The Blood-Testis Barrier and Blood Vessel Permeability in Rat Testis

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ACTH:	adrenocorticotropin hormone
ABP:	androgen-binding protein
EDL:	efferent duct ligation
EDS:	ethane dimethane sulphonate
EDTA:	ethylenediamine tetra-acetic acid
FSH:	follicle stimulating hormone
hCG:	human chorionic gonadotrophin
HPLC:	high-performance liquid chromatography
5-HT:	5-hydroxytryptamine
ITF:	interstitial tissue fluid
LCSF:	Leydig cell stimulatory factor
LH:	luteinizing hormone
PAH:	p-aminohippurate
PMN:	polymorphonuclear
PS:	permeability surface area product
RIA:	radioimmunoaasay
RTF:	rete testis fluid
SGP:	sulfated glycoprotein
STF:	seminiferous tubule fluid
TVB:	testicular venous blood plasma

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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ABSTRACT

The blood-testis barrier is composed of two components: a tubule barrier; and a capillary barrier. The former consists mainly of the tight junctions between Sertoli cells, whereas the latter comprises unfenestrated or continuous endothelial cells. The barrier function of the Sertoli cell is quite stable, while that of the endothelial cell can be destabilized by a number of factors including hormones, chemicals and age. Here, the tubule and capillary barriers will be considered from the point of view of anatomy, physiological function and possible factors which may cause the tubule barrier to be breached or influence substance exchange across the capillary wall.

Since the concept of the blood-testis barrier was proposed around the middle of 1960's, the tubule barrier has been explored from a variety of aspects. Ultrastructural studies revealed that the conjunction between Sertoli cells was a type of tight junction, which can exclude certain substances such as some dyes and drugs. Accordingly, the millieu within the adluminal compartment is unique. The idea of a special microenvironment was substantiated by analytic studies which demonstrated large concentration differences of some substances between seminiferous tubule fluid and interstitial tissue fluid. Therefore, it has been postulated that spermatogenesis may be impaired when the tubule barrier is disrupted. Further research stagnated owing to lack of a suitable animal model. In this study an animal model aimed at characterizing the effect of breaching the tubule barrier has been developed; three different injuries to seminiferous tubule—heat; efferent duct ligation, and exposure of rats to N_2O, were chosen as factors with which to challenge integrity of the tubule tarrier. The competence of the tubule barrier after these treatments was judged by measuring the ratio of interstitial ^{51}Cr-EDTA space to the morphometry volume. The latter was evaluated microscopically using cryostat sections. The results showed that a temporary breach of the tubule barrier was observed in the testes 40-48 hours after efferent duct ligation, but,

there was no effect after heating and exposing animals to N_2O. This finding provides a technique for further study of the function of tubule barrier.

Although there have been many studies of the tubule barrier, information involving blood flow and vascular permeability in testis is limited. However, both blood flow and permeability of the capillary are very important not only for uptake of peptides, such as pituitary gonadotrophins, but also for secretion of steroid hormones. Pituitary gonadotrophins, the major regulator of testicular function, are lipid-insoluble. It has been speculated that uptake of gonadotrophin by tissue will be influenced more by vascular permeability than by blood flow. In contrast, secretion of lipid-soluble testosterone from testis into blood stream should be affected by these parameters in the opposite manner. To confirm this inference, perfusion experiments were performed in isolated testes by using Krebs-Ringer's solution containing 1% bovine serum albumin. After perfusing the tissue at different flow rates, ^{125}I-albumin, a model molecule for pituitary gonadotrophins, was injected into the testis and measured in testicular parenchyma. In similar experiments, testosterone output across the testicular wall was investigated by assaying the effluent collected from testis which had been perfused at different protein concentrations and at different perfusion flow rates. The results indicated that the secretion of testosterone correlated positively with the flow rate *in vitro,* but increased only temporarily after elevating the protein concentration in the perfusate. In comparison, albumin transport across the capillary barrier was not affected by flow rate, especially when flow rates were above normal. In order to gain insight into the effect of vascular permeability on uptake of albumin by testis, an albumin transport experiment was designed to examine the effects *in vitro* of factors including human choriogonadotrophin (hCG), cadmium, and age, all of which influence blood vessel permeability *in vivo*. Two different calculations for permeability surface area product (PS), true PS and apparent PS, both made use of ^{125}I-albumin tracer. Results in this study showed that *in vitro* there were no significant changes of true PS in testis

during puberty and after hCG treatment although the apparent PS *in vivo* increased dramatically. However, the true PS in testes did increase after treatment with cadmium, a modality which damages capillary endothelium. The data support the suggestion that testosterone transport is mainly flow limited, whereas albumin transport is controlled by vascular permeability. Moreover, the increases of apparent PS in the testes after hCG and during puberty were mainly attributed to changes in net fluid flux.

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PREFACE

Aspects of the work presented in this thesis have been reported elsewhere:

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L.Tao and B.P.Setchell (1992) The blood-testis barrier after efferent duct ligation. Miniposter in 7th European workshop on molecular and cellular endocrinology of the testis. P 45 Castle Elmau, Germany

B.P.Setchell and L.Tao (1992) Effect of perfusate flow on protein uptake by the rat testis. Abstracts Ninth Int. Congr. Endocrin. P 457 Nice, France

B.P.Setchell, S.Maddocks, Tao L. and Zupp, J.L. (1992) Vascular function in the testis and infertility. In "Understanding male infertility: basic and clinical approaches", ed. R.W.Whitcomb, B.Zirkin, Serono Symposium, Rome, Italy, Raven Press, New York, pp 81-93.

CHAPTER 1: REVIEW OF LITERATURE

1. 1. SCOPE OF THE REVIEW

The concept of the blood testis barrier was formed around the middle of 1960's. Recently, we often use term "blood-testis barriers" instead of "blood-testis barrier", because there is more than one barrier. The following literature review will describe the blood testis barriers, the tubule barrier and capillary barrier, in anatomy and physiological function. The stress will be placed on the factors which may breach the tubule barrier or influence substance exchange across the capillary wall.

1. 2. THE BLOOD-TESTIS BARRIERS

1. 2. 1 Introduction

The testis is an organ with two functions: it produces male gametes (spermatozoa) and secretes steroid hormones. Early observation of these functions can be traced far back into the past, when people were aware that the testes played a role in male behaviour and were essential for male fertility.

It was not until the 17th century that the fine structure of the testis was first explored. Claude Aubry published a brief description of the seminiferous tubules in 1658, and ten years later, Regnier de Graaf published a fuller description of these tubules (Jocelyn and Setchell, 1972). In 1849, a hormonal role for the testis was demonstrated by Berthold (Berthold, 1849).

The structure of the testis, reflecting its two functions, consists of seminiferous tubules and interstitial tissue, which is present in the angular spaces between the tubules. The interstitial tissue contains blood vessels, lymphatic vessels, Leydig cells and other interstitial cells.

The Leydig cells in the interstitium are the major source of androgens, as was originally noted by Bouin and Ancel in 1903 (Bouin and Ancel, 1903) and confirmed by later research using separated testicular tissue cultures (Christensen and Mason, 1965; Hall et al., 1969; Parvinen et al., 1970) and histochemical techniques (Woods and

Domm, 1966; Bubenik et al., 1975). The importance of testosterone to spermatogenesis in rats has been shown by a classical experiment, the regression of the seminiferous tubules after hypophysectomy could be prevented by giving the animal a large dose of testosterone immediately after surgery (Clermont and Harvey, 1967).

Besides its relationship with androgens, normal spermatogenesis also depends on an unique microenvironment. This microenvironment is established and maintained by the blood-testis barriers, which consist of three separate components.

Although the concept of the blood-testis barrier has been developed mainly over the last 25 years, the early evidence for a capillary component to the barrier can be traced back to the beginning of this century. Bouffard (1906) found that the seminiferous tubules were excepted from staining when some intravital dyes were injected into an animal. Half a century later researchers turned their attention to this area again. Trypan blue (Kormano, 1968) and.the amino acid L-Dopa (Kormano, 1967a) were confined within the testicular capillaries when injected intravenously. Better tubule staining was seen with acriflavine when given by intratesticular injection than with intravenous injection (Kormano,1967a; 1967b; Kormano & Penttila, 1968). It seems likely, therefore, that there is a capillary component to the blood-testis barriers involving cell-cell junctions in the capillary wall.

Work in rat and guinea pig has provided evidence for a peritubular component to the blood-testis barriers. Junctions between myoid cells restrict the passage of larger markers such as carbon, thorium and ferritin, which pass through the capillary walls after intravenous injection. For smaller markers, like peroxidase and lanthanum, the junctions between myoid cells restrict the passage of these substances in most, but not all areas. Smaller markers can pass through some of the junctions between myoid cells and then reach the interspace between spermatogonia or primary spermatocytes and Sertoli cells in the basal compartment, but they never penetrate the specialized junctions between Sertoli cells (Dym & Fawcett, 1970; Fawcett et al., 1970). This was also

demonstrated by using an electron microscope with an immunocytochemical technique to observe the distribution of serum albumin in the rat testis (Christensen et al., 1985).

Briefly, it can be seen that the blood-testis barriers consist of three different components: the testicular capillary wall, the myoid cells and the Sertoli cells in the seminiferous tubules. Both the capillary wall and myoid cells only provide incomplete layers to the barriers. In other words, the unique cell-cell junctions between Sertoli cells are the key to the blood-testis barriers.

1. 2. 2. Anatomy of the blood-testis barriers

In the testis, blood vessels, lymph vessels and nerves are found in the interstitial spaces, and never penetrate into the seminiferous tubules. Therefore, for a substance to pass from the bloodstream into the tubules, it must cross the capillary wall, and then either the peritubular tissue and the Sertoli cells in the seminiferous tubules, or the epithelium in the rete testis and approach the lumen of the tubules.

1. 2. 2. 1. Capillaries

The testicular capillaries can be classified into two types. (1) Intertubular capillaries or Zwickelcapillaren, run into the intertubular connective tissue column, and supply blood to Leydig cells and other interstitial cells; (2) Peritubular capillaries, also called Quercapillaren, run into the peritubular connective tissue sheet, and supply blood to the seminiferous tubules (Murakami et al., 1989). Both types of capillaries stem from intertubular arterioles, and then converge into the intertubular venules. They form a capillary network which appears like a ladder adjacent to the seminiferous tubules, the peritubular vessels serving as the rungs around the tubules, and the intertubular vessels, the pillars of the ladders in the column spaces between tubules (Murakami et al., 1989). This vascular arrangement can supply sufficient nutrition, oxygen and necessary hormonal signals to interstitial cells and seminiferous epithelium under normal condition.

The testicular capillary wall is the first barrier restricting substances in the bloodstream from entering the tubules. It consists of unfenestrated endothelium and a definite basement membrane. Among all endocrine organs, only testicular capillaries possess this peculiarity (Fawcett et al., 1973b; Farquhar et al., 1961). In rats there is a cleft of 150 to 200Å width (Fawcett et al, 1970), rather than the specialized tight junction between adjacent endothelial cells. However, this cleft seems to be sealed by desomosomes in rabbits (Crabo, 1963). The endothelium is thick and rich in mitochondria with a well developed reticulum (Crabo, 1963; Gunn et al., 1966). It also possesses more microvillous processes than the endothelial cells in other organs (Gabbiani and Majno, 1969). The basement membrane surrounding the capillary endothelium is multi-layered and continuous (Crabo, 1963; Ross, 1963; Fawcett et al., 1969). It could represent a barrier obstructing the passage of large particles (Farquhar et al., 1961).

1. 2. 2. 2. The seminiferous tubules

The seminiferous tubules are a major component part of the testis, contributing 65-90% testis weight (Setchell,1978; 1990a, Fig. 1-1). The tubule diameter in most species is about 200-250 μm. In rat testis, each gram contains approximately 12 m of the tubules in length, with about 120 cm^2 in surface area (Wing and Christensen, 1982). As the tubules are slightly smaller in diameter in man, each gram of testis, will contribute a much greater length (20-25 m/g) and a larger surface area for the tubules (Bascom and Osterud, 1925; Lennox et al., 1970).

There are normally approximately 30 tubules in the rat testis (Clermont and Huckins 1961). More tubules, divided into around 300 lobules, have been found in humans (Lauth, 1830). In most mammals, each tubule has two ends (Clermont and Huckins, 1961), both opening into the rete testis (Roosen-Runge, 1961; Fig 1-2). Fluid and sperm from the seminiferous tubules are transported via the rete testis through the efferent ducts to the epididymis, where the sperm are stored until ejaculation. No tubular lumen can be found in newly born animals. It first appears on the 10th postnatal

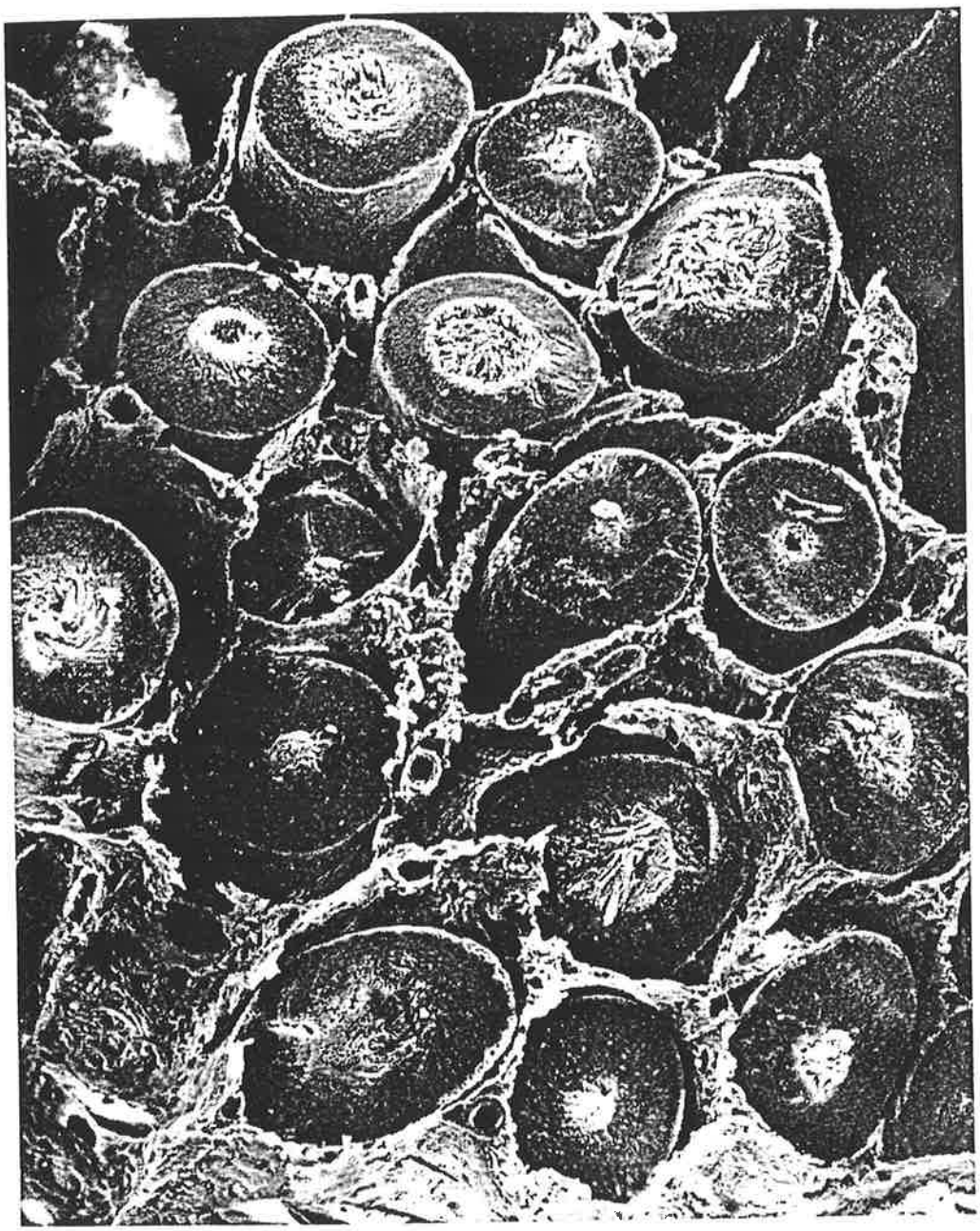


Fig. 1-1 A scanning electron micrograph of a cut surface of a rat testis (courtesy of Dr A. Kent Christensen from Setchell and Brooks, 1988).

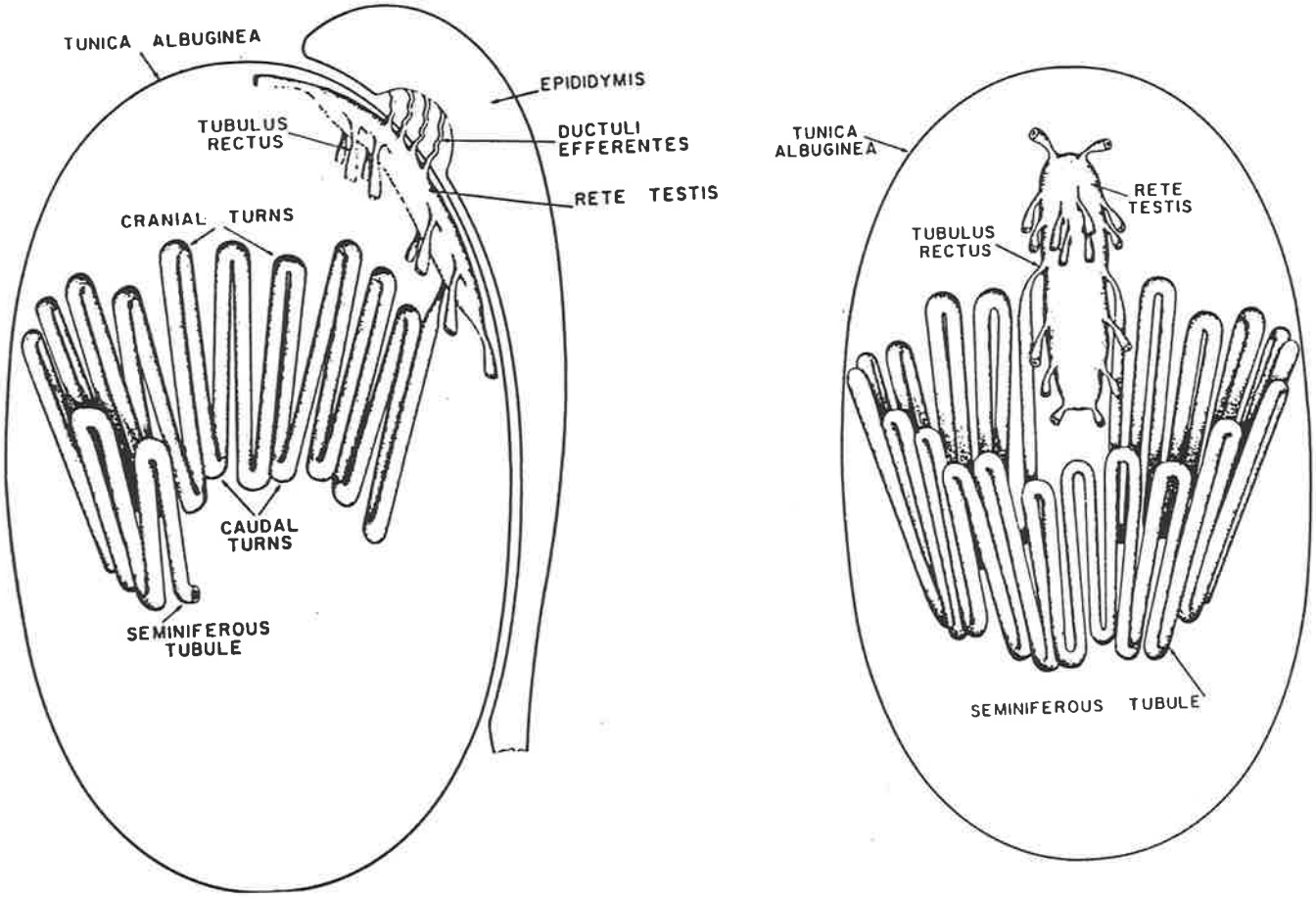


Fig. 1-2 A diagram of the anatomy of an individual seminiferous tubule and the rete testis of a rat. Left: Only part of one tubule is shown, and only some of the junctions of seminiferous tubules with the rete testis are depicted. (from Clermont and Huckins, 1961)

day in the rats, slowly increasing from the 10th to 30th postnatal day then rapidly increasing to the 50th postnatal day when it achieves adult levels (Russell et al., 1989). In the seminiferous epithelia the cells are formed in certain associations according to their stages of spermatogenic cycle (Kerr, 1988a; 1988b). The concept of the "spermatogenic wave" was introduced by Von Ebner in 1871 and confirmed by later researchers. Experiments in rats showed that each seminiferous tubule contains about 12 complete spermatogenic waves. Successive stages of the cycle move from the rete testis in descending order then change in order when the two waves meet (Perey et al., 1961; Hochereau, 1963). It is still unknown why the order of the stages appears this way, although the Sertoli cell structure and function appear to be related to various stages of the spermatogenic cycle (de Kretser, 1990).

(1) Peritubular tissue

The seminiferous tubules consist of germinal cells, somatic Sertoli cells, enclosed by a well-defined wall, called peritubular tissue.

In most species, the peritubular tissue consists of four layers (Fig. 1-3A), ordered from outside to inside as following: (1) a layer of endothelial cells, which is the outside cellular tissue with open junctions between cells and which also lines the lymphatic sinusoids in interstitial tissue (Clermont, 1958; Dym and Fawcett, 1970; Setchell and Waites, 1975; Setchell, 1978); (2) a layer of collagen fibres, which is the outside non-cellular tissue; (3) a layer of myoid cells, which is the inner cellular tissue; (4) a basement membrane, which is the inner layer of non-cellular material and consists of fibronectin and proteoglycans, both of which are produced by myoid cells in vitro (Tung et al., 1984; Skinner and Fritz, 1985a).

The myoid cells derived from mesenchyme play an important role in forming the boundary layer (Clermont, 1958; Lacy and Rotblat, 1960; Leeson and Leeson, 1963; Ross, 1967) and in maintaining the structural integrity of the seminiferous tubules. In golden hamsters with active spermatogenesis, myoid cells are squamous in shape (Kurohmaru et al., 1990) and most of the cytoplasm is packed with actin filaments

(Toyama, 1977). Few organelles and significant numbers of pinocytotic vesicle are found in the cytoplasm. In animals with inactive spermatogenesis, produced during short photoperiod conditions, myoid cells still appear flat in shape, but with highly irregular surface contours. Both actin filaments and pinocytotic vesicles decreased in myoid cells in inactive animals, particularly, the latter which were approximately three-fold less than in active animals (Kurohmaru et al., 1990). Pinocytotic vesicles are considered to be a vehicle for transporting substances from the lymphatic side of the myoid cell to the seminiferous epithelial side of the cell. Actin filaments, which are abundant in active animals, (Kurohmaru et al., 1990), are thought to be part of the actinomyosin contractile system (Vol and Soucy, 1985). This contractile property of the cells most likely aids in propulsion of fluid and sperm down the tubular lumen (Roosen-Runge, 1951; Ross and Long, 1966; Russell et al., 1989).

Dym and Fawcett (1970) found that both closed and open junctions are present at the boundaries of rat myoid cells (Fig. 1-3B). That is considered as a morphological base for smaller markers which were restricted outside the junctions between the myoid cells in some areas, but passed through it in other areas (see above). Little or no resistance to the passage of molecules was found in the myoid cells in monkeys, since these cells overlap each other without the presence of occluding junctions (Dym, 1973). Kurohmaru et al. (1990) recently found that there seem to be no occluding junctions between myoid cells in either gonadally active or inactive golden hamsters.

A large number of androgen receptors have been found in rat myoid cells (Verhoeven, 1980), which appear to be dependent on the presence of androgen for their differentiation (Hovatta, 1972; Bressler and Ross, 1972; de Kretser et al., 1975). It has been reported that androgens stimulate the myoid cells to secrete a paracrine factor PModS, which influences the function and differentiation of the Sertoli cells (Skinner and Fritz, 1985b; 1986). It is believed that FSH is a major modulator for RNA and protein synthesis in Sertoli cells (Ritzen et al., 1989). However, the action of PModS on Sertoli cell function is considered more important than that of FSH (Skinner et al.,

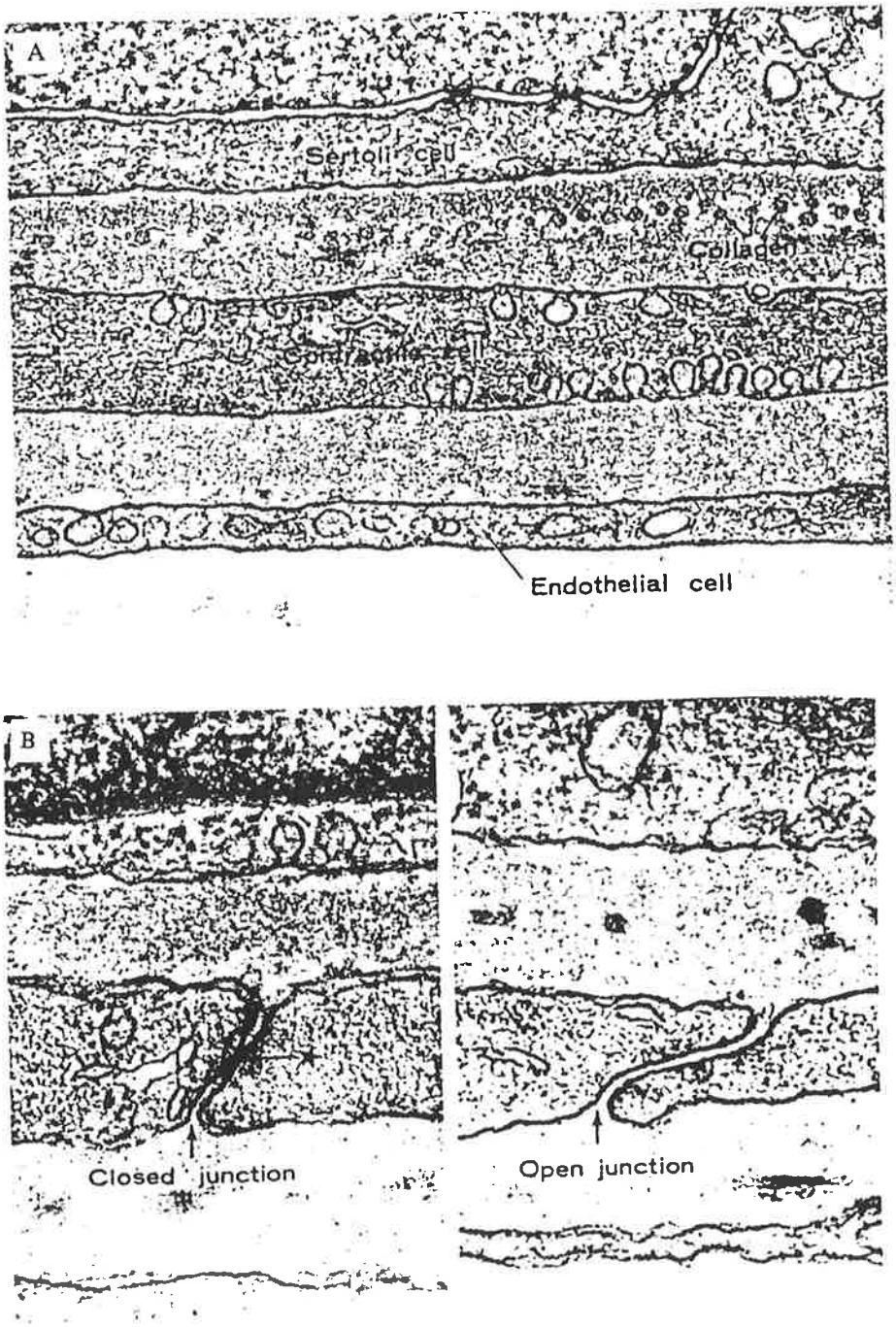


Fig. 1-3 Series of electron micrographs showing peritubular tissue surrounding seminiferous tubules. (A) Peritubular tissue consist of four layers. B) Both open and closed junctions are observed at the boundaries of moyid cells (courtesy of Fawcett from Setchell and Waites, 1975).

1988). It is likely that androgens from Leydig cells modulate the function of Sertoli cell indirectly through PModS secreted from the myoid cells.

(2) The Sertoli cells

The Sertoli cells, originally described by Enrico Sertoli in 1865, are somatic cells of the seminiferous tubules, and they reside on the inside basement membrane of peritubular tissue and extend cytoplasmic processes to the tubule lumen. Between adjacent Sertoli cells, germ cells of different stages are enveloped between the lateral surface of the Sertoli cells. They migrate from the basement to the lumen as they mature. As neither capillaries nor nerves have been found inside the tubules, germ cells are considered to be supported and nursed by Sertoli cells.

The primary Sertoli cell precursors are derived from a different embryo tissue than the germ cells. The mesenchymal cells (Jost,et.al., 1974) and the mesonephric ducts (Wartenberg,1978) are possible candidates, but further evidence is still needed to elucidate source of the Sertoli cells.

The shape and the distribution of the Sertoli cell in the seminiferous tubule in rat were described as the trees in an orchard by Elftman (1963). The basal part of the Sertoli cell was compared to the trunk of a tree and the many cytoplasmic ramifications were likened to the branches of a tree. Briefly, the overall shape of these cells is tall, irregular column with numbers of very thin lateral processes and cylindrical recesses (de Kretser and Kerr, 1988). Recent ultrastructural studies in the rat have shown continual shape changes in Sertoli cells to accommodate the structural transformations and mobilization of germ cells from the base to the tubule lumen (Kerr, 1988a). Cyclic variations in Sertoli cell shape have been demonstrated and there are also changes in Sertoli cell volume. The maximum volume of a single Sertoli cell was found during stage 12-14 (7700-8000μm^3) and the volume decreased in the first half of the cycle, reaching a nadir at stage 8 (5300-5500μm^3). The cyclic changes in Sertoli cell shape and volume are considered to relate to their functions of supporting and nursing.

The nucleus of the Sertoli cell in most species is large, irregular with a highly infolded nuclear membrane (Fawcett, 1975; Ploen and Ritzen, 1984) and is usually located within the basal aspect of the cell (Chiappa and Fink, 1977). These structural features make Sertoli cell distinguishable from early germ cell under the light microscope. However, the position and the shape of the Sertoli cell nucleus vary according to the stages of seminiferous cycle in rodents. They are usually triangular or polygonal following release of mature spermatids and elongated as the elongated spermatids penetrate into the recesses of the Sertoli cells (Elftman, 1950). Electron microscopy shows a homogeneous distribution of euchromation accompanied by a fine fibrillar-granular texture (Wong and Russell, 1983). The Sertoli cell in human and in rodent testes contains obvious nucleoli, which comprise a central dense, compact nucleolar mass or nucleolonema and two satellite karyosomes (Fawcett,1975). Autoradiographic studies (Mirre and Knibiehler, 1982) of Sertoli cell nucleoli have revealed fibrillar centres inside the central nucleolar mass together with an interconnecting nucleolar fibrillar network which constitute the site of RNA synthesis.

The organelles and inclusions of the Sertoli cell generally concentrate in the trunk region of the cells, with the apical projections containing few such structures; however, mitochondia, smooth endoplasmic recticulum and glucogen are present frequently in the apical extensions of Sertoli cell cytoplasm in the mouse, squirrel and human (Schulze, 1974; Ross, 1976; Vogl et al., 1983). Recent study of the rat Sertoli cell reveals that the shape of their mitochondia is not limited to slender and round forms. S shapes and irregular dumbbell or doughnut forms are also observed in this species (de Kretser and Kerr, 1988). The mitochondial volume of average Sertoli cell does not show clear changes throughout the stages of the spermatogenic cycle (Kerr,1988a).

The Golgi apparatus of Sertoli cell in rodents consists of many primary networks interconnected by narrow bridges. In stages 5-8 of the cycle, the Golgi apparatus has been described as a large, three-dimensional structural cylinder, which extends in the trunk cytoplasm of Sertoli cell from the juxtanuclear region toward the lumen of the

seminiferous epithelium (de Kretser and Kerr, 1988). Electron microscopy reveals few vesicles or condensing vacuoles, which are considered as a sign of synthesis of proteins in cell, in the Golgi membrane (Fawcett, 1975; 1977). Similarly, rough endoplasmic reticulum, which is another sign of protein synthesis in cell, appears only in limited amount in the Sertoli cell of many species (Fawcett, 1975). Although the Sertoli cell is capable of secreting many proteins and peptides, the histological evidence for this function is slight.

The smooth endoplasmic reticulum is usually abundant in Sertoli cell of various species, and the shape is quite different depending on species and the methods used for tissue fixation. In ram Sertoli cell, multiple concentric layers of smooth endoplasmic reticulum surround droplets of lipid as well as the deep recesses in the membrane in which developing acrosomes of spermatids are located (Fawcett, 1975; 1977; Russell, 1980). In rat Sertoli cell, the configuration of smooth endoplasmic reticulum changes from tubular to vesicular with the spermatogenic cycle (Kerr and de Kretser, 1974; Olvik and Dahl, 1981). A dramatic variation of smooth endoplasmic reticulum volume and surface area is also found associated with spermatogenic cycle in this species (Kerr, 1988a). The functional significance of Sertoli cell smooth endoplasmic reticulum is still unknown, although there is speculation that it is associated with metabolism or synthesis of certain steroid compounds.

The amount and size of lipid inclusions in Sertoli cell are highly variable between species and the stage of the cycle. The size and number of lipid inclusion are usually consistent with the reinitiation of spermatogenesis. Both increase significantly with spermatogenic arrest or in conditions that cause germ cell damage (Lincoln, 1981; Lofts, 1972; Johnson, 1970; Kerr et al., 1980; de Kretser et al., 1983). Because of these observations, there has been speculated that degenerating germ cell and residual bodies of spermatid are the source of the lipid inclusions (Lacy, 1967; Johnson, 1970), which in turn may be utilized by Sertoli cell for steroidgenesis.

Numerous dense bodies, which include primary lysosomes, multivesicular bodies and heterophagic vascules, are found in the trunk cytoplasm of Sertoli cell (Fawcett, 1975; 1977; Schulze, 1984), flanking the nucleus or close to developing spermatids (de Kretser and Kerr, 1988). Lysosomes contain strong hydrolytic enzymes, which can digest residual bodies of spermatids , degenerating germ cells (Sapsford et al., 1969; Russell, 1980) as well as exogenous substances (Clegg and MacMillan, 1965; Carr et al., 1968; Soares-Pessoa and David-Ferreira, 1980). Histological studies have shown that the lysosomes are able to fuse with residual bodies of spermatids and transform them into phagolysosomes which disintegrate at the base of the Sertoli cells (Morales et al., 1985). Because of their scavenger function, lysosome volume in a single Sertoli cell is high around the stages of releasing mature spermatids into the lumen and low in other stages (Kerr, 1988a).

Filaments, distributed throughout the Sertoli cell cytoplasm, play a major role in providing support for the columnar portions of the cell and in moving cytoplasm for displacing late spermatids. They are rich in actin and vimentin (Toyama, 1976; Franke et al., 1978; 1979) and are mainly concentrated in the trunk region. Those filaments close to ectoplasmic specializations are thought to participate in forming junctional complexes between neighbouring Sertoli cells in the basal aspects of the epithelium (de Kretser and Kerr, 1988).

Different types of specialized junctional regions are observed within the seminiferous epithelium. They include tight junctions, desmosomes, hemidesmosomes and gap junctions. Desmosomes, also called the macula adherens, occur on the lateral aspects of adjacent cells, and are thought to present between Sertoli and round germ cells (Pelletier and Friend, 1983; Russell, 1977a; Schulze, 1984; Altorfer et al., 1974). They not only anchor round germ cells against any force tending to dislodge them into the lumen (Russell, et al., 1983), but also assist the Sertoli cell to move maturing germ cells towards the lumen. Hemidesmosomes connect the base of the Sertoli cell to the basement membrane (Russell, 1977a; Connell, 1974). Gap junctions, which present

very narrow gaps (20Å) between adjacent cells but are usually permeable to ions and other small molecules, are found between Sertoli cells in laboratory animals (Gilula et al., 1976; McGinley et al., 1977) and between Sertoli and round germ cells (Bellve, 1979; McGinley, et al., 1979).

Tight junctions between Sertoli cells occur at puberty and occupy approximately 4% of the plasma membrane surface area in the rat, These junctions involve very complicated cell architecture in mammals. Weber et al. (1988) suggested that the maintenance of the junctions is related to the ectoplasmic specialization formed by the actin cytoskeleton. The longitudinal view of a tight junction as seen by electron microscopy shows: (1) Multiple focal fusions of the opposing membranes (up to 50 times) rather than fusion throughout the junction region, leaving a number of intercellular spaces separated by the Sertoli cell membrane contacts in this region; (2) Discrete filament bundles located in cytoplasm close to the opposing membrane fusions of both cells, which are orientated parallel to the cell surface; (3) cisternae of the endoplasmic reticulum, located deeper to that layer of filaments and orientated parallel to the cell boundary. These have irregular fenestrations so that the cisternae show a discontinuous profile. Ribsomes are borne on the cisternae surface towards the cell body but the cisternae are devoid of ribosomes on the other side (Setchell,1978; Fig. 1-4). These characteristics enable the junction not only to block permeability to substances from both sides but also to move maturing germ cells towards the tubule lumen.

The tight junctions between adjacent Sertoli cells divide the seminiferous epithelium into two compartments (Fig. 1-5). The basal compartment is occupied by spermatogonia, preleptotene and leptotene primary spermatocytes. The more differentiated germ cells reside in the adluminal compartment inside the tight junction (Fawcett, 1973a; Dym, 1973; Dym and Fawcett, 1970). Small markers (Dym and Fawcett, 1970; Fawcett et al., 1970) and serum albumin (Christensen et al.,1985) can enter the basal compartment and distribute between Sertoli cells and spermatogonia or primary spermatocytes, but they are stopped by the tight junctions between Sertoli cells. The tight junctions contribute to an unique physiological environment for the more

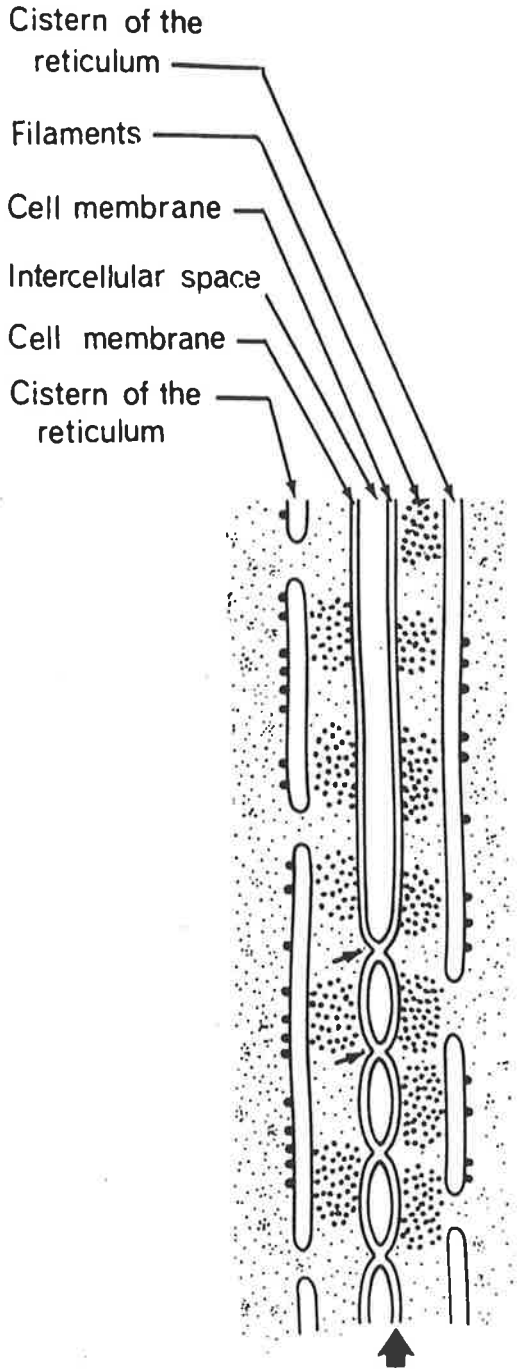


Fig. 1-4 Diagram presenting the components of the junctional complex between Sertoli cells. Details are described in text. (from Fawcett, 1975)

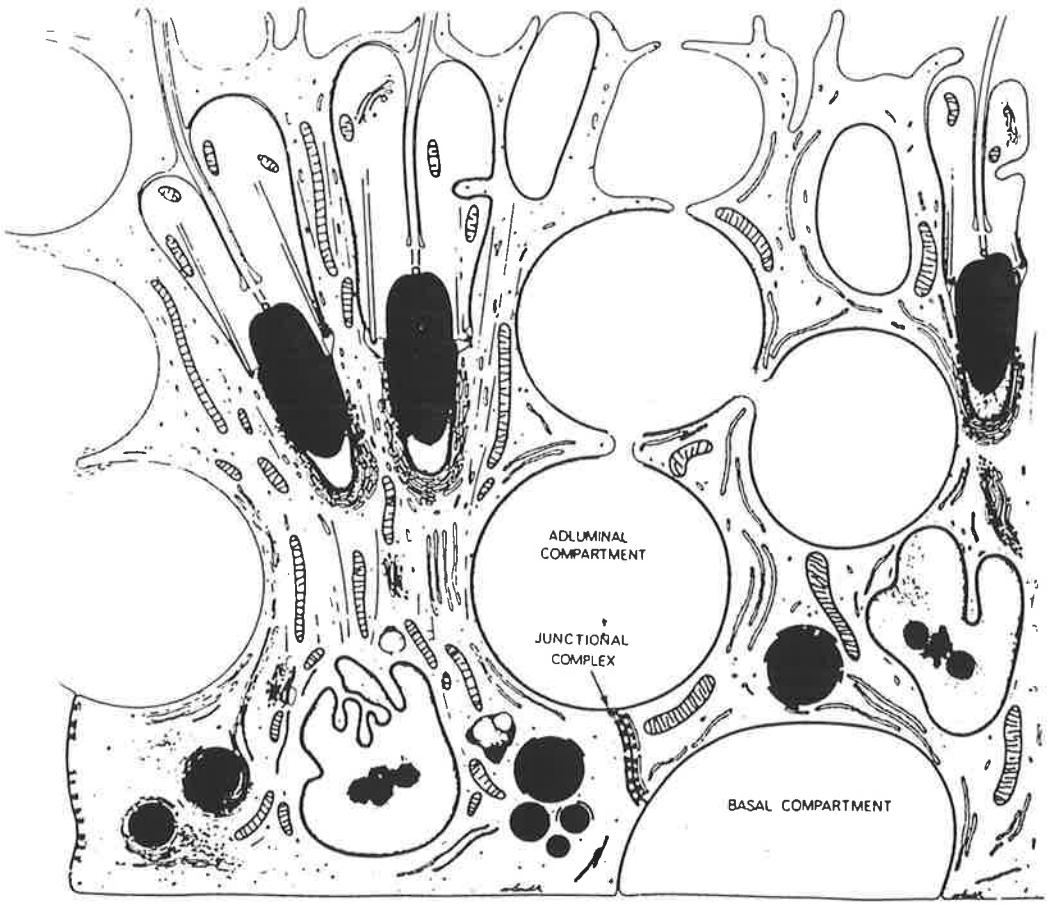


Fig. 1-5 Diagram of tight junctions between adjacent Sertoli cells. Details are described in text. (from Fawcett, 1975)

differentiated germ cells so that they are protected from immunoglobulin and other substances in the bloodstream.

How do maturing primary spermatocytes move from the basal compartment to the adluminal compartment? Dym and Caviccia (1977) observed the distribution of tracers around different developing germ cells by electron microscope and found that: (1) Tracers run around spermatogonia located on the basement membrane, but stopped at the tight junction above the spermatogonia; (2) When spermatogonia develop into primary spermatocytes, the cytoplasm of the Sertoli cells moves and envelops the whole primary spermatocyte. Junctions between Sertoli cells can then be found below, above, or both below and above those primary spermatocytes, but they still allow tracers to enter between Sertoli cells and distribute around the sperm cells (including those with tight junctions below or both below and above) which are connected via cytoplasmic bridges; (3).Tight junctions which exclude tracers form below spermatocytes entering the zygotene phase of meiotic prophase; (4) The tight junctions above the zygotene spermatocytes begin to dissociate into hemijunctions around those cells. Thus, the barrier is flexible and deformable and compatible with the movements of migrating germ cells.

The hemijunctions, composed of bundles of filaments sandwiched between the Sertoli cell membrane and cisternae of the endoplasmic reticulum, are found around germ cells from zygotene onward in primates and other species (Gondos and Conner, 1973; Schulze, 1984; Dym, 1973; Gravis, 1979). Their functions may by associated with: (1) providing flexibility for Sertoli cells during germ cell mobilization (Russell, 1980; 1977b); (2) participating in acrosome shaping and sperm release (Fawcett, 1979; Ross, 1976; Russell, 1984; Romrell and Ross, 1979); (3) a contractile role (Toyama, 1976; Gravis, 1978); (4) a site of intercellular attachment (Ross, 1976; 1977; Franke et al., 1978; Romrell and Ross, 1979). There are three inferences for the fate of hemijunctions. The bundles of filaments and cisternae of the endoplasmic reticulum (1) degenerate and are absorbed by the Sertoli cells (Ross, 1976; Russell et al., 1980; Gravis, 1978; Clermont et al., 1980; Cooper and Bedford, 1976); (2) are removed from

the Sertoli cell surface and reutilizated by the next generation of germ cells (Russell, 1977c; Russell et al., 1980; Gravis, 1980); (3) the hemijunction adheres to the cytoplasmic lobe of the sperm and then selectively retains the lobes of residual cytoplasm (Gravis, 1979). To date, the factors regulating formation and degeneration of the tight junctions remain to be determined.

The large surface area contact of round germ cells in the seminiferous epithelium is beneficial to the interaction between those germ cells and Sertoli cells. Although maturing spermatids which have ascended to the apical lumen have lost their relationship with hemijunctions, they still retain their contact with the seminiferous epithelium via an apical stalk of Sertoli cell cytoplasm which covers the head, and the neck of the elongated spermatids. There is also a cytoplasmic lobe extending from the spermatid neck and into the apical region of the Sertoli cells, called the tubulobulbar complex. This is enveloped by the membrane of the Sertoli cell (de Kretser and Kerr, 1988). It has been suggested that the tubulobulbar complexes possess the following functions: (1) to anchor the spermatid head and participate in its stabilization (de Kretser and Kerr, 1988); (2) to allow, by degeneration, the resorption of spermatid cytoplasm by the Sertoli cells (Russell, 1979); (3) to trigger the sperm release mechanism by the flow of a proportion of spermatid cytoplasm into the tubulobulbar complex (Gravis, 1980). The tubulobulbar complexes probably become part of the residual bodies after sperm release, and then are degraded by the digestive system in the Sertoli cells (de Kretser and Kerr, 1988).

The rete testis is lined by epithelial cells ranging from squamous to low columnar with few organelles. Some infolding is observed at their basal surface, and abundant microvilli are present on the luminal surface (Leeson, 1962). The specialized tight junctions between these epithelial cells are present near the lumen (Jones et al., 1979; Leeson, 1962). Because of morphological characterisations and the difference in ion concentration between tubule and rete fluid (see below), it is believed that the barrier in the rete testis is less complete than in tubules, specially with regard to immune privilege (Waksman, 1959; Johnson, 1970).

1. 2. 3. Functions of the blood-testis barriers

The blood-testis barrier does not appear in animals before puberty, but at the time spermatogenesis begins. Russell and co-workers (1989a) observed the shrinkage of germ cells and the Sertoli cells in rat testis following hypertonic fixation, indicating that the occluding junctions between Sertoli cells began development at about the 16th postnatal day, and progressively attained adult levels at about the 22nd postnatal day. However, using ^{51}Cr-EDTA as a marker, Setchell et al.(1988a) demonstrated that the formation of the Sertoli cell junctions occurs earlier than the formation of a functional blood-testis barrier. One hour ^{51}Cr-EDTA space began to fall after the 25th postnatal day and reached an adult value by the 33rd postnatal day (Setchell, 1986). This difference may be due to the use of different rat strain as well as variance of method. Although the forming time of the barrier is different in these two experiments, both showed that the beginning time of barrier development (16-25 days) is later than the time when the lumen appears (10-15 days) (Setchell et al., 1988a; Russell et al., 1989a). However, the barrier attains its adult level (22-33 days) much earlier than the lumen does (45-50 days) (Setchell et al., 1988a; Russell et al., 1989a). These observations suggest that the pressure within the tubule lumen against the tight junctions between Sertoli cells is the base for full development of the tubule lumen, though Sertoli cell secretion may be earlier than the barrier forming.

The significance of the barrier can be considered in two ways: (1) the milieu for spermatogenesis; (2) transmission of signals.

1. 2. 3. 1. Supply of a suitable microenvironment for spermatogenesis

Samples from different parts of the testis show different concentrations of ions, proteins and hormones (Table 1-1). Concentrations of substances in testicular venous blood plasma (TVB) are more similar to those in the interstitial tissue fluid (ITF) than those in the seminiferous tubule fluid (STF). This result demonstrates that the blood-testis barrier is located in the seminiferous tubules.

(1) Ion concentration

Seminiferous tubule fluid contains low sodium and high potassium levels compared with those in blood plasma and ITF. Potassium concentration in tubule fluid is approximately 10 times that in blood plasma and ITF. Experiments, inhibiting Na^+-K^+-translocating ATPase with ouabain (Cheung et al., 1977; Setchell and Singleton, 1971; Wong, 1976) have demonstrated that there is a Na^+-K^+-translocating ATPase on the basal membrane of the Sertoli cell which may be involved in the uptake of potassium and the discharge of sodium. This activity is, in turn, related to the rate of STF secretion. Considering the high intracellular potassium levels maintained by Na^+-K^+-ATPase, potassium diffusion down a cell electrochemical gradient could explain potassium movement from the Sertoli cells into the tubule lumen. Alternatively, sodium from ITF enters the Sertoli cell by passive diffusion. A low intracellular sodium concentration is maintained by an active sodium-potassium exchange mechanism, which moves sodium from the Sertoli cell into the lumen if its route of entry is through the Sertoli cell (Waites and Gladwell,1982). Besides potassium, magnesium and total phosphorus are also higher in STF (Jenkins et al., 1980), whereas chloride and sulphur concentration in STF is similar to blood plasma (Tuck et al., 1970; Jenkins et al., 1980). Some researchers suggest that maintenance of high potassium and low sodium in tubular fluid probably depends on the presence of germ cells in the seminiferous epithelium. This is evidenced by germ cell free tubules, induced by busulfan in embryos, that lack the difference of potassium and sodium concentration from that in blood plasma and lymph (Levine and Marsh, 1975; Tuck, 1969). Conversely, disruption of the blood-testis barrier by intratesticular injection of glycerol is also accompanied by severe degeneration of germ cells (Wiebe et al., 1991). It is still unclear whether changes of sodium and potassium concentration in the lumen can inhibit spermatogenesis.

The ion concentration in rete testis fluid (RTF) differs from that of both STF and blood plasma (Table. 1-1). Potassium content in RTF is less than in STF, but still more

Table 1-1

Concentrations of some constituents of different fluids in rat testis

	STF*	RTF*	ITF**	TVB**
Ions(mM)				
Na^+	108	143	155	144
K^+	50	14	4.5	4.7
Glucose(mM)	< 1	< 0.1		
Protein(mg/ml)	6	1.78***	75	58
Testosterone(ng/ml)	40-115	22-46	12	23

* data from Setchell,1988;

** data from Maddocks and Setchell, 1988;

*** data from Main and Waites, 1977.

than in blood plasma. However, RTF sodium concentration is similar to that in blood plasma. RTF has more chloride and less bicarbonate than many other fluids (Setchell, 1988). This ion composition of RTF may be explained by electrochemical gradients across the rete epithelium, which favour the exit of potassium and entry of sodium with the chloride in broad electrochemical balance (Waites and Gladwell, 1982). Experiments showed that seminiferous tubule epithelium is different from rete testis epithelium with regard to permeability to water, ions and other small molecules. The entry rate of $^{3}H_2O$ and ^{42}K is more rapid in tubules than in rete testis, and ^{24}Na enters the tubules more slowly (Main, 1976; Okumura et al., 1975; Setchell et al., 1978a; 1969).

(2) Protein concentration

Although protein content in STF is about 1/10 of that in blood plasma, the number of types of protein are not less. However, using acrylamide gel electrophoresis Koskimies and Kormano (1973) found that protein types in rat STF were different from those in blood plasma. Only 9 out of 21 protein types in serum could be observed weakly (except one protein) in STF. On the other hand, there were 14 protein types in STF that were not present in blood plasma. Most of these 14 proteins were of low molecular weight and constitute approximately 25% of the total protein content in STF. The bulk of them (about 2/3) are prealbumins. Research based on Sertoli cell culture in vitro found that Sertoli cells could secrete a number of proteins (Griswold, 1988). Some of these were only secreted into the tubule lumen, and were seen only in electrophoresis of STF or RTF (Koskimies and Kormano, 1973); others were secreted into both the tubule lumen and blood. Griswold (1988) classified those proteins secreted by the Sertoli cells into 5 groups; (1) transport proteins, which include Androgen-binding protein (ABP), transferrin, and ceruloplasmin; (2) hormones or growth factors, which include inhibin, seminiferous growth factor etc.; (3) enzymes, which include plasminogen activator etc.; (4) basement membrane components, which include type IV collagen and laminin; (5) products with unknown functions or activities,

which include sulfated glycoproteins (SGP) 1 and 2, cyclic protein 2 etc.. ABP was the first protein secretion product of Sertoli cells to be isolated and characterized biochemically, and its concentration is much higher in STF than in serum. The function of ABP is probably to transport androgens to the epididymal epithelium (Hansson et al.,1975; Lobl et al., 1980). Transferrin and ceruloplasmin are present mainly in serum. They are involved with Fe^{3+} and Cu^{2+} movement from serum to germ cells within the Sertoli cells (Griswold, 1988). Plasminogen activator may be required for the passage of preleptotene spermatocytes through the tight junctions and for the release of spermatids during spermiation (LaCroix et al.,1979). Recently, studies indicate that the function of SGP2 may involve in regulation of spermatogenesis and sperm maturation (Bardin et al., 1988) and this protein appears to be identical to clusterin found in ram rete testis fluid (Fritz et al., 1983).

The RTF protein content is approximately 1/4 - 1/5 that of STF (Waites and Gladwell, 1982). Most of the proteins in RTF come from serum and STF (Koskimies and Kormano, 1973). Some proteins of serum origin in RTF were at a higher concentration than in STF. Immunoglobulins are undetectable in STF, but present in very low concentration in RTF (Koskimies et al., 1971). Some authors suggest that albumin could be transferred into the tubule lumen by way of the rete testis rather than through the tubule (Main, 1976; Setchell and Wallace, 1972b).

(3) Testosterone

Testosterone concentration is about 40-115 ng/ml in rat STF, higher than in ITF collected with a push-pull cannula as well as in RTF and testicular blood plasma (see Table 1-1). Besides free testosterone, much testosterone in STF is bound to ABP, then transported to the epididymis. Testosterone is produced by Leydig cells in interstitial tissue and enters the tubule through the Sertoli cells. However, its entry rate cannot be explained by lipid-solubility and simple diffusion due to the facts that testosterone has lower lipid-solubility and a higher entry rate than other steroids in the testis. The entry rate of radioactive testosterone could be inhibited by an increase in non-radioactive

testosterone in the blood, so it is believed that testosterone enters the tubule by facilitated diffusion (Setchell et al., 1978b)

1. 2. 3. 2. Transmission of signals

Germ cells are enveloped by the Sertoli cells and then surrounded by peritubular tissue so that any signal capable of influencing spermatogenesis must involve either modification of the metabolism of the Sertoli cell causing significant effects on spermatogenesis via protein secretion by the Sertoli cell or an action on peritubular cells, which produce a factor by altering the function of Sertoli cell, thereby indirectly affecting germ cells.

Follicle stimulating hormone (FSH) is secreted by the pituitary, and carried to testis by the blood. This hormone is necessary for initiation of spermatogenesis in immature animals, but once the spermatogenesis is established, it can be maintained by androgens even in hypophysectomized rats (Dym et al., 1977; Walsh et al., 1934). The Sertoli cell is a major target for FSH (Hansson et al., 1978), that has been demonstrated by the evidence that autoradiographic and electro microscopic studies using FSH labelled with ^{125}I (Desjardins et al., 1974; Orth and Christensen, 1977) and ferritin (Castro et al., 1972; 1970) showed FSH accumulation on the Sertoli cells. Whether FSH accumulates on spermatogonium still needs to be confirmed.

FSH receptors on plasma membrane of Sertoli cells are saturable, high affinity, low capacity and hormone specific (Setchell, 1978; Ryan et al., 1988; Sprengel and Braun, 1992). The amount of FSH bound in the rat testis increases with age until the 15th postnatal day, and then remain constant (Fakunding et al., 1976). The effects of FSH on spermatogenesis are mainly attributable to stimulation of protein secretion by the Sertoli cells. The mechanism of effect of FSH is described follows: (1) FSH binds to receptors on surface of Sertoli cell; (2) This binding complex activates adenyl cyclase (Means, 1973) which, in turn, results in an increase of cAMP (Means et al., 1969); cAMP stimulates protein kinase activity (Means, 1973; Means et al., 1974), then

induces the phosphrylation of proteins. A cascade of subsequent events in the Sertoli cell ultimately leads to altered function.

Many studies confirm that ABP levels increase after chronic FSH treatment both in vivo and vitro (Vernon et al., 1974; Steinberger et al., 1975; Hansson et al., 1973); a number of other proteins such as: plasminogen activator (Fritz and Karmally, 1983), transferrin (Perez-Inffante et al., 1986), inhibin and some unknown functional proteins (Cheng et al., 1986) are also increased by FSH. In vitro FSH increases Leydig cell stimulatory factor (LCSF) secretion of Sertoli cells; LCSF seems to be a paracrine substance which stimulates steroidogenesis in Leydig cells (Verhoeven and Cailleau, 1985).

Besides protein secretion, FSH regulates Sertoli cell energy and fat metabolism through stimulation of glucose uptake (Hall and Mita, 1984), lactate production (Mita et al., 1982), the activity of glycogen phosphorylase (Slaughter and Means, 1983), and modulation of cholesterol esterase activity (Bardin et al., 1988). FSH also increases the synthesis of cell surface glycoproteins (Marzowski et al., 1985), which, in turn, serve as cell adhesion molecules. It seems likely that FSH influences division of Sertoli cells through increasing DNA synthesis and mitotic activity both in the pre- and postnatal period (Griswold et al., 1976; 1977).

Androgens are well known for their importance to spermatogenesis. Up to date, no androgen receptors have been found in the germ cells, but they are present in Sertoli cells. Therefore, the Sertoli cell is considered to be a pathway by which androgens exert their influence on the spermatogenic process. In adult hypophysectomized rats, androgens alone can maintain spermatogenesis and ABP production if given immediately after surgery. On the other hand, FSH and androgens act synergistically in stimulating ABP production by Sertoli cells in regressed testes after hypophysectomy (Hansson et al., 1975). In adult rats, transferrin production by Sertoli cells decreased markedly after administration of exogenous testosterone and oestradiol, which suppress endogenous LH and testosterone production, but no effect was observed in SGP2

production in vivo (Roberts et al., 1991). FSH induced inhibin secretion by Sertoli cells can be arrested with testosterone (Bardin et al., 1988). Besides an effect on protein secretion, testosterone also stimulates spermatid binding to Sertoli cells, but FSH only induces a junction-related structural "competency" of the Sertoli cell and is not directly involved in the cell attachment phenomenon in vitro (Cameron and Muffly, 1991). Seminiferous tubules in different spermatogenic cycle stages express different androgen-sensitivity. Seminiferous tubule culture showed that when the rats are treated with ethane dimethane sulphonate (EDS), to eliminate the Leydig cells, incorporation of ^{35}S-methionine into secreted protein declined only in stage VI-VIII of the tubules rather than in stage II-V and IX-XII of the tubules (Sharpe et al., 1991).

The direct effect of androgens on Sertoli cell function in vitro may be less than those obtained with FSH. However, researchers found that peritubular cells enhance the actions of androgens on Sertoli cells. Androgen receptors are present in peritubular cells and androgens can increase production of the paracrine factor PModS by peritubular cells, which, in turn, influences Sertoli cell function by stimulating transferrin and ABP production (Skinner and Fritz, 1985b). The PModS effect on some protein synthesis by Sertoli cells is as great as could be obtained by maximal stimulation with FSH and testosterone (Ritzén et al., 1989).

Recently, Onoda and co-workers (1991) found that in testicular cell co-culture, germ cells could modulate Sertoli cells to secrete a special protein, which, in turn, stimulates Leydig cell steroidogenesis.

1. 2. 4. Factors which influence the function of the blood-testis barriers

Generally, factors which probably influence the function of the blood-testis barriers can be divided into 3 categories: (1) physical factors, which include temperature, x-ray and pressure (efferent duct ligation); (2) chemical factors, which include vitamin A deficiency or excess vitamin B_6, and other chemicals, such as cadmium and glycerol; (3) physiological factors, such as age. Physical factors are the focus to be discussed in this section, whereas cadmium and age will be discussed in the following section.

1. 2. 4. 1. Elevated testicular temperature

In most mammalian species, the testes are located outside the body cavity in the scrotum, which supplies a specific milieu of low temperature for spermatogenesis. Early in 1922, Crew had already recognized the importance of temperature to spermatogenesis. Clinically, pathological changes induced by elevating testicular temperature occur in patients with cryptorchidism or varicocoele. The former is due to elevated testicular temperature directly to abdominal level; the latter may involve secondary hyperthermia induced by changes in testicular microcirculation.

Much attention has been paid to the effect of hyperthermia on the germ cells. This manifests mainly as a depletion of germ cells, specifically pachytene spermatocytes and spermatids in seminiferous tubules (Collins and Lacy, 1969; Chowdhury and Steinberger, 1970; Setchell et al., 1971; Gasinska and Hill, 1990), the decrease of testicular and epididymal weights (Au et al., 1987; Sharpe and Bartlett, 1987; Waites and Ortavant, 1968), and a low percentage of embryonic survival (Mieusset et al., 1991). Serum testosterone measurement showed no difference between heated or control animals (Main et al., 1978; Au et al., 1987; Galil and Setchell, 1987), but FSH levels increased dramatically (Main et al., 1978; Bartlett and Sharpe, 1987; Au et al., 1987; Galil and Setchell, 1987), accompanied with changes of ABP in ITF (Sharpe and Bartlett, 1987; Jegou et al., 1984) and inhibin secretion decreases (Au et al., 1987) in

heated animals. Those results suggest that Sertoli cells are injured during heating or are responding to changes in germ cell numbers.

Morphological changes were observed at testes exposed to 43°C at least 15 min (Collins and Lacy, 1969), and Kerr and colleagues (1979) found that Sertoli cells contain large number of vacuoles and lipid inclusions after heating. The former were usually observed at the basal aspects of the Sertoli cells and at the narrow intercellular spaces between opposing inter-Sertoli cell junctions. At longer times after heating, as germ cells are eliminated from seminiferous tubules, the cytoplasm of the Sertoli cells becomes compact, which probably contributes to a "concertina" of vacuoles along inter-Sertoli cell junctions and later membrane complexes. Peritubular tissues showed a thickening after heating.

These modifications of the the blood-testis barriers are accompanied by little change of their function. There is no change in the concentrations of ionic composition, inositol, glycine and protein in RTF during or after heating (Setchell et al., 1971; Setchell and Waites, 1972a; Main and Waites, 1977), and albumin transport from blood to RTF is also maintained at normal rates after heating (Main and Waites, 1977), although transfer constants for radioactive K, Rb, Na, inulin, horseradish peroxidase and lysine consistently increased during heating (Main and Waites, 1977; Turner et al., 1982) and uptake of gonadotrophin was reduced in the cryptorchid testes (Sharpe, 1983). Main and Waites (1977) concluded that heating sufficient to damage spermatogenesis probably was not associated with dramatic alterations in the integrity of the blood-testis barriers, but more with changes in testicular metabolism.

Heating could induce a series of biochemical changes in testis. Fujisawa and colleagues (1988) found that morphological changes were accompanied by a decrease in DNA polymerase beta and gamma activities in cryptorchid testes. However, the sensitivity of advanced germinal cells to hyperthermia may contribute to greater instability of their cellular and lysosomal membranes (Lee 1974; Vunder and Murashev, 1984). A study using cultured pieces of seminiferous tubules suggested that tubules

from cryptorchid testes secreted less lactate than scrotal tubules, whereas the FSH-stimulated tissue plasminogen activator secretion was greater from cryptorchid than from scrotal tubules (Bergh et al., 1987a). Lactate is involved in providing energy to germ cells (Jutte et al., 1981; Mita and Hall, 1982). Tissue plasminogen activator may be involved in the transfer of preleptotene spermatocytes into the adluminal compartment (Lacroix et al., 1982) and the release of spermatids (Parvinen,1982). It is speculated that the changes in local secretion of substances may influence the histological profile of the tubules. On the other hand, some studies have indicated that elongated spermatids are implicated in the control of secretion of inhibin and ABP by Sertoli cells in vivo (Main et al., 1976; Jégou et al., 1984; Pineau et al., 1989). However, a study using culture of isolated Sertoli cells demonstrated that inhibin secretion can be directly suppressed by elevated culture temperature (Steinberger, 1980). This finding indicates a possible direct damaging effect of elevated temperature on Sertoli cell secretion in vivo. Up to date, it is still unclear whether the changes of Sertoli cell functions in vivo were caused by direct heat-induced damage in the Sertoli cells, or were an indirect consequence of germ cell depletion and/or Leydig cell damage.

The effects on the testis after exposure to irradiation are quite similar to those after heating excepting that the decrease of testicular weight appeared later than after heating (Wang et al., 1983), since the most sensitive germinal cells are the A-spermatogonia (Hopkinson et al., 1978). The low ABP level in the epididymis and high levels of FSH and LH in the blood (Pinon-Lataillade et al., 1991) indicate damage of Sertoli cells. However, like testis heating, the study on ^{51}Cr-EDTA space and the morphometrical interstitial volume fails to confirm the breach of the blood-testis barrier in testes after exposure to irradiation (Wang et al., 1983).

1. 2. 4. 2. Efferent duct ligation (EDL)

It is well known that EDL can cause aspermatogenesis (Smith, 1962). However, the mechanism of disruption in spermatogenesis caused by EDL is still not clear. There might be a direct effect of EDL on germ cells and/or an indirect effect of

EDL through the change of the function of Sertoli cells or Leydig cells on germ cells. Singh and Abe (1987) found that the number of mature spermatozoa is markedly reduced in EDL testes with an appearance of giant cells, which are formed as a result of the fusion of spermatids due to alterations in the intercellular bridges. They considered that this injury of the intercellular bridges was probably a direct consequence of the increase in intratubular pressure in the testis after EDL. Pilsworth et al. (1981) observed that blocking a tubule by introducing a plug of latex into it caused a similar lesion to EDL, and speculated that the aspermatogenesis after EDL may by related to interruption of fluid flow.

As the steroidogenesis by Leydig cells is not damaged after EDL (Jansz and Pomerantz, 1986), and there is an increase of testosterone concentration in local tissue (Wang et al., 1985), the low testosterone output, then, is considered to be due to decreased blood flow.

A focus of controversy is the effect of EDL on Sertoli cells. Studies have showed that Sertoli cells after EDL are characterized by a marked increase in the number of microtubules and microfilaments, extensive inter-Sertoli cell junctions, vacuolated cytoplasm and considerably dilated intercellular space (Osman and Plöen, 1978). This implies that Sertoli cell function may be impaired. However, observation of the distribution of horseradish peroxidase (Anton, 1982) and lanthanum (Osman and Plöen, 1978) demonstrated the integrity of the blood-testis barriers. On the other hand, measurement of ^{51}Cr-EDTA space and interstitial volume after EDL suggested that the blood-testis barriers were broken 2-3 days after EDL, but re-established as the interstitial tissue volume increase (Setchell, 1986). Further study is required to settle the controversy regarding the effect of EDL on the blood-testis barriers.

1. 2. 4. 3. Exposure of animals to N_2O

Up to now, only two articles involved in effect of exposure of animals to N_2O on male gland. The degree of damage to the testes of rats exposed to N_2O are different according to N_2O dosage and time of exposure. Chromosome damage of

spermatogonia, such as exchange of figers, rings and miscellanenous markers,appeared in testes of rats exposed chronically to low concentration of halothane-nitrous oxide (Coate et al, 1979). Damage to seminiferous tubules was observed in testes of rats exposed continuously to high concentration of N_2O for short times (2 to 35 days) (Kripke et al., 1976). As mentioned above, the effect of N_2O on the blood-testis barriers has also been studied.

1. 3 BLOOD FLOW AND VASCULAR PERMEABILITY IN TESTIS

1. 3. 1. Introduction

Because the testis is responsible for spermatogenesis and steroidogenesis, much attention has been paid to the role of blood flow and vascular permeability in transporting substances into and out of the testis. Owing to the specialization of the testicular vascular anatomy, it is necessary to distinguish between two sorts of "testicular blood flow": total blood flow and capillary or nutrient blood flow. The former is the total flow which passes through the testicular artery but which may or may not pass through testicular capillaries. The latter is the fraction of the total flow which exchanges with tissue via the capillaries. Total blood flow includes flow through arterio-venous anastomoses not only in the testis, but also in the spermatic cord, and should be used for calculation of testicular secretion or uptake rate; it can be measured by flow meters applied to the artery or by the dilution of a suitable maker, such as p-aminohippurate (PAH) and ^{51}Cr-EDTA. The capillary blood flow determines what reaches the cells in the testis, and can be measured by microspheres or Doppler techniques.

As described above (see 1.2.2.1.), the testis is the only endocrine organ to contain unfenestrated endothelia (Fawcett et al.,1973b; Farquhar et al.,1961). This vascular peculiarity is responsible for preventing the passage of certain dyes which pass readily across other capillary endothelia (Kormano,1967b). However, high levels of enzymes involved in transport activity are found in testicular capillary endothelium (Kormano, 1967b; Niemi and Setchell, 1986). Besides this, studies have shown that testicular vasculature is insensitive to some vasoactive compounds, such as histamine and 5-hydroxytryptamine, which usually cause a marked increase in vascular permeability in a number of tissues (Setchell et al., 1988b). These peculiarities complicate substance transport across the testicular vascular wall.

1. 3. 2 Blood vessels

1. 3. 2. 1. Anatomy of testicular blood vessels

The testicular artery originates from the abdominal aorta at the level of the kidney. In mammals with scrotal testes, the testicular artery elongates to allow for the descent of the testis. The artery between the inguinal ring and testis is called the spermatic artery, the main branch of which enters the testis and forms the capsular artery. The spermatic artery also gives rise to 2 subordinate branches, the superior and inferior epididymal arteries, to supply the epididymis (Setchell and Brooks, 1988). In rats, as the capsular artery enters the testicular tissue, it gives off cranial, dorsal, lateral, medial and caudal intratesticular arteries to supply different parts of the testis. From the intratesticular arteries a number of radiate arteries run into the testicular blood vascular bed. The smallest arteries called intertubular arterioles divide from each radiate artery, and then form capillary networks surrounding the seminiferous tubules (Murakami et al., 1989).

Intertubular venules collect blood from capillary networks, then join to become radiate veins and then collecting veins. Both the subalbugineal veins and the intra-albugineal venous plexus receive blood from collecting veins, which then continue to the pampiniform plexus. The dense pampiniform plexus converges into 2 veins, the main (thick) and the accessory (thin) efferent vessels. The former continues into the common iliac vein, whereas the latter drains into the inferior vena cava (right side) or into the left renal vein (left side) (Murakami et al., 1989).

As the testicular artery leaves the inguinal canal, it becomes extensively coiled. In large domestic animals such as rams, bulls and boars, 5-7 metres of artery can be tightly wound into approximately 10 cm of spermatic cord (Setchell, 1978). The thickness of the wall and the number of layers of the smooth muscle cells decreases

along the length of the artery (Hees et al., 1984), but the caliber increases 1.6 fold at its distal end as compared with that at its origin (Weerasooriya and Yamamoto, 1985). The veins of the pampiniform plexus surround the testicular artery in the spermatic cord. Murakami and colleagues (1989) found that in rats, the total cross-sectional area of the most thickened segment of the pampiniform plexus is about 2 times larger than that of the efferent vessels of the pampiniform plexus. None of the vein in the plexus have valves in contrast to those immediately above it (Setchell and Waites, 1964).

1. 3. 2. 2. Function of testicular vasculature

(1) Counter-current exchange system

These anatomical specificities of the artery and vein point to a reduced blood flow velocity in the artery and venous plexus, and to an enlarged contact surface area in order to enhance the efficiency of the heat exchange between the artery and veins. A counter-current heat exchange system cools the arterial blood traveling to the testis to a temperature similar to that of the subcutaneous scrotal tissue by warming up the venous blood returning from the testis. The thick adipose tissue surrounding the pampiniform plexus may act as an insulator to keep the blood temperature of the pampiniform plexus in a constantly cooled condition. Besides this benefit, the vascular arrangement in the spermatic cord may reduce the pulsatility of the arterial flow through the cord, although the blood pressure is maintained at a high mean level (Setchell, 1978; Setchell and Brooks, 1988).

Clinical and experimental observation showed that the difference between scrotal and abdominal temperature was reduced in the patients with a large varicocoele (Hanley and Harrison, 1962) and in rats with surgically induced varicocoele (Saypol et al., 1981). The function of heat exchange is to maintain the lower temperature milieu for spermatogenesis.

(2) Vascular anastomoses

The arterio-venous anastomoses between the arteries and veins in the cord allow up to 50% of the blood in the artery to return directly to the veins without exchange in the tissue (Maddocks and Sharpe, 1989; Noordhuisen-Stassen et al., 1985). Murakami and colleagues (1989) also described many different vascular anastomoses present inside the testis. There are numbers of arterio-arterial and arterio-venous anastomoses at the radiate vessel level, and blood which has reached in the intertubular arterioles does not always pass through intertubular and peritubular capillaries, but can be redistributed between intertubular arterioles through arteriolo-arteriolar channels or directly enter intertubular venules through thick arteriolo-venular capillary channels. Between the intertubular venules, there are also venulo-venular capillary channels . These vascular anastomoses regulate the blood flow to ensure an homogeneous distribution in whole testis without changing total blood flow, and to eliminate blood congestion in the testis to enhance the efficiency of the heat exchange mechanism.

As described above (1.2.2.1.), intertubular capillaries nurture Leydig cells and other interstital cells, while peritubular capillaries nurture the seminiferous tubules. The presence of intertubular arteriolo-venular capillary channels allow testosterone secreted by Leydig cells to be directly conveyed to the intertubular venules, by-passing the peritubular capillaries which sustain the spermatogenesis in the seminiferous tubules (Murakami et al., 1989). However, the close contact of the testicular artery with the pampiniform plexus allows some testosterone exchange from the vein to artery (Jacks and Setchell, 1973; Hees et al., 1984). This exchange may allow the restoration of testosterone to the peritubular capillaries.

(3) Microvasculature

The microvasculature includes arterioles, capillaries and venules. The smooth muscle in arteriolar walls can limit or control blood flow to the interstital and tubular regions of the testis. It has been observed that the perfusion profile of intertubular and peritubular capillaries appears to change according to the stage of the cycle of the seminiferous endothelium (Ichev and Barkardjiev, 1971; Kormano, 1970). PO_2 values in the interstitium range from 11 to 22 mmHg, while those in seminiferous tubules are between 6 to 19 mmHg (Duling and Damon, 1987). Steroidogenesis requires much molecular oxygen (Hall, 1988). It may be that the PO_2 difference between interstitium and seminiferous tubule is related to this, hence the arteriolar blood flow to interstitium being greater than to the tubules.

Capillaries and post capillary venules are involved in substance exchange in tissue. In the testis they have a continuous endothelium like those of skeletal muscle. The structure of testis capillaries has been described in 1.2.2.1.. The high permeability to androgens in the testicular capillaries means that despite the relatively brief interval between serial testosterone pulses (Fox and Raichle, 1986), the pulses of testosterone secretion by the Leydig cells are reflected in pulses in testosterone concentration in the venous effluent (Setchell, 1986). In testis, the venular endothelium participates in mediation of inflammatory responses. The separation of luminal endothelial cells in post capillary venules has been observed in a transitory inflammatory-like response induced by hCG (Damber et al., 1985; Bergh et al., 1987b), leading to the suggestion that venular endothelial cells can play an active role in regulating the porosity of the microvascular membrane to macromolecules in the testis (Bergh et al., 1987b).

1. 3. 3. Relationship to lymph flow and interstitial fluid

In rat testis, there are large lymphatic spaces lined by discontinuous lymphatic endothelium. Within these, the capillaries are surrounded by Leydig cells. In the boar, however, lymphatic vessels with continuous endothelium are apparent, and they are separated from blood vessels by closely packed Leydig cells (Fawcett, 1973b). These anatomic arrangements are accompanied by a completely different lymph flow, which is about 0.15% of the testicular blood flow in rat, but as high as 6.67% in boar (Setchell, 1986).

The formation of testicular interstitial fluid follows the revised Starling's principle of fluid exchange (Levick, 1991). Fluid filters out of the capillary whenever the difference in hydrostatic pressure (P_c-P_i) facilitating filtration exceeds the difference of colloid osmotic pressure (π_p-π_i) facilitating fluid absorption, whereas fluid will be absorbed into the capillary when the conditions are reversed. Besides these, the hydraulic conductivity (L_p), surface area (S) of the microvascular bed, and the reflection coefficient (σ) for protein also influence movement of fluid across the capillary wall. Thus the amount of fluid being filtered per unit time (J_v) can be expressed by the following equation:

$$J_v = L_p S [P_c - P_i - \sigma(\pi_p - \pi_i)]$$

The surface area of the testicular capillary bed can be considered to be a relative constant factor, because it is unlikely to change in a short time. The interstitial fluid surrounding testicular capillary has a high protein concentration (Maddocks and Setchell, 1988). Early studies also indicate that testicular lymph contains a higher protein concentration than any other afferent lymph in the body except liver (Lindner, 1963; Cowie et al., 1964; Setchell et al., 1969). This suggests that the gradient in colloid osmotic pressure across the microvascular wall in testis does not retard fluid filtration from capillary to

interstitium although it is one of major forces resisting filtration and favouring fluid absorption at the venous end of capillaries in most tissues. Therefore, the difference in hydrostatic pressure becomes the major influence on fluid movement between the capillaries and interstitium in testis. A recent study using hamsters (Sweeney et al., 1991) showed that the capsular arterial pressure is about 50% of systemic pressure with the pulse pressure of 2 mmHg. This is remarkably small for an artery with a mean internal diameter in excess of 300 μm. The pulse is not detected in testicular arterioles. This may induce a different filtration rate from vessels with apparent vasomotion, because vasomotion has the potential to reduce greatly the net filtration rate over a period of time (Levick, 1991). In hamster, P_c is very low (about 10 mmHg) and appears to remain constant over a range of systemic blood pressures as a result of high arterial resistance (Sweeney et al., 1991). However, P_i is higher in testis (+2mmHg) than in most tissues (less than zero) (Setchell et al., 1990b). Although P_c -P_i in testis appears to be lower than that in other tissues, the testicular capillaries would still filter fluid because the colloid osmotic pressure gradient is nearly zero across the wall. Sweeney and colleagues (1991) indicated that the venular resistance in testis is lower than that in cheek pouch in the same animal. Generally, the lower the venular resistance, the higher the sensitivity of capillary pressure to any change in venous pressure (Davis and Gore, 1986). This mechanism may be responsible for the interstitial edema associated with varicocoele. On the other hand, P_i can be influenced by the tone of the capsule and the volume occupied by the seminiferous tubules. Contraction of the myoid elements in the capsule of many species (Setchell and Brooks, 1988) may cause a physiological increase in P_i which returns fluid to the vascular system. The accumulation of interstitial fluid accumulation in testes lacking spermatids (Sharpe et al., 1990b) may be due to a reduction in interstitial pressure as a result of a reduction in the tubular volume (Setchell et al., 1992).

1. 3. 4. Transport of substances into and out of bloodstream

Both blood flow and vascular permeability are involved in substance transport across the capillary wall. Generally, substances extracted quickly during a single passage through an organ are mainly limited by blood flow; whereas substances which are extracted slowly during a single passage are affected more by the permeability and/or transport rate across the microvasculature than by blood flow.

Recent studies have shown that the extraction of macromolecules such as albumin (radius=35.5Å) is determined by both microvascular permeability and net fluid flux in most tissues (Setchell et al., 1991a; Bent-Hanson, 1991). However, there are two ways of calculating albumin transport into a tissue. Apparent permeability surface area product (PS) which value is influenced not only by vessel wall permeability but also by net fluid flux (Setchell et al., 1991a), is calculated from the accumulation of radioactive albumin in the tissue from blood or perfusate:

$$PS\ (\mu l/g/min) = k.V_{final}$$

where V_{final} is the volume of distribution of albumin at equilibrium, k is the slope of the line of Ln $(1-V_t/V_{final})$ against time, and V_t is the albumin space at time t (Amtorp, 1980; Setchell et al., 1988a). When ^{125}I-albumin was emploied as a tracer, V (μl/g) can be calculated by the cpm in tissue such as testicular parenchym (cpm/g) divided by the cpm in blood or effluent (cpm/μl). True PS is determined only by the permeability of the walls of the microvasculature in the organ and can be measured by the uptake during a single pass of an appropriate tracer from blood or perfusate :

$$PS\ (\mu l/g/min) = -Q.\ Ln\ (1-E)$$

where Q is the perfusion flow rate (μl/g/min) and E is the single pass extraction of substance in the tissue (Bustamante and Setchell, 1981) and can be calculated by cpm in

the tissue divided by total cpm in injected solution in vitro study. After a single injection of hCG, apparent PS to albumin increased significantly (Setchell and Sharpe, 1981; Sowerbutts et al., 1985). There are no data available for true PS to albumin in the testes of intact animals or after injection of hCG. However, studies in other tissues show that macromolecular transport is probably dominated by convection through large pores even at very low lymph flow (Haraldsson et al., 1987). Therefore the transport of albumin is dependent on the net fluid flux (J_v) across the capillary wall, which in turn is dependent on both gradients of hydraulic pressure and colloid osmotic pressure (see 1.3.3.). Moreover, certain vasoactive substances such as histamine, 5-hydroxytryptamine and prostaglandin can increase albumin transport by changing the reflection coefficient (σ) in most tissues (Michel, 1988), but none of them have any effect on testicular vessels (Sowerbutts et al., 1985; Setchell et al., 1988b).

Unlike albumin transport across the vascular wall, the movement of steroid hormones is mainly flow limited. Early studies, changing the flow rate of blood perfused from the femoral artery into the spermatic artery in anaesthetized dogs (Eik-Nes, 1964) or using spontaneous variations in testicular blood flow in rats (Damber and Janson, 1978; Free and Tillson,. 1973), found a positive correlation of testosterone secretion with flow rate. Similar results were reported for corticosterone secretion in isolated perfused adrenal gland (Sibley et al., 1981; Hinson, 1984). The increase of steroid hormone output elicited by elevated blood flow seems to involve several mechanisms. Firstly, increased blood flow may provide more efficient delivery of stimulants to the tissue. At low ACTH concentrations (1-10μunits/ml) in blood, cortisol secretion increases as flow increases because more ACTH is carried to the perfused tissue per unit time although the stimulation of ACTH on cortisol secretion is limited by a saturation mechanism (Urquhart, 1965). Secondly, this mechanism does not explain why steroid hormone secretion increases as perfusion flow rate increases, even if no ACTH is added to the perfusion medium (Hinson et al., 1986). So increased flow was

suggested to remove hormone, which itself may serve as an inhibitor of steroid biosynthesis. Steroids are lipid-soluble substances and can be transported across the vascular wall in most tissues by diffusion. Kirwin and others (1981) found that testosterone secretion decreased as testosterone concentration increased in the perfusate. This indicates that testosterone production may be controlled locally via a short-loop testosterone negative feedback mechanism. This is also suggested by the fact that when testicular blood flow decreases to 1/3 of normal flow in efferent duct ligation, the testosterone content in testicular tissue does not reach threefold the control level (Wang et al., 1985). Thirdly, increased flow expands local vasculature, which might trigger the release of substances that stimulate steroidogenesis. This hypothesis is supported by the finding that peptide-processing activity and elements of the renin-angiotensin system have been demonstrated in adrenal capillary and gland respectively (Del Vecchio et al., 1980). However, direct evidence is necessary to confirm this speculation.

Steroid production and transport not only involve stimulation of gonadotrophins, local concentration of steroid, and the rate of blood flow through the tissue, but also are influenced by steroid-binding protein in the vascular system. With technique involving injection of a bolus of markers into the aorta, Sakiyama and colleagues (1988) found that accumulation of labelled testosterone in the testis in vivo is significantly greater when 5% bovine albumin is the steroid injection vehicle rather than Ringer's solution with 1% albumin. Testosterone secretion also markedly increased when the perfusate was changed from 3% dextran to 3% bovine albumin (Ewing et al., 1976). These results support Westphal's (1974) hypothesis that binding proteins facilitate the passage of the steroids through the endothelium of the capillary wall.

1. 3. 5. Factors which affect blood flow and blood vessel permeability in testis

Gonadotrophins, age and certain chemicals such as cadmium can influence both blood flow and vascular permeability in testis, whereas other factors such as season and aspermatogenesis mainly influence testicular blood flow. The former is the focus to be discussed in this section.

1. 3. 5. 1. Human chorionic gonadotrophin

It is well known that human chorionic gonadotrophin (hCG) is a glycoprotein mainly secreted from the placenta and its function is quite similar to that of luteinizing hormone (LH). The targets for hCG and LH are the gonadal tissues. In the male, a low dose of hCG (12.5 IU s.c. in rat) can stimulate Leydig cells in testes to secrete testosterone, but its function of increasing testicular interstitial fluid volume is less obvious (Bergh et al., 1986). However, a later study showed that the testicular microvessel permeability was increased significantly (Bergh et al., 1990). A high dose of hCG (50 IU or higher s.c. in rat) may cause a series of changes in testes. This dose of hCG not only increases intratesticular testosterone concentration to the maximal value (Bergh et al., 1986), but also changes testicular microcirculation dramatically (Setchell and Sharpe, 1981).

The studies using a high dose of hCG showed an inflammation-like response in testes, which involves: (1) an accumulation of polymorphonuclear (PMN) leucocytes in testicular blood vessels and migration of PMN leucocytes towards the interstitial space (Bergh et al., 1986); (2) a progressive increase in capillary permeability and lymph flow in the testes, accompanied by rises in the volume of interstitial tissue fluid (ITF); (3) a slight increase in testicular blood flow.

The studies of various inhibitors of vasoactive compounds and steroidogenesis confirmed that histamine, bradykinin, prostaglandins and testosterone are not involved

in this testicular response to hCG (Sowerbutts et al, 1986). It would appear that 5-HT was also eliminated as a medium to cause increase of permeability following hCG stimulation (Setchell et al., 1988b). The mechanism of increased testicular vascular permeability was mainly explained by an inflammation-like response to a substance produced by the interstitial cells following hCG stimulation. The effect of hCG on the testicular capillary permeability, deduced either from the radioactive albumin space (Sowerbutts et al, 1986) or from the slope of the graph of the albumin space as a fraction of ^{51}Cr-EDTA space against time (Setchell and Sharpe, 1981), was apparent at 8 hours after treatment and reaches the peak value at around 20 hours after treatment. However, the accumulation of PMN leucocytes in vessels appears earlier than the change in vascular permeability (at 4 hours after hCG) (Bergh et al., 1986). The hCG-induced increase in vascular permeability does not occur in leukopenic rats. This indicates that PMN leucocytes play a crucial role in this vascular response(Widmark et al., 1987; Veijola and Rajaniemi, 1989).

Experiments also showed that the Leydig cells are required for the vascular response to hCG, because no effect of hCG on vascular permeability, ITF volume and blood flow was observed in testes of rats pre-treated with ethane dimethyl sulphonate (EDS) which eliminates Leydig cells in testes (Setchell and Rommerts, 1985; Damber et al., 1987). At the same time, the phenomenon of hCG-stimulated PMN leucocyte accumulation in vessels, preceding the change in microcirculation, also disappeared in animals pre-treated with EDS (Damber et al., 1987).

Although the importance of the PMN leucocyte and Leydig cell involvement in the testicular vascular permeability response to hCG is definite, how they cause the change of microcirculation in the testis is still a controversy. Recently, a group of scientists in Finland has undertaken a series of experiments in order to answer this question. They found that an intratesticular injection of hCG, ITF or PMN-conditioned medium alone had no effect on permeability, but a combination of hCG with ITF or

PMN-conditioned medium caused a significant increase in permeability. This response disappeared in rats with depletion of circulating neutrophils and could be arrested by *p*-aminobenzamidine, which is a protease inhibitor and specifically inhibits urokinase-type plasminogen activator (Veijola and Rajaniemi, 1989; Anna-Kaisa Loukusa et al., 1990). Further experiments in rats treated with hCG plus HPLC (high-performance liquid chromatography) fractions of ITF or PMN-conditioned medium suggested that: (1) Two components (probably protein) from both ITF and PMN-conditioned medium were capable of interacting with hCG to produce the permeability response; (2) hCG itself modified by incubation with ITF or PMN-conditioned medium may give rise to the permeability response (Veijola and Rajaniemi, 1992).

The findings presented above suggest a possible mechanism for hCG-stimulated permeability: (1) hCG interacts with certain components in both ITF and PMN-conditioned medium to convert it into a modified hCG; (2) this modified hCG gives rise to the production of leucoattractant in the Leydig cells, the plasminogen activator system is probably also involved in generating this leucoattractant; (3) this leucoattractant elicits the permeability response via neutrophil extravasation.

1. 3. 5. 2 Cadmium

It has long been known that cadmium is a chemical which specifically damages testicular tissue and causes animal to become permanently sterile at a dose that has no apparent effect on other organs (Parizek,1956,1957). Following cadmium treatment, the testes became swollen, dark red or purple in colour. Shortly after this, the testes turn small, hard and yellowish as weight decreases (Parizek, 1957). Studies indicate that circulatory disturbances precede any morphologically detectable damage to the seminiferous tubules (Chiquoine, 1964; Gunn et al., 1963). Testicular permeability increased 1 hour after treatment , followed by a decrease in blood flow (Waites and Setchell, 1966). A study with trypan blue and electron dense tracers (Aoki and Hoffer,

1978) revealed that 1-2 hours after injection of cadmium, discontinuities in the endothelial lining could be detected by the presence of carbon particles in the walls of the testicular capillary and venules. As extravasation of carbon increased with extension of the time after cadmium treatment, the vascular lumen became tightly packed with erythrocytes, platelets and neutrophils so that the blood flow decreased, and at last almost entirely stopped. It was suggested that ischemia of the testis is the factor inducing degeneration of the seminiferous epithelium and all biochemical and physiological changes occurred in testis at later time following cadmium treatment.

The damage to the testicular vasculature induced by cadmium seems to be a direct effect of cadmium rather than an indirect action through the release of chemical mediators, because cadmium-induced testicular vascular injury cannot be blocked by various permeability inhibitors (Clegg and Carr, 1967; Maekawa et al., 1966) , vasodilators and anticoagulants (Maekawa et al., 1965). A study with immature rats found that cadmium fails to produce detectable changes in testes of rats 2 weeks old or younger (Clegg et al.,1969; Gunn and Gould, 1970; Wong and Klassen, 1980b). The mechanism of this age difference in susceptibility is unknown. Hepatic metallothionein probably provides protection against cadmium toxicity in adult rats by sequestering cadmium in liver and thus reducing the amount of cadmium reaching the testes (Yoshikawa, 1973). The level of the protein is 20 times higher in newborn rats than in adults (Wong and Klassen, 1979), but the concentration of cadmium in the testes of newborn rats is still 5 times higher than that in adults (Wong and Klassen, 1980a). Measurements of testicular metallothionein also fails to confirm that its concentration in newborn rats accounts for their low sensitivity to cadmium (Wong and Klassen, 1980b). Therefore, it is speculated that receptors on the vasculature of the testis that are vulnerable to cadmium do not appear until the animals are almost mature (between 2-3 weeks old) (Singhal et al.,1985).

By 20 days of age, alkaline phosphatase levels reach the adult value (Clegg et al., 1969); it is suspected that this enzyme is involved in active transport across the capillary wall. Studies found that alkaline phosphatase in testicular capillaries increased after the administration of cadmium (Maekawa et al., 1965), whereas succinic dehydrogenase (Peyre et al., 1968), lactic dehydrogenase (Dimow and Knorre, 1968) and glutathione peroxidase (Omaye and Tappel, 1975) decreased after cadmium. All enzymes mentioned above are not testis-specific, and they also present in a variety of mammalian organs. Therefore, the effects of cadmium on these enzymes cannot explain the testis-specific sensitivity to cadmium. However, Hodgen and colleagues (1970) described a testis-specific isoenzyme of carbonic anhydrase, which decreased in activity as early as 30 minutes after cadmium injection and disappeared 4 hours after cadmium. They suggested that this organ-specific carbonic anhydrase is the primary site of action of cadmium on testis.

1. 3. 5. 3 Age

The structure and function of the blood-tubule barrier that have been described above (see 1.2.2. and 1.2.3.), can only be demonstrated in animals after puberty (Setchell and Waites, 1975). Gondos (1980) in his literature review indicated that Sertoli cells undergo extensive changes in morphology and biochemistry during the postnatal period. These changes are mediated mainly by FSH. Administration of FSH to immature mice can increase numbers and cytoplasm of Sertoli cells (Davies, 1971) as well as protein synthesis (Davies et al., 1975). Studies both in vivo (Bressler, 1976) and in vitro (Tung et al., 1975) elucidate the specific effect of FSH on formation of intercellular junctions.

In 10-day rat testis, the membranes of the Sertoli cells are separated by an interspace of about 200Å but gap junctions appear occasionally. By 15 days, the closer contact membranes in gap junctions are more extensive, and are often accompanied by

short cisternal profile of endoplasmic reticulum coursing parallel to the surface on one or both sides of the cell boundaries. At 20 days, the membrane junctions of Sertoli cells in the basal region of the epithelium possess all of the characteristic features of these junctions in mature testis except that the focal sites of membrane fusion are fewer and the cisternal profiles are shorter and often more dilated (Gilula et al., 1976). In contrast to the establishment of anatomical barrier in seminiferous epithelium, the function barrier attains its adult level (22-33 days, see 1.2.3.) much later than anatomical barrier (Setchell, 1986; Russell et al., 1989).

Studies with dyes demonstrate the presence of an incomplete capillary barrier in the testis (Kormano, 1968; 1967a). Fluorescent gamma globulin injected into the bloodstream could not be detected in the interstitial tissue of the testes of adult rats before 8h after injection, and no albumin could be found there until 24 h (Gupta et al, 1967). After intravenous injection of radioactively labelled proteins, the volume of distribution increases with the time after injection for up to about 10h (Setchell et al., 1980; 1988a). The structural basis for this incomplete barrier is characterized by unfenestrated vessels with 150-200Å pores.

The gross architecture of the testicular vasculature in rat has already been determined at birth (Harrison, 1949; Kar et al., 1959; Suoranta and Kormano, 1970), but during the first 2 weeks of postnatal life the intertesticular blood vessel walls are fragile and poorly organized (Kormano, 1967c; Roosen-Runge and Anderson, 1959). At 15-20 days of age, synchronous with the onset of endocrine activity of the testis in rat (Niemi and Ikonen, 1963), the microvasculature of the testis begins to organize into two different networks—intertubular capillaries and pertubular capillaries (see 1.2.2.1.) (Kormano, 1967c). However, most of the peritubular capillaries appear around 25-35 days of age, at the time of spermatid formation and consequent tubular enlargement (Kormano, 1967c). The corresponding studies of vascular permeability, judged by accumulation of radioactive albumin (Setchell et al, 1988a) or IgG (Pöllänen and

Setchell, 1989), found that the apparent PS is quite different according to the age, the permeability to both tracers increases from 20 to 27-30 days of age, then decreases gradually and reaches the adult level at around 60 days. This temporary increase of PS during the time around puberty may relate to the changes of the surface area of testicular vasculature or surface charge of testicular endothelial cells between 20-30 days.

1. 4. THE PURPOSE OF THIS STUDY

The structure and functions of the blood-testis barrier have been well established according to the many studies of last three decades. However, there are still questions to be answered, (1) what will happen to spermatogenesis if the tubule barrier is broken down? (2) is blood flow rate and permeability surface area product (PS) the major factor influencing transport of testosterone and pituitary gonadotrophins respectively across the so called capillary or endothelial barrier in testis? (3) what are those factors that can affect PS if PS is the major influenceing upon the uptake of pituitary gonadotrophins? (4) is the true PS of the actual vessel wall the factor limiting protein entry into the testis, or is this controlled by convective transport of proteins linked to net fluid flux, which also contributes to the value of apparent PS?

To address the first question mentioned above, an experimental animal model needed to be established for the further study of barrier breakdown on spermatogenesis. Three different factors—EDL, heating testes and exposure of animals to N_2O, of physiological important were examined for possible involvement in breakdown of the tubule barrier. Although there ais evidence for Sertoli cell injury by heating and EDL and chromosome abnormality during spermatogenesis after N_2O treatment in rats, the

effect of these factors on spermatogenesis by breaking tubule barrier is still controversial.

Although study *in vivo* has suggested an important role for blood flow rate in testosterone secretion, there is still a dispute on whether the blood flow rate and protein content of the blood is the major factor controlling testosterone output. However, application of quantative analysis may shed light upon this matter. Effluents for testosterone assay can be collected in isolated testis perfusion with different flow rates and at different protein concentrations in the perfusate.

It is well known that testis is the major target of gonadotrophins from pituitary, but little attention has been paid to effects of blood flow rate and vascular permeability on their transport into testes. Albumin was chosen as the tracer to investigate the effects of blood flow rate and vascular permeability in this study, because albumin and gonadotrophin are biochemically similar in molecular weight and lipid solubility. Previous studies *in vivo* also suggested that the apparent PS, which is calculated from ^{125}I-albumin accumulation in testicular parenchyma, increased in testis after hCG or cadmium treatment and around puberty in rats. However, this apparent PS could be affected by other factors, such as net fluid flux, as well as by permeability *in vivo*. Experiment with isolated testis could much more appropriate for calculation of true PS, i.e. without the interference of changes in net fluid flux through the tissue.

CHAPTER 2: MATERIALS AND METHODS

2. 1. REAGENTS

Human Chorionic Gonadotrophin, Testosterone, Bovine Albumin, Vitamin B_{12}, Antifoam A, Basic Fuchsin, Acid Fuchsin, Resorcin and Dextran T_{70} were obtained from Sigma Chemical Co. USA; ^{125}I-albumin, ^{51}Cr-EDTA, ^{3}H-Testosterone and Biodegradable counting scintillant from Amersham (Australia) Pty Ltd; Gelatine, $MgSO_4$, KH_2PO_4, Hexane, Xylol Depex and 2-Methylbutan-2-ol from AJAX Chemical Ltd Australia: EDTA, Na azide,NaCl, Na_2HPO_4, D(+) Glucose and Toluene from BDH (Australia) Pty; Cadmium Sulphate from M&B Ltd England; Charcoal Norit-A from MC*B Ltd USA; Pentobarbitone Sodium (Nembutal, Abbott) from Ceva Chemicals Australia Pty; 2,2,2-Tribromoethanol and Amylene hydrate from Aldrich Chemical Co. USA.

2. 2. ANIMALS

Porton strain male rats bred in the Adelaide University Central Animal House were used for all experiments. One or two days before treatment, animals were transferred to the small animal holding area of the Department of Animal Sciences (temperature 25^{o}C; 12h light per day), and maintained on a standard pelleted diet (Milling Industries Limited, South Australia) with free access to food and water.

2. 3. ANIMAL TREATMENT

2. 3. 1. Heating of the testes

Adult male rats were anaesthetized with avertin (8ml/kg, body wt) injected intraperitoneally. Avertin is an anaesthetic prepared according to Dr. Dyer and colleague's method (Dyer et al.,1981). The avertin recipe is following:

2,2,2-Tribromoethanol	5g
Amylene hydrate	3ml
Ethanol (100%)	20ml

stirr until dissolved , then add 250 ml 0.9% Nacl

In order to avoid increasing whole body temperature, rat was laid on its abdomen on a suspended plate over a temperature controlled (± 0.5°C) water bath for 30 min so that only the scrota were immersed in the water. Testes were exposed to 43°C for treated rats and to 33°C for its controls , respectively. After treatment, animals were returned to the small animal room and allowed to recover until determination of ^{51}Cr-EDTA space.

2. 3. 2. Efferent duct ligation

Each adult male rat was anaesthetized with avertin (see 2.3.1.) and laid on its back with the tail towards the operator. An abdominal incision was made,and each testis was exposed in turn. EDL was performed on the left testis only, the right testis was exposed, then returned to the scrotum as a control. To tie the efferent ducts, the left epididymal head was carefully lifted from the testis. The joining connective tissue was removed in order to expose the efferent ducts. Two sutures (5/0 silk, Cyanamid, Australia) were laid around the efferent ducts avoiding the blood vessels. One ligature was tied distally along the duct away from the testis, and the other was tied close to the testis. After EDL, the testis was returned to the scrotum and the abdominal incision was closed. Animals were allowed to recover until determination of ^{51}Cr-EDTA space.

2. 3. 3. Exposure of animals to N_2O

Animals were housed in normal stainless steel cages in the small animal area of the Department of Animal Sciences. For treatment, they were brought to the cattle shed each day. The animals were placed in a controlled atmosphere chamber for exposure to N_2O and O_2 (treated group) or pumped fresh air (control group) for 1h/day, 7days/week for 6 weeks.

A polymethyl methacrylate chamber 60 x 32 x 40 cm was used as the controlled atmosphere room for the rats. There were two holes (0.5 cm in diameter) on the cover. Gas was pumped into the chamber through one hole and escaped from the other.

Because the experiment was performed together with a cattle experiment (by another student), a mixture of N_2O and O_2 entered into a cattle hood first, and then was pumped into the chamber. O_2 concentration in the hood was recorded by a Oxygen monitor (Hudson Model 5520, CA USA) with Hudson sensor (Model 5510) every 15 min and a 1 ml sample was taken from the chamber gas exhaust hole every 10 min. N_2O from the 1ml sample was measured with a N_2O monitor (Wilks Foxboro Analytical Specific Vapor Analyser 101, CT USA). Because the N_2O monitor only shows diluted N_2O concentration (1ml in 2400 ml) in ppm, the following formula was used for getting a calibrated N_2O concentration as a percentage:

$$N_2O\ (\%) = R \times 2400 \times 1/1000000 \times 100$$
$$= R \times 0.24$$

"R" is the reading from N_2O monitor. For the 6 week experiment, N_2O concentration in the chamber was 21.49 ± 0.608 (%) (Mean±SEM), and O_2 concentration in the hood was 18.57 ± 0.144 (%) (Mean±SEM) (Fig. 2-1).

2. 4. DETERMINATION AND CALCULATION OF ^{51}Cr-EDTA SPACE IN ORGANS

Because ^{51}Cr-EDTA (Ethylenediamine tetra-acetic acid labelled with radioactive chromium) distributes throughout the extracellular fluid in most tissues, but is normally excluded from the tubular fluid (Setchell et al.,1969), it is considered to be a suitable marker for determining the function of the blood-testis barrier. For measuring ^{51}Cr-EDTA distribution in testis, animals were brought to the laboratory at appropriate times after treatment with different factors and anaesthetized with avertin (see 2.3.1.). Two polyethylene catheters (Dural, N.S.W. Australia) were introduced; one (1.0 mm o.d., 0.5 mm i.d.) was inserted into a jugular vein for infusion ^{51}Cr-EDTA (Amersham, UK), and the other (0.8 mm o.d., 0.5 mm i.d.) was placed in a femoral artery in some animals for monitoring ^{51}Cr-EDTA levels in blood plasma.

It is well known that many substances injected into the blood can pass from the bloodstream into the organs and tissues of the body, and then be excreted through the

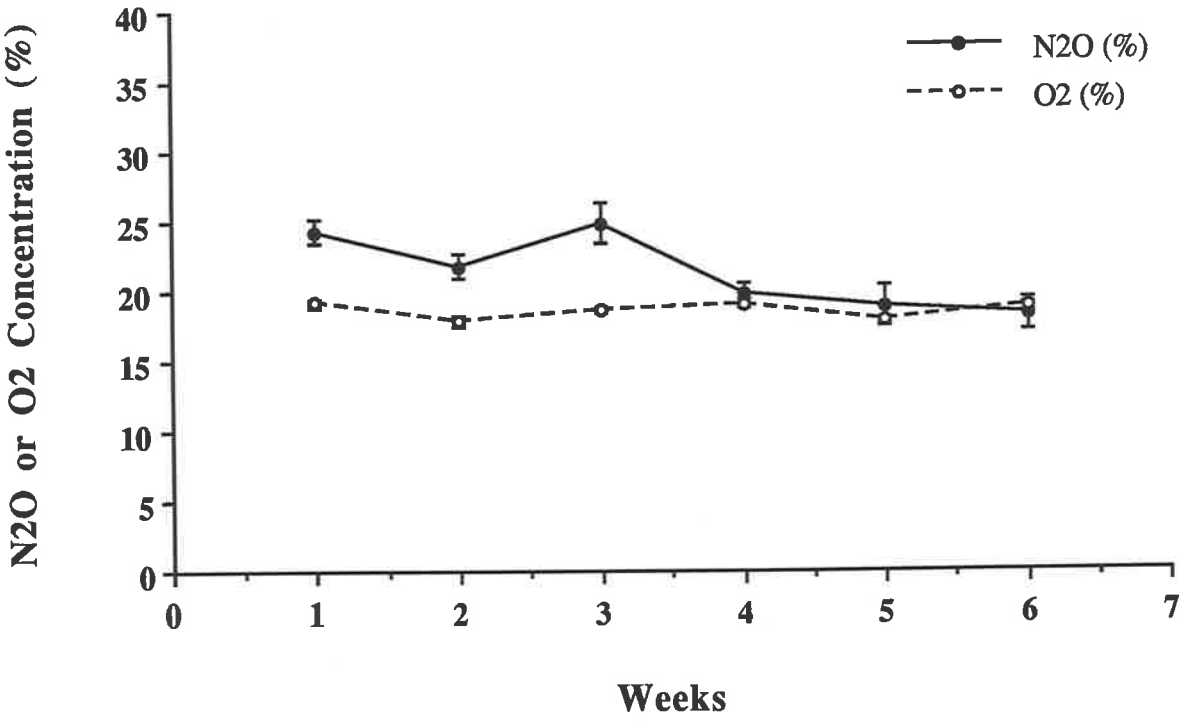


Fig. 2-1 The percentage concentration of N_2O and O_2 (Mean ±SEM) in the controlled atmosphere room at the times shown.

kidney or liver. A single injection results in the level of the substance in blood peaking and then decreasing rapidly, whereas a continuous constant speed injection results in a slow increase during the infusion period (Daniel et al., 1975). Different injection speeds were used in order to achieve an high ^{51}Cr-EDTA value quickly, and then to maintain that level during a period allowing marker equilibration in the tissues. For EDL rats, a priming dose of ^{51}Cr-EDTA (3μCi in 300 μl 0.9% Nacl) was injected intravenously by hand, and then a peristaltic pump (Minipuls 2, Gilson Medical Electronics, France) was employed for sustaining infusion (0.4μCi in 400μl 0.9% Nacl /min for 20 min). However, in rats exposed to N_2O and testis heating, an electronically controlled syringe (made in the workshop of department of Neuropathology, Institute of Psychiatry, London, England; to ref. see Pratt,1974; Daniel et al.,1974) was used for control the infusion speed. Loading time and time constant were set at 200 and 175, and resistor A and B were preset at 4 and 0 respectively so that the loading dose (3μCi in 300 μl) was infused into the blood within 20 seconds, and then the high level was maintained by an exponentially decreasing low speed infusion (2μCi in 200μl) over 20 minutes. In all experiments, 0.6 ml blood sample was taken at 2, 5, 10, 15, 20 min after injection start in order to get a ^{51}Cr-EDTA concentration curve in blood plasma for calculating ^{51}Cr-EDTA space in organs later. Each time after blood was withdrawn, the same volume of 0.9% Nacl was infused to replace the blood volume.

After a 20 minute ^{51}Cr-EDTA infusion, a blood sample was taken from the posterior vena cava and both testes removed quickly. The whole testis was weighed, then half was frozen in liquid nitrogen immediately for later histologic study, the other half was decapsulated. Each epididymis (divided into caput, corpus and cauda, Hinton et al, 1979), prostate and fat pad were separated and weighed in preweighed capped tubes, to prevent evaporative water loss. Plasma was separated from blood by centrifugation. ^{51}Chromium radioactivity in a known volume of plasma, weighed parenchyma , capsule and other tissues mentioned above were measured by using a gamma counter (1282 compugamma, Wallac-LKB, Turku, Finland).

The ^{51}Cr-EDTA spaces or distributions in organs were calculated according to following formula:

volume of distribution(μl/g)=organ cpm per g/plasma cpm per μl

Even though an electronically controlled syringe was employed in these experiments, the ^{51}Cr-EDTA level in blood still fluctuated during the infusion period. In order to correct the ^{51}Cr-EDTA space error caused by this fluctuation, the following formula was used:

Corrected space(μl/g)=volume of distribution(μl/g) / M/F

"F" is the blood plasma radioactive counts(cpm/ml) in the final sample during infusion. Here, F is equal to blood plasma counts in the 20 min sample, which was collected from all rats. "M" represents the mean (cpm/ml) of radioactive counts in blood plasma during the infusion period. Because one cannot take a blood sample every minute, only 5 samples has been taken during the infusion period. Therefore the following formula was used in order to approximate the mean during infusions:

$$M = \{T_1 \times [1th] + \Sigma(T_{1\text{-}f} \times ([L]+[F]/2))\} / T_t$$

"T_1" is the time (min) from the beginning of infusion to the first sample taken, here T_1 is 2 (min). "[1th]" is the blood plasma radioactivity counts (cpm/ml) in the first sample; here [1th] is equal to blood plasma counts in the 2 min sample. "$T_{1\text{-}f}$" is the interval time (min) from the former sample taken to the latter sample taken. "[L]" and "[F]" are the blood sample radioactivity counts (cpm/ml) in the latter and the former samples respectively. "T_t" is the total time (min) of infusion; here T_t is 20 (min). For those rats where 5 samples were taken, the calibrated value was achieved by using their own vzlue for M/F. For other rats, their own value for F and the mean of the M/F in the same batch of rats was used instead of individual M/F. In this way, all ^{51}Cr-EDTA space data presented are more comparable.

2. 5. HISTOLOGY AND MORPHOMETRY

2. 5. 1. Cryostat section

A refrigerated microtome (2800 Frigocut Cryostat, Reichert-Jung, Cambridge Instruments GmbH, Germany) was used to make cryostat sections. Before cutting, the box temperature was preset between -28 and -30°C and the object temperature was preset at around -16°C to -20°C according to the hardness of tissue with angle of the blade about 7.

Testis tissue previously frozen in liquid nitrogen was brought from the freezer and a selected 3 to 5 mm thick portion of the testis was cut. While still frozen, the tissue was attached to a specimen chuck by covering it with tissue freezing medium. Then the chuck was placed in a deep frozen station inside the cryochamber, so that the tissue was fixed to the chuck rapidly. Following that, the chuck was fixed to the object cylinder, and 10 μm sections were cut by turning the handwheel. These sections were stuck directly to slides treated with 10% poly-l-lysine solution (Sigma).

2. 5. 2. Fixing and staining

After cutting, the sections were fixed in Clarke's fixative for 2 min, then transferred to 80% alcohol until staining. Weigert and Van-Gieson stain solutions were used for testicular staining. The following staining precedures was used:

(1) The sections were stained in a special Weigert's resorcin-fuchsin solution for 40-60 min.

(2) The section was washed in 95% alcohol (just 2-3 dips).

(3) The sections were dipped via a series of gradually decreasing alcohol solutions and finally washing in water.

(4) The sections were stained in Van-Gieson solution 45 seconds for counterstaining.

(5) The sections were then blotted using soft tissue.

(6) They were then cleared in xylol for 2 min.

(7) They were then air dried

(8) The sections were covered by microscopic cover glasses which were mounted on slides by using DePex.

Solutions for fixing and staining

(1) Clarke's fixative

Absolute alcohol	3vols
Glacial acetic acid	1vol

(2) Weigert's resorcin-fuchsin solution

Basic fuchsin	2g
Resorcin	4g
Distiled water	to 200 ml

All the materials above were placed in a beaker and brought to the boil was stirred and boiled for another 3 min. After the solution had cooled, it was filtered through a fast filter paper. The filtrate was discarded and the paper with the precipitate was transferred into the original (unwashed) beaker which had been dried. 95% alcohol (200ml) was added to the original beaker, which was heated gently on the hot plate until the precipitate dissolved. The paper was removed and concentrated HCl (4ml) was added, then the solution was filtered again. This staining solution was made up to 200 ml in volume by adding 95% alcohol through the used filter paper.

(3) Van-Gieson's solution

1% aqueous acid fuchsin	10 ml
Saturated aqueous of picric acid	90 ml

This mixture was diluted with an equal volume of water and the solution was boiled on a hot plate for 3 min to ripen.

2. 5. 3. Measurement of interstitial area

A Leitz microscope connected to a Microbits computer and BQ system IV were employed in morphometry. A field area (A_1) was drawn as a box on the screen with the mouse, then tubular area (A_2) inside the box was drawn following the outside layer of each seminiferous tubule. The following formulas were used for calculating the interstitial tissue volume:

D_1= SUM (A_2) (SUM of tubular area)

$D_2 = D_1/A_1 \times 100\%$ (% of tubular area in the whole measured area)

$D_3 = 100\% - D_2$ (% of interstitial tissue in whole measured area)

interstitial volume ($\mu l/g$) = $D_3 \times 1000(\mu l)$

Normally, one area from each section and 3 sections per testis were measured. The size of measured area was around $2.535 \times 10^6 \pm 3938\ \mu m^2$ (from 300 sections).

2. 6. TESTIS PERFUSION IN VITRO

2. 6. 1. Treatment before perfusion

Animals being treated with hCG received a single injection of 50 i.u. hCG in 0.2 ml saline subcutaneously. Testes were removed for perfusion from these rats 8, 12, 16, 20, 24 hours after injection. For the cadmium salt experiment, 1.4% cadmium sulphate in saline (7mg/kg bw) was injected s.c. into the animal. The perfusion was performed 0.5, 1, 2, 3, 4 hours after injection. The control treatment for both of these experiments was saline injection (s.c.) of the appropriate volume .

2. 6. 2. Surgical procedures

Animals were anaesthetized with pentobarbitone sodium (50mg/kg bw) intraperitoneally. Both testes were exposed by cutting scrota and tunica vaginalis over the ventral longitudinal axis of the organ with scissors. The epididymis and connecting fat were separated by blunt dissection from the testis. Sutures were laid around both spermatic cords 3 cm above the upper pole of the testis. The spermatic cord was ligated and cut a few millimetres below the level of ligation proximally to the testis. The testicular artery running beneath the tunica albuginea was then pierced by a 26 gauge needle and cannulated with a fine tipped polythene tube. Perfusion was started around 20 seconds after interruptionof the circulation to the testis, which was covered by a piece of gauze moistened with 0.9% NaCl solution and placed in a small cup, 3.5 cm in diameter and 1.0 cm in height, filled with saline. The same procedure was repeated for the contralateral testis.

2. 6. 3. Perfusion

Krebs-Ringer-albumin solution was used as a major perfusate. This contains (mg/l): NaCl, 6890; KH_2PO_4,163; $MgSO_4.7H_2O$, 295; $NaHCO_3$, 2300; $CaCl_2$, 277; D-glucose, 2000; bovine serum albumin, 10000; cyanocobalamine (Vitamin B_{12}), 1.0. In the ^{125}I albumin space in vitro experiment, ^{125}I albumin was diluted in Krebs-

Ringer-albumin solution (6.25 x 10^{-3} μCi per ml) and this perfusate was used in order to obtain values for the apparent permeability of testis blood vessel which can be compared with in vivo data (Setchell et al, 1988). In two rats ^{125}I albumin was diluted in fresh sheep serum (0.25 μCi per ml) to investigate the reason for higher permeability found in vitro than that in vivo.

The perfusate was gassed with 95% O_2 -5% CO_2 for the entire time of perfusion to maintain pH around 7.3 - 7.4 and to provide O_2 to the perfused testes. Foaming was prevented by adding 2 drops of a silicone antifrothing agent (Antifoam A, Sigma) to the perfusate. The solution was delivered by a peristaltic roller pump (Minipuls II, Gilson Medical Electronics, France) via a pump tube (0.6cc/M), which was connected to a 110 cm length polythene tube (1.5mm o.d.,0.8mm i.d.) and then to a fine tipped tube of 30 cm length. There were 2 three-way junctions in the tube system. The middle junction was connected to a pressure transducer and recorder, which monitored the pressure for the whole perfusion time. However, it was not possible to estimate pressure near the tip of the catheter, and this may have been appreciably lower than the values recorded. A Y-shaped junction (Fig.2-2) was set close to the tip of the tube. A gas tight syringe was connected to this three-way junction so that the isotope inside the syringe was directed towards the testes (Fig. 2-3).

2. 6. 4. Effluent collection and ^{125}I-albumin injection

According to the evidence that in most animals the temperature of scrotal testes is around 33°C (Setchell,1978), the whole of the perfusion system was designed to keep inside of the temperature controlled chamber at 32.5± 0.5 °C during the experiment (Fig. 2-4). A fraction collector (ISCO golden retriever, Model 1200 pup, U.S.A.) (Fig. 2-5) was used for effluent collection, and was started at the same time as the peristaltic pump. Fractions were collected every 5 minutes and were frozen to be assayed for testosterone later. After 30 min of perfusion, 50μl ^{125}I-albumin in saline (5x10^4 to 20x10^4 cpm or around 0.01μCi) was injected through a gas-tight syringe connected to the second three-way junction (Fig. 2-6). The entire effluent was collected for radioactivity determination

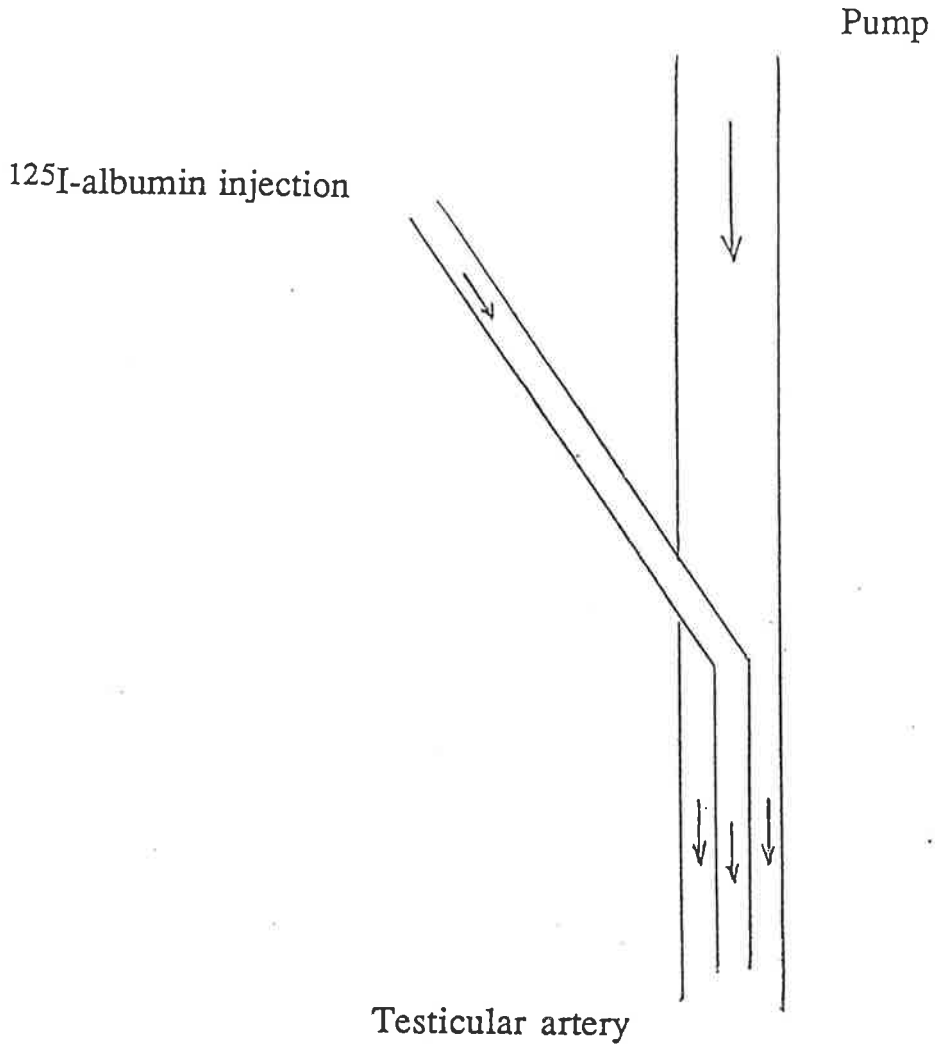
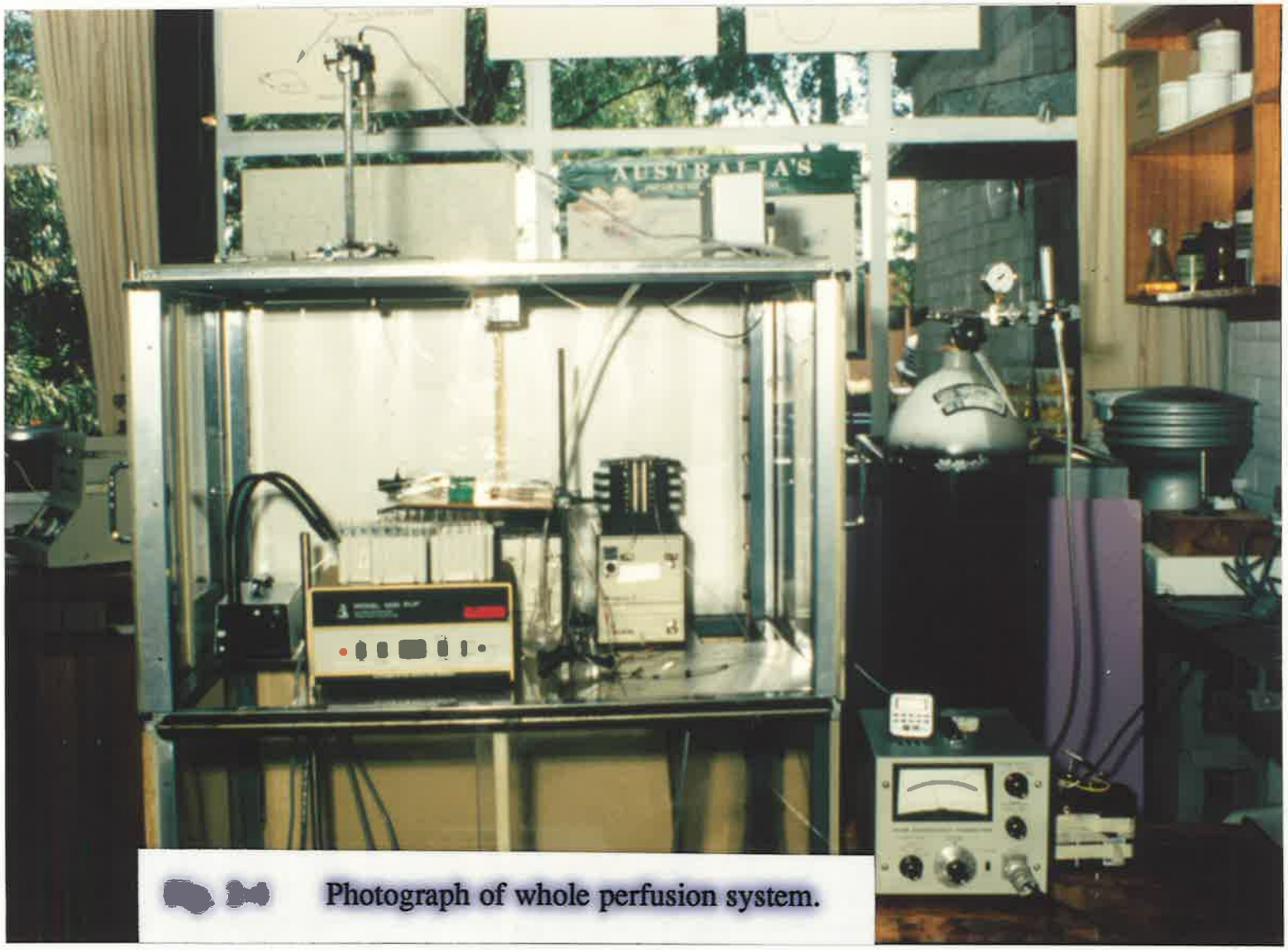
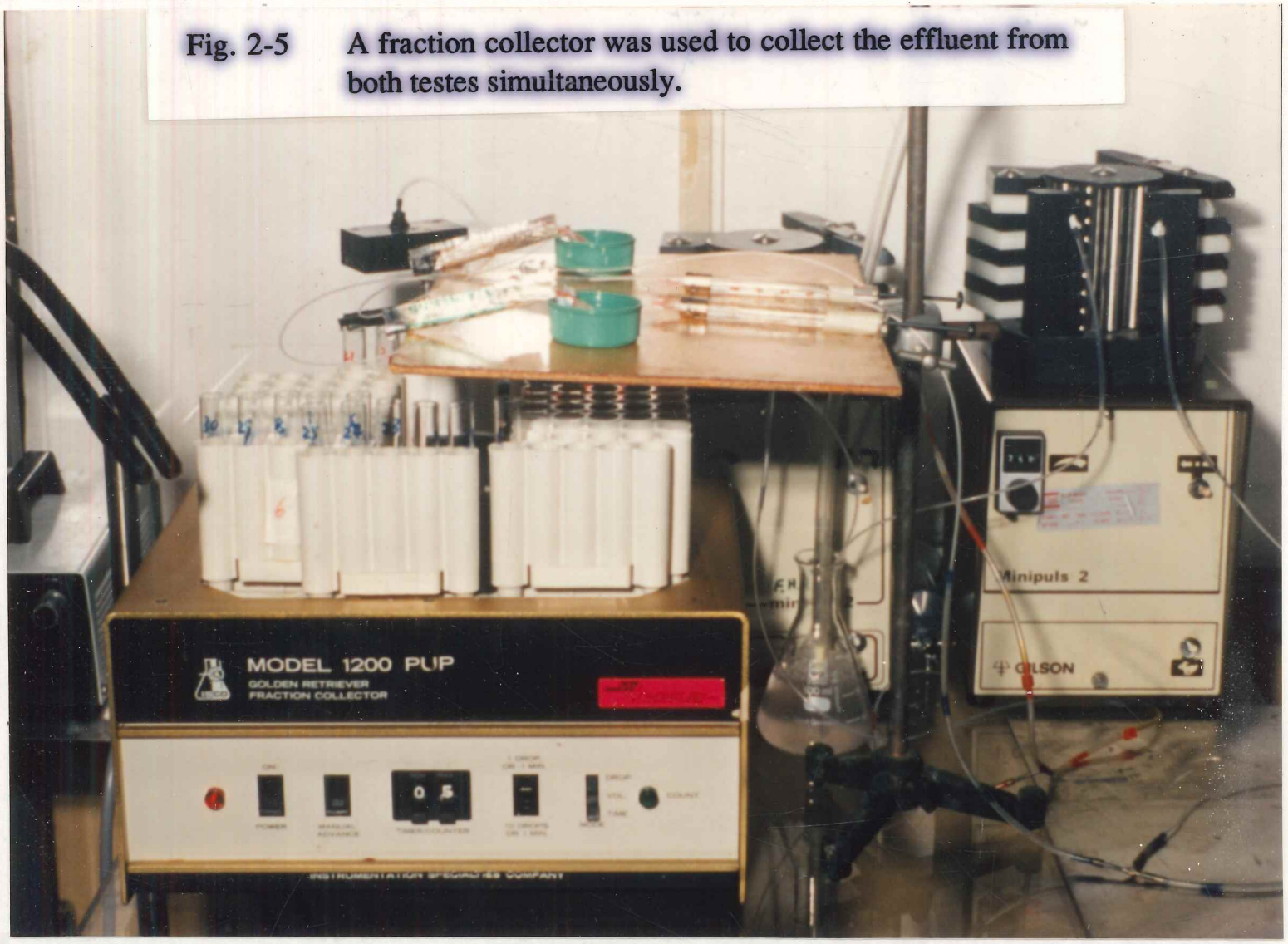


Fig. 2-2 A diagram of the Y-shaped junction for injecting ^{125}I-albumin to the testicular artery.



Photograph of whole perfusion system.

Fig. 2-5 A fraction collector was used to collect the effluent from both testes simultaneously.



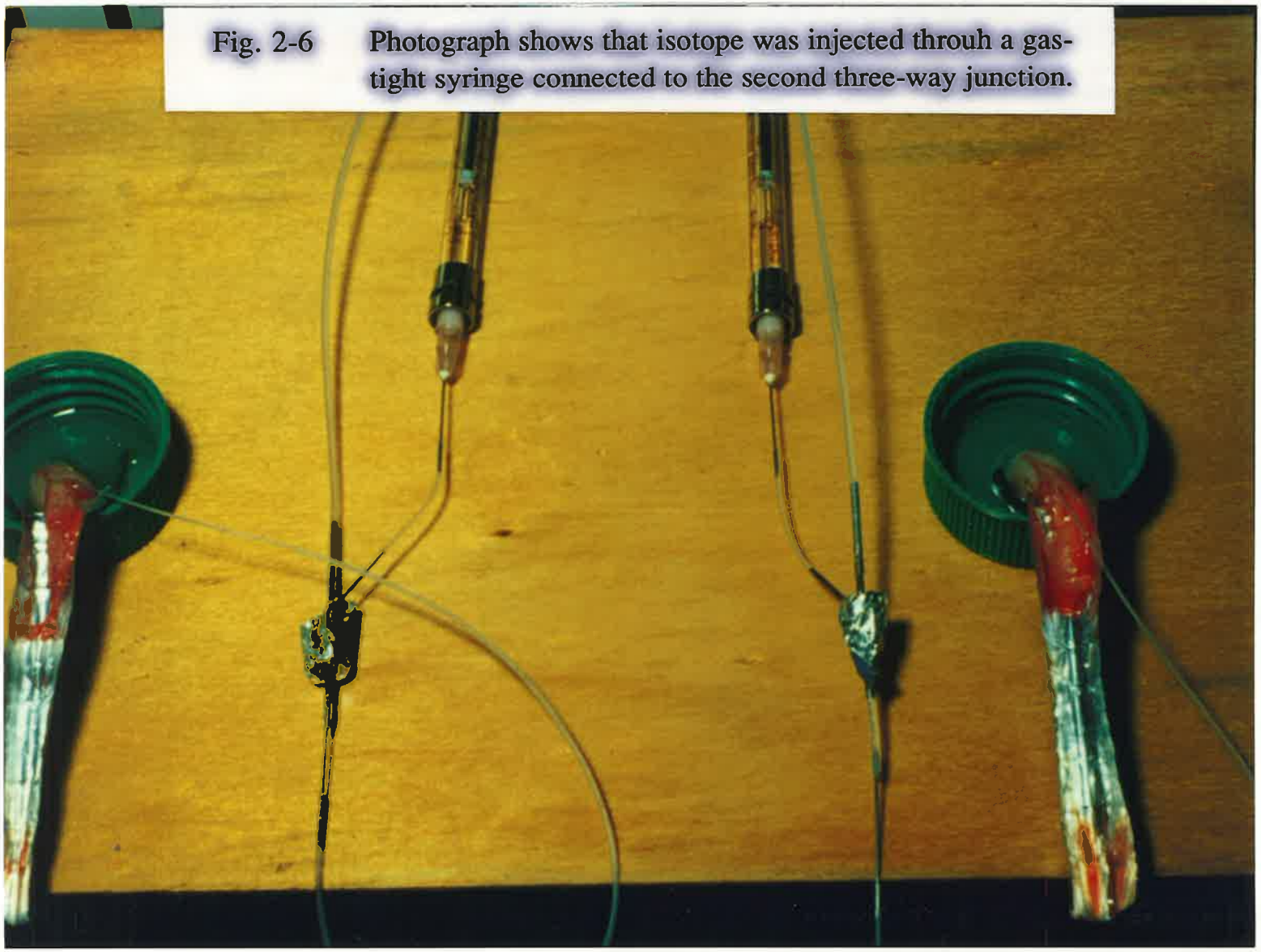


Fig. 2-6 Photograph shows that isotope was injected throuh a gas-tight syringe connected to the second three-way junction.

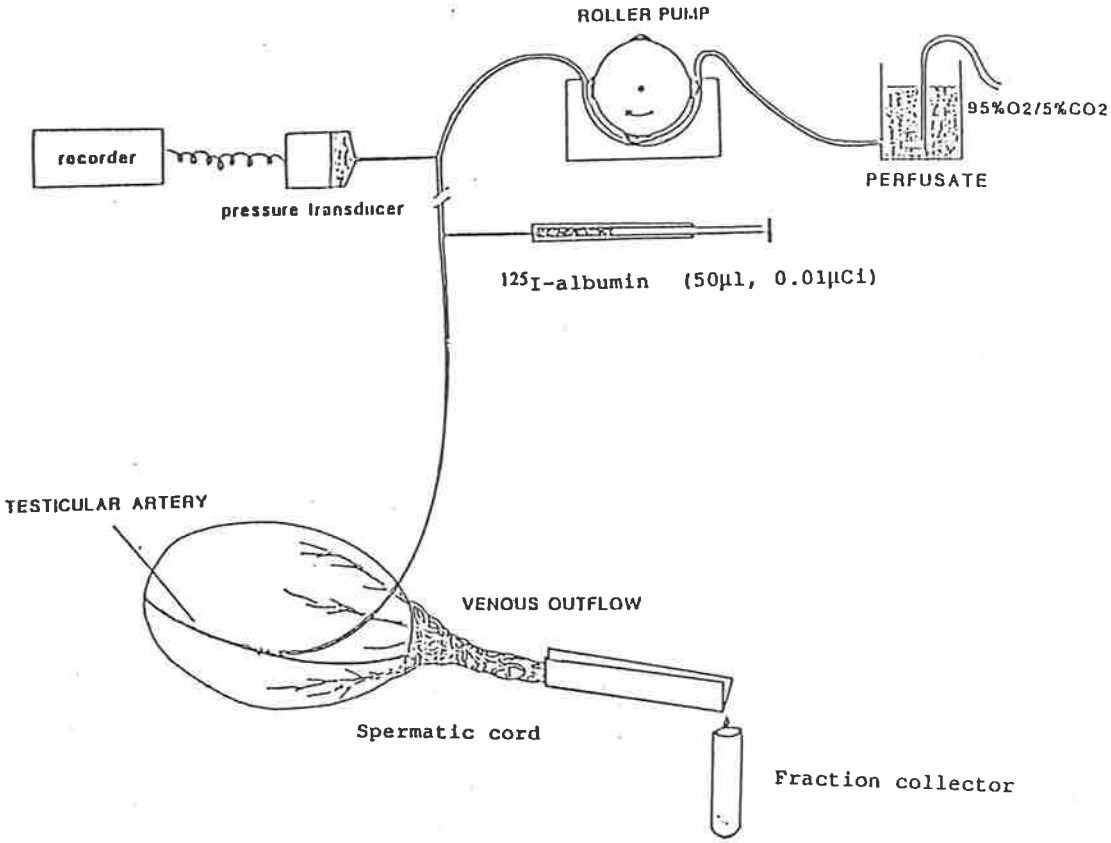


Fig. 2-3 A diagram of the perfusion system, based on that described by Bustamant and Setchell (1981).

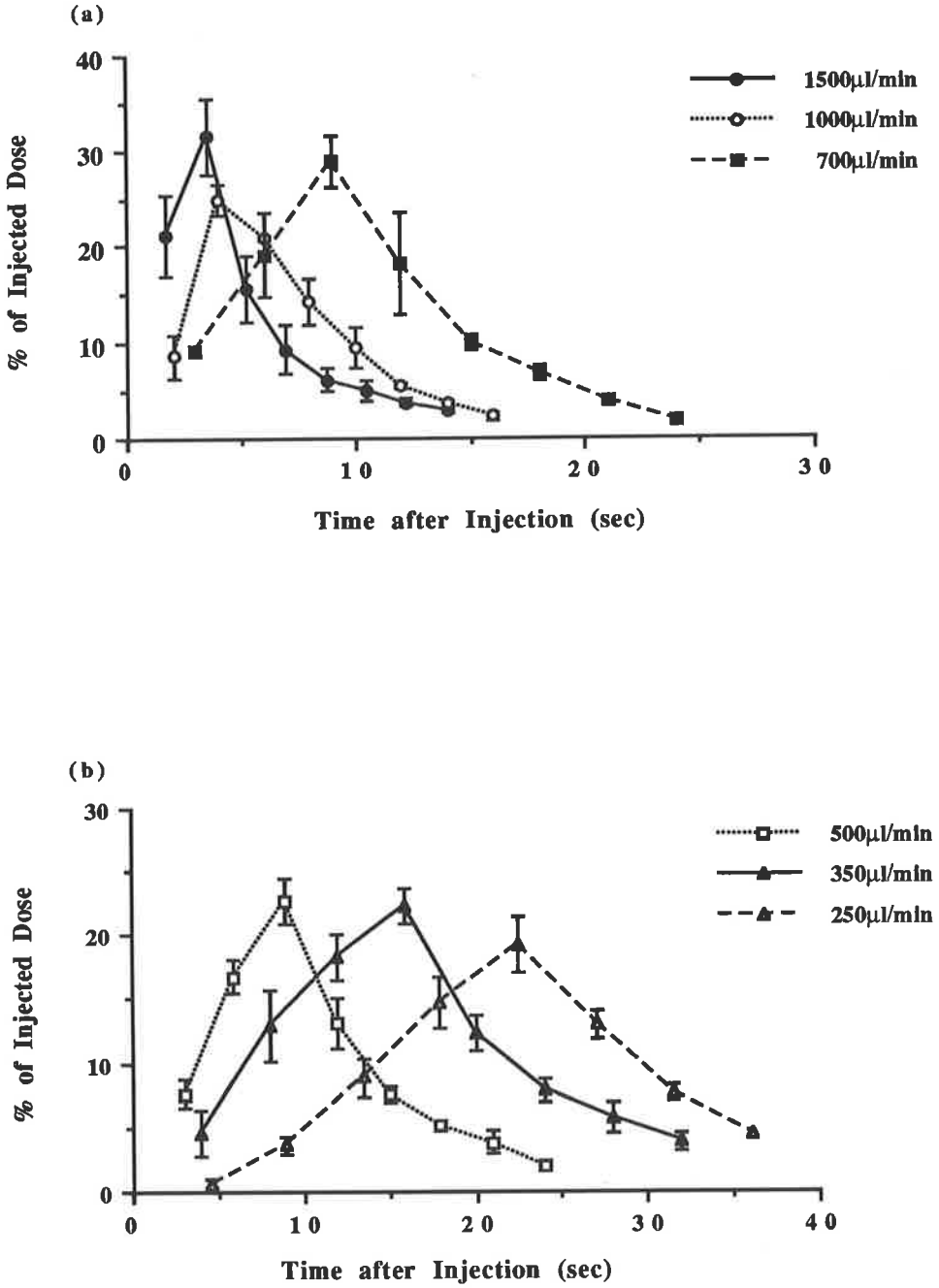


Fig. 2-7 Percentage of injected ^{125}I-albumin dose appeared in fraction samples taken by capillary tubes at different flow rates at time shown., (a) flow rates from 700µl/min to 1500µl/min ; (b) flow rates from 250µl/min to 500µl/min. Data were expressed as mean ± SEM., n=4.

for 1 min after injection, to enable calculation of the total amount injected. The reason for choosing 1 min as the effluent collected time based on the result from a preliminary tests, which showed that 80% of injected ^{125}I-albumin dose appeared in fraction samples taken by capillary tubes at 500µl/min flow rate during 25 seconds, and the effluent was essentially free of radioactivity by the end of 1 min. Even at the lowest flow rate (250µl/min), 70% of injected ^{125}I-albumin dose was collected by less than 40 seconds (Fig. 2-7).

2. 6. 5. Sample preparation and permeability surface area product (PS) calculation

After perfusion the spermatic cord was ligated close to the testis, then cut a few millimetres from the ligature. Both the testis and spermatic cord were weighed and then were placed in a gamma counter (LKB-Wallac) to measure their radioactivity. When different perfusion flow rates were used, the testis was decapsulated and the parenchyma placed onto a preweighed filter paper. The paper and parenchyma were centrifuged at 54G for 15 min in order to transfer the ITF onto the paper. ITF collected in this way would included the fluid in the microvesses, but as the radioactive bolus would have cleared from the vasculature of the testis (see above) and negligible amounts of radioactivity would have returned from the tissue to the the vessels, and the volume of the vascular fluid (approx. 10µl/g, Setchell & Sharpe, 1981) is much less than the volume of theITF, the error intoriduced would be negligible Seminiferous tubules, capsule, ITF (by subtraction of the weight of the dry paper from the weight of the paper plus fluid) and 1 min effluent (see previous section) were each weighed and counted in the gamma spectrometer. In other experiments, parenchyma was counted instead of seminiferous tubules and ITF.

The true PS of ^{125}I-albumin was calculated in all perfusion experiments using the equation (Renkin, 1959; Crone, 1963):

PS = - Q . Ln (1-E)

Q is the perfusion flow rate (µl/min/g); E is the testicular tissue fractional extraction which was calculated according to following equation:

E= cpm in tubules, capsule or ITF /cpm in whole testis, cord and effluent

To compare the PS obtained in vitro with the apparent PS which Setchell et al, (1988a) obtained in vivo, a different formula was used. Apparent PS was calculated by multiplying V_{final} by the slope (K) of the line Ln (1-V_t/V_{final}) against time. V_t was the albumin space at time *t* minus the albumin space at 2 min (se p 40); V_{final} was the albumin space at 120 min minus the albumin space at 2 min.

2. 7. TESTOSTERONE RADIOIMMUNOASSAY

2. 7. 1. Buffer and solutions

2. 7. 1. 1. Buffer

(1) Phosphate buffer solution (PBS) 0.01 M pH 7.5

Na_2HPO_4 (anhydrous)	1.42 g
EDTA	0.34 g
Na azide	1.00 g
NaCl	8.18 g

adjust pH to 7.5 with diluted HCl or NaOH and made up to 1.0 litre using double distiled H_2O

(2) 0.2% Gel PBS

Add 2.0 g gelatine to 1.0 L PBS

2. 7. 1. 2. Dextran coated charcoal mixture and scintillation fluid

(1) Dextran coated charcoal mixture

charcoal Norit-A	625 mg
dextran T70	62.5 mg

made up to 100 ml using PBS

(2) scintillation fluid

Biodegradable counting scintillant (BCS) (Amersham, U.S.A.) was used as a scintillation fluid in all testosterone assays.

2. 7. 1. 3. Stock solutions of ^{3}H-testosterone, testosterone standard and testosterone antiserum

(1) ^{3}H-Testosterone solution

^{3}H-Testosterone was purchased from Amersham and 100µl was dissolved in 5ml ethanol to make a stock solution. This solution was diluted in gel PBS to give a working solution with a radioactivity of 10,000cpm/200µl.

(2) Standard testosterone solution

Testosterone (17ß-Hydroxy-3-oxo-4-androstene, Lot: 74F-0711) was used for making stock solution (1µg/ml) which was kept in the freezer. For each standard curve, 64 µl (or ng) testosterone stock solution was added to 4.0 ml gel PBS to produce a 1600pg/100µl testosterone standard solution, of which two 100µl portions wer tranferred to two assay tubes. The standard solutions were then diluted in a twofold stepwise manner to produce a series of standards

for the preparation of a testosterone standard curve, with duplicate tubes containing each of the following amounts of testosterone in 100µl:

800, 400, 200, 100, 50, 25, 12.5 and 6.25pg.

Three tubes were prepared for estimation of non-specific binding (NSB) with 100µl testosterone stock solution diluted (100µl to 3.5ml) with gel PBS, and four blank tubes contained 100µl gel PBS.

(3) Testosterone antiserum

The testosterone antiserum (457, 13/9/76) was a gift from Dr. Ron Cox from CSIRO, Prospect, NSW Australia. The stock solution was prepared by dissolving 10mg antiserum powder into 10ml gel PBS, aliquots of this solution were then frozen for storage. A working solution was prepared such that 30-50% binding was achieved.

2. 7. 2. Assay procedures

2. 7. 2. 1. Extraction of the samples

(1) The standard solutions were prepared as above. Samples were pipetted into 12x75 glass culture tubes ,and adjusted to 100µl by adding gel PBS;

(2) Ten ml of Hexane-Toluene(1:2 vol:vol) extraction solution was added to each tube and vortexed for 30 seconds;

(3) Ethanol cooled with liquid N_2 was used to freeze the aqueous phase in each tube. The hydrocarbon phase was then decanted into assay tubes;

(4) The tubes were warmed to 37°C and dried by blowing air into them.

2. 7. 2. 2. Incubation

(1) After drying 200 µl of ^{3}H-Testosterone and 200 µl of antisera was added to each tube;

(2) The tubes were incubated overnight at 4°C.

2. 7. 2. 3. Adsorption of free ^{3}H-Testosterone

(1) 200 µl of Dextran-coated charcoal which was being stirred continuously at 4°C, was added to each tube. They were then shaken;

(2) The tubes were incubated for 15 min at 4°C;

(3) Samples were centrifuged at 800g(about 2500 rpm) for 20 min at 4°C.

2. 7. 2. 4. Counting

(1) The whole supernatant was decanted from the spun tubes into plastic counting vials;

(2) 1 ml of scintillation fluid was added to each plastic counting vial which was then capped and shaken for 30 min on a mechanical shaker;

(3) The plastic counting vials were placed in a liquid scintillation counter(LKB-Wallac 1215 Rackbeta II, Finland) for radioactivity determination.

2. 7. 3. Calculation of testosterone concentration

A Macintosh computer with program "Assayzap" was used to calculate testosterone concentration from the radioactivity counts. For 5 batches, the intra-assay coefficient of variation was 4.29-7.45%; the inter-assay coefficient of variation was 9.84% .

Because 1% albumin Krebs-Ringer solution was used in this experiment instead of blood, neither testosterone nor red blood cells were contained in this solution, so that testosterone production was calculated from the following formula:

T production(ng/g/min) = T conc.(ng/ml) x flow(ml/g/min)

2. 8. STATISTICAL ANALYSIS

All data are presented as mean ± S.E.M.(standard error of mean) except those stated. The probability of significant difference between treated and control groups was calculated by the appropriate Student's *t*-test. Linear regression was used to calculate K in permeability surface products (Setchell et al, 1988a).

CHAPTER 3: EFFECT OF EFFERENT DUCT LIGATION, HEATING OF TESTIS AND EXPOSURE OF ANIMALS TO N_2O ON THE BLOOD-TESTIS BARRIERS

3. 1. INTRODUCTION

It has been well established that the tubule barrier is mainly built up by Sertoil cells, which are joined together through tight junction. However, the tight junctions can be observed only after puberty (Setchell and Waites, 1975), and occupy approximately 4% of the plasma membrane surface area in rat. The tight junctions between the Sertoli cells divide the seminiferous epithelium into two compartments (Fawcett, 1973a; Dym, 1973; Dym and Fawcett, 1970), the basal and the adluminal. The basal one, located between the Sertoli cell barrier and the peritubular tissue, is occipied by spermatogonia and early stage spermatocytes. The adluminal compartment, on the luminal side of the Sertoli cell tight junctions, contains the more differentiated germ cells. Although many substances such as some tracers and serum albumin can enter the basal compartment from th interstitial tissue fluid, they are excluded by the tubule barrier formed by the Sertoli cells. Therefore, the adluminal compartment provides an unique physiological millieu for spermatogenesis, where the concentrations of many substances in STF are different from those in blood plasma. It is, thus, well recognised that the tubule barrier may be important in spermatogenesis. However, further studies stagnated owing to lack of a suitable animal model .

The aim here is to establish an animal model for studing the effects of breaking tubule barrier on spermatogenesis by three different injuries to seminiferous tubule—heat (Kerr et al., 1979; Sharpe and Bartlett, 1987; Jegou et al., 1984; Au et al., 1987), efferent duct ligation (Wang et al., 1985; Singh and Abe, 1987; Laurie and Setchell, 1980; Setchell, 1986) and exposure of animals to N_2O (Coate et al, 1979; Kripke et al., 1976).

In my study, the ratio of distribution of ^{51}Cr-EDTA in interstitial tissue to morphometry volume was used as an indicator of the integrity of tubule barrier. EDTA has a molecular weight about 340 and its molecular radius is larger than 0.5 nm. It does not enter cells, but can pass readily from blood to ITF and cannot pass through the tubule barrier into the adluminal compartment (Setchell et al, 1969). Therefore, the

distribution volume of ^{51}Cr-EDTA should be less than (because of exclusion from cells in the interstitium), or at most equal to the morphometry volume of interstitial tissue. Otherwise, breakdown of the barrier is indicated. The maximum volume of distribution of EDTA with a completely non-functional barrier woud be the above space plus the extracellular space between the tubular cells (which would be very small) and the volume of the luminal fluid (approximately 15% of the testis in normal animals, Setchell et al, 1988).

3. 2. EFFERENT DUCT LIGATION

3. 2. 1. Experimental procedures

Seventy-five adult male rats weighing 480 ± 8 g were used in this experiment. As described in 2.3.2., the efferent ducts of the left testis of each rat were ligated, with the contralateral testis serving as a control. ^{51}Cr-EDTA spaces in testes, epididymides and prostates was determined (see 2.4.) in animals 0, 2, 4, 8, 12, 16, 24, 32, 40, 48, 56, 64, 72 hours after EDL. Half of each testis was frozen for morphometrical study (see 2.5.)

3. 2. 2. Results

Organ weights:

Testicular weight gradually increased from 2 hours after EDL. It increased to a peak of 136% of the control value at 24 hours after EDL. The testis weight decreased after 24 hours, but did not return to normal levels until 56 hours after EDL (Table 3-1; Fig. 3-1).

Epididymal and prostatic weights are also shown in Table 3-1. The significant increase of organ weights was seen in caput at 12 hours after EDL and corpus at 48 hours after EDL, whereas a decrease of organ weights was found in cauda at 2 hours after EDL, caput at 64 hours after EDL and prostate at 48 hours after EDL. However,

Table 3-1

EFFECT OF UNILATERAL LIGATION OF THE EFFERENT DUCTS (EDL,left side) OF RATS ON TESTICULAR, EPIDIDYMAL AND PROSTATIC WEIGHTS (mg)

EDL Time (Hours)	Testis		Caput		Corpus		Cauda		Prostate	
	L	R	L	R	L	R	L	R	L	R
0	1808±38 (7)	1753±31 (7)	294±15 (7)	256±33 (7)	116±15 (7)	98±17 (7)	291±21 (7)	280±30 (7)	231±36 (7)	258±17 (7)
2	1641±140** (3)	1548±133 (3)	269±29 (3)	262±16 (3)	92±8 (3)	85±6 (3)	226±4* (3)	234±4 (3)	243±21 (3)	230±20 (3)
4	1862±47** (8)	1719±34 (8)	278±12 (8)	276±8 (8)	74±7 (8)	79±11 (8)	264±12 (8)	292±16 (8)	227±24 (8)	264±20 (8)
8	1864±51** (7)	1609±43 (7)	287±12 (3)	273±14 (3)	113±6 (3)	134±6 (3)	279±8 (3)	286±19 (3)	181±69 (3)	292±40 (3)
12	1972±84** (7)	1586±68 (7)	280±10** (7)	233±10 (7)	98±8 (7)	98±9 (7)	258±12 (7)	266±9 (7)	263±11 (7)	294±39 (7)
16	1794±112** (4)	1384±82 (4)	---	---	---	---	---	---	---	---
24	2198±80** (4)	1619±45 (4)	---	---	---	---	---	---	---	---
32	2215±67** (4)	1714±43 (4)	248±15 (4)	260±15 (4)	108±12 (4)	84±7 (4)	230±12 (4)	247±13 (4)	294±52 (4)	209±10 (4)
40	1781±154** (6)	1429±73 (6)	236±38 (2)	236±35 (2)	83±4 (2)	96±22 (2)	214±6 (2)	232±21 (2)	172±34 (2)	146±41 (2)
48	1838±140** (7)	1592±68 (7)	208±10 (3)	213±12 (3)	100±14* (3)	79±14 (3)	252±7 (3)	256±18 (3)	159±8* (3)	264±11 (3)
56	1789±98 (7)	1706±44 (7)	250±17 (7)	255±12 (7)	98±10 (7)	108±16 (7)	268±16 (7)	280±13 (7)	216±33 (7)	248±31 (7)
64	1713±58 (8)	1756±53 (8)	241±19* (6)	283±7 (6)	85±10 (6)	72±9 (6)	281±12 (6)	265±19 (6)	286±98 (6)	363±92 (6)
72	1593±52 (8)	1625±43 (8)	232±17 (8)	256±26 (8)	88±9 (8)	85±7 (8)	268±12 (8)	269±11 (8)	184±16 (8)	253±33 (8)

Values are expressed as mean ± S.E.M. with the number of determinations shown in parenthesis;
*P<0.05; **P<0.01 compared with R-side organs. by paired t-test

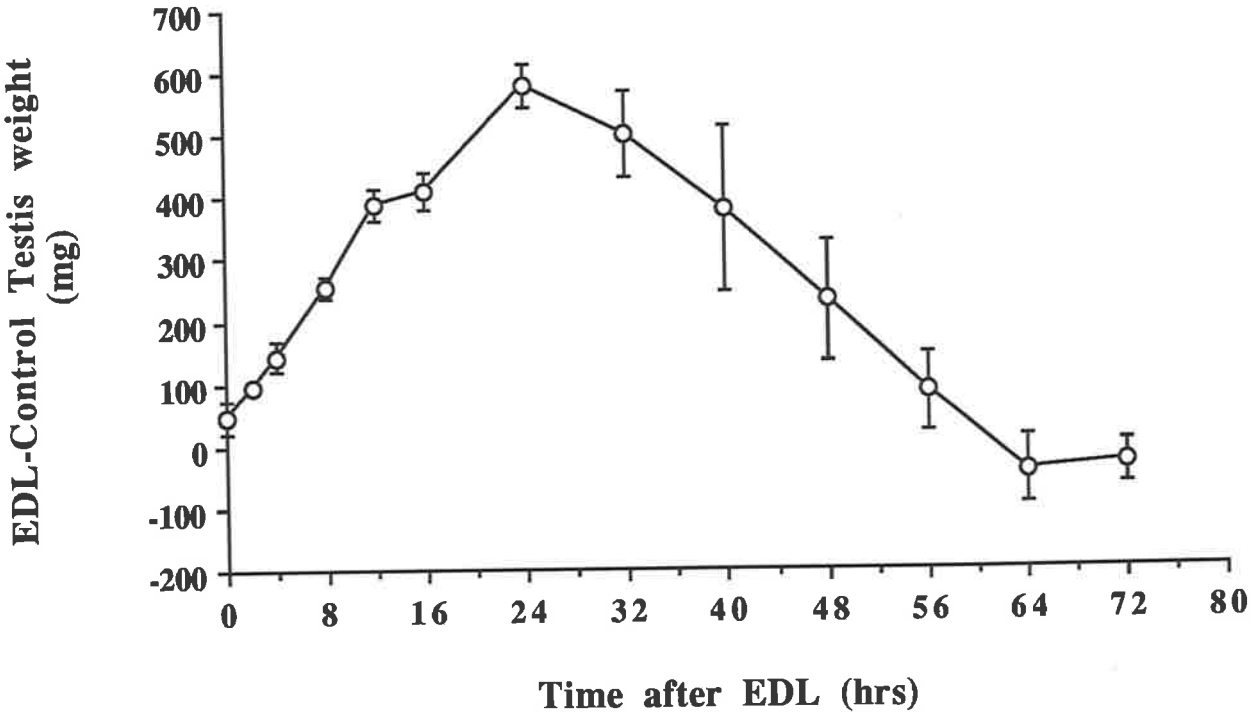


Fig. 3-1 The difference in weight (Mean ±SEM) between the ligated and control testes at various times after unilateral efferent duct ligation (EDL).

Table 3-2

^{51}Cr-EDTA space in testis,testicular parenchyma and capsule (μl/g)

EDL time (hours)	No.of rats	Testis		Parenchyma		Capsule	
		L	R	L	R	L	R
0	7	94± 6.66	86±12.03	69± 4.72	64± 4.97	484± 34.39	419± 57.07
2	3	59± 5.71	50±17.80	39± 5.74	38± 7.11	314± 53.69	271± 28.87
4	8	58± 4.97	58± 4.29	40± 3.33	41± 4.47	321± 23.69	327± 30.76
8	7	53± 2.71	54± 1.80	32± 1.44	34± 2.03	347± 27.97	362± 39.31
12	7	39± 4.35	46± 5.19	23± 2.42*	33± 4.13	308± 33.26	297± 38.17
16	4	91±19.30*	134±28.50	44± 6.42	91±19.96	696±192.00	675±129.00
24	4	120± 6.30*	146± 3.73	72± 5.88*	110± 6.66	764± 44.50	744± 85.00
32	4	89±14.98	53±13.17	74±12.93	44± 7.27	338± 68.50	251± 15.50
40	4	296±30.66**	160±18.87	231±34.17*	110± 9.18	780± 81.00	793±114.50
48	4	239±31.15*	144±12.32	198±33.04*	109±13.11	747± 63.00	734± 52.00
56	7	80±12.22**	51± 6.32	60± 9.68*	37± 5.93	284± 39.31	252± 36.28
64	8	185±28.48**	102±16.70	154±23.90**	80±10.89	561± 84.50	480±113.00
72	8	182±24.95**	104±14.67	137±20.40**	82±12.72	609± 86.27	463± 54.09

Values are expressed as mean ± S.E.M.; *P<0.05; **P<0.01 compared with R-side organs.

most of the groups showed no difference between the EDL and control organ weights, and the above differences are probably of no biological significance.

^{51}Cr-EDTA space in organs:

The ^{51}Cr-EDTA space for whole testis, testicular parenchyma and capsule are presented in Table 3-2. In the whole testis and parenchyma, ^{51}Cr-EDTA space decreased between 12 and 24 hours after EDL, but increased after 32 hours, reaching a peak at 40 hours after EDL and was still high 72 hours after EDL. In the testicular capsule, ^{51}Cr-EDTA space showed no significant difference between EDL and control groups at any time.

^{51}Cr-EDTA space in caput epididymidis increased 56 to 72 hours after EDL (see Table 3-3). The corpus and cauda epididymidis (Table 3.3) and also the prostate (Table 3.4) showed no significant differences for ^{51}Cr-EDTA space at any time.

Interstitial tissue volume:

Testicular interstitial tissue volume measured by morphometry is presented in Table 3-5. In the control testes (right side), the interstitial volume was between 99 and144 µl/g. However, the interstitial volume was decreased in EDL testes 8 hours after EDL ($P<0.01$ compared with control side) and then was increased at 56 hours and afterward. The interstitial tissue volume peaked around 40 hours after EDL, but because of the small numbers of animals, the high variability and the fact that the control interstitial tissue volume was slightly higher at this time , no significant difference was found between the two groups. There were large variations within the groups in interstitial volume in the ligated testes at 32, 40 and 48 h post EDL. The reason for this variability is not known. The greatest difference between ligated and control testes was seen at 56h post EDL.

The blood-testis barrier:

When ^{51}Cr-EDTA space and interstitial tissue volume are presented individually as above, they only show interstitial volume decreasing or increasing after EDL, and

Table 3-3

^{51}Cr-EDTA space in epididymis (μl/g)

EDL Time (hours)	No.of rats	Caput		Corpus		Cauda	
		L	R	L	R	L	R
0	7	227±18.45	192±16.18	256±28.92	234±13.71	162±11.11	151± 8.11
2	3	141±28.33	132±25.69	141±27.64	177±62.07	97±17.97	105±28.95
4	8	155±15.70	198±36.69	177±23.31	204±35.14	105±14.72	111±17.11
8	3	142±11.86	140±22.51	169±21.70	190±23.16	109±10.01	103± 6.66
12	7	140±21.90	123±17.05	171±27.21	140±19.89	91±15.17	94±13.47
32	4	176±41.56	104±19.70	185±44.12	124±26.43	84±16.55	71±10.49
40	2	129± 8.91	114± 1.49	183± 9.74	132± 0.27	90±15.12	102±14.55
48	3	166±48.92	110±13.51	159±36.49	172±36.56	99±20.29	92± 8.85
56	7	151±23.99*	108±14.95	158±23.48	156±14.78	117±10.01	80±10.88
64	6	259±25.97**	199±22.25	296±37.14	250±31.16	168±22.22	150±12.19
72	8	302±41.14**	228±35.75	299±41.55	321±60.84	174±22.51	173±25.35

Values are expressed as mean ± S.E.M.; *P<0.05; **P<0.01 compared with R-side organs.

Table 3-4

^{51}Cr-EDTA space in prostate (µl/g)

EDL Time (hours)	No.of rats	Prostate L	Prostate R
0	7	641± 91	586± 52
2	3	953±573	624±250
4	8	704±118	606± 96
8	3	559± 54	476±172
12	7	393± 72	447±139
32	4	529±226	442±166
40	2	265± 26	317± 82
48	3	498±273	360±244
56	7	402±132	337± 57
64	6	659±113	684±100
72	8	937±187	1029±162

Values are expressed as mean ± S.E.M.

Table 3-5

Volume of the Interstitial tissue (μl/g) of the rat testis by morphometry

EDL Time (hours)	No.of rats	Interstitial tissue volume L	R
0	6	119± 6.94	116± 3.84
2	3	120± 4.58	113± 1.33
4	7	93± 5.87	99± 2.51
8	7	87± 4.30**	107± 6.03
12	7	89± 8.33	105± 5.34
16	4	128± 7.54	105± 5.34
24	4	105± 2.86	128± 8.02
32	4	147±19.41	130± 8.67
40	4	191±27.60	144± 6.34
48	4	138±21.88	120± 8.99
56	7	162± 8.61**	104± 3.93
64	8	176±13.08**	122± 5.36
72	8	150± 4.56**	116± 1.57

Values are expressed as mean ± S.E.M.;

*P<0.05;**P<0.01 compared with R-side organs.

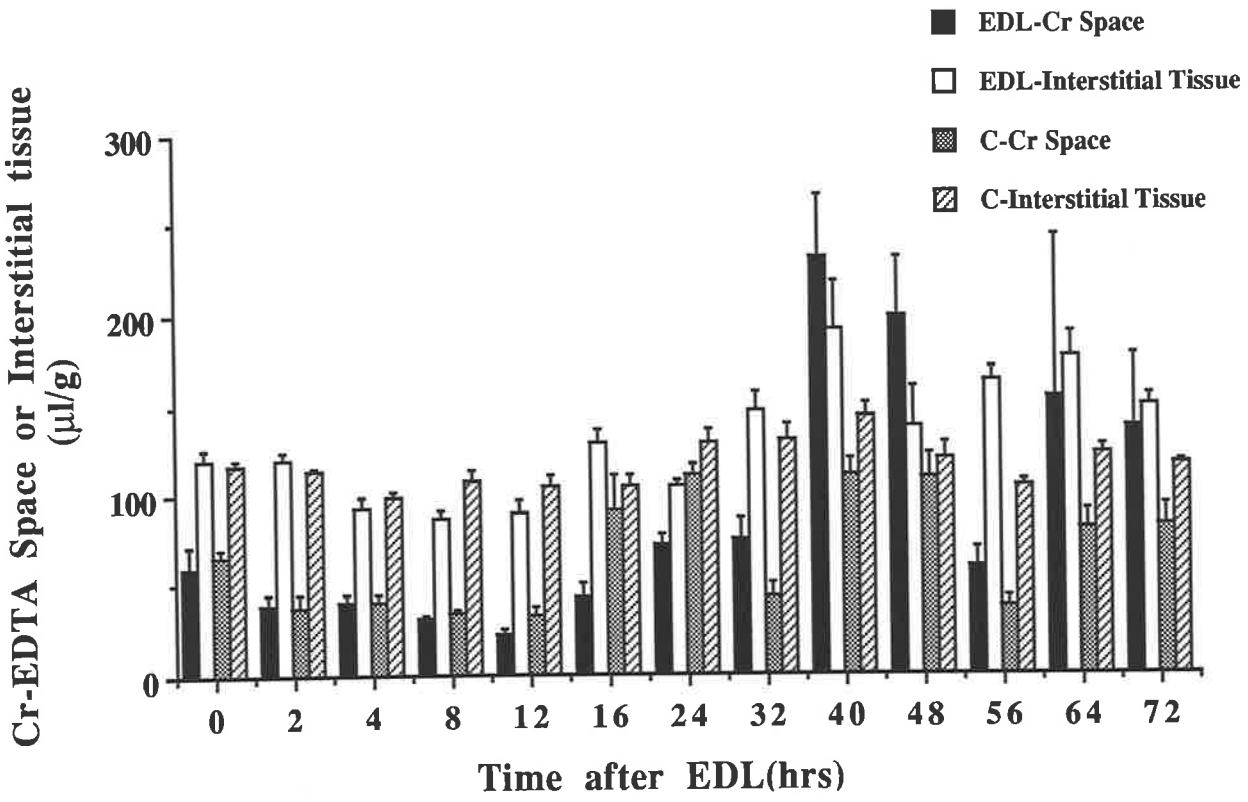


Fig. 3-2 The ^{51}Cr-EDTA space and the measured interstitial tissue volume (Mean ±SEM) in ligated and control testes at various times after EDL.

Table 3-6

THE RATIO OF ^{51}Cr-EDTA SPACE TO INTERSTITIAL VOLUME

EDL Time (hours)	No.of rats	EDTA space/Interstitial volume	
		L	R
0	6	0.50±0.10	0.57±0.05
2	3	0.33±0.04	0.34±0.06
4	7	0.43±0.05	0.40±0.05
8	7	0.37±0.02	0.32±0.02
12	7	0.27±0.02	0.31±0.03
16	4	0.35±0.15	0.71±0.18
24	4	0.70±0.07	0.87±0.10
32	4	0.56±0.15	0.35±0.08
40	4	1.22±0.13	0.77±0.10
48	4	1.52±0.33	0.90±0.09
56	7	0.36±0.05	0.36±0.05
64	8	0.85±0.10	0.67±0.09
72	8	0.91±0.12	0.71±0.11

Values are expressed as mean ± S.E.M.

cannot demonstrate whether the blood-testis barrier is broken or not. Since the blood-testis barrier is a physical barrier, its integrity can inhibit the entry of EDTA molecules into the seminiferous tubules. For this reason, the ^{51}Cr-EDTA space should always be less than the interstitial volume in the testis with an unbroken barrier. Fig. 3-2 shows that the ^{51}Cr-EDTA space was less than the interstitial volume (between 25% and 90% of the interstitial volume, Table 3-6) in EDL groups from 0 to 32 hours and in all control groups. However, the ^{51}Cr-EDTA space in the ligated testis was higher than the interstitial volume (between 120% and 150% of the interstitial volume) at 40 to 48 hours after EDL . At later times the ratio of the ^{51}Cr-EDTA space to interstitial volume in ligated testes returned to normal values.

3. 2. 3. Discussion

As the testis weight increased from 2 hours after EDL, the proportion of the testis parenchyma occupied by tubules (100-% interstitial tissue) was changed from less than 88% to more than 90% at around 4-12 hours after EDL. During this period the absolute interstitial volume remained unchanged (184±4.2μl/testis in EDL testes; 182±2.2μl/testis in control testes), with the increase in testis weights being mainly due to the accumulation of fluid inside the tubules. At 24 hours after EDL, testis weight reached its maximal value and at the same time, the percentage of interstitial volume returned to more than 10% between 16-24 hours after EDL. This was due to an absolute enlargement in both tubular and interstitial volume (231±2.2μl/testis). This increase in interstitial volume may be related to increased fluid transport from the tubules or reduced backflow from lymph.

At 32 hours after EDL, the testis weight began to decrease accompanied by an increase in ^{51}Cr-EDTA space and interstitial volume. This indicates the gradual atrophy of the tubules. ^{51}Cr-EDTA space significantly exceeded the interstitial volume 40-48 hours after EDL, suggesting the tubule barrier was broken, and ^{51}Cr-EDTA penetrated into the tubule . We cannot entirely exclude the possibility that tracer was entering cells after EDL, but this is most unlikely. However, once testis weight fell back to control

levels at 56 hours after EDL, the blood-testis barrier was re-established. As the breakdown of the barrier to ^{51}Cr-EDTA occurs after testis weight begins to fall, it can be concluded that the first loss of fluid from the tubules precedes the measurable breakdown of the barrier to ^{51}Cr-EDTA.

The reason for large variation in ^{51}Cr-EDTA space value between different time groups in control testis is unknown. Probably the low values in the unligated testes in the period 2 to 12h after EDL may be a post-anaesthetic effect, mediated by suppression of gonadotrophin secretion, but this does not explain the low values at 32 and 56h post-EDL. However, the important value is the ratio between the two testes of the same animal, and this figure would allow for the effects of any extraneous factors on the Cr-EDTA space. We have no explanation for the very high Cr-EDTA space in the capsule, but similar high values were reported for gamma-globulin in this tissue (Pollanen and Setchell, 1989).

Our results confirm and extend the finding of Setchell (1986), who observed the blood-testis barrier changes in EDL testes similar to ours, using the same tracer, but with fewer time points. The finding of no breakage in the barrier (Anton, 1982; Osman and Plöen, 1978) may by explained by the difference in EDL time and in the insensitivity of the methods employed to detect damage to the barrier. The results here show that the breakdown of the tubular barrier was only observed temporarily at 40-48 hours after EDL. Observation at any other time may give a different result. The position of ligation decides the amount of fluid accumulated inside the tubules. A recent study, cannulating the efferent duct at different positions shows that the rate of testicular fluid production was around 47.04 µl/h, but the rate of fluid entering the initial segment of the epididymis was 1.79 µl/h. This result indicates that the efferent ducts reabsorb 96.2% of the testicular fluid (Clulow et al.,1992). Soler et al.(1990) also found that the testis weight did not change after EDL near the epididymis. The tracer and animal species chosen may also influence the result.

The effects of EDL on the weight of epididymis and prostate were irregular, the differences may be due to variations in the technique of separation. However, ^{51}Cr-EDTA space in caput epididymidis increased significantly from 56 hour ($P<0.05$) after EDL, and was maintained at high levels for the remainder of the experiment ($P<0.01$). The anatomical position of caput may decide its vulnerability to EDL although there is no evidence available for this effect. Therefore, it is speculated that a temporary increase of epididymal blood flow and/or changes of pressure across the capillary wall may be involved in this increase of ^{51}Cr-EDTA space. In fact, the data from Wang and colleagues (1985) showed a tendency of increase in the epididymal blood flow at 24 hours after EDL and 7 days after EDL, unfortunately the measurement of the blood flow was not done between these times. The Cr-EDTA space in the prostate was hogh and variable, for reasons we cannot ecplain.

The importance of the integrity of the blood-testis barriers to spermatogenesis is well understood. Even temporary breakdown of the tubular barrier, could compromise the immunological privilege of the testis, and change ionic and protein composition, and hormone levels inside the tubules. These changes could influence spermatogenesis. The evidence is that reduction of mature spermatozoa (Wang et al., 1985) and appearance of giant cells (Singh and Abe, 1987) occur after tubular barrier breakdown. It may be speculated then that besides the effect of elevated pressure, the change of the milieu by breakdown of the tubular barrier is also involved in aspermatogenesis caused by EDL. However, the significance of the barrier dysfunction in producing the tubular lesions seen after EDL requires further clarification.

3. 3. HEATING OF TESTIS

3. 3. 1. Experimental procedures

Sixty adult male rats with body weight 534±9.85 g were divided randomly into 6 groups. Of each group 5 rats received testicular heating 43°C for 30 minutes, and another 5 rats 33°C for 30 minutes as the control (see 2.3.1). For each group the ^{51}Cr-

EDTA spaces and weights (see 2.4) of testes, epididymides and prostates at 2, 7, 14, 21, 35, and 56 days after testicular heating were determined. Testicular morphometry was performed for each frozen testis later (see 2.5).

3. 3. 2. Results

Organ weights:

The testis weight in the control group remained around 1700-1800 mg at all periods after exposing testes to 33°C. However, testis weight was significantly decreased ($P<0.01$) at 2 days after heating testes to 43°C, and at 14 days the testis weight was reduced to 58% of the control value and then sustained at this level until the last day (56th day) of the experiment (Table 3-7; Fig. 3-3). The epididymal weight, including caput, corpus and cauda, was not significantly affected in the treated groups until 14 to 21 days after heating. At 21 days, the epididymal cauda weight was reduced to 68% of the control value, and the caput and corpus weight was around 80% of the control value. The weight of epididymis was maintained at this lower level until day 56 of the experiment. However, prostate weight was not changed for either group over the time of the study (Table 3-7).

Table 3-7

EFFECT OF TESTICULAR HEATING OF RATS ON TESTIS, EPIDIDYMIS AND PROSTATE WEIGHT (mg)

Days after	Testis		Caput		Corpus		Cauda		Prostate	
heating	43°C	33°C	43°C	33°C	43°C	33°C	43°C	33°C	43°C	33°C
2	1596± 32**	1708±22	269±10	287± 8	117±18	108±6	273± 7	288±10	293±13	272±20
	(10)	(10)	(10)	(10)	(10)	(10)	(10)	(10)	(10)	(10)
7	1244± 50**	1757±36	244± 9	256± 8	62± 3*	73±2	213± 6	233±11	227±21	244±16
	(10)	(10)	(10)	(10)	(10)	(10)	(10)	(10)	(10)	(10)
14	974± 33**	1688±26	224±11*	279±19	113±10	125±9	236± 6**	296± 9	311±17	323±37
	(10)	(8)	(10)	(8)	(10)	(8)	(10)	(8)	(10)	(8)
21	1051± 61**	1742±49	221±10**	273±11	94± 5**	115±5	210±10**	311± 6	264±29	286±14
	(10)	(10)	(10)	(10)	(10)	(10)	(10)	(10)	(10)	(10)
35	1002± 99**	1700±15	206± 8**	274±10	74± 6**	106±6	205±14**	315± 8	303±13	294±24
	(10)	(10)	(10)	(10)	(10)	(10)	(10)	(10)	(10)	(10)
56	991±116**	1805±30	195±10**	255± 7	59± 5**	89±4	205±15**	323± 8	363±24	319±18
	(10)	(10)	(10)	(10)	(10)	(10)	(10)	(10)	(10)	(10)

Values are expressed as mean ± S.E.M. with the number of determinations shown in parenthesis;
*P<0.05; **P<0.01 compared with corresponding control (33°C).

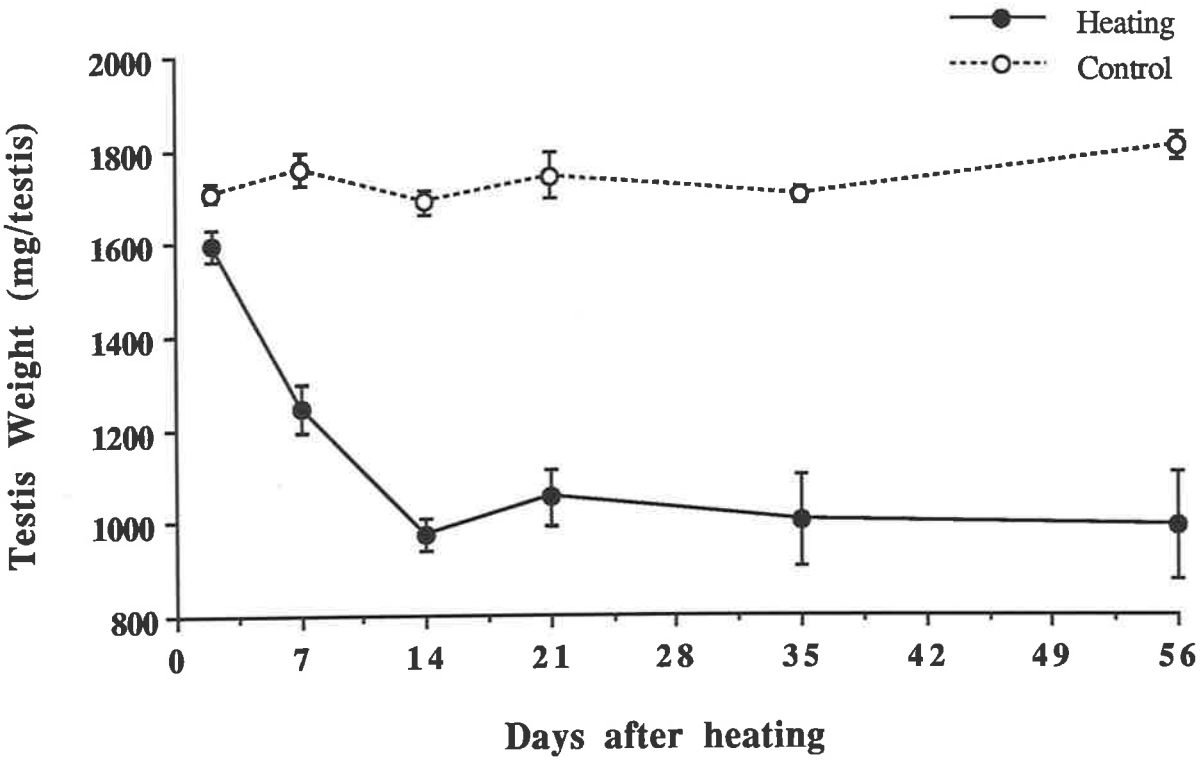


Fig. 3-3 Testis weights (Mean ±SEM) in heating (43°C) and control (33°C) animals at various times after heating of testes.

Table 3-8

EFFECT OF TESTICULAR HEATING ON TESTIS, TESTICULAR PARENCHYMA AND CAPSULE ^{51}Cr-EDTA SPACE (µl/g) IN RATS

Days after	Testis		Parenchyma		Capsule	
Heating	43°C	33°C	43°C	33°C	43°C	33°C
2	92± 8	105±13	72± 7	83±10	390± 43	414±49
	(8)	(8)	(8)	(8)	(8)	(8)
7	171±11**	129± 7	109±12	99± 5	726± 94	661±70
	(10)	(10)	(10)	(10)	(10)	(10)
14	151±14**	92± 5	122±13**	75± 4	452± 41	396±12
	(10)	(8)	(10)	(8)	(10)	(8)
21	155±28*	94± 7	128±24*	76± 4	376± 49	408±31
	(10)	(10)	(10)	(10)	(10)	(10)
35	237±24**	123±10	176±17**	96± 7	775±150	582±46
	(10)	(10)	(10)	(10)	(10)	(10)
56	226±18**	154±10	172±17*	120± 8	666± 87	722±51
	(10)	(10)	(10)	(10)	(10)	(10)

Values are expressed as mean ± S.E.M. with the number of determinations shown in parenthesis;
*P<0.05; **P<0.01 compared with corresponding control (33°C).

Table 3-9

EFFECT OF TESTICULAR HEATING ON EPIDIDYMIS ^{51}Cr-EDTA SPACE(μl/g) IN RATS

Days after	Caput		Corpus		Cauda	
heating	43°C	33°C	43°C	33°C	43°C	33°C
2	223±21	242± 42	270± 65	245± 52	199±23	190±36
	(8)	(8)	(8)	(8)	(8)	(8)
7	219±22	475±141	240± 18	436±120	235±13	231±42
	(10)	(10)	(10)	(10)	(10)	(10)
14	276±38	214± 23	319± 70	230± 36	227±26	185±11
	(10)	(8)	(10)	(8)	(10)	(8)
21	204±27	264± 7	418±115	238± 30	269±26**	170±12
	(10)	(10)	(10)	(10)	(10)	(10)
35	427±88	296± 64	584±124	295± 79	352±23**	222±36
	(10)	(10)	(10)	(10)	(10)	(10)
56	285±33	280± 26	266± 22	225± 15	311±21**	217±11
	(10)	(10)	(10)	(10)	(10)	(10)

Values are expressed as mean ± S.E.M. with the number of determinations shown in parenthesis;
*P<0.05; **P<0.01 compared with corresponding control (33°C).

Table 3-10

EFFECT OF TESTICULAR HEATING ON PROSTATE ^{51}Cr-EDTA SPACE (µl/g) IN RATS

Days after heating	Prostate 43°C	Prostate 33°C
2	606± 222 (8)	377± 108 (8)
7	464± 145 (10)	1105± 543 (10)
14	1618± 462 (10)	1666± 521 (8)
21	1788± 836 (10)	517± 142 (10)
35	1134± 536 (10)	1237± 711 (10)
56	4391±2054 (10)	1610±1218 (10)

Values are expressed as mean ± S.E.M. with the number of determinations shown in parenthesis.

The ^{51}Cr-EDTA space in organs:

The ^{51}Cr-EDTA space in whole testes and parenchyma increased from 7 and 14 days after testicular heating to 43°C respectively. It remained at a high level for the rest of the time of the experiment. The testicular capsule space was not significantly changed for either group during the study (Table 3-8).

The effect of heating the testes on the ^{51}Cr-EDTA space of the three parts of the epididymis was different. The space in the caput and the corpus was not affected by heating, although there was a great variability in the space in the heated corpus at 21 and 35 days post-heating; the space in the cauda was increased significantly from 21 days after heating and did not return to normal during the experiment (Table 3-9). The space in the prostate was unstable with a high SEM so that it was difficult to conclude whether the space of this organ was influenced (Table 3-10). On the other hand, the Cr-EDTA spaces in the prostate in both the treated and control groups in the heat experiment (Table 3-10) were much high than those in EDL experiment (Table 3-4). The reason is unknown, but there may have been a delayed effect of the anaesthesia in the heat experiment, as times only up to 72 h were studied in the EDL experiment.

Interstitial tissue volume and the integrity of the barrier:

While testis weight decreased in the treated groups, interstitial tissue volume as a percentage of the testis was increasing from 7 days after heating. It increased to 130% of the control value by the 7th day, then to 185% of the control value by day 14 and stayed at this high level for the remainder of the study (Table 3-11). As the increase occurred in both ^{51}Cr-EDTA space and interstitial tissue volume of testes in treated group, the ratios of ^{51}Cr-EDTA space to interstitial tissue volume were less than 0.85 during the whole period after heating (Table 3-12; Fig. 3-4). Although the seminiferous tubules atrophied with increasing of interstitial tissue volume, the blood-testis barrier in heated testes was still intact.

Table 3-11

INTERSTITIAL TISSUE VOLUME (μl/g) BY MORPHOMETRY

Days after heating	No.of rats	Interstitial volume 43°C	33°C
2	10	134± 6	140±5
7	10	168± 8**	129±5
14	10	229± 9**	124±5
21	10	270±42**	124±4
35	10	214±22**	116±5
56	10	274±36**	145±5

Values are expressed as mean ± S.E.M.;

**P<0.01 compared with corresponding control (33°C).

Table 3-12

THE RATIO OF ^{51}Cr-EDTA SPACE TO INTERSTITIAL VOLUME

Days after heating	Cr-EDTA space/Interstitial volume 43°C	33°C
2	0.56±0.06 (8)	0.57±0.06 (8)
7	0.73±0.05 (10)	0.78±0.05 (10)
14	0.53±0.05 (10)	0.61±0.04 (8)
21	0.49±0.05 (10)	0.61±0.03 (10)
35	0.85±0.09 (10)	0.83±0.05 (10)
56	0.68±0.06 (10)	0.84±0.07 (10)

Values are expressed as mean ± S.E.M. with the number of determinations shown in parenthesis.

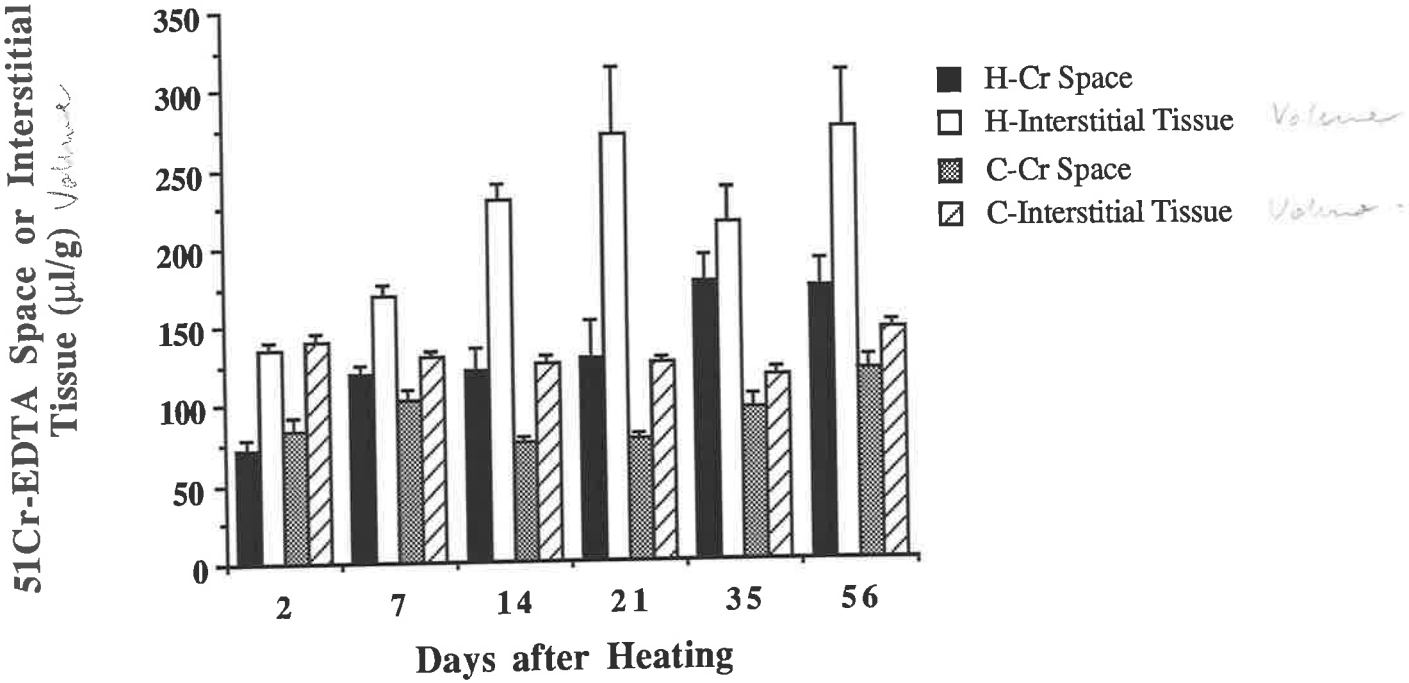


Fig. 3-4 The ^{51}Cr-EDTA space and the measured interstitial tissue volume (Mean ±SEM) in heated testes (H-) and control testes (C-) at various times after heating of testes.

3. 3. 3. Discussion

The main purpose of this present study was to investigate the function of the blood-testis barriers during aspermatogenesis produced by heat treatment. The results showed that although heating testes to 43°C for 30 min induced a dramatic decrease of testis and epididymis weight accompanied by a significant increase of testicular parenchyma ^{51}Cr-EDTA space and interstitial volume, the ratio of ^{51}Cr-EDTA space to interstitial volume always remained less than 0.85. These results suggest the atrophy of seminiferous epithelium with enlargement of the interstitium, but not the destruction of the integrity of the blood-testis barriers.

Testis weights have been reported to reach a minimal value at around 2 weeks after heating (Au et al., 1987; Galil and Setchell, 1987). This decrease is accompanied by the germ cell depletion in seminiferous epithelium, which is caused by the sensitivity of pachytene spermatocytes and early spermatids to heat (Collins and Lacy, 1969; Chowdhury and Steinberger, 1970; Kerr et al., 1979). A gradual recovery of testis weight occurred from 3 weeks after heating (Galil and Setchell, 1987), but in our study recovery only occurred in half of the heated testes. The other testes continued to lose weight, resulting in a high SEM for testis weight at days 35 and 56. An injury to the differentiating spermatogonia is probably involved in this failure of weight recovery.

At 14 days after heating ^{51}Cr-EDTA space was significantly increased (Table 3-8), but interstitial volume increased to 185% of the control value at the same time (Table 3-11). Nevertheless, in contrast to the observations after efferent duct ligation (see above 3.2.) and in prepubertal animals (Setchell et al., 1988a), the ^{51}Cr-EDTA space never exceeded the interstitial volume (Table 3-12). These results are similar to those of Wang et al. (1983) in irradiated testes and indicate that even in full aspermatogenesis caused by heating, the integrity of the blood-testis barriers is still maintained and support the previous suggestion that aspermatogenesis by heating does not involve a dramatic alteration of the blood-testis barriers but more in changes in testicular metabolism.

3. 4. EXPOSURE OF ANIMALS TO N_2O.

3. 4. 1. Experimental procedures

Eighteen adult male rats were divided randomly into N_2O and control groups. As described in 2.3.3., the rats in N_2O group were made to breathe 21.49±0.608 % (21.49 x 10^5 ±60.80 x10^3ppm) N_2O and 18.57±0.144 % O_2 1h/day for 42 days; pumped fresh air was used instead of N_2O and O_2 in control animals. The controlled atmosphere chamber described in 2.3.3. was used during the 1 hour treatment. After the treatment the rats were returned to their normal cages. After 6 weeks of treatment, the ^{51}Cr-EDTA space in testis, epididymis and prostate was determined (see 2.4). The testicular interstitial tissue volume of the frozen testis was measured later (see 2.5).

3. 4. 2. Results

For testis, epididymis and prostate, neither weight nor ^{51}Cr-EDTA space was different between N_2O and control groups (Table 3-13,3-14). The interstitial tissue volume was around 150 µl/g and the ^{51}Cr-EDTA space in testicular parenchyma only occupied about 45% of the morphometrical interstitial tissue volume (Figure 3-5).

3. 4. 3. Discussion

Nitrous oxide has long been used as an anaesthetic gas. Coate and colleagues (1979) reported the effect of chronic exposure (52 weeks) to low concentrations of halothane-nitrous oxide on male chromosomes in rats. Their results show that the proportions of aberrant cells and the number of marker chromosomes (exchange of figures, rings and miscellaneous markers) were significantly increased with dose in treated animals. Damage to seminiferous tubules was observed in testes of rats exposed continuously to a high dose of N_2O for short times (2 to 35 days). This damage was expressed as loss of germ cells accompanied by decrease of testicular

Table. 3-13

EFFECT OF N_2O ON TESTICULAR, EPIDIDYMAL AND PROSTATIC WEIGHTS

Organs	Numbers	N_2O (mg)	Control (mg)
Testis	18	1880±55	1876±51
Caput	18	296± 7	280± 9
Corpus	18	108± 4	115± 6
Cauda	18	323±15	293±10
Prostate	18	246±21	248±19

Values are expressed as mean ± S.E.M.

Table. 3-14

EFFECT OF N_2O ON TESTICULAR, EPIDIDYMAL AND PROSTATIC ^{51}Cr-EDTA-SPACE

Organs	Numbers	N_2O (µl/g)	Control (µl/g)
Testis	18	74± 1.98	81± 3.76
Parenchyma	18	62± 1.80	64± 3.40
capsule	18	316±20.69	353± 14.30
Caput	18	192± 8.33	199± 11.64
Corpus	18	228±25.85	207± 24.33
Cauda	18	155± 8.71	164± 12.28
prostate	18	508±99.76	493±126.89

Values are expressed as mean ± S.E.M.

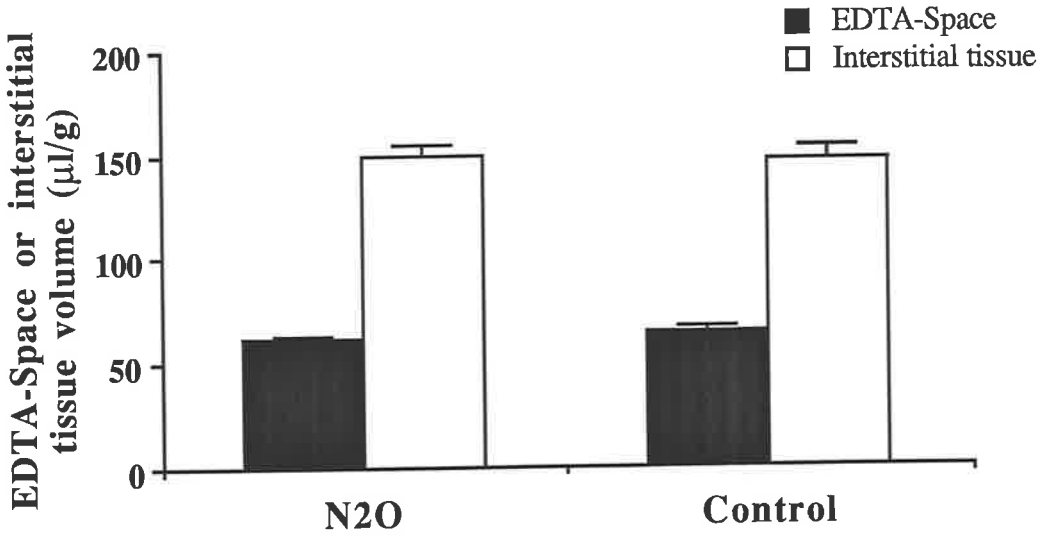


Fig. 3-5 The ^{51}Cr-EDTA space and the measured intersttial tissue volume (Mean ±SEM) in the testes of animal treated with N_2O and with fresh air (control).

weight, form of multinucleated giant cells and vacuolated cytoplasm in Sertoli cells (Kripke et al, 1976). However, no detectable effect of exposure to N_2O on the weight of sexual organs and the blood-testis barriers was observed in our study. Because the animals were only exposed to a high dose of N_2O 1h/day for 6 weeks, there is not sufficient reason to reject the effect of N_2O on the function of the blood-testis barriers. Further studies should involve exposure of animals to N_2O continuously for at least one month, as done by Kripke et al, (1976).

3. 5. CONCLUSION

Our results indicate that the Sertoli cell membrane, a major component of the blood-testis barriers, is very stable. Even temperature increases sufficient to damage spermatogonia do not disrupt the integrity of the barrier. Infertility caused by hyperthermia, as is the case with cryptorchidism and varicocoele, probably involves a change of Sertoli cell metabolism as well as direct damage to spermatozoa, but not the breakdown of the blood-testis barriers.

Efferent duct ligation alone among the 3 agents chosen for study can cause a temporary breakdown of the tubular barrier. Although clear evidence of barrier breakage occurs after EDL only when testis weight begins to fall, it is likely that the accumulation of tubular fluid in the tubule and the consequent high pressure is the major factor causing the breakdown of the blood-testis barriers. This experiment supplies a suitable animal model for further study on the effect of change of milieu on spermatogenesis.

CHAPTER 4: EFFECT OF PERFUSATE FLOW AND PROTEIN CONCENTRATION IN PERFUSION MEDIUM ON ALBUMIN AND TESTOSTERONE TRANSPORT ACROSS THE VASCULAR WALL IN TESTIS

4. 1. INTRODUCTION

Although blood flow and vascular permeability in testis are very important for both the uptake of peptide and the secretion of steroid hormones, there have been only a few studies relating to these. Pituitary gonadotrophins are the major regulators of testicular function . As these gonadotrophins are peptide hormones, which usually are not very lipid soluble, it is likely that their uptake by tissue will be influenced largely by vascular permeability, rather than by flow. However, for steroid hormones, which are much more lipophilic, movement from the testis into the general circulation should be affected more by blood flow than by vascular permeability. For our preliminary investigations into this question, the uptake of ^{125}I-albumin by the rat testis has been measured at different perfusion flow rates and testosterone output across the testicular vascular wall also studied at different protein concentrations in perfusion medium as well as at different perfusion flow rates.

4. 2. PERFUSION FLOW

4. 2. 1. Experimental procedures

Thirtyone mature male rats weighing 473±8 g were used in this experiment. Both testes from each animal were perfused simultaneously through the testicular artery on the surface of the testis (See 2.6.2) with Krebs-Ringer solution containing 1% bovine serum albumin gassed with 95%O_2-5%CO_2 (see 2.6.3). The effluent flowing from the cut end of the spermatic cord was collected in a fraction collector (see 2.6.4). During the first 30 min, both testicular perfusion flows were kept at 500µl/min, and then one was changed to a lower or higher flow rate (250, 350, 700, 1000 and 1500µl/min) for 15 min with the contralateral testis kept at 500µl/min as a control. Because it was not possible to weigh the testes before starting the perfusion, this value was chosen, based on an average testis weight of 1.67g, to give a flow of 300µl/g/min. After this 15 min

Table 4-1

Parenchyma %E and PS to Albumin

Flow rate (µl/testis/min)	Number of testes	%E	PS
250	6	9.86±1.45	13.97±2.11
350	6	7.11±1.71	15.66±4.01
500	31	7.35±0.60	21.20±1.85
700	7	6.87±0.85	27.04±3.01
1000	6	5.81±0.48	30.85±3.39
1500	6	4.49±0.35	36.79±3.77

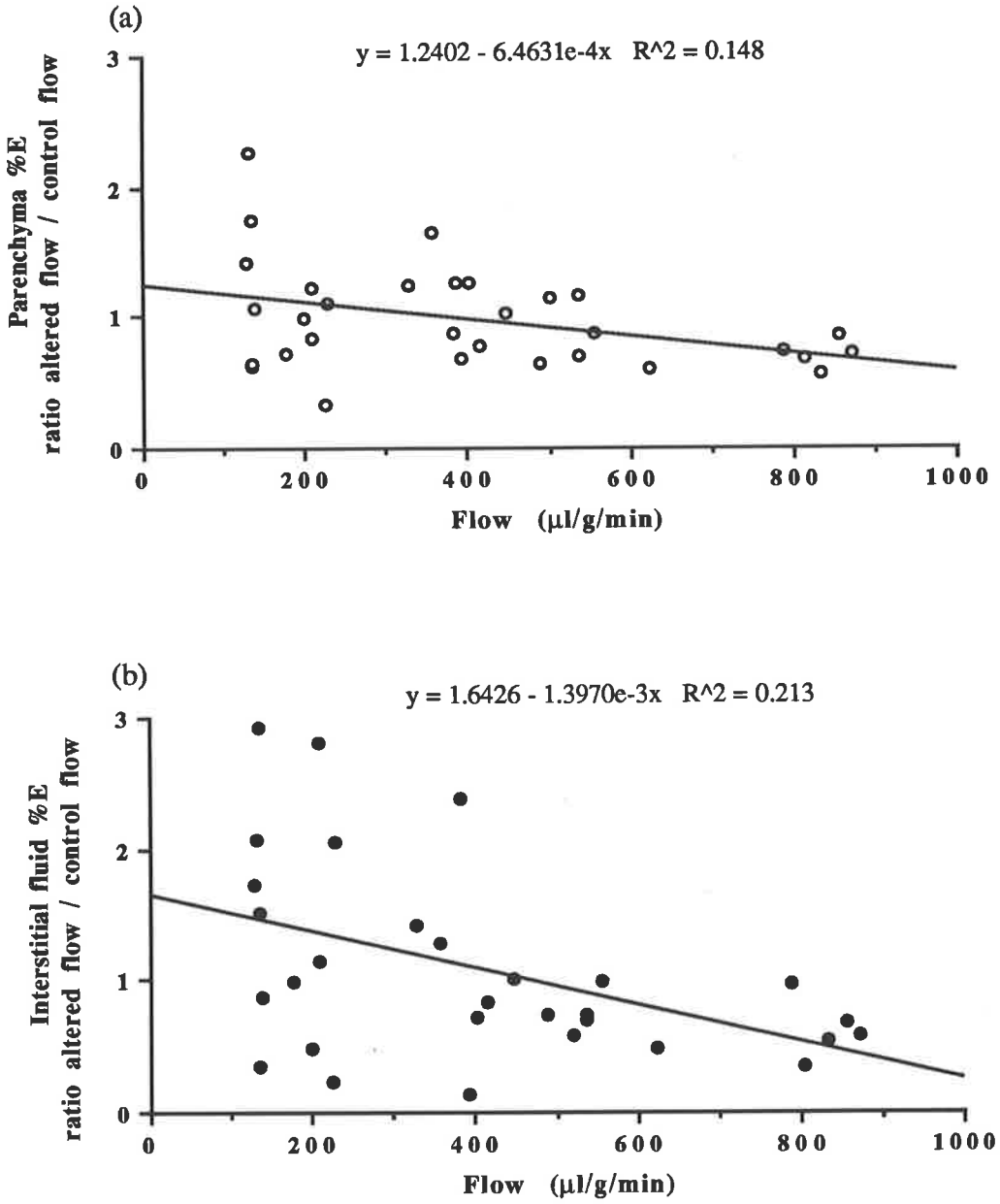


Fig. 4-1 The extraction (%E) of ^{125}I-HSA by the (a) parenchyma and (b) interstitial fluid of isolated perfused rat testes in relation to perfusion flow rate; the results are expressed as the ratio of the testis with altered flow to contralateral testis of the same animal, in which the perfusion was maintained at 500µl/testis/min.

perfusion, 50µl ^{125}I-albumin (around 0.01µCi) was injected through a three-way junction close to the testis (see 2.6.4) and effluent was collected over 1 min for radioactivity determination. At the end of the perfusion, the testis and separated capsule, seminiferous tubules and testicular interstitial fluid were weighed and counted in a gamma spectrometer (see 2.6.5). The effluent collected was also analysed by RIA for testosterone (see 2.7). The extraction and permeability surface area product to albumin were calculated according to the formulas shown in 2.6.5.

4. 2. 2. Results

Extraction (E) and permeability surface area product (PS):

Although testicular interstitial fluid, which does not include vascular fluid (see 2.6.5), weighed only 11% of the whole testis, it contained 48% of the counts taken up at the control flow rate. The corresponding values for the seminiferous tubules were 84% of weight and 34% of counts, and for the capsule 5% and 18% respectively. As perfusion flow increased from 250µl/min to 1500µl/min, the percentage extraction (%E) in testicular parenchyma, which includes seminiferous tubule and TIF, decreased gradually from 9.86±1.45% at 250µl/min to 4.49±0.35% at 1500µl/min (7.35±0.06% at 500µl/min as control) (Table 4-1). True PS was calculated by the formula:

$$PS = -Q \times Ln(1-E).$$

Because the percentage extraction in parenchyma and interstitial fluid decreased as flow rate increased (Fig.4-1a, b) , the testicular permeability surface product for albumin showed a significant, quadratic relation with flow rate, increasing only slightly as flow rate changed from 250 to 1500µl/min (134 to 800µl/g/min) (Fig.4-2a; Table 4-1). In fact, at flow rates above control, linear regression shows no significant effect of flow on PS (Fig.4-2b, r=0.089). Uptake of albumin, calculated as flow multiplied by extraction, showed a similar pattern to PS (Fig.4-3).

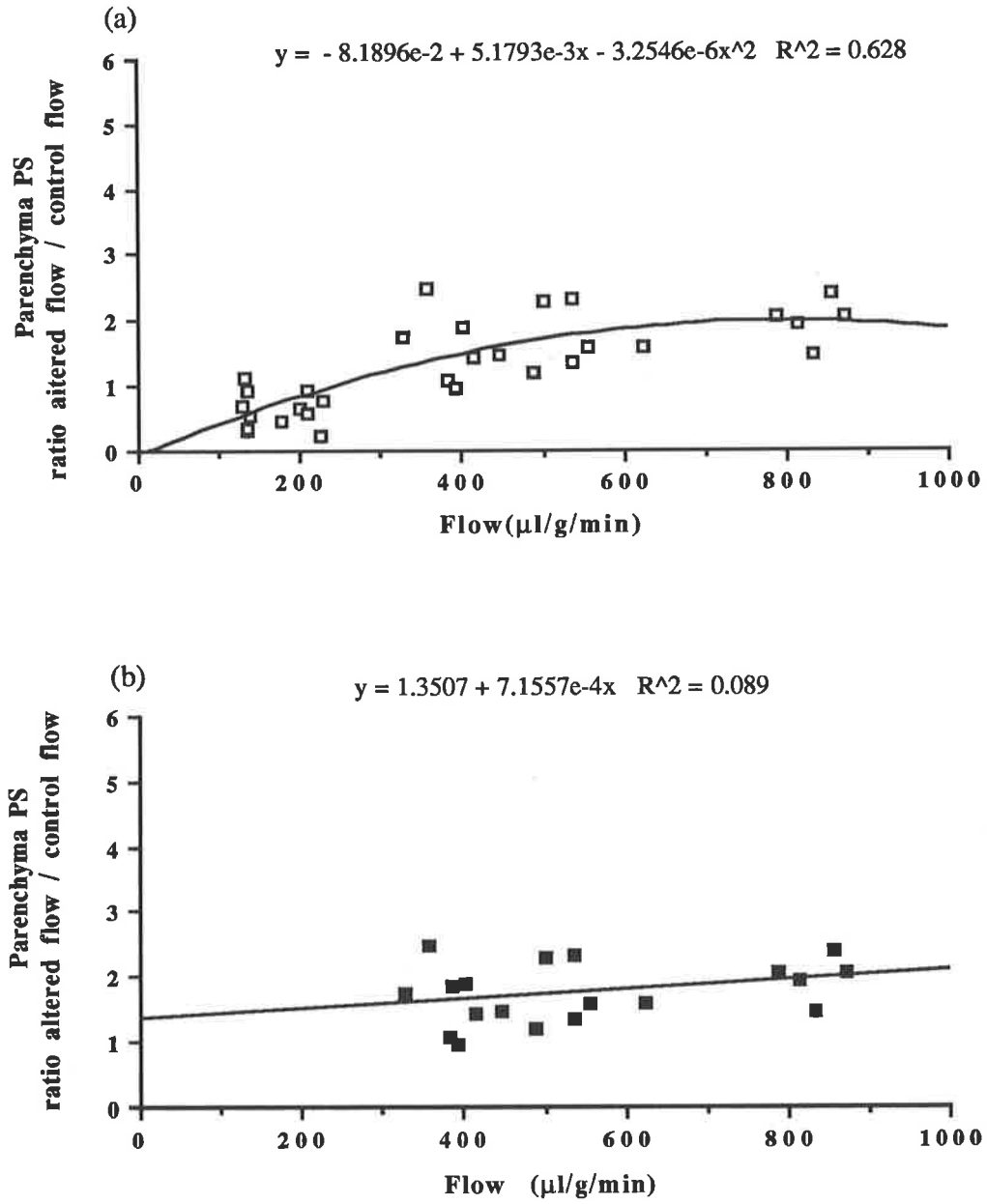


Fig. 4-2 PS of radioactive albumin by the parenchyma of isolated perfused rat testes, calculated by the formula PS=-Q x Ln (1-E) (see 2. 6. 5.) and expressed as a ratio of altered flow to control as in Fig. 4-1; (a) significant quadratic relationship for all flow rates used in this experiment; (b) the linear relationship for flow rates higher than normal, which was not significant (P>0.05).

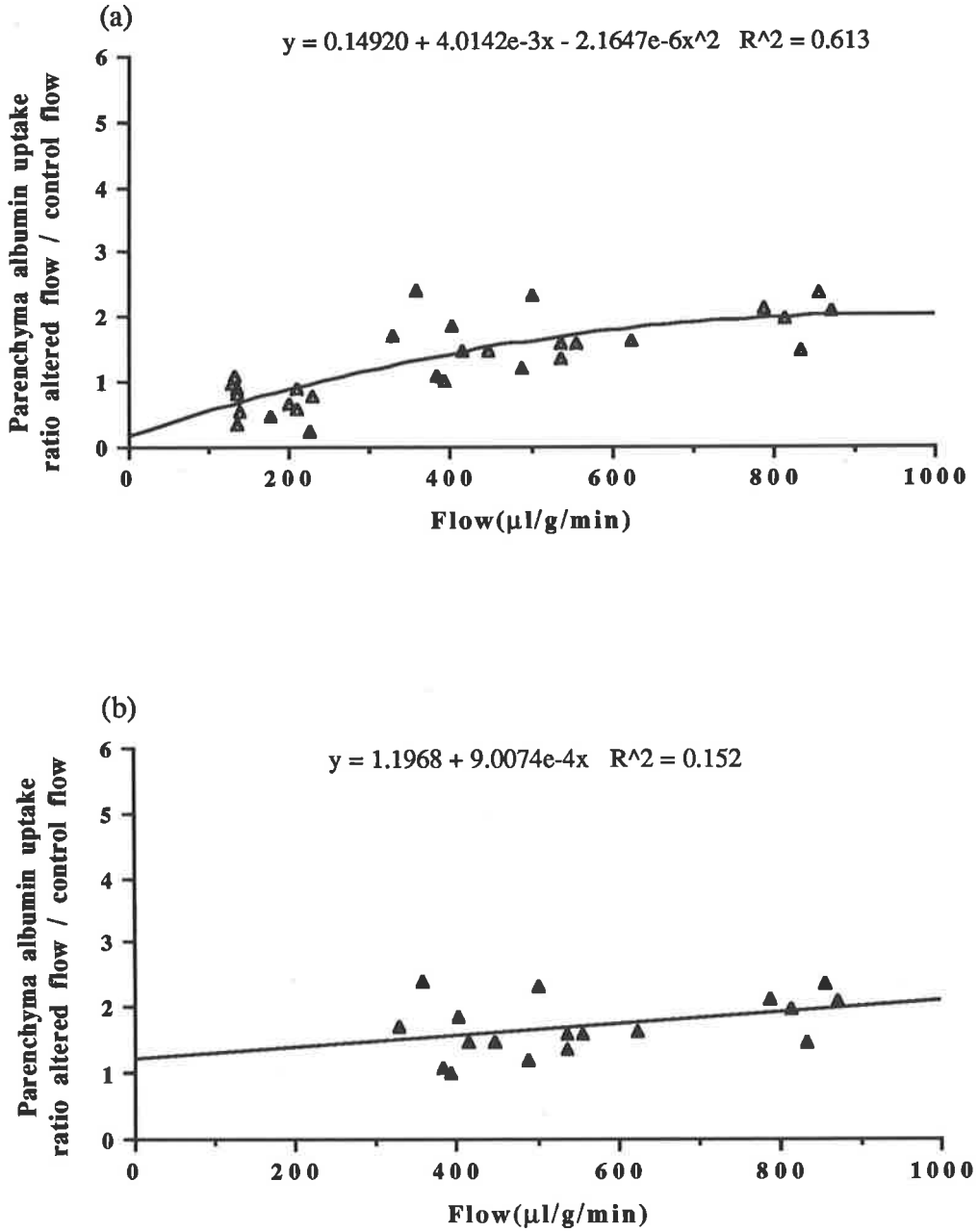


Fig. 4-3 Uptake of radioactive albumin by the parenchyma of isolated perfused rat testes, calculated as extraction of albumin multiplied by flow, and expressed as a ratio of altered flow to control as in Fig. 4-1, 4-2. (a) Significant quadratic relationship for all flow rates used in this experiment; (b) The linear relationship for flow rates higher than normal, which was not significant (P>0.05).

Table 4-2. Effect of perfusate flow (µl/min) on testosterone concentration (ng/ml) in the effluent.

Time	Control testis		Flow-altered testis		n
	Flow	T conc.	Flow	T conc.	
0-30'	500	29.9±13.5	500	39.0±20.6	6
31-45'	500	21.9±12.5	250	27.7±13.2	6
0-30'	500	20.0±5.5	500	18.9±5.9	6
31-45'	500	11.1±2.4	350	12.4±2.6	6
0-30'	500	20.3±5.1	500	19.6±5.0	6
31-45'	500	12.2±2.1	700	14.9±2.7	6
0-30'	500	46.7±17.3	500	47.7±17.3	6
31-45'	500	22.5±8.1	1000	21.3±5.7	6
0-30'	500	8.4±1.7	500	9.4±1.7	6
31-45'	500	9.0±1.7	1500	10.3±1.5	6

Testosterone concentration and production:

Testosterone concentration (ng/ml) in effluents from different flow rates were maintained in the same range as their controls for the whole experimental period. After 30 min of perfusion, testosterone concentrations in both control and flow-changed effluents were less than their effluents collected during the first 30 min. However, no difference was found between each flow-changed group and its corresponding control at the same perfusion time (Table 4-2).

Testosterone production (ng/g/min), calculated according to the formula in 2.7.3., increased linearly as the perfusion flow increased ($r=0.753$, $P<0.01$) (Fig.4-4; Table 4-3). During the first 30 min, when both groups were perfused at 500µl/min, no difference was found between control group and the corresponding flow altered group. During this period, the testosterone secretion ratio of flow-altered flow to control was maintained around 0.95-1.12. For the first 5 minutes (30-35'), when perfusion flow changed from 500 µl/min to 700 µl/min or higher, testosterone secretion increased significantly ($P<0.01$) and was maintained at the high levels for the remainder of the perfusion period (35-45'). At low perfusion flow rates (250-350µl/min), testosterone secretion appeared to decrease, but no significant difference was found between the lower flow groups and the control except at the third 5 minute period (40-45') at a flow rate of 350µl/min (Table 4-3; Fig.4-5).

There was considerable variation between animals, particularly with regard to testosterone production, as has been reported by other authors (Daber & Janson, 1978, Free & Tillson, 1973); consequently, all values in this experiment are presented as ratios of the value from testes with altered flow to the corresponding control testis from the same animal. The original data of percentage extraction and PS for albumin are presented individually in Table 4-1. The testosterone concentration in effluent and its secretion are presented as mean ±SEM in each group in Table 4-2 and 4-3, respectively.

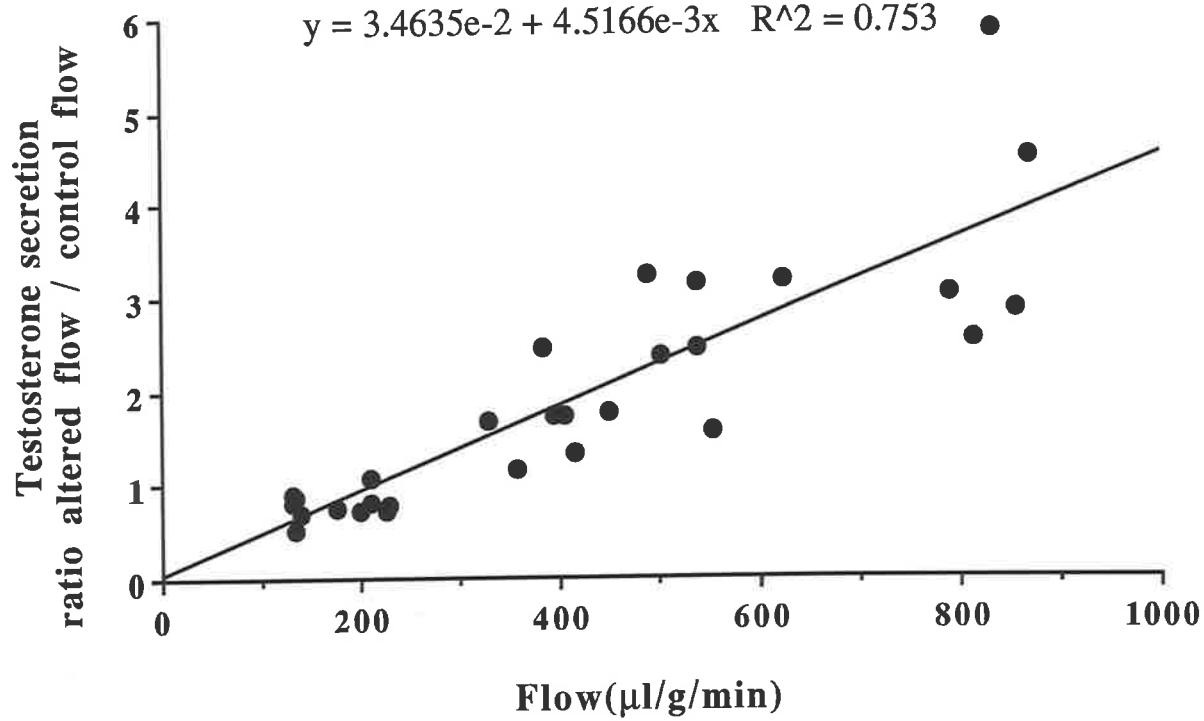


Fig. 4-4 Testosterone secretion in relation to perfusion flow in isolated perfused rat testes, the results are expressed as the ratio of the testis with altered flow rate to the contralateral testis of the same animal as in Fig. 4-1.

Table 4-3

Testosterone output in isolated perfused testes

Flow Rate (µl/min)	Control testis (ng/g/min)	Treated testis (ng/g/min)	n
250			6
0-30'	7.58±3.40	10.11±5.32	
30-35'	6.00±3.17	4.24±2.01	
35-40'	5.50±3.11	3.72±1.81	
40-45'	5.70±3.40	3.19±1.49	
350			6
0-30'	5.70±1.80	5.25±1.05	
30-35'	3.57±0.93	3.13±0.77	
35-40'	3.21±0.76	2.49±0.56	
40-45'	3.08±0.68	2.15±0.46*	
700			6
0-30'	5.31±1.43	5.34±1.49	
30-35'	3.71±0.83	6.39±1.70**	
35-40'	3.46±0.69	6.29±1.25**	
40-45'	2.91±0.63	5.09±0.96**	
1000			6
0-30'	11.51±4.10	11.53±4.11	
30-35'	6.99±2.35	11.83±3.23**	
35-40'	5.62±2.04	12.08±3.18**	
40-45'	4.99±1.87	7.99±2.03**	
1500			6
0-30'	2.25±0.42	2.46±0.47	
30-35'	2.52±0.44	8.16±1.46**	
35-40'	2.57±0.51	8.86±1.14**	
40-45'	2.57±0.52	6.25±1.02**	

* $P<0.05$; ** $P<0.01$

0-30': Both control and treated testes were perfused at a flow rate of 500µl/min/testis;

31-45': Control testis was perfused at the same flow rate as 0-30', treated testis was perfused at the flow rate marked for each group.

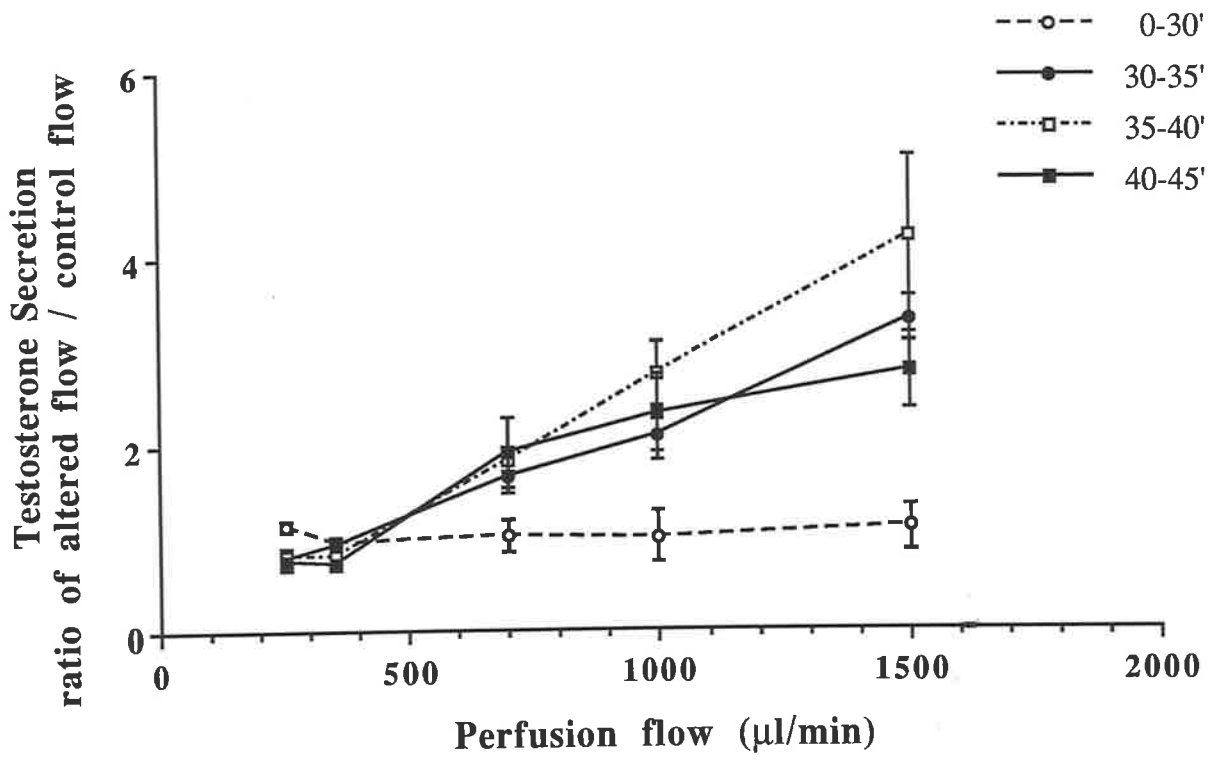


Fig. 4-5 Testosterone secretion in relation to perfusion flow in isolated perfused rat testes at different times after flow change. The results are expressed as the ratio of the testis with altered flow rate to the contralateral testis of the same animal as in Fig. 4-1. 0-30': both testes were perfused at 500µl/min, the ratio was around 0.95 to 1.12; 30-45': flow through one testis was changed to 250,350,700,1000 or 1500µl/min, testosterone secretion showed significant increase ($P<0.01$) when flow rate changed to 700µl/min or higher, and maintained in whole period of observation.

4. 2. 3. Discussion

Testicular blood flow measured by microspheres is around 500 µl/testis/min (about 320µl/g/min) in adult rats (Setchell and Brooks,1988). This value was chosen as the control perfusion flow rate used in control testes for the whole perfusion period and in flow altered rats for the first 30 minutes (0-30'). Even at a constant perfusion flow rate (0-30'), testosterone concentrations in effluents fluctuated over a very wide range (Table 4-2), which is greater than that found in an in vivo study using rats (Free and Tillson,1973), and might involve slight changes of perfusion condition such as temperature and perfusate pH. However, as both testes, one of which served as a flow changed and the other as control, were perfused at the same time influencing factors were similar for both the control and treated groups. Therefore, the mean of percentage difference between control and treated groups was 3±4 % at flow rate of 500µl/min.

The mean testosterone concentration (about 25 ng/ml) in effluent for the first 30 minutes was close to that of testicular venous blood in anaesthetized rats in vivo (28.5ng/ml) (Free and Tillson,1973), although these concentrations decreased with increased perfusion time. The change of perfusion flow rate without a corresponding change of testosterone concentration (Table 4-2) in the effluents contributed to a positive correlation between testosterone secretion and flow rate (Fig. 4-4). Thus the secretion of testosterone by the isolated perfused rat testis is largely determined by flow rate and this result is similar to the relationship between corticosterone secretion and blood flow in isolated perfused adrenal gland (Sibley et al., 1981; Hinson, 1984). It is also in accord with previous observations in vivo by Eik-Nes (1964) for the autoperfused dog testis and by Free &Tillson (1973) and Damber & Janson (1978) for the rat testis in vivo. Testicular blood flow decreases following testis heating (Galil and Setchell, 1987; Setchell et al., 1991b), efferent duct ligation (Wang et al., 1985) or exposing testis to irradiation (Wang et al., 1983) are always accompanied by a reduction of testosterone secretion, presumably as an effect of the reduced blood flow. Although no gonadotrophin was added to the perfusion medium, testosterone secretion still showed a positive correlation with perfusion flow. This indicates that blood flow itself is

regulating testosterone transport rather than hormone stimulants carried by the increased flow in this perfusion experiment.

In contrast to these findings with steroids, albumin uptake at higher than normal flows is only slightly affected by flow (Fig. 4-3b), Presumably this is because the time available for a single passage of labelled albumin through the testicular vasculature shortens as flow rate increase. Generally, albumin is transported mainly by convection through large pores. When the time of albumin passage through a vessel decreases, the chance of an albumin molecule passing through the pores is decreased, this in turn, results in lessened albumin extraction (Fig. 4-1). As flow rate increases, extraction of albumin decreases so that albumin uptake reaches a plateau at normal flow, and when flow is further increased, there is no further increase in uptake (Fig. 4-3). At lower than normal flows, increased albumin extraction cannot compensate for the effect of decreased flow so albumin uptake and PS are decreased (Fig. 4-2; 4-3), probably because some vessels are not being perfused at flows lower than normal. This research is the first one to study the effect of flow on the uptake of albumin in isolated perfused testis, and can be extended to study the effect of flow on uptake of gonadotrophins. It is expected that a similar result will be obtained.

4. 3. DIFFERENT PROTEIN CONCENTRATIONS IN PERFUSATE

4. 3. 1. Experimental procedures

Twenty male rats weighing 449±16.55 grams were used in this experiment. Both testes from each animal were perfused simultaneously through the capsular artery on the surface of the testis (see 2.6.2.) with Krebs-Ringer solution containing different concentration of bovine serum albumin (BSA) gassed with 95% O_2-5% CO_2 (see 2.6.3.). Perfusion flow rate was maintained around 500µl/min for the whole perfusion period. During the first 30 min, both testes were perfused with 1% BSA Krebs-Ringer solution (W/V), and then a lower or higher BSA concentration (0.25%, 0.5%, 2% or

4%) in Krebs-Ringer solution was used in one testis for another 30 min while the contralateral testis continued to receive 1% BSA Krebs-Ringer solution as the control. The effluent flowing from the cut end of the spermatic cord was collected by using beakers for the first 30 min, and a fraction collector (see 2.6.3.) for the remainder of the time. The testes and effluent were weighed to calculate the flow per unit weight of testis. The effluent was also stored at - 20 °C to be assayed for testosterone later.

4. 3. 2. Results

For the first 30 minutes, both testes were perfused with 1% BSA Krebs-Ringer solution, and testosterone concentration in the effluent was maintained at the same level in both testes. When protein concentration changed, testosterone concentration appeared to show initially a positive correlation with protein concentration in perfusate (Table 4-4). As all testes were perfused at constant flow rate (around 500µl/testis/min), testosterone secretion showed the same pattern (Table 4-5). When protein concentration in perfusate was increased to higher than control, significant increases only appeared during the first 5 minute (30-35') and /or second 5 minute (35-40') (Table 4-5; Fig. 4-6). In the third 5 minute (40-45') after change of protein concentration, significant difference of testosterone secretion between altered protein concentration and control protein concentration began to disappear, and the testosterone secretion ratio approached control (first 30 minutes), as perfusion time extended from 40 to 60 minutes even at the highest protein concentration in this experiment (Fig. 4-6, 4-7, 4-8b).

4. 3. 3 Discussion

This finding shows clearly that as protein concentration in the perfusion medium increases, testosterone uptake at constant flow rate only increases temporarily during the first 10 minutes after perfusate change. Moreover, the extent of increase in testosterone secretion is slight in contrast to the increase of protein concentration in the perfusate. This result only partly accords with previous observation by Ewing and colleagues (1976) in isolated perfused rabbit testes. They found when perfusion medium changed from 3% Dextran in Krebs-Ringer solution to 3% BSA in this solution, testosterone

Table 4-4

Effluent Testosterone Concentration

Albumin Conc.	Control(ng/ml)	Treated(ng/ml)	No. of Testes
0.25%			
0-30'	33.47±8.33	39.70±11.00	3
30-35'	35.02±8.67	23.62± 5.56	4
35-40'	23.20±5.16	20.55± 5.24	4
40-45'	21.30±5.07	19.28± 4.95	4
45-50'	21.42±3.82	18.11± 4.19*	4
50-55'	17.55±3.08	17.17± 4.65	4
55-60'	15.53±3.82	17.42± 4.89	4
0.50%			
0-30'	17.46±4.61	20.02±4.83	5
30-35'	13.38±3.22	11.46±2.73	5
35-40'	11.62±2.97	12.48±3.26	5
40-45'	10.66±2.63	11.62±3.09	5
45-50'	10.38±2.26	11.18±3.19	5
50-55'	10.30±2.43	9.54±2.35	5
55-60'	7.96±2.16	8.36±1.70	5
2.00%			
0-30'	16.10±6.06	17.66±6.49	5
30-35'	12.72±4.35	18.12±6.87	5
35-40'	11.20±4.05	18.56±6.48*	5
40-45'	10.60±3.49	14.00±5.12	5
45-50'	9.90±3.27	10.32±3.72	5
50-55'	8.64±2.94	8.58±2.92	5
55-60'	7.78±2.32	8.34±3.73	5
4.00%			
0-30'	29.35±9.10	28.51±9.29	6
30-35'	18.98±4.75	31.53±8.43*	6
35-40'	18.23±5.64	26.93±7.27	6
40-45'	17.88±4.95	18.92±5.85	6
45-50'	10.66±1.91	11.16±2.98	5
50-65'	9.08±1.36	8.68±2.02	5
55-60'	11.70±2.63	10.68±3.09	6

* $P<0.05$, compared with control side at same time.

All data are presented as mean ± SEM.

Table 4-5

Testosterone Secretion in perfused testes

Albumin Conc.	Control (ng/g/min)	Treated (ng/g/min)	No. of Testes
0.25%			
0-30'	9.48±2.49	10.93±3.04	3
30-35'	9.74±2.57	6.32±1.42	4
35-40'	6.39±1.41	5.50±1.36	4
40-45'	5.85±1.36	5.17±1.32	4
45-50'	5.91±1.31	4.85±1.08	4
50-55'	4.85±0.89	4.58±1.17	4
55-60'	4.30±1.10	4.68±1.32	4
0.50%			
0-30'	4.58±1.15	4.99±1.05	5
30-35'	3.50±0.72	2.85±0.56*	5
35-40'	3.03±0.63	3.11±0.69	5
40-45'	2.77±0.52	2.88±0.64	5
45-50'	2.69±0.53	2.76±0.65	5
50-55'	2.70±0.55	2.37±0.47	5
55-60'	2.05±0.96	2.09±0.71	5
2.00%			
0-30'	4.97±1.91	5.39±2.01	5
30-35'	3.93±1.37	5.51±2.12	5
35-40'	3.45±1.26	5.63±1.98*	5
40-45'	3.25±1.07	4.27±1.59	5
45-50'	3.03±0.99	3.13±1.01	5
50-55'	2.65±0.91	2.61±0.90	5
55-60'	2.39±0.71	2.54±0.97	5
4.00%			
0-30'	8.10±2.38	7.30±2.28	6
30-35'	5.25±1.23	8.12±2.06*	6
35-40'	5.00±1.41	6.91±1.73*	6
40-45'	4.96±1.30	4.84±1.45	6
45-50'	3.08±0.58	2.86±1.49	5
50-55'	2.62±0.45	2.24±0.44	5
55-60'	3.21±0.65	2.75±0.77	6

* $P<0.05$ compared with control side at same time.

All data are presented as mean±SEM.

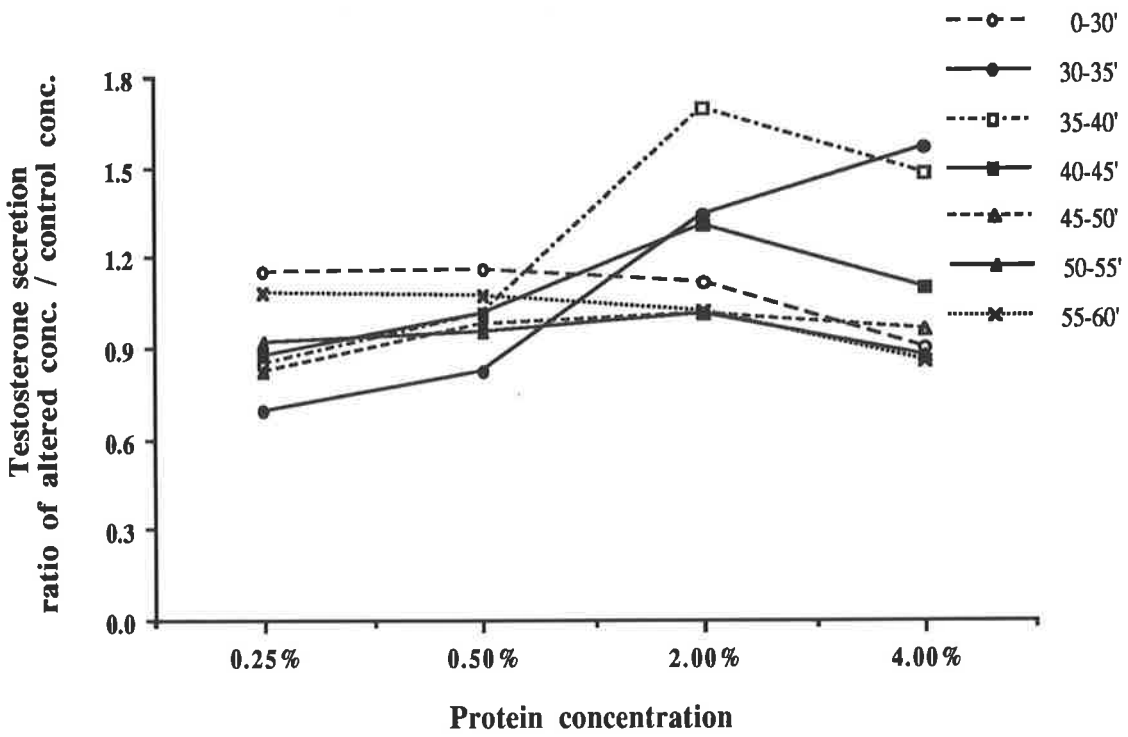


Fig. 4-6 Testosterone secretion in relation to protein concentration in perfusion medium in isolated perfused rat testes at different times after change of protein concentration in perfusate. The results are expressed as the ratio of the testis with altered protein concentration to the contralateral testis of the same animal with 1% BSA in perfusate. 0-30': both testes were perfused with 1% BSA in Krebs-Ringer's solution, the ratio was maintained around 0.9 to 1.16; 30-60': protein concentration was changed to 0.25%, 0.5%, 2% or 4%. Testosterone secretion showed significant difference ($P<0.05$) when protein concentration was 0.5% and 4% at first 5 minutes (30-35'), and 2% and 4% at second 5 minutes (35-40'). No difference was found after this time.

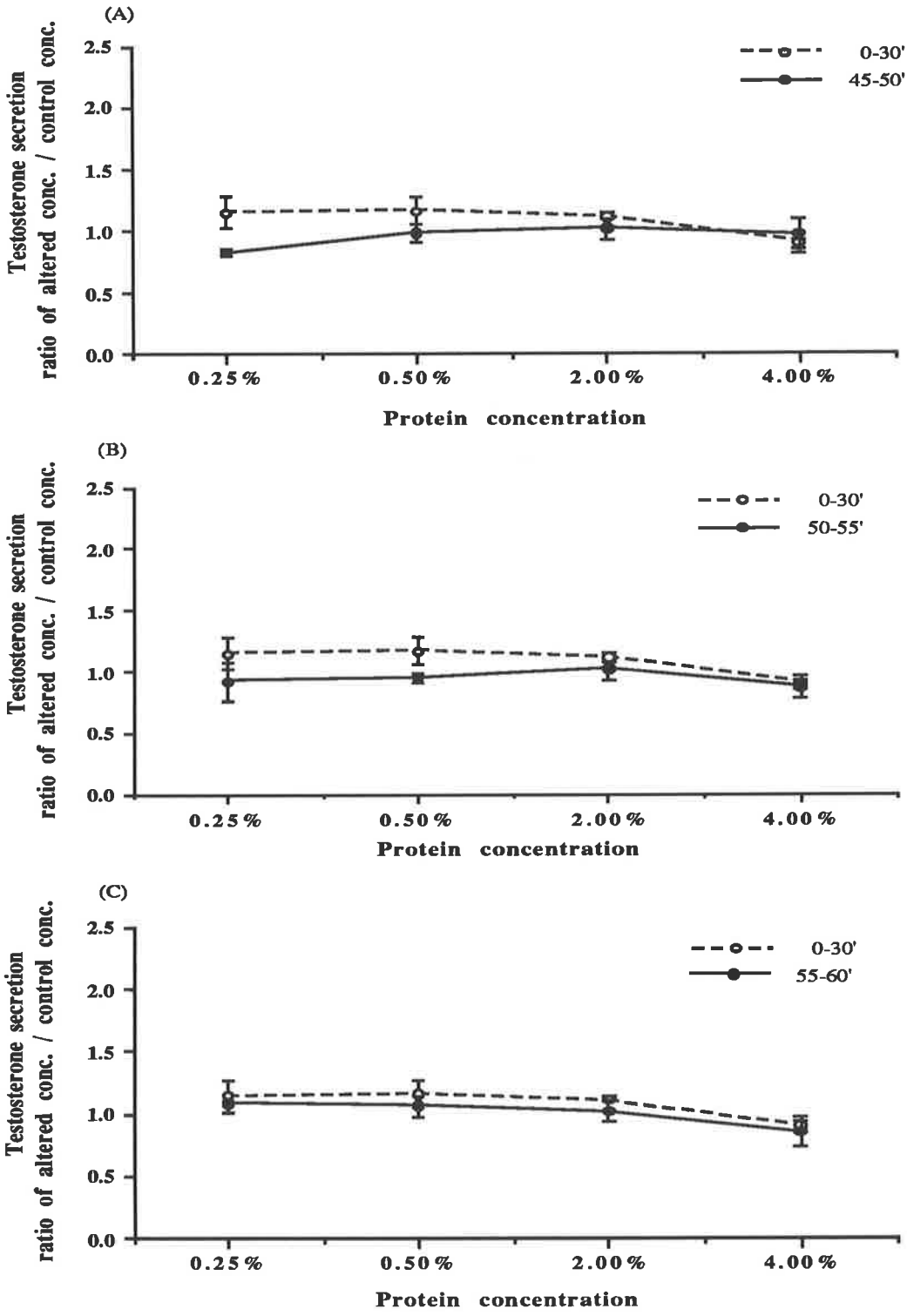


Fig. 4-7 Testosterone secretion in relation to protein concentration in perfusion medium in isolated perfused rat testes later than 15 minutes after the change of protein concentration in perfusate. The results are expressed as the ratio of the testis with altered protein concentration to the contralateral testis of the same animal with 1% BSA in perfusate. The curve of testosterone secretion ratio approached to control as time extended towards 60 minutes.

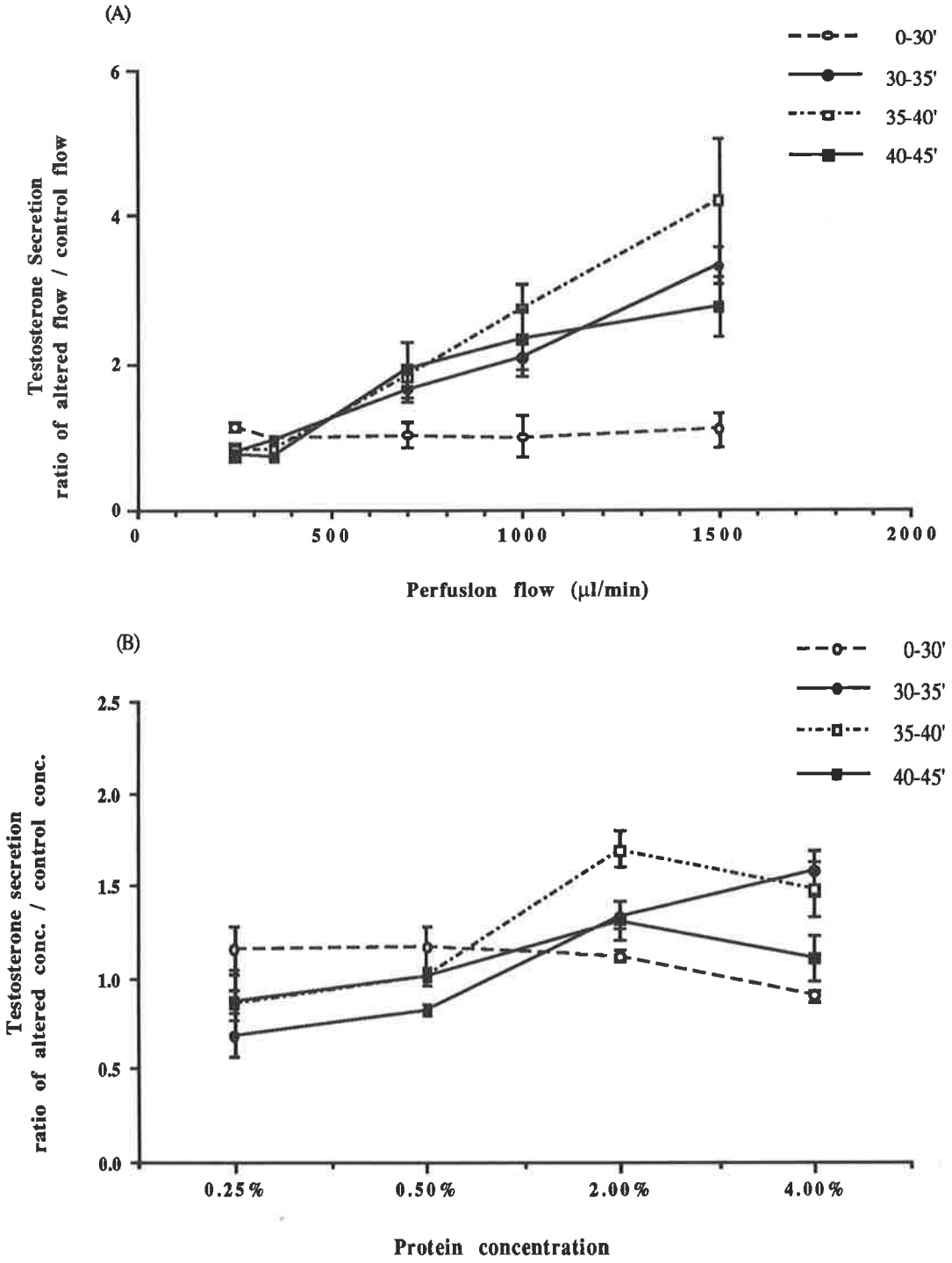


Fig. 4-8 Testosterone secretion in relation to (a) perfusion flow; (b) protein concentration in isolated perfused rat testes less than 15 minutes after flow or protein concentration change. Testosterone secretion showed significant increases as flow increased and this was maintained for at least 15 minutes after the flow change. In contrast to this, testosterone secretion only showed slight and temporary increase when protein concentration was elevated.

secretion increased dramatically and maintained at high level until the last hour (3 hours after change of perfusate) of observation. The difference between these two experiments might be contributed to by the perfusate used in control and different species. In our study, control perfusate already contained 1% albumin, and with any further increases of protein concentration in the perfusate, testosterone secretion only changed slightly. This indicates that testosterone secretion only depends on whether protein is present rather than on its concentration, which could be explained via a hypothesis that testosterone diffusion is enhanced by the presence of steroid-binding macromolecules (such as albumin) in the blood. Albumin not only serves as a carrier of steroid hormones (Pardridge and Landaw, 1988), but also modifies the endothelial membranes (Michel, 1988). There is no doubt that the former facilitates the passage of the steroids through the endothelium of the capillary wall, however, the effect of the later on increasing passage of testosterone needs further investigation.

4. 4. CONCLUSION

These results show clearly that the secretion of testosterone by the isolated perfused testes is largely determined by flow rate. Although stimulants are very important in testosterone secretion either in vivo or in vitro, the flow itself is effective in this perfusion experiment without any gonadotrophins in the perfusate. Therefore, the increase of testosterone output elicited by elevated blood flow may be related to at least two mechanisms: (1) a short-loop testosterone negtive feedback is eliminated by increased flow: (2) the high perfusion rate provides better supply of nutrients and O_2 to Leydig cell, which is the major source of androgens. Among the two mechanisms, the former is considered to be much more inportant one.

As testosterone secretion from isolated testis could maintain at a much high level and a longer duration (Fig. 4-8a) compared to the results from study with perfusate having elevated protein concentration (Fig. 4-8b), the temporary increase in testosterone

secretion by increased protein concentration in perfusate may not due to depletion of storage It is suggested that the increase of protein content is not a significant factor in regulation of testosterone secretion.

Unlike testosterone transport, albumin uptake by testicular tissue depends largely on vascular permeability and/or net fluid flux rather than flow rate. This result is demonstrated in vivo by a recent finding that a physiological dose of LH increases testicular vascular permeability rather than blood flow (Bergh et al., 1990), which in turn, facilitates LH itself to pass through capillary wall and elevates bio-utilization of stimulant.

CHAPTER 5: CHANGE IN VASCULAR PERMEABILITY TO ALBUMIN IN RAT TESTES.

5. 1 INTRODUCTION

Previous studies have showed that the calculated apparent permeability of the testicular vasculature to albumin as judged by the accumulation of radioactive albumin in the testis increases in rats about the time of puberty (Setchell et al., 1988a) and after treatment with cadmium salts (Setchell and Waites, 1970) or hCG (Setchell and Sharpe, 1981) in vivo. The mechanisms for these apparent increases in capillary permeability appear to involve endothelial barrier changes before puberty, capillary wall damage by cadmium and an inflammation-like response to a substance produced by the interstitial cells following stimulation with hCG. However, studies on other organs suggested that the net fluid flux across the capillary wall is another important factor in the transport of macromolecules such as albumin into tissues (Haraldsson et al., 1987). In the experiment mentioned above (testicular perfusion at different flow rates), the result showed that the measured true testicular permeability to albumin at normal perfusion flow rates in isolated testes is much higher than the values calculated from the accumulation of radioactive albumin in the tissue; this fact also supports the suggestion that the latter may be affected not only by permeability but also by net fluid flux, which in turn is affected by hydraulic pressure and colloid osmotic pressure gradients between the capillary lumen and the interstitium. In order to investigate whether other factors are involved in apparent permeability responses in rats during puberty and after administration of hCG or cadmium, isolated testis perfusion was used in animals at different ages and after treatment with hCG or cadmium.

5.2. HUMAN CHORIONIC GONADOTROPHIN

5. 2. 1. Experimental procedures

Adult male rats weighing 513±13 g (n=29) were used in this experiment. Animals were injected subcutaneously with human chorionic gonadotrophin 50 I.U. in 0.2ml saline or saline only for the control. The rats were anaesthetized 8, 12, 16, 20 and 24 hours after hCG injection. Both testes from each animal were removed and perfused through the testicular artery on the surface of the testis (see 2.6.2.) with Krebs-Ringer solution containing 1% bovine serum albumin gassed with 95% O_2 - 5% CO_2 (see 2.6.3.). Perfusion flow was maintained around 500µl/min for 30 min at 32.5±0.5°C and the effluent from the cut end of the spermatic cord was collected for checking the flow rate. After this 30 min perfusion, 50µl ^{125}I-albumin (around 0.01 µCi) was injected through a three-way junction close to the testis (2.6.3.) and the effluent was collected over 1 min for determination of radioactivity. At the end of the perfusion, the testis, the separated capsule, the parenchyma, the spermatic cord and 1 min effluent were weighed and counted in a gamma spectrometer. Extraction was calculated by dividing the amount of radioactivity in testicular parenchyma by the total counts recovered in tissues (capsule, parenchyma and cord) and effluent (see 2.6.5.).

5. 2. 2. Results

In the control group, although the testicular capsule weighed only 5.86% of the whole testis, it contained 40% of the counts in the whole testis, while corresponding values for the parenchyma were 94.14% and 60% respectively. At different times after hCG, the percentage of capsule and parenchyma weights and counts of whole testis remained in the same range as in controls. However, the percentages of extraction (%E) in parenchyma (Fig.5-1) and the whole testis were higher than that in control at 12 and 16 hours after hCG. The values in the whole testis were 7.59% at 12 hours and 5.58% at 16 hours after hCG (4.38% in control). Therefore, the permeability surface area product (PS) to ^{125}I-albumin, calculated from the formula PS=-Q.Ln(1-E), and the ^{125}I-

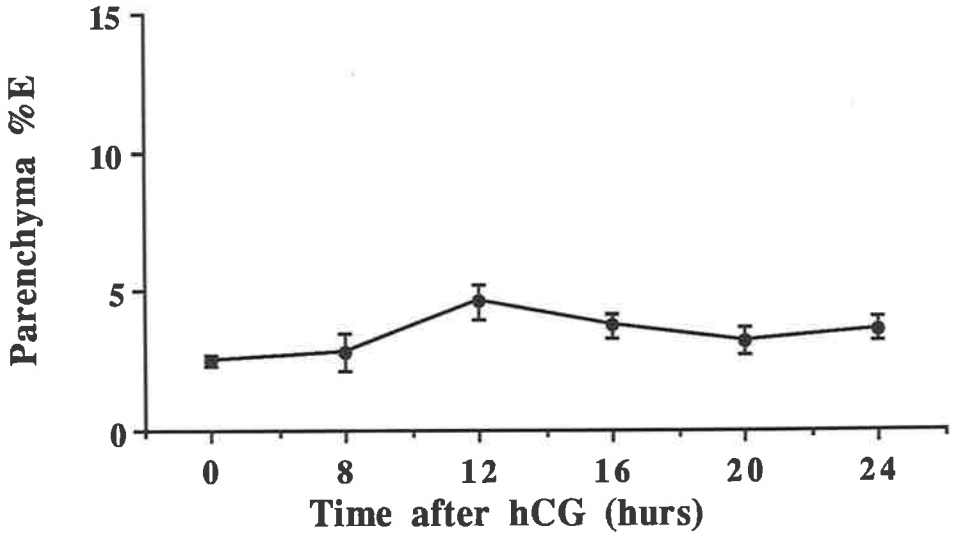


Fig. 5-1 The extraction (%E) of ^{125}I-HSA by the parenchyma of testes of rats injection with hCG (50 IU subcutaneously) at time 0 (Mean ±SEM). $P<0.01$ at 12 hours and $P<0.05$ at 16 hours after hCG compared with control value.

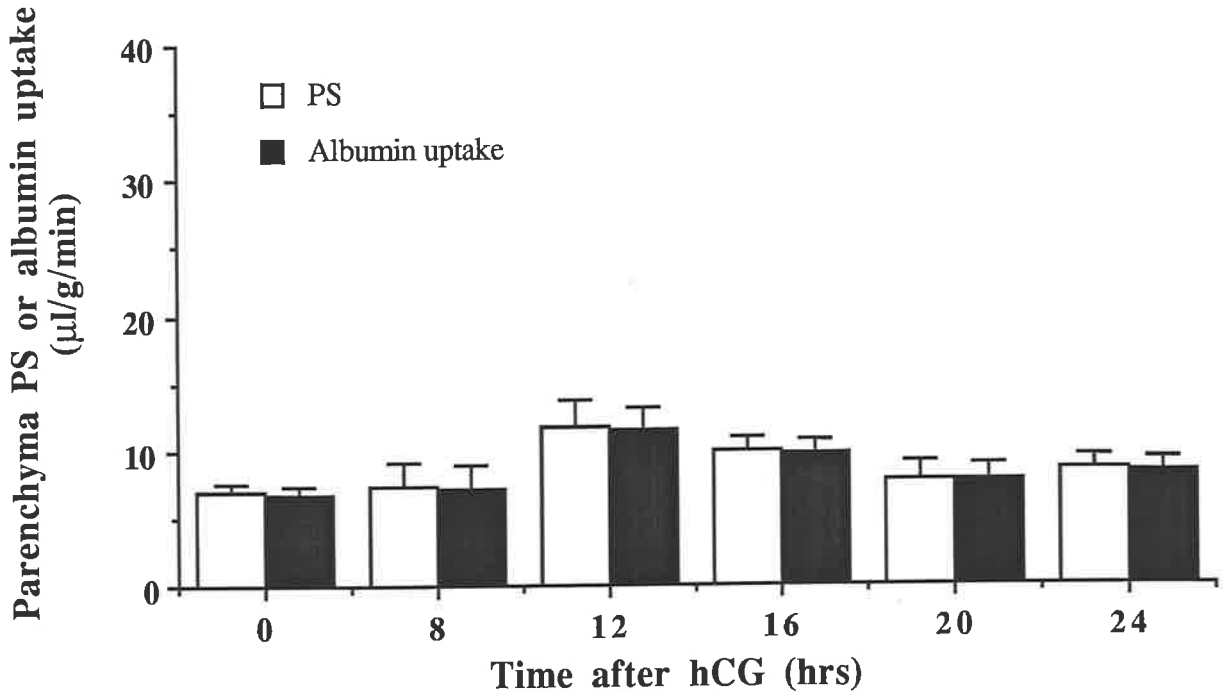


Fig. 5-2 The true PS, calculated from the formula PS=-Q.Ln(1-E), and uptake of radioactive albumin, calculated as the product of flow and extraction, by the parenchyma of testes of rats injected with hCG at time 0. Data were presented as Mean ±SEM.

albumin uptake, calculated as flow (μl/g/min) multiplied by extraction, showed a similar pattern to %E in parenchyma. The PS increase occurred only temporarily at 12 and 16 hours after hCG (Fig.5-2). This result was quite different from the result found in vivo (Setchell and Sharpe, 1981; Sowerbutts et al.,1986).

5. 2. 3 Discussion

It is surprising that the effect of hCG on testicular vascular permeability in isolated perfused testes did not involved a sustained increase in PS, since there is a dramatic and sustained increase in apparent permeability, as judged from the accumulation of radioactive albumin in the testis of animals treated with hCG (Setchell and Sharpe, 1981; Sowerbutts et al., 1986). In the present study, uptake of albumin in isolated perfused testes has been used to deduce true permeability (PS=-Q.Ln (1-E)) (see 1.3.4.). According to this formula, only a slight and temporary increase of true permeability to albumin was found in testes of rats treated with hCG. It seems likely that increase of apparent permeability to albumin in testes after treatment of the animals with hCG may be related to an increase of albumin transport through net fluid flux, because apparent permeability calculated by the formula PS= $k.V_{final}$ (see 1.3.4.) is influenced not only by vascular wall permeability but also by net fluid flux (Setchell et al.,1991a). Studies in other tissues indicate that albumin transport is largely dominated by convection through the pores presented in vascular wall, which depends on the net fluid flux (J_v) across the capillary wall (Haraldsson et al., 1987; Reed et al., 1985; Noer and Lassen, 1979).

The factors affecting net fluid flux have already been discussed in 1.3.3.; in summary, the gradients of hydraulic pressure and colloid osmotic pressure are major factors influenced net fluid flux in most tissues. However, the similar protein concentrations presented across the testicular vascular wall (Maddocks and Setchell,1988) mean that there is only a small gradient of colloid osmotic pressure in testis. There is no evidence that a change of hydraulic pressure in normal testes after hCG although the pressure in the interstitium was increased after hCG treatment in

abdominal testes (Hjertkvist et al.,1988). Observation of testicular blood flow with laser Doppler flowmetry found vasomotion was absent in the testes of rats treated with hCG (Widmark et al.,1987). Because vasomotion has the potential to reduce net filtration rate over a period of time (Levick,1991), abolished vasomotion induced by hCG might be considered as one of the mechanisms in increase of apparent permeability to albumin in testes of animals after hCG.

Moreover, net fluid flux involves the reflection coefficient (σ), which is influenced by some vasoactive substances such as histamine, 5-hydroxytryptamine, bradykinin and prostaglandins in most tissues. Although these vasoactive substances appear not to affect the testicular vasculature, the substance produced by the interstitial cells following stimulation with hCG (Veijola and Rajaniemi, 1992) probably plays a similar role in the testis to that played by these other vasoactive substances in other tissues. Change of reflection coefficient may be another mechanism in increase of apparent permeability to albumin in testis of animals treated with hCG. Whether other mechanisms or factors are involved in the testicular vascular permeability response to hCG needs further investigation.

However, the major difference between the results obtained with a method which involves transient exposure to the marker and one involving long-term exposure, is that the accumulation of marker in the tissue ubder the latter conditions would be affected by fluid movements through the extravascular compartment, which in turn would be affected by net fluid flux, whereas the former would be controlled only by the characteristics of the vessel wall.

5. 3. CADMIUM

5. 3. 1. Experimental procedures

Adult male rats weighing 450±13 g (n=31) were used in this experiment. Rats were injected subcutaneously with 1.4% cadmium sulphate (7mg/kg body weight) in saline or saline only for control and then anaesthetized 0.5, 1, 2, 3, 4 or 6 hours after injection. The subsequent procedures were the same as described in 5.2.1..

5. 3. 2. Results

A marked increase in %E of the whole testis and parenchyma occurred in cadmium treated rats (Fig.5-3). %E in the whole testis increased from 5.19% at 30 min after cadmium to 19.47% at 4 hours after cadmium (4.38% in control). Because of the constant perfusion flow rate, PS to ^{125}I-albumin and its uptake in parenchyma showed the same pattern as %E (Fig.5-4). The PS increase appeared as early as 30 min after cadmium injection ($P<0.05$) and became progressively greater over the period of observation ($P<0.01$ at 2 hours after cadmium and late times). The percentage weights and count distribution of parenchyma and capsule in whole testis were not different between treated and control rats (data not shown).

The perfusion pressure needed to maintain a normal flow rate was less than 70 mmHg (29-68 mmHg) in control rats. At one hour after cadmium injection, this pressure was still maintained at the control level. However, the perfusion pressure increased from 2 hours after cadmium (120-140 mmHg) and was around 324-454 mmHg at 4 hours after cadmium (Fig. 5-5). Little effluent from the cut end of the spermatic cord was collected in the testes at 6 hours after cadmium injection despite a perfusion pressure of over 730 mmHg., and no results were obtained at this time.

5. 3. 3. Discussion

The progressive increase in PS to albumin started from 30 min ($P<0.05$) after cadmium while the perfusion pressure remained around the normal level; PS became

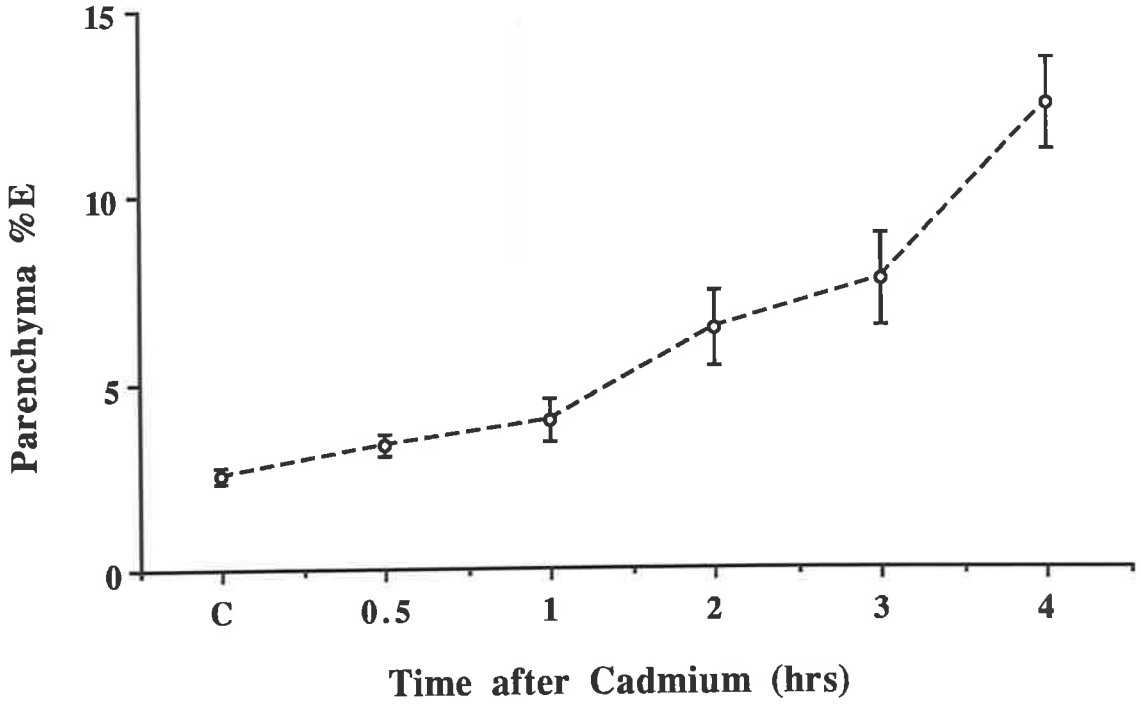


Fig. 5-3 The extraction (%E) of ^{125}I-HSA by the parenchyma of testes of rats injection with cadmium sulphate (7 mg/kg body weight subcutaneously) at the time shown. $P<0.05$ at 0.5 and 1 hour and $P<0.01$ at other times after cadmium compared with pooled controls. (c) Data were presented as Mean±SEM.

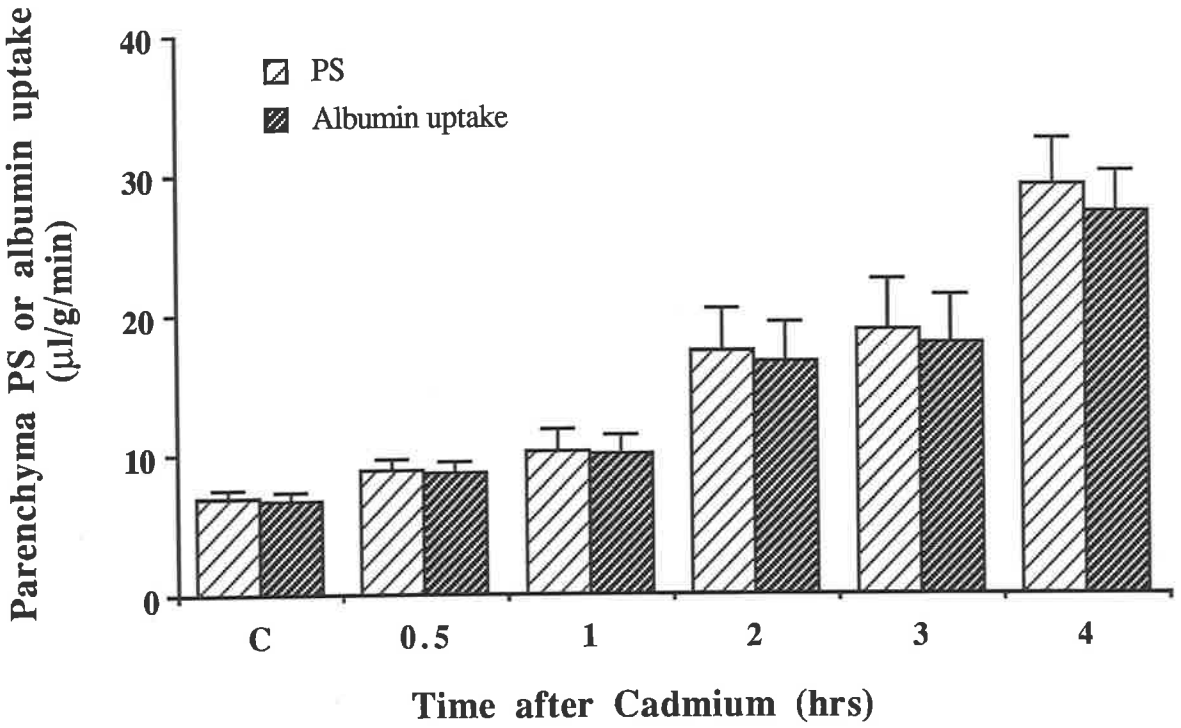


Fig. 5-4 The true PS and uptake of radioactive albumin (Mean ±SEM) determined as in Fig. 5-2 by the parenchyma of testes of rats after cadmium at the time shown.

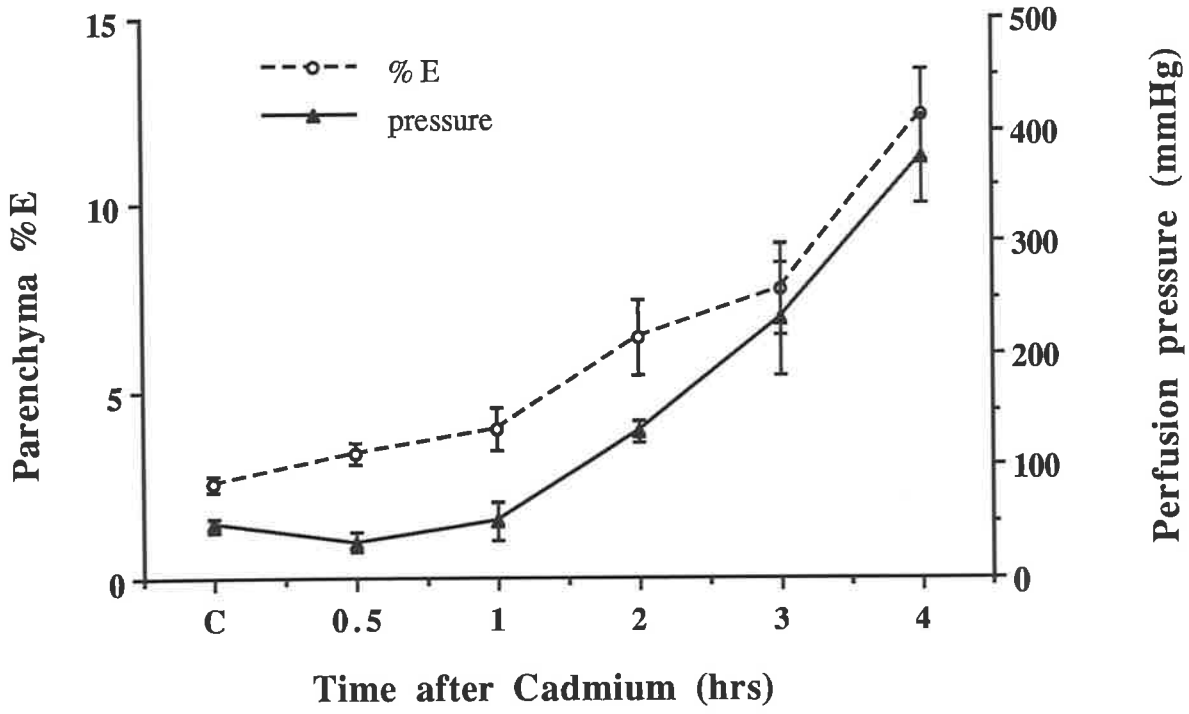


Fig. 5-5 Combined extraction and perfusion pressure graph showing increases in %E accompanied by elevated perfusion pressure.
Data were presented as Mean ±SEM.

even greater ($P<0.01$) at the time (2 hours after cadmium) when perfusion pressure increased dramatically. This result is quite similar to the previous finding in vivo (Waites and Setchell, 1966). Although the studies on albumin transport in other tissues (Haraldsson et al.,1987) and in isolated testes after animals treated with hCG (see 5.2.) indicate that the increase of albumin transport in tissues is mainly influenced by the net fluid flux, the true PS shows a increase comparable to that in apparent PS after cadmium and this suggests that the increase in radioactive albumin accumulation in the testes after treatment of the animals with cadmium was due to the damage of capillary endothelium rather than the change of net fluid flux in vivo.

The perfusion pressure indirectly reflected the vascular resistance in the testes after cadmium. An increase in perfusion pressure represented a significant increase of vascular resistance to perfusion flow, and would be expressed as a decrease of blood flow in testis in vivo because testicular perfusion pressure would be unchanged. In fact, the effluent volume decreased despite the perfusion pressure increase, it fell down to 86% of control value at 4 hours after cadmium even through the pump was set at constant velocity (500µl/min), presumably reflecting a failure of the pump to maintain the designated output at the high pressures. At earlier times after Cd, flow was not affected. This increase of vascular resistance to perfusion can also be explained by the finding of a disseminated intravascular coagulation n the testicular microvascular system which occurred after cadmium injection (Agustin and Hoffer, 1978). This could have happened in the present studies before the testes were removed for perfusion, butno histological studies were undertaken.

5. 4. AGE

5. 4. 1. Experimental procedures

Male rats aged 25, 27, 30, 35, 40, 50, 60 and 90 days were used in this investigation. Because of variation of the testicular weight in different age animals,

perfusate flow was set according to testicular weight, which was calculated by the following formula:

Testis weight (mg) $=1/6\pi ab^2$

Where "a" is the testicular length and "b" is the testicular width in mm. In order to get the estimated value of testicular weight, a vernier caliper was employed to measure testicular length and width after testes were exposed through scrotum incision. Isolated testes were perfused at 300µl/g/min for 30 min. The following procedures are the same as described in 5.2.1..

5. 4. 2. Result

As rat age increased from 25 days to 90 days, percentage weight of parenchyma in whole testis increased from 89.14±0.90% to 94.14±0.28%, the percentage of radioactivity in parenchyma was maintained around the same level except that at 60 days, it was increased to 68.94% ($P<0.05$ compared with 59.99% at 90 days). At rat ages from 25 to 40 days, % E and PS in parenchyma decreased from 2.87±0.93% and 8.82±2.74 (µl/g/min) at 25 days to 1.31±0.28% and 4.29±0.84 (µl/g/min) at 40 days ($P<0.05$). However, those values increased in 50 and 60 day rats, %E and PS reached 4.29±0.51% and 12.22±1.46 (µl/g) in 60 day rats ($P<0.01$). In 90 day rats, the values of %E and PS had fallen again to 2.53±0.22 and 6.87, these levels being quite close to those from 30 day rats (2.54±0.57 and 7.35±1.65) (Fig. 5-6; 5-7).

5. 4. 3 Discussion

The results from isolated perfused testes in this experiment are completely different from the results in vivo studies (Setchell et al., 1988a; Pöllänen and Setchell,1989), which showed that the apparent PS was significantly increased at rat age about 30 days and returned to the normal level after 40 day age. In present study, as rat age increased from 25 to 40 days, the true PS gradually decreased and it reached the lowest value (4.29µl/g) at 40 days in this study ($P<0.05$). This progressive decrease of true PS may involve maturation of the vasculature of the testis during this time.

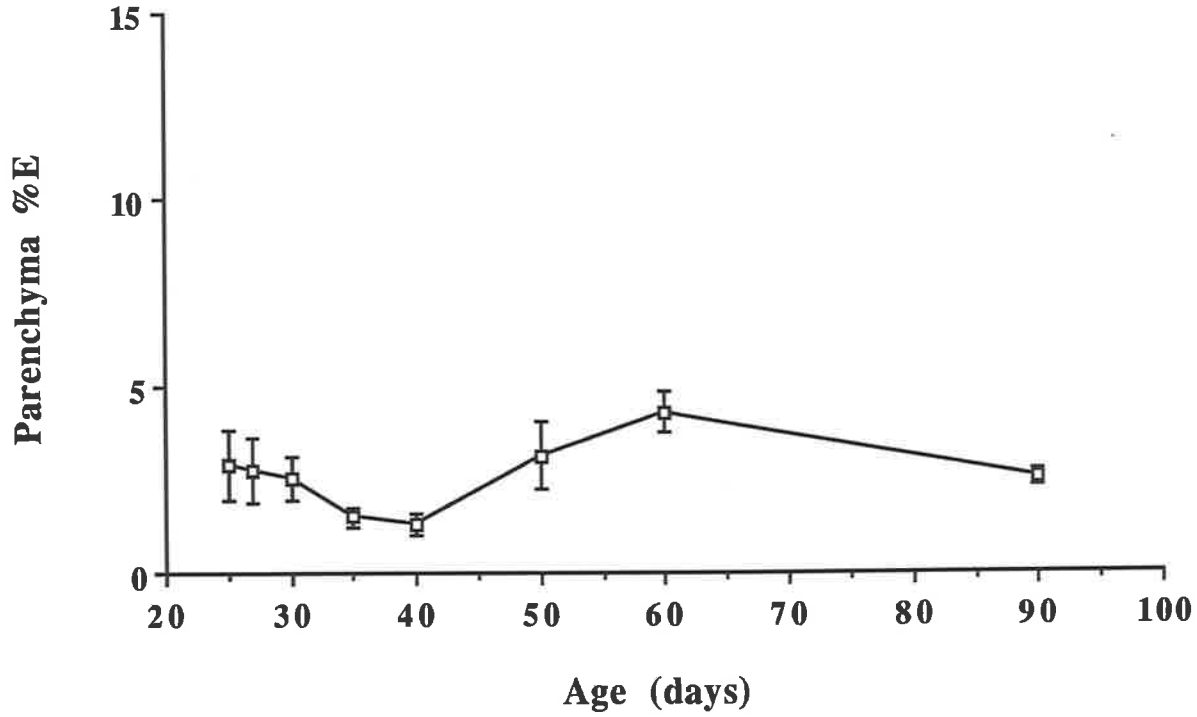


Fig. 5-6 The extraction (%E) of ^{125}I-HSA by the parenchyma of testes at different age rat shown. $P<0.05$ at 40-day-age testes and $P<0.01$ at 60-day-age testes. Data were presented as Mean ±SEM.

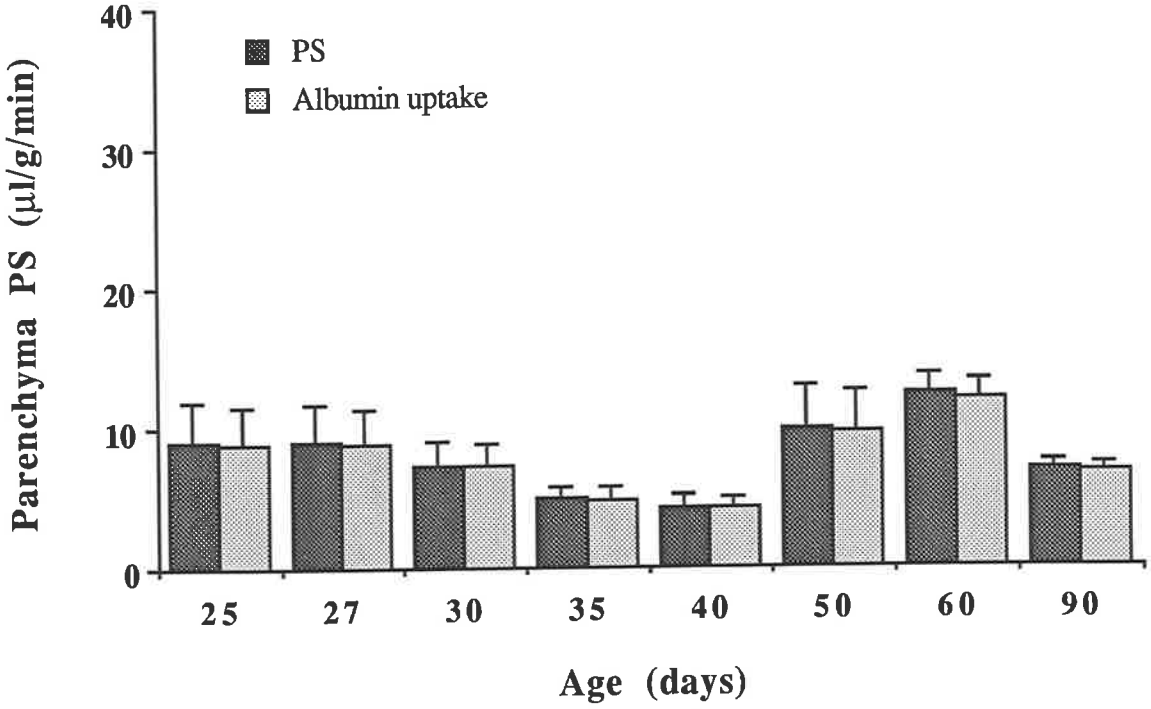


Fig. 5-7 The true PS and albumin uptake, calculated as in Fig. 5-2, by the parenchyma of testes at different age rats shown. Data were presented as Mean ±SEM.

Therefore, the accumulation of radioactive albumin and IgG in testicular interstitium at around 27-33 days in vivo studies (Setchell et al., 1988a; Pöllänen and Setchell,1989) is probably due to changes in net fluid flux, which may be induced by an increase of surface area of the vascular bed (Kormano, 1967c) and testicular susceptibility to LH (Ojeda and Urbanski, 1988) at around this age. Alternatively, it could be due to a change in hydraulic conductivity of the vessel walls.

The true PS showed a temporary increase in 60-day testes ($P<0.01$; Fig. 5-7). However, study with IgG in vivo showed that apparent PS in 60-day testis has achieved adult level (Pöllänen and Setchell,1989). Vascular anatomy and biochemistry also show adult profiles (Gunn and Gould, 1975) at this age. No reason can be found why the true PS in the testes increased at this age.

5. 5. PERMEABILITY MEASUREMENTS FROM ALBUMIN SPACES

The true PS was much higher than the apparent PS from in vivo studies (Setchell et al., 1988a) even in the testes from normal adult rats, the following experiment was designed to compare the permeability in isolated perfused testes with that in vivo.

5. 5. 1 Experimental procedures

Isolated testes from 10 adult rats were perfused with Krebs-Ringer solution containing 1% bovine serum albumin and ^{125}I labelled human serum albumin (6.3×10^{-3} μCi/ml) for 2, 30, 60, 90 and 120 min. At the end of the perfusion, the parenchyma was separated and counted in a gamma spectrometer. The albumin space (V) and PS in parenchyma were calculated according to the following formulas:

V (μl/g) = cpm in parenchyma(cpm/g) / cpm in effluent (cpm/μl)

$PS = K.V_{final}$

Where "K" was the slope of the line $Ln\ (1-V_t/V_{final})$ against time; V_t was the albumin space at time "*t*" minus the vascular volume,which was taken to be the albumin space at the beginning (2min); V_{final} was the albumin space at 2 hours minus by the

Table 5-1

(A) Albumin space (µl/g) in testicular parenchyma of testes perfused with Krebs-Ringer bicarbonate containing ^{125}I labelled human serum albumin and 1% bovine serum albumin.

Time (min)	Mean	SD	SEM	No. of testis
2	14.75	1.82	1.05	3
30	70.86	17.64	8.82	4
60	82.33	21.15	10.58	4
90	141.66	24.75	12.38	4
120	154.11	29.94	14.97	4

(B) Albumin space (µl/g) in testicular parenchyma of testes perfused with sheep serum containing ^{125}I labelled human serum albumin.

Time (min)	Rat 1	Rat 2
30	97.29	99.25
60	193.67	212.57

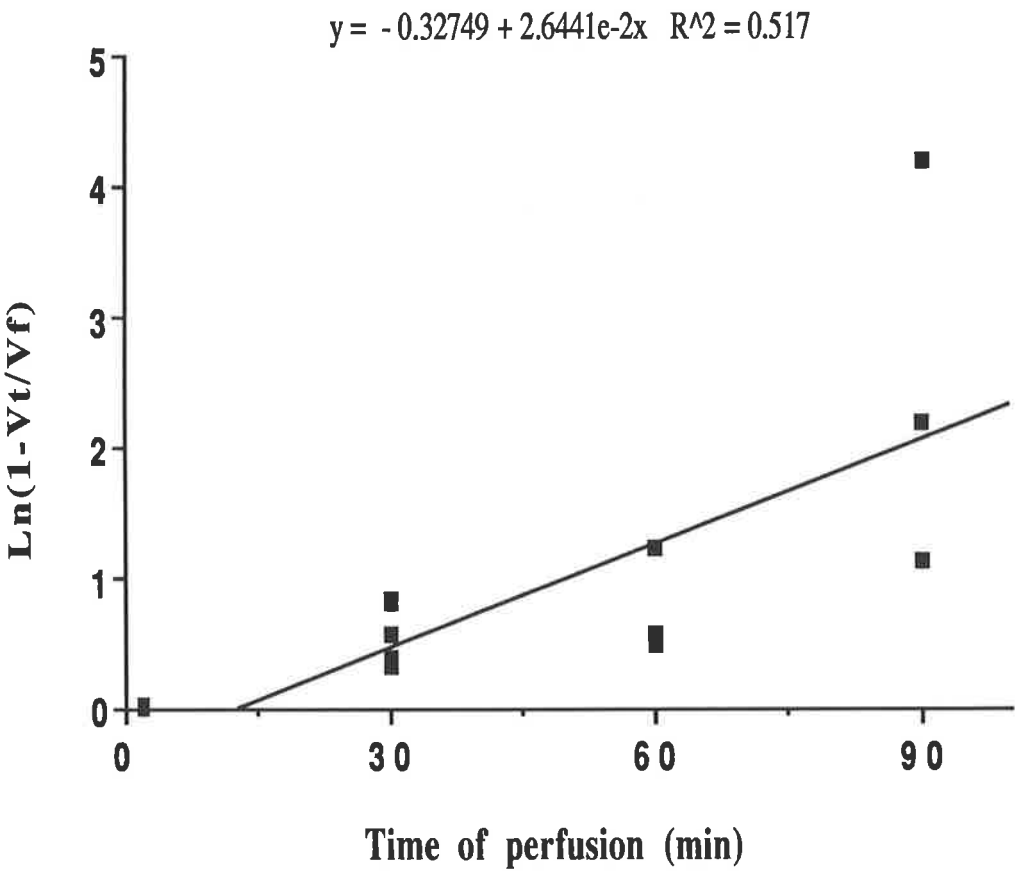


Fig. 5-8 The slope (k) of the line Ln (1-V_t/V_{final}) against time in isolated testes (n=3-4) perfused with radioactive Krebs-Ringer solution (see Table 5-1). In this study

k =0.02644;

PS = k.V_{final}

= 0.02644 x (154.11-14.75)

= 3.685 (µl/g)

vascular volume (albumin space at the beginning). The reason for using 2 minutes as the begining time is that the perfusate can pass through whole testis at a constant flow rate (500μl/min) in 2 minutes. Similar experiments were conducted with fresh sheep serum containing ^{125}I human serum albumin (6.3×10^{-3}μCi/ml) instead of the Krebs-Ringer solution in 2 adult rats. Albumin space and PS were calculated as above.

5. 5. 2 Results

The result from adult rat testes perfused with radioactive perfusate showed that the albumin space was higher in perfused testis (Table 5-1a) than in vivo (Setchell et al., 1988a). Also the slope (k) of the line $Ln(1-V_t/V_{final})$ against time in vitro (K=0.02644) (Fig. 5-8) was about 8 times higher than in vivo (K=0.00323, Setchell et at. 1988a). The PS value of adult rat testes in vitro was 3.685 μl/g/min, the corresponding value in vivo was 0.354 μl/g/min (Setchell et al. 1988a).

The result from the testes perfused with radioactive sheep serum showed in table 5-1b, the albumin space at 0.5 and 1 hour was even higher than that from testes perfused with radioactive Krebs-Ringer solution.

5. 5. 3. Discussion

In isolated adult rat testes perfused with radioactive Krebs-Ringer solution, PS calculated by the same formula as the in vivo study was about 8-fold higher than the level in vivo (Setchell et al., 1988a), but still less than the true PS. This difference between in vitro and in vivo study is not due to the the lower protein concentration in perfusate, because when radioactive sheep serum was used to instead of Krebs-Ringer solution to infuse testes, the accumulation of radioactive albumin in parenchyma was even higher (Table 5-1). It is unclear why the true PS or PS in isolated testis calculated by using the same formula as in vivo are higher than apparent PS in vivo in intact adult rat testis. Further investigation is necessary to answer these questions.

5. 6. CONCLUSION

Although apparent PS, calculated from the accumulation of radioactive albumin in the testis, increased in adult rats after treatment with hCG (Setchell and Sharpe, 1981) and in younger rats at around 30-days of age (Setchell et al.,1988a), the true PS, measured by the uptake of albumin in isolated perfused testes, showed only a slight temporary increase at around 12 hours after hCG, whereas the true PS in the testes of rats aged from 25 to 90 day showed a decrease, then an increase, and then a decrease again. The difference between apparent PS and true PS here supports the suggestion that the apparent PS is affected not only by permeability, but also by net fluid flux (Setchell et al., 1991a). The lack of an increase in true PS in rats after hCG or during puberty indirectly demonstrated that the accumulation of radioactive albumin in those rat testes is dominated by convective transport rather than true permeability.

In contrast to these results with hCG and at puberty, the true PS increased progressively starting from 30 min after cadmium, and became even greater at longer times after cadmium. This result is quite similar to the previous finding in vivo (Setchell and Waites, 1970) and confirms that the major mechanism of PS increase after cadmium is disruption of vascular endothelial barrier.

CHAPTER 6: GENERAL DISCUSSION

The blood-testis barriers include two barriers, which consist of three kinds of cell components: a tubule barrier composed of tight junctions between Sertoli cells and myoid cells in peritubular tissue; a capillary barrier composed of unfenestrated or continuous endothelial cells.

Because of open junctions at the boundaries of myoid cells (Dym and Fawcett,1970) and clefts of about 200Å width observed between adjacent endothelial cells of capillary (Fawcett et al.,1973; Farquhar et al.,1961), these two components form only incomplete barriers. Substances, even as large as albumin (Christensen et al.,1985), can pass through them. Tight junctions between Sertoli cells appear during puberty, and attain their adult level at around 22-33 days (Russell et al.,1989a; Setchell 1986). Once the tubule barrier is established, it can block the passage of most substances, even as small as EDTA molecules. Moreover, the function of the tubule barrier is quite stable, even during aspermatogenesis induced by elevated testicular temperature or irradiation, the integrity of the barrier still maintained. Previous studies of heated testes demonstrate a change in Sertoli cell metabolism as well as the damage to germ cells. Although the ectoplasmic specializations become a "concertina" of vacuoles along inter-Sertoli cell junctions due to the compacting of Sertoli cell cytoplasm (Kerr et al.,1979), little change was found in the function of barrier (Setchell and Waites, 1972a; Main and Waites,1977). Our study of heated testes also demonstrates the integrity of the tubule barrier, through the temperature increase was sufficient to damage spermatogenesis.

The finding of a breach of tubule barrier after EDL (Setchell, 1986) has be challenged by the observation of the distribution of horseradish peroxidase (Anton, 1982) and lanthanum (Osman and Plöen, 1978). In this thesis, the result judged by the ratio of interstitial ^{51}Cr-EDTA space to morphometrically determined volume confirms

the temporary breach of the tubule barrier after EDL. We believe that the high pressure in the tubule lumen and a loss of fluid from the tubule contribute to the breakdown. Because of the breach of the barrier is only temporary and the efferent ducts, if proximal to the ligature, are capable of reabsorbing large amounts of fluid, the opposite results from others might relate to observation time, ligation position and methods employed to detect damage of the barrier.

As the testis is also an endocrine organ, it becomes more important to stress effects of blood flow and vascular permeability on substance transport across the capillary barrier. Gonadotrophins released from pituitary have to pass through the capillary wall so that they could combine with the receptors on the target cells in testis, whereas testosterone produced by Leydig cells in the interstitium must enter the bloodstream through the capillary wall, then to be carried to its target organs.

Previous studies in vivo have demonstrated that secretion of testosterone positively correlates with the rate of blood flow through testicular vessels (Eik-Nes,1964; Free and Tillson,1973; Damber and Janson,1978), and the evidence from isolated perfused testes in this thesis confirms this suggestion. Moreover, the results also indicate that change of flow rate itself is the most important determinant in this perfusion study in vitro rather than the increase of stimulants and protein content carried by elevated flow. According to these results, the mechanisms of increase of testosterone secretion induced by flow rate could be because (1) small molecules are mainly transported by diffusion and the gradient of testosterone concentration across the wall is always maintained at a high level as flow increased so that the passage of testosterone across the capillary barrier increases per unit time; (2) testosterone transport into the bloodstream results in a decrease in its concentration in the interstitium, eliminating the short-loop testosterone negative feedback (i.e. inhibition of synthesis of testosterone in the Leydig cells by elevated local concentrations of testosterone) and facilitates testosterone production; (3) high perfusion flow rate provides better supply of nutrients

and O_2 to Leydig cells which is the major source of androgens. The increase in the amount of protein passing through the testicular vessels is not the major mechanism; the rate of testosterone secretion is affected by the presence of protein or albumin in the perfusate, but not the protein concentration. Although an increase of gonadotrophins carried by the elevated blood flow may contribute to the increase of testosterone secretion in vivo , the increase of testosterone secretion here is not due to this reason because the perfusate did not contain any gonadotrophin.

There have been few studies on the influence of blood flow and vascular permeability on the uptake of peptide hormones in testis. As a preliminary investigation into this question, the uptake of radioactive albumin by the isolated rat testes was measured at different flow rates in this thesis. The result demonstrates that albumin uptake by testis is dominated by vascular permeability rather than blood flow, especially at the flow rate over normal rate (500µl/testis/min).

Setchell and colleagues (1991a) according to the studies on albumin transport in other tissues speculated that the accumulation of radioactive albumin in testes in vivo may be influenced by net fluid flux as well as permeability, so they termed the earlier measurement of permeability "apparent permeability". Previous studies indicate that there is an increase in apparent permeability at testes after animals treated with hCG or cadmium and in rats around puberty. The results from uptake of radioactive albumin in isolated perfused testes in this thesis show that true permeability changed only slightly after hCG or around puberty. A comparison of these results suggests that the increase of apparent permeability mainly reflects the effect of net fluid flux on albumin transport in the testis after hCG or at puberty. On the other hand, true permeability in testes after cadmium changes in a similar way to apparent permeability, confirming that the increase of apparent PS is due to the damage to the capillary endothelium by cadmium.

CHAPTER 7: REFERENCES

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