MODEL SYSTEMS TO STUDY BIOCHEMICAL FUNCTIONS OF RAT SERTOLI CELLS

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BIOCHEMISCHE AKTIVITEITEN VAN SERTOLI CELLEN VAN DE RAT IN VERSCHILLENDE KWEEKSYSTEMEN

PROEFSCHRIFT

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PROMOTIECOMMISSIE

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Dit proefschrift werd bewerkt in het instituut Biochemie II (Chemische Endocrinologie) van de Faculteit der Geneeskunde, Erasmus Universiteit Rotterdam.

CONTENTS

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ABBREVIATIONS AND TRIVIAL NAMES

ABP	androgen binding protein
ATP	adenosine 5'-triphosphate
B/I ratio	ratio inhibin bioactivity/immunoreactivity
BSA	bovine serum albumin
1C	amount of DNA present in a haploid gamete
cDNA	complementary deoxyribonucleic acid
DNA	deoxyribonucleic acid
dpm	disintegrations per minute
ECM	extracellular matrix
ED_{50}	dose required for half maximal stimulation
EGF	epidermal growth factor
FCS	fetal calf serum
FIRT	FSH, insulin, retinol, testosterone
FSH	follicle-stimulating hormone or follitropin
g	gravity
glc	gas-liquid chromatography
GSH	glutathione
HIC	α-hydroxyisocaproate
hsp	heat shock protein
IGF-I	insulin-like growth factor-I
kb	kilo base
(k)Da	(kilo) Dalton
KIC	α-ketoisocaproate
LDH-C_4	lactate dehydrogenase-C_4
LH	luteinizing hormone or lutropin
MEM	Eagle's minimum essential medium
mRNA	messenger ribonucleic acid
MW	molecular weight
PBS	phosphate-buffered saline
P-mod-S	peritubular cells modulate Sertoli cells
RBM	reconstituted basement membrane
RIA	radioimmunoassay
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
SSC	standard saline citrate

testosterone	4-androstene-17β-ol-3-one
TGF-(α or β)	transforming growth factor (α or β)
U	units
v/v	volume/volume

CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction

The mammalian testis is composed of two compartments, the seminiferous tubules and the vascularized interstitial tissue (Fig. 1.1). In the interstitium many different cell types are present, including the Leydig cells which are the source of androgens. Androgens are involved in the development of primary and secondary male sex characteristics and are important for spermatogenesis.

Spermatogenesis is a complex process through which primitive stem cells develop into spermatozoa, via the intermediate formation of spermatogonia, spermatocytes and spermatids (Fig. 1.1). This process takes place in the tubular compartment of the testis. The developing germ cells are enfolded by the somatic components of the seminiferous epithelium, the Sertoli cells (Fig. 1.1). The intimate contact between Sertoli cells and germ cells suggests that Sertoli cells are important for spermatogenesis. Furthermore, adjacent Sertoli cells are connected through tight junctional complexes, thus forming a Sertoli cell or blood-testis barrier (Fig. 1.2). This barrier divides the tubules in a basal compartment, containing spermatogonia and early spermatocytes, and an adluminal compartment, containing spermatocytes and spermatids. The barrier excludes a number of substances from entering the adluminal compartment. Selective passage of compounds through this barrier combined with active secretion of compounds produced by Sertoli cells, probably results in an optimal environment for the developing germ cells.

Initiation and maintenance of spermatogenesis is dependent on the hormones follicle-stimulating hormone (FSH or follitropin) and testosterone. Sertoli cells contain receptors for both hormones, but these are more or less completely absent in spermatogenic cells, and it is generally accepted that effects of FSH and testosterone are mediated via Sertoli cells. It is largely unknown how these two hormones control spermatogenesis, although it is most likely that this control involves growth and maturation of Sertoli cells and regulation of the synthesis of various Sertoli cell secretion

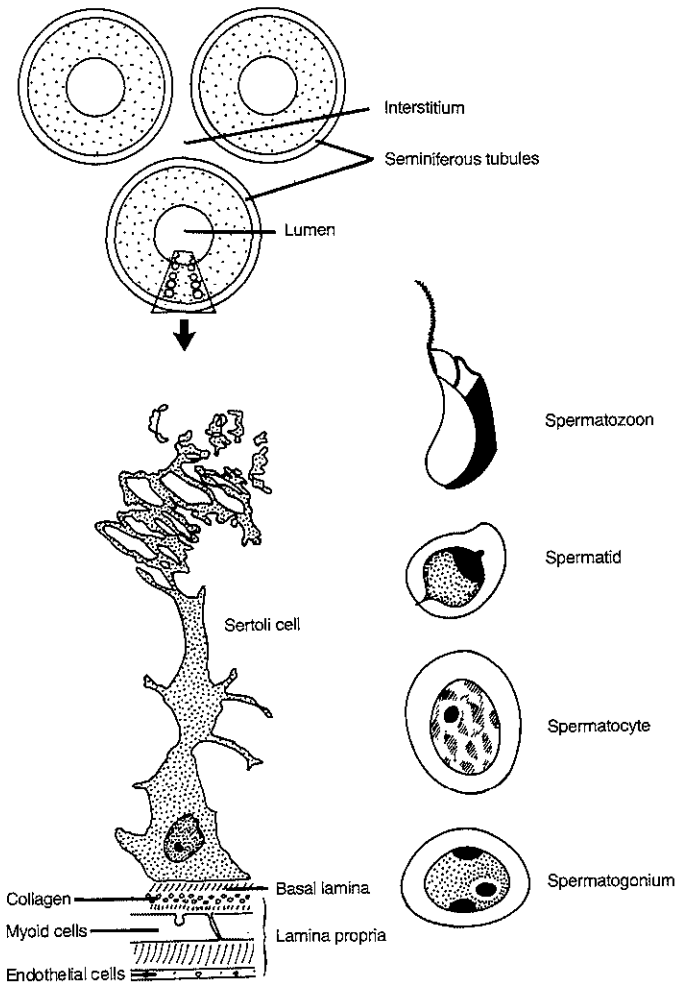
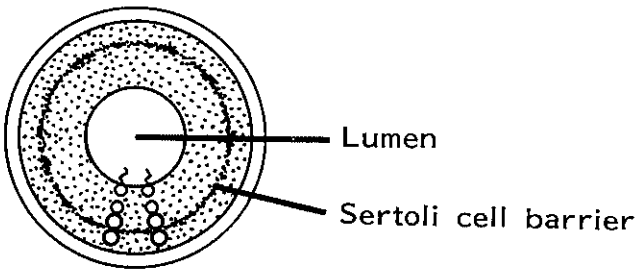


Figure 1.1

Schematic drawing of seminiferous tubules and the interstitium. A part of a seminiferous tubule is drawn in more detail. The germ cells at different steps of development are located in between Sertoli cells and are surrounded by Sertoli cell cytoplasm. The lining of the tubules is formed by the basal lamina and lamina propria which can be subdivided into a number of layers.



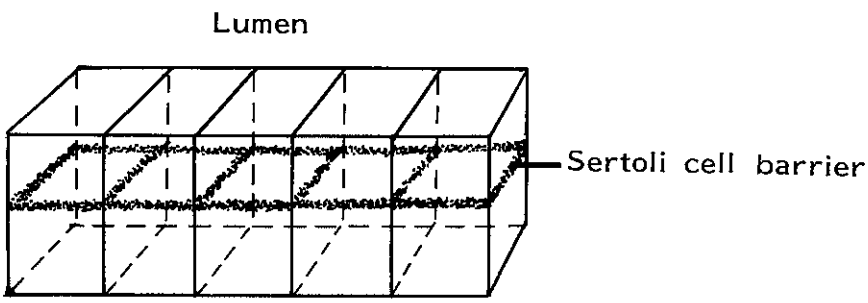


Figure 1.2

The Sertoli cell barrier is formed by tight junctions between neighbouring Sertoli cells. The tight junctions are located around each Sertoli cell. The seminiferous epithelium is thus divided in a basal and an adluminal compartment and substances from the interstitium are prevented to penetrate the adluminal compartment unless they pass the Sertoli cell cytoplasm.

products.

1.2 Aim and scope of this thesis

The aim of the work presented in this thesis was to try to improve our understanding of the hormonal regulation of Sertoli cell functions and the control of spermatogenesis. In this context, this thesis forms part of the research programme symbolized by Fig. 1.3.

The production and secretion of Sertoli cell products are subject to complex regulation (Fig. 1.4). Luteinizing hormone (LH or lutropin), a

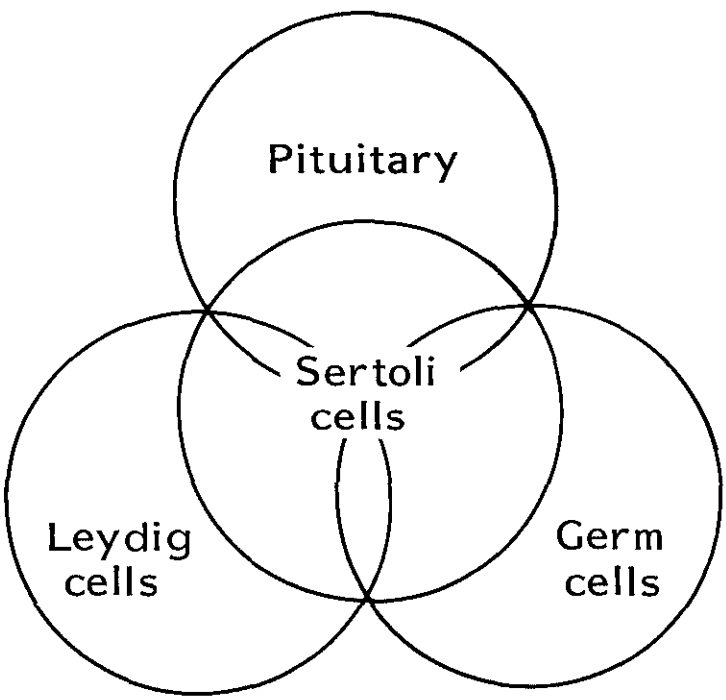


Figure 1.3
Symbol of a Dutch Foundation for Medical Research (Medigon) programme. Schematic presentation of the interactions between testicular cell types and between testis and the pituitary gland, which are the main topics of interest of the research programme of which this thesis forms a part of.

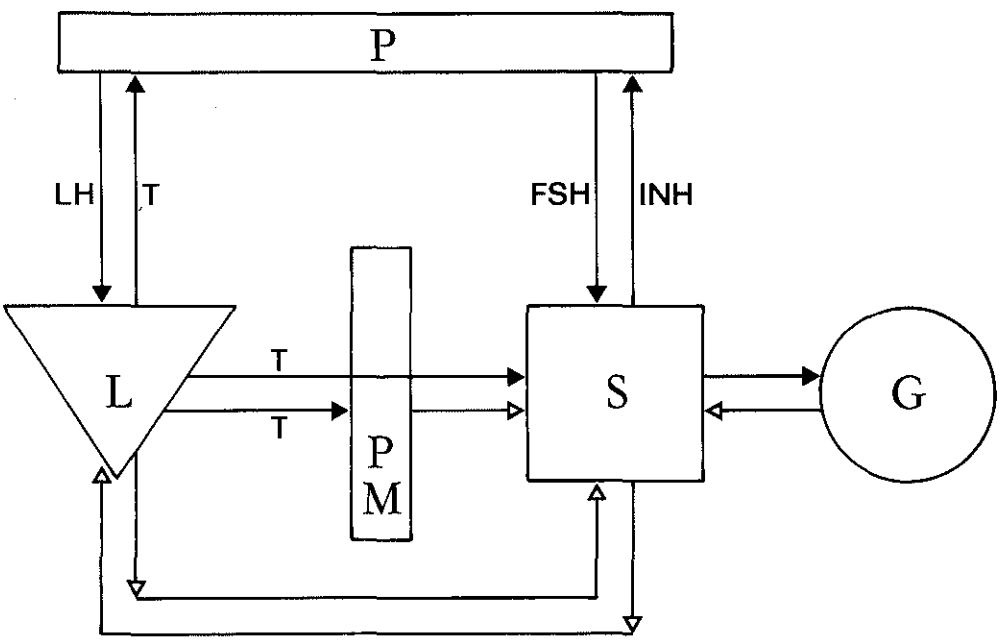


Figure 1.4

Schematic representation of possible interactions between different cell types in the testis.

Cells: G (germ cells), L (Leydig cells), PM (peritubular cells), S (Sertoli cells)

Factors: FSH, LH, INH (inhibin), T (testosterone), and unknown factors (⟶▷)

gonadotropin which like FSH is secreted by the pituitary gland, stimulates Leydig cells to produce androgens which are important for Sertoli cell function and spermatogenesis. In addition, FSH directly exerts multiple effects on Sertoli cells. One of these effects is the stimulation of the production of proteins such as androgen binding protein (ABP) and inhibin. The glycoprotein inhibin can inhibit the FSH release by the pituitary gland, thus possibly forming a feed-back system between testes and pituitary. Another important negative feed-back loop involves the inhibition of the release of gonadotropins by circulating testosterone. Many other hormones and growth factors may exert effects on Sertoli cells. For example, insulin and insulin-like growth factor I (IGF-I) receptors have been identified on rat Sertoli cells (Borland et al., 1984; Oonk and Grootegoed, 1987; 1988).

Although Sertoli cells are target cells for various hormones and growth factors, it cannot be excluded that a number of hormones and growth factors exert their effects indirectly, via other testicular cell types. Such indirect effects on the rate of synthesis of Sertoli cell products have been reported, and it is likely that some effects of testosterone on Sertoli cells can be mediated by peritubular cells (Skinner and Fritz, 1985a). Peritubular cells (mainly fibroblasts and myoid cells, but also endothelial cells) are cells which surround the seminiferous tubules. In cooperation with the Sertoli cells they produce a basement membrane (Tung and Fritz, 1980).

Multifarious actions of hormones and growth factors on different cell types in the testis make it difficult to study the hormonal regulation of Sertoli cell functions and the control of spermatogenesis in whole animals (in vivo).

In the experiments described in this thesis, the hormonal regulation of Sertoli cell functions, effects of Sertoli cell products on spermatogenic cells, and development of spermatids in the presence of Sertoli cells were studied using various in vitro incubation systems.

CHAPTER 2

SERTOLI CELLS AND SPERMATOGENESIS

Sertoli cells are named after Enrico Sertoli, the first author who described in 1865 these cells in the human testis and indicated their possible supporting and nutritive role for the developing germ cells.

Sertoli cells are a population of somatic cells which proliferate during the fetal and prepubertal period, but do not further divide after approximately 16 days of age in the rat (Steinberger and Steinberger, 1971; Orth, 1982). However, during the initiation of spermatogenesis, up to approximately sixty days of age in the rat, the Sertoli cells continue to grow in size as they mature and form ramifications, establishing their task as supporting and nutritive cells for the spermatogenic cells.

2.1 Spermatogenesis

The development of the male germ cells ("spermatogenesis") starts with stem cells, the undifferentiated spermatogonia. Spermatogenesis can be divided into three main phases. In the first phase, undifferentiated spermatogonia proliferate and give rise to differentiated spermatogonia and a new generation of undifferentiated spermatogonia. The differentiated spermatogonia give rise to spermatocytes. In the second phase, spermatocytes undergo meiotic divisions to form haploid spermatids. In the third phase, the spermatids differentiate into spermatozoa, the process of spermiogenesis (for a review see Clermont, 1972).

In more detail, spermatogonia give rise, in the rat, to differentiated type A_1 to A_4 spermatogonia, intermediate and type B spermatogonia, and to a new generation of undifferentiated type A spermatogonia, via a series of mitotic divisions. Type B spermatogonia, which are together with the other spermatogonia still located in the basal compartment of the tubules, develop via a mitotic division into preleptotene spermatocytes, which then enter meiosis. At the onset of meiosis, the DNA of the preleptotene spermatocytes is replicated resulting in primary spermatocytes with a 4C amount of DNA (1C represents the amount of DNA present in a haploid gamete). During the early prophase of meiosis, the spermatocytes are transported towards the tubular lumen, thus crossing the blood-testis barrier and entering the adluminal

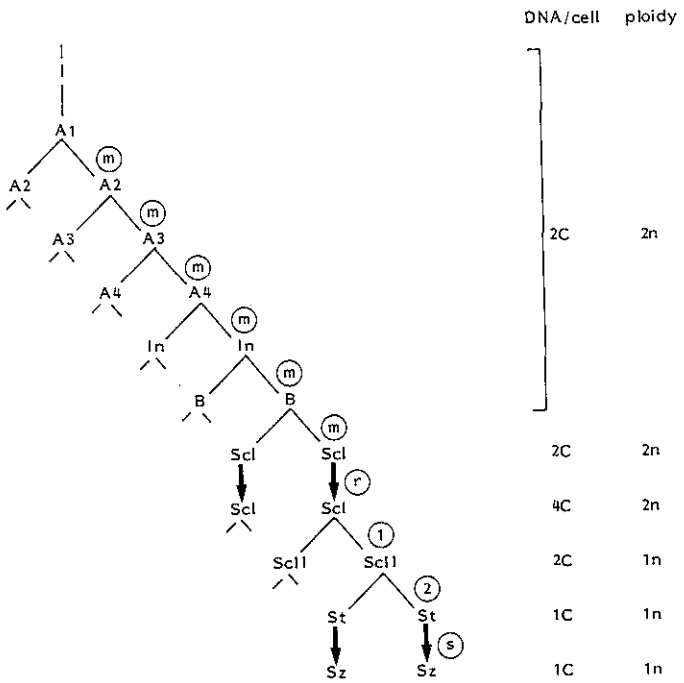


Figure 2.1

Schematic representation of spermatogenesis in the rat. Mitotic divisions, meiotic divisions, the amount of DNA per cell (1C represents the amount of DNA of a haploid gamete), and the ploidy of the cells (a single set of chromosomes per cell or haploid = 1n) are indicated.

A1-4 : spermatogonia A 1-4

B : spermatogonia B

ScI-II : primary and secondary spermatocytes

St : spermatids

Sz : spermatozoa

m : mitotic divisions

r : DNA replication, and meiotic prophase

1 and 2 : first and second meiotic division

s : spermiogenesis

compartment (Russell and Peterson, 1985). The leptotene spermatocytes develop via zygotene and pachytene stages into diplotene spermatocytes. During the first meiotic division the homologous chromosomes of the diplotene spermatocytes are separated, resulting in haploid secondary spermatocytes with a 2C amount of DNA. Without duplicating their DNA, the cells then undergo the second meiotic division, thus forming haploid spermatids with a 1C amount of DNA. Spermatids do not further divide but differentiate via Golgi-, cap-, acrosome- and maturation-phases into spermatozoa (Leblond and Clermont, 1952 a,b) (Fig. 2.1).

2.2 Cycle of the seminiferous epithelium

In a cross section through a tubule of a sexually mature animal, one or two generations of spermatogonia, spermatocytes, and spermatids can be observed in addition to the Sertoli cells. A generation is defined as a group of cells that are produced at approximately the same time and develop synchronously. The different germ cell types do not occur in random combinations, but certain developmental steps of spermatids are associated with defined generations of spermatocytes and spermatogonia. In rats, 14 of these associations, termed stages, have been described. The stages follow each other in time in a given sequence. A complete series of stages is termed a cycle of the seminiferous epithelium and lasts approximately 12 to 13 days in different strains of rats (12.9 days in the strain Sprague-Dawley) (Leblond and Clermont, 1952b; Dym and Clermont, 1970; Clermont, 1972; Parvinen, 1982). Complete spermatogenesis in rats lasts approximately 50 days (Clermont, 1972).

It is known that Sertoli cells and germ cells interact extensively, and it has been shown that Sertoli cell properties change in a quantitative way during the different stages of the cycle (for a review see Parvinen, 1982). Maximal binding of FSH to the Sertoli cells is observed at stage I, whereas there is a relatively low binding at stage VII. The secretion of a number of Sertoli cell proteins is also stage-specific. For example, plasminogen activator is secreted mainly in stages VII and VIII, and the secretion of a protein named Cyclic Protein-2 (CP-2) is maximal at stage VI, whereas it is secreted in negligible amounts at other stages. The secretion of ABP is high at stages VII to XI, with a maximum during stage VIII, but ABP is also produced in considerable amounts at the other stages of the cycle (Parvinen,

1982; Wright, 1988). The inhibin content in tubule segments and inhibin secretion into the medium was lowest at stages VII-VIII (Gonzales et al., 1989). The stage dependent rate of secretion of different Sertoli cell products may indicate a specific role of certain Sertoli cell proteins during the different steps of germ cell development and/or effects of germ cells on Sertoli cells.

2.3 Sertoli cell morphology

Using light microscopical techniques, Sertoli cells have been described as columnar cells extending from the basal lamina to the tubular lumen. The cells have a tree-like appearance. With the use of electron microscopical techniques, it was observed that the Sertoli cell morphology was very complex. Near their basis, adjacent Sertoli cells form tight junctional complexes (for a review see Russell and Peterson, 1985). These junctions participate in maintaining the Sertoli cell barrier or blood-testis barrier. At the adluminal side of this barrier, Sertoli cells have protrusions which surround the developing germ cells. Wong and Russell (1983) have used semiserial section techniques to investigate the three-dimensional structure of Sertoli cells, showing the tall and irregular columnar Sertoli cell morphology in much detail.

In addition to the stage-dependent secretion of a number of Sertoli cell products, cyclic and stage-dependent variations were observed in the ultrastructure of the Sertoli cells and it was suggested that these may reflect changes in the Sertoli cell activities which are necessary for the support of spermatogenesis (Kerr, 1988). This is discussed in more detail in chapter 7.

2.4 Sertoli cells in culture

It is generally accepted that with the present isolation procedures Sertoli cells *in vitro* have largely lost their typical configuration. Nevertheless, many different primary Sertoli cell cultures have been set up with different aims. In general, two main areas of interest can be distinguished.

The first considers the Sertoli cell itself, and concerns mainly the characterization of specific products, mechanisms of hormone action and the regulation of the secretion of Sertoli cell products. Usually simple

incubation systems are used which are easy to handle in quantities sufficient for biochemical analyses (see for example Steinberger et al., 1975; Oonk et al., 1985; appendix papers 1 and 2). For these experiments it is of importance that the Sertoli cell cultures are pure, or only minimally contaminated with other testicular cell types.

The second area of interest is to develop a culture system which more or less resembles certain aspects of the *in vivo* situation, either with or without germ cells. In order to achieve this, rather complicated culture systems have been developed, using total testis tissue, matrices, bicameral culture systems, intact tubules, tubular fragments, or cocultures of different cell types (Steinberger et al., 1964; Hadley et al., 1985; Toppari and Parvinen, 1985; Borland et al., 1986; Byers et al., 1986; Janecki and Steinberger, 1986; Kierszenbaum et al., 1986; appendix paper 4).

The ultimate aim of the investigators in both areas is to improve insight in the role of Sertoli cells and their products in spermatogenesis.

2.4.1 Sertoli cell-only cultures

A cell isolation method which involves treatment of testis tissue from immature rats with trypsin and collagenase yields cell aggregates which contain approximately 70% Sertoli cells (Tung et al., 1984). After incubation on plastic in chemically defined medium for three or more days this percentage is increased to approximately 90%, because most of the contaminating germ cells do not remain attached to the Sertoli cells. When serum is added to the culture medium, contaminating peritubular cells proliferate and can become the dominant cell type(s) (Tung et al., 1984). These peritubular cells initially grow underneath the Sertoli cells. A double collagenase treatment, followed by mechanical agitation, yielded Sertoli cell preparations which contained at least 94% Sertoli cells (Oonk et al., 1985). These cell preparations were routinely incubated for 48h in the presence of fetal calf serum (FCS) (Oonk et al., 1985), but it was not studied whether the contamination with peritubular cells increased during the incubation period.

Primary Sertoli cell cultures have proven useful in studies concerning the effects of hormones such as FSH, insulin, IGF-I, and androgens on the production and release of various Sertoli cell products. However, it was observed that contaminating peritubular cells, even a low number, can modify

the secretion of several Sertoli cell products. ABP and transferrin secretion by cultured Sertoli cells is enhanced by the presence of peritubular cells (Hutson and Stocco, 1981; Skinner and Fritz, 1985a). A positive correlation has been observed between the amount of secreted ABP and the number of peritubular cells which were added to the cultures (Hutson and Stocco, 1981). Furthermore, the decline of the rate of ABP production by Sertoli cells, which is observed during culture in hormone-free medium, can be partly prevented by coculture with peritubular cells (Skinner and Fritz, 1985b) (see also paragraph 7.2). Peritubular cells contain testosterone receptors (Verhoeven, 1980) and can respond to testosterone *in vivo* and *in vitro* (Bressler and Ross, 1972; Hovatta, 1972). Therefore, it is possible that androgens act directly on contaminating peritubular cells, thus causing indirectly the observed effects on Sertoli cells. Recently, a protein was isolated from peritubular cell-conditioned medium (Skinner et al., 1988). This protein stimulated ABP and transferrin production by Sertoli cells, and was therefore termed P-mod-S (P: peritubular cells; mod: modulate; S: Sertoli cells). The production of P-mod-S, and of possible other factors that modulate Sertoli cells, by peritubular cells is enhanced by testosterone (Skinner and Fritz, 1985a,b; Verhoeven and Cailleau, 1988b).

From the above it is concluded that, to investigate direct effects of androgens and probably also of other hormones on Sertoli cell function, it is essential to use cultures with a minimal number of contaminating peritubular cells. To achieve this, Tung et al. (1984) extended one of the conventional isolation procedures with a treatment using hyaluronidase. This resulted in a Sertoli cell preparation which contained less than 1% peritubular cells after 6 days of incubation in the absence or presence of serum (see also chapter 4 and appendix paper 2).

Sertoli cell function *in vitro* may be influenced also by contaminating germ cells. It has been shown that FSH-induced secretion of ABP was stimulated by direct contact between Sertoli cells and germ cells (Galdieri et al., 1984; Le Magueresse and Jégou, 1988 a and b) (see also paragraph 7.2). Since germ cells are very sensitive to changes in osmolarity, as compared with Sertoli cells, it is possible to apply an osmotic shock to selectively remove germ cells from the culture (Galdieri et al., 1981).

The response to FSH of the highly purified Sertoli cells, prepared with the extended isolation procedure which includes treatment with hyaluronidase and an osmotic shock, was comparable with that of the conventionally

prepared Sertoli cell cultures (chapter 4 and appendix paper 2). This implies that the highly purified Sertoli cells have preserved their FSH response, and could be very useful in studies on hormonal regulation of Sertoli cell function. Cocultures of Sertoli cells with a known amount of peritubular cells and/or germ cells can be used to investigate the direct effects of these cell types on Sertoli cells, and the indirect effects of hormones via the peritubular cells.

2.4.2 Sertoli cells on matrices

Sertoli cells which are cultured on glass or plastic do not maintain or restore their typical configuration as highly polarized cells, but form a monolayer of squamous cells (Fig. 2.2). The degree of flattening is dependent on the plating density of the cells. The Sertoli cells have lost their polarity and the cytoplasmic extensions which surround the developing germ cells in vivo have largely disappeared. In addition, many biochemical characteristics of the Sertoli cells may have changed (Borland et al., 1986). A lack of support, and the changed microenvironment, may cause the failure of germ cells associated with Sertoli cells to differentiate into spermatozoa in vitro. To try to improve Sertoli cell morphology in culture, the cells can be grown on a variety of matrices.

In tissues, cells produce the extracellular matrix (ECM) or basal lamina, a substrate upon which cells migrate, proliferate, and differentiate. Broadly outlined, ECMs are composed of different types of collagen, glycosaminoglycans, proteoglycans, and glycoproteins (Gospodarowicz et al., 1980). In the literature, a number of different matrices for the culture of cells has been described to improve cell morphology, growth, and differentiation.

Mammary epithelial cells are unable to maintain their differentiated state when cultured on plastic or on collagen gels which are attached to the growth area of culture wells (Emerman and Pitelka, 1977; Shannon and Pitelka, 1981). However, when the cells were grown on floating collagen membranes, maintenance and induction of differentiation was observed (Emerman and Pitelka, 1977; Shannon and Pitelka, 1981). In a 3-dimensional collagen lattice, cell proliferation and hormonal regulation of casein production occurred (Tonelli and Sorof, 1982; Haeuptle et al., 1983). This indicates that cells grown in a system which allows the cells to maintain

certain characteristics of the in vivo morphology, can maintain a more optimal response to hormonal signals. Collagen gels are easy to obtain. Usually, collagen fibers (Type-I collagen) from rat tails are prepared as described by Michalopoulos and Pitot (1975). Cells in or on collagen gels can be regained for further investigation by treatment of the gels with collagenase.

A more complex ECM can be obtained as follows: A plastic growth area can be coated with an ECM, by culturing corneal endothelial cells for a number of days on the plastic. Subsequently, the corneal cells are lysed and the ECM remains attached (Gospodarowicz et al., 1980). This corneal cell ECM can stimulate the proliferation of cells (Gospodarowicz et al., 1980), and can regulate the synthesis and deposition of collagen by corneal cells which are grown on their own ECM (Tseng et al., 1983).

Sertoli cells and peritubular cells in culture produce ECM components. It was observed by Skinner et al. (1985) that the components synthesized by Sertoli cells differed from those produced by the peritubular cells. Sertoli cells which are cultured in serum-free medium mainly release Type IV collagen and relatively low amounts of laminin, whereas peritubular cells release fibronectin, Type I collagen and Type IV collagen into the medium. Deposition of ECM components, from the monocultures, in the form of extracellular fibrils was not observed. However, when Sertoli cells were plated on a layer of peritubular cells, deposition of ECM components as fibrils occurred and structures were formed which histologically resembled cords (Tung and Fritz, 1980). The role of the ECM in these processes is unknown, but it is very well possible that the specific components produced by the co-cultured cells play a role in the formation and maintenance of Sertoli cell morphology. According to Tung and Fritz (1986), Sertoli cells are unable to retain a columnar shape during a number of days in culture when grown on one component of the ECM, such as Type-I collagen (rat tails). However, Borland et al. (1986) described that Sertoli cells, when cultured for a number of days on a floating Type-I collagen matrix, were taller than cells grown on plastic.

Tubule fragments from adult rats in culture produce an ECM with components which are also produced by Sertoli cell-peritubular cell co-cultures (Tung and Fritz, 1984; Skinner et al., 1985). Sertoli cells which are grown on this ECM retain a spherical, cuboidal or short columnar shape for up to 6 days in culture. The nuclei of Sertoli cells cultured on ECM are

spherical and located more apically as compared with the flat nuclei of cells cultured on plastic. The height of the Sertoli cells grown on ECM slowly decreased, and after 14 to 21 days the cells had become flattened with flattened nuclei. A cell structure which resembled the in vivo histology was evident for periods of 10-14 days after plating of Sertoli cells on ECM.

2.4.3 Bicameral culture systems

The culture media used in the systems described above may be too artificial and inadequate to allow survival and development of germ cells to a similar extent as compared with the in vivo situation. In vivo, the composition of the tubular fluid, or free-flow fluid, is mainly determined by Sertoli cells. This is indicated by the observation that there is a big difference between the composition of free-flow tubular fluid as compared with that of the testicular lymph which surrounds the tubules (for a review see Setchell and Waites, 1975). The testicular tubules end in the rete testis, so that rete testis fluid may also influence the composition of the free-flow fluid. This is concluded from the observation that the potassium concentration in newly secreted tubular fluid, the primary fluid, is higher as compared with the concentration in the free-flow fluid. It has been suggested that the primary fluid is diluted with rete testis fluid, which contains a lower concentration of potassium, by peristaltic actions of the tubules (Setchell and Waites, 1975).

Several authors have set up a new type of culture system to investigate the secretion of products by Sertoli cells at the adluminal side as well as at the basal side of the Sertoli cell barrier (Byers et al., 1986; Janecki and Steinberger, 1986; Handelsman et al., 1989). This system consists of a permeable membrane on which Sertoli cells are plated. After approximately 5 days of culture the Sertoli cells have formed tight junctions, thus decreasing the permeability of the monolayer for proteins. The membrane is fitted into a hollow cylinder and this chamber is put into a culture well. The medium in the well is in contact with the basal side of the Sertoli cells but not with the medium on top of the cells. Thus, a bicameral or two-compartment culture system is formed. The membrane used in this system can be plain (Janecki and Steinberger, 1986), or coated with ECM components or reconstituted basement membrane (RBM) (Byers et al., 1986; Janecki and

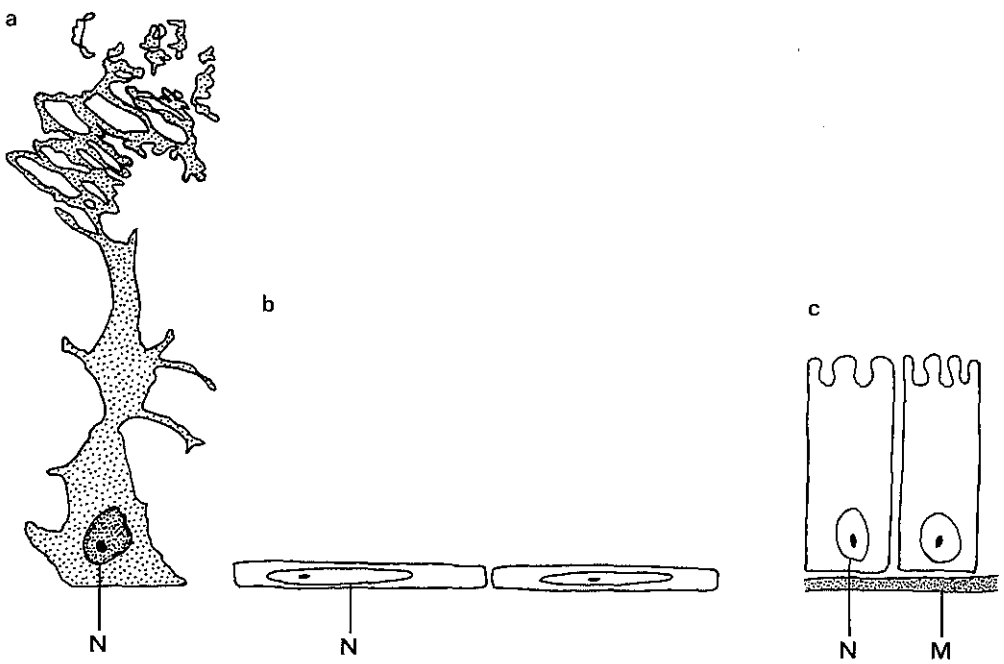


Figure 2.2

Schematic drawing of Sertoli cells in vivo (a) or during culture (b and c). Figure a represents a Sertoli cell in stage VIII of the cycle of the seminiferous epithelium (redrawn from Kerr, 1988), whereas Figure b and c represent Sertoli cells cultured on plastic and on a matrix, respectively.

N = Sertoli cell nucleus

M = matrix

Steinberger, 1987). In both systems the Sertoli cells showed a polarized columnar morphology, and the ABP and transferrin secretion were enhanced. The relative amounts secreted into the basal and the adluminal compartment (B/A ratio) were not changed by the presence of ECM components or RBM. There was no effect of serum or testosterone, as observed by Janecki and Steinberger (1987). However, Hadley et al. (1987) reported that the B/A ratio for ABP secretion increased when testosterone was added to the medium, whereas there was no effect of testosterone on transferrin secretion. It was suggested that these differences may be the result of incomplete saturation of the filters on which the Sertoli cells were cultured (Janecki and Steinberger, 1988). In co-cultures of Sertoli cells and peritubular cells, in which the peritubular cells were grown on the opposite side of the membrane and therefore not in direct contact with the Sertoli cells, high production rates of ABP and transferrin were observed (Janecki and Steinberger, 1987). The amount and direction of ABP and transferrin secretion were influenced by testosterone. It was suggested that soluble factors from the peritubular cells and/or cooperation between Sertoli cells and peritubular cells in the deposition of ECM are responsible for the observed effects. In contrast, however, Ueda et al. (1988) were unable to show effects of peritubular cells on ABP or transferrin secretion by Sertoli cells in a comparable bicameral culture system.

An extension of the two-compartment culture systems is the use of a superfusion system (Janecki et al., 1987). In such a system, the medium in the basal and adluminal compartment is changed with a certain flow-rate. The advantage of such a system is that hormones and other substances can be delivered to the cells in a continuous or pulsatile manner, while secretion products can be collected at any time during the culture (Kierszenbaum and Tres, 1987). Comparable results have been obtained with this system and the stationary cultures, although some Sertoli cell responses might be faster in the superfusion cultures (Janecki et al., 1987).

Several controversial results described above indicate that the bicameral culture system is very complex and needs to be improved. Further investigations should be performed to eliminate the most conspicuous experimental pitfalls. When standardized incubation conditions are obtained, the bicameral culture system could be very useful to investigate many aspects of the regulation of the secretion of Sertoli cell products and cell-cell interactions in the testis.

2.4.4 Spermatogenesis *in vitro*

Spermatocytes, isolated from the frog Xenopus laevis, are able to differentiate into spermatids under *in vitro* incubation conditions (Risley, 1983). However, isolated mammalian spermatocytes do not differentiate into spermatids but degenerate (Steinberger and Steinberger, 1966; Koulischer et al., 1982). Rat and hamster spermatocytes and spermatids are metabolically active after isolation, but maintain their biochemical integrity for at most 72h (Grootegoed et al., 1977; Jutte et al., 1981; Den Boer et al., 1989).

A number of attempts have been made to initiate or maintain spermatogenesis *in vitro* in the presence of Sertoli cells. Cultured testis fragments of Xenopus laevis were able to differentiate, and to produce motile and fertile sperm (Risley et al., 1987). However, in cultured immature rat testis tissue, with an intact tubular wall, differentiation of germ cells up to pachytene spermatocytes was observed, but no spermatids were formed, and there was a pronounced degeneration of germ cells (Steinberger et al., 1964; Steinberger and Steinberger, 1969). This degeneration may be caused by an insufficient transport of nutrients and waste products between medium and cells.

In Sertoli cell-germ cell co-cultures, some progress of developing germ cells was observed until late stages of the meiotic prophase (Tres and Kierszenbaum, 1983). However, in such an open system only a small number of germ cells remains attached to the Sertoli cells.

It has been suggested that the Sertoli cells on ECM possess characteristics which favour the survival of germ cells present in the cultures better as compared with Sertoli cells cultured on plastic surfaces (Tung and Fritz, 1984). Hadley et al. (1985) have used a reconstituted basement membrane (RBM) gel from a tumor as substrate. An advantage of this material is its porosity, so that the culture medium can reach the base of the Sertoli cells and also the germ cells which may be present near the base of the Sertoli cells. Sertoli cells plated on either of the matrices showed altered biochemical characteristics, including enhanced secretion of ABP and transferrin, compared with cells grown on plastic. However, whereas the cells cultured on ECM lost their polarity after approximately 2-3 weeks after plating, cells cultured on RBM retained their differentiated morphology for at least 8 weeks. Germ cells that were present at the start of the cultures (mainly spermatogonia), remained close to the basal region

of the Sertoli cells underneath the tight junctions which were formed during culture. However, further differentiation of the germ cells was not observed. Sertoli cells cultured within, rather than on top of, the RBM formed cords. In these cords, the apical surface of the cells was not in contact with the medium but located near the center of the cords. The spermatogonia were located in the periphery of the cords. Some of the spermatogonia differentiated up to pachytene spermatocytes and migrated into the central compartment, passing the tight junctions that were formed in between Sertoli cells. Thus, RBM seems to provide a model to study the early stages of spermatogenesis in vitro.

The culture system as described above may be too complex to be used to study biochemical aspects of spermatogenesis in vitro. Such biochemical studies require a simple and quantitatively defined culture system.

Toppari et al. (1986) have cultured tubular segments from adult rats, containing stages II-III of the cycle of the seminiferous epithelium. The number of pachytene spermatocytes and round spermatids was estimated using flow-cytometry and morphological techniques. It was observed that there was differentiation of a number of spermatocytes into spermatids (Parvinen et al., 1983; Toppari and Parvinen, 1985). However, the spermatids which were already present stopped differentiating when they approached the elongation and maturation phase (Toppari et al., 1986). Furthermore, a marked degeneration and loss of spermatocytes and spermatids was observed. Using a similar system, germ cell degeneration was most pronounced during the first two days of incubation (Parvinen et al., 1983).

In chapter 6 and appendix paper 4, a culture system consisting of open testicular tubules from immature rats, of which the testes are growing and developing rapidly (Mills et al., 1977), is described. Tubule fragments from 26-day-old rats were cultured, and the number of pachytene spermatocytes and round spermatids was estimated using DNA flow-cytometry. In addition, the activity of LDH-C_4, a germ cell specific isoenzyme, was estimated. It is suggested that the survival of spermatocytes and spermatids in this open incubation system is better as compared with closed tubules or testis tissue in culture (chapter 6 and appendix paper 4).

2.5 Conclusions

A number of in vitro incubation systems for Sertoli cells and spermatogenic

cells have been described above. Some systems are relatively easy to handle and may be useful to examine a number of biochemical processes. Other systems are very complicated to work with, but are suitable mainly for qualitative (e.g. histological) rather than for quantitative (e.g. biochemical) examination.

Most systems are based on the use of immature rats with an age from 10 up to 32 days, often 21 days. Trivial reasons for this include that Sertoli cells can be isolated much more easily from immature rat testis. In systems which include germ cells, the workload of the Sertoli cells is much lower in immature testes, because the number of advanced germ cells is relatively low. A more convincing reason for the use of immature rat testes is that, at this age, the testes are growing and developing rapidly and the first spermatocytes and spermatids start to develop (Mills et al., 1977; Ekwall et al., 1984). Moreover, the Sertoli cells, which are in the middle of their postnatal maturation phase, are sensitive to FSH and various other hormones including testosterone and IGF-I (Rich et al., 1983; Oonk and Grootegoed, 1988; Roberts and Griswold, 1989).

CHAPTER 3

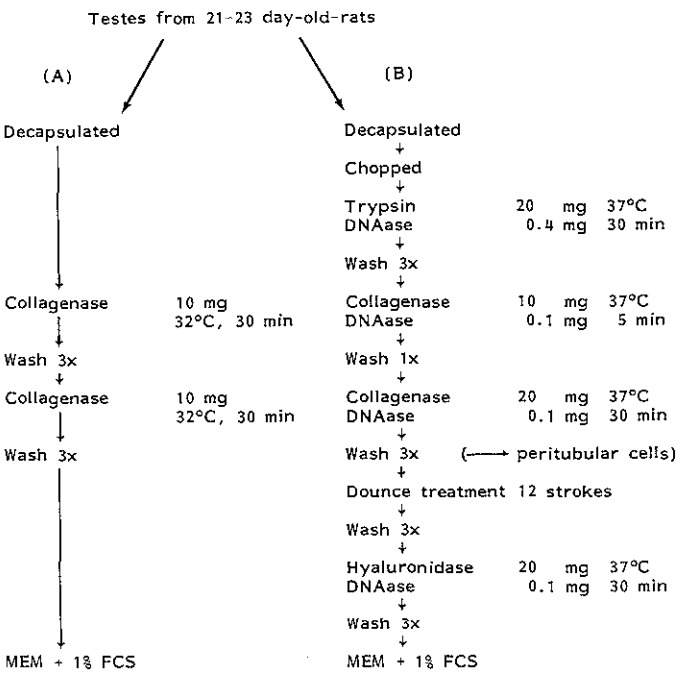
METHODS

In this chapter a number of methods are presented which are of special importance for the work which is discussed in this thesis, or which are not described in detail in the appendix papers.

In this thesis, *in vitro* incubation systems play an important role. The complex cell-cell interactions which occur *in vivo* necessitate the use of *in vitro* systems. However, it should be kept in mind that cell functions may change upon isolation and incubation and that observed effects may not represent the *in vivo* situation.

3.1 Isolation and incubation of Sertoli cells

In the studies described in this thesis two different Sertoli cell culture systems were used to investigate the regulation of inhibin production and secretion (chapter 4 and appendix papers 1 and 2). One of these Sertoli cell culture systems was of high purity and the other had some contamination with germ cells and peritubular cells. The highly purified Sertoli cells were used to exclude effects of other testicular cell types on the production of inhibin by Sertoli cells. The isolation procedures are described and compared in Fig. 3.1. The incubation protocol is outlined in Fig. 3.2. The short isolation procedure (A) was followed by an incubation period without hypo-osmotic shock treatment, and resulted in Sertoli cell preparations which were contaminated with peritubular cells and germ cells (Method A, in combination with a hypo-osmotic shock was used by Oonk et al., 1985). The extended isolation procedure (B), in combination with an incubation which included a hypo-osmotic shock, yielded Sertoli cell preparations with a contamination by peritubular cells and germ cells of less than 0.5% and 1-3%, respectively (Method B is a modification of published methods, for references see appendix paper 2). Using these highly purified Sertoli cell preparations, direct effects of hormones on the regulation of the production and secretion of Sertoli cell products can be examined, while indirect effects of hormones via other testicular cell types can be virtually excluded. However, a disadvantage of the latter isolation and incubation method (Method B) is the low recovery of Sertoli cells per testis, compared



The incubations were performed in 20 ml PBS in a 100 ml sylanized Erlenmeyer flask using a shaking waterbath (120 cycles/min).

Figure 3.1

Outline of isolation procedures for Sertoli cells. Sertoli cell preparations with some contamination with germ cells and peritubular cells, or very pure Sertoli cell preparations were obtained when methods A and B were used, respectively.

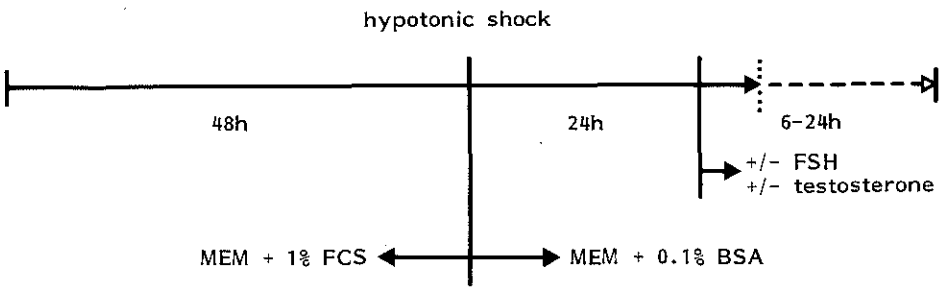


Figure 3.2
Schematic outline of the incubation procedure during culture of Sertoli cells.

with method A, due to large losses of cell material during several steps of the procedure.

3.2 Isolation and incubation of tubule fragments

Tubule fragments from 26-day-old rats were isolated and incubated as shown in Fig 3.3. The incubation medium which was used in the experiments was chosen after comparison of different media. The main difference with the other media was that this medium contained HEPES as an extra pH buffer (see chapter 6).

3.3 Isolation of spermatocytes and spermatids

Pachytene spermatocytes and round spermatids were isolated from 32-35-day-old rat testes by velocity sedimentation at unit gravity followed by Percoll

density gradient centrifugation, as follows: The testes were decapsulated and treated with collagenase (0.5 mg/ml) in phosphate-buffered saline supplemented with 10 mM DL-lactate (PBS-L), for 30 min at 32 °C, as described above and in appendix paper 4. The tubule fragments, thus obtained, were washed and subsequently treated with trypsin (0.5 mg/ml) in PBS-L, supplemented with 2.5 µg/ml DNAase. After 15 min, trypsin inhibitor (0.5 mg/ml) was added to stop the action of trypsin, and the cells were dispersed by pipetting the suspension (10 times in a plastic 10 ml pipet). Subsequently, the cell suspension was washed two times (12 x g) to remove Sertoli cell clumps, and the supernatant which contained spermatogenic cells was layered on a non-linear albumin gradient as described in detail by Grootegoed et al. (1977). Using such a gradient, cells with different sizes can be separated by sedimentation at unit gravity (the so-called Staput method) (Miller and Phillips, 1969). Pachytene spermatocytes sediment relatively fast, because they are larger than round spermatids. This method yielded preparations of pachytene spermatocytes and round spermatids with a purity of approximately 80% (results not shown). The spermatocyte and spermatid preparations were further purified using Percoll gradient centrifugation, which allows separation of cells with different densities, as described in detail by Jutte et al. (1985). The combination of both methods yielded preparations of pachytene spermatocytes and round spermatids with a purity of 90-95% or higher.

3.4 RNA isolation procedure

Testis tissue or cell cultures were frozen rapidly in liquid N_2 or in a solid CO_2/ethanol mixture, respectively, and stored at -80°C until assay.

Guanidinium thiocyanate (4.2 M, 0.7% 2-mercaptoethanol pH 7.0) (Chirgwin et al., 1979) was added, and the tissue or cells were homogenized immediately using an Ultra-Turrax (half speed for 1 min at room temperature) or a Vortex, respectively. The homogenates were centrifuged (1000 x g, 5 min, 4°C) to remove clumps and the supernatant was applied to polyallomer centrifuge tubes containing 3 ml 5.7 M CsCl, 0.1 M EDTA, (pH 7.0) (Glišin et al., 1974). The samples were centrifuged in a SW40 rotor (30,000 rpm for 16h) at room temperature. Subsequently, the supernatant was removed. The RNA pellet was dissolved in H_2O and extracted with a phenol-chloroform-isoamyl alcohol mixture (25:25:1). RNA was precipitated from the waterlayer at -20°C

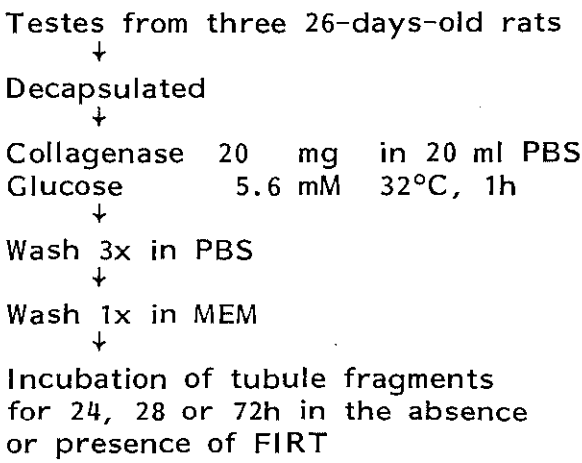


Figure 3.3

Schematic outline of the isolation and incubation of tubule fragments.

overnight, after addition of sodium acetate to a final concentration of 0.3 M and 2.2 volumes of ethanol.

In a number of experiments, total RNA fractions obtained as described above, were subjected to oligo-dT chromatography to separate poly(A)$^+$ RNA (polyadenylated mRNA) from rRNA (Aviv and Leder, 1972). The mRNA fraction was rechromatographed to increase the purity. The mRNA and rRNA fractions were ethanol precipitated as described above.

Total RNA, mRNA and rRNA samples were denatured in the presence of glyoxal and electrophoresed in 1% agarose (Thomas, 1980). The rRNA samples were included to establish the absence of RNA degradation and to act as a molecular weight marker. The RNA was transferred from the agarose to filters (Gene-Screen) by overnight diffusion, and subsequently the filters were baked for 2h at 80-100°C.

The rRNA lanes of the filters were fixed with acetic acid (5%) and stained with methylene blue in 0.05 mM sodium acetate buffer (pH 5.2). The other part of the filters was prehybridized for 6h at 42°C in a solution containing 44% (v/v) formamide, 5 x standard saline citrate (SSC; 1 x SSC contains 0.15 M NaCl and 0.015 M trisodium citrate), 10 mM sodium phosphate pH 6.5, 0.2% (v/v) polyvinyl pyrrolidone, 0.2% (v/v) Ficoll, 25 μg/ml salmon sperm DNA, and 5% v/v dextran sulphate. Subsequently, the blots were hybridized in the above solution, containing ^{32}P-oligo-labelled cDNA probes (Feinberg and Vogelstein, 1983). cDNA probes with specific activities of 0.3-1.6 x 10^9 dpm/μg were used.

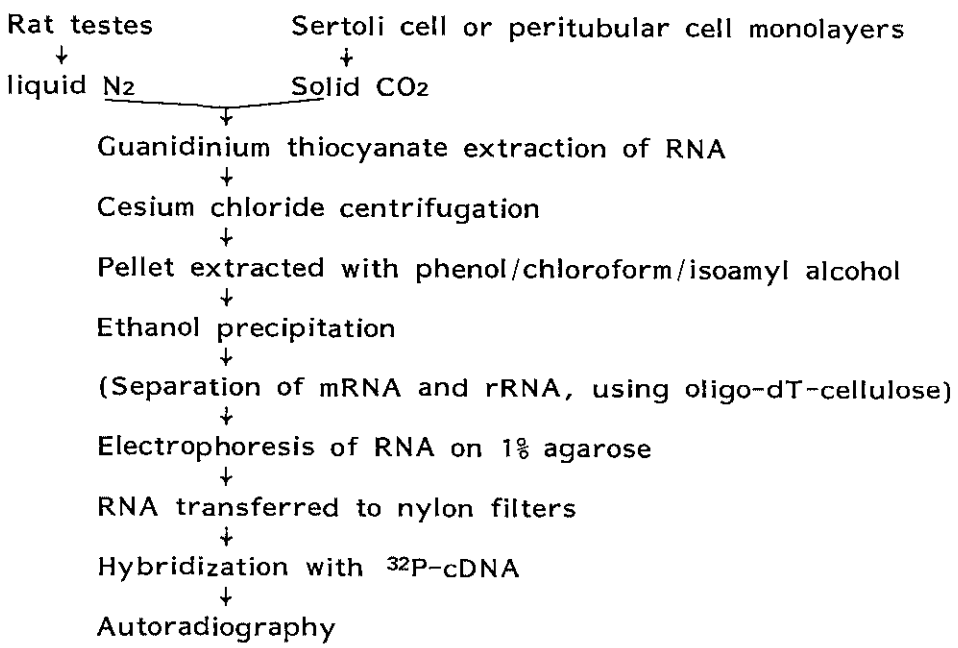


Figure 3.4

Schematic outline of the isolation and hybridization of testis and Sertoli cell RNA.

After hybridization, the filters were washed three to six times for 20 min at 50°C in 0.1 x SSC, containing 0.1% sodium dodecyl sulphate, airdried, and exposed to X-ray film (Hyperfilm-MP, Amersham) for 3-36h at -80°C, using intensifier screens. The above described Northern blotting method is outlined schematically in Figure 3.4.

The amounts of total RNA, mRNA, and rRNA were quantitated by A260 absorbance. The RNA samples were assessed for their purity by A260 nm/A280 nm and A260 nm/A230 nm absorbance ratios. A260/280 nm ratios of 1.5-1.8 and A260/230 nm ratios of 2.3-3.0 were routinely obtained. Purified calf liver RNA, used as quality control, gave corresponding ratios of 1.8 and 2.6, respectively. The staining pattern of 18S and 28S rRNA after electrophoresis was indicative for unexpected RNA degeneration during the extraction procedure.

The cDNA probes, used in the studies described in this thesis, were a 480 bp cDNA fragment corresponding to the α-subunit mRNA of bovine inhibin, a 360 bp cDNA fragment to the β_A-subunit mRNA of bovine inhibin, and a 920 bp cDNA fragment corresponding to the β_B-subunit of human inhibin. A hamster actin cDNA probe was used as a control, to estimate the relative abundance of mRNA applied to the different lanes of the gels (Dodemont et al., 1982).

CHAPTER 4

REGULATION OF PROTEIN PRODUCTION BY SERTOLI CELLS

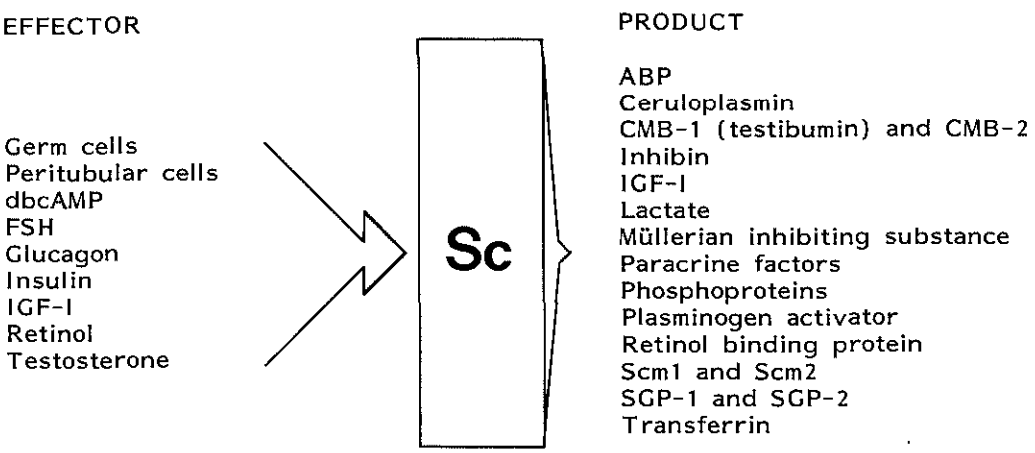
Introduction

Sertoli cells produce a large variety of compounds (Table 4.1). Some of these secretion products are relatively well known, and the regulation of their production has been studied by many investigators (for example ABP, transferrin, inhibin, lactate). Other secretion products have been detected only recently by using HPLC, gel electrophoresis, bioassays and other discriminating methods [for example Scm 1, Scm 2, CMB-1 or testibumin, CMB-2, sulphated glycoprotein-1 and -2 (SGP-1 and SGP-2, the latter also called clusterin), and unidentified factors which influence Leydig cell function] (DePhilip and Kierszenbaum, 1982; Cheng and Bardin, 1986; Lee et al., 1986b; Hugly et al., 1988; Papadopoulos et al., 1987, respectively). For most of the above-mentioned Sertoli cell products their function in the testis is not clear.

The production and secretion of many Sertoli cell products can be regulated by a number of hormonal factors including FSH and testosterone. This regulation may take place at different levels of the production process, in particular gene transcription, translation, and post-transcriptional modifications. Therefore, to be able to describe the hormonal regulation of a gene product, it is important to study all or as many as possible aspects of this process. The availabitily of cDNA probes, a radioimmunoassay, and a bioassay made it possible for us to investigate the hormonal regulation of inhibin production at the level of mRNA expression, protein production, and the secretion of biologically active inhibin. This could give us a base for further investigations of the regulation of other Sertoli cell products.

To exclude effects of other testicular cell types on inhibin production, two different methods to obtain Sertoli cell preparations were used yielding preparations containing either low or very low amounts of contaminating germ cells and peritubular cells. The results of these studies are discussed in this chapter and appendix paper 1 and 2.

TABLE 4.1 Products of Sertoli cells and factors which may influence their secretion



4.1 Sertoli cell mRNA expression

The isolation of cDNA probes corresponding to a number of Sertoli cell (secretion) products has made it possible to develop sensitive and specific methods to investigate the expression of genes encoding these products. Until now, the cDNA probes which have been isolated include the probes corresponding to ABP, transferrin, SGP-1 , SGP-2, cellular retinol binding protein, and inhibin. Using the isolated cDNA probes, a large number of investigators is working on the hormonal regulation of gene expression in Sertoli cells (See for example Griswold et al., 1986; Eskild et al., 1988; Hugly et al., 1988; Joseph et al., 1988; Meunier et al., 1988; Reventos et

al.,1988; Verhoeven and Cailleau., 1988a; appendix papers 1 and 2).

4.2 Regulation of ABP and transferrin production

Much attention has been paid to the secretion of ABP by Sertoli cells. ABP was one of the first Sertoli cell products which has been identified and many of the early investigations on Sertoli cell secretion products focussed on this compound.

It is generally accepted that both FSH and testosterone stimulate ABP secretion and mRNA expression in vivo. There is a good correlation between ABP mRNA expression and ABP secretion. In Sertoli cell cultures, however, FSH is the main regulator of ABP secretion and mRNA expression. The respons to testosterone in cultured Sertoli cells with respect to ABP production and mRNA expression was small or absent (Perez-Infante et al., 1986; Joseph et al., 1988). These results indicate that mRNA expression and protein secretion in vitro may be influenced by other factors as compared with the in vivo situation.

Transferrin is another major Sertoli cell secretion product. Transferrin secretion is stimulated by FSH, insulin and retinol (Skinner and Griswold, 1982). Perez-Infante et al. (1986) and Huggenvik et al. (1987) reported a small stimulatory effect of testosterone. Transferrin secretion by cultured Sertoli cells is stimulated most optimally by a combination of FSH, insulin, retinol and testosterone (FIRT) (Skinner and Griswold, 1982).

Transferrin mRNA expression is also stimulated by the above mentioned factors, and most by FIRT (Griswold et al., 1986; Huggenvik et al., 1987). This stimulatory effect on mRNA expression is quantitatively very similar to the stimulation of transferrin protein secretion (Griswold et al., 1986; Huggenvik et al., 1987).

4.3 Inhibin

Inhibin is a member of the TGF-β family of growth factors which includes also another Sertoli cell product, Müllerian inhibiting susbtance (MIS), with a known biological function (Cate et al., 1986). The MIS protein causes regression of the Müllerian ducts during the development of the male reproductive tract.

Inhibin consists of two dissimilar subunits, termed α and β. There are

two different forms of the β-subunit, β_A and β_B, thus forming two different inhibins. The two β-subunits show 70% homology in amino acid secquence. The α, β_A, and β_B-subunits are the products of separate genes.

In addition to the $\alpha\beta$-heterodimers, $\beta\beta$-homodimers, termed activins, have been identified which have a stimulatory effect on FSH release by pituitary cells (Ling et al., 1986; Vale et al., 1986). The activins are also members of the TGF-β family of growth factors (Massagué, 1987) (Fig. 4.1).

TGF-β consists of two subunits and it shows similarities with the β-chain

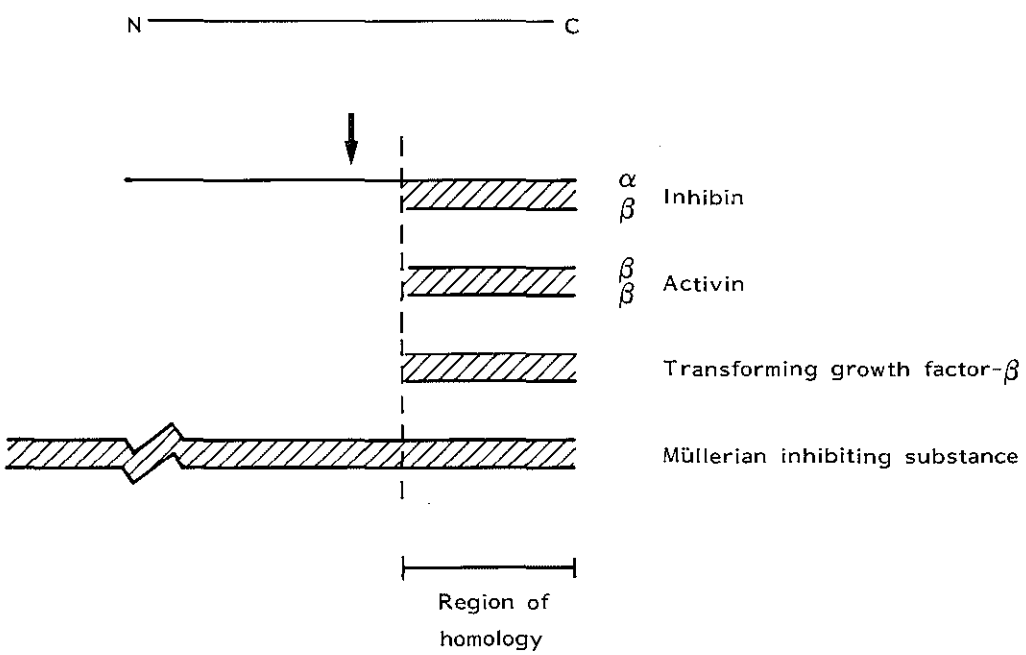


Figure 4.1

Schematic drawing of some members of the TGF-β family. The β-chains of the members of the TGF-β family show pronounced homology.

of inhibin. The action of activin on pituitary cells is mimicked by TGF-β (Massagué, 1987).

4.4 Estimation of inhibin

Until recently, methods to estimate inhibin activity, also termed inhibin-like activity, relied on the effect of inhibin on the pituitary gland. In other words, the estimation of inhibin bioactivity is based on its known effect which, however, does not imply that other bioactivities may not be prevalent. Currently, there are three main methods to study inhibin production. The inhibin *in vitro* bioassay system is used by many authors. This bioassay is based on the inhibition of FSH-release by pituitary cells in culture after addition of an inhibin containing sample. The degree of suppression is taken as a measure for the biological activity of inhibin. One major problem is that the bioassay is sensitive to the opposing effects of inhibin and activin. Hence, the presence of inhibin may be partly masked by activin, and *vice versa*. Furthermore, most *in vitro* bioassays are not sensitive enough to detect small amounts of inhibin, which are for example present in normal serum (for a review see de Jong, 1988).

To circumvent the above-mentioned problems, radioimmunoassays (RIAs) are being developed, which show specificity for inhibin and are much more sensitive. Antibodies raised against fragments of the α-chain (Bicsak et al., 1986; 1987) or against bovine 31 kDa inhibin (McLachlan et al., 1986; 1987; Robertson et al., 1988) have been used. In both cases it is essential to exclude cross-reactivity with single α- or β-chains. In our experiments, the latter antibody was used in the RIAs. However, it should be kept in mind that proteins which show immunoreactivity are not necessarily biologically active (see also below).

The third method to study inhibin production involves regulation at the level of transcription of the genes and mRNA expression, using cDNA probes corresponding to the different subunits (Mason et al., 1985; Forage et al., 1986; Esch et al., 1987).

From the results presented in this chapter and appendix papers 1 and 2 it is concluded that there is a correlation between the levels of inhibin α-subunit mRNA expression and the production of immunoreactive inhibin. However, it was observed that the ratio between inhibin bioactivity and immunoreactivity was not always the same (Risbridger et al., 1988; appendix

paper 1). It was suggested that larger forms of inhibin (58kDa) which have been detected in follicular fluid (Esch et al., 1987; Leversha et al., 1987; Robertson et al., 1985) may be produced by Sertoli cells and detected in the RIA. These and other forms of inhibin may have intrinsically different bioactivities. The levels of the different forms of inhibin may increase following FSH stimulation. However, the only form of inhibin which was detected by Grootenhuis et al. (1989) in total testis tissue form 22-day-old rats or in medium which was secreted by cultured Sertoli cells was a 30kDa protein. Single subunits which are obtained by reduction and alkylation of the heterodimeric inhibin do not cross-react in the RIA that was used in the present experiments. It is not certain, however, whether this treatment did not influence the immunoreactivity of the subunits, and whether untreated subunits do not cross-react in the RIA.
As yet, there are no RIAs available to estimate free α-subunits, β-subunits and activin.

4.5 Regulation of inhibin production

Factors which regulate the synthesis and release of inhibin by Sertoli cells have been studied by a number of investigators (Table 4.2). However, some of the studies are contradictory and only the most recent studies include information on regulation of the level of inhibin mRNAs.

In the present experiments, RNA was isolated from total testis tissue

TABLE 4.2 Effects of hormones on immunoreactive (I) or bioactive (B) inhibin secretion by cultured Sertoli cells

REFERENCES	EFFECTOR				
	FSH	T	DHT	A	E_2
Steinberger, 1981 (B)	+	+			
Le Gac & de Kretser, 1982 (B)	+				
Verhoeven & Franchimont, 1983 (B)	=	+	+		
Ultee-van Gessel et al., 1986 (B)	=/+	-			
Bicsak et al., 1987 (I)	+		=		=
Morris et al., 1988 (I)	+	=/-		-	
Appendix paper 2 (I)	+	=			

\+ stimulatory effect
\- inhibitory effect
= no effect

from 21-23-day-old rats as described in chapter 3.4, and hybridized with cDNA probes corresponding to the inhibin α- and β_B-subunit mRNAs. It was observed that there was specific hybridization with one α-subunit mRNA species of 1.6 kb and two β_B-subunit mRNAs of 4.2 kb and 3.5 kb (appendix paper 1). The 4.2 mRNA species was also detected when poly(A)$^+$ RNA from total testes was used (appendix paper 1). This confirms that the 4.2 kb band represents a mRNA species and does not reflect cross-hybridization with 28S rRNA.

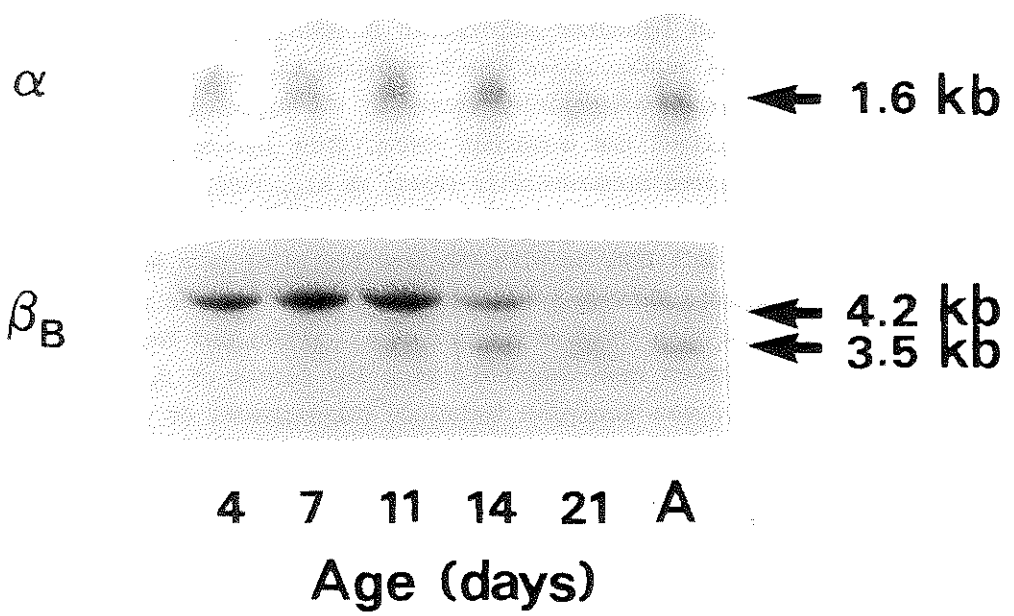


Figure 4.2

Northern blot analysis of RNA from testis tissue of rats of different ages. RNA was isolated from rats of 4, 7, 11, 14 and 21 days of age and from adult rats (A) and electrophoresed. The blots were hybridized with cDNA probes corresponding to the α- and the β_B-subunit of inhibin.

Total RNA was also isolated from testes of rats of various ages. The expression of the 1.6 kb α-subunit mRNA per total testes RNA remained constant during testis development (Fig. 4.2). This indicates that the α-subunit mRNA expression in Sertoli cells from older rats is higher as compared with the expression in younger rats, when the data would have been expressed per Sertoli cell, because total testis RNA from older rats contains a larger proportion of germ cell RNA.

Interestingly, the ratio between the amounts of the two β_B-mRNA species changed markedly with age (Fig. 4.2). The reason for this is unknown. The presence of two β_B-subunit mRNAs may be explained by alternative splicing events and/or different polyadenylation sites (Setzer et al., 1980; Tosi et al., 1981). This could lead to different control at the level of RNA stability and translation. Transcription and translation inhibitors had different effects on the expression of the three mRNAs in cultured Sertoli cells. The transcription inhibitor actinomycin D stabilized the α-subunit mRNA, but decreased the expression of the 3.5 kb and the 4.2 kb β_B mRNAs. The translation inhibitor cycloheximide increased the expression of the α and β mRNAs and stabilized the α-subunit mRNAs. This indicates that the subunit mRNAs are regulated differentially (Klaij et al., 1989).

Using a bovine β_A-subunit cDNA probe, no specific hybridization was observed with Sertoli cell RNA, or with RNA from testes of rats of various ages (results not shown). This is in agreement with observations from Esch et al. (1987), but not with those of Meunier et al. (1988) and Feng et al. (1989). The latter authors have detected very low levels of β_A mRNA in testes by the use of a more sensitive technique (S1-nuclease) or very long exposure time of the autoradiogram (7 days).

The effects of FSH and testosterone on inhibin production and secretion by Sertoli cells were investigated using cultures of highly purified Sertoli cells, this in order to exclude effects of other testicular cells. The isolation and incubation method used is described in detail in chapter 3. This method resulted in a Sertoli cell preparation with less than 0.5% peritubular cells and 1-3% germ cells. The responsiveness of the highly purified Sertoli cell cultures to FSH was comparable to that of less pure Sertoli cell cultures (not shown).

In the highly purified Sertoli cell cultures, the expression of inhibin α-subunit mRNA was enhanced by incubation with FSH for 24h, but not by testosterone (appendix paper 2). Addition of FSH to the culture medium

resulted already within 6h in a markedly increased expression of the α-subunit mRNA (not shown). There were no effects of FSH or testosterone on the expression of the β_B-subunit mRNAs (appendix paper 2).

FSH stimulated the amounts of immunoreactive inhibin, present in or secreted by the Sertoli cells, in a dose dependent manner with an ED_{50} of 5-50 ng/ml and a maximally stimulating dose of 500 ng/ml (Fig. 4.3). Incubation with FSH (500 ng/ml) firstly increased the amounts of intracellular inhibin, until a plateau of 100-125 units (U)/mg protein was reached after approximately 6-8h. During the first 6h, the amount of secreted inhibin was very low, but the rate of secretion became maximal after 6-8h of incubation in the presence of FSH (appendix paper 2).

Addition of testosterone to the medium, either alone or in the presence of FSH, did not influence the amounts of immunoreactive inhibin produced by the Sertoli cells (appendix paper 2). This indicates that, under the present conditions, there are no direct effects of testosterone on cultured Sertoli cells with respect to inhibin mRNA expression and inhibin protein synthesis.

It cannot be excluded that peritubular cells are needed to evoke a testosterone effect with respect to inhibin synthesis. Incubation of mixed Sertoli cell/peritubular cell cultures with different doses of FSH resulted in a dose-dependent increase of the inhibin α-subunit mRNA expression (appendix paper 1). As for the highly purified Sertoli cells, there was no effect of FSH on the expression of β_B-subunit mRNAs (appendix paper 1), and of testosterone on α- or β_B-subunit mRNA expression (not shown). Effects of testosterone on immunoreactive inhibin production were not estimated under these conditions.

In all experiments, using both Sertoli cell preparations, there was also production of *in vitro* bioactive inhibin. Bioactive inhibin levels in the culture medium were enhanced by FSH (appendix paper 1), in a dose-dependent manner (Fig.4.3). When doses of FSH higher than 5 ng/ml were used, the B/I ratio decreased approximately two-fold. This has also been observed by Risbridger et al. (1988). The possible explanations of this change have been described above (paragraph 4.4).

The measurement of inhibin in the samples containing high levels of testosterone resulted in a significant underestimation of inhibin bioactivity, due to the known stimulatory effects of testosterone on FSH secretion by pituitary cells (Campen and Vale, 1988). Removal of testosterone by charcoal treatment or repeated methanol extraction of the

lyophilised samples at -20°C, while successfully removing testosterone, also resulted in significant losses of inhibin bio- and immunoreactivity. The effects of testosterone on the secretion of inhibin bioactivity by Sertoli cells, therefore, have not been assessed.

It was observed by Iusem et al. (1984) that androgens can stimulate the glycosylation of glycoproteins in the epididymis. These authors suggested that this modulation takes place during early N-glycosylation at the level of dolichol nucleotide sugar transferases. In addition, it has been shown that deglycosylation of the α- or the β-subunit of glycoprotein hormones had differential effects on the biological activity of the protein (Sairam and Bhargavi, 1985). We cannot exclude that, in the present experiments, testosterone may have affected the glycosylation of the inhibin protein and hence its bioactivity.

The results of this paragraph are sumarized schematically in Fig. 4.4.

4.6 The possible role of the TGF-β family in the testis

Apart from a possible role in the brain-testis feed-back systems (Ultee-van Gessel et al., 1985; Culler and Negro-Vilar, 1988), an important role of testicular inhibin might involve paracrine actions in the testis. Inhibins and activins may modulate Leydig cell steroidogenesis (Hsueh et al., 1987) and spermatogonial divisions (van Dissel-Emiliani et al., 1988). An action of inhibin on germ cells is supported by observations from Spaliviero et al. (1988) and Handelsman et al. (1989), that Sertoli cells in a bicameral culture system mainly secrete inhibin from the apical side into the upper chamber. Hence, inhibin could act also on the germ cells in the adluminal compartment. A portion of the produced inhibin eventually is released into the circulation, via the rete testis (Maddocks and Sharpe, 1989). Data from Robertson et al. (1988) showed that inhibin serum levels decreased after gonadectomy of adult male rats.

Inhibin and activin may have an immunoregulatory role in the testis and in other tissues where inhibin and activin are produced. It has been observed that inhibin stimulates [^{3}H]thymidine incorporation by thymocytes in the presence of phytohaemagglutinin (PHA), whereas this was inhibited by activin and TGF-β (Hedger et al., 1989). Inhibin and activin could not affect thymocytes which had been stimulated with maximal doses of concanavalin A (ConA), while these thymocytes were inhibited by TGF-β (Hedger et al.,

1989). PHA, but not ConA, responsiveness appears to be restricted to differentating thymocytes, and it has been suggested that inhibin and activin may mainly act upon mature thymocytes, while TGF-β can act on both mature and immature thymocytes (Hedger et al., 1989). In addition, activin and TGF-β can stimulate the proliferation of 3T3 fibroblasts, whereas inhibin was ineffective (Hedger et al., 1989).

TGF-β gene expression has been detected in rat Sertoli cells and

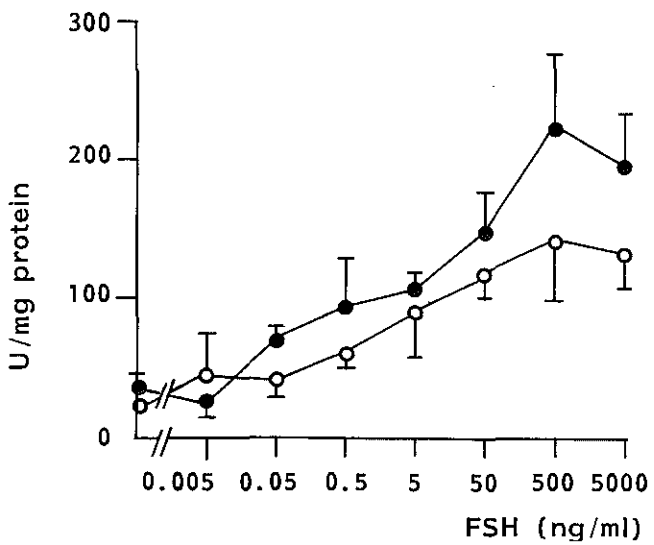


Figure 4.3

Dose-response curves of the effect of FSH on immunoreactive (●—●) or bioactive (○—○) inhibin secretion. After a 3-day preincubation period the cells were incubated for 24h with different doses of FSH. The data represent the mean ± s.d. of triplicate incubations.

peritubular cells (Skinner and Moses, 1989). These authors have not observed effects of exogenous TGF-β on the growth of Sertoli cells and peritubular cells of 20-day-old rats and on the secretion of transferrin by Sertoli cells. However, peritubular cell morphology and migration was changed after addition of TGF-β. It was suggested that TGF-β might play a role in the maintenance of peritubular cell functions (Skinner and Moses, 1989). The role of TGF-β during early testis development, in rats younger than 20 days of age, and possible effects of TGF-β on germ cells remain to be investigated. In the absence of contra-evidence, a role of TGF-β in germ cell development cannot be excluded.

TGF-β can act as a stimulator of cell growth, but also as an inhibitor (Massagué, 1987). The action of TGF-β depends on the cell type and on the presence of interacting growth factors (Sporn and Roberts, 1985). With most of the epidermal growth factor (EGF)-responsive epithelial cell types, it has been found that TGF-β inhibits the EGF-stimulated proliferation. This was also observed in granulosa cell cultures (Skinner et al., 1987). On the other hand, TGF-β stimulated the FSH-induced aromatase activity (Hutchinson et al., 1987; Adashi et al., 1989), and EGF receptor formation on granulosa cells (Feng et al., 1986). The number of LH receptors on granulosa cells was stimulated (in the presence of low doses of FSH) or inhibited (in the presence of high doses of FSH) by TGF-β (Knecht et al., 1987). From this it is concluded that TGF-β plays a role in granulosa cell growth and differentiation. In view of some similarities between granulosa cells and Sertoli cells, this may imply that Sertoli cells are also target cells for TGF-β. The observation that TGF-β has effects on cultured granulosa cells may indicate that there are also effects of inhibin and activin on cultured gonadal cells. It is not clear, however, to what extent inhibins and activins could be important for regulating growth and differentiation of gonadal cells *in vivo*.

4.7 Conclusions

It is concluded that there is a correlation between inhibin α-subunit mRNA expression and immunoreactive inhibin levels in Sertoli cells under different incubation conditions. Discrepancies between the secretion of immunoreactive inhibin and bioactive inhibin, however, require further investigation.

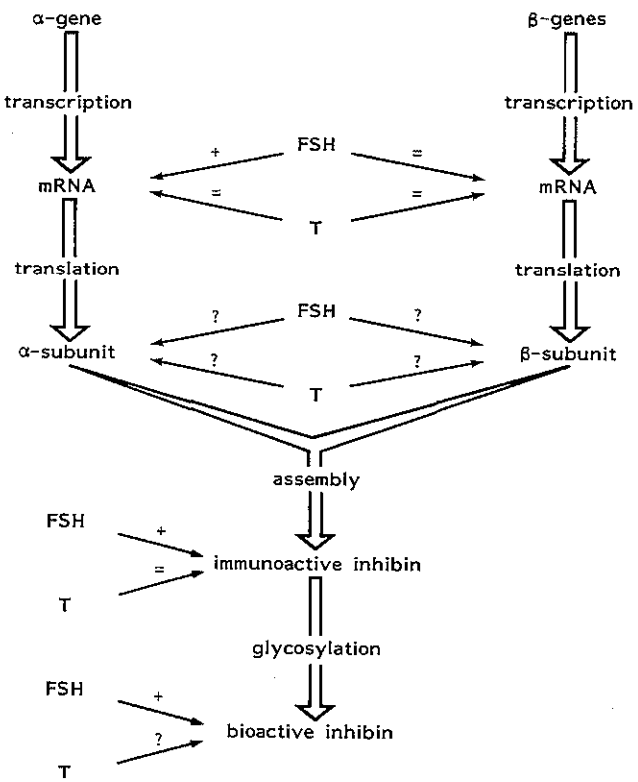


Figure 4.4

Schematic representation of the (possible) effects of FSH and testosterone on inhibin mRNAs, and on immunoreactive and bioactive inhibin levels.

The results of the experiments using highly purified Sertoli cells were comparable with those obtained using less pure Sertoli cell preparations. From this it can be concluded that the observed effects of FSH on Sertoli cells are not markedly affected by small numbers of germ cells or peritubular cells. Effects of testosterone on inhibin production by Sertoli cells were not found, either in the presence or absence of peritubular cells. However, an effect of testosterone may require a longer time period of exposure of the Sertoli cells to the hormone. Furthermore, the present results do not exclude effects of testosterone on glycosylation or other posttranslational modifications of the inhibin protein.

The results presented in this chapter were obtained using cultured Sertoli cells. The regulation of inhibin production and secretion *in vivo* may be influenced by additional factors, as has been described for other Sertoli cell products (see paragraph 4.2). A relatively small number of *in vivo* studies have been performed. The inhibin bioassay is not sufficiently sensitive to estimate circulating inhibin levels in plasma. Another problem is that the testicular level of inhibin activity is not by itself a measure of testicular inhibin production (Au et al., 1984). Using efferent duct ligation to estimate testicular inhibin production, it was observed that FSH, but not testosterone, was able to stimulate inhibin production in testes from adult hypophysectomized rats (Au et al., 1985). FSH injection of intact immature rats (no efferent duct ligation) increased testicular inhibin levels (expressed as U/paired testes) as compared with the levels in control rats (Ultee-van Gessel et al., 1988). These results are in agreement with the results presented in this chapter. However, more *in vivo* experiments need to be performed. Furthermore, sensitive techniques for the detection of single α- and β-subunits and the different dimers should be developed to correlate expression of subunit mRNAs and subunit protein synthesis, in order to compare the regulation of inhibin production and secretion by Sertoli cells in culture and in the testis. Knowledge about the regulation of inhibin secretion at the different steps of the production process could guide further investigations on the regulation of the synthesis of other Sertoli cell proteins.

CHAPTER 5

EFFECTS OF SERTOLI CELL PROTEINS ON SPERMATOGENIC CELLS

Introduction

Intercellular transport of diffusable compounds produced by Sertoli cells, such as lactate, may be essential for spermatogenesis.

In addition, proteins produced by Sertoli cells (for example ABP, transferrin, inhibin, see chapter 4) may exert effects on the developing germ cells. Specific binding sites for ABP have only been found for pachytene spermatocytes, with only a small number of sites per cell (approximately 1,000) (Steinberger et al., 1984). More convincingly, specific binding sites for transferrin have been shown on or in spermatocytes and spermatids by a number of authors (Holmes et al., 1983; Steinberger et al., 1984; Sylvester and Griswold, 1984; Brown, 1985; Vanelli et al., 1986). The possible presence of inhibin receptors on any cell type, including the germ cells, has not yet been documented.

The presence of transferrin receptors on germ cells and the accurate regulation of transferrin secretion by Sertoli cells suggest that Sertoli cell transferrin plays an important role for the developing germ cells, in particular because serum transferrin may not be transported across the blood-testis barrier.

5.1 Transferrin

Transferrin is a glycoprotein with a molecular weight of approximately 80,000 which can bind two atoms of iron (Fe^{3+}). The binding and transport of iron is the most important and best known function of transferrin, but it cannot be excluded that transferrin may also act as a circulating and local growth factor (Ekblom et al., 1983).

It has been suggested that there is no or very little production of transferrin by Sertoli cells *in vivo* (Lee et al., 1986a; Shabanowitz and Kierszenbaum, 1986). Lee et al. (1986a) suggested that Sertoli cells acquired the ability to produce transferrin during culture. However, this was contradicted by Griswold et al. (1987) and Morales et al. (1987). These authors reported that *in vivo* labeling with [^{35}S]methionine (intratesticular

injections), after removing the liver, resulted in labelled immunoprecitable transferrin in the testes. In addition, with *in situ* hybridization experiments, these authors could demonstrate that transferrin mRNA was expressed in the Sertoli cells. The highest expression of transferrin mRNA was found associated with stages XIII and XIV of the seminiferous epithelial cycle.

Sertoli cell transferrin resembles serum transferrin; there is only a slight difference in glycosylation (Skinner et al.,1984). Sertoli cell transferrin constitutes 7-15% of the total amount of proteins secreted by cultured Sertoli cells (Skinner and Griswold, 1980,1982; Skinner et al., 1984). Sertoli cell transferrin is probably involved in the transport of iron across the blood-testis barrier to the spermatocytes and spermatids, as follows: iron-loaded serum transferrin binds to transferrin receptors at the basis of the Sertoli cells, and subsequently, the iron-transferrin complex is taken up by the Sertoli cells via receptor mediated endocytosis (Wileman et al., 1985). Intracellularly, the iron atoms are released from the transferrin molecule, and the latter returns to the serum. The intracellular iron interacts directly or indirectly with Sertoli cell transferrin. It has been suggested that iron is released from the Sertoli cells in a low molecular weight form, and may become bound to transferrin after both the transferrin and the low molecular weight iron complex are released from the Sertoli cells (Wauben-Penris et al., 1988). In this way the Sertoli cells are involved in the transport of iron from the basal compartment to the adluminal compartment. Sertoli cell transferrin binds to receptors on the surface of the germ cells (Huggenvik et al., 1984).

From studies on transferrin, however, there was no evidence that iron, carried by transferrin, is actually taken up by the germ cells. The study described in this chapter and appendix paper 3 was undertaken to investigate whether spermatocytes and spermatids were able to take up iron from exogenous transferrin.

5.2 Ferritin

Iron can oxidize, hydrolyze and polymerize under physiological conditions, thus forming insoluble polymers (Octave et al., 1983). Furthermore, free iron is toxic to cells. Possibly, the protection against free iron is another important function of the production of iron-binding proteins by

Sertoli cells. It has been shown that transferrin is also present in rete testis fluid and epididymis (Djakiew et al., 1986) and this transferrin may protect spermatozoa against iron-induced lipid peroxidation (Braughler et al., 1986). To prevent the existence of free iron, transport and storage molecules for iron are produced by cells, including the extracellular transferrin but also the intracellular ferritin.

Ferritin consists of H and L subunits. Acidic ferritin mainly contains H subunits, whereas in basic ferritin the L subunit is dominant. Acidic ferritin can incorporate iron and release iron very rapidly. Basic ferritin is metabolically more stable and it mainly serves as an iron storage site. When a sufficient amount of iron is present in a tissue, ferritin mainly consists of L subunits, but when iron is deficient the H subunits will become dominant (Okuyama et al., 1985).

5.3 Iron transport into Sertoli cells in an *in vitro* incubation system

It was observed that cultured Sertoli cells can take up iron from exogenous transferrin. This uptake was linear with time for at least 20h (appendix paper 3; Fig. 5.1). The net uptake of ^{125}I-labelled transferrin by the Sertoli cells was less than 1 pmol/mg protein (appendix paper 3). This is in agreement with current models, that transferrin is secreted immediately after the iron is released (Wileman et al., 1985).

Experiments with ^{59}Fe-citrate showed that the uptake of iron from Fe-citrate was in the same range as the non-specific uptake of iron from transferrin (appendix paper 3). This confirms the role of transferrin-receptor interactions.

The hormone/vitamin cocktail FIRT is known to stimulate transferrin production by Sertoli cells (Skinner and Griswold, 1982). It was investigated whether this hormone cocktail had an effect on the iron uptake by the Sertoli cells. The cells were incubated, after a 3-day preincubation period in the absence or presence of FIRT, for different time periods (0-20h) with or without FIRT in the presence of double-labelled transferrin, and subsequently iron and transferrin uptake was measured. Fig. 5.1 shows the iron uptake by the Sertoli cells. In this experiment, the net uptake of iron was 5-7 pmol/mg protein per h, whereas the net uptake of transferrin was less than 1 pmol/mg protein. There was no pronounced effect of FIRT on the iron uptake by the cells. The amount of transferrin, produced by the

Sertoli cells, was not estimated. The above results indicate that the endogenous transferrin concentration is probably not rate-limiting in iron uptake by the Sertoli cells. Other factors, including the number of

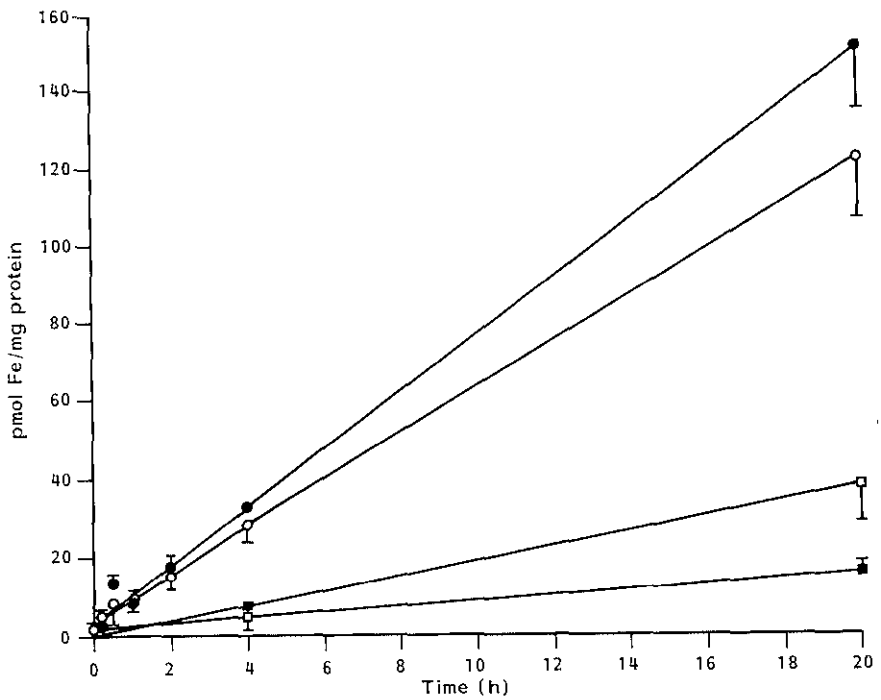


Figure 5.1

Uptake of ^{59}Fe from exogenous transferrin by Sertoli cells in culture. The Sertoli cells were incubated for 72h in the absence or presence of FIRT, and incubations were then continued for various time periods in the presence of double labelled (^{125}I, ^{59}Fe) transferrin. The total (○—○) and non-specific (□—□) uptake of iron by control cells and the total (●—●) and non-specific (■—■) uptake of iron by cells which had been incubated with FIRT are shown. Each point is the mean ± s.d. of four incubations.

transferrin receptors and the cellular ferritin concentration, may play a role in the regulation of iron uptake.

5.4 Iron uptake by isolated spermatocytes and spermatids

Incubation of freshly isolated spermatocytes and spermatids with double-labelled transferrin did not result in specific iron uptake by the cells but there was specific uptake of iron after a 16h preincubation period. The loss and recovery of transferrin receptors as a consequence of enzyme treatment during the cell isolation procedure may explain the above results. For this reason, iron uptake by isolated spermatogenic cells was estimated at different time periods after a 16h preincubation period. The results are in agreement with the participation of transferrin receptors in the uptake of iron also by spermatogenic cells.

In the present experiments, the time period that was needed to obtain complete recovery of the transferrin receptors after isolation of the cells, and thus of iron uptake, was not investigated in detail. Calculation of the rate of iron incorporation was not performed, because it was observed that iron incorporation into round spermatids was not linear with time, whereas Sertoli cells showed linear uptake. Rather, iron uptake in spermatids reached a plateau after approximately 2h of incubation with double-labelled transferrin (appendix paper 3). In addition, it was observed that the iron:transferrin ratio in spermatids was increased approximately six-fold after 10 min of incubation, whereas this ratio remained 2:1 in Sertoli cells after 10 min (appendix paper 3). This indicates that there was a very rapid initial uptake of iron by the spermatogenic cells as compared with the Sertoli cells. This implies that the kinetics of iron uptake by spermatids differs from that by Sertoli cells.

To test the viability of the germ cells, in all experiments the ATP content of the cells was measured in parallel with estimations of iron and transferrin uptake. It was observed that cells with very low ATP levels (lower than 1-3 pmol/10^6 cells) were unable to take up iron or transferrin specifically (i.e. there was no difference between total and non-specific uptake). It seems possible that the specific uptake of iron by the spermatogenic cells requires a certain amount of ATP. It has been suggested that ATP is needed for the removal of iron from transferrin (Egyed, 1982). In addition, mitochondria can also accumulate iron (Egyed, 1982). Moreover,

leakage of the cell membrane of damaged cells may allow free in- and outflow of iron-bound transferrin, thus increasing the non-specific uptake.

5.5 Complexation of intracellular iron to ferritin

In the present study, it was investigated whether the iron, taken up by Sertoli cells and spermatids, remained complexed to transferrin or was transferred to another protein, in particular intracellular ferritin. This was performed by separating cellular proteins, followed by precipitation of the separated proteins using specific antibodies against transferrin and ferritin, as described in appendix paper 3.

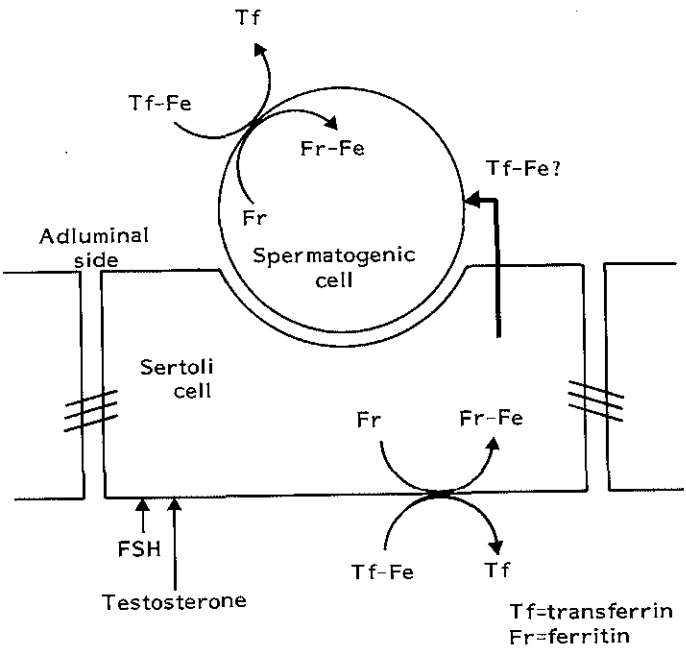
Most of the iron, taken up by Sertoli cells and spermatids was complexed to ferritin (appendix paper 3). After isoelectric focussing of the Sertoli cell proteins from the ferritin fractions (8 and 9), the ^{59}Fe label was found to co-migrate with a basic rat-ferritin (not shown). From these results it was concluded that most of the iron was complexed to basic ferritin. This may suggest that sufficient amounts of iron are present in the cultured Sertoli cells, and that the iron is stored until it is needed, rather than used immediately following uptake (see paragraph 5.2).

5.6 Conclusions

From the data presented above, it is concluded that Sertoli cells, spermatocytes and spermatids are able to take up iron from exogenous transferrin via specific transferrin binding sites (cell surface receptors). The incorporated iron is complexed to ferritin in both Sertoli cells and spermatids. The kinetics of iron uptake by Sertoli cells and spermatids, however, are different.

These results, and those of other authors (Morales et al., 1987; Wauben-Penris, 1988), are in favour of iron transport across the blood-testis barrier according to the model described by Huggenvik et al. (1984). Our data (Fig. 5.2) indicate that ferritin is also involved in the overall scheme of iron transport and storage in the testis.

Although the present results imply that there is uptake of iron by spermatogenic cells, the role of transferrin and the incorporated iron is unclear. It has been observed that iron-bound transferrin can stimulate the growth of many cell types, including two mouse testis-derived cell lines



Scheme of iron uptake by spermatogenic cells in vitro

Figure 5.2

Model of iron incorporation into Sertoli cells and spermatogenic cells in cultured tubule fragments. Sertoli cells and spermatogenic cells take up iron from exogenous transferrin, which then becomes bound to intracellular ferritin. It is as yet not known whether the spermatogenic cells take up iron from Sertoli cell transferrin in situ.

(non-spermatogenic cells), whereas iron-free transferrin cannot (Perez-Infante and Mather, 1982). The latter can be explained by the fact that cells require iron for cell division. The observation that "free" iron (i.e. in the presence of a chelator) can partly substitute iron-transferrin in stimulating cell growth, supports the idea that the growth-promoting effect

of transferrin is due to its ability to deliver iron to the cells (Thesleff et al., 1985). The role of transferrin, either alone or in combination with iron, as a requirement for growth and development of spermatogenic cells is unknown as yet (see also Discussion appendix paper 3).

Erythroid cells and rapidly dividing cells require much iron. Spermatids do not further divide, but iron will be required for turn-over of iron-containing proteins which, most likely, also occurs in these cells. Incorporated iron can serve, for example, in prosthetic groups in cytochromes, oxidases, catalase, ribonucleotide reductase, etc. (Bergeron, 1986).

The presence of transferrin receptors on germ cells may imply that transferrin and/or iron are important for the spermatogenic cells. However, further investigations concerning the different aspects of the role of transferrin in the testes as a possible growth factor, a protective agent, and/or transport protein for iron should be performed.

CHAPTER 6

CULTURED TUBULE FRAGMENTS

Introduction

Apart from transferrin, there is little information on the possible role of Sertoli cell proteins for interactions between Sertoli cells and germ cells which may support the survival and development of the germ cells. Since it is very difficult to investigate these interactions *in vivo*, most studies are performed using *in vitro* incubation systems, which have been discussed in chapter 2. In this chapter, an *in vitro* incubation system is described, in which development of spermatids is estimated using flow cytometric analysis and estimation of LDH-C_4 activity, a germ-cell specific lactate dehydrogenase (LDH) isoenzyme. Furthermore, a number of other biochemical parameters have been tested.

6.1 Tubule fragments *in vitro*

Different incubation systems have been described in chapter 2. In the present study we used an open tubule system from 26-day-old rats to evaluate germ cell development *in vitro*. A scheme of the isolation and incubation procedure is described in chapter 3.

In the present system, the peritubular cells, which surround the tubules, were largely removed by collagenase treatment. This was performed to allow the Sertoli cells in the tubule fragments to attach to and to spread out on the plastic surface, so that an open system was formed, with a relatively large number of spermatogenic cells still attached (Fig. 6.1). This in view of the pronounced degeneration of germ cells in closed tubule segments or tissue fragments, which could be explained by limited transport of nutrients and waste products. Morphological observations indicated that there was still a number of peritubular cells present, mainly underneath the Sertoli cells (not shown). These cells may contribute to a matrix, which may result in a better preservation of Sertoli cell functions (Tung and Fritz, 1984).

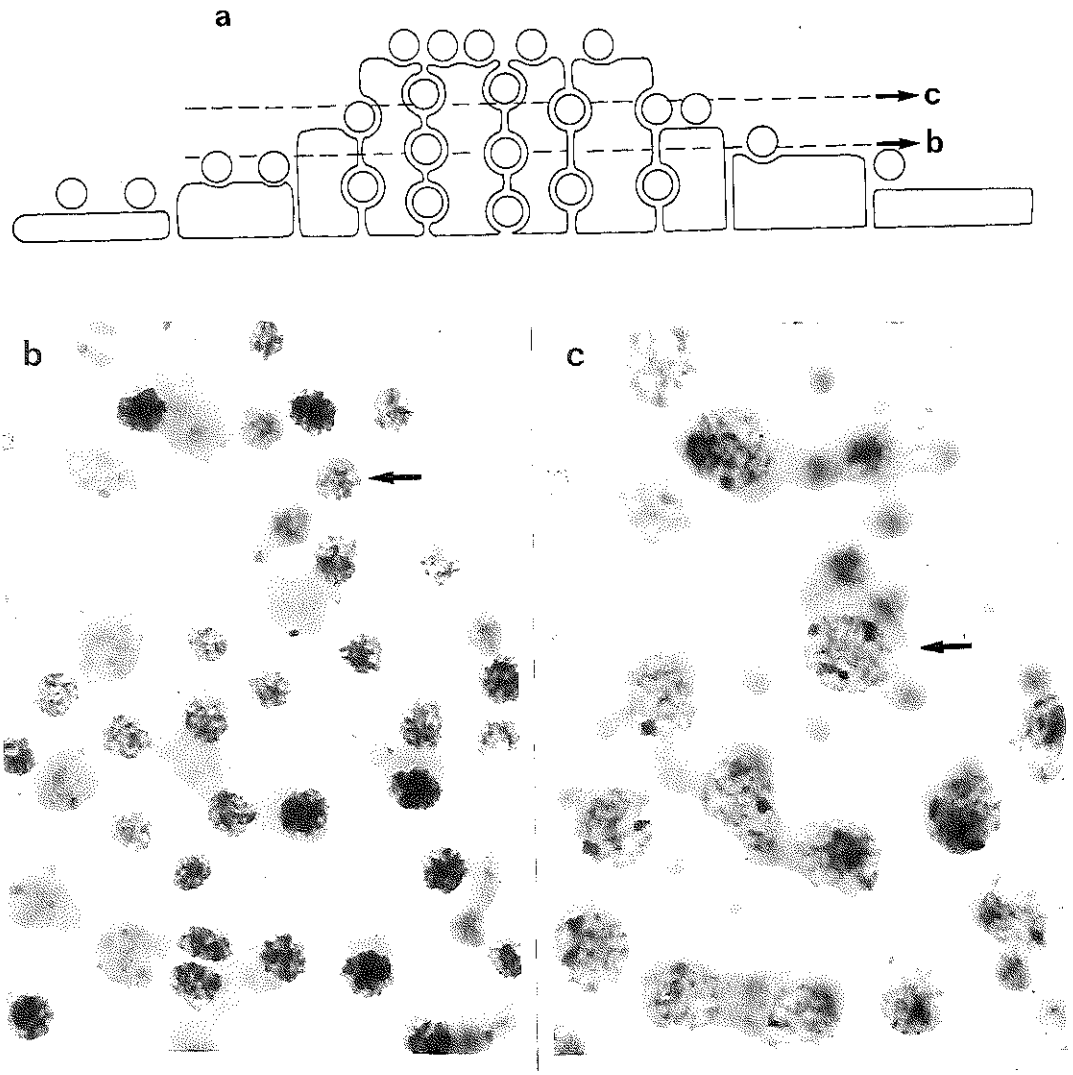


Figure 6.1

Tubule fragment in culture. A schematic drawing of a tubule fragment, isolated from a 26-day-old rat and cultured on plastic for 2 to 3 days, is presented in a. Figures b and c are photomicrographs, focussed in plane b or c (see a), showing early (b) and middle-late (c) pachytene spermatocytes as indicated by the arrows.

6.2 The incubation medium

Flow cytometry and gas-liquid chromatography (the latter to detect the formation of α-hydroxyisocaproate, HIC; see appendix paper 4) were performed in order to test the survival and development of spermatocytes and spermatids in tubule fragments in a number of incubation media. In Eagle's Minimum Essential Medium (MEM) which contained HEPES to obtain a stable pH, spermatocytes and spermatids survived in higher numbers, compared with incubations in MEM without HEPES (not shown).

Addition of fetal calf serum (FCS) (2% v/v) to the incubation medium had no effect on the survival of the germ cells in tubule fragments and did not affect HIC formation (not shown).However, Tres and Kierszenbaum (1983) observed that spermatogenic cells which were co-cultured with Sertoli cells disintegrated very fast when serum (10% v/v) was present in the incubation medium. Because serum did not improve the survival of the germ cells, and in order to use a chemically defined incubation medium, MEM containing HEPES was used in all other experiments. To examine effects of hormones on germ cell survival and development in vitro, the hormone/vitamin cocktail FIRT, which contains FSH, insulin, retinol and testosterone (appendix paper 4) was used. FIRT is known to effectively stimulate secretion of a number of proteins by Sertoli cells, during culture for a number of days (Skinner and Griswold, 1982; Skinner and Fritz, 1985a).

6.3 Development of germ cells present in tubule fragments in vitro

In all experiments 26-day-old rats were used with a body weight which was within the normal range (appendix paper 4). Tubule fragments were isolated from the testes as described in appendix paper 4 and chapter 3 and incubated for up to 72h (chapter 3).

DNA flow cytometric analysis in combination with DNA estimations showed that the percentage of cells with a 1C amount of DNA, representing spermatids, and the actual number of spermatids increased approximately 2-fold during incubation of tubule fragments from 26-day-old rats (appendix paper 4). However, the percentage of cells with a 4C amount of DNA, representing primary spermatocytes, and the actual number of spermatocytes decreased during culture. This decrease is probably mainly caused by disintegration of the spermatocytes and only partly by conversion of

spermatocytes into spermatids. The present system does not give information on the number of spermatogonia that enter the meiotic prophase.

There was no effect of FIRT on the number of spermatids. In all experiments, the decrease in the number of spermatocytes was slightly less pronounced when the tubule fragments were cultured in the presence of FIRT (appendix paper 4).

In order to test whether the spermatogenic cells in the tubule fragments were metabolically active, a number of parameters have been tested. To distinguish Sertoli cell activity from spermatogenic cell activity, either spermatogenic cell specific activities can be tested, or the spermatogenic cells need to be isolated from the fragments after incubation. Both methods have been used.

LDH-C_4 is a LDH isoenzyme which is specific for spermatocytes, spermatids, and spermatozoa (Blanco et al., 1976; Hintz and Goldberg, 1977; Meistrich et al., 1977). LDH-C_4 has a broad substrate specificity. This involves that not only pyruvate can be converted into lactate and vice versa, but that also a number of other 2-oxo and 2-hydroxy acids can serve as a substrate (Blanco et al., 1976). In the present experiments, α-ketoisocaproate was used as a substrate. The LDH-C_4 activity increased during the incubation period (Fig. 6.2; appendix paper 4). This increase was most pronounced during the first 24h of incubation. A significant ($p<0.01$) difference was observed after 48h and 72h between incubations in the absence or presence of FIRT.

In another experiment, tubule fragments were isolated from 32-day-old rats and the LDH-C_4 activity and protein and DNA contents per well were estimated at t=0, 24h, 48h, and 72h. Comparing these data with those obtained using younger rats (26-day-old), the LDH-C_4 activity per mg of protein was higher in the older rats (32-day-old) (Fig. 6.2.a). However, there was a larger fold increase in LDH-C_4 activity in tubule fragments from 26-day-old rats during culture, when the data were expressed as percentage of the starting value (Fig. 6.2.b). This may be explained by a higher number of degenerating germ cells in tubule fragments from 32-day-old rats, but it is also possible that this is related to a more rapid development of the testis of rats of 26 days of age as compared with the testis of rats of 32 days of age (Mills et al., 1977).

Sertoli cells contain a high activity of the enzyme branched-chain amino acid aminotransferase (Grootegoed et al., 1985). This enzyme can convert

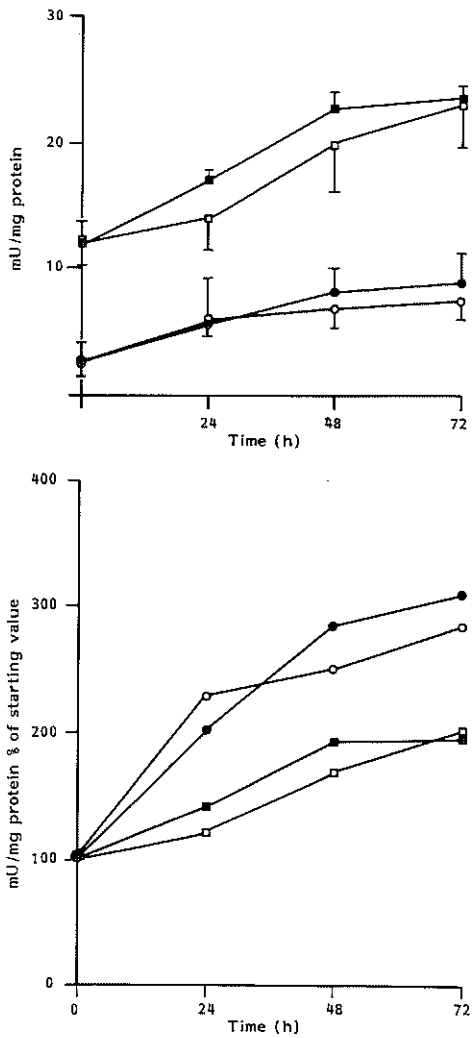


Figure 6.2

LDH-C_4 activity of tubule fragments isolated from testes of 26- or 32-day-old rats and incubated for 24, 48 or 72h. The tubule fragments from the 26- and 32-day-old rats were incubated in the absence (○ and □, 26- and 32-day-old, respectively) or presence of FIRT (● and ■, 26- and 32-day-old, respectively). The data are expressed as U per mg protein (a) or as U per mg protein as a percentage of the starting value (b)

branched-chain amino acids (leucine, isoleucine and valine) into the corresponding branched-chain α-oxo acids (leucine into α-ketoisocaproate, KIC). Spermatogenic cells do not contain this enzyme (Grootegoed et al., 1983) but spermatocytes and spermatids can convert branched-chain α-oxo acids into branched-chain α-hydroxy acids (KIC into α-hydroxyisocaproate, HIC) and vice versa via a NADH-dependent reaction. This reaction is catalyzed by LDH-C_4, which is not present in Sertoli cells. It was observed that both KIC and HIC were formed by the tubule fragments (appendix paper 4). This indicates that the Sertoli cells converted leucine into KIC and that this compound diffused into the germ cells, which converted KIC into HIC. From this it can be concluded that metabolic interactions between Sertoli cells and spermatogenic cells were still present after 48h-68h of incubation of the tubule fragments, and that there was a certain degree of biochemical integrity of the cells.

It was observed in previous experiments that isolated spermatids are able to take up Fe from exogenous transferrin. The ^{125}I-labelled transferrin was not incorporated into the cells (chapter 5 and appendix paper 3). In the experiments described in this chapter, it was studied whether the spermatogenic cells in incubated tubule fragments from 29- to 32-day-old rats were also capable of incorporating Fe. The specific uptake of Fe by spermatids freshly isolated from total testis and incubated for 20h is shown in Table 6.1. Spermatids isolated from tubule fragments which had been incubated with double-labelled transferrin during 0-20h or 48-68h showed a comparable specific uptake of Fe (Table 6.1). The uptake of Fe by the spermatogenic cells was in the same range (4.2 ± 2.2 pmol/10^6 cells per 20h) as described previously (appendix paper 3). From these results it was concluded that spermatogenic cells in tubule fragments showed biochemical integrity with respect to active Fe uptake, even after 48h of incubation of the tubule fragments.

6.4 LDH-C_4 activity in isolated spermatocytes and spermatids

In the experiments previously described, LDH-C_4 was used as a quantitative marker for spermatogenic cells. It was observed that the LDH-C_4 activity of tubule fragments in culture increased most markedly during the first 24h of incubation. It is possible that this increase reflects an unscheduled increase of the enzyme activity per cell, rather than cell development or an

TABLE 6.1

Fe uptake by isolated spermatids and by spermatids in tubule fragments in culture

	Fe uptake (pmol/10^6 cells per 20h)			
	Exp. 1		Exp. 2	
	Total	Nonspecific	Total	Nonspecific
Isolated at t=0	3.0	0.8	nd	nd
Isolated at t=20h	3.0	0.2	5.5	0.3
Isolated at t=68h	nd	nd	5.6	0.3

Total and nonspecific Fe uptake was estimated in spermatids which were isolated at t=0, and incubated for 20h with double-labelled ($^{125}I;^{59}Fe$) transferrin. In addition, total and nonspecific Fe uptake was estimated in spermatids isolated from cultured tubule fragments at t=20h or t=68h which had been incubated with double-labelled transferrin from 0-20h or 48-68h, respectively. The specific (total minus nonspecific) uptake of transferrin was less than 0.1 pmol/10^6 cells per 20h. Spermatids from Exp.1 were isolated by the so-called Staput method and by Percoll gradient centrifugation and those from Exp.2 by Percoll gradient centrifugation. The data represent the mean from 2 incubations per experiment. (nd; not determined)

increased number of LDH-C_4 containing cells. Therefore, it is important to demonstrate that the enzyme activity per cell is constant also during in vitro manipulation of the cells. In order to investigate this, a number of experiments have been carried out using isolated spermatocytes and spermatids.

Isolation of spermatocytes and spermatids as described elsewhere (appendix paper 4), but at 4°C, resulted in cells with a LDH-C_4 activity which was approximately 50% lower compared with cells isolated at room temperature (22-24°C) (appendix paper 4). From this, it is clear that the LDH-C_4 activity per cell can change under the influence of the isolation procedure. To examine whether preferential synthesis of LDH-C_4 occurred during the isolation procedure, this procedure was carried out in the presence of the protein synthesis inhibitor cycloheximide. The LDH-C_4 activity of spermatocytes and spermatids after isolation in the presence of 100 μM cycloheximide was in the same range compared with that of cells

isolated in the absence of cycloheximide (appendix paper 4). These data showed that the enzyme activity measured after isolation at room temperature was not the result of accelerated protein synthesis during isolation but may represent the activity as present in the cells in the testis. The results furthermore indicate that LDH-C_4 in the isolated spermatids has a long half-life.

From the above, it seems likely that a low temperature can affect the LDH-C_4 activity in spermatogenic cells. To investigate this further, the cells were isolated at room temperature and subjected to a cold-shock for 1h. It was observed that the LDH-C_4 activity of the spermatocytes (not shown) and spermatids (appendix paper 4) was decreased after the cold-shock. The cells were, however, able to restore this activity to control levels when the cells were subsequently incubated for 2h at 32°C (appendix paper 4). Addition of cycloheximide during this 2h incubation had almost no effect, indicating that the recovery of enzyme activity at 32°C was not dependent on de novo synthesis of the enzyme (appendix paper 4). The cold shock and addition of cycloheximide did not affect the ATP and GSH levels in the cells. From this it was concluded that the cells were still intact and that the lowered enzyme activity at 4°C was not caused by leakage of the enzyme out of the cells.

It is known that the cellular heat shock proteins (hsps) can modulate the conformation of other proteins (Pelham, 1986). The most prominent hsps have molecular weights of approximately 70,000 and 90,000 and are termed hsp70 and hsp90, respectively. These hsps have been highly conserved in all organisms. Heat shock proteins are synthesized during normal development and, at an increased rate, by cells upon exposure to high temperatures or other environmental stresses such as heavy metals, anoxia, or toxic agents (Allen et al., 1988a and b). Also a cold shock can induce hsp formation (Burton et al., 1988). Heat shock proteins may protect cells against environmental stresses (Petersen and Mitchell, 1981; Li and Werb, 1982; Pelham, 1986). In addition, they may be involved in other events, such as regulation of the functional state of steroid receptors (Sanchez et al., 1987).A cell-specific member of the hsp70 family was detected in mouse spermatogenic cells. This protein, P70, is present in large amounts in pachytene spermatocytes and round spermatids, but at much lower levels in early spermatocytes, and is primarily synthesized in pachytene spermatocytes (Allen et al., 1988a and b). This indicates that P70 is expressed in a

developmental stage-dependent manner in male germ cells. In contrast, hsp70 was not detected in unstressed cells, but was induced in all stages of spermatogenic cell development after a heat shock. In spite of the presence of P70, pachytene spermatocytes and round spermatids are more sensitive to heat (37°C) than other developing germ cells in the testis (Chowdhury and Steinberger, 1970). Allen et al. (1988b) suggested that P70 may not be involved in the protection of the germ cells to heat stress. It is even suggested that P70 interferes with other members of the hsp70 family, thus leaving spermatocytes and spermatids particularly sensitive to heat and other stresses (Allen et al., 1988b). The major role of P70 and hsp70 may not be to enhance the thermostability of the developing germ cells (Allen et al., 1988a). P70 and LDH-C_4 are produced and expressed during the same stages of spermatogenic cell development (Meistrich et al., 1977; Allen et al., 1988b). In this respect, it can be suggested that P70 has a function in germ cell development (Allen et al., 1988a). In addition, the hsps, including P70, may stabilize and restore, if necessary, the conformation of a number of cellular proteins (Pelham, 1986), including LDH-C_4. Activities of hsps in spermatogenic cells may explain the above described recovery of LDH-C_4 activity after cold-shock.

6.5 Conclusions

Using tubule fragments from immature rats, it is possible to develop an incubation system with a relatively small decrease in the number of spermatocytes and net appearance of a small number of spermatids. This is the first time that an actual increase in the number of spermatids during culture of testicular tissue has been described. DNA flow cytometry provides an accurate method to quantitate the numbers of primary spermatocytes and round spermatids in the tubule fragments.

Additional experiments showed that after 48-72h of incubation of the tubule fragments some Sertoli cell-germ cell metabolic interactions were still preserved, indicating that the spermatogenic cells enclosed in the fragments were metabolically active.

LDH-C_4 activity in germ cells is very sensitive to cold-shock, and possibly also to other stresses inflicted upon the cells, which implies that the activity per cell can vary during in vitro manipulation. On the other hand, LDH-C_4 has a low turn-over rate and the cellular enzyme activity

remains constant during several hours of incubation of the spermatids at 32°C (appendix paper 4). In conclusion, LDH-C_4 activity can be used as a marker for the presence of spermatocytes and spermatids *in vitro*, but changes in the activity due to exposure of the cells to stresses should be taken into account. Therefore, it is suggested that LDH-C_4 activity should be used as marker in combination with other parameters such as DNA estimation and DNA-flow cytometry.

CHAPTER 7

GENERAL COMMENTS

The results described in the present thesis concern several aspects of the hormonal regulation of spermatogenesis which were investigated using a number of different incubation systems. In this study, the age of the animals is an important variable, because testis development is partly a postnatal event. In all experiments, immature rats were used with an age from 21 up to approximately 32 days. At these ages, the Sertoli cells are in the middle of their postnatal maturation phase, and are responsive to different hormones, including FSH, testosterone and IGF-I (Rich et al., 1983; Oonk and Grootegoed, 1988; Roberts and Griswold, 1989). Concomitantly, the testis as a whole is developing and growing very rapidly, which involves the appearance of spermatids and a dramatic increase of the workload of the Sertoli cells (Mills et al., 1977; Ekwall et al., 1984).

In this chapter the role of Sertoli cells in spermatogenesis will be discussed. Some properties of cultured Sertoli cells undergo a change, as a consequence of impairment of Sertoli cell maturation. In this respect, the maturation of Sertoli cells *in vitro*, as well as possible markers to examine the degree of maturity of the Sertoli cells, are discussed in paragraph 7.2. In addition, some notes are made about the relevance of *in vitro* studies for our understanding of the *in vivo* situation.

7.1 The role of Sertoli cells in spermatogenesis

7.1.1 Sertoli cells support, rather than direct, germ cell development

Isolated mammalian spermatocytes cannot complete the meiotic divisions and differentiate into round spermatids, under the *in vitro* conditions which are generally used. Possibly, the germ cells are damaged during the isolation procedures, consequent on rupture of Sertoli cell-germ cell plasma membrane junctions or the cytoplasmic bridges which interconnect the germ cells of the same developmental stage (Weber and Russell, 1987). This may prevent their further development. On the other hand, continuous support by Sertoli cells is probably essential for germ cell survival and development. During the studies described in this thesis we have found that, in tubule fragments from immature rats containing mainly Sertoli cells, spermatocytes,

spermatids, a limited number of spermatocytes gave rise to spermatids via the meiotic divisions (chapter 6 and appendix paper 4). Possibly, this result was obtained due to supporting and protecting activities of the Sertoli cells in the cultured tubule fragments.

The role of Sertoli cells in spermatogenesis most likely includes a great variety of cellular activities, which by no means are completely clear and fully investigated. In this respect the question appears relevant whether germ cells are highly dependent upon the Sertoli cells for the regulation of their development, or may perhaps autonomously follow an intrinsic developmental programme which involves a supporting and protecting function of Sertoli cells. In other words, do Sertoli cells support, rather than direct, germ cell development?

An indication for the occurrence of an intrinsic programme in germ cells is given by the fact that the number of spermatogonial divisions and the duration of the development from early primary spermatocyte up to spermiation is constant for a given species and is the same for all germ cells (for a review see Parvinen, 1982). This temporal control of synchronous germ cell development may also rely on the syncytial nature of the germ cells, caused by incomplete cytokinesis and the continued existence of cytoplasmic bridges.

7.1.2 Mechanisms of support of germ cell development by Sertoli cells

Structural aspects of the contacts between Sertoli cells and germ cells in the seminiferous tubules have been described in much detail. The observations have led to the suggestion that support of the germ cells by Sertoli cells may be mediated in part by cell surface interactions (Ziparo et al., 1980). It has been shown that there is specific adhesion of isolated pachytene spermatocytes, and to a lesser extent of round spermatids, to monolayers of cultured Sertoli cells (Ziparo et al., 1980). It was suggested that glycoproteins expressed at the cell surface of the germ cells were involved in the adhesion between the cell types (D'Agostino et al., 1984; D'Agostino and Stefanini, 1988).

Support of the developing germ cells by Sertoli cells may occur also via diffusable compounds, including lactate. Glucose is converted to lactate in large amounts by cultured Sertoli cells, and lactate is essential to support

ATP production by isolated spermatogenic cells (see for a review Grootegoed and Den Boer, 1989). However, many other diffusable compounds may play a role. Co-culture of Sertoli cells and germ cells resulted in an enhanced incorporation of precursors into RNA and DNA of the spermatogenic cells, which was not mimicked by lactate (Rivarola et al., 1985). The authors did not provide evidence that the enhanced incorporation of precursors actually represented an increased rate of RNA and DNA synthesis, but the results indicate an effect of an unidentified diffusable compound. Another possible example concerns the conversion of leucine into HIC by tubule fragments, which involves transport of a metabolic intermediate from Sertoli cells to the germ cells (Grootegoed et al., 1985, chapter 6 and appendix paper 4).

In the spermatogenic epithelium, numerous growth factors are produced. Some of these growth factors (seminiferous growth factor, IGF-I, interleukin 1α, Sertoli cell secreted growth factor, TGF-α, inhibin) have been localized to, or are known to be secreted by, Sertoli cells. Germ cells, however, may not produce so many different factors, although production of β-nerve growth factor by primary spermatocytes and spermatids has been reported (Bellvé and Zheng, 1989). It has been suggested that testicular growth factors are important for the regulation of testicular development and functions, and that such factors might be involved in the temporal and spatial co-ordination of germ cell development (Bellvé and Zheng, 1989). However, there is as yet no experimental evidence that growth factors are somehow involved in the developmental scheme of the spermatogenic cells. In addition, for most of the growth factors no specific effects and/or the presence of specific binding sites have been shown on germ cells, although it should be noted that this type of effect may involve long-term effects via low-abundance receptors which have gone unnoticed in experiments carried out thusfar.

7.1.3 Effects of germ cells on Sertoli cells

The communication between Sertoli cells and germ cells is probably not a one-way route, and may include effects of compounds from germ cells on Sertoli cells. It has been observed that germ cells can further enhance FSH-stimulated ABP secretion by Sertoli cells (Galdieri et al., 1984; Le Magueresse and Jégou, 1988a and 1988b). Furthermore, transferrin secretion by cultured Sertoli cells was stimulated (Le Magueresse et al., 1988),

whereas the FSH-stimulated 17β-estradiol production (i.e. aromatase activity) (Le Magueresse and Jégou, 1988a) was inhibited after addition of germ cells. Finally, it has been suggested that germ cells may influence the basal versus apical secretion ratio of ABP secretion by Sertoli cells, but not that of transferrin (Janecki et al., 1988). It is thought that the action of spermatocytes and spermatids on Sertoli cells is achieved through production and secretion by the germ cells of one protein or different proteins (Le Magueresse and Jégou, 1988a). However, the characterization and the mechanism of action of the protein(s) need further investigation. Also, it remains to be substantiated whether, and via what mechanism, spermatogenic cells can secrete proteins. Elongating spermatids of transgenic mice expressing human growth hormone (hGH) do not secrete hGH. This may indicate that the spermatids do not contain a constitutive secretory pathway (Braun et al., 1989).

Parvinen (1982) and Kerr (1988) have suggested that Sertoli cells support the germ cells, as an interpretation of the cyclic and stage-dependent variations in the activities and ultrastructure of Sertoli cells. However, it is not clear whether these changes in Sertoli cell activities and properties are primary events and are a prerequisite for germ cell development. Possibly, these variations in Sertoli cell properties are a consequence of effects of germ cells on Sertoli cells (with or without feedback action on the germ cells). Relevant in this respect, reflecting consequence rather than cause, is the production of plasminogen activator by Sertoli cells. Phagocytosis of the residual bodies from late spermatids by Sertoli cells during spermiation is probably one of the stimuli which increase the production of plasminogen activator by Sertoli cells (Lacroix et al., 1982). The secretion of plasminogen activator is highest during stages VII and VIII of the cycle of the seminiferous epithelium, the stages at which spermiation occurs and the primary spermatocytes move across the Sertoli-cell barrier. It has been suggested that plasminogen activator may play a role in restructuring the spermatogenic epithelium during these processes (Lacroix and Fritz, 1982). In this case, the late spermatids might be the primary cause of changes in the Sertoli cells, which would then have a consequence for the development of early primary spermatocytes. The cascade of events may continue even further, because spermatogonial divisions are likely to be influenced by the transport of spermatocytes across the barrier.

7.2 Sertoli cell maturation and spermatogenesis in vitro

In the rat testis, after approximately 16 days of age, the Sertoli cells do not further divide, although growth and maturation of the Sertoli cells continue. This growth and maturation may subside after approximately 60 days of age, when spermatogenesis is fully established. In order to investigate whether a certain degree of maturity is maintained in vitro, a marker for Sertoli cell maturation would be useful. Possible markers for Sertoli cell maturation are the rate of ABP production, aromatase activity, and inhibin mRNA expression.

The initial rate of secretion of ABP by isolated Sertoli cells was 20-fold higher when the Sertoli cells were isolated from 25-day-old rats as compared with 10-day-old rats. A further 2-fold increase by 35 days of age was observed (Rich et al., 1983). After prolonged culture of Sertoli cells from rats of different ages (below 20 days) it was observed that the ABP secretion in the cultures increased until the total Sertoli cell age (i.e. the age of the animal plus the number of days in culture) was 20 days (Rich et al., 1983). This increase was not dependent on the presence of germ cells. This indicates that immature Sertoli cells in culture continue to mature for a short time, when ABP secretion is taken as an indicator of Sertoli cell maturation. Addition of a combination of non-steroid and steroid hormones, growth factors, and vitamin E to the culture medium enhanced and prolonged the ABP secretion and viability of Sertoli cells from young rats. Coculture of Sertoli cells with peritubular cells or germ cells also increases the secretion of ABP (Hutson and Stocco, 1981; Galdieri et al., 1984). Furthermore, the secretion of ABP by Sertoli cells which are cultured on a membrane or a matrix and have a more polarized morphology, is higher as compared with the secretion by Sertoli cells which are cultured on plastic (Byers et al., 1986; Janecki and Steinberger, 1986; 1987). This may indicate that Sertoli cell maturation is partly dependent on polarization of the cells. Furthermore, progression of Sertoli cell maturation in vivo may, in addition to hormones, be partly dependent on the presence of spermatogenic cells and activities of other testicular cell types.

Aromatase activity has been considered as another potential marker for Sertoli cell maturation. Sertoli cell aromatase activity decreases with the age of the rats. The synthesis of estradiol-17β by Sertoli cells of 30-day-old rats is very low and cannot be stimulated with FSH, in contrast with the

high levels of estradiol-17β production by 5- to 20-day-old rats which can be stimulated with FSH (Dorrington and Armstrong, 1979). An increase in FSH- or dibutyryl cAMP-stimulated aromatase activity during culture may result from Sertoli cell de-differentiation (Rommerts et al., 1978; Dorrington and Armstrong, 1979).

From the above it can be concluded that the rate of ABP secretion and aromatase activity change in a reciprocal manner, *in vivo* and in culture. A combined use of these parameters may give a reliable impression about the degree of maturity of Sertoli cells. The mentioned markers are not suitable to estimate small changes in the degree of maturity of Sertoli cells from rats older than approximately 30 to 40 days of age because aromatase activity is very low at that age and ABP secretion starts to reach a plateau.

In addition to the above markers, the ratio of the expression of the two inhibin β_B-subunit mRNAs changes with the age of the rats (chapter 4). Further investigations should point out whether this ratio-change could be used as a marker for Sertoli cell maturation.

It is difficult to demonstrate that responses to hormones which occur in culture also occur to the same extent and in the same fashion *in situ*, and *vice versa*. Ironically, the incubation systems which are most different from the *in vivo* situation, in particular cultures of an isolated cell type, can be analyzed and interpreted most easily. For example, when FSH is added to cultures of highly purified Sertoli cells, FSH acts directly on the Sertoli cells to increase the inhibin production and secretion (chapter 4 and appendix papers 1 and 2). Using cultures containing Sertoli cells and other cell types, it cannot be excluded that hormones act indirectly on the Sertoli cells via the other cell types. A clear example of the latter is the production of P-mod-S by peritubular cells which is enhanced by testosterone (see chapter 2). P-mod-S is produced by peritubular cell cultures, containing peritubular myoid cells and endothelial cells. P-mod-S stimulates inhibin production (Skinner et al., 1989) and the production of many other proteins (see chapter 2) by Sertoli cells. Addition of P-mod-S to, or co-culture of peritubular cells with, highly purified Sertoli cell cultures has aided to give more insight in Sertoli cell-peritubular cell interactions. However, the degree of interaction *in vivo* and in culture may differ significantly.

In all available culture systems, there is no quantitative survival of

germ cells, nor pronounced progression of spermatogenesis. A partial loss of differentiated functions of Sertoli cells in various culture systems, may result in an impaired capacity of the Sertoli cells to support and protect the developing germ cells. As a prerequisite for spermatogenic cell development *in vitro* it will be essential to prevent that partial loss of differentiated functions of the Sertoli cells. Therefore, it seems particularly important to improve culture methods, to try to maintain Sertoli cell differentiated functions (maturity). The level of Sertoli cell maturity during culture can possibly be estimated using the parameters discussed herein.

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Regulation of inhibin α- and β_B-subunit mRNA levels in rat Sertoli cells
Molecular and Cellular Endocrinology, in the press.

SUMMARY

Development of male germ cells (spermatogenesis) takes place in the testicular seminiferous tubules. The hormones follicle-stimulating hormone (FSH) and testosterone are essential for the initiation and maintenance of spermatogenesis.

The germ cells in the tubules (spermatogonia, spermatocytes and spermatids; in order of development) are in intimate contact with the Sertoli cells which are attached to a basal lamina. Adjacent Sertoli cells are connected through tight junctions, thus forming a Sertoli cell barrier. The barrier divides the tubules in a basal and an adluminal compartment. Germ cells enter the adluminal compartment when they have reached a certain stage of development, and are thereafter not in direct contact with the environment outside the tubules. Consequently, Sertoli cell secretion products are thought to play an important role during spermatogenesis.

Sertoli cells contain FSH and testosterone receptors, and are target cells for these hormones. The germ cells, however, do not contain receptors for FSH and testosterone. Hormonal control of spermatogenesis therefore involves effects of hormones on Sertoli cells. However, the peritubular cells, surrounding the tubules, may also take part in this hormonal regulation.

In chapter 1, a short outline of the structure of the testes and of spermatogenesis is given. In addition, the aim of the present thesis has been described. The aim was to try to contribute to a better insight in the hormonal regulation of spermatogenesis and the role of Sertoli cells in this process. For this purpose, one possible experimental approach involves the isolation and culture of Sertoli cells and germ cells.

Several factors involved in the regulation of spermatogenesis, and the possible function of Sertoli cells, are discussed in chapter 2. Biochemical properties of Sertoli cells are described, as well as a number of Sertoli cell culture systems with their advantages and disadvantages.

Chapter 3 gives a description of the cell isolation and incubation procedures which have been used in the present study, as well as the RNA isolation and analysis procedures which have been used for the investigations described in chapter 4 and appendix papers 1 and 2.

The regulation of the production and secretion of a number of Sertoli cell products is discussed in chapter 4. Recent developments, including

studies at the level of gene transcription and mRNA translation, have made it possible to investigate the regulation of the production and secretion of several proteins. The results of such investigations on inhibin are described in chapter 4 and appendix papers 1 and 2. Inhibin is a member of the TGF-β family of growth factors, and consists of two dissimilar subunits, α and β. One of the possible bioactivities of inhibin involves inhibition of FSH release by the pituitary gland *in vitro*. It was observed that the expression of the α-subunit mRNA in Sertoli cells was increased by FSH, and the production of immunoreactive inhibin and the secretion of bioactive inhibin were also increased, in a dose-dependent way. There was no effect of FSH on the expression of β-subunit mRNA. Furthermore, we have not observed effects of testosterone, neither on the expression of the mRNAs nor on the production and secretion of immunoreactive inhibin. Effects of testosterone on the secretion of bioactive inhibin could not be assessed because testosterone interfered with the *in vitro* bioassay. It cannot be excluded that testosterone may affect the glycosylation of the inhibin protein moiety, and thereby its bioactivity. The possible role of inhibin in the testes is discussed (chapter 4).

Germ cells probably do not contain a variety of specific binding sites for different hormones, growth factors and Sertoli cell secretion products. One exception is the transferrin receptor. The main function of extracellular transferrin is the intercellular transport of iron (free iron is toxic, but iron is bound to iron transport/storage proteins). Transferrin produced by Sertoli cells may serve this function in the spermatogenic epithelium. It was examined whether transferrin is involved in the uptake of iron by the developing germ cells. The results are described in chapter 5 and appendix paper 3. It was concluded that not only Sertoli cells, but also spermatocytes and spermatids are able to take up iron from exogenous transferrin. In the cells, the incorporated iron was subsequently bound to the iron storage protein ferritin. It is evident that transferrin can play a role in iron transport into spermatogenic cells, but possible other functions of transferrin production by Sertoli cells are discussed.

In chapter 6 and appendix paper 4, Sertoli cell-germ cell interactions are discussed. A culture system containing Sertoli cells and germ cells, which may be used to study these interactions, was evaluated. The results of the experiments indicated that in the culture system there was a small increase of the number of spermatids during three days of culture, although

there was no optimum quantitative survival of spermatocytes. A biochemical interaction between Sertoli cells and germ cells was observed with respect to amino acid metabolism. A certain degree of biochemical integrity of the germ cells was further indicated by an increase in the total lactate dehydrogenase-C_4 activity (LDH-C_4; an isoenzyme which is found exclusively in male germ cells) of the cultured tubule fragments. The possibility to use LDH-C_4 as a marker for the number of germ cells was investigated, and it was concluded that LDH-C_4 activity should be used for this purpose only in combination with other parameters.

In chapter 7 it is discussed whether germ cell development is directed by regulatory effects of Sertoli cells, or whether Sertoli cells provide mainly support and protection to the germ cells which may have an autonomous and fixed developmental programme. It is discussed that the latter possibility appears to be the most likely type of interaction of spermatocytes and spermatids with Sertoli cells.

In all available culture systems, there is no quantitative survival of spermatogenic cells, and no pronounced progression of spermatogenesis during prolonged culture. This could be explained by direct negative effects of culture conditions, which may be appropriate for many cell types but sub-optimal for testicular cells. It is also known that the degree of Sertoli cell maturation decreases during culture. This may result in a partial loss of differentiated functions, and impair the supporting and protecting capacity of the Sertoli cells. In this context, the secretion of androgen-binding protein (ABP), aromatase activity, and the expression of inhibin mRNAs as markers for Sertoli cell maturation are discussed in chapter 7.

The aim of the experiments described in this thesis concerns the mechanisms which are involved in the regulation of spermatogenesis. However, it should be kept in mind that results obtained using isolated and cultured cells may only approximate, rather than represent, the *in vivo* situation. The validity and relevance of *in vitro* model systems, to study the complex process of spermatogenesis, need to be supported by future work.

SAMENVATTING

De ontwikkeling van mannelijke zaadcellen (de spermatogenese) vindt plaats in de zaaddragende buisjes (de tubuli seminiferi) van de testikels. Voor het opstarten en het in stand houden van de spermatogenese zijn de hormonen follikel-stimulerend hormoon (FSH) en testosteron noodzakelijk.

De zich ontwikkelende zaadcellen (germinale cellen) (spermatogonia, spermatocyten en spermatiden; in volgorde van ontwikkeling) staan in de buisjes in nauw kontakt met steuncellen, Sertoli cellen genaamd. De Sertoli cellen zorgen door middel van een zogenaamde Sertoli cel-barrière ervoor dat de germinale cellen, nadat ze een bepaald ontwikkelingsstadium gepasseerd zijn, niet in direkt kontakt staan met het milieu buiten de buisjes (bloed-testis barrière). Uitscheidingsprodukten van Sertoli cellen kunnen derhalve een belangrijke rol spelen tijdens de spermatogenese.

De Sertoli cellen zijn door middel van specifieke hormoon-bindende eiwitten (receptoren) doelwit-cellen voor FSH en testosteron. De germinale cellen zelf hebben geen receptoren voor deze hormonen. De regulatie van de spermatogenese vindt waarschijnlijk plaats via effekten van hormonen op de Sertoli cellen, mogelijk ook met tussenkomst van de om de buisjes heen liggende peritubulaire cellen.

In hoofdstuk 1 is een korte beschrijving van de opbouw en de organisatie van de testikels en de spermatogenese gegeven en het doel van het werk beschreven in dit proefschrift wordt uiteengezet. Het doel is een beter inzicht te verkrijgen in de regulatie van de spermatogenese en de rol van Sertoli cellen hierbij. Hiervoor kan mogelijk gebruik gemaakt worden van uit de testikels geisoleerde en gekweekte Sertoli cellen en germinale cellen.

In hoofdstuk 2 wordt ingegaan op de regulatie van de spermatogenese en de mogelijke rol van de Sertoli cellen bij de spermatogenese. Een aantal biochemische eigenschappen van Sertoli cellen zijn beschreven, evenals een aantal mogelijke manieren om deze cellen te kweken, met hun voor,- en nadelen.

Een beschrijving van de isolatie en kweek van de in dit onderzoek gebruikte celpreparaten is gegeven in hoofdstuk 3. Tevens is een methode beschreven om RNA (ribonucleinezuur) uit testikelweefsel en Sertoli celkweken te isoleren en te analyseren. Deze techniek werd gebruikt bij het onderzoek zoals beschreven in hoofdstuk 4 en in de bijgevoegde artikelen 1 en 2.

De hormonale regulatie van de produktie en sekretie van een aantal Sertoli celprodukten is beschreven in hoofdstuk 4. De produktie en sekretie van een aantal van deze eiwitten kan dankzij recente ontwikkelingen bestudeerd worden op de niveaus van boodschapper-RNA (mRNA), eiwitproduktie en de aanwezigheid van biologisch aktieve eiwitten. De resultaten van dergelijk onderzoek betreffende inhibine, zijn beschreven in hoofdstuk 4 en bijgevoegde artikelen 1 en 2. Inhibine, een lid van de TGF-β (transformerende groeifactor-β) familie van groeifactoren, is opgebouwd uit twee verschillende subeenheden, α en β. De verkregen resultaten suggereren dat FSH in de Sertoli cellen de hoeveelheid van het mRNA van de α-subeenheid, de hoeveelheid immunologisch aktief inhibine en de hoeveelheid inhibine die daadwerkelijk biologisch aktief is kan stimuleren op een dosis-afhankelijke manier. Er werd geen effekt gevonden van FSH op de hoeveelheid β-subeenheid mRNA. Testosteron had geen aantoonbaar effekt op de hoeveelheid mRNA en het immunologisch aktief inhibine. Effekten op de hoeveelheid biologisch aktief inhibine konden niet worden bepaald, aangezien testosteron storend werkt in de bepalings-methode. De mogelijke rol van inhibine in de testikels is bediscussieerd.

Er zijn vrijwel geen receptoren voor gesekreteerde Sertoli cel-eiwitten op germinale cellen waargenomen. Een uitzondering hierop is de transferrine-receptor. De bekendste funktie van transferrine, dat zich buiten de cellen bevindt, is het transport van ijzer tussen verschillende cellen. Transferrine dat geproduceerd wordt door Sertoli cellen zou deze funktie in het spermatogenetisch epitheel kunnen vervullen. Experimenten om te bepalen of transferrine een rol speelt bij het transport van ijzer van buiten de Sertoli cel-barriere naar de germinale cellen zijn beschreven in hoofdstuk 5 en bijgevoegd artikel 3. Er werd gekonkludeerd dat niet alleen Sertoli cellen, maar ook germinale cellen (spermatocyten en spermatiden) ijzer op kunnen nemen als dat gebonden aan transferrine aangeboden wordt. Het opgenomen ijzer wordt gekoppeld aan een ijzer opslageiwit, ferritine. Het is duidelijk dat transferrine mogelijk een rol speelt bij ijzertransport naar germinale cellen. Eventuele andere funkties van het Sertoli cel transferrine zijn bediscussieerd.

In hoofdstuk 6 en bijgevoegd artikel 4 worden Sertoli cel-germinale cel interakties besproken. Om deze interakties te kunnen bestuderen werd een kweeksysteem met Sertoli cellen en germinale cellen in detail uitgewerkt. De resultaten van de experimenten, uitgevoerd met dit kweeksysteem, lieten een

geringe toename zien van het aantal spermatiden gedurende drie dagen in kweek. Echter, het aantal spermatocyten nam af. Een interaktie tussen Sertoli cellen en de germinale cellen op stofwisselingsgebied (aminozuur metabolisme) werd waargenomen. Een zekere mate van biochemische integriteit van de germinale cellen werd nog eens benadrukt door een toename in de laktaatdehydrogenase-C_4 ($LDH-C_4$) aktiviteit tijdens de kweek ($LDH-C_4$ is een isoenzym dat uitsluitend in mannelijke germinale cellen voorkomt). De mogelijkheid om $LDH-C_4$ aktiviteit te gebruiken als maat voor het aantal germinale cellen werd onderzocht en er werd gekonkludeerd dat $LDH-C_4$ als zodanig alleen in kombinatie met andere gegevens gebruikt mag worden.

In hoofdstuk 7 wordt beredeneerd of de germinale cellen voor hun ontwikkeling afhankelijk zijn van regulerende effekten uitgeoefend door Sertoli cellen, of dat de Sertoli cellen voornamelijk als steun en bescherming dienen voor de germinale cellen die mogelijk een vaststaand en autonoom ontwikkelingsprogramma doormaken. Het is bediscussieerd dat deze laatste interaktie tussen spermatocyten en spermatiden het meest waarschijnlijk lijkt.

In alle tot nu toe bekende kweeksystemen is er geen kwantitatieve overleving van het aantal spermatogene cellen, noch een duidelijke vooruitgang van de spermatogenese tijdens langdurig kweken. Dit zou verklaard kunnen worden door direkte negatieve effekten van kweekomstandigheden, die mogelijk optimaal zijn voor veel andere celtypen, op de germinale cellen. Het is echter ook bekend dat de mate van rijping van de Sertoli cellen afneemt tijdens kweek, hetgeen een gedeeltelijk verlies van gedifferentieerde Sertoli cel funkties kan betekenen. Dit kan het ondersteunende effekt van Sertoli cellen op de germinale cellen aantasten. In hoofdstuk 7 wordt deze Sertoli celrijping besproken, aan de hand van de produktie van het androgeen-bindend eiwit (ABP), de aktiviteit van het aromatase enzym en de expressie van inhibine mRNAs.

De in dit proefschrift beschreven experimenten kunnen mogelijk een bijdrage leveren aan de opheldering van de mechanismen betrokken bij de regulatie van de spermatogenese. Men dient echter rekening te houden met het gegeven dat de resultaten, gevonden met geisoleerde en gekweekte cellen, de werkelijke situatie <u>in vivo</u> nooit meer dan ten dele benaderen. Het belang van kweekmodellen, zeker waar het zoals hier een zeer complex systeem (de spermatogenese) betreft, zal door toekomstige experimenten bepaald moeten worden.

CURRICULUM VITAE

De schrijfster van dit proefschrift werd op 28 januari 1960 te Eindhoven geboren. Het diploma Gymnasium-β werd behaald in 1978 aan het Van Maerlant Lyceum te Eindhoven. In hetzelfde jaar werd begonnen met de studie Biologie aan de Rijks Universiteit te Utrecht, alwaar in 1981 het kandidaatsexamen Medische Biologie (B5*) werd afgelegd. Het doctoraalexamen, afgelegd in 1984, omvatte de hoofdvakken Functionele Neuromorfologie en Histologie (Dr. J. Diederen en Dr. W. Flight) en Immunologie (Prof. Dr. J. Willers) en het bijvak Hematologie en Medische Enzymologie (Prof. Dr. G. Staal en Dr. J.-W. Akkerman). Van 1 juli 1984 tot 1 december 1987 was zij als wetenschappelijk assistent aangesteld op de afdeling Biochemie II (Chemische Endocrinologie), alwaar het onderzoek, beschreven in dit proefschrift, werd uitgevoerd. Vanaf juli 1989 is zij als medisch bioloog betrokken bij vruchtbaarheidsonderzoek aan de afdeling gynaecologie, Diaconessenhuis Eindhoven.

Rapid Communication

Effects of FSH and IGF-I on immature rat Sertoli cells: inhibin α- and β-subunit mRNA levels and inhibin secretion

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Summary

Effects of follicle-stimulating hormone (FSH) and insulin-like growth factor-I (IGF-I) on inhibin production by cultured Sertoli cells from 21- to 23-day-old rats were studied. The expression of inhibin α- and β-subunit mRNAs, and inhibin immunoreactivity and in vitro bioactivity were estimated. Using a cDNA probe corresponding to the α-subunit of bovine inhibin, specific hybridization with a 1.5–1.7 kilobase (kb)mRNA species was observed. Addition of FSH to the cultured Sertoli cells for 24 h markedly increased the level of this mRNA in a dose-dependent way. IGF-I had no effect on the intensity of the hybridization. Using a cDNA probe corresponding to the β_B-subunit of human inhibin, 3.5 and 4.2 kb mRNA species were detected. FSH and IGF-I had no effect on the hybridization signal. No hybridization was observed with a cDNA probe corresponding to the β_A bovine inhibin subunit. Inhibin activity was detected in cells and medium by immunoassay, and in the medium by in vitro bioassay. FSH stimulated both immunoreactivity and in vitro bioactivity, whereas IGF-I had no effect at all. The present effect of FSH on inhibin α-subunit mRNA expression in cultured Sertoli cells indicates that regulation of inhibin production by FSH includes an effect at the transcriptional level. However, this does not exclude additional translational and posttranslational effects.

Introduction

Inhibin can be defined as a protein hormone which acts on the pituitary, preferentially inhibiting the production of FSH. Inhibin is produced and secreted by Sertoli cells (Steinberger and Steinberger, 1976) and granulosa cells (Erickson and Hsueh, 1978). Inhibin is composed of two partially homologous subunits, α and β, linked through disulfide bridges. The α-subunit is the product of one gene, whereas there are two separate genes encoding two forms of the β-subunit, β_A and β_B. Dimers of inhibin β-subunits, $\beta_A\beta_A$ and $\beta_A\beta_B$, have been shown to be present in gonadal fluids. These dimers were termed activins, because they can stimulate the release of FSH from cultured pituitary cells (Ling et al., 1986; Vale et al., 1986).

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Factors which regulate the synthesis and the release of inhibin by Sertoli cells have been studied (see for review de Jong, 1988). However, these studies do not include information on the levels of the inhibin subunit mRNAs.

cDNA probes corresponding to the mRNAs of the α-, β_A- and β_B-subunits of inhibin have become available recently (Mason et al., 1985; Forage et al., 1986), providing a tool for estimation of the expression of the α- and β-subunit mRNAs.

Sertoli cells are target cells for FSH and insulin-like growth factor-I (IGF-I). Effects of FSH and IGF-I on Sertoli cells are mediated via specific binding sites for FSH and IGF-I (Means et al., 1980; Borland et al., 1984; Oonk and Grootegoed, 1988).

The aim of the present study was to obtain information on the effects of FSH and IGF-I on inhibin production. The study involves estimation of the mRNA levels of the α- and β-subunits in cultured Sertoli cells, and estimation by RIA and bioassay of the amounts of intracellular and secreted inhibin.

Materials and methods

Isolation and incubation of Sertoli cells. Sertoli cells were isolated from testes of 21- to 23-day-old rats (Wistar, substrain RI Amsterdam), using collagenase as described by Oonk and Grootegoed (1988). The Sertoli cell preparations were incubated for 48 h at 37 °C in Eagle's minimum essential medium (MEM) supplemented with 1% fetal calf serum (FCS). Subsequently, the cells were incubated for 24 h at 37 °C in MEM without FCS but containing 0.1% (w/v) bovine serum albumin (BSA). After this 3-day incubation period the cells were incubated for 24 h in MEM with 0.1% BSA, in the absence or presence of ovine FSH (NIH S16) or IGF-I (Amersham International, Amersham, U.K.). Subsequently, the media were collected and the cell monolayers were frozen and stored for RNA extraction. Cells from parallel incubations were solubilized using MEM containing 0.1% BSA and 0.1% Triton X-100. The media and the Triton-dissolved cells were freeze-dried, and the inhibin immuno- and bioactivities were estimated as described below.

RNA isolation and analysis. Cells or tissues were lysed and RNA was separated from other cellular components as described by Glišin et al. (1974).

Poly(A)$^+$ RNA from total testis RNA was prepared by oligo(dT) chromatography (Aviv and Leder, 1972). RNA was denatured using the glyoxal and dimethyl sulfoxide (DMSO) method, and analysed by agarose gel electrophoresis (Maniatis et al., 1982). The RNA was transferred to nylon filters (Gene-Screen, NEN-Dupont, Boston, MA, U.S.A.) by overnight diffusion as described by the suppliers. The blots were hybridized overnight at 42 °C with ^{32}P-oligo-labeled cDNA probes (Feinberg and Vogelstein, 1983) in the presence of formamide (44%, v/v), using standard conditions. Filters were washed at 50 °C with several changes of 0.1 × SSC (standard saline citrate; 1 × SSC contains 0.15 M NaCl and 0.015 M trisodium citrate), containing 0.1% sodium dodecyl sulfate, and exposed to X-ray film (Hyperfilm-MP, Amersham International, Amersham, U.K.) for 3–36 h at −80 °C, using intensifyer screens.

The cDNA probes, used in these studies, were a 480 bp cDNA fragment corresponding to the α-subunit mRNA of bovine inhibin, a 360 bp cDNA fragment corresponding to the β_A-subunit mRNA of bovine inhibin, and a 920 bp cDNA fragment corresponding to the β_B-subunit of human inhibin. Control hybridizations were performed using a hamster actin cDNA probe (Dodemont et al., 1982). Before rehybridization, the hybridized probe was removed from the blot as described by Thomas (1980).

Radioimmunoassay and in vitro bioassay of inhibin. The RIA of inhibin in Sertoli cell media and cell lysates was performed essentially as described by McLachlan et al. (1987). Inhibin in vitro bioactivity was estimated using a rat pituitary cell culture system as described by Scott et al. (1980). A rat ovarian extract (JU-3) obtained from PMSG-stimulated immature rats was used as standard in both RIA and in vitro bioassay. Results are expressed in terms of an ovine testicular lymph standard preparation with an arbitrary unitage of 1 unit per mg protein.

Estimation of lactate and cellular protein. The method of Hohorst (1970) was used to estimate the amount of lactate in the spent incubation

medium. Sertoli cell protein was measured using BSA as standard (Lowry et al., 1951).

Results

Using total RNA from cultured Sertoli cells, a specific hybridization to a 1.5–1.7 kb mRNA species was observed with the inhibin α-subunit cDNA probe, and to 3.5 and 4.2 kb mRNA species with the inhibin β_B-subunit cDNA probe. Kidney RNA did not contain detectable levels of α- and β-subunit mRNA (Fig. 1*a* and 1*b*). Using the bovine β_A-subunit probe, no specific hybridization with testis RNA was detected. For total RNA from bovine granulosa cells, a specific hybridization of the β_A-probe was observed to a 1.5 kb mRNA species (results not shown).

Poly(A)$^+$ RNA was not isolated from the cultured Sertoli cells, Using poly(A)$^+$ RNA from total testis tissue, it was observed that the α- and β_B-probes hybridized with the same RNA species described above. The 4.2 kb β_B-mRNA species was also detected in the poly(A)$^+$ RNA fraction (Fig. 1*c*), indicating that the 4.2 kb band does not merely reflect cross-hybridization with 28S rRNA.

In the present experiments the cultured Sertoli cells were responsive to FSH and IGF-I, as indicated by a 2- to 3-fold stimulation of lactate production after 6 h of incubation with the hormones (not shown). Similar effects of FSH and IGF-I have been described by Oonk and Grootegoed (1987).

A dose-dependent increase in the amount of 1.5–1.7 kb α-subunit mRNA was observed after incubation of the Sertoli cells in the presence of FSH for 24 h (half-maximal stimulation at approximately 5 ng FSH/ml). IGF-I (100 ng/ml) had no effect on the level of 1.5–1.7 kb α-subunit mRNA, either in the absence (Fig. 2*a*) or presence (data not shown) of 5 ng/ml FSH. Addition of FSH and IGF-I to the Sertoli cell culture medium for 24 h did not influence the amount of β_B-mRNA (Fig. 2*b*).

All blots were also probed with actin. This control showed that approximately equal amounts of mRNA were present in the different lanes of the blots (data not shown). Therefore, the observed effect of FSH on the amount of α-subunit mRNA represents a rather selective effect of the

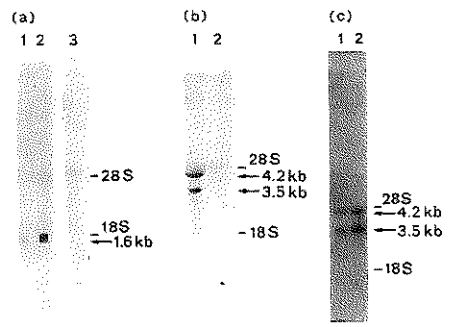

Fig. 1. (*a*) Northern blot analysis of RNA using the inhibin α-subunit cDNA probe. Lanes 1 and 2 contain 40 μg total RNA from Sertoli cells, which had been incubated for 24 h either in the absence or presence of 500 ng/ml FSH, respectively. Lane 3 contains 40 μg total RNA from kidney tissue. (*b*) Northern blot analysis of RNA using the inhibin β_B-subunit cDNA probe. Lane 1 contains 40 μg total RNA from cultured Sertoli cells and lane 2 contains 40 μg total RNA from kidney tissue. (*c*) Hybridization of the inhibin β_B-subunit cDNA probe to 5 μg (lane 1) or 10 μg (lane 2) poly(A)$^+$ RNA from total rat testis tissue.

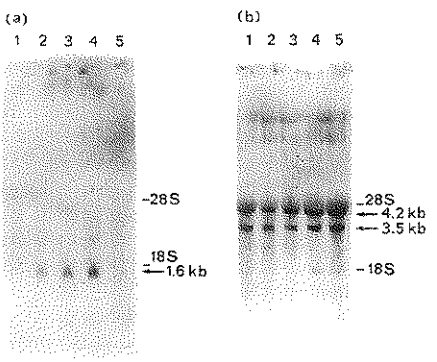

Fig. 2. (*a*) Effects of FSH and IGF-I on inhibin α-subunit mRNA levels. Sertoli cells were incubated for 24 h either in the absence (lane 1) or presence of 5, 50 and 500 ng/ml FSH (lanes, 2, 3 and 4), or in the presence of 100 ng/ml IGF-I (lane 5). Each lane contained 40 μg of total RNA from the cultured Sertoli cells. (*b*) Effects of FSH and IGF-I on inhibin β_B-subunit mRNA levels. Sertoli cells were incubated for 24 h without hormones (lane 1), in the presence of 5 and 500 ng/ml (FSH (lanes 2 and 3), 100 ng/ml IGF-I (lane 4), or 5 ng/ml FSH plus 100 ng/ml IGF-I (lane 5). Each lane contained 40 μg of total RNA from the cultured Sertoli cells.

TABLE 1

IMMUNOREACTIVE AND BIOACTIVE INHIBIN PRESENT IN OR SECRETED BY CULTURED SERTOLI CELLS

Sertoli cells were cultured for 72 h in the absence of hormones, and subsequently for 24 h in the absence or presence of the indicated hormones (500 ng/ml FSH or 100 ng/ml IGF-I). At the end of this incubation, aliquots from medium and cells were assayed for inhibin immunoreactivity and in vitro bioactivity (see Materials and Methods). The data represent the mean ± SD of multiple incubations (the number of incubations is shown between brackets).

Treatment	Immunoreactive inhibin (U/mg protein)		Bioactive inhibin (U/mg protein)	
	Secreted (I)	Intracellular	Secreted (B)	B : I ratio
Control	50.3 ± 6.4 (12)	14.0 ± 5.3 (7)	94.7 ± 31.6 (12)	1.9 ± 0.8
FSH	217.0 ± 49.1 (9) *	113.5 ± 14.0 (7) *	155.6 ± 31.0 (9) *	0.7 ± 0.2 *
IGF-I	50.9 ± 15.2 (6)	11.1 ± 1.8 (8)	64.9 ± 21.6 (6)	1.2 ± 0.6

* Significantly different ($P < 0.01$) from control group (Student's t-test).

hormone treatment on the expression of specific mRNAs.

The cultured Sertoli cells secreted immunoreactive and in vitro bioactive inhibin. Stimulation of the secretion of bioactive inhibin by FSH but not by IGF-I was observed (Table 1). Moreover, FSH strongly stimulated the amounts of intracellular and secreted immunoreactive inhibin, whereas there was no effect at all of IGF-I (Table 1). The ratio bioactive inhibin/immunoreactive inhibin (B : I ratio) of the secreted inhibin was lowered by FSH treatment (Table 1).

Discussion

Very little α-subunit mRNA was detected in Sertoli cells incubated in the absence of FSH, whereas marked hybridization was observed using the β_B-probe. Because heterologous probes were used for hybridization there is as yet no information with respect to the absolute amounts of the α- en β-subunit mRNAs.

Using the inhibin β_A-subunit cDNA probe, no specific hybridization was observed in the present experiments for testis RNA. This is in agreement with data from Esch et al. (1987). The β_A-cDNA was isolated from an ovarian cDNA library, indicating that the β_A-gene is expressed in ovaries. It can be suggested that there are differences between the production of the inhibin β_A- and β_B-subunits in female and male mammals. Furthermore, it has been described that both α- and β_A-subunit mRNA levels in ovaries are stimulated by pregnant mare serum gonadotrophin (Davis et al., 1986) and by FSH (Meunier et al., 1987). This indicates that, at the transcriptional level, β_A- and β_B-subunits of inhibin are regulated in a different way in ovaries and testis, respectively.

A stimulatory effect of FSH but not of IGF-I on the secretion of immunoreactive and in vitro bioactive inhibin was observed. For granulosa cells, however, it has been found that inhibin secretion, determined using an α-subunit directed RIA and an in vitro bioassay, was increased by IGF-I (Bicsak et al., 1986; Zhiwen et al., 1987). These results indicate different effects of IGF-I on inhibin secretion by granulosa cells and Sertoli cells.

The decrease in B : I ratio following FSH stimulation can be attributed either to an increased production of activin which interferes with the inhibin in vitro bioassay, or to an increased production of immunologically active substances with reduced biological activities. Bovine inhibin subunits and activin do not cross-react in the RIA (McLachlan et al., 1987). A larger form of inhibin (58 kDa) with a N-terminally extended α-chain has been identified in bovine (Robertson et al., 1985) and ovine (Leversha et al., 1987) follicular fluids. Based on the sequence of rat inhibin (Esch et al., 1987) an inhibin molecule of similar size could be present in rat gonads. This large form of inhibin may have an intrinsically B : I ratio, and its level may increase following FSH stimulation.

The present data clearly indicate that FSH stimulates the inhibin α-subunit mRNA expression. This could involve enhanced transcription and/or stabilization of the mRNA. The β-gene is expressed constitutively, under the conditions

used, and there was no effect of FSH on the expression of β-subunit mRNA. The selective effect of FSH on inhibin α-subunit mRNA expression does not exclude, however, that FSH also regulates inhibin production at the translational or posttranslational level. Further studies will include quantitative aspects of transcription, translation, and subunit assembly.

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APPENDIX PAPER 2

Effects of FSH and testosterone on highly purified rat Sertoli cells: inhibin α-subunit mRNA expression and inhibin secretion are enhanced by FSH but not by testosterone

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ABSTRACT

The effects of FSH and testosterone on inhibin mRNA expression and inhibin production by highly purified Sertoli cell preparations were examined. Sertoli cells were isolated from testes of 22-day-old rats by sequential trypsin, collagenase and hyaluronidase treatments, with subsequent osmotic shock treatment on day 3 of culture. Contamination by peritubular and germ cells was <0·5 and 1–3% respectively. Intracellular and secreted inhibin levels were measured by radioimmunoassay, using Sertoli cells which were incubated for 24 h in the absence or presence of FSH and testosterone from days 4 to 5 of culture. FSH stimulated the cellular inhibin content and the secreted inhibin level by four- and sevenfold respectively, with a half-maximal effective dose of 5–50 ng/ml. Under the present incubation conditions, testosterone (1 μmol/l) had no effect on immunoreactive inhibin levels in either the presence or absence of FSH. Similarly, the expression of inhibin α-subunit mRNA was increased following FSH stimulation, whereas testosterone had no effect. The expression of inhibin β_B-subunit mRNAs was not influenced by FSH or testosterone.

It is concluded that highly purified Sertoli cell preparations, with a very low number of peritubular or germ cells, are fully responsive to FSH with respect to inhibin mRNA expression and inhibin production. *Journal of Endocrinology* (1989) **122,** 757–762

INTRODUCTION

Inhibin, which can suppress secretion of follicle-stimulating hormone (FSH) by pituitary gonadotrophic cells, has been isolated from ovarian and testicular sources (Robertson, Foulds, Leversha *et al.* 1985; de Jong, Grootenhuis, Sander *et al.* 1988) and its sequence has been determined by cloning techniques (Mason, Hayflick, Ling *et al.* 1985). Structurally, inhibin is a heterodimeric glycoprotein of molecular mass 30–32 kDa, consisting of the partially homologous α- and β-subunits. Inhibin ββ-subunit dimers have been identified in follicular fluid, and were termed activins because these homodimers stimulate FSH release by pituitary cells (Ling, Ying, Ueno *et al.* 1986; Vale, Rivier, Vaughan *et al.* 1986). Production of activins by Sertoli cells has also been reported (Grootenhuis, Steenbergen, Timmerman *et al.* 1989).

Sertoli cells and seminiferous tubule segments in culture secrete inhibin, as estimated by in-vitro bioassay and immunoassay, and the secretion can be stimulated by FSH (Gonzales, Risbridger & de Kretser, 1988; Toebosch, Robertson, Trapman *et al.* 1988). Observations on the effects of testosterone on inhibin production, however, are controversial, with testosterone either stimulating (Verhoeven & Franchimont, 1983) or inhibiting (Ultee-van Gessel, Leemborg, de Jong & van der Molen, 1986) bioactive inhibin levels. Bicsak, Vale, Vaughan *et al.* (1987) did not observe an effect of testosterone on the production of immunoreactive inhibin by cultured Sertoli cells.

It has been reported that the testicular peritubular cells, which are found as contaminating cells in Sertoli cell cultures, are able to produce factors (P-Mod-S) under androgen stimulation which influence Sertoli cell function (Skinner & Fritz, 1985*a*). In addition, contaminating germ cells may influence the secretion

Journal of Endocrinology (1989) **122,** 757–762 *Printed in Great Britain*
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of certain Sertoli cell products. Secretion of androgen-binding protein by Sertoli cells, for example, is enhanced when Sertoli cells are co-cultured with germinal cells (Galdieri, Monaco & Stefanini, 1984; Le Magueresse & Jégou, 1988). From this, it would appear that the presence of contaminating peritubular and germinal cells may influence the effects of hormones on cultured Sertoli cells.

We have previously reported the effects of FSH on inhibin production and the expression of inhibin mRNAs in partly purified Sertoli cells (Toebosch *et al.* 1988). The aim of the present study was to re-examine the effects of FSH and testosterone on inhibin mRNA expression and inhibin production by highly purified Sertoli cell preparations. A procedure for isolating Sertoli cells has been used which results in Sertoli cell preparations of high purity, with low numbers of contaminating peritubular and germ cells.

MATERIALS AND METHODS

Materials

Collagenase (174 U/mg) and trypsin from bovine pancreas (24 U/mg) were obtained from Worthington (Freehold, NJ, U.S.A.). Eagle's Minimal Essential Medium (MEM) was obtained from Gibco (Grand Island, NY, U.S.A.). DNase (code DN-25), bovine serum albumin fraction V (BSA) and hyaluronidase from bovine testes (Type 1-S) were from Sigma (St Louis, MO, U.S.A.). Ovine FSH (NIH-S13 or -S16) was a gift from NIH (Bethesda, MD, U.S.A.). Testosterone was purchased from Steraloids (Wilton, NH, U.S.A.). All other chemicals (analytical grade) were obtained from commercial sources.

Sertoli cell isolation

Highly purified Sertoli cells were isolated from 21- to 23-day-old Wistar rats (substrain RI; Amsterdam, The Netherlands) using a combination and modification of the methods of Galdieri, Ziparo, Palombi *et al.* (1981), Oonk, Grootegoed & van der Molen (1985) and Skinner & Fritz (1985*b*) as follows. Twelve testes were decapsulated and chopped at 0·5 mm in two perpendicular directions. The fragments were treated for 30 min with 20 mg trypsin and 0·4 mg DNase in 20 ml Dulbecco's phosphate-buffered saline (PBS) (Dulbecco & Vogt, 1954). Subsequently, the fragments were allowed to sediment at unit gravity, washed three times in PBS containing 0·5% (w/v) DNase and subjected to a double collagenase treatment with 10 and 20 mg collagenase in 20 ml PBS containing 0·1 mg DNase, for 5 and 30 min respectively. After the first collagenase treatment the fragments were allowed to settle and the supernatant was removed. After the second treatment the fragments were allowed to settle and washed three times in PBS with DNase. The supernatant of the second collagenase treatment yielded the peritubular cell cultures. Subsequently, a Dounce treatment (12 strokes) of the fragments was performed to remove most of the spermatogenic cells (Oonk *et al.* 1985). The resulting smaller fragments were washed three times in PBS containing DNase for 2 min at 100 *g*. Finally, the dispersed fragments were treated for 30 min with 20 mg hyaluronidase and 0·1 mg DNase in 20 ml PBS. All enzyme digestions were carried out in a 100 ml sylanized Erlenmeyer flask in a shaking water bath (120 cycles/min) at 37 °C.

The final preparation was washed five times in PBS with DNase (centrifugations for 2 min at 100 *g*) and the cells were seeded in MEM in 12- or 24-well plates, or in 25 cm^2 (50 ml) culture flasks, at a density of 25–30 μg protein/cm^2. The cells were cultured for 48 h in MEM supplemented with 1% (v/v) fetal calf serum (FCS). Subsequently, most of the remaining spermatogenic cells were removed by hypotonic shock treatment with 10% MEM in water for 2·5 min (Galdieri *et al.* 1981), unless otherwise indicated, and the culture was continued for another 24 h in MEM supplemented with 0·1% (w/v) BSA. At the end of the 3-day preincubation period the cells were incubated for different times in MEM with 0·1% BSA in the absence or presence of FSH and/or testosterone.

Quantitative and qualitative analysis of the cultured cells

After 4 days of culture the Sertoli cells were fixed with Bouin's fixative and stained with haematoxylin for 5 min. Sertoli, peritubular and germ cell nuclei were identified by their distinctive nuclear morphology (Tung, Skinner & Fritz, 1984).

Peritubular cell contamination of the Sertoli cell preparations was estimated using alkaline phosphatase activity, which can be detected in peritubular cells but not in Sertoli cells isolated from rat testis (Chapin, Phelps, Miller & Gray, 1987; Blok, Mackenbach, Trapman *et al.* 1989), according to the method of van Duyn, Pascoe & van de Ploeg (1967).

Estimation of lactate and cellular protein and DNA

The amount of lactate in spent medium of Sertoli cells was estimated according to the method of Hohorst (1970). Sertoli cell protein was measured by the method of Lowry, Rosebrough, Farr & Randall (1951) using BSA as a standard. The DNA content was measured using the fluorescent dye 4′,6-diamidino-2-phenylindole as described previously (Toebosch, Kroos & Grootegoed, 1987).

RNA isolation and analysis

Total RNA from Sertoli cells was isolated using a guanidinium thiocyanate–phenol extraction procedure as described previously (Toebosch *et al.* 1988). The RNA samples were electrophoresed and analysed by Northern blotting using a 480 bp cDNA fragment corresponding to the α-subunit of bovine inhibin and a 920 bp cDNA fragment corresponding to the β_B-subunit of human inhibin (Toebosch *et al.* 1988).

Radioimmunoassay (RIA) and bioassay of inhibin

Inhibin immunoactivity was measured in Sertoli cell media and Triton-lysed cells by RIA (Robertson, Hayward, Irby *et al.* 1988). Media and cell extracts were lyophilized and reconstituted with water before assay. The RIA consisted of a rabbit antiserum raised against purified bovine 31 kDa inhibin, and iodinated 31 kDa bovine inhibin as tracer. This RIA showed minimal cross-reactivity with activin and isolated α- and β-subunits following reduction and alkylation of inhibin. Data are expressed as U/mg protein. Inhibin bioactivity was estimated in the media by an in-vitro bioassay as described by Scott, Burger & Quigg (1980).

In both RIA and bioassay, a rat ovarian extract obtained from pregnant mare serum gonadotrophin-stimulated rats was used as standard, which was calibrated in terms of an ovine testicular lymph standard preparation with an arbitrary unitage of 1 unit/mg protein (Robertson *et al.* 1988).

Statistical procedure

Student's *t*-test was used to calculate *P* values.

RESULTS

Sertoli cell cultures

The purity of the Sertoli cell cultures was assessed using light microscopic techniques (Tung *et al.* 1984) and histochemical localization of alkaline phosphatase activity (Blok *et al.* 1989). Using these procedures, it was observed that peritubular cell contamination was $<0{\cdot}5\%$ and germ cell contamination 1–3% after 4 days of culture.

A decrease in the total amounts of cellular protein and DNA per well ($90{\cdot}3 \pm 17{\cdot}3$ and $78{\cdot}7 \pm 10{\cdot}7\%$ from the starting value respectively; mean ± S.D. of three different experiments; not significantly different and $P<0{\cdot}05$ respectively) was observed after 5 min of osmotic shock treatment but not after 1 or 2·5 min ($104{\cdot}1 \pm 2{\cdot}4$, $100{\cdot}1 \pm 14{\cdot}9$ and $95{\cdot}2 \pm 9{\cdot}9$, $93{\cdot}8 \pm 15{\cdot}9\%$ respectively).

Basal lactate production as well as the responsiveness of the Sertoli cells to half-maximal doses of FSH (50 ng/ml) were unaffected by the osmotic shock treatment (not shown). Testosterone (1 μmol/l) had no effect on lactate production (not shown).

On the basis of these results, the 2·5-min period of osmotic shock was used in all further experiments.

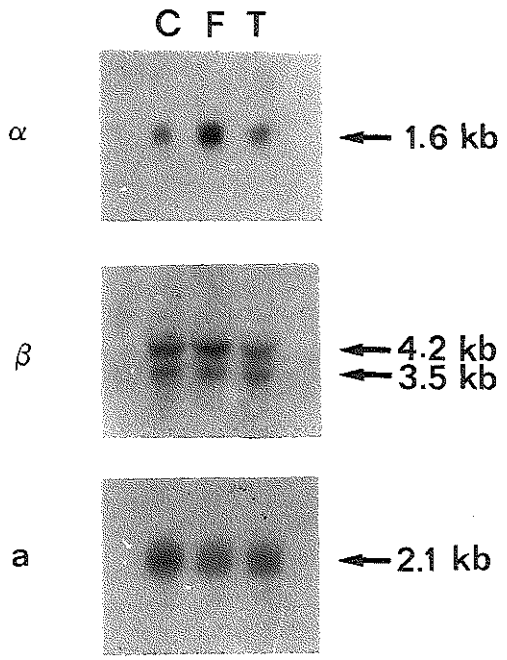

FIGURE 1. Northern blot analysis of the expression of inhibin (*a*) α-subunit mRNA, (*b*) β_B-subunit mRNA and (*c*) actin in rat Sertoli cell cultures. After a 3-day preincubation period the cells were incubated for 24-h in the absence (C) and presence of FSH (500 ng/ml; F) or testosterone (1 μmol/l; T).

Inhibin mRNA expression

The expression of inhibin α-subunit mRNA by the highly purified Sertoli cells was enhanced by FSH, whereas testosterone had no effect (Fig. 1*a*). The response to FSH shown in the figure was observed after 24 h, but an increased mRNA level was also detected 6 h after addition of FSH (not shown). There

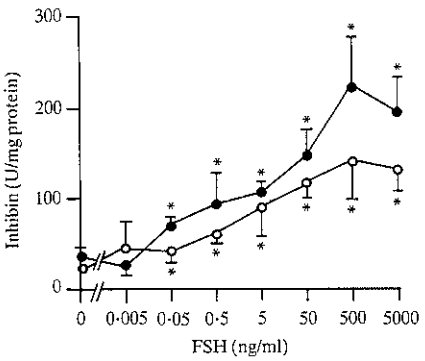

FIGURE 2. Dose–response curves of the effect of FSH on immunoreactive inhibin levels present in (○) or secreted by (●) cultured rat Sertoli cells. After a 3-day preincubation period the cells were incubated for 24 h with various doses of FSH. The data represent means ± S.D. of triplicate incubations. $^*P < 0{\cdot}01$ compared with incubations in the absence of FSH (Student's *t*-test).

were no effects of FSH and testosterone on β_B-subunit mRNA expression (Fig. 1*b*).

Inhibin immunoreactivity

The amounts of immunoreactive inhibin which were present in or secreted by the highly purified Sertoli cells were increased by FSH in a dose-dependent manner. The half-maximal effective dose was between 5 and 50 ng/ml and the maximally stimulating dose was 500 ng/ml (Fig. 2).

After 6–8 h of incubation with 500 ng FSH/ml the cellular inhibin content reached a plateau of approximately 100–125 U/mg protein (Fig. 3). The rate of inhibin secretion was relatively low during the first 6 h and became maximal after 6–8 h (Fig. 3). The cellular and medium concentrations of inhibin in the control cultures after 24 h were 40·1 ± 8·7 and 77·1 ± 14·0 U/mg protein respectively. The intracellular and medium concentrations in the unstimulated cells varied considerably in different experiments. The estimated values were 29·8 ± 8·0 and 54·3 ± 19·3 U/mg protein for the intracellular and medium concentrations respectively (mean ± S.D., $n = 5$).

Addition of testosterone to the medium, alone or in combination with FSH, did not change the levels of immunoreactive inhibin in the Sertoli cell cultures (Fig. 4).

DISCUSSION

Using histological and histochemical techniques, it was shown that the Sertoli cell preparations used in the present experiments were only minimally contaminated with peritubular and germ cells (less than 0·5 and 1–3% respectively). Peritubular cell contamination was similar to that in the study of Tung *et al.* (1984).

Results on the expression of inhibin α- and β_B-subunit mRNAs after addition of FSH or testosterone in the present experiments were comparable with those obtained previously using Sertoli cell preparations with a higher contamination of peritubular

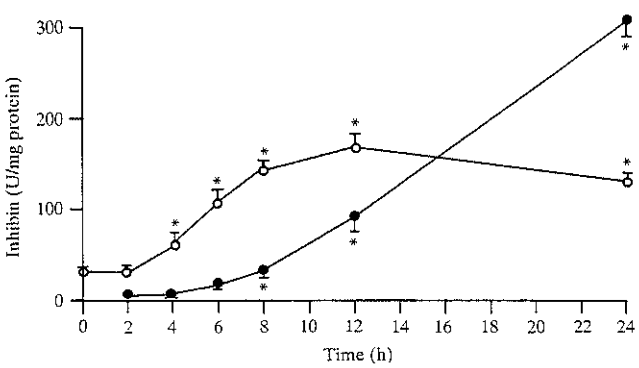

FIGURE 3. Time-course of the effect of FSH on immunoreactive inhibin levels present in (○) or secreted by (●) rat Sertoli cells. After a 3-day preincubation period the cells were incubated with 500 ng FSH/ml for different time-periods. The data represent means ± S.D. of triplicate incubations. $^*P < 0{\cdot}01$ compared with time 0 (Student's *t*-test).

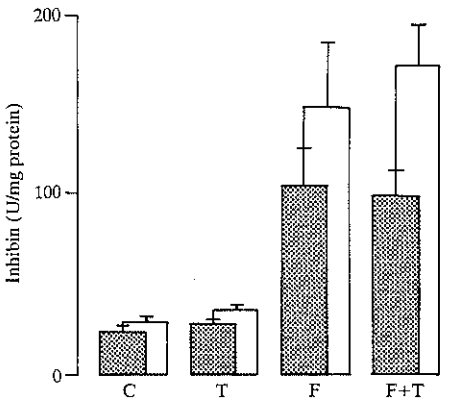

FIGURE 4. Effects of FSH and testosterone on immunoreactive inhibin levels present in (stippled bars) or secreted by (open bars) rat Sertoli cells. After a 3-day preincubation period the cells were incubated for 24 h with 500 ng FSH/ml (F), 1 μmol testosterone/l (T), a combination of both hormones (F + T) or in the absence of hormones (C). The data represent means ± S.D. of triplicate incubations.

and germ cells (Toebosch *et al.* 1988; A. M. W. Toebosch, unpublished results). This indicates that the observed effects of FSH on Sertoli cells are not modified by interactions with germ or peritubular cells.

Inhibin immunoreactivity in the Sertoli cells was stimulated within 6 h of addition of FSH, coinciding with the increased expression of inhibin α-subunit mRNA. A correlation between expression of inhibin α-subunit mRNA and inhibin immunoreactivity is further indicated by the FSH dose–response curves of mRNA expression and immunoreactive inhibin levels (Toebosch *et al.* 1988; present results).

During the first 8 h of FSH stimulation, the intracellular levels of immunoreactive inhibin increased, but there was almost no secretion of inhibin before 6 h. The relatively high intracellular inhibin concentration, compared with the low secretion rate, indicates that there was a delay in the secretion of newly synthesized inhibin. This delay could be associated with post-translational processing of the protein.

The present data do not provide evidence that the expression of α-subunit mRNA is the rate-limiting factor in the production of the inhibin αβ-heterodimer protein moiety. There is as yet no information on the efficiency of translation of the α- and β-subunit mRNAs. Furthermore, it cannot be excluded that developmental changes in the relative expression of the 4·2 and 3·5 kb β_B-subunit mRNAs (Klaij, Toebosch, Shimasaki *et al.* 1988) also play a critical role in inhibin production. Finally, nothing is known about the assembly of the heterodimer from the individual subunits and the possible production of single subunits.

Au, Robertson & de Kretser (1985) have suggested from bioassay data that FSH is the sole regulator of testicular inhibin levels *in vivo* after hypophysectomy of adult rats. Furthermore, in the present experiments there was no effect of testosterone on inhibin mRNA expression and the production of inhibin immunoreactivity by cultured Sertoli cells from immature rats. This observation is in accordance with data from Bicsak *et al.* (1987) on inhibin immunoreactivity. Other studies, however, have indicated that androgens may regulate bioactive inhibin production by cultured Sertoli cells (Steinberger, 1981; Verhoeven & Franchimont, 1983; Ultee-van Gessel *et al.* 1986). These discrepancies may not be explained by the use of different cell isolation methods, resulting in different purities of the cell preparations, because the present experiments indicate that there is no difference between highly purified and less pure Sertoli cell preparations. Possibly, differences in the in-vitro bioassay conditions (de Jong, 1988) may markedly influence the outcome of the experiments. In the present experiments the effects of testosterone on the secretion of bioactive inhibin by Sertoli cells could not be assessed, due to interference of testosterone with the inhibin in-vitro bioassay (not shown). Possible effects of testosterone on inhibin synthesis and secretion need to be further investigated. Effects of testosterone may require long-term exposure of the cultured cells to the hormone. Furthermore, the available data do not exclude the possibility that testosterone has an effect on inhibin bioactivity through regulation of post-transcriptional events involved in processing, assembly and glycosylation of the inhibin subunits.

ACKNOWLEDGEMENTS

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APPENDIX PAPER 3

Transport of transferrin-bound iron into rat Sertoli cells and spermatids

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Summary

Transferrin (Tf), a major secretory protein of Sertoli cells, may transport iron to spermatogenic cells. This was assessed by measuring the uptake of Fe from ^{59}Fe-^{125}I-labelled rat Tf by Sertoli cells and round spermatids *in vitro*. Uptake of Fe from labelled Tf by Sertoli cells after a 72-h pre-incubation period was linear for 20 h (approximately 18 pmol/10^6 cells/20 h), whereas the uptake of Fe from labelled Tf by round spermatids after a 16-h pre-incubation period reached a plateau by 2 h (approximately 5 pmol/10^6 cells/2 h). The corresponding net uptake of Tf by both cell types was <0.1 pmol. High speed supernatants prepared from Sertoli cells and spermatids labelled with ^{59}Fe-^{125}I-Tf were fractionated by gel permeation chromatography. Separate peaks of protein-bound ^{59}Fe and ^{125}I-Tf were observed. Protein bound ^{59}Fe could be precipitated with an antiserum to rat ferritin. It is concluded that iron from exogenous Tf is transported into Sertoli cells and round spermatids *in vitro*, and is complexed to intracellular ferritin. However, the present results do not exclude the possibility that Sertoli cell Tf may serve purposes other than iron transport.

Keywords: iron transport, transferrin, Sertoli cells, spermatids.

Introduction

The biochemical interactions between Sertoli and germ cells, which could play essential roles in spermatogenesis, are largely unknown. These cell–cell interactions could involve intercellular transport of diffusable compounds such as lactate, which is produced by Sertoli cells and may support ATP production by spermatocytes and spermatids (Robinson & Fritz, 1981; Jutte *et al.*, 1981, 1982). Likewise, proteins from Sertoli cells may exert important effects on germ cells. Sertoli cells from rats in culture produce a number of proteins, including androgen binding protein (ABP) and transferrin (Tf) (Wright *et al.*, 1981; Skinner & Griswold, 1982; Perez-Infante *et al.*, 1986).

Serum Tf transports iron to cells that require iron, i.e. rapidly growing cells and erythroid cells (Putnam, 1975; Egyed, 1982; May & Cuatrecasas, 1985). The uptake of iron by the cells involves binding of Tf to a specific Tf receptor at the cell

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surface (Egyed, 1982). Amino acid analysis and tryptic peptide mapping indicate that Sertoli cell Tf and serum Tf are essentially identical (Huggenvik, Sylvester & Griswold, 1985). The secretion of Tf by Sertoli cells can be stimulated by different hormones and also by retinol (Skinner & Griswold, 1982; Perez-Infante *et al.*, 1986).

Transferrin produced by Sertoli cells might be involved in the transport of iron from serum to the germ cells situated at the adluminal side of the blood–testis barrier. Sylvester & Griswold (1984) have suggested that serum Tf binds to receptors on the basal surface of Sertoli cells, while Tf from the Sertoli cells binds to surface receptors on the germ cells. This is supported by recent data, which indicate that Sertoli cells in culture secrete Tf from the adluminal (apical) part of the cell surface (Byers *et al.*, 1986; Janecki & Steinberger, 1986), and that specific binding sites for Tf are present on pachytene spermatocytes and round spermatids (Holmes *et al.*, 1983; Steinberger, Dighe & Diaz, 1984; Sylvester & Griswold, 1984; Brown, 1985; Vanelli *et al.*, 1986).

The possible function of an iron transporting mechanism in the seminiferous epithelium could be connected with the presence in mature sperm of an appreciable amount of iron that is accumulated during spermatogenesis (Gunn & Gould, 1970). Receptors for Tf on spermatids become lost during the elongation phase of spermiogenesis, indicating that iron is supplied to the spermatogenic cells at some earlier stage of their development (Brown, 1985). There is no evidence, however, that iron from extracellular Tf is transported into spermatogenic cells and becomes complexed to intracellular proteins.

The aim of the present study was to investigate the actual uptake of iron from Tf by Sertoli cells and isolated round spermatids, and the possible binding of iron to ferritin, an intracellular iron storage protein.

Materials and methods

Chemicals and reagents

Trypsin inhibitor (type I) and bovine serum albumin, fraction V (BSA) were purchased from Sigma (St Louis, MO, U.S.A.). Collagenase (CLS-I) and trypsin from bovine pancreas (TRL, 24 U/mg) were obtained from Worthington (Freehold, NJ, U.S.A.). Eagle's minimum essential medium (MEM) containing 25 mM Hepes was obtained from Gibco (Grand Island, NY, U.S.A.). Sephadex G-100 and PD-10 columns were purchased from Pharmacia (Uppsala, Sweden). Human serum Tf was obtained from Kabi, Sweden. Rat Tf was purified as described by Van Eijk & Van Noort (1976) and labelled with ^{125}I according to Katz (1961). ^{125}I-Tf was loaded with ^{59}Fe as described by Van der Heul, Kroos & Van Eijk (1978). Unbound Fe was removed by chromatography of the Tf–Fe complexes on PD-10 columns. ^{59}Fe-citrate and Na-^{125}I were purchased from the Radiochemical Centre (Amersham, U.K.). All other chemicals (analytical grade) were obtained from commercial sources.

Isolation and incubation of tubular fragments, Sertoli cells and round spermatids

Sertoli cells were isolated from rats aged 21–23 days (Wistar, substrain RI, Amster-

dam) and incubated for 72 h in MEM as described by Oonk & Grootegoed (1987). The medium was then removed and fresh MEM, supplemented with 0.1% (w/v) BSA containing 0.75 μM diferric, double labelled (^{125}I; ^{59}Fe) rat-Tf, was added. To determine non-specific uptake of the labelled Tf, cells were incubated in parallel in medium containing labelled Tf plus a 100-fold excess of unlabelled human Tf (approximately 75% saturated with Fe). At different time periods after the addition of Tf, the Sertoli cells were washed three times with ice-cold phosphate-buffered saline (PBS) (Dulbecco & Vogt, 1954) and the amount of incorporated ^{125}I and ^{59}Fe was estimated using a three-channel Packard autogamma 500-C spectrometer. Counts for ^{125}I were corrected for ^{59}Fe cross-over (13% of ^{59}Fe counts).

In another experiment, the Sertoli cells were incubated in MEM supplemented with 0.1% BSA containing 1.5 μM ^{59}Fe-citrate (20-fold excess of citrate). After different time periods, the cells were washed and ^{59}Fe-uptake was estimated as described above.

In order to obtain isolated pachytene spermatocytes and round spermatids, cell suspensions from the testes of rats aged 32–35 days were separated by velocity sedimentation at unit gravity, and the fractions containing spermatocytes or spermatids were further purified by Percoll gradient centrifugation (Jutte *et al.*, 1985). The germ cells were incubated for different time periods in MEM supplemented with 0.1% BSA, in the presence of either labelled Tf alone or labelled Tf plus a 100-fold excess of unlabelled Tf, as described above for Sertoli cells. After incubation, the cells were washed three times with ice-cold PBS (centrifugations for 5 min at 150 × *g*) and the amounts of ^{125}I and ^{59}Fe incorporated were measured as described above.

Tubular fragments, containing Sertoli cells, spermatocytes and round spermatids were isolated from the testes of rats aged 29–32 days using collagenase, as follows. Testes were incubated in 20 ml PBS containing 10 mg collagenase for 60 min at 32°C, in a shaking water-bath at 120 cycles per min. This treatment removed most of the tubule wall. The tubule fragments were incubated for 20 h in MEM containing 0.1% (w/v) BSA, in the presence of either labelled Tf alone or labelled Tf plus a 100-fold excess of unlabelled Tf, as described above. At the end of incubation, the round spermatids were isolated from the tubule fragments by sedimentation at unit gravity, followed by Percoll centrifugation as described above, and the amounts of ^{125}I and ^{59}Fe incorporated were measured.

Analysis of intracellular iron-binding proteins

After measurement of the content of ^{125}I and ^{59}Fe, the Sertoli cells and spermatids were suspended in a diluted (1/10) PBS solution and sonicated. The lysate was then centrifuged for 5 min at 100 000 × *g*, and the supernatant fractionated on a Sephadex G-100 column (90 × 1.2 cm), using 0.1 M Tris, 0.5 M NaCl, pH 8.1 for elution. Fractions of 5 ml were collected at a flow rate of 10 ml/h. Fractions 8 and 9, containing most of the ^{59}Fe activity, were then pooled and the proteins precipitated with antibodies to rat ferritin and rat Tf bound to CNBr-activated Sepharose 4B (Van Eijk & Van Noort, 1976; Josic *et al.*, 1986). The antibodies used were raised in rabbits and characterized as described by Van Eijk & Van Noort (1976), using

purified ferritin (Van Kreel, Van Eijk & Leijnse, 1972) and purified Tf (Van Eijk & Van Noort, 1976) as antigens.

Measurement of cellular protein and DNA

The protein content of Sertoli cells was measured according to Lowry *et al.* (1951). The DNA content was measured by a fluorometric assay using 4′,6-diamidino-2-phenylindole (DAPI) as a fluorescent dye (Kapuscinski & Skoczylas, 1977; Brunk, Jones & James, 1979), as follows. Samples were dissolved in 1 M NaOH and neutralized with 1 M HCl. A 100 µl portion of the neutralized sample was then mixed with 900 µl Tris-EDTA buffer (0.4 M Tris, 1 mM EDTA, adjusted to pH 7.8 with acetic acid) containing 10 µg/ml BSA and 0.2% (w/v) sodium-azide, and 1 ml DAPI solution (400 ng/ml). Fluorescence of the samples (0.25 ml portions) was measured using a Perkin Elmer fluorometer at wavelengths of 362 nm and 450 nm, representing maxima of excitation and emission, respectively.

Flow cytometry

Flow cytometric DNA-analysis was carried out as described by Vindeløv, Christensen & Nissen (1983). In this procedure, the frozen samples were treated with trypsin and ribonuclease A. The single nuclei thus obtained, was stained with propidium iodide. The suspension was filtered through a 30-µm nylon mesh and analysed using a FACS II cell sorter (Becton Dickinson, Sunnyvale, CA, U.S.A.).

Results

The cellular composition of the preparations of Sertoli cells and spermatids was analysed by DNA-flow cytometry (Fig. 1). The Sertoli cell preparations contained 90.5–91.7% of cells with a 2C amount of DNA, which could represent spermatogonia, secondary spermatocytes and peritubular cells, in addition to Sertoli cells. The contamination with germ cells was very small, because only 0.5–0.7% of the cells contained a haploid 1C amount of DNA (spermatids) and only 3.4–3.7% of the cells contained a 4C amount of DNA (primary spermatocytes). The isolated spermatid preparations contained 90.0–94.5% of haploid cells, 2.2–4.5% of cells with a 2C amount of DNA and 2.4–4.6% of cells with a 4C amount of DNA.

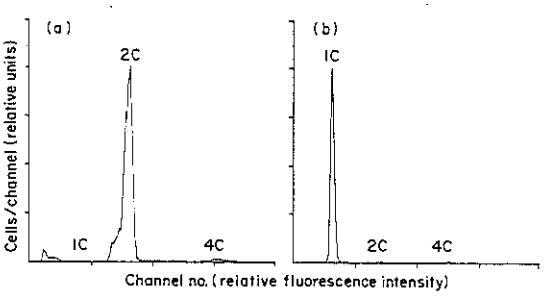

Fig. 1. Flow cytometric analysis of preparations of Sertoli cells and spermatids. The fluorescence intensities of single nuclei of the Sertoli cell preparations (a) and the isolated spermatids (b) were estimated after staining of the DNA with propidium iodide.

The total and non-specific uptake of Tf and Fe by Sertoli cells were estimated after incubation of the cells for different time periods in the presence of ^{125}I- and ^{59}Fe-labelled Tf. This results in Fig. 2 indicate that an increasing amount of ^{59}Fe was incorporated, whereas a much smaller amount of ^{125}I remained bound to the cells. The Fe uptake was linear with time (correlation coefficient r=0.999). After 20 h of incubation in the presence of the labelled Tf, still no saturation of Fe uptake was observed. The non-specific uptake of ^{59}Fe in the presence of a 100-fold excess of unlabelled Tf was five to ten times lower than the total ^{59}Fe uptake. After incubation of the Sertoli cells for different time periods in the presence of ^{59}Fe-citrate, only small amounts of Fe became associated with the cells. After 1, 2, 4 and 20 h, the uptake of Fe was 2.5 ± 0.4, 4.2 ± 0.4, 8.4 ± 0.8 and 26.6 ± 3.4 pmol/mg protein, respectively. These data are comparable to those obtained after incubation of the cells with ^{125}I- and ^{59}Fe-labelled Tf in the presence of a 100-fold excess of unlabelled Tf. This result indicates that iron uptake by Sertoli cells requires interaction of the Tf–iron complex with Tf receptors.

Isolated spermatids did not incorporate ^{59}Fe from exogenous Tf within the first 2 h after completion of the isolation procedure (results not shown). However, specific uptake of ^{59}Fe from labelled Tf was observed after pre-incubation of the spermatids for 16 h in the absence of labelled Tf (Fig. 3). Similar to the Sertoli cells, ^{59}Fe was also accumulated by the round spermatids, and much less ^{125}I remained bound to the cells. However, Fe incorporation into round spermatids was not linear with time, but reached a plateau of 5 pmol/10^6 cells after 2 h of incubation. The non-specific uptake of ^{59}Fe after 2 h was 32–38% of the total ^{59}Fe uptake.

In order to compare the present results on Sertoli cells and spermatids, the cellular protein:DNA ratio of the cultured Sertoli cells was estimated. It was found that 1 mg of Sertoli cell protein represents approximately 14 × 10^6 cells and the Sertoli cell data were expressed as pmol/10^6 cells. After 2 h of incubation in the

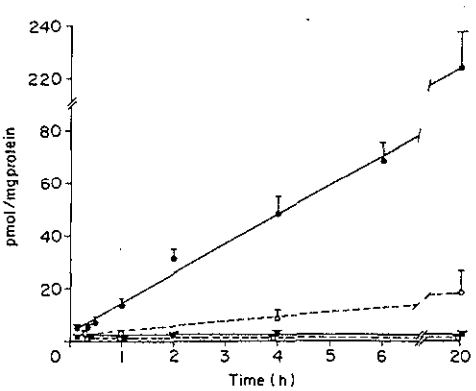

Fig. 2. Uptake of ^{125}I–transferrin and ^{59}Fe by Sertoli cells in culture. The Sertoli cells were incubated for 72 h before the addition of labelled Tf, and incubations were then continued for various time periods. The total (■—■) and non-specific (□---□) uptake of ^{125} I–Tf, and the total (●—●) and non-specific (○---○) uptake of ^{59}Fe are shown. Each point is the mean ± SD of four incubations.

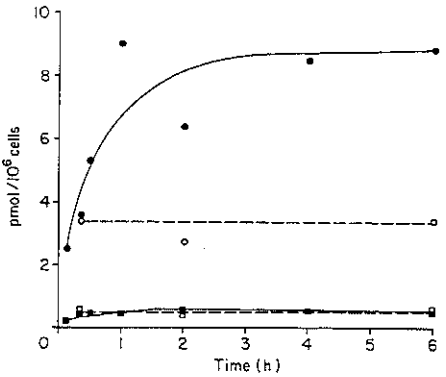

Fig. 3. Uptake of ^{125}I-transferrin and ^{59}Fe by isolated round spermatids. Spermatids were incubated for 16 h before the addition of labelled Tf, and incubations were then continued for various time periods. The total (■——■) and non-specific (□--□) uptake of ^{125}I–Tf, and the total (•——•) and non-specific (○---○) uptake of ^{59}Fe are shown for one experiment. Total minus the non-specific uptake of ^{59}Fe after 20 h was 4.2 ± 2.2 pmol/10^6 cells (data from four experiments).

presence of labelled Tf, the net uptake of Fe by 10^6 Sertoli cells was 2 pmol/2 h, and that by 10^6 spermatids was 5 pmol/2 h. Moreover, in spermatids the Fe:Tf ratio was 12:1 after 10 min of incubation, whereas the Fe:Tf ratio of the Tf added to the medium was 2:1. This indicates a high initial rate of Tf uptake and/or binding by the spermatids, and a rapid dissociation of the iron–Tf complex in the cells. The cultured Sertoli cells do not show such a rapid initial binding and uptake, because after 10 min of incubation these cells contained Fe and Tf at a 2:1 ratio.

As described above, the Fe uptake by isolated spermatids reached a plateau of 5 pmol/10^6 cells within 2 h, following a pre-incubation period of 16 h. When round spermatids were incubated for 20 h (starting on isolation) in the presence of labelled Tf, the specific (total minus non-specific) uptake was 4.2 ± 2.2 pmol Fe/10^6 cells and 0.08 ± 0.09 pmol Tf/10^6 cells (mean ± SD of four different experiments using four different cell preparations). In another series of experiments, spermatids were also incubated for 20 h in the presence of labelled Tf or labelled Tf plus a 100-fold excess of unlabelled Tf under completely different conditions, namely *in situ* in seminiferous tubule fragments. The total and non-specific uptake of ^{59}Fe and ^{125}I were then measured in round spermatids that were isolated from the tubule fragments at the end of the 20-h incubation period. The total uptake per 10^6 cells were 3.0 pmol Fe and 0.05 pmol Tf, whereas the non-specific uptake per 10^6 cells were 0.2 pmol Fe and 0.02 pmol Tf. The results indicate that, during prolonged incubations with labelled Tf, the Fe uptake by spermatids did not exceed the plateau that was reached after 2 h of incubation.

The uptake of Tf-bound iron was also studied for pachytene spermatocytes. After incubation of the isolated spermatocytes for 20 h (starting on isolation) in the presence of ^{59}Fe-^{125}I-labelled Tf, the specific (total minus non-specific) uptake was 7.6 ± 3.9 pmol Fe and 0.3 ± 0.1 pmol Tf per 10^6 cells (mean ± SD of four different experiments using four different cell preparations). These results are similar to

those obtained for the round spermatids, showing net uptake of iron in the absence of accumulation of Tf in the cells.

After incubation of Sertoli cells and spermatids for 20 h in the presence of labelled Tf, the intracellular proteins were fractionated on a Sephadex G-100 column. The ^{59}Fe-labelled proteins were eluted from the Sephadex column in fractions 7–10, whereas ^{125}I-labelled proteins and a human Tf standard were eluted in fractions 11–14 (Fig. 4). Recovery of the ^{59}Fe-radioactivity from the column was 60–85%. This low recovery of ^{59}Fe is in agreement with data from the literature (Jacobs, 1977; Bakkeren *et al.*, 1985), which indicate that a low molecular weight fraction of undefined components containing ^{59}Fe cannot be eluted from the column. The ^{59}Fe-labelled proteins from fractions 8 and 9 were treated with Sepharose-bound anti-rat ferritin antibodies and Sepharose-bound anti-rat Tf antibodies. The anti-ferritin antibodies, but not the anti-Tf antibodies, effected precipitation of most of the radioactivity from fractions 8 and 9 (Table 1). These results indicate that ^{59}Fe incorporated into Sertoli cells and spermatids was mainly complexed to ferritin.

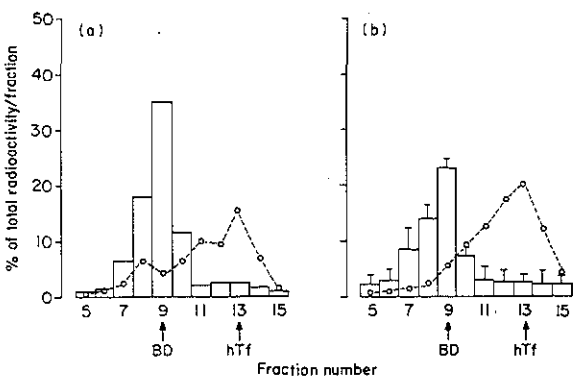

Fig. 4. Fractionation of proteins labelled with ^{125}I and ^{59}Fe on Sephadex G–100. Cellular proteins labelled with ^{126}I(○-○)or ^{59}Fe (bars) from (a) Sertoli cells or (b) round spermatids were fractionated on Sephadex G–100. Human Tf(hTf) and blue dextran (BD) were loaded onto the column in separate runs (arrows). For each fraction the data are expressed as the percentage of the total amount of radioactivity loaded onto the columns, and represent either the mean of two experiments (a) or the mean ± SD of three experiments (b).

Discussion

DNA-flow cytometric analysis of the isolated cell preparations used in the present study indicated that the purity of the Sertoli cells and the round spermatids was more than 90%. The possible effects of Sertoli cells and other cell types on iron uptake by the isolated round spermatids should, therefore, have been very small. Similarly, it is unlikely that in the present studies germ cells had any marked effect on iron uptake by the cultured Sertoli cells. The purity of the cell preparations is also supported by the kinetics of iron uptake by Sertoli cells and spermatids, which were very different.

The cultured Sertoli cells incorporated iron from extracellular Tf with a constant rate, and the ^{59}Fe content of the Sertoli cells increased linearly up to 20 h. A

Table 1: Immunoprecipitation of cellular proteins, labelled with ^{59}Fe following fractionation on Sephadex G–100

Antibody used	Sertoli cell proteins (fractions 8 and 9)	Spermatid proteins (fractions 8 and 9)
Anti-Tf	–	11
Anti-ferritin	88	74 ± 9*

Proteins from the void volume (fractions 8 and 9), obtained after chromatography of supernatants from Sertoli cells and spermatids (see Fig. 4), were immunoprecipitated. The data represent the percentages of the ^{59}Fe radioactivity from fractions 8 and 9, which were precipitated using antibodies to Tf or ferritin. These were prepared and characterized as described by Van Eijk & Van Noort (1976) and Van Kreel *et al.* (1972).

*Mean ± SD of three different cell preparations.

linear uptake of ^{59}Fe with time (for 2–3 h) has been reported also for other cell types, such as hepatocytes (Thorstensen & Romslo, 1984), bone marrow cells and reticulocytes (Martinez-Medellin & Schulman, 1972), and K-562 cells (Slørdahl, Romslo & Lamvik, 1984; Mattia *et al.*, 1986).

It has been shown that Sertoli cells possess Tf receptors (Holmes *et al.*, 1983; Brown, 1985), although there is one report that no Tf receptors were detected on human Sertoli cells (Vanelli *et al.*, 1986). Immunohistochemical observations show that the number of Tf binding sites on Sertoli cells is relatively small when compared to the numbers on germ cells (Brown, 1985).

The present results indicate that there was a rapid initial uptake of iron by spermatids. The uptake of ^{59}Fe by round spermatids reached a plateau after 2 h. This kinetics of iron uptake is similar to that described for teratocarcinoma cells, which showed a linear uptake of ^{59}Fe for 1 h, followed by a plateau (Karin & Mintz, 1981).

The presence of Tf receptors on spermatocytes and spermatids has been demonstrated previously by measurement of the specific binding of Tf to isolated cells and Scatchard analysis of the binding data (Holmes *et al.*, 1983; Steinberger *et al.*, 1984). The results of these authors indicate that the number of binding sites on spermatocytes was higher than that on round spermatids. Using immunohistochemical methods, it was observed that Tf receptors may be located on the cell surface of the spermatogenic cells, but also in the juxtanuclear area (Sylvester & Griswold, 1984). It has been described for epidermoid carcinoma cells that the intracellular routing of Tf receptors involves a juxtanuclear compartment in addition to the peripheral recycling system for the Tf receptor–ligand complex (Hopkins, 1983). Although it remains to be shown if this model is applicable to all other cell types, it may explain the location of the Tf receptors in the juxtanuclear area of spermatocytes and spermatids.

Within the first 2 h after completion of the isolation procedure, round spermatids did not incorporate ^{59}Fe from exogenous Tf. However, specific uptake of ^{59}Fe

was observed when radiolabelled Tf was added after 16 h of pre-incubation in the absence of labelled Tf. The Tf receptors may have been largely removed by collagenase or trypsin treatment during the cell isolation procedure (May & Cuatrecasas, 1985). Recovery of Tf receptors on hepatocytes has been observed after 2 h of incubation at 37°C, subsequent to treatment of the cells with collagenase (Kishimoto & Tavassoli, 1986a). The loss of Tf receptors by enzyme treatment followed by recovery may explain our results on isolated spermatids.

The present data indicate that the net uptake of Tf by Sertoli cells and spermatids was very small. This is in agreement with recent observations by Djakiew *et al.* (1986) and Wauben-Penris, Strous & van der Donk (1986), which show that only picomolar amounts of Tf remain associated with Sertoli cells. The present experiments were carried out in the presence of albumin. Iron uptake involves high affinity Tf receptors and is not influenced by inhibition of low-affinity Tf binding sites by albumin (Thorstensen & Romslo, 1984; Kishimoto & Tavassoli, 1986b). The low net uptake of Tf may reflect rapid release of the Tf from the cells subsequent to receptor binding and internalization.

The present results indicate that the Tf-bound iron that was incorporated by Sertoli cells and spermatids became associated mainly with the intracellular iron storage protein ferritin. The transfer of iron from extracellular Tf to intracellular ferritin has been described for a number of cell types, including K-562 erythroleukemia cells (Bottomley, Wolfe & Bridges, 1985; Josic *et al.*, 1986) and hepatocytes (Young, Roberts & Bomford, 1985). Association of iron with ferritin in Sertoli cells and spermatids may be an important part of iron handling after the Tf-bound iron has been taken up by these cells. Recently it has been shown that some Tf-bound ^{59}Fe, added to Sertoli cells in culture, was transferred to endogenous Sertoli cell Tf (Djakiew *et al.*, 1986). However, intracellular ferritin could be involved in the transport of iron from exogenous Tf to Sertoli cell Tf.

The present results on iron uptake by spermatids, namely a high initial rate followed by a plateau, could reflect a high number of Tf receptors and a relatively low ferritin content, as compared with Sertoli cells. This is in agreement with immunohistochemical data on testicular Tf binding sites (Brown, 1985). However, the cellular uptake of Tf-bound iron by different cell types can be influenced in a complex manner by marked effects of Tf and iron on the number of Tf receptors and the ferritin content (Rhyner *et al.*, 1985; Mattia *et al.*, 1986; Rao *et al.*, 1986). Such effects may result in altered kinetics of iron uptake by isolated and cultured cells.

It may be concluded from the present results that there is an actual uptake of iron from exogenous Tf by spermatids. However, the role of incorporated iron in spermatids remains unclear. Much iron is required for erythroid cells and rapidly proliferating normal and transformed cells. Spermatids do not further divide, but iron uptake by spermatids may be required to supply the iron content of spermatozoa.

Tf receptors are mainly present on immature, undifferentiated cells and the number of receptors decline when cells differentiate (Rhyner *et al.*, 1985; Petraki *et al.*, 1986). In this context, spermatogenic cells may be considered as immature cells. This is indicated also by the observation that peanut agglutinin (PNA)

binding sites that are not masked by sialic acid residues are present on spermatogenic cells (Watanabe *et al.*, 1981; Grootegoed *et al.*, 1982; Maekawa & Nishimune, 1985). It has been suggested that such unmasked PNA binding sites are a differentiation-dependent marker of immature cells (Reisner *et al.*, 1977, 1979). Furthermore, binding of Tf to Tf receptors could generate an intracellular signal via a mechanism that does not involve iron transport (Trowbridge & Lopez, 1982; May & Cuatrecasas, 1985). In this respect, it cannot be excluded that binding of Sertoli cell Tf to spermatogenic cells may serve a purpose other than iron transport.

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APPENDIX PAPER 4

Quantitative evaluation of the maintenance and development of spermatocytes and round spermatids in cultured tubule fragments from immature rat testis

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Summary

Maintenance and development of spermatocytes and round spermatids was studied in an in-vitro incubation system. This system consisted of open tubule fragments from 26-day-old rat testes, obtained after collagenase treatment. The tubule fragments contained Sertoli cells and spermatogenic cells up to and including a small number of early round spermatids. The number of primary spermatocytes and round spermatids in the tubule fragments was estimated using flow-cytometric analysis, immediately after isolation and after 72 h of incubation. In addition, the activity of LDH-C_4 in the tubule fragments was measured. After 72 h of incubation, the percentage of spermatocytes was reduced by 70–80%, but the percentage of spermatids was doubled. The total LDH-C_4 activity per well was increased 2–3-fold during 72 h of incubation of the fragments. A modest improvement of the culture results was observed when a combination of FSH, insulin, retinol and testosterone was added to the medium. LDH-C_4 activity was investigated to see whether it could be used as a quantitative marker of isolated and cultured spermatocytes and spermatids. It was observed that LDH-C_4 activity per cell was decreased when spermatocytes and spermatids were isolated and/or incubated at 4°C. However, the cellular enzyme activity returned to control values during subsequent incubation of the cells at 32°C, either in the absence or presence of a protein synthesis inhibitor. Cellular LDH-C_4 activity may be influenced not only by temperature, but possibly also by other cell isolation conditions. It is concluded that LDH-C_4 activity may not be a reliable quantitative marker for the presence of spermatocytes and spermatids in culture, but should be used in combination with other analytical methods such as DNA estimation and DNA flow cytometry.

Keywords: testis, Sertoli cells, spermatids, flow cytometry, LDH-C_4.

Introduction

Spermatocytes and spermatids are located in the adluminal compartment of the testicular tubules, separated from extratubular macromolecular factors by the

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the lysed cell suspension was centrifuged at 4°C (10 000 *g*) for 15 min. The LDH-C_4 activity in the supernatant was assayed at room temperature (22–24°C), essentially as described by den Boer & Grootegoed (1988), but using 0.25 mM α-keto-isocaproate (KIC) as the substrate. Using the rat cells, the enzyme activity was maximal at a concentration of 0.25 mM KIC and the reaction rate was linearly dependent on the amount of enzyme (not shown). The results are expressed as units (U) per well, per mg protein, or per mg DNA.

Flow cytometry

Flow cytometric analysis of tubule fragments and isolated germ cells was performed essentially as described by Vindeløv, Christensen & Nissen (1983), as outlined previously (Toebosch, Kroos & Grootegoed, 1987).

Analysis of leucine metabolism

After 48 h incubation of tubule fragments, the medium was removed and fresh medium containing 2 mM L-leucine was added. After a subsequent 20 h incubation period the culture medium was analysed to estimate the formation of α-hydroxyisocaproate (HIC) using gas-liquid chromatography as described by Grootegoed, Jansen & van der Molen (1985).

Estimation of ATP content

Incubations of spermatocytes and spermatids were terminated by adding 20 µl perchloric acid (PCA) to the cells. The ATP content of the cells was then measured in the PCA precipitates as described by Grootegoed, Jansen & van der Molen (1984).

Estimation of GSH content

The GSH content of spermatocytes and spermatids was estimated according to the methods of Hissin & Hilf (1976) and Grosshans & Calvin (1985) with slight modifications. After incubation of the spermatocytes and spermatids, the cells were washed once at room temperature in PBS with lactate (1500 *g*). Subsequently, the supernatant was removed and the cell pellet was frozen and stored at −80°C, until assay. The cells were lysed in 100 µl water and the cellular proteins were precipitated by addition of 50 µl 3 M PCA/1 mM EDTA. The homogenate was kept on ice for 10 min and subsequently centrifuged. The supernatant was neutralized by addition of 2 M KOH/0.3 M HEPES and centrifuged to remove $KClO_4$. A 25 µl portion of the neutralized PCA supernatant was mixed with 450 µl sodium phosphate buffer (0.1 M; 5 mM EDTA, pH 8.0) and 25 µl of a solution of *o*-phthaldialdehyde (1 mg/ml methanol). The mixture was incubated for 15 min at room temperature and the fluorescence at 420 nm (excitation 350 nm) was measured using a fluorescence spectrophotometer (Kontron Instruments, SFM 25; Zürich, Switzerland).

Estimation of leucine incorporation

Leucine incorporation into isolated spermatocytes and spermatids was measured as follows. The cells were incubated for different time periods with L-[1-^{14}C]leucine (0.10 µCi/incubation) and unlabelled leucine (final concentration 28 µM). Subsequently, the cells were centrifuged (1500 *g* for 5 min at 4°C) and the supernatant removed. The cell pellet was then frozen and stored at −80°C until assay. The frozen cell pellets were mixed with 500 µl H_2O and the lysates were precipitated on

leucine-saturated filters, placed in an Amicon VFM-1 vacuum filtration apparatus, using ice-cold trichloroacetic acid (TCA, 15% w/v). The filters were washed twice with 10 ml ice-cold TCA and once with ethanol (70%, v/v). Subsequently, the filters were dried at 50°C and dissolved in 10 ml Filtercount (Packard Instrument Company Inc., Downers Grove, U.S.A.) and the radioactivity estimated using an Isocap-300 liquid scintillation counter (Searle Analytic, Des Plains, U.S.A.).

Estimation of cellular protein and DNA

The tubule fragments from one well were collected by flushing with a plastic pipette, washed once with 10 ml PBS (centrifugation for 5 min at 150 *g*) and dissolved in 1 ml 1 M NaOH.

The amount of cellular protein was measured as described by Lowry *et al.* (1951), using BSA (fraction V) as standard. The DNA content was measured using the fluorescent dye DAPI (Kapuscinski & Skoczylas, 1977; Brunk, Jones & James, 1979) as described previously (Toebosch *et al.*, 1987).

Statistical procedures

The results on cultured tubule fragments represent the mean ± SD for three or four experiments using different cell preparations, unless indicated otherwise. In each experiment, the data are the mean of four to six different incubations. Probability values were calculated using the paired Student's *t*-test for dependent data.

The results on isolated spermatids represent the mean ± SD for one or two experiments, with triplicate incubations per experiment unless indicated otherwise. Probability values were calculated using the Student's *t*-test.

Results

Protein and DNA content during incubation of tubule fragments

Changes in the total amounts per culture well of protein and DNA during 72 h

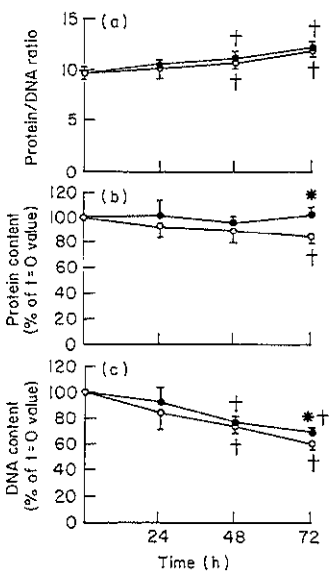

Fig. 1. Protein and DNA content, after 24, 48 or 72 h incubation, of tubule fragments isolated from the testes of rats aged 26 days. Incubations were in the absence (○) or presence (●) of FIRT. (a) The protein/DNA ratio, the data represent the mean ± SD of three different experiments (three incubations per experiment). The data represent the total amounts per well of protein (b) and DNA (c) expressed as a percentage of the starting value (t=0). *Significantly different from the incubations in the absence of FIRT ($P<0.02$). †Significantly different from t=0 ($P<0.01$).

incubation of tubule fragments are presented in Fig. 1. The protein/DNA ratio, shown in Fig. 1a, was increased slightly during incubation, without an effect of FIRT. This increase in the protein/DNA ratio reflected a relatively large loss of DNA when compared to the loss of protein (Fig. 1b, c). There was no loss of protein during incubation in the presence of FIRT, and the loss of DNA was prevented to some extent by this hormone/vitamin mixture.

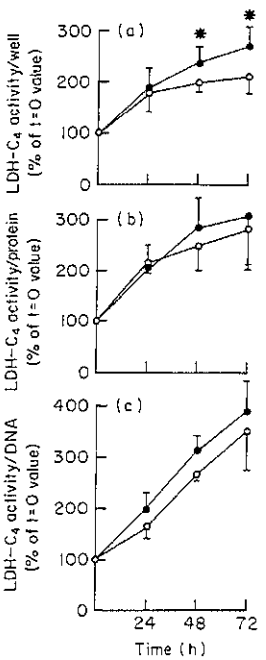

Fig. 2. LDH-C_4 activity, after 24, 48 or 72 h incubation, of tubule fragments isolated from the testes of rats aged 26 days. Incubations were in the absence (○) or presence (●) of FIRT. The data represent the total activity per well (a), per mg of protein (b), or per mg of DNA (c), and are expressed as a percentage of the starting value (t=0). The data are the mean ± SD of three different experiments (three incubations per experiment). *Significantly different from incubations in the absence of FIRT ($P<0.01$). All values at t=24, 48 and 72 h are significantly different from t=0 ($P<0.01$).

LDH-C_4 activity

The mean specific activity of LDH-C_4 in freshly isolated tubule fragments from rats aged 26 days was 2.9 ± 1.0 mU/mg protein. LDH-C_4 activity during 72 h incubation in the absence or presence of FIRT is presented in Fig. 2. A marked increase in total LDH-C_4 activity per well (approximately 1.8-fold) was observed after 24 h incubation (Fig. 2a). After 48 h and 72 h, a significant difference was observed in total LDH-C_4 activity per well between incubations in the absence or presence of FIRT. This was connected mainly with the absence of a further increase of LDH-C_4 activity from 24 to 72 h in the absence of FIRT (Fig. 2a). The increase in LDH-C_4 activity during the incubation was also manifest when the activity was expressed per mg protein or DNA (Fig. 2b,c). The mean specific activity of LDH-C_4 in isolated tubule fragments from rats aged 29 days was 11.8 ± 5.8 mU/mg protein (mean ± SD of seven experiments).

Flow cytometry

The method of tissue solubilization for flow cytometric analysis of cellular DNA content, as described by Vindeløv *et al.* (1983), has been used widely for somatic

cells from normal and tumour tissues. To evaluate if this is also a reliable method for the analysis of spermatogenic cells, an experiment was carried out to estimate the recovery of isolated spermatocytes and spermatids using the flow cytometric method. The number of cells with a 1C amount of DNA (1C is the amount of DNA in a haploid gamete) was taken as a measure of the number of haploid spermatids. Cells with a 2C amount of DNA were mainly Sertoli cells, but also included spermatogonia, secondary spermatocytes and peritubular cells. The cells which contained a 4C amount of DNA were primary spermatocytes following the meiotic S-phase. The purity of the cell preparations is indicated in Table 1 and in Fig. 3a,b. The isolated spermatids and spermatocytes were counted using a haemocytometer and mixed in a ratio of 4:6. Table 1 and Fig. 3c show the results of the flow cytometric analysis of this mixture. The data indicated that the recovery of spermatocytes and spermatids was virtually identical.

Table 1. DNA-flow cytometric analysis of single cell suspensions of spermatids and spermatocytes

	DNA content (%)		
Cell preparation	1C	2C	4C
Spermatids	92.5(90.0–94.5)	3.1(2.2–4.5)	3.6(2.4 –4.6)
Spermatocytes	1.6(1.6 –1.7)	4.1(3.9–4.3)	93.1(92.5–93.7)
Mixture*	41.2(39.9–42.3)	2.2(2.2–2.3)	55.9(54.8–57.2)

*Mixture of spermatids:spermatocytes (4:6).
Flow cytometric analysis was performed using isolated and purified spermatids and spermatocytes, and a 4:6 mixture of these cell preparations. The calculated composition of this mixture was 39.9% 1C and 60.1% 4C. The results represent the mean and range of two or three estimations.

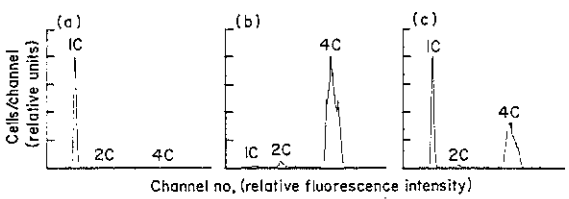

Fig. 3. DNA-flow cytometric analysis of isolated spermatids (a), isolated spermatocytes (b), and a 4:6 mixture of these cell types (c). The fluorescence intensities of single nuclei were estimated after staining the DNA with propidium iodide.

The results of flow cytometric analysis of freshly isolated tubule fragments from rats aged 26 days are presented in Table 2. The mean percentages of cells with a 1C, 2C and 4C amount of DNA were 2.8 ± 1.8, 53.8 ± 3.7, and 39.2 ± 1.9, respectively. The percentage of cells with a 1C amount of DNA was in the range 0.9–4.6% (Table 2). This range reflects that the testes in rats aged 26 days undergo rapid development. The mean percentage of cells with a 1C amount of DNA in tubule fragments from rats aged 29 days was 10.2 ± 1.9. The mean percentages of cells with a 2C and 4C amount of DNA were 47.0 ± 4.5 and 40.1 ± 4.0, respectively (mean ± SD of eight experiments).

Table 2. DNA-flow cytometric estimation of the cellular composition of tubule fragments from rats aged 26 days

Experiment no.	DNA content (%)		
	1C	2C	4C
1	4.6±0.5	52.1±1.9	39.4±1.8
2	1.7±0.6	55.3±1.1	40.5±1.3
3	3.9±0.4	49.6±1.6	36.4±1.1
4	0.9±0.2	58.0±0.9	40.3±1.1
Mean ± SD	2.8±1.8	53.8±3.7	39.2±1.9

The results of each experiment represent four or six determinations.

Typical DNA-distribution patterns for tubule fragments from rats aged 26 days immediately after isolation (t=0) and after 72 h incubation are shown in Fig. 4. The results of incubation of the tubule fragments are presented in Table 3. The total number of cells per well with a 1C, 2C, or 4C amount of DNA was calculated from the flow cytometric DNA-distribution patterns and the total amount of DNA per well at time zero and after 72 h incubation. In all experiments the number of spermatocytes was decreased after 72 h incubation ($P<0.01$). This decrease was slightly less pronounced, but not significantly different, after incubation in the

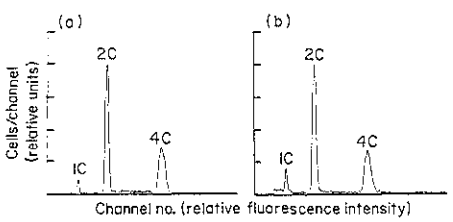

Fig. 4. DNA-flow cytometric analysis of tubule fragments from rats aged 26 days (a) directly after isolation or (b) after 72 h incubation.

Table 3. DNA-flow cytometric analysis and DNA content of tubule fragments isolated from rats aged 26 days before and after incubation

Parameter	At time zero	After 72 h incubation	
		Control	FIRT
Percentage 1C	2.8±1.8	6.2±2.8	5.4±2.8
Percentage 4C	39.2±1.9	28.6±2.7	31.0±4.3
Spermatids/well (10^6)	0.17±0.11	0.28±0.14	0.25±0.14
Spermatocytes/well (10^6)	2.28±0.17	1.25±0.17	1.40±0.11
DNA content/well	46.7±3.8	30.9±2.0	33.5±1.4

Tubule fragments isolated from rats aged 26 days were incubated in the absence (Control) or presence of FIRT. Flow cytometric analysis and estimation of the total DNA content of the tubule fragments were performed immediately after isolation (time zero) or after 72 h incubation. The actual number of spermatids and spermatocytes per well was calculated from these data. The data represent the mean ± SD of four experiments (four or six determinations per experiment).

presence of FIRT. The total number of spermatids per well at time zero was in the range 0.05–0.28 × 10^6 cells. This number was increased by a factor of 1.8 ± 0.5 after 72 h incubation ($P<0.01$). The percentage of 1C cells was increased by a factor of 2.3 ± 0.6 ($P<0.02$). There was no effect of FIRT on the number and percentage of spermatids.

Leucine metabolism

In incubated tubule fragments, leucine is converted to KIC by the Sertoli cells which contain a high activity of branched-chain amino acid transferase (Grootegoed *et al.*, 1985). The KIC is subsequently converted to HIC by spermatocytes and spermatids, via an NADH-dependent reaction catalysed by LDH-C_4 (Grootegoed *et al.*, 1985). In the present experiments an HIC peak was formed during a 48–68 h incubation period (Fig. 5). This provides evidence that the germ cells present in the cultured tubule fragments had an active metabolism which involved NADH production.

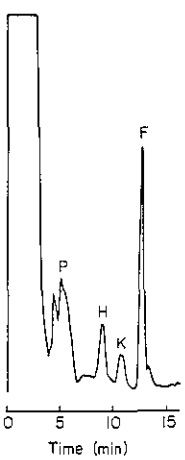

Fig. 5. Production of α-hydroxyisocaproate by tubule fragments isolated from rats aged 30 days. After 48 h pre-incubation, the tubule fragments were incubated for 20 h in medium containing 2 mM L-leucine. The spent medium from this 20 h incubation was analysed by gas-liquid chromatography after addition of the standard fumaric acid, as described by Grootegoed *et al.* (1985). The positions of pyruvate (P), α-hydroxyisocaproate (H), α-ketoisocaproate (K), and fumaric acid (F) are indicated.

LDH-C_4 activity in isolated spermatocytes and spermatids

In order to use LDH-C_4 activity as a quantitative marker for germ cells it is essential to demonstrate that the enzyme activity per cell is constant during isolation and incubation of the cells. Spermatocytes and spermatids were therefore isolated, not only as described above at room temperature, but also at 4°C, to try to prevent any possible changes in enzyme activity due to increased synthesis or breakdown of the enzyme protein. It was observed that the LDH-C_4 activity of spermatocytes and spermatids was approximately 50% lower after isolation at 4°C, when compared with the activity after isolation at room temperature (Table 4). No statistical analysis of this difference was performed because there was a large variation in enzyme activity in the different experiments, as is discussed below.

From these results it would appear that LDH-C_4 activity either increased during isolation at room temperature or decreased during isolation at 4°C. To study the

spermatids. The present data do not give information on the entry of spermatogonia into meiotic prophase, but it is concluded that changes in the number of spermatocytes in the cultured tubule fragments are mainly caused by disintegration of spermatocytes. The decrease in the number of spermatocytes correlates with a decrease in total DNA content, but not in total protein content of the cultures. It seems likely that cellular proteins from degenerating spermatocytes are not eliminated rapidly. When tubule fragments, isolated from rats aged 29 days were compared with those from rats aged 26 days it was observed that there was a 3–4-fold increase in both LDH-C_4 activity and the percentage of 1C cells. However, the increase in these parameters in cultured tubule fragments from rats aged 26 days was approximately twofold after 72 h.

Secretion of a number of proteins by Sertoli cells during culture for several days can be stimulated most efficiently by a combination of FSH, insulin, retinol and testosterone (FIRT) (Skinner & Griswold, 1982; Skinner & Fritz, 1985). The present results show that the loss of spermatocytes and the decrease in DNA content in the cultures was partly prevented when the tubule fragments were incubated in the presence of FIRT. This indicates that hormonal stimulation of Sertoli cells may result in a better maintenance of spermatocytes. There was, however, no effect of FIRT on the number of spermatids in the cultured tubule fragments.

In the present experiments, tubule fragments converted leucine into HIC after 48 h of incubation. This metabolism involves production of KIC from leucine by Sertoli cells, and NADH-dependent LDH-C_4 activity which catalyses the reduction of KIC to HIC in the germ cells. The results indicate that the spermatocytes and spermatids which were present in the tubules after 2–3 days of culture, had maintained the capacity to reduce NAD^+. Moreover, the formation of HIC from leucine represents a metabolic interaction between Sertoli cells and germ cells which does not require intact structural interactions, but which points to a certain degree of biochemical integrity of the cells.

Biochemical integrity of the germ cells in the cultured tubule fragments is also supported by the observation that the total LDH-C_4 activity per well was increased, mainly during the first 24 h of incubation, and was maintained up to 72 h. After 48 h there was a significant difference in this total LDH-C_4 activity between incubations in the absence and in the presence of FIRT. The effect of FIRT can be explained by the larger number of spermatocytes that were maintained in the presence of the hormone/vitamin mixture. However, it cannot be excluded that secondary spermatocytes, with a 2C amount of DNA, are formed but do not undergo the second meiotic division to form spermatids. These 2C cells, which are not distinguished from Sertoli cells using the flow cytometer, may have a high LDH-C_4 activity, thus contributing to the total amount of LDH-C_4 activity.

The rapid increase in LDH-C_4 activity during the first 24 h of incubation of the tubule fragments might reflect a rapid increase in the number and/or a rapid advancement of the developmental stage of the LDH-C_4-containing cells. However, this conclusion relies on a constant LDH-C_4 activity per cell in a given developmental stage. It was therefore investigated if there might be changes in the LDH-C_4 activity per cell as a consequence of the isolation procedures and in-vitro

manipulations. LDH-C_4 activity was not influenced by isolation of the cells in the presence of cycloheximide. The cellular enzyme activity, however, was decreased by cold-shock, although this activity could be restored without synthesis of new proteins. It was observed that LDH-C_4 activity in isolated germ cells varied considerably between the different experiments. A high sensitivity of the cellular enzyme activity to external factors, which can induce distinct kinds of shock, possibly plays a role in this respect.

From the present experiments, it is concluded that LDH-C_4 activity measured after isolation of the cells at room temperature may reflect the true cellular activity *in vivo*. This may also apply to LDH-C_4 activity in the tubule fragments.

It has been suggested that heat shock proteins (hsp), which are produced by cells under stress conditions, can influence the conformation of proteins (Pelham, 1986). The most prominent hsp has a molecular weight of approximately 70 kD and is termed hsp70. A germ cell-specific member of the hsp70 family, P70, has been detected in mouse pachytene spermatocytes and round spermatids (Allen *et al.*, 1988a; 1988b). P70 and LDH-C_4 are produced and expressed during the same stages of spermatogenesis (Meistrich *et al.*, 1977; Allen *et al.*, 1988b). It has been suggested that P70 is involved in germ cell differentiation, whereas hsp70 in spermatocytes and spermatids is produced in response to stress (Allen *et al.*, 1988b). It seems possible that the maintenance and recovery of the conformation and enzymic activity of LDH-C_4 is influenced by the hsp which are present in the germ cells. This might explain the present observations on the variability of cellular LDH-C_4 activity.

From the above it is concluded that LDH-C_4 activity should be used with caution as a quantitative marker for the presence of spermatocytes and spermatids in culture. In the present experiments, LDH-C_4 activity was therefore used in combination with DNA estimation and DNA-flow cytometry.

Acknowledgment

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