contrast, the role of IGF-I in the alteration in anion composition is less clear. Although IGF-I infusions mimicked the alterations in anion composition observed following superovulatory treatments, anti-IGF-I antibody failed to restore these alterations (Figs. 4-3 and 4). However, this does not rule out a role
for IGF-I in regulating levels of anions in uterine luminal fluids. Alterations in anion composition of the uterine luminal fluids may be regulated by multiple factors which include IGF-I. Therefore, inhibition of IGF-I action alone may not be able to restore the altered anion composition, caused by the superovulatory treatment. This is in direct contrast to the regulation of cation composition, where IGF-I appears to play a major regulatory role.

It is unclear how superovulation or IGF-I causes alterations in the electrolyte composition of uterine luminal fluid. The uterine flushings from superovulated rats often contains desquamated cellular debris (Miller and Armstrong, 1981a). In the present study, a visible amount of cellular debris was observed in some of the uterine luminal flushes from the superovulation and the IGF-I infusion groups; the amount of cellular debris in the IGF-I group was considerably less than that observed in the superovulation group. Intracellular fluid has a greater K⁺/Na⁺ ratio than extracellular fluid. Together, cell disruption of the endometrial epithelial cells may be, at least in part, responsible for the increase in the K⁺/Na⁺ ratio caused by superovulation. IGF-I may mediate this detrimental effect of superovulation. However, the levels of HPO₄²⁻, determined as total inorganic phosphate, were constant throughout these experiments (Figs. 4-3 and 4). Since intracellular fluid contains high levels of inorganic phosphate, this may suggest that disruption of the uterine luminal epithelial cells may not contribute significantly to the increase in the K⁺/Na⁺ ratio observed in the present study.

Superovulation may increase uterine luminal fluid volume. Generally, the sum of the four cations that were examined in this study can represent total cation levels in body fluid, and total anion levels are equivalent to those of cations. Thus, changes in the total cation contents of uterine luminal flushes, determined in the present study, may reflect changes in the volume of uterine luminal fluid. Total cation levels of the superovulation group increased by approximately 2-fold compared to those of the non-infusion or 4 IU PMSG groups (Table 4-4). This may indicate a comparable increase in the volume of
uterine luminal fluid. The increase in the uterine luminal fluid volume is consistent with an increase in uterine wet weight of up to 2-fold that is observed after superovulatory treatment in immature rats (Miller and Armstrong, 1981a). Superovulation may increase fluid accumulation in the uterus and the uterine lumen. Such an increase in uterine luminal fluid volume may adversely affect embryonic development.

In contrast, total cation content in the uterine luminal fluid of the IGF-I infusion and superovulation + anti-IGF-I antibody groups were indistinguishable from that of the non-infusion group. This may suggest that the increase in the volume of uterine luminal fluid caused by superovulatory treatment is not mediated by IGF-I. Local regulators, other than IGF-I, may mediate this change following superovulation.

The ratio of Na\(^+\) to K\(^+\) in the non-infusion control group of the present study (5.2:1) is different from that observed in uterine flushes obtained on day 5 of pregnancy (1.4:1, Setty et al., 1973). This may be caused by differences in the experimental designs of the two studies. In the present study, rats were unilaterally ovariectomized on day 1 and uterine luminal fluid collected on day 3 of pregnancy. Differences in the method of uterine luminal fluid collection may also be responsible for differences in the ratio of the two cations. The previous experiment (Setty et al., 1973) used deionized water (0.5 ml/horn), while the present study used an isotonic solution (0.25 ml/horn). Large volumes of hypotonic solution may increase K\(^+\) content by causing cell disruption during uterine luminal fluid collection.

Electrolyte environment appears to be critical for preimplantation embryonic development as previously discussed in chapter one. Superovulatory treatments and IGF-I infusions decreased the percentage of Na\(^+\) and Cl\(^-\), and increased those of K\(^+\) and HCO\(_3\)\(^-\) (Figs. 4-3 and 4). These changes have been shown to be detrimental to preimplantation embryonic development. High levels of K\(^+\) reduce the rate of blastocyst formation in the mouse (Wiley, 1984; Wiley, 1987). Substitution of either Na\(^+\) or Cl\(^-\) in the embryo culture media reduced the rate of blastocoele expansion in the mouse (Manejwala et al., 1989).
Low levels of NaCl in the culture medium allow glutamine, a preferred energy substrate for early preimplantation embryonic development, to impair embryonic development (Chatot et al., 1989; Lawitts and Biggers, 1992).

Evidence suggests that the inhibitory effects of K⁺, Na⁺, and Cl⁻ on the formation of blastocoele are exerted through their ability to modulate Na⁺/K⁺-ATPase activity (Cohen et al., 1976; Wiley, 1984; Wiley, 1987; Dumoulin et al., 1993). Na⁺/K⁺-ATPase plays a central role in formation of the blastocoele that morphologically defines the blastocyst stage (Wiley, 1987). This is consistent with the observation that the uterine luminal fluids obtained from superovulated rats decreased the rate of embryonic development to the blastocyst stage (Table 4-2). Thus, the observed alterations in electrolyte composition following superovulation appear to be critical for preimplantation embryonic development.

Locally enhanced actions of IGF-I, caused by superovulatory treatments, may affect implantation and the post-implantational development of embryos. Uterine luminal fluids from the IGF-I infused uterine horns and uterine luminal fluids of the superovulation group not only reduced the developmental rate of embryos to the blastocyst stage but also reduced the number of cells in the blastocysts (Table 4-2). As discussed in chapter two, blastocoele cavity formation appears to reflect the development of fluid transport systems, which is related to time after fertilization (Smith and McLaren, 1977; Winston et al., 1991). Therefore, embryos with blastocoelic cavity that have fewer cells are not necessary capable of implanting. Furthermore, a substantial proportion of blastocysts which contain a fewer number of cells may have a numerically deficient or absent ICM and are not capable of post-implantation development (Hardy et al., 1989).

Data from IGF-I infusion experiments performed in study one of this chapter led to some interesting observations. IGF-I, infused at 1 μl/h at all concentrations examined, elevated uterine IGF-I levels comparable to those found after superovulation (Fig. 4-2A). The total amount of IGF-I infused during a 48-hour period at 10 to 50 nM hr-IGF-I, at 1 μl/h, is approximately 3.7 to 18.5 ng, respectively. These amounts are not enough to
achieve increases in uterine IGF-I levels by simple accumulation of infused hr-IGF-I. This may indicate that IGF-I up-regulates its own synthesis in the uterus. Synthesis of IGF-I in the uterus is primarily under estrogen control (Murphy et al., 1987c). GH, a principal regulator of IGF-I synthesis in most of organs, has been reported to stimulate, inhibit, or to have no effect on uterine IGF-I synthesis under various conditions (Murphy et al., 1987b; Murphy and Friesen, 1988; Norstedt et al., 1989). Further evidence indicates that IGF-I may self-regulate its own synthesis. IGF-I infusions into rats decreased hepatic IGF-I mRNA levels when rats were maintained on an energy-restricted diet (Schalch et al., 1989). Insulin, a closely related peptide which shares its receptors with IGF-I, enhanced IGF-I synthesis in the liver and other tissues (Fagin et al., 1989b; Johnson et al., 1989). Clearly, further investigations are needed to determine whether IGF-I self-regulates its own synthesis in the uterus. Examining the levels of IGF-I mRNA in the IGF-I infusion model may give us insight into the mechanisms regulating IGF-I expression in the uterus. Another explanation for the increase in IGF-I levels after IGF-I infusions is that the IGF-I infusions may increase blood flow by some modification in the local vascular system. Since serum IGF-I levels are considerably greater than extractable-uterine tissue IGF-I levels (Figs. 3-3 and 4), an increase in blood flow may result in a significant increase in IGF-I tissue levels. The effect of IGF-I on blood flow regulation has not been determined.

Another interesting finding in this study is that IGF-I seems to directly regulate IGFBP levels in the rat uterus. Some hormones, such as ovarian steroid hormones, that stimulate the secretion of specific IGFBP subtypes can inhibit the secretion of other IGFBP subtypes (Girvigian et al., 1994). Although the effect of IGF-I and its related peptides on IGFBP secretion also differs between tissues and cell types, IGF-I and insulin have been reported to inhibit IGFBP-1 secretion in human decidual cells (Thrailkill et al., 1990). In the present study, the ability of IGF-I to regulate total IGFBP levels differed depending on the concentration of hr-IGF-I (Fig. 4-2). When hr-IGF-I was infused at 1 μl/h, IGF-I at lower concentrations (10 and 25 nM) suppressed IGFBP levels, while IGF-I at higher
concentration (50 nM) increased IGFBP levels (Fig. 4-2B).

These results are interesting since uterine IGF-I levels were at the same level in these three infusion groups at the end of the 48-h infusion period. There are at least two possible explanations for this apparent discrepancy. First, there may be differences in the time course of IGF-I regulating its expression in the different IGF-I infusion groups. For example, uterine IGF-I levels in the 10 nM-1 µl/h group might achieve the elevated IGF-I levels at the end of the 48 h infusion period, while uterine IGF-I levels of the 50 nM-1 µl/h group might reach the same levels earlier and maintain the elevated levels for an extended period. Elevated IGF-I levels over an extended period of the time may be responsible for switching the IGF-I regulation on IGFBP levels from a inhibitory or stimulatory mode.

Second, IGFBP levels were determined as a total amount of binding proteins, rather than the amount of specific IGFBPs. As discussed above, the levels of IGFBP subtypes appear to vary differently to changes in ovarian steroid hormone levels (Girvigian et al., 1994). Subtypes of IGFBP would respond differently to IGF-I. Such differential regulation may also be responsible for the biphasic response in the total uterine IGFBP levels observed in the different IGF-I infusion groups. Examination of the effects of IGF-I on the levels of the specific IGFBP subtypes at different time points would provide insight into the role of IGF-I in the regulation of IGFBPs in the uterus.

V. SUMMARY AND CONCLUSIONS

This chapter demonstrates a role for the uterine IGF system in increasing early embryonic loss following superovulatory treatments. It is likely that pharmacological dosages of exogenous gonadotropins create superphysiological levels of estradiol-17β in the circulation. Superphysiological levels of estradiol-17β enhance IGF-I actions in the uterus by increasing IGF-I levels and by decreasing IGFBP levels. Enhanced IGF-I actions render a uterine environment hostile to preimplantation embryonic development.
The alterations in electrolyte composition of the uterine luminal fluids may reflect, at least in part, changes in uterine microenvironment for preimplantation embryonic development following superovulation. These findings also suggest that the uterine IGF system is an important mediator of estrogen action in the regulation of uterine function.

Superovulatory treatment may change a volume of uterine luminal fluid. This may also adversely affect embryonic development. This detrimental effect of superovulation appears to not be mediated by IGF-I. Apparently, IGF-I is one of a number of autocrine and paracrine factors that regulate uterine function. Studies involving other growth factors and cytokines may provide further understanding on the mechanisms through which superovulatory treatment causes the changes in uterine environment observed in this study.
CHAPTER FIVE

THE EFFECT OF IGF-I ON DECIDUALIZATION

I. INTRODUCTION

Decidualization, like other uterine functions, is primarily regulated by ovarian steroid hormones. Endocrine requirements that achieve uterine sensitization for the decidual response have been well characterized in ovariectomized rats (De Feo, 1967; Finn and Martin, 1972; Psychoyos, 1976). It is noteworthy that only a small amount of estrogen, after the uterus has been exposed to progesterone for at least 2 days, is required for uterine sensitization (Psychoyos, 1976). This small amount of estrogen is called "nidatory estrogen" in naturally occurring pregnancy. A role for the pituitary hormones in decidualization has also been demonstrated in hypophysectomized rats (Kennedy and Doktorcik, 1988). Hypophysectomy impaired the decidual response in ovariectomized-steroid hormone treated rats. Treatment with GH or thyroxine (T₄, a substitute of TSH) restored the poor decidual response after hypophysectomy (Kennedy and Doktorcik, 1988). Thus, both estrogen and GH, which regulate the uterine IGF system, are required to achieve a maximal decidual response.

IGF-I has been shown to mediate the action of GH. This has led to the hypothesis that IGF-I mediates the stimulatory action of GH and T₄ on decidual tissue formation (Kennedy and Doktorcik, 1988). However, evidence suggests that expression of IGF-I mRNA during decidualization is not dependent on pituitary hormone action (Croze et al., 1990a). It has been also suggested that IGF-I may inhibit the differentiation of stromal cells, since decidual tissue contains low levels of IGF-I mRNA. Furthermore, high levels of IGFBP-1, which inhibits IGF-I action, is found in the decidualized uterus. In contrast, GH and T₄ appear to stimulate uterine IGF-I expression during the sensitization period (Croze et al., 1990a). Treatments with GH and T₄ during the sensitization period stimulate
decidual tissue formation (Kennedy and Doktorcik, 1988). No further effect was observed by continuous treatment with GH and T₄ during the formation of decidual tissue. In addition, IGF-I receptor levels increase during the sensitization period and of the time of decidual tissue formation (Chandrasekhar et al., 1990; Katagiri et al., 1996). These observations suggest that IGF-I may mediate the action of GH and T₄ in the uterine sensitization process and at the onset of the decidual cell reaction.

Chapter three demonstrated that there are changes in the uterine IGF system following superovulation. This has led to the hypothesis that changes in the uterine IGF system, caused by superovulation, may perturb the process of uterine sensitization for a deciduogenic stimulus. This may partially explain the failure of implantation associated with superovulation. This chapter first examines the effect of enhanced and suppressed IGF-I action on decidualization. Then, the effect of IGF-I on deciduoma formation and alkaline phosphatase (ALP) activity, a well used marker for decidualization, in relation to the GH and T₄ actions in hypophysectomized-ovariectomized rats will be examined. Since PGs have been shown to stimulate the ALP activity in the rat uterus (Daniel and Kennedy, 1987; Yee and Kennedy, 1988), the effect of IGF-I on the PG-stimulated ALP activity was determined in cultured endometrial stroma cells.

II. MATERIALS AND METHODS

Study One

Animal Preparation

Female Sprague-Dawley rats (180-210 g BW) were purchased from the Animal Care Centre of The University of British Columbia. All rats were bilaterally ovariectomized and kept for 5 to 7 days prior to steroid hormone treatment. Rats were primed with three daily injections of 300 ng estradiol-17β (Sigma), followed by no steroid treatment on the following day (day 1 of pseudopregnancy). The rats were then given five
daily injections of 6 mg progesterone (Sigma). On the third day of progesterone treatments, females were given a single dose of 100 ng estradiol-17β in addition to progesterone at 13:00 h. All steroid injections were prepared in 0.1 ml sesame oil and given subcutaneously at 9:00 h unless otherwise stated.

*IGF-I and Anti-IGF-I Antibody Infusions*

Infusions were performed with an Alzet osmotic pump model 1003D or model 2001. For IGF-I infusions, reservoirs were filled with 1 and 10 nM hr-IGF-I in 100 mM phosphate buffer, pH 7.4, containing 0.1% gelatin and 20 IU heparin. The vehicle alone was infused in the control group for IGF-I infusions. Anti-IGF-I antibody was prepared as described in chapter four and infused at a protein concentration of 100 μg/ml in 100 mM phosphate buffer, pH 7.4 containing 20 IU heparin. As a control, anti-IGF-I antibody was replaced by 100 μg/ml of rabbit IgG in the control group for the anti-IGF-I antibody infusion. Seven rats were used in each infusion group. The osmotic pump was implanted as described in chapter four (Fig. 4-1). Infusions were from 9:00 h on day 1 to 9:00 h on day 3 and from 9:00 h on day 3 to 11:00 h on day 5 (the time that the deciduogenic stimulus was applied) with a model 1003D pump and from 11:00 h on day 5 to 11:00 h on day 9 (the time that the degree of the decidual response was determined) with a model 2001 pump. The osmotic pump and delivery tubule were removed at the end of each infusion period.

The ability of the IGF-I infusion to increase IGF-I action in the uterus was demonstrated for the infusion period from day 1 to day 3 in chapter four (Fig. 4-2). The ability of the IGF-I infusion to increase uterine IGF-I action during the other infusion periods was also examined. Groups of three rats were infused with 10 nM hr-IGF-I from day 3 to day 5 and from day 5 to day 9. Rats were sacrificed at the end of each infusion period. Uterine and serum levels of IGF-I, total IGFBP and IGF-I receptor were determined as described in chapter four. The levels of IGF-I and total IGFBPs in the
serum of rats which had not received any treatment were used as base line values.

**Deciduoma Induction**

The formation of deciduomal tissue was induced by a bilateral intrauterine injection of 100 μl mineral oil at 11:00 h on day 5 of pseudopregnancy. Five rats were ovariectomized, treated with ovarian steroid hormones, and deciduoma formation induced without receiving an infusion (non-infusion group). Another group of five rats were treated with steroid hormones but were not given an infusion or a deciduogenic stimulus (non-stimulated group). All the rats were sacrificed at 11:00 h on day 9 to examine the extent of deciduoma formation. Each uterine horn was removed, cleaned and weighed. The degree of decidual tissue formation was determined by the weight of each uterine horn. Uterine horns were longitudinally opened for visual observation of uterine endometrium to confirm decidual tissue formation.

**Study Two**

**Animal Preparation**

Hypophysectomized female Sprague-Dawley rats (180-220 g BW) were purchased from Charles River Canada Inc. (St-Constant, PQ) and pituitary intact Sprague-Dawley rats (180-210 g BW) purchased from the Animal Care Centre of The University of British Columbia. Hypophysectomized rats were given 2% glucose water throughout the experimental period. The pituitary intact and hypophysectomized rats were bilaterally ovariectomized and primed with steroid hormones for maximal uterine sensitization for a deciduogenic stimulus as described above (intact and HYPOX groups, respectively). Some hypophysectomized rats were subcutaneously injected with 200 μg porcine growth hormone (pGH, Sigma) twice daily at 9:00 h and 21:00 h and with 1 μg thyroxine (T₄, Sigma) daily at 9:00 h from day 1 to day 5 of pseudopregnancy (HYPOX-GH, T₄ group).
**IGF-I and Anti-IGF-I Antibody Infusions**

hr-IGF-I was prepared at concentrations of 0.1, 1, and 10 nM in 100 mM phosphate buffer, pH 7.4, containing 0.1% gelatin and 20 IU heparin. Fourteen rats of each treatment group; intact, HYPOX, and HYPOX-GH, T₄ groups, were infused with hr-IGF-I at an infusion rate of 1 μl/h at each hr-IGF-I concentration from 9:00 h on day 3 to 11:00 h on day 5 (prestimulation period) with an osmotic pump model 1003D. Seven rats from each treatment group were infused with hr-IGF-I at an infusion rate of 1 μl/h from 9:00 h on day 3 till rats were sacrificed at 11:00 h on day 9 (pre- and poststimulation period) with an osmotic pump, model 2001. The vehicle alone was infused in five rats of each treatment group as controls. Anti-IGF-I antibody was infused in five HYPOX-GH, T₄ rats from 9:00 h on day 3 to 11:00 h on day 5 with a pump model 1003D. Anti-IGF-I antibody was replaced by 100 μg/ml of rabbit IgG in the control group.

**Decidual Formation and ALP Activity Assay**

Decidual formation was induced by a bilateral intrauterine injection of 100 μl mineral oil at 11:00 h on day 5 of pseudopregnancy. Seven out of the fourteen rats that received hr-IGF-I infusions during the prestimulation period alone, were sacrificed 14:00 h on day 5. The other seven rats and seven rats given hr-IGF-I infusions throughout the pre- and poststimulation period were sacrificed at 11:00 h on day 9. All rats given anti-IGF-I antibody and IgG infusions were induced to undergo decidual formation as above, and sacrificed 11:00 h on day 9 of pseudopregnancy. The uterine horns were weighed and the endometrial tissue removed from the rest of the uterus by mechanical scraping. Separated endometrial tissue was homogenized in 0.25 M sucrose solution at 4°C and stored at -20°C until being used for ALP activity assay. ALP activity was determined by the method of Lowry (Lowry, 1957). The ALP activity is presented as unit activity which is defined by an amount (nmole) of substrate, p-nitrophenol phosphate (Sigma), hydrolyzed/hour/μg protein in each sample.
Study Three

Uterine Endometrial Stromal Cell Culture

All media and reagents for cell culture were purchased from GIBCO except for indomethacin and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) which were purchased from Sigma. Pituitary intact rats were ovariectomized and received a series of ovarian steroid hormone treatment for uterine sensitization as above. hr-IGF-I (1 nM) was infused into the right uterine horn through the ovarian and uterine artery from day 3 to day 5 of pseudopregnancy. Rats were sacrificed on day 5, at the end of the infusion, and the uterus removed. As IGF-I infusion showed no systemic effect <i>in vivo</i> (Figs. 4-2 and 5-1), cells were prepared from the right uterine horns. Uterine horns were opened longitudinally and washed in three changes of Hanks' balanced salt solution supplemented with 20 mM HEPES, 100 units/ml penicillin, 100 µg/ml streptomycin and 1.25 µg/ml fungizone (HBSS). Uterine horns of the same group (usually from 4 to 6 rats) were pooled and treated with 0.5% trypsin and 0.25% pancreatin in HBSS for 2 h at 4°C, followed by 30 min digestion at room temperature. The endometrial tissue was mechanically removed with forceps and incubated in HBSS containing 0.05% trypsin and 0.02% EDTA for 5 min at 37°C. Tissue digestion was terminated by adding an excess amount of Dulbecco's modified Eagle's medium:Ham's F-12 nutrient mixture supplemented with 20% IGF-I free FCS, 100 units/ml penicillin, 100 µg/ml streptomycin and 1.25 µg/ml fungizone (DMEM:F-12). Endometrial cells were dispersed by pipetting and centrifuged at 800 x g for 5 min. All pellets were resuspended in DMEM:F-12 at a cell density of 5 x 10<sup>5</sup> cells/ml and seeded in 24-well cell culture plate containing 1 ml of culture medium. Cells were cultured at 37°C with 5% CO<sub>2</sub> in humidified air. The culture medium was changed with fresh DMEM:F-12 2 h after seeding to remove most of the epithelial cells from the cell culture.

Cell Treatments and Harvest

Uterine endometrial stroma cells were cultured for 12 h after which they were
treated with 0.01, 0.1, 0.5, 1.0 or 10 nM hr-IGF-I in DMEM:F-12 for 48 h. The medium was changed after the first 24 h of the culture period to minimize the degradation of hr-IGF-I. Some cells were treated with 10 μM indomethacin for the first 12 h of the treatment period, followed by treatment with 1 μM PGE\(_2\) plus hr-IGF-I for 48 h in the presence of 10 μM indomethacin. At the end of the treatment period, cells were washed with HBSS and lysed with deoxycholate, pH 9.5, and stored at -20°C before being used for the ALP activity assay. At least four independent cell preparations were used in each treatment groups. To examine the effect of IGF-I on cell proliferation, the DNA content of 3-5 wells in each treatment group were determined by the method of LaBarca and Paigen (LaBarca and Paigen, 1980).

Statistical analysis

The uterine horn weight and ALP activity, both in vivo and in vitro, were logarithmically transformed prior to statistical analysis. Data are presented as the mean ± SEM for each group. For the ALP activity assay, decidual tissues from two animals were combined but considered as one sample in some treatment groups due to a limited tissue volume. The means among different treatment groups were compared by ANOVA, followed by Tukey’s test. The means between the control and infused horns within animals were compared by paired Student’s t-test. Difference of means was defined by a P-value of 0.05 unless otherwise stated. Analysis was conducted using the computer software ‘SYSTAT’.

III. RESULTS

Study One

Infusion of 10 nM hr-IGF-I increased uterine IGF-I levels in the infused horn by approximately 40% and 35% from day 3 to day 5 and from day 5 to day 9 of pregnancy
period, respectively (Fig. 5-1A). Uterine levels of total IGFBP were suppressed in the infused horn by approximately 20% in both infusion periods (Fig. 5-1B). The hr-IGF-I infusion did not affect the levels of IGF-I and total IGFBP in the control horns or in the serum of the IGF-I infused rats. IGF-I receptor levels in both the control and infused horns were not affected by IGF-I infusions (Fig. 5-1C).

Infusion with hr-IGF-I from day 1 to day 3 inhibited deciduoma formation in the infused horns, compared with that of the control horn in the same animal (P<0.001, Fig. 5-2). The mean weight of the infused horns was less than that of the non-infusion group but greater than that of the non-stimulated group (P<0.001). The same IGF-I infusion had no effect on deciduoma formation in the control horns of the infusion group or in the control horns of all the groups (Fig. 5-2). The hr-IGF-I infusion had no effect on deciduoma formation in the other two infusion periods. Vehicle infusions had no effect on the deciduoma formation in any infusion period (Fig. 5-2).

When anti-IGF-I antibody was infused during the prestimulation period (between day 1 and day 3, and between day 3 and day 5), the antibody infusions suppressed decidual tissue formation in the infused horn, but had no effect on the control horn in the same animal (P<0.001, Fig. 5-3). The weight of the infused horn of both infusion groups was less than that of the non-infusion group but greater than that of the non-stimulated group (P<0.001). Anti-IGF-I antibody infusion during the poststimulation period had no effect on decidual tissue formation. Infusions with IgG had no effect on deciduoma formation at any infusion period (Fig. 5-3).

The degree of inhibition of the decidual response by the hr-IGF-I infusion during day 1 to day 3 greatly varied from almost no effect to complete inhibition (Fig. 5-4). Visible decidual tissue formation was not observed in the infused horn of three rats. The tissue weight of the infused horns, in these three rats, were 94, 115 and 120 mg, respectively (Figs. 5-4). These weights were not significantly different from those observed in the non-stimulated group (Figs. 5-2 and 4). The degree of inhibition of
Figure 5-1  The effect of IGF-I infusions at different time period on the uterine IGF system. IGF-I (10 nM, 1 μl/h) was infused into the right uterine horn during the indicated time period by an osmotic pump that was implanted in the abdominal cavity (Fig. 4-1). The levels of uterine and serum IGF-I (A) were determined by RIA and the levels of IGFBP (B) and IGF-I receptor (C) determined by ligand binding assays using $^{125}$I-IGF-I. The levels of IGF-I, IGFBP, and IGF-I receptor in the control uterine horn and the infused uterine horn were determined separately. The levels of IGFBP and IGF-I receptor are presented as percentages of the levels of the control horn. The levels of IGF-I and IGFBP in the serum of rats which had not received any treatment were used as the control. Values represent the means and SEM for three animals in each group. Asterisks indicate statistical differences (P<0.05) of the means between the control and infused uterine horns within the animals.
Uterus
Control horn
Infused horn

Infused horn

Serum
Non-infusion
Infusion

(A) IGF-I

(B) IGFBP

(C) IGF-I receptor

(day 3-day 5)

(day 5-day 9)
The effect of IGF-I infusions at different time period on deciduoma formation. IGF-I (10 nM, 1 μl/h) or vehicle alone was infused into the right uterine horn in the ovariectomized-steroid hormone treated rats during the indicated time period. A bilateral deciduogenic stimulus was applied on day 5 of pseudopregnancy. The degree of deciduoma formation was determined separately on the infused and control horns by weighing each uterine horn on day 9. A group of five rats were treated as above and deciduoma formation induced without receiving an infusion (non-infusion). Another five rats were treated as above but were not given an infusion or a deciduogenic stimulus (non-stimulation). Values represent the means and SEM of the uterine horn weight in each group. Letters on the top of bars indicate statistical differences across the different infusion periods within the same uterine horn group (a>b, P<0.01).
- Non-infusion
- Non-stimulation
- Vehicle-control horns
- Vehicle-infused horns
- IGF-I-control horns
- IGF-I-infused horns

Infusion periods:
- day 1-day 3
- day 3-day 5
- day 5-day 9

LOG Uterine horn weight (mg)
Figure 5-3  The effect of anti-IGF-I antibody infusions at different time period on deciduoma formation. Anti-IGF-I antibody (100 μg/ml, 1 μl/h) or rabbit IgG (100 μg/ml, 1 μl/h) was infused into the right uterine horn in the ovariectomized-steroid hormone treated rats during the indicated time period. A bilateral deciduogenic stimulus was applied on day 5 of pseudopregnancy. The degree of deciduoma formation was determined separately on the infused and control horns by weighing each uterine horn on day 9. A group of five rats were treated as above and deciduoma formation induced without receiving an infusion (non-infusion). Another five rats were treated as above but were not given an infusion or a deciduogenic stimulus (non-stimulation). Values represent the means and SEM of the uterine horn weight for seven rats in each group. Letters on the top of bars indicate statistical differences across the different infusion periods within the same uterine horn group (a>b, P<0.01).
Non-infusion
Non-stimulation
IgG-control horns
IgG-infused horns
Anti-IGF-I-control horns
Anti-IGF-I-infused horns

LOG10 Uterine horn weight (mg)

- 2.0 - 2.5 - 3.0 - 3.5 -

day 1-day 3  day 3-day 5  day 5-day 9

Infusion periods

Legend:

- Non-infusion
- Non-stimulation
- IgG-control horns
- IgG-infused horns
- Anti-IGF-I-control horns
- Anti-IGF-I-infused horns
Figure 5-4  Distribution of the uterine tissue weight of the IGF-I-infused horns on day 9 of pseudopregnancy. IGF-I (10 nM, 1 μl/h) or vehicle alone was infused into the right uterine horn in the ovariectomized-steroid hormone treated rats during the indicated time period. A bilateral decidualogenic stimulus was applied on day 5 of pseudopregnancy. The weight of the infused uterine horn was determined on day 9 and shown on the logarithmic scale.
A decidual response by the anti-IGF-I antibody infusion was relatively consistent in the two infusion periods (Fig. 5-5). A visible decidual tissue formation was observed in all rats. The smallest uterine weight of the infused horn was 267 and 335 mg for the infusion between day 1 and day 3, and between day 3 and day 5, respectively (Fig. 5-5).

Study Two

All infusions of hr-IGF-I and anti-IGF-I antibody, as well as the control infusions, had no effect on deciduoma formation and uterine ALP activity in the control horn. Therefore, the remainder of this section will only describe the effect of infusions in the infused horn, unless otherwise stated. Hypophysectomy suppressed deciduoma formation and ALP activity in the endometrial tissue (P<0.01, Figs 5-6, 7, 8, and 9). Treatment with pGH or T₄ in hypophysectomized rats restored the formation of decidual tissue and ALP activity to the same levels of pituitary intact rats (P<0.01). However, hr-IGF-I infusions were not capable of restoring the reduced deciduoma formation and ALP activity, caused by hypophysectomy, at any concentration (Figs 5-6, 7, 8, and 9). Anti-IGF-I antibody infusions had no effect on the ability of pGH or T₄ to restore the reduced deciduoma formation and ALP activity in hypophysectomized rats (Fig. 5-10).

hr-IGF-I infusion of 0.1 nM during the prestimulation period stimulated deciduoma formation in the intact and HYPOX-GH, T₄ groups (P<0.01, Fig. 5-6). However, when the infusion was performed throughout the pre- and poststimulation periods, hr-IGF-I infusion at the same concentration suppressed deciduoma formation in the two groups (Fig. 5-7). The hr-IGF-I infusion at greater concentrations (1 and 10 nM) had no effect on deciduoma formation. Interestingly, hr-IGF-I infusions had no effect on deciduoma formation in the HYPOX group (Figs. 5-6 and 7).

The effect of hr-IGF-I infusions during the prestimulation period on uterine ALP activity was dependent on the concentration of infused hr-IGF-I and the day of examination (Figs. 5-8). The hr-IGF-I infusions at 1 and 10 nM increased ALP activity in intact and
Distribution of the uterine horn weight of the anti-IGF-I antibody-infused horns on day 9 of pseudopregnancy. Anti-IGF-I antibody (100 μg/ml, 1 μl/h) or rabbit IgG (100 μg/ml, 1 μl/h) was infused into the right uterine horn in the ovariectomized-steroid hormone treated rats during the indicated time period. A bilateral deciduogenic stimulus was applied on day 5 of pseudopregnancy. The weight of the infused uterine horn was determined on day 9 and shown on the logarithmic scale.
Anti-IGF-I antibody

IgG
Figure 5-6 The effect of IGF-I infusions during the prestimulation period on deciduoma formation. Seven rats in the pituitary intact (intact), hypophysectomized (HYPOX), and hypophysectomized-porcine GH and T4 treated (HYPOX-GH, T4) groups were infused with 0 (vehicle), 0.1, 1, or 10 nM IGF-I into the right uterine horn from day 3 to day 5, till a bilateral deciduogenic stimulus was applied. Rats were sacrificed on day 9 of pseudopregnancy. Since IGF-I infusions had no effect on deciduoma formation on the control horn in all infusion groups, only the weights (mean ± SEM, n=7) of the infused horn are presented. Letters on the top of bars indicated statistical difference across the experimental groups (a>b, P<0.01).
Figure 5-7  The effect of IGF-I infusions during the pre- and poststimulation periods on deciduoma formation. Seven rats in the pituitary intact (intact), hypophysectomized (HYPOX), and hypophysectomized-porcine GH and T4 treated (HYPOX-GH, T4) groups were infused with 0 (vehicle), 0.1, 1, or 10 nM IGF-I throughout the pre- and poststimulation periods into the right uterine horn. A bilateral deciduogenic stimulus was applied on day 5 and sacrificed on day 9 of pseudopregnancy. Since IGF-I infusions had no effect on deciduoma formation on the control horn in all infusion groups, only the weights (mean ± SEM, n=7) of the infused horn are presented. Letters on the top of bars indicated statistical difference across the experimental groups (a>b, P<0.01).
Intact
HYPOX
HYPOX-GH, T4

- LOG10 Uterine horn weight (mg)
- IGF-I (nM)

Vehicle 0.1 1 10
Figure 5-8 The effect of IGF-I infusions during the prestimulation period on the levels of uterine alkaline phosphatase (ALP) activity. Fourteen rats in the pituitary intact (intact), hypophysectomized (HYPOX), and hypophysectomized-porcine GH and T4 treated (HYPOX-GH, T4) groups were infused with 0 (vehicle), 0.1, 1, or 10 nM IGF-I into the right uterine horn from day 3 to day 5 of pseudopregnancy. Seven rats in each group were sacrificed on day 5 (A), and the other seven rats were given a bilateral deciduogenic stimulus on day 5 and sacrificed on day 9 (B). Since IGF-I infusions had no effect on ALP activity on the control uterine horn, only the levels of ALP activity (mean ± SEM, n=7) on the infused horn are presented. Letters on the top of bars indicated statistical difference across the experimental groups (a>b>c, P<0.01).
The effect of IGF-I infusions during the pre- and poststimulation periods on the levels of uterine alkaline phosphatase (ALP) activity. Seven rats in the pituitary intact (intact), hypophysectomized (HYPOX), and hypophysectomized-porcine GH and T4 treated (HYPOX-GH, T4) groups were infused with 0 (vehicle), 0.1, 1, or 10 nM IGF-I into the right uterine horn from day 3 to day 9. Rats were given a bilateral decidualogenic stimulus on day 5 and sacrificed on day 9 of pseudopregnancy. Since IGF-I infusions had no effect on ALP activity on the control uterine horn, only the levels of ALP activity (mean ± SEM, n=7) on the infused horn are presented. Letters on the top of bars indicated statistical difference across the experimental groups (a>b>c>d, P<0.01).
Intact
HYPOX
HYPOX-GH, T4

LOG10 ALP activity (nmole/h/µg protein)

Vehicle 0.1 1 10

IGF-I (nM)

a
b
b
a a

Vehicle 0.1 1 10
The effect of anti-IGF-I antibody on the deciduoma formation (A) and uterine alkaline phosphatase (ALP) activity (B). Anti-IGF-I antibody was infused in five hypophysectomized-porcine GH and T₄ treated rats from day 3 to day 5. Rats were given a bilateral decidualogenic stimulus on day 5 pseudopregnancy. Anti-IGF-I antibody was replaced by rabbit IgG in the control group. The degree of deciduoma formation (the uterine horn weight) and ALP activity were determined on day 9. The uterine horn weights and ALP activity of the non-infusion/stimulation and non-infusion groups from study one (Figs. 5-2 and 3) were adapted for comparison. Values represent the means and SEM for five rats in each group.
HYPOX-GH, T₄ groups on day 6 (P<0.01, Fig. 5-8A). ALP activity in the 1 nM hr-IGF-I infusion group remained high until day 9. However, ALP activity levels in the 10 nM hr-IGF-I infusion group decreased below base levels by day 9 (P<0.01, Fig. 5-8B). Continuous infusions with hr-IGF-I (0.1 and 1 nM) throughout the pre- and poststimulation period increased ALP activity, but decreased ALP activity at 10 nM, compared to that of the vehicle infusion group in intact and HYPOX-GH, T₄ groups (P<0.01, Fig. 5-9). In intact and HYPOX-GH, T₄ groups, the continuous infusion of hr-IGF-I (0.1 and 1 nM) achieved greater levels of ALP activity than those of the corresponding infusion groups of the prestimulation period (P<0.01, Figs. 5-8 and 9). The infusion of hr-IGF-I had no effect on ALP activity in the HYPOX group.

Study Three

All of the hr-IGF-I treatments had no effect on total DNA and protein content in cultured endometrial stromal cells. Treatments of endometrial stromal cells with hr-IGF-I stimulated basal ALP activity at 0.1 and 0.5 nM (P<0.01). In contrast, higher levels of IGF-I had no effect on ALP activity in endometrial cells in vitro (Fig. 5-11). The uterine hr-IGF-I infusion during the sensitization period, prior to the cell culture preparations, enhanced the effect of IGF-I on basal ALP activity in the cultured endometrial stromal cells (P<0.01). PGE₂ increased the unit ALP activity in cultured endometrial stromal cells (P<0.01, Fig. 5-11). Treatments of endometrial stromal cells with hr-IGF-I suppressed PGE₂-stimulated ALP activity at 0.5, 1, and 10 nM (P<0.01, Fig. 5-4). hr-IGF-I infusion during the sensitization period, prior to the cell culture preparations, did not alter the effects of the IGF-I treatment on PGE₂-stimulated ALP activity in endometrial stromal cells.
Figure 5-11  The effect of IGF-I on alkaline phosphatase (ALP) activity in cultured uterine endometrial stroma cells. All the rats were ovariectomized and treated with estradiol-17β and progesterone to sensitize the uterus to a deciduogenic stimulus. Some rats were given additional IGF-I infusion from day 3 to day 5 of pseudopregnancy (IGF-I infused). Endometrial stroma cells were obtained from the uterus on day 5 of pseudopregnancy. The cells were treated with IGF-I at the indicated concentrations in the absence (Basal) and presence of indomethacin and prostaglandin E₂ (PGE₂-stimulated). Values represent the means and SEM of ALP activity for at least four preparations of cells in each point.
IV. DISCUSSION

Changes in the uterine IGF system following superovulation appear to be detrimental to decidualization in rats. Changes in the uterine IGF system following superovulation are characterized by an enhanced uterine IGF-I action between day 1 and day 3 of pregnancy and by a suppressed IGF-I action thereafter (Fig. 3-3). Deciduoma formation was suppressed in uterine horns which had been exposed to high levels of IGF-I from day 1 to day 3 and reduced IGF-I action from day 1 to day 3 and from day 3 to day 5 of pseudopregnancy (Figs. 5-2 and 3).

Comparisons of the time and degree of inhibition of deciduoma formation by hr-IGF-I and anti-IGF-I antibody infusions give rise to some interesting explanations. First, the increased and reduced IGF-I actions may disturb the sensitization process which in turn inhibits decidual tissue formation. Rats were given a series of ovarian steroid hormone treatment to sensitize the uterus for a maximal decidual response in the present study. The hr-IGF-I and anti-IGF-I antibody infusions during the prestimulation period inhibited deciduoma formation, while both infusions during the poststimulation period had no effect on deciduoma formation (Fig. 5-2 and 3).

The exact timing that the antibody infusion becomes ineffective to inhibit the decidual response was not examined in the present study. However, it appears that anti-IGF-I infusions become ineffective when the uterine sensitization for the decidual response has been achieved. IGF-I action may no longer be necessary or become less critical for decidual tissue formation, once the uterine endometrium has been sensitized. This hypothesis is comparable to the previous observations. Expression of IGF-I mRNA and extractable IGF-I levels in the uterus increases towards the time of decidualization, followed by a decline during the decidualization period (Fig. 3-3A) (Croze et al., 1990a; Kapur et al., 1992).

Secondly, the mode of inhibition by the enhanced and suppressed IGF-I action may
be different. The enhanced IGF-I action may be capable of inhibiting decidual tissue formation completely. However, suppressed IGF-I action may only partially inhibit the decidual response (Figs. 5-4 and 5). The visual inspection of the uterine luminal surface found no deciduoma formation in the infused horn in three out of seven rats following hr-IGF-I infusion from day 1 to day 3. In contrast, all rats given the antibody infusion during the prestimulation period developed a visible decidual tissue mass in the infused horn. The uterine IGF system is an integral part of the growth factor/cytokine network which regulates uterine growth and differentiation. Other members of the regulatory network may respond differently to increased or reduced IGF-I action. The distinct responses of the other growth factors and cytokines may cause inhibition of the decidual reaction at a different step of the decidualization process and to a various extent.

Decidualized endometrial tissue has low levels of IGF-I mRNA transcripts but has high levels of IGFBP-1 mRNA (Croze et al., 1990a). IGFBP-1 has been localized to the luminal and glandular epithelium in the antimesometrial region, but not the decidual cells (Croze et al., 1990a; Sadek et al., 1994). Since IGFBPs, in general, inhibit IGF-I actions, these observations led to the hypothesis that IGF-I may even inhibit decidual tissue formation (Croze et al., 1990a; Sadek et al., 1994). The ability of IGF-I to stimulate cell multiplication may prevent uterine endometrial stroma cells from differentiating into decidual cells. Therefore, suppressed IGF-I actions, as the result of increased IGFBP-1 levels and reduced IGF-I expression in the endometrial tissue may enhance differentiation of the uterine endometrial stromal cells into decidual cells (Croze et al., 1990a). During the poststimulation period, the inhibition of decidual tissue formation appears to occur at relatively low levels of IGF-I. The continuous infusion of 0.1 nM hr-IGF-I throughout the pre- and poststimulation period suppressed deciduoma formation (Fig. 5-7). It is noteworthy that the levels of IGF-I receptor increase during the sensitization period and at the time of decidualization (Chandrasekhar et al., 1990). In the present study, uterine IGF-I receptor levels increased by 60% between day 5 and day 6 in the control ras (Fig. 3-3C).
Together with the reduction in IGF-I mRNA and the high levels of IGFBP-1 mRNA transcripts, a small increase in IGF-I levels, accompanied by a decline in IGFBP-1 levels, may serve as an effective inhibitory mechanism.

The effect of GH and T₄ on decidual tissue formation in hypophysectomized rats has been previously demonstrated (Kennedy and Doktorcik, 1988). The present study confirmed the effect of GH and T₄ on decidual tissue formation. In addition, the present study showed that GH and T₄ may also be required for maximal stimulation of ALP activity, associated with decidualization. Treatment with pGH and T₄ may increase total uterine ALP activity due to an increase in decidual tissue mass. However, more importantly, the treatment appears to increase ALP activity/total protein content of the decidual tissue (Figs. 5-8 and 9). This leads to the hypothesis that the effect of GH may be exerted through the GH-IGF-I axis, since IGF-I mediates GH action in many tissues. One of objectives of the present study was to determine whether IGF-I mediates the effect of GH and T₄ on decidual tissue formation. However, IGF-I appears not to mediate the action of GH and T₄ as it does not restore decidual tissue formation and ALP activity in hypophysectomized rats. Infusions with hr-IGF-I failed to restore decidual tissue formation and ALP activity in hypophysectomized rats (Figs. 5-6, 7, 8, and 9). Furthermore, anti-IGF-I infusions in hypophysectomized-pGH and T₄ treated rats did not suppress decidual tissue formation (Fig. 5-10).

Although IGF-I may not mediate action of GH and T₄ in decidual formation in hypophysectomized rats, IGF-I appears to modulate GH and T₄ action on decidual tissue formation and ALP activity levels. IGF-I may also require the presence of GH and T₄ to exert its effect on decidualization. The hr-IGF-I infusions modulated formation of decidual tissue and ALP activity in the presence of GH and T₄ (intact and HYPOX-GH, T₄ groups) but had no effect in the absence of GH and T₄ (HYPOX group, Figs. 5-6, 7, 8, and 9). In addition, hr-IGF-I increased ALP activity levels in cultured endometrial stroma cells obtained from the sensitized uterus of pituitary intact rats in the absence of GH and T₄ (Fig.
5-11). Together, GH and T₄ may render the uterus capable of responding to IGF-I during the prestimulation period (i.e. the sensitization period). The exact period of time when the uterus requires the actions of GH and T₄ for maximal decidual response remains to be determined.

The mechanisms through which GH and T₄ enable IGF-I to regulate the process of decidualization has not been determined. One possible mechanism may be the regulation of IGF-I receptor levels in the endometrial cells by GH and T₄. The effects of GH and T₄ on IGF-I receptor levels in the uterus have been studied (Yallampalli et al., 1992). Hypophysectomy increased uterine IGF-I receptor levels and treatment with GH, but not T₄, reversed the increase in receptor levels. This study, however, utilized hypophysectomized-ovariectomized rats without ovarian steroid hormones. Ovarian steroid hormones are known regulators of IGF-I receptors in the uterus (Ghahary and Murphy, 1989) and as a primary regulator of uterine sensitization for decidualization. The uterine IGF-I receptor response to GH regulation may be different, if rats were treated with ovarian steroid hormones for maximal uterine sensitization, as in the present study. Nevertheless, this finding may suggest that GH plays a role in maintaining an optimal IGF-I receptor level in the uterus during the sensitization period, preceding the deciduogenic stimulus. Such regulation of IGF-I receptor levels by GH may be involved in the process of uterine preparation that enables IGF-I to modulate the decidualization process.

IGF-I appears to stimulate ALP activity throughout the pre- and poststimulation period. Continuous infusion of hr-IGF-I throughout the pre- and poststimulation period increased the unit ALP activity to a greater extent than the infusion of hr-IGF-I during the prestimulation period in both intact and HYPOX-GH, T₄ groups (Figs. 5-8 and 9). A differential effect of IGF-I on ALP activity was observed in cultured endometrial cells. Endometrial stroma cells isolated from uteri previously exposed to hr-IGF-I during the sensitization period showed a greater response in basal ALP activity than their non-infusion counterparts following IGF-I treatment in vitro (Fig. 5-11). It is likely that the hr-IGF-I
infusion during the sensitization period enhances the subsequent response of endometrial stromal cells to IGF-I. Total DNA and protein content/well were not significantly different in any of the groups, regardless of in vitro treatment conditions. The prolonged effect of the IGF-I infusion during the prestimulation period may be specific to the regulation of ALP activity rather than the result of an altered cell metabolism.

IGF-I has been reported to either stimulate, inhibit, or have no effect on basal ALP activity in bone cells in vitro and in vivo (Ohlsson et al., 1992; Fournier et al., 1993; Pirskanen et al., 1993; Tanaka et al., 1994). IGF-I also suppressed tri-iodothyronine-stimulated and carcitriol-stimulated ALP activity in cultured rat epiphyseal chondrocytes and human osteosarcoma cells, respectively (Ohlsson et al., 1992; Pirskanen et al., 1993). In contrast, IGF-I had no effect on the levels of basal ALP activity in cultured rat epiphyseal chondrocytes, but decreased basal ALP activity in human osteosarcoma cells. Thus, the effect of IGF-I on ALP activity appears to be diverse and under the influence of other factors. The mechanisms by which IGF-I exerts these effects on ALP activity remain to be elucidated.

In the present study, the effect of IGF-I on uterine PGE2-stimulated ALP activity in vivo was unable to be determined due to a technical difficulty. The decidural response varied to a great extent in the vehicle group during the preliminary study. This was probably due to a high degree of intervention associated with dual pump implantation for simultaneous infusions with IGF-I and PGE2. However, IGF-I appears to inhibit PGE2-stimulated ALP activity in cultured endometrial stroma cells (Fig. 5-11). The exact mechanism of this IGF-I action is not known. IGF-I may down-regulate receptors for PGE2 or interfere with an intracellular signaling mechanisms, such as the cAMP pathway, which may mediate the effect of PGE2 on ALP activity in the rat uterus (Yee and Kennedy, 1988; Yee and Kennedy, 1991). Further studies are needed to define the regulatory mechanism that allows IGF-I to stimulate basal ALP activity and simultaneously suppress PGE2-stimulated ALP activity in the rat uterus.
PGs have drawn a large amount of attention in the regulation of ALP activity and other processes in decidualization. PGs increase ALP activity, vascular permeability, and extracellular fluid volume in the uterus and stimulate subsequent decidual tissue formation (Hoffman et al., 1977; Kennedy and Lukash, 1982; Yee and Kennedy, 1988; Hamilton and Kennedy, 1994). Modulation of PG synthesis by local uterine products has also been described. IL-1α, EGF, and platelet activating factor stimulate PG synthesis in uterine endometrial stromal cells (Smith and Kelly, 1988; Paria et al., 1991; Bany and Kennedy, 1995). These local factors may play a role in the regulation of ALP activity through PG synthesis. Leukotrienes may also have a potential regulatory role on the uterine ALP activity associated with decidualization in rats. The levels of leukotrienes in the uterus increase prior to implantation in a similar production pattern observed with PGs in response to a nidatory estrogen surge (Malathy et al., 1986; Tawfik et al., 1987). Leukotrienes inhibit ALP activity in cultured uterine endometrial stromal cells (Cejic and Kennedy, 1991a). The inhibition of ALP activity by leukotrienes may be due to a mechanism independent of PG production (Cejic and Kennedy, 1991b). The present study demonstrated an inhibitory effect of IGF-I on PGE2-stimulated ALP activity (Fig. 5-11). This may indicate that IGF-I plays a role in the regulation of ALP activity by counteracting the stimulatory action of other regulatory factors that act through PG synthesis. However, interactions between these factors and IGF-I in the regulation of ALP activity remains to be determined.

The present study highlights the complexity of the regulatory mechanisms involved in the decidualization process. The effect of IGF-I infusions on deciduoma formation and ALP activity often did not correlate in the present study. For example, in intact and HYPOX-GH, T4 rats, the continuous infusion of 0.1 nM hr-IGF-I throughout the pre- and poststimulation periods inhibited deciduoma formation and stimulated ALP activity, which is often used for a marker of decidualization (Figs. 5-7 and 9). These observations, however, do not necessarily conflict with each other. An increase in ALP activity was
observed before the penetration of the blastocyst of the endometrial epithelium and occurs without application of an artificial deciduogenic stimulus in the pseudopregnant rat uterus, sensitized for the decidual reaction (Fig. 5-10). In contrast, decidual tissue forms in response to either naturally occurring, or artificial deciduogenic stimuli. Thus, these two events are regulated differently.

The infusion of 10 nM hr-IGF-I during the prestimulation period increased the levels of uterine ALP activity on day 6 in intact and HYPOX-GH, T4 treated rats (Fig. 5-8). However, when examined on day 9, ALP activity of the same infusion group as well as ALP activity following the infusion of 10 nM hr-IGF-I throughout pre- and poststimulation periods were lower than that of the vehicle group (Figs. 5-8 and 9). These observations suggest that excessive IGF-I action first increases and then decreases ALP activity in the uterus. However, deciduoma formation in these infusion groups remained at control levels (Figs. 5-6 and 7). This is another example of disagreement in the IGF-I regulation of the two markers associated with decidualization. Examination of the decidual tissue mass may be the simplest, single criterion available to evaluate the degree of uterine sensitization for the decidual reaction. However, the observations made in the present study strongly suggest that the use of multiple makers may be necessary to evaluate the effect of the treatments on decidual tissue mass in a practical setting.

Together with the aforementioned concentration- and time-dependent effects of IGF-I on the decidual tissue formation, and the differential effect of IGF-I on basal and PG-stimulated ALP activity, the potential role for IGF-I in the regulation of the decidualization process appears to be complex. Clearly, further studies are needed to define the role for the IGF system in this complex regulatory mechanism.
V. SUMMARY AND CONCLUSIONS

Studies in this chapter demonstrated that IGF-I may regulate decidualization at different levels such as decidual tissue formation and ALP activity. In particular, the uterine IGF system appears to play an important role in the uterine sensitization process required for the decidual response. Enhanced and suppressed IGF-I actions during the sensitization period may be detrimental to subsequent decidual tissue formation. Therefore, changes in the uterine IGF system during the preimplantation period following superovulation appears to have a significant effect on the decidual response. This may, at least in part, be responsible for the failure of implantation following superovulation in the rat.

The present study also demonstrated that GH and T₄ are involved in the regulation of uterine ALP activity during the uterine sensitization period, in addition to the decidual tissue formation that has been demonstrated previously. However, IGF-I was shown not to mediate the actions of GH and T₄ on the decidual tissue formation and ALP activity. Instead, IGF-I appears to regulate the decidualization process in the GH and T₄-dependent manner. The mechanisms by which GH and T₄ enable IGF-I to regulate the decidualization process remains to be determined.
CHAPTER SIX

SUMMARY AND GENERAL CONCLUSIONS

I. SUMMARY

The present study demonstrated that IGF-I plays a central role in the establishment of a successful pregnancy. IGF-I appears to be beneficial to preimplantation embryonic development in the rat. IGF-I stimulated embryonic development to the blastocyst stage in vitro and improved the viability of blastocysts (chapter two). This is compatible with the hypothesis that maternally derived IGF-I plays a role as a signaling factor. In this role, IGF-I regulates the coordinated growth observed between the uterine endometrium and the preimplantation embryos.

The role of IGF-I in the uterine function was determined in conjunction with the adverse effects of superovulation. Superovulatory treatments altered the uterine IGF system in the immature rat superovulation model (chapter three). Alterations of the uterine IGF system following superovulation are characterized by:

1. enhanced IGF-I actions by increased IGF-I levels and decreased total IGFBP levels during the first three days of pregnancy, and
2. reduced IGF-I actions by decreased IGF-I levels and increased total IGFBP levels during the second half of the preimplantation period.

Alterations in the levels of IGF-I action appears to mediate the detrimental effects of superovulation on the establishment of pregnancy through several distinct mechanisms. First, an increase in IGF-I action in the uterus during the first three days of the preimplantation period render the uterine luminal fluids detrimental to embryonic development in vitro (chapter four). This may result in an increase in early embryonic loss which is associated with superovulation. The detrimental effect of the uterine luminal fluid
on embryonic development may be attributed, at least in part, to a distortion of electrolyte balance of the uterine luminal fluids, obtained from the uterus that has been exposed to high levels of IGF-I (chapter four).

Secondly, enhanced IGF-I action is also likely to have detrimental effects on uterine sensitization to the decidualogenic stimulus. Decreased IGF-I action during the second half of the preimplantation period (the uterine sensitization period), may adversely affect decidualization. IGF-I promoted the uterine sensitization process and inhibition of IGF-I action at this time, by an anti-IGF-I antibody, resulted in a poor decidual response (chapter five). Blastocysts obtained from the culture with the uterine luminal fluids, obtained from the IGF-I infused horn and superovulated rats, contained a fewer number of cells, suggesting that these blastocysts may have reduced capability of implanting and developing into fetuses (chapter four). These detrimental effects on decidualization and viability of embryos, caused by alterations in the uterine IGF system, may partially explain the failure of implantation associated with superovulation.

Furthermore, superovulatory treatment may affect the levels of IGF-I and IGFBPs in the uterine luminal fluids in the same manner that is found in the uterine endometrium. If this happens, the levels of IGF-I action on preimplantation embryonic development is reduced during the blastocyst formation period. Since IGF-I appears to stimulate blastocyst formation and improve viability of the blastocysts, a decrease in the levels of IGF-I action may reduce the rate of blastocyst formation and their viability. This will result in a pregnancy with a smaller litter size.

Superovulation may also increase uterine luminal fluid volume (chapter four). Although this effect may not be mediated by IGF-I, increases in the volume of uterine luminal fluid may be detrimental on embryonic development. It is not known how superovulatory treatment causes this change in the rat uterus.

Many cytokines and growth factors, including IGF-I, are believed to interact with each other, form a complex regulatory network in the uterine endometrium, and provide
redundant systems to increase the chances for a successful pregnancy. Expression of these cytokines and growth factors in the uterus is regulated primarily by ovarian steroid hormones, such as estrogen and progesterone. Therefore, hyperestrogenemia, caused by superovulatory treatment, affects the entire local regulatory system for the regulation of uterine function.

The present study focused on the role for IGF-I, a member of the regulatory network, in mediating detrimental effects of hyperestrogenemia caused by superovulatory treatment. IGF-I appears to mediate, at least in part, the detrimental effects of hyperestrogenemia, which include an increase in early embryonic loss and a failure of implantation. It is apparent that IGF-I exerts these effects by interacting with other local regulators and modulating actions of hormonal regulators. For example, electrolyte composition of the uterine luminal fluids, especially that of anions, appears to be regulated by multiple factors that include IGF-I (chapter four). IGF-I modulated the actions of GH, T4, and PG in the uterine sensitization process for the decidual reaction (chapter five). However, mechanisms of these interactions remain to be defined.

Evidence suggests that the role for IGF-I in the implantation process is not as critical as those for some growth factors and cytokines, such as IL-1β, EGF, LIF, and CSF-1 (Pampfer et al., 1991; Pollard et al., 1991; Arceci et al., 1992; Johnson and Chatterjee, 1993b; Cross et al., 1994; Simon et al., 1994a). These cytokines have been shown to be regulated by ovarian steroid hormones in the uterus and play a critical role in the process of blastocyst implantation. However, the present study demonstrated that alterations in the uterine IGF system have a great impact on blastocyst implantation, by regulating the viability of implanting blastocysts and the uterine sensitization process for deciduogenic stimuli. This suggests that many local regulators other than these four cytokines can also affect implantation.

The present study also demonstrated the complexity in the regulatory mechanisms of the uterine IGF system in the uterus. For example, IGF-I infusions (10-50 nM, 1 μl/h)
up-regulated the levels of IGF-I in the uterus. In contrast, these IGF-I infusions decreased or increased the levels of total IGFBPs, depending upon the concentration of IGF-I (chapter four). As discussed previously, each subtype of IGFBPs may play a different role in the uterine IGF system, and therefore respond differently to IGF-I. IGF-I at varying concentrations may regulate expression of IGFBPs in a inhibitory or stimulatory mode. A condition of IGF-I infusion that up-regulates some subtypes of IGFBPs may down-regulate other subtypes. Levels of total IGFBPs are determined as the sum of these changes in all IGFBP subtypes. These differential regulations of IGF-I on expression of IGFBPs may partially explain the biphasic response of IGFBP levels to IGF-I infusions observed in the present study. Examination of the effects of IGF-I on the levels of the specific IGFBP subtypes would provide insight into the role of IGF-I in the regulation of IGFBPs in the uterus.

The present study showed that superovulatory treatment has a great impact on the functional aspect of uterine endometrium. Implantation is one of critical steps for the establishment of successful pregnancy. The adverse effects of ovarian hyperstimulation, resulting from conventional superovulatory treatments with exogenous gonadotropins, have been recognized since the early use of IVF-ET. Many studies aimed at improving superovulatory treatments have been focused on the ovarian functional aspects, in order to obtain maximum number of fertilizable oocytes. Aspects of uterine function have been largely disregarded. The present study examined the role for IGF-I in mediating the detrimental effects of superovulation with special reference to uterine endometrial function. The results from this study are consistent with the hypothesis that alterations in uterine function following superovulation are mediated by growth factors and cytokines in the uterus. This emphasizes that examination of function of the uterine endometrium is essential for improving superovulatory treatment.
II. CONCLUSIONS

The uterine IGF system appears to play a central role in the regulation of uterine functions which include maintenance of a receptive uterine environment for preimplantation embryonic development and uterine sensitization for the decidual reaction. In addition, IGF-I derived from the uterus appears to promote embryonic development during the preimplantation period in the rat. Therefore, a normally functioning uterine IGF system is essential for successful preimplantation embryonic development, blastocyst implantation, and subsequent fetal development. Early embryonic loss and failure of implantation associated with superovulation may be, at least in part, attributed to disturbances of the uterine IGF system, caused by conventional superovulatory treatments.


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