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APOPTOSIS IN PRIMARY CULTURES OF RAT LEYDIG CELLS

by

Mireille Limoges

Submitted in partial fulfillment
for the degree of Doctor of Philosophy

at

Dalhousie University
Halifax, Nova Scotia
September, 1998

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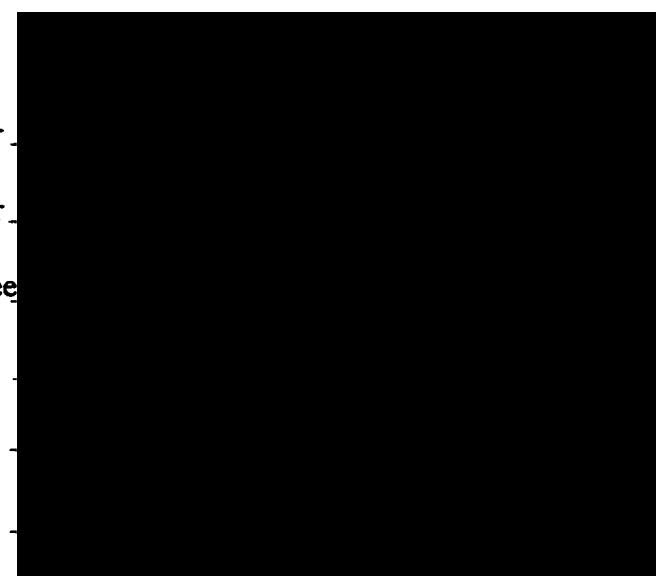
The undersigned hereby certify that they have read and recommend to the Faculty of Graduate Studies for acceptance a thesis entitled "Apoptosis in Primary Cultures of Rat Leydig Cells"

by Mireille Limoges

in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

Primary cultures of Leydig cells are an important tool in the study of steroidogenesis regulation. Unfortunately, Leydig cells, especially of rat origin, are fragile and short-lived once isolated from their intratesticular environment. The present studies showed that more than 99% of the freshly isolated cells were viable and had no signs of apoptotic DNA fragmentation. However, once in culture, rat Leydig cells soon presented biochemical evidence of internucleosomal DNA fragmentation. The number of apoptotic cells increased with the duration of the culture (3-72 h). The higher rate of apoptotic cell death, observed between 0-3 h of incubation, is believed to be induced by the lack of cell-cell contact between Leydig cells following cell plating. The addition of physiological doses of LH (0.1 ng/ml) to the culture media significantly increased androgen production from 0-72 h, but, had no significant effect on the onset of apoptosis from 0 to 24 h. Thereafter, LH at 0.1 ng/ml significantly inhibited the onset of apoptosis. On the contrary, the addition of supraphysiological doses of LH (10.0 ng/ml) promoted the onset of apoptosis in Leydig cells during the initial 24 h of incubation. Apoptotic cell death could also be induced *in vivo* in Leydig cells by injecting animals with large doses of hCG (100 IU/rat) 12 to 24 h prior to cell preparation. These results implicated the importance of reactive oxygen intermediates, generated during steroidogenesis, in the induction of apoptosis both *in vivo* and *in vitro*. Indeed, the addition of exogenous antioxidants, more specifically superoxide dismutase and catalase, was shown to inhibit the onset of apoptosis induced by supraphysiological stimulation of steroidogenesis. Finally, Western analysis of pro- and anti-apoptotic proteins, Bcl-2 and Bax, respectively, showed no evidence of their involvement in the regulation of physiological cell death in Leydig cells. However, these results do not rule out the involvement of other apoptosis-related proteins. In conclusion, although apoptosis in Leydig cells is complex, these results give a better understanding of the process, help to improve culture conditions and provide better insights on data obtained from *in vitro* studies.

LIST OF ABBREVIATIONS

AC	adenylate cyclase
AEC	3-amino-9-ethyl-carbazole
Asc. Ac.	ascorbic acid
ATP	adenosine triphosphate
bFGF	basic fibroblast growth factor
Biotin-14-dATP	biotinylated deoxyadenosine triphosphate
bp	base pair
BSA	bovine serum albumin
CAM	cell adhesion molecule
cAMP	cyclic adenosine 3',5'-monophosphate
Cat	catalase
Caly	calyculin A
cGMP	cyclic guanosine monophosphate
Cyt.c	cytochrome c
dATP	deoxyadenosine triphosphate
DMEM	Dulbecco's minimal essential medium
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylene diamine tetraacetic acid

EGF	epidermal growth factor
EGTA	ethylene glycol-bis (β -aminoethyl ether)
FCR	folin ciacalteau reagent
FCS	fetal calf serum
GH	growth hormone
G _s	G protein stimulatory subunit
GSH-Px	glutathione peroxidase
hCG	human chorionic gonadotropin
HEPES	N-2-hydroxyethyl-piperazine-N-2-ethane sulfonic acid
H ₂ O ₂	hydrogen peroxide
HS	horse serum
ICE	interleukin-1 β converting enzyme
IGF-I	insulin-like growth factor-I
IU	international unit
ISEL	<i>in situ</i> end labeling
Kbp	kilobase pair
KDa	kilo Dalton
LH	luteinizing hormone
M199	modified medium 199
MW	molecular weight
NAD	nicotinamide adenine dinucleotide

NAC	N-acetyl-L-cysteine
Oka	okadaic acid
PBS	phosphate buffered saline
PFA	paraformaldehyde
PKA	protein kinase A
PKC	protein kinase C
PP-1 or PP-2	phosphoprotein phosphatase-1 or -2
PMSF	phenylmethyl sulfonyl fluoride
Prl	prolactin
P450 _{scc}	cytochrome P450 side-chain cleavage
ROI	reactive oxygen intermediates
ROS	reactive oxygen species
r_s	correlation coefficient
RT	room temperature
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
sER	smooth endoplasmic reticulum
SOD	superoxide dismutase
Streptavidin-HRP	streptavidin-horseradish peroxidase
TBS	tris-buffered saline
TdT	terminal deoxynucleotidyl transferase

TE	tris-EDTA
TESPA	3-aminopropyl thiethoxysilane
TGF	transforming growth factor
TNF	tumor necrosis factor
TTBS	tris buffered saline containing tween 20
3 β -HSD	3 β -hydroxysteroid dehydrogenase
5 α -DHT	5 α -dihydrotestosterone
11 β -HSD	11 β -hydroxysteroid dehydrogenase
17 β -HSD	17 β -hydroxysteroid dehydrogenase
$\Delta\psi_M$	mitochondrial transmembrane potential
OH \cdot	hydroxyl radical
O $_2^-$	superoxide anion

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INTRODUCTION

I. THE LEYDIG CELLS

I.1. Historical Aspects

The study of the structure and function of human and animal testes goes back a very long time. The first evidence, recognizing the origin of testicular physiology, dates as early as the 15th century B.C. The Assyrians then recorded on rock tablets that castration of men was used as punishment for sexual offences. This record implies that the effects of castration on male reproductive abilities was recognized, at least by that time (Bremner, 1981).

Although Aristotle (400 B.C.) provided accurate descriptions of testicular anatomy and functions, the first convincing evidence of the role of the testis in the maintenance of male sexual characteristics was given in the middle of the 19th century. In 1849, Berthold demonstrated that the loss of male characteristics, following castration in fowl, could be prevented if the testes were transplanted to an abnormal site, for example, the abdominal cavity (Berthold, 1849; cited in Bremner, 1981; Saez, 1994). From these experiments, Berthold concluded that something must be transmitted from the testes, through the bloodstream, to affect other tissues. This conclusion was reached over 50 years before the terms "hormone" and "endocrine" were created by Bayliss and Starling (1902) (cited in Saez, 1994).

Concurrently, the interstitial cells of Leydig, named after Franz Leydig who

first identified them, were described in the mammalian testis as specialized connective tissue containing lipid inclusions (Leydig, 1850; cited in Saez, 1994). Some fifty years later, Bouin and Ancel (1903) suggested that the masculinizing factor, described by Berthold, originated from Leydig cells. Moreover, they offered convincing evidence that Leydig cells provided the hormonal stimulus for the production of sperm and for the maintenance of secondary sexual characteristics (Bouin and Ancel, 1903; cited in Bremner, 1981).

While testosterone was isolated in 1935, the steroidogenic function of Leydig cells could only be confirmed following the development of experiments performed on isolated testicular compartments (seminiferous tubules and interstitial tissue). By the late 1960s and early 1970s, the main hormonal interactions forming the hypothalamus-pituitary-testis axis, as well as the principal biosynthetic and metabolic pathway for steroids in Leydig cells, had been delineated. Since then, researchers have mainly focused on studying the endocrine, paracrine and autocrine regulation of Leydig cell functions (reviewed in Saez, 1994).

Although Leydig cell lines are now available, primary cultures are still indispensable. While culture conditions have been studied and improved considerably in the past decade, Leydig cell death *in vitro*, which remains inevitable, has not been studied in relation to steroidogenic activity. The main objective of this research project was to establish, in primary cultures of rat Leydig cells, some basic relations between the onset of apoptosis, steroidogenesis, and the culture conditions.

1.2. General Structure of the Testis

Anatomically, the testes are divided into two compartments, the interstitium and the seminiferous tubules (Figure 1). While the seminiferous tubules are responsible for the production and transport of sperm to the excretory ducts, the interstitium, more specifically the interstitial cells of Leydig, is responsible for the production of reproductive hormones. Intricately related, spermatogenesis and steroidogenesis are essential for the maintenance of normal reproductive functions in adult males.

a. The Seminiferous Tubules

The seminiferous tubule compartment is avascular and mainly composed of differentiating germ cells, in various stages of the spermatogenic cycle, engulfed in between Sertoli cells (Figure 1). The tubules so formed are wrapped into peritubular myoid cells and a basement membrane. Tight junctions, found in between Sertoli cells, as well as in between peritubular myoid cells, form the blood-testis barrier at the base of the tubules (Denef, 1998; Jegou, 1992). This permeable, but highly selective barrier, is essential for the isolation of meiotic and postmeiotic germ cells from the interstitium, premeiotic germ cells and potentially harmful blood borne agents (Jegou, 1992). Moreover, the blood-testis barrier induces the polarization of the Sertoli cells and is necessary for the creation of the specific microenvironment required for normal meiosis and spermiogenesis (Jegou, 1992). As male primordial germ cells differentiate into spermatozoa, they migrate

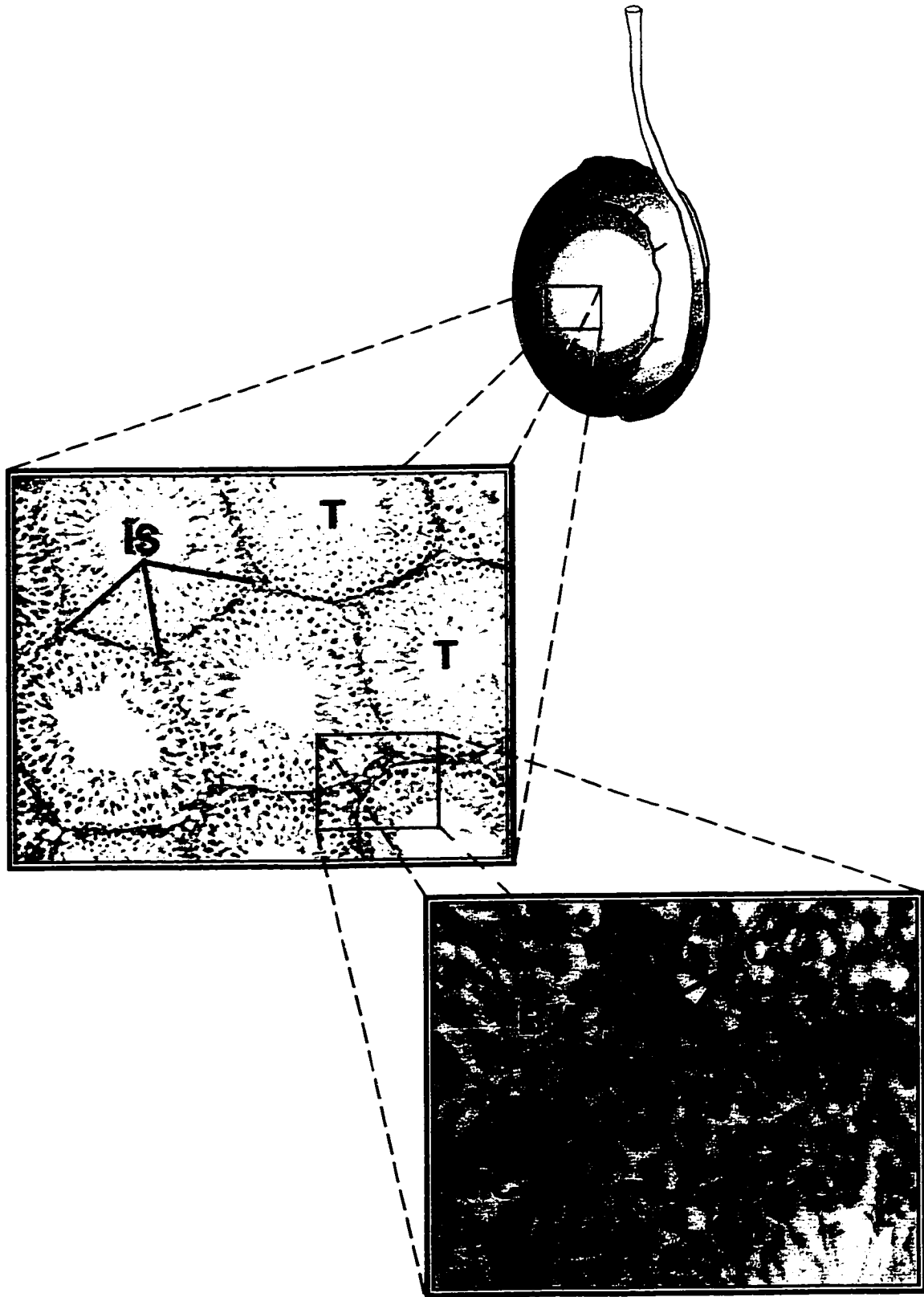


Figure 1. Intratesticular morphology of the testis. Interstitial space (IS), Seminiferous Tubules (T), Leydig cells (LC), Spermatids (Sp), Myoid cells (MC) and Blood vessels (BV)

from the outermost layer of the seminiferous tubule to the lumen, where they are eventually released.

In rats, about 80% of the testicular mass consists of highly coiled seminiferous tubules which can represent, once stretched, as much as 12 m of tubules per gram of testis (Hall, 1994).

b. The Interstitium

The interstitial tissue fills up the space in between seminiferous tubules. It is mainly composed of Leydig cells, macrophages, fibroblasts, myoid cells, lymphocytes, and plasma cells. The interstitium also nests blood and lymph vessels as well as nerves of the testicular parenchyma (Figure 1).

In testes, the interstitium volume is species specific and varies as a function of steroidogenic activity. In rats, the interstitium represents approximately 15% of the testis (Hall, 1994). Among interstitial cells, the adult population of Leydig cells, estimated between 25-34 million Leydig cell per testis in rats, occupy up to 49% of the interstitial space (Duckett *et al.*, 1997; Hardy *et al.*, 1989)

While little is known of the functions of the other cell types in the interstitial space, Leydig cells have been thoroughly studied. Leydig cells are responsible for the synthesis and secretion of most of the testicular androgens, with testosterone being, by far, the most important. Hormones released by the interstitial cells of Leydig are crucial for the regulation and maintenance of sperm production (spermatogenesis) and secondary sex characteristics in adult males.

c. Cell-Cell Contact in the Testis

The anatomical organization of the testis with two distinct compartments, one of which is avascular and separated from the other by a selective blood barrier, suggests the presence of active interaction between the different testicular cells and compartments (Saez, 1994). In the testes, cell communication is now known to involve both paracrine factors released in the extracellular space and affecting nearby cells, and cell-cell contact via intercellular junctions (Peluso *et al.*, 1996; Perez Armendariz *et al.*, 1994; Saez, 1994; Varanda and de Carvalho, 1994). The later have been shown to be an important prerequisite for steroidogenesis, spermatogenesis and sperm maturation (Miller *et al.*, 1983; Saez, 1994).

In vivo and *in vitro* immunolabeling studies have revealed the abundant expression of connexin-43 (Cx43), a typical marker for gap junctions, in testes. More specifically, gap junctions were localized between Leydig cells and/or macrophages in the interstitium as well as between Sertoli cells in the seminiferous tubules (Perez Armendariz *et al.*, 1994; Risley *et al.*, 1992; Saez, 1994; You *et al.*, 1998). Gap junctions are intercellular protein channels which provide a pathway for the exchange of ions and small molecules (M.W. <1000), such as cAMP and calcium (You *et al.*, 1998). This exchange of material allows metabolic coupling of cells. The study of Cx43 gene expression and regulation in rat Leydig cells revealed the time- and dose-dependent inhibitory effect of hCG on Cx43 mRNA. Therefore, while hCG was shown to increase Leydig cell steroidogenesis, it

inhibited Cx43 mRNA expression (You *et al.*, 1998). Moreover, cell adhesion molecules (CAMs) such as N-CAM, E-cadherin and N-cadherin were also shown to be expressed in Leydig cells (Byers *et al.*, 1994; Denduchis *et al.*, 1996; Peluso *et al.*, 1996). Cadherins are cell surface proteins which mediate intercellular calcium-dependent adhesion forming homotypic cell interactions in most epithelia.

Recently, Hutson (1992) demonstrated that, in Leydig cells, intercellular junctions do not form until postnatal day 30, just prior to the major increase in rat Leydig cell secretory activity associated with the onset of puberty (Hutson, 1992). These developments in the study of intercellular junctions in the testis suggest that paracrine factors, produced and secreted by Leydig cells, Sertoli cells, germ cells and/or macrophages, are actively involved in cross-talking among testicular cells. Intercellular communication in the testes is therefore believed to be essential for the precise regulation of normal testicular functions including cell proliferation and differentiation, Leydig and Sertoli cell metabolism as well as transport of binding proteins (Saez, 1994).

1.3. Leydig Cell Ontogeny and Life Cycle

In mammals, Leydig cell ontogenesis involves the generation of at least two distinct cell populations: the fetal-type and adult-type Leydig cells. Although both populations release testosterone as their main secretory product, they differ in a number of features such as morphology, hormone production, tropic and paracrine regulation, and physiological functions (reviewed in Huhtaniemi, 1994; Huhtaniemi

and Pelliniemi, 1992). The following sections will briefly present some basic facts about fetal- and adult-type Leydig cell ontogenesis and life cycle.

a. Fetal-type Leydig Cells

In the embryo, fetal-type Leydig cells differentiate relatively late in the course of testis formation. In the rat primordial gonads, the first sign of gonadal sex differentiation appears on embryonic day 13, as the formation of the seminiferous cords is initiated. Within 24 h, newly differentiated Sertoli cells, aggregating and surrounding the germ cells, along with peritubular myoid cells, slowly build the seminiferous tubules (Hall, 1994; Huhtaniemi, 1994; Huhtaniemi and Pelliniemi, 1992).

Soon after, on embryonic day 15, fetal Leydig cells begin to differentiate in the rat testicular interstitium. Fetal-type Leydig cells are believed to originate from a population of undifferentiated interstitial mesenchymal cells (Huhtaniemi, 1994; Huhtaniemi and Pelliniemi, 1992). In the rat, the number of fetal Leydig cells increases from 0.25×10^5 cells per testis on prenatal day 16.5 to a maximum of 10^5 cells per testis on prenatal day 20.5 (Kerr and Knell, 1988; Tapanainen *et al.*, 1984). Mitotic cell labeling studies, used to assess the importance of proliferation in the generation of a cell population, showed that fetal-type Leydig cells remain unlabeled following injections of [3 H] thymidine (Saez, 1994). These results suggested that the fetal-type Leydig cell population have no or very low mitotic activity and must, therefore, almost exclusively originate from primordial cell

differentiation.

As early as fetal day 15.5, the steroidogenic enzyme system becomes functional in Leydig cells and the testis begins to secrete increasing amounts of testosterone (Huhtaniemi, 1994; Huhtaniemi and Pelliniemi, 1992). The net increase in testicular steroidogenesis from day 15.5 to birth is mostly the result of the increasing number of differentiating Leydig cells. From day 18.5 to the end of the fetal life (day 21.5), the amount of testosterone contained in the testis *in vivo*, or secreted by the testis *in vitro*, remains constant or decreases (Saez, 1994; Tapanainen *et al.*, 1984). Since the number of Leydig cells increases between days 18.5 and 20.5, this temporal change in testicular steroidogenesis suggests that the steroidogenic activity of individual Leydig cells decreases.

Fetal-type Leydig cell volume clearly decreases during the first and the second postnatal weeks (Kerr and Knell, 1988; Kuopio *et al.*, 1989; Mendis Handagama *et al.*, 1987; Zirkin and Ewing, 1987), beyond that period, their fate is still uncertain. Histological and ultrastructural observations suggest that they either degenerate through physiological cell death (Kuopio *et al.*, 1989; Prince, 1990) or are transformed into adult-type Leydig cells (Mendis Handagama *et al.*, 1987). However, the suggestion that fetal-type Leydig cells persist as a stable population throughout the whole postnatal life (Kerr and Knell, 1988) is the most widely accepted hypothesis. It must be kept in mind that, according to the latter suggestion, fetal-type Leydig cells would represent less than 0.4% of the total

Leydig cell population since the adult testis contains approximately 25 to 34 million Leydig cells (Kerr and Knell, 1988).

b. Adult-type Leydig Cells

Adult-type Leydig cells can be identified in the interstitium by postnatal day 10 in the newborn rat. Their number increases significantly following day 14, to reach 25 to 34 million per testis by the end of puberty (day 56 postpartum) (Hardy *et al.*, 1989; Saez, 1994).

The adult-type Leydig cell population is believed to arise from a combination of mesenchymal cell differentiation (Fouquet and Kann, 1987; Hardy *et al.*, 1989; Mendis Handagama *et al.*, 1987; Tapanainen *et al.*, 1984) and mitotic division of newly differentiated Leydig cells (Fouquet and Kann, 1987; Hardy *et al.*, 1989). In support of this hypothesis, kinetic studies and [³H]thymidine pulse-chase labeling of interstitial cells in prepubertal rats revealed a proportional decrease of the mesenchymal cell population, between postnatal day 2 and 56, associated with an increasing Leydig cell population (Hardy *et al.*, 1989). According to Hardy *et al.* (1989), this reciprocal pattern was indicative of a precursor-product relationship between the two cell types. Interestingly, differentiation of mesenchymal cells into Leydig cells could not solely account for the rapid rise of the steroidogenic cell population following day 28 postpartum. Distribution of [³H]thymidine, following pulse-chase studies, suggested that Leydig cells, newly differentiated from mesenchymal cells during days 14 to 28 postpartum, must divide once or twice

between days 28 to 70 postpartum to complete the adult-type Leydig cell population (Fouquet and Kann, 1987; Hardy *et al.*, 1989).

It is generally accepted that, once full sexual maturity is achieved, adult Leydig cells form a static cell population (Saez, 1994; Teerds *et al.*, 1989c). Accordingly, under normal physiological conditions, adult-type Leydig cells are virtually never found in mitosis nor undergoing physiological cell death. Histological and pulse-chase studies have nevertheless demonstrated the proliferative ability of adult-type Leydig cells (Fouquet and Kann, 1987; Mendis Handagama, 1991; Teerds *et al.*, 1989b). The incidence of mitosis in Leydig cells in the adult testis is extremely rare, the turnover rate has been estimated to be anywhere from 142 to 2823 days (more than 7 years) by pulse-chase studies (Teerds *et al.*, 1989b). Similarly, a second study evaluated Leydig cell mitosis rate at around 1 in 17,500 Leydig cells in guinea pigs (Mendis Handagama, 1991). However, in adult rats, new Leydig cells can be formed at a higher rate under specific conditions. For example, chronic hCG treatment stimulates both proliferation of Leydig cells and development of new Leydig cells from precursor cells (Christensen and Peacock, 1980; Mendis Handagama, 1997).

1.4. Testicular Steroid Synthesis

Men are men because of testosterone. The effects of this hormone are evident in virtually every organ or tissue in normal adult men. In males, androgens are responsible, among other functions, for the development and maintenance of

internal and external genitalia, appearance of secondary sexual characteristics, development of the musculo-skeletal system and feedback inhibition on the hypothalamo-pituitary axis. However, the primary role of testosterone is not endocrine but paracrine, as it is the local stimulatory action of testosterone on spermatogenesis which determines fertility (Sharpe, 1990). In the fetus, androgens are essential for morphogenesis of normal masculinization of the primary sex characteristics (Huhtaniemi and Pelliniemi, 1992; Saez, 1994) and for the onset of spermatogenesis. In the adult, however, they function more in the maintenance of male sexual characteristics (Huhtaniemi, 1994).

In testes, androgen production takes place almost exclusively in Leydig cells. The principal steroids secreted by the testis are androgens, with testosterone being the most abundant secretory product. Androstenedione and dihydroepiandrosterone are also secreted by the testis but, in comparison to testosterone, show lesser degrees of androgenicity (Hall, 1994). Besides the production of the above androgens, Leydig cells can also convert testosterone further, although in small amounts, to another potent androgen, 5α -dihydrotestosterone (5α -DHT), and to the female sex hormone, estradiol (Hall, 1994). Although the seminiferous tubules, more specifically Sertoli cells, have been shown to secrete some androgens and estradiol, their contribution is of minor importance (Hall, 1994).

a. Steroidogenic Substrate

The synthesis of all steroid hormones requires a substrate providing a steroid ring backbone. In Leydig cells, as in all steroidogenic tissues, cholesterol is the main substrate (Figure 2.a.). Cholesterol, can be either synthesized *de novo* from acetyl CoA or derived from the plasma pool by receptor-mediated endocytosis of low-density lipoprotein particles. In either case, the precursor molecules are stored as esterified steroids in cytoplasmic lipid droplets. Both sources of cholesterol seem to be used during steroidogenesis (Hall, 1994). Moreover, the relative contribution of each of the two sources of cholesterol varies according to cell type, species, physiological state and substrate availability.

b. Steroidogenic Pathway

The conversion of cholesterol (C27) to testosterone (C19) involves a cascade of hydroxylation (C17, 20, 22), cleavage (C20-22, C17-20), dehydrogenation (3β , 17β), and isomerization Δ^{4-5} reactions. The pathway of testosterone synthesis from cholesterol and the conversion of testosterone to active androgen and estrogen metabolites is depicted in Figure 2.b.

Testosterone production in Leydig cells begins with luteinizing hormone (LH) binding to its plasma membrane receptor. Following intracellular second messenger system activation, LH binding promotes the translocation of cytoplasmic cholesterol to the inner mitochondrial membrane (Figure 2.b.). There, cholesterol is converted to pregnenolone (Simpson, 1979). This conversion is completed once

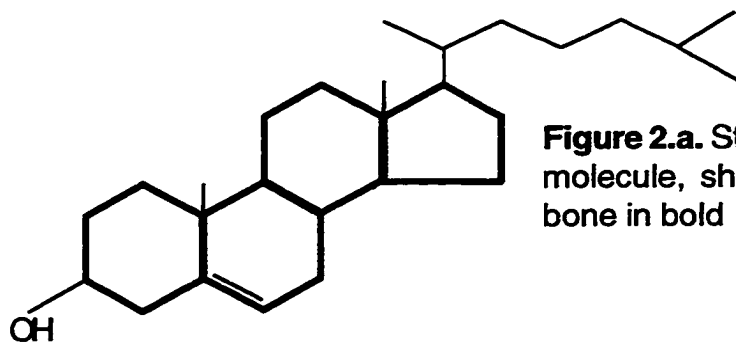


Figure 2.a. Structure of the cholesterol molecule, showing the steroid backbone in bold

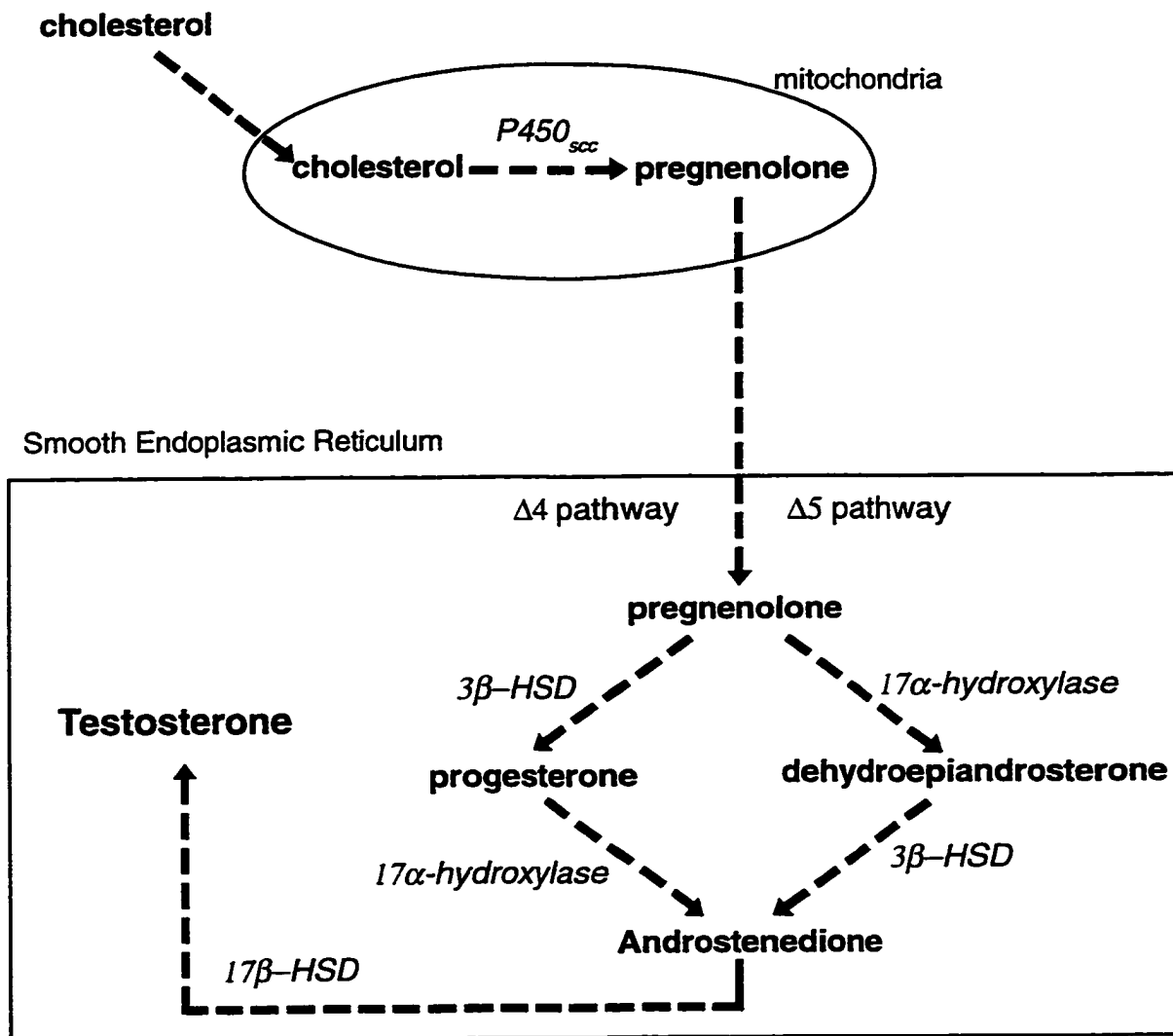


Figure 2.b. Steroidogenic pathway. Note that steroidogenic enzymes appear in *italic*

part of the cholesterol side chain (C21-27) is cleaved in a multistep reaction catalyzed by one enzymatic system, the cytochrome P-450 side-chain cleavage enzyme (P450_{scc}) system. This enzymatic system is composed of the cytochrome P450_{scc} enzyme, adrenodoxin, adrenodoxin reductase, and molecular oxygen (Simpson, 1979). In order to cleave the cholesterol side chain, P450_{scc} sequentially hydroxylates cholesterol at C22 and C20 to produce 22R-hydroxycholesterol and 20 α ,22R-dihydroxycholesterol, respectively (Burstein and Gut, 1976; Dixon *et al.*, 1970). Then, cytochrome P450_{scc} cleaves the 20,22-dihydroxycholesterol side chain to release pregnenolone, a 21 carbon compound, and isocaproic-aldehyde, a 6 carbon by-product. The high affinity of P-450_{scc} for 22R-hydroxycholesterol and 20,22-dihydroxycholesterol, along with the very high conversion rate, prevent the accumulation of these cholesterol intermediates in the mitochondria. Moreover, cholesterol intermediates remain tightly bound to the substrate binding site on P450_{scc} until the cholesterol side chain is cleaved (Orme-Johnson *et al.*, 1979). Pregnenolone then dissociates from the enzyme active site, and the whole process starts again with the binding of a new cholesterol molecule.

Meanwhile, the pregnenolone so released rapidly reaches the smooth endoplasmic reticulum (SER) where androgen synthesis is completed (Figure 2.b.). In the SER, three enzymatic reactions, catalysed by 3 β -hydroxysteroid dehydrogenase (3 β -HSD), P-450 17 α -hydroxylase/ lyase and 17 β -hydroxysteroid dehydrogenase (17 β -HSD), take place. The biosynthetic pathway of androgen formation from pregnenolone can follow either the Δ^5 - or Δ^4 -pathways depending

on whether 3β -HSD or P-450 17α -hydroxylase/ lyase acts first (Figure 2.b.). For example, in the rat, the Δ^4 - pathway is preferentially if not exclusively used (Browning *et al.*, 1982), while in pigs, rabbits and dogs the Δ^5 - pathway is used to varying degrees (reviewed in Hall, 1994). The choice of pathway is not random and could be determined by the arrangement and affinity of the steroidogenic enzymes in the SER membrane.

In rat SER, pregnenolone is therefore preferentially converted to progesterone, by 3β -HSD and then to androstenedione, by P-450 17α -hydroxylase /lyase (Figure 2.b.). 3β -HSD acts both as a dehydrogenase and an isomerase since it oxidizes the A ring 3β hydroxyl group to a ketone and rearranges the double bond at C5. The progesterone so formed must lose two more carbons to generate androgens (C19). Similar to the action of P-450_{scc}, P-450 17α -hydroxylase/lyase first catalyzes progesterone hydroxylation on C17 to generate 17α -hydroxyprogesterone and then cleaves the C17-C20 bond to finally release androstenedione. Recent evidence indicates that, like P450_{scc}, the 17α -hydroxylase and 17,20-desmolase activities are accomplished by a single cytochrome P450 and that a single active site is required for the two reactions to occur (Hall, 1994; Saez, 1994)

Finally, 17β -HSD catalyzes the interconversion of androstenedione to testosterone. This reaction is freely reversible. The enzyme possesses two substrate binding sites, one for reductase activity and the other for oxidase activity. Therefore, 17β -HSD activity *in situ* may be greatly influenced by the availability of

the two substrates and by the rate of secretory product removal (Hall, 1994).

Decreased serum testosterone levels with age have been reported in rats. This has been explained by the decreasing steroidogenic capacities of Leydig cells (Lin *et al.*, 1983). However, other studies have shown that it was not the case (Schafer *et al.*, 1982).

1.5. Regulation of Androgen Synthesis

In adults, Leydig cell steroidogenesis is primarily controlled by the gonadotropin luteinizing hormone (LH). In the absence of LH tropic action, significant production of testosterone is not possible and the testes start to degenerate (Reviewed in Saez, 1994). However, while LH is the main stimulator of steroidogenesis, several other blood-borne hormones, and intratesticular paracrine, autocrine and intracrine factors, modulate LH action, often without much influence on their own (Huhtaniemi and Toppari, 1995). Recent studies suggest that, under physiologically normal hormonal stimulation, intratesticular paracrine, autocrine and intracrine factors may play a key role in the fine tuning of LH stimulation of Leydig cell steroidogenesis (Saez *et al.*, 1991; Skinner, 1991; Skinner *et al.*, 1991).

a. LH Regulation of Testicular Steroidogenesis

LH is secreted in a pulsatile fashion by the adenohypophysis (Dufau *et al.*, 1984). In the testis, LH binds to the LH/hCG plasma membrane receptor, a

member of the seven span plasma membrane receptor family. Similar to the other members of this receptor family, LH binding to its receptor initiates the sequential activation of a cascade (Figure 3), beginning with G_s , and followed sequentially by adenylyl cyclase, cyclic AMP, and protein kinase A (PKA). In turn, PKA induces serine/threonine phosphorylation of a variety of proteins which, in turn, stimulate Leydig cells steroidogenesis. Other Leydig cell functions, such as cholesterol transfer from the cytoplasmic pool to the inner mitochondrial membrane and increased gene transcription of steroidogenic enzymes, are also stimulated (Kumar *et al.*, 1994).

Although the role of cAMP as the second messenger of LH is not challenged, there is increasing evidence that this second messenger does not explain all LH actions. In view of the putative ability of one receptor to use multiple signal transduction systems, other second messengers such as intracellular free calcium and protein kinase C (PKC) may also be involved. More precisely, the study of cytosolic free calcium changes in LH/hCG stimulated Leydig cells have revealed the importance of calcium as a second messenger system (Kumar *et al.*, 1994). This investigation allowed Kumar and colleagues to conclude that binding of hCG to its receptors would transmit the signal through G proteins to adenylyl cyclase to increase cAMP which would increase Ca^{2+} influx into cytosol across plasma membrane Ca^{2+} channels. Therefore, it would appear that the primary action of hCG is to increase cytosolic cAMP which would then regulate intracellular calcium concentrations as well as steroidogenesis (Kumar *et al.*, 1994). Chloride

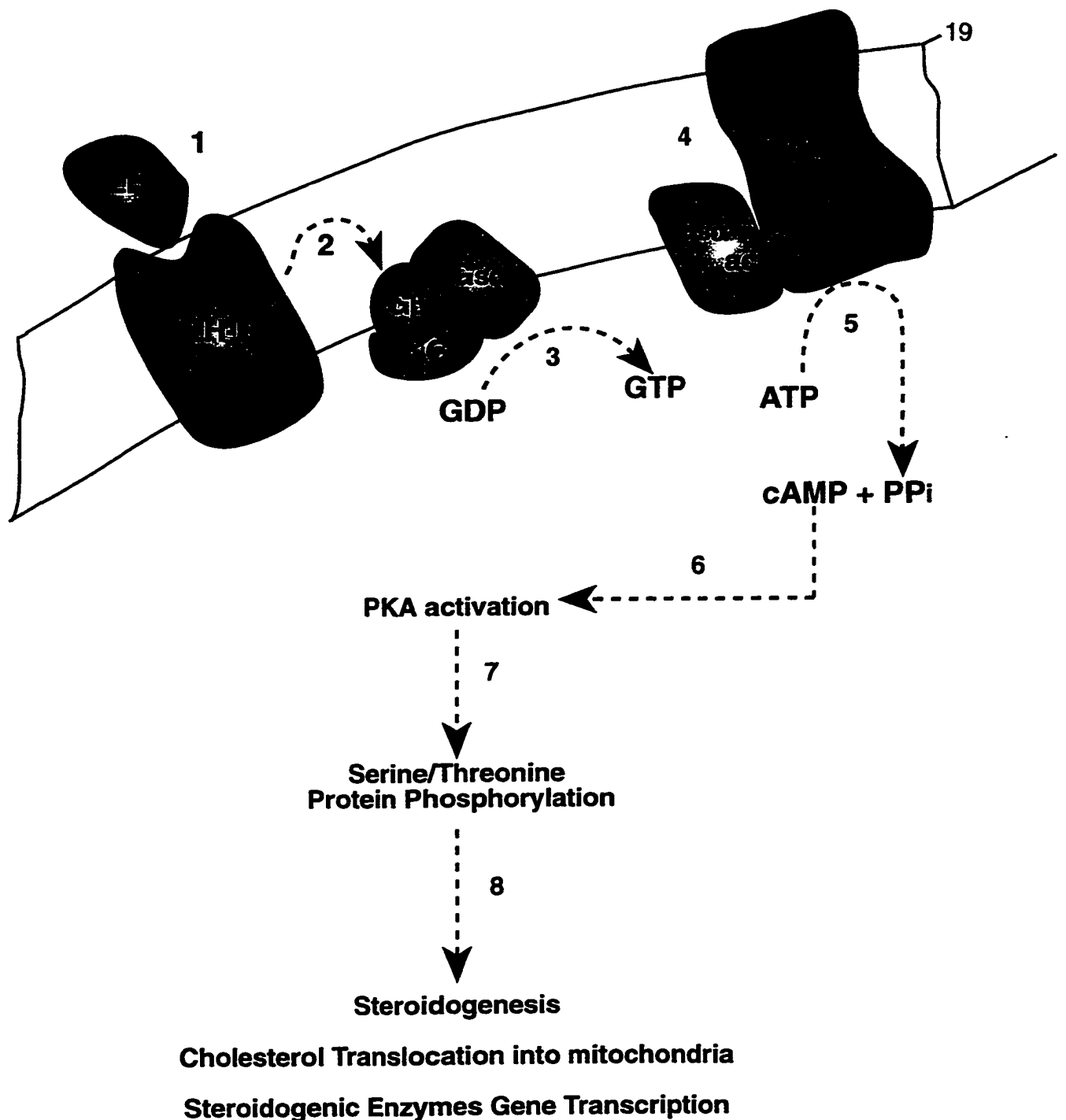


Figure 3. The mechanism of LH action on Leydig cells begins with LH binding to a specific plasma membrane receptor (1). The LH ligand-receptor complex then interact with the G protein complex (2). Following activation of the G_{α} unit (3) adenylyl cyclase (AC) can rapidly be activated (4). AC activation induces ATP dephosphorylation to cAMP (5). The interaction between newly formed cAMP-dependent protein kinase A (PKA) leads to the activation of PKA regulatory subunit (6). PKA then phosphorylates proteins and transcription factors regulating steroidogenesis (7-8).

channels and cGMP are also believed to modulate steroidogenesis in Leydig cells (Hall, 1994; Huhtaniemi and Toppari, 1995). The activation of alternative transduction systems following LH binding might be important especially when the LH concentration is low in the testes, for example, between LH pulses (Hall, 1994; Huhtaniemi and Toppari, 1995; Saez, 1994). Finally, active crosstalking between these signal transduction systems undoubtedly play an important role in LH action modulation.

b. Effects of Hormones Other than LH on Leydig Cell Function

An increasing body of evidence suggests a role for hormones, other than LH, in the physiological regulation of Leydig cell function. Among the most potent candidates, we find prolactin (Prl), growth hormone (GH), insulin and steroid hormones (reviewed in Huhtaniemi and Toppari, 1995). However, whether these factors should be qualified as endocrine or paracrine remain to be established since recent studies showed that some of these extragonadal hormones can also be locally produced in the testes (Saez, 1994).

First, prolactin has been shown to upregulate Leydig cell function in several species, including rodents (reviewed in Huhtaniemi and Toppari, 1995), both *in vitro* and *in vivo*. At physiological concentrations, Prl significantly amplifies the response of rat Leydig cells to LH stimulation. However, supraphysiological doses of Prl can inhibit steroidogenesis by blocking progesterone metabolism.

The pituitary GH plays a critical role in Leydig cell function, especially during

pubertal maturation. GH deficiency and/or resistance is associated with delayed puberty and/or poor Leydig cell response to LH/hCG (Chatelain *et al.*, 1991). GH stimulation of steroidogenesis is mediated by insulin-like growth factor IGF-1 (Lin *et al.*, 1987).

In Leydig cells, the pancreatic hormone insulin can stimulate both basal and LH-stimulated steroidogenesis (Rigaudiere *et al.*, 1988). Interestingly, both insulin and LH reciprocally up-regulate their receptors (Abele *et al.*, 1986).

Leydig cell function is also modulated by glucocorticoids. Glucocorticoids of adrenal origin have direct inhibitory effects on Leydig cell steroidogenesis. The inhibitory action of glucocorticoids is mediated, at least partly, through inhibition of the P450 cholesterol side chain cleavage (Hales and Payne, 1989) and 3β -HSD (Payne and Sha, 1991; Srivastava *et al.*, 1993) enzyme activity. Stress and other conditions that elevate levels of circulating glucocorticoids lead to depressed serum testosterone in all known animal species (reviewed in Gao *et al.*, 1996). Leydig cells are the primary targets of glucocorticoid action in the testis through glucocorticoid receptors expressed in these cells. Besides their inhibitory action on Leydig cell steroidogenesis, glucocorticoids, and more specifically corticosterone, induce intracellular 11β -HSD activity. This enzyme inactivates glucocorticoid by converting corticosterone to 11-dehydrocorticosterone, thereby protecting the testes against corticosterone-mediated inhibition of testosterone production (Gao *et al.*, 1996).

c. Paracrine and Autocrine Modulation of Steroidogenesis

In the late 1970's, Aoki and Fawcet (1978) reported that testicular implants of anti-androgens caused localized disruption of the rat seminiferous epithelium which was accompanied by hypertrophy of the adjacent Leydig cells. These authors proposed that cells of the seminiferous epithelium could produce either stimulatory or inhibitory factors that acted on Leydig cells. Today, a growing number of intratesticular factors are suspected of playing an important role in the local regulation of testicular function (reviewed in Deneff, 1998; Saez, 1994).

Among these, locally produced steroids, estrogens and androgens are believed to modulate Leydig cell steroidogenic activity as well as Sertoli and peritubular myoid cell function. The presence of androgen receptors in these cells supports their involvement in the androgenic control of spermatogenesis. (Vornberger *et al.*, 1994). While sex steroids can exert paracrine or autocrine effects in the testes, the physiological importance of such effects remain to be established.

Several growth factors, including epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), IGF-1, transforming growth factor- α and - β (TGF- α , TGF- β) and tumor necrosis factor (TNF) have been shown to be produced in the testis and play a role in the regulation of Leydig cell function (reviewed in Saez, 1994; Skinner, 1991; Chuzel *et al.*, 1996). Moreover, the presence of growth factor receptors in Leydig cells, Sertoli cells, macrophages and peritubular myoid cells support a putative role for these growth factors in the regulation of

testicular functions (reviewed in Saez, 1994; Saez *et al.*, 1991; Saez *et al.*, 1989; Huhtaniemi and Toppari, 1995; Risbridger, 1992). Recently, Chuzel and co-workers (1996) demonstrated by nuclear run-on assays the long term inhibitory (EGF, bFGF, TGF β 1) or stimulatory (IGF-1) effects transduced by these growth factors. Their results supported a role for growth factors in the modulation of the transcription rates of genes for gonadotropin receptors, as well as for cytochrome P-450_{scc} and 17 α -hydroxylase enzymes.

However the physiological importance of the paracrine and modulatory influences of the different hormones and growth factors still remains controversial mostly because of conflicting results obtained from *in vitro* studies.

1.6. What Is The link Between Leydig Cells And Apoptosis?

As mentioned earlier, LH is believed to stimulate Leydig cell steroidogenesis mainly via the cyclic AMP second messenger system, which, in turn, results in serine/threonine phosphorylation of a variety of proteins. In an effort to elucidate the sequence of events involved in the termination of this signal, protein dephosphorylation was studied in this laboratory. In this regard, the ubiquitous PP-1 and PP-2 phosphoprotein phosphatases were believed to play a key role in this process. Therefore, PP-1/2A inhibitors calyculin A (Caly) and okadaic acid (Oka) treatments on primary cultures of rat Leydig cells were used to further examine the role of the phosphatases in Leydig cell steroidogenesis (Moger, 1992). Results showed that when Leydig cells were incubated along with a maximum stimulatory

concentration of LH and PP inhibitors, androgen production was inhibited to control levels in a dose-dependent manner. The potential toxic effect of Caly and Oka were also studied on isolated Leydig cell DNA. Interestingly, electrophoresis studies revealed the presence of DNA fragmentation in control cells, following 24 h culture, as well as in Caly treated cells, following 6 h culture. The distinctive DNA fragmentation pattern observed following electrophoresis, is recognized as a hallmark of cells undergoing physiological cell death, or apoptosis.

In view of the relative absence of physiological cell death in Leydig cells *in vivo*, these results stimulated our curiosity. In an effort to better understand how physiological cell death is initiated and unfolds in primary cultures of rat Leydig cells, we have specifically pursued the study of apoptosis in this cell model.

II. APOPTOSIS: A PROCESS OF PHYSIOLOGICAL CELL DEATH

II.1. The Study of Cell Death : A Historical Perspective

Until recently, the study of cell death had aroused far less interest in the scientific community than the study of cell proliferation and differentiation. This relative neglect was probably the result of a misconception since, until less than thirty years ago, cell death was considered as a degenerative phenomenon produced by injury (Wyllie *et al.*, 1980). Although morphological descriptions of physiological cell death had been reported, for example by Flemming (1885) (cited in Hsu and Hsueh, 1998) in ovarian follicles and Glücksmann (1951) (cited in Wyllie *et al.*, 1980) in embryonic morphogenesis, the concept of controlled cell death was not recognized in the scientific community.

A new era in the study of physiological cell death began with the work of Kerr, Wyllie, Searle and Currie (Kerr *et al.*, 1972; Kerr and Searle, 1973). Based on their work and reports from other investigators (reviewed in Kerr *et al.*, 1972; Wyllie *et al.*, 1980) these scientists proposed the existence of a type of cell death morphologically distinct from necrosis. On Professor J. Cormack's (Department of Greek, University of Aberdeen, Scotland) suggestion, Kerr and colleagues named this process of physiological cell death "apoptosis" (Kerr *et al.*, 1972). In Greek, the word apoptosis describes the "dropping off" or "falling off" of individual petals from flowers, or leaves from trees.

While very little attention had been devoted to the study of physiological cell

death until the early 1970s, this new "apoptotic cell death" concept opened the way for a new field in basic research. In recent years, an exponentially growing interest for the study of physiological cell death has been observed, resulting in a better understanding of tissue homeostasis.

II. 2. Morphological Characteristics Associated with Apoptosis

By definition, apoptosis refers to a sequence of morphological changes that are clearly different from those seen in necrosis (Kerr *et al.*, 1972; Searle *et al.*, 1975). Whereas necrosis is caused by physical, chemical, or osmotic damage with consecutive disruption of internal and external membranes, apoptosis involves the active participation of endogenous cellular enzymes in the mediation of cell death, which occurs well before membranes lose their integrity (Bursch *et al.*, 1990a; Kroemer *et al.*, 1995). Physiological cell death is a unique feature of multicellular organisms. Moreover, it is essential for tissue renewal and maintenance of tissue homeostasis as it creates a balance between cell death and cell proliferation (Raff, 1992; Schwartzman and Cidlowski, 1991; Wyllie *et al.*, 1980). The physiological relevance of apoptosis is underscored by its description in all types of higher eukaryotes, namely, plants, molds, nematodes, insects and vertebrates (reviewed in Vaux *et al.*, 1994).

Morphologically, examination of necrotic tissues usually reveals early changes in mitochondrial shape, extensive cellular swelling and subsequent membrane damage. These changes soon result in cell disintegration inducing

spilling of the cell content into the surrounding extracellular space. The necrotic cell content leaking into the extracellular space rapidly triggers an acute inflammatory response which, in turn, promotes cell debris clearance by phagocytic cells (Wyllie *et al.*, 1980). Because injuries of this type are usually extensive, involving millions of cells simultaneously, the inflammatory response is necessary to rapidly clear away necrotic cell debris and to allow tissue repair (Cohen, 1996; Wyllie *et al.*, 1980).

In contrast, apoptosis, which generally occurs spontaneously in normal tissue, typically affects scattered single cells in an asynchronous fashion. Morphologically, the earliest evidence of the onset of apoptosis is usually found in the nucleus. Early apoptosis can be characterized by compaction and segregation of chromatin in sharply circumscribed masses which aggregate on the inner surface of the nuclear envelope. Concomitant with these early nuclear changes, cells committed to apoptosis rapidly begin to present overall condensation and promptly separate from their neighboring cells. Moreover, the apoptotic cells lose their cell membrane specializations, such as microvilli, if those were originally present in the healthy cells (Kerr *et al.*, 1972; Searle *et al.*, 1982; Wyllie *et al.*, 1980). The cell undergoing apoptosis can lose up to a third of its volume. During the early phases of apoptosis, organelle condensation is associated with the maintenance of their morphological and functional integrity (Kerr *et al.*, 1995; Wyllie *et al.*, 1980).

Cytoplasmic condensation is usually associated with extensive protrusion

of the cell surface. This phenomenon of plasma membrane ruffling and blebbing has been called "zeiosis" (Kerr *et al.*, 1972). The zeiotic protuberances formed at the surface of the cell soon break up into apoptotic bodies. Apoptotic bodies separate from the bulk of the apoptotic cell by sealing the plasmalemma to produce membrane-bounded vesicles of varying size and composition. Characteristically, apoptotic bodies maintain an intact and energy-dependent osmotic gradient preventing any cellular leakage (Kerr *et al.*, 1972).

In vivo, apoptotic bodies are rapidly phagocytized by nearby cells and resident macrophages (Searle *et al.*, 1975). Following phagocytosis, apoptotic bodies are degraded by lysosomic enzymes derived from the cells in which they lie. Until lysosomic degradation, the preservation of organelle integrity and absence of apoptotic cellular leakage prevents the onset of any inflammatory response in the surrounding tissue (Cohen, 1996; Wyllie *et al.*, 1980).

Cells growing in monolayer cultures tend to become detached and to float into the medium when they undergo apoptosis. Contrary to the *in vivo* situation, apoptotic bodies formed *in vitro* frequently escape phagocytosis and spontaneously degenerate in the culture media within a few hours. Therefore, when studying apoptosis *in vitro*, researchers must fix cells in such a way to include in their sample the floating apoptotic cells. Exclusion of the detached cells from the sample would most likely result in an underestimation of the number of apoptotic cells (Kerr *et al.*, 1995). The study of apoptosis in cultured cells is therefore more accurate when cells are grown in suspension. In such cases, the culture media

retain every cell so that, following centrifugation, viable and apoptotic cells as well as apoptotic bodies can be collected and assayed for apoptotic markers (Kerr *et al.*, 1995).

Today, electron microscopy still provides the most reliable method to morphologically recognize apoptotic cells. However, this technique does not allow the study of biochemical events associated with the early phases of apoptosis. Recent advances in molecular biology have provided an array of new techniques, markers, and antibodies which facilitate the study and the early identification of apoptosis in normal and malignant tissues.

II. 3. Biochemical Characteristics Associated with Apoptosis

Most, if not all, of the biochemical characteristics associated with apoptotic cell death occur prior to the visible morphological changes described in the previous section. Among the many biochemical events involved with the regulation and execution of apoptosis, modulation of gene and protein expression, increase in cytosolic calcium concentrations, disruption of anabolic functions, activation of Ca^{2+} - Mg^{2+} endonucleases, and progressive degradation of essential macromolecules are the most prominent (Cohen, 1996; Kroemer *et al.*, 1995; Reed *et al.*, 1996). Despite their paramount importance, the molecular mechanisms regulating and executing apoptosis are poorly understood. Although several hypotheses have been studied, no definite answers are available at this time.

a. Apoptotic DNA Fragmentation

Within a few minutes following the biochemical onset of apoptotic cell death, nuclear DNA is promptly cleaved into nucleosome-size fragments (Wyllie, 1980). With over a million such breaks induced in a cell, the process soon becomes irreversible and gene transcription is interrupted (Cohen, 1996). Irreversible DNA fragmentation is rapidly associated with the morphological changes observed by microscopy (Bursch *et al.*, 1990a).

Although the biochemical sequence responsible for the initiation and execution of DNA fragmentation is still unknown, evidence suggests that specific proteases and endonucleases are primarily involved. According to Neamati *et al.* (1995), a possible sequence would involve the nuclear intermediate filament lamins and a two-step cleavage of genomic DNA. Lamins are known to provide a framework for the attachment of chromatin to the inner nuclear envelope. They bind to genomic DNA at specific matrix attachment regions regularly spaced every 20-50 kbp. During apoptosis, lamins are believed to be irreversibly disassembled by proteolytic cleavage. Consequently, as lamins dissociate from the DNA, they reveal matrix attachment regions to activated endonucleases which then cleave the DNA into large fragments (Neamati *et al.*, 1995).

Following the release of large DNA fragments of 50 to 300 kbp, endogenous Ca^{2+} , Mg^{2+} -dependent endonucleases, activated by the cell death program, would then be responsible for the extensive internucleosomal DNA cleavage. The specificity of these nucleases results in the formation of numerous DNA fragments

of oligonucleosomal size (180-200 bp lengths) (Figure 4.a). The DNA fragments so generated can be visualized as a distinctive ladder pattern on agarose gels following electrophoretic analysis (Wyllie *et al.*, 1980) (Figure 4.b). This characteristic ladder pattern appears to occur in virtually all systems in which apoptosis has been identified (Bursch *et al.*, 1990a; Kyprianou *et al.*, 1988).

Interestingly, although apoptotic DNA fragmentation and the development of the ladder pattern in agarose gels came to be regarded as a biochemical hallmark for this process, typical apoptosis can be observed in the absence of internucleosomal DNA cleavage (Cohen *et al.*, 1992; Jacobson *et al.*, 1994; Kroemer *et al.*, 1995).

b. Gene and Protein Expression Involved in the Regulation of Apoptosis

Apoptosis is tightly controlled by a number of genes that either promote or inhibit cell death. These regulatory genes were first discovered in the nematode *Caenorhabditis elegans* (*C.elegans*) where the developmental fate of every cell is genetically programmed. In *C. elegans*, the *ced-3* and *ced-4* genes are necessary for cell death to occur (Ellis and Horvitz, 1986; Yuan and Horvitz, 1990), while the *ced-9* gene is essential to protect cells from premature cell death (Hengartner *et al.*, 1992).

In mammalian systems, various families of genes, homologous to the programmed cell death regulating genes in *C. elegans*, have recently been

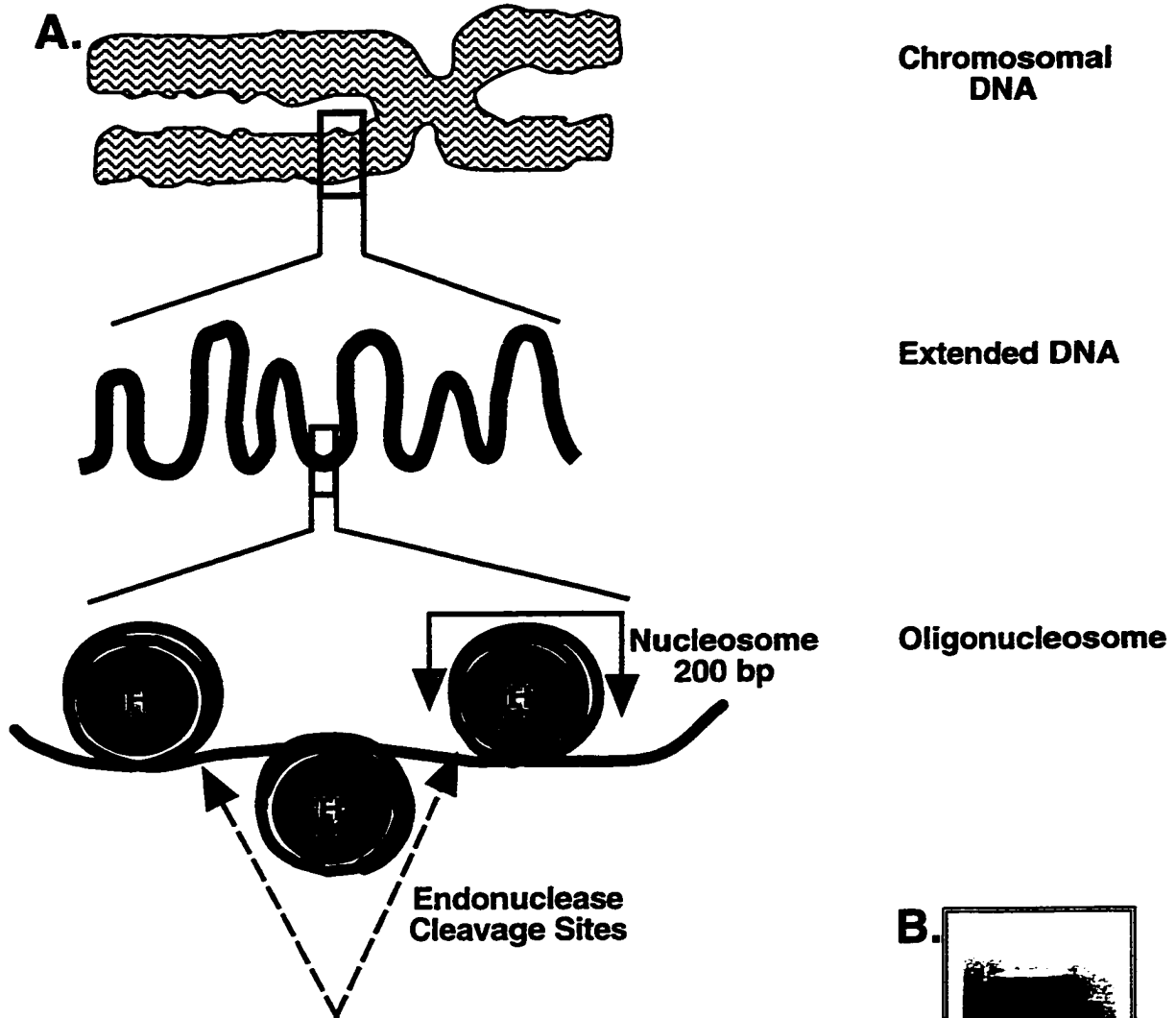


Figure 4. A. Apoptotic DNA fragmentation process. This figure representing the general structure of genomic DNA shows the internucleosomic sites of endonuclease cleavage.

B. Leydig cell DNA electrophoresis in ethidium bromide stained agarose gel. The oligonucleosomal size DNA fragments generated by the endonuclease action can be visualized as a distinctive ladder pattern following electrophoresis.

recognized (reviewed in Chinnaiyan and Dixit, 1996; Chinnaiyan *et al.*, 1996; Golstein, 1997; Reed *et al.*, 1996). Mammalian homologs of *ced-3* have been identified and found to form a family of at least ten cysteine proteases formerly called the interleukin-1 β converting enzyme (ICE) family, now called caspases (Alnemri *et al.*, 1996; Yuan *et al.*, 1993). Caspase activation is responsible for the characteristic morphological lesions associated with apoptotic cell death (Golstein, 1997). Mammalian homologs of *ced-9* correspond to a family of proteins referred to as the Bcl-2 related proteins (Hengartner *et al.*, 1992; Shaham and Horvitz, 1996). Recently, a mammalian homolog of *ced-4*, called Apaf-1, has been identified (Zou *et al.*, 1997).

Bcl-2 is the prototypic member of the Bcl-2-related proteins, a growing family of cell death regulators (Hale *et al.*, 1996a; Nunez and Clarke, 1994; Vaux *et al.*, 1992). The human Bcl-2 gene was first discovered by virtue of its involvement in the t(14;18) chromosomal translocations commonly found in non-Hodgkin's B-cell lymphomas. In these patients, overproduction of Bcl-2 was found to contribute to neoplastic B cell expansion. Bcl-2 was found to promote neoplasm formation by preventing cell turnover rather than by accelerating cell division rate (Vaux *et al.*, 1992).

Although some studies have suggested that Bcl-2 is associated with the inner mitochondrial membrane (Hockenbery *et al.*, 1990; Tanaka *et al.*, 1993), the bulk of recent studies using cell fractionation, confocal microscopy or immunoelectronmicroscopy, have found that Bcl-2 is located in the outer mitochondrial

membrane, the cytoplasmic surface of the nuclear envelope, and the endoplasmic reticulum (Akao *et al.*, 1994; Jacobson *et al.*, 1993; Lithgow *et al.*, 1994).

Following Bcl-2 isolation and sequencing, several homologous proteins were subsequently identified, thereafter forming the Bcl-2-related protein family. Interestingly, within this family of structurally homologous proteins, some block apoptosis while others promote cell death (reviewed in Boise *et al.*, 1995; Hale *et al.*, 1996b; Reed *et al.*, 1996). To date, at least nine mammalian homologs of Bcl-2 have been described, including the anti-apoptotic proteins Bcl-X_L (Boise *et al.*, 1993), Mcl-1 (Kozopas *et al.*, 1993), A1/Bfl-1 (Lin *et al.*, 1993) and Nr13 (Gillet *et al.*, 1995), and the pro-apoptotic proteins Bax (Oltvai *et al.*, 1993), Bcl-X_S (Boise *et al.*, 1993), Bad (Yang *et al.*, 1995), Bak (Chittenden *et al.*, 1995a; Farrow *et al.*, 1995), and Bik (Boyd *et al.*, 1995). Several of these homologous proteins are known to interact with each other, thus constituting a network of homo- and hetero-dimers that regulate physiological cell death (Oltvai *et al.*, 1993; Sato *et al.*, 1994; Sedlak *et al.*, 1995). For example, Bcl-2 forms homodimers and heterodimerizes with Bcl-x_L, Bcl-x_S, Bax, Mcl-1, and Bad. Similarly, Bcl-x_L forms homodimers and heterodimerizes with Bcl-x_S, Bax, Mcl-1, and Bad as well as with Bcl-2.

It has been proposed that the onset of apoptosis is controlled by the ratio of apoptosis-inhibiting to apoptosis-promoting proteins present in a given cell (Miyashita and Reed, 1995; Oltvai *et al.*, 1993; Sato *et al.*, 1994). In such a case, the Bcl-2/Bax ratio in a cell would act as a rheostat which regulates susceptibility

to apoptosis (Korsmeyer *et al.*, 1993). Therefore, an excess of Bax/Bax homodimers would initiate the apoptotic cascade while an excess of Bcl-2/Bcl-2 homodimers and Bcl-2/Bax heterodimers would maintain cell viability (Miyashita and Reed, 1995; Oltvai *et al.*, 1993).

Recently, the molecular link between Bcl-2s and caspases have been shown to involve CED-4/Apaf-1 (Figure 5). In mammalian cells, CED-4/Apaf-1 overexpression was shown to induce physiological cell death (Chinnaiyan *et al.*, 1996). Moreover, overexpression of Bcl-X_L, an anti-apoptotic protein, as well as caspase inhibitors were shown to inhibit CED-4/Apaf-1 induced mammalian cell death (Chinnaiyan *et al.*, 1996; Golstein, 1997). Based on these and from CED-3/CED-4/CED-9 co-precipitation studies, the authors established that Bcl-2/CED-9, located at the mitochondrial membrane, binds and modulates CED-4/Apaf-1, which, in turn, binds caspases/CED-3. Binding of CED-4/Apaf-1 to caspases/CED-3 may lead to caspases activation if CED-4/Apaf-1 is not bound to Bcl-2/CED-9 (Chinnaiyan *et al.*, 1996) (Figure 5). This simple, but promising pathway, is still incomplete since the involvements of many other proteins, growth factors and oncogenes remain to be elucidated.

CED-4 may not be the only intermediate between Bcl-2s and caspases. Cytochrome c (Cyt.c), released from the mitochondrial intermembrane space, may also be critical. In mammalian cells, there is a detectable shift of Cyt.c from mitochondria to cytosol, as soon as one hour after the induction of cell

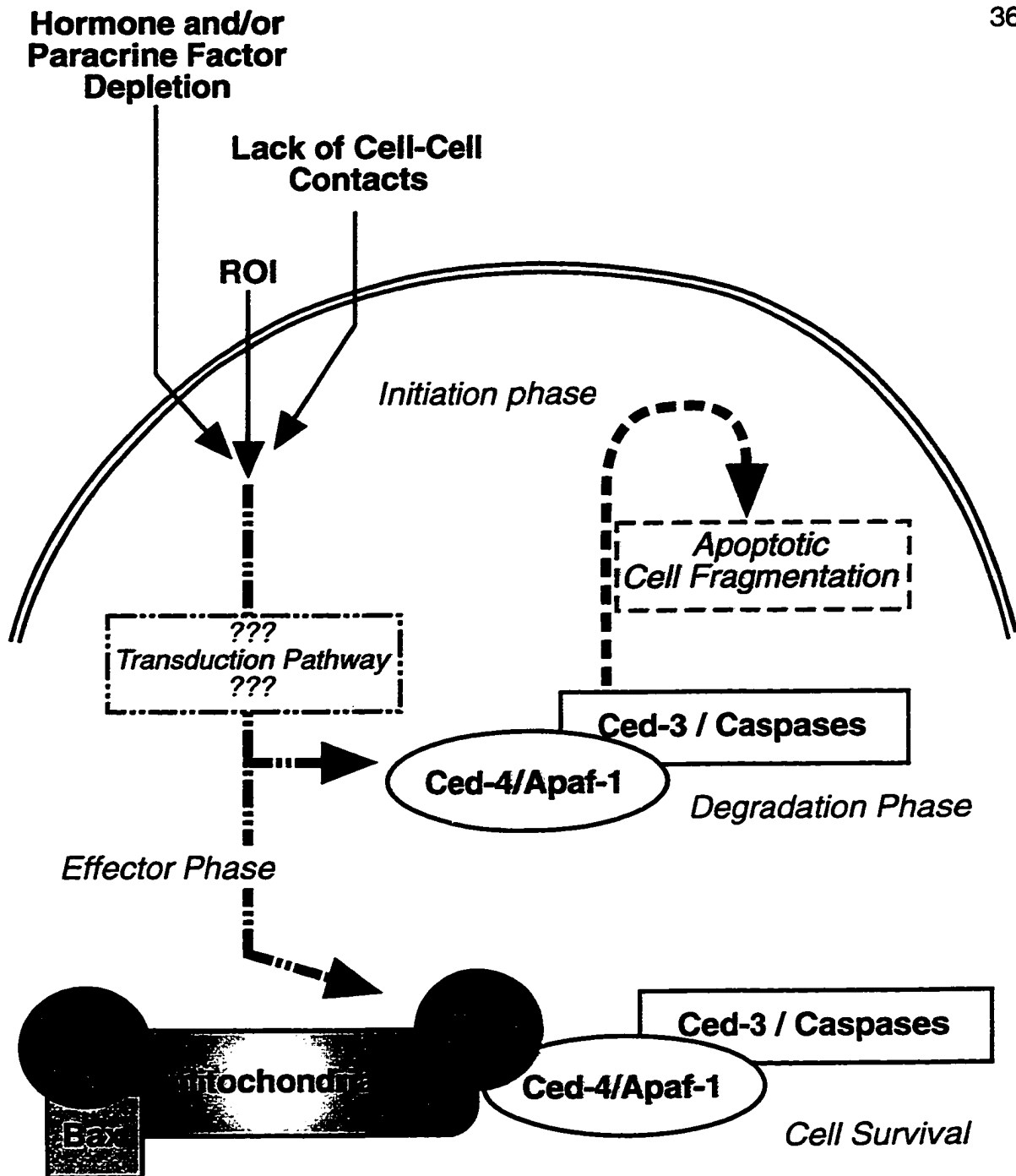


Figure 5. Regulation of Apoptosis: An Hypothetical Model. First, during the initiation phase, pro- and/or anti-apoptotic stimuli activate an unknown transduction pathway to convey information to the cell. Secondly, during the effector phase, Ced-9/Bcl-2 can interact with Ced-4/Apaf-1 and block the activation of Ced-3/Caspases by Ced-4/Apaf-1 to inhibiting cell death; or, Ced-9/Bcl-2 can interact with Bax and allow free Ced-4/Apaf-1 to bind to Ced-3/Caspases and induce apoptotic cell death. ROI:reactive oxygen intermediates

death. Translocation of Cyt.c from mitochondria to cytosol was blocked by overexpression of Bcl-2. Inhibitors of caspase activation did not block the release of Cyt.c from the mitochondria but blocked subsequent caspase activation and cell death (Golstein, 1997; Kluck *et al.*, 1997; Yang *et al.*, 1997).

Besides its involvement with caspases and CED-4 in the regulation of the onset of apoptosis, evidence suggests pleiotropic effects of Bcl-2 on different organelles. These include inhibition of calcium efflux from the endoplasmic reticulum (Lam *et al.*, 1994), blockade of nucleocytoplasmic trafficking of cell-regulatory proteins including p53, cdc2, and CDK2 (Meikrantz *et al.*, 1994), interaction with R-Ras, a GTP-binding protein, and the serine/threonine kinase Raf-1, neutralization of Bax, enhancement of the mitochondrial membrane potential (Hennet *et al.*, 1993) and ATP synthesis (Smets *et al.*, 1994), and inhibition of the generation and/or the deleterious action of reactive oxygen species (Hockenbery *et al.*, 1993; Kane *et al.*, 1993; Oltvai and Korsmeyer, 1994).

c. Mitochondrial Control of Apoptosis

Mitochondria play a crucial role in the generation of ATP. These organelles host an array of metabolic enzymes and regulatory proteins including proteins of the Bcl-2 protein family. New evidence suggests that mitochondria might not only host apoptosis regulatory proteins but also play an active role in the onset and commitment of the cell to die. In apoptotic cells, a significant reduction in

mitochondrial transmembrane potential ($\Delta\psi_M$) was found to occur before any nuclear morphological change could be observed (Kroemer *et al.*, 1995; Skulachev, 1997). Mitochondrial transmembrane potential reduction is induced by the asymmetrical distribution of charges between the inner and the outer side of the inner mitochondrial membrane. As a result, the sub-optimal electrochemical proton gradient across the membrane impedes mitochondrial ATP synthesis. Mitochondrial electron transport uncoupling from ATP synthesis then becomes responsible for the excessive generation of adverse reactive oxygen intermediates (ROI). Reduction in $\Delta\psi_M$ and consequent mitochondrial generation of ROI is normally prevented under conditions in which apoptosis is inhibited. Treatment with the apoptosis-inhibitory drug linomide, or the glutathione precursor N-acetyl-L-cysteine, concomitantly prevent mitochondrial alterations and physiological cell death induction in T Lymphocytes (reviewed in Kroemer *et al.*, 1995). Moreover, transfection-enforced overexpression of Bcl-2 prevents the breakdown of mitochondrial function and consequently apoptosis (Hennet *et al.*, 1993; Zamzami *et al.*, 1995).

d. Time-Course Associated with the Apoptotic Cascade

The apoptotic cascade can be divided into at least three functionally distinct phases (Figure 5) (Bursch *et al.*, 1990b; Kroemer *et al.*, 1995). First, the "initiation", or induction phase, refers to the heterogeneous signalling events leading from death-triggering extracellular agents to the activation of a central

control within the target cell. Second, the "sentencing", or effector phase, encompasses the intracellular events that irreversibly commit the cell to physiological death. During this phase, the newly committed apoptotic cell is subject to regulatory proteins such as Ced-9/Bcl-2 proteins. Moreover, CED-3/caspases activation occurs during this period. Third, the "execution", or degradation phase, includes the overt morphological changes associated with apoptotic cell death induced by effector molecules, for example nucleases and proteases (caspases). Caspase activation leads to the characteristic "apoptotic" structural lesions accompanying cell death. During this phase, an increase in the overall entropy, including activation of catabolic enzymes, precludes further regulatory effects (Bursch *et al.*, 1990b; Kroemer *et al.*, 1995).

Studies *in vivo* showed that no more than 2-3% of the cells undergo apoptosis at any one time. This particular feature of apoptosis makes it difficult to establish a precise chronology of the apoptotic sequence (Wyllie *et al.*, 1980). Despite the relative absence of studies focusing on the chronology of apoptotic cell death, a general consensus among the scientific community suggests a rapid process. Phase contrast microscopy showed that the onset of apoptosis is abrupt and fragmentation of the cell, into apoptotic bodies, is completed within several hours (Kerr *et al.*, 1995; Wyllie *et al.*, 1980). Based on tissue section observation, Bursch and colleagues (1990) estimated that, while the length of the initiation phase was variable, the effector phase was very rapid (2-5 minutes). Finally, the

execution phase was completed in no more than 3 hours.

More recently, Farschon and colleagues (1997) estimated 60 to 90 minutes as the time required for the initiation phase in a cell-free system developed from *Xenopus laevis*. From 90 to 120 minutes following initiation, the effector proteins of the sentencing phase were actively and irreversibly preparing the cell for apoptosis. Soon after, from 1.5 to 2 hr from time zero, the execution phase began.

In cerebellar granule neurons, Nardi and colleagues (1997) estimated 6 h as the time lapse between time zero, when cells were deprived of potassium, an essential trophic factor, and the first signs of apoptotic DNA fragmentation.

II. 4. Cellular Stimuli Triggering Apoptosis

The intricate complexity associated with the regulation of apoptotic cell death is also observed in its triggering mechanisms. Indeed, a wide array of intra- and extra-cellular stimuli can, depending on the cell system, irreversibly activate the apoptotic machinery. For example, reactive oxygen intermediate damage, trophic or growth factor deprivation, shortage of metabolic supply, DNA damage, binding of certain ligands to cell surface receptors, modification in cell-cell interactions, disruption of the cytoskeleton, irradiation, and glucocorticoid treatment are only a few, among a large number of stimuli by which cell death by apoptosis can be induced (Hsueh *et al.*, 1994; Kroemer *et al.*, 1995).

For our purpose, the following section will focus on the generation of reactive oxygen intermediates (ROI) and their roles in cell damage, potentially

leading to the onset of apoptosis.

a. Reactive Oxygen Intermediates

It is well established that a range of molecules, loosely grouped together as reactive oxygen intermediates (ROI), is normally generated *in vivo*. By definition, ROI are short-lived molecules or ions containing unpaired electron(s) and produced by the reduction of oxygen (Buttke and Sandstrom, 1994; Riley and Behrman, 1991). Two forms of oxygen intermediates are recognized, namely, the oxygen radicals, also called free radicals, and the reactive oxygen species (ROS). The addition of an unpaired electron to molecular oxygen results in the formation of oxygen radicals such as hydroxyl radicals ($\text{OH}\cdot$) and superoxide anions (O_2^-). On the other hand, ROS are oxidizing agents easily converted into oxygen radicals. These are found in the form of hydrogen peroxide (H_2O_2), singlet oxygen, lipid hydroperoxides, hypochlorous acid (HOCl), hypobromous acid (HOBr) and ozone (O_3).

ROI can be deliberately produced *in vivo*. For example, activated phagocytes can generate superoxide anions and hydrogen peroxide in order to kill foreign organisms. However, ROI often appear to arise by "accidents of chemistry". Indeed, O_2^- and H_2O_2 are produced by the chemical reaction of O_2 with such molecules as adrenaline, dopamine, and more importantly, some components of the cytochrome P-450 electron-transport chains (Buttke and Sandstrom, 1994; Halliwell, 1996). Such ROI release can be regarded as unavoidable in aerobic

organisms where molecular oxygen acts as the terminal electron acceptor for oxidative phosphorylation. Oxygen's nature as a double-edged sword manifests itself best in mitochondria where most of the oxygen utilized by eukaryotic cells is reduced.

The deleterious effects of ROI is observed in cells where they can readily react with cellular macromolecules. In such a case, ROI either damage the macromolecule directly or initiate a chain reaction wherein free radicals are passed from one macromolecule to another, resulting in extensive damage to cellular structures (Halliwell, 1996). Generally, the damaging effects of ROI can be summarized in three distinct categories. These include DNA and RNA oxidative base damage, lipid peroxidation, and protein damage (Halliwell, 1996; Riley and Behrman, 1991).

In order to exploit the effectiveness of molecular O₂ as a terminal electron acceptor for ATP production, the deleterious effects of ROI are controlled by antioxidants. This defense mechanism includes an elaborate arsenal of antioxidant enzymes working in concert to neutralize the radicals. Therefore, enzymes such as superoxide dismutase (SOD), catalase, glutathione peroxidase and thioredoxin scavenge and detoxify ROI while tocopherol, carotenoids and ascorbic acid principally block free-radical chain reactions. Moreover, lactoferrin, caeruloplasmin and transferrin are responsible for sequestering transition metals which can serve as a ready source of free electrons (Buttke and Sandstrom, 1994; Halliwell, 1996).

For example, in such a system, SOD catalyzes the conversion of the superoxide radical to hydrogen peroxide, while glutathione peroxidase and catalase clear peroxide therefore preventing the production of hydroxyl radicals, the initiators of lipid peroxidation and macromolecule damage.

Consequently, aerobic existence is accompanied by a persistent state of oxidative siege, wherein the survival of a given cell is determined in part by the balance between ROI and antioxidants. Therefore, apoptotic cell death can be induced by the addition of ROI and/or the depletion of cellular antioxidants (Larrick and Wright, 1990; Zhong *et al.*, 1993). Physiological cell death induction can be reversibly blocked by the addition of compounds with antioxidant abilities (Iwata *et al.*, 1992; Ramakrishnan and Catravas, 1992).

b. Generation of ROI in Leydig cells

As mentioned earlier, molecular oxygen is essential for electron transport during steroidogenesis in the interstitial cells of Leydig (reviewed in Hall, 1994). Although oxygen is required for P-450 activity, it is also a source of destructive ROI (Chabre *et al.*, 1993). Indeed, in steroidogenic cells, reactive oxygen intermediates initiate lipid peroxidation in cell membranes and are responsible for mitochondrial and endoplasmic cytochrome P-450 activity reduction (Chabre *et al.*, 1993; Georgiou *et al.*, 1987; Kukucka and Misra, 1993; Peltola *et al.*, 1992) resulting in steroidogenesis inhibition both *in vitro* and *in vivo* and possibly infertility.

The chronic generation of ROI in steroidogenic tissues is further supported by the presence of high levels of antioxidants. In testes, high concentrations of antioxidant vitamins, such as tocopherol, ascorbate and β -carotene, are observed in concert with high concentration of antioxidant enzymes. Among the latter, superoxide dismutase (SOD) reduces the superoxide anion to hydrogen peroxide, while catalase and glutathione peroxidase (GSH-Px) metabolize hydrogen peroxide (Peltola *et al.*, 1992).

In recent years, it has been reported that stimulation and maintenance of testosterone producing cells in culture is dependent on the oxygen tension *in vitro* (Abney and Meyers, 1987; Hornsby and McAllister, 1991; Myers and Abney, 1988; Payne *et al.*, 1987; Quinn and Payne, 1985). Moreover, Leydig cell viability and steroidogenic activity *in vitro* have been observed to decrease over a period of 24 h due to the excessive formation of ROI (Abney and Meyers, 1987; Klinefelter and Ewing, 1989). However the mechanism of oxygen toxicity in isolated Leydig cells has not been investigated in detail.

II. 5. Objectives of the Present Research Project

Although numerous immortal Leydig cell lines are available today, the study of steroidogenesis regulation still relies largely on primary cultures of Leydig cells, mainly from pig or rat origin. Even though primary cultures of Leydig cells can only be maintained for short terms in culture, to a maximum of 5-6 days, they still represent the closest model to the *in vivo* condition. For this reason, since Leydig

cells were first isolated and cultured in defined culture media, endocrinologists have never stopped trying to provide improved culture conditions.

The observation that cultured Leydig cells rapidly initiate apoptotic cell death following cell plating has prompted us to study this process in more detail. The ultimate objective of this research project was to better understand how apoptotic cell death is initiated and unfolds in primary cultures of rat Leydig cells. Indeed, a better appreciation of the importance of apoptotic cell death in this cell model might help improve the culture conditions so that Leydig cell metabolism, including steroidogenesis, is as close as possible to the physiological system in order to provide a more reliable model.

The onset of apoptosis in primary cultures of rat Leydig cells was initially quantified over time under control culture conditions. Then, the effect of various treatments were tested to determine their pro- or anti-apoptotic effects. More specifically, the role of LH/hCG as survival factors was studied in more detail. Indeed, the dose- and time-dependent effect of these gonadotropins on steroidogenesis and on the onset of apoptosis *in vitro* was studied.

The importance of ROI in triggering apoptosis in primary cultures of rat Leydig cells was assessed indirectly. For this purpose, specific antioxidants were added to the culture media and the effects on both steroidogenesis and the onset of apoptosis were measured.

Finally, the expression of the anti- and pro-apoptotic proteins Bcl-2 and Bax was measured by Western blotting to assess their putative role in the regulation

of apoptotic cell death in primary cultures of rat Leydig cells.

MATERIALS AND METHODS

I. MATERIALS

Ovine luteinizing hormone, NIADDK-oLH-26 (2.3 X NIH-LH-S1) was a generous gift from Dr. AF Parlow and the National Hormone and Pituitary Distribution Program, University of Maryland, School of Medicine (Baltimore, MD). Percoll was purchased from Pharmacia Biotech Inc. (Baie d'Urfé, Québec). Sodium bicarbonate, HEPES, penicillin-streptomycin, Dulbecco's minimal essential media, F-12 media, horse serum, proteinase K, streptavidin-horseradish peroxidase (HRP) conjugate, and ϕ X174 RF DNA/Hae III fragments were purchased from Canadian Life Technologies (Burlington, Ontario). 32 P-dATP and donkey anti-rabbit-HRP secondary antibodies were purchased from Amersham (Oakville, Ontario) while ribonuclease A was purchased from United States Biochemicals (Cleveland, Ohio, USA). Terminal deoxynucleotidyl transferase was purchased from Promega (Madison, WI, USA). BCL-2 and BAX antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). SuperSignal substrate for Western blotting was purchased from Pierce (Rockford, IL, USA). CO₂ and special gas mix composed of 5% CO₂, 2% O₂ and 93% N₂ were purchased from Medigas (Dartmouth, Nova Scotia, Canada).

Unless otherwise specified, all other chemicals were purchased from Sigma Diagnostics Canada (Mississauga, Ontario).

II. ANIMALS

Adult Sprague-Dawley rats (275-300 g) were purchased from Charles River (St-Constant, Québec). The animals were kept in the animal care facilities under controlled humidity, temperature, and a constant dark-light cycle. The animals received animal chow and water *ad libitum*. Housing and handling of the animals were in compliance with the guidelines of the Canadian Council on Animal Care (CCAC) and the University Committee on Laboratory Animals of Dalhousie University.

III. LEYDIG CELL PREPARATION AND CULTURE

III.1. Leydig Cell Isolation

Leydig cell isolation from adult Sprague-Dawley rats was done according to the method described by Rommerts *et al.* (1985) with modifications. Adult Sprague-Dawley rats were killed by CO₂ inhalation, and testes were immediately collected in ice-cold sterile saline. A maximum of six rats were used per experiment. Limiting the number of rats processed at together, at any one time, allowed keeping Leydig cell preparation time as short as possible, therefore resulting in optimal cell quality. Following dissection, cell preparation was performed in a clean air environment to prevent cell contamination. Unless specified otherwise, cells and solutions were kept on ice at all times.

Blood cells were washed away by perfusing the testes with ice-cold sterile saline (0.9% w/v NaCl) (1-2 ml per testis) through the testicular artery. Testicular

perfusion was done using a 30G1/2 needle.

The remainder of this procedure was carried out in Medium 199 modified to contain 10 mM N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES), 27 mM sodium bicarbonate, 0.7 mM glutamine, 1 mM pyruvate, and 1 mg/ml bovine serum albumin fraction V (BSA). Modified medium 199 (M199) was sterilized by positive pressure filtration through 0.22 μ m filters and stored at 4 C.

Following perfusion and decapsulation, the testes were enzymatically dispersed at 34 C with gentle shaking (80 cpm) for 20 minutes. Cell dispersion was carried out in a 50 ml conical tube (Falcon) containing 135 IU collagenase/ml, isolated from *Clostridium histolyticum*, and 0.5% BSA in M199 (3.5 ml / testis). A maximum of 4 testes were digested per tube.

The resulting cell suspension was diluted with 2 volumes of M199 and the tubules and other tissue fragments were allowed to settle for 5 min. The supernatant was then coarsely filtered through a 74 mesh filter superimposed on a 44 mesh filter. The sediment was washed with an additional volume of M199 and the supernatant was filtered, as above, to reach a final volume of 45 ml of interstitial cell suspension. The filtrate so obtained was centrifuged at 260 \times g for 5 min at 4 C in a PR-6000 Damon centrifuge (IEC Division, Needham Hts, MA, USA). The supernatant was discarded, and the cell pellet was gently resuspended in 3 ml M199.

A Leydig cell enriched population was isolated by centrifugation of the interstitial cell's fraction into a two-step Percoll gradient. According to the

manufacturer's protocol, nine parts of Percoll were mixed with one part of 10-fold concentrated HEPES buffer (100 mM HEPES, 1.45 M NaCl, 50 mM KCl, 50 mM glucose, 10 mM CaCl₂, pH 7.4), to give a final osmolarity of 300 mOsmol/kg. From this isotonic solution, a two-step Percoll gradient, consisting of 40% (1.06 g/cm³) and 80% (1.11 g/cm³) Percoll, was prepared by diluting the isotonic solution with M199. First, the 40% solution (8 ml isotonic Percoll + 12 ml M199) was poured in a 50 ml conical tube. The 80% solution (16 ml isotonic Percoll + 4 ml M199) was then gently layered at the bottom of the tube using a 20 ml syringe fitted with a 3 inch long, 16G catheter needle (Critikon Canada Inc, Markham, Ontario, Canada).

The cell suspension (3 ml per gradient) was slowly pipetted on top of the two-step Percoll gradient (Kuhn-Velten et al., 1982) and the latter was centrifuged at 420×g for 15 min at 4 C. Following centrifugation, the top layer of the gradient, mainly composed of non-Leydig interstitial cells, was discarded. A Leydig cell enriched fraction could then be recovered from the Percoll gradient's interphase. This interphasic cell layer was collected (10-15 ml per gradient) with a 20 ml syringe fitted with a 3 inch long, 16G catheter needle. These cells were then washed with 3 volumes of M199 and centrifuged at 260×g for 5 min at 4 C. In order to eliminate residual Percoll, the supernatant was discarded and the cell pellet was washed twice with 10 ml of M199 and centrifuged as above. When more than one gradient was used, cell pellets were combined following the first wash to obtain an homogeneous cell population. After the last wash, the cell pellet was gently resuspended in 2-4 ml of M199, and the number of cells quantified using

a hemacytometer. Sperm cells were excluded from the count.

This Leydig cell isolation protocol typically yielded a cell suspension containing 80-85% Leydig cells as assessed by 3β -hydroxysteroid dehydrogenase (3β -HSD) staining (Molenaar *et al.*, 1986) (see below). A percent cell viability was determined following cell preparation by trypan blue exclusion (see below). This test revealed that 95 - 97% of the freshly isolated cells excluded trypan blue and were therefore viable.

III.2. Leydig Cell Culture Conditions and Treatments

For most experiments, primary cultures of rat Leydig cells were incubated at 34 C in a humidified atmosphere of 95% air and 5% CO₂. Freshly isolated Leydig cells were promptly plated at a specific density according to the culture dish used and the experimental purpose (for details, see Table 1).

Cells were cultured in M199 supplemented with insulin (5 μ g/ml), vitamin E (200 μ mol/l), dimethyl sulfoxide (DMSO) (100 mmol/l), streptomycin (100 μ g/ml) and penicillin (100 U/ml). Note that DMSO was used both as a solvent for vitamin E and as an antioxidant (Murphy and Moger, 1982). Cells were cultured for 3-72 h with or without treatment. Those treatments included luteinizing hormone (LH) (0.1-10 ng/ml), superoxide dismutase (SOD) (100 U/ml), catalase (Cat) (50 ng/ml), ascorbic acid (Asc. Ac.)(1 mM), zinc (Zn)(1-5 mM), epidermal growth factor (EGF) (10 ng/ml), fetal calf serum (FCS)(15%), 5α -dihydrotestosterone (DHT) (30-500 ng/ml), cycloheximide (25-75 μ M), actinomycin D (4-40 nM), vitamin E (200-400

Culture Plate	Cell Density	Total Volume	Experimental Purpose
24 well plate	1x10 ⁵ cells/well	1 ml / well	Androgen Assay DNA Assay Trypan Blue Exclusion 3 β -HSD Assay
15 ml PP tube	1.5x10 ⁵ cells/tube	1.5 ml /tube	ISEL
60 mm dish	3x10 ⁵ cells/dish	5.0 ml /dish	DNA Isolation and Labeling Protein Isolation and Western Blotting

Table 1. Overview of the culture plate, cell density, culture media volume and experimental purpose used in primary cultures of rat Leydig cells.(PP=polypropylene)

μ mol/l) or N-acetylcysteine (NAC)(2.5-25 mM).

In some experiments, we substituted supplemented M199 either with M199 conditioned by preincubation for 24 h with testicular cells plated at high density (1.6×10^6 cells/cm²) (Murphy and Moger, 1982) or with Dulbecco's Minimal Essential Medium (DMEM)- F12 media (1:1, v/v).

In order to study the importance of oxygen on the onset of apoptosis in primary cultures of rat Leydig cells, the incubation conditions were modified. Normally, Leydig cells are cultured in an atmosphere composed of 19% oxygen and 5% CO₂, the remaining 76% being mainly composed of nitrogen. For two experiments, a custom-made gas mixture, composed of 2% O₂, 5% CO₂ and 93% N₂, was connected to the cell incubator. Gas flow was adjusted so that the level of CO₂, measured with a fyrite test kit (Bacharach, Pittsburgh, PA, USA), reached 5% in the culture chamber. Maintained at 5%, the CO₂ level was therefore equivalent to the CO₂ content of the special gas mixture. Consequently, the

determined gas flow rate also created the required low oxygen (2%) environment.

For each experiment where apoptotic DNA fragmentation or BCL-2 and BAX expression were investigated, some freshly isolated Leydig cells (basal cells) were also processed. Immediately after cell isolation, basal Leydig cells were lysed for total DNA extraction, total protein isolation, or were cytospun onto silinated glass slides (see below). These basal cells provided insights into the conditions of the cells *in vivo*. They also allowed studies on the effect of Leydig cell culture and treatments on the onset of apoptosis and on the expression of Bcl-2 and Bax proteins.

III.3. Long Term Incubation

The effects of incubation time and steroidogenesis on the onset of apoptosis were also studied. For those experiments, freshly isolated Leydig cells were plated and cultured for 24 to 72 hrs. During culture, these cells were treated with various combinations of LH (0, 0.1 and/or 10.0 ng/ml). Note that, when cell culture extended beyond 24 hours, the culture medium was replaced daily with fresh supplemented M199. Similarly, respective treatments were replaced daily. The spent media so collected was stored at -20 C until ready for androgen assay. For a complete description of daily LH treatments as a function of the total incubation time, refer to Table 2.

TOTAL INCUBATION TIME	LH TREATMENT		
	From: 0 - 24 h	From: 24 - 48 h	From: 48 - 72 h
24 hours	No LH	n/a	n/a
	LH 0.1 ng/ml	n/a	n/a
	LH 10.0 ng/ml	n/a	n/a
48 hours	No LH	No LH	n/a
	No LH	LH 0.1 ng/ml	n/a
	LH 0.1 ng/ml	LH 0.1 ng/ml	n/a
	No LH	LH 10.0 ng/ml	n/a
	LH 0.1 ng/ml	LH 10.0 ng/ml	n/a
72 hours	No LH	No LH	No LH
	No LH	No LH	LH 0.1 ng/ml
	LH 0.1 ng/ml	LH 0.1 ng/ml	LH 0.1 ng/ml
	No LH	No LH	LH 10.0 ng/ml
	LH 0.1 ng/ml	LH 0.1 ng/ml	LH 10.0 ng/ml

Table 2. LH treatment of primary cultures of rat Leydig cells incubated for 24 to 72 hours. Spent medium was collected every 24 h, under sterile conditions. Fresh supplemented M199 and respective LH treatments were added before the samples were returned to the incubator. For ISEL, cells were cultured in suspension with identical LH treatments. n/a= non applicable

III.4. In Vivo hCG Pretreatment

For some experiments, Sprague-Dawley rats were treated with a supra-physiological dose of human chorionic gonadotropin (hCG) 3, 12, 18, or 24 hours prior to sacrifice and Leydig cell isolation. For each time group, 3 rats received 100 IU hCG in 0.2 ml sterile saline injected subcutaneously with a 1ml syringe fitted with a 25G1 needle. One rat received 0.2 ml of sterile saline and was used as control. At the end of the hCG pretreatment, each rat was sacrificed and Leydig

cells were isolated as described above. Leydig cells were then incubated for 24 h with LH (0-10.0 ng/ml) as described above. In order to maximize our data points, Leydig cells from each rat were maintained separately during cell preparation and culture. Freshly isolated Leydig cells and cells cultured in suspension were cytopun and stained by ISEL (see below) for apoptosis quantification. Leydig cells cultured in 24 well plates for 24 h were used for androgen production determination and DNA quantification. As described in Table 1, Leydig cells cultured in 60 mm petri dishes for 24 h were used for total protein isolation and Western blotting analysis of Bcl-2 and Bax expression.

III.5. Trypan Blue Exclusion

Trypan blue exclusion is a cell viability assay based on the ability of live cells to exclude the vital dye trypan blue (Rommerts *et al.*, 1985).

For each well, the culture medium was discarded and the cell layer was gently washed with PBS. The cell layer was covered with 50 μ l of 0.5% trypan blue solution in saline and 100 μ l saline. Cells were incubated for 5 min at room temperature (RT) to allow for dye uptake. The trypan blue solution was then replaced with 150 μ l saline to facilitate cell counting. The percent viable cells (see eq.1), which excluded trypan blue (blue cells = dead cells) was estimated by counting the cells under an inverted microscope fitted with a gridded ocular. A minimum of 200 cells in three separate fields were counted per well.

$$\text{eq. 1. \% Viable Cells} = \frac{\text{number of cells excluding dye}}{\text{total number of cells}} \times 100$$

For cells in suspension, an aliquot of the cell suspension was mixed 1:1.5 (v:v) with a 0.5% trypan blue solution (w/v in sterile saline). Using a hemacytometer, the number of stained (non-viable) versus unstained (viable) cells was determined under the microscope.

III.6. 3 β -Hydroxysteroid Dehydrogenase Assay

Generally, 3 β -hydroxysteroid dehydrogenase (3 β -HSD) is used as a marker enzyme to estimate the percentage of Leydig cells in cell preparations (Molenaar *et al.*, 1986; Niemi *et al.*, 1986; Yee and Hutson, 1983).

This simple procedure for 3 β -HSD staining is usually performed on cells cultured for a minimum of 24 h. Following culture media removal, the cell monolayer was washed twice with PBS. A few drops of nitroblue tetrazolium solution (0.003% 3 β -hydroxy-5 α -androstane-17-one, 0.017% nitroblue tetrazolium, 0.017% nicotinamide, 0.017% nicotinamide adenine dinucleotide (NAD) in PBS) were then added to each well, enough to cover the cell monolayer. Since the cell membrane must be permeable for NAD⁺ to successfully detect the enzyme activity, the culture dishes were stored at -70 C for about 30 minutes to induce cell membrane damage and promote internalization of the dye. Cells were then thawed and a few more drops of the same solution was added to each well before cells were incubated at 34 C for 60 minutes.

The percent of stained cells, blue in color, which identified Leydig cells, was estimated by counting cells with an inverted microscope fitted with a gridded ocular. A minimum of 300 cells were counted per well in at least 2 separate wells. Eighty to eighty five percent of the cells in each cell preparation typically stained positive for 3 β -HSD activity.

IV. APOPTOTIC DNA FRAGMENTATION: QUALITATIVE AND QUANTITATIVE STUDY

IV.1. DNA Isolation and Quantification

Leydig cell total DNA was isolated following a method described by Sambrook *et al.* (1989), with a few modifications.

For each sample, total DNA was isolated from 1.5 million Leydig cells either immediately after cell preparation for basal cells or following primary cell culture. In each case, the culture media was discarded and the cells were washed twice with ice-cold PBS. Cells were lysed in 2 ml ice-cold 100 mM Tris-HCl (pH 8.0) containing 10 mM EDTA, 10 mM sodium chloride, 1 mg/ml proteinase K and 1% sodium dodecyl sulfate (SDS). The lysate was then scraped into a 15 ml polypropylene (PP) tube and incubated in a shaking waterbath at 37 C for a minimum of 6 h with gentle shaking (60 cpm). Note that this step could be carried on overnight. The lysate was subsequently extracted with an equal volume of water-saturated phenol pH 7.2: chloroform: isoamyl alcohol (25:24:1 v:v:v), gently vortexed, and centrifuged at 3500 \times g for 20 min at 18 C in a J2-21M/E Centrifuge

(Beckman Canada, Mississauga, Ontario, Canada). Vigorous vortexing was avoided to prevent DNA shearing.

The aqueous phase, containing the genomic DNA, was collected into a 15 ml PP tube containing ribonuclease A (100 IU/ml) isolated from bovine pancreas. The solution was mixed gently by inversion and incubated for 60 min in a shaking waterbath as described above. This digested solution was extracted with an equal volume of water-saturated phenol pH 7.2: chloroform: isoamyl alcohol (25:24:1 v:v:v) , vortexed, and centrifuged as above. Again, the resulting aqueous phase was collected. The latter was extracted one last time with an equal volume of chloroform: isoamyl alcohol (24:1 v:v), vortexed, and centrifuged as above. This last step was necessary to eliminate any residual trace of phenol. Finally, the aqueous phase was collected and the DNA was precipitated with 2.5 volumes of absolute ethanol and 0.25 M NaCl. The solution was mixed gently by inversion and stored at -70C until ready for further processing.

For sample quantification, DNA was recovered by centrifugation at 7500×g for 20 min at 4 C. Each DNA pellet was washed once with 70% ice-cold ethanol and centrifuged for 10 min in the conditions just described. Before its dissolution into 15 µl of Tris-EDTA (TE) (10 mM Tris-HCl pH 7.4, 1 mM EDTA) the pellet was allowed to air dry, on ice, for 10 min. For each sample, a 1:100 dilution in water was prepared and the optical density (OD) was measured by spectrophotometric analysis at 260, 280 and 300 nm in a DU 640 Spectrophotometer (Beckman Canada, Mississauga, Ontario, Canada). The concentration of DNA can be

determined from the absorbance at 260 nm (A_{260}). An absorbance of 1 unit at 260 nm corresponds to 50 μg of DNA per ml. Equation 2 shows the calculation of DNA concentration from the A_{260} and the dilution factor.

$$\text{eq. 2. DNA Concentration} = 50 \mu\text{g /ml} \times A_{260} \times \text{dilution factor}$$

The ratio between the readings at 260 nm and 280 nm (A_{260} / A_{280}) provided an estimate of the purity of DNA with respect to UV-absorbing contaminants such as protein. Pure DNA has an A_{260} / A_{280} ratio of 1.8 - 2.1. Finally, the readings at 300 nm provided information about possible phenol and protein contamination in DNA samples. The A_{300} was therefore expected to be as low as possible. Typically, the A_{300} averaged 0.007 in the DNA samples used for this study.

IV.2. Ethidium Bromide Analysis

The determination of DNA fragmentation by electrophoresis with ethidium-bromide stained agarose gels was the first technique we used to confirm the occurrence of apoptosis in primary cultures of rat Leydig cells. For each sample, the equivalent of 5 μg of DNA in 10 μl TE, pH 7.4, was mixed with 2 μl of 5X gel loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol in water). The samples were then loaded into dry wells and were electrophoretically separated on 1.8% agarose gels containing ethidium bromide (0.4 $\mu\text{g/ml}$) for 90 min at 80V in Tris-borate-EDTA buffer (TBE) (45 mM Tris base, 45 mM boric acid,

1 mM EDTA, pH 8.0). Finally, DNA was visualized by a UV transilluminator and the gels were photographed with a polaroid camera using Polaroid 400 film.

IV.3. DNA 3' End Labeling Analysis

DNA 3' ends were labeled with radioactive nucleotides using a method adapted from Tilly and Hsueh (1993) and Rosl (1992). For each sample, 0.75 µg of DNA was labeled per 50 µl of reaction mixture. DNA 3'ends were elongated with ³²P-dATP (15 µCi/reaction) by the addition of terminal deoxynucleotidyl transferase (TdT) (0.4 U/µl), purified from calf thymus. The reaction buffer was composed of 100 mM cacodylate buffer pH 6.8, 5 mM CoCl₂, 0.5 mM dithiothreitol (DTT) and 0.5 mg/ml BSA. Following 60 min incubation in a recirculating waterbath at 37 C, the reaction was stopped with 25 mM EDTA, pH 8.0 (2.5 µl from a 0.5 M stock solution). Carrier salmon testis DNA (2.0 µg/sample from a 0.48 µg/µl stock solution) was then added to promote labeled-DNA recovery during DNA precipitation. The unincorporated nucleotides were removed by three consecutive DNA precipitation cycles. For each cycle, DNA was precipitated by the addition of 2.5 volumes of absolute ethanol and 0.25 M NaCl. DNA samples were then stored at -70 C for at least 3 h before they were centrifuged at 25,000×g for 20 min at 4 C. Each DNA pellet was then resuspended in 25 µl TE, pH 7.4, and was ready for a new precipitation cycle or for electrophoresis. DNA samples (300 ng/lane) were electrophoresed on 1.8% agarose gels containing ethidium bromide (0.4 µg/ml) at 80V for 90 min in TBE buffer. Gels were then dried for 90 min in a slab gel dryer

(Model 583, Bio-Rad Canada, Mississauga, Ontario, Canada) with no heat and exposed to Kodak X-OMAT AR films.

IV.4. Leydig Cell Cyto centrifugation

Cyto centrifugation gently transfers cells in suspension from a sample chamber onto glass microscope slides. *In situ* end-labeling (ISEL) works best on cells equally dispersed over a small surface area. For this reason, suspensions of freshly isolated and cultured Leydig cells, destined for ISEL analysis, were centrifuged onto microscope slides using a cytopspin centrifuge (Shandon Cytospin 2, Johns Scientific, Toronto, Ontario). For most cell types, trypsin can be used to collect cells cultured in monolayer. Since Leydig cells attach strongly to plastic, it is very difficult to collect these cells by trypsinization. This difficulty has prompted us to culture, in suspension, the Leydig cells destined for ISEL.

Therefore, following cell isolation (for basal cells) or 3-72 h incubation (for cells cultured in suspension), Leydig cell samples (1.5×10^5 cells/sample) were centrifuged at 260xg for 10 min at 4 C. For each sample, the cell pellet was gently resuspended in 300 μ l PBS and kept on ice. Rapidly, Leydig cells were cytopspun onto silinated glass slides (2% 3-aminopropyl triethoxysilane (TESPA) in acetone). TESPAs-coated slides were used to prevent excessive cell detachment from the glass during staining. Moreover, cell adhesion was maximized by prewetting the silinated glass slides with 20 μ l of PBS and centrifuging the slides for 2 min at 900 rpm at RT. For each slide, 150 μ l of cell suspension (7.5×10^4 cells/sample) was

centrifuged for 7 min at 900 rpm at RT. Slides were allowed to air dry for 5 min before they were fixed in ice-cold acetone for 2 min and stored at -70 C until ready to use for ISEL.

IV.5. In Situ End Labeling (ISEL)

DNA fragmentation was quantitated *in situ* using a non-radioactive detection method developed by Gavrieli *et al.* (1992) and adapted to our cell system.

First, cells were fixed in paraformaldehyde (PFA) (4% in phosphate buffer; 0.1 M sodium phosphate monobasic, 0.1 M sodium phosphate dibasic, pH 7.4) for 5 min at RT. Following this step, cells were washed four times in nanopure water, for one minute in each bath. Unless specified otherwise, cells were similarly washed in water after each step of this protocol.

Next, nuclei were stripped to expose DNA 3'ends by incubating cells with either proteinase K (20 µg/ml in 10 mM Tris-HCl, pH 8.0) for 15 min at RT or 0.5% pepsin (in water, pH 2.0) for 20 min at 37 C. In order to reduce possible background staining, endogenous peroxidases were inactivated by immersing the cells in 2% H₂O₂ for 10 min at RT.

Before DNA fragments were elongated with biotinylated deoxyadenosine triphosphate (biotin-14-dATP), cells were equilibrated with 50 µl of TdT buffer (30 mM Tris, pH 7.2, 140 mM sodium cocadylate, 1 mM cobalt) per slide, for 5 min at RT. Once cells were equilibrated, TdT buffer was drained and replaced with 50 µl of elongation solution (12.5 pmol/µl biotin-14-dATP, 0.4 U/µl TdT enzyme in TdT

buffer). Cells were incubated for 1 h at 37 C in a humidified chamber.

The reaction was stopped by immersing slides in terminating buffer (300 mM sodium chloride, 30 mM sodium citrate, pH 8.0) for 15 min at RT. Non-specific staining was prevented by blocking cells, previously equilibrated in PBS for 5 min at RT, with 5% horse serum (HS) diluted in PBS for 10 min at RT. The excess HS was removed by washing for 5 min, in PBS at RT, before cells were washed three more times for one minute in PBS. At this point, we omitted washing the cells in water before they were incubated with streptavidin-horseradish peroxidase conjugate (streptavidin-HRP).

The detection sensitivity of the apoptotic DNA fragments, now elongated with biotinylated nucleotides, was enhanced by a signal-amplification method. The amplification system used here, exploited the exceptionally high binding affinity of biotin (a small water-soluble vitamin) for streptavidin (a bacterial protein).

Therefore, following HS treatment, the cells were incubated in streptavidin-HRP diluted 1:1000 in PBS. Approximately 60 μ l of streptavidin-HRP per slide was used to cover the cells which were then incubated for 45 min at 37°C in a humidified chamber. Following incubation, the samples were washed in PBS once and then in water before they were stained with 3-amino-9-ethyl-carbazole (AEC). Since AEC is not soluble in aqueous solution, 5 mg of AEC were dissolved in 5 ml dimethylformamide (DMF) before it was diluted to 50 ml in acetate buffer (0.1 M sodium acetate, 0.1 M acetic acid). Before use, the staining solution was filtered through a Whatman no.1 filter and 100 μ l of 30% H₂O₂ was added. To ensure

optimal staining, the filtration was carried out in an incubator at 37 C to warm up the solution and to protect this light-sensitive dye. Slides were immersed in the AEC staining solution and incubated for 40 min at 37 C.

In order to facilitate cell visualization and quantification, cells were finally counterstained with Harris' hematoxylin for 2 min, immersed in Scott's tap water (36.9 mM sodium bicarbonate, 72.2 mM magnesium sulfate in water) for 2 min and immersed in water until ready to mount with a cover slip.

Aquamount, a homemade water-soluble mounting media, was used to mount cover slips on the glass slides. The use of aquamount (10% polyvinol alcohol in 30% glycerol diluted in PBS) as opposed to a xylene-containing mounting media was necessary to protect the AEC staining against premature fading. After mounting, the covered slides were stored at RT for several months.

For each group of slides stained by ISEL, positive and negative controls were prepared. Negative control slides were obtained by omitting the TdT enzyme in the DNA elongation mixture. These slides were therefore incubated for 60 min with TdT buffer supplemented only with biotin-14-dATP.

Prior to DNA elongation positive control slides were digested with DNase I. This enzymatic digestion induced extensive DNA cleavage mimicking the apoptotic DNA degradation. Therefore, positive control slides were equilibrated in DNase buffer (30 mM Tris-HCl, pH 7.2, 140 mM sodium cocadylate, 4 mM magnesium chloride, 0.1 mM dithiothreitol) for 5 min at RT. The DNase buffer was then drained and replaced with 75 μ l of DNase I (1 ug/ml) diluted in DNase buffer.

Positive control slides were incubated for 10 min at RT, and washed as described above, before they were incubated with the elongation solution.

Apoptotic Leydig cells, displaying intensely stained red nuclei, and non-apoptotic Leydig cells, displaying a bluish color, were quantified by microscopy (magnification: X400). A minimum of 300 cells per slide were counted over at least four fields. The percent of apoptotic versus non-apoptotic cells was then calculated. For each Leydig cell sample, duplicate slides were prepared by cytospin, stained and quantified.

V. WESTERN BLOTTING STUDY OF BCL-2 AND BAX EXPRESSION IN LEYDIG CELLS

V.1. Total Protein Isolation

Among our objectives for this project, we wanted to study, by Western blotting analysis, the modulation of Bcl-2 and Bax expression in primary cultures of rat Leydig cells. The first step to meet this objective was to collect total protein from freshly isolated and cultured Leydig cells.

Following cell incubation, the culture media were discarded and the cell layer was washed with ice-cold PBS. Premature protein breakdown was prevented by processing cells and lysates on ice and using ice-cold buffers. Leydig cells (1.5×10^6 cells/sample) were lysed in 150 μ l cell lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EGTA, 1% Nonidet-40, 0.25% deoxycholic acid) containing protease inhibitors, namely, 1 mM PMSF, and 3 μ g/ml each of pepstatin

A, leupeptin and antipain. Considering their short half-life, the protease inhibitors were always freshly added, from stock solutions stored in aliquots at -20 C, to the lysis buffer shortly before use.

Leydig cells were then scraped and collected in autoclaved 1.5 ml eppendorff tubes. Cell lysates were sonicated for 20 sec at 20 W with a Sonifier Cell Disruptor (Heat Systems Ultrasonics, Plain View, NY, USA), microfuged in a MSE Micro Centaur (Johns Scientific, Toronto, Ontario) for 10 min at 6700×g and the supernatant was aliquoted in new eppendorff tubes. The samples were stored at -70C until ready to use.

Before freshly isolated Leydig cells could be lysed, cell suspensions were centrifuged for 10 min at 260×g and 4 C and the supernatant was discarded. As for cell monolayers, the freshly isolated cell pellets were gently washed once, with ice-cold PBS, and centrifuged as above before the cells were lysed.

V.2. Total Protein Quantification (Lowry)

Total cell protein was quantified using a simple colorimetric assay developed by Lowry *et al.* (1951) and adapted by Adams (1980). This technique allows the extrapolation of the total protein concentration of a given sample based on a standard curve.

First, the standard curve was prepared from a BSA stock solution (1 mg/ml in 0.1 N NaOH). This stock solution was diluted 1:4 (v/v) in 0.7N NaOH before the standard curve was determined. Standard concentrations ranged from 6.25 to 75.0

μg BSA per cuvette. Note that a blank cuvette, containing only the buffer solutions (0.7 N NaOH and Solution A) and dye (FCR) was also included. Table 3 describes more specifically the set up procedure.

For each sample, 5 μl was assayed per cuvette. Standards and samples were assayed in duplicate in 4.5 ml disposable polystyrene cuvettes (Fisher Scientific, Nepean, Ontario). Once all standards and samples were added to their respective cuvettes, the volume was adjusted to 1.5 ml with 0.7 N NaOH.

Next, 1.5 ml of freshly prepared Solution A (100 ml 13% Na_2CO_3 mixed with 3 ml of 4 % sodium tartrate and 3 ml of 2 % cupric sulfate), was added to each cuvette. The cuvettes were then mixed and incubated for 10 min at RT.

	Std / sple (μl)	Standard (μg)	0.7N NaOH (ml)	Solution A (ml)	FCR (ml)
BLANK	0.00	0.00	1.500	1.50	0.50
STD 1	25.00	6.25	1.475	1.50	0.50
STD 2	50.00	12.50	1.450	1.50	0.50
STD 3	75.00	18.75	1.425	1.50	0.50
STD 4	100.00	25.00	1.400	1.50	0.50
STD 5	200.00	50.00	1.300	1.50	0.50
STD 6	300.00	75.00	1.200	1.50	0.50
Sples	5.00	n/a	1.495	1.50	0.50

Table 3. Set up procedure for Lowry's protein assay. The type of solutions used are specified in the first row and the cuvette identification is described in the first column. The volume of each solution to be dispensed in each cuvette is specified in the body of the Table. The thick line between column 5 and 6 indicate an incubation of 10 min at RT. STD=standard; sple=sample; n/a=non applicable.

Following this incubation, 0.5 ml of Folin-Ciocalteu reagent (FCR) was added to each cuvette and mixed thoroughly. The assay was incubated for 30 min at RT before the absorbance was measured at 625 nm in a Beckman DU 640 Spectrophotometer.

Standard curve measurements were fitted in a linear regression analysis (GraphPad) and protein sample concentration was calculated.

V.3. Total Protein Electrophoresis and Transfer

Following total protein quantification by the method of Lowry, protein samples were separated on sodium dodecyl sulfate (SDS)-polyacrylamide gels by electrophoresis.

For each sample, 50 µg of total protein in 15 to 20 µl of water were diluted 1:5 (v/v) in ImmunoPure Lane Marker Reducing Sample Buffer (Pierce, Rockford, IL, USA).

For size estimation of protein bands, prestained SDS-polyacrylamide gel electrophoresis (PAGE) standards were electrophoresed alongside protein samples. Typically, broad range molecular weights of prestained SDS-PAGE standards included eight bands ranging between 208 to 7.2 KDa (Bio-Rad, Mississauga, Ontario, Canada). Prestained markers did not require any preparation prior to gel electrophoresis. Therefore, just before use, SDS-PAGE markers were thawed and 10 µl aliquots per lane were loaded, as required, onto SDS-polyacrylamide gels.

Similarly to protein samples, synthetic peptide standards were diluted in sample buffer. Samples and synthetic peptide standards were then boiled for 5 min before loading on a gel. The presence of DTT and SDS in the sample buffer and boiling allowed protein denaturation prior to electrophoresis.

Samples and standards were loaded onto 4-20% Tris-glycine linear gradient polyacrylamide gels fitted in a Mini-Protean II Dual Slab Cell system from Bio-Rad (Mississauga, Ontario, Canada). Electrophoresis was then carried out in Tris-glycine buffer (25 mM Tris base, 190 mM glycine, 3.5 mM SDS, pH 8.3) for 35 to 45 min at 140 V.

Following SDS-PAGE, samples and standards were transferred to 0.45 μ m nitrocellulose membranes (Hybond ECL, Amersham, Mississauga, Ontario) using a Trans-Blot cell (Bio-Rad). Protein electroblotting was carried out in cold transfer buffer (25 mM Tris-HCl, 192 mM glycine, 20% methanol, 0.02% SDS, pH 8.3) at 150 mA for 90 minutes. Nitrocellulose membranes were wrapped and stored at 4 C until ready to use for Western blotting analysis.

V.4. Bcl-2 and Bax Western Blotting Analysis

Among the expanding list of proteins involved in the regulation of apoptosis, Bcl-2 and Bax are believed to play a key role. Modulation of Bcl-2 and Bax expression was therefore studied, by Western blotting analysis, in freshly isolated and cultured Leydig cells.

Nitrocellulose membranes, containing electroblotted proteins (see above),

were first blocked in Blotto (Tris-buffered saline [TBS: 10 mM Tris-HCl, 150 mM NaCl] mixed with 1% Tween-20 and 5% [w/v] non-fat skim milk powder) for 60 min at RT with gentle shaking. Occasionally, blocking was carried on overnight at 4 C.

Rabbit Bcl-2 polyclonal antibodies were raised against a synthetic peptide corresponding to amino acids 4-21 mapping at the amino terminus of Bcl-2 of human origin. These antibodies react with Bcl-2 of mouse, rat and human origin by Western blotting analysis and do not cross-react with Bax. Rabbit Bax polyclonal antibodies were raised against a synthetic peptide corresponding to amino acids 43-61 mapping at the amino terminus of Bax of mouse origin. Bax antibodies specifically react with mouse, rat and human Bax proteins by Western blotting analysis and do not cross-react with Bcl-2.

Membranes were then incubated in Blotto containing 0.75 µg/ml of Bcl-2 or Bax antibody for 1-2 h at RT on an orbital shaker. In order to maximize the antibody usage, membranes and primary antibody solution were sealed in polyester heat sealable bags (Kapak Corp., Minneapolis, MN, USA). A maximum of two membranes, placed back to back, were sealed per bag. The bags were inverted every 15 min.

Following primary antibody binding, the nitrocellulose membranes were washed twice for 7 min, with gentle shaking, in TBS-1% Tween-20 (TTBS) and then incubated with the secondary antibody. Horseradish peroxidase-linked donkey anti-rabbit immunoglobulins were used as the secondary antibodies at a 1:2000 dilution in Blotto. The membranes were incubated for 45 min, with gentle shaking

at RT. Finally, the membranes were washed three times for 5 min in large volumes of TTBS and once more more in TBS for 5 min.

Immunoreactive bands were detected by enhanced chemiluminescence (ECL). SuperSignal substrate for Western blotting was used for that purpose. According to the manufacturer's protocol, an equal volume of luminol / enhancer solution was combined with stable peroxide solution and added to the surface of each membrane. The membranes were then allowed to react with this revealing solution for 5-7 min at RT, gently blotted to remove excess solution, and exposed to autoradiographic film for 5 min and up to 3 h.

VI. ANDROGEN RADIOIMMUNOASSAY (RIA)

For most experiments, when rat Leydig cells were cultured for 3-72 h, androgen levels in the spent media were determined by radioimmunoassay. Total androgen content was assayed by using the antibody β -8, raised against testosterone. Since this antibody cross-reacts extensively with other androgens (see Table 4), the results obtained represent total androgen content of the spent media rather than testosterone content. The antibody β -8 was a generous gift from Dr. D. Armstrong (University of Western Ontario, London, Ontario, Canada). The assay was done on unextracted samples according to the procedure described by Anakwe and Moger (1986).

The androgen radioimmunoassay protocol used here required 5 components, namely, androgen standards or samples, steroid assay buffer,

STEROID	% BINDING
Testosterone	100.00
5 α -Dihydrotestosterone	98.00
5 α -Androstane-3 α , 17 β diol	58.00
5 α -Androstane-3 β , 17 β diol	24.00
Androstenedione	1.60
Androsterone	1.20
Estradiol	0.16
Progesterone	<0.001
Cortisol	<0.007

Table 4. Percent cross reaction of the antibody β -8 with various steroids.

tritiated testosterone ($^3\text{H-T}$), β -8 anti-testosterone antibody, and finally a charcoal-dextran suspension. Table 5 describes the specific volumes, used in each tube, for each solution.

For each assay, duplicate aliquots of 5-100 μl of spent media were transferred to 12 \times 75 mm glass tubes. The volume was then adjusted to 500 μl with steroid assay buffer (0.1 M phosphate buffer, pH 7.4, 61.4 mM Na_2HPO_4 , 38.6 mM $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 0.247 mM thimerosal; supplemented with 154 mM NaCl and 0.1% gelatin).

The testosterone standard curve, also assayed in duplicate, contained 10 to 500 pg testosterone in 500 μl steroid assay buffer. In order to reduce interassay variability, a testosterone standard stock solution (100 ng testosterone/ml in absolute ethanol) was previously prepared and used for all assays. Prior to use in the assay, the testosterone stock solution was diluted 1:100 (v/v) in steroid assay

buffer to obtain a working concentration of 1 pg testosterone/ μ l.

Each androgen assay also included duplicate tubes to measure total ^3H -testosterone ($^3\text{H-T}$) counts (TC), duplicate tubes to measure non-specific binding (NSB) of the $^3\text{H-T}$ to the coated charcoal, and, finally, duplicate tubes to measure total $^3\text{H-T}$ binding to the antibody (TB) in the absence of any unlabelled steroid.

Following the addition of the standard or samples and the steroid assay buffer to their respective glass tubes, 100 μ l of a diluted $^3\text{H-T}$ solution was added to each tube. This $^3\text{H-T}$ solution was prepared from a stock solution stored in benzene:ethanol (9:1) solvent. A sufficient aliquot of this solution was air dried

Tube id.	T. Conc. (pg/tube)	Std / sple (μ l)	Buffer (μ l)	$^3\text{H-T}$ (μ l)	Ab β -8 (μ l)	Charcoal (μ l)
TC	0.0	0.0	800.0	100.0	0.0	0.0
NSB	0.0	0.0	600.0	100.0	0.0	200.0
Std 0 (B_0)	0.0	0.0	500.0	100.0	100.0	200.0
Std 1	10.0	10.0	490.0	100.0	100.0	200.0
Std 2	25.0	25.0	475.0	100.0	100.0	200.0
Std 3	50.0	50.0	450.0	100.0	100.0	200.0
Std 4	100.0	100.0	400.0	100.0	100.0	200.0
Std 5	200.0	200.0	300.0	100.0	100.0	200.0
Std 6	300.0	300.0	200.0	100.0	100.0	200.0
Std 7	400.0	400.0	100.0	100.0	100.0	200.0
Std 8	500.0	500.0	0.0	100.0	100.0	200.0
samples	unknown	5-100	SV to 500	100.0	100.0	200.0

Table 5. Set up procedures for androgen radioimmunoassay. The type of solutions used are specified in the first row while the tube identification (tube id.) and the testosterone concentration (T. Conc.) of the different standards (Std) is described in the first and second column respectively. The volume of each solution to be dispensed is specified in the body of the Table. TC=Total count; NSB=non-specific binding; SV=sufficient volume; Ab=antibody.

and re-dissolved in steroid assay buffer to obtain a final count of 14,000 cpm per 100 μ l.

Next, the β -8 anti-androgen antibody was added to every assay tube, with the exception of the TC and the NSB tubes. For the assay, a working solution was prepared by diluting the β -8 anti-androgen antibody stock solution (0.67% antibody w/v in 0.9% NaCl) 1:150 (v/v) in steroid assay buffer.

The tubes were then vortexed, covered and stored at 4 C overnight in a refrigerator certified for radioisotope storage. The next day, the unbound steroids were separated by adsorption with a suspension of charcoal and dextran (250 mg Norit A charcoal, 25 mg dextran T-70 in 40 ml steroid assay buffer). The charcoal-dextran suspension was freshly prepared, placed in an ice bath and allowed to mix on a magnetic stirrer for 15 minutes before use. Each assay tube, except the TC tubes, received 0.2 ml of the charcoal-dextran suspension.

Following a 10 min incubation on ice, every tube was centrifuged at 1000 \times g for 15 min at 4 C. The resulting supernatants, containing the antibody-bound androgens, were decanted into 7 ml polyethylene scintillation vials. The samples were then mixed with 5 ml of Ecolite scintillation fluid and the radioactivity was measured in a 1215 Rackbeta II Liquid Scintillation Counter (Wallac Oy, Finland).

The androgen content of the samples was extrapolated from the standard curve using the computer program RIAPC Radioimmunoassay Calculation designed by Donald Rieger (Ph.D thesis, 1988). The program transforms the raw counts (in cpm) for the standard curve according to the following equation:

$$\text{logit (Y)} = \log [100/(100-\% \text{ bound Y})]$$

Where: $\% \text{ bound Y} = 100 \times [(\text{cpm Y} - \text{cpm NSB}) / (\text{cpm B}_0 - \text{cpm NSB})]$

And: Y = the standard being calculated

Specific binding for the assay, $(\text{cpm B}_0 / \text{cpm TC}) \times 100$, was generally around 40%. The assay sensitivity, corresponding to 90% B_0 , was approximately 8 pg/tube.

VII. FLUORESCENT DNA ASSAY

In order to determine the rate of steroidogenesis per cell in our culture model, the amount of DNA in the cultures was assayed. The procedure used was that reported by Labarca and Paigen (1980) with only slight modifications. This assay is based on the ability of the fluorescent dye bisbenzamide to emit a fluorescent signal once bound to DNA. Following the addition of the dye, the assays were handled under diffuse light to reduce dye bleaching.

Following Leydig cell culture, the culture medium was collected for androgen RIA and the cell monolayer was washed twice with 0.5 ml of washing buffer (0.15 M NaCl, 5.0 mM Na_2HPO_4 , pH 7.4) per sample. Each sample then received 0.5 ml of phosphate buffer (50 mM Na_2HPO_4 , 2 mM EDTA, 0.02% sodium azide, pH 7.4) and were sonicated for 10 sec at 20 watts using a Sonifier Cell Disruptor (Heat Systems Ultrasonics, Plain View, NY, USA). The samples were collected in

1.5 ml eppendorf tubes and stored at -20 C until ready for assay.

The DNA standard curve was prepared by diluting a DNA stock solution (500µg calf thymus DNA per ml in nanopure water, stored at 4 C for up to 5 months). Note that the precise DNA concentration of this stock solution was determined by spectrophotometry (DU 640 Beckmann, Mississauga, Ontario) using a 1:100 dilution read at 260 nm. The precise DNA concentrations could then be calculated according to the following equation:

$$\text{mg DNA/ml} = (\text{optical density} \times \text{dilution}) / 18.5$$

where $18.5 = A_{260}$ for 1 mg calf thymus DNA / ml

and optical density = $A_{260} \times C_{\text{stock}} \times 1/\text{dilution}$

The DNA standard curve was obtained by diluting the stock solution approximately 1:100 in phosphate buffer and pipetting 10, 20, 50, 100, 200, 300, 400 µl, in duplicate, in disposable polystyrene cuvettes respectively, to precisely obtain a standard curve ranging from 50 to 2000 ng DNA per cuvette. A blank cuvette containing only the buffers and dye was also prepared to blank the spectrophotometer. For each sample, a 200 µl aliquot was assayed.

The volume in all cuvettes (blank, standards and samples) was completed to 1.5 ml with NaCl/phosphate buffer (4M NaCl, 50 mM Na_2HPO_4 , 2 mM EDTA, 0.02% sodium azide, pH 7.4). A fresh solution of bisbenzamide was prepared by diluting 1:100 in NaCl/phosphate buffer a 200 µg/ml in nanopure water stock solution. Five hundred microliters of this diluted dye solution was added to each cuvette using a repetitive pipettor. When necessary, the assay could be stored at

4 °C in the dark for up to 16 h before reading. The samples were read in a spectrofluorometer at 356nm excitation and 458nm emission, keeping the slit width at 10 μm. The amount of DNA in the samples was determined by comparison to the standard curve with correction for dilution. The standard curve was calculated by linear regression analysis. The volume of each sample was adjusted so that DNA content fell in the mid-range of the standard curve.

VIII. DATA ANALYSIS

Most experiments were repeated at least three times. Representative autoradiograms and light microscope pictures are presented for the analysis of DNA fragmentation. Quantitative analysis of apoptotic Leydig cells (% apoptotic cells vs non-apoptotic cells ± SD) were obtained by microscopy (magnification: X400). Statistical differences ($P < 0.05$) between mean values were determined by one-way analysis of variance (GraphPad InStat, Intuitive Software for Science, San Diego, CA, USA) or two-way analysis of variance (Systat 7.0, SPSS Inc.) according to the experimental design. Correlation between steroidogenesis rate, incubation time or treatment on the onset of apoptosis was statistically assessed by Spearman rank-order correlation coefficient analysis (Systat 7.0, SPSS Inc.).

RESULTS

I. SPONTANEOUS ONSET OF APOPTOSIS IN PRIMARY CULTURES OF RAT LEYDIG CELLS

I.1. Ethidium Bromide-Stained Agarose Gel Analysis

In preliminary experiments, rat Leydig cells (1.5×10^6 cells/sample) were cultured for 0-48 h in supplemented M199 with no additional treatment. Total DNA, isolated and purified from these cells, was analyzed by agarose gel electrophoresis (5 μ g DNA/lane) and stained with ethidium bromide. Sizes of DNA fragments (kbp) were estimated by comparison to migratory distances of ϕ X174 RF DNA/Hae III fragments visualized in Figure 6.

DNA extracted from freshly isolated (basal) cells (Figure 6, 0 h) revealed no apoptotic DNA fragmentation. This observation suggested that, in the normal adult rat testis, apoptosis in Leydig cells is a rare event.

Moreover, DNA extracted from Leydig cells cultured for 3 h (Figure 6) showed no obvious difference from basal cell DNA. However, DNA extracted from cells cultured for 6 h revealed the first signs of internucleosomal DNA cleavage associated with apoptosis. Following 12 h of incubation, or longer, DNA electrophoretic analysis clearly displayed the characteristic DNA ladder pattern of apoptosis (Figure 6).

In primary cultures of Leydig cells, while cell death is expected to occur with increasing incubation time, these results suggested that the onset of apoptosis was

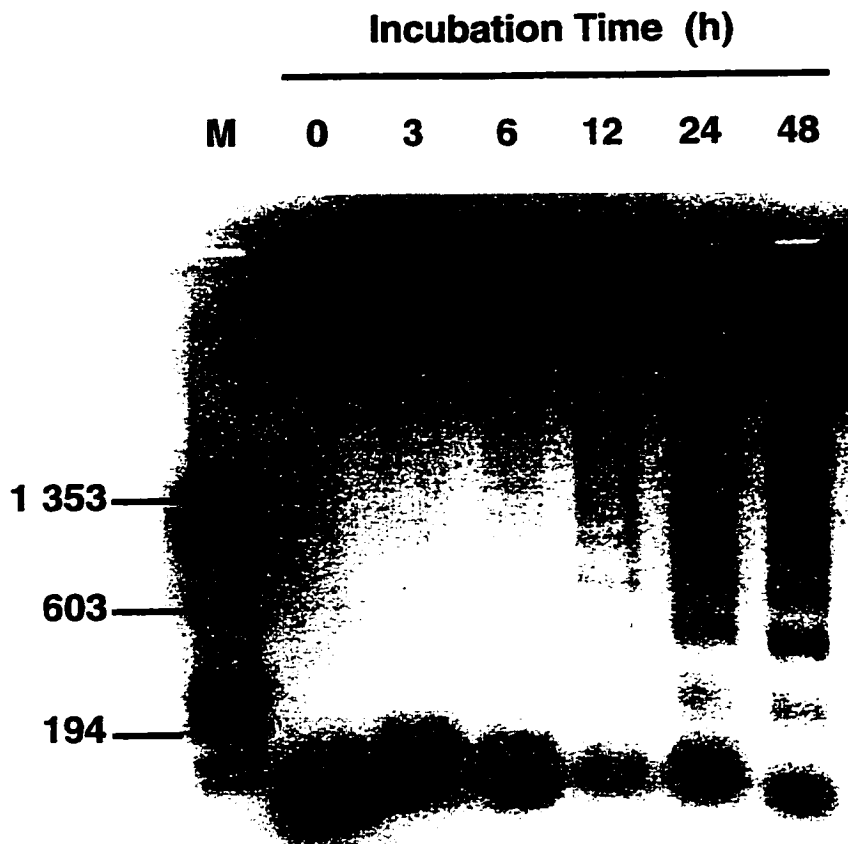


Figure 6. Leydig cell DNA electrophoresis in an agarose gel containing ethidium bromide. Rat Leydig cells (1.5×10^6 cells/sample) were cultured for 0 - 48 h in supplemented M199 with no additional treatment. Total DNA, isolated and purified from these cells, was analyzed by agarose gel electrophoresis ($5 \mu\text{g}$ DNA/lane). DNA visualization by UV transillumination revealed that no apoptotic DNA fragmentation could be detected in freshly isolated Leydig cells. However, DNA extracted from cells cultured for 6 h showed the first signs of internucleosomal DNA fragmentation. Following 12 h of incubation, or longer, DNA samples clearly displayed the characteristic apoptotic ladder pattern. M refers to molecular weight marker expressed in base pairs.

rapidly initiated *in vitro*. We therefore became interested in studying the onset of apoptosis in more detail, hoping to slow down this process by improving the culture conditions.

1.2. DNA ³²P- 3' End Labeling

The first step was to adapt, to our cell model, a DNA labeling technique that would detect apoptotic DNA fragmentation with greater sensitivity. Modification of the DNA ³²P-3' end labeling technique developed by Tilly and Hsueh (1993) allowed us to label apoptotic DNA fragments with a tail of ³²P-adenosine nucleotides.

Similar to the ethidium bromide DNA analysis (Figure 6), DNA 3' end labeling revealed no apoptotic DNA fragmentation in freshly isolated cells (Figure 7, 0 h). However, this radioactive labeling technique convincingly detected the spontaneous onset of apoptosis by 3 h in culture. Indeed, DNA extracted from Leydig cells cultured for 3 to 18 h displayed the characteristic internucleosomal fragmentation of DNA into 180 - 200 bp multiples (Figure 7).

This DNA ³²P-dATP-3' end labeling technique allowed analysis of minute amounts of DNA; 0.3 µg DNA was loaded per sample as opposed to 5 µg for ethidium bromide analysis. Moreover, its extreme sensitivity also permitted detection of apoptotic DNA fragmentation much earlier during culture than could be detected by ethidium bromide staining.

Considering the sensitivity of this technique, the absence of apoptotic DNA



Figure 7. Leydig cell DNA (0.3 μg DNA/lane) electrophoresis in an agarose gel following ^{32}P -3' end labeling. Rat Leydig cells were cultured in 60 mm petri dishes for 0 to 18 h with no additional treatment. Total DNA was isolated and elongated at the 3' end with ^{32}P -dATP using the TdT enzyme. Following drying, the agarose gel was exposed to X-ray film (Kodak X-OMAT) for 5 min. Gel autoradiography revealed no detectable apoptotic DNA fragmentation in freshly isolated Leydig cells. However, following 3 h of incubation, the DNA fragmentation pattern, specific to physiological cell death, was evident.

fragmentation observed in basal cells became even more significant, supporting the rarity of apoptosis in Leydig cells *in vivo*.

The major disadvantage arising from DNA ^{32}P -dATP-3' end labeling was the impossibility to quantify apoptosis. The use of deoxyadenosine triphosphate sustained a continuous elongation process where an unknown number of radioactive nucleotides bound to DNA fragments. Since the length of the radioactive nucleotide tail undoubtedly differed from fragment to fragment, we had to restrict our analysis to qualitative assessment. Moreover, the radioactive signal emitted by the ^{32}P adenosine nucleotide tail was so strong that it was often difficult to prevent overexposure of the X-ray films (Figure 7).

1.3. Quantification of Apoptosis by In Situ End Labeling (ISEL)

Even though we had resolved the problem arising from detection sensitivity, we were still unable to quantify apoptosis in primary cultures of rat Leydig cells. This was finally solved with ISEL. This technique, which combines both high sensitivity and quantitative attributes, induces DNA 3' end elongation with biotinylated nucleotides; here, biotinylated d-ATP was used. Elongated DNA fragments were detected by AEC staining. Inasmuch as cells remained intact during and after the labeling procedures, it was possible, using microscopy, to count apoptotic versus non-apoptotic cells (Figure 8). Results were then expressed as the percent apoptotic cells as a function of the total number of cells counted (Figure 9).



Figure 8.a. Immediately following cell isolation, Leydig cells were cytospun on glass slides and fixed. Less than 1% of these cells stained positive for apoptosis. The sample presented to the left shows no apoptotic cell since all the cell nuclei remained blue following ISEL.

Figure 8.b. Leydig cells were incubated for 3 h with no additional treatments before processed for ISEL. Cell analysis by microscopy revealed the presence of 12.3% apoptotic cells as shown by the red nucleic staining in this typical sample.

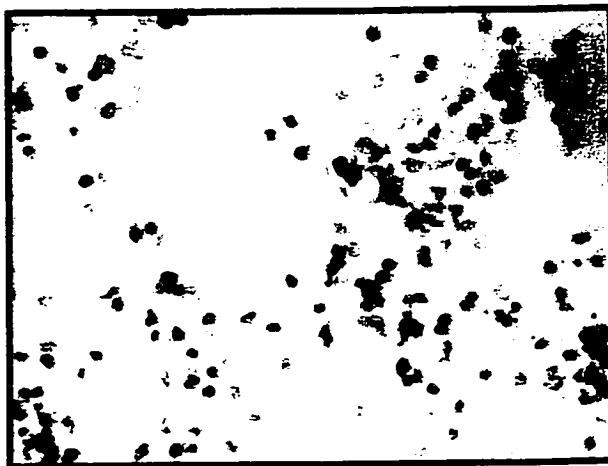
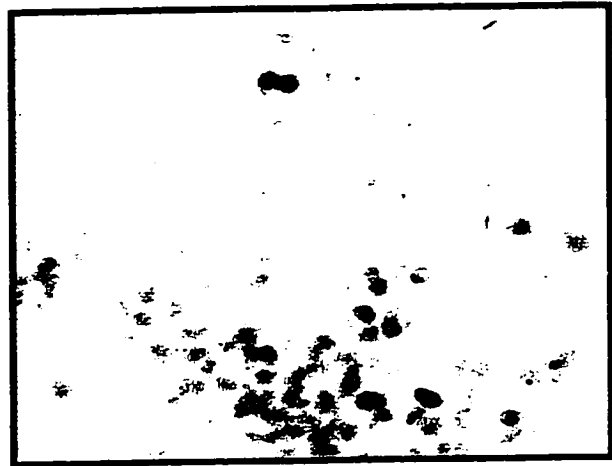


Figure 8.c. Following 24 h of incubation with no additional treatments, the number of apoptotic cells increased to 32.3% in primary cultures of rat Leydig cells.

Figure 8. ISEL stained Leydig cells visualized by microscopy (400 X).

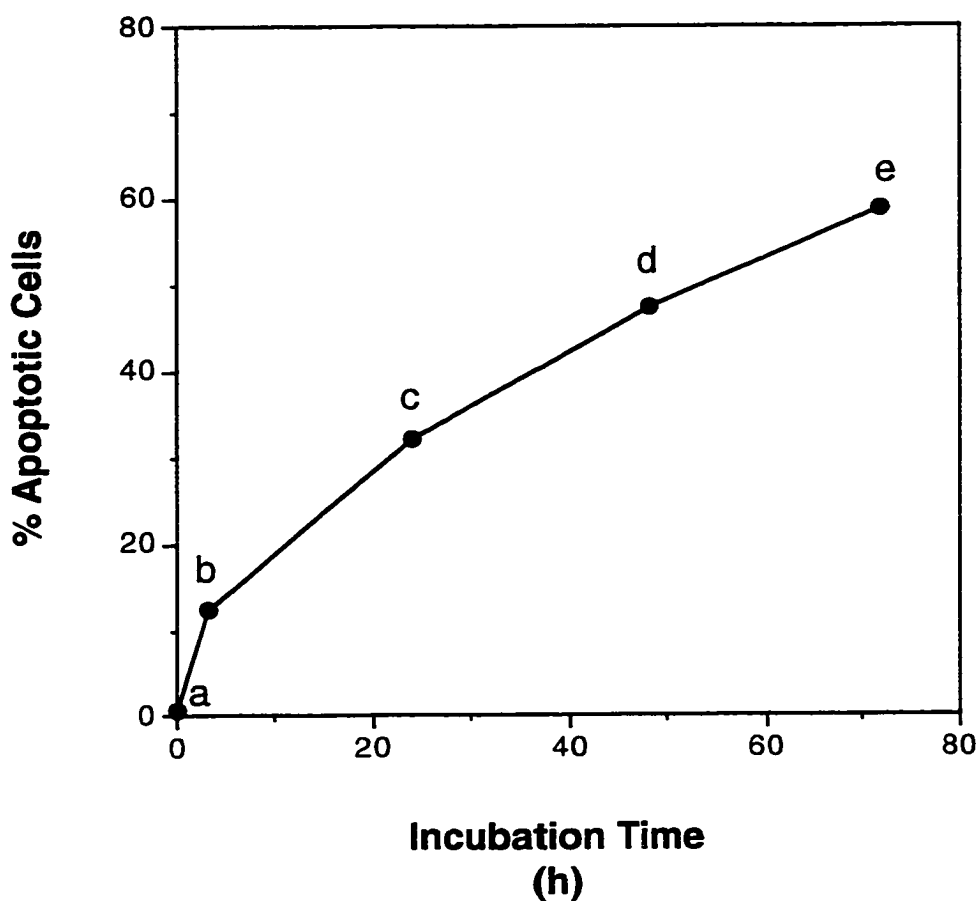


Figure 9. Time-dependent spontaneous onset of apoptosis in primary cultures of rat Leydig cells. Leydig cells were cultured in suspension from 0 to 72 h with no additional treatment, cytospun on glass slides and processed for ISEL. Apoptotic versus non-apoptotic cells were quantified by microscopy. Results show that the number of apoptotic Leydig cells increased steadily with the duration of the culture. Results are expressed as a percent of the total number of cells counted per sample and are presented as the mean of two to nine experiments. Groups with different letters are significantly different from each other as determined by a Scheffe multiple comparison test. $p < 0.0001$. (Note that for any group the SEM was less than 1.0)

ISEL performed on rat Leydig cells, cultured in suspension for 0 to 72 h in supplemented M199 with no additional treatment, was used to quantify the spontaneous onset of apoptosis. Leydig cells cytopun and fixed on glass slides immediately following cell preparation presented hardly any apoptotic cells (Figure 8.a). On average, $0.69\% \pm 0.22$ (mean \pm SEM, $n=3$) Leydig cells were apoptotic following cell preparation (Figure 9). Although early results presented evidence supporting the rarity of apoptosis in freshly isolated Leydig cells, these results provided a quantitative assessment. Like DNA 3'end labeling analysis, ISEL allowed early detection of apoptotic DNA fragmentation as indicated by the presence of red nuclei (indicating apoptotic cells) in cells incubated for 3 h or longer (Figures 8.b and 9). The time-dependent spontaneous onset of apoptosis, in untreated primary cultures of Leydig cells, became manifest following quantitative ISEL analysis; the number of labeled nuclei steadily increased with the duration of the culture (Figures 8.c and 9). Indeed, while results showed that freshly isolated cells, immediately fixed on glass slides, were virtually apoptosis-free, $12.3\% \pm 0.21$ (mean \pm SEM, $n=3$) of the rat Leydig cells stained positive for apoptosis following 3 hours in culture (Figure 9). The number of apoptotic cells increased to $32.28\% \pm 0.81$ (mean \pm SEM, $n=9$), $47.40\% \pm 0.62$ (mean \pm SEM, $n=2$) and $58.94\% \pm 0.42$ (mean \pm SEM, $n=2$) following 24, 48 and 72 h in culture, respectively (Figure 9). These results supported the observation that primary cultures of terminally differentiated cells do not survive long incubations. Moreover, Spearman rank-order correlation analysis corroborated the existing positive relation

between the extent of apoptosis and the duration of the incubation. This statistical analysis presented a very significant correlation coefficient (r_s) value equivalent of 0.97 ($p < 0.0001$).

The localization of ISEL staining was not the result of an artifactual reaction since the deliberate induction of DNA nicks with DNase I resulted in intensive labeling of all the cell nuclei. For each experiment, the ISEL reaction was tested against Leydig cells cytopun on glass slides and pretreated with DNase I (1 $\mu\text{g/ml}$) for 10 min at RT to induce extensive DNA fragmentation. These positive controls displayed uniform staining of all nuclei, while omission of the terminal transferase consistently led to completely negative results (data not shown).

II. EFFECTS OF LUTEINIZING HORMONE ON PRIMARY CULTURES OF RAT LEYDIG CELLS

In vivo, LH is principally responsible for stimulating steroidogenesis in Leydig cells. The essential role of LH in protecting the Leydig cell population from apoptosis was revealed in several studies where hypophysectomy and/ or LH antagonists were used (Saez, 1994; Tapanainen *et al.*, 1993). *In vitro*, we expected to observe similar trophic effects of LH on primary cultures of rat Leydig cells. For this purpose, the importance of LH in supporting Leydig cell viability and function *in vitro* was studied as a function of LH concentration over time.

II.1. Effects of LH on Primary Cultures of Rat Leydig Cells Cultured for 0 - 72

h

a. Effects of LH on the Production of Androgens

Primary cultures of rat Leydig cells (10^5 cells/ ml M199/ well) were incubated from 0 - 72 h in the absence or presence of LH (0.1 - 10.0 ng/ml). As mentioned earlier, LH stimulates steroidogenesis in Leydig cells when it binds to its plasma membrane receptors. Following cell culture, androgens, released by Leydig cells into the culture media, were quantified by RIA.

Rat Leydig cells cultured for 24 h with no further hormonal treatment typically produced 13.7 ng androgen/ 10^5 cells \pm 0.62 (mean \pm SEM, n=13) (Figure 10). LH treatments, either at a physiological level (0.1 ng LH/ml), or at a supra-physiological concentration (10.0 ng LH/ml), markedly stimulated steroidogenesis over this period of incubation. The androgen production increased more than 3-fold in Leydig cells treated with LH at 0.1 ng/ml, as 46.1 ng/ 10^5 cells \pm 1.86 (mean \pm SEM, n=11) could be measured in the culture media. Moreover, Leydig cells treated with LH at 10.0 ng/ml secreted 103 ng androgen/ 10^5 cells \pm 6.1 (mean \pm SEM, n=11). The latter represented more than 7-fold increase compared to control conditions (Figure 10). Statistical analysis of the androgen RIA data confirmed the significant increase ($p < 0.0001$) in steroid production induced by increasing LH stimulation.

Leydig cells, cultured for more than 24 h, are known to gradually lose their

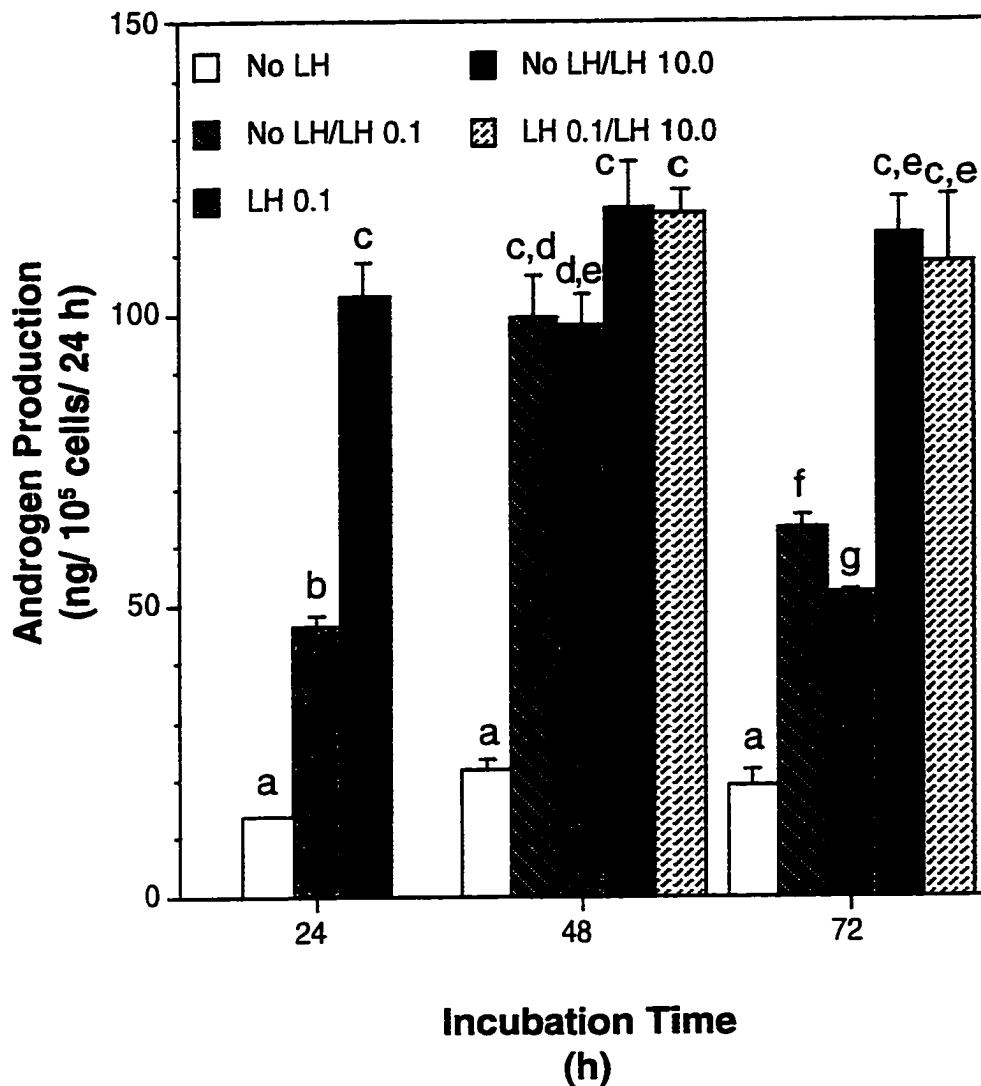


Figure 10. *In vitro* androgen production in rat Leydig cells cultured for 24 to 72 h in absence or presence of LH (0.1 - 10.0 ng/ml). Leydig cells cultured for 48 or 72 h were treated with no LH or LH at 0.1 ng/ml from 0 - 24 h or 0 - 48 h, respectively. LH 0, 0.1, or 10.0 at ng/ml was then added, as specified in Table 2, for the last 24 h of incubation. Results represent mean \pm SEM of two to eleven experiments. Groups with different letters above the error bars are significantly different from each other as determined by a Scheffe multiple comparison test. $p < 0.05$.

steroidogenic abilities (Rommerts, 1981). In an effort to test the protective role LH on Leydig cell survival and steroidogenic abilities *in vitro*, we treated cultured Leydig cells with various combinations of LH (0, 0.1 and 10.0 ng/ml) over 48 and 72 h incubation periods (see Table 2 for treatment description, Section III.3).

Leydig cells, cultured for a total of 48 h, were treated with no LH or LH at 0.1 ng/ml from 0 - 24 h of incubation. Similarly, Leydig cells cultured for a total of 72 h were treated with no LH or LH at 0.1 ng/ml from 0 - 48 h of incubation. The culture medium was collected and replaced with fresh medium with or without LH each day. Twenty-four hours before the end of cell cultures, the media was replaced with fresh M199 supplemented with no LH, or with LH at 0.1 ng/ml or with LH at 10.0 ng/ml until the total incubation time was reached. Each group of culture medium collected over 24 h was assayed separately for androgen content.

Rat Leydig cells, cultured in M199 with no additional treatment, released 21.8 ng androgen/ 10^5 cells \pm 1.99 (mean \pm SEM, n=2) following 48 h incubation and 19.3 ng androgen/ 10^5 cells \pm 2.62 (mean \pm SEM, n=2) following 72 h incubation (Figure 10). Statistical analysis of these androgen RIA data did not show any significant decrease ($p > 0.05$) of steroid production rate over time in cell samples cultured with no hormonal treatment for 24 to 72 h. The rather limited sample number for cells cultured for 48 or 72 h could have contributed to these statistically non-significant results.

Leydig cells, treated with no LH from 0 - 24 h and LH at 0.1 ng/ml from 24 - 48 h, released 99.5 ng androgen/ 10^5 cells \pm 6.85 (mean \pm SEM, n=2) over the last

24 h of culture (Figure 10). Similarly, cell samples, treated with LH at 0.1 ng/ml from 0 - 48 h, released 98.1 ng androgen/ 10^5 cells \pm 5.32 (mean \pm SEM, n=2) over the last day of culture (Figure 10). Statistical analysis revealed no significant difference ($p>0.05$) between those two treatments. Interestingly, Leydig cells cultured for a maximum of 48 h in the presence of no LH from 0 - 24 h and LH at 0.1 ng/ml from 24 - 48 h, or in the presence of LH at 0.1 ng/ml from 0 - 48 h, secreted almost twice as much androgens ($p<0.01$) than Leydig cells cultured for a maximum of 24 h in presence of LH at 0.1 ng/ml. These figures suggested an increased steroidogenic response to LH at 0.1 ng/ml following 24 h incubation. The potentially slower steroidogenic rate, affecting Leydig cells in culture from 0 - 24 h, can possibly be explained by the cell requirements, following cell isolation, for an adaptation period *in vitro*.

Moreover, prolonged exposure to LH usually induces LH receptor desensitization. Although Leydig cells were cultured in the presence of LH at 0.1 ng/ml for 24 to 48 h, androgen release over the last 24 h of incubation did not show the effects of LH receptor down regulation on steroidogenesis.

Leydig cell samples, cultured for 72 h and treated with no LH from 0 - 48 h and LH at 0.1 ng/ml from 48 to 72 h, produced 63.1 ng androgen/ 10^5 cells \pm 2.48 (mean \pm SEM, n=2). Cell samples, treated with LH at 0.1 ng/ml for 72 h, produced 52.0 ng androgen/ 10^5 cells \pm 0.61 (mean \pm SEM, n=2). Statistical analysis revealed a significant difference ($p<0.05$) between these results. In this case, the evidence suggests that cell exposure to LH 0.1 ng/ml for 72 h, induced

LH receptor desensitization which resulted in a reduced steroidogenic rate. Moreover, in comparison to LH (0.1 ng/ml)-treated cells cultured for 48 h, Leydig cells cultured for 72 h under analogous conditions secreted significantly ($p < 0.01$) less androgens. Besides the involvement of LH receptor desensitization, incubation length might have contributed to a reduced steroidogenic rate. As mentioned earlier, Leydig cells are known to lose their steroidogenic abilities over time.

Over the last 24 h of incubation, some cell samples were incubated in the presence of LH at 10.0 ng/ml. Similar to cells cultured in the presence of LH at 10.0 ng/ml for 24 h, Leydig cells treated with no LH from 0 - 24 h and LH at 10.0 ng/ml from 24 - 48 h secreted 118 ng androgen/ 10^5 cells \pm 8.28 (mean \pm SEM, $n=2$). Cell samples incubated in the presence of LH at 0.1 ng/ml from 0 - 24 h and LH at 10.0 ng/ml from 24 - 48 h released 117 ng androgen/ 10^5 cells \pm 3.71 (mean \pm SEM, $n=2$) into the culture medium (Figure 10). Statistically, no significant difference ($p > 0.05$) existed in the amount of androgens released by Leydig cells, treated with LH at 10.0 ng/ml over the last 24 h incubation and cultured for a total of 24 h or 48 h.

Leydig cells cultured in the presence of no additional treatment from 0 - 48 h, and then in presence of LH at 10.0 ng/ml from 48 - 72 h, secreted 113 ng androgen/ 10^5 cells \pm 6.48 (mean \pm SEM, $n=2$) into the culture media over the last 24 h of incubation. When Leydig cells were cultured in the presence of LH at 0.1 ng/ml from 0 - 48 h and LH at 10.0 ng/ml from 48 - 72 h, 109 ng androgen/ 10^5

cells ± 11.58 (mean \pm SEM, n=2) were released into the culture media (Figure 10). Again, no statistically significant difference ($p>0.05$) existed between these values. Moreover, statistical analysis revealed no significant difference ($p>0.05$) between any of the cell samples incubated in the presence of LH at 10.0 ng/ml during the last 24 h of incubation, regardless of the incubation length or early LH treatments.

Interestingly, in cell samples treated with LH at 10.0 ng/ml over the last 24 h of incubation, no apparent LH receptor desensitization could be observed. Furthermore, under these conditions, androgen production did not seem to be affected by incubation length. Evidence suggests that the supra-physiological dose of LH, present in the culture media for the last 24 h of incubation, was sufficiently potent to maintain a high steroidogenic rate.

b. Effect of LH on the onset of apoptosis

Rat Leydig cells, cultured in suspension from 0 to 72 h and treated with various combinations of LH (see Table I for treatment description, Section III.3), were processed for ISEL. While LH was shown to be essential for Leydig cell survival *in vivo*, the results shown below suggest a difference *in vitro*.

As mentioned earlier, $32.3\% \pm 0.81$ (mean \pm SEM, n=9) of Leydig cells, cultured for 24 h under control conditions, underwent apoptotic cell death (Figure 11). The addition of LH to the culture media, at a physiological dose (0.1 ng/ml), had no significant effect ($p>0.05$) on the onset of apoptosis in 24 h cultures. Indeed, $34.9\% \pm 1.09$ (mean \pm SEM, n=7) of the Leydig cell population displayed

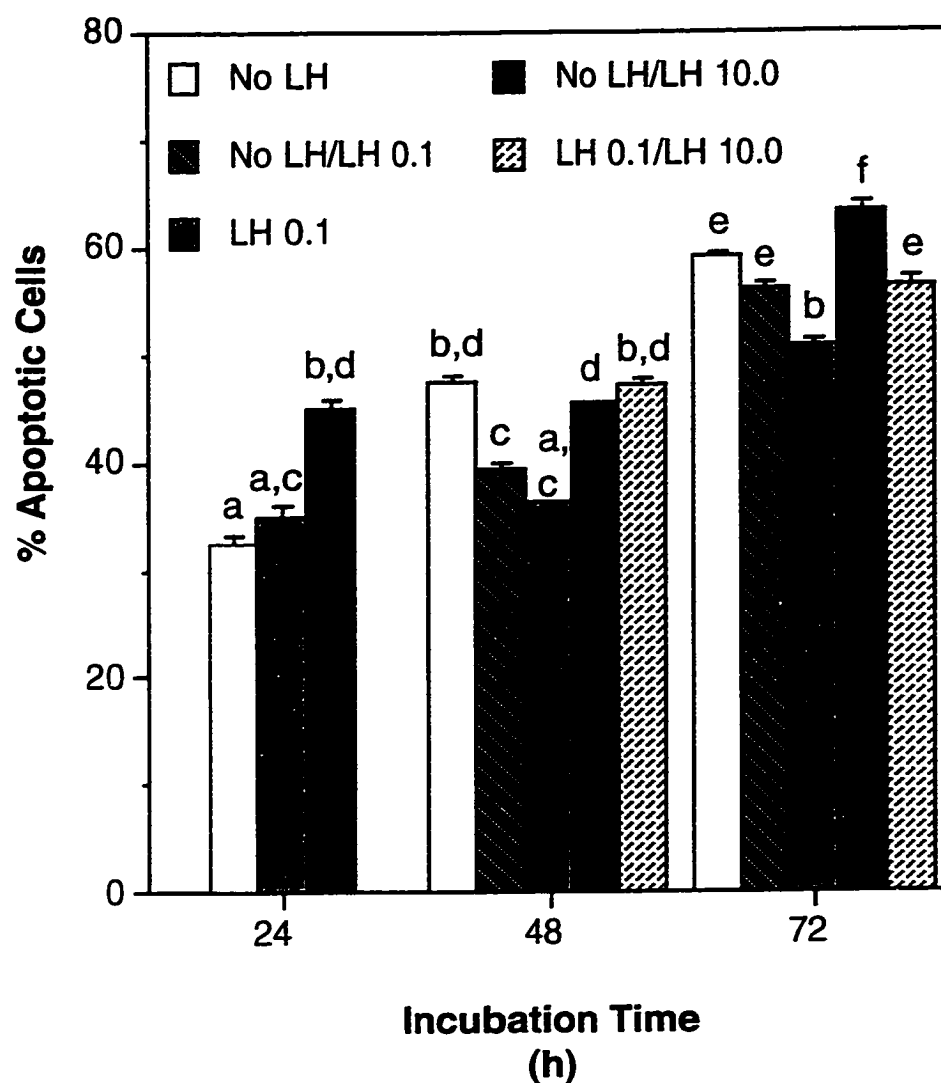


Figure 11. Percent apoptotic cells in primary cultures of rat Leydig cells incubated for 24 to 72 h. Leydig cells were cultured in absence or presence of LH (0 - 10.0 ng/ml). Cell samples incubated for 48 or 72 h were treated with no LH or LH 0.1 at ng/ml from 0 - 24 h or 0 - 48 h, respectively. LH 0, 0.1 or 10.0 at ng/ml was then added to the media, as specified in Table 2, for the last 24 h of incubation. Results represent mean \pm SEM of two to eleven experiments. Groups with different letters above the error bars are significantly different from each other as determined by a Scheffe multiple comparison test. $p < 0.05$.

the characteristic red nuclei following ISEL. However, Leydig cell stimulation with LH at 10.0 ng/ml from 0 - 24 h, triggered a significant increase ($p < 0.0001$) in the number of apoptotic cells. Results from ISEL quantitative analysis showed that, under these conditions, $44.8\% \pm 0.98$ (mean \pm SEM, $n=9$) of the Leydig cells were apoptotic after 24 h incubation (Figure 11).

In contrast to its neutral effect on the onset of apoptosis following 24 h incubation, LH at 0.1 ng/ml notably protected ($p < 0.0001$) cells cultured for 48 h. Figure 11 reveals that $47.4\% \pm 0.62$ (mean \pm SEM, $n=2$) Leydig cells, cultured for 48 h with no LH, were apoptotic. The addition of LH at 0.1 ng/ml slowed down the apoptotic machinery since $39.4\% \pm 0.48$ (mean \pm SEM, $n=2$) of Leydig cells were apoptotic when LH was added for the final 24 h incubation. The onset of apoptosis was not further inhibited ($p > 0.05$) when LH at 0.1 ng/ml was continuously present in the culture media for 48 h. Under these conditions, $36.4\% \pm 0.14$ (mean \pm SEM, $n=2$) of the Leydig cell population cultured with LH at 0.1 ng/ml for 48 h were apoptotic. Nevertheless, in comparison to control Leydig cells cultured for 48 h with no added treatment, 23% less cells underwent apoptotic cell death when treated with LH at 0.1 ng/ml (Figure 11). Interestingly, there was no significant difference ($p > 0.05$) between the number of apoptotic Leydig cells incubated for 24 h or 48 h in the presence of LH at 0.1 ng/ml.

Contrary to its apoptotic effect in cells cultured for 24 h, LH at 10.0 ng/ml treatments added from 24 - 48 h to the culture media did not promote additional apoptosis ($p > 0.05$). Results showed that, similar to control cells cultured for 48 h,

45.5% \pm 0.14 (mean \pm SEM, n=2) of cells were apoptotic when cultured with no LH from 0 - 24 h followed by LH at 10.0 ng/ml treatment from 24 - 48 h. Similarly, the addition of LH at 0.1 ng/ml from 0 -24 h followed by LH at 10.0 ng/ml from 24 - 48 h induced apoptosis in 47.1% \pm 0.49 (mean \pm SEM, n=2) of the Leydig cells (Figure 11).

Following 72 h incubation, 58.9% \pm 0.42 (mean \pm SEM, n=2) of the Leydig cells, cultured with no additional treatment, were apoptotic. Statistically, no significant difference ($p>0.05$) could be observed when cells were incubated with no added treatment from 0-48 h and LH at 0.1 ng/ml from 48-72 h. Consequently, 56.0% \pm 0.49 (mean \pm SEM, n=2) of the Leydig cell population was apoptotic following 72 h incubation under these culture conditions (Figure 11). Contrary to its effect on cells cultured for 48 h, these results suggested that LH, present in physiological doses from 48 to 72 h incubation, could not significantly ($p>0.05$) prevent the onset of apoptosis. However, the anti-apoptotic effect of LH at 0.1 ng/ml could be observed in samples where the gonadotropin was continuously present for 72 h. For these Leydig cells, 50.8% \pm 0.45 (mean \pm SEM, n=2) of the population was apoptotic when cultured in the presence of LH at 0.1 ng/ml for 72 h (Figure 11). In comparison to cells cultured for 72 h, either with no added treatment or with no LH followed by LH at 0.1 ng/ml, the continuous presence of LH at 0.1 ng/ml for 72 h significantly prevented ($p<0.002$) the onset of apoptosis in primary cultures of rat Leydig cells.

The greatest number of apoptotic cells was seen in Leydig cells cultured in the presence of no added treatment from 0 - 48 h followed by the presence of LH at 10.0 ng/ml from 48 - 72 h. In these samples, $63.3\% \pm 0.82$ (mean \pm SEM, n=2) Leydig cells were apoptotic following 72 h cultures. Early treatment with LH at 0.1 ng/ml, from 0 - 48 h, significantly ($p < 0.0001$) protected Leydig cells from LH at 10.0 ng/ml induced apoptotic cell death since $56.3\% \pm 0.76$ (mean \pm SEM, n=2) of the cell population was apoptotic following 72 h culture (Figure 11). Statistically, the onset of apoptosis in these samples was not different ($p > 0.05$) from either cells cultured with no added treatment for 72 h, or cells cultured with no added LH from 0 - 48 h and with LH at 0.1 ng/ml from 48 - 72 h.

c. Correlation Between Steroidogenesis and the Onset of Apoptosis

In vitro

The importance of the rate of steroidogenesis on the onset of apoptosis was statistically assessed by Spearman rank-order correlation coefficient (r_s) analysis. When analyzed all together, Spearman correlation coefficient ($r_s = 0.12$, $p > 0.05$) supported no dependence of the onset of apoptosis on androgen production in primary cultures of rat Leydig cells. However, once the data were grouped by incubation time (24, 48, or 72h), the analysis presented a strong correlation ($r_s = 0.90$, $p < 0.0014$) between androgen production and the onset of apoptosis over the first 24 h of incubation. Thereafter, when Leydig cells were incubated for 48 to 72 h, the importance of steroidogenesis on the onset of apoptosis became non-

significant ($r_s < 0.26$, $p > 0.05$). Unfortunately, the limited number of samples, treated with physiological doses of LH and incubated for more than 24 h, precluded the calculation of correlation coefficients specific for LH 0.1 ng/ml treatments. With a larger sample size, this study might have supported the anti-apoptotic effect of LH at 0.1 ng/ml over long term incubation. Nevertheless, detailed analysis of LH treatments versus the onset of apoptosis suggest that the continuous presence of physiological doses of LH became more important with increasing incubation time. While LH at 0.1 ng/ml had no protective effect on the onset of apoptosis in Leydig cells cultured for 24 h, its presence in samples cultured for 48 h significantly delayed the cell death process. In 48 h cultures, the potentially protective effect of LH at 0.1 ng/ml was apparently overwhelmed by Leydig cell stimulation with supra-physiological doses of LH from 24 to 48 h. As mentioned above, the cogent effect of androgen production on the onset of apoptosis had essentially vanished by 72 h incubation. In cell samples cultured for 72 h, the early presence of LH at 0.1 ng/ml from 0 - 48 h or 0 - 72 h reduced the onset of apoptosis, regardless of the androgen production rate, most likely by somehow improving the culture conditions.

Overall, these results suggest that, over the first 24 h incubation, the onset of apoptosis in primary cultures of rat Leydig cells is essentially dependent on the cell quality following cell isolation, culture conditions and steroidogenic rate. Subsequently, the culture conditions, conjugated with the cell's differentiation state and their ephemeral life *in vitro*, become the major inducers of apoptosis in primary

cultures of rat Leydig cells.

II.2. Effects of Luteinizing Hormone on Primary Cultures of hCG Pretreated Rat Leydig Cells

The results presented in the previous section showed that, contrary to its role *in vivo*, LH could not invariably prevent Leydig cells from triggering the apoptotic machinery *in vitro*. This observation prompted us to investigate the hypothetical protective effect of a high dose of gonadotropin, in this case LH's alter ego hCG, given at different times prior to animal sacrifice. This hypothesis was based on the potential ability of LH to increase Leydig cell resistance against the stress arising from cell isolation and culture.

a. Effect of *In Vivo* hCG Pretreatment on the Production of Androgens

Adult Sprague-Dawley rats were injected subcutaneously with hCG (100 IU/rat) or saline (control animals) 3, 12 or 24 h prior to sacrifice. Following Leydig cell isolation and 24 h culture, androgens released in the culture media were quantified by RIA. Results showed that both LH and hCG had very significant effects ($p < 0.0001$) on the androgen production in cultured Leydig cells (Figure 12).

In control animals, which received saline injection in place of hCG, *in vitro* LH treatments significantly increased steroidogenesis ($p < 0.05$). Leydig cells, isolated from control animals and cultured for 24 h with no additional LH, released

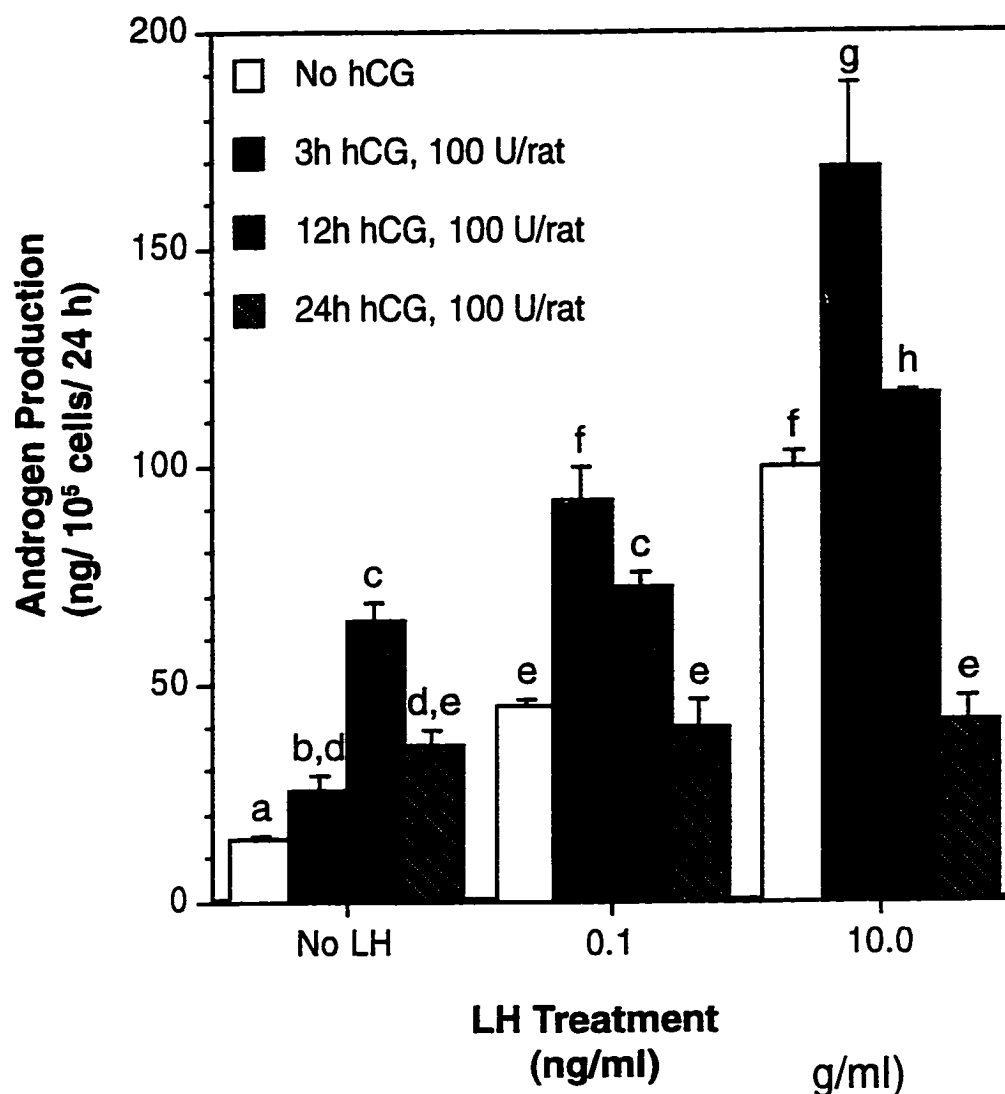


Figure 12. Androgen production in primary cultures of rat Leydig cells isolated from rats treated with hCG (0 or 100 IU) for 3 to 24 h. Following isolation, Leydig cells were cultured for 24 h in presence or absence of LH (0.1 - 10.0 ng/ml). Results represent mean \pm SEM of 3 experiments. Groups with different letters above the error bars are significantly different from each other as determined by Scheffe multiple comparison test. $p < 0.05$.

14.0 ± 0.71 ng androgens/ 10^5 cells (mean \pm SEM, n=3) (Figure 12). The addition of LH at 0.1 ng/ml stimulated androgen production so that 44.6 ± 1.70 ng androgen per 10^5 cells (mean \pm SEM, n=3) were released into the culture media. Moreover, the addition of a supra-physiological dose of LH (10.0 ng/ml) had the most dramatic effect on androgen production. Indeed, Leydig cells, isolated from control rats and treated with LH at 10.0 ng/ml, secreted 100 ± 3.7 ng androgen/ 10^5 cells (mean \pm SEM, n=3) over 24 h in culture.

Over 24 h incubation, Leydig cells isolated from rats pre-injected with hCG for 3 h, secreted significantly more androgens ($p < 0.05$) than Leydig cells isolated from control animals. This observation was true for cells cultured with or without additional LH treatments (Figure 12). Androgen RIA results showed that Leydig cells, isolated from rats pre-treated with hCG for 3 h, secreted 25.7 ± 3.00 ng androgen/ 10^5 cells (mean \pm SEM, n=3). Therefore, in the absence of *in vitro* LH treatment, the hCG treated Leydig cells secreted 1.8 times more androgens than did the control cells under similar culture conditions. When these Leydig cells were further stimulated with LH 0.1 - 10.0 ng/ml *in vitro*, androgen RIA revealed a proportionally equivalent increase of the steroidogenic response. As shown in Figure 12, Leydig cells, isolated from rats pretreated with hCG for 3 h, secreted 92.0 ± 7.79 ng androgen/ 10^5 cells (mean \pm SEM, n=3) when incubated for 24 h in the presence of LH at 0.1 ng/ml. Steroidogenesis reached a maximum *in vitro*, when these cells were stimulated with LH at 10.0 ng/ml for 24 h. Under these conditions, 169 ± 19.48 ng androgen/ 10^5 cells (mean \pm SEM, n=3) were secreted

in the culture media.

Overall, these results suggested that hCG, given 3 h prior to rat sacrifice, binds to Leydig cell plasma membrane receptors and initiates the steroidogenic response *in vivo*. HCG-stimulated steroidogenesis was apparently maintained *in vitro* since Leydig cells, isolated from 3 h hCG-treated rats, typically secreted about twice as much androgen than did their respective controls. Moreover, these results allow us to disregard possible LH/hCG receptor desensitization induced by prolonged exposure to gonadotropins.

Interestingly, Leydig cells isolated from rats treated with 100 IU hCG, 12 h prior to sacrifice, released as much as 64.6 ± 4.13 ng androgen/ 10^5 cells (mean \pm SEM, n=3) when cultured with no additional treatment (Figure 12). This significant augmentation in androgen production ($p < 0.02$), compared to non-hCG, non-LH treated cells, represented a 3-fold increase in steroidogenesis. Similar to Leydig cells isolated from 3 h hCG treated rats, these results suggested that, when injected 12 h prior to animal sacrifice, a single dose of 100 IU hCG effectively stimulated steroidogenesis in testicular interstitial cells. Once in culture, the Leydig cell's steroidogenic machinery was still stimulated by hCG and could therefore produce androgens at a higher rate.

In comparison to Leydig cells isolated from 3 h hCG-treated animals, LH (0.1-10.0 ng/ml) stimulation *in vitro* did not further stimulate steroidogenesis in Leydig cells isolated from 12 h hCG-treated animals. Actually, RIA results showed

that LH-stimulated Leydig cells isolated from 12 h hCG-treated rats consistently secreted less ($p < 0.05$) androgens than their 3 h hCG-treated counterparts. In Leydig cells isolated from 12 h hCG treated rats and further treated with LH *in vitro*, the prolonged exposure to LH/hCG had most likely induced hCG/LH receptor desensitization. This process partially inhibited steroidogenesis. In Figure 12, Leydig cells isolated from rats pretreated with hCG for 12 h, released 72.3 ± 3.28 ng androgen/ 10^5 cells (mean \pm SEM, $n=3$) when incubated in presence of LH 0.1 ng/ml. While this value represented a 20% reduction compared to androgen production in 3 h hCG - LH (0.1 ng/ml) treated cells, it still marked a 1.6-fold increase ($p < 0.05$) compared to control cells treated with LH 0.1 ng/ml alone.

LH/hCG receptor desensitization, in Leydig cells isolated from 12 h hCG-treated rats, became more obvious following LH at 10.0 ng/ml treatment *in vitro*. A maximum of 116 ± 1.2 ng androgen/ 10^5 cells (mean \pm SEM, $n=3$) was secreted in the culture media, when Leydig cells isolated from 12 h hCG-treated rats were incubated in the presence of LH at 10.0 ng/ml. In comparison to 3 hCG - LH (10.0 ng/ml)-treated cells, statistical analysis revealed an important decrease ($p < 0.01$) of *in vitro* steroidogenesis, while a barely significant difference ($p < 0.05$) existed between the later value and the level of androgen released by control cells stimulated with LH at 10.0 ng/ml.

In vitro androgen production was markedly reduced in Leydig cells isolated from rats pretreated with 100 IU hCG for 24 h prior to sacrifice. Androgen RIA

revealed that these cells released 36.1 ± 3.02 ng androgen/ 10^5 cells (mean \pm SEM, n=3), 40.2 ± 6.13 ng androgen/ 10^5 cells (mean \pm SEM, n=3), and, 41.2 ± 5.66 ng androgen/ 10^5 cells (mean \pm SEM, n=3) when cultured for 24 h with no LH or with LH (0.1-10.0 ng/ml), respectively. Statistical analysis showed no significant difference ($p > 0.05$) in androgen production between any of these treatments and with Leydig cells isolated from control animals and cultured with LH at 0.1 ng/ml. LH/hCG receptor desensitization was strongly suspected to induce the dramatic inhibition of steroidogenesis measured in Leydig cells isolated from 24 h hCG-treated animals.

b. Effect of *In Vivo* Pretreatment with Human Chorionic Gonadotropin on the Onset of Apoptosis

In view of the apoptotic effects of LH on cultured Leydig cells and its known protective effect *in vivo*, the objective of this study was to assess the possibility of reinforcing Leydig cells, prior to Leydig cell isolation and culture, by injecting the animals with 100 IU hCG 3 to 24 hours before sacrifice.

Although our results were contrary to the above hypothesis, very interesting observations could be drawn from quantification of apoptotic Leydig cells isolated from hCG treated rats. LH deprivation *in vivo* has been known to induce apoptosis in Leydig cells. The results described below suggest that, similar to LH deprivation, LH abundance can also be detrimental to Leydig cells.

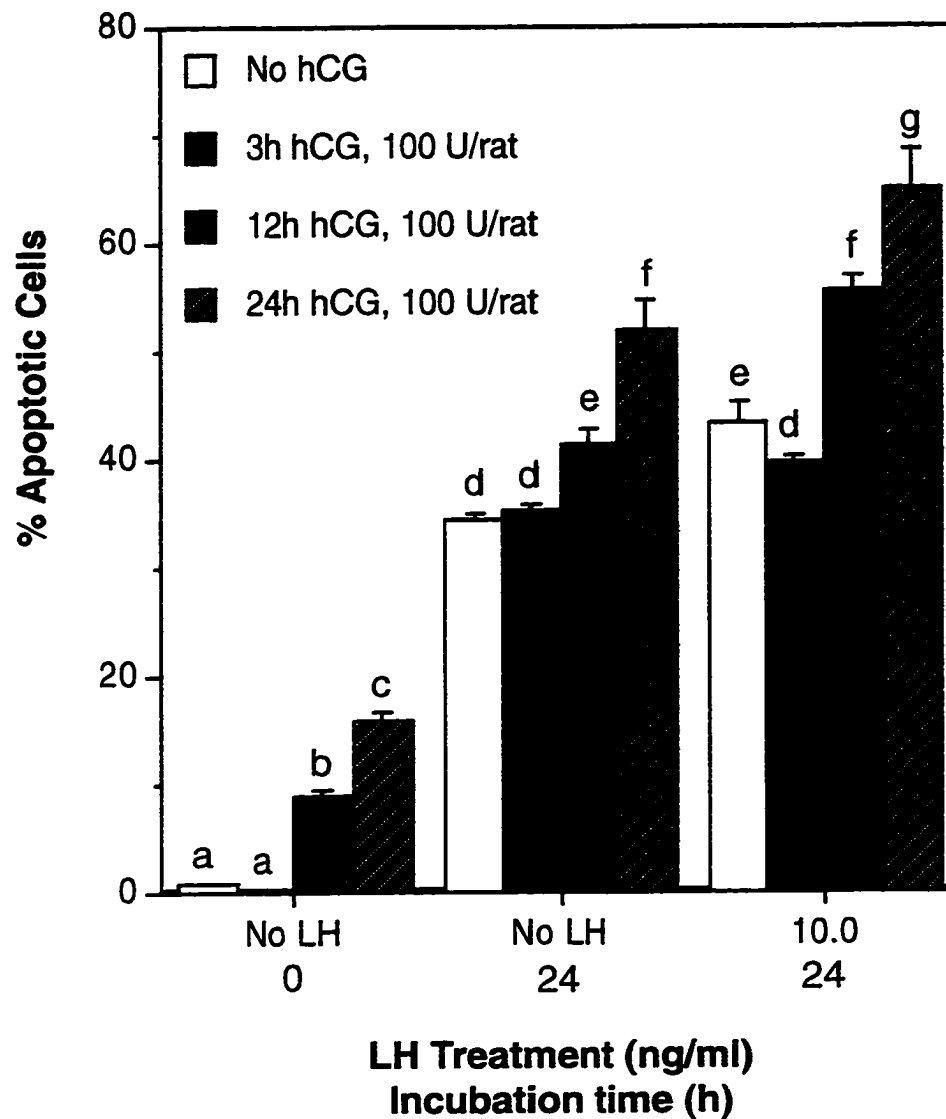


Figure 13. Apoptotic cell quantification in primary cultures of Leydig cells isolated from hCG (0 - 100 IU) treated rats. Cells were cultured from 0 - 24 h in the presence or absence of LH 10.0 ng/ml. Following cell cytospin on glass slides and ISEL, apoptotic cells were quantified by microscopy. Results are expressed as a percent of the total number of cells counted per sample and are presented as the mean \pm SEM of three replicate experiments. Groups with different letters above the error bars are significantly different from each other as determined by a Scheffe's multiple comparison test. $p < 0.05$.

Leydig cells, freshly isolated from control animals or rats pretreated with 100 IU hCG for 3 h, presented very few apoptotic cells (Figure 13). Respectively, $0.69\% \pm 0.22$ (mean \pm SEM, n=3) and $0.11\% \pm 0.07$ (mean \pm SEM, n=3) Leydig cells were apoptotic in each cell group. This slight difference was shown to be statistically non-significant ($p > 0.05$). On the other hand, freshly isolated cells, from rats treated with 100 IU hCG for 12 or 24 h prior to sacrifice, presented significantly more ($p < 0.0001$) apoptotic cells (Figure 13). Indeed, $8.88\% \pm 0.35$ (mean \pm SEM, n=3) Leydig cells were found to be apoptotic in freshly isolated cell preparations from rats treated with hCG for 12 h; $15.8\% \pm 0.650$ (mean \pm SEM, n=3) Leydig cells were found to be apoptotic in freshly isolated cell preparations from rats treated with hCG for 24 h (Figure 13). Although unexpected, these results reinforced the crucial importance of tight hormonal regulation in the testes since sub-physiological levels of LH/hCG, as induced by LH deprivation, seemed to be as detrimental as supra-physiological levels.

Leydig cells, isolated from rats treated with 100 IU hCG for 3 to 24 h prior to animal sacrifice were cultured in suspension for 24 h in presence or absence of LH at 10.0 ng/ml. Cells were then cytopun on glass slides and processed for ISEL. Similar to the results obtained in freshly isolated cells, cell samples isolated from control animals or 3 h hCG-treated rats and cultured with or without LH 10.0 ng/ml disclosed no significant difference among themselves ($p > 0.05$) in the percent apoptotic cell number. For these cell samples, respectively, $34.4\% \pm 0.55$ (mean

\pm SEM, n=3) and $35.1\% \pm 0.52$ (mean \pm SEM, n=3) of the Leydig cell populations were apoptotic following 24 h culture with no additional LH. *In vitro* cell stimulation with LH at 10.0 ng/ml proportionally promoted the onset of apoptosis in these Leydig cells. While $42.3\% \pm 1.86$ (mean \pm SEM, n=3) Leydig cells isolated from control animals were apoptotic following LH 10.0 ng/ml stimulation for 24 h, $39.7\% \pm 0.44$ (mean \pm SEM, n=3) of Leydig cells isolated from 3 h hCG treated animals were apoptotic following culture under identical conditions (Figure 13).

However, statistically significant ($p < 0.05$) increases in the number of apoptotic figures were observed in Leydig cells isolated from rats pretreated with hCG for 12 or 24 h and cultured with or without LH at 10.0 ng/ml for 24 h (Figure 13). Following 24 h incubation in the absence of additional LH, $39.2\% \pm 1.44$ (mean \pm SEM, n=3) and $47.6\% \pm 2.98$ (mean \pm SEM, n=3) of the Leydig cells, isolated from rats pretreated with hCG for 12 and 24 h respectively, were apoptotic. The addition of LH at 10.0 ng/ml to the culture media further promoted the onset of apoptosis in these cells so that, $53.1\% \pm 1.52$ (mean \pm SEM, n=3) and $69.7\% \pm 3.57$ (mean \pm SEM, n=3) of Leydig cells, isolated from rats pretreated with hCG for 12 and 24 h, respectively, were apoptotic.

c. Correlation Between Steroidogenesis and the Onset of Apoptosis Following hCG Treatments

Except for Leydig cells isolated from 3 h hCG treated rats and regardless

of the post-isolation cell treatment, proportionally more Leydig cells initiated their apoptotic cell death program following longer *in vivo* hCG treatments. In addition, except for Leydig cells isolated from 3 h hCG treated rats, LH at 10.0 ng/ml stimulation further induced apoptosis following 24 h incubation.

Correlation coefficients (r_s), between androgen production and apoptosis were calculated by Spearman rank-order analysis. The positive correlation coefficient obtained for the hCG study, namely $r_s=0.31$, supported a significant ($p<0.02$) cause and effect relation between increasing androgen production and the onset of apoptosis in rat Leydig cells. Correlation coefficients for the hCG study were also calculated for each hCG group. Correlation analysis by hCG groups revealed that, for hCG treatments administered for 0, 3 and 12 hours, the percent apoptotic cells significantly ($p<0.01$) increased relative to androgen production stimulation. The correlation between apoptosis and androgen production was non-significant ($p>0.05$) in samples treated with hCG for 24 h. In those samples, excessive steroidogenesis stimulation *in vivo* induced prominent LH receptor desensitization. The effects of this hyperstimulation were visualized *in vitro* as an important reduction in the level of androgens released in the culture media. Although our data showed a low androgen production paired with a high apoptosis level, androgen RIA did not account for the androgens released *in vivo*. Total androgen production (*in vivo* and *in vitro*) in these samples would no doubt fit the correlation where, in primary cultures of rat Leydig cells, incubated for a maximum of 24 h, the onset of apoptosis is somehow associated with the steroidogenic rate.

III. EFFECTS OF OTHER TREATMENTS ON PRIMARY CULTURES OF RAT LEYDIG CELLS

The next logical step in this study was to amend the culture media in an effort to improve the culture conditions and therefore limit the onset of apoptosis in our cell model. For this purpose, we investigated the effects of an array of agents, including fetal calf serum (FCS), epidermal growth factor (EGF) and zinc (Zn), on androgen production and the onset of apoptosis in primary cultures of rat Leydig cells.

III.1 Effects of Fetal Calf Serum (FCS) on Steroidogenesis and on the onset of Apoptosis in Primary Cultures of Rat Leydig Cells

Fetal and adult mammalian serum have been used for decades as a source of macromolecules and growth factors in cell culture. In addition to supplying nutrients, serum provides hormones, vitamins, trace elements and binding proteins which may be required for optimum cell function. *In vitro*, the presence of serum in the culture media can affect a host of processes including cell anchorage, nutrient uptake, phospholipid metabolism and synthesis of RNA, DNA and protein (Temin *et al.*, 1972).

However, the undefined hormonal milieu of serum-supplemented medium can induce unwanted reactions and greatly confuses the interpretation of individual hormone effects.

Primary cultures of rat Leydig cells were cultured in suspension in the presence or absence of 15% FBS and in the presence or absence of LH (0.1 - 10.0 ng/ml). Following 24 h of incubation, Leydig cells cultured with no additional treatments typically secreted 16.7 ng androgen/ 10^5 cells \pm 1.61 (mean \pm SEM, n=2) (Figure 14). At a physiological level, LH (0.1 ng/ml) stimulated steroidogenesis in Leydig cell cultures so that 43.4 ng of androgen/ 10^5 cells \pm 7.0 (mean \pm SEM, n=2) was released in the culture media. Leydig cells cultured in the presence of supra-physiological doses of LH produced 161 ng of androgen/ 10^5 cells \pm 0.9 (mean \pm SEM, n=2). In accordance with the results described earlier (section II.1.a and II.2.a), *in vitro* LH treatments significantly ($p < 0.0001$) stimulated androgen production in Leydig cells.

In the presence of 15% FCS, primary cultures of rat Leydig cells secreted 12.3 ng androgen/ 10^5 cells \pm 0.87 (mean \pm SEM, n=2), 25.2 ng androgen/ 10^5 cells \pm 4.74 (mean \pm SEM, n=2) and 111 ng androgen/ 10^5 cells \pm 8.2 (mean \pm SEM, n=2) when the LH concentration was 0, 0.1 ng/ml or 10.0 ng/ml, respectively (Figure 14). Statistically, the addition of 15% FCS to the culture media significantly ($p < 0.0001$) inhibited steroidogenesis in those cells. Therefore, although FCS contained a myriad of trophic factors that may have improved cell survival, these results suggest that potential steroidogenesis inhibitors were also present. In the absence of additional LH, basal androgen production did not show any significant difference in the presence or absence of 15% FCS. However, the limited population size in this study did not allow us to determine if this inhibitory effect of

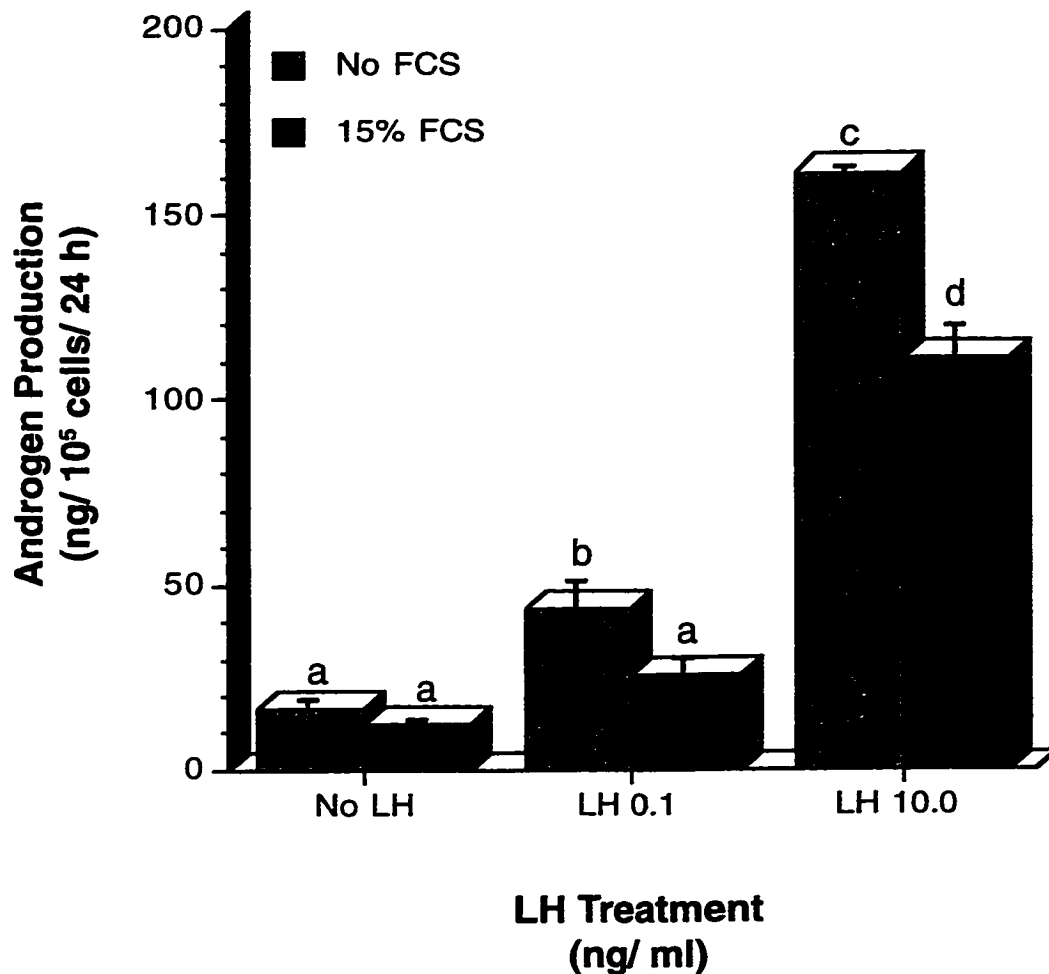


Figure 14. Effect of FCS on androgen production in cultured Leydig cells. Primary cultures of rat Leydig cells were incubated for 24 h in M199 with or without FCS (15%) and with or without LH (0.1 - 10.0 ng/ml). FCS treatment was found to partially inhibit LH stimulated steroidogenesis. Results represent mean \pm SEM of two replicate experiments. Groups with different letters above the error bars are significantly different from each other as determined by Scheffe multiple comparison test. $p < 0.05$.

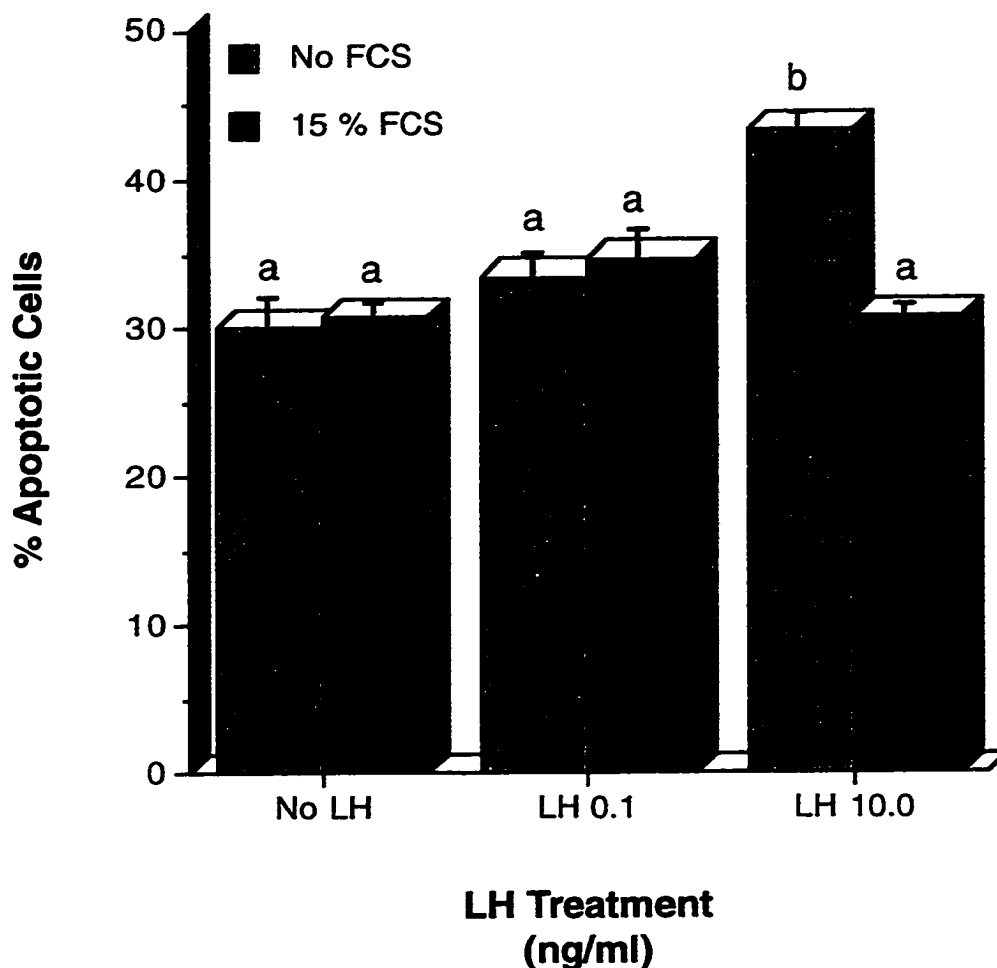


Figure 15. Effect of FCS on the onset of apoptosis in rat Leydig cells. Primary cultures of Leydig cells were incubated in suspension for 24 h in M199 with or without FCS (15%) and with or without LH (0.1 - 10.0 ng/ml). FCS had no significant effect on the onset of apoptosis in cells cultured with LH (0 - 0.1 ng/ml). However, in the presence of 10.0 ng LH/ ml, FCS significantly reduced the onset of apoptosis. Results represent the mean \pm SEM of three replicate experiments. Groups with different letters above the error bars are significantly different from each other as determined by a Scheffe multiple comparison test. $p < 0.05$.

FCS on steroidogenesis was limited to LH-stimulated androgen production.

Leydig cells incubated for 24 h in the absence or presence of FCS (15%) and in the absence or presence of LH (0.1 - 10.0 ng/ml) were also labeled for apoptosis quantification (Figure 15). Results showed that $30.0\% \pm 2.02$ (mean \pm SEM, n=3) and $33.4\% \pm 1.50$ (mean \pm SEM, n=2) of the Leydig cell population stained positive for apoptosis when cultured without any additional treatment or with LH at 0.1 ng/ml, respectively. The addition of LH at 10.0 ng/ml to the culture media significantly promoted the onset of apoptosis since $43.4\% \pm 1.02$ (mean \pm SEM, n=2) of the Leydig cells cultured into those conditions were apoptotic (Figure 15).

The addition of 15% FCS to the culture media had no significant effect ($p>0.05$) on the onset of apoptosis when Leydig cells were co-treated with no added LH or with LH at 0.1 ng/ml (Figure 15). For these cell samples, $30.8\% \pm 0.92$ (mean \pm SEM, n=2) and $34.7\% \pm 1.89$ (mean \pm SEM, n=2) of the Leydig cells were apoptotic, respectively. Interestingly, FCS (15%) effectively protected ($p<0.01$) Leydig cells from undergoing apoptosis when these cells were maximally stimulated with LH at 10.0 ng/ml. Results showed that among Leydig cells cultured in the presence of LH at 10.0 ng/ml and 15% FCS, $30.8\% \pm 0.65$ (mean \pm SEM, n=2) of the cell population was apoptotic following 24 h of incubation. In comparison to Leydig cells cultured in the presence of LH at 10.0 ng/ml only, the presence of 15% FCS reduced the number of apoptotic cells by 25 percent.

III.2. Effects of EGF on Steroidogenesis and on the Onset of Apoptosis in Primary Cultures of Leydig Cells

In the testes, the steroidogenic responsiveness of Leydig cells to stimulation by gonadotropin can be modulated by many circulating peptides or local factors secreted by cells within the testis. Among the local factors known to affect androgen production by Leydig cells are growth factors. Four growth factors seem particularly important in the local control of Leydig cell steroidogenesis: transforming growth factor β (TGF β), basic fibroblast growth factor (bFGF), insulin-like growth factor I (IGF-I), and epidermal growth factor (EGF). For this study, the effects of EGF supplementation on steroidogenesis and on the onset of apoptosis in primary cultures of rat Leydig cells were studied.

Similar to the FCS supplementation study, the addition of EGF in the absence of additional LH had no significant effect on androgen secretion in cell samples incubated for 24 hours. In these samples, 16.7 ng androgen/ 10^5 cells \pm 1.61 (mean \pm SEM, n=2) and 18.76 ng androgen/ 10^5 cells \pm 1.02 (mean \pm SEM, n=2) were secreted in the culture medium in the presence of no EGF and 10 ng EGF/ml, respectively. The addition of a physiological dose of LH significantly stimulated steroidogenesis ($p < 0.0001$) in the presence or absence of EGF. Leydig cells incubated with LH (0.1 ng/ml) and no added EGF secreted 43.36 ng androgen/ 10^5 cells \pm 7.0 (mean \pm SEM, n=2) and 53.36 ng androgen/ 10^5 cells \pm 4.93 (mean \pm SEM, n=2) when EGF was present in the culture medium.

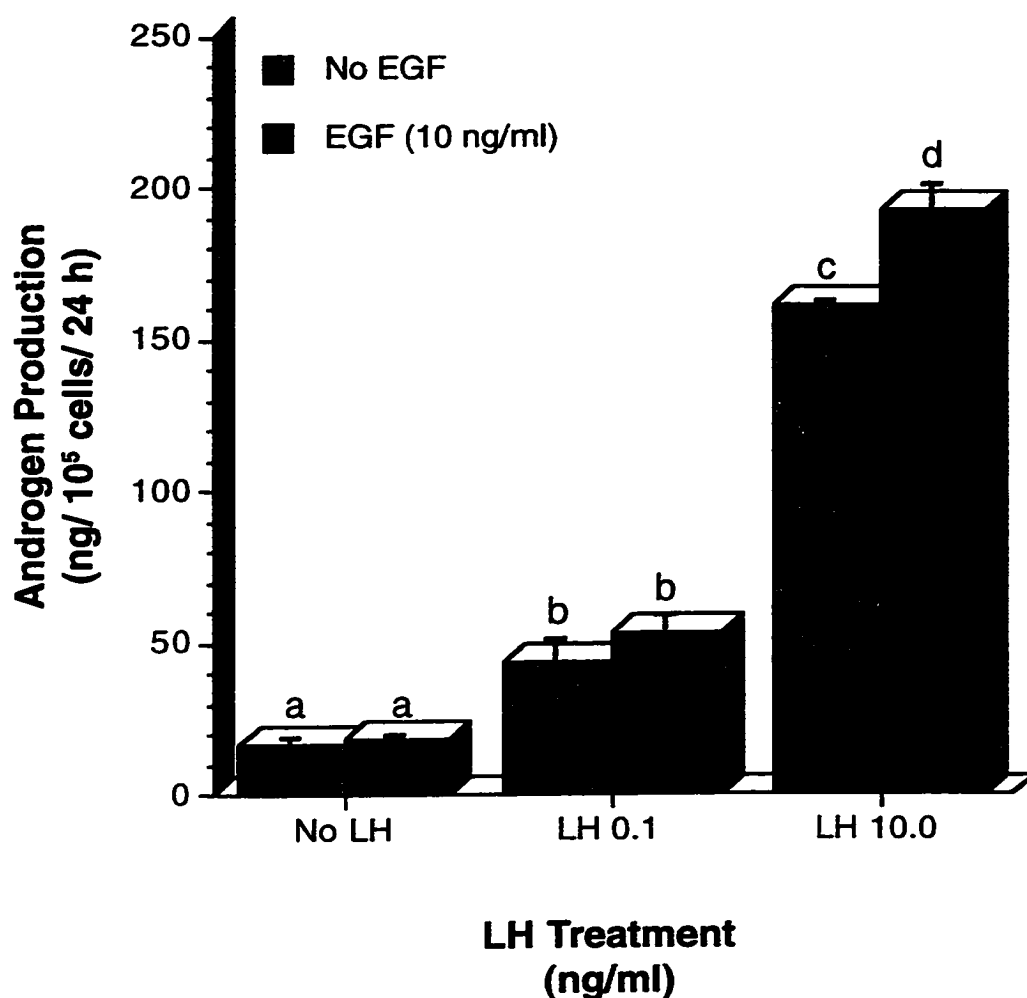


Figure 16. Effect of EGF on androgen production in cultured Leydig cells. Primary cultures of rat Leydig cells were incubated for 24 h in M199 with or without EGF (10 ng/ml) and with or without LH (0.1 - 10.0 ng/ml). EGF specifically promoted steroidogenesis when present in conjunction with 10.0 ng/ml LH. Results represent mean \pm SEM of two replicate experiments. Groups with different letters above the error bars are significantly different from each other as determined by a Scheffe multiple comparison test. $p < 0.05$.

However, in cell samples incubated in the presence of LH at 10.0 ng/ml, EGF had a significant stimulatory effect on steroidogenesis. Androgen RIA revealed that while 161 ng androgen/ 10^5 cells \pm 0.9 (mean \pm SEM, n=2) were released in the culture medium following 24 h of incubation in the presence of 10.0 ng/ml LH, 192 ng androgen/ 10^5 cells \pm 18.2 (mean \pm SEM, n=2) were secreted when LH at 10.0 ng/ml and EGF 10.0 ng/ml were present in the culture medium. Although the sample number was rather small and the SEM value rather large for these latter samples, the difference remained statistically significant ($p < 0.05$).

Apoptotic cell quantification revealed that EGF protected Leydig cells in culture although this effect was only significant in cells incubated in the presence of both LH at 10.0 ng/ml and EGF at 10.0 ng/ml. Therefore, 27.4% \pm 3.10 (mean \pm SEM, n=2) and 29.97% \pm 1.81 (mean \pm SEM, n=2) of the Leydig cell population was apoptotic following 24 h of incubation in the presence of no added EGF or EGF at 10.0 ng/ml, respectively. In agreement with results described earlier, Leydig cells cultured in the presence of 0.1 ng LH/ml presented no changes in their cell death rate compared to control cells. Results showed that 33.4% \pm 1.50 (mean \pm SEM, n=2) and 28.4% \pm 2.14 (mean \pm SEM, n=2) Leydig cells were apoptotic at the end of 24 h of incubation in the presence of LH at 0.1 ng/ml alone or with EGF at 10.0 ng/ml. Similar to FCS, the antiapoptotic effect of EGF could only be observed in cell samples treated with a combination of a supra-physiological concentration of LH along with EGF. In these samples, 43.4% \pm 1.02

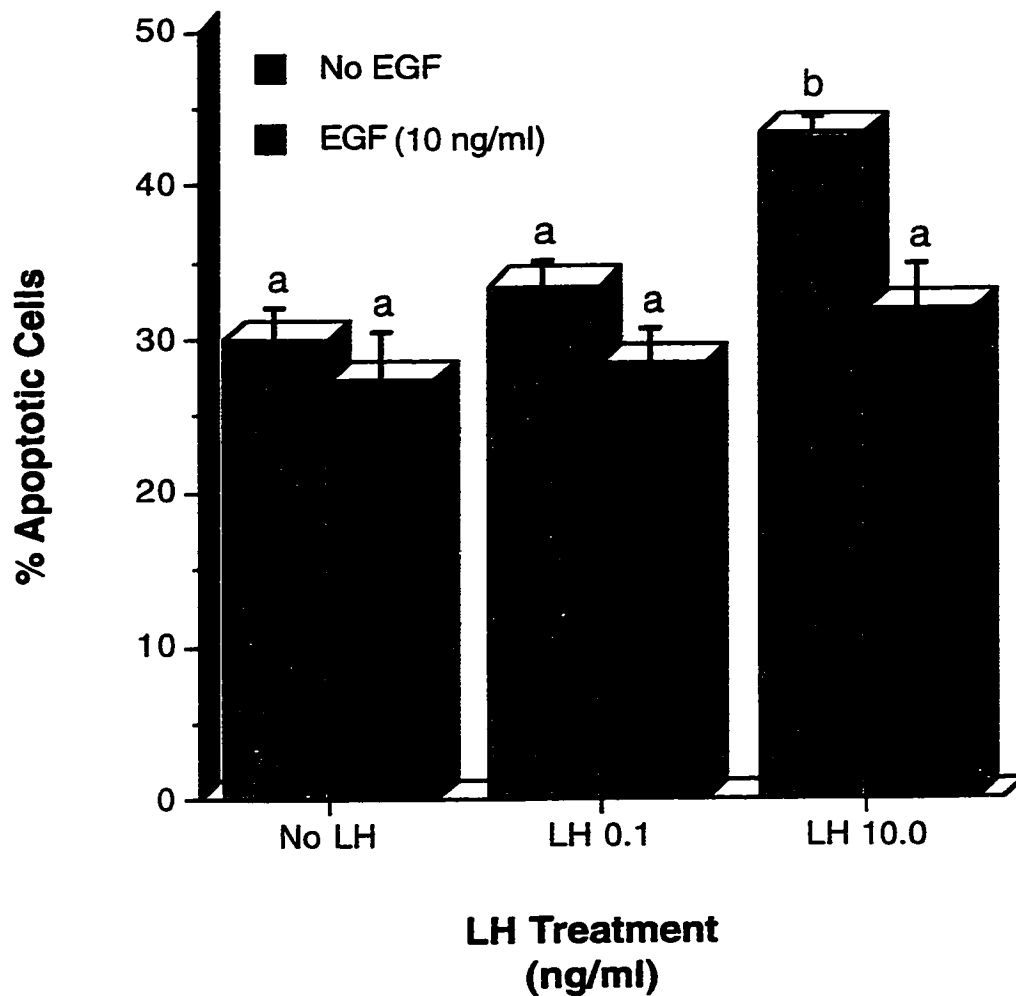


Figure 17. Effect of EGF on the onset of apoptosis in rat Leydig cells. Primary cultures of Leydig cells were incubated in suspension for 24 h in M199 with or without EGF (10 ng/ml) and with or without LH (0.1 - 10.0 ng/ml). EGF had no significant effect on the onset of apoptosis in cells cultured with LH (0 - 0.1 ng/ml). However, in the presence of 10.0 ng LH/ml, EGF significantly reduced the onset of apoptosis. Results represent the mean \pm SEM of duplicate experiments. Groups with different letters above the error bars are significantly different from each other as determined by a Scheffe multiple comparison test. $p < 0.05$.

(mean \pm SEM, n=2) and 31.9% \pm 2.85 (mean \pm SEM, n=2) of the Leydig cell population was apoptotic when incubated with LH at 10.0 ng/ml alone or in combination with EGF at 10.0 ng/ml, respectively.

Statistical analysis confirmed the significant stimulatory effect ($p < 0.001$) of LH on the onset of apoptosis in Leydig cells and significant inhibitory effect ($p < 0.01$) of EGF.

III.3. Early Work Medley

a. Effect of Zinc on the Onset of Apoptosis and Steroidogenesis *in vitro*

In some experiments, primary cultures of rat Leydig cells were treated with zinc (1-5 mM) and cultured with or without LH for 0-24 h in supplemented M199. Zinc is known to inhibit the $\text{Ca}^{2+}/\text{Mg}^{2+}$ -dependent endonucleases responsible for apoptotic DNA fragmentation (Barbieri *et al.*, 1992; Gaido and Cidlowski, 1991). However, although zinc blocks endonucleosomal DNA fragmentation, it does not inhibit chromatin condensation or DNA cleavage into 300 to 50 kb fragments (Oberhammer *et al.*, 1993a; Oberhammer *et al.*, 1993b) nor does it shut off the apoptotic machinery.

In this regard, ISEL results confirmed that zinc significantly prevented endonucleosomal DNA fragmentation. In the presence of 1-5 mM zinc with or without additional LH, the number of apoptotic Leydig cells was significantly ($p < 0.001$) reduced compare to Leydig cells cultured with no zinc, reaching over

70% inhibition in comparison to control conditions (data not shown).

However, androgen RIA performed on the spent media reveal that androgen production was almost non-existent in zinc-treated Leydig cells. These results suggested that, while inhibiting internucleosomal DNA fragmentation, zinc also significantly interfered with the steroidogenic pathway in cultured Leydig cells.

b. The Possible Effect of Collagenase on the Onset of Apoptosis in Primary Cultures of Rat Leydig Cells

Mid-way through this research project, a new stock of collagenase had to be purchased in order to isolate Leydig cells. ISEL studies performed on primary cultures of rat Leydig cells suggested that batch to batch variations of collagenase enzyme might modulate the survival ability of our cell model *in vitro*.

Surprisingly, ISEL results obtained from Leydig cell dispersed in the older collagenase revealed that up to $76.7\% \pm 1.28$ (mean \pm SEM, n=3) of the Leydig cell population was apoptotic following 24 h of incubation in the presence of a supra-physiological dose of LH (10.0 ng/ml) (data not shown). Under the same conditions, no more than $44.8\% \pm 0.98$ (mean \pm SEM, n=9) of the Leydig cells dispersed in the newer collagenase were apoptotic following 24 h of incubation.

c. Preliminary Studies Using DNA 3' End Labeling to Assess Apoptosis

Prior to ISEL, DNA 3' end labeling performed on isolated genomic DNA was used for the study of apoptosis in primary cultures of rat Leydig cells (data not

shown).

For these experiments, Leydig cells were typically incubated for 3 h in the presence of LH (0-10.0 ng/ml) and one of the following treatments: N-acetyl-L-cysteine (NAC) (2.5-25mM) to inhibit endonucleases, cycloheximide (0-75 μ M) or actinomycin D (0-40nM) to inhibit protein synthesis and mRNA transcription, respectively, or, vitamin E (0-400 μ mol/l) to optimize ROI neutralization. Moreover, in some experiments, M199 was replaced by M199 conditioned by preincubation for 24 h with testicular cells plated at high density (Murphy and Moger, 1982) or by DMEM-F12 media (1:1 v/v).

DNA 3' end labeling followed by electrophoresis and autoradiographic analysis revealed no evident inhibition of DNA fragmentation from any of the treatments used. However, although we could not find any treatment to inhibit the onset of apoptosis in primary cultures of Leydig cells, we could not confirm they had no effects on the level of DNA fragmentation since this DNA labeling method is strictly qualitative.

IV. EFFECTS OF SPECIFIC EXOGENOUS ANTIOXIDANTS IN PRIMARY CULTURES OF RAT LEYDIG CELLS

Reactive oxygen intermediates (ROI), are known to induce apoptotic cell death. In a state of homeostasis, cellular metabolism normally release ROI that are immediately neutralized by a series of antioxidation enzymes. In testes,

enzymes such as superoxide dismutase (SOD) and catalase, are on the first line of defence to protect Leydig cells from the ROI they themselves generate .

In an effort to limit apoptotic cell death induced by ROI *in vitro*, we investigated the effect of three antioxidants, namely SOD, catalase and ascorbic acid (Asc. Ac.), on the level of steroidogenesis and the onset of apoptosis in primary cultures of rat Leydig cells.

IV.1. Effects of Specific Antioxidants on the Androgen Production In Vitro

Primary cultures of rat Leydig cells were incubated in the presence or absence of an antioxidant and in the presence or absence of LH (0.1-10.0 ng/ml). The antioxidants used for this study included SOD (100 U/ml), catalase (50 ng/ml) and Asc. Ac. (1mM). Following 24 h incubation, the culture media was collected and radioimmunoassayed for androgen content (Figure 18).

Leydig cells, cultured in supplemented M199 with no additional treatments for 24 h, typically secreted 12.3 ng androgen/ 10^5 cells \pm 0.47 (mean \pm SEM, n=3). The addition of LH at 0.1 ng/ml significantly stimulated steroidogenesis since 52.2 ng androgen/ 10^5 cells \pm 4.5 (mean \pm SEM, n=3) were released into the culture media. Finally, the addition LH at a supraphysiological dose, namely 10.0 ng/ml, stimulated Leydig cell steroidogenesis so that 108 ng androgen/ 10^5 cells \pm 9.5 (mean \pm SEM, n=3) could be measured in the culture media. As expected, the addition of LH (0-10.0 ng/ml) to the culture media had a significant stimulatory effect on Leydig cell steroidogenesis ($p < 0.0001$).

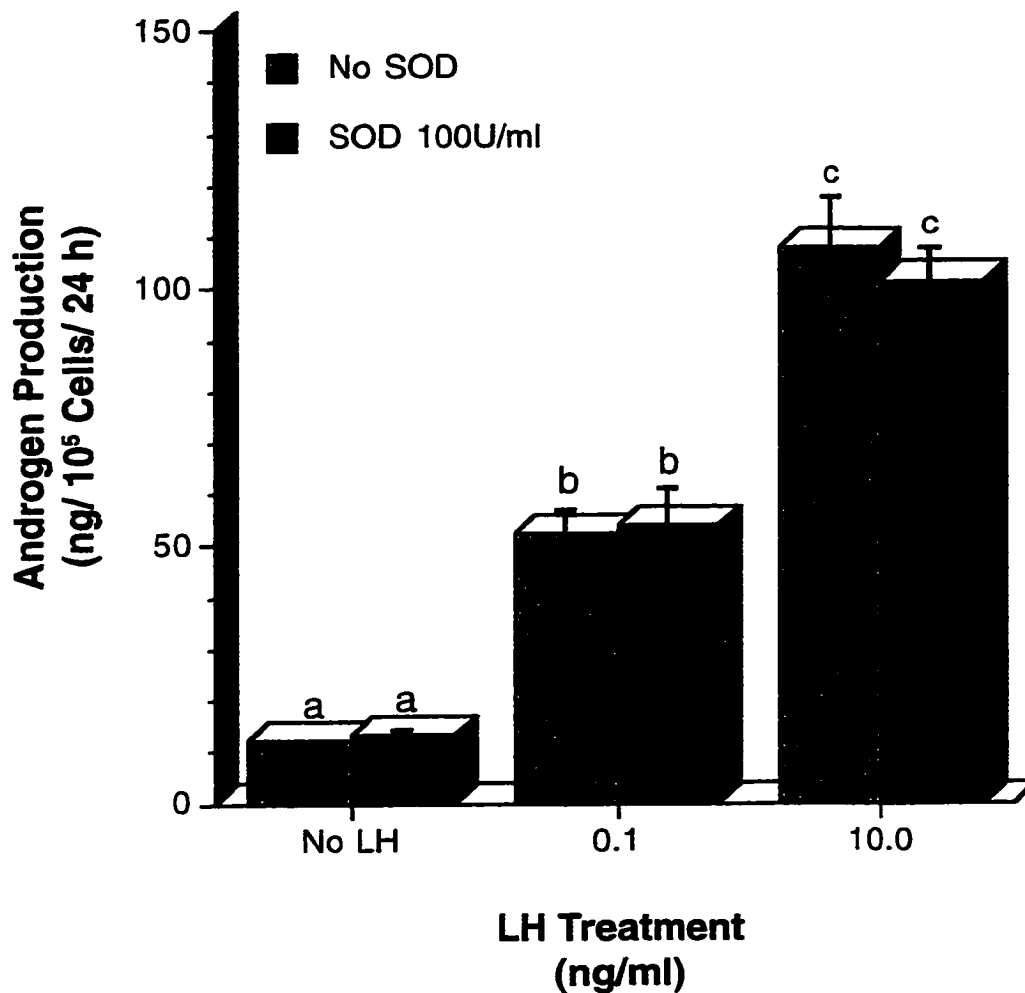


Figure 18. *In vitro* effect of the antioxidant SOD on androgen production in rat Leydig cells. Primary cultures of Leydig cells were incubated for 24 h in M199 with or without SOD (100 U/ml) and with or without LH (0.1-10.0 ng/ml). While SOD did not modulate androgen production, LH (0.1-10.0 ng/ml) had a significant stimulatory effect. Results represent mean \pm SEM of three replicate experiments. Groups with different letters above the error bars are significantly different from each other as determined by a Scheffe multiple comparison test. $p < 0.002$.

Leydig cell steroidogenic rate was not affected ($p>0.05$) when primary cultures of rat Leydig cells were treated with SOD (100 IU/ml) (Figure 18). Results showed that Leydig cells cultured in presence of 100 IU SOD/ml released 13.0 ng androgen/ 10^5 cells \pm 0.72 (mean \pm SEM, $n=3$). Moreover, SOD did not interfere with the stimulatory effect of LH since, androgen production, in Leydig cells treated with LH (0.1 -10.0 ng/ml) alone, was not different ($p>0.05$) from androgen production in Leydig cells treated with a combination of LH (0.1 -10.0 ng/ml) and SOD (100 IU/ml). Effectively, SOD treated Leydig cells stimulated with LH at 0.1 ng/ml or LH 10.0 ng/ml released 53.9 ng androgen/ 10^5 cells \pm 6.57 (mean \pm SEM, $n=3$) and 101 ng androgen/ 10^5 cells \pm 5.8 (mean \pm SEM, $n=3$) respectively (Figure 18).

Similar to SOD, the antioxidant catalase had no significant effect on steroidogenesis in primary cultures of rat Leydig cells ($p>0.05$) (Figure 19). As mentioned above, primary cultures of rat Leydig cells were incubated with or without catalase (50 ng/ml) and with or without LH (0.1-10.0 ng/ml). Androgen RIA results, performed on the culture media collected subsequently to 24 h of incubation, revealed that Leydig cells treated with catalase 50 ng/ml released 11.0 ng androgen/ 10^5 cells \pm 0.60 (mean \pm SEM, $n=3$). In presence of LH at 0.1 ng/ml or LH at 10.0 ng/ml, catalase treated Leydig cells, respectively, secreted 54.7 ng androgen/ 10^5 cells \pm 4.95 (mean \pm SEM, $n=3$) and 101 ng androgen/ 10^5 cells \pm 4.5 (mean \pm SEM, $n=3$) in the culture media (Figure 19).

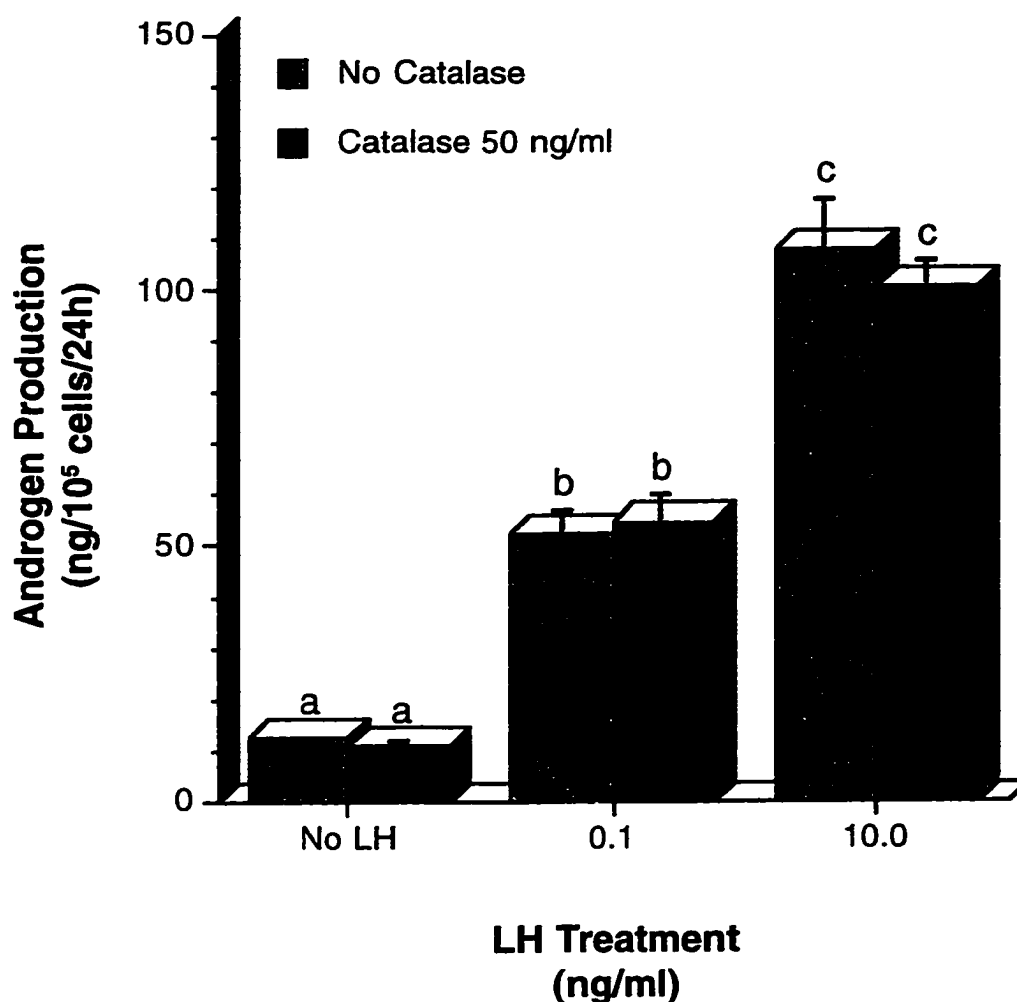


Figure 19. Effect of the antioxidant catalase on androgen production in cultured Leydig cells. Primary cultures of rat Leydig cells were incubated for 24 h in M199 with or without catalase (50 ng/ml) and with or without LH (0.1-10.0 ng/ml). While catalase did not modulate androgen production, the addition of LH (0.1-10.0 ng/ml) had a significant stimulatory effect. Results represent mean \pm SEM of three replicate experiments. Groups with different letters above the error bars are significantly different from each other as determined by a Scheffe multiple comparison test. $p < 0.0001$

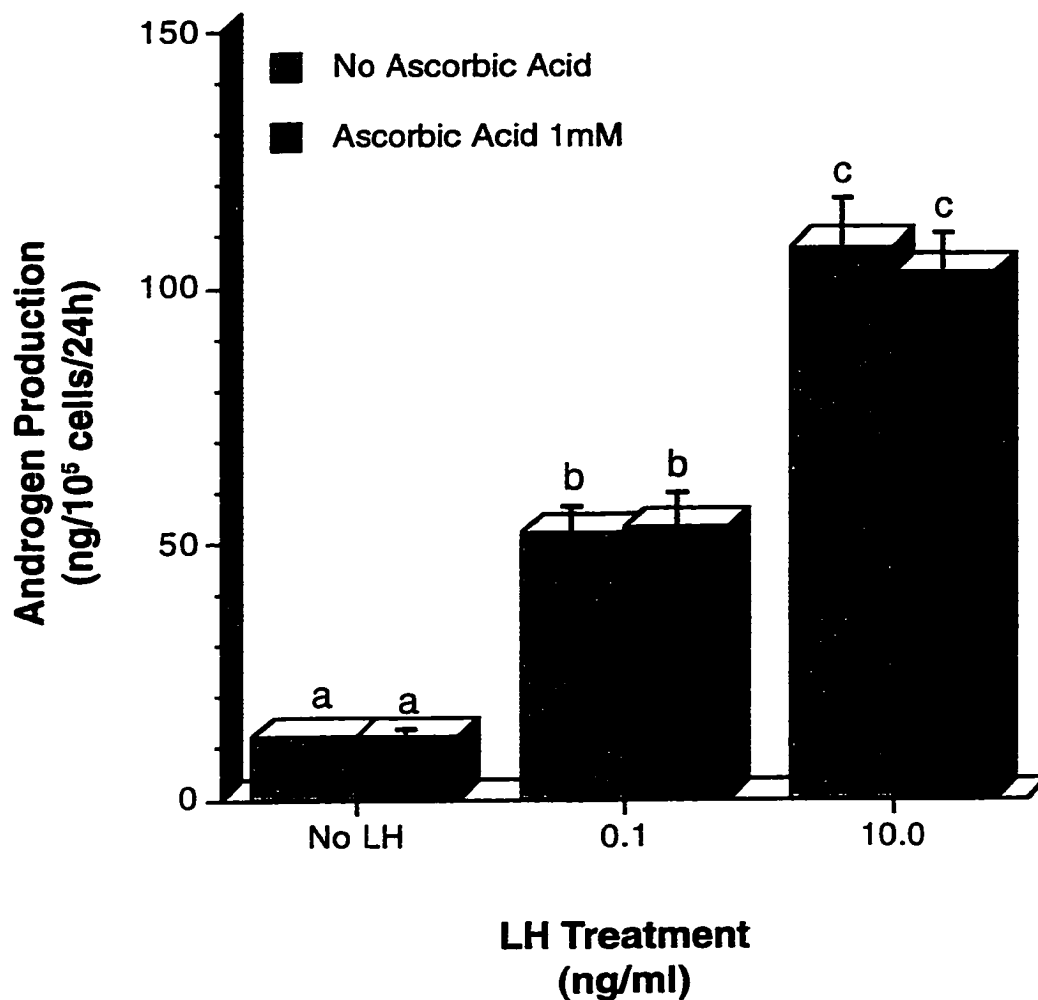


Figure 20. *In vitro* effect of ascorbic acid (Asc. Ac.) on androgen production in Leydig cells. Primary cultures of rat Leydig cells were incubated for 24 h in M199 with or without Asc. Ac (1 mM) and with or without LH (0.1-10.0 ng/ml). While the addition of Asc. Ac had no significant effect on androgen production the addition of LH (0.1-10.0 ng/ml) had a significant stimulatory effect. Results represent mean \pm SEM of three replicate experiments. Groups with different letters above the error bars are significantly different from each other as determined by a Scheffe multiple comparison test. $p < 0.003$

As mentioned above, LH significantly stimulated steroidogenesis ($p < 0.0001$) in Leydig cells and this positive effect of LH was not affected by the presence of catalase ($p > 0.05$).

The effect of Asc. Ac. on steroidogenesis was also studied. Primary cultures of rat Leydig cells incubated for 24 h with or without Asc. Ac. (1 mM) revealed no significant change ($p > 0.05$) in basal androgen production (Figure 20). Indeed, 12.3 ng androgen/ 10^5 cells \pm 1.26 (mean \pm SEM, $n=3$) were measured in the media following 24 h incubation in presence of 1 mM Asc. Ac. Moreover, similarly to SOD and catalase, Asc. Ac. did not interfere with the steroidogenesis stimulatory effect of LH ($p < 0.0001$) since Leydig cells, treated with Asc. Ac. and LH (0.1 - 10.0 ng/ml) presented equipotent steroidogenic ability ($p > 0.05$) as Leydig cells treated with LH alone. In presence of Asc. Ac (1 mM), LH at 0.1 ng/ml stimulated primary cultures of rat Leydig cells secreted 53.0 ng androgen/ 10^5 cells \pm 6.29 (mean \pm SEM, $n=3$) while LH at 10.0 ng/ml stimulation induced the release of 103 ng androgen/ 10^5 cells \pm 7.1 (mean \pm SEM, $n=3$) (Figure 20).

IV.2. Effects of Specific Antioxidants on the Onset of Apoptosis in Leydig Cells

The effects of SOD (100 IU/ml), catalase (50 ng/ml) and Asc. Ac. (1 mM) on the onset of apoptosis in primary cultures of rat Leydig cells was studied.

Under control conditions, where Leydig cells were cultured in supplemented M199 with no added treatment, 31.4% \pm 1.73 (mean \pm SEM, $n=3$) of the cell

population stained positive for apoptosis following 24 h incubation (Figure 21). As described earlier, the addition of LH 0.1 ng/ml had no significant effects ($p>0.05$) on the onset of apoptosis *in vitro*. Under these culture conditions, $34.0\% \pm 2.14$ (mean \pm SEM, $n=3$) of the cell population was found to be apoptotic. However, supra-physiological doses of LH significantly promoted the onset of apoptosis since $45.9\% \pm 2.38$ (mean \pm SEM, $n=3$) Leydig cells were apoptotic following 24 h incubation (Figure 21).

Based on the role of ROS on the onset of apoptosis and the putative accumulation of these free radicals during steroidogenesis *in vitro*, we hoped to observe some inhibition of the onset of apoptosis in cell samples treated with antioxidants.

Interestingly, although puzzling, quantitative analysis of apoptosis by ISEL revealed that SOD (100 IU/ml) had no significant ($p>0.05$) protective effect in Leydig cells cultured for 24 h with LH 0-0.1 ng/ml (Figure 21). In those samples, ISEL analysis showed that $29.7\% \pm 1.25$ (mean \pm SEM, $n=3$) and $32.0\% \pm 1.42$ (mean \pm SEM, $n=3$) of the Leydig cell population was apoptotic when cells were, respectively, treated with SOD (100 IU/ml) alone or with LH 0.1 ng/ml. However, in Leydig cells stimulated with LH 10.0 ng/ml, SOD effectively ($p<0.001$) reduced apoptotic cell death. Quantitative analysis revealed that, similar to cells cultured in control conditions, $32.2\% \pm 1.25$ (mean \pm SEM, $n=3$) of the Leydig cell population was apoptotic following 24 h incubation in presence of SOD 100 IU/ml and LH 10.0 ng/ml (Figure 21). This value represent a reduction of more then 29%

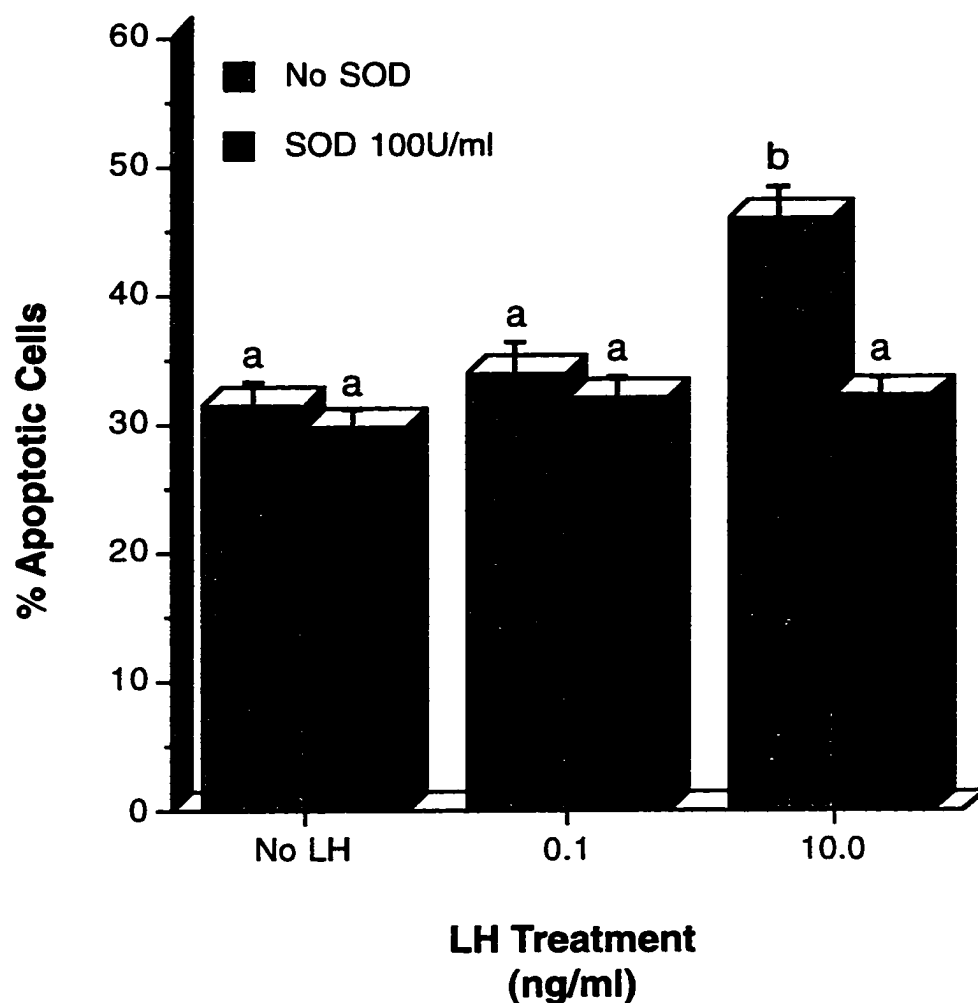


Figure 21. *In vitro* effect of the antioxidant SOD on the onset of apoptosis in rat Leydig cells. Primary cultures of Leydig cells were incubated in suspension for 24 h in M199 with or without SOD (100 U/ml) and with or without LH (0.1 - 10.0 ng/ml). SOD had a marked inhibitory effect on the onset of apoptosis in Leydig cells stimulated with LH 10.0 ng/ml. Results are expressed as a percent of the total number of cells counted per sample and are presented as the mean \pm SEM of three replicate experiments. Groups with different letters above the error bars are significantly different from each other as determined by a Scheffe multiple comparison test. $p < 0.05$.

in the number of apoptotic cells compared to Leydig cells incubated with LH 10.0 ng/ml alone.

These results suggested that, although SOD could inhibit apoptosis induced by supraphysiological stimulation of steroidogenesis, it was inefficient in preventing the onset of basal apoptosis. Moreover, these results also suggested that apoptosis, in primary cultures of rat Leydig cells, can be triggered by at least two distinct stimuli. The first stimulus could be identified as the culture conditions, on which SOD has no influence. The second, which can be neutralized by SOD, could be identified as the effect of ROS most likely released by excessive steroidogenesis.

Very similar data were obtained when Leydig cells were treated with catalase (50 ng/ml) in the presence of LH (0-10.0 ng/ml). Following 24 h of incubation, $35.7\% \pm 1.14$ (mean \pm SEM, n=2), $31.1\% \pm 0.93$ (mean \pm SEM, n=2) and $33.2\% \pm 0.44$ (mean \pm SEM) Leydig cells were apoptotic when treated with catalase (50 ng/ml) alone, or with catalase and LH at 0.1 or at 10.0 ng/ml, respectively (Figure 22). Like SOD, catalase had a significant protective effect ($p < 0.001$) against the onset of apoptosis in Leydig cells, exclusively when these cells were treated with both catalase and LH at 10.0 ng/ml (Figure 22). The action of catalase in LH at 10.0 ng/ml treated Leydig cells allowed a 27% reduction in the number of apoptotic cells compared to Leydig cells treated with LH at 10.0 ng/ml alone.

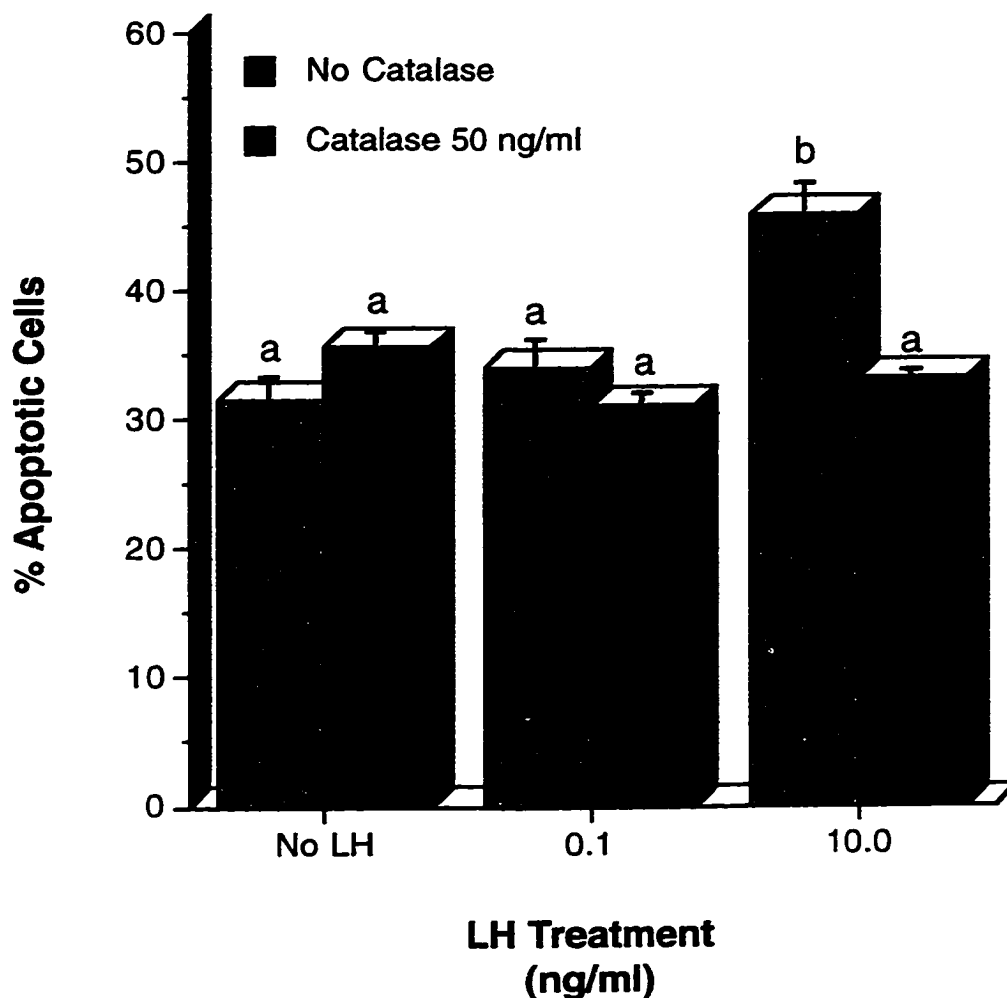


Figure 22. Effect of the antioxidant catalase on the onset of apoptosis in cultured Leydig cells. Primary cultures of rat Leydig cells were incubated in suspension for 24 h in M199 with or without catalase (50 ng/ml) and with or without LH (0.1-10.0 ng/ml). Catalase had a significant inhibitory effect on the onset of apoptosis in Leydig cells stimulated with LH 10.0 ng/ml. Results are expressed as a percent of the total number of cells counted per sample and are presented as the mean \pm SEM of 2 replicate experiments. Groups with different letters above the error bars are significantly different from each other as determined by a Scheffe multiple comparison test. $p < 0.05$.

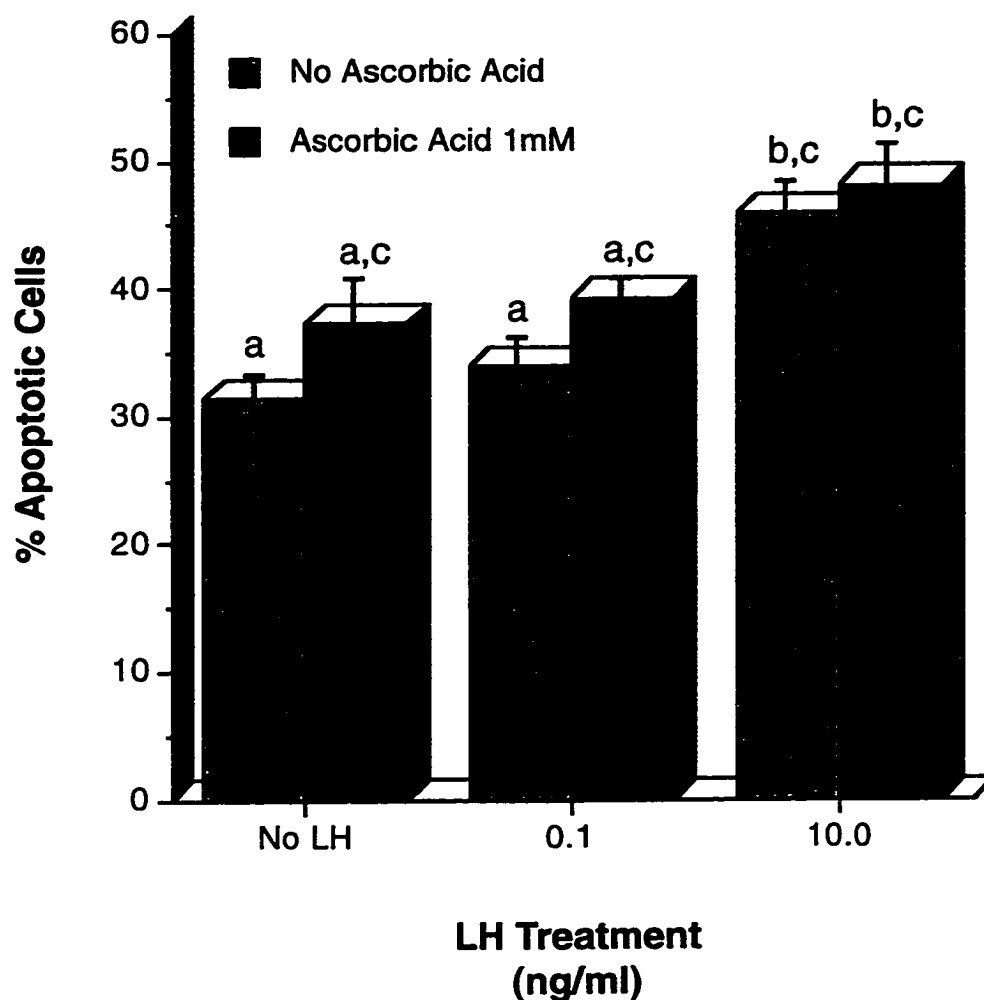


Figure 23. Effect of ascorbic acid (Asc. Ac) on the onset of apoptosis in cultured rat Leydig cells. Primary cultures of Leydig cells were incubated in suspension for 24 h in M199 with or without Asc. Ac (1 mM) and with or without LH (0.1-10.0 ng/ml). These results show that Asc. Ac had no effect on the onset of apoptosis in Leydig cells in presence or absence of LH. Results are expressed as a percent of the total number of cells counted per sample and are presented as the mean \pm SEM of 3 replicate experiments. Groups with different letters above the error bars are significantly different from each other as determined by a Scheffe multiple comparison test. $p < 0.05$

In order to test the importance of using specific antioxidants to prevent the onset of apoptosis, the anti-apoptotic potential of Asc. Ac., a broad spectrum antioxidant, was also studied. Primary cultures of rat Leydig cells, incubated in the presence of 1mM Asc. Ac. and LH (0 - 10.0 ng/ml) revealed no changes ($p>0.05$) in the onset pattern of apoptosis (Figure 23). Similar to cells cultured with LH (0-10.0 ng/ml) alone, $37.4\% \pm 3.32$ (mean \pm SEM, $n=3$), $39.3\% \pm 1.38$ (mean \pm SEM, $n=3$) and $48.1\% \pm 2.98$ (mean \pm SEM, $n=3$) of Leydig cells, cultured with Asc. Ac. (1 mM) and LH at 0, 0.1 or 10.0 ng/ml, respectively, were apoptotic following the 24 h incubation (Figure 23). Again, the addition of LH at 0.1 ng/ml, in presence or absence of Asc. Ac. (1 mM), had no significant ($p>0.05$) effect on the onset of apoptosis, compared to control conditions, following 24 h of incubation. However, the addition of LH at 10.0 ng/ml, with or without Asc. Ac. (1 mM), significantly ($p<0.05$) promoted the onset of apoptosis. Interestingly, statistical analysis of the isolated effect of Asc. Ac. (1 mM) revealed an overall significant effect ($p<0.05$) of the antioxidant on the onset of apoptosis. This stimulating role of Asc. Ac. on the onset of apoptosis was not related to the isolated effect of LH since the overall p -value for Asc. Ac. \times LH was greater than 0.05.

IV.3. Correlation Between Steroidogenesis and the Onset of Apoptosis in Antioxidant Treated Leydig Cells

Long term incubation studies performed on LH/hCG-treated Leydig cells (see Results, Section II.1.c. and II.2.c) enabled us to establish a significant

correlation between the rate of androgen production and the onset of apoptosis over 0 - 24 h of incubation.

The antioxidant study described here indirectly aimed at supporting the possible role of ROI, generated during steroidogenesis, on the onset of apoptosis. The importance of the rate of steroidogenesis on the onset of apoptosis was statistically assessed by Spearman rank-order correlation coefficient analysis. When analyzed all together, regardless of SOD (0 - 100 IU/ml) treatments, results from the SOD study revealed a significant ($p < 0.002$) correlation between the rate of steroidogenesis and the the onset of apoptosis ($r_s = 0.51$). However, once the data were grouped according to the SOD treatments, the importance of androgen production on the onset of apoptosis became non-significant ($p > 0.05$) in presence of SOD 100 IU/ml ($r_s = 0.40$). The statistical analysis presented here suggests that the anti-apoptotic effect of SOD in Leydig cells co-treated with a supra-physiological dose of LH was more important than the pro-apoptotic effect of supra-physiological rate of steroidogenesis. Based on these results, it is conceivable that the excessive generation of ROI, induced by supra-physiological stimulation of steroidogenesis in Leydig cells *in vitro*, was effectively neutralized by the addition of exogenous SOD, therefore preventing the onset of apoptosis in these cells.

The correlation between androgen production and the onset of apoptosis was even more affected by catalase treatments. In the absence of catalase, the correlation coefficient ($r_s = 0.80$) supported a strong cause and effect relation

between androgens and apoptosis. The addition of catalase (50 ng/ml) to primary cultures of rat Leydig cells, neutralized this relation. With a specific correlation coefficient of -0.18 ($p>0.05$), the androgen production rate significantly lost its induction ability over the apoptotic machinery.

Finally, as shown in the above results, Asc. Ac. treatments had no protective effects on the onset of apoptosis in primary cultures of rat Leydig cells. These results were reflected in the correlation analysis ($r_s=0.62$) since, regardless of the Asc. Ac. treatment, the androgen production rate maintained its strong influence ($p<0.0001$) over the onset of apoptosis.

V. EXPRESSION OF BCL-2 AND BAX IN PRIMARY CULTURES OF RAT LEYDIG CELLS

In an effort to better understand the cellular regulation of the onset of apoptosis in primary cultures of rat Leydig cells, modulation in the expression of two proteins, Bcl-2 and Bax, was studied by Western blotting analysis. In most tissues, the anti-apoptotic protein Bcl-2 is known to play a key role in cell survival contrary to its homolog, Bax, which counters Bcl-2 and promotes apoptosis. Although our understanding of the apoptotic regulatory mechanism is still limited, it is believed that the balance between anti-apoptotic and pro-apoptotic proteins determines a cell's fate.

The effect of incubation time, LH (0-10.0 ng/ml) treatments and exogenous

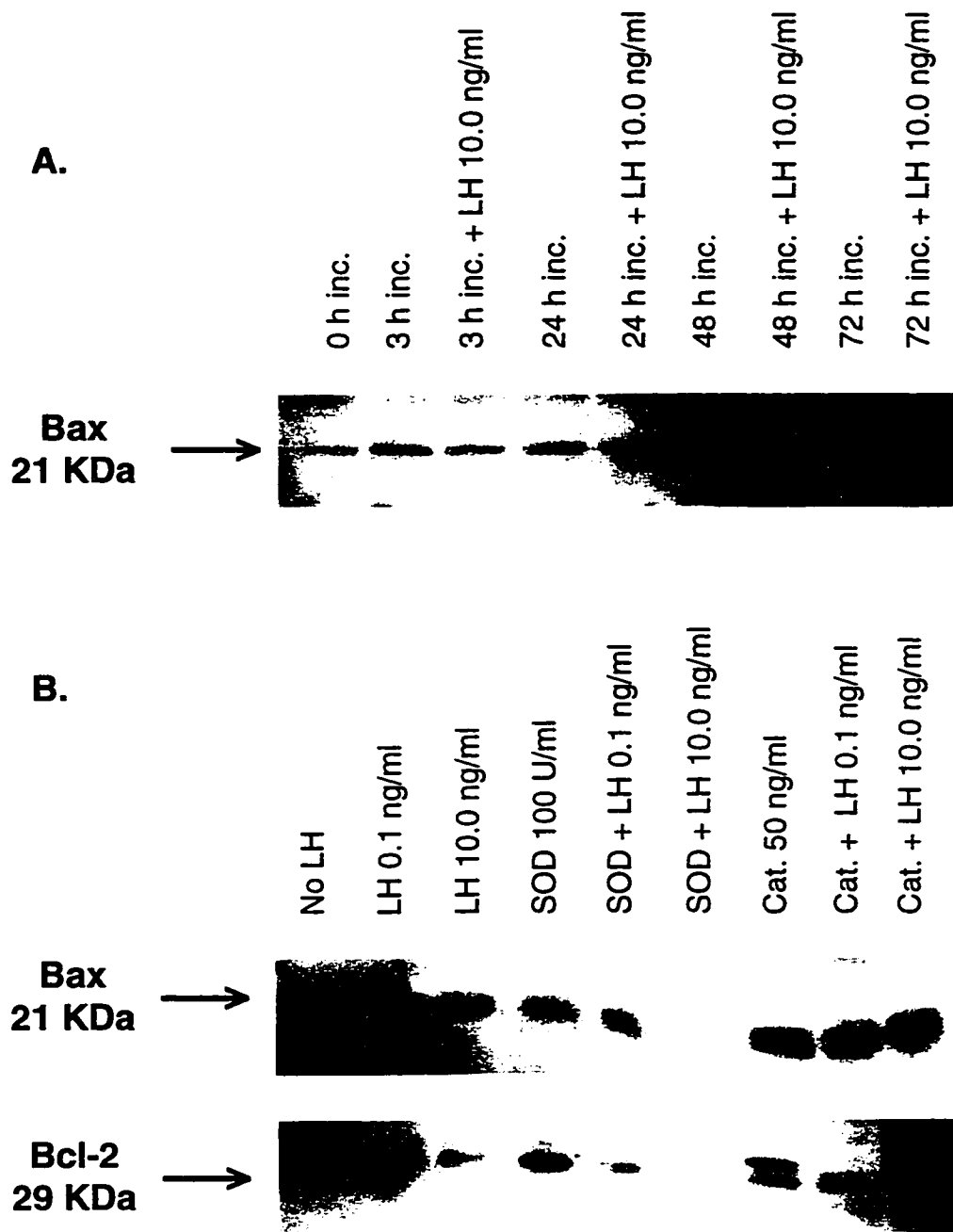


Figure 24. Bcl-2 and Bax Western blotting analysis. Total protein isolated from freshly isolated or cultured rat Leydig cells were separated by electrophoresis on SDS-PAGE. Following transfer, proteins were analyzed by Western blotting. Panel A. shows the expression of Bax in Leydig cells incubated from 0 to 72 h with or without LH 10.0 ng/ml. Panel B. shows the expression of Bax (upper band) and Bcl-2 (lower band) in Leydig cells incubated for 24 h in presence or absence of SOD, catalase, and/or LH (0-10.0 ng/ml).

antioxidant (SOD, Cat. or Asc.Ac.) supplementation on the expression of Bcl-2 and Bax was investigated. Following cell culture, protein electrophoresis and Western blotting analysis, we successfully detected immunoreactive bands of 29 KDa corresponding to Bcl-2 proteins as well as immunoreactive bands of 21 KDa corresponding to Bax proteins.

Even though most experiments were repeated in duplicate or triplicate, the densitometric analysis results, expressed as percent changes in comparison to control, could not be averaged between experiments. This was mainly caused by the large variations in the intensities of the Bcl-2 and Bax immunoreactive bands from one autoradiograph to the other. However, the results presented below represent the general trend in the level of expression of Bcl-2 and Bax in Leydig cells cultures with or without any additional treatment.

Densitometric analysis of the autoradiographic films revealed that, in Leydig cells cultured in supplemented M199 with no additional treatment, the level of Bax expression slightly increased with increasing incubation time (Figure 24 A). In these samples, the level of Bcl-2 expression decreased over time in cells cultured for 0 to 72 hours (data not shown). Interestingly, modulation in Bcl-2 and Bax expression did not reflect the apoptotic cell death rate observed earlier in Results section 1.3. Indeed, considering the high apoptosis rate at 3 h of incubation we expected to see a drastic drop in the expression of Bcl-2 along with a significant upregulation of Bax expression. The results presented here therefore suggest that regulation of apoptotic cell death in Leydig cells may involve anti- and pro-

apoptotic proteins other than Bcl-2 and Bax.

The addition of LH, either at a physiological concentration (0.1 ng/ml) or at a supraphysiological concentration (10.0 ng/ml) to Leydig cells cultured for 3 to 24 hours, showed no evidence of significant changes in Bcl-2 or Bax protein expression in comparison to cells cultured with no additional treatment. This observation was also true for Leydig cell samples cultured for 48 to 72 h in the presence of LH at 0, 0.1 and/or 10.0 ng/ml, as described in Table 2, (Figure 24).

The potential effect of exogenous antioxidant supplementation on the expression of Bcl-2 and Bax was also studied in primary cultures of rat Leydig cells. Immunoblotting analysis of Bax expression in Leydig cells cultured for 24 h in the presence of SOD (100 IU/ml), catalase (50 ng/ml) (Figure 24 B) or Asc. Ac. (1 mM) (data not shown) with or without LH (0.1-10.0 ng/ml) revealed interesting results. Indeed, while the level of Bax expression appeared to be inhibited by the addition of SOD (100 U/ml)(Figure 24 B), the addition of catalase (50 ug/ml)(Figure 24 B) or Asc. Ac. (1 mM) had no significant effect on the expression of this pro-apoptotic protein (data not shown). In these cells, although the results obtained did not allow to draw any definitive conclusion, Bcl-2 expression appeared to be up-regulated by SOD supplementation. Based on these results, it is surprising that the onset of apoptosis could not be inhibited in Leydig cells cultured in the presence of SOD and no LH or LH at 0.1 ng/ml as in cells cultured in the presence of SOD and LH at 10.0 ng/ml. These results support the complexity of apoptotic cell death regulatory system and suggest that other proteins may be involved in the process.

DISCUSSION

Over the past fifteen years, the growing interest in the study of physiological cell death has reached enormous proportions. In 1997 alone, more than 5700 papers were published on apoptosis! As in most other fields in basic research, andrology has been affected by the apoptosis epidemic.

In this regard, while it was generally accepted that Leydig cells constitute a stable population of terminally differentiated cells, a few researchers have persisted in the study of adult-type Leydig cell population status. Some of these studies have demonstrated the ability of the adult-type Leydig cells to undergo physiological cell death (Furuta *et al.*, 1994; Gaytan *et al.*, 1995; Shikone *et al.*, 1994; Tapanainen *et al.*, 1993). These studies, along with the research presented here, suggest that Leydig cells can engage in physiological cell death rather than die by necrosis, both *in vivo* and *in vitro*. Moreover, in Leydig cells, as in most cell types, the onset of apoptosis was shown to be induced in response to various stimuli, including LH and/or trophic factor depletion. Overall, these observations suggest that, in Leydig cells, apoptosis may be a more general phenomenon than previously suspected, therefore justifying a detailed investigation of the process. Indeed, a better understanding of apoptosis in primary cultures of rat Leydig cells might help to improve culture conditions and provide new insights on any results obtained from *in vitro* studies.

I. SPONTANEOUS ONSET OF APOPTOSIS IN PRIMARY CULTURES OF RAT LEYDIG CELLS

Although numerous immortal Leydig cell lines are available today, the study of steroidogenesis regulation still relies largely on primary cultures. Unfortunately, Leydig cells, especially from rat origin, are fragile and have been shown to be particularly sensitive to culture conditions as well as mechanical distortions induced by the cell isolation procedures (Rommerts *et al.*, 1985; Sharpe and Cooper, 1982). Moreover, cells in primary cultures are known to lose some functional properties over time. For isolated adult Leydig cells, changes in steroidogenic activities have been observed as early as 24 h of incubation (Browning *et al.*, 1982; Hedger *et al.*, 1990; Rommerts *et al.*, 1985).

Unfortunately, since cell death was expected in primary cultures, little consideration has been given to this process. The study of the spontaneous onset of apoptosis in cultured Leydig cells presents new aspects to the importance of optimal culture conditions and cell isolation procedures. Apoptotic cell quantification, as a function of the total cell number, has provided a sensitive method to evaluate the quality of Leydig cell preparations as well as cell survival over time *in vitro*. The study of apoptotic cell death in primary cultures of Leydig cells has also brought up a potential problem associated with the expression of androgen production in our cell model. Indeed, the study of apoptosis in steroidogenic cells have provided evidence of compartmentalization of the steroidogenic organelles during the initial steps of apoptosis. This compartmentali-

zation process plays an important role in the preservation of steroidogenesis in apoptotic cells and apoptotic bodies for at least 24 h (Amsterdam *et al.*, 1997). Although we believed that expressing androgen production as a function of viable, non-apoptotic cells, would provide a more accurate interpretation of the results, the ability of apoptotic Leydig cells and bodies to produce androgens suggests otherwise. How long can apoptotic cells maintain their steroidogenic potential? How significant is the rate of steroidogenesis in apoptotic cells? These questions remained unanswered at this time.

The study presented here showed that, following cell preparation and ISEL staining, more than 99% of the freshly isolated cells were viable and revealed no signs of apoptotic DNA fragmentation (Figure 8 and 9). However, once in culture, rat Leydig cells soon presented biochemical evidence of internucleosomal DNA fragmentation. Following 3 to 72 h of incubation in supplemented M199 with no additional treatment, the number of apoptotic cells increased with the duration of the culture (Figure 8 and 9). The relative absence of physiological cell death in freshly isolated Leydig cells reflected the stability of this cell population *in vivo*. Moreover, these results undoubtedly confirmed the acquired fragility of the Leydig cells, once removed from their natural environment in the testicular interstitium.

1.1. Apoptotic Cell Death Rate is Time-Dependent

Determination of the apoptotic cell death rate in cultured Leydig cells (Figure 9) suggested that, per unit of time, the number of Leydig cells undergoing

apoptosis was the highest between 0 to 3 h in culture. Such a rapid rate of apoptosis onset would have decimated the cell sample in less than 26 hours if maintained throughout the total culture period. However, following 3 h of incubation, although the total number of apoptotic cells kept increasing with the incubation time, we could observe a drastic decrease in the rate of apoptosis onset.

The precise chronology associated with the different phases of physiological cell death is not well established. However, the rare occurrence of apoptotic cells and/or apoptotic bodies observed by microscopy analysis in normal tissue, favors a rapid course for apoptosis. From the beginning of the induction phase, many scientists believe that no more than 3 to 6 hours are required for complete cell degeneration (Farschon *et al.*, 1997; Kerr *et al.*, 1995; Wyllie *et al.*, 1980). In primary cultures of rat Leydig cells originating from an apoptosis-free population, the rapid onset of apoptosis observed following 3 h of culture correlates with this temporal concept.

The significant drop in the apoptotic cell death rate following 3 h of incubation, strongly suggests the presence of variations either in the source and/or the strength of the pro-apoptotic stimuli triggering the apoptotic machinery. This dual apoptotic cell death rate could also be explained by the differential potential of the target cells to cope with pro-apoptotic stimuli.

Among those stimuli that could affect Leydig cell survival *in vitro*, we suspect the detrimental shock induced by the cell isolation procedures, the sudden

change in cellular environment once the cells are maintained *in vitro*, and finally, the heterogeneity of the Leydig cell enriched cell samples.

1.2. Pro-Apoptotic Stimuli Triggering Cell Death in Leydig Cell Cultures

a. Impact of the Cell Isolation Procedures on the Onset of Apoptosis

The preparation of an enriched Leydig cell suspension requires the disruption of the extracellular matrix (ECM) and intercellular junctions holding testicular cells together. While the ECM plays a role in regulating cell behavior, influencing their development, migration, proliferation, shape and metabolic functions, the intercellular junctions play a crucial role in paracrine communication and regulation of testicular functions (Peluso *et al.*, 1996; Perez Armendariz *et al.*, 1994). In the interstitium, numerous gap junctions and adhesion-type junctions connect Leydig cells to adjacent Leydig cells and macrophages (Hutson, 1992; Perez Armendariz *et al.*, 1994; Risley *et al.*, 1992). This complex intercellular network is believed to play an essential role in the regulation of Leydig cell functions (Hutson, 1992; Saez, 1994; Saez *et al.*, 1991).

The cell isolation procedures used here (Materials and Methods, section III.1) utilize collagenase to dissociate the interstitial cells from the bulk of the testes, along with gentle mechanical shaking to allow cell dispersion. This protocol has been shown to be the best method to isolate Leydig cells in order to maintain cell structure and functions (Rommerts *et al.*, 1985; Sharpe and Cooper, 1982).

However, the high rate of apoptotic cell death during the early stage of the

culture strongly suggests that the cell isolation procedure might indirectly induce apoptosis. Considering the importance of paracrine communication in Leydig cell maintenance (Denef, 1998; Saez, 1994), it is conceivable that, as interstitial cells dissociate from the bulk of the testicular tissue and cell-cell contacts are severed, the sudden interruption of intercellular communication could induce apoptosis. In support of this hypothesis, two studies have demonstrated the essential role of cell-cell contacts in cell survival.

Similar to Leydig cells, normal human colonic epithelial cells are notoriously difficult to grow in culture and limited success has been reported for short term primary cultures (2 to 4 days) (Hague *et al.*, 1997). In a recent study, human colonic epithelial cell survival *in vitro* was shown to be dependent on cell-cell contacts and specific survival factors including insulin, IGF-I and II, hydrocortisone and EGF. More specifically, the authors demonstrated that cell-cell contact disruption, rather than the lack of trophic factors, was responsible for irreversibly inducing apoptotic cell death in these premalignant cells (Hague *et al.*, 1997).

Similarly, in granulosa cells isolated from immature rats and cultured in serum-free media, cell-cell contacts were shown to be essential to block the onset of apoptosis in a progesterone-independent manner (Peluso *et al.*, 1996). These authors demonstrated that adhesion-type junctions, but not gap junctions, were essential to maintain granulosa cell viability *in vitro*. The importance of adhesion-type junctions in granulosa cell survival was confirmed by the inhibition of their

formation with either an antibody to N-cadherin or a synthetic N-cadherin peptide. Following N-cadherin inhibition, cell aggregation was attenuated and the percentage of apoptotic cells increased. These observations suggested that homophilic binding of N-cadherin molecules, located on the surface membranes of adjacent granulosa cells, initiated a signal transduction cascade that ultimately inhibited apoptosis (Peluso *et al.*, 1996).

Together, these studies suggest that following cell isolation and seeding, Leydig cell viability might depend on their ability to promptly restore cell-cell contacts. Shortly following cell plating, Leydig cells begin to attach to the culture dishes and to adopt a spindle shape in order to touch neighboring cells. It is therefore conceivable that the high cell death rate observed during the first three hours of culture might reflect the period of time required for Leydig cells to restore the cell junction network necessary for paracrine communication.

The importance of cell-cell contacts for Leydig cell survival and function *in vitro* was also reflected in cell density studies. These studies demonstrated that increasing the cell density to up to 100 000 cells/16 mm well caused a significant increase in basal and hCG-stimulated testosterone secretion per cell (Hedger and Eddy, 1990; Murphy and Moger, 1982). These data suggested that, at high cell density, the incidence of cell-cell junction formation was significantly increased resulting in an improved cooperation between Leydig cells *in vitro*. Indeed, cell-cell junctions provide a pathway for the exchange of ions and small molecules (M.W. <1000), such as cAMP and calcium, known to stimulate Leydig cell

steroidogenesis *in vitro* (You *et al.*, 1998).

In the present study, a cell density of 10^5 Leydig cells/ml/16 mm well, or 10^5 Leydig cells/ml/15 ml tube was used for cell monolayer and cell suspension culture, respectively. Cells cultured in monolayer rapidly attached to the dish and formed cell-cell contacts. Leydig cells, cultured in suspension, were not allowed to attach to the culture dish. Nonetheless, the presence of calcium in the culture media allowed cell clumping and cell-cell contact formation (Sato *et al.*, 1982).

In order to confirm the importance of cell-cell contacts in the early phase of primary cultures of rat Leydig cells, immunohistochemical, Western blot, and microscopic analysis could be performed to establish the time required to restore cell-cell contact, identify the different cell junction types formed between Leydig cells *in vitro* and determine their importance for cell survival. Therefore, studying the effects of cell-cell contact inhibition *in vitro* would help to evaluate any correlation between early apoptotic cell death in primary cultures of rat Leydig cells and deficient cell-cell communication.

b. Impact of the Culture Conditions on the Onset of Apoptosis

The availability of supplemented serum-free media, which sustain basal and LH stimulated androgen production in Leydig cells *in vitro*, has made possible the study of steroidogenesis regulation under controlled conditions. Modified medium 199, as used in this study, and Ham's F12 medium and Dulbecco's Modified Eagle's Medium (DMEM) mixed in a 1:1 (v/v) ratio are among the most common

media used for primary cultures of Leydig cells. Both media have been shown to support acceptable levels of steroidogenesis *in vitro* (Murphy and Moger, 1982; Risbridger, 1992). Primary cultures of Leydig cells represent an invaluable model to study the regulation of steroidogenesis. Actually, much of the evidence supporting a role for paracrine factors in the regulation of Leydig cell steroidogenesis comes from *in vitro* studies using isolated Leydig cells (Denef, 1998; Lin *et al.*, 1989; Lin *et al.*, 1986; Risbridger, 1992; Saez, 1994; Sharpe and Cooper, 1982). It is therefore essential that the Leydig cell response to any stimulus closely reflects the *in vivo* response.

In this regard, multiple amendments have been proposed to improve primary culture conditions and Leydig cell steroidogenic response. For example, the addition of antioxidants, such as α -tocopherol (Vitamin E), was shown to enhance cell survival by protecting oxygen-sensitive components of the medium and preventing ROI cellular damage (Mather *et al.*, 1983; Risbridger and Hedger, 1992). In primary cultures of Leydig cells, Vitamin E has also been shown to be important to maintain the high affinity of LH receptors for its ligand (Akazawa *et al.*, 1987). A number of other antioxidants were tested to replace Vitamin E, including ascorbic acid, although much higher concentrations were required to obtain equivalent protection (Hornsby and McAllister, 1991; Mather *et al.*, 1983). Moreover, a variety of factors have been investigated as culture media supplements. For example, FCS, growth factors (IGF-I, EGF), transferrin, selenous acid, as well as diluted spent media have all been used to improve the culture

media's ability to support steroidogenesis (Browning *et al.*, 1983; Haour *et al.*, 1983; Lin *et al.*, 1986; Moger and Murphy, 1987; Murphy and Moger, 1982; Myers and Abney, 1988). Also, low density serum lipoprotein (LDL) supplementation was shown to enhance both basal and LH-stimulated testosterone production *in vitro* by providing the Leydig cells with additional steroidogenic substrate (Benahmed *et al.*, 1981; Benahmed *et al.*, 1983; Risbridger, 1992). Finally, physiological doses of LH, continuously present in the culture media, have also been shown to improve Leydig cell survival and metabolism *in vitro* (Risbridger, 1993). Considering the essential role of LH to maintain Leydig cell volume and function *in vivo* (Keeney and Ewing, 1990), it was reasonable to expect a similar effect *in vitro*.

However, improving the culture conditions by amending the media with various hormones and/or trophic and growth factors complicate the interpretation of any results so obtained.

c. Impact of the Leydig Cell Population Heterogeneity on the Onset of Apoptosis

Besides cell-cell contact requirements and optimal culture conditions, the apoptotic cell death rate in primary cultures of rat Leydig cells might also vary as a function of the differential potential of the target cells to cope with pro-apoptotic stimuli.

In testes, different populations of Leydig cells are believed to co-exist in the adult male (Bergh, 1982). In isolated Leydig cells, it is clear that within a

morphological homogenous population of testicular cells, functional differences may occur. For example, distinct cell fractions, isolated by density fractionation, showed quantitatively different responses to LH stimulation (Browne *et al.*, 1990; Laws *et al.*, 1985; Rommerts and Brinkman, 1981).

In an elaborate study, Dehejia and colleagues (1982) analyzed five distinct interstitial cell fractions isolated on a continuous metrizamide gradient. Among those fractions, three were responsive to LH/hCG stimulation. Cells from fraction density 1.085 and 1.105 g/cm³ (fraction III and IV) were the most responsive, expressing the highest number of hCG receptors on the membrane surface (Dehejia *et al.*, 1982). While lower density cell fractions (fraction I and II) mostly contained damaged Leydig cells and/or cells expressing fewer LH/hCG receptors, the higher density cell fraction (fraction V) mostly contained red blood cells.

These studies suggest that cell isolation does not yield perfectly homogenous Leydig cell samples. Therefore, functional differences observed among isolated Leydig cells might result in variations in cell responses to a given stimulus and cell survival *in vitro*. For example, if some Leydig cells are more dependent on paracrine factors and cell-cell contacts, the steroidogenic response following basal and/or LH stimulated androgen production would be expected to differ significantly. Moreover, a maximum of 1-1.5 million Leydig cells are typically recovered per testis following Leydig cell isolation. These numbers represent less than 10% of the total Leydig cell population per testis (Molenaar *et al.*, 1983; Rommerts *et al.*, 1985; Sharpe and Cooper, 1982). Therefore, it is possible that

the isolated Leydig cells might not constitute a representative sample of the total population. These functional differences among Leydig cells might transduce into variations in Leydig cell metabolism and resistance to undergoing apoptotic cell death *in vitro*.

Finally, Leydig cell preparations may be of varying degrees of purity, ranging from 5-10% Leydig cells in crude interstitial cells to 95% in elutriated and Percoll separated Leydig cell preparations (Risbridger, 1992). Primary cultures, although predominantly of one cell type, usually are contaminated to some degree with other cell types. In the study presented here, cell suspensions typically contained 85% Leydig cells.

II. EFFECTS OF LH/HCG ON STEROIDOGENESIS AND ON THE ONSET OF APOPTOSIS IN LEYDIG CELLS

With the concept of physiological cell death there have arisen words such as "cell survival factor". Although no strict definition has been established at this time, the term "cell survival factor" broadly refers to secretory products, such as hormones and growth factors, that are essential to prevent cell death *in vivo* and/or *in vitro*. In testes, pituitary gonadotropins and testicular testosterone fit such a description. Indeed, LH, FSH and androgens have been shown to be essential for the survival, growth and differentiation of somatic cells, as well as for the initiation and maintenance of spermatogenesis (Tapanainen *et al.*, 1993).

II.1. Effects of Gonadotropin Deprivation on Leydig Cells.

The essential role of gonadotropins, more specifically LH, for the maintenance of Leydig cell differentiated state, viability and function have been demonstrated *in vivo* in several studies.

Typically, gonadotropin deprivation can be induced either by hypophysectomy (Keeney and Ewing, 1990; Tapanainen *et al.*, 1993; Teerds *et al.*, 1989a), administration of high doses of steroids, GnRH antagonists, or by neutralization of LH and/or FSH by specific antibodies (reviewed in Saez, 1994). Regardless of the method used, pituitary gonadotropin deprivation was shown to cause Leydig cell atrophy, decreased steroidogenic enzyme activity (in particular P-450_{scc} and P-450-17 α) as well as steroidogenesis inhibition (Keeney and Ewing, 1990; Mendis Handagama, 1997; Saez, 1994).

Within 24 h of LH deprivation *in vivo*, distinct morphological alterations could be observed in Leydig cells. Following 6 to 8 days, gonadotropin-deprived Leydig cells presented total cell volume reduction, pleiomorphic nuclear profiles, decreased organelle volume, including smooth and rough ER, mitochondria, peroxisomes and Golgi, as well as decreased peroxisomal catalase content and fewer cell-cell junctions (Mendis Handagama, 1997; Russel *et al.*, 1992).

The direct involvement of LH in the maintenance of Leydig cell function was confirmed following LH/hCG replacement therapy. In hypophysectomized rats, LH/hCG replacement was shown to rapidly restore the structure and function of Leydig cells (Keeney *et al.*, 1990; Saez, 1994; Tapanainen *et al.*, 1993).

Tapanainen and colleagues (1993) also demonstrated that hypophysectomy-induced testicular cell degeneration was mediated by apoptotic cell death. Their results showed that while seminiferous tubules and interstitial cells of intact animals contained predominantly non-apoptotic, high molecular weight DNA, cells isolated from hypophysectomized animals presented substantial apoptotic DNA fragmentation. In addition, these authors presented evidence that pituitary gonadotropins and androgens play an essential role in the inhibition of apoptotic cell death in testicular cells. Indeed, FSH replacement was shown to block hypophysectomy-induced apoptotic DNA cleavage by more than 90% in both seminiferous tubules and interstitial cells. Furthermore, hCG replacement reduced hypophysectomy-induced DNA cleavage by 76.7% in interstitial cells. Finally, testosterone replacement could suppress hypophysectomy-induced apoptotic cell death by only 55.4% in interstitial cells (Tapanainen *et al.*, 1993).

II.2. Effect of LH Treatments on Steroidogenesis and the Onset of Apoptosis in Cultured Leydig Cells

The *in vitro* effects of LH treatments on androgen production and cell survival were studied in primary cultures of rat Leydig cells. Overall, the results presented in this study (Results, Section II.1.a and b) demonstrate that the potent stimulatory effect of LH on steroidogenesis as well as its effects on the onset of apoptosis in Leydig cells were time- and dose-dependent.

a. Time- and Dose-Dependent Effect of LH in Primary Cultures of Rat Leydig Cells.

Detailed analysis of the role of LH at 0.1 ng/ml as a survival factor in Leydig cells suggest a time-dependent effect. During the initial 24 h of incubation, while responsive to LH stimulation as shown by elevated androgen production (Figure 10), primary cultures of rat Leydig cells presented no sign of an essential requirement for LH at 0.1 ng/ml as a survival factor (Figure 11). Thereafter, ISEL quantification results support an anti-apoptotic effect of LH at 0.1 ng/ml on *in vitro* Leydig cell survival (Figure 11).

The non-significant effect of LH on cell survival during the initial 24 h of incubation was also observed in cell samples incubated for 48 h in the presence or absence of additional LH. Figure 11 shows that there was no detectable difference in the number of apoptotic cells in Leydig cells incubated from 0 to 24 h in the absence of LH followed by 24 h of incubation in the presence of LH at 0.1 ng/ml and the number of apoptotic cells in samples incubated for 48 h in the presence of LH at 0.1 ng/ml. The similar number of apoptotic cells between these samples suggest that the observed anti-apoptotic effect of LH (0.1 ng/ml) becomes significant strictly following 24 h of incubation.

Time-dependent LH action as a survival factor is further supported by ISEL results obtained from Leydig cells incubated for 72 h. In these cells, LH 0.1 ng/ml could prevent the onset of apoptosis if present in the medium over the whole incubation period. When LH 0.1 ng/ml was only added following 48 h of incubation,

i.e. for the last 24 h of incubation, Leydig cells could not be rescued from apoptotic cell death. Moreover, in cell samples stimulated by LH 0.1 ng/ml for 48 h and then with LH 10.0 ng/ml for 24 h, physiological LH concentration had a dramatic protective effect which was not seen when cells were incubated for a maximum of 48 h in similar conditions.

b. Analysis of the Anti-Apoptotic Effect of LH in Leydig Cells

The results presented here undeniably support a role for physiological doses of LH in Leydig cell survival. However, the inability of LH to prevent the onset of apoptosis during the initial 24 h of incubation suggests a more complex regulatory system, involving several survival factors acting on Leydig cells. This hypothesis implies that, like LH secretion *in vivo*, LH supplementation *in vitro* is necessary, although not sufficient, to maintain Leydig cell survival.

In support of this concept, recent studies, including the study published by Tapanainen *et al.* presented above, have demonstrated the anti-apoptotic effect of LH, FSH and androgens in the testes (Furuta *et al.*, 1994; Heiskanen *et al.*, 1996; Tapanainen *et al.*, 1993). Among those survival factors, FSH had the most dramatic anti-apoptotic effect on interstitial cell survival (Furuta *et al.*, 1994; Tapanainen *et al.*, 1993). In testes, since FSH receptors are exclusively expressed in Sertoli cells (Bernier *et al.*, 1986; Bortolussi *et al.*, 1990; Heckert and Griswald, 1991; Misrahi *et al.*, 1996; Orth and Christensen, 1977), these results imply that the observed effect of FSH on interstitial cell survival must be indirect. Recently,

purified and recombinant FSH have been shown to stimulate Leydig cell functions *in vivo*, in immature hypophysectomized rats, by increasing LH/hCG receptor content and androgen biosynthetic potential (Vihko *et al.*, 1991). Furthermore, other studies present evidence of testicular factors, derived from the seminiferous tubules, that may influence Leydig cell differentiation and function through paracrine mechanisms (Denef, 1998; Kerr and Sharpe, 1985; Lecerf *et al.*, 1993; Russell *et al.*, 1993; Saez, 1994; Sharpe *et al.*, 1990; Verhoeven and Cailleau, 1990).

The onset of hypophysectomy-induced apoptosis was also significantly blocked by hCG replacement (Tapanainen *et al.*, 1993). Although hCG was not as effective as FSH in suppressing apoptosis, it significantly inhibited apoptotic DNA fragmentation in both the interstitium and the seminiferous tubules. Since Leydig cells are thought to be the only testicular cells expressing LH/hCG receptors (Bernier *et al.*, 1986; Bortolussi *et al.*, 1990; Misrahi *et al.*, 1996), it is likely that the effect of LH/hCG on cell survival in the seminiferous tubules was mediated by testosterone and/or paracrine factors (Tapanainen *et al.*, 1993).

In perspective, the ISEL results presented in this study fit with this concept where multiple survival factors must exert their anti-apoptotic effect to maintain Leydig cell viability. While physiological concentration of LH present evidence of some anti-apoptotic action, its limited influence leaves room for other survival factors, including FSH and androgens, to inhibit physiological cell death.

The complex regulation of steroidogenesis and spermatogenesis, along with the essential requirements of spermatogenesis for androgens, support multiple functions for regulatory hormones and paracrine factors in the testes, involving both cell metabolism and viability. Indeed, since germ cell development depends so closely on Sertoli and Leydig cells and Leydig cells and Sertoli cells depend on each other's survival and secretory functions, the control of testicular cell viability is expected to reflect this symbiosis.

Although presenting a limited potential, physiological doses of LH do inhibit the onset of apoptosis in long term cultures of rat Leydig cells. How is this protective effect transduced in Leydig cells? This question remains unanswered at present. However, the increasing importance of physiological doses of LH as a survival factor over time suggests that LH's protective action is not necessarily transmitted through the steroidogenic pathway.

c. Analysis of the Pro-Apoptotic Effect of LH in Leydig Cells.

Contrary to the effect of LH 0.1 ng/ml, supraphysiological doses of LH significantly induced apoptotic cell death in Leydig cells during the initial 24 h of incubation. Although we had hoped to observe enhanced anti-apoptotic potential, the results presented in this study showed otherwise differently (Figure 10 and 11).

These results suggest that excessive stimulation of the steroidogenic pathway following LH treatment could promote the formation and release of

abnormally high levels of ROI. The inability of the Leydig cells to neutralize these ROI most likely leads to irreversible oxidative cell damage, eventually turning the apoptotic machinery on.

Following 24 h of incubation, the decreased correlation between supraphysiological stimulation of steroidogenesis and the onset of apoptosis in cultured Leydig cells might reflect the reacquired ability of the cells to cope with elevated rate of steroidogenesis. For example, although Leydig cells can neutralize physiological levels of ROI generated by basal and LH at 0.1 ng/ml stimulated steroidogenesis, they might not be able to cope with excessive ROI generated by supraphysiological stimulation of steroidogenesis. Thereafter, the potential ability of the cells to upregulate antioxidant expression and activation, might provide cells with adapted antioxidant defence mechanisms.

II.3. Effect of Human Chorionic Gonadotropin (hCG) Pretreatment on the Onset of Apoptosis in Cultured Leydig Cells.

In view of the anti-apoptotic effects of LH (0.1 ng/ml) on cultured Leydig cells in long term cultures and its known protective effect on Leydig cells *in vivo*, the objective of this experiment was to assess the possibility of reinforcing Leydig cells *in vivo*, by injecting the animals with 100 IU hCG / animal for 0-24 h prior to animal sacrifice and Leydig cell isolation.

a. Pro-Apoptotic Effect of High Doses of hCG on Leydig Cells.

The results presented in this study indicate that, similar to gonadotropin depletion *in vivo*, supraphysiological doses of hCG could activate the apoptotic cell death mechanism (Results, Section II.2.b). Indeed, freshly isolated Leydig cells, isolated from animals pretreated with hCG for 12 to 24 h prior to sacrifice, presented a significant increase in the number of apoptotic cells (Figure 13). Once in culture, and despite evident signs of LH receptor desensitization, the pro-apoptotic effect of LH at 10.0 ng/ml could still be observed (Figure 12 and 13).

b. Analysis of the Pro-Apoptotic Effect of hCG.

In testes, although LH/hCG are considered as essential survival factors at physiological level, supraphysiological doses might actually initiate physiological cell death. Similar to the effect of supraphysiological doses of LH administered *in vitro*, hCG treatment given to rats 0-24 h prior to Leydig cell isolation might indirectly promote the excessive production and intracellular release of damaging ROI through the maximal activation of the steroidogenic pathway. These results suggest that with time, LH excess becomes as detrimental for Leydig cells as LH deprivation, both *in vitro* and *in vivo*.

ROI have been shown to initiate lipid peroxidation in cell membranes. Moreover, they are responsible for mitochondrial and endoplasmic cytochrome P-450 activity reduction (Chabre *et al.*, 1993; Georgiou *et al.*, 1987; Kukucka and Misra, 1993; Peltola *et al.*, 1992) resulting in steroidogenesis inhibition and

possibly apoptotic cell death. It is possible that the sudden exposure of Leydig cells to supraphysiological stimulation by hCG *in vivo* did not allow Leydig cells to upgrade their antioxidant system nor to down-regulate LH receptors quickly enough to limit the harmful side-effects generated by steroidogenesis over-stimulation.

The significant time-dependent increase in the number of apoptotic cells in freshly isolated Leydig cells from hCG-treated rats could not be attributed to an artifactual cell isolation process. Since Leydig cell density has been shown to decrease following chronic LH/hCG stimulation (Dehejia *et al.*, 1982) it was possible that Percoll gradient separation was unable to isolate functional, yet lighter Leydig cells, from less responsive, broken and/or apoptotic Leydig cells. However, analysis of the steroidogenic capacity of these cells *in vitro* tends to obviate this hypothesis. Indeed, in Leydig cells isolated from hCG pretreated animals, an acceptable level of basal and LH/hCG-stimulated androgen production, along with evident signs of LH receptor desensitization, could be measured by RIA (Figure 12).

In order to confirm the pro-apoptotic effect of long term hCG stimulation on rat Leydig cells *in vivo*, whole testis sections from hCG treated rats could be processed for ISEL. This would discard any doubt regarding the origin of apoptosis in Leydig cells and confirming that it is not an artifact created by the cell isolation process.

c. LH/hCG Receptor Down-Regulation Effect on the Onset of Apoptosis.

In rat Leydig cells, prolonged exposure to increased LH/hCG levels *in vivo* and *in vitro* results in LH/hCG receptor down-regulation and, subsequently, to reduced androgen production (Saez, 1994). The time course of LH receptor down-regulation appears to be time- and dose-dependent as well as species specific. In rat Leydig cells, LH receptor down-regulation is rather slow and appears after a lag time of several hours (Chasalow *et al.*, 1979; Dufau *et al.*, 1984; Sundby *et al.*, 1983) despite the fact that internalization of the hormone-receptor complex starts very rapidly after the binding of LH/hCG (Mather *et al.*, 1982).

Early studies have shown that hCG induced Leydig cell desensitization is a complex phenomenon (Chasalow *et al.*, 1979). LH/hCG receptor desensitization is induced by distinct cellular modifications including decreased number of membrane receptors following internalization along with decreased LH receptor mRNA (Pakarinen *et al.*, 1990), changes in the coupling system between receptor and adenylate cyclase, and, blockade of steroidogenesis beyond cAMP formation due to decreased activity in both lyase and 17 α -hydroxylase activities (Chasalow *et al.*, 1979).

Quantification of the onset of apoptosis in LH/hCG-treated Leydig cells (Figure 13) demonstrated that, in these cells, apoptosis is not inhibited by LH/hCG receptor desensitization. Indeed, Leydig cells isolated from rats pretreated with hCG for 12 or 24 h and cultured with LH 10.0 ng/ml for 24 h presented significantly

more apoptotic Leydig cells than other sample groups. The high level of apoptotic cell death occurred regardless of the ongoing LH receptor desensitization process as shown by decreased androgen production in these samples. These results suggest that LH/hCG binding to its receptor is probably not directly responsible for the activation of the apoptotic machinery. However, we believe that following LH/hCG stimulation, activation of the steroidogenic transduction pathway might stimulate messengers, or oncogenes, common to the apoptotic pathway. Therefore, as steroidogenesis reaches unphysiological levels or induces sufficient ROI, common second messenger(s) could rapidly induce apoptotic cell death to control these abnormally functioning cells.

In this regard, supraphysiological elevations of cAMP by administration of the cAMP analog 8-bromo-cAMP was shown to induce apoptosis in more than 90% of the population in primary cultures of granulosa cells (Aharoni *et al.*, 1995). It is therefore conceivable that, in Leydig cells, excessive levels of cAMP in the cytosol could inhibit anti-apoptotic proteins and/or stimulate pro-apoptotic proteins.

III. EFFECTS OF EXOGENOUS ANTIOXIDANTS IN CULTURED LEYDIG CELLS

Cultured steroidogenic cells are known to be susceptible to oxygen-mediated free radical damages (Hornsby, 1980; Hornsby and McAllister, 1991). *In vivo*, Leydig cells are normally protected by an elaborate arsenal of antioxidants. However, cell isolation procedures and subsequent culture are believed to partly deplete steroidogenic cells of antioxidants (Kukucka and Misra, 1993). In an effort

to reduce the level of apoptosis in our cell culture model, we investigated the effect of exogenous antioxidant supplementation on the level of steroidogenesis and the onset of apoptosis following 24 h of incubation.

The results presented in this study (Results, Section IV.2) showed that while superoxide dismutase (100 U/ml) or catalase (50 ng/ml) supplementation had no effect on the onset of apoptosis in basal and LH (0.1 ng/ml)-stimulated Leydig cells *in vitro*, both antioxidants inhibited the onset of apoptosis in LH (10.0 ng/ml) stimulated Leydig cells (Figure 21 and 22). The addition of ascorbic acid (1mM) did not have any significant effect on the onset of apoptosis in basal or LH-stimulated Leydig cells cultured for 24 h.

These results also suggest that, in the absence of supraphysiological stimulation of steroidogenesis, endogenous antioxidants coupled with the antioxidants present in the culture media adequately prevented the damaging effects of ROI on the onset of apoptosis or steroidogenesis. Indeed, the addition of SOD, catalase or Asc. Ac. could not further inhibit the onset of apoptosis in basal or LH 0.1 ng/ml stimulated Leydig cells nor could it improve the steroidogenic capacity of the cells.

Recently, LH has been linked to the regulation of antioxidants in steroidogenic cells (Peltola *et al.*, 1992; Peltola *et al.*, 1996). In rat testes, although LH stimulation causes ROI generation via activation of the steroidogenic pathway, it is also responsible for the stimulation of peroxide-metabolizing enzymes, such

as SOD, in the interstitial tissue. Moreover, rat ovarian antioxidants were also shown to be regulated by LH action *in vivo* (Aten *et al.*, 1992). In that study, the authors showed that ovarian levels of antioxidant vitamins E, C and A and of glutathione peroxidase are subject to marked changes during the ovarian cycle. More specifically, increased LH secretion was responsible for inducing a prominent rise in ovarian vitamin E. The authors concluded that the control of the antioxidant vitamin reserve in ovaries is under endocrine regulation and represent therefore a dynamic regulatory process.

In the ovaries and the testes, where gametogenesis depends so closely on the production of reproductive steroids, physiological cell death seems to play an important role in the elimination of potentially harmful ROI-producing cells. Indeed, apoptosis would be responsible for purifying the gonads from those cells which fail to control excessive ROI production in spite of the fact that all the cellular defence mechanisms have already been activated (Skulachev, 1997).

In this regard, steroidogenic cells could present three lines of defence against ROI-induced damages. First, following LH stimulation, steroidogenic cells upregulate and activate antioxidants to neutralize newly generated ROI. Second, as ongoing steroidogenesis indirectly maintain a high rate of ROI production, the antioxidant system is overwhelmed and ROI accumulate in the cytosol. In attempting to slow down cell functions, excess ROI are then responsible for inducing cellular damages and inhibit steroidogenic enzymes and subsequently

inhibit steroidogenesis. Finally, as a last resort, physiological cell death is initiated, probably via ROI action, in order to protect the remaining cell population.

IV. WESTERN BLOTTING ANALYSIS OF BCL-2 AND BAX EXPRESSION IN PRIMARY CULTURES OF RAT LEYDIG CELLS

In rat Leydig cells, the expression of the anti-apoptotic protein Bcl-2 and its pro-apoptotic homolog Bax was studied by Western blotting analysis. Although some research groups have found no evidence of Bcl-2 expression in Leydig cells (Taylor *et al.*, 1998), the results presented here (Results, Section IV) demonstrate the presence of both Bcl-2 and Bax proteins. However, while Bcl-2 and Bax were detected, we could not conclusively associate Bcl-2 and/or Bax expression with the regulation of apoptotic cell death in Leydig cells.

Western blotting analysis in cultured Leydig cells showed that the incubation time and the addition of SOD significantly affected the expression of the pro-apoptotic protein Bax. Unfortunately, although the expression of the anti-apoptotic protein Bcl-2 seemed to decrease with increasing incubation time (data not shown), we could not draw any definite conclusion on the effect of LH (0-10.0 ng/ml) stimulation and/or exogenous antioxidants supplementation (Results, Section IV). Overall, these results suggested that, in primary cultures of rat Leydig cells, modulation in the expression of Bcl-2 and Bax did not reflect the onset of apoptotic cell death, especially not following 3 h of incubation. While Bcl-2-dependent inhibition of apoptosis is not impossible in rat Leydig cells, these results

suggest the involvement of other anti-apoptotic members of the Bcl-2 family of proteins, for example Bcl-x_{Long}.

As seen above, extracellular signals and cellular receptors important for apoptosis and survival of different tissues are extremely diverse. Likewise, redundant genes in the apoptotic pathway may have developed during evolution, and different subsets of death and anti-death genes may be responsible for apoptosis and survival of cells in a tissue-specific manner (Hsu and Hsueh, 1998). Indeed, the expression patterns of Bcl-2 homologs in different tissues appear to overlap and vary greatly; some are widely distributed while others are restricted. For example, the Mcl-1 protein is expressed mainly in epithelial cells in the prostate, breast, endometrium, epidermis, stomach, intestine, colon, and respiratory tract, whereas expression of Bcl-x is restricted to bone marrow and thymus (Boise *et al.*, 1993; Kozopas *et al.*, 1993). In contrast, Bax and Bak are found in most tissues (Chittenden *et al.*, 1995b; Farrow *et al.*, 1995; Hsu and Hsueh, 1998). Thus the composition of Bcl-2 homologs in any given cell type is likely to differ.

The notion that different Bcl-2 homologs regulate apoptosis in a tissue-specific manner is supported by immunohistochemical studies and work in knock-out mice deficient in different members of the *bcl-2* gene family (reviewed in Hsu and Hsueh, 1998). In Bcl-2-deficient mice, most tissues developed normally, with only cells in hair follicles, kidney, and the lymphoid system showing apparent defects in apoptosis (Kamada *et al.*, 1995). Likewise, mice deficient in Bax

appeared healthy, although cells of lymphoid and gonadal lineages showed aberrations in cell death (Knudson *et al.*, 1995). Finally, Bcl-x_{Long} proteins were shown to be expressed in neurons, cells of the hematopoietic system, epithelial cells and reproductive tissues including the testes (Krajewski *et al.*, 1994). These immunohistochemical and knock-out studies, along with the result presented in this study, support a role for members of the Bcl-2 family of homologs, other than Bcl-2 and Bax, to play a key role in the regulation of apoptotic cell death in rat Leydig cells.

In testes, as well as in primary cultures of rat Leydig cells, the identity and role of the proteins involved in the regulation of physiological cell death should be further investigated. Since apoptotic cell death affect singles cells in a non-synchronized fashion, the study of apoptosis should ideally be completed in intact cells. Indeed, tissue and cell extracts, containing a minority of apoptotic cells, could not precisely reflect the protein and/or gene expression in single apoptotic cells. Moreover, *in situ* immunostaining should probably be coupled with a morphological assessment of the apoptotic cells in order to associate protein expression with the different stages of physiological cell death.

V. PROSPECTIVE RESEARCH

Future direction of research would benefit from a detailed study of the importance of cell-cell contacts among Leydig cells as well as between Leydig cells and macrophages. Indeed, a better understanding of cell-cell contact type,

chronology of formation *in vitro* and importance for Leydig cell survival and function, might help to improve cell culture conditions and limit cultured-induced apoptotic cell death.

Also, as mentioned above, recent studies suggest an important role for paracrine communication in the regulation of steroidogenesis and cell survival in the testes. Although the studies presented here demonstrate that apoptosis can be induced by different pro-apoptotic stimuli, the use of co-culture might provide Leydig cells with essential paracrine factors that could limit the onset of apoptosis induced by one or more of those stimuli. Therefore, the study and identification of paracrine factors capable of improving Leydig cell viability *in vitro* might be informative.

Our ultimate objective was to isolate and culture Leydig cells capable of maintaining their physiological metabolic function and regulation *in vitro*. In view of the high rate of apoptotic cell death during the initial 3 h of incubation, it might be informative to study the effect of allowing freshly isolated Leydig cells to recover from the cell isolation procedures and severing from their ideal intratesticular environment. Cell recovery might be possible during an initial 24 h of incubation using ECM-coated culture plates and maximally supplemented culture media including, for example, serum, antioxidants and trophic factors. Thereafter, Leydig cells could be returned to a minimal requirement medium in order to control experimental parameters.

Finally, although for a long time the absence of mitochondrial changes was

considered as a hallmark of apoptosis, mitochondria appear today as the central executioner of physiological cell death. For example, mitochondria are now known to contribute to the production of cell-death-signaling ROI and host members of the Bcl-2-related protein family. They are also involved in the control of the activation of the cell death machinery by docking at their surface, via Bcl-2s, execution Apaf-1 and caspases. In steroidogenic cells, mitochondria also play a central role in steroid synthesis as the primary site of cholesterol processing. Future work should focus on the important role of mitochondria in apoptosis and steroidogenesis, addressing the connection between cell death signals, mitochondrial alterations and modulation in steroidogenic rate.

CONCLUSION

The biochemical mechanisms involved in the execution of cell death are poorly understood. Cells respond to aberrant combinations of physiological signals and subnecrotic chemicals or physical damages, generally via apoptosis, in order to minimize the risk of perpetuating or expanding mutated, aberrantly differentiated, or transformed cells.

The study presented here demonstrates Leydig cells' ability to undergo physiological cell death. While adult-type Leydig cells form a stable population with little turnover *in vivo*, they can rapidly undergo apoptosis once isolated from their intratesticular environment.

With regards to Leydig cells, their ability to undergo physiological cell death might be related to the dependence of the male reproductive system and more specifically germ cells on testosterone. If the extracellular environment is not adequate, Leydig cells would rather die than risk supporting germ cell development in sub-optimal conditions.

In primary cultures of rat Leydig cells, apoptosis occurs spontaneously *in vitro*. Although the stimuli triggering cell death are still unclear, the results presented here suggest that the cell isolation procedures and/or incubation conditions could disrupt cell homeostasis and trigger apoptosis in primary cultures of rat Leydig cells.

This study also provided insight on a variety of stimuli, including lack of cell-cell contact, gonadotropin depletion and ROI damage, which are potentially responsible for the induction of apoptosis in primary cultures of rat Leydig cells.

Expressions of the anti-apoptotic protein Bcl-2 and its pro-apoptotic homolog Bax were also studied, although with limited success. Indeed, no evidence of a regulatory function for either Bcl-2 or Bax could be observed in this cell model. However, since members of the Bcl-2 family of proteins have been shown to be differentially expressed in tissues, we suggest that other Bcl-2 homologs might be responsible for the regulation of apoptotic cell death in Leydig cells .

In order to improve Leydig cell viability and function *in vitro*, future research should focus on identifying the stimuli involved in the onset of apoptosis as well as the proteins responsible for its regulation. A better understanding of apoptotic cell death *in vitro* would allow improvement and standardization of primary cell cultures of Leydig cells and enable different laboratories to compare results with more objectivity.

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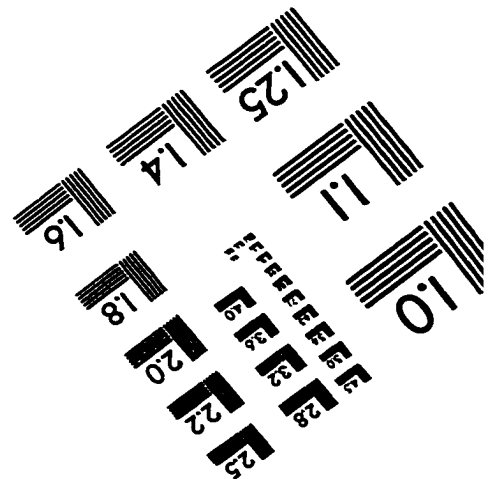
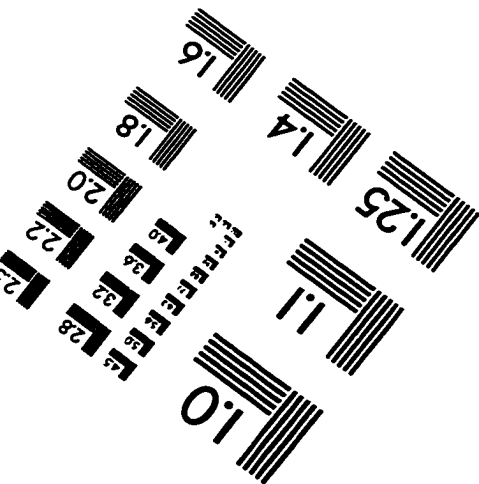
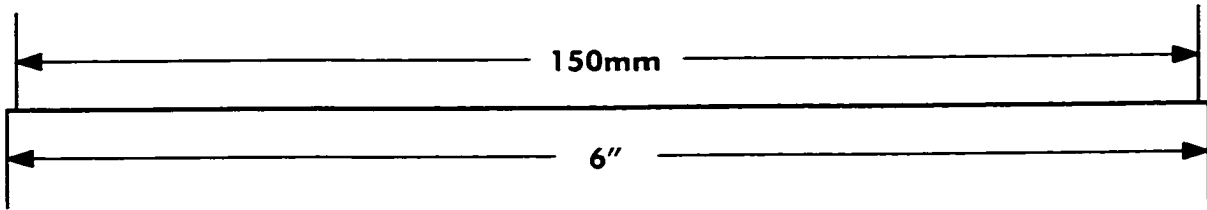
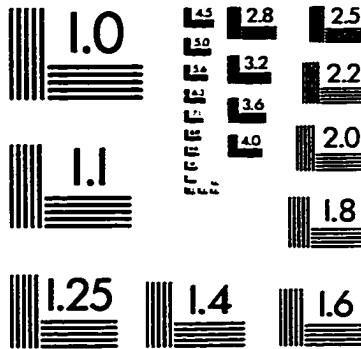
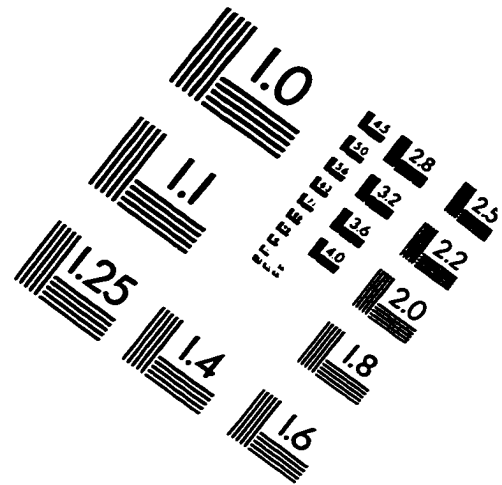
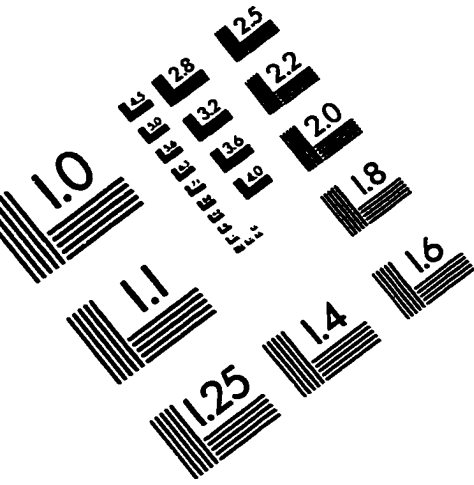
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IMAGE EVALUATION TEST TARGET (QA-3)



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