STUDIES ON THE REGULATION OF CHORIONIC GONADOTROPIN PRODUCTION IN EXPLANT CULTURES OF HUMAN PLACENTA

by
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ABSTRACT

The effects of various steroid hormones on human chorionic gonadotropin (hCG) production and placental viability were investigated using an explant culture model of human placenta.

Placental hCG production was assessed using two different methods: a) hCG concentrations in media recovered from cultures were measured by radioimmunoassay and b) tissue levels of hCG in cultured placentae were determined immunohistochemically; both were evaluated before and after exposure to steroid hormones. In first trimester placentae, progesterone and dehydroepiandrosterone (DHEA) increased hCG concentrations both in collected medium and levels in cultured placentae. Estradiol increased the levels of hCG in tissues but not in media. Cortisol increased concentrations in media but did not alter tissue levels. Testosterone decreased hCG levels in media, but had no effect on hCG placental content. In third trimester cultures, progesterone and DHEA were the only hormones studied which increased concentrations of hCG in media; estradiol, cortisol and testosterone had no effect. Progesterone, estradiol and DHEA, alone or in combination, extended the viability of first trimester placental explant cultures from approximately 7 to 30 days. There was a significant relationship between placental viability and tissue hCG levels ($r = 0.73$, $P < 0.001$). The concentrations of hCG, progesterone and estradiol in human placentae were determined at various times through gestation. These studies suggest that a temporal relationship exists between the placental levels of hCG and these steroids, and that they may be significant determinants of growth and differentiation of the placenta in vivo. Furthermore, these investigations support the hypothesis that hCG production by the placenta is subject to paracrine regulation by steroid hormones.

RESUME

L'effet d'hormones stéroidiennes variées sur la production de gonadotropine chorionique humaine (hCG) et la viabilité placentaire ont été investigués en utilisant un modèle de culture d'explant de placentas humains.

La production placentaire de hCG a été quantifiée en utilisant deux méthodes différentes: a) les concentration de hCG dans les milieux recouverts des cultures de placentas ont été mesurées par radioimmunoessai et b) les taux tissulaires de hCG dans les placentas en culture ont été déterminés de façon immunohistochimique . Les deux paramètres ont été évalués avant et après exposition aux hormones stéroidiennes. Dans les placentas du premier trimestre, la progestérone et la déhydroépiandrostérone (DHEA) ont augmenté les concentrations de hCG dans les milieux collectés ainsi que les taux dans les placentas en culture. L'estradiol a augmenté les taux de hCG dans le tissu main non dans le milieu. Le cortisol a augmenté les concentration dans le milieu mais n'a pas altéré les taux tissulaires. La testostérone a diminué les taux de hCG dans les milieu, mais a été sans effet sur la contenu placentaire. Dans les cultures du troisième trimestre, la progestérone et la DHEA furent des hormones étudiées les seules qui ont augmenté les concentrations de hCG dans le milieu; l'estradiol, le cortisol et la testostérrone furent sans effet. La progestérone, l'estradiol et la DHEA seules ou en combinaison, ont prolongé la viabilité des cultures d'explant de placentas du premier trimestre d'environ 7 à 30 jours. Il y avait un rapport significantif entre la viabilité placentaire et les taux tissuelaires de hCG ($r = 0.73$, $P < 0.001$). Les concentrations de hCG, progestérone et d'estradiol dans le placenta humain ont été déterminées à différents temps durant la gestation. Ces études suggèrent qu'un rapport temporel existe entre les taux placentaires de hCG et ces stéroides, et suggèrent que ces hormones sont des déterminants importants de la coissance de la différentiation du placenta in vivo. De plus, ces investigations supportent l'hypothèse que la production de hCG par la placenta est sujette à une régulation paracire impiquant les hormones stéroidiennes.

For my parents
and
for the memory of Brinda and Arti

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PREFACE

This thesis is presented as a series of published or submitted manuscripts, in accordance with the "Guidelines Concerning Thesis Preparation" of the Faculty of Graduate Studies and Research. The following is quoted directly from these guidelines.

> "The candidate has the option, subject to the approval of the Department, of including as part of the thesis, the text, or duplicated published text (see below), of an original paper, or papers. In this case the thesis must still conform to all other requirements explained in the Guidelines Concerning Thesis Preparation. Additional material (procedural and design data as well as descriptions of equipment) must be provided in sufficient detail (e.g. in appendices) to allow a clear and precise judgement to be made of the importance and originality of the research reported. The thesis should be more than a mere collection of manuscripts published or to be published. It must include a general abstract, a full introduction and literature review and a final overall conclusion. Connecting texts which provide logical bridges between different manuscripts are usually desirable in the interests of cohesion.
>
> "It is acceptable for theses to include as chapters authentic copies of papers already published, provided these are duplicated clearly on regulation thesis stationary and bound as an integral part of the thesis. Photographs or other

material which do not duplicate well must be included in their original form. In such instances, connecting texts are mandatory and supplementary explanatory material is almost always necessary.

"The inclusion of manuscripts co-authored by the candidate and others is acceptable but the candidate is required to make an explicit statement on who contributed to such work and to what extent, and supervisors must attest to the accuracy of claims, e.g. before the Oral Committee. Since the task of Examiners is made more difficult in these cases, it is in the candidate's interest to make the responsibilities of authors perfectly clear. Candidates following this option must inform the Department before they submit the thesis for review."

Each of the manuscripts included in this thesis constitutes a chapter. In the interests of continuity and clarity a preface precedes each chapter and the relevant references are collected at its conclusion. The salient features of the Materials and Methods appear as a separate chapter (Chapter 2).

I am the first author of the manuscripts presented in chapters 3,4 and 5. All three manuscripts were co-authored by Dr. Beverley Pearson Murphy, my research supervisor, and the third (Chapter 4) was co-authored as well by Dr. Mary Senterman, who supervised the histological aspects of this work.

The investigations described in this thesis were designed and conducted by myself with the advice and consultation of Dr. Murphy. I formulated the protocols and conducted all the tissue culture

experiments described. As well, I cultured and prepared all tissues for H/E and immunohistochemical staining. These staining techniques were carried out by the technicians of the Pathology Department at the Montreal General Hospital. All assay determinations regarding concentrations of hormones given in this thesis were done by me. The contributions to original knowledge arising from these investigations appear at the conclusion of the thesis in Chapter 9.

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LIST OF ABBREVIATIONS

Å	angstrom unit
Ab	antibody
ACTH	adrenocorticotropin
Ag	antigen
AgAb	antigen-antibody complex
5'AMP	5' adenosine monophosphate
5'ATP	5' adenosine triphosphate
b	bovine
C	carbon
°C	degrees centigrade
cAMP	cyclic adenosine monophosphate
CBG	corticosteroid-binding-globulin or transcortin
cDNA	complementary deoxyribonucleic acid
CG	chorionic gonadotropin
cGMP	cyclic guanosine monophosphate
cm	centimeter
CO2	carbon dioxide
Con-A Sepharose	Concanavalin-A Sepharose
cpm	counts per minute
CRF	corticotropin releasing factor
D	dalton
dbcAMP	dibutyrl cyclic adenosine monophosphate
dbcGMP	dibutyrl cyclic guanosine monophosphate
DEAE-Sephadex	diethylaminoethyl-sephadex
DHEA	dehydroepiandrosterone
DHEA(S)	dehydroepiandrosterone-sulphate
DNA	deoxyribonucleic acid
E	cortisone
EGF	epidermal growth factor
F	cortisol
FBS	fetal bovine serum
FSH	follicle stimulating hormone
g	gram
GH	growth hormone
GnRH	gonadotropin releasing hormone
h	human
hCG	human chorionic gonadotropin
hMG	human menopausal gonadotropin
hr	hour
HSD	hydroxysteroid dehydrogenase

Ig.....immunoglobulin
i.m.....intramuscular(ly)
IU.....international unit (1IU/ml=1279 ng/ml)
i.v.....intravenous(ly)

l.....litre
LDL.....low density lipoprotein
LH.....luteinizing hormone
LMP.....last menstrual period

µ.....micron
µCi.....microcurie
µg.....microgram
µl.....microlitre
µM.....micromolar
M.....molar
mCi.....millicurie
MCR.....metabolic clearance rate
mg.....milligram
min.....minute
mIU..... milli international unit
ml..... millilitre
mm.....millimeter
mRNA.....messenger ribonucleic acid

N2.....nitrogen
ND.....no data
ng.....nanogram
nM.....nanomolar

o.....ovine
O2.....oxygen

p.....porcine
P.....probability
PBS.....phosphate buffered saline
pg.....picogram
PO2.....partial pressure of oxygen

r.....correlation coefficient
RIA..... radioimmunoassay
RNA.....ribonucleic acid

S.D..... standard deviation
S.E.....standard error

TSH.....thyroid stimulating hormone

wks.....weeks
wt.....weight

x g..... times gravity

GLOSSARY OF TERMS

androstenedione.....4-androstene-3,17-dione

^{14}C.....radioisotope of carbon, atomic weight 14
cortisol.....11β,17,21-trihydroxy-4-pregnene-3,20-dione
cortisone.....17α,21-dihydroxypregn-4-ene-3,11,20-trione

dehydroepiandrosterone.....3β-hydroxy-5-androstene-17-one
dehydroepiandrosterone-sulphate....17-Oxo-5-androstene-3-β-yl-sulphate

estradiol.....1,3,5(10)estratriene-3,17 β-diol
estriol.....1,3,5(10)estratriene-3,16α,17βtriol
estrone.....3-hydroxy-1,3,5(10)estratriene-11-one

^{3}H.....radioisotope of hydrogen, atomic weight 3

^{125}I.....radioisotope of iodine, atomic weight 125
international unit.....the international unit of chorionic gonadotropin is defined as the (bio)activity contained in 0.001279 mg of the second international standard. This was determined by quantification of bioactivity of purified hCG in various laboratories. Bioactivity was measured using seminal vesicle weight, prostate weight, uterine weight, ovarian weight, vaginal smear, spermiation and immunological assays.

Pergonal.....menotropins (hMG), parentral: 75 IU FSH, 75 IU LH, activity per 2 ml, ampule: 150 IU FSH, 150 IU LH activity per 2 ml.
prednisolone.....17α,21-dihydroxypregn-1,4-diene-3,11,20-trione
pregnenolone.....3β-hydroxy-5-pregnen-20-one
progesterone.....4-pregnen-3,20-dione

testosterone.....17β-hydroxy-4-androsten-3-one

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CHAPTER 1

Introduction and Historical Review

1.1: THE PLACENTA

1.1.1 Introduction:

In the non-pregnant woman, the ovarian steroids estrogen and progesterone undergo cyclical changes with an average periodicity of 28-32 days (1). At any given time, their plasma concentrations are dictated by the complex interactions of the hypothalamic-pituitary-gonadal axis. This complex system ideally causes proliferation of the uterine endometrium and differentiation of its glandular epithelium, stimulates production of adequate and appropriate cervical mucus and induces the punctual ovulation of a mature and fertilizable oocyte. It thus produces a physiologically conducive environment for gamete transport, fertilization, implantation and placentation. The end-point is the establishment and maintenance of pregnancy, which ensures the continued propagation of the species.

1.1.2 Embryogenesis:

Fertilization is a complex process (2). The pivotal event of syngamy entails the fusion of the male and female pronuclei and the establishment of the diploid state. The resultant single celled organism is called a zygote, which cleaves following syngamy to form an embryo. Early development of the embryo involves morulation and blastocyst formation. The cells of the blastocyst are divided into the trophectoderm, which lie in a single-layered rim surrounding a blastoceolic cavity and the inner cell mass which is eccentrally placed against the trophectoderm within the cavity. The majority of cells

(~ 90%), are trophectodermal and will give rise to extra-embryonic structures. The inner cell mass differentiates into the tissues of the embryo proper (2,3).

Prior to implantation, the blastocyst is dependent upon uterine secretions for nutritional support (2,3). It actively accumulates organic molecules and ions by specific cellular transport mechanisms, whilst the exchange of CO2 and O2 is passively diffusional. However, such exchange rapidly becomes inadequate as the embryo increases in size.

Generally, 6 days after fertilization, implantation is initiated with removal of the zona pellucida by proteolytic enzymes of embryonic and maternal origin (3,4). The implanting embryo initially draws from the abundant glycoproteins and glycogen stores of the secretory maternal endometrium and utilizes metabolic substrates released from the local digestion of the decidua and rupture of maternal blood vessels, occurring as a result of implantation. This type of nutritional support is known as histotrophic and is mediated by the differentiation of a distinct and highly vascularized region of the extra-embryonic surface.

The first macroscopically visible link between mother and conceptus is attachment; the 2 organisms adhere via close apposition of trophectodermal and endometrial cells with interlocking microvilli (3,4). Attachment induces decidualization, a process in which the maternal stromal endometrium becomes transformed into the maternal component of the placenta, the decidua. This process is likely mediated by signals released by the invading trophoblast;

prostaglandins and histamine have been implicated. In the mature decidua 3 components can be distinguished: the decidua basalis which lies directly beneath the site of implantation, the decidua capsularis which grows to surround the entire embryo and separate it from the uterine cavity and the decidua parietalis which lines the remainder of the pregnant uterus. The conceptus becomes entirely embedded in the maternal endometrium by 9 days post fertilization. Integrity of the maternal blood supply at the site of implantation is lost, thus forming blood filled vacuoles which coalesce to give rise to lacunae; these bathe the trophoblast directly. However, it is not until 14-15 days after conception that maternal arterial blood enters these lacunae. Fetal and maternal blood vessels are functional by the 17th day.

Implantation and placentation are 2 distinct but closely linked processes. The first allows the conceptus to establish contact with the maternal organism and derive nutritional support until the second process is complete. With placentation, the trophectoderm and decidua develop into a zone of adjacent and highly vascularized contact between mother and conceptus.

Between 9-20 days after fertilization, the trophectoderm differentiates into the fetal component of the placenta, the chorion (3-6). This begins with the fusion and differentiation of some of these cells into a syncitium, the syncytiotrophoblasts; whilst others retain their cellularity and differentiate into cytotrophoblasts. These 2 cell types organize themselves such that the syncytial cells cover the underlying cytotrophoblasts and together form finger-like projections called primary villi which extend into the decidua basalis.

Embryonic mesenchyme progressively differentiates into pre-umbilical blood vessels and blood elements. Secondary chorionic villi are formed as a result of invasion into primary villi by cores of embryonic mesoderm. Vascularization with fetal capillaries of these secondary villi form tertiary villi, which extend into maternal blood-filled lacunae. Thus the terminal villus is the definitive organ of exchange between mother and fetus. This type of nutritional support is known as haemotrophic or hemochorial in rodents and primates and in the human is firmly established by 3 weeks of fetal age.

The amnion begins to develop just after implantation (3,5). It begins as a small vesicle derived from the extra-embryonic mesenchyme and differentiates into a thin membrane which contains the embryo and its amniotic fluid. It directly opposes the chorion and is normally slightly adherent, though easily separated from it. Chorionic villi become organized into placental lobules with continued growth and differentiation. Between the 3rd and 4th month of pregnancy, part of the chorion loses its villi and differentiates into a smooth membrane, the chorion-laeve. In the early months of pregnancy, the embryo does not occupy the entire uterine cavity, however as the conceptus grows, it bulges into this cavity and by the 4th month the decidua capsularis and decidua parietalis become opposed. Morphological changes are associated with contact of the two membranes; these are reviewed by Wynn (3). Until the 4th month, the placenta grows both laterally and circumferentially, after this time however, the placenta grows only in circumference.

It should be noted that implantation and therefore placentation

can occur only into a receptive endometrium: estrogen-primed and progesterone-dominant as is the case during the luteal phase of the normal menstrual cycle (1).

1.1.3 Anatomy, histology and ultrastructure of the placenta:

The placenta lies within the uterine cavity attached to the decidua basalis by inter-digitated structures of the chorion (5-8). The fetus is attached to the placenta via the umbilical cord which contains 2 umbilical arteries arising from the dorsal aorta and a single umbilical vein which returns blood from the placenta to the fetal heart. The decidua is vascularized by the uterine artery which gives rise to the spiral artery which empties into blood sinuses called lacunae; these in turn are emptied by the spiral and the uterine veins.

Decidual cells are large and polygonal with round, vesicular nuclei. They contain clear basophilic cytoplasms and house an extensive endoplasmic reticulum, but otherwise have few organelles.

The chorionic villus contains as its core a fetal capillary, surrounded by a double cell-layer of inner cytotrophoblasts and then outer syncytiotrophoblasts (5-8). As gestation advances beyond the first trimester, cytotrophoblasts diminish and become discontinous in areas, although they persist to some extent throughout pregnancy. The syncytial cells thin out except at the tips of villi, thus bringing the maternal and fetal circulations into closer proximity.

Ultrastructurally, syncytiotrophoblasts are multinucleated, and devoid of mitotic figures (5-8). These cells are electron dense,

contain abundant mitochondria, well-developed smooth and rough endoplasmic recticuli and Golgi apparati. Numerous secretory granules, both proteinaceous and lipoidal are apparent. Exocytotic and endocytotic vesicles are seen at the luminal surface. Ribonucleoproteins, free ribosomes and lysosomes are present. Morphologically, these cells are well adapted for protein and steroid hormone synthesis. Syncytial cells secrete both classes of hormones into the maternal blood space. In contrast, cytotrophoblasts contain large nuclei, prominent nucleoli and can often be observed with mitotic figures. They house large mitochondria and numerous polysomes, but are otherwise under-developed ultrastructurally. Such morphology is common to embryonic or neoplastic tissue and is well suited for growth and differentiation. Indeed, cytotrophoblasts serve as stem cells, renewing the overlying syncytial layer. In conditions of fetal stress such as hypoxia, there is a reflexive formation of new syncytium from cytotrophoblasts (4).

1.1.4 Physiology of the placenta:

1.1.4.1 Transfer and exchange functions

Throughout pregnancy, the placenta fulfills fetal requirements for respiration, digestion, metabolism and excretion, thereby maintaining a steady-state *in utero* environment (9). Indeed, fetal growth and development are largely dependent upon the adequacy of these functions. Low molecular weight and non-polar substances cross the placenta largely by passive diffusion, the rate of which is determined by Fick's Law (appendix 2), whereas polar and large molecular weight substances are transported by active or factilitated transport.

Oxygen is required in continuous supply by the fetus as it stores only enough to sustain approximately two minutes of hypoxia. Oxygen transfer from mother to fetus is passive diffusion. This transfer is facilitated by the greater oxygen affinity of fetal haemoglobin than its maternal homologous counterpart. Under non-pathological conditions, neither maternal arterial PO2 nor placental perfusion limit fetal oxygenation; this is regulated almost entirely by fetal demand.

The amnion and chorion are freely permeable to water. The impressive accumulation of water by the fetus is likely mediated by bulk flow, which in turn is determined by small and intermittent changes in hydrostatic pressure between maternal and fetal compartments. Carbon dioxide is quite soluble in plasma and moves across the placenta in response to its concentration gradient. Its transfer is made more efficient by the Bohr effect. The transport of sodium, potassium and chloride ions, as well as urea and bilirubin is passive.

Glucose and fructose are required for fetal growth and development and are transported by facilitated diffusion. Amino and nucleic acids, calcium, iron, folic acid and vitamin B-12 reach the fetus by active transport. Amino and nucleic acids are crucial for anabolic processes. Calcium is required, especially in the final trimester, for skeletal ossification. Folic acid and vitamin B-12 are co-enzymes/factors essential for normal growth.

The maintenance of adequate concentration gradients between maternal and fetal compartments and the intracellular mechanisms which mediate active transport across the placenta are the subject of a

review by Johnson and Everitt (9).

1.1.4.2 Immunological functions

Teleologically, the function of sexual reproduction is to produce a genetically distinct individual. Histocompatability antigens appear in embryonic cells as early as implantation. Throughout pregnancy, the maternal immune system remains competent but tolerant of the fetal allograft. The placenta acts as a barrier separating fetal and maternal circulations. Convincing but not definitive evidence exists that syncytiotrophoblasts either lack entirely or do not express, because of masking, their histocompatability antigens (9,11,12). Additionally, the placenta produces and is bathed in fluids containing high concentrations of progesterone, corticosteroids and hCG. These hormones may act locally to reduce cytotoxic effects of maternal immune cells (12). The placenta functions as a filter, precluding the passage of maternal immune cells or antibodies to the fetus. IgG is a notable exception. It is actively transported to the fetus, conferring on it some degree of passive immunity against bacterial and viral infections in the early post-natal period.

1.1.4.3 Endocrine functions

The placenta is an endocrinologically diverse organ capable of synthesizing both steroid and peptide hormones and releasing factors (107). Many of these have been localized autoradiographically in the chorion; most of these, except GnRH and CRF in syncytiotrophoblasts.

Placental steroid hormones arise from the interaction of anatomically distinct but biochemically complementary structures in the mother, fetus and placenta (reviewed in section 1.3).

1.2: HUMAN CHORIONIC GONADOTROPIN

1.2.1 Introduction:

Successful placentation ensures adequate nutritional support of the embryo-fetus, as described in section 1.1. The establishment of the pregnant state and survival of the conceptus is critically dependent on progestational support of the uterine endometrium; thus luteolysis and consequent hormonal cyclicity must be prevented. In sexually reproducing species this may occur in a variety of different ways (for review see 3,4). In the equine and primate, this is accomplished by a luteotrophic hormone of chorionic origin, which in the case of the human is called human chorionic gonadotropin (hCG).

In 1905, Halban (14) observed that the endocrine and metabolic changes associated with pregnancy occurred only in the presence of a fertilized ovum, and was the first to implicate the placenta as a source of a then unknown stimulating substance. Almost a decade later, it was reported that treatment of immature guinea pigs with placental extracts resulted in ovarian hyperemia and corpus luteum formation(15). Hirose (16) transplanted placental tissue under the skin of female rabbits and observed stimulation of the reproductive organs. He called the active substance 'prolan'. However it was Ascheimm and Zondek in 1927 who were the first to directly demonstrate the presence and provide detailed characterization of gonadotrophic activity in the urine of gravid females, and for these studies they are credited with the discovery of hCG (17).

1.2.2 Molecular Structure:

HCG was initially purified in the 1950's and 60's from pregnancy urine by the tedious efforts of Got and Burrillion; being the first to propose its glycoprotein nature (18,19). It is a hormone of 38,000 daltons (D) molecular weight (20), consisting of two dissimilar (21) non-covalently linked peptide chains, designated as α and β. The mature glycosylated peptide chains are readily dissociable with 1.0 M urea buffer and can then be separated on DEAE-Sephadex (20,21). Characterization and sequencing of these subunits has revealed the α subunit to comprise either 89 (20) or 92 (22,23) amino acids, the difference arising from amino terminal heterogeneity. The molecular weight of α-hCG is approximately 14,700, with approximately 10,200 and 4,500 D derived from peptide and carbohydrate moieties respectively (20,24,25). It contains 5 disulphide bonds (20) and has a Stokes-Adams radius of approximately 23.2 Å (24). The β subunit consists of between 139-147 amino acids. Approximately 2/3 of its 23,000 D molecular weight is attributable to the protein component and 1/3 to carbohydrate moieties (20,22,23). It contains 6 disuphide bonds (20), and has a calculated Stokes-Adams radius of 30.2Å (24).

The α peptide shares considerable sequence homology with the α subunits of the human pituitary glycoproteins (FSH, LH, TSH); however much less similarity is apparent between α-hCG and its bovine, porcine or ovine counterparts. β-hCG exhibits sequence homology with the β subunits of human, bovine or porcine luteinizing hormone (hLH, bLH and pLH). Its amino acid sequence was not similar to the β subunits of other human glycoproteins (20,23,26,27). The amino terminal 115 amino

acids of β-hCG and β-hLH are nearly identical (20). β-hCG differs in primary structure from other glycoprotein β peptides in containing an additional 30 serine and proline-rich residues at its carboxyl terminal. The considerable sequence homology between β peptides of different species and α peptides of the same species implies the existence of a single ancestral gene for each subunit.

Approximately 30% of the molecular weight of hCG derives from monosaccharides (19), which include sialic acid, L-fucose,, D-galactose, D-mannose, N-acetylglucosamine and N-acetylgalactosamine (20). There are 7 (20) or possibly 8 (25) oligosaccharide chains attached to the protein component of hCG; 4 at asparagine and 3-4 at serine residues. The α chain contains 2 asparagine-linked carbohydrate chains at positions 52 and 78; the β chain harbours the remaining 5-6 at positions 13 and 30 (asparagine-linked), and 118, 129, and 131 (serine-linked). Each oligosaccharide chain contains 1-2 sequential sialic acid residues at its non-reducing terminus; thus there are 20 sialic acids per molecule (20,25,28). Sequence determination of these chains using specific tryptic glycosidases has met with partial success (29).

1.2.3 Biosynthesis:

1.2.3.1 Sources

It was originally thought that normal biosynthesis and secretion of hCG occurred only in the placenta. It has become increasingly evident however, that this was an over-simplification. HCG was localized to syncytiotrophoblasts using fluorescein-labelling and

immunohistoenzymological techniques (30-33). More recently, hCG activity was precisely localized to the placental villous border and within intracellular secretory vesicles (32,34). The free β subunit was also found within these cells (31). Under conditions of prolonged culture, cytotrophoblasts were shown to synthesize α-hCG (31). Other studies of fresh tissue excluded hCG and β-hCG from these cells (32). Detection of small quantities of hCG was reported in some cells of the amniotic epithelium (32).

The possibility of normal extra-placental expression of hCG genes has recently been explored. Acevedo et al (35) and Asch et al (36) used fluorescein-labelled double antibody technology to independently demonstrate β-hCG activity in 5-7% of human spermatozoa in every tested semen sample. Such activity was not demonstrable in samples of other species (36). Extracts of human testis (37) and pituitary (38) contained a substance chromatographically, electrophoretically and bioactively similar to hCG. Yoshimoto et al (39) documented significant quantities of hCG in normal human kidney, liver, lung, stomach and colon (ie. in every tissue tested) by radioreceptor and radioimmunoassay. Interestingly, hCG extracted from these tissues did not bind to Con-A Sepharose columns, indicating the absence of carbohydrate in this species (40). These authors proposed that hCG is a ubiquitous cellular protein; the trophoblast is unique in its capacity to glycosylate and secrete physiologically active hCG (39,40). Testicular and pituitary tissue may also express this capacity.

Peri-implantation embryos of rabbit, mouse and rat contained radioreceptor-assayable and immunofluorescent CG (41-43). The

relevance of this finding remains speculative.

Immuno and bioreactive hCG is secreted in vast quantities from all trophoblastic tumors, both molar and choriocarcinoma (44,45). Ectopic hCG secretion was found in 42 and 51% of ovarian adenoma and testicular tumor patients respectively (46). Five percent of testicular seminomas were associated with hCG production (47,48). Non-gonadal neoplasias (malignant tumors of the pancreas, stomach, lung, bowel and liver) have been frequently associated with detection of peripheral hCG (49). In fact, 10% (50) or perhaps more (49) of all patients presenting with non-endocrine tumors showed hCG in their blood or urine. Remarkably, urine of cancer patients contained hCG-producing bacteria (51,52).

1.2.3.2 Molecular Aspects

Physiologically functional biosynthesis of hCG involves transcription, mRNA processing, translation and glycosylation of the peptide chains. The genes encoding the α peptide have been localized to human chromosome 18 (53). The α-hCG message is a 10S fragment (54), and its 621 base pair cDNA has been cloned (55). Efforts to localize the β-hCG gene have thus far remained unsuccessful.

Transcription or protein synthesis inhibitors such as puromycin or cycloheximide decreased α-hCG, β-hCG and hCG secretion from first trimester placental organ cultures (56). The α and β peptides are translated from separate mRNAs and the biosynthesis of the two chains occurs independently (57). The metabolic stability of the α and β messages was approximately equal, but much less than most other protein transcription products (54). Messenger RNA was isolated from first trimester (54) and term placenta (57), and when transplanted into cell

free systems, directed the synthesis of a 14,000 (58) or 16,000 D (54) apoprotein, which was subsequently cleaved to the mature unglycosylated 10,600 D (54) α-peptide. The β message was translated into a 18,000 D precursor (58) which, when processed, resulted in an unglycosylated 15,000 D peptide. Beta subunit synthesis was the rate-limiting step in hCG production.

The synthesis of carbohydrate moieties appears to occur independently of the peptide chains, within the Golgi apparatus. Glycosylation was a membrane dependent phenomenon (57) which required glycosyltransferase (59). Some investigators conceive this to occur co-translationally (57), while others propose it to be a post-translational event (59). Trimming of the carbohydrate moieties by an endogenous glycosidase, likely mannosidase, is the rate-limiting step in the synthesis of hCG and occurs in the Golgi apparatus (60). See (28) for review.

1.2.3.3 Patterns of placental synthesis

Placental hCG synthesis and secretion change qualitatively and quantitatively through gestation. In relation to the α subunit, its β counter-part is synthesized with far less heterogeneity and in much less quantity; thus in its free form it was detectable only in minute amounts in tissue, peripheral serum or medium collected from organ or monolayer cultures (61-64). Several immature, non-secreted forms of the α-peptide have been described (60,65,66,72); it is secreted in its mature form as two different species (61,67). The first is physically and immmunologically indistinguishable from the dissociated hCG α-subunit and is detectable in free form in placental tissue, urine

and plasma throughout gestation. The alternate form is secreted free and unassociated with the β subunit, and differs slightly from the first form in molecular weight, carbohydrate content and electophoretic mobility (67), but appears only after 9 weeks gestation (61).

In recent years, the changing profile of placental biosynthetic capability of the maturing placenta has become evident; this change is especially peculiar in the case of hCG. For instance, in organ cultures, immunofluorescent labelling of hCG and β-hCG was greater in immature (8-10 week) than mature (term) placental villi. In contrast, α-hCG immunofluorescence was greater at term (31). Concurrent with these findings, placental concentrations of β-hCG and hCG were greatest in first trimester; however in second and third trimester tissue, α-hCG exceeded hCG levels 10 fold (61). Messenger RNA isolated from first trimester placenta directed the synthesis of 8 fold more α-hCG than did the message isolated from term placenta (58). In organ cultures, α-hCG exceeded hCG secretion 3-6 times in first trimester and 10 times in third trimester placenta (62,60). Incubation of placental polyribosomes in cell free systems showed an increase in the ratio of α-hCG:β-hCG synthesis from first term (1.2) to third term (2.3) (68). α and β-hCG accounted for 5 and 4% of total protein synthesized by first trimester placenta but only 1.3 and 0.6% by term placenta respectively (69).

1.2.3.4 Immuno and Bioreactivity

The biological potency of a hormone preparation is often used as an indication of its purity. In the case of hCG, the tremendous

biochemical heterogeneity of urinary and plasma fractions complicated efforts to establish standard preparations. Urinary extractions of hCG generally expressed low in vivo bioactivity in rat ventral prostate, ovarian weight or ovarian ascorbic acid depletion assay (20,70). These preparations varied tremendously with respect to electrophoretic mobility, carbohydrate content and molecular weight, but proved to have similar immunological potencies by haemagglutination or complement fixation assay (71). In addition, hormone preparations originating from plasma were immunologically indistinguishable from urinary preparations, but possessed greater in vivo bioreactivity and less molecular heterogeneity (71). Conversely, both urinary and plasma purified preparations expressed high and near-equal in vitro steroidogenic stimulating capacity in rat testicular or ovarian bioassay systems (72,74,75). Purification procedures which selectively separated molecularly heterogeneous variants demonstrated that, whereas immunoreactivity of the preparation plateaued near 500 IU/mg, (1 IU = 1297 ng) bioreactivity increased up to 15-20,000 IU/mg (73).

Through the late 1960's until the mid 1970's investigators perplexed by these conflicting observations, worked furiously to resolve them into a cohesive understanding of the structural requirements for bioreactivity and immunodetection. Such investigations revealed that neuraminidase-treated asialo-hCG possessed reduced in vivo bioactivity without significant alteration in its immunodetection. 25% desialation resulted in a 75% loss of bioactivity, whereas 100% desialation resulted in only a 10% loss of immunoreactivity (20). Complete removal of all carbohydrate moieties

by glycosidase treatment was associated with total loss of bioactivity and a 30% decrease in immunoactivity *in vivo* (71,72). ^{125}I-labelled asialo-hCG was concentrated less efficiently by pseudopregnant rat ovaries than the labelled native hormone (76). Indeed the greater the electrophoretic mobility of the purified preparation, the greater its sialic acid content and the higher its *in vivo* activity. Additionally, the greater the sialic acid content of the preparation, the longer its plasma half-life and slower its metabolic clearance rate in intact mice or rats (72,77,183).

These findings contrasted sharply with *in vitro* studies, in which desialation did not detectably alter steroid hormone output by homogenates of rat testis or ovaries (75). However, removal of significant portions of the remaining carbohydrate moieties was associated with a decreased bioactivity under similar conditions (40,75,76).

Experiments such as these have confirmed that the attached carbohydrate residues, especially sialic acid, of hCG are essential for neither antigenic determination nor *in vitro* biological effect; rather this portion of the molecule protects hCG from hepatic and renal degradation and thus is necessary for biological action in the intact animal. That the antigenic specificity of hCG derives from its polypeptide content was confirmed by tryptic digestion (20,183).

The dissociated individual subunits possessed low bioreactivity both *in vivo* and *in vitro*. Injection of the α or β-subunit into rats was associated with 1 and 6% respectively of the bioactivity of intact hCG as assessed by rat ventral prostate assay (78). *In vitro* activity

of the subunits was also low (79,183).

1.2.3.5 The hCG receptor and biological actions

Early investigations defined the gonads, both male and female, as target organs by demonstrating that ^{3}H or ^{125}I-labelled hCG injected into rats was specifically concentrated in these sites (76,78). In vitro experiments confirmed that specific, saturable, high affinity (Kd = 10^{-9}-10^{-10} M/l) and biologically coupled receptors existed only in the gonads; binding in other tisues was non-specific (20). More recently, the hCG receptor was localized to the plasma membrane of human and rat Leydig, luteal and granulosa cells (79) as well as homogenates of human fetal testis (30,36).

^{125}I-hCG binding to rat and human testicular or ovarian homogenates was reversible and inhibited by hLH, bLH and hCG (70,20). Binding was greatest under physiological conditions (37°C, pH 7) and achieved a steady state in 10-15 minutes (83). HCG and LH are considered to act at the same receptor, which appears to lack species specificity (20). This receptor was purified to homogeneity from membrane fractions of bovine corpora luteal cells. The purified receptor has properties similar to the membrane-bound species. It is a largely proteinaceous macromolecule with an estimated molecular weight between 70-200,000 D, and exists in primarily two classes: high affinity, low capacity and low affinity, high capacity. Lipids present in the cell membrane may affect receptor binding activity (83). Negative cooperativity was demonstrated. Receptor concentrations were increased in the presence of FSH, prolactin and sex steroids; whereas they decreased in response to LH and hCG (down regulation) (81,84). A

reflex recovery of receptor numbers was observed after the removal of the stimulus (84).

Early *in vivo* experiments with rats demonstrated lower target organ incorporation of injected labelled asialo-hCG or its fully glycosylated subunits than with the native hormone (76). The labelled β-subunit showed greater ovarian uptake than its α counterpart.

In vitro experiments using gonadal homogenates or purified target cell membrane fractions however proved more useful in strictly defining the structural requirements of the hCG receptor. The desialated hCG derivative displayed equal (74) or greater (75,20) binding affinity than the intact molecule. The greater binding affinity of asialo-hCG reported by some authors may be attributable in part to the resultant decrease in molecular negativity, which increases its attraction to the negatively charged membrane (20). Asialo-agalacto and asialo-agalacto-ahexosamine derivatives displayed 160 and 85% respectively of the binding affinity of the intact molecule. However, a 20% reduction in mannosyl residues dramatically decreased hCG affinity for its receptor. This may be attributable to either a loss of conformational structure or molecular stability, or perhaps to the existence of a mannosyl-specific binding site on the receptor molecule. The individual subunits failed to compete effectively for receptor occupancy; however, binding was restored with subunit recombination (79). These data imply that the low target organ uptake of desialated hCG derivatives *in vivo* is not due to a lack of receptor recognition or binding but rather to its diminished metabolic stability. In fact, the aforementioned experiments confirm that with the possible exception of mannosyl

residues, neither sialic acid nor other monosaccharides are necessary for receptor binding activity. The combined subunit structures fulfil the molecular requirements for receptor binding (20,183).

HCG has been shown to stimulate adenylate cyclase activity and thus cause cAMP accumulation in all target tissues tested (20,83,181). Interestingly however, asialo-hCG and the other glycosidase-treated derivatives failed to increase intracellular concentrations of cAMP in isolated Leydig cells (20). Thus, the carbohydrate moiety of hCG which was not important for receptor recognition or binding may be required for stimulation of post-receptor binding events.

HCG stimulated testosterone production from isolated rat and human Leydig cells, progesterone from luteal and granulosa cells and androgens or estrogens from thecal tissue. These stimulatory effects on steroidogenesis are thought to be due to an hCG induced increase in cholesterol conversion to pregnenolone (85,86). The intermediary role of cAMP has been implicated but not proved (20). Interestingly, auto-antibodies to the hCG-LH receptor have been described in patients suffering from infertility of idiopathic origin (83).

1.2.3.6 Metabolism

In 1971, Van Hall (87) demonstrated that progressive desialation of hCG was associated with an increase in its clearance from plasma. He and his co-workers showed that, in pseudopregnant rats, 25% desialation of an hCG preparation resulted in a 50% reduction in its plasma half-life; similarly 62% desialation caused half-life to decrease 90%; there was no change in half-life with increasing desialation beyond 62%. ^{3}H-hCG injected into animals was recovered

almost entirely from the gonads, whilst the labelled individual subunits or asialo-hCG was recoverd almost entirely from the liver or urine (74,78). However, only 5-25% of injected activity was excreted into urine (83), thus implicating hepatic mechanisms as the major site of extra-renal hCG clearance. The removal of sialic acid exposes the underlying galactose residues. Recently it became evident that hepatic uptake mechanisms possess high affinity for this monosaccharide, thus facilitating hepatic metabolism of asialo-hCG (83).

The plasma half-life of native hCG and its individual subunits has been calculated, based upon rates of disappearance in intact rats (78). In each case, the half-lives were described by a rapid and slower component; for hCG: 141 and 725 minutes respectively; for the α subunit: 6.2 and 58 minutes respectively and the β subunit: 11.5 and 81 minutes respectively. Similar studies in man revealed that intact hCG possessed a plasma half-life of 5-9 hours (88,89) for the rapid component and 23-37 hours for the slower component. Rates of disappearance were slightly prolonged in females (88).

The metabolic clearance rate of hCG was estimated at 2-6 ml of plasma per minute; the greater estimations arising from bioassay versus RIA (83,88). These values are approximately 1/10 the rates of clearance reported for LH; this is thought to be due to the presence of a greater number of sialic acid residues in hCG. Clearance was slightly greater in females (88) and the α subunit cleared more quickly than did the β (90). Renal clearance was estimated at 0.4-1.0 ml plasma/minute (83). Clearance was similar for pregnant and non-pregnant subjects (88), after normal delivery or induced labour (91).

1.2.4 Methods of detection:

1.2.4.1 In vivo bioassay

In vivo bioassays for hCG are generally based upon its gonadotrophic property. Ascheimm and Zondek in 1927 (17) devised the first test of pregnancy based upon this finding in immature female mice. Similar assays in rats, rabbits and male mice were described in the 1940's (92,93,94). The in vivo biological assay can detect hCG concentrations of 1.0 IU/ml of urine or 0.4 IU/ml of plasma, and thus can diagnose pregnancy 6 weeks after the last menstrual period (LMP). A later method involved injection of urine or serum into male toads or frogs. If the injected sample contained hCG, there was reflex discharge of sperm into the animals' urine within 1-5 hours. This method confirms pregnancy 2-4 weeks after the first missed period with an accuracy of approximately 90%. Its sensitivity was reported at 1.5 IU/ml (1 IU = 1279 ng).

1.2.4.2 In vitro bioassay

Measurements of testosterone production by isolated rat testis in response to increasing concentrations of hCG was described by Dufau and co-workers (85) in 1972 as a sensitive method of determining biological activity at the target cell level. Similar assays have been described for other species including the human. An in vitro bioassay which measured progesterone secretion from isolated porcine granulosa cells in the presence of hCG was also reported (95). Although these assays distinguish biological from immunological activity, they do not discriminate between LH and hCG activity.

1.2.4.3 Immunological methods

The groups of Daniel Mishell (96) and Samuel Brody (97) were successful in their independent efforts to couple purified hCG to sheep red cell antigens or latex particles. This pivotal development formed the basis of complement fixation and haem-agglutination inhibition assays. Immunological tests provide a sensitivity of 500-1500 mIU of hCG per ml and thus can confirm pregnancy 6 weeks after LMP with an accuracy of approximately 93% (98).

1.2.4.4 Radioimmunoassay

A radioimmunoassay (RIA) is based upon the competitive binding of labelled and unlabelled antigen (hCG) to a fixed concentration of antibody (anti-hCG). Initial RIA techniques did not distinguish between hCG and LH. However, in 1972, Vaitukaitis et al (99) described a technique which selectively measured hCG in the presence of physiological concentrations of LH, using antisera to the β-hCG subunit. More recently, specificity of this assay was increased by chemical modification of the carboxyl-terminal β-peptide or its artificial synthesis (100,101). Antigenic specificity arises from residues 133-137 of the carboxyl-terminal β-peptide (102). These antisera however, lacked high affinity and failed to neutralize the biological activity of hCG. It is therefore postulated that biospecific antibody production is related to the conformational structure of the molecule rather than its specific amino acid sequence. This hypothesis is supported by the finding that antisera raised against asialo-β-hCG displayed lower sensitivity for free β-subunit and did not cross-react with the native hormone (103). Unfortunately,

carboxyl-terminal β-hCG-specific antisera do not distinguish free β-peptide from the intact molecule, thus precluding assessment of biological activity. These antisera possessed tremendous sensitivity; detecting hCG concentrations as low as 6 mIU/ml which permits the diagnosis of pregnancy only 1 week after ovulation.

1.2.4.5 Radioreceptor assay

Specific high affinity LH receptors are present in thecal, granulosa, luteal and Leydig cells of the human, rat, pig, cow and sheep. This receptor binds only the intact molecule and thus combines the sensitivity of the β-subunit RIA and the specificity of the bioassay. Although receptor affinity was 2-5 fold greater for hCG than hLH, significant cross-reactivity was reported by Saxena (105) who first quantitated this method. The radioreceptor assay can detect 5-10 mIU hCG per ml, thus allowing confirmation of pregnancy 1 week after conception. A receptor-immunoassay based upon competitive binding of anti- β-hCG and purified receptors to ^{125}I-hCG was recently described (104). Crossreactivity with ^{125}I-hCG was low.

1.2.5 Concentrations in pregnancy:

HCG concentrations reach the limit of detection (5-10 mIU/ml) in maternal urine or plasma at about the time of implantation (6-7 days post-conception). Concentrations are generally higher in urine than plasma and early detection is usually more successful in this medium (106). Saxena et al (107) demonstrated the capacity of hCG to cross from the human post-ovulatory endometrium into the maternal circulation within 6 hours. This finding explains the presence of hCG in the

peripheral circulation in the absence of placental vascularization (111,112). Heavy glycosylation of hCG imparts to it a relatively long half-life, thus facilitating its transfer and presence in detectable quantities so early in pregnancy. Braunstein et al (108), calculated an hCG production rate of 1.4 X 10^{-2} IU/cell/day in the first 2 weeks following conception. A pulsatile pattern in maternal serum levels between 5-8 weeks of pregnancy, with a pulse occurring every 4 hours was documented by Odell and his collegues (109).

The first significant rise in hCG levels occurs between 9-13 days post-ovulation (110); thereafter levels rise exponentially with a doubling time of 1.7-2 days, to reach peak concentrations of 100,000-200,000 mIU/ml (1-2 μg/ml), between 8-11 weeks of gestation (113-115). This early rise in hCG concentration was recently described to fit a precise logarithmic function by independent laboratories (113,116). Peak immunological activity usually plateaus at approximately 5,000 IU/ml (~5,000 μg/ml), whereas bioactivity increases to reach 10,000-15,000 IU/ml (83,117).

Following the initial surge, in plasma hCG levels decrease sharply despite an increase in placental weight, and reach a nadir at approximately 14 weeks; second and third trimester values range between 10,000-20,000 mIU/ml (83,117). A small third trimester increase has been observed by some investigators (118,123), but this was not confirmed by others (117).

Fetal serum and amniotic levels mirror maternal urinary and plasma concentrations, although their absolute values are between 10-20 fold lower, confirming reports of preferential secretion into the

maternal compartment (91,119). Maternal α-hCG concentrations increase after first trimester, whilst β-hCG remains low and constant (120); concentrations of the intact hormone remain greater than either subunit alone (121).

HCG levels are generally greater and plasma doubling time more rapid in multiple pregnancies (122,114). Women bearing male fetuses excrete smaller concentrations of hCG into urine than do women bearing female fetuses (123,124). Ectopic pregnancies are associated with normal concentrations until the 10th post-ovulatory day; thereafter levels are consistently 2-3 fold lower than normal for the same gestational period (125). HCG concentrations of less than 50% of normal are commonly associated with spontaneous abortion (126). The radioassays allow detection of pregnancies which abort prior to the first missed period; these previously unrecognized pregnancies are now known as subclinical or occult.

1.2.6 Physiological functions and clinical aspects:

Rescue of diminishing corpora luteal activity seen in the normal cycle has been attributed to hCG since the 1940's (127). Ovariectomy or lutectomy prior to the 7th gestational week was incompatible with the normal continuation of pregnancy (128). Administration of hCG to non-pregnant subjects extended the life of the corpus luteum, was associated with luteal plasma progesterone concentrations and postponed shedding of the endometrium by 10-15 days. This finding suggests that the progesterone-synthesizing capacity of the corpus luteum is intrinsically limited (128). *In vitro* experiments confirmed

hCG-induced progesterone secretion from human luteal cells via specific receptor mediated mechanisms, which resulted in the accelerated conversion of cholesterol to pregnenolone (83,95).

HCG has been shown to cause Leydig cell proliferation in the human male fetus (130). Specific, saturable and high affinity binding of ^{125}I-hCG was demonstrated in human fetal testicular homogenates (14 -20 weeks gestation). Physiological concentrations of hCG caused a dose-dependent stimulation of testosterone output in the same system (82). Fetal testicular hCG concentrations were greater than most other fetal tissues, supporting the concept that the fetal testis is a target organ for hCG (131). Testosterone production in the first trimester male fetus is essential for normal male sexual differentiation and maturation. A role for hCG in the regulation of fetal adrenal DHEA synthesis has been postulated (132,188), although this was not borne out by culture studies of fetal adrenal tissue (133). Recently, Wolf et al (134) reported that supraphysiological concentrations of hCG stimulated aromatase activity and conversion of estrone to estradiol in perfused human placenta. These effects were inhibited by the presence of DHEA or DHEA(S).

Immunological tolerance of the antigenically distinct fetal allograft during pregnancy defies precise explanation. A role for hCG in this process has been postulated. HCG was found to be completely inhibitory in lymphocyte responses to phytohaemagglutinin, antigen stimulation or in mixed lymphocyte cultures (135). Therefore investigators have proposed that hCG represents trophoblastic surface antigens and thus blocks the action of maternal lymphocytes. This

hypothesis is supported by the coincident detection of hCG production and blastocyst implantation (111), which infers direct contact between maternal and fetal organisms. The recent finding of hCG in human spermatozoa and peri-implantation rabbit blastocysts lends credence to this hypothesis (41,42).

Interestingly, Whyte (34) has suggested that the association of hCG with the syncytial border may alter the conformation of plasma membrane proteins such as antigens, thus rendering them unrecognizable to maternal lymphocytes. It is quite likely that syncytial cells lack major histocompatibility antigens; however the presence of organ specific antigens has been demonstrated (83). In conflict with these observations is the finding that highly purified hCG preparations were less inhibitory to lymphocyte function than were cruder preparations (136). This suggests that previous reports of hCG induced lymphocyte inhibition were in fact due to an unrecognized contaminant of pregnancy urine, rather than hCG itself.

HCG has been detected in a significant proportion of tumorigenic tissues (49). The aforementioned characteristics of hCG may protect neoplastic growth from immune mechanisms of foreign tissue irradication. In the clinical setting, hCG is used extensively as a tumor marker.

HCG is easily purified in large amounts from pregnancy urine; as such it is preferred over LH, which is available in much smaller quantities, to induce ovulation in women suffering from anovulatory failure (137). More recently, it has been used to cause superovulation, in women with fallopian tube blockage, to facilitate

the initial collection of mature oocytes required for subsequent in vitro fertilization and embryo transfer (138). HCG is also used as an adjunct to Pergonal therapy to stimulate adequate sperm production in oligospermic men (139). The effectiveness of antibodies raised against β-hCG as contraceptive agents has been considered. Immunization of baboons and marmoset monkeys with β-hCG prevented pregnancy without disturbing normal cyclicity (140). In a preliminary clinical trial 3 of 4 salphingectomized women immunized with β-hCG coupled to tetanus toxoid produced anti-β-hCG and continued to have normal cycles without evidence of metabolically or endocrinologically discernable side effects for a period of 1 year. The fourth woman produced antibodies and remained healthy but was amenorrheic, perhaps secondary to lactation (141).

1.2.7 The regulation of hCG production:

The regulation of hCG is poorly understood. It is particularly intriguing that maternal plasma hCG levels fall precipitously after 11 weeks gestation, despite a tremendous increase in placental weight. No satisfactory feedback control for hCG has ever been described; its waxing and waning in first trimester is thought to reflect the normal growth and maturation of trophoblastic tissue. Early investigators suggested that it was the decrease in the ratio of cytotrophoblasts to syncytiotrophoblasts through pregnancy which led to this curious pattern in maternal plasma (142). However this theory was disproved with the discovery that the syncytiotrophoblast and not the cytotrophoblast was the origin of this hormone (30).

In malignant trophoblastic cell lines, inhibitors of mitosis such as deoxyuridine, hydroxyurea, cytosine arabinoside, actinomycin D, vincristine, colchicine and vinblastine stimulated hCG synthesis (28). The relevance of these findings remains unclear.

The effects of culture conditions on hCG production has been examined. HCG secretion from first trimester explants was greater in an environment of 95% air, 5% CO2 than in environments of 90% N2, 5% CO2 and 5% O2 or 45% N2, 50% O2 and 5% CO2. The mixture of 95% O2 and 5% CO2 was incompatible with hCG secretion (143). However, no effect of oxygen partial pressure was noted in term explant cultures (144).

Fetal calf serum augmented hCG secretion from first trimester (145) and term explants (143), as well as choriocarcinoma cell lines (146,147). HCG secretion into medium initially decreased prior to a secondary increase in both monolayer (148) and explant cultures (149). The existence in vivo of an inhibitory factor from which hCG synthesizing mechanisms are released as a result of placental culture is implied. The nature of this factor remains elusive. The role of prolactin in this regard was investigated. A significant negative correlation existed between maternal plasma hCG and prolactin throughout pregnancy (150). In a single third trimester female with a prolactinoma, bromocriptine (a prolactin inhibitor) treatment was followed by an augmentation of plasma hCG levels. Cessation of treatment resulted in a fall in hCG concentrations (152). Explants of human term placenta responded to physiological concentrations of prolactin with a decrease in hCG secretion. In contrast to these findings, Ranta and colleagues (151) reported that in several first

trimester females, acute or moderate changes in serum prolactin by bromocriptine or chlorpromazine treatment was not associated with any change in hCG levels.

The stimulatory effect of 1-20 mM dbcAMP on hCG and its subunits has been confirmed in term explant or monolayer systems (147,153,154,182), and in 4 hour incubations of midterm or term tissue (155). The effect of this cyclic nucleotide was diminished but still significant in choriocarcinoma or other hCG producing cell lines (156). Theophylline augmented this response (156,157). Several cAMP dependent phosphoproteins have been identified and partially characterized in human term placenta (158). 5'AMP, 5'ATP, adenosine, cGMP, dbcGMP had no effect on hCG production (28).

The biosynthesis and secretion of pituitary glycoproteins is partially controlled by hypothalamic GnRH; by analogy, investigators have considered its role in placental glycoprotein or, more specifically, hCG production. Khodr and Siler Khodr (159) documented stimulation of hCG synthesis in first trimester placental explants. This effect was dose-dependent in term placenta for hCG and β-hCG but not for α-hCG (160). Belisle et al (161) confirmed this finding in midterm and term monolayers and reported low hCG responsiveness of midterm placentae of anencephalic fetuses. No effect of GnRH was observed in 4 hour incubation studies of midterm and term placenta (155), or as a result of continuous (18-20 μg/hr for 6 hrs) or bolus (200 μg i.v.), administration to pregnant females (14-38 weeks) (162). In midterm or term explant cultures, GnRH-induced hCG secretion was abolished by a GnRH antagonist (163), DHEA, low density lipoprotein

(LDL) (154) or by filter sterilization of medium added to term cultures following GnRH enrichment (144).

Immunofluorescent techniques localized GnRH in cytotrophoblasts and excluded it from syncytiotrophoblasts in first term placenta (164). Immunoreactive GnRH levels in placenta at various times through pregnancy were reported (12-23 wks - 36.6 pg/mg; 24-29 wks - 31.6 pg/mg; 30-36 wks - 15.7 pg/mg and term 25.7 pg/mg) (165). Binding sites for GnRH in human term placenta were first reported by Currie (166) and further characterized by Iwashita (167). Decreased GnRH binding was demonstrated in placenta of anencephalic fetuses (168).

In continuous cultures of choriocarcinoma cell-lines, EGF stimulated hCG and α-hCG production; this effect was dependent upon the presence of fetal calf serum (146,169). However, there was no effect of EGF in first trimester explants (169). HCG secretion was slightly enhanced in the presence of primozide, concanavalin A, cholergen and sodium butyrate (28). No effect of somatostatin on hCG or cAMP-stimulated hCG output was noted in term explants (170). Sodium fluoride, dopamine and porcine follicular fluid were similarly ineffective (28).

In the last decade, some investigators have attempted to evaluate the importance of various steroids to the regulatory mechanisms of hCG production. Unfortunately, these findings are neither complete nor consistent.

The addition of cholesterol, LDL, epinephrine, norepinephrine or arachidonic acid to term explants was without effect on hCG levels in collected medium (28,149). In 4 hour incubations of midterm or term

placenta, prostaglandins were similarly ineffective (155). In vivo, the administration of DHEA(S) to second and third trimester women or in vitro exposure of midterm or term tissue to DHEA or DHEA(S) did not alter hCG concentrations in peripheral plasma or collected medium (154,172,173). Plasma hCG levels of pregnant women were unresponsive to 20α-dihydroprogesterone (172). In contrast, in vitro experiments demonstrated the inhibitory effect of this progestin (174).

Explant cultures of term placenta were unresponsive to testosterone and dihydrotestosterone or estriol (171,174). Cortisol was ineffective (171) or stimulatory in term explants (175). However, in a first trimester simian virus-transformed placental cell-line, cortisol greatly inhibited hCG synthesis and induced α-hCG synthesis; it had no effect in term-virally transformed or choriocarcinoma cells (176).

The addition of 1-10 ng/ml of estradiol increased tissue levels of mRNA-α-hCG and collected medium concentrations of α-hCG, but was without effect on β-hCG or hCG secretion in first trimester placental explants (177). Higher concentrations, however, did not alter tissue or medium hCG levels (178). Pharmacological doses (10^{-7}) of estradiol were either ineffective or suppressive of hCG output from term tissue (179). In vivo, estriol-succinate caused an increase in peripheral levels of hCG in pregnant females (172). More confusing is the finding that an aromatase inhibitor decreased hCG secretion from term explants; this effect was independent of its effect on estrogen synthesis (149,154).

Tissue concentrations of progesterone and pregnenolone decreased

hCG output in a dose-dependent manner from term placental explants (174,179). Additionally, Marou and co-workers (178) reported that 5-20 μg/ml of progesterone added to explants of first trimester decreased tissue mRNA-α-hCG, mRNA-β-hCG, α-hCG and β-hCG as well as α-hCG and β-hCG secretion into medium. These findings were not confirmed with lower concentrations of progesterone in term placenta (171). In addition, during 4 hour incubations or following in vivo administration to pregnant women, progesterone was without effect on placental hCG secretion (155,172). In contrast, a significant positive correlation was reported between progesterone and β-hCG concentration in medium recovered from placental explants (149). Recently, Yosef et al (180) showed that 3 successive i.m. injections (100 mg) of progesterone to women between 11-15 weeks gestation resulted in a significant increase in peripheral β-hCG levels. Additionally, these authors noted a moderate correlation between the increment of progesterone and β-hCG.

1.3: SYNTHESIS AND FUNCTION OF STEROID HORMONES PRODUCED IN THE FETO-PLACENTAL UNIT

1.3.1 Introduction:

Until the 7th week of pregnancy, steroid hormones arise largely from maternal sources: ovaries, adrenals and liver. Thereafter, steroid hormone production becomes increasingly dependent upon the interaction of anatomically distinct but biochemically complementary compartments involving the placenta, maternal and fetal livers and adrenals (184,185,186).

1.3.2 Progestins:

The placenta lacks the enzymes necessary to synthesize cholesterol de novo from acetate; therefore, progesterone is produced in the placenta from cholesterol derived mostly from maternal serum and to a smaller degree from the fetal liver. Pregnenolone-sulphate(S) and 17α-hydroxypregnenolone(S) are produced in the fetal zone of the adrenal and are metabolized by placental sulphatase and 3β-hydroxysteroid-dehydrogenase(3βHSD) to progesterone and 17α-hydroxyprogesterone. Pregnenolone(S) is hydroxylated at the 16 position in the fetal liver which is subsequently converted to 16-hydroxyprogesterone in the placenta.

1.3.3 Androgens:

The placenta lacks C17-20-desmolase and thus cannot produce C19 androgens from C21 progestins. LDL-cholesterol is produced in the

fetal liver and is metabolized to pregnenolone and pregnenolone sulfate in the fetal zone of the adrenal. This fetally produced pregnenolone as well as a fraction of placental pregnenolone are efficiently converted to DHEA and then DHEA(S) in the fetal adrenal. Androstenedione arises from desulphation and subsequent Δ^5-Δ^4 conversion of fetally produced DHEA(S) in the placenta. Testosterone is produced from mid-first trimester in the fetal testis and is an intermediate product of placental estrogen synthesis.

1.3.4 Estrogens:

Placental sulphatase, 3β-HSD and aromatase metabolize DHEA(S) into estrone which is reduced in part to 17β-estradiol. A considerable portion of fetal adrenal DHEA(S) in diverted to the fetal liver, where it is 16-hydroxylated to give 16-hydroxy-DHEA(S). This androgen is subsequently desulphated and aromatized to estriol. Estetrol is formed by 16α and 15α hydroxylation of placental estradiol in the fetal liver.

1.3.5 Corticoids:

The large and very active fetal zone of the fetal adrenal gland cannot produce Λ^4-3 ketones such as progesterone and cortisol. However, the small definitive zone may produce a small amount of progesterone from cholesterol which arises in the fetal liver. Thus cortisol is produced in the definitive zone mostly from progesterone of placental origin. The quantitative importance of this pathway remains uncertain. The organs of the fetus including the

placenta represent an important site for the metabolism of cortisol to its inactive form cortisone (187). This activity is present from 12 weeks to term and is most prominent in the chorionic villi (about 85%). The only fetal tissues that reduce cortisone to cortisol are the fetal liver, intestine and chorio-decidua. Reductive activity in the chorio-decidua increased with advancing gestation. Activity in the pregnant uterus showed the same pattern as the chorio-decidua (189).

1.3.6 Trophic functions of steroid hormones:

The actions of estrogens, progestins, androgens and corticoids are diverse. In general, they cause stimulation of cellular protein synthesis and possess trophic effects. Progesterone stimulates proliferation of stromal epithelium, causes glandular development of mammary tissue and induces synthesis of glycoproteins, amino acids and carbohydrates from cervical and endometrial cells. Estrogens are mildly anabolic, uterotrophic, stimulate stromal and ductal growth in breast and induce the synthesis of progesterone receptors, estrogen receptors, cervical mucus, melanin, corticosteroid-binding-globulin (CBG) and other hepatic proteins (190). Testosterone exerts negative feedback effects on pituitary LH secretion, maintains secondary sex characteristics and exerts important protein anabolic and growth promoting effects. Along with FSH, testosterone is responsible for maintenance of spermatogenesis (191).

Cortisol is generally catabolic in the short-term, but is required for normal growth and maturation of all organ systems in the long-term. It is known to accelerate development of hepatic and gastro-intestinal

enzyme systems (191) and promote DNA accumulation in fetal lung monolayers (192,193). It may also induce 11β-hydroxysteroid dehydrogenase (11β-HSD) function in fetal lung (193).

DHEA levels decrease in maternal plasma and increase in fetal plasma through the normal course of pregnancy. DHEA functions primarily as a precursor for estrogen biosynthesis; its other functions have not been well studied (191).

1.4 REFERENCES TO CHAPTER 1

1. Goldfien and Monroe SE, The Ovaries In: Basic and Clinical Endocrinology, Greenspan FS and Forsham PH (eds.), Lange Medical Publications, Los Altos, CA, p. 363, 1983.

2. Johnson MH and Everitt BJ, Coitus and Fertilization, In: Essential Reproduction, Blackwell Scientific Publications, Oxford, p. 192, 1984.

3. Wynn RM, Principles of placentation and early placental development, In: The Placenta, Gruenwald, P (ed.), University Park Press, Baltimore, p.18, 1975.

4. Johnson MJ and Everitt BJ, Implantation and establishment of the placenta, In: Essential Reproduction, Blackwell Scientific Publications, Oxford, p. 215, 1984.

5. Stevens DH, Structure and development: Concepts of the trophoblast and its role in placentation, In: Biology of the Trophoblast, Loke YW and Whyte A (eds.), Elsiever Science Publictions, p. 3, 1983.

6. Junqueira LC and Carneiro J, The female reproductive system, In: Basic Histology, Lange Medical Publications, Los Altos, p. 468, 1983.

7. Wynn RM, Fine Structure of the Placenta, In: The Placenta , Gruenwald P (ed.), University Park Press, Baltimore, p. 56, 1975.

8. Deaden L and Ocklefold CD, Structure of the human trophoblast: correlations with function In: Biology of Trophoblast, Loke YW and Whyte A (eds.), Elseiver Science Publications, p. 70, 1983.

9. Johnson MJ and Everitt BJ, Maternal recogniton and support of pregnancy, In: Essential Reproduction, Blackwell Scientific Publications, Oxford, p. 243, 1984.

10. Dancis J and Schneider H, Physiology: transfer and barrier functions, In: The Placenta, Gruenwald P, (ed.), University Park Press, Baltimore, p. 98, 1975.

11. Lanman JT, Immunological functions of the placenta, In: The Placenta, Gruenwald P. (ed.), p. 145, 1975.

12. Fauk WP and Hsi BL, Immunobiology of human trophoblast membrane antigens, In: Biology of Trophoblast, Loke YW and Whyte A (eds.), Elsiever Science Publications, p. 535, 1983.

13. Contractor SF, Metabolic and enzymatic activity of human trophoblast, In: Biology of trophoblast, Loke YW and Whyte A (eds.), Elsiever Science Publications, p. 23, 1983.

14. Halban J, Die innere secretion von Ovarium und Placenta und ihre Bedeutung fur die Function der Milchdruse, Arch Gynaekol, 75: 353, 1905.

15. Aschner B, Ueber brunstartige Ersheinungen nach subkuntaner Injection von Ovarial-oder placentarextrakt, Arch Gynaekol, 99: 534, 1913.

16. Hirose T, Stimulation of corpus luteum for maturation in rabbits by the administration of various factors, Jap J Gynecol, 16: 1055, 1920.

17. Aschheim S and Zondek B, Hypophysenvorderlappenhormon und Ovarial hormon im Harn von Schwangeren, Klin Wochenschr, 6: 1322, 1927

18. Got R, Gonadotropine choriale humaine; isolement et caracterization, Path Biol 8: 1583, 1960.

19. Got R, Burrillon R and Michon J, Bull Soc Chim Biol (Paris), 42: 31, 1960.

20. Bahl OMP, Human Chorionic Gonadotropin, its receptor and mechanisms of action, Fed Proc 38: 2118, 1977.

21. Swanithan N and Bahl OMP, Dissociation of human chorionic gonadotropin and its subunits, Biochem Biophys Res Commun, 40: 412, 1970.

22. Bahl OMP, Carlsen RB and Bellisario R, Human chorionic gonadotropin, amino acid sequence of α and β subunits, Biochem Biophys Res Commun, 40 : 416, 1972.

23. Morgan FJ, Birken S and Canfield RE, The amino acid sequence of α and β hCG, J Biol Chem, 250: 5247, 1974.

24. Morgan JF and Canfield RE, Nature of the subunits of human chorionic goandotropin, Endocrinology, 88: 1045, 1971.

25. Canfield RE, Morgan FJ, Kammerman S, Bell J and Agosto M, Studies of human chorionic gonadotropin, Rec Prog Horm Res, 27: 121, 1971.

26. Vaitukaitis JL, Ross GT, Reichert LE, Word DN, Immunologic Basis for within and between cross-reactitivty of lutenizing hormone, Endocrinology, 91: 1337, 1972.

27. Pierce JG, Bahl OMP, Cornell JS and Swaminthan N, Bioactive hormone preparations by recombination of α-hCG and hormone specific chain of β-thyrotropin of bovine LH, J Biol Chem, 246: 2321, 1971.

28. Hussa RO, Biosynthesis of human chorionic gonadotropin, Endocrine Rev, 19: 268, 1980.

29. Kesler MJ, Reddy MS, Shah RH and Bahl OMP, Studies of the sturcture of the carbohydrates of hCG, J Biol Chem, 254: 7901, 1979.

30. Midgley AR and Pierce S, Immunohistochemical localization of human chorionic gonadotropin, J Exp Med, 115: 289, 1961.

31. Gaspard UL, Hustin J, Reuter AM, Lamboite R and Franchimont P, Immunoflourescent localization of placental lactogen and chorionic gonadotropin and its α and β subunits in organ cultures of human placenta, Plac, 1: 315, 1980.

32. Ikonicoff LK and Cedard L, Localization of human chorionic gonadotropin and sommatomamotropin by peroxidase and immunohistoenymological method in villi and amniotic epithelium of human placentas, Am J Obstet Gynecol, 116: 1124, 1973.

33. Taberilli M, Kofler R and Wick G, Placental hormones, Plac, 4: 379, 1983.

34. Whyte A, HCG and trophoblast antigenicity, Lancet, 2: 1003. 1978.

35. Acevedo HF, Slifken M, Pouchet GR and Rashsan M, Identification of the β subunit of chorionic gonadotropin in human sperm, In: The Testis in Normal and Infertile men, Troen P and Nankin HR (eds.), Raven Press, New York, p. 185, 1977.

36. Asch RH, Fernandez EO and Parkerstein CJ, Immunodetection of hCG-like substance in human sperm, Fertil Steril, 28: 1258, 1977.

37. Braunstein GD, Raser J and Wade ME, Presence in normal testis of CG-like substance distinct from hLH, N Engl J Med, 293: 1339, 1975.

38, Robertson DM, Sugingami H, Montes-Hernandes H, Puri CP, Choi SK and Diczfaulsy E, Studies on hCG-like material present in non-pregnant subjects, Acta Endocrinol, 89: 492, 1978.

39. Yoshimoto Y, Wolfsen, AR, Hirose F and Odell WD, Human chorionic gonadotropin like material: Presence in normal tissue, Am J Obstet Gynecol, 134: 729, 1979.

40. Yoshimoto Y, Wolfsen AR and Odell WD, Human chorionic gonadotropin like substance in non-endocrine tissues of normal subjects, Science, 197: 575, 1977.

41. Varma SK, Dawood YM, Haouri F, Channing C and Saxena BB, Gonadotropin-like substance in the perimplantation rabbit blastocyst, Fetil Steril, 31: 68, 1979.

42. Wiley LD, Presence of gonadotropin on the surface of peri-implanted mouse embryos, Nature, 252: 715, 1974.

43. Asch RH, Fernandez EO, Magnansco LA and Paurstein CJ, Demonstration of a chorionic gonadotropin-like substance of rabbit morula, Fetil Steril, 29: 444, 1978.

44. Vaitukaitis JL and Ebersol ER, Evidence for altered synthesis of human chorionic gonadotropin in gestational trophoblastic tumours, J Clin Endocrinol Metab, 42: 1048, 1970.

45. Dawood YM, Saxena BB and Landesman RL, Human chorionic

gonadotropin and its subunits in hydatiform mole and choriocarinoma, Obst Gynecol, 50: 172, 1977.

46. Rochman H, Tumour associated markers in clinical diagnosis, Ann Clin Lab Sci, 8: 167, 1978.

47. Javadpour N, McIntyre KR, Waldman TA and Bargman SM, The role of alpha-fetoprotein and human chorionic gonadotropin in seminoma, J of Urol, 120: 687, 1978.

48. Javadpour N, McIntyre KR and Waldman TA, Human chorioinc gonadotropin and alpha-fetoprotein in sera and tumour cells of patients with testicular seminoma, Cancer, 42: 2768, 1978.

49. Braunstein GD, Vaitukaitis JL, Carbone PP and Ross GT, Ectopic production of hCG by neoplasms, Ann Int Med, 78: 39, 1973.

50. Hattri M, Fukase M, Yoshimo H, Matsaura S and Immua H, Ectopic production of hCG in malignant tumours, Cancer, 42: 2328, 1978.

51. Cohen H and Stampp A, Bacterial synthesis of a substance similar to hCG, Proc Soc Exp Biol Med, 152: 408, 1976.

52. Acevedo H, Slifken M, Pouchet GR and Pardo M, Immunohistochemical localization of a chorionic gonadotropin-like protein in bacteria isolated from cancer patients, Cancer, 41: 1217, 1978.

53. Bordelon MR and Kohler PO, Tentative assignment of hCG to chromosome number 18, Abst. No. 114. 59th Annual Meeting of the Endocrine Society, 1977.

54. Landefield TD, McWilliams DR and Biome I, Isolation of mRNA encoding the α subunit of hCG, Biochem Biophys Res Commun, 72: 381, 1976.

55. Fiddes JC and Goodman HM, Isolation, cloning and sequence analysis of the cDNA for the α subunit of human chorionic gonadotripin, Nature, 281: 351, 1979.

56. Loberboum H, Schneider T, deGroot N and Hochberg AA, The effects of protein and RNA synthesis inhibitors on the synthesis and secretion of α-hCG and β-hCG in organ cultures, Eur J Obstet Gynecol Reprod Biol, 18: 57, 1984.

57. Belinski M and Biome I, Molecular characterization of human chorionic gonadotropin, Proc Natl Acad Sci USA, 75: 1768, 1978.

58. Daniels-McQueen S, McWilliams D, Birken S, Canfield R, Landefield T and Biome I, Identification of mRNA's encoding the α and β subunits of human chorionic gonadotropin, J Biol Chem, 252: 7109, 1978.

59. Sakakibara R, Tominga N and Ishiguro M, Intracellular molecular species of hCG from normal but non-cultured first trimester placental tissue, Biochem Biophys Res Commun, 137: 443, 1986.

60. Ruddon RW, Hartle RJ, Peters BJ, Andersen C, Hout RI and Stomberg K, Biosynthesis and secretion of chorionic gonadotropin subunits by organ cultures of first trimester human placenta, J Biol Chem, 256: 11389, 1981.

61. Vaitukaitis JL, Changing placental concentrations of hCG and its subunits through gestation, J Clin Endocrinol Metab, 38: 755, 1974.

62. Marou T, Studies on in vitro synthesis of human chorionic gonadotropin and its subunits, Endocrinol Jap, 23: 119, 1976.

63. Mochizwei M and Hoshina M, Recent progress in placental hormones, molecular regulation in the synthesis and secretion of placental protein hormones, Acta Obst Gynec Jpn, 37: 1949, 1985.

64. Marou T and Mochizuki M, Functional differentiation of trophoblast and its implications for self-regulatory mechanisms of placental protein hormones, Acta Obst Gynec Jpn, 38: 610, 1986.

65. Graesslin D, Weise HE, Brandile W and Czygen PJ, Isolation and partial characterization of 6 different molecular forms of human chorionic gondotropin, Int Congr Ser, Abst 256, 1972.

66. Posillico EG, Handwerger S and Tyrey L, Human chorionic gonadotropin α subunit of normal placenta; characterization of synthesis and association with the β subunit, Biol Reprod, 32: 1101, 1985.

67. Chen R, Burnea E and Benviniste R, Characertization of glycoprotein hormones free α-subunit from human pituitary and placental extracts, Horm Res, 23: 38, 1986.

68. Chatterjee M and Monroe HN, Changing ratios of hCG subunits synthesized by early and full term placental polyribosomes, Biochem Biophys Res Commun, 77: 426, 1977.

69 Ashitaka Y, Tokuro Y, Tane M, Mochizuki M and Tojo S, Studies on the biochemical properties of highly purified hCG, Endocrinology, 87: 2333, 1970.

70. Schuurs AH, deJager E and Homan JDH, Studies on human chorionic gonadotropin, II: Immunochemical characterization, Acta Endocrinol, 59: 120, 1988.

71. Goverde BC, Veenkamp FJN and Homan JDH, Studies on human chorionic gonadotropin, II: Chemical composition and its relation to bioactivity, Acta Endocrinol, 59: 105, 1968.

72. Posillico EG, Handwerger S and Tryey L, Demonstration of intracellular and secreted forms of large hCG α-subunit in cultures of normal placenta, Plac, 4: 439, 1983.

73. Vaitukaitis JL and Ross GT, Recent advances in evaluation of gonadotrophic hormones, Ann Rev Med, 24: 295, 1973.

74. Tsuruhara T, VanHall EV, Dufau ML and Catt KJ, Characterization of binding of intact and desialated hCG in vivo and in vitro, Endocrinology, 91: 463, 1972.

75. Dufau ML, Catt KJ and Tsuruhara T, Retention of in vitro biological activity by desialated of LH and hCG, Biochem Biophys Res Commun, 44: 1032, 1971.

76. Kammerman S and Canfield RE, The inhibition of binding of iodinated hCG to mouse ovary in vivo, Endocrinology, 90: 384, 1972.

77. VanHall EV, Vaitukaitis JL, Ross GT, Hukman JW and Ashwell G, Immunological and biological action of hCG following progressive desialation, Endocrinology, 88: 456, 1971.

78. Brausntein GD, Vaitukaitis JL and Ross GT, The in vivo behavior of hCG after dissociation into subunits, Endocrinology, 91: 10310, 1972.

79. Lundenberger F and Reicher CE, Studies on the uptake of human chorionic gonadotropin by rat testicular homogenates and interstitial tissue, Endocrinology, 91: 135, 1972.

80. Catt KJ, Kesteleger JM and Dufau ML, Receptors for gonadotropin hormones, In: Methods in Receptor Research, Blecher M (ed.), Marcel Dekker, New York, p. 175, 1976.

81. Davies TF, Dufau ML and Catt KJ, Gonadotropin receptors: characterization and clinical application, In: Clinics in Obstetrics and Gynecology, vol 5, p. 329, 1978.

82. Huhtaniemi IL, Koberbrot CC and Jaffe TC, Human chorionic binding and stimulation of testosterone biosynthesis in human fetal testis, J Clin Endocrinol Metab, 44: 963, 1977.

83. Saxena BB, Human chorionic gonadotropin, In: Endocrinology of Preganacy, Fuchs F and Klopper A (eds.), Harper Row, Philadelphia, p. 50, 1983.

84. Morris P and Saxena, Dose and age-dependent effects of prolactin on LH and prolactin binding sites in rat Leydig cell homogenates, Endocrinology, 107: 1639, 1980.

85. Dufau ML, Catt KJ and Tsuruhara T, A sensitive gonadotropin response system, Endocrinology, 90: 1032, 1972.

86. Ashitaka Y and Koide SS, Interaction of human chorionic gonadotropin with rat gonads, Fertil Steril, 25: 177, 1979.

87. VanHall EV, Vaitukaitis JL, Ross GT, Hukman JW and Ashwell G, Effects of progressive desialation on the rate of disappearance of immunoreactive human chorionic gonadotropin from plasma in rats, Endorinology, 89: 11, 1971.

88. Rizallah T, Gurpide E and Wiele RLV, Metabolism of human chorionic

gonadotropin in man, J Clin Endocrinol Metab, 29: 92, 1969.

89. Midgley R and Jaffe RB, Regulation of human chorionic gonadotropin, II: Disappearance of hCG following delivery, J Clin Endocrinol Metab, 28: 1712, 1968.

90. Wide L, Johansson E, Tillinger KG and Diczfalusy E, Metabolic clearance rate of human chorionic gonadotropin, Acta Endocrinol (Kbn), 59: 579, 1968.

91. Bruner J, Distribution of chorionic gonadotropin in mother and fetus at various stages of pregnancy, J Clin Endocrinol, 11: 360, 1951.

92. Friedman MH and Lapman ME, A simple, rapid procedure for the laboratory diagnosis of early pregnancy, Am J Obstet Gynecol, 21: 4501, 1931.

93. Frank RT and Berman RL, 24 hour prenancy test, Am J Obstet Gynecol, 42: 492, 1941,

94. Kupperman HS, Greenblatt RB and Troback CR, Two and six hour pregnancy tests: A preliminary report, J Clin Endocrinol Metab, 36: 548, 1943.

95. Channing CP, The use of ganulosa cell cultures and suspensions as an assay system for measuring LH and hCG, In: Gonadotropins and Gonadal Function, Mondgal NP (ed.), New York, Academic Press, p. 135, 1974.

96. Mishell DP, Wide L and Gemzell CA, Immunological determination of hCG in serum, J Clin Endocrinol Metab, 23: 125, 1963.

97. Brody S and Carlstrom G, Estimation of hCG in biological fluids by complement fixation, Lancet, 2: 99, 1960.

98. Wide L and Gemzell CA, An immunological pregnancy test, Acta Endocrinol, 35: 261, 1960.

99. Vaitukaitis JL, Braunstein GD and Ross GT, A radioimmunassay which specifically measures hCG in the presence of hLH, Am J Obstet Gynecol, 113: 751, 1972.

100. Bahl OMP, Muralidhar K, Chaudhuri G and Lippes J, Preparation and properties of a specific antibody against reduced and alkylated β-subunit of human chorionic gonadotropin, 60th Annual Meeting of the Endocrine Society, Abst 248, 1980.

101. Matsuura C, Chen HO and Hodgen GD, Antibodies to he carboxyl terminal fragment of β-hCG: Characterization of antibody recogniton sites using synthetic peptide analogues, Biochem, 17: 575, 1978.

102. Birken C and Canfield RE, Structural and immunochemical properies of human chorionic gonadotropin, In: Structure and Function of

gonadotropins, McKerns KW (ed.), New York, Plenum Press, p. 47, 1978.

103. Birken S, Canfield RE, Agosto G and Lewis J, Characterization of hCG specific antisera elicited to sialic acid containig β-hCG COOH terminal peptides, 60th Annual Meeting of the Endocrine Society, Abst. 249, 1980.

104. Jibiki K and Saxena BB, A receptorimmunoassay for the deterimination of specificity of anti-β-hCG sera, Acta Endocinology, 87: 838, 1978.

105. Haour F and Saxena BB, Charaterization and solubilization of gonadotropin receptor of bovine corpus luteum, J Biol Chem, 249: 2195, 1974.

106. Beling CG, Cederquist LI and Fuchs F, Demonstration of gonadotropin during the second half of the cycle in women using intrauterine contraception, Am J Obstet Gynecol, 125: 855, 1966.

107. Saxena BB, Kaali S and Landesman R, The transport of chorionic gonadotropin through the reproductive tract, Europ J Obstet Gynecol Reprod Biol, 7: 1, 1977.

108. Braunstein GD, Grodin JM, Vaitukaitis JL and Ross GT, Secretory rates of hCG by normal trophophoblast, Am J Obstet Gynecol, 115: 447, 1973.

109. Odell W, Owens M, Ryan KJ and Tulchinsky D, Episodic secreton of hCG in early pregnancy, J Clin Endocrinol Metab, 53: 1307, 1981.

110. Catt KJ, Dufau ML and Vaitukaitis JL, Appearance of hCG in pregnancy plasma following the initiation of implantation of the blastocyst, J Clin Endocrinol Metab, 40: 537, 1975.

111. Mishell DR, Nakanura RM, Barberra JM and Thorneycroft IH, Initial detection of HCG in serum in normal gestation, J Clin Endocrinol Metab, 118: 990, 1972.

112. Michell DR, Thorneycroft IJ, Nagaka Y, Murata T and Nankamura RM, Serum gonadotropin and steroid patterns in early human gestation, Am J Obstet Gynecol, 117: 631, 1973.

113. Marshall JR, Hammond CB, Ross GT, Jacobson A, Rayford P and Odell W, Plasma and urinary chorionic gonadotropin during early pregancy, Obstet Gynecol, 32: 760, 1968.

114. Fritz M and Gou S, Doubling time of human chorionic gonadotropin in early normal pregnancy, Fertil Steril, 47: 584, 1987.

115. Jaffe RB, Lee PA and Midgley AR, Serum gonadotropins before at the inception of and following human pregnancy, Am J Obstet Gynecol, 29: 1238, 1969.

116. Daya S, Human chorionic gonadotropin increase in normal early

pregnancy, Am J Obstet Gynecol, 156: 280, 1987.

117. Braunstein GD, Rasor J, Alder D, Dannier H and Wade ME, Serum hCG levels throughout normal pregnancy, Am J Obstet Gynecol, 126: 678, 1976.

118. Dattatreymurty B, Seth AR, Joshi LR and Rao SS, Changes in the ratio between serum and specific levels of hCG in different trimesters of pregnancy, Am J Obstet Gynecol, 121: 300, 1973.

119. Clements JA, Reyes FI, Winter JSD and Fairman C, Studies on human sexual development, J Clin Endocrinol Metab, 42: 9, 1976.

120. Ashitaka Y, Mishumura R, Endoh Y and Tojo S, Subunits of human chorionic gonadotropin and their radioimmunoassay, Endocriol Jap, 21: 429, 1974.

121. Hagen C and McNeily AS, The gonadotrophic hormones and their subunits in human maternal and fetal circulations at delivery, Am J Obstet Gyncecol, 121: 926, 1975.

122. Javanovic I, Landesman R and Saxena BB, Screening for twin pregnancies, Science, 198: 738, 1977.

123. Brody S and Carlstrom G, Human chorionic gonadotropin patterns in serum and its relation to sex of the fetus, J Clin Endocrinol, 25: 792, 1965.

124. Boroditsky R, Reyes S, Winter J and Fairman CV, Serum hCG and progesterone patterns in the last trimester of pregnancy: Relation fetal sex, Am J Obstet Gynecol, 121: 238, 1978.

125. Landesman R, Saxena BB, Diagnosis of ectopic pregnancy by radioreceptorassay of chorionic gonadotropin, Lancet, 8079: 772, 1975.

126. Rosal L, Saxena BB and Landesman R, Application of radioreceptorassy of human chorionic gonadotropin in the diagnosis of early abortion, Fertil Steril, 26: 1105, 1975.

127. Salvtierra V. Uber den Gonadotronstrolfwechel wahrend der Schwergeshaft, Arach fur Gynaekolie Bd, 184: 617, 1954.

128 Tulsy AS and Koff AK, Some observations on the role of the corpus luteum in early human pregnancy, Fertil Steril, 8: 118, 1957.

129. Yoshemi T, Strote CA, Marshall JR and Lipsett MB, Corpus luteum function in early pregnancy, J Clin Endocrinol, 29: 225, 1969.

130. Albert A, Underhalhl LU, Greene LF and Lorenz N, Male hypogonadism, In: The normal testis, Proc Staff Mtgs Mayo Clinic, 28: 409, 1953.

131. Huhtaniemi IT, Kobenbroft CC and Jaffe R, Content of chorionic gonadotropin in human fetal tissues, J Clin Endocrinol Metab, 46: 994, 1978.

132. Lauritez CH and Lehman WD, Levels of chorionic gonadotropin in the newborn and their relation to DHEA, Acta Endocrinol (Suppl), Kbn, 100: 112, 1965.

133. Abu-Hakima M, Branchaud CL, Goodyer CG and Pearson Murphy BE, The effects of human chorionic gonadotropin on growth and steroidogenesis of the human fetal adrenal gland in vitro, Am J Obstet Gynecol, 156: 681, 1987.

134. Wolf AS, Henricks I and Lauritzen C, Influence of chorionic gonadotropin and prostaglandins on steroid metabolism of the perfused human placenta, Contr Gynecol Obstet, 131: 162, 1985.

135. Adcock EW, Teasdale, August CS, Cox S, Meschia G, Batiaglia FC, and Naugton MA, Human chorionic gonadotropin: its possible role in maternal lymphoctye suppression, Science, 118: 845, 1973.

136. Morse JH, Stearns G, Arden J, Agosto GM and Canfield RE, The effect of crude and purified hCG on in vitro stimulated lymphocyte cultures, Cell Immun, 25: 178, 1976.

137. Burn JP, Pituitary control of ovarian function, Aust NZ J Obstet Gynecol, 18: 47, 1978.

138. Talbot TM, Dooley M, Leston J, Lopater A, MacMastor R, Wood C, Brown JB and Evans JH, Gonadotropin stimulation of oocyte recovery on in vitro fetilization, Aust NZ J Obstet Gynecol, 16: 111, 1976.

139. Lunenfeld B, What can one expect from treatment of male infetility? In: Clinical Applications of human chorionic gonadotropin, Bellendorf G and Insler V (eds.), Stuttgard Georg Theiem Verlarg p. 167, 1970.

140. Stevens VC, Immunization with hormones, In: Reproduction Research, Amsterdam North Holland Publications, New York, p. 217, 1975.

141. Kumar S, Sharma NC, Bajaj JS, Talwar GP and Hingorami V, Clinical profiles and toxicity studies of 4 women immunized with Pr-β-hCG-TT, Contraception, 13: 53, 1976.

142. Wislocki GB and Bennett HS, The histology and cytology of the human and monkey placenta with special reference to the trophoblast, Am J Anat, 73: 335, 1948.

143. Huot RI, Foidarde JM and Stromberg K, Effect of culture conditions on the synthesis of human chorionic gonadotropin by placental cultures, In vitro, 15: 497, 1979.

144. Hanning RV, Gribble RK, Meuir S and Leifheit TL, Effects of dbcAMP and lutenizing hormone releasing hormone added prior to filter sterilization of the media and variation of oxygen pressure on the output of hCG from term explants, Gynecol Obstet Invest, 20: 74, 1985.

145. Loke YW, Human trophoblast in culture, In: Biology of Trophoblast, Loke YW and Whyte A (eds.), Elseiver Science Publications, p. 633 1983.

146. Benveniste R, Speeg KV, Carpenter G, Cohen S, Linder J and Rabinovitz D, Epidermal growth factor stimulates secretion of hCG by cultured human choriocarinoma cells, J Clin Endocrinol Metab, 46: 169, 1978.

147. Story MT, Hussa RO and Pattilo RA, Independent dibutryl cyclic adenosine monophosphate stimulation of human chorionic gonadotropin and estrogen secretion by malignant trophoblast cells in vitro, J Clin Endocrinol Metab, 39: 877, 1974.

148. Hall CSG, James TE, Goodyer C, Branchaud C, Guyda H and Giroud CJP, Short term tissue cultures of human miderm and term placenta: Parameters of hormonogenesis, Steroids, 2159: 569, 1977.

149. Hanning RV, Choi L, Kiggens AJ, Kuzma DL and Summerville JW, Effects of dibutryl cyclic mononphophate, LHRH and aromatase inhibitor on simulataneous outputs of PGF2α by term explants, Prostaglandins, 23: 29, 1982.

150. HoYoen B, Cannon W, Lewis J, Sly L and Wooley S, A possible role for prolactin in the control of hCG and estrogen secretion by the fetoplacental unit, Am J Obstet Gynecol, 136: 286, 1980.

151. Ranta T, Lehtorrta P, Steriman UH, Laatikainen T and Seepla M, Acute changes in serum prolactin concentrations have no effect on the secretion of progesterone, estradiol or chorionic gonadotropin during early pregnancy, J Clin Endocrinol Metab, 51: 544, 1980.

152. HoYoen B, Moon YS and Shin DH, Inhibition of hCG production by prolactin from term human trophoblast, Am J Obstet Gynecol, 154: 336, 1986.

153. Handwerger S, Barrett J, Tyrey L and Schomberg D, Differential effects of cyclic adenosine monophosphate on the secretion of human placental lactogen and human chorionic gonadotropin, Clin Endocrinol Metab, 36: 1268, 1973.

154. Hanning RV, Choi L, Kiggens AJ, Kuzma DL and Summervill W, Effects of dibutyrl cyclic adenosine monophosphate, LHRH and aromatse inhibitor on simulataneous outputs of progesterone, estradiol and human chorionic gonadotropin by term placental explants, J Clin Endocrinol Metab, 55: 213, 1982.

155. Hanning RV, Choi L, Kiggens AJ, Kuzma DL, Effects of dbcAMP, LHRH, estrogens, progesterone and potassium on PGF2α, human chorionic gonadotropin, estradiol and progesterone by placental minces, Prostaglandins, 24: 495, 1982.

156. Hussa RO, Pattilo RA, Ruchert ACF and Schuureman KW, Effects of butyrate and dbcAMP on hCG secretion from trophoblast and

nontrophoblast cell, J Clin Endocrinol Metab, 46: 69, 1978.

157. Hussa RO, Story MT and Pattillo RA, Cyclic adenosine monophophate stimulates secretion of human chorionic gonadotropin and estrogens by human trophoblast in vitro, J Clin Endocrinl Metab, 38: 338, 1974.

158. Moore E and Cardman KJ, Cyclic adenosine monophophate dependent phosphoproteins in human placenta, Endocrinology, 116: 288, 1985.

159. Khodr G and Siler-Khodr TM, The effects of LHRH on human chorionic gonadotropin secretion, Fertil Steril, 30: 301, 1978.

160. Siler-Khodr TM and Khodr GS, Dose response analysis of LHRH stimulation of hCG release from human term placenta, Biol Reprod, 25: 353, 1981.

161. Belisle S, Bellabarba D, Payet-Gallo N, Lehoux JG and Guevin JL, On the role of LHRH on the in vitro synthesis of bioactive human chorionic gonadotropin in human pregnancies, Can J Physiol Pharm, 64: 1229, 1986.

162. Tamada T, Akabon A, Komima S and Araki S, Lack of release of hCG by lutenizing hormone releasing hormone, Endocrinol Jap, 23: 531, 1976.

163. Siler-Khodr TM Khodr GS, Vickery BH and Nestor JJ, Inhibition of hCG, α-hCG and progesterone release from human placental tissue in vitro by a LHRH antagonist, Life Sci, 32: 2741, 1983.

164. Khodr GS and Siler-Khodr TM, Localization of lutenizing hormone releasing hormone in human placenta, Fertil Steril, 29: 523, 1978.

165. Siler-Khodr TM and Khodr GS, Content of LHRH in human placenta, Am J Obstet Gynecol, 130: 216, 1978.

166. Currie AJ, Fraser HM nd Sharpe RM, Human placental receptors of LHRH, Biochem and Biophys Res Commun, 99: 332, 1981.

167. Iwashita M, Evans MI and Catt KJ, Characterization of a LHRH receptor site in term placenta and chorionic villi, J Clin Endocinol Metab, 62: 127, 1986.

168. Besile S, Leroux JG, Bellabarba D, Gallo-Payet N and Gueiva JF, Dynamics of GnRH binding to human placental cells from normal and anencephalic gestations, Mol Cell Endocrinol, 49: 195, 1978.

169. Huot RF, Foidart JM and Stomberg K, Differential modulation of human chorionic gonadotropin by epidermal growth factor in normal and malignant placental cultures, J Clin Endocrinol Metab, 53: 1059, 1981.

170. Macaron C, Kyncl M, Rutsky L, Halpern B and Brower J, Failure of sommatostatin to affect human chorionic gonadotropin and sommatomamotropin secretion in vitro, J Clin Endocrinol Metab, 47: 1141, 1978.

171. Belleville F, Lasbennes A, Nabet P and Paysant P, Human chorionic gonadotropin and human chorioinc sommatomamotropin regulation in cultured placenta, Acta Endocrinol, 88: 169, 1978.

172. Lauritzen CH, Intraplacental regulation between steroid and peptide hormones (hCG and hPL) in pregnancy, 21 Symp Dtsch Ges Endocrin, Acta Endocrinol (Kbn), Supp 193, Abst 46, 1975.

173. Voutilainen K, Kahhi AI and Laahteenmaki P, The effects of DHEA and its sulphate on human midterm placental secretion of hCG, progesterone, estrone and 17β-estradiol in tissue culture, J Steroid Biochem, 14: 1153, 1980.

174. Wilson EA and Jawad MJ, Stimulation of human chorionic gonadotropin by glucocorticoids, Am J Obstet Gynecol, 142: 344, 1982.

176. Marou T and Chou YJ, Regualtion of hCG synthesis in cultured human placental cells by glucocorticoids, Endocrinology, 109, 888, 1981.

177. Matsuo H, Maruo T, Hosimo M and Mochizuki M, Selective augmentation of cellular α-mRNA-hCG and immunoreactive α-hCG secretion by 17β-estradiol in normal placenta, Folia Endocrinol Jap, 62: 1315, 1986.

178. Marou T, Matsou H, Ohtani T, Hoshina M and Mochizuke M, Differential modulation of chorionic gonadotropin subunit mRNA levels and chorionic gonadotropin by progesterone in normal placenta and choriocarcinoma cultured in vitro, Endocrinology, 119: 855, 1986.

179. Wilson EA, Jawad MJ and Powell DE, Effects of estradiol and progesterone on hCG secretion in vitro, Am J Obst Gynecol, 149: 143, 1984.

180. Yosef Sm, Nesha R, Navot D, Hadani E and Anteby SO, Effects of administration of progesterone on β-hCG blood levels in early pregnancy, Gynecol Obstet Invest, 18: 113, 1984.

181. Marsh Jm, Butcher RW, Savard K and Sutherland EW, The stimulatory effect of LH on cyclic adenosine monophophate accumulation in corpus luteum slices, J Biol Chem, 241: 5436, 1966.

182. Nulsen JC, Woolkais MJ, Kopt GS and Strauss JF, Adenylate cyclase in human cytotrophoblasts: characterization and its role in modualting hCG secretion, J Clin Endocrinol Metab, 66: 258, 1988.

183. Franchimont P, Gaspard OL, Reuter A and Heynen G, Polymorphism of proteins and polpypeptide hormones, Clin Endocinol, 1: 315, 1972.

184. Siiteri PK and MacDonald PC, Placental estrogen biosynthesis during human pregnancy, J Clin Endocrinol, 26: 715, 1966.

185. Siiteri PK and MacDonald PC, The utilization of dehydroepiandrostereone for estrogen biosynthesis during pregnancy, Steroids, 2: 713, 1963.

186. Maternal-Fetal Endocrinolgy, Tulchinsky D and Ryan KJ (eds.), Saunders, Philadelphia, 1980.

187. Murphy BEP, Ontogeny of cortisol-cortisone interconversion in human fetal tissues: a role for cortisone in fetal development, J Steroid Biochem, 14: 811, 1981.

188. Jaffe RB, Seron-Ferre M, Huhtaniemi I and Korenbrot C, Regualtion of the primate fetal adrenal glands and testis in vitro and in vivo, J Steroid Biochem, 8: 479, 1977.

189. Murphy BEP, Conversion of cortisol to cortisone by the human uterus and its reversal in pregnancy, J Clin Endocrinol Metab, 44: 1214, 1977.

190. Goldfien A and Monore SE, The Ovaries In: Basic and Clinical Endocrinology, Greenspan FS and Forsham PH (eds.), Lange Medical Publications, Los Altos, CA, p. 368, 1983.

191. Ganong WF, The Gonads: Development and Function of the Reproductive System, In: Review of Medical Physiology, Lange Medical Publications, Los Altos, CA, p. 346, 1985.

192. Torday JS, Glucocorticoid dependence of fetal lung maturation in vitro, Endocrinology, 107: 839, 1980.

193. Smith BT, Torday JS and Giroud CJP, The growth promoting effect of cortisol on human fetal lung cells, Steroids, 22: 515, 1973.

1.5: AIMS OF THE THESIS

A review of hCG-related literature reveals that, since its discovery in 1927 and purification in 1943, tremendous strides have made in our knowledge of this hormone. However, a comprehensive understanding of the mechanisms regulating gene expression, biosynthesis and secretion of hCG, is still lacking. The aims of my investigations are delineated below.

Firstly, studies were carried out to resolve the conflicting data regarding the effects of steroid hormones on hCG production and to further characterize these effects using an *in vitro* explant culture model of human placenta. HCG production was compared in first and third trimester placenta. The responsiveness of placental tissue to steroidal stimulations was compared at both gestational times.

The role of steroids in the regulation of hCG production was further characterized using immunohistochemical techniques. With this method, I was able to study the effect of steroid hormones on tissue levels of hCG. To evaluate the possibility of autocrine or paracrine regulation by steroids on hCG synthesis or secretion, placental concentrations of hCG, progesterone and estradiol through gestation were ascertained. Furthermore, I conducted studies to determine the effects of steroid hormones on placental viability in culture.

CHAPTER 2

Materials and Methods

2.1 PREFACE

This thesis is presented as a series of submitted or published manuscripts. To avoid being repetitive, only a succinct description of the 'materials and methods' is included with each. However, a discussion of the underlying principles and detailed characterization of procedures is warranted. This chapter is intended to elaborate on these aspects with regards to the major methods used in key experiments.

2.2 EXPLANT CULTURE TECHNIQUES

Placental function can be studied under various conditions. Recently, optimum conditions for perfusion of placental cotyledons were described (1-3). Although these systems are valuable for the study of materno-fetal transfer mechanisms, tissue viability under such conditions (2-3 hrs) greatly limits the investigation of more complex phenomena. Morrish and Sly (4) described a monolayer system of human term placenta in which they were able to maintain responsiveness to secretagogues for up to 21 days. Subculture of monolayers may allow the maintenance of a human placental cell-line for up to 100 days (5). These systems are well-suited for the study of individual cell types and their histogenetic properties; current innovations have allowed the separation of cytotrophoblasts from syncytiotrophoblasts (6). However, monolayers may not accurately reflect in vivo morphology, physiology or biochemistry. The use of trypsin treatment which is required for the preparation of monolayers results in the destruction of normal exopolysaccharides; these are important for cell:cell recognition and interaction. Additionally, cell surface proteins such as receptors may be inactivated (7). With such limitations in mind, I opted to use an explant culture model of human placenta for the study of hCG production. Such a system involves only minimal disruption of cellular integrity or organo-typic architecture. Tissue proliferation is minimal and organ functions can be studied under close to physiological conditions.

The experiments of Strangeways, Fell and Robinson marked the

modern era of organ or explant culture (8-10). Organ culture is defined as 'the maintenance or growth of tissues, organs, primordia or whole parts of an organ in vitro, in a way that may allow differentiation and preservation of the architecture and or function'(11), and have been used as research tools for nearly 70 years. Early experiments were conducted mostly with embryonic animal tissues using the hanging drop method (9). In 1924, TSP Strangeways devised the tube and watch glass methods. These were used for the investigation of embryonic chick tissues supported in a coagulum of either plasma and embryo extract, or a solidified substrate agar medium (8,9,12). These systems however were limited by the inadequacy of diffusion, resulting in a local exhaustion of metabolites and an accumulation of waste products in the immediate vicinity of the tissue. These problems were overcome by the development of fluid media containing organic and inorganic substrates required for tissue growth. Although some tissues survived when partially or wholly submerged in the liquid phase others failed to thrive. Faced with this problem James Chen (13) used lens paper rafts upon which to float tissue for his studies of embryonic rat organs. In 1954, Trowel (14) introduced the 'grid' technique whereby a platform of perforated metal gauze (tantalum) was used to support the tissue at the surface of the fluid media. This allowed simultaneous contact with both aqueous and gaseous phases and greatly improved the viability of tissues in culture. In the experiments described in this thesis, I utilized this original design with some modifications to maintain human placenta in culture.

The first paper published regarding the effects of a hormone, which was studied using organ culture methodology (Gaillard and deJongh, 1938), investigated the effect of estrogen on uterine muscle (15).

Several biological changes are associated with removal of tissue from its in vivo environment (16). These changes fall into 3 phases referred to as the initial 'shock' phase, the intermediary 'stable' phase and the final 'hydration' phase. My experiments were conducted 2-3 days after the preparation of cultures to allow sufficient time for the acclimatization of tissue and prior to the onset of significant histologically detectable degenerative changes. Explants were prepared with careful manipulation using sharp instruments to minimize tissue trauma. The optimum size of explants is a function of tissue metabolism and oxygen diffusing capacity. It has been found that 0.5-1.0 mm^3 pieces of placenta are suitable. Throughout the culture period, tissues were maintained at physiological temperature (37°C) and pH, in a humidified environment of 95% air and 5% CO2. This CO2 concentration approximates its partial pressure in the alveolar space (17), and was found to be most suitable for the culture of placental tissue (18). The acidic effects of CO2 were buffered by bicarbonate present in the culture medium (Appendix 1), thus the pH of the fluid surrounding the tissue remained between 7.2-7.6.

Organic and inorganic substrates required for tissue survival were provided by the addition of a commercially available medium (Appendix 1). This medium was supplemented with 10% fetal bovine serum (FBS) to provide serum proteins and growth factors, as well as with antibiotics to prevent contamination with infectious agents. The cultures were

prepared using sterile technique. Our tissue culture experiments yielded reproducible results (Chapters 3 and 4) and with the exception of a single incident were free from infection.

2.3 RADIOIMMUNOASSAY FOR HCG

In 1972, Vaitukaitis et al (19), described a radioimmunoassay (RIA) which measured hCG in the presence of physiological concentrations of hLH. The essential feature of this assay was an antibody raised in rabbits against the carboxyl tail of the β-hCG molecule. The hCG values reported in the following investgations were quantitated using a commercially available specific β-subunit RIA based upon the antisera developed by Vaitukaitis (Bio-Mega Diagnostics, Montreal, Que.).

The fundamental basis of a RIA is the ability of a fixed amount of antibody (Ab) to bind to a specific antigen (Ag) in a competitive manner according to the Law of Mass Action. The antigen may be either radiolabelled (Ag*, added in fixed amounts) or unlabelled (Ag, variable amounts).

$$Ag^* + Ag + Ab \rightleftharpoons AbAg^* + AbAg$$

If the concentration of Ag* and Ag exceeds that of the Ab, then they will compete for Ab binding sites in proportion to their relative concentrations. If the concentrations of Ag* and Ab are constant, and unlabelled Ag is added in increasing amounts, it will displace Ag*, and the proportion of AgAb complex will increase as the proportion of AbAg* decreases. After allowing the reaction to reach equilibrium, AgAb complexes can be precipated by the addition of an anti-gamma globulin; this is raised in an animal different from that in which the

Ab is raised. AgAb complexes are separated by centrifugation and the radioactivity of the bound fraction is counted. Quantification of the unknown samples is accomplished by comparison of the activity of the sample with a standard curve prepared with known concentrations of hormone.

The assay for hCG used in my experiments is known to cross-react to a significant degree with hLH (see Appendix 3). This relatively high degree of cross-reactivity did not present a problem in my experiments however, since the placenta produces hCG in far greater quantity than hLH (21).

Reagents:

A β-subunit hCG RIA was used in my experiments to measure hCG levels in the medium collected from tissue culture experiments and in human placenta. All reagents used were purchased from Bio-Mega Diagnostics (Montreal, Que.). All hCG assay measurements were calibrated against the Second International Standard (20) (1 IU = 1279 ng, see Glossary of Terms). A standard curve for the assay was set up as follows: 3 mIU/ml, 6 mIU/ml, 12 mIU/ml, 25 mIU/ml, 50 mIU/ml, 100 mIU/ml and 200 mIU/ml. The standards contained 0.1% soduim azide as a preservative, they were prepared from human sera, and were reconstituted in 2.0 ml of glass-distilled water. Six ng (2 micro-curies) of ^{125}I-hCG was reconstituted in 12.5 ml of glass- distilled water and was used as the tracer. After reconstitution, the tracer solution contained 0.01 M phosphate buffer with 0.021 M EDTA, 0.01% sodium azide and 0.23% bovine serum albumin at pH 7.5. Rabbit anti-β-hCG was used as antiserum. This was reconstituted in 12.5 ml of

glass-distilled water and contained 0.01 M phosphate buffer, 0.01% sodium azide, 0.021 M EDTA and 0.25% bovine serum albumin at pH 7.5. One hundred µl of this antiserum binds 35-50% of 120 pg of β-hCG. A freeze dried preparation of goat-anti-rabbit gamma globulin was used to separate free and bound fractions of hCG in the sample. This was reconstituted in 12.5 ml of glass-distilled water and contained 0.01 M phosphate buffer, 0.021 EDTA, 0.01% sodium azide, 0.25% bovine serum albumin at pH 7.5. One mg of rabbit gamma globulin is precipitated by 2.2 mg of this antiserum. A positive control was provided by a specified amount of pooled human pregnancy serum containing sodium azide as a preservative.

Sample Preparation:

Medium collected from tissue culture experiments was frozen immediately at -20°C until the date of the assay. Samples were thawed at room temperature and 100 µl was assayed directly or in dilutions of 1/100-1/1000 as required. Samples were assayed in duplicate and repeated freezing and thawing of samples was avoided.

Protocol:

The assay was set up as follows: 2 tubes for total counts
2 tubes for blank counts
2 tubes for zero
14 tubes for standard curve
2 tubes for positive control
2 tubes for each unknown sample.

200 µl of each stock solution was pipetted into test tubes for the standard curve, followed by 100 µl of each unknown sample. 200 µl of

human serum was added to the blank and zero tubes. 100µl of anti-β-hCG serum was added to all tubes except total counts and blank tubes. This was followed by gentle vortexing and a 30 minute incubation period at 22°C, after which 100 µl of ^{125}I-βhCG tracer was added to each tube. The tubes were gently mixed and incubated for 1.5 hours at 22°C. Following the incubation, 100 µl of goat-anti-rabbit gamma globulin was added to all tubes except total counts. 500 µl of a precipitating reagent was added to all tubes except total counts, the tubes were mixed and then incubated for 5 minutes at 22°C. All tubes except total counts were centrifuged at 1250 x g for 15 minutes at 4°C and then decanted. The radioactivity remaining in each tube was counted using a gamma counter. Duplicate values were averaged and the average background radioactivity was subtracted.

Per cent binding was calcuated as:

$$\frac{\text{CPM of zero tube}}{\text{CPM of total counts}} \times 100$$

This value usually fell between 30-50%.

The B/Bo (bound fraction of the unknown/bound fraction of the zero tube) for each standard control and unknown was calculated by dividing their corresponding mean counts by those of the zero and then multiplying the result by 100.

Therefore,

$$\frac{B}{Bo} \times 100 = \frac{\text{CPM of unknown}}{\text{CPM of the zero tube.}}$$

The results were automatically plotted on 3 cycle semi-log graph paper with B/Bo x 100 on the ordinate and β-hCG concentration

(concentrations of standards) on the abscissa. The results were corrected manually for dilution.

Sensitivity, defined as the amount of hCG distinguishable from zero with 95% confidence is 0.4 mIU. The inter-assay coefficient of variation was calculated to be 15.4% and the intra-assay coefficient of variation was calculated to be 8.0%. The cross-reactivity data are given in Appendix 3.

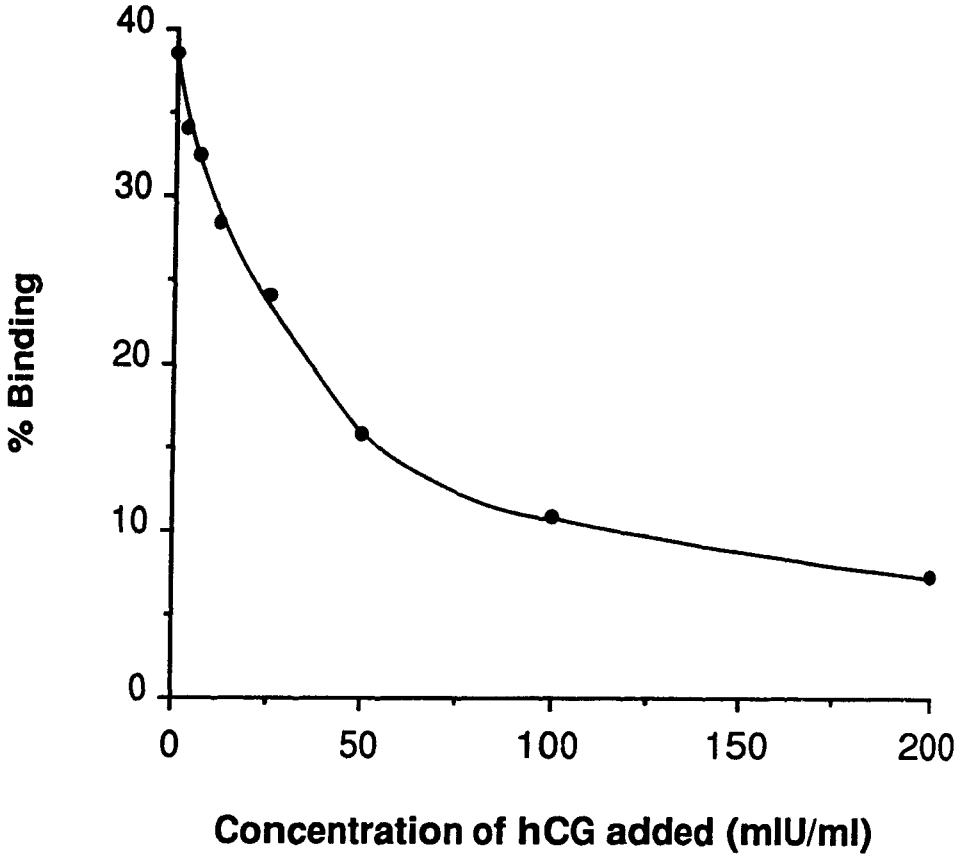

Figure 2.1 An example of a standard curve for the radioimmunoassay for hCG.

2.4 IMMUNOHISTOCHEMICAL STAINING TECHNIQUES FOR HCG

Immunohistochemical staining techniques are designed for localization and visualization of human antigens that survive tissue fixation, embedding and sectioning (22). Such antigens may include cell surface proteins, hormones, enzymes or receptors (22,23). In the experiments described in Chapter 4, placental tissues were stained for hCG using a commercially available Histogen immunohistological peroxidase-antiperoxidase (PAP) system (Bio-Genex Laboratories, San Ramon, CA.). The PAP method was intially introduced as an alternate to direct labelling antibody techniques (24,25). Techniques involving direct labelling of antibodies are limited by the steric hinderance caused by large labelling groups which may interfere with antibody-antigen binding. The use of an unconjugated primary antibody in the PAP system is an effective and sensitive technique which gives reliable and consistent visualization of specific antigens in fixed and paraffin embedded sections. This method is limited by choice of fixative, over-fixation, over-heating or other denaturants (26,27).

In this method, deparaffinized and rehydrated tissue sections are first treated with hydrogen peroxide solution to destroy endogenous peroxidase activity. Sections are then treated with normal serum to block non-specific binding sites and incubated with a primary antibody specific for the antigen, in this case hCG. The primary antibody binds specifically to antigenic sites in the tissue. The sections are then incubated with a linking antibody. Under these conditions, one of the combining sites on the link antibody binds to the primary antibody

and the second binding site remains free. The labelling reagent which is subsequently applied is a soluble peroxidase-antiperoxidase complex. It binds to the free binding site of the link antibody, which itself is bound to the primary antibody, with the primary antibody being bound to the antigen. The entire complex is visualized using the chromogenic substrate, aminoethylcarbazole. This substrate forms a water insoluble red-brown stain at the antigenic site when reacted with horse-radish peroxidase.

Reagents:

All reagents discussed were provided in the purchased assay kit. 3% hydrogen peroxide was used as the first blocking reagent. Normal serum, which originated from the same species as the link antibody was used as the second blocking reagent. The primary antibody, specific to β-chains of the hCG molecule, was dissolved in phosphate buffered saline (PBS) with carrier protein and sodium azide added. Non-immune or pre-immune human, goat or rabbit serum or hybridoma tissue culture supernatant was used as negative control. This was diluted with PBS and contained a carrier protein and sodium azide. The link antibody used was a preparation of anti-immunoglobulin serum diluted in PBS with a carrier protein and sodium azide. Peroxidase-antiperoxidase solution in PBS with sodium azide was used as the labelling antibody. The chromogen-visualizing reagent was a 2.2% solution of 3-amino-9-ethyl-carbazole in N,N-diethyl tormamide.

Protocol:

After the appropriate time in culture, tissue was dissected free from the wire mesh explant grid using a sharp scalpel while grasping

tissue with fine forceps. Sections were fixed in 10% formalin until processed. Sections were prepared in the usual manner and mounted on glass slides. These slides were then incubated in the presence of hydrogen peroxide for 10 minutes at room temperature, rinsed well with PBS and blotted dry. Human serum was then added and the samples incubated for 20 minutes at room temperature. Excess liquid was removed by shaking and the sections were again blotted dry. Anti-hCG-antibody was added to each specimen. Positive and negative controls were added to the appropriate slides. The slides were rinsed and blotted dry. The slides were then incubated in the presence of a link antibody for 20 minutes at room temperature. Slides were rinsed and again blotted dry. Finally, the labelling antibody was added and the samples were incubated as mentioned previously. Slides were rinsed again and blotted to remove excess liquid. A solution of hydrogen peroxide and chromogen was added to each slide. This was followed by a 20 minute incubation at room temperature. Sections were rinsed with deionized water, counterstained with hematoxylin and fixed in ammonia.

The sensitivity of this assay system as reported by Bio-Genex Laboratories (San Ramon, CA) was 0.5 mIU of hCG. Cross-reactivity data for the antibody are given in Appendix 3.

2.5 RADIOTRANSINASSAY FOR PROGESTERONE

In the human, progesterone is bound in serum most strongly by corticosteroid-binding-globulin (CBG) it is also bound to a lesser degree to albumin and to orosomucoid (28). In the experiments described in this thesis, progesterone was measured by a competitive protein binding assay using pregnant guinea pig transprogestin. The radiotransinassay is similar in principle to the radioimmunoassay, but in this technique, a plasma borne, naturally occurring transport protein is utilized rather than an antibody. One of the drawbacks of RIA techniques is that antibodies vary to some extent from one animal to another and from batch to batch, thus making it necessary to characterize each batch. In contrast, within a given species, a serum binding protein such as transprogestin represents a specific and homogenous class of proteins with a consistent set of binding characteristics. The binding characteristics of guinea pig transprogestin have been studied and it was found to be highly specific for progesterone (29).

Reagents:

Progesterone was extracted from samples into redistilled hexane (American Chemicals Co., Montreal, Que.). Extracted steroids were stored at -20°C in ethanol until the time of the assay. Mid to late pregnancy guinea pig serum was used as a source of specific binding protein. The tracer used was progesterone, $(1,2,6,7\text{-}^{3}H(N))$, 50 μCi/ml (New England Nuclear Boston, Mass.). A charcoal solution (Norit A, Fisher Scientific Co., Pittsburg, PA.: phopshate buffer-0.075 M;

1:20) was used to absorb the unbound fraction of progesterone in the sample. The standard curve was prepared from stock solution available in the laboratory; these were made from progesterone (4-pregnene-3,20-dione, Sigma Chemical Co., St. Louis, MO.), and stored in ethanol. The assay was done in glass test-tubes (16 X 100 mm, Fisher Scientific Co., Pittsburg, PA.).

Protocol:

Aliquots of the extract in ethanol were pipetted into test tubes and evaporated to dryness under air at 45°C. A standard curve was pipetted in duplicate as follows: 0.0, 0.2, 0.4, 0.8, 1.2, 2.4, and 4.8 ng; the ethanol was evaported. A protein tracer solution was prepared as follows: 130 µl of the tracer was pipetted into a glass test-tube and the ethanol evaporated, to which was added 9 ml of gelatin water, 1 ml of phosphate buffer and 10 µl of pregnant guinea pig serum. The solution was mixed thoroughly. The test-tubes were arranged in a rack as follows:

-total counts (in duplicate)

-first standard curve

-unknown samples (in duplicate)

-second standard curve

An aliquot (100 µl) of the protein tracer solution was added sequentially to each tube using a 5000 µl dispenser (Hamilton, Reno, Nev.). The assay tubes were incubated at 45°C for 5 minutes to redissolve the progesterone in the protein tracer solution and then at 4°C for approximately 90-120 minutes. Incubation at low temperture enhances the binding by increasing the affinity of the binding protein

for the steroid. Following the incubation peroid, 1 ml of the charcoal solution was added to each tube except total counts; this was done at 4°C. The tubes were again incubated at 4°C for 4 minutes and then shaken vigorously in a horizontal shaker at high speed at 22°C for 1 minute. Immediately following this, the charcoal and unbound progesterone were removed by centrifugation at 2800 x g for 5 minutes at 4°C. A fraction of the supernatant (0.5 ml) was transferred to a counting vial, scintillator was added and the radioactivity of each sample was counted using an LKB-Wallac Rack-Beta liquid scintillation counter (Turku, Finland). The concentration of progesterone in each sample was determined automatically using an LKB program from a plot of counts/minute of each standard versus concentration of progesterone in each standard. The results were corrected manually for dilution.

The inter-assay coefficient of variation for this assay has been established in this laboratory to be approximately 10.0% and the intra-assay coefficient of variation was calculated from my data to be 7.5%. The results of specificity studies are given in Appendix 4.

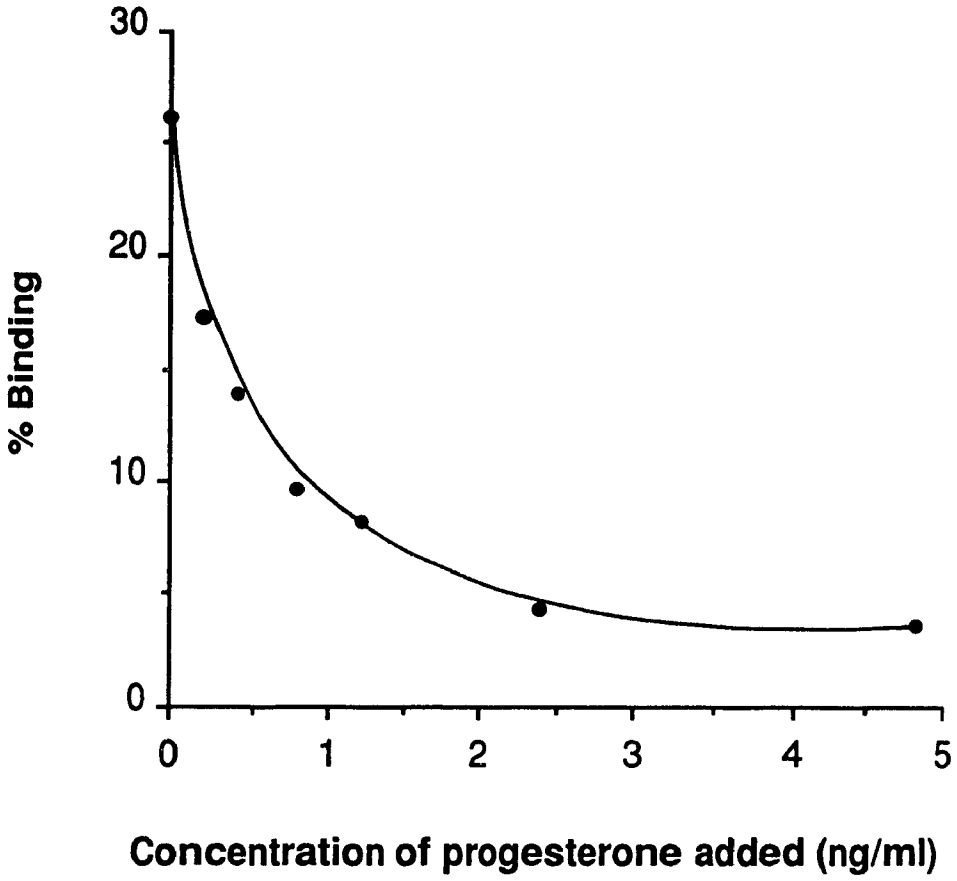

Figure 2.2 An example of a standard curve for the radiotransinassay for progesterone.

2.6 RADIOIMMUNOASSAY FOR ESTRADIOL

In principle the radioimmunoassay for estradiol is essentially the same as previously described for hCG (section 2.2). For the studies described in Chapter 5, estradiol concentrations were determined using a specific antiserum kindly donated by Dr. Hamish Robertson (Ottawa, Ont.).

Reagents:

Estradiol was extracted from samples into diethyl-ether (Anachemia Chemicals Ltd., Lachine, Que.) and was stored in ethanol at -20°C until the time of the assay. Estradiol, $(2,4,6,7\text{-}^{3}H(N))$, 50 μCi/ml was used as the tracer. A charcoal solution (Norit A, Fisher Scientific Co., Pittsburg, PA.: phosphate buffer-0.075 M; 1:20) was used to absorb the unbound fraction of estradiol in the sample. A standard curve was prepared from stock solutions available in the laboratory. These were made from 1,3,5(10)estratriene-3,17β-diol (Sigma Chemical Co., St. Louis, MO.). The assay was conducted in polypropylene (Fisher Scientific Co., Pittsburg, PA.) instead of glass test tubes due to the better recovery of ^{3}H-estradiol in this system.

Protocol:

Aliquots of the estradiol extracts were pipetted into test tubes and evaported to dryness at 45°C. A standard curve was pipetted in duplicate as follows: 0, 20, 40, 120 and 200 pg and the ethanol was evaporated. A tracer solution was prepared by evaporating 200 μl of ^{3}H-estradiol to dryness in a test tube and then redissolving the radioactive steroid in 10 ml of phosphate buffer. In a separate test

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tube, 200 µl of the antiserum was thoroughly mixed with 10 ml of phosphate buffer. Test tubes were arranged in a rack as follows:

-total counts in duplicate

-first standard curve

-unknown samples in duplicate

-second standard curve.

One hundred µl of the tracer solution and the antiserum solution were added sequentially to each tube, with the exception of total counts which did not receive the antiserum solution. The tubes were incubated for 60 minutes at 37°C and then at 4°C for 30 minutes. Following the incubation period, 1 ml of the charcoal solution was added to each tube, with the exception of total counts. The tubes were again incubated at 4°C for 4 minutes and then shaken at high speed in a horizontal shaker at 22°C for 1 minute. Immediately following this, the charcoal and unbound fraction of estradiol were removed by centrifugation at 4°C for 5 minutes at 2800 x g. A fraction of the supernatant (0.5 ml) was transferred to a counting vial and the radioactivity of each sample counted using an LKB-Wallac Rack-Beta liquid scintillation counter (Turku, Finland). The concentration of estradiol in each sample was determined automatically using an LKB program from a plot of the counts/minute of each standard versus the concentration of estradiol in each standard. The results were corrected manually for dilution.

The inter-assay coefficient of variation for this assay has been established in this laboratory to be approximately 10.0% and the intra-assay coefficient of variation was calculated from my data to be 8.3%. The results of specificity studies are given in Appendix 5.

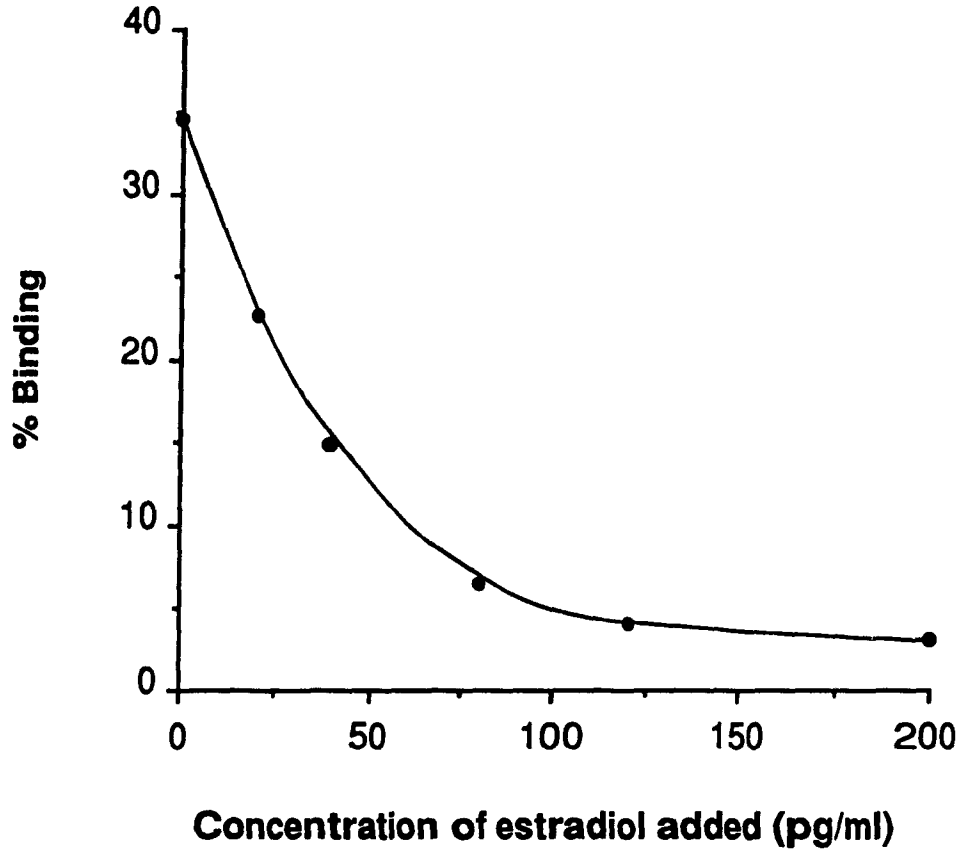

Figure 2.3 An example of a standard curve for the radioimmunoassay for estradiol.

2.7 REFERENCES

1. Bloxam DL and Bullen BE, Conditions and performance of the perfused human placental cotyledon, Am J Obstet Gynecol, 155: 383, 1986.

2. Brandes JM, Tavonoli N, Potter BJ, Sarkozi L Shepard MD and Benk PD, A new recyling technique for human placental cotyledons perfusion: Applications to study feto-maternal transfer of glucose, insulin and antipyrine, Am J Obstet Gynecol, 146: 800, 1983.

3. Contractor SF, Eaton BM, Firth JA and Bauman KF, A comparison of the effects of different perfusion regimes on the structure of isolated human placenal lobules, Cell Tissue Res, 237: 609, 1984.

4. Morrish DW and Sly O, Critical factors in establishing monolayer cultures of normal human placental cells in serum-free medium, Endocrine Res, 229, 1986.

5. Hunt PA and Jacobson PA, In vitro growth and chromosome constitution of placental cells, Cytogen Cell Genet, 39: 1, 1985.

6. Kuman HJ, Nestler JE, Sermansi E, Sanger JM and Jerome FS III, Purification characterization and in vitro differentiation of cytoptrophoblasts from human term placenta, Endocrinology, 118: 1567, 1986.

7. Hodges GM, A review of methodology in organ culture, In: Organ Culture in Biomedical Research, Cambridge University Press, London, Ball M and Monnickedam MA (eds.) p 15, 1976.

8. Strangeways TPS and Fell HB, Experimental studies on the differentiation of embryonic tissues growing in vitro and in vivo, Proc Roy Soc Ser B, 99: 340, 1926.

9. Strangeways TPS and Fell HB, Experimental studies on the differentiation of embryonic tissues growing in vitro and in vivo, Proc Roy Soc Ser B, 100: 273, 1927.

10. Fell HB and Robinson R, The growth, development and phosphotase activity of embryonic avain femoral and limb buds cultivated in vitro, Biol J, 23: 767, 1929.

11. Federoff S, Proposed usage of animal tissue culture terms, J Natl Inst, 38: 607, 1967.

12. Wolff E and Halken K, Sur une method de culture d'organes embryonaire in vitro, Texas J Repro of Biol Med, 10: 463, 1952.

13. Chen JM, The cultivation in fluid media of organized liver, pancreas and other tissues of fetal rats, Exp Cell Res, 7: 518, 1954.

14. Trowell OA, A modified technique for organ culture in vitro, Exp Cell Res, 6: 246, 1954.

15. Gallard JP and deJongh, Die Wirkurg des Oestrons auf den uterus de Maus in vitro, Acta Brevia Neelandica 8: 172, 1938.

16. Schilling JA, Wound Healing, Physiol Rev, 48: 374, 1968.

17. McLimans WF, The gaseous environment of the mammalian cell in culture, In: Growth, Nutrition and Metabolism of Cells in Culture Rothblatt GH and Cristofalo VJ (eds.), New York and London, Academic Press, 137, 1792.

18. Hout, Foidard JM and Stromberg K, Effects of culture conditions on the synthesis of hCG by placental organ cultures, In Vitro, 15: 497, 1979.

19. Vaitukaitis JL, Braunstein GD and Ross GT, A radioimmunoassay which specifically measures human chorionic goandotropin in the presence of lutenizing hormone, Am J Obstet Gynecol, 113: 751, 1972.

20. Bangham DR and Grab B, The second international standard for chorionic gonadotropin, Bull Wld Hlth Org, 31: 111, 1964.

21. Vaitukaitis JL, Changing placental concentrations of hCG and its subunits through gestaion, J Clin Endocrinol Metab, 38: 755, 1974.

22. Mukai K and Rossai J, Immunoperoxidase techniques in pathology. In: Progress in Surgical Pathology, Wolff CM (ed.) Masson Publishing USA, Inc., New York p. 15, 1980.

23. Mason DY, Farrell C and Taylor CR, The detection of intracellular antigens in human leukocytes by immunoperoxidase staining, Brit J Haematol, 31: 361, 1975.

24. Sternberger LA, The unlabelled antibody peroxidase-antiperoxidase (PAP) method, In: Immunocytochemistry, 2nd edition, John Wiley & Sons Hughes J (ed.), New York, p. 109, 1979.

25. Sterberger, LA, Hardy PH and Cuclis JJ, The unlabelled antibody-enzyme method of immnohistochemistry. J Histochem Cytochem, 18: 315, 1970.

26. Taylor, CR, Immunoperoxidase techniques, Practical and theoretical aspects, Arch Pathol Lab Med, 102: 113, 1978.

27. Taylor CR, Kledzik GS, Immunohisotlogical techniques in surgical pathology: a spectrum of new special stains, Human Pathology, 12: 7, 1981.

28. Murphy BEP, Protein binding and radioassay of estrogens and progestins, Handbook of Physiology and Endocrinology II, 29: 631, 1981.

29. Tan SY and Murphy BEP, Specificity of the progesterone-binding-globulin of the guinea pig, Endocrinology, 94: 122, 1974.

CHAPTER 3

The Effects of Various Hormones on HCG Production in Early and Late Placental Explant Cultures[1]

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3.1 PREFACE

Although much is known about the biochemistry and physiology of hCG, very little is understood about the factors which initiate and sustain its secretion in pregnancy. Most *in vitro* studies regarding the regulation of hCG have considered its production from third trimester placenta. However, hCG production is highest and probably of greatest physiological significance during the first trimester of pregnancy. Recent advances in the detection of hCG have allowed earlier diagnosis of pregnancy. In the case of an unwanted pregnancy, it is customary to terminate it as soon after its detection as possible. This usually occurs between 6-10 weeks of gestation and has afforded easier access to first trimester human placental tissues.

In the following study, I investigated the effects of various steroid hormones on the output of hCG from explant cultures of first trimester placenta. Additionally, parallel studies were performed in third trimester explants in order to compare the regulatory effects of these steroids at both intervals of gestation.

Some of the hormones which I chose to study are known to regulate the production of the pituitary gonadotropins FSH and LH and therefore were investigated with regards to the production of the placental gonadotropin, hCG.

3.2 ABSTRACT

Although hCG production is greatest in the first trimester of pregnancy, very little is known about its regulation at this critical time. We have compared hCG output and its response to various hormones in placental explants of 6-12 (early) and 37-40 weeks (late) gestational age. Human chorionic gonadotropin (hCG) production was studied in response to various treatments: progesterone, dehydroepiandrosterone (DHEA), cortisol, progesterone + DHEA, progesterone + cortisol, gonadotropin releasing hormone (GnRH), testosterone and 17β-estradiol. Hormones were added on days 4 and 5 of the 8 days of culture.

HCG production was seven-fold higher from early versus late cultures. In early cultures, hCG output was increased 3-4 fold by progesterone, DHEA and cortisol, while late cultures responded only to progesterone and DHEA (5-10 fold). The combination of progesterone + DHEA resulted in an additive stimulation of hCG output, while progesterone + cortisol caused an output of hCG that was greater than additive in both early and late cultures (up to 15-fold). GnRH increased hCG production slighly (1-2 fold) by both early and late explants; however early placental cultures showed a greater sensitivity. Testosterone was inhibitory (early) or ineffective (late). Estradiol failed to affect hCG secretion.

These studies demonstrate firstly, that explants of placenta provide a useful model for the study of hCG production. Secondly that, there are many similarities but some clear differences between early and late placental secretion of hCG and finally, that steroids exert significant effects on hCG secretion.

3.3 INTRODUCTION

HCG is a heavily glycosylated peptide hormone, consisting of non-identical α and β subunits. It is produced by the syncytiotrophoblast cells of the placenta and is detectable in maternal serum as early as 8 days after conception. Maternal plasma levels peak around 10 weeks of gestation; thereafter hCG concentrations begin to decline to relatively low values and plateau throughout the remainder of pregnancy (1).

Early secretion of hCG ensures maintenance of corpus luteum function and therefore progesterone production until placental steroidogenesis is well established. HCG supports early gonadal differentiation by stimulating testosterone production by the fetal testes. An immunosuppressive role in preventing rejection of the fetal allograft has also been suggested (1).

Although there is a wealth of literature regarding hCG, very little is known about the factors that initiate and support the production of this hormone by the trophoblast. It has been hypothesized that the changing profiles of hCG in maternal blood and urine are related to the changing ratios of cytotrophoblast to syncytiotrophoblast in the developing placenta. This theory has fallen into disfavor since the discovery that syncytiotrophoblasts and not cytotrophoblasts are the source of hCG. More recently, regulatory factors for hCG have been reported. Human placenta produces GnRH, which is immunologically indistinguishable from the hypothalamic species (2). It stimulated the <u>in vitro</u> production of hCG from

placenta of various ages (3), and GnRH receptors were localized on syncytiotrophoblast cells (4).

The effects of the various steroids of pregnancy on hCG have been studied in midterm and term placental cultures (5-8). However, hCG production by the placenta is maximal in first trimester, and there are few reports of steroid effects at this time.

In this study we compared the effects of various hormone treatments, both steroidal and non-steroidal, on hCG production in placental explant cultures of 6-12 and 37-40 weeks gestational age.

3.4 MATERIALS AND METHODS

Materials

Hams's F-10 powdered culture medium and heat inactivated fetal bovine serum were purchased from Grand Island Biological Company (Burlington, Ont.). Amphotericin was obtained from Squibb, Inc. (Montreal, Que.), and Gentamicin from Scherring (Pointe Claire, Que.). Wire mesh was obtained from Johnson Wire Works (Montreal, Que.) and 60 X 15 X 2 mm culture dishes were purchased from Sigma Chemical Co. (St. Louis, MO.). Cycloheximide was obtained from Fluka Chemical Co. (New York, NY.). The GnRH used was a commercially available clinical preparation, Factrel, from Ayerst (Montreal, Que.).

Placental tissue was obtained under aseptic conditions from women undergoing either dilatation and evacuation for socio-medical reasons (6-12 weeks), or after elective cesarean section (37-40 weeks).

Preparation of explant cultures

The tissues were immediately transferred to 0.9% saline and kept on ice until explant cultures were prepared. Placental villous tissue was rinsed thoroughly with culture medium to remove excess blood and dissected free of membranes using forceps and blade. 0.5-1 mg pieces of villous tissue were placed onto wire mesh grids. Two prepared grids were placed into each culture dish, with 3 ml of culture medium. The average total weight of tissue per dish was 10-15 mg. Cultures were incubated for 8 days in a humidified environment of 95% air, 5% CO2 at 37°C, in Ham's F-10 + 10% fetal bovine serum. Further details of this procedure are given in Chapter 2.

Treatments

Each treatment group consisted of 6 culture dishes, which were compared to 6 control dishes. Culture medium was collected daily from each dish, the dishes were rinsed and the medium replaced. Collected medium was frozen immediately at -20°C until analysis. Each experimental protocol was carried out at least twice.

Stock solutions of steroids were prepared in ethanol and the appropriate amount evaporated and redissolved in culture medium to give concentrations of 2, 10, and 25 μg/ml. Estradiol was prepared in concentrations of 0.05 0.5 and 25 μg/ml. GnRH was added directly to the medium to yield final concentrations of 2, 10 and 25 μg/ml. The treatment groups received steroid-enriched medium on days 4 and 5, while controls received unenriched medium. Cycloheximide-treated cultures received cycloheximide-enriched medium (1 mM) daily for the 8 day incubation period.

Analysis

Radioimmunoassay (RIA) of β-hCG was carried out on 100 μl aliquots of appropriately diluted (1/100-1/1000) culture medium using a Bio-Mega Diagnostic assay, Bio-Mega Diagnostics Inc. (Montreal, Que.). Further details of the assay procedure are provided in Chapter 2.

Statistical Analysis:

The probabilities of statistical differences between control and treatment groups were calculated using Student's t-test.

3.5 RESULTS

Explant cultures of early and late placenta were successfully maintained for 8 days. Toxicity of hormone treatments was excluded using histological analysis (see Chapter 4).

Early explant cultures produced hCG in concentrations 7 $\pm$ 0.8 S.E. times greater than late cultures under control unstimulated conditions (Figure 3.1). Patterns of secretion under both conditions were remarkably similar. HCG levels decreased 30-40% in the first 24 hours of culture and thereafter increased 35-45% to attain maximal values on day 4. After day 4, hCG levels declined steadily. The addition of 1 mM cycloheximide abolished the secondary rise in hCG seen after day 2 (Figure 3.2).

Progesterone and DHEA stimulated hCG output in a dose-dependent manner in both early and late cultures (Figure 3.3 and 3.4). These effects were significant at all doses used ($p < 0.05$). The simultaneous addition of progesterone and DHEA resulted in a dose-dependent response which was additive at both ages (Figure 3.5).

Cortisol alone increased the production of hCG in early cultures at the 3 doses tested; in late cultures, however, cortisol alone failed to have any effect on the production of this hormone (Figure 3.6). The combination of progesterone and cortisol resulted in hCG output that was greater than additive in both early and late cultures (Figure 3.7). It is interesting to note that in late cultures, the presence of cortisol greatly enhanced the effect of progesterone, while cortisol alone was without effect.

GnRH increased concentrations of hCG assayed in medium from both early and late explants (Figure 3.8). This effect was dose-dependent and significant in early cultures at 10 and 25 but not 2 μg/ml. In late cultures, this effect reached significance only at the highest dose used (25 μg/ml).

Testosterone was the only hormone studied which inhibited the production of hCG (Figure 3.9). This effect was present only in early cultures and showed clear dose-dependence. Estradiol in concentrations ranging from 0.05-25 μg/ml failed to have any effect on hCG production in early or late cultures (Figure 3.10).

A comparison of the effects of all experimental treatments at the highest dose used (25 μg/ml), is presented in Figure 3.11. At the time of maximal stimulation (day 6), progesterone was more potent than DHEA, which was more potent than cortisol, which was more potent than GnRH in stimulating hCG production from early cultures. The effects were similar in late cultures, with the exception that cortisol alone had no effect.

Although hCG production by early cultures greatly exceeded that of late cultures, on average, production by late cultures increased to a relatively greater extent with progesterone, DHEA and progesterone + DHEA treatments. However, late cultures did not respond to cortisol and were less responsive to the combination of progesterone + cortisol.

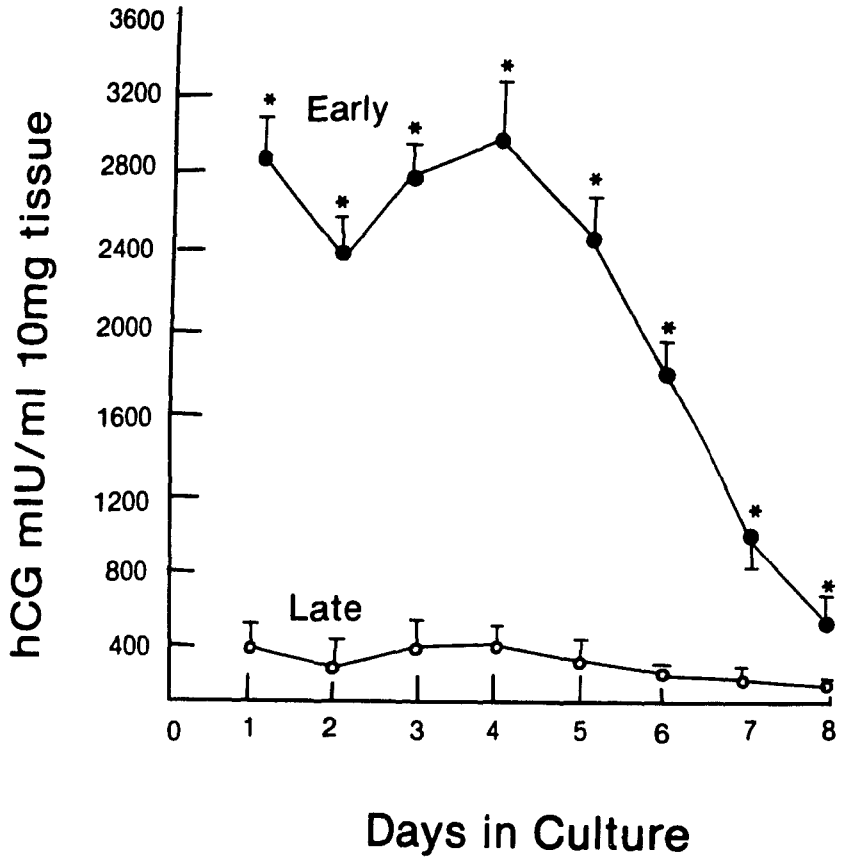

Figure 3.1: A comparison of the basal patterns of hCG secretion in early and late cultures. Each point is the mean ± S.E. of 12 determinations. Significant effects ($p < 0.05$) are denoted by an asterisk. (1 IU = 1279 ng)

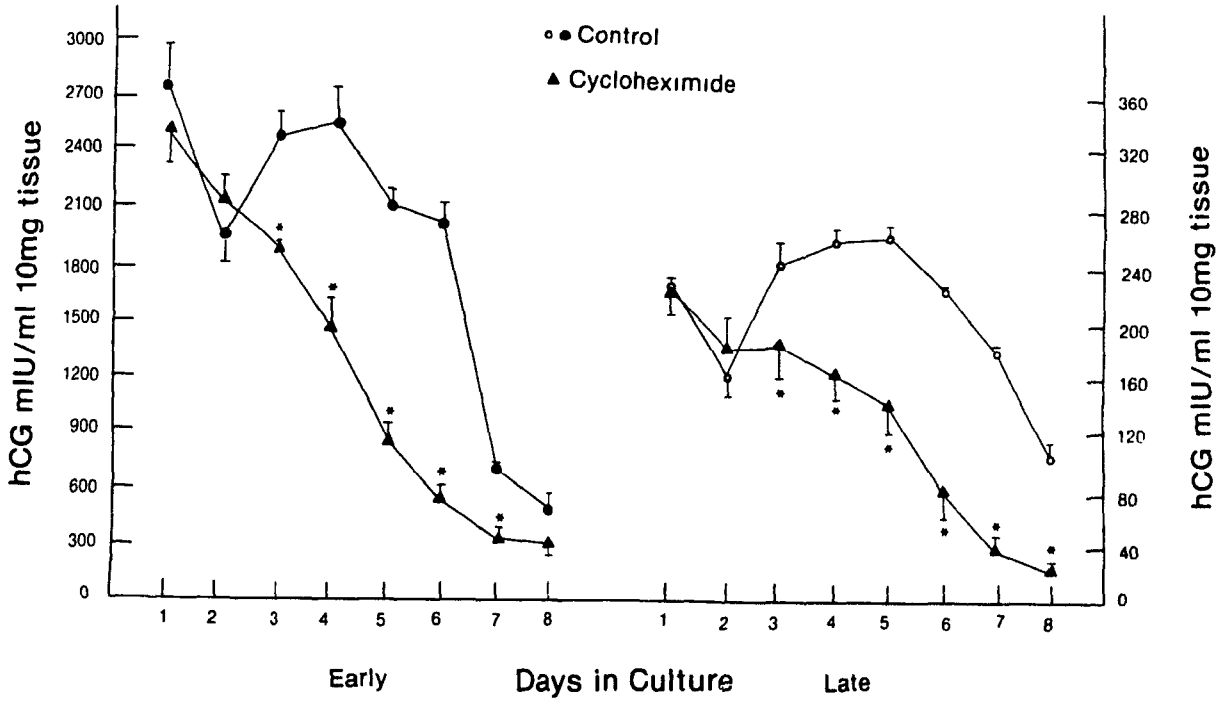

Fig 3.2: The effects of daily addition of 1 mM cycloheximide on hCG production. The left panel shows the results of treatment in early cultures, the right panel, the results in late culture. Each point is the mean ± S.E. of 6 determinations. Significant effects ($p < 0.05$) are denoted by an asterisk. (1 IU = 1279 ng)

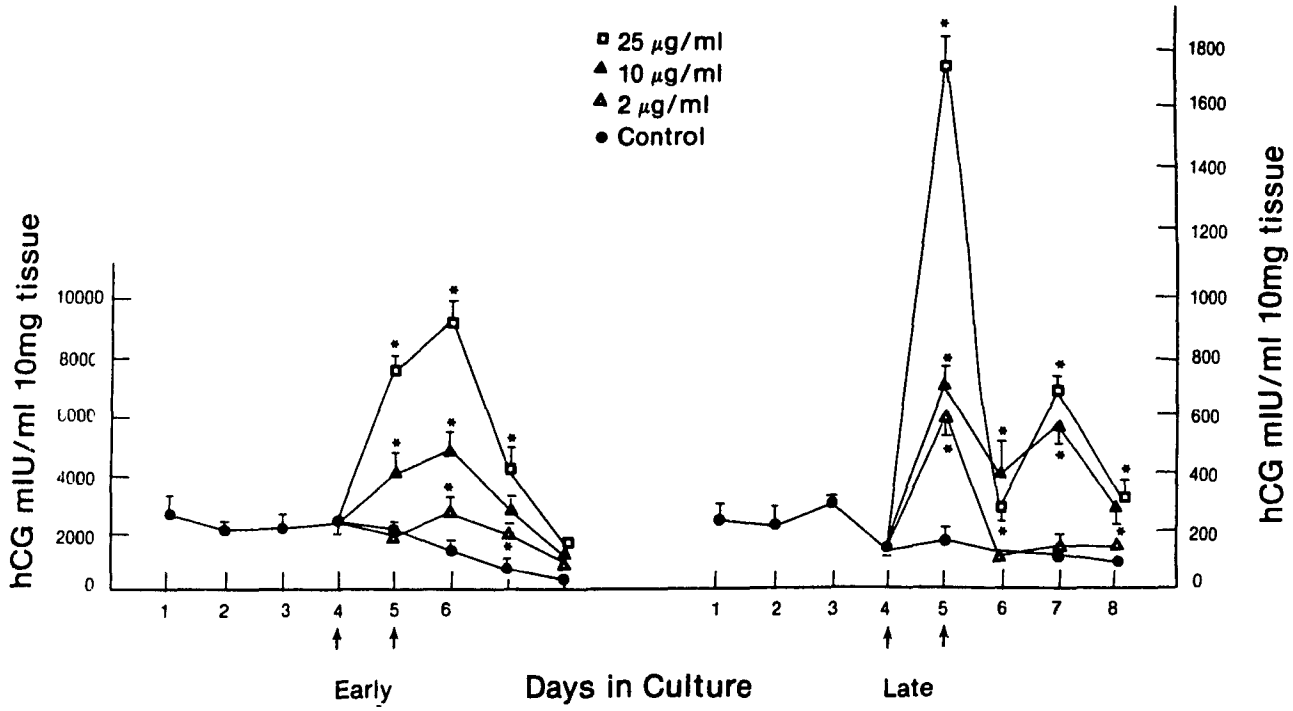

Figure 3.3: The effect of progesterone on hCG output on early (left panel) and late cultures (right panel), at concentrations of 2, 10 and 25 μg/ml. Progesterone was added on days 4 and 5 as denoted by the arrows below the horizontal axis. The data points corresponding to days 1, 2 and 3 are the means ± S.E. of 36 determinations. The data points corresponding to days 4-8 are the mean ± S.E. of 6 determinations. Significant effects ($p < 0.05$) are denoted by asterisks. (1 IU = 1279 ng)

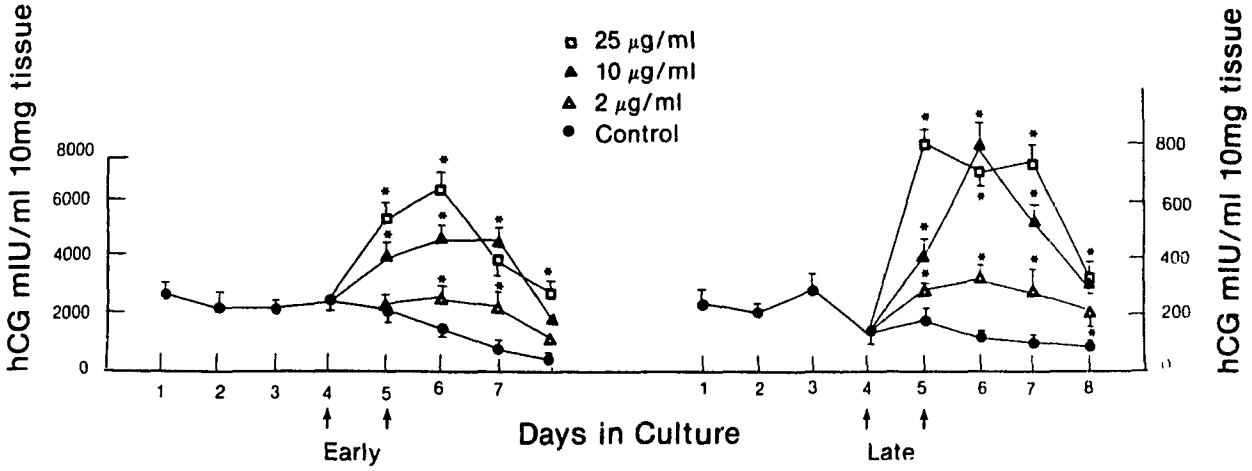

Figure 3.4: The effect of DHEA on hCG output in early (left panel) and late cultures (right panel), at concentrations of 2, 10 and 25 μg/ml. DHEA was added on days 4 and 5 as denoted by the arrows appearing below the horizontal axis. The points corresponding to days 1, 2 and 3 are means ± S.E. of 36 determinations, while those corresponding to days 4-8 are means ± S.E. of 6 determinations. Significant effects are denoted by asterisks. (1 IU = 1279 ng)

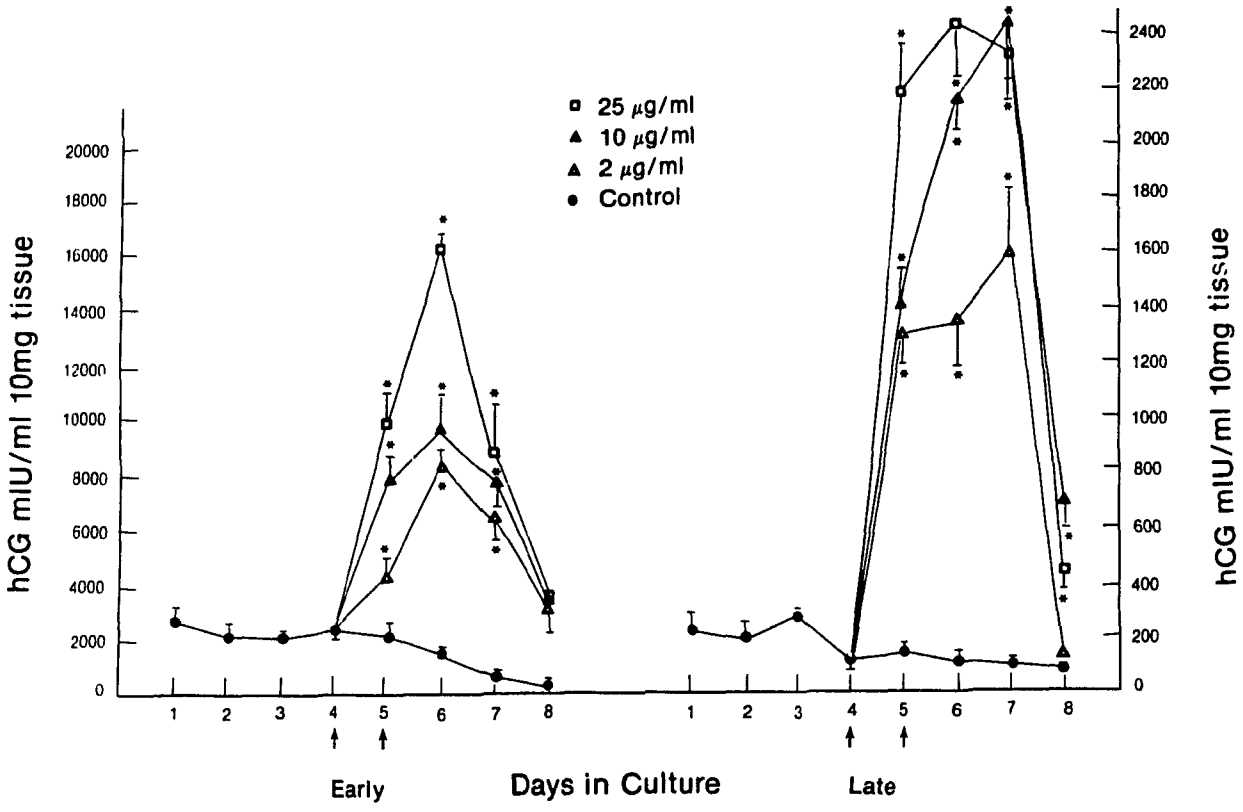

Figure 3.5: The effect of the simultaneous addition of progesterone + DHEA on hCG production in early (left panel) and late cultures (right panel), at concentrations of 2, 10 and 25 μg/ml each. The hormones were added on days 4 and 5 as denoted by the arrows below the horizontal axis. The points corresponding to days 1, 2 and 3 are means ± S.E. of 36 determinations. The points corresponding to days 4-8 are means ± S.E. of 6 determinations. Significant effect are denoted by asterisks. (1 IU = 1279 ng)

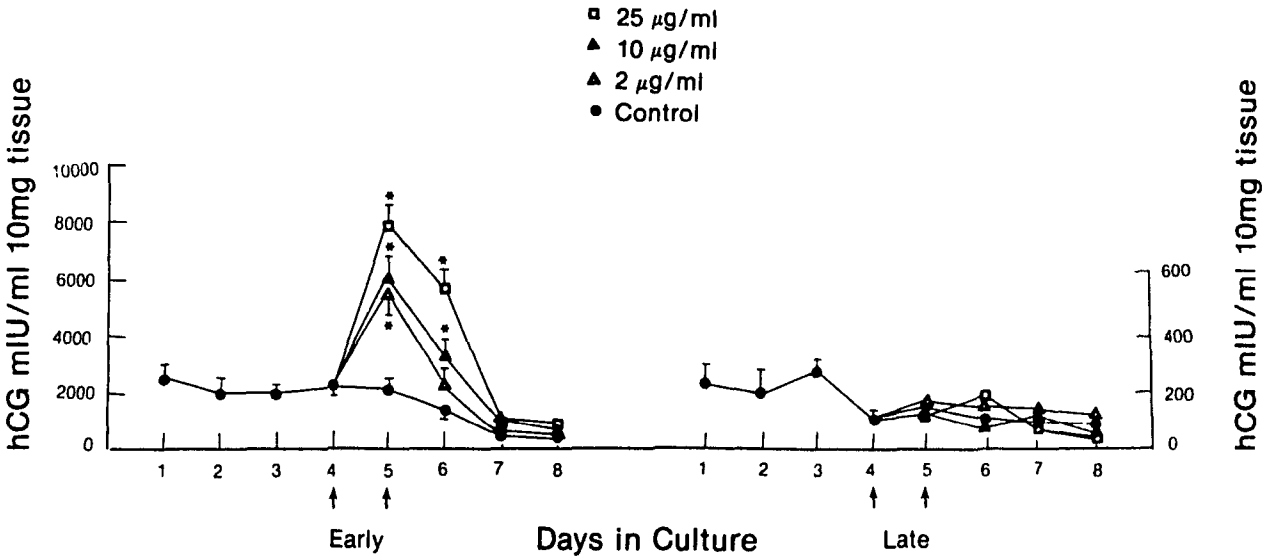

Figure 3.6: The effect of cortisol on hCG output in early (left panel) and late cultures (right panel), at concentrations of 2, 10 and 25 μg/ml. Cortisol was added on days 4 and 5 as denoted by the arrows below the horizontal axis. The points corresponding to days 1, 2 and 3 are means ± S.E. of 36 determinations, while those corresponding to days 4-8 are means ± S.E. of 6 determinations. Significant effects are denoted by asteriks. (1 IU = 1279 ng)

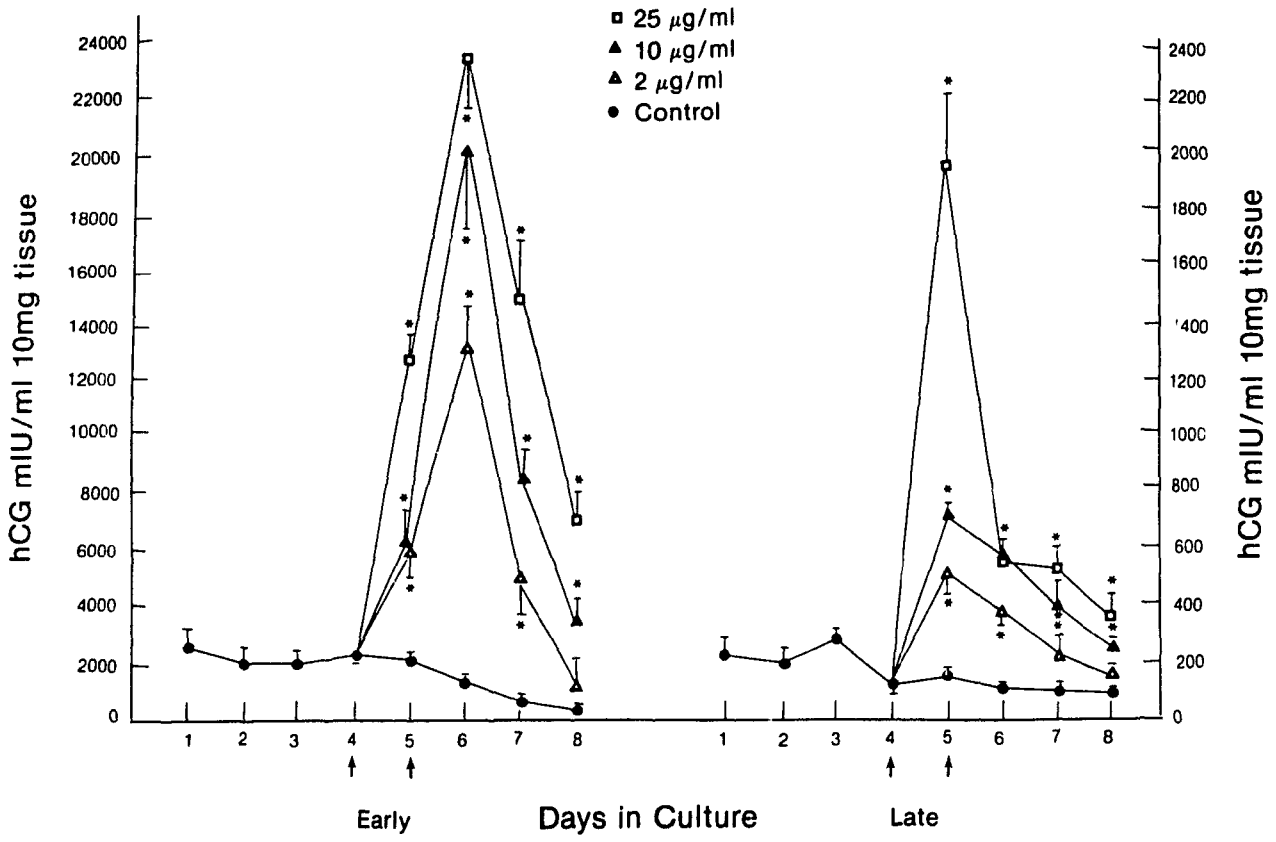

Figure 3.7: The effects of the simultaneous addition of progesterone + cortisol on hCG production in early (left panel) and late cultures (right panel), at concentrations of 2, 10 and 25 μg/ml each. The hormones were added on days 4 and 5 as denoted by the arrows below the horizontal axis. The points corresponding to days 1, 2 and 3 are means ± S.E. of 36 determinations. The points corresponding to days 4-8 are means ± S.E. of 6 determinations. Significant effects are denoted by asterisks. (1 IU = 1279 ng)

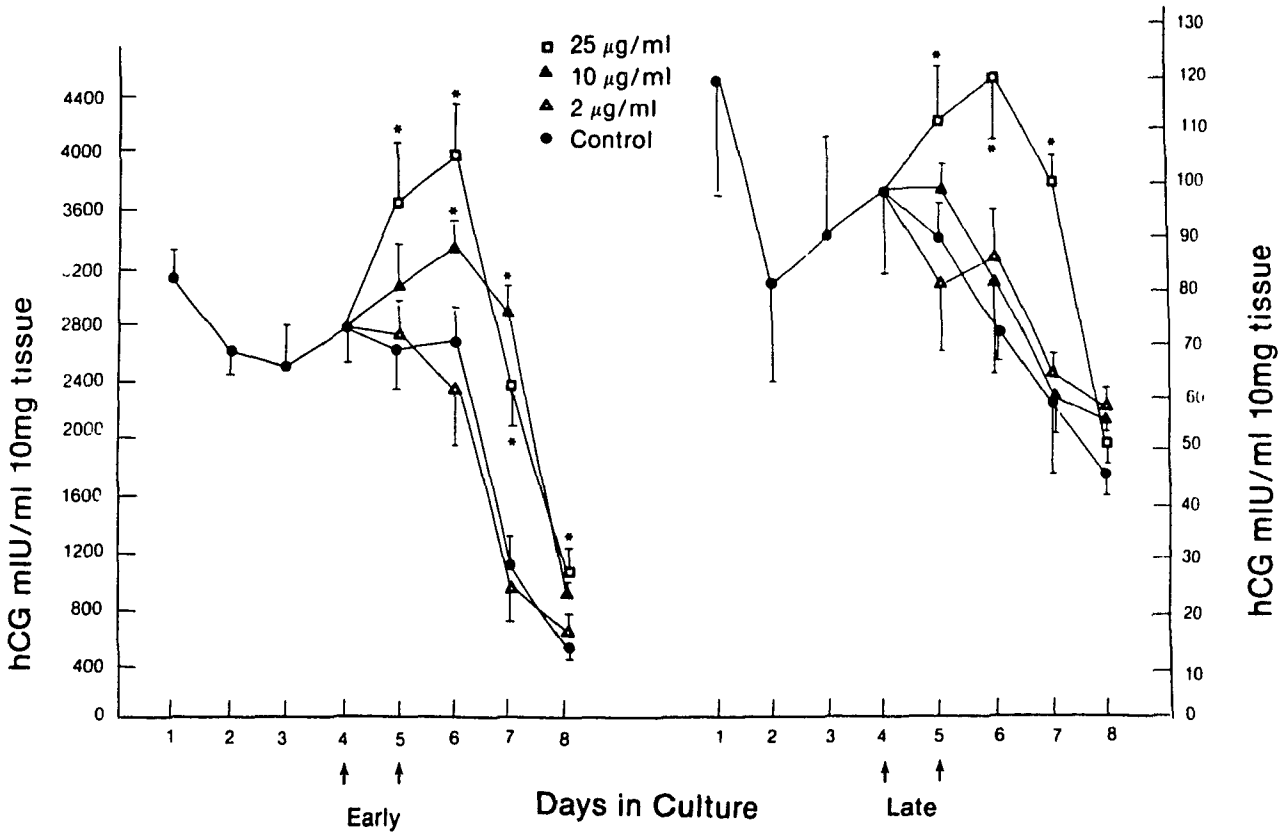

Figure 3.8: The effects of GnRH at concentrations of 2, 10 and 25 μg/ml on hCG production by early (left panel) and late cultures (right panel). The stimulation produced by GnRH is smaller than other hormones tested, and the scale is adjusted accordingly. GnRH was added on days 4 and 5 as denoted by the arrows below the horizontal axis. The points corresponding to days 1, 2 and 3 are means ± S.E. of 36 determinations, while those corresponding to days 4-8 are means ± S.E. of 6 determinations. Significant effects are denoted by asterisks. (1 IU = 1279 ng)

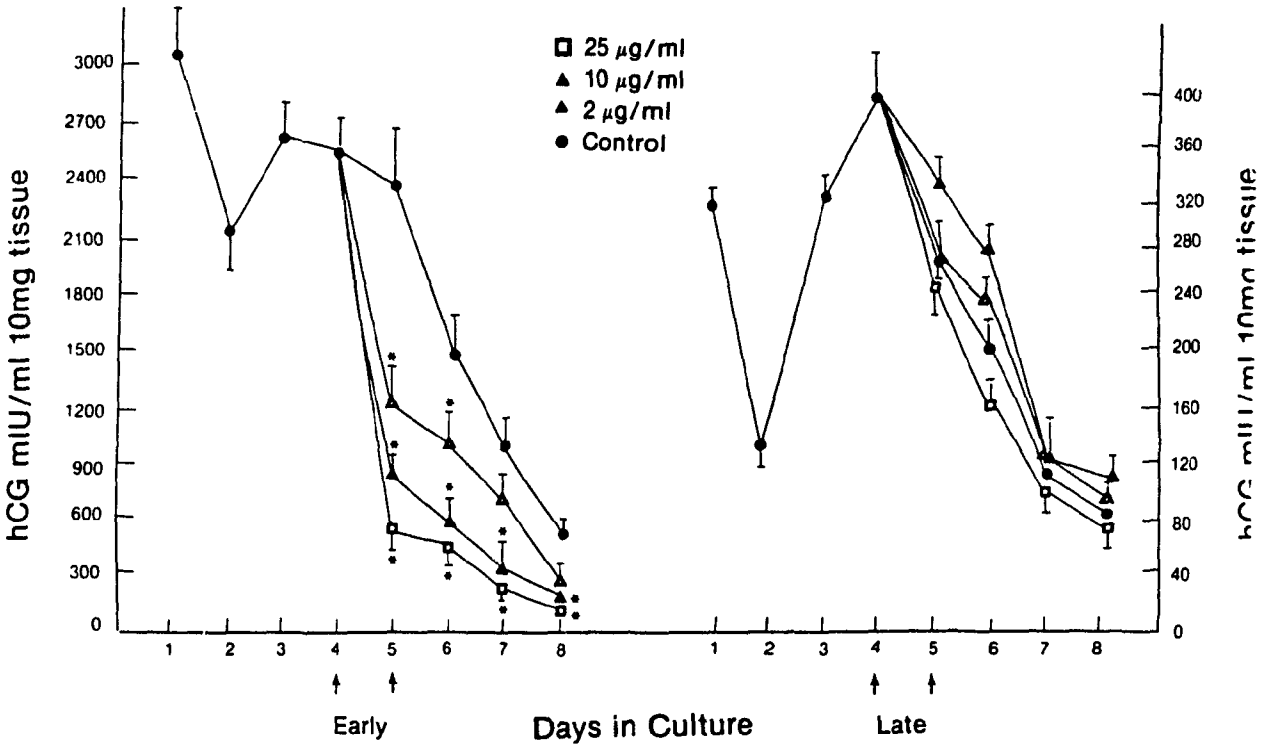

Figure 3.9: The effect of testosterone on hCG production in early (left panel) and late cultures (right panel) at concentrations of 2, 10 and 25 μg/ml. Testosterone was added on days 4 and 5 as denoted by the arrows below the horizontal axis. The data points corresponding to days 1, 2 and 3 are means ± S.E. of 36 determinations, while those corresponding to days 4-8 are means ± S.E. of 6 determinations. Since testosterone produced an inhibiton of hCG production, these data are presented on a smaller scale than the other figures. Significant effects are denoted by asterisks. (1 IU = 1279 ng)

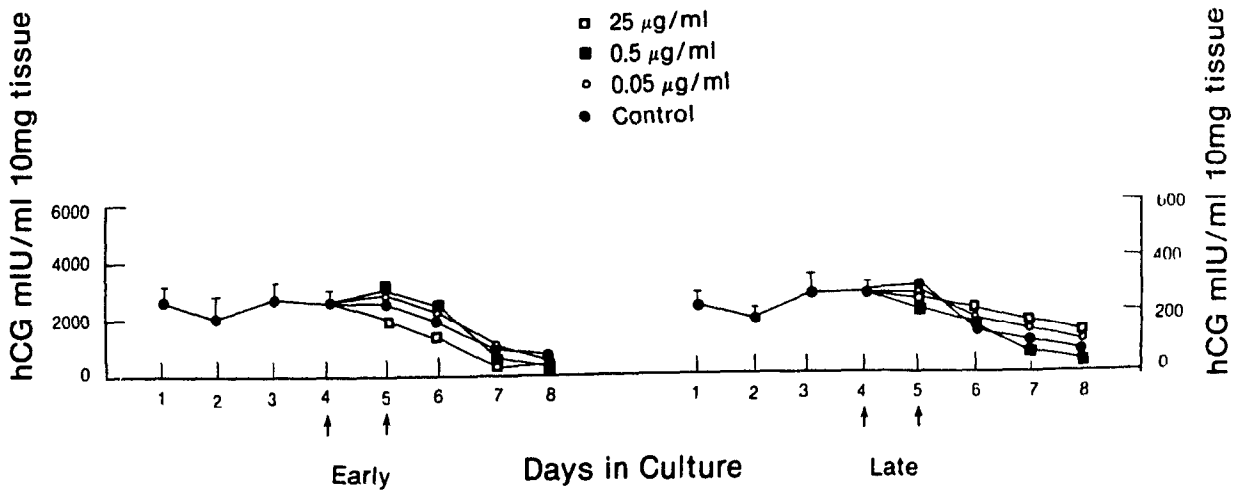

Figure 3.10: The effect of 17β-estradiol on hCG output in early (left panel) and late cultures (right panel), at concentrations of 0.05, 0.5 and 25 μg/ml. Estradiol was added on days 4 and 5 as denoted by the arrows below the horizontal axis. The points corresponding to days 1, 2 and 3 are means ± S.E. of 36 determinations, while those corresponding to days 4-8 are means ± S.E. of 6 determinations. Significant effects are denoted by asterisks. (1 IU = 1279 ng)

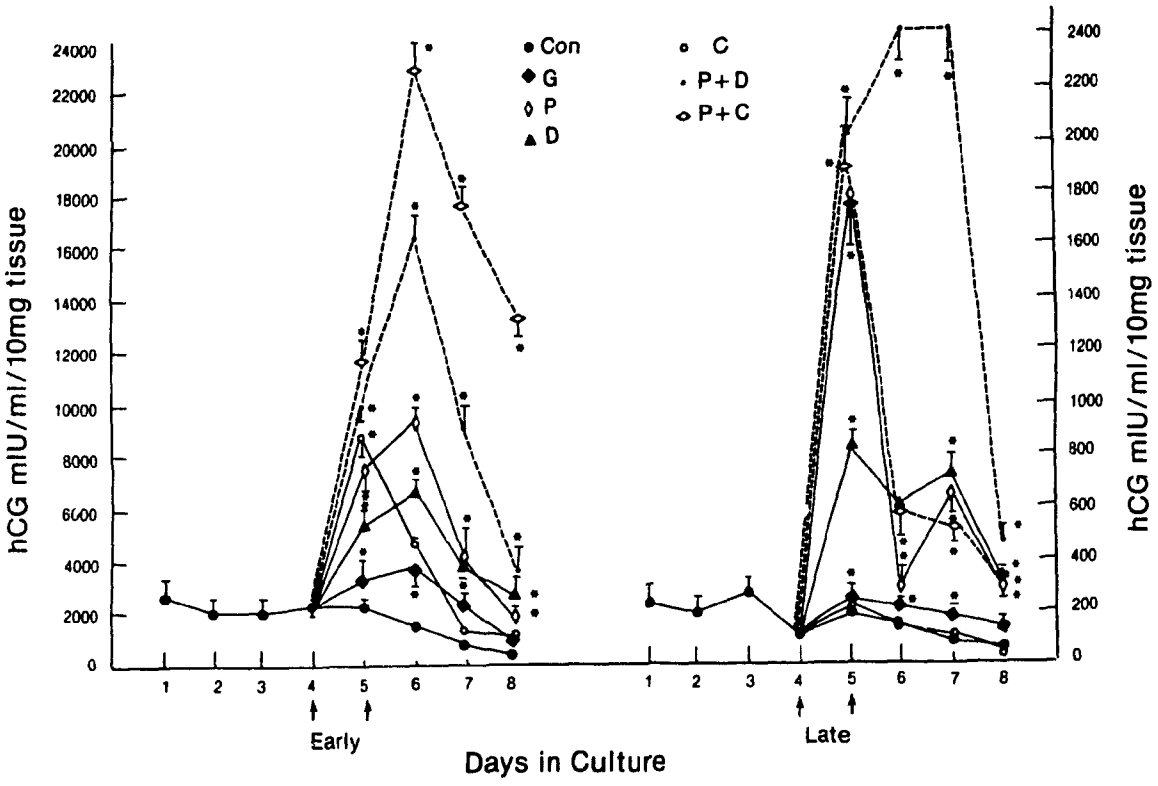

Figure 3.11: A comparison of the effects of all treatments tested in the study, at the highest concentration used (25 μg/ml). The left panel shows the results in early and the right panel the results in the late cultures. All hormones were added on days 4 and 5 as denoted by the arrows below the horizontal axis. The points corresponding to days 1, 2 and 3 are means ± S.E. of 36 determinations, while those corresponding to days 4-8 are means ± S.E. of 6 determinations. CON = control; P = Progesterone; D = DHEA; G = GnRH; C = cortisol; P+D = progesterone + DHEA; P+C = progesterone + cortisol. Significant effects are denoted by asterisks. (1 IU = 1279 ng)

3.6 DISCUSSION

At term, placental weight is about 500 g, whereas at 9 weeks it is about 5 g. Although maternal levels of hCG at term are only 1/10th those at 9 weeks, term placental weight is about 100 times greater. This discrepancy between placental weight and maternal plasma concentrations may be explained by either, a greater per unit weight production or a longer half-life of hCG in early pregnancy. Our studies of the unstimulated tissue (i.e. without the addition of hormones) showed a 7-fold greater hCG output in early compared with late pregnancy; the difference in vivo may be much more. It is possible that we remove other stimulating factors present in maternal or fetal serum by subjecting the tissue to our culture system.

The daily addition of 1 mM cycloheximide inhibited basal hCG output on days 3-8 and abolished peak hCG production on days 4 and 5. Therefore it is concluded that, hCG assayed in the medium from days 1 and 2 was preformed and that the increase in hCG secretion seen subsequently requires the translation of new messenger ribonucleic acid (mRNA).

In both early and late placental cultures, progesterone and DHEA stimulated the output of hCG in a dose-dependent manner. Until the 7th week of pregnancy, progesterone is derived exclusively from the ovaries; thereafter, placental steroidogenesis becomes the most important source of progesterone in the pregnant female (1). DHEA arises from both the maternal and fetal adrenal glands. The fetal adrenal is identifiable as early as 4 weeks gestation and steroidogenic

capacity is detectable as early as the 7th week (10).

Cortisol stimulated hCG output in a dose-dependent fashion in early but not in late cultures. Term placenta very rapidly and efficiently coverts cortisol to its inactive metabolite cortisone (11). This rapid inactivation may account for the lack of effect seen with cortisol in term explants. The efficiency of this reaction has not yet been documented in early placental tissue. Wilson and Jawad (7) reported that cortisone was only half as potent a stimulus of hCG output as cortisol in organ cultures of term placenta. Their finding of a stimulatory effect with cortisol in term cultures may be attributable to the higher concentrations of hormone used in their studies.

Testosterone inhibited hCG output in a dose-dependent manner in early cultures but was without effect in late cultures. This may be explained by the loss of testosterone receptors in the placenta or a decrease in their affinity through advancing gestation. HCG stimulates testosterone production from the fetal testes beginning around 5 weeks gestation (11). Fetal testosterone levels are rising at the time when hCG concentrations are beginning to decline (around 11 weeks). Testosterone arising from the fetal testes may reach critical concentrations at this time and possibly induce a negative feedback effect to stem hCG secretion following establishment of placental steroidogenesis sufficient to maintain pregnancy. This hypothesis is supported by the finding that hCG concentrations are greater in peripheral sera of women bearing female fetuses than those bearing males (12).

The addition of GnRH resulted in a stimulation of hCG output. This effect showed a clear dose-dependence in early cultures, but reached statistical significance only at 25 μg/ml in late cultures. Siler-Khodr and Khodr (3) found that placental tissue showed the greatest responsiveness to GnRH between 13-17 weeks gestation. In contrast to our findings, they reported that cultures of 6-9 weeks were less responsive to GnRH than were cultures 39-40 weeks. The discrepancy between their results and those presented here cannot be explained at present.

17β-estradiol in concentrations ranging from 0.05-25 μg/ml did not alter hCG output at either placental age. This is consistent with the findings of Wilson and Jawad (5) in organ cultures of term placenta.

Our studies show that early placental tissue responds to various stimuli by increasing its hCG production. Progesterone, DHEA, cortisol and GnRH are important factors which determine the hormonal milieu of maternal and fetal plasma as well as placental tissue in early gestation. The exponential rise in hCG levels observed in early pregnancy may possibly be accounted for by the combined stimulatory effects of these 4 hormones (progesterone, DHEA, cortisol and GnRH) on the developing trophoblast. This contention is supported by the presently reported findings that the combination of progesterone and DHEA stimulates hCG output additively, and that progesterone and cortisol enhance hCG production synergistically.

Maternal plasma hCG concentrations begin to decline around the 10th or 11th gestational week. At this time, the rise in maternal plasma progesterone begins to plateau while fetal testosterone, which

as we have shown here inhibits hCG production in early explants, is climbing. Placental GnRH content falls slightly from first to second trimester (4). Maternal unbound cortisol rises slightly in early pregnancy. It is possible that the onset of placental cortisol inactivation may coincide with the fall in maternal plasma hCG. We have previously shown that this conversion (cortisol to cortisone) is active by 11 weeks gestation (13). The rapid fall in maternal plasma hCG at 10-11 weeks may possibly be due to the combined effects of plateauing progesterone, rising fetal testosterone, declining placental GnRH content and the onset of placental cortisol inactivation.

3.7 REFERENCES

1. Tulchinsky D and Ryan KJR, Maternal-Fetal Endocrinology, W.B. Saunders Co., Philadelphia, p. 17, 1980.

2. Siler-Khodr TM and Khodr G, Localization of LHRH in the human placenta, Fertil Steril, 29: 523, 1978.

3. Siler-Khodr TM, Khodr G, Valenzuela G and Rhode, GnRH effects of placental hormones during gestation: α-hCG, hCG, hCS, Biol Reprod, 34: 245, 1986.

4. Currie AJ, Fraser HM and Sharpe RM, Human placental receptors for GnRH, Biochem Biophys Res Commun, 99: 332, 1981.

5. Wilson EA, Jawad MJ and Powell DE, Effects of estradiol and progesterone on hCG secretion in vitro, Am J Obstet Gynecol, 149: 143, 1984.

6. Voutilainen R, Kahrai AI and Lahteenmaki P, The effects of DHEA and its sulphate on human midterm placental secretion of hCG, progesterone, estrone and estradiol-17β in tissue culture, J Steriod Biochem, 14: 1153, 1981.

7. Wilson EA and Jawad MJ, Stimulation of hCG by glucocorticoids, Am J Obstet Gynecol, 143: 344, 1982.

8. Wilson EA, Jawad MJ and Dickson LR, Suppression of hCG by progestational steroids, Am J Obstet Gynecol, 138: 708, 1980.

9. Daniels-McQueen S, McQueen D, Buken S, Canfield R, Landerfield T and Biome I, Identification of mRNA's encoding the α and β subunits of hCG, J Biol Chem, 253: 7109, 1978.

10. Greenspan FS and Forsham PH, Basic and Clinical Endocrinolgy, Lange Medical Publications, Los Altos, CA, p. 436, 1983.

11. Murphy BEP, Clarke SJ, Donald IR, Vedady D and Pinsky M, Coversion of cortisol to cortisone during placental transfer to the human fetus, Am J Obstet Gynecol, 118: 538, 1974.

12. Boroditsky RS, Reyes FI, Winter JSD and Fairman C, Serum hCG and progesterone patterns in last trimester of pregnancy: relationship to fetal sex, Am J Obstet Gyncol, 121: 238, 1974.

13. Murphy BEP, Chorionic membranes as an extra-adrenal source of foetal cortisol in human amniotic fluid, Nature, 266: 179, 1977.

CHAPTER 4

Explant Cultures of Human Placenta: The Effect of Steroids on Viability and Tissue HCG Content[1]

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4.1 PREFACE

In an effort to further characterize the effects of steroid hormones on hCG production, I used an immunohistochemical method to detect the presence of hCG in cultured placenta after exposure to various treatment regimes. This protocol enabled an indirect assessment of the effect of steroid hormones on hCG production at the intra-tissue level. Furthermore, I studied the effects of steroid hormones on histologically assessed placental viability in culture.

4.2 ABSTRACT

Explant cultures of placenta provide a useful tool for the exploration of physiological and biochemical aspects of this functionally diverse organ. However, investigations utilizing this technique are limited by the unavoidable necrosis of tissue under culture conditions. We have characterized the viability of placental tissue at 6-9 weeks gestation under control conditions and studied the effects of various hormones on this parameter in an effort to delineate the optimal environment for in vitro cultivation of these tissues. Additionally, we have followed the pattern of immunoreactive hCG in cultured placenta over time and in response to various steroid hormones in order to characterize placental function under these conditions.

We found a significant correlation ($r = 0.73$, $p < 0.001$) between placental viability and the presence of immunoreactive hCG in cultured tissue. Immunodetection of hCG was limited to syncytiotrophoblasts; cytotrophoblast and decidual cells showed little or no reactivity. Under control conditions (Hams F-10 medium + 10% fetal bovine serum, antibiotics, 95% air and 5% CO2), the tissue survived 5-7 days as indicated by appearance and function. Cytotrophoblasts survived longer than did syncytiotrophoblasts and decidual components deteriorated rapidly in culture. The complete absence of serum decreased the duration of tissue viability in culture as compared to control. Cultures studied in the presence of 100% serum also displayed a similar decrease in viability. The addition of various steroids alone or in

combination tripled or quadrupled the longevity of placental cultures. In control cultures, immunohistochemically detectable hCG was present in tissue up to the 5th day, but not after the 10th day. Progesterone, estradiol or dehydroepiandrosterone (DHEA) alone or in combination prolonged the presence of immunoreactive hCG in cultured placenta up to 20 days. We suggest that these steroid hormones may be important regulators of placental growth and endocrine function in vivo.

4.3 INTRODUCTION

Human placenta is widely available and easily cultured. Explant or organ cultures of this tissue survive well in chemically defined media containing proportionally adequate quantities of inorganic salts, trace elements, organic acids, amino acids, carbohydrates and vitamins, usually supplemented with serum and antibiotics (1). Cultured human placenta was reportedly quite tolerant to changes in oxygen tension; although values as low as 6% or as high as 95% were toxic and did not support protracted survival (2-4). Syncytiotrophoblasts were more vulnerable than cytotrophoblasts to hypoxic or hyperoxic conditions. Hypoxia was associated with the differentiation of cytotrophoblasts to syncytiotrophoblasts and the gradual loss of syncytial elements (2). Trophoblast cells were able to withstand dramatic alterations in temperature and hypotonic stress, although they were exquisitely sensitive to proteolytic enzymes (5).

In vitro cultures are important tools for studying placental function, especially in the human where many in vivo experiments are not ethically permissible. Therefore, it is important to define optimal culture conditions for survival of this tissue. In this study we report the effects of steroid hormones on placental viability and hCG production in culture, assessed by both histological and immunohistochemical criteria.

4.4 MATERIALS AND METHODS

Materials:

Ham's F-10 powdered culture medium and heat inactivated fetal bovine serum (FBS) were both purchased from Grand Island Biological Company (Burlington, Ont.). Amphotericin was obtained from Squibb Inc. (Montreal, Que.), Penicillin from Glaxo Laboratories (Montreal, Que.) and Gentamicin from Scherring (Pointe Claire, Que.). Wire mesh was obtained from Johnson Wire Works (Montreal, Que.) and 60 X 15 X 2 mm culture dishes were purchased from Falcon Plastics (Los Angeles, CA.). Steroids were obtained from Sigma Chemical Co. (St. Louis, MO.). Placental tissues were obtained under aseptic conditions following abortion by dilatation and evacuation (6-9 weeks gestational age).

Immunohistological staining was performed using the Histogen immunohistology kit obtained from Bio-Genex Laboratories (San Ramon, CA.) and rabbit anti-β-hCG was obtained from DAKO immunoglobulins (Denmark).

Preparation of Explant Cultures:

The tissues were immediately transferred to 0.9% saline and kept on ice until explants were prepared. Placental villous tissue was rinsed with culture medium and dissected free of membranes. Six 1 mm^3 chunks of tissue were placed on wire mesh grids; two such grids were placed into each culture dish with 3 ml of medium. Cultures were incubated in a humidified environment of 95% air, 5% CO2 at 37°C in one of several types of media (see below).

Treatments:

Culture medium was collected and replaced with fresh solutions daily. Control cultures received Ham's F-10 nutrient mixture supplemented with 10% fetal bovine serum and antibiotics. Treated cultures received media enriched with 15 μg/ml of progesterone, estradiol, DHEA, testosterone or cortisol alone, or in various combinations, beginning 2 days after commmencement of culture until the termination of the experiment. Additionally, some cultures were studied in media consisting of 0 or 100% FBS. Each protocol was carried out in duplicate.

Histological Analysis:

Tissue specimens were removed on appropriate days of culture and fixed immediately in 10% formalin. Specimens were processed by routine histological methods and stained with hemotoxylin and eosin (H/E). Based on the following criteria, a morphological score was assigned to each randomly chosen specimen by a pathologist (Dr Senterman) who was unaware of its identity:

5 - cytotrophoblasts, syncytiotrophoblasts and villous interior appear completely viable as indicated by strong staining.

4 - slight degenerative changes in cytotrophoblast and syncytiotrophoblast cells are evident.

3 - syncytiotrophoblasts are mostly necrotic with pyknotic nuclei and acidophilic cytoplasms, while cytotrophoblasts show slight to moderate changes, with the centers of the villi retaining viability.

2 - a few cytotrophoblasts persist, but show ischemic changes of pyknotic nuclei and acidophilic cytoplasms; the centers of the villi show mild degenerative changes.

1- all layers of the villi are completely ischemic as indicated loss of nuclear staining and strongly acidophilic cytoplasms.

Immunohistochemical staining:

Placental sections were stained for hCG using the peroxidase-antiperoxidase (PAP) technique. This staining technique involved the use of specific antibodies which react with specific tissue antigens. The tissues were then incubated with a link antibody and then a labelling reagent (soluble peroxidase-antiperoxidase complex). The addition of a chromogenic substance (aminoethylcarbazole) allows the visualization of the antigen-link antibody-enzyme complex. Positive areas appeared as red or red-brown.

The intensity of staining of each specimen was ranked between 1-5 in a manner similar to that which was previously described. A rank of 5 represented the greatest staining intensity (as found in fresh samples) and 1 represented a complete lack of staining (indicating the absence of hCG). Further details of this technique are provided in Chapter 2.

4.5 RESULTS

Table 4.1 shows the morphological scores of tissue specimens at various times after the initiation of culture. Under control conditions, tissue morphology was well preserved for 3 days, there was a small decrease in tissue integrity noted by the 5th day, and complete necrosis was evident 10 days after culture. Tissue viability was decreased in the presence of 0 or 100 % fetal bovine serum. The addition of testosterone did not alter the longevity of the cultures.

The presence of estradiol, progesterone or DHEA nearly tripled the length of viable cultures (20 days) as compared to control. Cortisol also extended tissue viability, but was less effective. Estradiol was the most effective of the tested hormone treatments for maintaining tissue viability. The simultaneous addition of progesterone and estradiol increased tissue longevity four-fold, to 30 days. The combinations of progesterone and DHEA or progesterone and cortisol also increased tissue viability; however, this effect was less evident.

Table 4.2 shows the results of immunohistochemical hCG staining of placental tissue following various treatments at 5, 10 and 20 days in culture. Under control conditions, staining was present after 5 days but was undetectable after 10 days in culture. The presence of 0 or 100% FBS in the culture medium caused a reduction in the intensity of hCG staining as compared to control. Cortisol or testosterone treatments did not differ from control. With the addition of progesterone, DHEA or estradiol to the cultures, hCG was detectable in tissue up to 20 days. This effect was increased with the combined

addition of at least two of these hormones.

Syncytiotrophoblasts displayed strong immunoreactivity for hCG, while cytotrophoblasts occassionally showed minimal staining and decidual cells never gave a reaction. Further examination of the tissues revealed, that in culture, decidual components remained morphologically well preserved for less time than did corresponding samples of trophoblastic tissue. Furthermore, decidual tissue was apparently unresponsive to the preserving effects of the hormone treatments. Degenerative changes were evident in syncytiotrophoblasts before cytotrophoblasts.

There was good correlation between placental viability and function as evidenced by hCG staining ($r = 0.73$, $p < 0.001$). These results are displayed pictorially in figures 4.1-4.12.

Table 4.1: The effects of various hormone treatments on placental viability under conditions of explant culture[1].

Single treatments[2]:

Day[4]	Control	Prog	17βE2	DHEA	Cort	Testo	0%FBS	100%FBS
1	5,5	5,5	5,5	5,5	5,5	5,5	5,5	5,5
3	5,5	5,5	5,5	5,5	5,5	5,5	4,4	5,4
5	5,5	5,5	5,	5,5	4,4	5,4	3,3	2,3
7	3,3	5,4	5,5	4,	4,3	3,2	2,1	2,1
10	2,1	4,3	5,4	4,3	3,3	1,1	1,1	1,1
20	1,1	3,3	3,3	3,3	1,1	1,1	1,1	1,1

Combined treatments[3]:

Day[4]	Control	Prog + 17βE2	Prog + DHEA	Prog + Cort
1	5,5	5,5	5,5	5,5
3	5,5	5,5	5,5	5,5
5	4,3	5,5	5,	5,5
7	4,3	5,5	5,4	5,4
10	1,1	4,5	4,4	4,3
20	1,1	4,5	4,3	4,2
30	1,1	3,4	1,1	3,2

[1] The numbers in the table are the duplicate scores assigned to each tissue sample according to the scale below.
[2] The result of adding each hormone alone (15μg/ml).
[3] The result of adding the hormones in combinations (15μg/ml each).
[4] The number of days after the initiation of culture that the specimens were fixed for histological analysis.

Prog = progesterone, 17βE2 = estradiol, DHEA = dehydroepiandrosterone, cort = cortisol, testo = testosterone, FBS = fetal bovine serum. ND = no data.

The numerical rating scale used to assess tissue viability is described below:

5 - cytotrophoblasts, syncytiotrophoblasts and villous interior appear viable and indicated by strong staining of tissue.
4 - slight degenerative in cytotophoblasts and syncytotrophoblasts are evident.
3 - most syncytiotrophoblasts are ischemic with pyknotic nuclei and acidophilic cytoplasms, while cytotrophoblasts show slight to moderate changes; the centers of villi retain viability.
2 - a few cytotrophoblasts persist, but show ischemic changes of pyknotic nuclei and acidophilic cytoplasms; the centers of the villi show mild degenerative changes.
1 - all layers of the villi are completely necrotic as indicated by loss of nuclear staining and strongly acidophilic cytoplasms.

Table 4.2: The effect of various hormone treatments on the intensity of immunohistochemical staining of hCG in placenta under conditions of explant culture[1].

Single treatments[2]:

Day[4]	Control	Prog	17βE2	DHEA	Cort	Testo	0%FBS	100%FBS
5	3,3	3,4	5,4	3,3	3,3	3,3	3,2	1,2
10	2,1	4,	3,4	2,	1,2	1,2	2,1	1,
20	1,1	3,4	3,3	4,5	1,1	1,	1,1	ND

Combined treatments[3]:

Day[4]	Control	Prog + 17βE2	Prog + DHEA	Prog + Cort
5	3,3	ND	ND	ND
10	1,1	3,	4,	4,5
20	1,1	5,4	4,4	4,4

[1] The numbers in the table are the duplicate scores assigned to each tissue sample according to the scale below.
[2] The result of adding each hormone alone (15μg/ml).
[3] The result of adding the hormones in combinations (15μg/ml each).
[4] The number of days after the initiation of culture that the specimens were fixed for immunohistochemical staining.

Prog = progesterone, 17βE2 = estradiol, DHEA = dehydroepiandrosterone, Cort = cortisol, testo = testosterone, FBS = fetal bovine serum, ND = no data.

The numerical rating scale used to assess staining intensity is described below:

5 - greatest staining intensity, as found in fresh samples, indicating a large tissue reserve of hCG.
1 - a complete lack of staining, indicating the absence hCG in tissue.
4,3 and 2 - indicate levels of staining between 5 and 1 and reflect intermediate levels of tissue hCG concentrations.

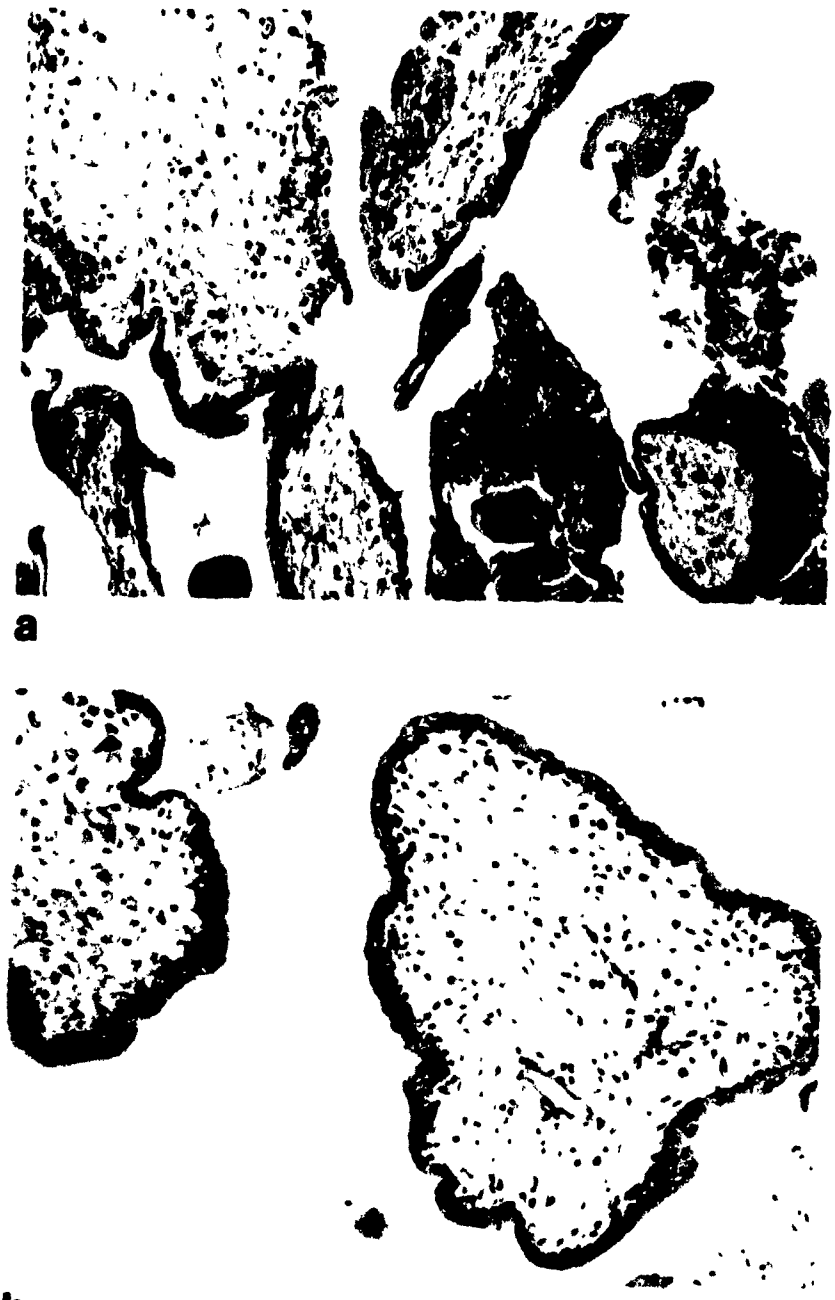


Figure 4.1: Viability of first trimester human placenta under control conditions. Tissue specimens removed from culture after 1 day. Tissue morphology is well maintained and there is no evidence of focal necrosis. (H/E stain, 100X magnification)

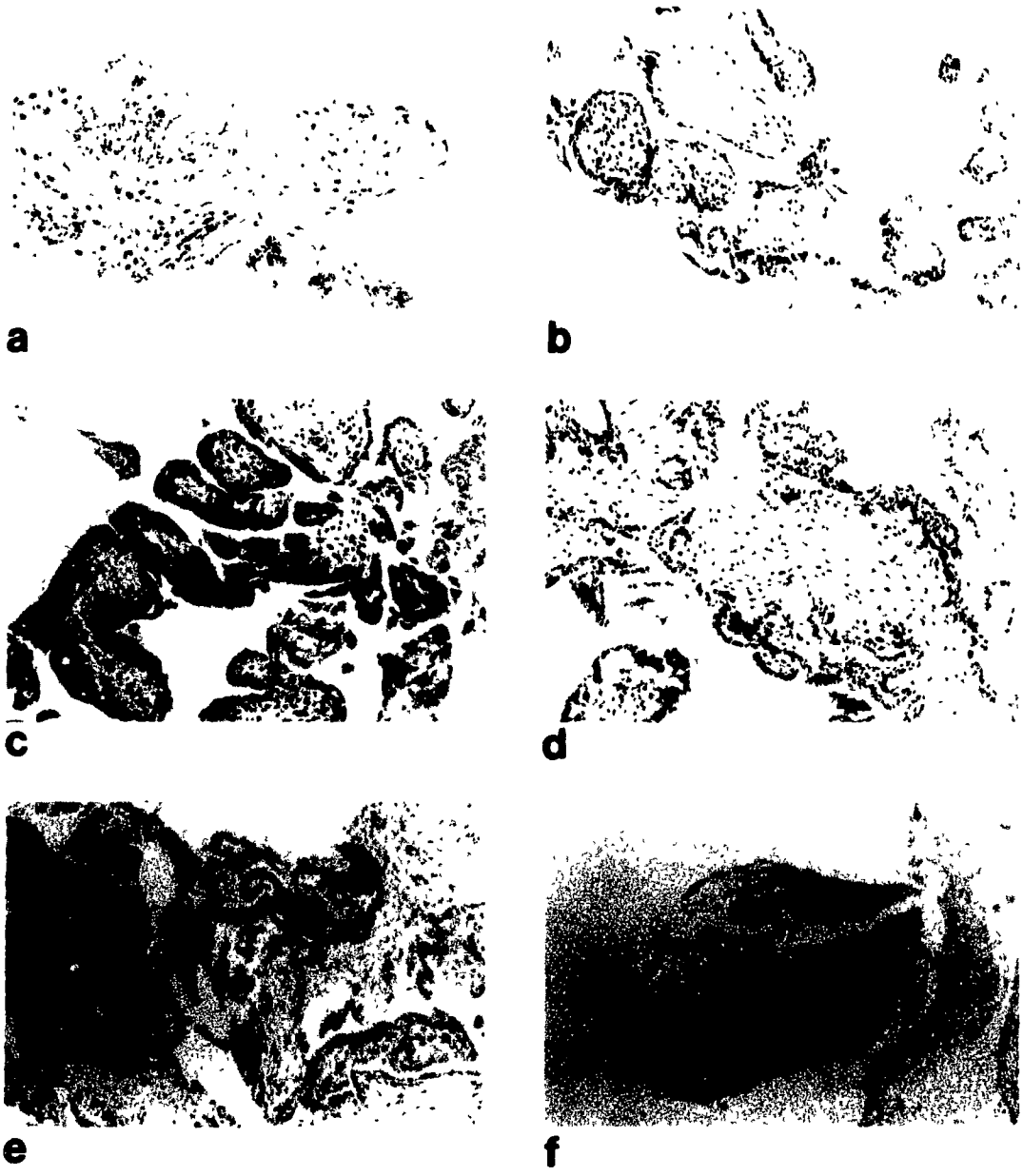


Figure 4.2: The effect of single hormone treatments on viability of first trimester human placenta. Tissue specimens removed after 10 days in culture. The control specimen is necrotic (a), while tissue cultured in the presence of progesterone (b), estradiol (c) or DHEA (d) are well preserved. Cortisol increased viability only slightly (e). Testosterone had no effect (f). (H/E stain, 100X magnification)

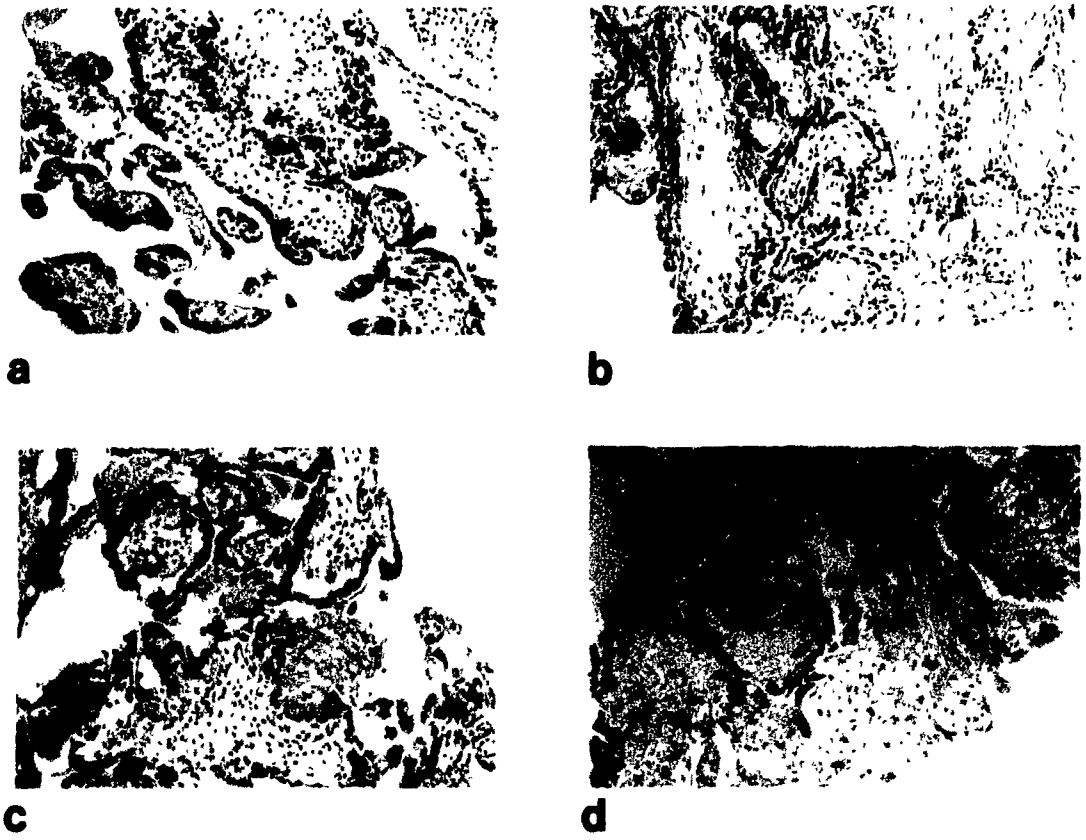


Figure 4.3: The effect of single hormone treatments on viability of first trimester human placenta. Tissue specimens removed after 20 days in culture. The presence of progesterone (a), estradiol (b) or DHEA (c) increased the duration of tissue viability in culture from less than 10 days to 20 days. Cortisol increased viability only minimally (d). (H/E stain, 100X magnification)

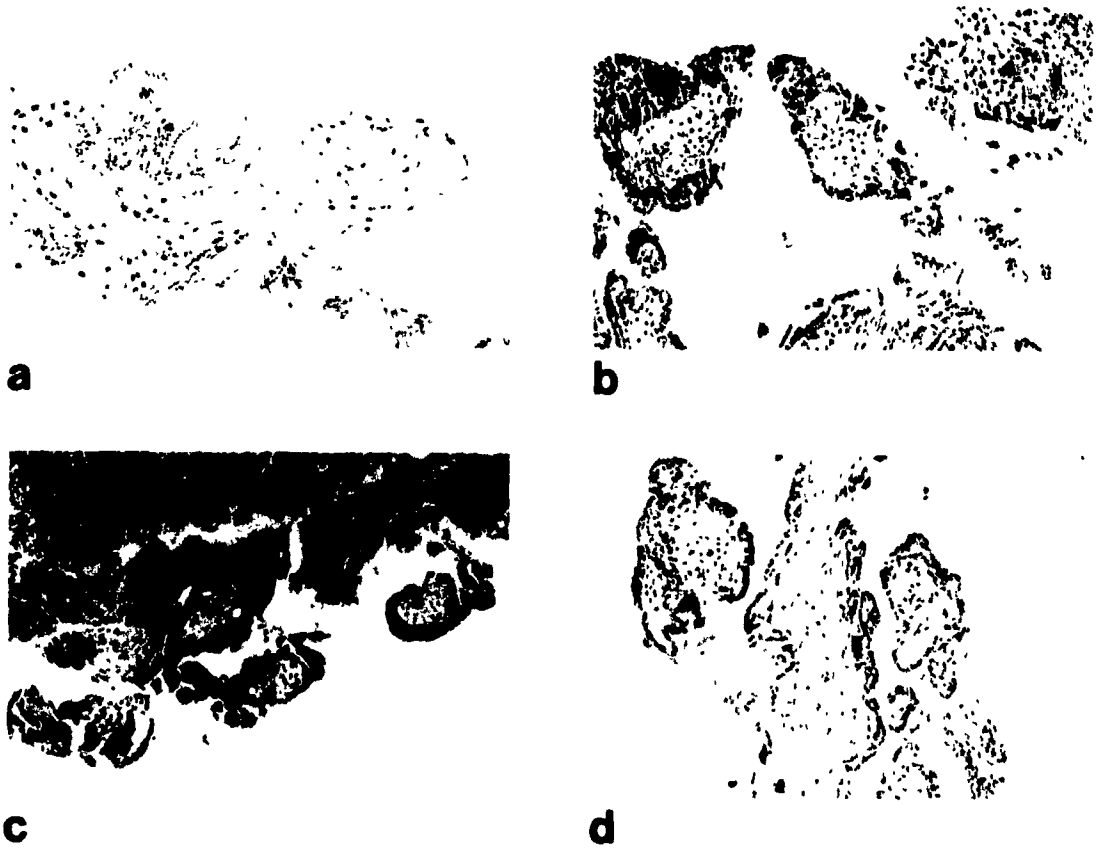


Figure 4.4: The effect of combined hormone treatments on viability of first trimester human placenta. Tissue specimens removed after 10 days in culture. Control specimens appeared necrotic (a). Progesterone + estradiol added together (b) had the greatest effect. Progesterone + DHEA (c) and progesterone + cortisol (d) also increased the duration of viable culture. (H/E stain, 100X magnication)

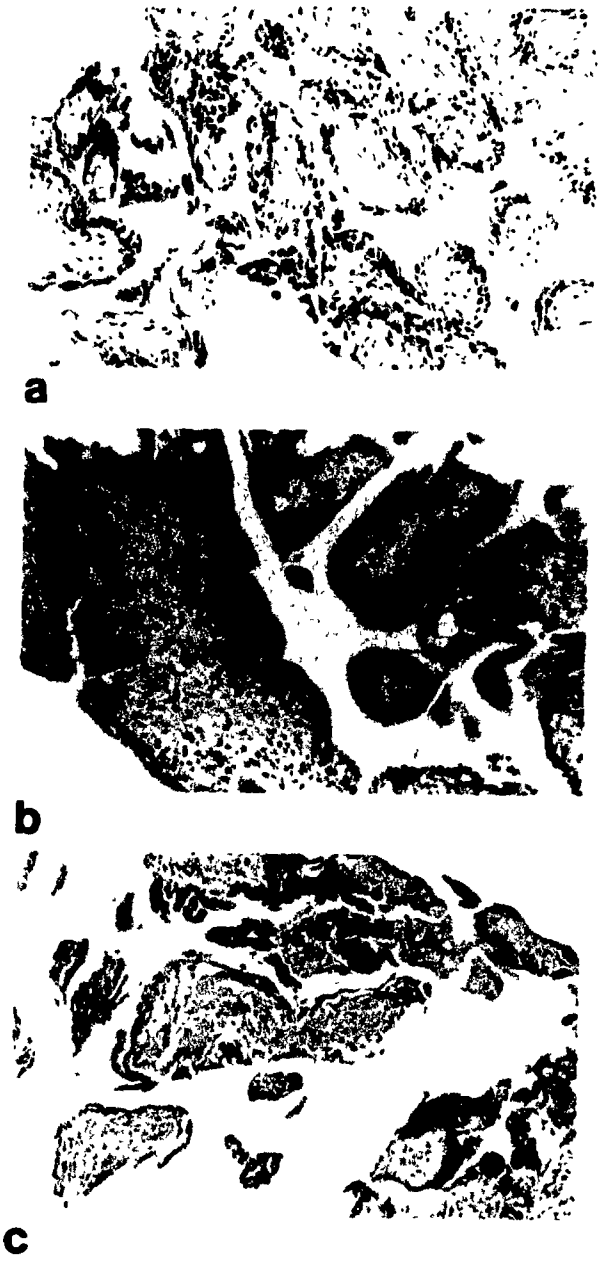


Figure 4.5: The effect of combined hormone treatments on viability of first trimester human placenta. Tissue specimens removed after 20 days in culture. With progesterone + estradiol treatment (a) tissue morphology is maintained. The tissue remained viable in the presence of progesterone + DHEA (b) although areas of focal necrosis are evident. The effect of progesterone + cortisol (c) was found to be least potent as tissue appears mostly necrotic. (H/E stain, 100X magnification).

Figure 4.6: The effect of combined hormone treatments on viability of first trimester human placenta. Tissue specimens removed after 30 days in culture. With progesterone + estradiol treatment (a) cultured tissue to remained viable up to 30 days. A high degree of tissue integrity is maintained, although evidence of focal necrosis can be found. Progesterone + DHEA treatment (b) failed to preserve the tissue until the 30th day. Note the complete necrosis of decidua (D) while the villi (V) retain some tissue architecture. This indicates the differential effects of hormone treatments on these 2 types of tissue. (see figure 4.9). With 30 days of progesterone + cortisol treatments (c), tissue architecture was maintained better than with progesterone & DHEA but not as well as with progesterone + estradiol. (H/E stain, 100X magnification)

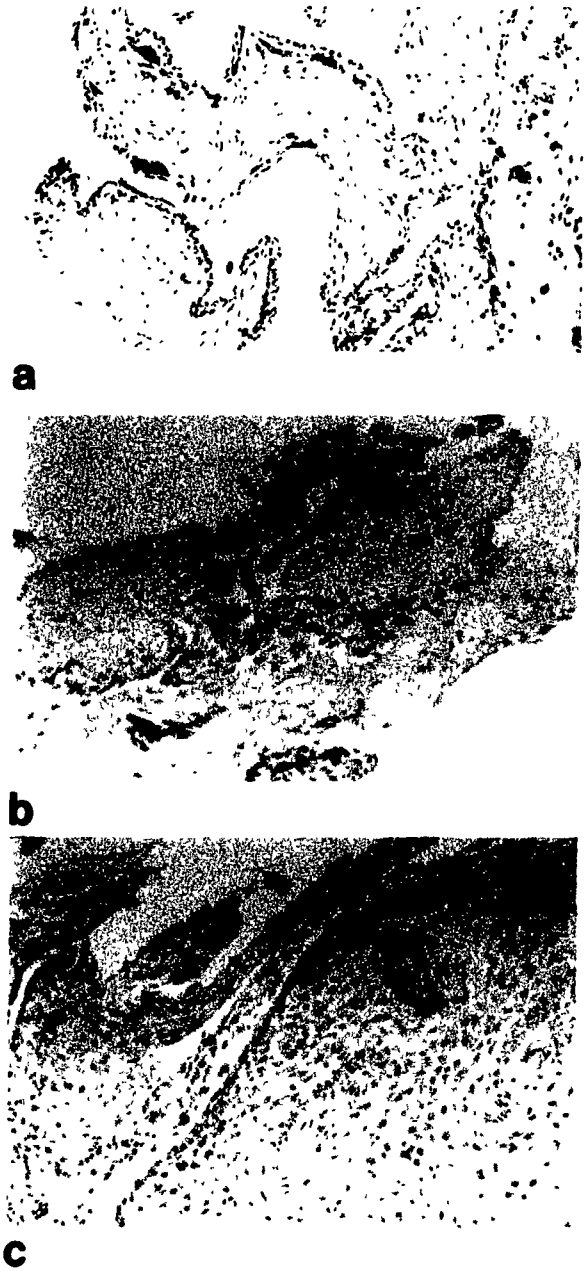


Figure 4.7: The effect of FBS on viability of first trimester human placenta. Tissue specimens removed after 7 days in culture. Control (a) shows evidence of focal necrosis, although as a whole the tissue is viable. When cultured in the absence of FBS (b) or in 100% FBS (c), the tissue is necrotic after 7 days in culture. (H/E stain, 100X magnification)

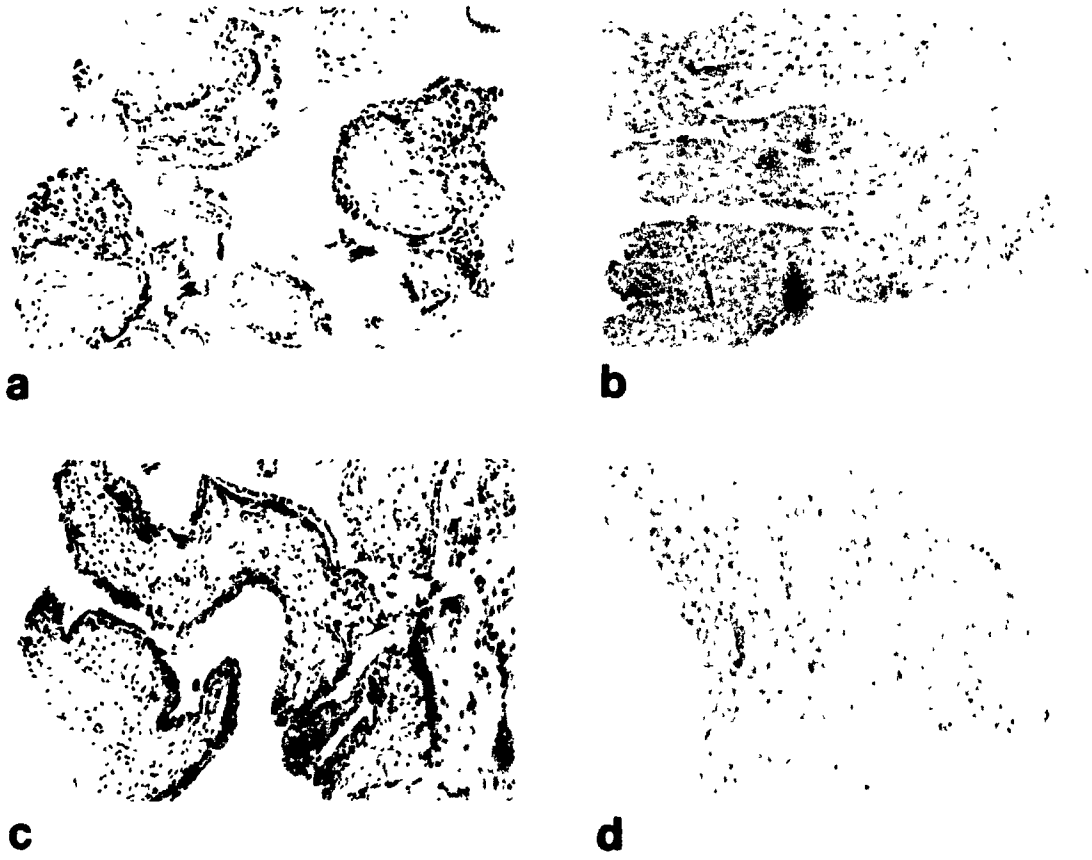
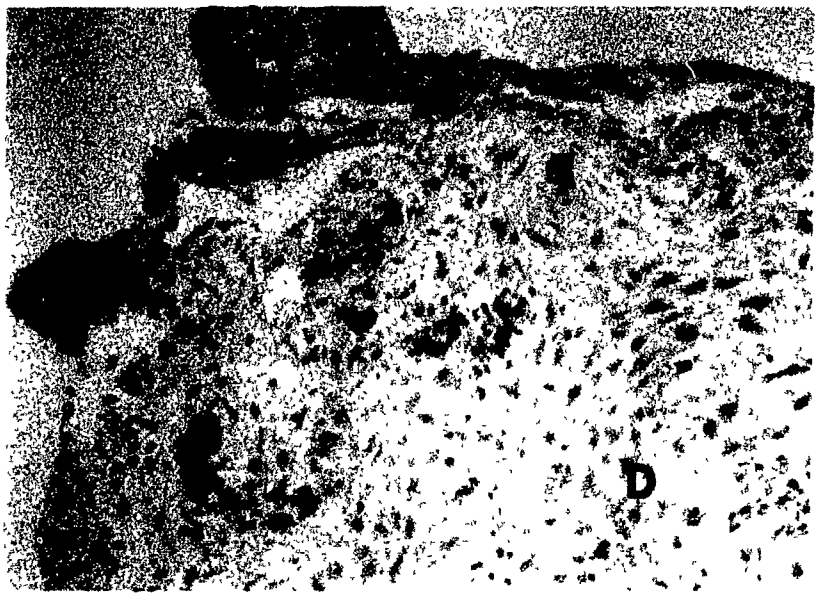
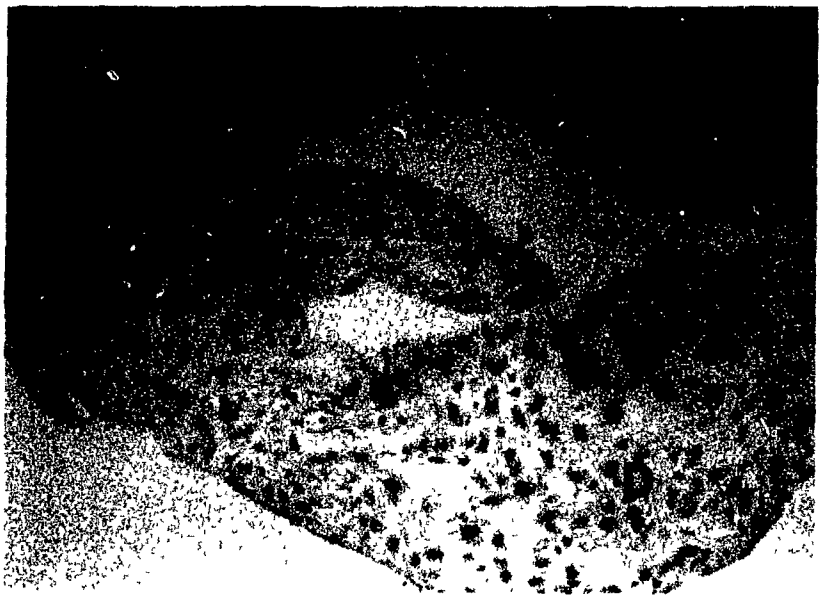


Figure 4.8: The differential viability of decidual and trophoblastic tissue under explant culture conditions. First trimester human placenta removed from control culture conditions after 5 (a,b) or 7 (c,d) days. After 5 days, trophoblastic elements (a) are well preserved, while decidual tissue (b) is mostly necrotic. After 7 days in culture the same pattern is evident (c,d). (H/E stain, 100X magnifiation)



a



b

Figure 4.9: The differential effects of hormones on the viability of first trimester decidual and trophoblastic tissue. Tissue specimens removed after 30 days in culture. In the presence of progesterone + estradiol (a) or progesterone + DHEA (b) trophoblastic villi (V) remain recognizable and retain some degree of tissue architecture; whereas decidual tissue (D) is completely necrotic. This indicates that decidual components are unresponsive to the preserving effects of these steroids. (H/E stain, 100X magnification)

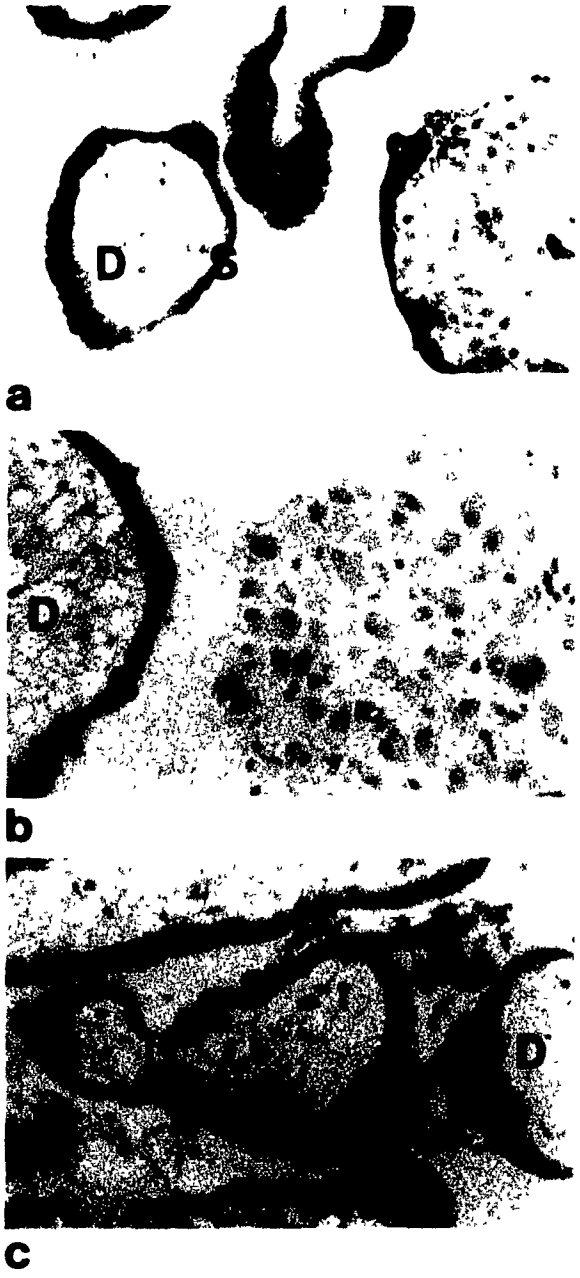


Figure 4.10: Immunohistochemical staining of hCG in first trimester human placenta. Plates demonstrate fresh villi (a), fresh villi and decidua (b). Note the intense staining of syncytiotrophoblasts (S) and the absence of staining from decidua (D). In placenta removed after 5 days in culture (c) staining intensity is less than in the fresh specimen but is still significant. (250X magnification)

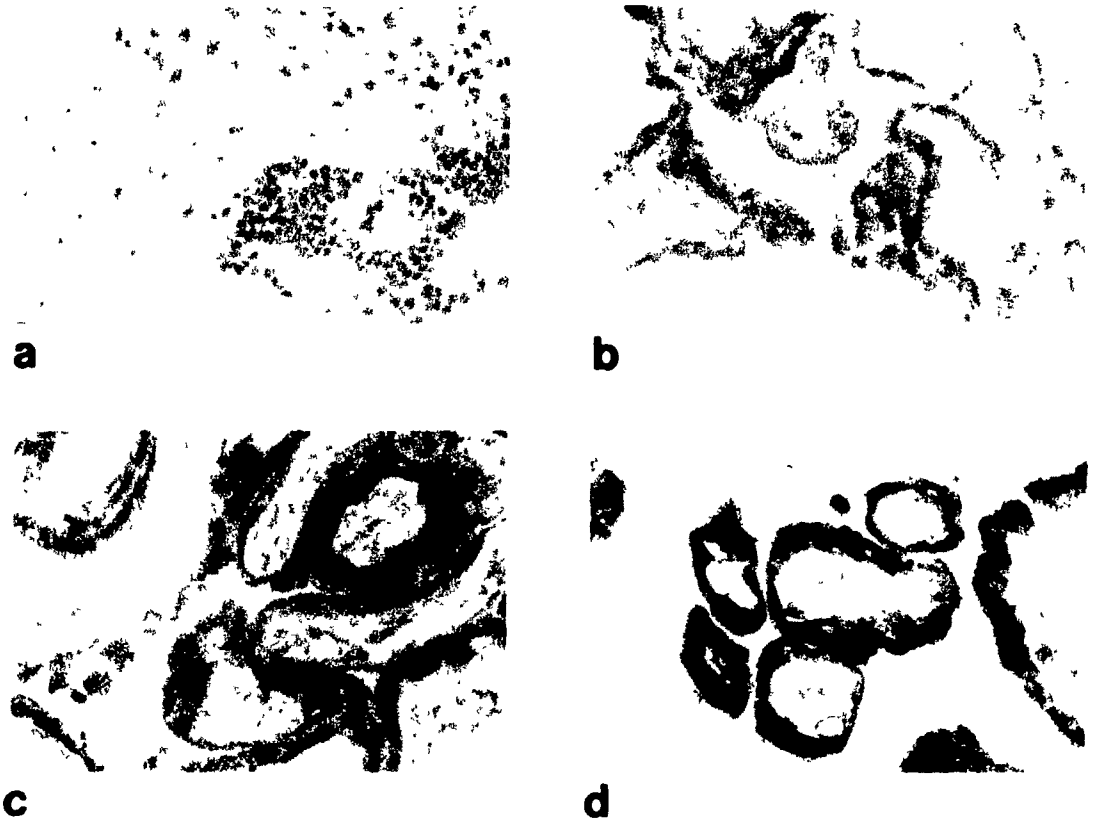


Figure 4.11: The effect of single hormone treatments on immunohistochemical hCG staining of first trimester human placenta. Specimens removed after 20 days in culture. Under control conditions (a), staining is completely absent. The presence of progesterone (b), estradiol (c) or DHEA (d) increased staining intensity, which indicates maintained hCG production in tissue until the 20th day. DHEA had the most potent effect. (250X magnification)

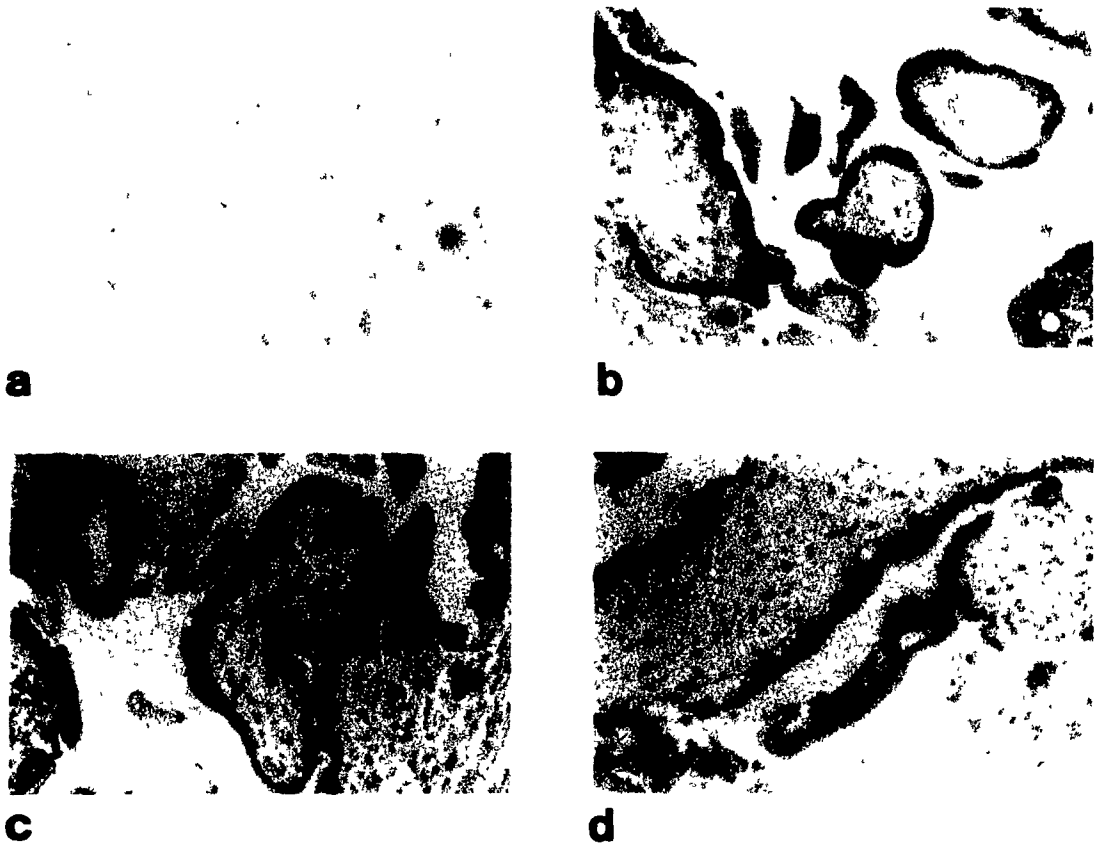


Figure 4.12: The effect of combined hormone treatments on immunohistochemical hCG staining of first trimester human placenta removed after 20 days in culture. Under control conditions (a) there is no detectable staining. Tissue cultured in the presence of exogenous progesterone + estradiol (b), progesterone + DHEA (c) or progesterone + cortisol (d) showed evidence of immunoreactive hCG in tissue after 20 days (250X magnification)

4.6 DISCUSSION

The present study showed that viability of first trimester placental explant cultures, assessed histologically and histochemically can be extended in the presence of various steroid hormones. Under control conditions, we noted no significant histological changes before 3 days and gradual necrosis after 5 days. Ogino et al (6), reported similar findings.

We demonstrated a greater longevity of villous versus decidual components; this is in accord with the greater acclimatizing capacity of fetal versus adult tissues. In addition, decidual tissues appeared to be unresponsive to hormonal stimulation. The presence of decidual progesterone and estradiol receptors has been documented in term placenta (7). The presence of such receptors earlier in gestation has not been investigated. As well, we found that degenerative changes in syncytiotrophoblasts preceded those of cytotrophoblasts. Similar findings were reported by Ogino et al (6). Cytotrophoblasts are thought to be stem cells which continually give rise to overlying syncytiotrophoblasts (5). The greater longevity of cytotrophoblasts in culture that we and others (6) report may be related to the greater intrinsic life-span of these cells.

The absence of fetal bovine serum decreased the viability of our cultures; this is in agreement with the reports by Loke (5). Fetal serum likely provides growth factors or additional substrates for tissue hyperplasia and maintenance. However, some investigators (8), have dispensed with the addition of serum to placental cultures

entirely and claimed no evidence of focal necrosis before 14 days. Tissue maintained in 100 % fetal serum did not survive as well as controls. This may be due the increased osmolarity or lack of adequate hydration of tissues under such conditions. Embryonic tissues of the rat and rabbit were shown to thrive best under conditions of low osmolarity (9).

Immunohistochemical techniques have been used previously to localize hCG in human placenta (10-13). We found detectable levels of hCG only in syncytiotrophoblsts. This finding is consistent with other reports (10-13) and strengthens the theory that these cells are the only placental source of hCG. Recently, hCG was localized to specific secretory granules and large dense bodies within syncytiotrophoblast cells (14).

Supplementation of medium with progesterone, estradiol or DHEA extended the duration of immunohistochemically detectable hCG within cultured tissue at least three-fold, as compared to control. This is in agreement with our previous report that these steroids stimulate hCG output from placental explant cultures as measured by RIA of collected medium (15). However, cortisol, testosterone and estradiol had different effects on radioimmunoassayable hCG concentrations in collected medium (15) and immunoreactive levels of hCG in tissue. This difference may be due to the differential effects of these hormones on hCG secretion, as measured by levels in collected medium (15), or synthesis and storage as measured by tissue levels.

We found a good correlation ($r = 0.73$, $p < 0.001$) between histological morphology and the intensity of immunohistochemical hCG

staining. This is in contrast to the findings of Loke (5) who showed no consistent correlation between histological morphology and radioimmunoassayable hCG production in term explants.

The effects of various factors on viability of different types of organ cultures has been explored. Epidermal growth factor, nerve growth factor, mesenchymal factor and erthropoietin were demonstrated to be important for growth of epithelial cultures (1). Insulin was implicated as an essential factor for the maintenance of prostate, mammary gland, uterus, thyroid, liver and fetal heart cultures (16-19). Increased viability was demonstrated as a result of the addition of testosterone, cortisol, prolactin and somatomammotropin to cultures of mouse mammary gland, rodent salivary and rat ventral prostate cultures (1). Supplementation of medium with zinc and insulin supported growth and differentation of embryonic pancreas (20). Thyroxine was a regulator of growth and differentiation of embryonic skin cultures. The importance of ascorbic acid, vitamins, nucleic acids, amino acids and sugars for the survival of various organ cultures has been demonstrated (1,21).

Steroid hormones generally act via intracellular receptors to induce the transcription of a specific gene product. They may also have specific effects upon the permeability of certain cell membrane systems (22). The increased viability of placental tissues exposed to steroids, as observed in this study, may be due to the induction of growth factors. Although we did not assess growth itself (thymidine incorporation), no significant out-growth of tissue or detectable increase in size or mass were noted; thus indicating that perhaps we

were not observing growth *per se*, rather the maintenance of tissue architecture and cellular integrity. Therefore, it may be more likely that in this case steroids induce the synthesis or function of factors required for tissue repair rather than hypertrophy or hyperplasia. Progesterone, estradiol and DHEA are synthesized and/or metabolized by the placenta; therefore a physiological role for these steroids in placental growth and maintenance is supported.

This is the first report of the effects of steroid hormones on the morphological and functional viability of human placenta in culture. This may be an important step towards accurately defining optimal conditions for the *in vitro* maintenance of this tissue for extended periods of time. In light of these findings, steroids may prove to have important functions with respect to placental growth and development *in vivo*. Furthermore, the finding that some steroids increased immunoreactive tissue levels of hCG, speaks strongly for their role in the regulation of hCG production from placenta.

4.7 REFERENCES

1. Hodges GM, A review of methodology in organ culture, In: Organ Culture in Biomedical Research, Cambridge University Press, London, Ball M and Monnickedam MA (eds.), p. 15, 1976.

2. Ishimara T, A study of synthesis and secretion of hCG in trophoblastic cultures, Acta Obstet Gynecol Jpn, 18: 172, 1971.

3. Hout RT, Foidart JM and Stromberg K, Effects of culture conditions on the synthesis of hCG by placental organ cultures, In Vitro, 15: 497, 1979.

4. Tominaga T and Page EW, Accomodation of the human placenta to hypoxia, Am J Obstet Gynecol, 94: 679, 1966.

5. Loke YW, Human trophoblast in culture, In: Biology of the Trophoblast, Loke YW and Whyte A (eds.), Elsiever Science Publications. p. 663, 1983.

6. Ogino M, Satoh M, Kinoshita K, Satoh K, Mizuno and Sakamoto S, Viability of human placental villi in organ culture, Arch Gynecol, 2234: 235, 1984.

7. Khan-Dawood FS and Dawood MY, Estrogen and progesterone receptors and hormone levels in human myometrium and placenta in term pregnancy, Am J Obstet Gynecol, 150: 510, 1984.

8. Hustin and Gaspard U, Comparison of histological changes seen in placental tissue cultures and in placentae obtained after fetal death, Brit J Obstet Gynecol, 84: 201, 1977.

9. Brinster RL, Studies on the development of mouse embryos in vitro I: The effects of osmolarity and hydrogen ion concentration, J Exp Zoology, 158: 49, 1965.

10. Midgley AR and Pierce GB, Immunohistochemical localization of human chorionic gonadotropin, J Exp Med, 115: 1, 1962.

11. Ikonicoff LK and Cedard L, Localization of human chorionic gonadotrophic and somatomammotrophic hormones by peroxidase immuno-ezymological methods in villi and amniotic epithelium of human placentas, Am J Obstet Gynecol, 116: 1124, 1973.

12. Hoshina M, Ashitake Y and Tojo S, Immunohistochemical interaction of antisera to hCG and it subunits with chorionic tissue of early gestation, Endocrinol Japon, 26: 175, 1979.

13. Dresken RB, Spicer SS and Greene WB, Ultrastructural localization of chorionic gonadotropin in human term placenta, J Histochem and Cytochem, 18: 862, 1970.

14. Hamaski K, Ueda H, Okamura Y and Fujimoto S, Double immunoelectron microscopic labelings of human chorionic gonadotropin and human placental lactogen in human chorionic villi, Sangyo Ika Daiguku Zasshi (Japan), 10: 171, 1988.

15. Ahmed NA and Murphy BEP, The effects of various hormones on human chorionic goandotropin production in early and late placental explant cultures, Am J Obstet Gynecol, 159: 1220, 1988.

16. Franks LM, Nutritional and other factors influencing the balance between function and proliferation in organ culture, National Cancer Institute Monographs, 11: 83, 1963.

17. Elias JJ, Response of mouse mammary duct end buds to insulin in organ culture, Exp Cell Res, 27: 601, 1962.

18. Benzo CA and Nemeth AM, Factors controlling development of chick embryo liver cells during organ culture, J Cell Biol, 48: 235, 1971.

19. Windethal K, Substrate requirements of fetal mouse hearts maintained for long periods in organ culture, J Physiol, 217: 56, 1971.

20. Parsa I, Marsh WH and Fitzgerald PJ, Chemically defined medium for organ culture differentiation of rat pancreas anlage, Exp Cell Res, 59: 171, 1970.

21. Brinster RL, Cultivation of mammalian embryo, In: Growth, Nutrition and Metabolism of Cells in Culture, Rothblat GH and Cristotalo VJ (eds.), Vol 2, p. 251, New York Academic Press, New York, 1972.

22. Westphal U, Notes on Steroid Interactions with Enzymes, In: Steroid-Protein Interactions, Monographs in Endocrinology, Vol. 4, Srpinger-Verlang, New York, p. 463, 1971.

CHAPTER 5

Concentration of Chorionic Gonadotropin, Progesterone and Estradiol in Human Placenta Through Gestation and in Medium Collected from First Trimester Explant Cultures[1].

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A manuscript in preparation

5.1 PREFACE

The studies presented in Chapter 3 showed that progesterone increased radioimmunassayable levels of hCG in medium collected from placental explant cultures. In agreement with this, I found that progesterone increased immunoreactivity for hCG in cultured placenta (Chapter 4). However, estradiol was found to have no effect on hCG concentrations in collected medium (measured by RIA, Chapter 3), but it caused a marked increase in tissue levels of hCG under culture conditions (Chapter 4). These data suggest that in cultured human placenta, progesterone increases both the synthesis and secretion of hCG, while estradiol increases its synthesis but does not affect its secretion.

Measurements of maternal levels of these hormones has provided no insight into the mechanisms of hCG stimulation or local changes in placental concentrations which may be occurring. Therefore, to further characterize the interactions between hCG, progesterone and estradiol, *in vivo*, their placental concentrations were determined through gestation. These concentrations were compared with the effective concentrations required for hCG stimulation *in vitro* (Chapters 3 and 4) and with their production rates in culture under control conditions.

The data presented in this chapter regarding hormone concentrations in human placenta are only preliminary findings. Although they cannot be considered conclusive, they reveal interesting trends which may be important to the mechanisms of hCG regulation.

5.2 ABSTRACT

Previous studies originating from our laboratory suggest that steroids such as progesterone and estradiol may be important in the control of hCG production in human placenta (1,2). The aim of the present study was to compare the levels of these steroids in human placenta at various times through pregnancy with the concentrations required for their *in vitro* effectiveness (1,2) and with their output from first trimester explant cultures.

The concentrations of hCG, progesterone and estradiol were determined in placental villous tissue at various times through gestation. Tissue concentrations of hCG and progesterone increased in early pregnancy to peak at 10 and 11 weeks respectively and decreased thereafter. Estradiol concentrations were low in early pregnancy, quadrupled between 6-11 weeks and increased steadily thereafter.

In culture, under control conditions (Ham's F-10 + 10% fetal bovine serum in the absence of added steroids), the placental output of progesterone and estradiol was much lower than might have been expected on the basis of their respective tissue concentrations. The concentrations of these hormones were found to be 10-fold greater in medium collected from cultures than in fresh control medium. Placental production of these steroids *in vitro* peaked between 48-72 hours after the initiation of culture and then began to decline.

We suggest that alterations in the placental concentrations of these steroid may be important paracrine determinants of placental and maternal plasma hCG levels through pregnancy and may be implicated in the first trimester hCG surge.

5.3 INTRODUCTION

Many studies have documented the maternal serum and urinary patterns through pregnancy of placental hormones such as hCG, progesterone and estradiol. However, there are few reports of these hormone levels in placental tissue and fewer still of placental tissue levels in first trimester. Such studies are of interest since, as we have documented in this report, there are differences in the patterns of tissue and plasma concentrations. Knowledge of the placental concentrations of these hormones may shed light on their interactions and may increase our understanding of the regulatory mechanisms governing hCG production.

Progesterone and estradiol are important regulators of hCG synthesis and secretion (1,2). HCG, progesterone and estradiol all originate from the placenta during pregnancy and changes in the local concentrations of these steroids may have important endocrine implications on placental functions, such as the production of hCG.

5.4 MATERIALS AND METHODS

Materials:

Human placentae were obtained following various obstetrical procedures. Placentae at 6-12 weeks gestation were obtained from the Family Planning Unit at the Montreal General Hospital following dilatation and evacuation. Placentae at 14-20 weeks were obtained following dilatation and curettage or hysterotomy. Placentae of 30-36 weeks were obtained following premature delivery. Term placentae were obtained following cesarean section for maternal indications, or as a result of spontaneous or induced labour at 38-42 weeks.

All organic solvents used in the extraction of steroids were purchased from American Chemical Co., (Montreal, Que.). The scintillator, Beta-Max, was purchased from ICN Chemicals (Irvine, CA.). Radioactive tracers were purchased from New England Nuclear (Boston, MA.).

Homogenization and Extraction of Hormones from Tissue:

All placentae were obtained within 1 hour of the obstetrical procedure and were immediately dissected to separate chorio-decidual membranes from trophoblastic villous tissue. Trophoblastic tissue was rinsed three times with saline to remove excess blood and frozen in saline until extraction of the hormones.

For the extraction of hCG, the tissue was frozen and thawed three times. Aliquots (0.5 g) of this tissue were then homogenized in five volumes of saline at high speed using a glass homogenizer and automatic rotator. The tissue homogenate was centrifuged for 20 minutes at

2500 x g. The supernatant was removed and frozen at -20°C until assayed.

For the extraction of steroids, 0.5 g aliquots of tissue were thawed at room temperature, homogenized in two volumes of saline and extracted twice with five volumes of the appropriate organic solvent. Progesterone was extracted into hexane and 17β-estradiol into diethyl ether. Extracted steroids were stored in 1 ml ethanol at -20°C until assayed.

Extraction of Steroids from Medium:

Explant cultures of first trimester human placenta were prepared as previously described (1,2). Culture medium was collected and replaced daily. Collected medium was frozen until extracted and assayed. Fresh culture medium (Ham's F-10 + FBS, both from Grand Island Biological Co., Burlington, Ont.) was stored at 4°C until assayed. Progesterone and estradiol were extracted from aliquots of fresh medium (500µl) and medium collected from cultures (100 µl) using the same solvents as those used to extract steroids from tissue.

Assay Procedures:

Human chorionic gonadotropin was assayed using a specific β-subunit assay (Bio-Mega Diagnostics, Montreal, Que.) as described previously (1). Progesterone was measured by radiotransinassay using pregnant guinea pig serum (3,4). Estradiol-17β was quantitated by radioimmunoassay using an antiserum kindly provided by Dr. Hamish Robertson (Ottawa, Ont.). Further details of assay procedures are provided in Chapter 2.

5.5 RESULTS

The concentrations of hCG, progesterone and estradiol in placental villi are shown in Table 5.1. HCG reached a peak concentration of 845 IU/g at 9 weeks gestation (about 100-fold greater than that at term) and thereafter decreased to very low levels in the second and third trimesters. Estradiol levels were low in early gestation, almost quadrupled between 6-10 weeks and then rose steadily thereafter. Progesterone levels were 8-fold higher at 10-14 weeks than at 6 weeks and term.

The pattern of hormone output from first trimester explant cultures is given in Table 5.2. Output of progesterone and estradiol was lower in the first 24 hours of culture than in the subsequent 24-48 hours. The production of these steroids began to decrease after the third day in culture. Estradiol output was better maintained than progesterone output; its levels declined more rapidly. The concentrations of progesterone and estradiol in fresh medium were approximately 10-fold less than in medium collected from placental cultures.

TABLE 5.1: Concentrations of hCG, progesterone and estradiol in human placenta at various gestational ages[1].

GESTATIONAL AGE[2] weeks	n	HCG IU/g[3] X ± S.E.	n	PROGESTERONE ng/g X ± S.E.	n	ESTRADIOL pg/g X ± S.E.
6	6	183 ± 23	4	617 ± 61	5	323 ± 57
7	3	168 ± 70	3	970 ± 150	3	600 ± 240
8	3	473 ± 58	2	1785 ± 257		ND
9	3	602 ± 86	2	926 ± 121		ND
10	5	845 ± 61	2	5323 ± 233	4	1119 ± 135
12		ND	2	3520 ± 350	2	1656 ± 216
14	2	257 ± 26	1	4941	1	2787
15		ND	1	1386	1	3900
16	4	70 ± 12	3	1150 ± 189	3	5093 ± 109
17	2	57 ± 10		ND	1	5600
20	5	43 ± 10	4	883 ± 215	3	8470 ± 160
30	3	11 ± 4	2	646 ± 211		ND
34	4	14 ± 2	3	1006 ± 195		ND
36	2	15 ± 8	3	693 ± 112		ND
38	3	7 ± 3		ND		ND
40	3	9 ± 4	4	991 ± 146	3	8093 ± 60
42	4	11 ± 5	3	675 ± 119	2	14401 ± 775

[1] Values are given as means ± standard errors.
[2] gestational age as determined by menstrual history and medical exam.
[3] 1 IU = 1279 ng.
n = the number of determinations (in duplicate), each from a different placenta.
ND = no data.

TABLE 5.2: Daily production rates of progesterone and estradiol from explant cultures of first trimester human placenta and their concentrations in fresh medium[1].

# OF DAYS AFTER INITIATION OF CULTURE	PRODUCTION RATE	
	PROGESTERONE ng/ml/10mg/24hrs	ESTRADIOL pg/ml/10mg/24hrs
1	8.9 ± 2	175 ± 42
2	12.8 ± 3	318 ± 23
3	8.2 ± 3	335 ± 70
4	5.0 ± 1	265 ± 37
5	6.7 ± 2	205 ± 72
6	5.5 ± 1	288 ± 48
CONCENTRATION IN FRESH MEDIUM[2]	1.8 ± 1 ng/ml	48 ± 13 pg/ml

[1] Results are expressed as means ± standard error of six determinations.
[2] As determined by assay of Ham's F-10 + 10% FBS.

5.6 DISCUSSION

Our determinations of second and third trimester placental concentrations of hCG, progesterone and estradiol are in general agreement with previously reported findings (5-10). We are unaware of any studies which report first trimester concentrations of these hormones. These are preliminary findings which cannot be considered complete, but do give useful indications as to trends.

Placental hCG concentrations (IU/g) increased approximately 4-fold between 6-10 weeks and then decreased approximately 17-fold by 20 weeks, remaining relatively constant thereafter. This pattern is closely mirrored in maternal plasma (11). Our findings of greater hCG concentrations in early tissue, concur with in vitro experiments that showed hCG output to be 32 times greater from midterm than term placental explant cultures (12). Furthermore, in four hour incubations, hCG secretion was greater from 12 than 16 week placentae; term tissue had the lowest secretion rate (13). Explant cultures of first trimester human placenta produced hCG in concentrations 7-fold greater than term placenta under similar conditions (1).

We report significant progesterone concentrations in placenta as early as the 6th gestational week, with peak levels at 10-14 weeks. This would allow the placenta to be an important determinant of maternal plasma progesterone in early gestation, despite its relatively small size at this time. In accord with this, Ogino (14), demonstrated that the placenta can convert radio-labelled pregnenolone to progesterone at a signficant rate by 7 weeks. Furthermore, although

the contribution of the corpus luteum is thought to be negligible after 8 weeks (15), maternal plasma progesterone levels rise progessively from 3-13 weeks of pregnancy. Our confirmation of significant progesterone concentrations in the placenta by 6 weeks concur with the suggested timing of the luteo-placental shift (16).

After 14 weeks, placental progesterone concentrations (ng/g) declined and remained relatively low until term. This is in agreement with Siler Khodr (17) who reported that progesterone production by placental explants was greater at 13 weeks than at term. Thus the increase in maternal plasma progesterone beyond 14 weeks must be attributed to an increase in placental mass rather than per gram production.

Estradiol levels in placenta were relatively low at 6-7 weeks, then increased rapidly to almost quadruple between 6-10 weeks. This rapid increase in placental concentration coincides with the increase in tissue and maternal hCG levels. Aromatase activity has been demonstrated in the placenta at 11 weeks (18), 8 weeks (19) and 12-13 weeks (20). Smith and Axelrod (21), documented a steady increase in aromatizing capacity per gram of placental tissue from 5-24 weeks. Other investigations showed that aromatase function almost doubled between second and third trimester (19). In blighted ovum pregnancies, estradiol levels rise normally until 8 weeks but fail to increase as expected thereafter, suggesting that the placenta becomes a source of estrogens exclusive of the corpus luteum only after 8 weeks (22). In contrast to our findings, Ogino et al (23) showed that in organ cultures of human placentae, productivity of estrogens was greater

before 10 weeks than at any other time through gestation. A two-component membrane bound molecule (molecular weight 165,000), possessing aromatase activity, was recently isolated from term human placenta (24).

Ultra-microscopic and tissue culture studies confirmed the capacity for fetal adrenal steroidogenesis as early as 7 weeks (25); this suggests that the fetal adrenal becomes a source of estrogen precursors after 7 weeks. Endogenous dehydroepiandrosterone (DHEA) production is precluded due to the lack of C21-C19 metabolizing enzymes in the placenta (19,26). It is interesting that our data show estrogen synthesis to increase between 6-11 weeks, at about the time that the fetal adrenal is thought to become an important source of DHEA (25). Preliminary studies from this laboratory suggest that DHEA may be detectable in placenta only in early gestation and that it is undetectable after approximately 12 weeks. If this finding is confirmed, it would agree with our present data which show relatively low placental concentrations of estradiol in early gestation. The presence of DHEA in early placenta may also be functionally important because, as we have shown, it stimulates the synthesis and secretion of hCG (1,2).

The finding that the patterns of placental hCG and progesterone concentrations are temporally correlated and that placental estradiol concentrations rise most sharply within the same time frame as maximal placental hCG levels are being attained (Table 5.2), support our hypothesis that these steroids may be important regulators of hCG production (1,2). A relationship between placental steroid and hCG

production has been reported by other authors. Pang et al (27), noted a negative correlation between maternal plasma levels of progesterone and hCG during pregnancy. Their study, however, did not consider the levels of these hormones at their site of action or synthesis and therefore hinders the comparison of their data with ours. In contrast to the findings of Pang et al (27), is the observation that in vivo administration of progesterone to women between 11-15 weeks of pregnancy resulted in a consistent and significant increase in serum β-hCG concentrations (28). Furthermore, it was reported that in pregnancies eventually ending in spontaneous abortions, an early decline in serum progesterone levels was closely followed by a drop in plasma hCG levels (22).

In accord with our findings, Hanning et al (29) showed a positive correlation between levels of progesterone and hCG measured in medium collected from placental explants and between hCG and progesterone levels in maternal plasma, as expressed as a function of placental weight (30). Siler Khodr et al (17) demonstrated a relationship between progesterone concentrations in collected medium and the magnitude of an hCG response to GnRH stimulation in placental explants. In contrast to our findings, progesterone suppressed (31) or failed to have any effect (12) on hCG secretion under tissue culture conditions. These discrepancies may be attributable to differences in assay techniques used to measure hCG.

Estradiol increased cellular levels of α-hCG and mRNA$_\alpha$-hCG but had no effect on β-hCG or mRNA$_\beta$-hCG in term placenta (5). Similarly, explants of term placenta did not respond to estradiol stimulation with

a change in hCG secretion (12,32). We are the only group to report an effect of DHEA on hCG secretion by placenta (1). No demonstrable effect of DHEA was reported in either 11 week placental explants (33), term explants (29), or as a result of *in vivo* administration, except in the presence of low estrogen levels (34).

Progesterone and estradiol may have trophic functions in placental growth and development *in vivo* (2). It is interesting that their placental concentrations rise most rapidly in early pregnancy when mitogenic activity in the placenta is the greatest (35).

The results presented in Table 5.2 show that steroid output by placental explants was maintained over the first 7 days in culture. These data are consistent with our studies of placental viability in culture, which showed that morphology and hCG production remained well-preserved over the same time-peroid. (2). Hormone production in culture was less than what might be expected from an extrapolation of the observed tissue concentrations. It is difficult to compare these two parameters however, as tissue concentrations reflect an instantaneous content whereas placental production in culture measures hormone output over 24 hours. Even when considering the difference in time-frame, it appears that the production of these steroids is much less *in vitro* than *in vivo*. This is presumably due to a relative deficiency of substrates which are normally supplied to the tissue *in vivo*. The concentrations of progesterone and estradiol in fresh control medium were approximately one-tenth those in medium recovered from cultures, suggesting that substrates required for their production are present in fresh medium in at least the amounts required

to sustain the observed level of hormone production.

The observation that placental production of progesterone and estradiol in culture appears to be substrate-limited and is much less than their concentrations in fresh placenta, explains our previous report that their exogenous addition stimulated hCG production and tissue viability under similar conditions (1,2).

The relatively high concentrations of progesterone in first trimester placenta may also have immunological significance. This hormone is thought to function as a local immunosuppressant during pregnancy. Stites and Siiteri (36), found that the progesterone concentrations required to block T-cell activation, inhibit lymphocyte DNA synthesis or block the mitogenic activity of interleukin-1 ranged between 5-20 μg/ml. However, these concentrations are several orders of magnitude above plasma levels, even during pregnancy. Our tissue concentrations of first trimester placenta are within the effective range reported by these authors. It seems an interesting speculation that such high tissue concentrations of progesterone may act in early pregnancy to stem local maternal immune responses directed against the implanting embryo.

Recent reports from our laboratory (1,2) showed that progesterone increased both hCG secretion (as measured by RIA of medium collected from cultures) and tissue levels (as measured by immunohistochemical techniques) of hCG in first trimester explants of human placenta. Estradiol increased tissue content but did not affect secretion. We therefore hypothesize that these hormones act as paracrine signals to stimulate hCG synthesis and secretion <u>in vivo</u> and that they may in part

be responsible for the huge hCG surge occurring in the first trimester. This surge cannot be explained by other factors such as epidermal growth factor (37), prolactin (38), dibutryl-cAMP (39-42) or GnRH (1,43) which have all been shown to affect placental hCG secretion. Interestingly, the concentrations of progesterone required for in vitro stimulation of hCG (1,2) correlate well with its presently reported concentrations in placenta. We propose that local changes in tissue concentrations of progesterone and estradiol contribute to the observed pattern of maternal plasma hCG. This report of the levels of these hormones in placenta through gestation lends credence to this hypothesis.

5.7 REFERENCES

1. Ahmed NA and Murphy BEP, The effects of various hormones on hCG production in early and late placental explant cultures, Am J Obstet Gynecol, 159: 1220, 1988.

2. Ahmed NA, Senterman MK and Murphy BEP, Placental explant cultures: Effect of steroids on viability and tissue hCG content, Submitted for publication.

3. Pearson Murphy BE and Antonipillai I, Serum oestrogens and progesterone in mother and infant at delivery, Br J Obstet Gynaecol, 84: 179, 1977.

4. Tan SY and Murphy BEP, Specificity of progesterone binding globulin of guinea pig, Endocrinology, 94: 122, 1974.

5. Matsou H, Maruo T, Hoshina M and Moanizuki M, Selective augmentaion of cellular hCGαmRNA levels and immunoreactive αhCG secretion by 17β-estradiol in normal placenta, Folia Endocrinol Jap, 62: 1315, 1986.

6. Diczfaulsy E, Chorionic gonadotropin and estrogens in human placenta, Acta Endocrinologica, Suppl 12 vol 12: 1, 1953.

7. Wilson EA, Jawad MJ and Powell DE, Effects of estradiol and progesterone on hCG secretion in vitro, Am J Obstet Gynecol, 149: 143,1984.

8. Ferre F, Jansens Y, Tanguy G, Breuiller M, dePariente D and Cedard L, Steroid concentrations in human myometrium and placental tissue at 39 weeks of pregnancy, Am J Obstet Gynecol, 131: 500, 1978.

9. Runnebaum B, Runnebaum H, Stober I and Zander J, Progesterone, 20α-dihydroprogesterone and 2β-progesterone levels in different compartments from the human foeto-placental unit, Acta Endocrinologica, 80: 558, 1973.

10. Khan-Dawood FS and Dawood YM, Estrogen and progesterone receptor and hormone levels in human myometrium and placenta in pregnancy, Am J Obstet Gynecol, 150: 501, 1984.

11. Saxena BB, Human chorionic gonadotropin, In: Endocrinology of Pregnancy, Fuchs F and Klopper A (eds.), 3rd editon, Harper and Row Publications, 1983.

12. Belleville F Lasbennes A, Nabet P and Paysant P, hCS and hCG regulation in cultured placenta, Acta Endocrinologica, 88: 169, 1973.

13. Hanning RV, Choi L, Kiggens AJ and Kuzma DL, Effects of prostaglandins, dbcAMP, LHRH, estrogens, progesterone and potassium on output of prostaglandin F2α, hCG, estradiol and progesterone by placental minces, Prostaglandins, 24: 495, 1982.

14. Ogino, Kinoshita K, Satoh K, Mizunom M, and Sakamoto S, Metabolism of ^{14}C-pregnenolone in the placenta throughout pregnancy in organ culture, Endocrinologica Japonica, 30: 631, 1984.

15. Tulchinsky D and Hobel CJ, Plasma hCG, estrone, estradiol estriol progesterone and 17α-progesterone in human pregnancy, Am J Obstet Gynecol 117(7): 885, 1973.

16. Johnson MH and Everitt BJ, Coitus and Fertilization, In: Essential Reproduction, Blackwell Scientific Publications, Oxford, p. 192, 1984.

17. Siler-Khodr TM, Khodr GS, Valenzuela G and Rhode J, GnRH effects on placental hormones during gestation II: progesterone, estrone, estradiol and estriol, Biol Reprod, 34: 255, 1986.

18. Rao B, Effects of prostaglandins on in vitro synthesis of progesterone and estradiol in placenta from early pregnancy, Prostaglandins, 18: 93, 1979.

19. Fukuda M, Okuyama T and Furuya H, Growth and function of the placenta with reference to various enzymes involved in the biosynthesis of steroids om the human placenta, Acta Obstet Gynecol Jpn, 38(3): 411, 1986.

20. Cedard and Lehman, Estrogen biosynthesis in perfused human placenta at different stages of pregnancy, Second International Congress on Hormonal Steroids, Exerptica Medica, International Congress Series No. 111: 173, 1966.

21. Smith SW and Axelrod LR, Studies on the metabolism of steroid hormones and their precursors in human placenta at various stages of gestation II: In vitro metabolism of 3β-hydroxyandrost-5-ene-17-one, J Clin Endocrinol, 29: 1182, 1969.

22. Schweditsch NO, Dubin NH, Jones GS and Wentz AC, Hormonal considerations in early normal pregnancy and blighted ovum syndrome, Fertil Steril, 31: 252, 1979.

23. Ogino M, Productivity of estrogens by human placental organ cultures at different stages of gestation, Endocrinol Japon, 32: 607, 1985.

24. Muto N and Tan L, Purification of oestrogen synthetase by high pressure liquid chromatography, J Chromat, 326: 137, 1985.

25. Thau RB and Lanman JT, Endocrinological aspects of placental function, In: The placenta, Gruenwald P (ed.), University Park Press, Baltimore, p. 98, 1975.

26. Telegdy G, Weeks JW, Archer DF, Wilquist N and Diczfaulsy E, Acetate and cholesterol metabolism in the human foeto-placental unit at mid-gestation, Acta Endocrinologica, 63: 119, 1970.

27. Pang SF, Tang Pl, Tang GWK, Yam AWC and Ng KW, Plasma levels of immunoreactive melatonin, estradiol, progesterone, follicle stimulating hormone and βhCG during pregnancy and shortly after parturition in the human, J Pineal Res, 4: 21, 1987.

28. Yosef MS, Nasher R, Navot D, Hadani PE and Antenby SO, Effect of administration of progesterone on βhCG blood levels in early pregnancy, Gynecol Obstet Invest, 18: 113, 1984.

29. Hanning RV, Choi L, Kiggens AJ, Kuzma DL and Summerville JW, Effects of dbcAMP and aromatase inhibitor on the simultaneous outputs of progesterone, 17β-estradiol and hCG by term placental explants, Prostaglandins, 23: 29, 1982.

30. Hanning RV, Choi L, Curet LB, Henderson PA, Kiggins AJ, Leifet TL, Olson RW, Swabe MG, Swartz DB and Traver M, Relationships among hCG, 17β-estradiol, progesterone and estriol in maternal serum; evidence for an inhibitory effect of the fetal adrenal on secretion of hCG, J Clin Endocrinol Metab, 56: 1188, 1981.

31. Wilson EA, Jawad MJ, Dickson LR, Supression of hCG by progestational steroids, Am J Obstet Gynecol, 138: 708, 1980.

32. Marou T, Matsuo H, Ohtari T, Hoshino M and Mochizuki M, Differential modulation of chorionic gonadotropin (CG) subunit mRNA and CG secreton by progesterone in normal placenta and choriocarcinoma cultured in vitro, Endocrinology, 119: 855, 1986.

33. Voutilainen R, Kahari AI and Lahteenmaki P, The effects of DHEA and its sulphate on human mid-term placental secretion of hCG, progesterone, estrone and estradiol in tissue culture, J Steroid Biochem, 14: 1153, 1981.

34. Lauritzen Ch, Intraplacental regulations between steroid and peptide hormones (hCG and hPL) in pregnancy, Acta Endocrinologica suppl 193, Abst no. 46, 46, 1975.

35. Stevens DH, Structure and development, Concepts of the trophoblast and its role in placentation, In: Biology of the Trophoblast, Loke YW and Whyte A (eds.), Elsiever Science Publications, p. 3, 1983.

36. Stites DP and Siiteri PK, Steroids as immunosuppressants in pregnancy, Inmmunol Rev, 75: 117, 1983.

37. Benvineste R, Speeg RV, Carpenter G, Cohen S, Lender J and Rabinowitz D, EGF stimulates hCG by cultured choriocarinoma cells, J Clin Endocrinol Metab, 46: 169, 1978.

38. Yuen BH, Moon and Shin DH, Inhibition of hCG production by prolactin from term human trophoblast, Am J Obstet Gynceol, 154: 336, 1986.

39. Hanning RV, Choi L, Kiggens AJ, Kuzma DL and Summerville JW, Effects of dbcAMP, GnRH and aromatase inhibitor on the simultaneous outputs of progesterone and 17β-estadiol and hCG by term placental explants, J Clin Endocrinol Metab, 55: 213, 1982.

40. Hussa RO, Story MT, Pattilo RA, CAMP stimulates the secretion of hCG and estrogens by human trophoblast in vitro, J Clin Endocrinol Metab, 55: 214, 1982.

41. Handwerger S, Barrett J, Tyrey L and Schomberg D, Differential effects of cyclic adenosine monophosphate on the secretion of hPL and hCG, J Clin Endocrinol Metab, 36: 1268, 1973.

42. Story MT, Hussa, and Patillo RA, Independent stimulation of hCG and estrogens secretion by malignant trophoblast cells, J Clin Endocrinol Metab, 39: 877, 1974.

43. Khodr G and Siler Khodr TM, Effects of LHRH on hCG secretion, Fertil Steril, 30: 301, 1978.

CHAPTER 6

General Discussion and Conclusions

6.1 PREFACE

Human chorionic gonadotropin is a unique hormone in that it is involved in the communication between two distinct organisms. In a reproductive sense hCG is a crucial signal, the magnitude of which is an important determinant of successful embryo implantation. Therefore, defining its regulation would have important applications in the direction of fertility control. Clinically, an understanding of the regulation of hCG production could aid in the treatment of early pregnancy loss in cases where a deficiency of hCG has been implicated. Furthermore, controlling the production of hCG may contribute to advances in the field of contraceptive technologies.

The studies described in this thesis investigate the nature of hCG regulation by the steroid hormones known to characterize pregnancy. In this chapter, I shall integrate my results and based upon these, present a hypothesis regarding the endocrine regulation of hCG production.

6.2 GENERAL DISCUSSION AND CONCLUSIONS

The studies presented in Chapter 3 show that hCG output by placenta in culture was responsive to progesterone, DHEA, cortisol and testosterone. Progesterone and DHEA stimulated hCG output in both first and third trimester explants. Testosterone inhibited, while cortisol stimulated first trimester hCG production; both these steroids were ineffective in third trimester cultures. The combinations of progesterone + DHEA and progesterone + cortisol caused an additive or greater than additive stimulation of hCG secretion from both early and late placental cultures. Basal hCG production was quantitatively greater from first trimester than third trimester cultures (Fig. 3.1). This is in accord with the first trimester surge and subsequent decline of hCG concentrations in placental tissue (Table 5.1). In contrast however, term explants showed a greater responsiveness than early explants to progesterone or DHEA stimulation, alone or in combination. This is evidenced by the observation that in response to the aforementioned hormones, the average increase in hCG output from term placental cultures was greater than from early cultures (Figs. 3.3, 3.4 and 3.5). These finding may be explained by an increase in receptor number or affinity in placenta with advancing gestation.

Cortisol stimulated hCG production in a dose-dependent manner in first trimester explant cultures of human placenta; interestingly however, it was without effect in third trimester cultures. (Fig. 3.6). It is well established that after about 12 weeks gestation, the placenta rapidly metabolizes cortisol to its inactive metabolite

cortisone (1). Some preliminary experiments in this laboratory suggest that in early placenta (less than 12 weeks), tissue capacity to inactivate cortisol to cortisone is lower than in later gestation. It is possible although yet to be proved that, in the human, an alteration in cortisol metabolism in first trimester may account for the difference between the effect of cortisol on hCG secretion in early and late explants (Fig. 3.6).

The studies presented in Chapter 4 extended the concepts regarding steroidal regulation of hCG production in the placenta. Immunoreactive levels of hCG in cultured first trimester tissue were increased by the presence of progesterone, estradiol and DHEA, alone or in various combinations (Table 4.2). Radioimmunoassayable hCG concentrations in medium collected from similar cultures, however, were unaffected by the presence of estradiol (Fig. 3.10). This is an interesting finding which may indicate that estradiol increases the synthesis and storage of hCG, but does not affect its secretion. Thus immunohistochemically detectable placental hCG levels rise (Table 4.2) while the quantity secreted into medium does not change from controls (Fig. 3.10). In fact, the pituitary synthesis, storage and secretion of luteinizing hormone (LH) may be subject to similar regulation by estradiol (2-6).

The data presented in Chapter 5 suggest that placental concentrations of estradiol increase four-fold between 6-10 weeks of gestation, showing the greatest rate of rise in this time interval (Table 5.1). It is hypothesized that during the menstrual cycle, estradiol concentrations > 200 pg/ml if sustained for greater than 48

hours will trigger the rapid release of stored LH from the pituitary; this is thought to account for the LH surge (4-6). It is conceivable that in the placenta, in a manner analogous to the pituitary, secretion of hCG remains sub-maximal until tissue concentrations of estradiol reach a critical level, at which time there is a massive release of previously synthesized and stored hCG. Synthesis and storage of hCG may continue under the direction of progesterone, DHEA, cortisol and estradiol, but its complete secretion is blocked by sub-optimal levels of estradiol. Thus rising placental estradiol concentrations in association with high placental levels of progesterone could account for the huge surge in maternal plasma hCG concentrations observed in first trimester (7). The decrease in placental concentrations of progesterone between 14-15 weeks (Table 5.1) may be an important signal which diminishes placental production of hCG.

HCG concentrations in medium recovered from first trimester cultures (Figs. 3.3 and 3.4) and in cultured tissue (Table 4.2) were both increased by progesterone and DHEA, alone or in combination. These data suggest that the synthesis, storage and secretion of hCG is stimulated by both progesterone and DHEA. In the pituitary, levels of progesterone that approximate those of the luteal phase of the menstrual cycle appear to have an inhibitory effect on gonadotropin production. Investigators have attributed this finding to an antagonistic effect of progesterone on estradiol-stimulated gonadotropin synthesis (8,9). However, in estrogen-primed women, progesterone clearly has the ability to induce the discharge of LH (4,10,11). Furthermore, low levels of circulating progesterone

(1 ng/ml) facilitate estrogen-induced gonadotropin surges by increasing peak concentrations of LH or FSH and by advancing the onset of their release (9,12). Moreover, the failure of estrogen alone to induce FSH surges in the human is corrected by the addition of progesterone (9,12). My preliminary findings (Table 5.1) suggest a tight association between hCG and progesterone concentrations in placenta through pregnancy. These findings speak strongly for a regulatory role of progesterone in hCG production.

Cortisol did not alter tissue levels of hCG (measured immunohistochemically, Table 4.2), however it increased hCG secretion from first trimester explants (measured by RIA, Fig. 3.6). It is possible therefore, that cortisol stimulates the release of pre-synthesized hCG from tissue but is without effect on intracellular translation or transcription of this hormone. This contention is supported by the observation that the increase in radioimmunoassayable hCG in medium occurs within the first 24 hours of the cortisol stimulus; cortisol failed to demonstrate significant effects thereafter (Fig. 3.6).

Testosterone caused a potent decrease in radioimmunoassayble hCG ouput from placenta (Fig. 3.9), but did not effect the levels of hCG in cultured tissue (Table 4.2). From these data, I infer that testosterone does not affect synthesis but rather decreases the secretion of hCG from placenta. In parallel, testosterone is known to decrease pituitary output of LH from gonadotrophes <u>in vivo</u> and <u>in vitro</u> (13,14).

Studies regarding placental function have shown that this organ rapidly aromatizes androgens to estrogens (15). However, testosterone

and estradiol affected placental hCG content, secretion and viability in different ways; therefore, placental aromatization of androgens cannot account for the effects of testosterone reported in this thesis. DHEA can be aromatized to estrogens by the placenta (16). The importance of this metabolic route to placental hCG output or viability remains to be addressed.

With regards to the investigations in Chapter 3, the antiserum for the RIA that was used to measure hCG in medium recovered from cultures was raised against a portion of the β-subunit (17). Similarily, the antibody used to measure immunoreactive levels of hCG in cultured placenta was raised against isolated β-chains of the hCG molecule (18). Therefore it is unlikely that the discrepant effects of steroids on hCG levels in collected medium and in tissue (measured immunohistochemically) could be a result of a methodological inconsistency with respect to specificity of the antisera used in these two studies. The anti-β-hCG antibody cross-reacts with the intact hCG molecule and the free β-subunit, but not with the free α-subunit. Therefore, the studies presented in Chapters 3 and 4 do not distinguish between the effect of the steroids on β-hCG or hCG production. The effect of steroid hormones on α-hCG synthesis cannot be assessed from the data presented.

The studies presented in Chapter 4 provide insight into optimal conditions for the <u>in vitro</u> cultivation of human placenta. Progesterone, estradiol and DHEA were found to dramatically increase the viability of placenta in culture (from 7 to 30 days, Table 4.1). This suggests that these steroids may be involved in the growth and

differentiation of this organ *in vivo*. Steroids are known to have trophic effects in many tissues. This subject is reviewed elsewhere (Section 1.3.6).

The finding that the exogenous addition of progesterone and estradiol prolonged tissue survival (Table 4.1), suggested that *in vitro* rates of synthesis of these steroids was less than *in vivo* rates and that this was likely due to a relative deficiency of substrates (cholesterol and DHEA respectively). This hypothesis was confirmed by subsequent experiments which showed the production rates of these steroids in culture to be approximately 10-fold less than their fresh tissue concentrations (Tables 5.1 and 5.2). Furthermore, calculations based upon placental weight and documented production rates of progesterone and estradiol in mid-first trimester, demonstrate that the production of these steroids is at least 10-100 times less *in vitro* (Table 5.2) than *in vivo* (19,20). The finding that the concentrations of these hormone in control medium (Ham's F-10 + 10% FBS) was approximately 100-fold less than their respective fresh tissue concentrations (Table 5.2) suggests that this medium is inadequate for the prolonged survival ($\approx$ 30 days) of placenta in culture; this is presumably due to the lack of trophic effects *in vitro*, which are provided by progesterone and estradiol *in vivo*.

From these findings, I infer that commercial medium supplemented with 10% FBS does not approximate the admixture of maternal and fetal plasma to which the human placenta is exposed during pregnancy, at least with regard to the concentrations of these two hormones and their precursors. It is known that the placenta secretes mostly into the

maternal compartment; therefore fetal levels of progesterone and estradiol are only a fraction of maternal levels (7). It is not surprising therefore, that the addition of fetal bovine serum proved inadequate for protracted tissue survival. The addition of pregnant maternal serum to cultures may be more appropriate in this regard.

Under cultures conditions, the absence of fetal bovine serum was incompatible with prolonged tissue survival or maintained levels of hCG in cultured placenta (Tables 4.1 and 4.2). This is an expected finding in light of the fact that fetal bovine serum is the only source of steroid hormones or their substrates available to the tissue in vitro. In this regard, it is interesting that the stimulatory effect of epidermal growth factor on hCG and α-hCG production from choriocarcinoma cell cultures was dependent upon the presence of fetal calf serum (21).

The synthesis and secretion of hCG in the placenta appears to be dependent upon the complex interaction of many factors. Steroids may be important regulators which target specific biochemical events involved in the production of this important hormone. I hypothesize that the steroid hormones under investigation in my experiments, may interact to deterimine the level of hCG output by the placenta in the following manner. In early pregnancy, placental hCG levels rise sharply in response to increasing local concentrations of progesterone and estradiol and the transitory presence of DHEA. When placental estradiol concentrations reach a critical value (≈ 1000-1500 pg/gm), they initiate a strong stimulus for the secretion of hCG; this would account for the huge increase in maternal plasma hCG levels between

8-11 weeks gestation (22). The decrease in progesterone and possibly the onset of potent cortisol oxidation and the disappearance of detectable levels of DHEA in the placenta would contribute to a fall in placental and maternal hCG levels. Therefore it is the evolving nature of the tissue and the interaction between hormones at any particular time which determines the level of hCG secretion.

Immunofluorescence techniques have localized hCG, progesterone and estradiol within the cytosolic fraction of syncytiotrophoblast cells (23,24). These cells also contain the enzymes of steroidogenesis and a transcortin-like molecule (24,25). Cytotrophoblasts are the likely source of GnRH (26). Furthermore, trophoblast cells were shown to express receptors for GnRH (27), progesterone (28,29), estradiol (28-30), androgens (31,32) and glucocorticoids (33,34). It is a plausable hypothesis therefore, that the regulation of hCG production is subject to paracrine and autocrine signals, which may include progesterone, estradiol, DHEA, cortisol and GnRH.

6.3 REFERENCES

1. Murphy BEP, Ontogeny of cortisol-cortisone interconversion in human tissues: a role for cortisone in human fetal development, J Steroid Biochem, 14: 811, 1981.

2. Hotchkiss J, Dierschke TJ, Butler WR, Fritz GR and Knobil E, Relationship between levels of circulating ovarian steroids and pituitary gonadotropin content during the menstrual cycle of the Rhesus monkey, Biology of Reprod, 26: 421, 1982.

3. Hoff JD, Lasley BL, Wang CF and Yen SSC, Two pools of pituitary gonadotropin: Regulation during the menstrual cycle, J Clin Endocrinol Metab, 44: 302, 1977.

4. Leyendecher G, Wardlaw S, Noocke W and Plotz EJ, Experimental studies on the endocrine regulations during the periovulatory phase of the human menstrual cycle, Acta Endocrinol, 71: 160, 1976.

5. Monroe SE, Jaffe RB and Midgley AR, Jr, Regulation of human gonadotropins XII. Increase in serum gonadotropin in response to estradiol, J Clin Endocrinol Metab, 34: 342, 1972.

6. Yen SSC and Tsai CC, The biphasic pattern in the feedback action of ethinyl estradiol on the release of pituitary FSH and LH, J Clin Endocrinol Metab, 33: 298, 1972.

7. Martin MC and Hoffman PC, The endocrinology of pregnancy, In: Basic and Clinical Endocrinology, Greenspan FS and Forsham PH (eds.), Lange Medical Publications, Los Altos, California, p. 457, 1983.

8. Dierschke DJ Yamaki T, Karsch FJ, Weike RF, Weiss G and Knobil E, Blockade by progesterone of estrogens induced by LH and FSH release in the Rhesus monkey, Endocrinology, 92: 1426, 1973.

9. Helmond FA, Simons PA and Hein PR, Strength-duration characteristics of the facilitory and inhibitory effects of progesterone on the estrogen-induced gonadotropin surge in the female Rhesus monkey, Endocrinology, 108: 1837, 1981.

10. Nellus SSJ and Wide L, Effect of progesterone on serum levels of FSH and LH in postmenopausal women treated with estrogens, Acta Endocrinol, 67: 302, 1971.

11. Odell WD and Swerdloff RS, Progestin-induced luteinizing and follicle stimulating hormone surge in post-menopausal women: A stimulated ovulatory peak, Proc Natl Acad Sci, USA, 61: 529, 1968.

12. Chang RJ and Jaffe RB, Progesterone effects on gonadotropin release in women treated with estradiol, J Clin Endocrinol Metab, 47: 119, 1978.

13. Santen RJ, Is aromatization of testosterone to estradiol required for the inhibition of LH secretion in men? J Clin Invest, 50: 1555, 1975.

14. Winters SJ, Janick JJ Loriaux and Sherins RJ, Studies on the role of sex steroids in the feedback control of gonadotropin concentrations in men II: use of an estrogen antagonist, clomiphene-citrate, J Clin Endocrinol Metab, 48: 222, 1979.

15. Sitteri PK and MacDonald PC, Placental estrogen biosynthesis during human pregnancy, J Clin Endocrinol Metab, 26: 751, 1966.

16. Dicfalusy E, Steroid metabolism in the foeto-placental unit, In: The Foeto-placental Unit, Excerpta Medica, International Congress Series, Pecile A and Finizi C (eds.), No. 183, Excerpta Medica, Amsterdam, p. 65, 1968.

17. Instruction Manual, ^{125}I-β-hCG radioimmunoassay kit, BioMega Diagnostics, Montreal, Canada, 1986.

18. Histogen Immunohistology Peroxidase-Antiperoxidase System, Instruction manual, BioGenex Laboratories, San Ramon, California.

19. Estrogens, Progesterone and related neutral steroids, In: Endocrinology of Pregnancy, Fuchs F and Klopper A (eds.), Harper and Row, New York, 1972.

20. Wynn RM, Fine Structure of the Placenta, In: The Placenta, Gruenwald P, (ed.), Unviersity Park Press, Baltimore, p. 56, 1975.

21. Benveniste R, Speeg RV, Carpenter G, Cohen S, Lander W and Rabinovitz D, Epidermal-growth-factor stimulates secretion of hCG by cultured choriocarcinoma cells, J Clin Endocrinol Metab, 46: 169, 1978.

22. Saxena BB, Human chorionic gonadotropin, In: Endocrinology of Pregnancy, Fuchs F and Klopper A (eds.), Harper Row Publications, Philadelpia, p. 50, 1983.

23. Midley AR and Pierce JG, Immunohistochemical localization of hCG, J Exp Med, 115: 289, 1961.

24. Dobashi K, Ajika K, Kambegawa A and Akai K, Localization and distribution of unconjugated steroid hormones in normal placenta at term, Plac, 6: 445, 1985.

25. Werthamer S, Govirdraj S and Amaral L, Placenta, transcortin and localized immune response, J Clin Invest, 57: 1000, 1976.

26. Khodr GS and Siler Khodr T, Localization of gonadotropin releasing hormone in the human placenta. Fertil Steril, 29: 523, 1978.

27 Iwashita M, Evans MI and Catt KJ, Characterization of a gonadotropin releasing hormone receptor site in term placenta and chorionic villi, J Clin Encocrinol Metab, 62: 127, 1986.

28. Younes G,, 17β-estradiol and progesterone binding in human term placental cytosols, Am J Obstet Gynecol, 141: 170, 1981.

29. Khan Dawood FS and Dawood Y, Estrogen and progesterone receptors and hormone levels in human myometrium and placenta in term pregnancy, Am J Obstet Gynecol, 150: 501, 1984.

30. Laminski NA and Hammond KD, Receptors for estradiol in human placenta, Plac, 6: 383, 1985.

31. Grill HJ, Knichell A, Schweikhart G, Beck T, Mam B and Pollow K, Characterization of a high affinity low capacity androgen binding protein in human term placental cytosols, Exp Clin Endocrinol, 58: 3331, 1985.

32. Younes MA, Besch NF and Besch PK, Evidence for an androgen binding component in human cytosols, J Steroid Biochem, 16: 311, 1982.

33. Speeg W and Harrison RW, The ontogeny of the human placental glucocorticoid receptor and inducibility of heat stable alkaline phosphatase, Endocrinology, 104: 1346, 1974.

34. Lageson JM, Spelberg TC and Coulam CB, Glucocorticoid receptors in human placenta, Studies of concentration and functional differences of preterm and term tissue, Am J Obstet Gynecol, 145: 515, 1983.

CHAPTER 7

The Summary of Conclusions

7.1 PREFACE

In the interest of clarity and for the convenience of the reader, the major findings regarding the effects of steroid hormones on hCG production and placental viability, as well as some conclusions which may be drawn from these findings, are presented in this chapter in tabular form.

7.2 THE EFFECT OF VARIOUS TREATMENTS ON PLACENTAL HCG PRODUCTION AND VIABILITY IN FIRST TRIMESTER EXPLANT CULTURES[1].

	CONCENTRATION OF HCG IN MEDIUM[2]	CONCENTRATION OF HCG IN TISSUE[3]	TISSUE VIABILITY[4]
PROGESTERONE	↑↑↑	↑↑	↑↑
ESTRADIOL	NE	↑↑	↑↑↑
DHEA	↑↑	↑↑↑	↑↑
CORTISOL	↑	NE	↑
TESTOSTERONE	↓↓↓	NE	NE
0% FBS	-----	↓↓	↓↓
100% FBS	-----	↓↓	↓↓

[1] A semi-quantitative summary of the results presented in chapters 3 and 4.

[2] As determined by β-subunit RIA of medium recovered from explant cultures.

[3] As determined by immunohistochemical techniques using an anti-β-hCG antibody.

[4] As determined by histological analysis of placenta after removal from culture.

↑ - denotes an increase as compared to control, the number of arrows indicates the magnitude of the effect.

↓ - denotes a decrease as compared to control, the number of arrows indicates the magnitude of the effect.

----- indicates that the effect of this parameter was not evaluted.

NE - no effect.

7.3 THE HYPOTHESIZED EFFECTS OF STEROID HORMONES ON HCG PRODUCTION IN FIRST TRIMESTER HUMAN PLACENTA[1].

	SYNTHESIS[2]	STORAGE[2]	SECRETION[3]
PROGESTERONE	↑↑	↑↑	↑↑↑
ESTRADIOL	↑	↑	NE
DHEA	↑↑↑	↑↑↑	↑↑
CORTISOL	NE	NE	↑
TESTOSTERONE	NE	NE	↓↓

[1] based upon the findings presented, it is possible that steroid hormones target different processes in the synthesis, storage and secretion of hCG. The hypothesized role of each of the hormones studied is presented in the table above.

[2] evaluated by relative tissue content of cultured tissue as determined immunohistochemically, using a β-hCG antibody.

[3] evaluated by β-hCG radioimmunoassay of medium collected from cultures.

↑ - denotes an increase as compared to control, the number of arrows indicates the magnitude of the effect.

↓ - denotes a decrease as compared to control, the number of arrows indicates the magnitude of the effect.

NE - no effect.

CHAPTER 8

Future Directions

FUTURE DIRECTIONS

This work establishes the importance of steroids in the production of hCG from first trimester placenta. The studies on placental concentrations of progesterone and estradiol suggest a significant parallel with the placental and maternal serum levels of hCG. It would be interesting to extend these studies and document the placental concentrations of other steroids such as androstanediols which may affect the production of hCG.

Further investigations addressing the effects of steroid hormones on the placental concentrations of mRNA of the α and β subunit of hCG as well as its intact form are required. These studies would provide a more comprehensive understanding of the role of steroid hormones in the molecular aspects of hCG synthesis, storage and secretion.

My research demonstrated that progesterone, estradiol, DHEA and cortisol are important for the maintenance of human placenta in culture. However, the mechanisms underlying these effects remain to be defined. Indeed it remains unresolved whether these are direct effects or effects secondary to production of growth factors from placenta. Further investigations in this direction may prove fruitful.

The studies in this thesis showed that in explant cultures of first trimester placenta, cortisol stimulated the secretion of hCG as measured by RIA; however it was without effect in third trimester explants. Murphy (1), showed that after 14 weeks, the placenta has a potent capacity to convert cortisol to its inactive metabolite cortisone. Preliminary findings in this laboratory suggest that early

placenta is much less potent in this regard. The completion of these investigations would prove an interesting epilogue to this thesis. Abramovitz et al (2), showed that in fetal lung monolayers, estradiol potentiated the activity of 11β-hydroxysteroid dehydrogenase, the enzyme which converts cortisol to cortisone. The effect of estrogens on placental 11β-hydroxysteroid dehydrogenase remains to be addressed.

Growth and differentiation of the fetus is a complex and still little understood area. Given the effects of the steroids reviewed in this thesis, they may be important for the development of many fetal organ systems. Future investigations should involve a study of the effects of varying concentrations of FBS on placental viability and hormone production. The effects of pregnant and non-pregnant maternal serum on these parameters remains to be evaluated.

The studies herein described, may prove to have important clinical implications in the area of fertility control and tumour biology. Investigations such as these may help to uncover the mechanisms whereby hCG is produced from neoplasia. A more comprehensive understanding of placental hCG synthesis and its regulation may enable the development of better methods which increase or decrease the efficacy of reproduction.

REFERENCES

1. Murphy BEP, Ontogeny of cortisol-cortisone interconversion in human tissues: A role for cortisone in human fetal development, J Steroid Biochem, 14: 811, 1981.

2. Abramovitz M, Carriero R and Murphy BEP, Investigation of factors influencing 11β-hydroxysteroid dehydrogenase (EC 1.1.146) activity in midgestational human fetal lung monolayers and explant cultures, J Steroid Bioichem, 21: 677, 1984.

CHAPTER 9

Claims to Original Research

CLAIMS TO ORIGINAL RESEARCH

This is the first comprehensive study of the effects of steroid hormones on the production of hCG in first trimester explant cultures of human placenta.

1. DHEA, cortisol and progesterone were shown to have a stimulating effect on the production of hCG by first trimester explants, as measured by RIA.
2. Testosterone was shown to have an inhibitory effect under the same conditions.
3. Estradiol had no effect on hCG production as measured by RIA.
4. Progesterone, DHEA and estradiol were shown to have a stimulatory effect on the production of hCG, as measured immunohistochemically.

The in vitro viability of first trimester placenta was evaluated in the presence and absence of various hormones and varying concentrations of fetal bovine serum.

5. It was shown that the exogenous addition of progesterone, estradiol and DHEA increased the duration of viable culture, as defined by morphological and histological criteria.
6. Testosterone had no effect on this parameter.
7. Placental cultures maintained in an evironment of 0 or 100% FBS also showed diminished survival as determined by histological analysis.

The placental concentrations of progesterone, estradiol and hCG were documented at various times in gestation.

8. The first trimester concentrations of progesterone, estradiol and hCG were determined.

Appendices

APPENDIX 1: FORMULATION OF HAM'S F-10 CULTURE MEDIUM [1]

Inorganic salts	mg/l
Calcium chloride (anhydrous)	33.29
Cuperic sulphate	0.0025
Iron sulphate	0.834
Potassium chloride	285.0
Potassium hypophosphate	83.0
Magnesium sulphate (anhydrous)	74.64
Sodium chloride	7400.0
Sodium hypophosphate (anhydrous)	153.7
Zinc sulphate	0.0288
Other components	
Glucose	1100.0
Hypoxanthine (Na salt)	4.68
Lipoic acid	0.2
Phenol Red	1.2
Sodium pyruvate	110.0
Thymidine	0.7
Amino Acids	
L-alanine	9.0
L-arginine HCl	211.0
L-asparagine	15.01
L-aspartic acid	13.0
L-cysteine	25.0
L-glutamic acid	14.7
L-glutamine	46.0
Glycine	7.51
L-histidine	23.0
L-isoleucine	2.6
L-leucine	13.0
L-lysine HCl	29.0
L-methionine	4.48
L-phenylalanine	5.0
L-proline	11.5
L-serine	10.5
L-threonine	3.57
L-tryptophan	0.6
L-tyrosine (disodium salt)	2.62
L-valine	3.5
Vitamins	
Biotin	0.024
D-Ca pantothenate	0.715
Choline chloride	0.698
Folic acid	1.320
i-Inositol	0.541
Niacinamide	0.615
Pyridoxine HCl	0.206
Riboflavin	0.376
Thiamine HCl	1.000
Vitamin B-12	1.360

[1] Manufactured by Grand Island Biological Co., (Burlington, Ont.).

APPENDIX 2: FICK'S LAW OF DIFFUSION [1]

In the case of two solutions of non-uniform concentration, the diffusion of the solute from the more to the less concentrated region takes place in accordance with Fick's Law as expressed by the equation:

$$\frac{dm}{dt} = -DS\frac{dc}{dx}$$

This equation expresses the mass of a solute diffused per unit time through a cross-section S, in terms of the concentration gradient dc/dx in the direction x, perpendicular to the cross-section. The diffusion coefficient D, is constant for a given solute and solvent at a given temperature. For any one pair of substances, D is found to be proportional to the absolute temperature. These statements apply only to non-electrolyte substances.

[1] Reproduced from Van Nostrand's Scientific Encyclopedia, 7th edition, 1976, Litton Educational Publishing Inc., p. 793.

APPENDIX 3: SPECIFICITIES OF THE β-HCG ANTISERA.

Cross-reactivity data for the antibody used for RIA determinations of medium recovered from placental explant cultures[1].

POLYPEPTIDE HORMONE	% CROSS-REACTIVITY
hCG	100
hLH	55
hFSH	0.01
hTSH	0.02
hGH	0.0001

[1] Cross-reactivity between β-hCG and various other polypeptides was assessed by calculating the ratio at 50% B/Bo binding of the amount of each hormone compared with the amount of unlabelled hCG.

Data from Vaitukaitis, 1972.

The antibody used to measure tissue levels of hCG in cultured tissue cross-reacted approximately 3% with luteinizing hormone. None of the other tested compounds cross-reacted greater than 0.5%. (Personal communication: Mr. L. Roth, Product Manager, Dimensions Laboratories, Toronto, Canada).

APPENDIX 4: SPECIFICITY OF GUINEA PIG TRANSPROGESTIN[1].

TRIVIAL NAME	SYSTEMATIC NAME	% CROSS-REACTIVITY
Progesterone (P)	Pregn-4-ene-3,20-dione	100
Medrogestone	6,17 α-Dimethylpregn-4,6-diene-3,20-dione	350
5α-Dihydro-P	5α-Pregnane-3,20-dione	65
5β-Dihydro-P	5β-Pregnane-3,20-dione	30
20α-Dihydro-P	20α-Hydroxypregn-4-ene-3-one	37
20β-Dihydro-P	20β-Hydroxypregn-4-ene-3-one	6
Desoxycorticosterone	21-Hydroxypregn-4-ene-3,20-dione	43
11-Keto-P	Pregn-4-ene-3,11,20-trione	1.65
11β-OH-P	11β-Hydroxypregn-4-ene-3,20-dione	0.95
17α-OH-P	17α-Hydroxypregn-4-ene-3,20-dione	0.06
16-Dehydro-P	Pregn-4,16-diene-3,20-dione	2.1
18-OH-P	18-Hydroxypregn-4-ene-3,20-dione	5.5
Provera	6α-Methylpregn-4-ene-3,20-dione-17α-yl	1.35
11-Desoxy-cortisol	17α,21-Dihydroxypregn-4-ene-3,20-dione	0.9
Pregnenolone	3β-Hydroxypregn-5-ene-20-one	2.3
Testosterone (T)	17β-Hydroxyandrost-4-ene-3-one	8.0
1-Dehydro-T	17β-Hydroxyandrost-1,4-diene-3-one	0.7
Dihydrotestosterone (DHT)	17β-Hydroxy-5α-androstan-3-one	20
7αCH3-DHT	17β-Hydroxy-7α-methyl-5α-androstan-3-one	0.5
17αCH3-DHT	17β-Hydroxy-17α-methyl-5α-androstan-3-one	26
7α,17α-Dimethyl-DHT	17β-Hydroxy-7α-17α-dimethyl-5α-androstan-3-one	2.2
9α-Flouro-11βOH-17αCH3-DHT	9α-Flouro-11β,17α-dihydroxy-17α-methyl-5α-androstan-3-one	3.6
Androstenediol	Androst-4-ene-3β,17β-diol	0.5
	5α-Androstan-3,17-dione	12
	5β-Androstan-3,17-dione	0.65
Androstenedione	Androst-4-ene-3,17-dione	2.0
	Androst-1,4-diene-3,17-dione	0.5
	5α-Androstane-3β-ol	1.0
19-Nor-testosterone (19-nor-T)	17β-Hydroxyestr-4-ene-3-one	17
2α-Methyl-19-nor-T	17β-Hydroxy-2α-methylestr-4-ene-3-one	13
4-Methyl-19-nor-T	17β-Hydroxy-4-methylestr-4-ene-3-one	53
7α-Methyl-19-nor-T	17β-Hydroxy-7α-methylestr-4-ene-3-one	2.1
7β-Methyl-19-nor-T	17β-Hydroxy-7β-methylestr-4-ene-3-one	46
17α-Methyl-19-nor-T	17β-Hydroxy-17α-methlyestr-4-ene-3-one	26
7α,17α-Dimethyl-19-nor-T	17β-Hydroxy-7α,17α-dimethylestr-4-ene-3-one	15
17α-Ethinyl-7α-methyl-19-nor-T	17β-Hydroxy-17α-ethinyl-7α-methylestr-4-ene-3-one	32
6-Dehydro-19-nor-T	17β-Hydroxyestr-4,6-diene-3-one	19
19-Nor-DHT	17β-Hydroxy-5α-estran-3-one	29

TRIVIAL NAME	SYSTEMATIC NAME	% CROSS-REACTIVITY
7α-Methyl-19-nor-DHT	17β-Hydroxy-7α-methyl-5α-estran-3-one	1.2
7α,17α-Dimethyl-19-nor-DHT	17β-Hydroxy-7α,17α-dimethyl-5α-estran-3-one	3.2
	17β-Hydroxy-4,4-dimethylestr-5-ene-3-one	160

[1] All other tested compounds cross-reacted less than 0.5%. Such compounds included androgens and estrogens.

Data from Tan and Murphy, 1974.

APPENDIX 5: SPECIFICITY OF THE ESTRADIOL ANTISERUM[1].

STEROID HORMONE	% CROSS-REACTIVITY
17β-estradiol	100
estrone	4
estriol	1.8
cortisol	<0.05
testosterone	<0.05
androstenedione	<0.05
dehydroepiandrosterone	<0.05

[1] All other tested compounds cross-reacted less than 0.05%.

Data from Dr. Murphy's laboratory.