A STUDY OF SPERMATOGENESIS IN THE DOG, WITH SPECIFIC REFERENCE TO THE ROLE OF THE LEYDIG CELL

Thesis submitted
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SUMMARY

The work included in this thesis describes studies in the spermatogenic process in the dog, and the approach used was to attempt supression of LH and testosterone in the testis and study the mechanisms affected by this interference to the normal testicular environment.

The first two dogs were treated with an unsuccessful progesterone implant and then with subcutaneous injections of Medroxyprogesterone acetate, the results did not show an effect in the circulating blood plasma levels of either LH or testosterone, or in the testicular tissue. However it appeared to have an effect at epididymal level as seen by changes to the epithelium and the spermatozoa found in this section.

Therefore GnRH agonists were considered. A Nafarelin derivative administered by osmotic subcutaneous pumps was used in two dogs, however, due to problems with the pumps, the treatment was ineffective until changed to daily subcutaneous injections. The results of this treatment showed that it did produce testicular changes which were reflected on the supression of LH but more so of testosterone. There was a length of treatment related change in the testicular tissue, as evidenced by the partial or total elimination of germ cells from the seminiferous tubules.

To further study the changes at testicular level, the isolation, purification and culture of Leydig cells was also undertaken. For this purpose testicular tissue from dogs, rats and mice was used. The results indicated that the procedure for handling the cells was producing viable cells capable of responding to exogenous treatments when assessed by their testosterone producing capacity.

Subsequently the techniques thus developed were applied to the Leydig cells that were obtained from dogs and mice that were treated with daily subcutaneous injections of Nafarelin acetate. As far as the testosterone produced by the Leydig cell cultures from the two treated dogs the results were inconclusive as they had opposite responses to what was expected. However the results on the detection of testicular GnRH-like receptors suggests that the GnRH agonist acted directly on the pituitary and not in the gonad as is the case in the rat.

It was concluded that a larger animal sample is required to further understand the mechanisms involved in testicular function in the dog.

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LIST OF ABBREVIATIONS.

20α-HSD20	αhydroxysteroid dehydrogenase
22ROHC	22R-hydroxycholesterol
17 βOHSD	17 β hydroxi steroid dehydrogenase
3β-HSDδ^5	3βhydroxysteroid dehydrogenase
ABP	Androgen binding protein
ACP	Acepromazine maleate
ATP	Adenosine triphosphate
BSA	bovine serum albumin
cAMP	Cyclic adenosine monophosphate
cm	centimeters
CO_2	carbon dioxide
CV	coefficient of variance
DAR	donkey anti-rabbit
dbcAMP	dibutyryl cyclic adenosine monophosphate
dia	diameter
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ea.	each
EDTA	Ethylenediamine tetracetic acid
ELISA	enzyme-linked immunosorbent assay
FSH	Follicle stimulating hormone
g	relative centrifugal force
G	gauge
GnRH	Gonadotrophin releasing hormone
g	gram
H & E	Haematoxylin and Eosin
HCG	Human chorionic gonadotrophin
HCl	Hydrochloric acid
HHG	Hypothalamic-hyphophyseal-gonadal axis.
i.e.	same as
iu	international unit
iv	intravenous
kg	kilogram
LH	Luteinizing hormone
l	litre
M	molar
mg	milligrams

ml	millilitres
mM	millimolar
MPA	Medroxyprogesterone acetate
MSB	Martius, Scarlet & Blue
MW	molar weight
$Na\ H_2\ PO_4$	Sodium di-hydrogen orthophosphate
$Na_2\ HPO_4$	Di-sodium hydrogen orthophosphate
$Na_2HOPO_4.12H_20$	Di-sodium hydrogen orthophosphate dodecahydrate
$NaH_2PO_4.2H_20$	Sodium dihydrogen orthophosphate dihydrate
NaOH	Sodium hydroxide
ng	nanogram
nmol	nanomol
NRS	normal rabbit carrier serum
NSB	non specific binding
^oC	degree Celsius
P_4	Progesterone
PBS	Phosphate buffer saline
rpm	revolutions per minute
s/c	subcutaneous
TCs	total counts
ug	micrograms
ul	microliters

DEDICATION

I wish to dedicate this work to my family

My parents: Jose and Esther

My sisters and brothers: Silvia, Gloria, Ignacio, Jaime, Jorge.

My sisters in law: Rocio, Pilar, Alma.

And the little ones: Bricia, Diego, Daniela, Jorgito.

I know they will all understand if I make a special dedication to my sister Silvia because it was following her steps to school that I found my way.

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DECLARATION

I hereby declare that the work presented in this thesis was carried out by the author under supervision except for the following:

The ultrasound scanning of the dogs used in this thesis was carried out by Professor J.S. Boyd of the Department of Anatomy.

The processing of tissue samples for histology was undertaken by the staff technicians of the Department of Surgery and Reproduction.

The iodination of GNRH was carried out by Dr. I. Jeffcoate of the Department of Veterinary Physiology.

I certify that the work included in this thesis is original and no part of it has been submitted previously for the award of a degree to any university.

Rosa Maria de Guadalupe Paramo Ramirez.

CHAPTER 1

GENERAL INTRODUCTION

I. INTRODUCTION

A male's reproductive potential depends on his capacity to produce and deposit in the female, semen capable of achieving good fertility.

The production of the male gamete, throughout the process of spermatogenesis, involves the interaction of man y factors. How this is achieved has been the subject of much research undertaken mainly in the rat and mouse. In domestic animals, such as ruminants, the pig and the horse there have been many studies, but fewer in the dog.

The male reproductive tract can be divided into the gamete producing organ, the testis, the epididymides where maturation and storage of the spermatozoa takes place and the accessory glands. In the dog there is only one such gland, the prostate, which produces seminal plasma which is the vehicle for transport of the gametes through the ejaculatory duct, namely the penis.

In the embryo the germinal cells develop initially in the yolk sac, from where they migrate towards the undifferentiated genital ridge positioned behind the kidneys. In the male, the arrival of these cells triggers the transformation of the ridges into testes by changing the original arrangement of its somatic cells into seminiferous tubules which will accommodate the germ cells, known as gonocytes. The gonocytes are present until puberty is reached (Ashdown, 1987).

TESTES

There are 2 main components of the testis. Firstly the seminiferous tubules which contain the Sertoli cells and the various stages of germ cells from which the spermatozoa are produced. The second component is the interstitial tissue around the seminiferous tubules which contains the blood vessels, nerves and the Leydig cells which produce testosterone.

In the dog, the testes are located in the abdomen at birth, but normally within a few days they migrate to their normal position outside the body by passing downwards through the internal and external inguinal rings coming to rest in the scrotum which is a distended pouch of skin. The scrotal sac is composed of an outer layer of thin pigmented skin, within which lies the capsule of the testis known as the *tunica albuginea* which consists of 3 layers, the external one the *tunica vaginalis* of visceral peritoneum, the *tunica albuginea* proper and the *tunica vasculosa* (Setchell and Brooks, 1988). In the dog, the testes are ovoid shaped and their axes are directed dorsocaudally. There is considerable variation in size of testes according to breed, with average dimensions of 3 x 2 x

1.5 cm. weighing approximately 11 grams (Christiansen, 1984). In the dog it is not unusual to find one testis lying directly behind the other (Allen *et al.* 1979).

The vascular supply to the testis is provided by the testicular arteries which originate from the aorta proximal to the kidneys and from the renal arteries. These reach the testis within the spermatic cord which also contains the veins draining blood from the testes which are at a lower temperature than that of the rest of the body (Waites and Setchell 1990). Within the spermatic cord, these veins coil around the arteries, thereby providing a temperature control mechanism which cools the arterial blood before it reaches the testes. This cooling is further aided by the cremaster muscle which, on contraction or relaxation, raises or lowers the testes from its position near the body wall (Christensen 1979; Christiansen 1984; Ashdown 1987, Setchell and Brooks, 1988; Waites and Setchell, 1990).

Innervation of the testes is provided by the superior spermatic nerves which arise from the spermatic ganglion close to the origin of the testicular artery. The nerves and arteries run in parallel until they reach the testes. In addition, there are the inferior spermatic nerves which originate in the pelvic plexus. These latter nerves, together with branches of the hypogastric nerve, also supply the cauda epididymis (Christensen, 1979; Setchell and Brooks, 1988).

SEMINIFEROUS TUBULES

Each seminiferous tubule is a double-ended convoluted loop, surrounded by peritubular cells, myoid cells and fibroblasts. The number of layers of myoid cells can vary according to species. Spermatogenesis occurs within seminiferous tubules and several of these are grouped into lobules by thin connective tissue. Both ends of each tubule are connected to the rete testis, a network of channels through which the spermatozoa and fluid that accompanies them pass to the efferent ducts which link with the epididymis (Dym and Fawcett, 1970; Setchell and Brooks, 1988).

The spermatogenic cycle that takes place in a given area of the seminiferous tubule refers to the series of transformations that the germ cells must undergo to become mature spermatozoa . It starts as the spermatogonia found closest to the basal membrane, by successive transformation move toward the lumen of the tubule where they arrive as spermatids and are released into the lumen as spermatozoa. This is also occurring all along the length of the tubule and this

sequential chain of changes is known as the spermatogenic wave (Garner and Hafez, 1987).

SERTOLI CELLS

As well as the developing germ cells, the seminiferous tubules contain the Sertoli cells which also originate from the somatic cells. The Sertoli cells lie between the spermatogonia on the basement membrane and provide the support and nutrition required by the spermatogonia while they undergo transformation. It is a triangular shaped complex structure whose base lies on the basement membrane of the seminiferous tubule with the remainder of the cell extending into the lumen of the tubule (Setchell, 1982). There is extensive ramification of the cytoplasm of the Sertoli cell which allows close association of the different sizes and shapes of the various stages of spermatogenesis (de Kretser and Kerr, 1988).

There is a physiological relationship between the Sertoli cells and adjacent germ cells which is suggested by the fact that changes occur both in the Sertoli cell shape and the nucleus depending on the stages of the spermatogenic cycle. The nucleus may often be polygonal in appearance following the release of spermatids which occupy the lower edges of the Sertoli cell during their development. At other times elongated when the germ cells penetrate into the basal aspects of the epithelium or flattened when lying along the basal lamina of the seminiferous tubules, interspersed between spermatogonia (Ritzen *et al.* 1981; Setchell 1982; de Kretser and Kerr, 1988).

Sertoli cells contain only limited amounts of rough or granular endoplasmic reticulum and only a few condensed vacuoles which are usually associated with cells actively engaged in the synthesis of proteins. This is surprising in the light of the evidence that Sertoli cells synthesise inhibin and the androgen binding protein ABP (Ritzen *et al.* 1981; de Kretser and Kerr, 1988). However they contain large amounts of smooth endoplasmic reticulum associated in many species with lipid inclusions (Setchell, 1982).

To date, neither the precise role of the smooth endoplasmic reticulum of the Sertoli cell is clear nor the role of the numerous lipid inclusions present in their cytoplasm (Setchell, 1982). It is believed that they may represent the metabolism or synthesis of certain steroid compounds, known to occur in isolated Sertoli cells. They may also represent phagocytised material that the Sertoli cells have removed from the developing germ cells. Sertoli cells are known to

be active in phagocytosis and have strong hydrolytic enzyme properties. They are thus equipped to deal with the removal of degenerating germ cells and excess spermatid cytoplasm. In several species, Sertoli cells are involved in the absorption of fluid from the immature spermatozoa. This involves the formation of a tubule which lies in the cytoplasm of the head of the spermatid and ends as a bulbous region in the cytoplasm of the Sertoli cell. This connection is broken shortly before spermiation, when the spermatid is released into the lumen of the seminiferous tubule (de Kretser and Kerr, 1988).

The desmosome-like junctions which exist between Sertoli cells and the basement membrane, form part of the blood testes barrier. They divide the seminiferous tubular epithelium into separate compartments, the basal or exterior compartment where spermatogonia and preleptotene spermatocytes are found and the interior or adluminal compartment containing spermatocytes and spermatids. The primary spermatocytes pass from the basal into the adluminal compartments by way of these desmosome-like junctions. Another type of junction, the gap junction, exists between Sertoli cells and also between Sertoli and germ cell. The germ cells which communicate with the Sertoli cell by this type of junction are the primary spermatocytes in the pachytene stage (Waites and Setchell, 1990).

FSH receptors have been identified on the cell membrane of both Sertoli cells and spermatogonia. Both these types of cell are located in the basal compartment of the seminiferous tubules, thus making them more accessible to this hormone which reaches them via the blood vessels located in the interstitial tissue (Waites and Setchell, 1990). Evidence that this is the case is the fact that cultured Sertoli cells produce an FSH inhibiting factor known as inhibin, indicating that the cell itself controls the amount of FSH which it requires (Steinberger and Steinberger, 1976).

LEYDIG CELLS

In 1850, Franz Leydig reported that the spaces between the seminiferous tubules were occupied by conspicuous masses of cells which contained fatty vacuoles and pigments. He suggested that these cells were modified connective tissue. In 1903, Bouin and Ancel showed evidence that these Leydig cells provided the hormonal stimulus for the production of sperm and the maintenance of male sexual characteristics. However it was not until Gallagher *et al.* (1929) named the testicular hormone "testosterone " and later was associated with the lipid

inclusions in the Leydig cells that the true role of this cell in the synthesis of testosterone was established.

In most laboratory species, Leydig cells are located close to the blood vessels in the interstitial tissue. However, in other species including the dog this is not the case as they are scattered in groups throughout the tissue. In the dog, there are relatively large numbers of Leydig cells which lie closely packed together. Why this species develops such large masses of Leydig cells is not yet understood. The size and structure of Leydig cells varies according to the stage of the spermatogenic cycle (de Kretser and Kerr, 1988).

In mammals, Leydig cells are polygonal and have round or oval dark-staining nuclei containing two nucleoli (de Kretser and Kerr, 1988) and a thin rim of peripheral heterochromatin and a dark staining cytoplasm (Themmen *et al.* 1986). In studies in which isolated Leydig cells are used and examined under the phase contrast microscope, they have been identified by a characteristic luminous yellowish halo around them (Schumacher *et al.* 1978; Gale *et al.* 1982; Joffre *et al.* 1984).

The membrane of the Leydig cell contains the LH receptors coupled to adenylate cyclase. These receptor sites are receptive to both LH and hCG (Reeves, 1987). Smooth endoplasmic reticulum is present in abundant quantities in the cytoplasm of the adult Leydig cell. This is a feature of cells that specialise in lipid metabolism, being large enough to accommodate the enzymes required to transform the cholesterol into steroid hormones (Alberts *et al.* 1989). The endoplasmic reticulum of some species including the dog can present concentric formations which surround lipid droplets. Their functional significance remains unknown, although it is believed that their presence may provide specific requirements in those species in which they are found (de Kretser and Kerr, 1988).

The cytoplasm of dog Leydig cells contain a relatively high proportion of lipid inclusions which represent sites of storage of cholesterol. The transformation of cholesterol into pregnenolone occurs inside the mitochondria, possibly on the surface of the *christae*. This appears to be associated with the arrival of LH from the pituitary, as demonstrated by the fact that stimulation of such cells with LH or GnRH results in a rapid disappearance of the bulk of the lipid bodies and an increase in testosterone in the circulating plasma (de Kretser and Kerr, 1988).

The Golgi apparatus of the Leydig cells of mammalian species is well defined, but as yet its role is not fully understood, although it appears that it plays an important part in the secretion of glycoproteins by Leydig cells. The manner in which testosterone is released from the Leydig cells appears to be just speculative and it is suggested that similar to progesterone from the luteal cells of the ovary, testosterone is released as granules (Gemmel and Stacy, 1979).

In most mammals there appears to be two generations of Leydig cells, the first appearing during late foetal life or very early in the postnatal period (Wartenberg, 1981) and they secrete the androgens that masculinize the foetus. The development of this first generation of cells appears to be controlled by the foetal pituitary hormones. At the initiation of puberty, the second adult generation of Leydig cells makes its appearance. These derive from interstitial mesenchymal cells and are fibroblastic in appearance until they differentiate and acquire the Leydig cell characteristics, such as development of mitochondria, smooth endoplasmic reticulum and Golgi apparatus. The development of the mature Leydig cells appears to be mostly dependent on LH (Sharpe, 1982). It is possible that FSH might be involved in the process of transforming macrophage and mesenchymal cells into Leydig cells (de Kretser and Kerr 1988).

Some interesting findings associated with the study of the Leydig cell, are that although they are thought to disappear, for the most part, from the testes of young men from about one year old until just before puberty, the levels of testosterone in the spermatic vein is five times that of the circulating plasma at this particular time. If these prepubertal boys are injected with hCG, there is a marked increase in their circulating plasma testosterone. How this can occur in the absence of a population of Leydig cells is not clear (Rivarola *et al.* 1970).

ENDOCRINE CONTROL OF TESTICULAR FUNCTION.

The Sertoli and Leydig cells which govern testicular function are controlled by the higher brain centres by way of a long loop feed back mechanism which involves the hypothalamic-hypophyseal-gonadal (HHG) axis. The GnRH, produced by the hypothalamus, is transported rapidly to the pituitary via a local short feedback mechanism. The pattern of GnRH secretion is oscillatory and the frequency is low (Vickery and McRae, 1984). The stimulated pituitary then produces the gonadotrophins, LH and FSH, which are transported in the circulating plasma to their target organs. In the male there is a constant pulsatile pattern of the two gonadotrophins which continues throughout adult life. There

are no surges of LH as are found in the female preovulatory peaks (Reeves, 1987; Waites and Setchell, 1990).

FSH and LH affect both the Leydig and Sertoli cells of the testes. The major role of LH is to stimulate the Leydig cells to produce testosterone and that of FSH is to stimulate the Sertoli cells thus controlling their ability to produce the nutrition, enzymes, proteins and ABP (Garner and Hafez, 1987) required for the developing spermatozoa. Some of the testosterone produced by the Leydig cells diffuses into the interstitial fluid and by crossing the blood testis barrier reaches the Sertoli cell, where in some species a proportion becomes aromatised into oestrogens (Pudney *et al.* 1985; Rosselli *et al.* 1992). These oestrogens are used by the Sertoli cells in the process of nurturing the developing spermatozoa. In the rat, aromatization of testosterone by the Sertoli cells only occurs during the immature stage of development of the male. However once it has reached maturity, the Leydig cell takes over as the main producer of oestrogens in the testis (Sachs *et al.* 1988). This aspect of testicular function in the dog has not as yet received much attention.

Most of the testosterone reaching the Sertoli cell becomes attached to the androgen binding protein (ABP), which transports it to the epididymis, where it is required for maturation of the spermatozoa. A proportion of the testosterone enters the circulating plasma where it plays a role in regulating normal libido, prostatic function and development of other male characteristics, such as muscular development in certain areas of the body for example neck (Sharpe, 1987). The concentration of testosterone in the circulating plasma is less than that of the interstitial fluid. This is due mainly to the testicular levels being diluted in the higher volume of circulating plasma. However, it suggests the importance of higher levels of testosterone required locally for testicular function (Maddocks and Sharpe, 1988).

PARACRINE CONTROL

Interaction between the Leydig, Sertoli and germinal cells integrates part of a local extra short loop or paracrine control. This appears to involve complicated mechanisms which are the subject of many studies, some of which are mentioned here (Sharpe, 1984; Tahka, 1986; Papadopoulus *et al.* 1987; Findlay, *et al.* 1987; de Kretser, 1990). At present, the relationship whereby the Sertoli cells control the production of testosterone by the Leydig cells, is thought firstly to involve the germinal cells within the tubules. These cells control Sertoli cell

function by exchange of substances through the gap junctions (Kasuga *et al.* 1989; Ojeifo, *et al.* 1990; Waites and Setchell, 1990). One of these is a Sertoli cell produced GnRH-like substance which is received by GnRH-like receptors in the Leydig cells (Sharpe, 1984).

The myoid cells which are in very close contact with the Sertoli cells, may also play a role in the intercommunication between Leydig and Sertoli cells as it appears that they have androgen receptors. Indeed, it has been demonstrated that communication takes place between myoid and Sertoli cells by the production of a very large molecule by the former cells which appears to aid the Sertoli cell in modulating the production of ABP. Myoid cells receive oxytocin from the Leydig cell which allows them to contract, whereby they also contribute to the transport of the seminiferous tubule fluids containing the spermatozoa (Sharpe, 1986; Rosselli *et al.* 1992).

TESTICULAR FUNCTIONS

SPERMATOGENESIS

At puberty, the gonocytes in the testes start transforming into spermatogonia just before puberty is reached and they are found in the basal compartment of the seminiferous tubules which is the one closest to the basal membrane. Three types of spermatogonia have been classified namely A, Intermediate and B spermatogonia. A subsequently subdivide into A_0 (also known as A_S) A_1, A_2, A_3 and A_4. The mitotic division of these spermatogonia is at random for A_0. But for A_1 to A_4, intermediate and B, their divisions are co-ordinated to the spermatogenic cycle (Setchell, 1982). Type B spermatogonia are transformed into preleptotene primary spermatocytes containing double the amount of nuclear DNA where the chromosomes are as yet unidentifiable within the nucleus. This proceeds to the long meiotic prophase with leptotene, which involves the pairing of homogeneous chromosomes. During zygotene, pairs of chromosomes undergo synapses, and in pachytene the crossing over of chromosomal material occurs, such that on separation in diplotene, the chromosomes now contain different genetic material from those in the parent cell. Anaphase I now follows, when each member of the bivalent chromosome moves to the opposite end of the cell. In telophase I, the cell divides resulting in daughter cells which contain the haploid number of chromosomes and the haploid amount of DNA., this ends meiosis I. Meiosis II begins without duplication of DNA with the very short prophase II leading to metaphase II where the chromatids become broader, coiled

and shorter. Anaphase II follows with the separation of the chromatids and equal numbers of chromatids moving to opposite poles. The cell now divides during telophase II and produces two haploid daughter cells, the spermatids (Dym, 1977; Setchell, 1982; de Kretser and Kerr, 1988; Alberts *et al*, 1989).

SPERMIOGENESIS

Spermiogenesis is the transformation of spermatids into immature spermatozoa. It is a process by which a conventional cell is converted into a highly organised motile structure. The changes include formation of the acrosome, changes in the nuclear position, development of the tail, reorganisation of the organelles, separation of the spermatids from the supporting Sertoli cells and entry into the freedom of the lumen of the seminiferous tubule (de Kretser and Kerr, 1988).

In the nucleus the chromatin condenses to form larger and more dense particles which eventually fuse to form a homogeneous mass whose density varies between species. During condensation of the chromatin, dramatic changes in the shape of the nucleus occur to end in the typical elongated head shape, with various minor differences according to the characteristics of the spermatozoa of each specific mammalian species. It is also at this stage that, in the majority of the mammalian species, the nucleus changes from its central position within the round spermatid to an eccentric position (de Kretser and Kerr, 1988; Alberts *et al.* 1989).

The acrosome arises from *cisternae* containing granules produced by the Golgi complex of the newly formed spermatid. Both *cisternae* fuse to form just one vesicle and the granules move toward the nucleus of the cell which will become the head of the sperm, where they become attached. As more material is produced by the Golgi apparatus, the acrosome is enlarged (Setchell, 1982) and depending on the species it can cover between 25-60% of the nucleus surface. The acrosome contains a variety of lysosome enzymes for example hyaluronidase, which are used in the penetration of the *zona pellucida* of the ovum. The protein acrosin is also involved in this process (Bedford, 1982). At the equatorial region which is round the middle of the sperm, the acrosome becomes accentuated, and it is this aspect of the acrosome which remains after the rest of the acrosomal contents have been released, just prior to penetration of the ovum (de Kretser and Kerr, 1988).

The neck region contains the proximal and distal centrioles and its truncated apex points distally and from it emerges the tail of the sperm. Anteriorly there is the implantation *fossa*. From the neck region arise the nine longitudinal cross striated fibres, which fuse together to varying degrees to form the rest of the tail.

The tail of the spermatozoa originates from the centrioles of the spermatids and moves to the periphery of the cell. The axial filament which positions itself at the opposite pole to that of the acrosome which arises from the centriole lying nearest to the cell membrane. The axial filament consists of nine microtubules which are arranged concentrically at whose centre are two single microtubules. An invagination of the cell membrane at this same area then occurs forming a cleft, into which the tail of the sperm fits like a ball and socket joint, and the initial region of the flagellum or tail forms the neck region (de Kretser and Kerr, 1988).

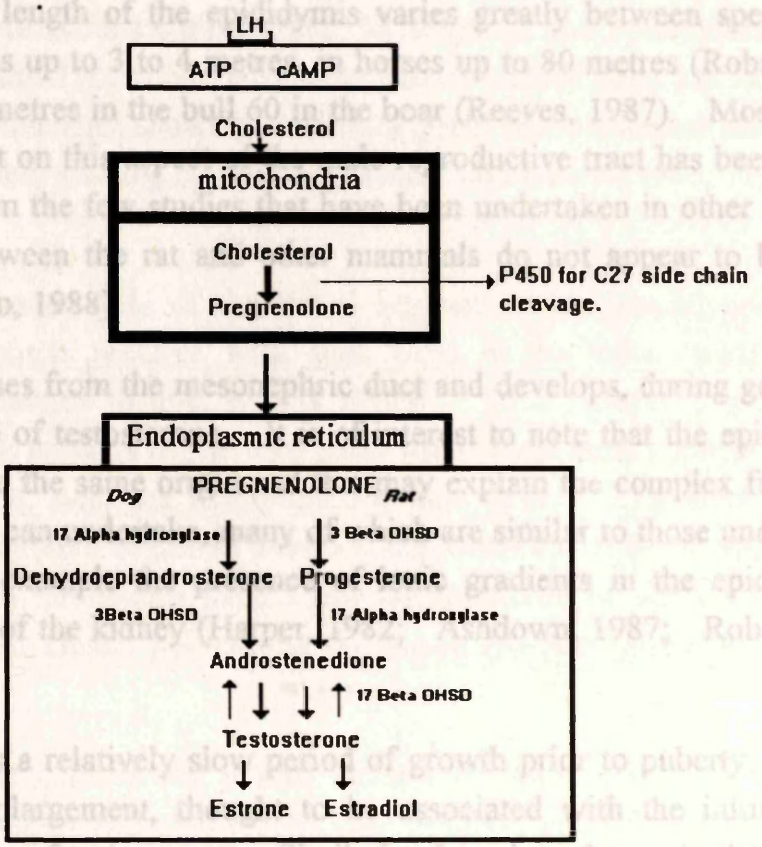
Studies of the detail of the axial filaments forming the sperm tail have been published in man, mouse and rat but little research of this aspect has been undertaken in the dog. In the mammalian species, the mitochondria form a spiral sheath of variable length in the midpiece neck region. The number of spirals forming the sheath apparently varies between species for example the mouse 90, the bat 15 and the rat 350 (de Kretser and Kerr, 1988). However no reference is made to this aspect in the dog.

Associated with the final stages of spermiogenesis is the shedding of a portion of cytoplasm which contains the remnants of the Golgi apparatus. This cytoplasm extrusion is phagocytised by the Sertoli cells (de Kretser and Kerr, 1988). A small portion of this cytoplasm remains connected to the spermatid and once the connection is broken it gives rise to the proximal cytoplasmic droplet (Garner and Hafez, 1987). Surrounding the spermatozoa and the acrosome is a complete outer membrane namely the plasma membrane and thus any displacement of the acrosome indicates a change in the outer membrane of the sperm and thus an abnormality (Setchell, 1982).

STEROIDOGENESIS

Although there are species differences in the steroidogenic process of testosterone production by the Leydig cells, the basic steps seem to be as follows.

DIAGRAM



LH is a protein hormone which binds to specific receptors on the cell membrane of Leydig cells. It activates adenylate cyclase which catalyses the conversion of adenosine triphosphate (ATP) into cAMP (the second cell messenger) which in turn produces specific mRNA. This mRNA enters the cytoplasm and is translated to steroidogenic enzymes which regulate steroid production and the regulation of the passage of cholesterol from the cytoplasm into the mitochondria which controls the rate of steroid sunthesis. It is then transformed into

pregnenolone by the C27 side chain cleavage enzyme. The pregnenolone is then taken up by the endoplasmic reticulum and in the dog is transformed by a 17 α hydroxylase into dehydroepiandrosterone which is transformed by 3βHSD into androstenedione, and then into testosterone by 17βOHSD (Hall, 1988; di Zerega and Sherins, 1981).

EPIDIDYMIS

The spermatozoa from the seminiferous tubules leave from the rete testes, travel through the efferent ducts which are a series of tubules that vary in number from 4 to 20 according to the species and then enter the single convoluted duct, the epididymis. The length of the epididymis varies greatly between species for example in man it is up to 3 to 4 metres, in horses up to 80 metres (Robaire and Hermo, 1988), 40 metres in the bull 60 in the boar (Reeves, 1987). Most of the research carried out on this aspect of the male reproductive tract has been in the rat. However, from the few studies that have been undertaken in other species, the differences between the rat and other mammals do not appear to be great (Robaire and Hermo, 1988).

The epididymis arises from the mesonephric duct and develops, during gestation, under the influence of testosterone. It is of interest to note that the epididymis and the kidney have the same origin and this may explain the complex functions that the epididymis can undertake, many of which are similar to those undertaken by the kidney for example the presence of ionic gradients in the epididymis, analogous to those of the kidney (Harper, 1982; Ashdown, 1987; Robaire and Hermo, 1988).

The epididymis has a relatively slow period of growth prior to puberty, when it undergoes rapid enlargement, thought to be associated with the initiation of testicular production of testosterone. Similarly, there is a change in the content of the epididymal fluid, in association with the appearance of ABP, the levels of which are also greatly increased around puberty. ABP is the transport mechanism for testosterone from the seminiferous tubule to the epididymis (Lobl *et al.* 1983; Robaire and Hermo, 1988).

A muscle layer surrounds most of the tube and is thickest in the lower regions of the epididymis, as it approaches the vas deferens. External to the muscle layer there is loose connective tissue which contains the nerves and blood vessels. Anatomically the epididymis can be divided into three regions, the head or caput,

the body and the tail or caudal region. Maturation of the spermatozoa takes place in the first two regions and storage is the main function of the caudal region, the tail of the epididymis is palpable in normal adult male domestic animals (Ashdown, 1987), including the canine.

The histology of the epididymis identifies three separate regions, which do not coincide with the anatomical divisions. The epididymal walls are composed of pseudostratified columnar epithelium resting on a basal lamina and a lamina propia surrounded by smooth muscle. The epithelium lining each area is composed of basically the same types of cell but in differing proportions and in each region the cells have some aspects specific to that part. There are three types of epithelial cells found throughout the length of the epididymis. The three types are principal columnar cells, clear narrow shaped cells and halo cells, which have a dense nucleus, showing patches of peripheral condensed chromatin, surrounded by a palely staining cytoplasm (Robaire and Hermo, 1988; Hamilton 1990).

The principal columnar cells of the initial segment of the epididymis, while sharing many common features with such cells in the other areas of the epidiymis, show features distinct to this region. In the initial segment, the principal cells make up 80% of the total cells while basal cells contribute 12%, 3% are narrow cells, with the remaining 5% halo cells. Lymphocytes with pycnotic nuclei are found scattered between the epithelial cells of this region. Most of the cells in this region have a few tall microvilli or stereocilia which form a brush border, almost obliterating the lumen (Robaire and Hermo 1988; Hamilton 1990).

The majority of the cells in the middle section are clear cells with a highly vacuolated apical region with numerous dense granules present both above and below the nucleus. The nucleus is elongated and palely staining and shows a prominent nucleolus. In this area the percentage of principal cells declines, while the numbers of clear and halo cells increase to 30%. The lumen of the epididymis is greater than in the initial section because the height of the principal cells is decreased (Orsi, 1983; Robaire and Hermo, 1988; Hamilton, 1990).

In the terminal segment, the lumen is at its widest and the main characteristics of the epithelial cells lining this section are that they have ovoid nuclei and their apical aspects have microvilli (Orsi, 1983; Hamilton, 1990).

It is now accepted that the blood testis barrier existing between the seminiferous tubules and the interstitial tissue continues into the epididymis. The actual site of this barrier in the epididymis would appear to be at the luminal surface of the principal cells, where the typical tight junctions are found. The exact role of this barrier has not yet been defined, but it is obvious that it protects the spermatozoa from foreign or toxic substances, for example serum albumin does not pass the barrier (Robaire and Hermo, 1988).

The epididymis itself can undertake *de novo* synthesis of testosterone.(Robaire and Hermo, 1988) However this activity must be minimal as epididymal atrophy occurs after castration. Thus, most of the androgen required for epididymal function is delivered from the seminiferous tubules attached to ABP (Lobl *et al.* 1983). Some epithelial cells of the epididymis can also take up proteins and other particulate matter, most probably by endocytosis. The epididymis is also engaged in spermiophagy, the removal of dead sperm from the lumen (Ashdown, 1987). An interesting finding concerning the contents of the epidiymis in the rat, is that there is a substance present in quantity in the caput region which has an immobilising effect on the spermatozoa, thereby maintaining their energy whilst in storage, and only on release from this region do they become highly motile. However, this substance has as yet not been found generally in mammals (Robaire and Hermo, 1988). The epithelial cells of the epididymis are also capable of the uptake of small molecules from the circulation. Some of these are secreted directly into the lumen of the duct. Others are involved in the synthesis and metabolism of steroids and prostaglandins. On the other hand, some of the epithelial cells can themselves synthesise and secrete small molecules and an array of glycoproteins such as carnitine and inositol (Robaire and Hermo, 1988; Hamilton ,1990).

It is a matter of some controversy as to the exact role of the epididymis in spermatozoa achieving fertilising potential whether it is a passive role which may come about simply because of the time that the spermatozoa are exposed to the epididymal environment as they go through this convoluted tube. On the other hand, the maturation process may actually involve changes to the spermatozoa which require stimuli produced by the epididymis. It is interesting to note, that even though the spermatozoa, once they have passed through the head and body of the epididymis, have the ability to penetrate ova, and it is not until they actually reach the cauda of the epididymis, that they have acquired motility and are able to fertilise eggs (Robaire and Hermo, 1988).

As in the ram, (Lino, *et al.* 1967) it appears likely, that the sperm in the cauda of the epididymis of the dog are all voided to the exterior. This can take place in one of three ways, namely by ejaculation, masturbation, or urination (Harper, 1982; Ashdown, 1987). It has been shown that in the fertile male dog, spermatozoa are always present in urine. Therefore, it would seem that there is a constant excretion of spermatozoa. This tends to suggest that absorption of dead sperm from the epididymis does not tend to occur in the dog (Ferguson and Renton, 1988).

An important function of the epididymis is to absorb some of the large volume of fluid accompanying the sperm. However in the bull and hamster and possibly in man, this takes place in the *efferent ducts.* Most of the fluid emerging from the testes is absorbed in the head and body regions mainly by passive diffusion. Absorption of this fluid and the transport of the ions is dependent on testosterone, probably by a method very similar to that of the kidney (Harper, 1982; Hamilton 1990). Changes in ion concentration takes place in the different regions of the epididymis, with variations between species. In the rat, potassium rises steadily throughout the epididymis, while chloride levels remain stable, the lowest concentrations being found are those of calcium, whereas sulphur and magnesium are more concentrated in the body of the epididymis (Harper, 1982).

The spermatozoa are transported from the seminiferous tubules into the *rete testis* in a passive way suspended in the fluid secreted by the Sertoli cells and assisted by the re-absorption of the fluid in the *efferent ducts* which pulls them into the head of the epididymis. Once they reach this point the transport through the epididymis becomes more active as it involves hydrostatic pressure, muscular contractions and cilliary movement. This journey takes about 10-12 days days in most mammals, with more than 50% of this time being spent in the tail (Courot, 1981; Harper 1982; Ashdown, 1987) where they retain their fertilising ability for 30 days (Robaire and Hermo, 1988).

ENDOCRINE CONTROL OF EPIDIDYMAL FUNCTION.

Knowledge to date suggests that the main hormone involved in epididymal function is testosterone. Withdrawal of androgens results in atrophy of the epididymis, degeneration of the epithelial cells and their function including transport of ions across the epididymal epithelium. All these functions such as the transport of spermatozoa are dependent on androgens. And in particular dihydrotestosterone plays an important part in the spermatozoa's acquisition of

fertilising ability. Testosterone is transformed into dihydrotestosterone in the epididymis and LH and FSH are needed for transport of testosterone which is bound to the ABP produced by the Sertoli cell (Lobl *et al.* 1983; Robaire and Hermo, 1988).

ACCESSORY GLAND THE PROSTATE

The prostate in the dog is a symmetrical globular gland, that weights between 1.7 and 14.5 grams depending on the size of the dog and is normally found in the middle of the pelvis, over the cranial part of the symphysis pelvis, surrounding the pelvic urethra (Christiansen, 1984). Its glandular epithelium is simple columnar or pseudo stratified, with a few basal cells found among the columnar cells. In the segment closest to the urethra, the epithelium changes to the transitional type found in the bladder and the prostatic urethra (Dym, 1977).

LIBIDO AND EJACULATION:

In the male, the desire to seek sexual contact is defined as sexual motivation, or libido. His ability to copulate is referred to as performance or potency. This male behaviour, is controlled by androgens transported in the circulating plasma, acting on brain centres. In animals with previous sexual experience, behaviour will remain unchanged for some considerable time, after removal of the gonads. However, progressive loss in intensity and frequency and total disappearance does eventually occur (Sachs and Meisel, 1988).

Libido, manifests itself by the intensity of the animals arousal to the visual and olfactory stimuli, provided by the oestrous female, by her presence and willingness to cooperate with the male's mounting attempts, and by how successfully copulation and ejaculation are achieved. There are time variations between species in initiation of copulation and actual ejaculation. In dogs and other canids, ejaculation commonly begins prior to intromission and before the swelling of the *bulbus* of the penis results in the locking or tying of the male to the female. This may last for 30 minutes or more (Sachs and Meisel, 1988).

Ejaculatory organs

The emission of spermatozoa, suspended in epididymal fluid, from the *cauda epididymis* through the *vas deferens*, is by contractions produced by increased sympathetic nerve activity, which also closes the neck of the bladder this prevents retrograde ejaculation, once the sperm reach the *coliculus seminalis*,

they mix with the secretion from the accesory glands, in the case of the dog, prostatic fluid only, thus producing the ejaculate (Christiansen, 1984; Robaire and Hermo, 1988). The ejaculate proceeds to enter first the pelvic urethra into the penile urethra. The penis of the dog, is made up of two areas of cavernous tissue, which surround the urethra one is the *corpus spongiosum penis* and *glandis*, and the *corpus cavernosum penis*, which when filled with blood, provides the turgidity necessary for intromission and copulation (Christensen, 1979; Christiansen, 1984). In the, dog part of the penile *corpus cavenosusm* ossifies to produce the os penis, which facilitates penetration of the vagina by the erected penis, and also aids to its retention within the vagina (Williams-Ashman, 1988). The penis is housed in the prepuce, which is a layer of skin covered with hair (Christiansen, 1984).

Ejaculate fractions

The ejaculate of the dog is made up of three fractions, the first of which is clear and watery originates from the prostate and is voided during the thrusting movements made while attempting to gain intromission this first fraction is a means of flushing out the urethra. Once the thrusting movements have ceased the second a sperm rich fraction is then ejaculated and by this time, due to enlargement of the bulbus of the penis, the "tie" to the female has occurred. The third and last fraction from the prostate is again clear and continues to be ejaculated in a pulsatile fashion for as long as the "tie" is maintained, which is until ejaculation has been completed and the bulbus is relaxed. This last fraction of the ejaculate is produced by the prostate and is similar to the first fraction, except that it contains the residue of the second or sperm rich fraction (Christiansen, 1984; Allen *et al.* 1979).

MANIPULATION OF MALE FERTILITY.

Many of the original studies of male fertility in both humans and domestic animals, were attempting firstly to develop methods of assessing and improving fertility and secondly storage of the male gamete by freezing in liquid nitrogen for future use in artificial insemination. However recently, interest has arisen in the human field in methods of male contraception. This aspect of manipulating male fertility is also of special interest in the dog as the stray dog problem and the spread of rabies is now of world wide importance (Faulkner, 1975; Wildt and Seager, 1977; Christiansen, 1984; Gonzalez *et al.* 1989; Carter, 1990; Eng and Fishbein 1990; Olson and Moulton, 1993).

One of the main lines of research is the development of contraception methods in the human male has been to attempt to suppress gonadal steroid production which was already being used as a means of treatment for steroid-dependent prostatic cancer. Another approach investigated has been to suppress the production of viable sperm, either temporarily or permanently by interference with the transport and/or maturation of sperm in the epididymis. Most of these studies into male contraception have been in the man and only a few have been carried out in the dog.

METHODS AIMED AT REDUCING CIRCULATING PLASMA

GONADOTROPHIC HORMONES.

The hormonal approaches investigated have included the use of progestogens and androgens, used either singly or in combination. With the development of GnRH analogues, the use of both agonist and antagonists as a means of contraception have also been investigated.

PROGESTOGENS

In the canine species progestogens were used initially in the bitch as a means of oestrus control (Van Os, 1982; Evans , 1978; Evans and Sutton 1989). However they are now routinely used in the dog for control of undesirable sexual and social behaviour (Gerber and Sulman 1964; Gerber *et al.* 1973; Evans and Sutton 1989). Most of these studies have been of a clinical nature with few references as to the changes in circulating plasma concentrations achieved in the animals treated. However Wright *et al.* (1979), did measure testosterone concentrations in a group of 4 mixed sheep-dogs treated with a single 4 mg/kg injection of Medroxyprogesterone acetate and found that testosterone levels had been reduced by 58%, measured 7 weeks after the injection. However this was the mean of the dogs and there was not enough information as to the number of samples taken. They also reported no evidence of testicular degeneration or changes in semen characteristics. The action of progestogens is presumed to be achieved by the drug producing a negative feedback on the hypothalamus, resulting in suppression of gonadotrophin release from the pituitary and consequently a suppression of gonadal function.

ANDROGENS

In the human, androgens such as testosterone, were thought to be an ideal method of achieving an increased negative feed back to the hypothalamus whilst maintaining male libido. However, the long acting ester, testosterone oenanthate, which has been the most widely tested, was considered not to be very practical as it required weekly injections, as did testosterone propionate and other testosterone esters used. This, added to the fact that the results were not totally satisfactory as not all of the men in which it was used achieved azoospermia, resulted in this method not being widely used. In addition, undesirable side effects, such as weight gain and skin problems such as acne, occurred quite frequently in patients treated this way (Paulsen *et al.* 1982; Knuth *et al.* 1987; Wu, 1988).

In an attempt to avoid these problems, androgens were combined with progestogens. This resulted in azoospermia not always being achieved, even when high doses were used and the undesirable side effects still occurred. The most effective combination used, in the human to date, is testosterone plus depot Medroxyprogesterone acetate, which has produced azoospermia in 60% of the men treated. The incidence of side effects of a similar nature to those resulting from testosterone treatment, was low (Paulsen *et al.* 1982; Knuth *et al.* 1987; Wu, 1988).

Oral administration of androgens has also been tried in humans but blood levels are not continuously suppressed by this route of administration, therefore the intermittent stimulation of Leydig cells could be enough to allow spermatogenesis to continue (Knuth *et al.* 1987).

GnRH ANALOGUES (Agonists and Antagonists).

GnRH antagonists work by causing pituitary desensitisation but they must be constantly present in the circulation. This means that the cost of the high doses required has somewhat restricted their use. However, their inhibitory effect is superior to that achieved with agonists (Rivier *et al.* 1985).

More of the information available is on the effects of GnRH agonists in suppressing production of gonadotrophins. The original idea behind the development of synthetic analogues of GnRH both agonist and antagonist, was to use them to stimulate gonadotrophin production in cases of human infertility. However it was found that their long term use had the opposite effects because the initial response was indeed an increase of gonadotrophin production but , the

treatment eventually led to a reduction in the release of gonadotrophins. This effect is thought to be due to a desensitisation process at hyphophyseal level due to down-regulation of GnRH receptors. Reducing the hyphophyseal gonadotrophin secretion directly affects the gonads but the mechanisms involved in this down-regulation are as yet not fully understood (Kastin *et al.* 1972; Cusan *et al.* 1979; Bambino *et al.* 1980; Akhtar, *et al.* 1984; Doelle, 1984; Corbin *et al.* 1985; Fraser, 1985; Vickery, 1986).

There is also evidence from work in the human, rat, mouse and monkey that the effect of GnRH agonists differs according to the species. For example, in the rat the effects are both at hypothalamic and gonadal level, due to the presence of GnRH like receptors in the rat testis. In the human and primates, the effect appears to be solely at the hypothalamic level (Hsueh *et al.* 1981; Bhasin *et al.* 1986). Mice appear not to respond to the inhibitory effects (Bex, *et al.* 1982), rats are partially responsive (Vickery *et al.* 1983) and primates are also somewhat resistant to treatment (Bhasin *et al.* 1986).

On the other hand, dogs appear to be extremely sensitive to the effects of GnRH agonists (Vickery, 1986). In intensive studies into the use of GnRH agonists in the dog, Vickery using several different GnRH agonist formulations including Nafarelin acetate, found the same initial increase in the circulating plasma levels of LH, FSH and testosterone that occur in other species, indicating that this initial increase disappears as treatment progressed. He also reported a decline in prostatic size, testicular volume with alterations to the testicular structure, erection period was shorter and lower ejaculated volume. The degree of response varied according to either the potency or the dose of the agonist (Vickery and McRae, 1984; Vickery *et al.* 1984; Vickery *et al.* 1985; Vickery, 1986; Vickery *et al.* 1989; Lincoln, 1992).

In the human, there seems to be an age related difference in response to GnRH agonists. In elderly human males, suffering from prostate cancer, who are usually weakened by their illness, the effect of GnRH agonist treatment is drastic enough to suppress circulating testosterone to levels similar to those seen after castration. This, as expected, results in cessation of spermatogenesis. On the other hand, in younger men receiving the same treatment for contraception purposes, the response in suppressing circulating plasma testosterone concentrations is not as good (Vickery, 1986).

Anabolic steroids, such as 19-nortestosterone (19-NT) and 19-NT-hoxyphenylpropionate, are known to suppress male fertility, but even though they have been in clinical use for many years, their potential as possible contraceptives has only recently received attention. This has come about because of their wide use amongst male athletes and their subsequent effect on fertility. However, further studies are required before any substantial evidence of their capability as antifertility drugs can be reported (Wu, 1988).

METHODS AFFECTING EPIDIDYMAL FUNCTION

Another approach to manipulation of fertility is by affecting epididymal function by interfering either with sperm maturation or sperm transport. Drugs such as alpha chlorohydrin have been investigated, which act mainly on the epididymis. They cause changes in the vasculature and alter the composition and amounts of epididymal fluids. This drug also has a direct effect on sperm during their transit through the epididymis reducing their motility, altering the spermatozoal membrane and so reducing the sperm fertilising capabilities. The species in which this drug has been studied are the rat, guinea pig, hamster, mice, ram and pig (Ratnasooriya 1982; Wu 1988).

Another group of substances which were also investigated were 6 CDG sugars, such as 6-Chloro-6 deoxyglucose. When given to marmoset monkeys they diminished the motility of their spermatozoa, apparently by interfering with their energy producing metabolism (Wu, 1988).

Oestrogens inhibit spermatozoa maturation and increase their rate of transport through the epididymis. Some research has been undertaken on this aspect in rats, hamster, mice and it appears that part of their effect is due to an increase in the peristaltic contractions in the epididymal tubule. However, the use of oestrogens has been associated to the thromboembolic phenomena in the human and so far no reliable method of their use has been published (Ratnasooriya, 1982).

Methoxamine, a sympathomimetic drug, temporarily reduces the fertility in male rats, but there seems to have no effect on their ability to mate. The route of administration was by implants placed adjacent to the epididymis. Almost no spermatozoa were seen at 3-7 days after insertion and there is an increase in the number of tailless spermatozoa in the epididymis. Methoxamine appears to have a direct toxic effect on the spermatozoa and apparently creates a hostile

environment at this site by reducing the blood supply to the epididymis . It also accelerates transport of sperm through the epididymis (Ratnasooriya 1982).

USE OF NON-HORMONAL PREPARATIONS

In China, the use of a variety of drugs have been investigated. These include gossypol, isolated from the cotton plant, and *Trypterigium wilfordii*. *In vitro*, gossypol inhibits the motility of epididymal and ejaculated spermatozoa. Histologically it appears to degenerate spermatids, Sertoli cells and late pachytene spermatocytes. However its use *in vivo* produces many side effects such as fatigue, decreased libido, hypokalemia and gastrointestinal problems. In about 10% of the human study subjects it caused irreversible infertility which does not make it an ideal method in the human. The total glycosides extracted from *Trypterigium wilfordii*, were found to decrease epididymal spermatozoa viability, but it is still being studied in order to identify the active constituents (Wu, 1988).

METHODS AFFECTING EJACULATION

In the human, inhibition of ejaculation has been reported as secondary effects, of the administration of sympatholytic drugs such as Guanethidine, Reserpine, Bretyliam and Bethimedine which are used in antihypertensive therapy. There were no apparent changes to libido, but during treatment azoospermic ejaculates were produced. Guanethidine when given to rats caused damage to "short adrenergic neurones" that innervate the *vas deferens* and epididymis. Another find was that it appeared to cause spermatoceles in the *cauda epididymides*, with many non motile and tailless spermatozoa being found in the rest of the epididymis (Ratnasooriya, 1982).

A group of anti-psychotic drugs (Reserpine, Tetrabenazine, Haloperidol), are known to cause disturbances in ejaculatory function and caused suppression of ejaculation in dogs. This was thought to be associated with interference of the transmitters in the brain's monoaminergic systems involved in ejaculation (Ratnasooriya, 1982).

Oxytocin and prostaglandins are involved in producing the epididymal contractions required to expel the spermatozoa through ejaculation, therefore blocking their action should result in azoospermia. Methallibure, an oxytocin blocking drug, when used in rabbits inhibited the amount of spermatozoa in the

ejaculates. A similar effect was observed, with prostaglandin synthesis inhibitors, like Indomethacin (Ratnasooriya, 1982).

An anti-androgen drug, Cyproterone acetate, has also been tried in humans. Normal epididymal function is androgen dependant and it was assumed that local interference would disrupt epididymal metabolism and cellular arrangement. However, this anti androgen drug required two months to take effect and it not only decreased testosterone produced locally, but in the general circulation as well, thus affecting libido which makes it unacceptable for human use (Ratnasooriya, 1982; Paulsen *et al.* 1982; Knuth and Nieschlag, 1987).

INMUNOLOGICAL APPROACH

At present a great deal of research is being directed towards finding effective contraceptive vaccines for female use against, gonadotrophins, inhibin and spermatozoa. Inactivation of peptides involved in sperm-zona adhesion, sperm activation, sperm oocyte fusion and sperm activated oocyte cleavage, GnRH (Wu, 1988; Bhasin *et al.* 1986; Lincoln, 1992). But so far this approach is still in the realms of research and no commercial vaccines are as yet available.

In the male dog, some work has been done in immunisation against LH and GnRH, with varied results (Gonzalez *et al* 1989). Chemical occlusion of the epididymis by chemical substances applied directly to the tail of the epididymis (Pineda, 1978) has also been attempted or directly into the testis (Naz and Talwar, 1981). However, so far the only method known to effectively suppress male dog fertility is the surgical removal of gonads.

AIM OF THE THESIS.

The aim of the work was to attempt to alter spermatogenesis in the dog by suppressing the release of gonadotrophins from the pituitary and to endeavour to identify the role of the Leydig cell in the production of spermatozoa. This thesis initially describes the administration of either Medroxyprogesterone acetate or GnRH agonists to several adult male dogs and records the clinical, histological and circulating plasma hormonal changes recorded during and at the end of each treatment period. It then describes and discusses the investigations undertaken to determine a suitable method for the isolation and culture of dog Leydig cells. The final section describes the findings after the administration of a GnRH agonist to two male dogs incorporating the changes determined by the results from the first experiments. It then describes the subsequent studies of Leydig cell cultures, using cells collected from normal and from the dogs treated with GnRH agonists.

CHAPTER 2-A

MEDROXYPROGESTERONE ACETATE (MPA) EXPERIMENTS

1. INTRODUCTION

As spermatogenesis is controlled by the hormones FSH and LH released from the pituitary and transported to the testes by the circulating plasma, it seems reasonable to attempt to manipulate spermatogenesis in male dogs, by suppression of these gonadotrophins. Initially, a progestogen was used for this purpose, as it is known to suppress gonadotrophin release from the pituitary. Adult male beagles were treated for varying periods of time and their circulating plasma LH and testosterone concentrations monitored prior to, and throughout the treatment period. In addition, semen was collected as often as possible, in advance of, and throughout the experimental period. Following sacrifice, samples were collected from scrotal contents and prostate for subsequent examination by histological and histochemical methods.

2. MATERIALS AND METHODS

Animals used

Animals used				
Name	Breed	Age (years)	Weight (kgs)	Body condition
Fred	Beagle	7	25	good
Gus	Beagle	4	17	good

Animals were maintained in colony accommodations, fed a diet of canned meat and meal. They were exercised twice daily for about half an hour and their external genitalia was examined frequently prior to attempts at semen collection.

2.1. EXAMINATION OF REPRODUCTIVE ORGANS

TESTIS

Size: A piece of string was cut to the dorsoventral length of each testis another to the width, determined by passing the string around the middle of the scrotum. Each piece of string was then measured against a ruler and the results recorded in centimetres.

Consistency. The consistency of the gonads was evaluated by manual palpation and any variations from normal noted.

EPIDIDYMIS

The presence of each tail of epidiymis was determined by manual palpation as well as evidence of the body and tail areas on the medial aspect of each testis noted.

PROSTATE

The size and position of the prostate was determined by introducing a lubricated gloved finger into the rectum

2.2. ULTRASOUND SCANNING OF SCROTAL CONTENTS AND PROSTATE

The scanner used had a 9.5 MHz sector transducer with annular phased array capability (Apogee CX, Interspec Inc. Aldermaston, Berks. UK). Prior to scanning the dogs were prepared by first clipping the hair of the scrotum and posterior abdominal region. Medical ultrasound gel was applied to the skin. Scanning was done with the dogs in a standing position.

Measurements made

Prostate. Long axis: length of right and left lobes .

Cross section: height and middle of right and left lobes.

Testes. The testes were scanned through scrotal wall and maximum width and height recorded in cross section. Measurements were taken after scanning in the different planes and the maximum measurement for each parameter was recorded.

2.3. LIBIDO

Libido was evaluated as follows :-

0 Male not interested either in the female or in the manual stimulation, no signs of erection.

1 A delayed response either to the presence of the bitch or to manual stimulation. Semen was only collected after a delay of at least five minutes stimulation.

2 Response to manual stimulation produced an erection within a few minutes of handling the penis. Semen was ejaculated almost immediately afterwards.

3 Normal response with a bitch, male very happy and excited, mounted very quickly. Erection and ejaculation occurred within two minutes.

2.4. SEMEN COLLECTION

Material required for collection.

Latex cone
Glass centrifuge tube attached to cone (Figure 14)
Plastic thermos bottle
(To transport semen from collection to evaluation area).

1.-Stimulation of the male .

A: Using a teaser bitch in oestrus.

B: Without a bitch.

A) Using a teaser bitch in oestrus.

The male dog was presented to a bitch in oestrus and allowed to sniff around the vulva for a few minutes. When the *bulbus glandis* started to enlarge, the penis was extruded such that the enlarged *bulbus glandis* was exteriorised from prepuce. The penis was then held behind the *bulbus glandis* and directed towards the collecting latex cone (Figure 14). After ejaculation of the first fraction when the dog showed violent thrusting movements, the penis was directed backward between the animal's hind legs, in the same position as in natural mating. Some first fraction was collected along with the entire second fraction and as soon as the third fraction started to be produced the collection was stopped.

B) Without a bitch present.

In order to collect from the dogs when a bitch was not available, the animals were always taken to a specific site of collection where a bitch had previously been used. The penis was stimulated by digital manipulation by rubbing over the prepuce with forward and backward movements of the hand over the area of the

bulbus glandis. As soon as the *bulbus* started enlarging, the penis was extruded and the ejaculate collected as discussed above.

2.5. SEMEN EVALUATION

Immediately after collection the volume and colour of ejaculate were evaluated.

Colour classified as observation	
White	Normal, not contaminated.
Yellow	Abnormal urine contamination
Red, pink	Abnormal, blood contamination.

Motility

.Material required to evaluate motility.

Prewarmed glass slides (Gold Star, Chance Propper Ltd. England)

Pasteur pipettes (Bilbate, Ltd., Daventry, England)

Glass cover slips (Chance Propper Ltd. England)

Microscope

Procedure. Immediately upon arrival in the laboratory, motility was evaluated by placing a drop of ejaculate on a prewarmed glass slide and a cover slip was placed over it. The movement of the sperm was observed under the 10 x objective and scored for the percentage of progressive motility seen in the slide.

The score was as follows

Motility scale	Limits %
5	90-100
4	80-90
3	70-80
2	60-70
1	50-60

Live dead spermatozoa evaluation.

Material required

Nigrosine-Eosin stain

Glass slides

Pasteur pipettes.

Reagents for Nigrosine-Eosin stain	
Nigrosine	5.0 grams
Eosin (yellowish)	0.84 grams
Sodium citrate	1.45 grams
Distilled water	50.00 mls

All reagents were purchased from BDH Merck, Thornliebank Glasgow, Scotland.

The Nigrosine-eosin was prepared as follows:

1.- Dissolve the sodium citrate in water.

2.- Dissolve the eosin in 8-10 ml of the citrate solution.

3.- Heat the remainder of the citrate solution by placing into a 100 °C

water bath and then adding the nigrosine.

4.- Add the eosin/citrate solution.

5.- This was then heated for 20 minutes and the mixture occasionally mixed.

6.- The beaker was covered when not stirring to prevent evaporation.

7.- The solution was then filtered while still warm.

Smear preparation: A nigrosine-eosin smear was prepared as follows: Using a Pasteur pipette one drop of semen was placed on a clean dry microscope glass slide to which an equal amount of nigrosine-eosin stain was added. The stain had been prewarmed to 32°C in a water bath. Using the tip of a clean dry pipette, both the semen and the stain were thoroughly mixed and left to settle for 30 seconds. Then a smear was made and air dried. Once dried, a drop of immersion oil was placed over the slide for examination under the 100 x objective. One hundred cells were counted and the number of dead sperm was recorded as those that absorbed the stain and therefore looked pink. Those that did not stain and appeared clear were recorded as live.

Concentration

Concentration was determined using a hemocytometer by the method of Zaneveld and Polakoski (1977).

1. A 1:20 dilution was prepared by adding 20 microlitres of ejaculate to 400 microlitres of a 10% formalin solution in an Eppendorf vial.

2. The suspension was thoroughly mixed by shaking the vial 10 microlitres of this suspension was placed in both chambers of a standard haemocytometer.

3. The spermatozoa settled on the bottom of the chamber and once they stopped drifting the count was made.

4. The spermatozoa found in the 4 small corner squares and the central ones in the large central square were counted. The multiplication factor was thus 50,000.

The formula used to calculate sperm concentration was:

1. -Sperm concentration = no. of sperm counted x multiplication factor x dilution factor.

2. -The dilution factor was 20.

3. -The multiplication factor was 50,000, because 5 squares were counted.

4. - Therefore: Sperm concentration = Number of sperm x 50,000 x 20.

SPERMATOZOA ABNORMALITIES

Using a nigrosine-eosin stained smear and the 100 x magnification, one hundred individual sperm were carefully examined for abnormalities of the head, neck and tail, as well as for changes in the acrosome.

Acrosome evaluation.

Smear preparation: A nigrosine-eosin smear was prepared as follows: Using a Pasteur pipette one drop of semen was placed on a clean dry microscope glass slide to which an equal amount of Nigrosine-eosin stain was added. The stain had been prewarmed to 32°C in a water bath. Using the tip of a clean dry pipette, both the semen and the stain were thoroughly mixed and left to settle for 30 seconds. Then a smear was made and air dried. Once dried, a drop of immersion oil was placed over the slide for examination under the 100x objective. One hundred cells were counted and the some evaluation, Spermac stain (Stain Enterprises, P.O. Box 152, Wellington, Rep. S. Africa 7655) was used according to the method of Oettle (1986). A smear was prepared by placing a drop of semen on a clean glass slide and by spreading it across with the aid of another glass slide, allowed to dry in air and stained as according instructions.

2.6. PROCEDURES FOR BLOOD SAMPLING :

Blood samples were collected by:

a) *Venepuncture*: A sterile 21 gauge 1 inch needle attached to a 10 ml lithium heparin monovette tube (Sarstedt, Leicester England) was used and 5 ml. samples were taken.

b) *Cannulation.* Prior to cannulation the animal was sedated with 0.5 ml of ACP (C.Vet Ltd. 2 mg/ml). The skin over the jugular vein was clipped, washed with an antiseptic solution (Pevidine BK Vet. Prods. Ltd.) and rinsed with absolute alcohol. The skin around the point of entry of the cannula was infiltrated with 2ml of Lignocaine-A (Univet Ltd.).The jugular vein was then cannulated using a G18 Intraflon 2 trochar catheter iv length 38 mm x diameter 13 mm. (Teflon, Vygon). Once inserted, the cannula was secured by adhesive tape and a bandage. Finally the cannula was flushed in situ with 2 ml of 0.5% solution of heparin in warmed saline.

Procedure for collection of blood sample from testicular vein :

This was done according to the method of Sharpe & Maddock (1988). The animal was premedicated with ACP and general anaesthesia induced with Thiopentone sodium (Thiovet. C. Vet. UK) The *tunicae albuginea* and *vaginalis* were incised exposing the veins on the surface of the testis. Either a 26 or a 30 gage needle was inserted into one of the larger testicular veins and 1-2 ml of blood were collected using a 2ml heparinised syringe.

Subsequent treatment of blood samples.

Blood samples were centrifuged at 3000 g for 10 minutes and the separated plasma stored in 2 ml Eppendorf tubes, and stored at -20 ^{o}C.

2.7. DOG TREATMENT

1st Treatment. 100 mg progesterone subcutaneous implant (Organon Laboratories, Ltd. Cambridge, England UK). The implant was inserted

through a small incision in the neck. The skin around the area was previously infiltrated with 2 ml of Lignocaine-A..

2nd Treatment. 75mg/ml subcutaneous injection of Depoprovera, Medroxyprogesterone Acetate (MPA), (Upjohn Ltd, Crawley UK) administered once.

3rd Treatment. 100 mg/ml subcutaneous injection of Depoprovera, Medroxyprogesterone Acetate (MPA), (Upjohn Ltd. Crawley UK), was administered as a single dose.

2.8. HORMONE ASSAYS

The analysed hormones were progesterone, LH and testosterone.

PROGESTERONE ESTIMATION

Plasma progesterone concentration was determined using a progesterone enzyme-linked immunosorbent assay kit (Ovucheck Bovine Plasma, Cambridge Life Sciences PLC.) as described by Eckersall and Harvey (1987). All samples were assayed in duplicate.

10 µl of standard or sample was added to wells precoated with an antibody, followed by the addition of 200 µl of progesterone labelled with phosphatase alkaline. After 30 minutes incubation at room temperature, the wells were washed 3 times with distilled water and 200 µl of a solution (pH 9.8) containing 1M diethylanolamine and 0.5 mM magnesium chloride acting as the enzyme substrate added and incubation continued for a further 30 minutes in the same conditions. The reaction was stopped by adding 100 µl of a solution containing 0.5mM di-potassium hydrogen orthophosphate and 5mM ethylenediamine tetracetic acid (EDTA), (pH 10.0). A spectrophotometer (Titertek Multiscan Plus) was utilised to read the absorbance at 405nm. The curve was plotted and results calculated using a personal computer connected to the spectrophotometer and programmed for calibration of data from Elisa assays (Titersoft, Flow Laboratories). Cross reactivity with steroids other than progesterone has been calculated by the manufacturer and was found to equal 66, 16, 4.5, 3.3 and 3.0% with 11-alpha-hydroxyprogesterone, 5-pregnan-3-B-ol-20-one,5-B-pregnan-3,20-dione,5-alpha-pregnane-3,20-dione and deoxycorticosterone acetate respectively

and was insignificant (<1%) with other steroids. Limit of detection calculated by Eckersall and Harvey (1987) was 0.5 ng/ml. The inter-assay coefficient of variation calculated after assaying the same control sample in 16 assays was 12.34% at 4 ng/ml. The intra-assay coefficient of variation estimated by assaying the same sample in 10 replicates was 11.3% at 4 ng/ml.

LUTEINIZING HORMONE ESTIMATION.

Preparation of buffers.

General purpose buffer 0.5M phosphate buffer (pH7.4) was prepared and stored at room temperature.

358.15g di-sodium hydrogen orthophosphate dodecahydrate (Na_2HPO_4. $12H_2O$, MW=358.15 (Fison Laboratory Supplies, Loughborough, England) were dissolved in 2 litres distilled water.

39g sodium dihydrogen orthophosphate dihydrate ($NaH_2PO_4.2H_2O$, MW= 156.01: Formachem Ltd. Strathaven, Scotland) were dissolved in 500 ml distilled water.

When both solutions were homogeneous and clear, 475 ml of solution 2 was added to 2 litres of solution 1 and pH was checked.

LH assay buffer: Fresh assay buffer was made weekly by dissolving 5g bovine serum albumin (Sigma Chemical Co., St. Louis, USA) and 2g sodium azide (BHD Chemicals Ltd. Poole, England) into the general purpose buffer followed by the addition of 1 ml iso-octylphenoxy-polyethoxyethanol (Triton X-100, BDH Chemicals Ltd. Poole, England).

Procedure: Plasma LH was measured by the double antibody radio-immunoassay method of Boyns *et al.* (1972) with modifications as follows. Iodination, was described by Renton *et al.* (1991) 100 µg purified ovine LH (LER 1374A, kindly donated by Dr. L Reichert, Albany Medical College) was reconstituted with 500 ul $0.5M^{-1}$ phosphate buffer and aliquots of 100 µl (2µg LH) each were put into conical iodination tubes (Sarsted Ltd, Leicester, England) and stored at -80°C. A freshly prepared solution of chloramine T (10 mg in 10 ml $0.05M^{-1}$ phosphate buffer pH 7.4) was added and left for 30 seconds. The reaction was stopped by the addition of a freshly prepared solution of sodium metabisulphite (20 mg. in 10 ml 0.05 M phosphate buffer (0.05M phosphate buffer containing 0.9% (w/v)

sodium chloride, 0.5% (w/v) bovine serum albumin (BSA) and 0.1% (w/v) sodium azide). For every assay, the working solution was prepared by further diluting the label with assay buffer to give 12000 to 17000 counts per minute per 100 µl.

The first antibody used, rabbit anti-rat LH serum (CSU120) at a dilution of 1:20 in assay buffer (0.05 M^{-1} phosphate buffer containing 0.25% w/v BSA, 0.1% (w/v) sodium azide and 0.05% (v/v) triton X-100) was supplied by G.D. Niswender who has successfully used this antibody for canine LH assays (personal communication). The standard used was purified pituitary canine LH (LER 1685-1; Dr. L. Reichert) to which it was found necessary to add LH-free serum (prepubertal bitch blood) and serial dilutions were made at concentrations ranging from 50 to 0.788 ng /ml. Recovery studies revealed that the addition of 12.5 ng. of canine LH/ml gave a recovery value of 81% and the addition of 6.25 ng of canine LH/ml gave a recovery of 68%.

The second antibody used was Dynospheres donkey anti-rabbit provided by the Royal Infirmary, Glasgow (McConway *et al.* 1989).

The assay was set up to include 4 tubes for total counts (TCs), two tubes for non-specific binding (NSB), and two tubes for each of the standards and samples. In all assays, 100 µl of assay buffer was added to the (0) standard tubes and 100 µl of every standard or plasma samples was added to the other identified tubes. The first antibody was then added (100 µl) to all tubes except the TCs and NSB tubes. Assay buffer (100 µl) was added to the NSB tubes.

The reagents were mixed by gentle shaking of all tubes and incubation was carried out overnight at 4°C. The following day, 100 µl of the radioactive label was added to all tubes, reagents were mixed and incubation continued overnight at 4°C. Separation of the bound and free fractions was achieved by addition of 100 µl of second antibody to all but the total counts (TCs) tubes followed by a further incubation at room temperature for at least one hour.

Physiological saline (1 ml) was added to all tubes except the TCs. Centrifugation at 2000 g at 4°C for 25 minutes was carried out and the supernatant aspirated and decanted. The precipitated pellet was counted using an auto gamma scintillation spectrometer (Packard 5230, Berks England). The curve was plotted and results calculated using a computer program (SAS Immunoassay Program 632014).

Plasma samples of known high and low LH were included twice in every assay to act as quality controls.

The limit of detection defined as twice the standard deviation of the blank values (i.e.maximum binding) was 2.96-50 ng/ml assessed over 10 assays. The inter assay coefficient of variance calculated from 10 assays was 2.6% at 12.67 ng LH/ml and 13.55 % at 3.018 ng LH/ml. The intra-assay coefficient of variance was 3.89 % and was assessed by the duplicate samples of a randomly selected assay and was calculated by the method of Chard, 1982 as follows.

$$CV = \sqrt{\frac{\sum(\frac{d}{\bar{x}} \times 100)^2}{2N}}$$

Where: d= the difference between duplicate estimates

X= the mean of duplicate estimates.

N= the number of duplicate estimates.

The composite standard curve from 10 assays is shown in Figure 13.

TESTOSTERONE EVALUATION.

Plasma testosterone was measured by the double antibody ether extraction radio immunoassay (RIA) as previously described by Cook and Beastall (1987). The ^{125}I Histamine-testosterone prepared by chloramine-t method (Cook and Beastall, 1987), was kindly provided by Dr.C.E.Gray of the Department of Pathological Biochemistry, Royal Infirmary, Glasgow UK. The rabbit-antibody to testosterone (AB-1030) was obtained from Bioclinical Services Ltd. (Cardiff, UK.) According to the suppliers, the testosterone antibody was raised against a testosterone-3-(O-carboxy methyl) oxime-bovine serum albumin conjugate and shows the following cross-reactivity: testosterone, 100%; 5 alpha dihydrotestosterone, 16%; 5 alpha-androstane-3 alpha 17- diol, 5.8%; 5 alpha-androstane-3 beta,17 beta diol, 3.7%;androstenedione, 2.1% dihydroepiandrosterone, 0.04% and cortisol <0.01%. Testosterone standards ranging from 0-34.8 nmol l^{-1}, were prepared by serial dilutions in donkey serum (9SO77-220) which was provided by SAPU. This donkey serum was obtained

from a pool of blood obtained from a gelding and two female donkeys and contained non-detectable levels of testosterone in RIA. Donkey anti-rabbit (DAR) and normal rabbit carrier serum (NRS) were provided by SAPU.

100 microlitre of sample or standard in duplicate was pipetted into 13 x 100 mm clean borosilicate glass tubes (Ciba-Corning, Essex, UK.) 3 ml di-ethyl ether (Analar grade, Merck, Thornliebank, Glasgow, Scotland) was added and tubes were vortexed for 5 min in a Baird and Tatlock Multivortex Shaker (Searle Instrument, Harlow, England). A methanol-dry ice bath was used to freeze the aqueous layer and the ether phase containing testosterone was carefully decanted into clean 10.5 x 70 mm borosilicate assay tubes. Ether was evaporated in a fume cupboard using a Tecam SC-3 sample concentrator (Techne, Cambridge, UK) connected to a vacuum pump.

The dried extraction residues were re-dissolved in 300 ul assay buffer (0.05M phosphate buffered saline, 0.25% w/v bovine serum albumin, pH 7.4) vortexed and 100 ul of the primary anti-testosterone antibody (1:6000) and 100 µl of the tracer (containing approx. 10,000 cpm) were added. The tubes were vortexed again and incubated at room temperature for 2 h. Thereafter, 500 µl of the double antibody reagent containing DAR and NRS (1:80 and 1:1000 respectively) was added and the tubes were incubated overnight at 4°C. The antibody-bound fraction was separated from the unbound by centrifugation at 1,500 g for 25 min at 4°C using a MSE Mistral 6L Centrifuge (Fisons Instruments, Sussex, UK) and the supernatant was aspirated using a finely drawn metal pipette connected to an aspirator pump attached to a running water tap. Counting and calculation was done in a Gamma counter Minaxi, Auto Gamma 5000 series (United Technologies Packard UK).

Using this technique, the efficiency of testosterone ether extraction was found to be over 96% following vortexing for 2-4 min. (Cook and Beastall 1987). This was verified by Mutayoba,1994 who used 100 µl aliquots of donkey serum spiked with a known amount of standard testosterone (1.45, 12.5 and 32.6 nmol l), which were extracted using 3 and 10 ml di-ethyl ether for 4 and 10 min and run in a normal RIA. Extraction using 3 ml of ether and vortexing for 4 min produced similar testosterone recoveries (range 96.3-101.4%) as those obtained by using 10 ml of ether and extraction for 4 (range 95.2-102.9%) or 10 min (range 97.8-104.1%). In subsequent assays the volume of 3 ml ether and extraction of 5 min was used and no recoveries were monitored.

The limit of detection was 1.39 to 34.80 and was defined from the point where the standards CV changes to more than 22%. The inter assay coefficient of variance calculated from 8 assays was 21.37% at 7.58 nmol/l and 28.2 % at 2.78 nmol/l. The intra-assay coefficient of variance was 9.8% and was assessed by the duplicate samples of a randomly selected assay by the same method and formula as indicated for LH. The composite standard curve from 8 assays is shown in Figure 12.

RIA Buffer (pH 7.4)

Reagents.
Stock solutions were prepared as follows:
A.- 0.25M Sodium dihydrogen orthophosphate Made by dissolving 9.8 grams NaH_2PO_4. $2H_2O$ (MW 156.01) in distilled water to make up to 250 ml
B.- 0.5M Disodium hydrogen orthophosphate Made by dissolving 35.5 grams Na_2 HPO_4 anhydrous (MW 141.96) or 89.54 grams Na_2HPO_4. $12H_2O$ (MW 358.17) in distilled water and make up to 500 ml.

RIA Buffer was prepared as follows:
Stock solution:
1.- Dissolve 17.5 g sodium chloride + 0.5 g Merthiolate (Thiomersal) in 250 ml distilled water.
2.- Add 30 ml solution **A** and 120 ml solution **B** and mix.
3.- Check the pH and if necessary adjust to pH 7.4 with NaOH or HCl solution.
4.- Make the solution up to 2 litres with distilled water, mix and recheck the pH.
5.- Store at 4 ^{o}C for up to 6 months.

Diluent buffer

RIA buffer plus 0.25I% BSA (Sigma). This buffer is prepared fresh for each assay

2.9. HEMICASTRATION

Premedication: 0.5 ml of 10 mg/ml Acepromazine Maleate BPC (ACP- C-Vet. Ltd) was injected intramuscularly. General anaesthesia was induced with iv Thiopentone sodium at a dose of 30 mg/kg.

1. The dog was positioned in dorsal recumbency, with his hind limbs stretched out and tied to the operating table.

2. The hair between the thighs and the posterior abdomen was clipped and thoroughly washed with an antiseptic solution (Pevidine-A) and then rinsed with 70% alcohol.

3. The testicle to be removed was pushed forwards out of the scrotum and an incision made over the tensed testis cutting through the skin and subcutaneous tissue

4. The testis and spermatic chord were exteriorised.

5. The exposed spermatic cord was ligated with gut and then severed.

6. The testis was then removed.

7. The subcutaneous tissue was then sutured with simple interrupted sutures .

8. The skin was also sutured with simple interrupted sutures with silk.

9. After the procedure, 1.7 ml of Duphapen LA (Duphar Veterinary Limited) was injected s/c for the following 2 days.

2.10. TISSUE PROCESSING FOR HISTOLOGY

Fixative used for all histology samples was Bouin's fluid (Culling, 1974).

Reagents used for Bouin's fluid	
Picric acid, saturated aqueous solution	75 ml
Formalin (40% formaldehyde)	25 ml
Glacial acetic acid.	5 ml

Preparation of Bouin's fluid.

The picric acid was placed into a flask into which the formaldehyde and glacial acetic acid were added and then mixed. This solution will last indefinitely without degrading.

Tissue fixation. Pieces of tissue of approximately 0.5 cm^3 were immersed in Bouin's fluid for 12- 24 hours.

Processing of fixed tissue samples: These samples were processed in an automatic Histokine 24 hour tissue processor (Shandon Elliot Ltd.) The complete process took 29 hours.

The process stages were	
2 Hours	70% methylated spirit.
2 Hours	70% methylated spirit + 5% phenol.
2 Hours	90% methylated spirit + 5% phenol
2 Hours	absolute alcohol + 5% phenol
1 Hour	absolute alcohol + 5% phenol
1 Hour	absolute alcohol + 5% phenol
4 Hours	2% celloidin in methyl benzoate.
1 Hour	histoclear
1 Hour	histoclear
1 Hour	histoclear
6 Hours	paraffin wax
6 Hours	paraffin wax

Preparation of Histological Sections

Using an optical microtome (Spencer AO 821, American Optical Company, Buffalo 15, NY, USA) 5 micron thick sections were cut from the paraffin blocks containing the samples, mounted on glass slides and dried in an oven at 56°C for one hour.

2.11. HISTOCHEMISTRY

I.-Haematoxylin and eosin (H & E)

Haematoxylin and eosin stain were prepared and used according to the method of Mayer (1903).

Reagents.	
Haematoxylin	1.0 grams
Potassium alum	50.0 grams
Sodium iodate	0.2 grams
Citric acid	1.0 grams
Chloral hydrate	50.0 grams
Distilled water	1000.0 ml

Preparation procedure:

Haematoxylin

1.-The haematoxylin was dissolved in the distilled water at 60 ^{o}C.

2.-The alum was then added and dissolved, again at 60 ^{o}C.

3.-The sodium iodate, citric acid and chloral hydrate were then added.

4.-The mixture was boiled for five minutes, then cooled and filtered.

Eosin:

Reagents	
Eosin	3 grams
Absolute alcohol	100 ml

Preparation procedure:

The saturated eosin solution was prepared by adding the eosin to the absolute alcohol.

Staining of histological sections:

1. Sections were placed and agitated in xylene for 1-2 minutes.
2. Transfer to absolute alcohol and leave for 1 minute.
3. Remove and place in methylated spirits for 1 minute.
4. Rinse in water.
5. A few drops of iodine were added and left in the solution for 1-2 minutes.
6. The sections were rinsed in water for 30 seconds and hypoed for 1 minute with 5% sodium thiosulphate.
7. Washed again in water and immersed rapidly three times in acid alcohol.
8. Washed again in water for 30 seconds.
9. Blued in Scott's tap water (2g potassium bicarbonate and 20 g magnesium sulphate in 1 litre distilled water) for 90 seconds.
10. Washed in water for two minutes and rinsed in methylated spirit.
11. Dehydrate, cleared and mounted in DPX.

II.- Martius, Scarlet, Blue (MSB).

This stain was prepared and used as described by Lendrum et al (1962).

Reagents for Martius yellow	
Martius yellow	0.5 grams
Phosphotungstic acid	1.0 grams
95% Alcohol	100 ml

Preparation :

The Martius yellow and the phosphotungistic acid were dissolved in the 95% alcohol.

Reagents for Brilliant crystal scarlet (1%)	
Brilliant crystal scarlet (acid red 44)	1.0 grams
Glacial acetic acid	2.0 ml
Distilled water	100 ml

Preparation:

Brilliant crystal scarlet was dissolved in glacial acetic acid and water was added .

Reagents for Methyl blue (0.5%)	
Methyl blue (acid blue 93)	0.5 grams
Glacial acetic acid	1.0 ml
Distilled water	100 ml

Preparation :

Methyl blue was dissolved in glacial acetic acid and water was added to make up this solution.

Staining of histological sections:

1. Sections were dewaxed and washed in water.
2. They were taken through the preliminary stages as described for haematoxylin staining up to the acid alcohol treatment stage.
3. Following a good wash in tap water, sections were rinsed in 95% alcohol.

4. Stained in 0.5% Martius yellow/phosphotungstic acid for two minutes.

5. Rinsed in distilled water and stained in 1% brilliant crystal scarlet for ten minutes.

6. Rinsed in distilled water.

7. Treated with 1% phosphotungstic acid in distilled water for ten minutes.

8. Rinsed in distilled water.

9. Stained in 0.5% methyl blue solution.

10. Rinsed in 1% acetic acid.

11. Rinsed in water.

12. Dehydrated.

13. Cleared an mounted in DPX.

2.12. EXAMINATION OF HISTOLOGICAL SECTIONS.

Sections of testis and epididymis (head, body, tail) were examined under light microscope at magnifications of x 125, x 250, x 500 and x 1250. All the fields in each slide were examined to determine changes in the cell population of seminiferous tubules, the main findings reported were in relation to:

Testicular tissue:

1 Generation of germinal cells present in the seminiferous tubules.

2 Presence or absence of spermatids and spermatozoa in the lumen of the seminiferous tubules.

Epididymis:

1.Changes to the number of cell layers

2. Changes in the cellular arrangement .

3. RESULTS

Table 1. Protocol for the Medroxyprogesterone acetate (MPA) treatment of an adult male beagle (Fred) for two months.

Days	Procedure
-7	Semen collection
-6 to -4	Daily bleeding
-3	Bleeding every 15 min. for 1 hour. 3 Samples collected at: 2,3,5 hours later.
0	Semen collection Insertion of a 100 mg·progesterone implant (Organon Laboratories Ltd. Cambridge UK).
1-60	Daily bleeding.
2 27	Depot injection of 1/2 ml Depot injection of 1 ml Of 150 mg/ml of Medroxyprogesterone acetate (MPA) (Depoprovera, Upjohn Ltd. West Sussex UK)
+7,14,21	Semen collection
30	Hemicastration

Table No. 2 Protocol for the Medroxyprogesterone acetate treatment of an adult male beagle (Gus) for 3 months.

Days	Procedure
-7	Semen collection
-7 to -2	Daily bleeding
-1	Bleeding every 15 min. for 2 hours
0	1st 50 mg. MPA depot injection
2-4	Daily bleeding
20, 34, 48, 62, 76	100 mg Depot injection of MPA
18-20 & 21-23 32-34 & 35-37 46-48 & 49-51 59-62 & 63-65 74-76 & 77-79	Daily bleeding
+ 7, 14, 21, 28, 35, 42, 49, 56, 63, 70, 77, 84.	Semen collection
20, 34, 48, 62, 76	Scanning
90	Bleeding every 15 min. for 2 hours

Table 3. Examination of the external genitalia of this animal (Fred) during the first month of treatment revealed little evidence of any change in scrotal contents. Several unsuccessful attempts to collect semen took place before the first ejaculate was obtained on the 28th of June. The initial two ejaculates were yellowish in colour but the subsequent ones were cloudy. On the 23rd of July the ejaculate evaluation showed an increase in tailless sperm. On the 5th of August before the 2nd injection was given, the ejaculate collected contained blood. On the day the injection was given a slight enlargement of the left epididymis was noticed. It felt soft as if fluid filled. On August 13th the left testis was removed. After removal of the left gonad no attempt was made to collect semen, but the dog did attempt to mount a bitch in oestrous.

Table 3. Clinical findings, libido and semen evaluation of a dog (Fred) treated for two months with Medroxyprogesterone acetate.

		CLINICAL EVALUATION			SEMEN EVALUATION					ABNORMALITIES				
Date	Treatment route and dose in mgs	Libido	Test cons.	Epid.	Volume in ml	Color	Progess motl. %	Live:dead in 100	Head %	Acrosome %	Neck %	Tail %	Total	Comments
12/06/91		0	firm	normal	0									
13/06/91														Clinical examination normal
17/06/91		0	firm	normal	0									
24/06/91		0	firm	normal	0									
25/06/91		0	firm	normal	0									
28/06/91		2	firm	normal	1	yellow	4	97:3	2	normal	1	4	7	Collection with no bitch
29/06/91		1	firm	normal	0									Erection when checking dog
04/07/91		0	firm	normal	0									
09/07/91	P4.implant 100 mg	2	firm	normal	1	yellow	4	97:3		normal	8		8	Collection without teaser
12/07/91	Depot injection 75 mg													
17/07/91		2	firm	normal	1	cloudy	4	98:2	5	normal	7	6	18	Collection without teaser
23/07/91		2	firm	normal	1	cloudy	4	92:8	11	normal	7	21	39	Collection without teaser
30/07/91		2	firm	normal	1	cloudy	4	86:14	7	normal	8	5	20	Collection with teaser
05/08/91		2	firm	normal	1	bloody	0							Collection without teaser
09/08/91	Injection 150 mg		firm	enlarged										Enlarged left epididymis
13/08/91														Hemicastration
14/08/91		0	swollen											
15/08/91		0	swollen											
16/08/91		0	swollen											
17/08/91		0	sore											
18/08/91		0	sore											
19/08/91		0	sore											
20/08/91		0	sore											
21/08/91		0	sore											
22/08/91		1	sensitive											Attempted to mount bitch
28/08/91		0	sensitive											
07/09/91		0												
12/09/91		0												
13/09/91														End of experiment

Table 4. Presents the results of clinical examination of the reproductive tract of an adult beagle dog (Gus) as well as the frequency with which the treatment was given at fortnightly intervals for a total of three months. In addition, the seminal characteristics in ejaculates collected whenever possible during the same period are shown. Note that semen was only collected when there was a bitch in oestrus used as a teaser and that semen was only collected on two occasions towards the end of the treatment period. There was no clinical evidence of changes in any aspects of the external genitalia during the treatment period. On the two occasions that semen was collected, libido was excellent and colour and percentage live were within normal range.

Table 4. Clinical findings, libido and semen evaluations of a dog (Gus) treated with Medroxyprogesterone acetate for 3 months.

	CLINICAL EVALUATION				SEMEN EVALUATION.					ABNORMALITIES				
Date	MPA mg	Libido	Test cons	Epid	Ejac in ml	Color	Progress motility %	Concentration x (10)6 /ml	Live/dead	Head	Acrosome Neck	Tail	Total	Comments
05/09/91			firm	normal										Clinically evaluated, healthy
10/09/91		0	firm	normal										
17/09/91		0	firm	normal										
20/09/91		0	firm	normal										
24/09/91		0	firm	normal										
27/09/91		0	firm	normal										
30/09/91		0	firm	normal										
04/10/91		0	firm	normal										
08/10/91		0	firm	normal										
14/10/91			firm	normal										
17/10/91	50													
21/10/91		0	firm	normal										
24/10/91		0	firm	normal										
30/10/91	100													
31/10/91		0	firm	normal										
04/11/91		0	firm	normal										
08/11/91		0	firm	normal										
13/11/91	100													
14/11/91		0	firm	normal										
21/11/91		0	firm	normal										
25/11/91		0	firm	normal										
28/11/91	100													
30/11/91		0	firm	normal										
02/12/91		3	firm	normal	4.5	white	4	485	92/8	3	0	0	0	3	Collection with teaser
04/12/91		3	firm	normal	3	white	4	380	98/2	7	0	1	0	8	Collection with teaser
11/12/91	100													
07/01/92		0	firm	normal										
09/01/92														End of experiment

Figure 1. The frequent bleeding carried out prior to Fred's treatment indicated that the circulating plasma concentrations of LH ranged between (2-75ng/ml) and those of testosterone between (5-22 nmol/l). The concentrations of both hormones were greater in blood samples collected in the afternoon compared to those collected in the morning. Notice the unusually high (55.24 ng /ml) LH in the sample taken at 17 hours.

Fig. 1 Circulating plasma testosterone & LH in blood samples taken every 15 min. for 1 hour and at 2,3,5 hours. (Fred)

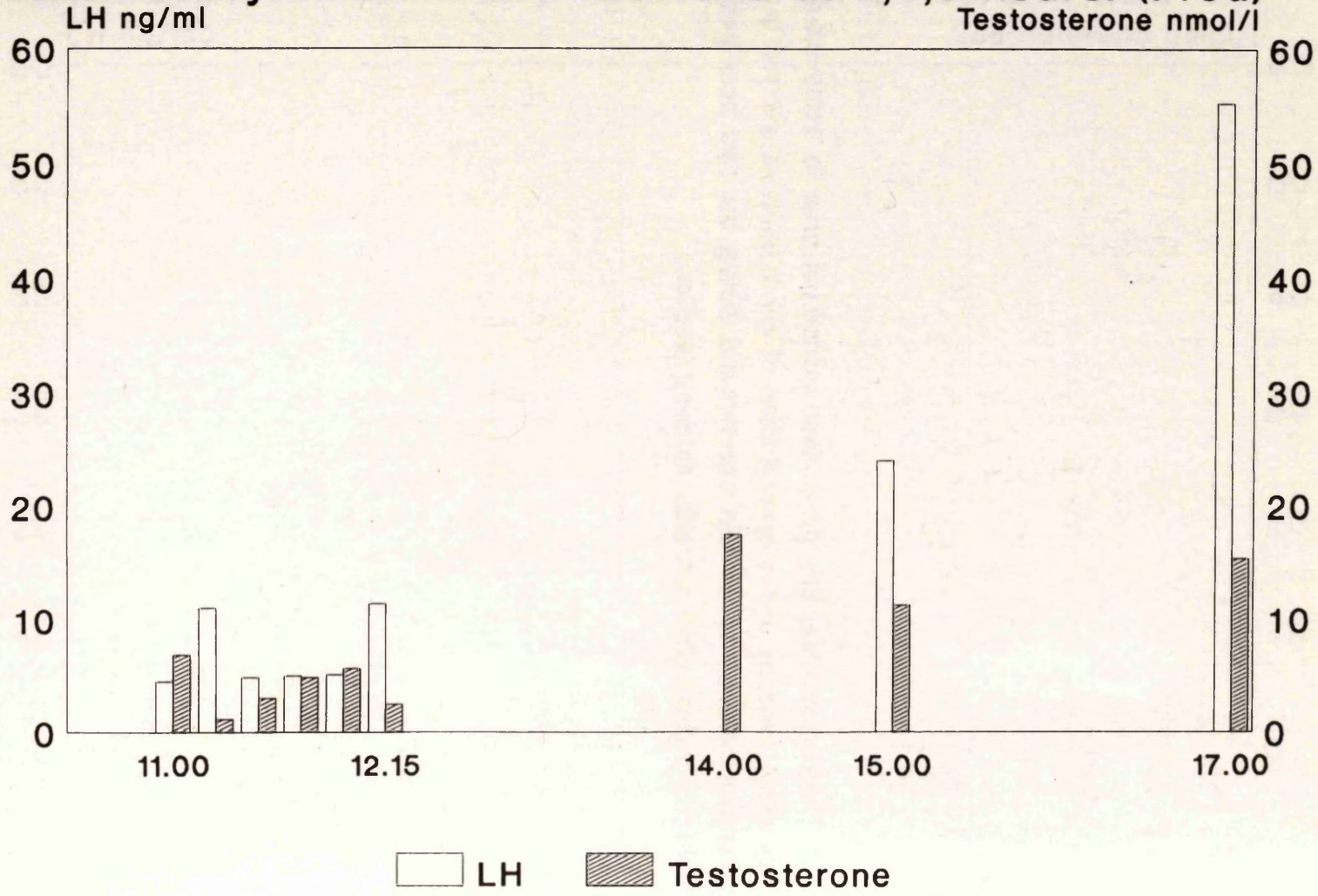


Figure 2. The frequent bleeding programme carried out prior to treatment does not show much variation in the concentration of either hormone assayed in the blood samples collected at 15 min intervals.and during the two hour period. Notice that the higher values are in the morning samples.

Fig. 2. Circulating plasma testosterone & LH in blood samples taken every 15 minutes for 2 hours in Gus.

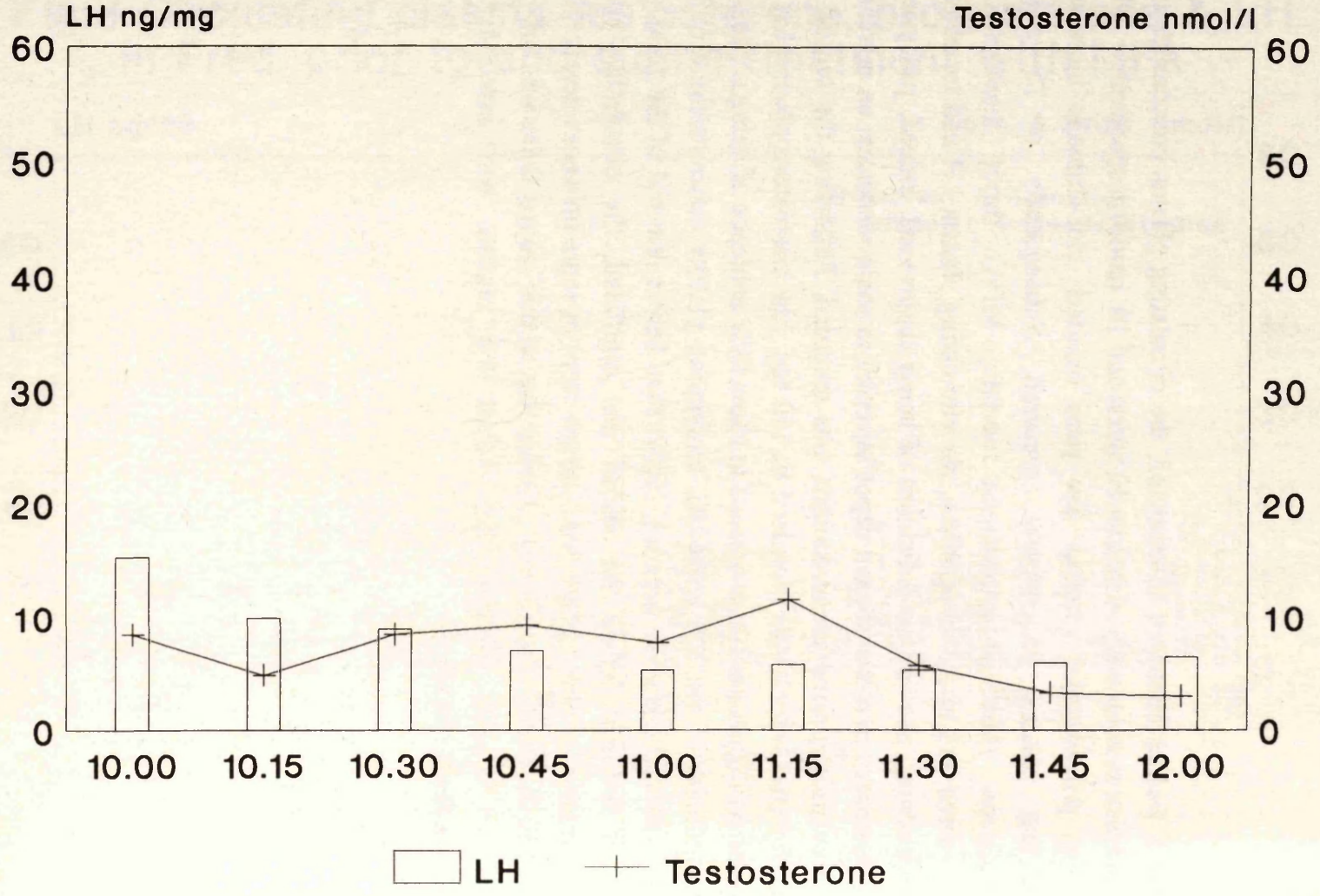


Figure 3. Before treatment commenced, the circulating plasma concentrations of LH and testosterone were all below 10 ng/ml and 10 nmol/l respectively. After a 100 mg. progesterone implant (**im**) was inserted no dramatic increase in circulating plasma progesterone occurred. Subsequently a 75 mg.(**in**) Depoprovera (Medroxyprogesterone acetate, MPA) depot injection was administered and after this injection, the circulating plasma LH and testosterone concentrations, showed no indication of being suppressed. Indeed, just prior to the administration of the second depot injection in some instances, an increase in the circulating plasma concentrations was recorded. Following the second (**in**) depot injection which was increased to 150 mg., the circulating plasma LH and testosterone concentrations continued to show little evidence of being suppressed. After removal of the left testis (**h**), circulating plasma concentrations of both testosterone and LH were generally higher than before removal of the gonad. In the final fortnight before the animal was sacrificed, the circulating plasma concentrations of both LH and testosterone were in some instances nearly 20 LH ng/ml and 20 nmol/l respectively. Notice that in this animal there was only one period of frequent bleeding (**w**) before the implant was inserted, thus corresponding to figure 1.

Fig.3 Circulating plasma testosterone, progesterone & LH in Fred, prior to and during treatment with MPA

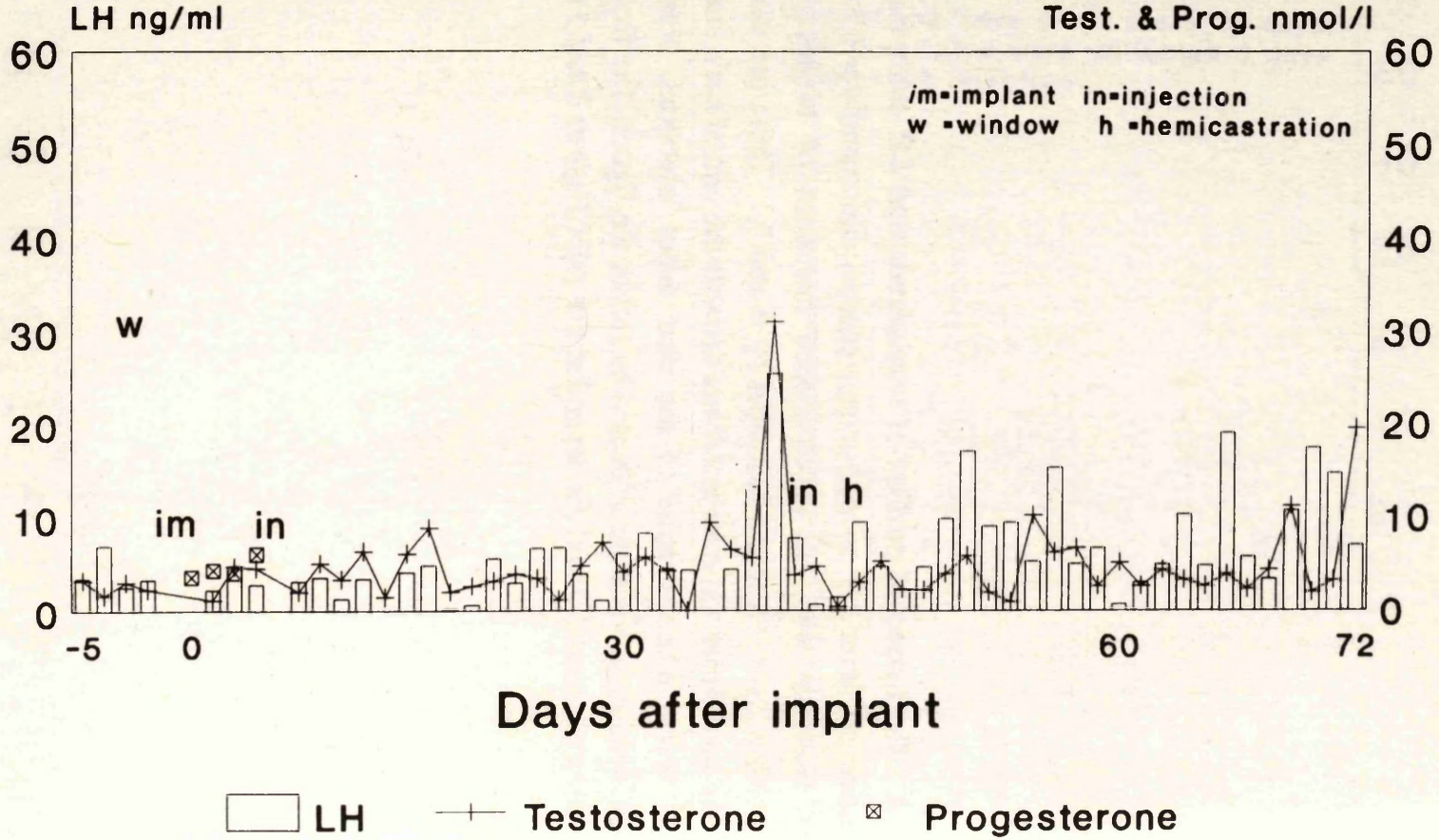


Figure 4. The hormonal profiles of testosterone and LH show no evidence of suppression of either LH or testosterone plasma concentrations during the three months treatment with six subcutaneous injections of Medroxyprogesterone acetate (MPA) and these are indicated by **0** and *. There are indications of a rise in the circulating LH concentrations towards the end of each monthly period, i.e. just before administration of the next depot injections. Notice that two frequent bleeding periods are indicated by (w) in the figure. The first was on the day the treatment started and the second at the end (Figures 2 and 5).

Fig. 4 Circulating plasma testosterone & LH concentrations prior to and during treatment with MPA for 3 months in Gus.

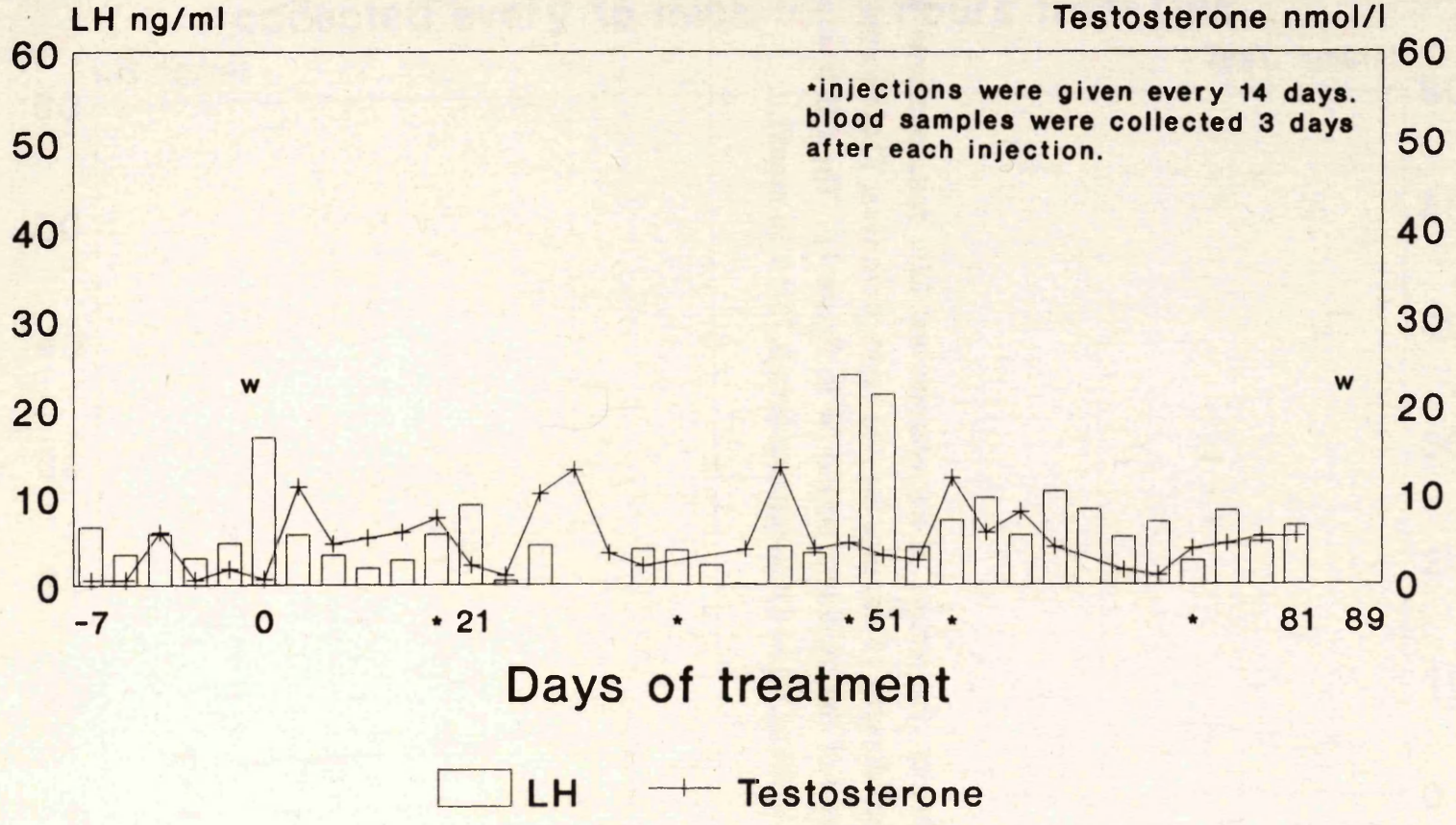


Figure 5. Presents the results of testosterone and LH concentrations in blood samples collected every 15 minutes for two hours from Gus. These samples were taken at the end of the treatment period (w in figure 4). The LH levels ranged between 4.4-10.60 ng/ml and testosterone from 3.75-16.16 nmol/l.

Fig.5 Circulating plasma testosterone & LH in blood samples collected every 15 mins for 2 hours from Gus

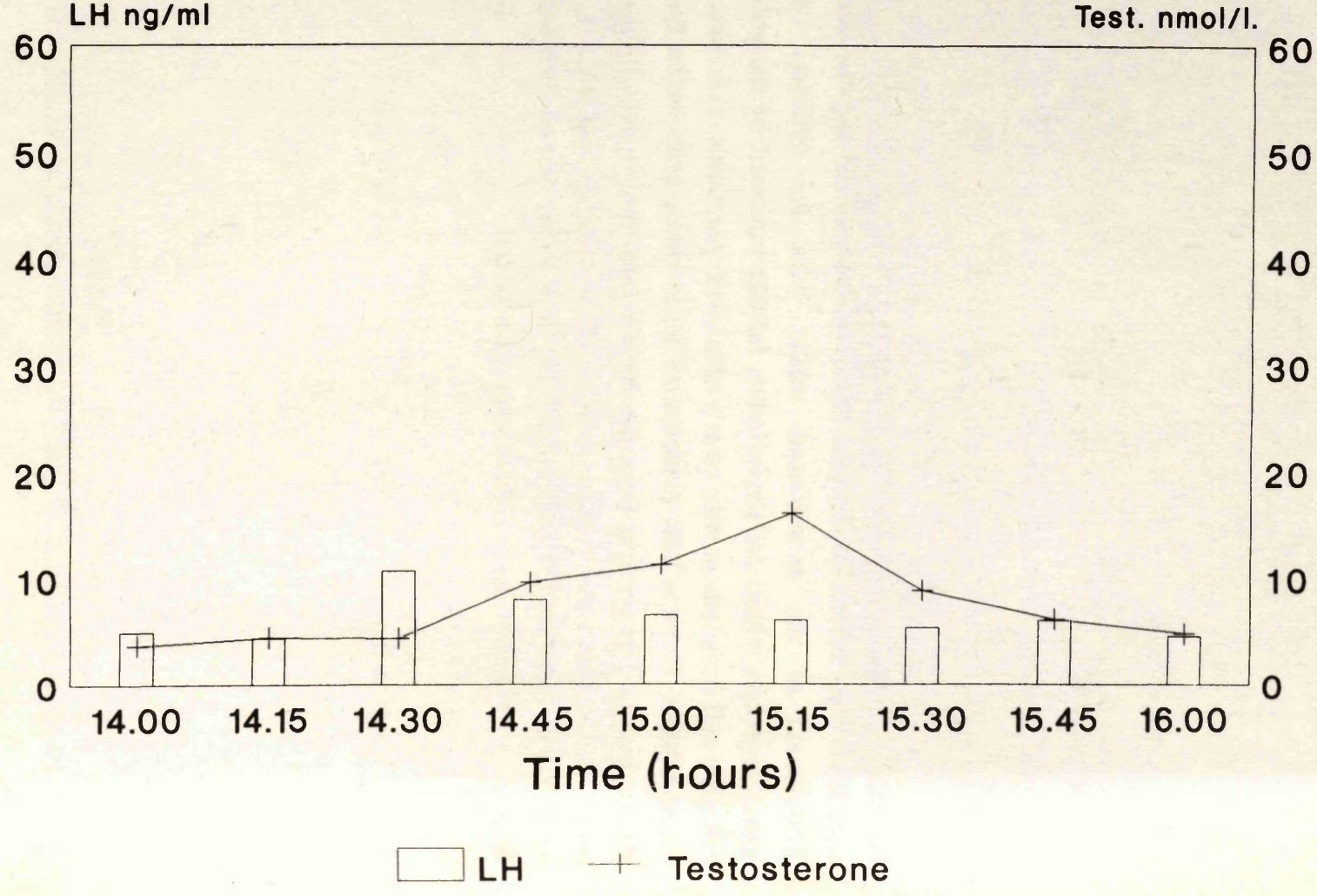
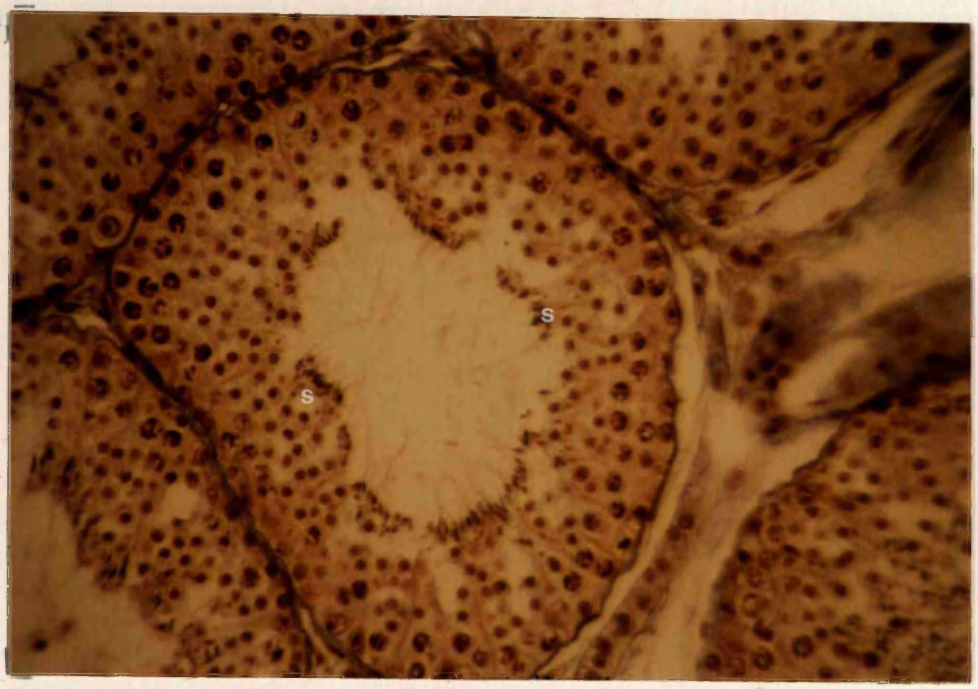


Figure 6. Histological sections of testes from a) normal dog and b) a dog treated with Medroxyprogesterone acetate. Notice in the normal dog, the very uniform arrangement of the seminiferous tubule. Also the evidence of ongoing spermatogenesis within the seminiferous tubules indicated by the presence of a full germ cell complement with clearly identified spermatids (s) around the lumen of the tubules. There is little evidence of degenerating cells within the lumen of these tubules. The section from the treated dog reveals very little difference, other than an increase in the amount of cell debris within the lumen of seminiferous tubules. Magnification 54 x. Both slides stained with MSB. A blue microscope filter was used in the bottom photograph.

a



b

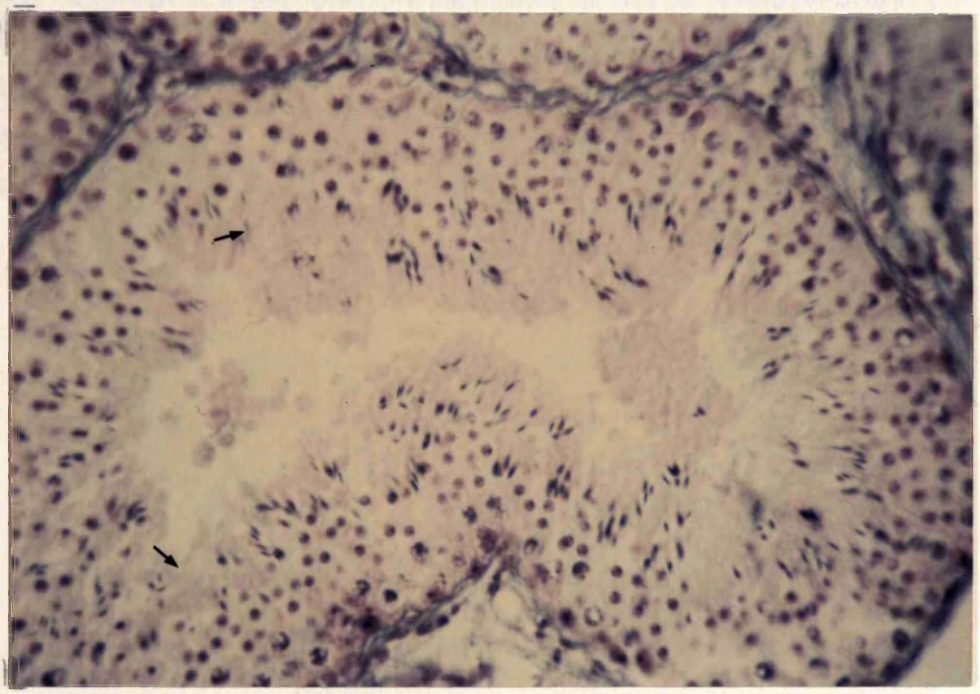
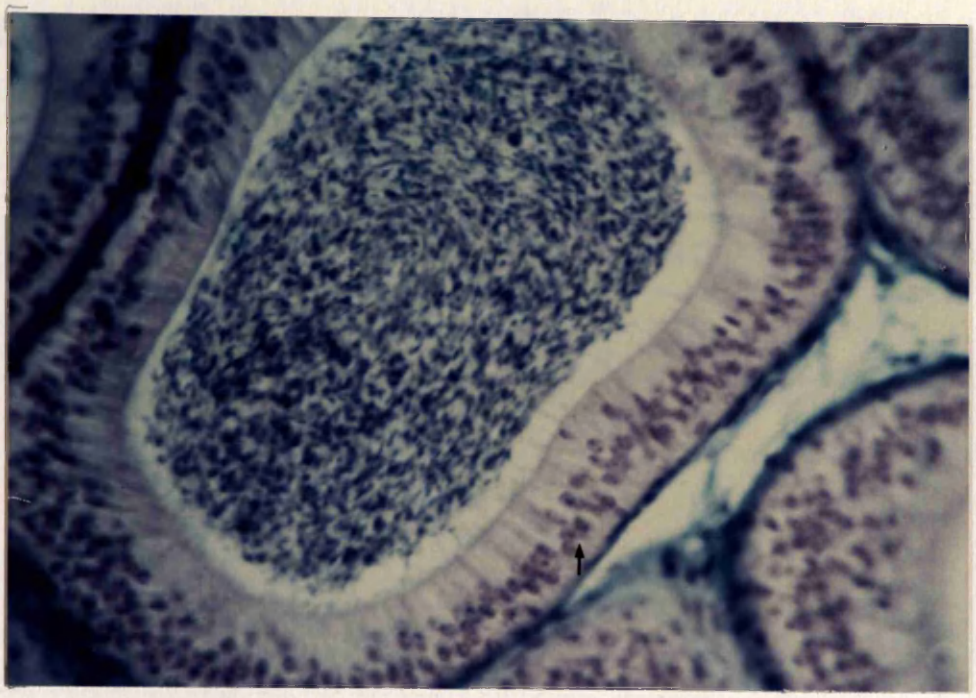


Figure 7. Histological sections of epididymis from a) normal dog and b) dog treated with Medroxyprogesterone acetate. Note, in the section from the normal dog, the regular arrangement of the cells lining the epididymis and the position of the nucleus with regards to the basement membrane. In comparison, the nuclei in the cells lining this tubule in the treated dog are much more irregular in position with the majority of them lying much closer to the basal membrane than those of the normal dog. In addition, it is obvious that the cytoplasm of the cells of the treated tissue has lost its normal homogeneity, with numerous vacuoles present within the cytoplasm. Magnification 100 x. Both slides stained with MSB. A blue microscope filter was used to take both photographs.

a



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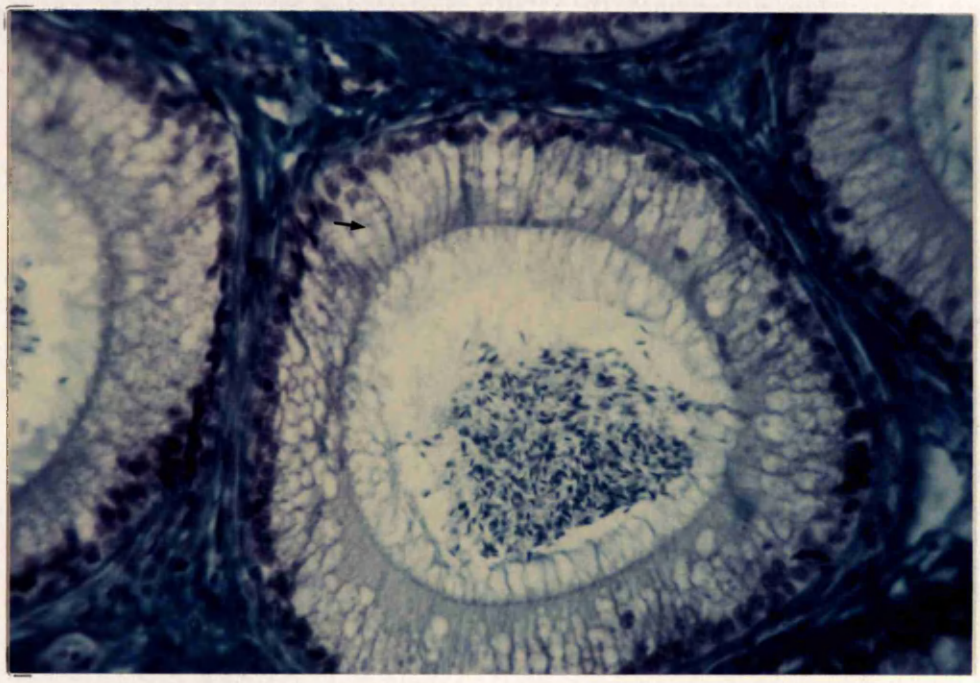
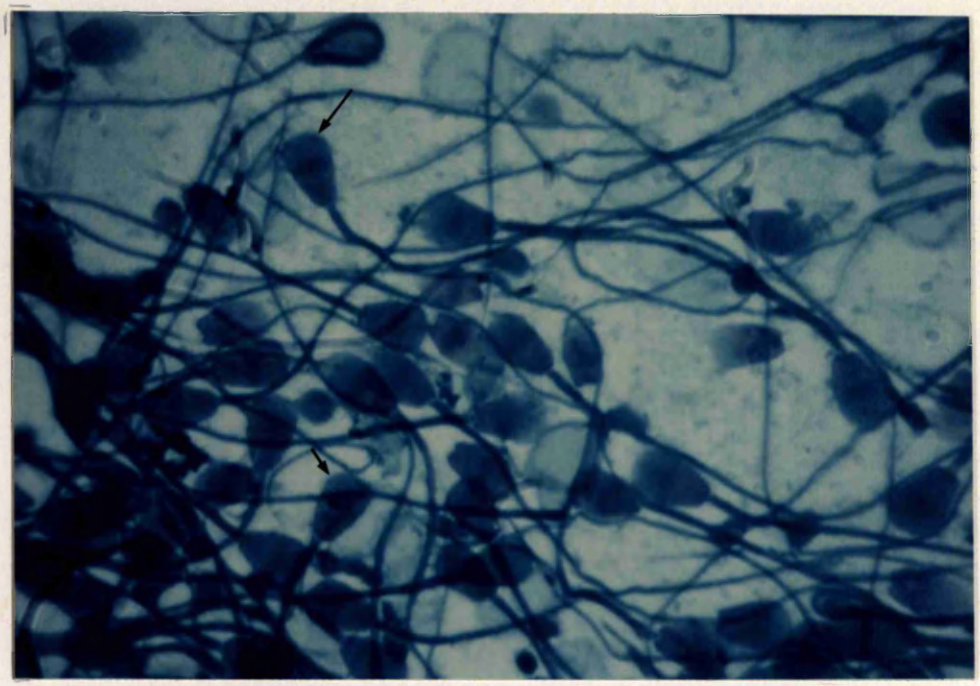


Figure 8. Presents an example of spermatozoa removed from the head of the epididymis of a) normal dog and b) from the head of the epididymis of the dog (Fred) treated with Medroxyprogesterone acetate. In the sperm from the normal dog, the acrosome is closely and firmly attached to the head of the sperm, whereas in the sperm from the treated dog, the acrosome has a fan like appearance due to the separation of the cap from the underlining sperm allowing it to form folds. Magnification 100 x. Both slides were stained with Spermac.

a



b

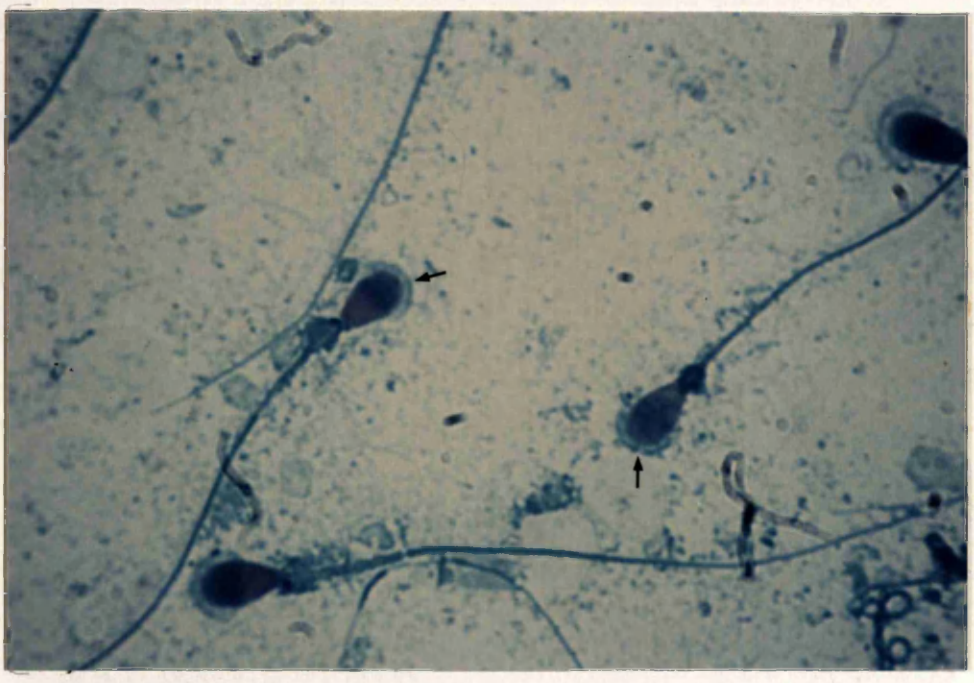
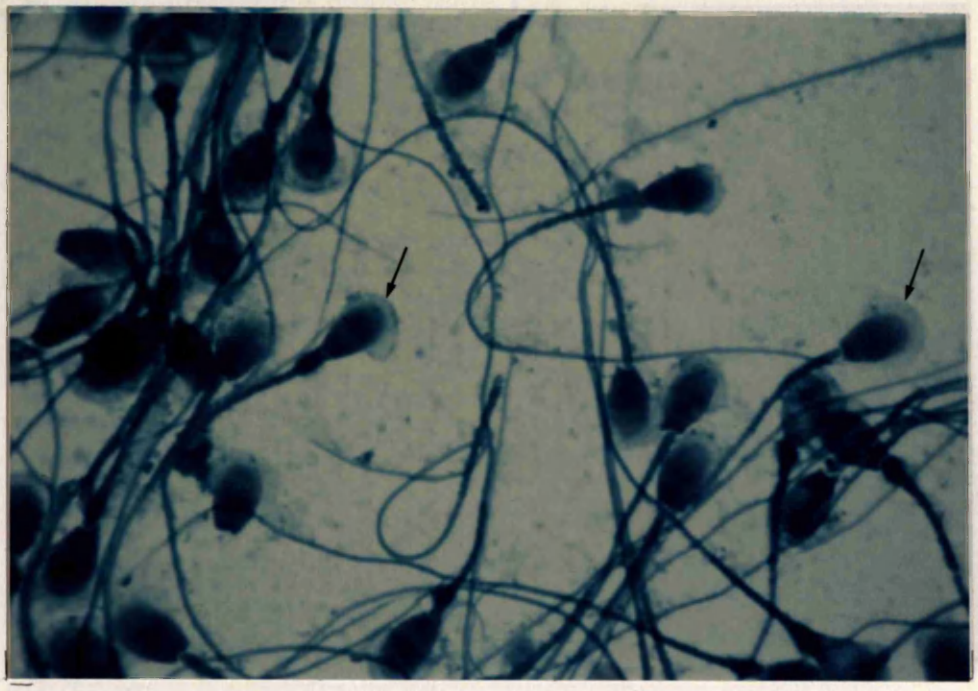


Figure 9. Presents an example of spermatozoa removed from: a) head of the epididymis of a normal dog b). From the head, c) body, d) tail, e) from the ejaculate of a dog (Gus) treated for three months with MPA. Notice in the normal dog the acrosome is closely attached to the head of the sperm, whereas in the sperm from the treated dog, the acrosome has a fan like appearance in the samples taken from sections of the head,body and tail of the epididymis. Notice also the presence of sperm with no acrosome which begin to appear in the body section. The numbers of spermatozoa with no acrosome increases in the samples taken from the tail section. In the ejaculate sample (e) the equatorial region looks wider and is seen here as a pale band surrounding the edge of the acrosome. Magnification used 100 x. Both slides were stained with Spermac.

a



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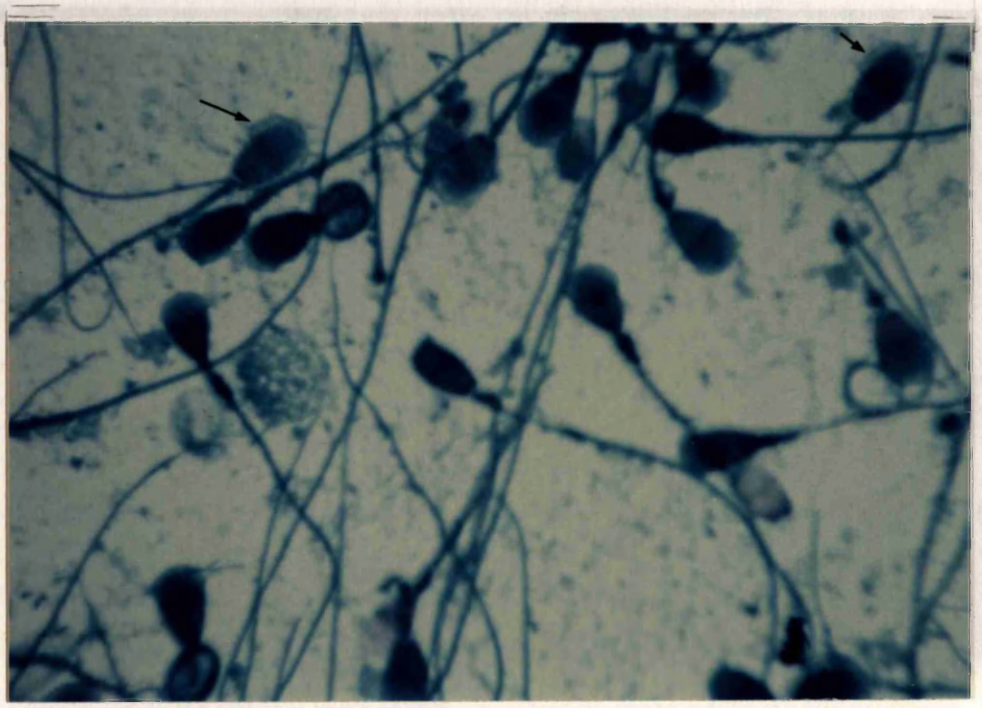
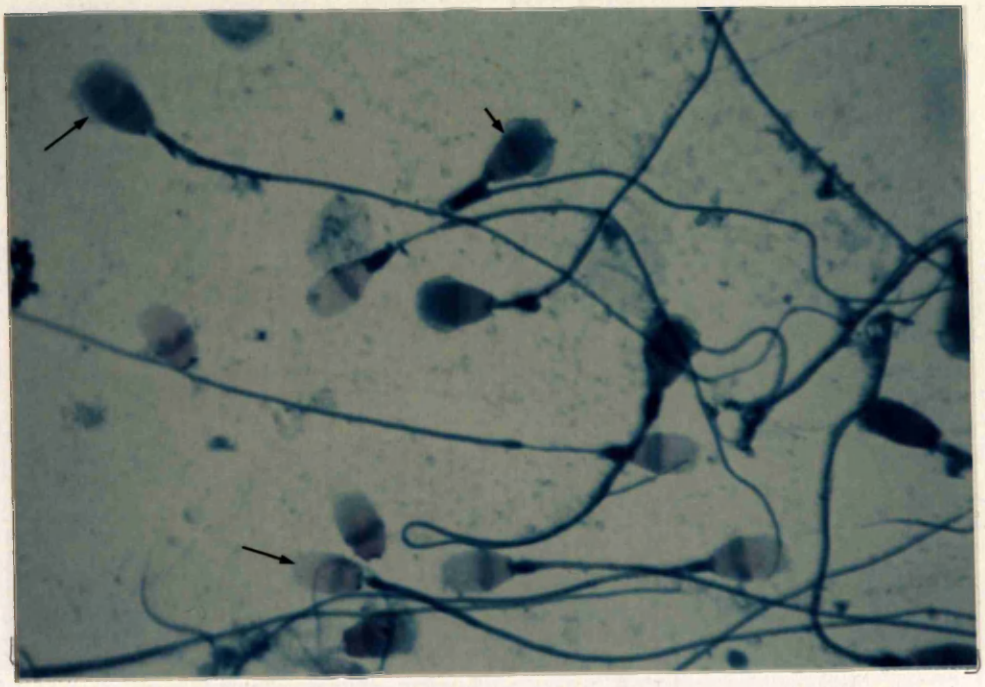


c



d

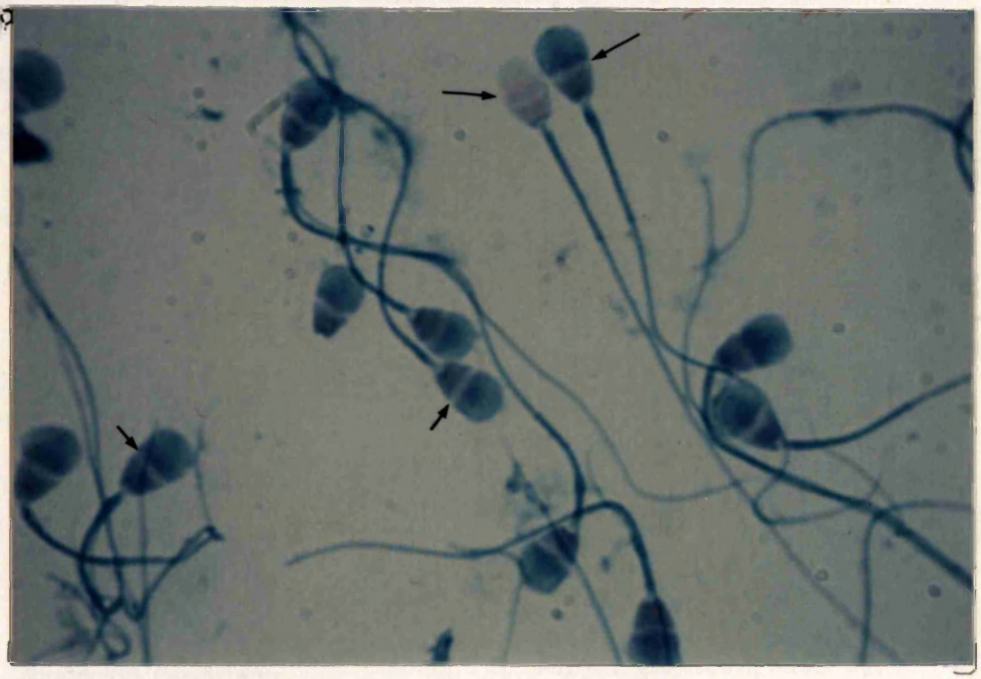
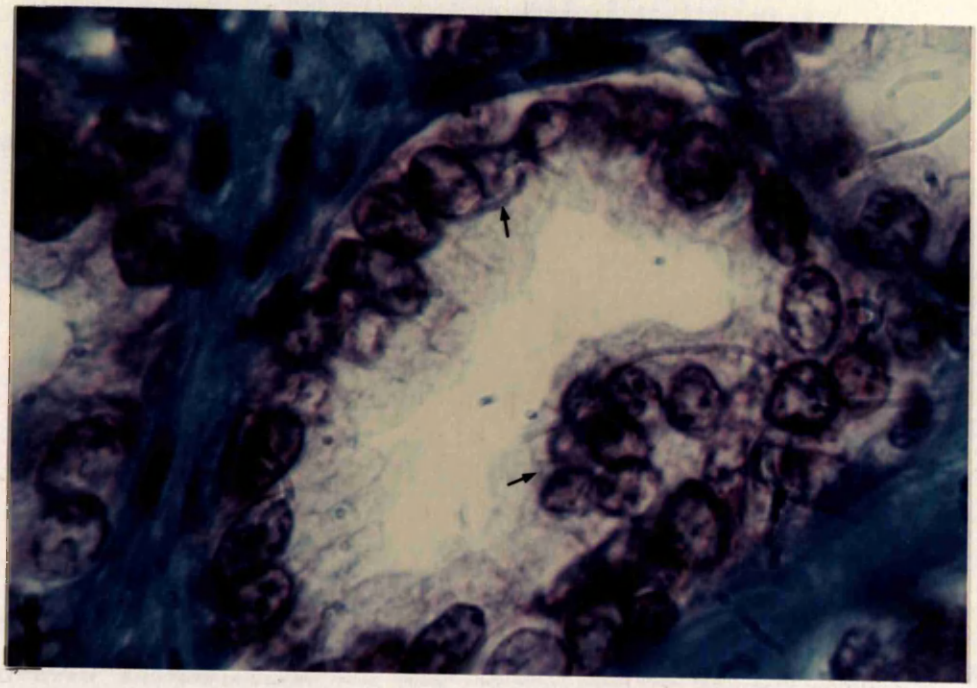


Figure 10. Presents the histological sections of prostate taken from a) normal dog, b) beagle Fred treated with Medroxyprogesterone acetate. There is a slight hyperplasia of the gland as the secretory cells from the treated dog have a very large cytoplasm and a flattened nucleus in the proximity of the membrane. In the normal dog, the cells cytoplasm is smaller with a round nucleus. Magnification 100 x . Both slides stained with MSB.

a



b

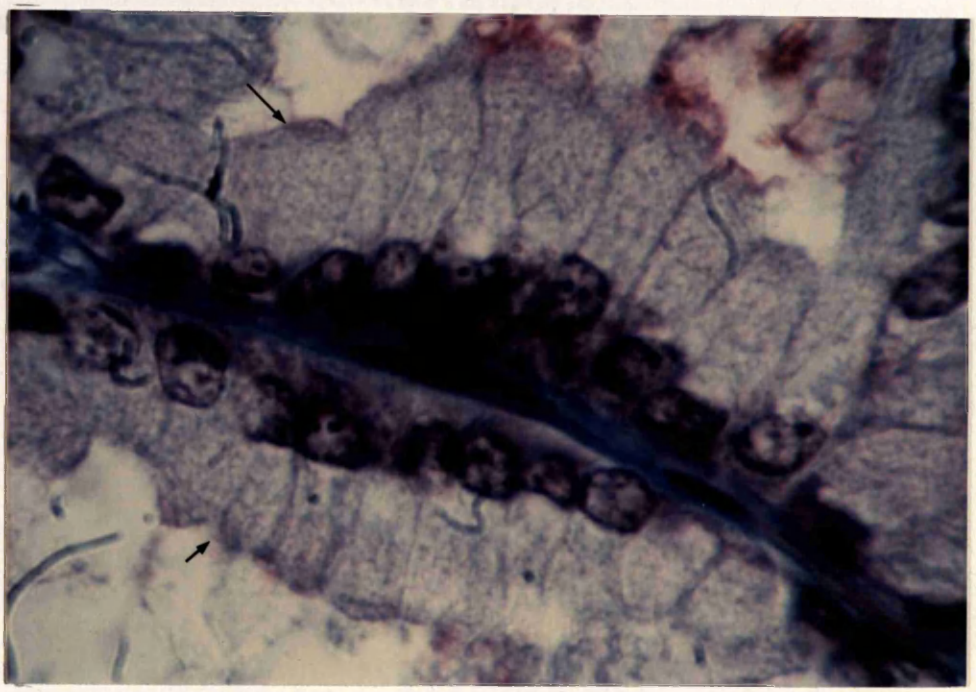
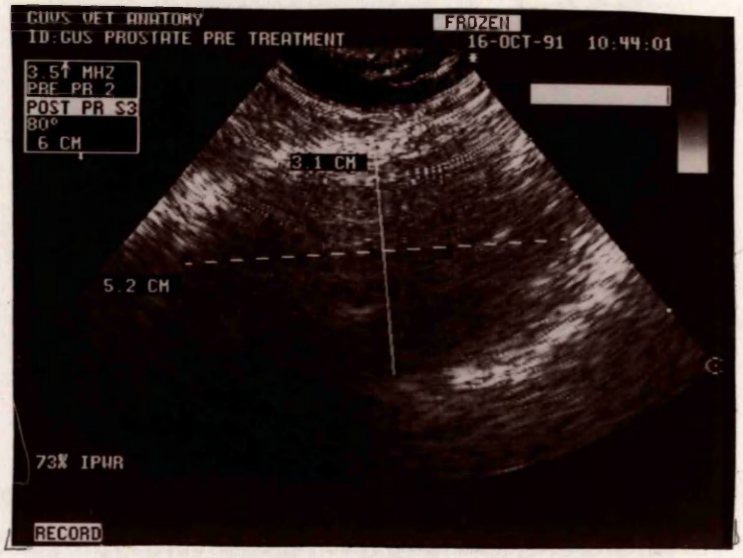


Fig. 11. Ultrasonographs of the prostate taken from Gus, the second dog treated with Medroxyprogesterone acetate. Notice that the measurements were taken longitudinally. The first measurements were made prior to treatment on October 16 and the length at that time was 5.2 cm and the width 3.1 cm. In the second photo taken on November 13, both measurements show a decrease, the width being 2.6 cm and the length 4.8 cm.

a


GUVS VET ANATOMY
ID:GUS PROSTATE PRE TREATMENT
FROZEN
16-OCT-91 10:44:01
3.5↑ MHZ
PRE PR 2
POST PR S3
80°
6 CM
3.1 CM
5.2 CM
73% IPWR
RECORD

b

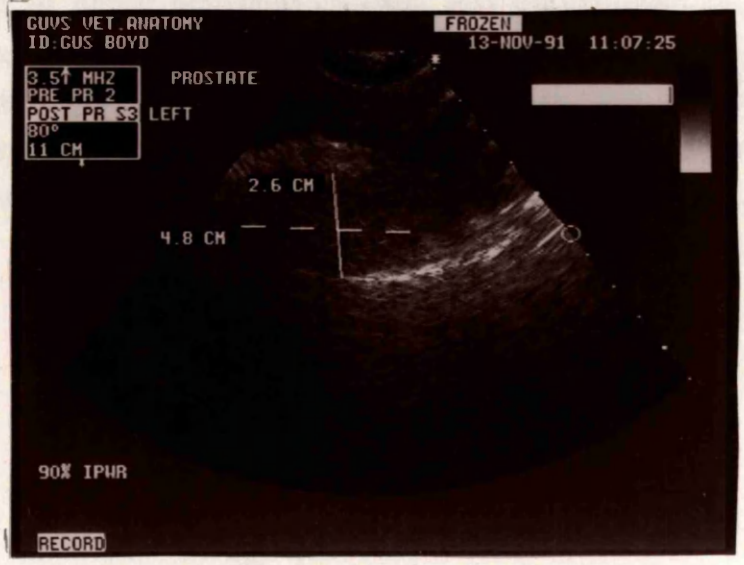

GUVS VET ANATOMY
ID:GUS BOYD
FROZEN
13-NOV-91 11:07:25
3.5↑ MHZ
PRE PR 2
POST PR S3
80°
11 CM
PROSTATE
LEFT
2.6 CM
4.8 CM
90% IPWR
RECORD

Table 5. Morphological characteristics of spermatozoa collected from the head of the epididymis of a dog (Fred) treated with Depoprovera (Medroxyprogesterone acetate, MPA) after one month of treatment and at *post mortem* after a further two month's treatment. Sperm with abnormal acrosomes were removed from both epididymides. The degree of separation of the acrosome from the head of the sperm varied. In some cases it was completely absent. After one month's treatment, the commonest abnormality was swollen acrosome found in sperm removed from the body and tail region whereas, in the sperm removed from the same area of the epididymis after two months the most common was tailless sperm. Although the sperm were recovered after different lengths of treatment, the percentage of spermatozoa with either detaching acrosomes, or absence of acrosomes was virtually the same in the epididymides.

Table 5. Evaluation of acrosomal changes, presence of cytoplasmic droplets in spermatozoa from epididymides from a dog (Fred) treated for 2 months with Medroxyprogesterone acetate

Source of spermatozoa		Acrosome swelling %	Detaching Acrosome %	No Acrosome %	Tailless sperm %	Cytoplasmic droplet proximal %	Cytoplasmic droplet distal %	Normal
Epididymis after 1 month of treatment	Head	0	31	23	5	6	0	35
	Body	60	0	1	17	0	0	22
	Tail	50	0	0	0	0	0	50
Epididymis after 2 months of treatment	Head	0	26	19	51	4	0	0
	Body	0	0	0	66	0	0	34
	Tail	0	0	0	100	0	0	0

Table 6. Looking at the results of the abnormalities found in the spermatozoa removed from the epididymides after 3 months treatment with MPA, it is of interest to see that the commonest abnormality was that of detaching acrosomes, a change which was present in all the spermatozoa removed from the head region, in 30 % from the body and 15% from the tail. Tailless sperm were found in both the body and tail.

Table 6. Evaluation of acrosomal changes, presence of cytoplasmic droplets in spermatozoa from a dog (Gus) treated for 3 months with Medroxyprogesterone acetate.

Source of spermatozoa		Acrosome swelling %	Detaching acrosome %	no acrosome %	Tailess spermatozoa %	Prox droplet %	Normal %
From left epididymis	Head	0	100	0	0	0	0
	Body	0	30	0	50	0	20
	Tail	15	0	0	30	0	55
From right epididymis	Head	0	100	0	0	0	0
	Body	63	0	15	15	7	0
	Tail	20	0	0	0	0	80

Figure 12. This figure presents the standard curve, for the standards used in the radioimmunoassay for the evaluation of testosterone in plasma samples. It presents the repeatability of the radioimmunoassay used in the present work, and is described in material and methods in this chapter.

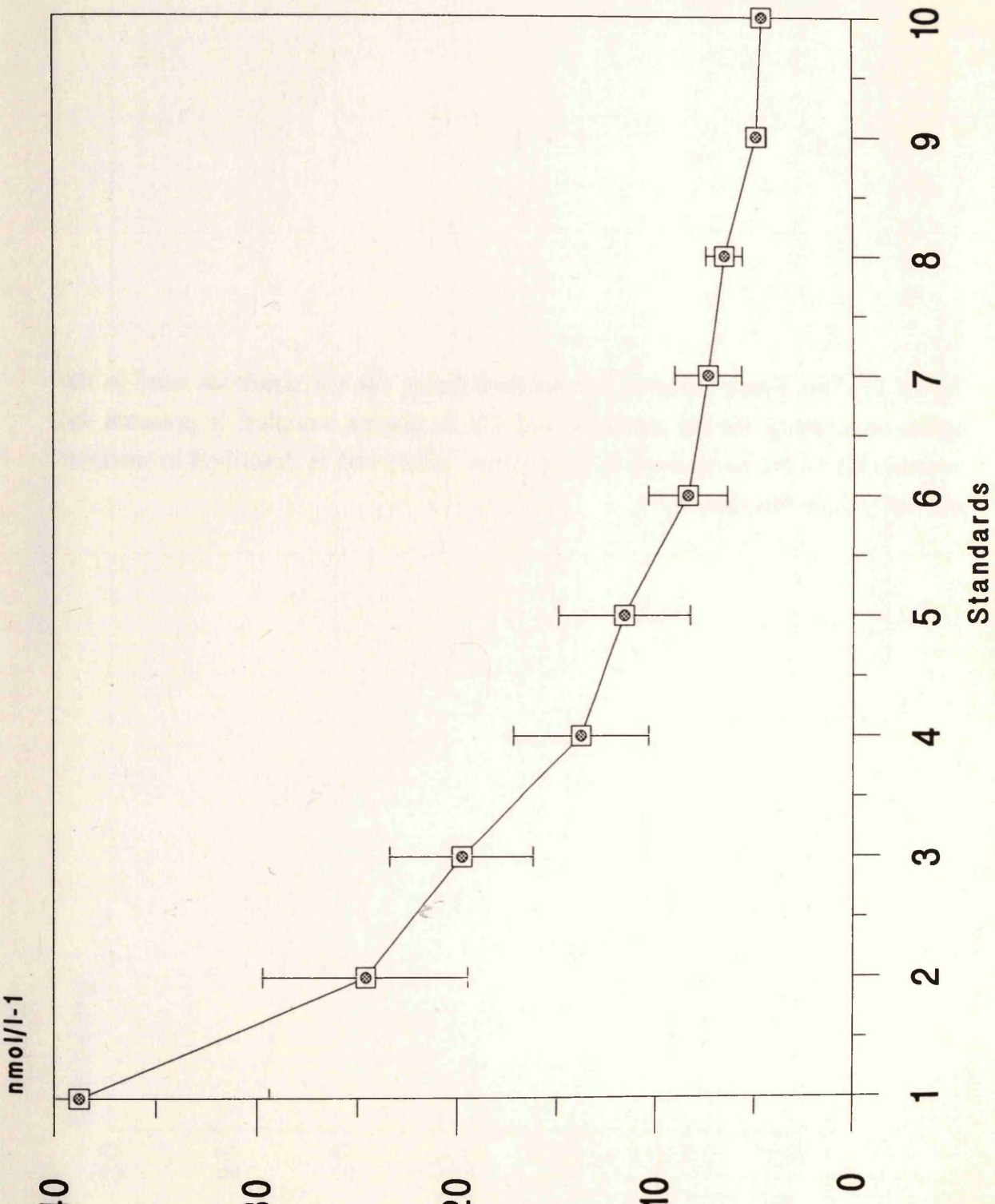

nmol/l-1
40
30
20
10
0
1
2
3
4
5
6
7
8
9
10
Standards

Figure 13. This figure presents the standard curve, for the standards used in the radioimmunoassay for the evaluation of LH in plasma samples. It presents the repeatability of the assay used in the present work, and is described in material and methods in this chapter.

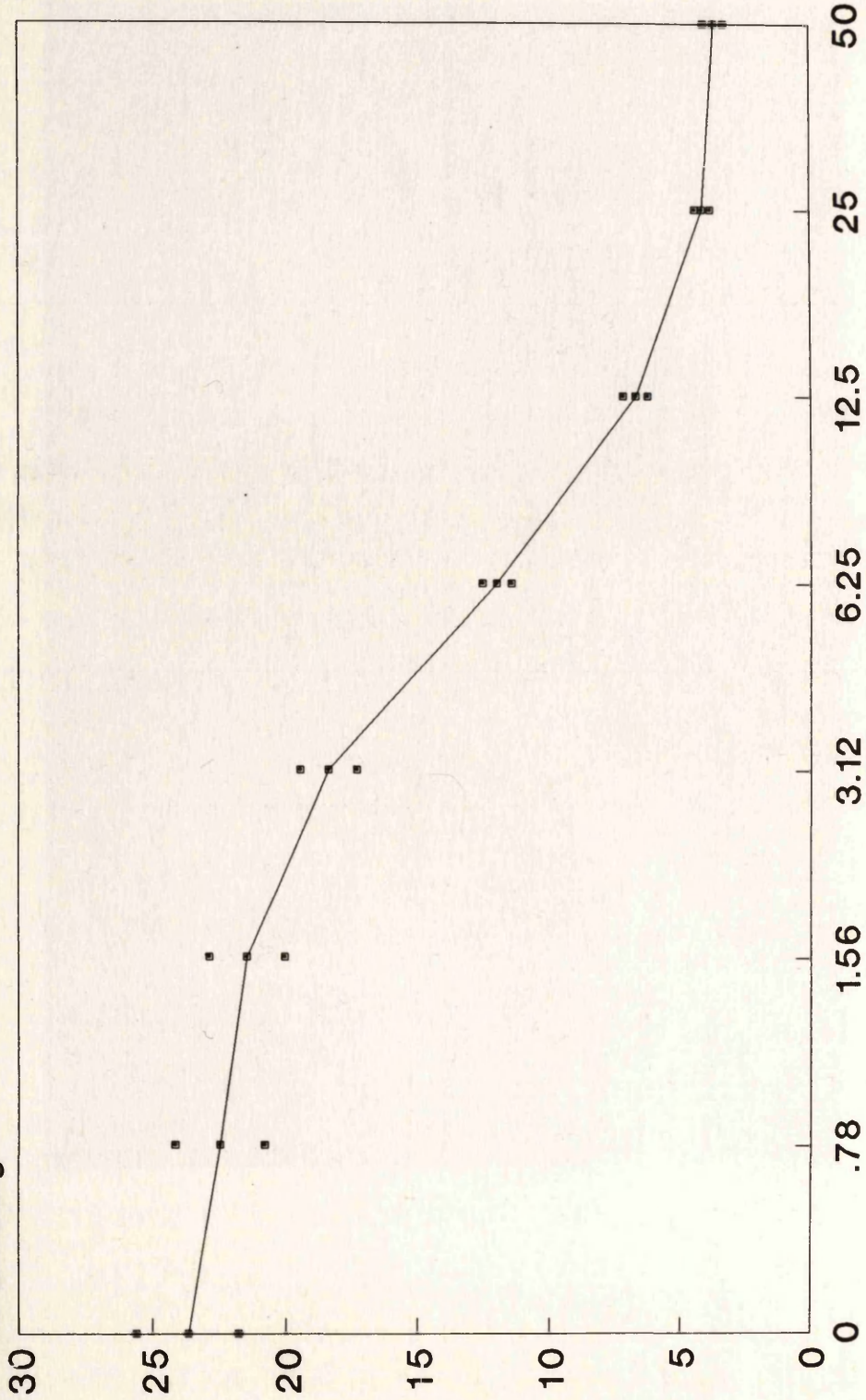


Figure 13. Repeatability of standard curve for LH in blood (10 assays)

Figure. 14 This is a photograph of the material used for semen collection in the dog, showing the latex cone with a plastic centrifuge attached to it as ready to be used.

4. DISCUSSION

The main reason for using a progestogen for hormonal manipulation of fertility in the male dog was because of recent interesting findings in man (Wu and Aitken 1989). In this instance, the progestogen Medroxyprogesterone acetate (MPA) was used in conjunction with testosterone, but it was felt that the use of testosterone in the human was mainly as a means of maintaining normal libido and that it would be of interest to investigate the effect of progestogens alone on male fertility.

Natural and synthetic progestogens are supplied in various forms, such as depot injections, compressed pellets, or silastic implants. The first treatment chosen was an implant of progesterone (Tables 1 and 3) as it was then possible to monitor the rate of absorption of the progesterone relatively rapidly by determining the circulating plasma progesterone concentrations with a commercial ELISA kit, using the method described by Eckersall and Harvey (1987). Several days after initiation of this treatment, there was no apparent increase in circulating plasma progesterone concentrations (Figure 3) although the implant used contained a relatively large amount of progesterone (100 mgs).

Little is published in the literature about the concentrations achieved by giving hormones as implants in any species, but the results of the monitoring carried out indicated that this method of administration was of little use in increasing the amounts of the preparation in the plasma (Figure 3). This warrants further investigation, as even the firms who produced the implants were unable to supply information regarding the levels being released into the blood, even in humans, and there was absolutely no information regarding this aspect in dogs.

It was therefore decided that this means of administering progesterone was of no use for the purposes intended, so, at that point, a depot injection of Depoprovera (Medroxyprogesterone acetate, MPA) was administered (Table 3).

The amounts of MPA and testosterone administered by depot injection to the human male was calculated on a weight basis of 2.85 mg per kg and this achieved suppression of circulating plasma LH in these patients after 7 days (Wu and Aitken 1989). In the initial canine experiment, Depoprovera was administered as depot injections at a dose calculated on a similar weight basis to that adopted in the human males (3 mg/kg). The subsequent results of daily

monitoring of the circulating plasma LH concentrations. (Figures 3 and 4) indicated that there had been no apparent suppression of the circulating LH or testosterone concentrations. Even though there is some discrepancy in the levels reported in previous studies they are still within those limits (1.38-20.76 nmol /l) reported by de Palatis *et al.* (1978); (5.55-11.07 nmol/l in Tremblay and Belanger (1985); (10.38 -13.84 nmol/l) in Vickery *et al* (1984), (3.08 ±0.21 to 1.57 ±0.25) Taha *et al.* 1981.

The same applies to the LH levels found throughout the present study (3.2-15.29 ng/ml) (Figures 1, 2, 16, 24, 30) which are just slightly higher than those reported by de Palatis et al. (1978), (0.2-12 ng/ml). The only exception was the one sample in figure 1 of 55.24 ng/ml, which may well have been due to faulty processing of the sample. Unfortunately there was not enough sample to retest the sample.

This lack of suppression may have been due to the absence of testosterone in the treatment. This is confirmed by later studies in which MPA is still being used with androgens in man as reported by the World Health Organisation (1993). In a study in Indonesian men, 43 of 45 men achieved consistent azoospermia when MPA was combined with T enanthate and 44 out of 45 when the combination was made with a 19-nortestosterone ester. Testosterone also acts via the hypothalamus to reduce the release of GnRH and so the release of gonadotrophins. On the other hand, it might well be that metabolism of progesterone is much more rapid in the dog than in the human. Interestingly in the bitch in which MPA is given to suppress oestrus, 50 mg /kg the doses used namely (Sokolowski et al.1973; Sokolowski et al. 1974) and 75 mg/kg (McCann et al. 1987), are relatively much higher than those given to humans, for example 150 mg used by Shrimanker et al. (1978).

In retrospect, it would have been extremely interesting to monitor the uptake of MPA in the dogs treated, as there is an assay available to do so (Shrimanker et al. 1978). This would have allowed more accurate deductions to be made as to the levels obtained and the half life of this substance in this species. However in these experiments, this was not undertaken and it was decided to double the amount given by depot injection (Table 3).

Just before the increased amount of progesterone was administered, an attempt was made to determine whether there were any changes in the testes, epididymides and developing spermatozoa. Hemicastration of one of the animals

was undertaken. (Table 3). Up to this stage, semen samples had been obtained on several occasions and was obvious that this dog's libido was not affected by the progestogen treatment.

In the male, sexual activity such as libido and ejaculation requires testosterone to manifest itself and to be maintained. Proof that this is so arises from the fact that in castrated dogs, although this behaviour does not disappear immediately, it does slowly decline. Therefore in this dog, the fact that semen could be collected could indicate that testosterone production in this animal was still sufficient to maintain this aspect of normal male fertility (Sachs and Meisel, 1988). Progestogens are initially administered to male dogs to suppress unwanted sexual behaviour (Gerber and Sulman ,1964; Gerber *et al.* 1973; Evans, 1976; Wright *et al.* 1979). However there are no evaluations as to the degree of suppression of testosterone required to produce an effect on sexual behaviour.

Even before commencing the progestogen treatment in this dog (Fred), his libido was scored as less than normal (Table 3). This was most probably due to the animal being collected from by a stranger, as semen had been obtained from him on previous occasions by other people. However, within a fortnight his libido improved markedly and a semen collection was achieved fortnightly thereafter.

After a certain amount of training, semen collection from normal male dogs is usually successfully carried out even without the presence of a bitch in oestrus. However as mentioned before the libido in this particular animal (Fred) was never very high and after hemicastration, no attempt was made to collect from him since, as anticipated, there was a fair degree of scrotal swelling. However he did attempt to mount an oestrous bitch (Table 3).

The profiles of both the circulating plasma LH and testosterone throughout the treatment period in both dogs treated with Depoprovera (Tables 3 and 4), suggest that initially in Fred, there may have been some suppression of the two hormones due to the presence of the progestogen depot. When the depot became reduced in amount toward the end of the first month of treatment, it allowed the concentrations of LH and testosterone to increase as indicated by the peak of both hormones around this time (Figure 3).

The window bleedings demonstrated that the normal concentration of both hormones in the pre-treatment samples (Figures 1 and 2) could be as high as any of the concentrations recorded during the treatment period (Figures 3 and 4).

Thus one must accept that all that can be deduced from the hormone results is that the amount of Medroxyprogesterone acetate (MPA) given did not produce any maintained suppression of LH or testosterone in either of the male dogs, although Gus was treated with 6 subcutaneous injections in a time period that was just 17 days longer than Fred (Table 2 Figure 4). These results are in agreement with the findings of Bamberg and Forsberg (1992) who also reported that dogs treated with MPA at doses ranging between 3-8 mg/kg twice weekly did not show any reduction in their circulating plasma LH or testosterone. However they did not agree with those of Wright et al (1979), who reported a 58% reduction in testosterone concentrations in dogs after 7 weeks of receiving a Depoprovera injection of 4 mg/kg. However, the 58% figure quoted represents the mean of the results from 4 dogs, and it does not take into account the fact that there is a great deal of variations between dogs, which has also been reported by others (Taha *et al* 1981) or that there are oscillations in the testosterone secretion pattern, as can be seen in Figures 3 and 4.

Before the treatment began the colour of the ejaculate in one of the dogs, Fred (Table 3) was recorded as yellow. This yellow colouration was due to the presence of a few urine drops which occurred in this dog even when he urinated immediately prior to collection. With time, this problem was overcome as the dog became more used to the collector and subsequently uncontaminated semen was collected. On another occasion, the ejaculate from this same dog contained blood. This was probably of urethral origin since the integrity of the penis was checked, as it is sometimes possible to cause trauma to the erect penis by contact with the collecting receptacle. No evidence of penile trauma was found.

No spermatozoa was found in this ejaculate, although they had been present 5 days previously (Table 3). This was because, as soon as the bloody ejaculate was seen, further collection ceased and so only the first fraction containing the blood was collected and the sperm rich fraction was not obtained.

It has been suggested that blood may be detrimental to semen when collected for artificial insemination as it is always recommended not to use semen of an abnormal colouring as it indicates contamination either by urine, or blood (Hafez, 1987). However, the engorged state of the erect penis makes it very prone to bleed, so that it is almost certain that in many instances of successful natural matings there is a degree of bleeding from the penis, besides the fact that mating occurs when the bitch is bleeding due to oestrus.

An interesting finding in this dog (Fred) was that, at the end of the experimental period, he was found to have a very enlarged prostate which when examined histologically revealed benign hyperplasia (Figure 10). Prostatic hyperplasia is relatively common in the older male dog and can lead to blood being voided in both urine and semen. It is not clear if the progestogen played a role in the occurrence of this abnormality in this first dog, as no examination of this structure was undertaken before the start of the experiment. However, because of this finding, in the animals subsequently treated, ultrasound scanning of the prostate gland was included as part of the clinical investigations.

It was thanks to the scanning photos of the second dog (Gus) that it was possible to determine an interesting effect of the treatment, which was the apparent shrinking of the gland. The difference can be appreciated in the first photo taken prior to treatment (Figure 11a) and the last photo taken almost at the end of treatment (Figure 11b). This finding would agree with Bamberg and Forsberg (1992) who also reported a decrease in size with one 3 mg/kg body weight dose of MPA, which they used for treating benign prostatic hyperplasia in the dog. The *post-mortem* examination in this second dog (Gus) also revealed a hyperplastic condition but to a lesser degree than that of the first animal.

Before hemicastration was carried out, palpation of the scrotal contents suggested that there was some swelling of the left epididymis(Table 3). This finding indicated that this would be the testis removed, as this would allow a thorough histological examination of this structure. No obvious macroscopic changes of this epidiymis were recorded at *post-mortem* and subsequent histological examination failed to reveal any explanation for the swelling. Manual examination of scrotal contents is very subjective and only very obvious changes tend to indicate meaningful changes.

In the studies reported here, attempts were made to collect the second ejaculated fraction on its own, but this is almost impossible to achieve, as the second fraction tends to be ejaculated immediately following expulsion of the first fraction. A noticeable pause exists between the ejaculation of the second and third fraction (Allen et al. 1979) and thus, the ejaculate collected from these dogs represented a mixture of the first and second fractions and therefore, not a great deal of notice can be taken of the volume recorded. However, the more sperm produced, the more dense the sample is and so the description of cloudy tends to suggest that the concentration in the ejaculate in one of the dogs (Table 1) was

on the low side, as generally a good stud dog will produce a milky to creamy second fraction.

Routinely, sperm concentration is assessed by the number of spermatozoa present in the drop when semen is examined microscopically for initial motility and only if there is indication that there is a very low count, is a scientific method then adopted. In the first animal used in these studies, only visual examination was carried out (Table 3).

Histological examination of sections from the testes removed at hemicastration from Fred, the dog treated with MPA for two months, revealed an increase in the amount of debris occupying the lumen of several of the seminiferous tubules (Figure 6). Some workers have suggested that this sloughing of cells is due to dysfunction of the Sertoli cells as during the final stages of spermatogenesis they "pull off " the excess cytoplasm from the spermatids and retain this residual body within their own cytoplasm (de Kretser and Kerr, 1988). The sloughed material could represent accumulation of these residual bodies or on the other hand it could represent spermatids which were degenerating because the Sertoli cells had lost their ability to remove the excess cytoplasmic droplets.

The most obvious histological changes in both dogs were in the head of the epididymis (Figure 7). If these changes were associated with a suppression of testosterone which is transported to the epididymis by ABP, then this would tend to suggest that these changes were the culmination of exposure for two months to the two injection of progestogen. However, if the changes were brought about by the action of the progestogens directly on the epididymis then it would appear that the head region is more sensitive to progesterone than are body or tail of the epididymis. The changes were an obvious displacement of the nucleus of the epithelial cells lining the head of the epididymis and an increase in the number of vacuoles present within these cells. The functions of these cells include the reabsorption of some of the large amounts of fluid which accompanies the spermatozoa from the seminiferous tubules and occurs mainly in the head regions. A second function is the exchange of glycoproteins, ions and small organic molecules to and from the epididymal lumen, and finally by means of the microvilli located in the luminal surface of the cells, propulsion of the spermatozoa during ejaculation (Robaire & Hermo,1988). Thus the alterations found in the cells lining the head of the epididymides could have affected any of these functions.

However, the most dramatic change identified in both dogs was that in the spermatozoa removed from the lumen of the head of the epididymis (Figure 8). This involved the swelling and loosening of the acrosome and in some sperm the acrosome completely separated from the head. In the dog which was hemicastrated after one month's treatment, the acrosomal changes were present in the head region as the acrosome was either beginning to detach or had already detached. This particular acrosome defect appears in a study of sperm abnormalities in Spermac stained spermatozoa (Oettle, 1986) where it is classified as a secondary acrosomal change in which acrosomal contents have been lost (Oettle and Soley, 1988).

Under normal circumstances the acrosome containing the enzymes acrosin and hyaluronidase is shed immediately prior to penetration of the mature egg by the sperm (Bazer *et al*. 1987). This event takes place in the Fallopian tube of the female (Yanagimachi, 1988) and at this particular time, there is an increase in the circulating plasma progesterone in the bitch. Therefore the acrosomal changes seen in both dogs (Figures 8 and 9) after the progestogen treatment, could suggest that although the amount of progesterone given was not sufficient to cause a determinable change in the circulating plasma LH levels and testosterone concentration, it was sufficient to affect the epididymal environment and subsequently to have an affect on the spermatozoa during their passage through this area of the male reproductive tract. Again, as with the cellular changes in this region, this could be by one of two routes, either by direct action of the progesterone on the epididymis or by the progesterone affecting the amount of testosterone reaching the epididymis (Lobl *et al*,1983) where it is essential for the normal function of this region.

At the end of the experimental period, LH concentrations were still not suppressed in either dog and sperm were still being produced (Figures 3, 4, 5). Therefore, it is unlikely that the testicular testosterone production was reduced nor its transport to the epidiymis affected by the progestogen administration. Furthermore, as this type of acrosomal change occurs normally in sperm while within the female whilst under the influence of raised concentrations of progesterone, it is more likely that the acrosomal changes found in the dogs treated with MPA were the result of a direct action of progestogen on the epididymis itself. This seems to be likely as the rate of fluid absorption is highest in the head of the epididymis, which would thus allow for greater concentrations of MPA in this region.

This, however appeared to be a cumulative effect as after one month's treatment only the sperm in the head region of the epididymis showed these changes (Figure 8). It would be of interest to study sperm kept in an environment of elevated progesterone to investigate whether acrosomal changes would be induced, or if the progesterone required other factors present in the epididymis to bring about those changes.

There is another possibility that would explain the absence of acrosome in some of the sperm. It has been reported (de Kretser and Kerr, 1988) that, as the acrosome contains enzymes, it is possible that they can digest the acrosome in dead or dying spermatozoa. This is known as false acrosome reaction (Yanagimachi, 1988) and it could explain why some sperm had no acrosome, as they may have been dying due to the different environment created by the MPA.

In the hemicastrated dog, there was still the presence of sperm with the acrosomal defect already described in the material removed from the epididymis at *post-mortem*, also noted was the presence of tailless spermatozoa. (Table 5). This was probably due to the change in environment of the epididymis because of the trauma to this region after the removal of the left testis. Similar samples taken from Gus's epididymides showed the highest percentage of sperm with acrosomes in the process of losing their acrosome and reported as detaching acrosome in Table 6, which may be related to the increased number of injections given.

As scrotal enlargement was very obvious for quite some time after the operation, this would lead to degeneration of the epididymal sperm. This would include those spermatozoa already present and those arriving in this region during this time. Spermatogenesis would almost certainly have been affected during the day immediately following the operation due to the inflammation and the consequent increased temperature of the scrotum, so that sperm concentration would also have been affected.

Unfortunately the dog in which hemicastration was not carried out could only be collected from when a bitch in season was available, as only in these circumstances was his libido such as to allow semen to be collected (Table 4). A bitch in oestrus was only available towards the end of the treatment period and so no real deductions can be drawn from the semen picture obtained in this animal, as no semen had been collected before the treatment began. However, after the three months of treating this animal with MPA, the sperm in the ejaculate had an increased equatorial region (Figure 9). This type of change was also reported by

Oettle and Soley (1988) in their study on sperm abnormalities in the dog. Furthermore, the swollen acrosomal change seen in the first dog was present in a relatively large percentage of the sperm removed from all three areas of the epididymis at *post-mortem* (Figures 9, a, b, c). These findings tend to suggest that the extended length of time over which this animal was treated, increased the acrosomal abnormalities, thus supporting the hypothesis of a cumulative affect of MPA.

In conclusion, the results of these two experiments showed that the Depoprovera (MPA) treatment did not suppress either LH or testosterone production and thus spermatogenesis continued in an apparently normal way. However the MPA regime did affect the epididymal environment therefore indicating a very interesting potential for a contraceptive mechanisms, by damaging the fertilising capacity of the spermatozoa during maturation in the epididymis.

However, as the aim of this project was to study the changes in the spermatogenic cycle by inhibiting the LH release from the pituitary, with the subsequent reduction in the production of testosterone, another method of achieving this required to be investigated.

CHAPTER 2-B

GnRH AGONISTS

1. INTRODUCTION

A great deal of information about the use of synthetic GnRH agonists and antagonists as a means of manipulating fertility in both humans and laboratory animals is available in the literature. In the human they were used initially for treatment of infertility cases where abnormal amounts of gonadotrophins were detected. However it was established that GnRH desensitisation occurs at the pituitary level and in the rat at gonadal level as well since GnRH-like receptor sites have been identified in their Leydig cells (Sharpe,1986). Although there have been several reports of the use of both agonists and antagonists in the dog, most of the work has been associated with contraception in the bitch. However Vickery and colleagues (Vickery, *et al.* 1982; Vickery *et al.* 1983; Vickery and McRae, 1984; Vickery *et al.* 1984; Vickery *et al.* 1985; Vickery *et a*l. 1986; Goodpasture, Bergstrom and Vickery, 1988; Vickery *et al.* 1989), have done extensive studies of their use in the male dog and their results demonstrated the ability of the GnRH agonist to suppress circulating plasma concentrations of both testosterone and LH. It was therefore decided to undertake preliminary investigations of the use of a Nafarelin derivative kindly donated to us by Dr. Patrick Concannon (Cornell University) and subsequently Nafarelin acetate donated by Syntex (Palo Alto, California). And the aim was to determine the optimum conditions for administration of the drug in the dog, such as the length of treatment required to produce continued suppression of circulating plasma testosterone and the subsequent changes in testes, epididymis and prostate

2. MATERIALS AND METHODS.

Animals used				
Name	Breed	Age, years	Weight, kgs.	Body condition
Adam	Beagle	1	17`	good
Baxter	Beagle	1	17	good
Dexter	Beagle	1	17	good

2.1. MAINTENANCE

As discussed in chapter 2-A

2.2. TESTICULAR MEASUREMENTS

As discussed in chapter 2-A

2.3. SCANNING

As discussed in chapter 2-A

2.4. LIBIDO EVALUATION

As discussed in chapter 2-A

2.5. SEMEN COLLECTION

As discussed in chapter 2-A

2.6. SEMEN EVALUATION

As discussed in chapter 2-A

2.7. ALKALINE PHOSPHATASE EVALUATION

100 microliters of ejaculate were analysed for alkaline phosphatase contents, using a commercial kit code no. 980941, by the DEA buffer, and 37°C. by the method of the (Empfehlunger der Deutschen Gesellschaft fur Klinische Chemie, 1970 and 1972) and measured in a Dynamic selective analyser from Kone Instruments. The results are expressed as units/litre.

2.8. BLOOD SAMPLE COLLECTION AND PROCESSING.

As discussed in chapter 2-A

2.9. MEDICATION USED

Nafarelin derivative No.RS 49947-298 supplied by courtesy of Dr. Patrick Concannon (Cornell University). This was administered by osmotic pumps which were subcutaneously implanted and by daily subcutaneous injections.

Nafarelin acetate supplied by courtesy of (Syntex Research, Palo Alto, Ca. USA). This was used as subcutaneous daily injections only.

2.10. ADMINISTRATION OF MEDICATION

By subcutaneous osmotic pumps and subcutaneous injections

The pumps used were Alzet model no. 2ML4 (Alzet Charles River UK. Ltd) for subcutaneous implant.

1. The empty pump was weighted together with the flow moderator.

2. Filling the pump was achieved by means of a syringe and a blunt-tipped filling tube as follows: Drawing the solution into the syringe to which the loading tube was already attached, making sure that both syringe and tube were free of air bubbles.

3. With the flow moderator removed and holding the pump in an upright position, the filling tube was inserted thorough the opening at the end of the pump until it could go no further, to the bottom of the pump reservoir.

4. By slowly pushing the plunger of the syringe, whilst holding the pump in an upright position until the solution appeared at the outlet.

5. Excess solution was wiped off and the flow moderator inserted until the cap was flush with the top of the pump. The insertion of the flow moderator displaced some of the solution from the filled pump.

6 The filled pump was weighed and the difference in the weights from steps 1 and 6 was taken as the net weight of the solution loaded. For most dilute aqueous solutions, the weight in milligrams is approximately the same as the volume in microlitres. The total volume should be over 90% of the reservoir volume specified. If so, the filled pump is then ready for use. If not, there may be some air trapped inside the pump. Evacuate the incompletely filled pump and refill.

2.11. PUMP INSERTION

a) Under local anaesthesia.

b) Under general anaesthesia.

a). With local anaesthesia.

Premedication, 0.8 ml of ACP (0.004 ml /kg)

The incision site was the area in the back between the shoulder blades, and the skin over this area was clipped and washed with an antiseptic solution (Pevidine A BK. Veterinary Prods Ltd.), rinsed with 70% alcohol and then infiltrated with Lignocaine-A

b) With general anaesthesia.

Pre-Medication 0.8 ml ACP (0.004 ml/kg)

Anaesthesia was induced with 5.0 ml iv Thiopentone (Intraval sodium, May & Baker pharmaceutical) followed by administration of Halothane, Oxygen and Nitrous oxide through a face mask.

Subcutaneous implantation:

1. An incision was made in the neck between the shoulder blades. By inserting artery forceps through the incision and opening the jaws, the subcutaneous tissue was dilated to create a pocket for the pump, until sufficiently large to allow movement of the

pump. The pocket was not made too large, as this allowed the pump to turn around or slip down towards the flank of the animal.

2. The filled pump was inserted into the pocket portal end first .This minimises interaction between the compound delivered and the healing of the incision.

3. The subcutaneous tissue was then sutured with simple dissolvable interrupted stitches. The skin was closed with continuous stitches and silk.

4. After either procedure, the dog received an intramuscular injection of 1.7 ml Duphapen LA (Duphar Veterinary Limited)

2.12. HORMONE ASSAYS

Same as discussed in chapter 2-A

2.13. TISSUE PROCESSING FOR HISTOLOGY.

Same as discussed in chapter 2-A

3.- RESULTS

Table 7 Protocol for the Nafarelin derivative treatment of an adult male beagle (Adam) treated for 75 days

Days	Procedure
-3	Daily bleeding
-2 +5, then weekly	Semen collection
-1	Bleeding every 15 min. for 3 hours
26	Bleeding every 15 min. for 2 hours
0-28 28-41 41-54 54-75	Nafarelin derivative administration First pump inserted releasing 17 ug/day Second pump releasing 17 ug /day Third pump releasing 34 ug/day Daily injection 24 ug.
15,36,53,78.	Scanning of prostate Weight taken .
Daily	Body temperature taken
-2+4 then weekly	Genitalia evaluation.

Table 8. This table presents details of the experimental protocol in Adam. The Nafarelin derivative treatment to this dog was initially given by means of osmotic pumps inserted subcutaneously, the initial dose of 17 ug/day being changed in the third pump to twice that dose. Even when some problems arose with the use of the osmotic pump, this did not cause any systemic reaction as gauged by body temperature and the animal's well-being. The treatment was changed to daily subcutaneous injections with doses which were modified several times until the final dose of 50 ug/day was selected. Notice that there was a small decrease in the size of the testis toward the end of the treatment period. There was also an associated change in consistency of the gonads, these changes in the testes being reflected by similar changes in the epidydimides.

Table 8. Clinical findings and external genitalia evaluation, prior to and during treatment of a dog (Adam) with a Nafarelin derivative administered by osmotic pump and daily subcutaneous injections

		Testicular measurements				Clinical evaluation				
Date	Nafarelin derivative	Left Width (cms)	Left Length (cms)	Rigth Width (cms)	Right Length (cms)	Testicular Consistency	Epididymis	Temperature o F	Weight kgs	Comments
02/03/92		7	8	7	8	resilient	normal		16.8	
06/03/92	1st. pump 17 ug/day	7	8	7	8	resilient	normal			
07/03/92								101.2		
08/03/92								101.4		
09/03/92								101.2		
10/03/92								101.6		
11/03/92		7	9	7	8	resilient	normal		16.9	
13/03/92								101		
18/03/92		7	8.5	7	8	resilient	normal			
21/03/92								101.2		
22/03/92								101.2	17	
25/03/92		7	8	7	8	resilient	normal			
01/04/92		7	8	7	8.5	resilient	normal			
02/04/92	2nd. pump 17 ug/day							101		
03/04/92								101		slight swelling area pump insertion
04/04/92								101		
08/04/92		7	8.5	7	8	resilient	normal			
15/04/92	3rd. pump 34 ug. day	7	8	7	8	resilient	normal	101		
16/04/92								101.4		slight swelling area pump insertion
17/04/92								101.2		
21/04/92										
23/04/92		7	8	7	8	resilient	normal			
28/04/92	inject. 34 ug	7	8	7	8	resilient	normal		17	
29/04/92	inject. 68 ug									
30/04/92	inject.150 ug							101		
01/05/92	"							101.2		
02/05/92	inject 100 ug/day							101	17	
03/05/92	"									
04/05/92	"									
05/05/92	"									
06/05/92	"	6.8	7.8	6.8	7.8	softer	smaller			
07/05/92	inject 50 ug day									
08/05/92	"									
09/05/92	"									
10/05/92	"								16.9	
11/05/92	"									
12/05/92	"	6.3	7.2	6.2	7.3	softer	smaller			
13/05/92	"									
14/05/92	"									
15/05/92	"									
16/05/92	"									
17/05/92	"									
18/05/92	"									
19/05/92	"	6	7	6	7	flabby	smaller			
20/05/92	"	5.5	6.5	5.5	6.5	flabby	smaller		16.9	
21/05/92	"	5.5	6.5	5.5	6.5	flabby	smaller			

Table 9 The libido in this animal only twice reached the normal score (3), but it was sufficient to allow semen to be collected on numerous occasions. Note that even before administration of the GNRH agonist, this dog's ejaculate contained a high percentage of abnormal sperm, the highest percentage of which were sperm with bent necks. An interesting point is the decrease in ejaculate produced at the end of the treatment and the high spermatozoal concentration in the last sample in which it was possible to evaluate concentration.

Table 9. Libido and semen evaluation of a dog (Adam) treated with a Nafarelin derivative by osmotic pump and daily subcutaneous injections

							Abnormalities						
Date	Libido	Ejac. vol in ml	Color	Progress motility %	Concent. x (10)6/ml	Live:dead	Head %	Acrosome %	Bent neck %	Tailles sperm %	Total %	Alkaline phosph. UI/L	Comments
25/02/92	3	2	white	4	348	96:4		normal	28	6	34	230508	Semen collection before treatment
02/03/92	2	2	white	4	220	97:3	2	normal	20	1	23	208500	Semen collection before treatment
06/03/92													1st. pump inserted
10/03/92	2	1	white	4	157	98:2	1	normal	48	4	53	253600	
13/03/92	2	1	white	4	320	98:2		normal	40	1	41	220500	
17/03/92	2	1	white	4	48	99:1		normal	36	3	39	998020	
24/03/92	2	2	white	4	442	98:2	1	normal	20	19	40	56600	
31/03/92	2	1	white	4	196	97:3	3	normal	25	6	34	269150	
02/04/92													2nd. pump inserted
07/04/92	2	1	white	4	174	93:7	2	normal	31	4	37	156400	
14/04/92	2	2	white	4	620	93:7	2	normal	28	5	35	259000	
15/04/92													3rd . pump inserted
21/04/92	1	1	white	4	488	95:5	1	normal	40	3	44	233100	
28/04/92	2	2	white	4	673	100:0	6	normal	14	8	28	201750	Subcutaneous injections begin
06/05/92	2	drop	white	3	not done	98:2		normal	24	6	30	200750	
12/05/92	3	drop	white	3	833	93:7	2	normal	37	14	53	not done	
19/05/92	2	drop											
20/05/92	2	drop											
21/05/92													End of experiment

Figure 15. This figure presents the results of the frequent bleedings undertaken prior to treatment which indicated that the circulating plasma concentrations of both LH and testosterone showed similar variations at 15 minute intervals to those found during daily bleeding. All LH and testosterone values were below 10 ng/ml nmol respectively. It is the first such window taken during the experimental period and is indicated in figure 16 by a (w).

Fig 15 Circulating plasma testosterone & LH in blood samples collected every 15 minutes for 3 hours from Adam

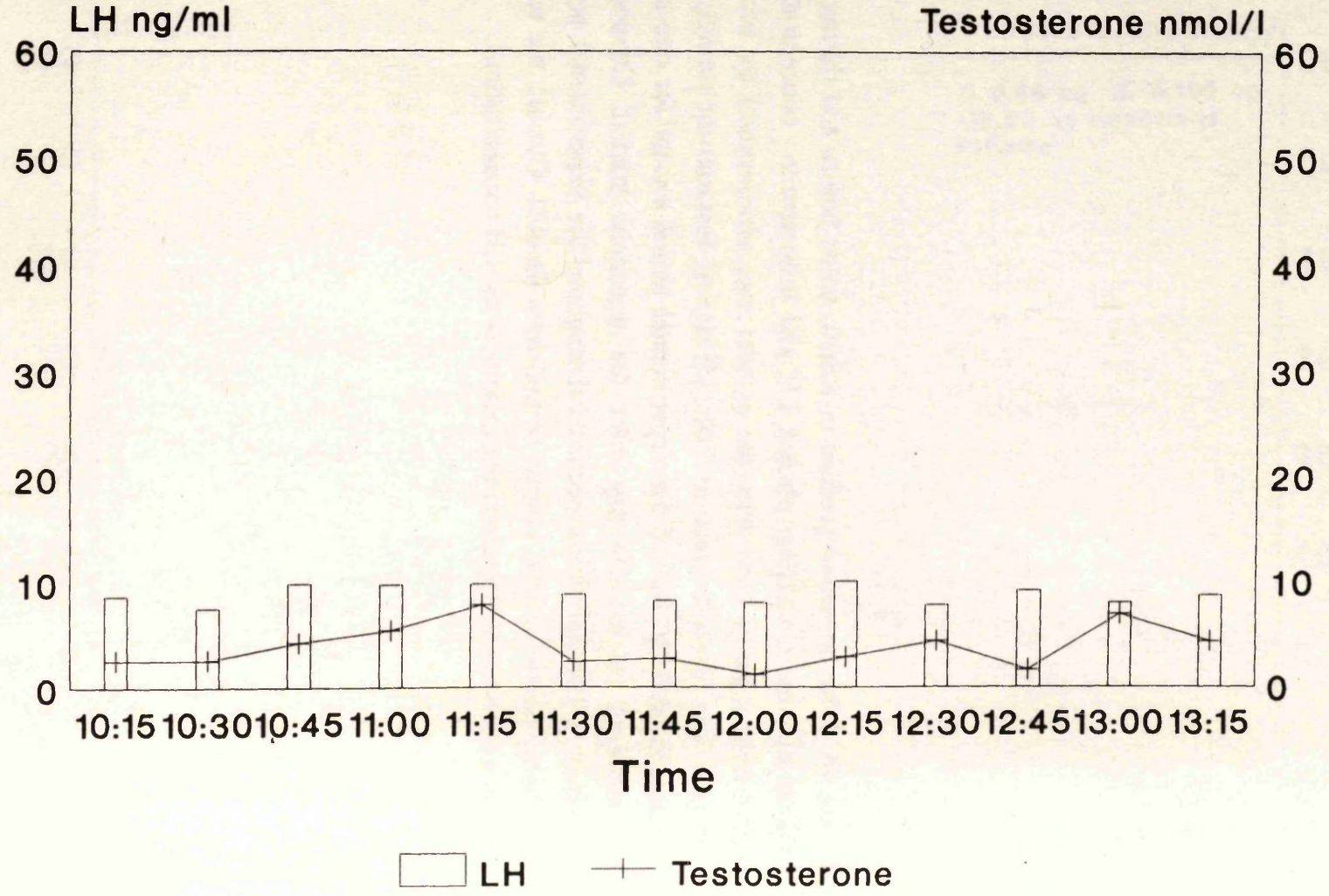


Figure 16. The hormonal profiles in Adam, taken before and during treatment indicate that the circulating plasma LH and testosterone concentrations were within normal range until after the agonist was administered by subcutaneous injection. The levels dropped to 0.01 (LH ng/mg, testosterone nmol/l) and were maintained until the end of the experimental period except for one small peak that occurred on the 4th day after the injections started. Otherwise, wide variations in concentrations occurred throughout the experimental period, with most being below 10 (LH ng/mg, testosterone nmol/l). Overall, the testosterone concentrations tended to mimic the pattern of the LH concentrations.

Fig. 16 Circulating plasma LH & testosterone concentrations in samples collected prior to and during treatment with Nafarelin derivative by injection and pumps in Adam

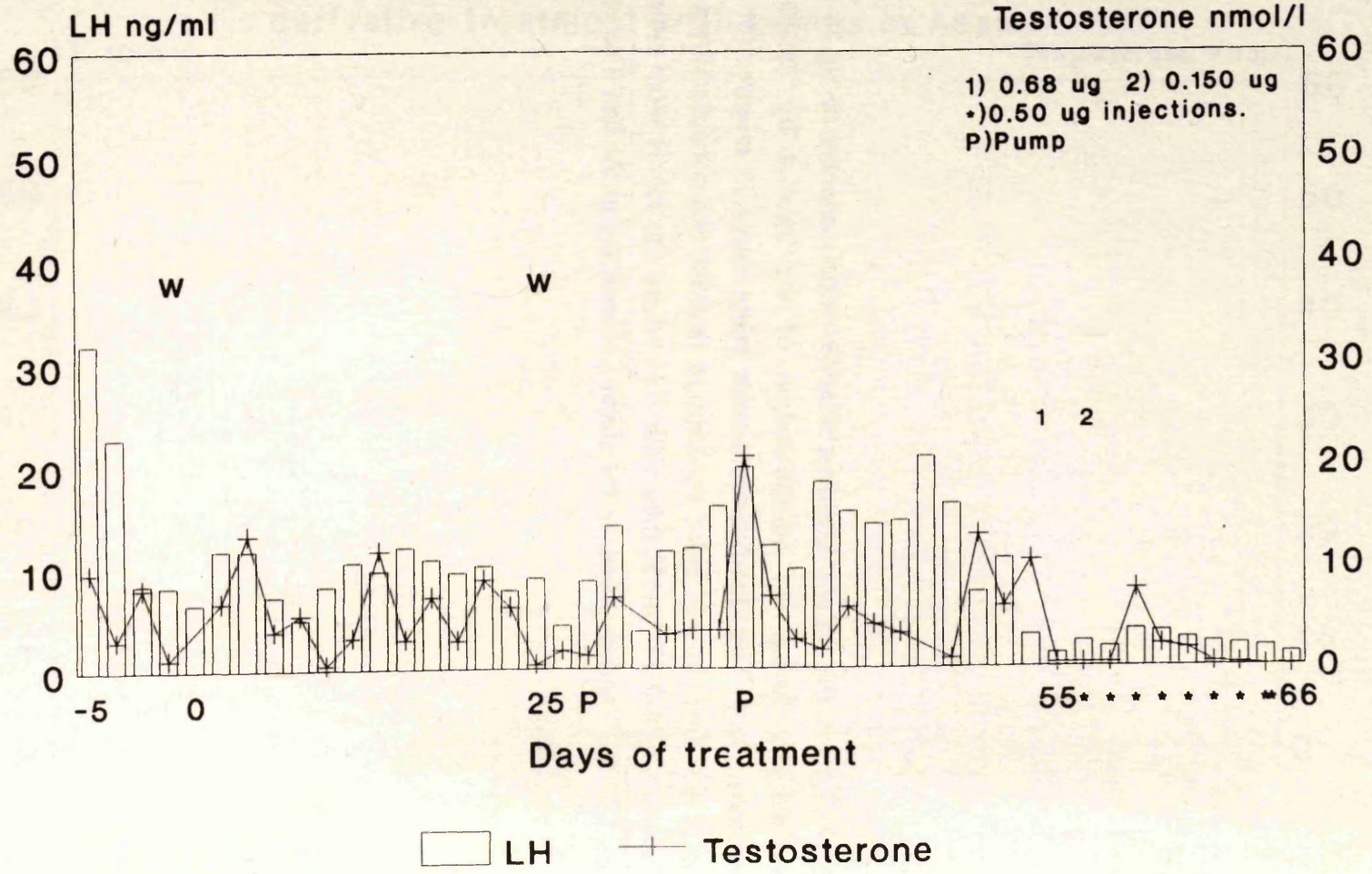


Figure 17. This is the second bleeding window which appears in fig.16 (w). It was carried out during the administration of the agonist by means of a subcutaneous pump. The blood samples were taken every 15 minutes for a total of 2 hours and they showed more variation in testosterone concentrations which were between 1.04-8.27 nmol/l than with LH where the levels were maintained between 3.28-8.77 ng/ml. Observe the slight LH increase in the last 2 samples.

Fig. 17 Circulating plasma LH & testosterone concentrations in samples collected every min. for 2 h. during Nafarelin derivative treatment with pumps in Adam

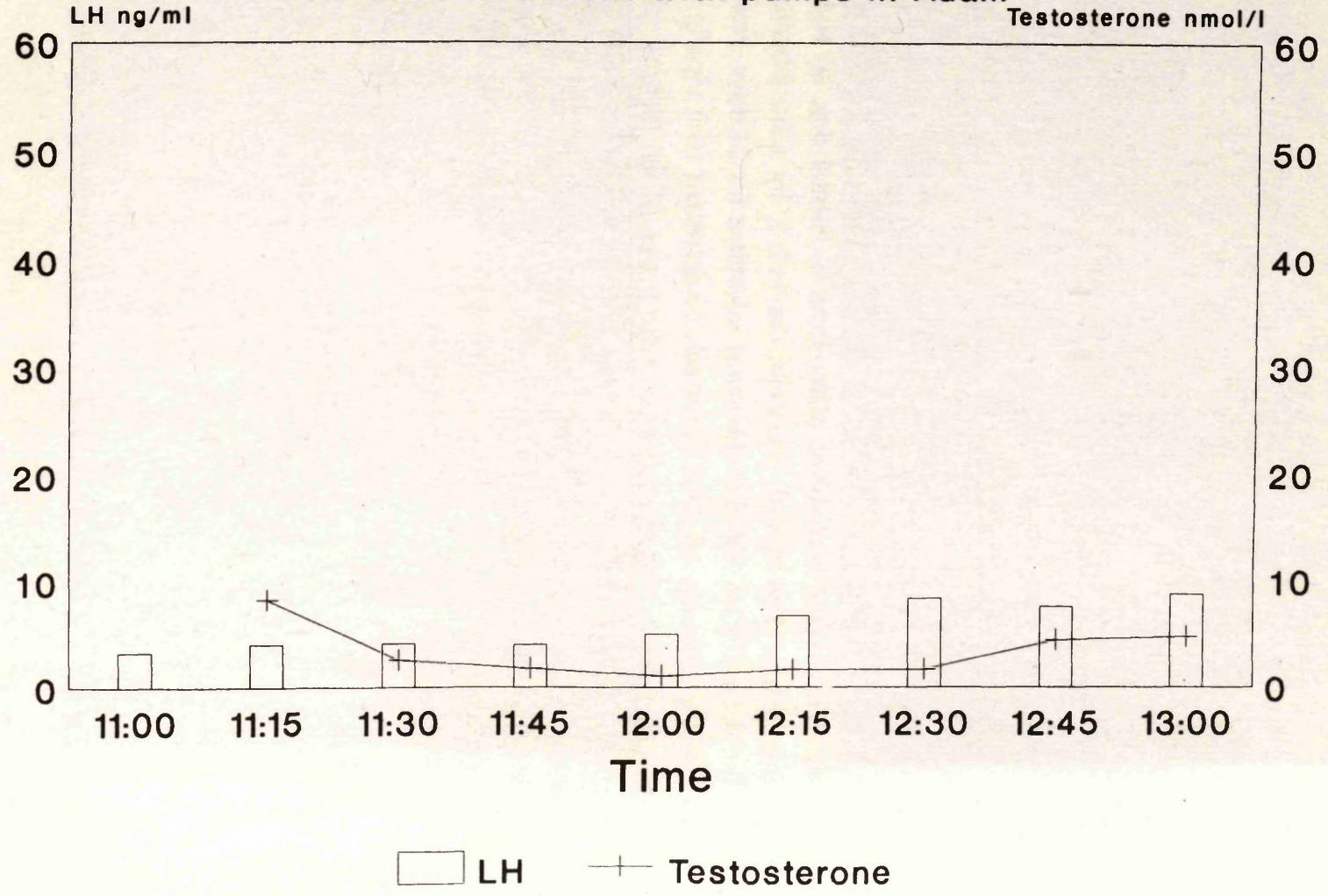
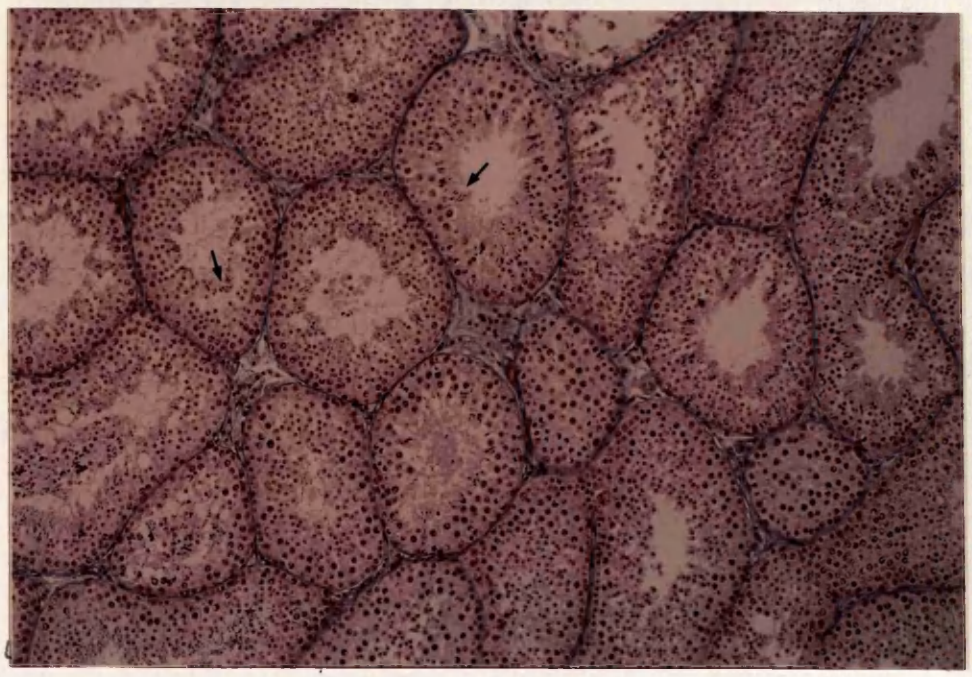


Figure 18. Histological sections of testes from a) normal dog, b) and an adult beagle dog (Adam) treated with Nafarelin derivative, by subcutaneous osmotic pumps for 54 days plus daily subcutaneous injections for 21 days. Notice in the normal dog the presence of a full germ cell complement with clearly identified spermatids (pointed by the arrows) around the lumen of the tubules, which have almost totally disappeared from the treated dog testis, where neither spermatids or spermatozoa can be seen. The tubular lumen has disappeared as well. The only cells found in most of the tubules are Sertoli cells and the spermatogonia closest to the membrane. Magnification10 x. Both slides stained with MSB. A blue microscope filter was used to take both photographs.

a



b

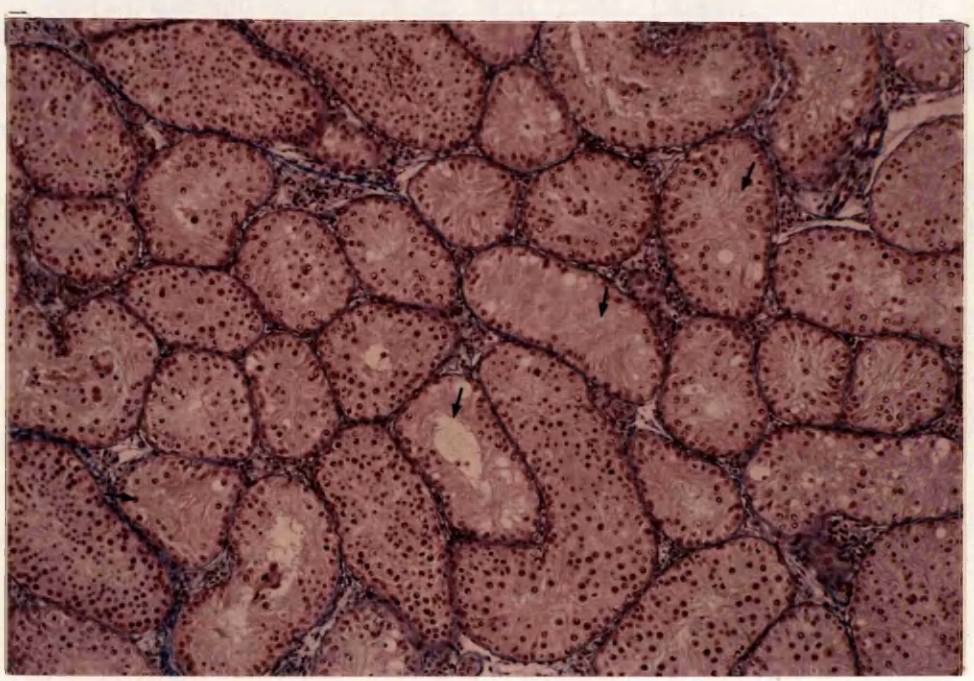
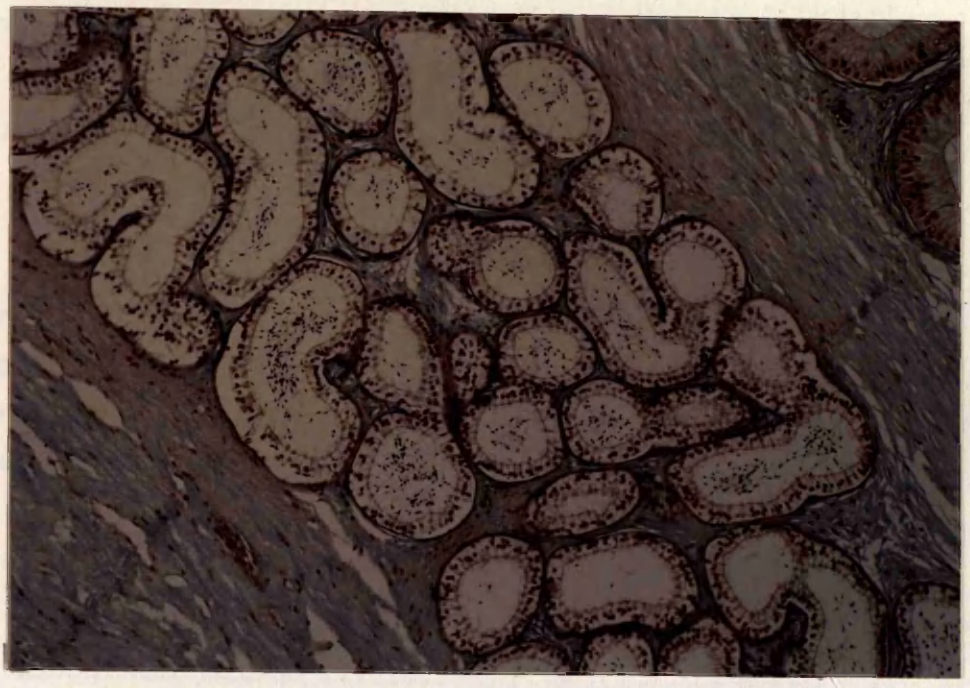


Figure 19. Histological sections of a different area of the head of the epididymis from a) normal dog and b) beagle dog treated with Nafarelin derivative (Adam). Notice the presence of vacuoles in an area of the tubule in the treated dog, whereas in the normal dog nothing similar was found. Also note the absence of spermatozoa from the lumen of the tubule in comparison to the normal dog. Magnification10 x. Both slides stained with MSB. A blue microscope filter was used to take the top photograph .

a



b

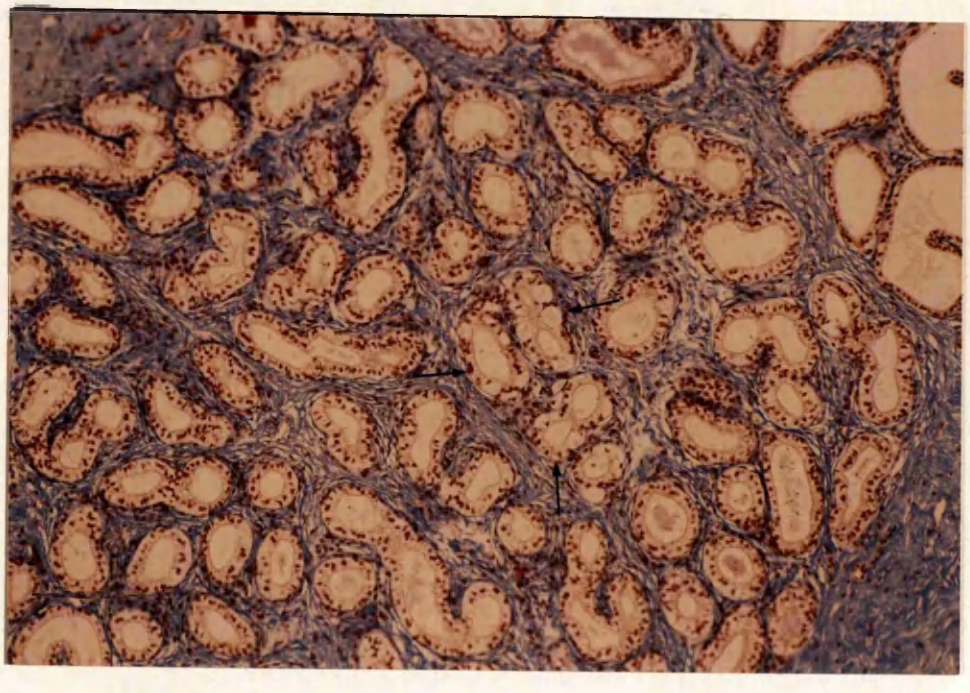
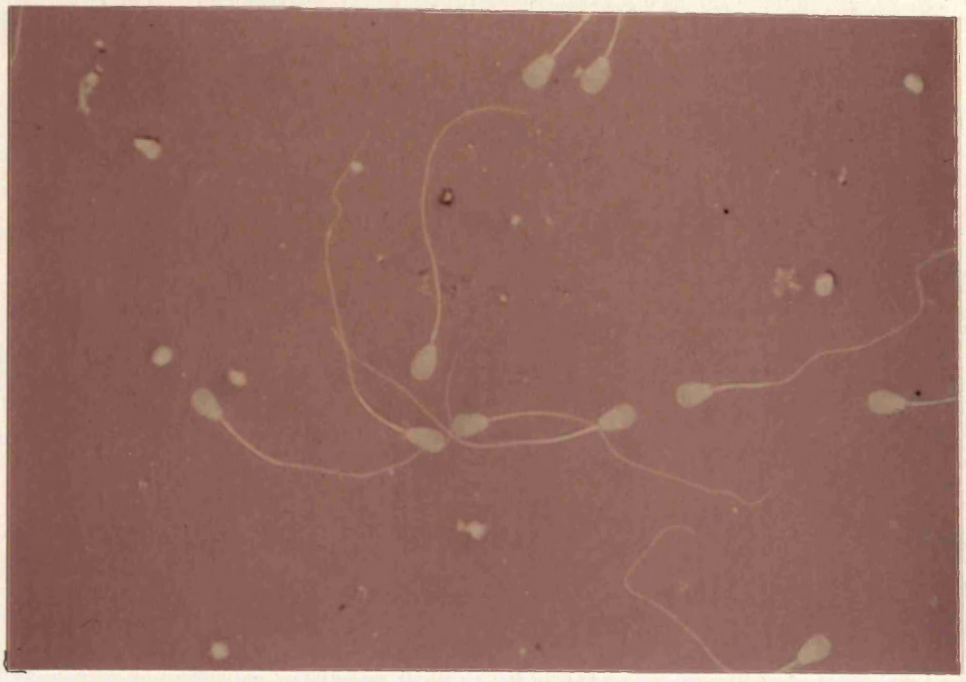


Figure 20. Semen sample from a) normal dog b) beagle dog treated with Nafarelin derivative (Adam). Note that the neck of the sperm in the normal dog is as straight as the tail, whereas in Adam the neck is bent and in some instances it looks almost broken. This defect was noticed even before treatment started. Magnification 100 x. Both slides stained with nigrosine-eosin.

a



b

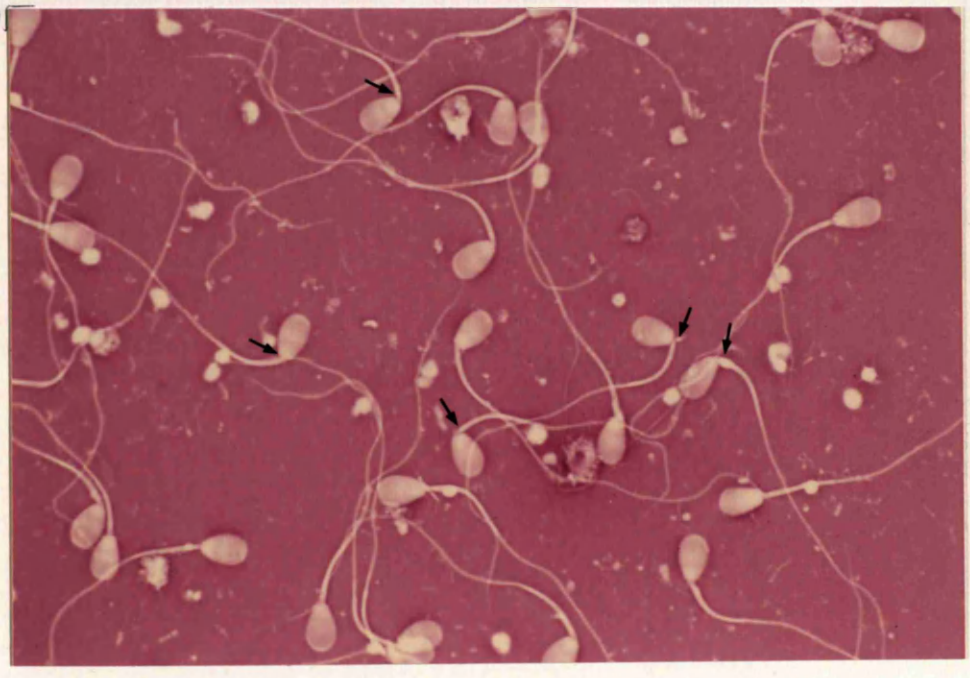
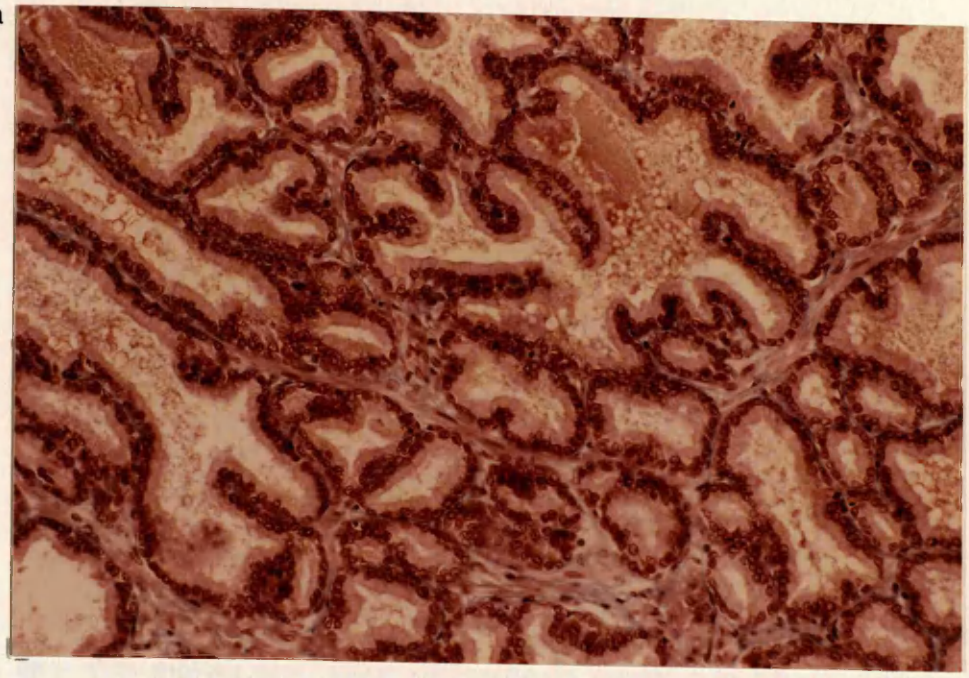


Figure 21. This is an example of a histological section of a prostate, taken from a) normal dog, b) beagle dog treated with Nafarelin derivative (Adam). In the treated animal, the prostatic alveoli have a shrunken appearance and an increase in the connective tissue that surrounds the alveoli indicative of the gland's atrophy. Notice also the absence of secretion in Adam which is abundant in the normal dog's prostate. Magnification 54 x. Both slides stained with MSB.

a



b

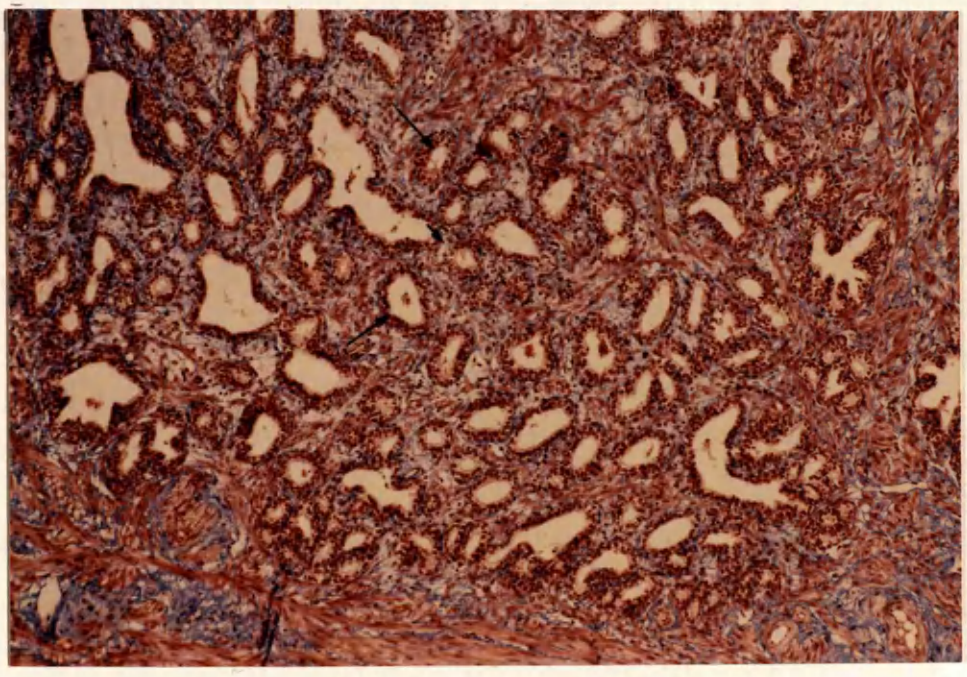
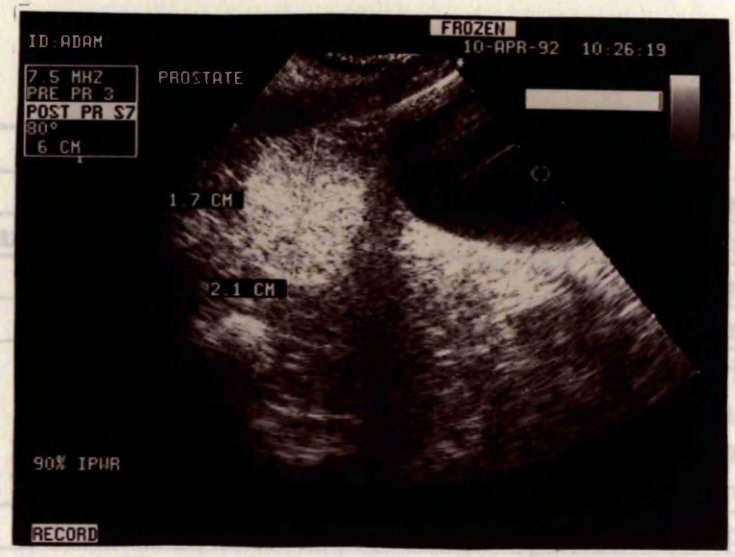


Figure 22. Ultrasonographs of Adam's prostate on April 10 (width 1.7 cms and length 2.1 cms) shows is a small decrease by May 21st (width 0.9 cms and length 1.2 cms) indicating a difference of 0.8 cms in the width and 0.9 cms in the length at the end of treatment.


ID:ADAM
FROZEN
10-APR-92 10:26:19
7.5 MHZ
PRE PR 3
POST PR S7
80°
6 CM
PROSTATE
1.7 CM
2.1 CM
90% IPWR
RECORD

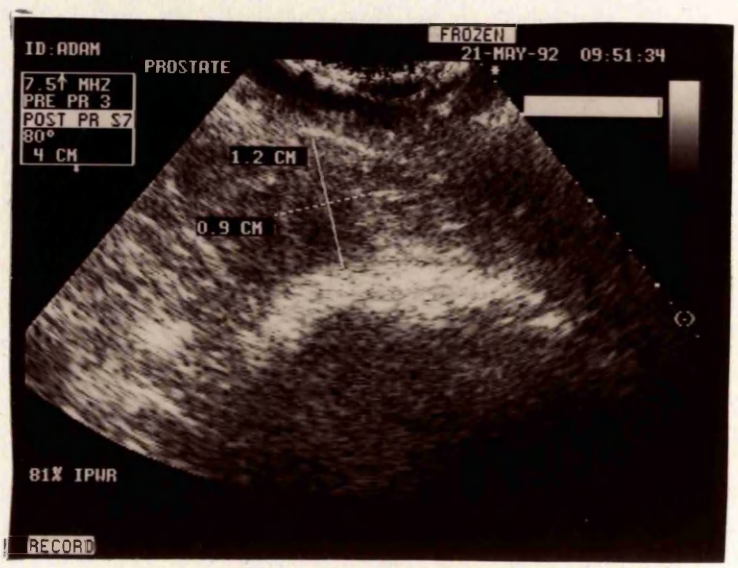

ID:ADAM
FROZEN
21-MAY-92 09:51:34
7.5↑ MHZ
PRE PR 3
POST PR S7
80°
4 CM
PROSTATE
1.2 CM
0.9 CM
81% IPWR
RECORD

Table 10 Protocol for the Nafarelin derivative treatment of an adult male beagle (Baxter) treated for 48 days

Days	Procedure
-3	Daily bleeding
-2+6, and then weekly	Semen collection
-1	Bleeding every 15 min. for 3 hours
26	Bleeding every 15 min. for 2 hours.
0-28 28-41 41- 48	Nafarelin derivative administration First pump inserted releasing 17 ug/day Second pump releasing 17 ug/day Third pump releasing 34 ug/day
15, 36	Scanning of prostate
Daily	Temperature taken
-2 +4 and then weekly	Genitalia evaluation.

Table 11. This table shows details of the experimental protocol in Baxter. The Nafarelin derivative treatment to this dog was administered by subcutaneous osmotic pumps only. The results indicate that, even when problems arose with the use of the osmotic pump, this did not cause any systemic reaction as gauged by body temperature with the first two pumps. However, complications with the insertion of the third pump made it necessary to end this experiment. The testicular measurements taken during treatment, except for small differences in the length, do not indicate a marked or continuous decrease in size. There were no changes to the consistency of either testis or epididymides.

Table 11. Clinical findings and external genitalia evaluation, prior to and during treatment of a dog (Baxter) with a Nafarelin derivative by osmotic pump

Date	Nafarelin derivative	Testicular measurements: Left width (cms)	Left Length (cms)	Right Width (cms)	Right Length (cms)	Clinical evaluation: Testicula consistency	Epididymis	Temperature o F	Body weight kgs	Comments
02/03/92		9.8	8.7	8.7	8	resilient	normal		17.2	
06/03/92	1st pump 17 ug/day	9.8	8.5	8.7	8	resilient	normal			Pump insertion
07/03/92										Slight swelling of wound
11/03/92		9.8	8.6	8.7	8	resilient	normal			
17/03/92								101		
18/03/92		9.8	8.7	8.7	7.5	resilient	normal	101.1		
19/03/92								101.1	17.2	
20/03/92								101.1		
23/03/92								101		
24/03/92								101.1		
25/03/92		9.8	8.7	8.7	8	resilient	normal	101.2		
01/04/92		9.8	8.5	8.7	7.5	resilient	normal			
02/04/92	2nd pump 17 ug/day							101.6	17.1	Pump insertion
03/04/92								101.1		Inflamed incision
04/04/92								101.6		
05/04/92								101.6		
07/04/92								101.1		
08/04/92		9.8	8.5	8.7	7.5	resilient	normal	101.1		
09/04/92								101.1		
10/04/92								101.1		
11/04/92								101.2	17.2	
14/04/92								101.2		
15/04/92	3rd pump 34 ug/day	9.8	8.5	8.7	7.5	resilient	normal	101.2		Inflamed incision
16/04/92								101.2		Inflamed incision
17/04/92								101.4		"
18/04/92								101.3		"
19/04/92								101.4		"
20/04/92								101.1	17.1	"
21/04/92								101.3		Wound open
22/04/92								101.1		End experiment

Table 12. The libido in this animal reached the normal score (3) three times only , but it was sufficient to allow semen to be collected on numerous occasions, even without the aid of a teaser bitch. No alterations to any of the parameters can be seen except for a small decrease in the amount of the last ejaculate.

Table 12 Libido and semen evaluation of a dog (Baxter) prior to and during treatment with a Nafarelin derivative by osmotic pump

								Semen abnormalities						
Date	Treatment	Libido	Ejac. vol in ml	Color.	Progress mot %	Concent x (10)6 /ml	Live:dead	Head %	Acrosome %	Neck %	Tail %	Droplet %	Total	Alkaline phosphatase u/l	Comments
25/02/92		3	2	white	4	275	93:7		normal		7	3	10	253,000	Teaser in oestrus
02/03/92		2	2	white	4	200	96:4	4	normal				4	267,000	Teaser in anestrus
06/03/92	1st. pump 17 ug day														
10/03/92		2	2	white	4	190	92:8	1	normal		4		5	98,010	Teaser in anestrus
13/03/92		2	2	white	4	260	98:2		normal		2		2	149,250	No teaser
17/03/92		3	3	white	4	170	96:4		normal		4		4	121,200	No teaser
24/03/92		2	2	white	4	380	92:8	1	normal		8		9	69,560	Teaser in anestrus
31/03/92		3	3	white	4	18	97:3	1	normal		2		3	130,150	No teaser
02/04/92	2nd pump 17 ug day														
07/04/92		2	2	white	4	673	97:3		normal	5		1	6	135,820	No teaser
14/04/92		2	2	white	4	602	98:2	1	normal	17	3		21	169,650	No teaser
15/04/92	3rd.pump 34 ug/day														
21/04/92		2	1.5	white	4	393	97:3		normal		23		23	153,000	Teaser in anestrus

Figure 23. This figure presents the results of samples that were taken every 15 minutes for a total of 3 hours. It was undertaken prior to treatment with Nafarelin derivative by osmotic pumps. It indicates higher circulating plasma levels of testosterone during the morning samples. The LH profile does not show much variation during the same period with a range of 3.02-4.52 ng/ml, whereas testosterone values show a wider fluctuation from 0.72-17.64 nmol/l. This is the first of the two window (w) bleedings indicated in figure 24.

Fig. 23 Circulating plasma LH & testosterone concentrations in samples collected every 15 minutes for 3 hours in Baxter

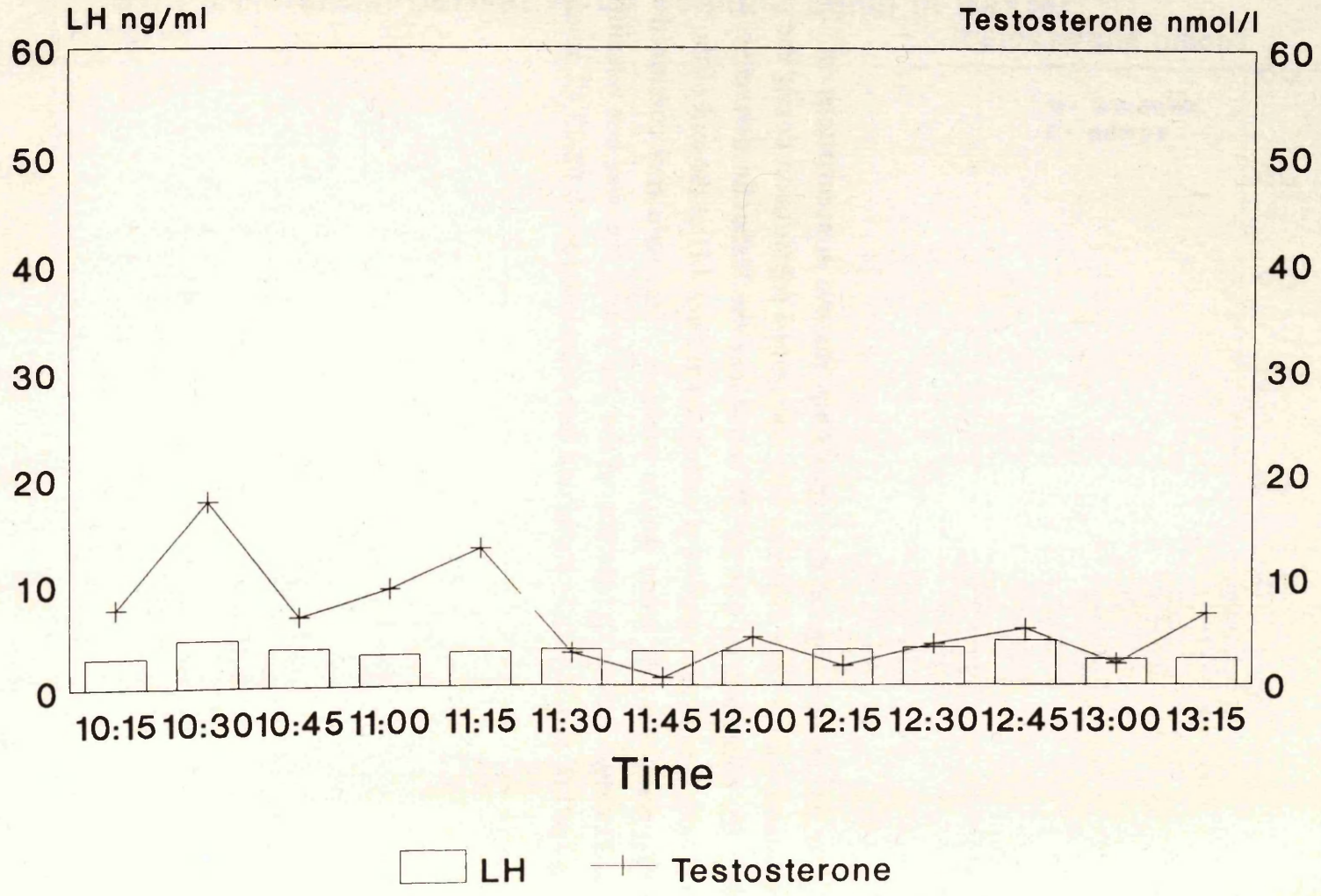


Figure 24. In this figure the circulating plasma concentrations of LH and testosterone show little evidence of a maintained suppression during the periods when the subcutaneous pumps (**P**) containing the Nafarelin derivative were in situ. Note the reduced amount of circulating plasma LH at the end of the 25 days during which the first pump was in position. This reduction continued for the first few days following insertion of the 2nd pump, but was not sustained. The range of LH was 2.4-21.84 ng/ml and that of testosterone 0.02-23.82 nmol/l.

Fig. 24 Circulating plasma LH & testosterone concentrations in samples collected prior to and during 2 months treatment with a Nafarelin derivative by osmotic pump in Baxter

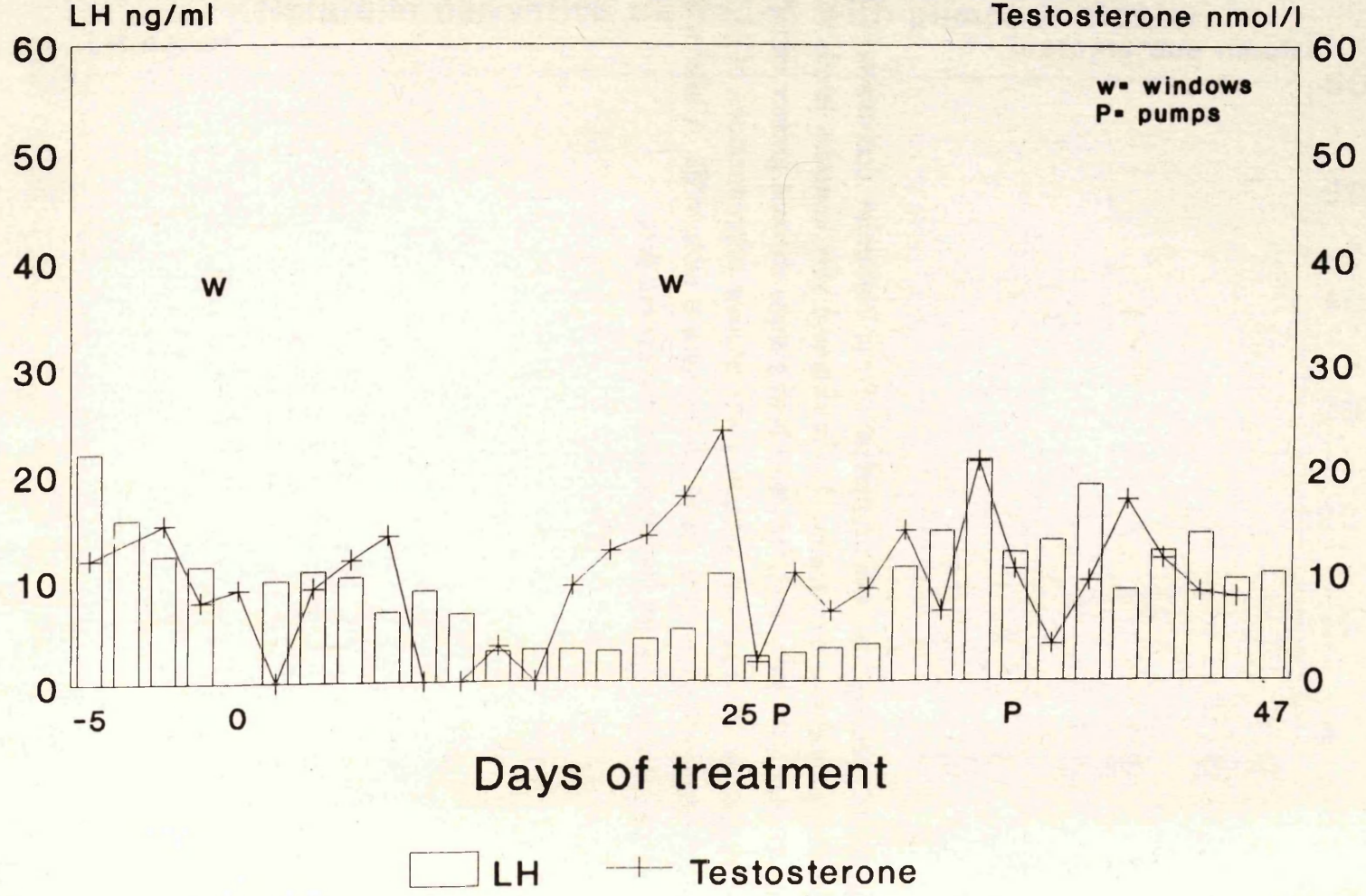


Figure 25. During the administration of the Nafarelin derivative, a frequent bleeding period was undertaken. LH maintained very constant levels (2.04-3.85 ng/ml) during the two hours, whereas testosterone showed greater variations and an increase in the afternoon samples, with plasma concentrations of (3.64-12.72 nmol/l). Magnification 54 x . Both slides stained with MSB. A blue microscope filter was used to take the photograph of the normal dog.

Fig. 25 Circulating plasma LH & testosterone concentrations in samples collected every 15 minutes for 2 hours at end of Nafarelin derivative treatment with pumps in Baxter

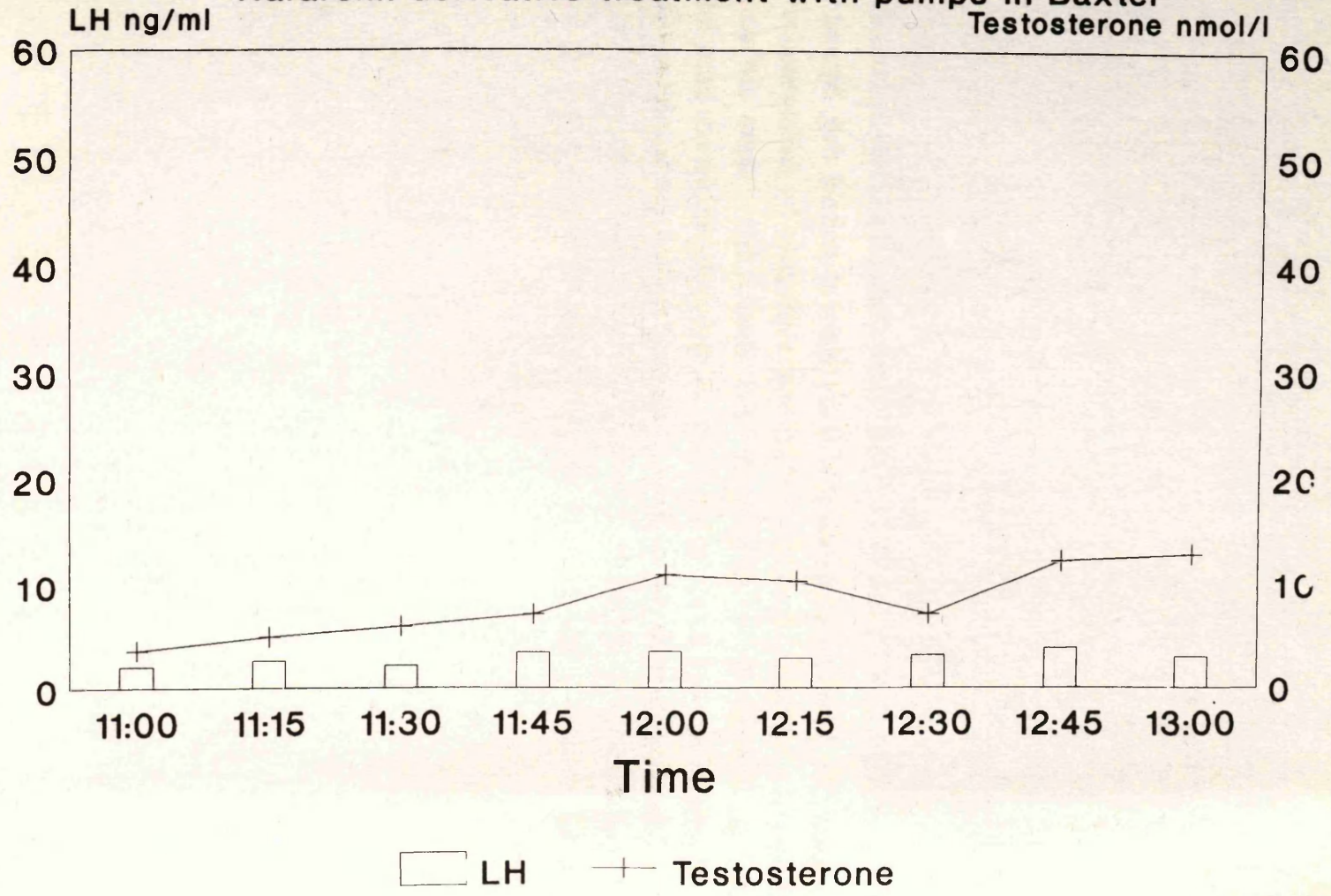
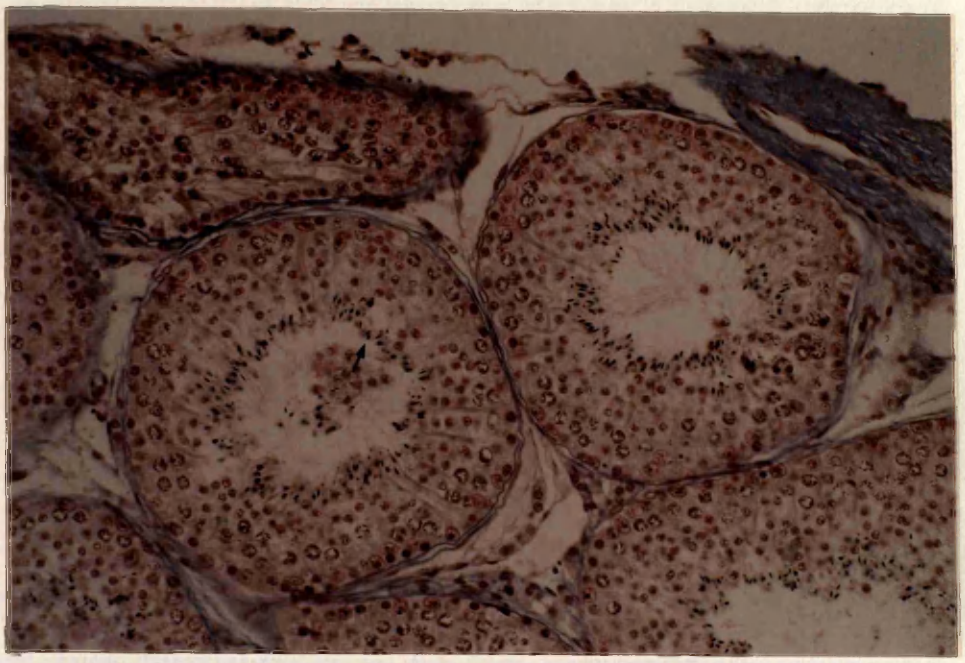


Figure 26. Histological sections of testes from a) normal dog, b) and an adult beagle dog (Baxter), treated with Nafarelin derivative by subcutaneous osmotic pumps. Comparing the normal with the treated dog, there are no obvious differences between them, as evidently spermatogenesis has not been interrupted in either sample. The only difference appears to be a slight increase in cellular debris in the lumen of the tubules. Magnification 54 x . Both slides stained with MSB. A blue microscope filter was used to take the photograph of the normal dog.

a



b

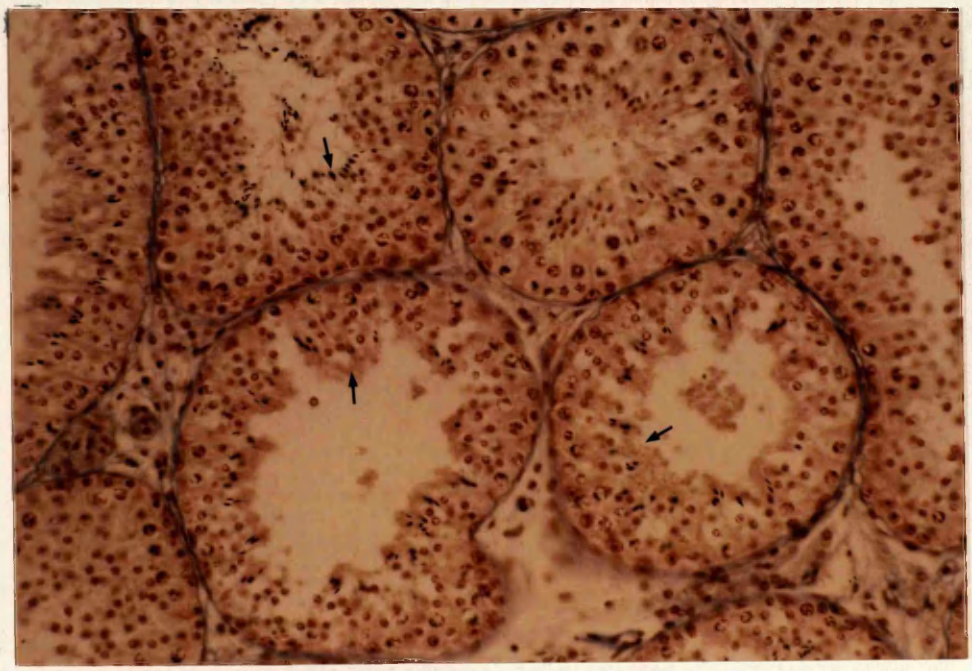
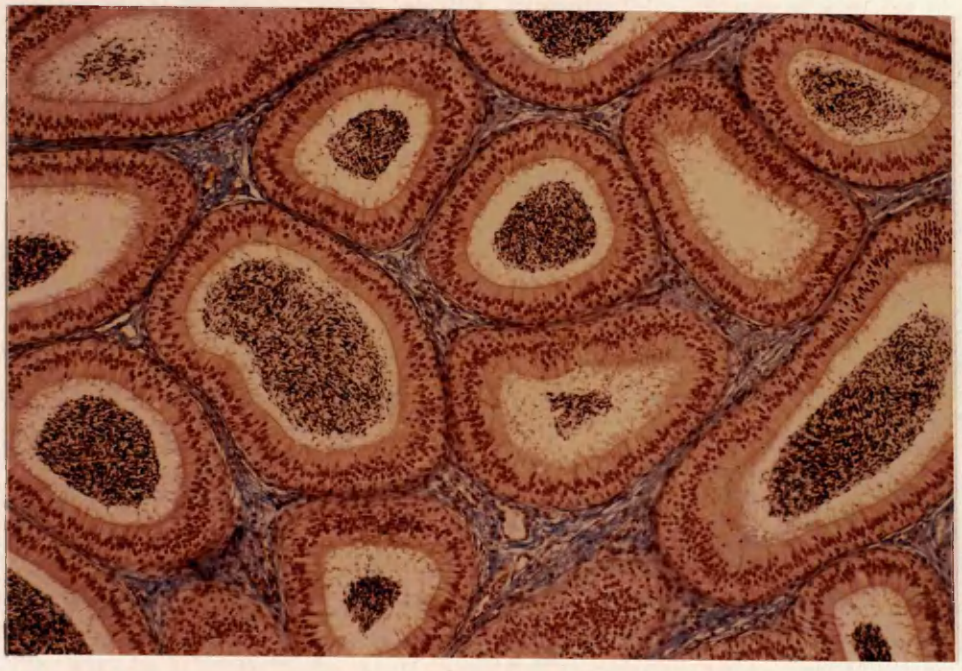


Figure 27. Example of a histological section of the head of the epididymis from a) normal dog and b) dog treated with Nafarelin derivative (Baxter) administered by subcutaneous pump for 48 days. Both samples appear similar, as there is neither evidence of alteration to the homogeneity of the cell's cytoplasm nor to the regular arrangement of the cells lining the epididymis. Notice the presence of spermatozoa in the lumen of the tubules in both dogs. Magnification 10 x. Both slides stained with MSB. A blue microscope filter was used to take the photograph of the treated dog.

a



b

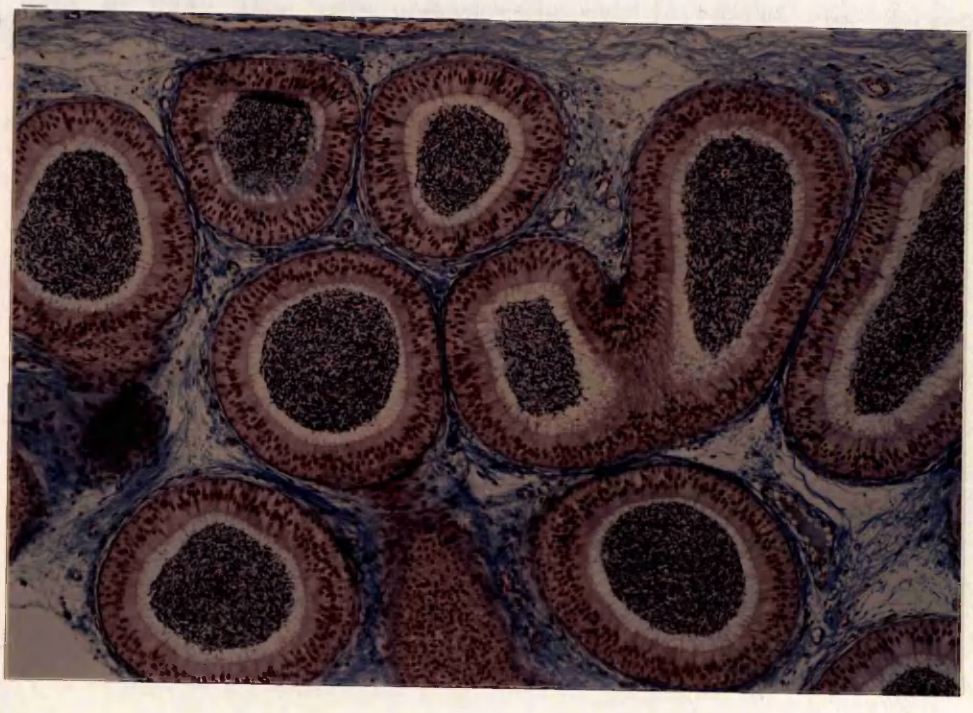
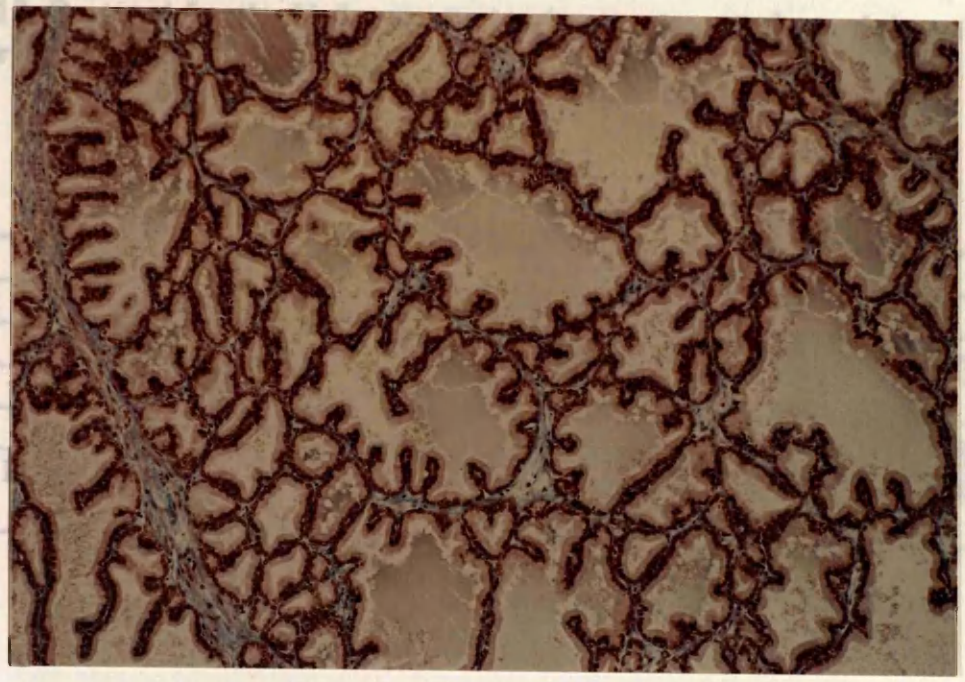


Figure 28. Histology sections from prostate of a) normal dog, b) beagle dog treated with Nafarelin derivative (Baxter). Both samples show functioning cells with a full cell cytoplasm. The comparison of both samples does not show much difference between the normal and the treated dog. Magnification 10 x. Both slides stained with MSB. A blue microscope filter was used to take the photograph of the treated dog.

a



b

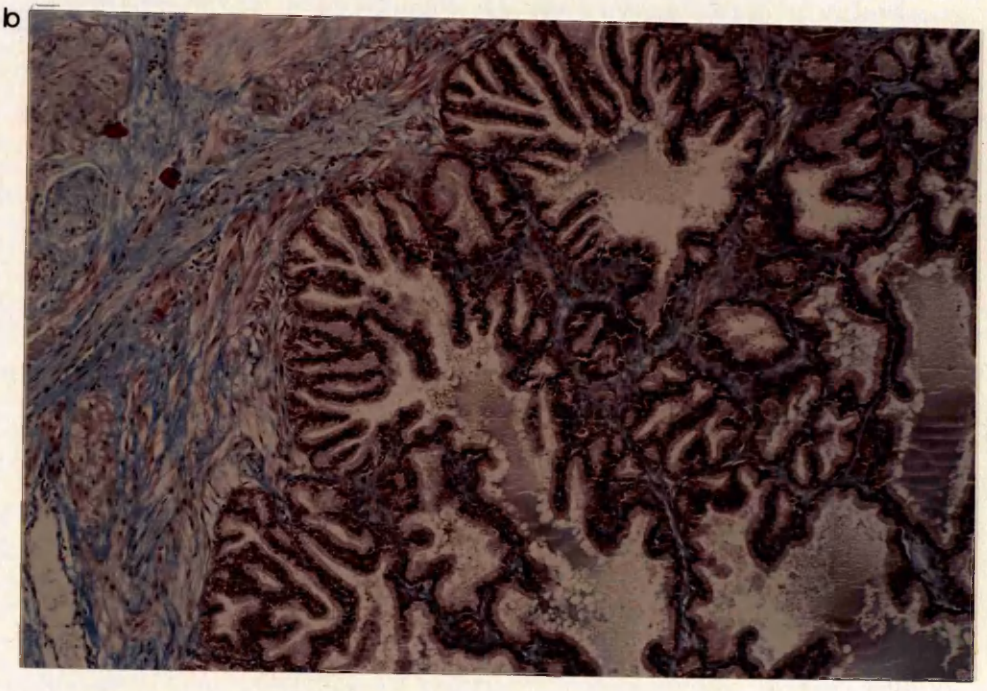


Table 13 Protocol for the Nafarelin derivative treatment of an adult male beagle (Dexter) treated for 23 days.

Days.	Procedure
-5 to -2	Daily bleeding
-1	Bleeding every hour for 6 hours
1 to 23	Daily bleeding
1 to 23	Daily injection of 50 ug. Nafarelin acetate
-7, +7, 14, 21	Semen collection, genitalia evaluation and body temperature taken.

Table 14. Presents details of the clinical findings in the external genitalia in Dexter, in whom the Nafarelin acetate was administered by daily subcutaneous injections for 23 days. Temperature and weight remained within the normal limits with no major changes. Notice the slight decrease in testicular size and consistency changes towards the end of the 23 day treatment period. The only apparent change was in the testicular consistency of the gonad at the end of treatment.

Table 14 Clinical findings and external genitalia evaluation, prior to and during treatment of a dog (Dexter) treated by daily subcutaneous injections of Nafarelin acetate for 23 days

		Testicular measurements				Clinical evaluation			
Date	Nafarelin acetate	Left width (cms)	Left length (cms)	Right width (cms)	Right length (cms)	Testicular consistency	Epididymis	Temperat. o F	Weight. Kg
03-Jun-92		6.5	6.2	6.5	6.2	resilient	normal	101	17.1
05-Jun-92									
06-Jun-92									
07-Jun-92		6.5	6.2	6.5	6.2	resilient	normal	101.1	17
08-Jun-92									
09-Jun-92									
10-Jun-92									
11-Jun-92	50								
12-Jun-92	"	6.5	6.2	6.5	6.2	resilient	normal	101	17.1
15-Jun-92	"								
16-Jun-92	"								
17-Jun-92	"								
19-Jun-92	"								
22-Jun-92	"								
25-Jun-92	"								
26-Jun-92	"	6.4	6.1	6.4	6.1	resilient	normal	101.2	17
29-Jun-92	"								
01-Jul-92	"								
03-Jul-92	"	6.4	6.1	6.4	6.1	less resilient	normal	101	17

Table 15. The libido of this animal (Dexter) was normal (3) but only in the presence of a teaser bitch. The first ejaculate was collected on the same day that treatment started, and was collected before the first injection was given. Note that only one drop of ejaculate was produced 21 days after start of treatment, but the libido score was normal (3) with a prolonged erection.

Table 15 Libido and semen evaluation of a dog (Dexter) treated with Nafarelin acetate by daily subcutaneous injections

Date	Nafarelin acetate	Libido	Ejac. vol. in ml	Color	Progress mot %	Concent x (10)6 /ml	Abnormalities Live:dead	Acrosome %	Neck %	Tail %	Total	Alkaline phospatase u/l	Comments
11/06/92	1st. injection	3	3	white	80	144	99:1	normal	4	1	5	93,740	Semen collected before injection.
22/06/92		3	4	clear	0	5	96:4		17	18	35	2,650	
01/07/92		3	1 drop	clear	0	not done						not done	
03/07/92		1	0										

Figure 29. This figure presents the hourly bleeding period undertaken prior to treatment and shows LH values which ranged between 3.31 to 7.64 ng/ml with testosterone showing variations in the range of 5.35-15.36 nmol/l within the 6 hour period. This window is indicated in figure 30 (w).

Fig. 29 Circulating plasma LH & testosterone concentrations in samples collected every hour for 6 hours in Dexter

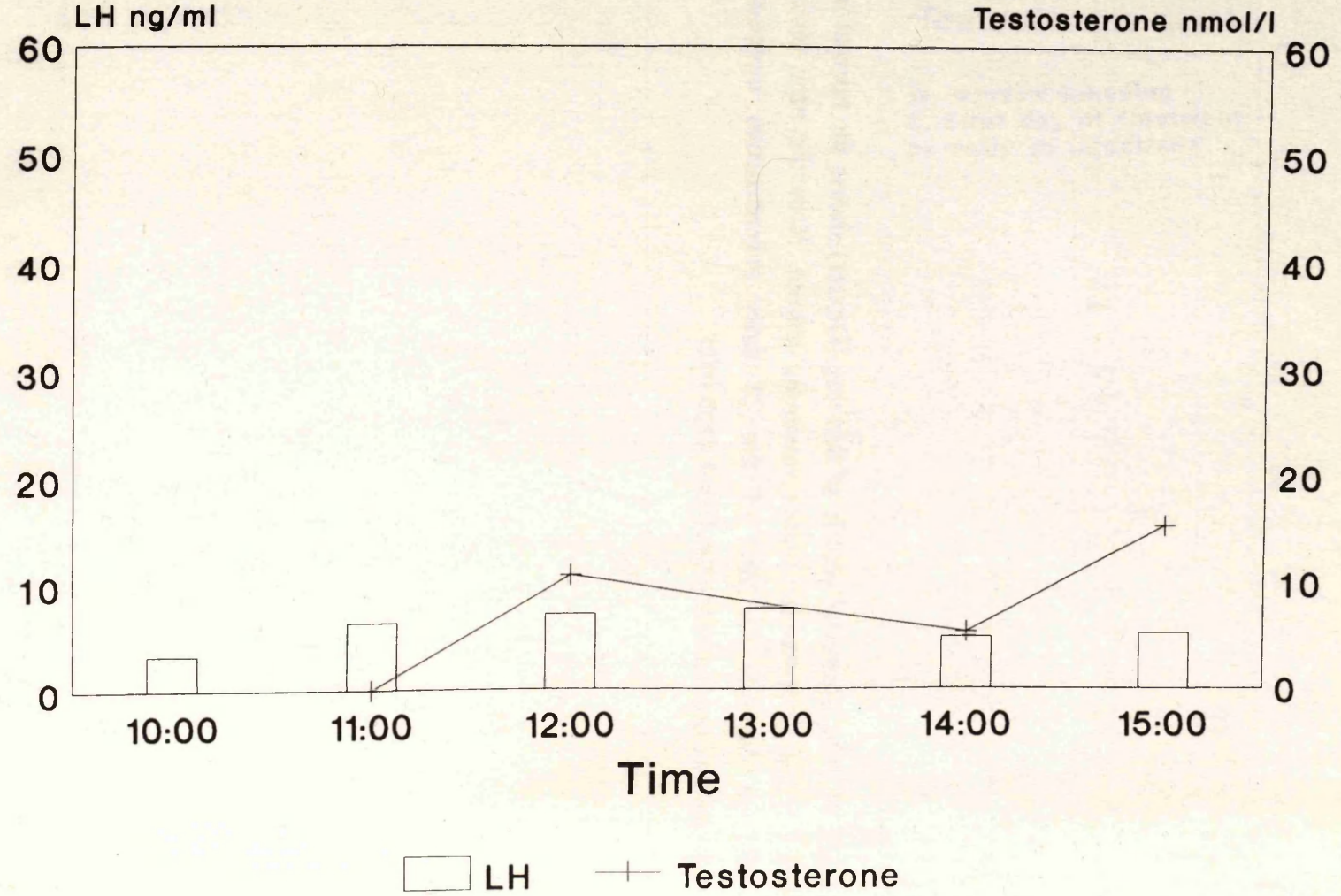


Figure 30. The hormonal profile of this dog (Dexter) shows the normal levels of both LH and testosterone before treatment started. Note the high testosterone levels on the day the first of the 23 daily subcutaneous injections was administered and the sharp decline 4 days later.

Fig. 30 Circulating plasma LH & testosterone concentrations in samples taken prior to and during treatment with Nafarelin acetate by daily injection in Dexter

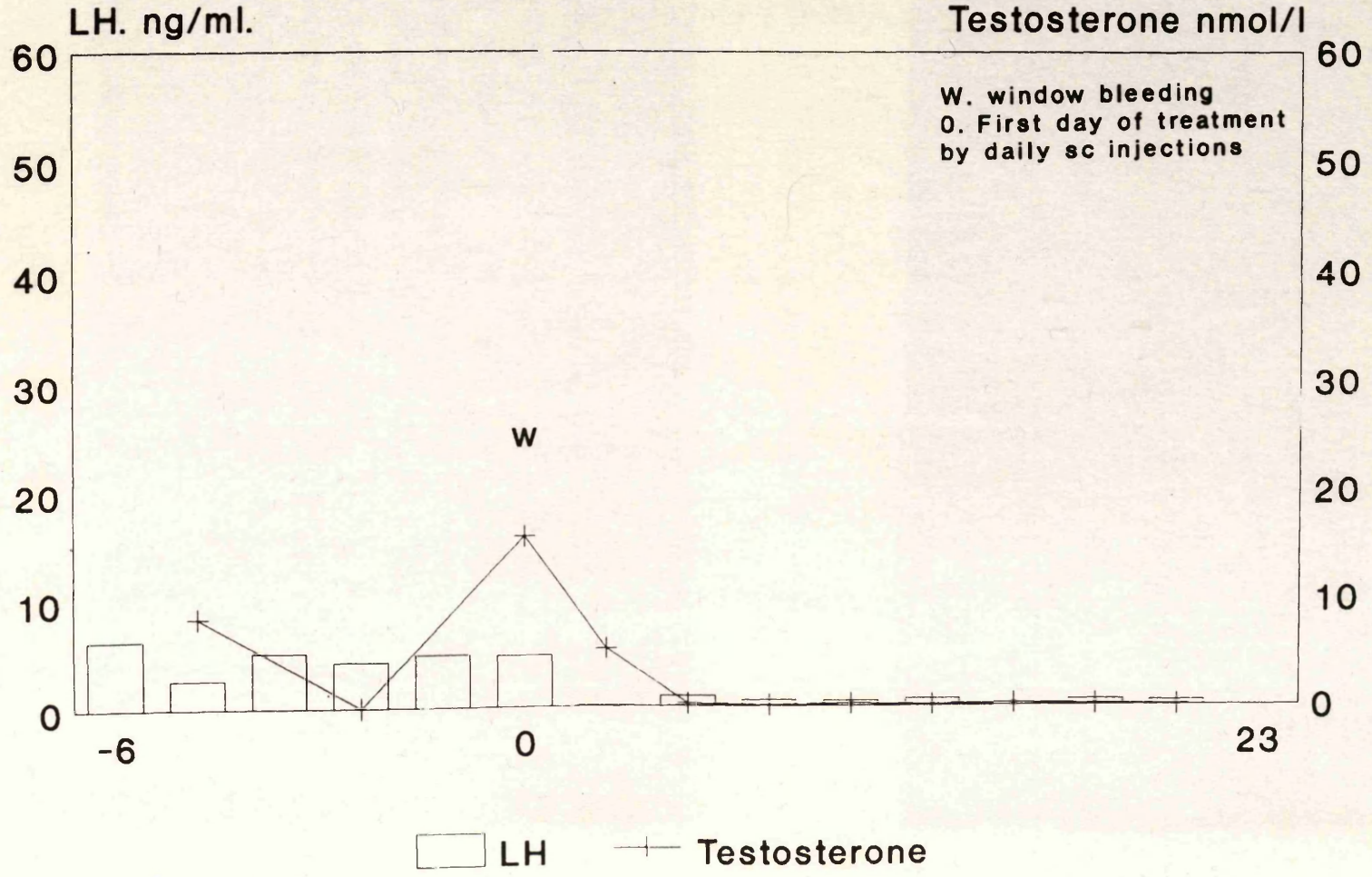
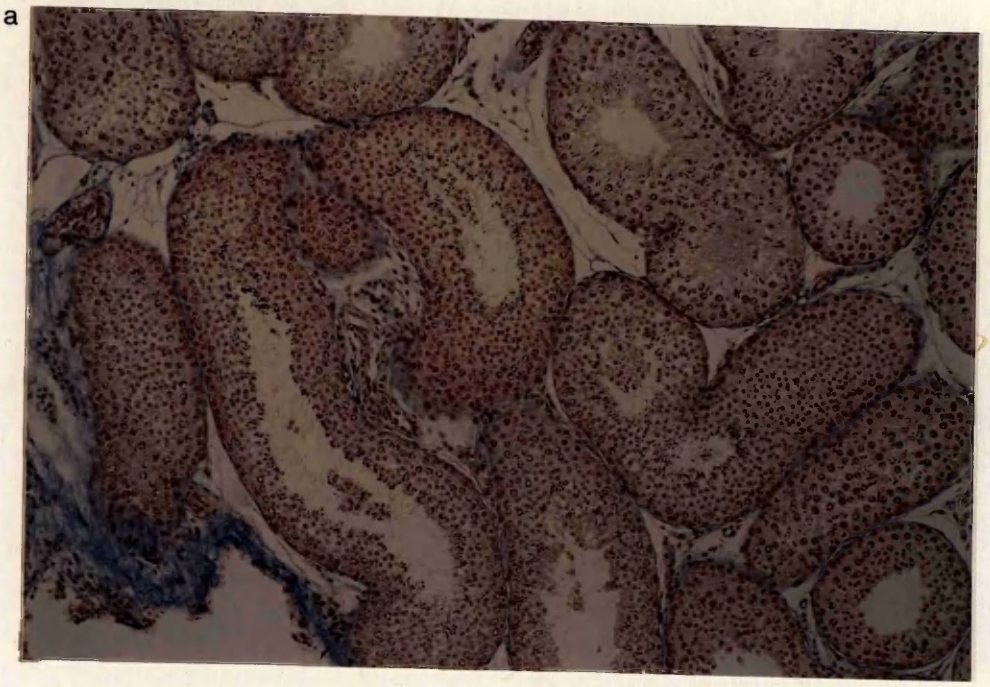


Figure 31. Histological sections of the testes from a) normal dog b) beagle dog treated with Nafarelin acetate (Dexter). In the normal dog, the seminiferous tubule has a very uniform arrangement and the evidence of ongoing spermatogenesis is indicated by the presence of a full germ cell complement with clearly identified spermatids (indicated by arrows) around the lumen of the tubules. These have begun to disappear in some of the treated seminiferous tubules but where some spermatids can still be seen (see arrows). Magnification 10 x. Both slides stained with MSB. A blue miscroscope filter was used to take the normal dog photograph.

a



b

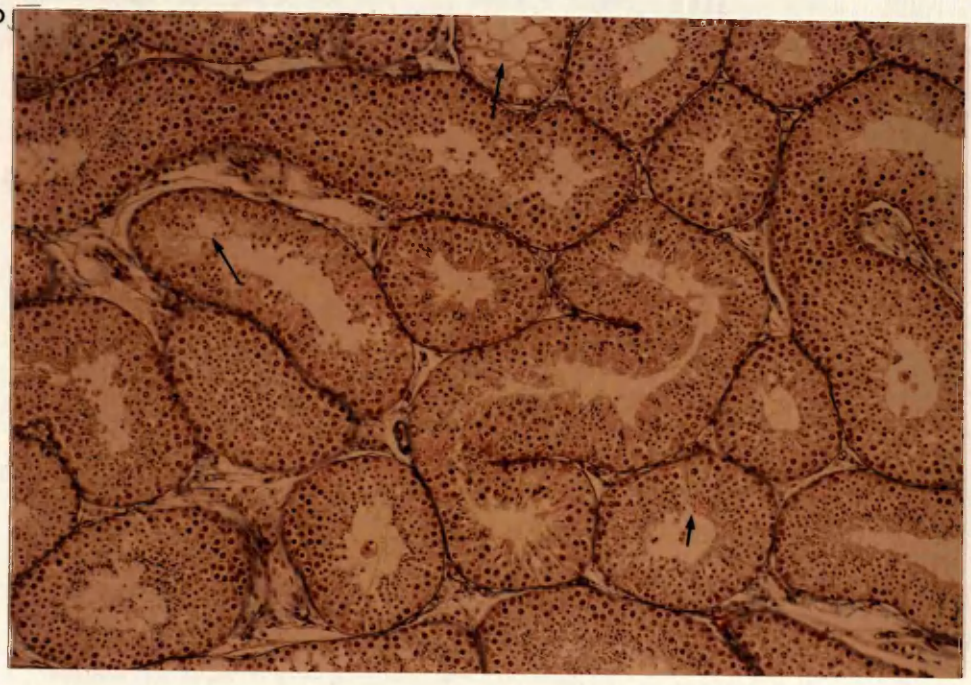
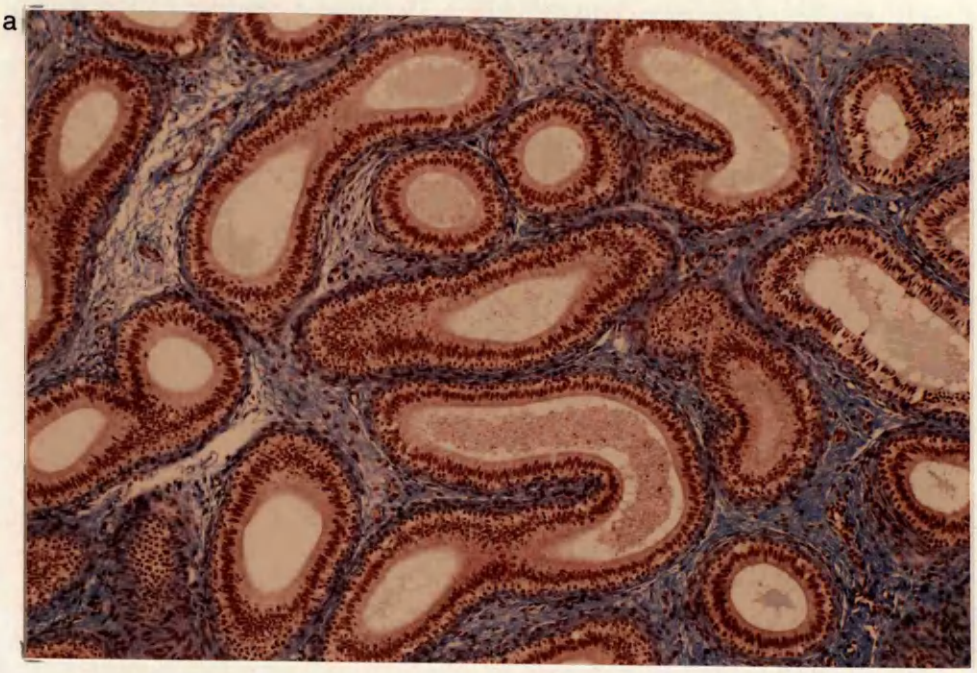


Fig. 32. Histological sections of the body of epididymis from a) normal dog, b) beagle dog (Dexter), treated with Nafarelin acetate. Notice a few empty spaces in between the cells in the treated beagle which gives it an irregular appearance, whereas in the normal dog the cells have a very even and uninterrupted arrangement.

a



b

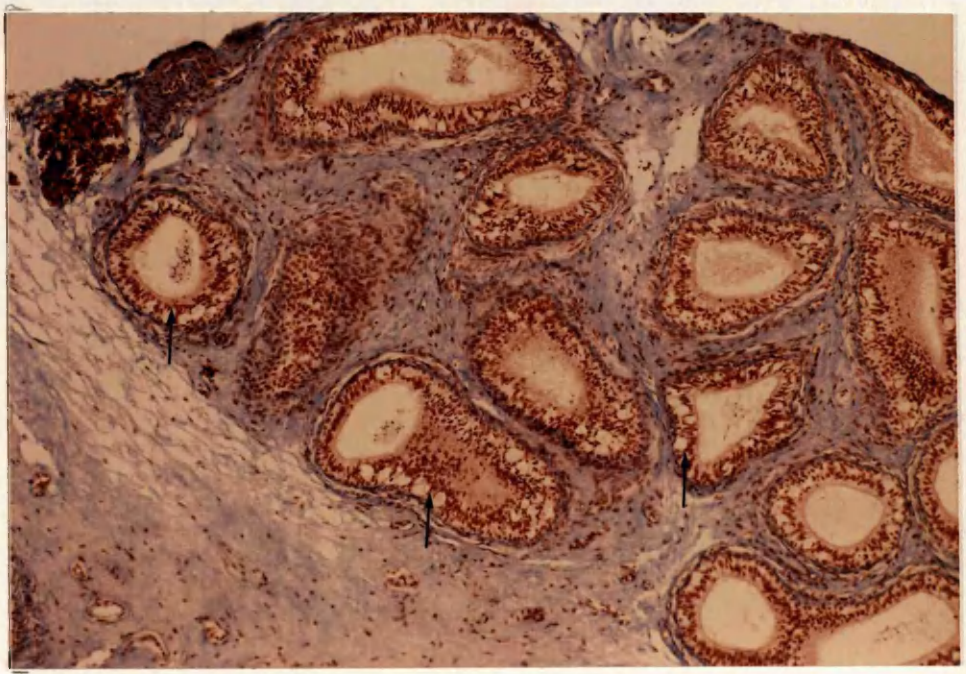
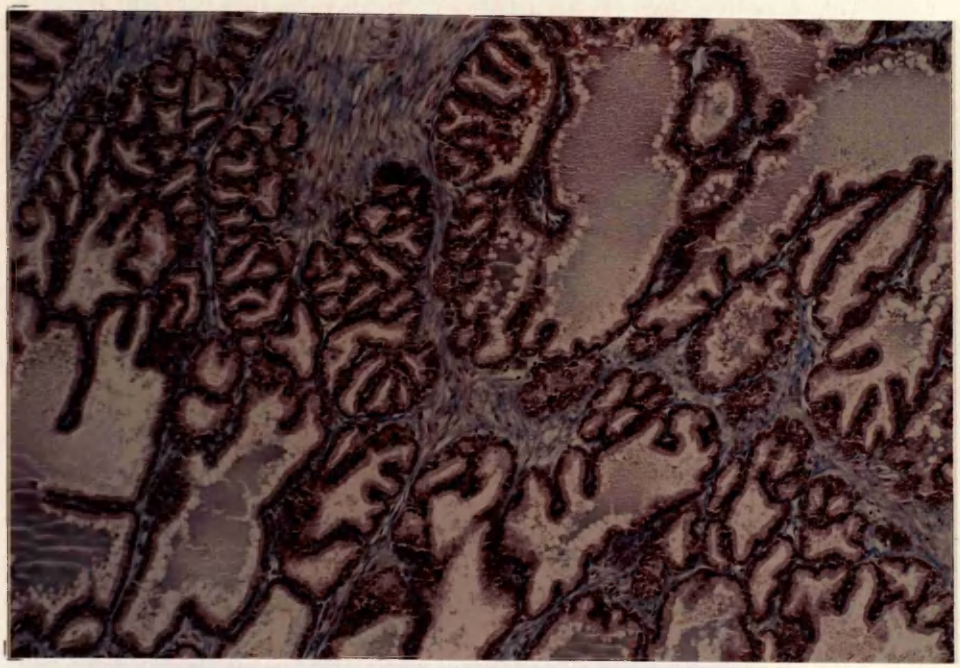


Figure 33. Histological sections of prostate from a) normal dog, b) beagle dog (Dexter) treated with Nafarelin acetate. Notice that the size of the alveoli in the treated dog look smaller and there is an increased amount of connective tissue indicative of a slight atrophy in the treated animal in comparison with the very wide lumina in the normal dogs. Note also the presence of abundant section in the lumen of the alveoli in the normal dog whereas in the treated dog it is absent. Magnification 10 x . Both slides stained with MSB

a



b

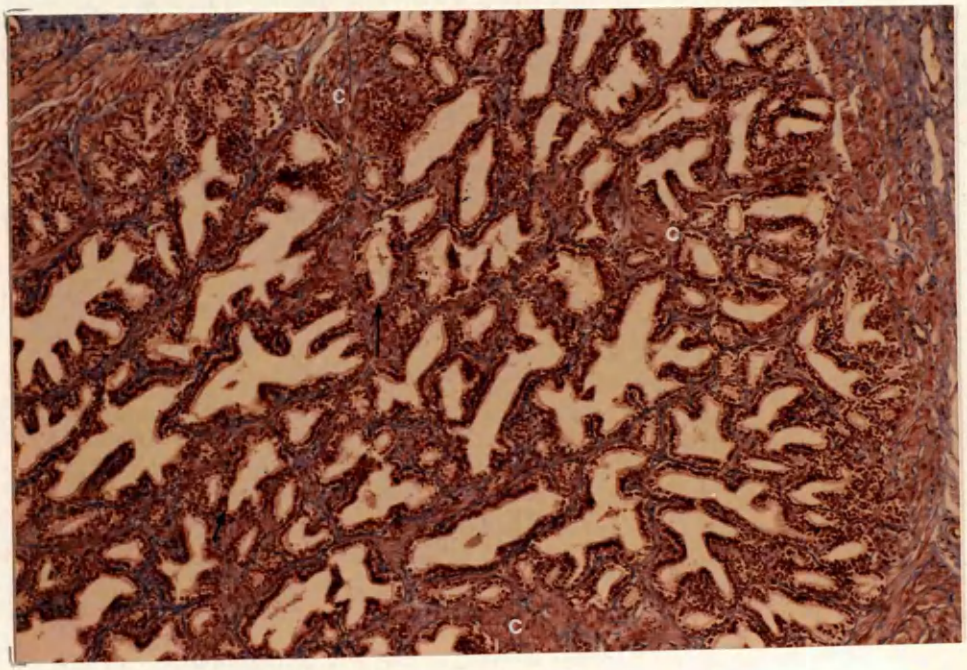
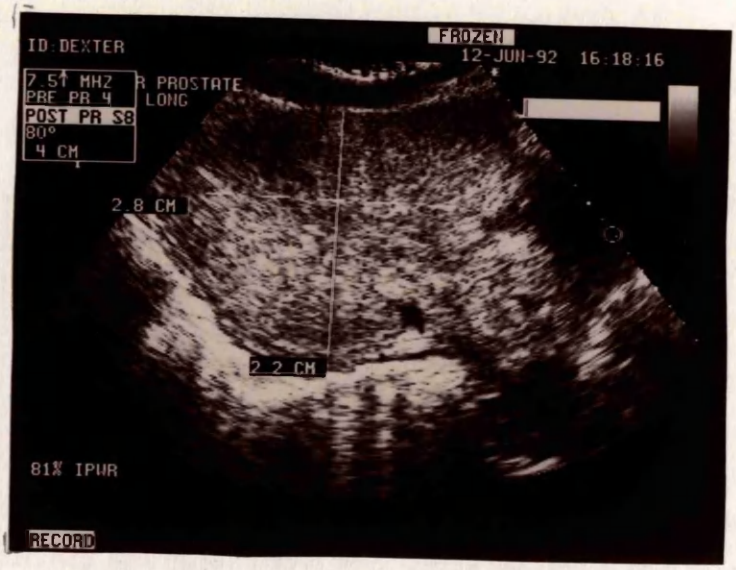


Figure 34. A slight decrease in the measurements of the prostate can be appreciated in Dexter's ultrasonographs There is a 0.1 cm difference in both length and width between those taken on June 12th and those taken 13 days later on the 25th.

a


ID:DEXTER
FROZEN
12-JUN-92 16:18:16
7.5↑ MHZ
PRE PR 4
POST PR S8
80°
4 CM
R PROSTATE
LONG
2.8 CM
2.2 CM
81% IPWR
RECORD

b

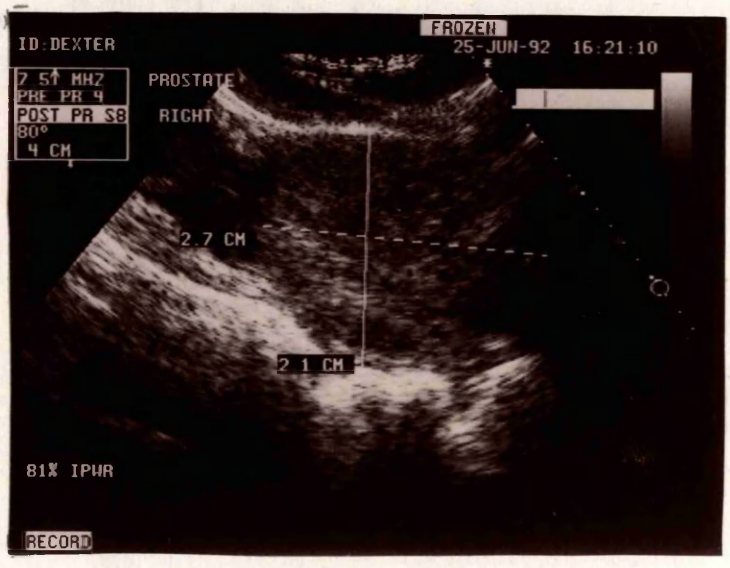

ID:DEXTER
FROZEN
25-JUN-92 16:21:10
7.5↑ MHZ
PRE PR 4
POST PR S8
80°
4 CM
PROSTATE
RIGHT
2.7 CM
2.1 CM
81% IPWR
RECORD

Table 16 This table presents the testosterone concentrations in blood samples taken from both the jugular and testicular veins from a control group. Baxter and Adam which were treated with Nafarelin derivative and Dexter which was treated with Nafarelin acetate. The mean found in testicular veins is 62.8 ±30.5 nmol/l which means that the 0.3 nmol/llevels found in the treated dogs, is well below the lowest limit of 32.3 nmol/l. And the jugular vein testosterone levels of 12.2±6.47nmol/l means that the 0.3 nmol/l levels found in Adam and Dexter are below the lowest limit of 5.73 nmol/l. Note that Baxter who had treatment problems has jugular levels similar to those in control animals.

Table 16. Testosterone concentration from jugular and testicular veins blood from controls and three beagle dogs treated with Nafarelin derivative and acetate

Control dog number	Testosterone nmol/l-1	
	Testicular veins	Jugular vein
1	81	2
2	106	36
3	47	3
4	51	4
5	29	16
	62 8±30.5	12.2±6.47
Treated dogs		
Baxter	not done	8
Adam	<0.3	<0.3
Dexter	<0.3	<0.3

4 DISCUSSION

In order to achieve long term suppression of LH and testosterone by administering GnRH agonists, the treatment would appear to require to be given in a defined amount on a daily basis (Auclair *et al.* 1977; Conn, 1986; Handelsman and Swerdolff, 1986; Vickery, 1986). Initially, it appeared that the use of subcutaneous pumps would be the ideal method to use as they removed the need for daily administration and the amount released was reported to be at a slow, consistent rate (Concannon *et al.* 1988; Concannon, 1989) reported that this was an efficient method for administration of such drugs in bitches over an extended period.

However, in Baxter, who was one of the first three dogs in which this method was attempted, problems arose as can be seen in Table 11. These included allergic reaction, irritation and oedema around the site of insertion of the pump and it was assumed that these changes were preventing the drug from being absorbed as desired. Furthermore, preliminary results of circulating plasma LH concentrations indicated that no suppression of this hormone was being achieved in either of the first two dogs (Baxter and Adam) treated by this method (Figures 16, 17, 24, 25). It was not possible to determine whether this lack of suppression was due to an insufficient amount of drug being given or to faulty use of the subcutaneous pumps. With more experience, the use of the osmotic pumps could have proved successful. However, economics and the lack of available research animals made it impossible for this sophisticated method to be successfully adopted.

In the case of Baxter (Table 10) who had the problems, it was decided to terminate the experiment and determine whether the treatment given had resulted in any identifiable changes in the genital tract from histological examination of sections from testes and epididymides obtained at *post-mortem examination* (Figures 26 and 27). With regard to Adam (Table 8), it was decided to continue administration of the agonist by daily subcutaneous injections, whereby the amount given could be controlled. The animals subsequently treated this way showed neither physical nor physiological reactions to this approach.

Pre treatment blood samples were collected in the three animals (Figures 15, 23, 29). The baseline levels of LH show a very flat profile where the amplitude of the oscillations is very similar in all the samples, whereas the testosterone shows a slightly higher amplitude and shorter frequency pulses. Both hormone levels in normal dogs have been mentioned in section A and the concentrations found in these pre treatment samples are within the reported range.

When treatment with an increased amount of the drug was administered by subcutaneous injection both to the remaining dog already treated by osmotic pumps (Adam, Table 7) and one other dog (Dexter, Table 13) the circulating plasma testosterone and LH concentrations subsequently dropped . This occurred almost immediately after commencing the injections in Adam (Figures 16 and 17). In Dexter, who only received treatment by injection (Table 14), this happened some 4 days after the injection (Figure 30) a similar finding to that of Vickery *et al.* (1989). The very rapid response in Adam was probably due to the cumulative action of the drug from the subcutaneous pumps, which in conjunction with the additional amounts given by injection brought about the marked reduction in the circulating plasma hormone concentrations.

Maddocks and Sharpe (1988) suggested from work in the rat that the circulating plasma concentrations of testosterone need not necessarily be related to the situation at testicular level. Their results indicated that the testis maintained the amounts of testosterone necessary for its requirements and only released the surplus into the general circulation. However, the amount of testosterone required for the continuation of spermatogenesis has as yet not been established (Sharpe, 1987). In a later report, Sharpe, *et al.* (1988) indicated that to maintain normal spermatogenesis, testosterone levels must be at least 24-46% of the concentrations normally found within the testis, but it is argued by the same workers that these levels could be underestimated (Sharpe *et al.* 1988). Were this aspect determined, then obviously the manipulation of spermatogenesis and male fertility would become much more accessible.

In an attempt to identify changes in the concentration of testosterone at testicular level, blood samples were collected from the superficial testicular veins (Maddocks and Sharpe, 1988) just prior to euthanasia in the treated animals and before castration in the control dogs (Table 16). However, in the existing circumstances where collection of semen was an essential part of the work, it was not possible to collect similar testicular blood samples prior to the

commencement of the studies. It was not possible to say therefore whether there was any real evidence of a change in the amount of testosterone in the testicular veins following the treatment given. However when comparing the results from the normal dogs, it would appear that there was indeed a drop in the amount of testosterone being produced, as the concentrations were markedly lower in the blood samples from the treated animals than from those of the normal dogs (Table 16). Whilst levels in spermatic venous blood (62 8 $\pm$ 30.5 nmol/l) in the normal dogs, the 0.3 nmol/l values in the treated animals are markedly below the lowest normal value in the untreated dogs. The same applies to the findings in the jugular vein values.

There were also individual variations in these testicular testosterone values, similar variations in human testicular testosterone production have also been reported by Qureshi and Sharpe, (1993). These testicular blood concentrations in comparison are similar to those recorded in the rat where the higher testosterone concentrations were also found in the testis by Maddocks and Sharpe (1988) with spermatic venous blood levels of 269.50 $\pm$ 30.63 nmol/l and in the circulation 4.55 $\pm$ 0.55 nmol/l. The decrease in circulation levels due to dilution. However it also indicates that a high testosterone environment is required by the testicle to maintain the ongoing spermatogenesis function of the seminiferous tubules.

The first indication that the administration of the agonist by s/c injection was potentially affecting each dog's fertility was a decrease in the volume of ejaculate. This tended to be accompanied by changes in testicular size and consistency, but libido was not affected in either dog. This was similar to what happened with the dogs given MPA and the explanations presented there would apply in this situation.

During both types of treatment namely MPA and GnRH agonists, difficulties were experienced in recording changes in external genitalia. The method devised for measurement of the testes was not very precise, especially when the gonads began to lose their resilience and became flabby. At this point it became difficult to hold the measuring tape around the testicle without applying such pressure as to introduce a variation in the measurements recorded. However, in Adam, 15 days after initiation of the subcutaneous injections of the agonist, the change in the gonads was very obvious, this being more of a change in the consistency of the testicle, which by then had become flabby (Table 7).

The consistency of the testes depends mainly on the cellular content of seminiferous tubules but partly on its fluid content which comprises blood supply, the fluid contained within the seminiferous tubules. An interesting finding at the end of the treatment was that the testes appeared paler than normal and the blood vessels on their surface were much less obvious and reduced in size, when compared to those of normal male dogs. Thus, these small flabby testes appeared to have a reduced blood supply which would reduce the amount of LH reaching the Leydig cells and this particular find agrees with some studies where it is suggested that testicular weight and blood flow are closely associated (Wang *et al.* 1983; Damber *et al.* 1985; Boujrad *et al.* 1992; Steele and Leung, 1992; Leung and Steele, 1992; Verhoeven *et al.* 1992). This in turn would affect the transfer of fluid to the interstitial tissue. As the communication between Sertoli and Leydig cells is through the interstitial fluid, the amount of testosterone transferred to the seminiferous tubules, especially to the Sertoli cells, would be less than normal, thereby affecting their function. This would subsequently result in ever decreasing amounts of testosterone being produced by the Leydig cells and eventually producing a cessation of spermatogenesis.

Sertoli cells are also involved in the production of ABP which transports the necessary testosterone to the epididymis and also produces the fluid which carries both the ABP and the immature spermatozoa forward into the epidiymis. This could explain the changes which were recorded in the only clinically palpable part of the epididymis the tail, which became less and less easily palpable as treatment continued such, that by the end of the study, this aspect of the male genital tract was almost indiscernible in both dogs. Therefore the changes recorded, other than that of libido, would tie in with the decrease in circulating plasma LH and testosterone.

During treatment, although libido was maintained, the volume of ejaculate decreased such that by the end of the treatment, only a few drops were expelled. These findings would suggest that as well as cessation of spermatogenesis, a decrease in the production of seminal plasma was also taking place. Several possible explanations for this finding need to be considered. Firstly, that there was reduction in the amount of fluid being produced in the seminiferous tubules by the Sertoli cells, which transports the sperm into the epididymis. This could have happened because of the treatment affecting these cells as well. Even though only LH was actually measured the desensitization effect of the pituitary

by the GnRH treatment would also affect the release of the FSH required by the Sertoli cells to function properly.

Another possibility could be that there was either a reduction in the amount of fluid produced by the epididymides themselves, or an occlusion at this level caused by cell debris or dead sperm, which reduced the passage of fluid. The epididymis depends on testosterone to function properly, (Moore and Bedford 1979; Lobl *et al.* 1983) and as the levels of testosterone had indeed been affected by the treatment it may explain the fact that when attempting to make smears of the epididymal sperm in the same way as with the MPA dogs, which was done by gently pressing the epididymis and extracting the epididymal fluid over glass slides it was not possible in the case of the the GnRH treated dogs, as the epididymis sections were apparently dry.

Finally, as most of the seminal plasma is produced by the prostate, these findings would tend to indicate abnormal prostatic function. This would appear to be the case as the prostate histological sections (Figures 21 and 33) showed that both Adam and Dexter had an atrophied gland, with the lobules of the prostate shrunk in size, the secretory cells hardly visible and there was an increase in connective tissue around the alveoli. However, in Baxter similar changes were not observed in any of the histological preparations (Figure 28).

Attempts to monitor the changes in the prostate during treatment, and to associate these with the reduction in the production of seminal plasma, were made by carrying out ultrasound scanning of this region at frequent intervals during the experimental period. Again, experience is required in devising the best method of measuring and recording the various parameters of this organ in order to accurately determine meaningful changes. Making certain that the same aspects were being compared on the different occasions proved difficult and time consuming. However, in both dogs there did appear to be a reduction in the size of the prostate by the end of the treatment period (Figures 22 and 34). Because of this effect on the prostate, GnRH agonists have been used as a treatment both of benign prostatic hyperplasia in the dog to produce the regression of the gland (Vickery, *et al.* 1982; Dube *et al.* 1984). And in man for in the treatment of prostatic cancer (Atala and Amin, 1991; Chrisp and Goa, 1990; Prevost *et al.* 1993; Harris *et al.* 1993; Kakar, *et al.* 1992).

In order to investigate the role of the epididymis in the decrease of ejaculate, the alkaline phosphatase content of the ejaculates were evaluated. Alkaline

phosphatase is produced mainly in the epididymis with smaller amounts produced by the testes and prostate (Frenette *et al.* 1986). Olson (1991), used this information to detect abnormalities or obstructions in the epididymis, whereby any dramatic drop in 27,000 iu/L levels in the alkaline phosphatase contents of the ejaculate, would indicate a problem in the epididymis. However, in that report, the actual method by which alkaline phosphatase was measured is not indicated and in the present work the levels obtained were 10 times higher (Tables 8,11,14) than those reported by Olson. There are several methods of analysing this enzyme, which use different temperatures when processing the samples, so it would seem that the differences found in this work and Olson's may be due to the temperature treatment, the samples in the present studies being processed at 37°C. Therefore, the results presented here cannot be compared to Olson's as far as the actual amounts are concerned, but the fact that any drop from the pre-treatment levels would indicate a problem and could be used for a comparison in this case.

No drop in the concentration of alkaline phosphatase was found in any of the ejaculates examined from Baxter or Adam (Tables 9 and 12). However in Dexter, there was a very low concentration of alkaline phosphatase in the ejaculate collected eleven days after initiation of the treatment (Table 15). This animal would only allow collection of semen when in the presence of an oestrus bitch and so, the number of ejaculates obtained were fewer (Table 14) than in Adam and Baxter who did not require much training (Tables 9 and 12). Thus, no further results of the alkaline phosphatase concentration in the ejaculate of this animal are available, as only one further collection was possible, and that was obtained towards the end of the experimental period when only a few drops of ejaculate were expressed. This was not sufficient to carry out any laboratory tests. However, in this particular case it may indicate that the reason why the alkaline phosphatase was so low was not due to an obstruction of the epididymis but more to a lack of fluid arriving from the rete testes, because of an impaired Sertoli cell function as previously suggested.

Before commencing the treatment, the initial clinical examinations of the external genitalia of the three dogs revealed no major abnormalities. However, in one of the dogs (Adam), a percentage of sperm with curved mid pieces (Figure 20) was noted on the several occasions that semen was collected. This type of abnormality is not one commonly reported in the dog although there is a published photograph of one such sperm (Allen *et al* 1979). As the abnormality

apparently did not affect the morphology of the sperm in that the attachment of the head and neck seemed normal, it would appear that this change in the sperm occurred after spermiogenesis or during maturation in the epididymis. The degree of curvature was such that those particular sperm were not capable of achieving normal progressive motility rather going round in circles. As there were repeatedly high percentages of this type of sperm in this animal's ejaculates, he should perhaps have been excluded from the experimental programme. However, it was decided that as he was both amenable to collection of semen and was an easily handled animal, he was included in the treatment. It also seemed possible that the treatment might produce some interesting and enlightening findings on spermatogenesis and/or changes in epididymal histology. During the treatment period, the frequency of these abnormal sperm did not appear to increase (Table 8) except that in some sperm the neck appeared to be close to breaking. No other obvious type of abnormality was seen in the ejaculates obtained from the other two dogs, either prior to, or during the treatment period (Tables 12 and 15).

Histological examination of sections from the testes of Adam and Dexter revealed that in both cases spermatogenesis had been affected to varying degrees (Figures 18 and 31). In Baxter who had only received treatment with the osmotic pump and in which there had been no apparent decrease in circulating plasma testosterone or LH concentration, there was no real evidence that spermatogenesis had been affected, as sperm was still present in the last ejaculate prior to sacrifice (Table 12).

In Adam and Dexter, who became azospermic before treatment was stopped, the degree of change present in their seminiferous tubules was not uniform. In Dexter (Figure 31) some tubules were devoid of most of the germ cell stages, containing only Sertoli cells and spermatogonia, whilst others contained various stages of developing cells. However, only a few elongated spermatids and secondary spermatocytes were seen in any of the sections examined. On the other hand in Adam who had been treated the longest, all the tubules were affected to the same degree, as these seminiferous tubules are almost totally devoid of germ cells, except for the presence of the spermatogonia closer to the basal membrane and the Sertoli cells (Figure 18). The degree of spermatic arrest was obviously determined by the length of the treatment period (Figures 18 and 31). Dube *et al.* (1987) reported the presence of seminiferous tubules containing only Sertoli cells and spermatogonia and only a very few primary spermatocytes,

following four months treatment with GnRH agonist. A similar atrophy to the seminiferous tubules in dogs was also reported by Labrie *et al.* (1985). However, these spermatogonia apparently are in a state of suspended animation and are capable of resuming spermatogenesis, even after treatment lasting for at least a year (Vickery *et al.* 1984; Vickery *et al.* 1989). Thus, treatment with GnRH agonists appears to be reversible (Sandow *et al.* 1980; Dube *et al.* 1987; Cavitte, *et al.* 1988).

Several reports (Parvinen, 1982; Sharpe, 1983; Parvinen *et al.* 1986), outlined the intricacies and the particular requirements of each stage of the spermatogenic cycle. A mini-environment is created around each seminiferous tubule which depends on the interaction of germ, myoid and Sertoli cells inside the tubule and which varies according to the stage of the cycle taking place at a particular time (Ritzen, *et al.* 1981; Kasuga, *et al.* 1989; Ojeifo *et al.* 1990; de Kretser, 1990) It is these requirements which are transmitted to the Leydig cells in apposition in the interstitial tissue.

The need for a local mechanism must first consider that the seminiferous tubules lack blood vessels to directly supply their needs and that therefore they must rely on the interstitial blood vessels to do so. However, the continuous transformation of germ cells in the tubules which demands the creation, of a mini environment around each particular stage of the seminiferous cycle, which must be constantly changing to adjust to the tubule's ever-changing demands. Such requirements would need a faster mechanism than that provided by the general circulation (Sharpe, 1983; Sharpe and Cooper, 1983; Spiteri *et al.* 1993).

Evidence that such a local control exists and is provided by the interaction between the seminiferous tubule and the interstitial tissue is suggested by earlier work showing that Leydig cell cultures increased their testosterone production when they were co-cultured with Sertoli cells, or with cell medium removed from Sertoli cell cultures (Papadopoulus *et al.* 1987). Other evidence suggested that Sertoli cells could convert androgen precursors into oestrogen in the absence of Leydig cells. In rats oestrogens reduced testosterone synthesis independently of LH, which pointed to the possible role of oestrogens as the mediator between Sertoli and Leydig cells (diZerega and Sherins, 1981). However, more recent information arises from studies on the effects on the germ cell stages of androgen withdrawal caused by Leydig cell destruction. The observed damage was a shrinkage of the nucleus of cell in stages VII, IX and XI, which began to return to

their normal size within two days after supplementation with testosterone (Kerr, Millar, Maddocks and Sharpe, 1993). A similar experiment showed that the withdrawal of androgens altered the Sertoli cell junctions by dilating the intercellular spaces, but without alteration to the tight junctions (Kerr, Savage, Millar and Sharpe,1993).

There is evidence that stages VII and VIII are under androgen control (Parvinen, 1982), but some researchers believe that testosterone acts only at stage VII (Sharpe 1990). Thus, if testosterone production is suppressed, the tubules containing these stages of the spermatogenic cycle, namely pachytene spermatocytes, round and elongated spermatids, would tend to be the first tubules to show signs of degeneration (Figures 18 and 31). Such tubules would thus be seen only to contain developing cells, up to the primary spermatocyte stage. Furthermore Parvinen, (1982) and Sharpe (1990) indicated that FSH requirement of the Sertoli cells is highest in the earlier stages, I to V, and as the treatment used suppressed both gonadotrophins, this could explain the appearance of the tubules in which this was the situation. It would have been of interest to determine the concentration of FSH in both the testicular vein blood samples and the circulating plasma in these dogs, but this was not undertaken in the studies reported here.

Histological examination of sections from the epididymides and sperm therein contained, did not reveal the more dramatic changes which were seen in the dogs treated with MPA (Figure 8). Although some vacuolization of the epithelial cells lining the epididymis (Figures 19 and 32) in all areas was seen, it was nothing like the marked degree seen in the MPA treated dogs.

These findings in the dogs treated with the GnRH agonist would tend to support the hypothesis, presented in the case of the MPA dogs, that the changes in the epididymal sperm removed from the MPA treated dogs were due to the progestogens having a direct action on the epididymis .

These preliminary experiments with live dogs served to identify a suitable drug for manipulation of spermatogenesis and the best route of administration. It also identified the best parameters to be monitored during such studies and formed a basis for further studies of the histological changes which can be expected to occur at testicular level when such treatment is used. They also provided information regarding the effects of GnRH agonist in the hormonal profile and in the tissue changes in their reproductive organs which was part of

the aim of this section. However, in order to find out more about the role the Leydig cells played in causing these changes, it was necessary to work directly with the cells themselves isolated from the interstitial tissue, as this has been the approach used by researchers studying Leydig cell function (Dufau *et al.* 1974; Janszen *et al.* 1976; Conn *et al.* 1977; Payne *et al.* 1980; Hsueh, 1980; Browning *et al.* 1981; Rommerts *et al.* 1982).

CHAPTER 3

ISOLATION AND CULTURE OF LEYDIG CELLS

1. INTRODUCTION

Preparations of freshly isolated Leydig cells, obtained from the testis of both human males and animal species, mainly rat and mice, have been used extensively in the investigation of testicular function (Conn, *et al.* 1977; Browning, *et al.* 1981; Browning, *et al.* 1983; Aquilano & Dufau, 1984; Bhalla, *et al.* 1987).

Many techniques have been developed, to isolate, purify and identify Leydig cells in the above mentioned species. However very little similar work has been reported to any extent in the dog.

The purpose of this work was to attempt to develop the necessary techniques that would allow similar work to be done in dog Leydig cells.

2. MATERIALS AND METHODS

2.1. TISSUE COLLECTION

Rats and mice.

Testes from rats and mice were obtained immediately after euthanasia and transported to the laboratory for immediate processing.

Dogs.

Testes removed at castration were transferred to the laboratory where they were immediately placed in plastic lidded containers and covered with swabs moistened with normal saline until they were processed.

2.2. FREEZING OF TESTICULAR TISSUE;

Method 1.

5 Centimeters. cubes of testicular tissue were placed in labelled cryotubes and plunged into liquid nitrogen where they were stored until required for processing.

Method 2.

5 Centimeters. cubes of testicular tissue were placed in labelled cryotubes containing Medium 199 with Hanks salts, L-glutamine and 24 mM Hepes (Gibco BRL UK) and then stored in liquid nitrogen until required for processing.

Method 3

5 Centimeters. cubes of testicular tissue were placed in labelled cryotubes containing M199 plus 1.5 Dimethyl sulfoxide (DMSO) and immersed in liquid nitrogen, where they were stored until required.

Method 4

Testicular homogenates (see below) were placed in labelled cryotubes containing Medium 199 with Hanks salts, L-Glutamine and 24mM Hepes. (Gibco BRL UK) and immersed in liquid nitrogen in which they were stored until required.

2.3. THAWING:

Method 1.

Without removing the frozen pieces of testes from the cryotubes they were left to thaw at room temperature.

Method 2.

Without removing the frozen pieces of testes from the cryotubes they were placed in a beaker of water at 37°C for two minutes, or until thawed.

Method 3.

Without removing the frozen pieces of testes from the cryotubes they were placed in a beaker of water at 32°C for two minutes or until thawed.

Method 4.

Without removing the frozen pieces of testes from the cryotubes they were left to thaw at refrigeration temperature of 4°C.

2.4. TISSUE DISSOCIATION.

1st.method.

A 2 cm cube of fresh testicular tissue was placed in a Petri dish containing Hepes medium (Gibco Life Technologies Ltd, Paisley Scotland). The dish was placed on a hot plate and the collagenase added in very small quantities at a time, until dissociation was obvious.

2nd Method.

Required cell medium preparation

Reagents		
100 ml	Cell culture medium 199	(ICN Flow Herts UK)
.05 g.	Bovine serum albumin	Sigma Chemical Co. Ltd. Dorset England

1 ml	200 mM (1--X)L Glutamine	Gibco Life Technologies, Paisley Scotland UK

Medium B.		
40 ml	Medium A	Sigma Chemical Co.Ltd D Dorset England.
.800 ml	Trypsin inhibitor	"
57 mg	Collagenase C-9881	"

Tissue dissociation procedure:

Testicular tissue was cut into small pieces with scissors and approximately 2 grams were then placed in universal bottles containing 6 ml medium B. The universals and contents were placed in a shaking water bath incubator (Gallenkamp, Fisons Scientific Equipment, Loughborough, England) at 37° C at a speed of 80 cycles until all tissue had been dissociated. The solution was then filtered through a mesh filter of 45 um pore diameter (Millipore, UK Ltd, Research Park Riccarton, Edinburgh), centrifuged at 500 g for 10 minutes (Figure 55).

Third method

After thoroughly drying the testes (in the rats and mice) and removing all traces of blood, the whole testes were decapsulated. In the dog, two circular incisions were made around the middle of the testicle and the capsule was removed from this by holding the cut edge of the capsule from one side with a needle and scraping the testicular tissue off with a scalpel blade. The tissue was then cut into 12 segments of 0.5 g ea. Two pieces of the tissue were then placed in universal bottles containing 7 ml of medium B.

The universals and contents were placed in the shaking water bath incubator (Gallenkamp, Fisons Scientific Equipment, Loughborough, England) at 37° C and a speed of 80 cycles and left until in the rat the thread like tubules were seen to have separated. In the dog, when the tissue had dissolved into a thin homogenate with no large lumps, the bottles were removed from the water bath. To stop the digestion

process, cold medium A was added to fill the universals and then the mixture was allowed to stand for 10 minutes to allow settling of debris to the bottom.

The supernatant was then decanted and passed through a nylon mesh to eliminate the debris and then the solution was passed through an 80 um filter (Millipore, UK Ltd, Research Park Riccarton, Edinburgh). Both filters were washed with additional medium A, to free any cells retained in them. Additional medium was also added to the debris that had settled in the bottom of each universal bottle, and filtered again. The resulting filtrates were centrifuged for 10 minutes at 800 g.

The resulting pellets were resuspended in 0.5 ml of medium A by drawing them in and out of a Pasteur pipette, the additional 4.5 ml of medium A was added. This final 5 ml was used for the Percoll gradient purification

2.5. CELL VIABILITY AND IDENTIFICATION

1st. Method.

The identification of cells resulting from the 1st. method of tissue dissociation (see below) were stained with Diff Quick staining set (Baxter, Dade. Thetford, Norfolk England). Smears were made by placing a drop of the tissue homogenate on glass slides (Gold Star, Chance Propper Ltd. England), left to dry at room temperature, and then fixed and stained according to the manufacturer's instructions. However this method was just used for the initial crude collagenase tissue dissociations.

2nd. Method.

A small volume (10 ul) of cell suspension was added to an equal volume of 0.4% trypan blue in 0.9% saline 10 ul of this solution was placed in a hemocytometer chamber (Newbauer, Weber, England) and viewed under a light microscope. Cells excluding the dye were considered to be live, and were therefore counted.

Third Method.

The cells were prepared with the trypan blue as above but were viewed under an Olympus BH2 phase contrast microscope. Cells excluding the dye and showing the bright yellow halo around as reported by Schumaker et al (1978), Joffre et al. (1984), Gale et al. (1982), Hunter et al. (1982) were considered to be live Leydig cells and were therefore counted as such before culture.

Viability and identification was performed at various stages of the cell purification process in order to determine whether damage was occurring to the cells at each step.

1. After collagenase dispersion.

2. After each rinsing before placing in the Percoll gradient.

3. Using each band collected from the Percoll gradient.

4. After rinsing the Percoll off the cell suspension.

5. During different incubation times.

2.6. CELL PURIFICATION BY PERCOLL GRADIENT

Method A. Continuous gradient.

Continuous gradient was prepared by the method described by Schumacher, Schafer, Holstein and Hilz (1978) with some modifications, as follows.

1.- Preparation of solutions

2.- Gradient Preparation

3.- Pump loading.

Reagents

For the following solutions the reagents were purchased from Gibco Life Technologies, Ltd., Paisley Scotland UK.and from Sigma Chemical Co. Ltd. Dorset England

Solution preparation

Solution 1	
10 ml	10 fold concentrated. Earl's balanced salt solution.
7 mg	BSA
2.4 mg	Hepes 20 mM /l

Solution 2
To 1 part of solution 1 add 9 parts of Percoll to make Percoll solution.

Solution 3	
20 ml	Earl's balanced salt solution
0.07 g.	BSA

Solution 4
To 9 ml of 2nd solution add 1 ml of 3rd. solution

Pump preparation

Pump used was a LKB Bromma 2120 Varioperpex II (LKB Bromma Sweden)

Rinse pump wells with distilled water.

Solution 3 is placed in the left pump well

Solution 4 is placed in the right pump well.

The hose is placed against the tube wall and held there until the whole gradient is finished, the density of this gradient is 0-90 %. The cell suspension is then placed in top of the gradient and centrifuged at 3000 g for 30 minutes.

Collecting cells from the gradient:

A cannula attached to a syringe was placed in the centre of each band, collecting the topmost layer first, and in the same way proceeding to collect the others

Rinsing Percoll off the cells

Each collected band was then transferred to a 14 ml centrifuge tube, to which more medium A was added. The tubes were then centrifuged at 800 g for 10 minutes, the supernatant was removed. The resulting pellets were collected from all the tubes by rediluting in 0.5 ml medium and transferred to a 14 ml centrifuge tube, which was then filled with A. medium and centrifuged again for 800 g for 10 minutes. The supernatant was removed and the resulting pellets were collected from all the tubes by rediluting in 0.5 ml medium and rediluted again for cell culture. After this procedure the cells were ready for the next step of culture or staining.

METHOD B. Discontinuous gradients:

1st. Method.

This method as described by Simpson, Wu and Sharpe, (1987) was used with some modifications.

Medium B. Isotonic Percoll.	
2.5 ml	Medium A (as described before).
22.5 ml	Percoll, density 1.13 +- 0.005 (Pharmacia LKB, Biotechnology, Uppsala Sweden).

Gradient preparation.

Formula 1			
No.	Density	Isotonic Percoll (ml.)	Medium A (ml)
1	1.00	0	10.0
2	1.03	2.55	7.45
3	1.05	4.3	5.7
4	1.07	6.0	4.0
5.	1.09	7.7	2.3

The following formulas were variations of this first formula

Formula 2.		
No.	Isotonic Percoll (ml)	Medium A (ml)
1	1 1	9 9
2	2	8
3	3	7
4	4	6
5	5	5

Formula 3		
No.	Isotonic Percoll (ml).	Medium A (ml).
1	6	4
2	7	3
3	8	2
4	9	1
5	10	0

Formula 4		
No.	Isotonic Percoll (ml)	Medium A (ml).
1	7.7	2.3
2	6.0	4.0
3	4.3	5.7

4	2.55	7.45
5	0	10.0

2.7. GRADIENT PREPARATION

Material required for gradient preparation.	
50 ml Plastic centrifuge tube	Greiner Labortechnik, Ltd.(Dursley UK)
12 Ga X 2" Intravenous cannula	Aloe Medical Mfg. (St. Louis Mo. USA)
10 ml. Syringe	

Preparation of gradient.

The loaded syringe with the cannula attached was placed firmly in the bottom of the tube and the Percoll concentrations starting with no. 1, were deposited by slowly pushing the syringe plunger.

Cell purification

5 ml were removed from the topmost layer of the prepared gradient to make room for the 5 ml of cell suspension that was placed on top. The tube containing the gradient and the cell suspension was then centrifuged at 3000 g. for 30 min.

Collecting cells from the gradient:

The cell bands were collected from the gradient with the same type of material as used for setting the gradients, but for each band a different syringe and cannula were used. The cannula was placed in the centre of each band, thus collecting the topmost layer first and, in the same way, proceeding to collect the others.

Rinsing Percoll off the cells.

As indicated before.

2.8. HISTOCHEMISTRY 3

HSD

First method

The method used was as described by Lobl and Levy (1968) and modified by Salaheddine (1993)

All reagents were purchased from Sigma (Chemical Company Ltd, Poole, Dorset, England),

First the following five stock solutions were prepared .

Solution 1	
6.35 g.	Polyvinyl pyrrolidone (PVP)
45 ml	0.1 M Phosphate buffer pH 7.4
Solution 2	
20 mg	Nitro-tetrazolium blue
20 ml	Distilled water.
Solution 3	
30 mg	B- Nicotinamide Adenosine Dinucleotide (NAD)
5 ml	Distilled water
Solution 4.	
15 mg	5-androsten 3B-ol7-one
5 ml	Dimethyl formamide
Solution 5.	
30 mg	NADH-2 (Grade III)

5 ml	Distilled water

These solutions were stored at 4o C for up to 2 weeks as this was found not to interfere with the reactions when compared with freshly made solutions. Final incubation media contained:

Incubation medium is made up of	
Solution 1	4.7 ml
Solution 2	2.0 ml
Solution 3	1.0 ml
Solution 4	0.3 ml

Staining procedure for cell preparations.

10 ul cell suspension samples were placed in a glass slide and dried at room temperature and stored at 20^{o} C .

Slides were stained by adding drops of the incubation medium to the previously prepared slides, and an equal number of slides were stained with solution 5 to verify the presence of the 3βHSD enzyme.

2.9. CELL CULTURE

Material used.

24 & 12 multiculture dishes

60 x 15 mm individual bacteriological Petri dishes

140 x 20 mm individual bacteriological Petri dishes

The culture dishes were from Nunc, Flow Laboratories, Herts England.

Cell culture medium M199 with 25mM Hepes, Earle's salts and L-Glutamine, from (Gibco BRL, Life Technologies, Ltd., Paisley, Scotland UK.)

Procedure

Not all the wells were used but the ones that were, were filled with 1 ml medium + 1ul cell suspension. To avoid evaporation during incubation some wells were filled with distilled water which were interspersed in between the wells containing cells. This measure reduced evaporation considerably.

The multi well culture dishes were changed to individual 60 x 15 mm bacteriological Petri dishes 3 each of this dishes were placed in a larger Petri dish 140 x 20 mm, the bottom of these larger dishes was covered with distilled water.

Incubation.

Initially the cultures were incubated in a LTE incubator (Laboratory Thermal Equipment Ltd, Greenfield, NR. Oldham UK) at 37°C.

This was changed to 32 °C and incubated in an automatic CO_2, model 160 incubator (ICN, Flow Laboratories, Herts England).

Third step, examination of the cultures.

Initially 100 ul aliquots were removed from the dishes and viewed under the light microscope. But this was changed to examination of the culture dishes with an inverted Will-Wetzlar GMBH-D-6330-21 microscope (Wetzlar W. Germany).

Fourth step, viability evaluation.

The evaluation of viability was initially made by removing small aliquots of the cell suspension after 3, 5, 8 hours, stained with trypan blue to verify viability in the light microscope. Also by removing the supernatant and staining with trypan blue the bottom of the culture dish to evaluate the viability of the cells that remained in the bottom.

As well as by examination of the whole culture dish without removal of supernatant under the inverted Will-Wetzlar microscope.

2.10. HORMONE DETERMINATION IN THE CELL CULTURE SUPERNATANT

Method used as described in Chapter 2-A.

Except that in these samples the ether extraction step was omitted.

The limit of detection was 0.35 to 7.0 nmol/l and was defined from the point where the standards CV changes to more than 22%. the interassay coefficient of variance calculated from 8 assays was 8.82% at 0.3 nmol/land 18.03 % at 4.0 nmol/l. The intra-assay coefficient of variance was 6.54% and was assessed by the duplicate samples of a randomly selected assay and was calculated by the same method and formula as indicated in Chapter 2-A. The repeatability of the standard curve is presented in Figure 58.

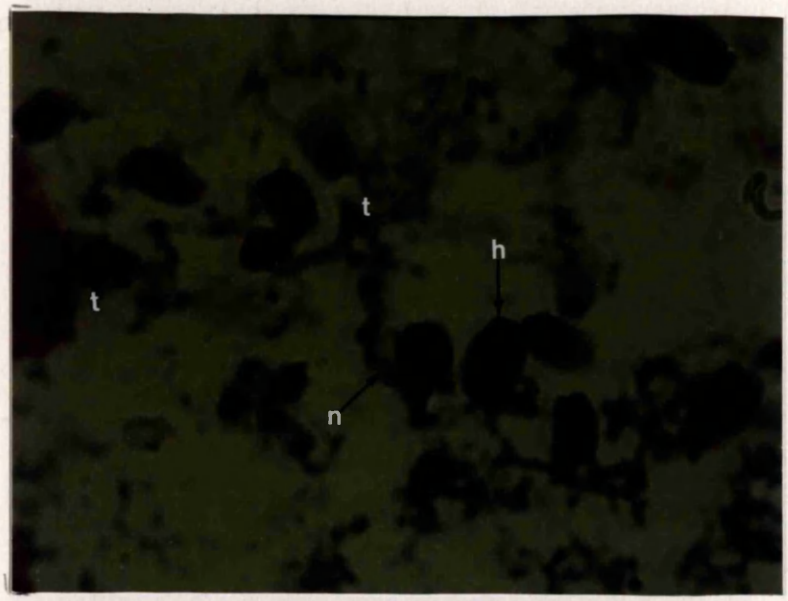
3. RESULTS

Figure 35. This figure presents some of the initial attempts at separating and identifying Leydig cells from dog testis.

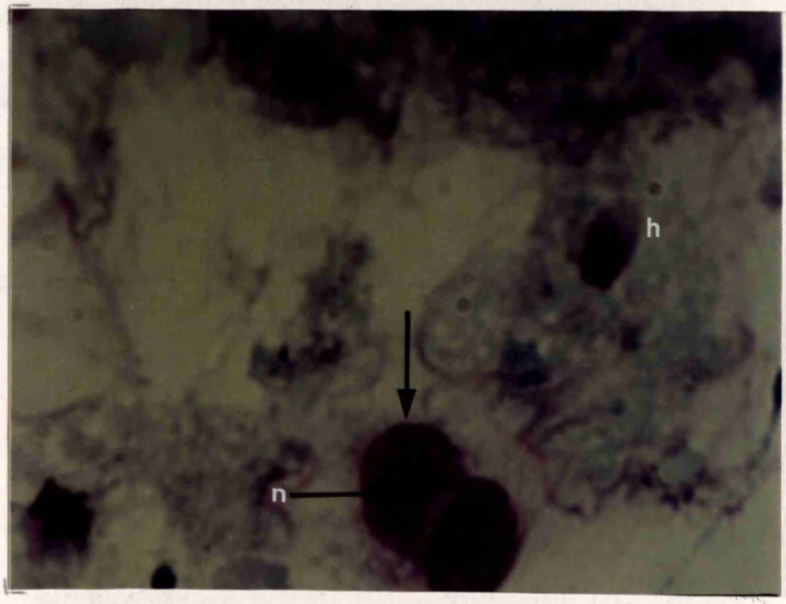
(a,b,c). These show different aspects of the cell suspension contents.The three smears were stained with Diff Quick and are seen at 100 x magnification.

a) and b) Are an example of a cell suspension which is not very clean, where tissue debris has not been eliminated (**t**), where there are sperm heads (**h**), and what may be cell nuclei (**n**) c) Another type of cell that was occasionally observed in these initial unidentified cell preparations.

a



b



c

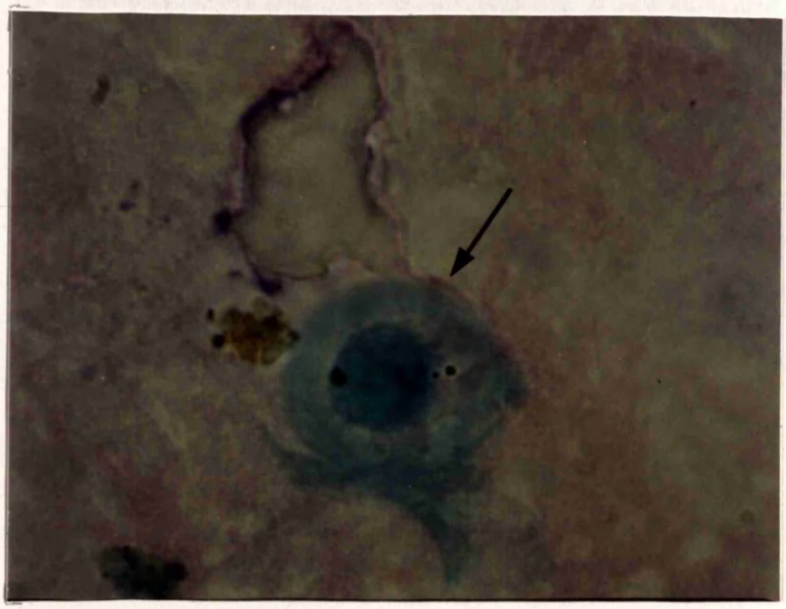
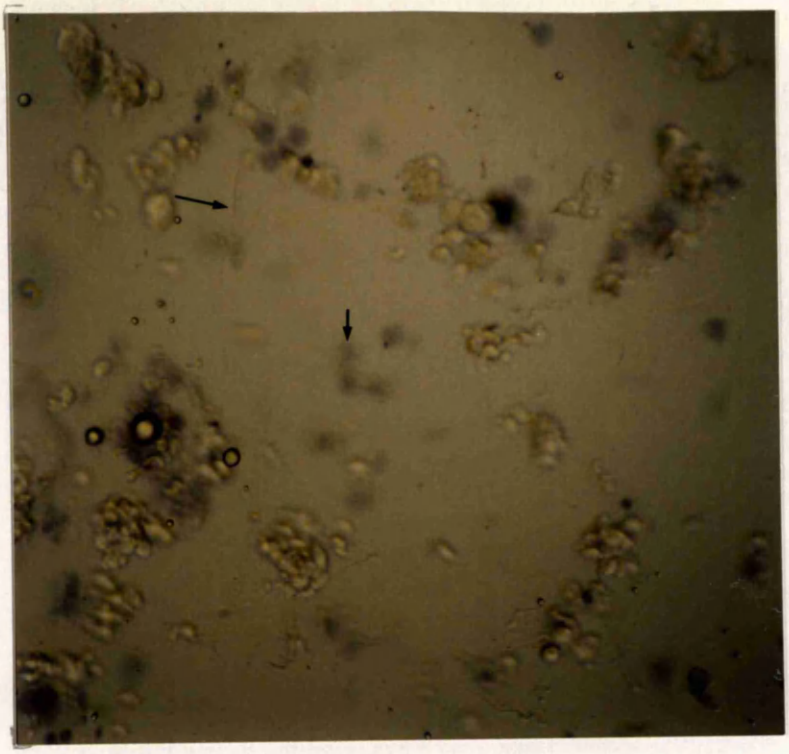


Figure 36. Fresh cell preparations stained with trypan blue.

a).Notice the dead blue cells in the background, and the unstained cells and spermatozoa which appear to be floating in the surface(10x) b) Shows the detail of a whole cell, and a sperm tail (100x).

a



b

Figure 37. Represents the attempts at detecting the presence of the 3βHSD enzyme. Notice first the indiscriminate staining where everything stains blue, debris, cells. The only difference is the variation in staining intensity. Notice also that however progressively cleaner the suspensions are, the staining preparation continues to be totally blue.

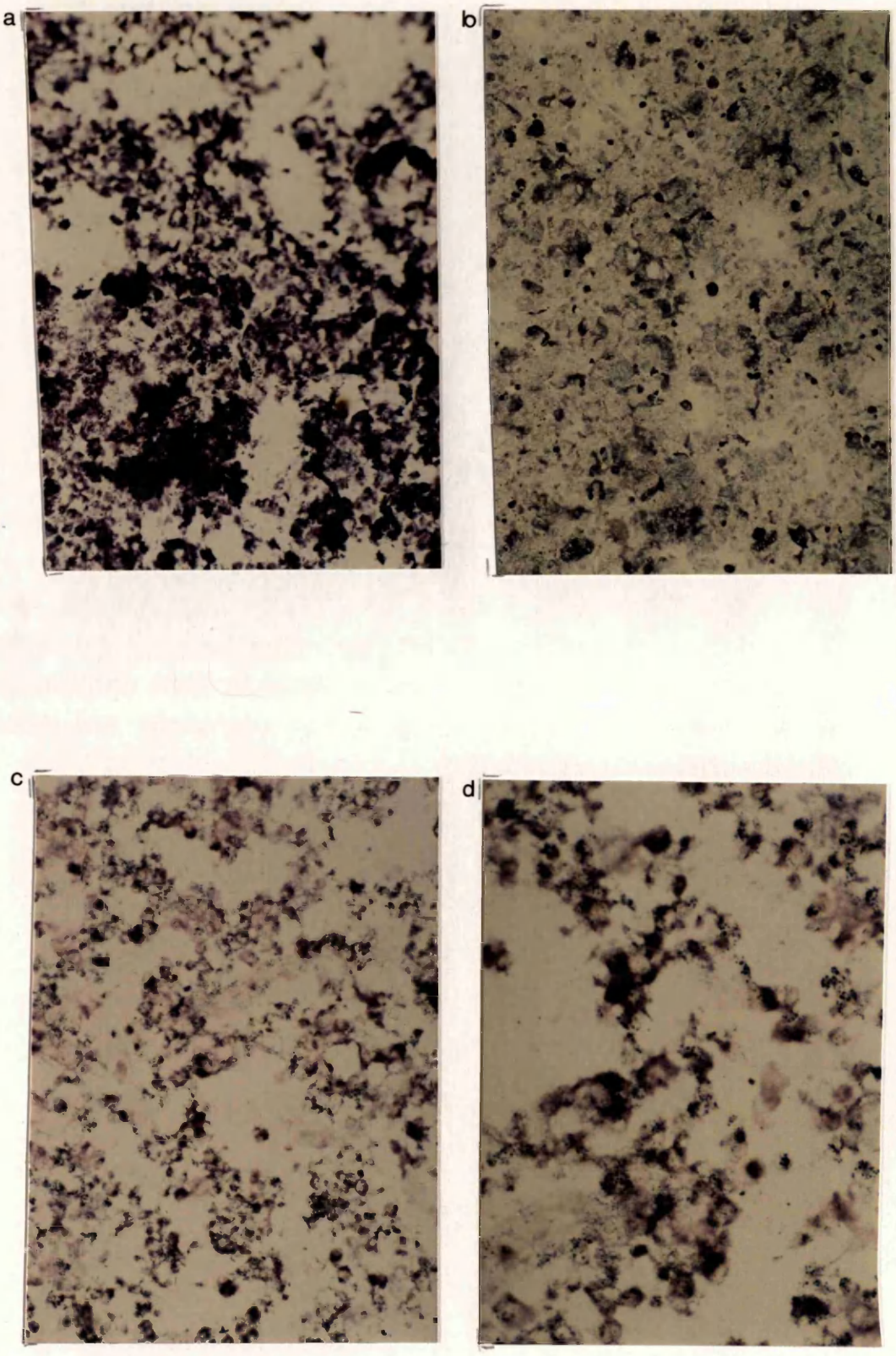
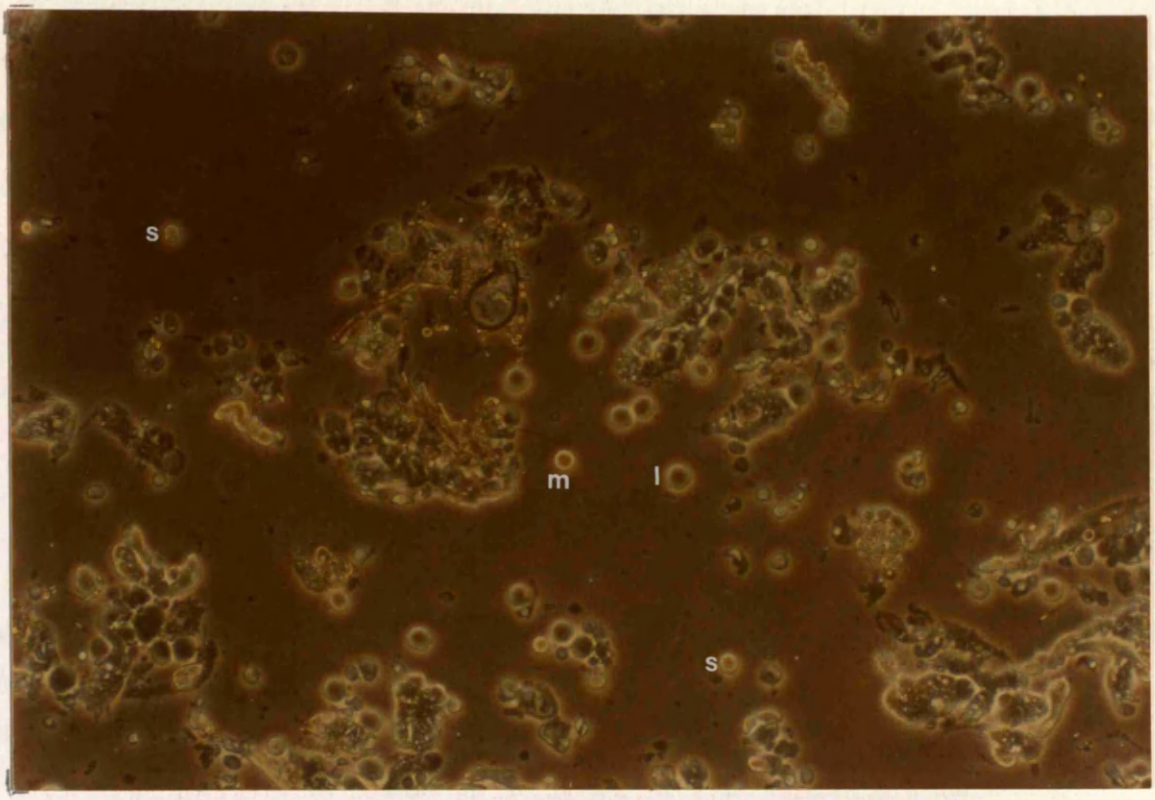

a
b
c
d

Figure 38. (a and b). Percoll purified dog Leydig cells showing some contaminating spermatozoa and debris in the background forming clumps. Notice that the characteristic Leydig cell yellow halo is shared by three different size cells, small (s), medium (m), large (l) Phase contrast microscope and stained with trypan blue before (magnified x 100).

a



b

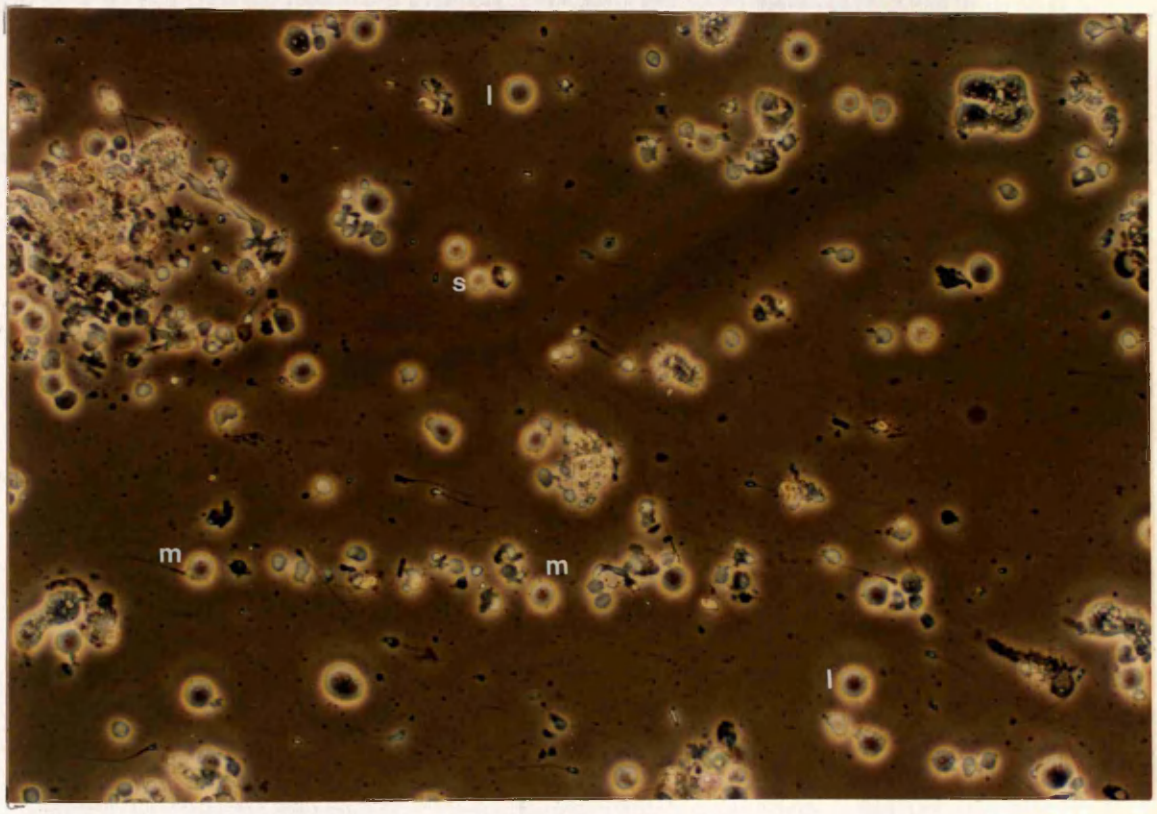
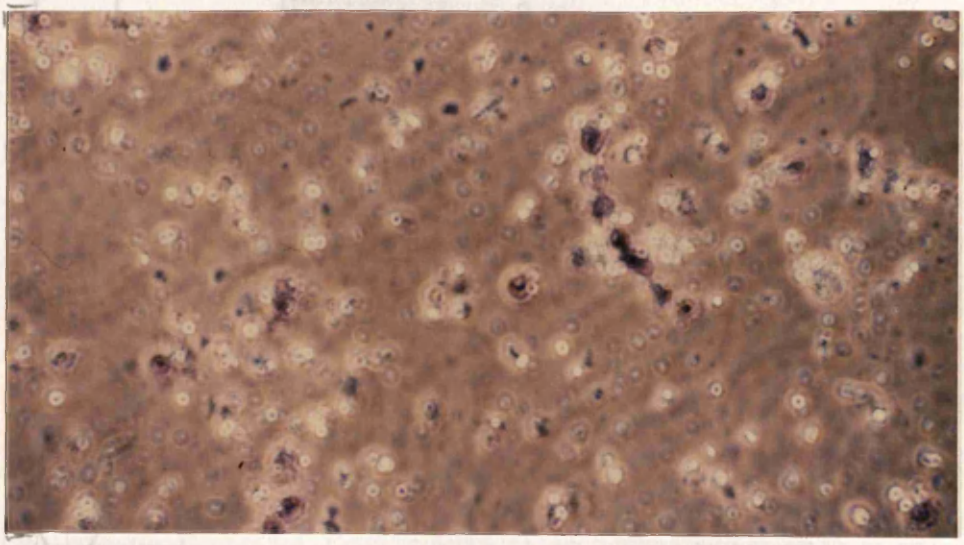
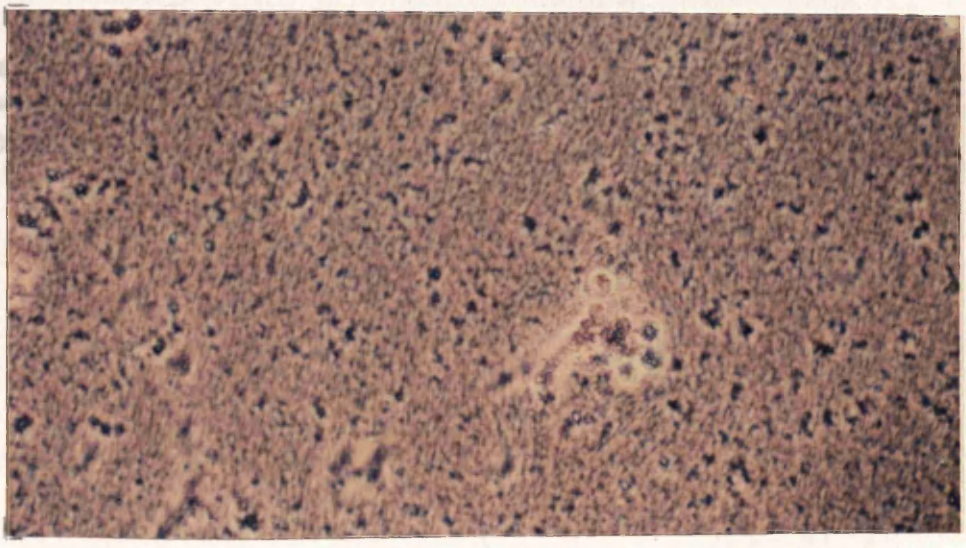


Figure 39 (a , b, c.). This figure presents an example of culture dishes showing the varying numbers of Leydig cell attachment to the bottom of cell culture dishes, a), before removal of supernatant. b) and c) after removal of supernatant.

a



b



c

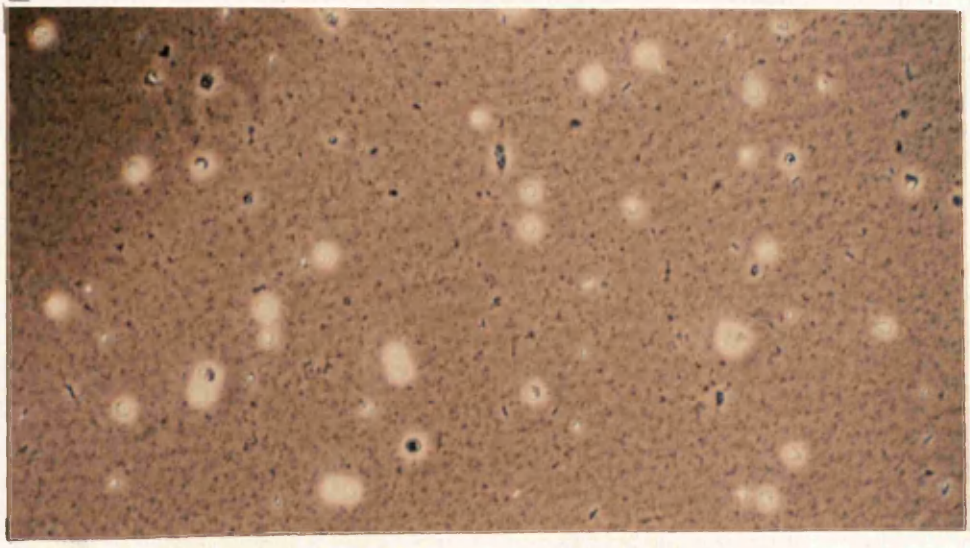
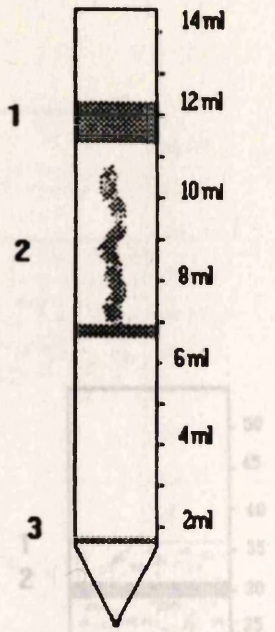


Fig. 40



Discontinuous method.

1.- Large & medium cells unidentified cells and spermatozoa

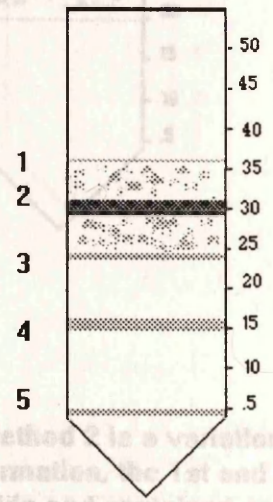
2.- Large and medium unidentified cells, some dead cells, many live spermatozoa.

3.- Erythrocytes.

This is an example of the discontinuous method, notice the cell formation between the 1st and 2nd bands. The separation between the bands is not very clear and all sizes of still unidentified cells as well as tissue debris are found in all the bands.

Fig. 41

Formula 1



1.- Wide cloudy band, few Leydig cells, many germ cells of different sizes, some tissue debris.

2.- Thick band, mostly Leydig cells, some germ cells, very little tissue debris.

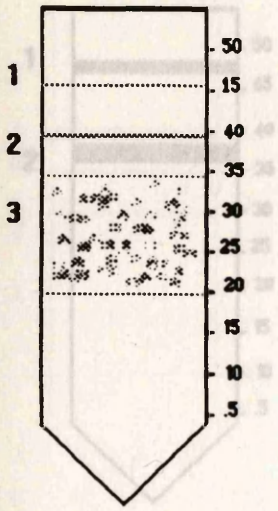
3.- Many live spermatozoa and tissue debris.

4.- Erythrocytes.

5.- Debris.

Fig 41 This is an example of the method of Simpson et al (1987) and it shows the number of resulting bands. The first and third bands looked cloudy and very close to the second band in which the Leydig cells were found, but this band was very clean of tissue debris. The cleanest and best separated band was the fifth which contained only erythrocytes.

Fig. 42



Formula 2

1.- Small, medium, large germ cells, many dead some sperm, tissue debris

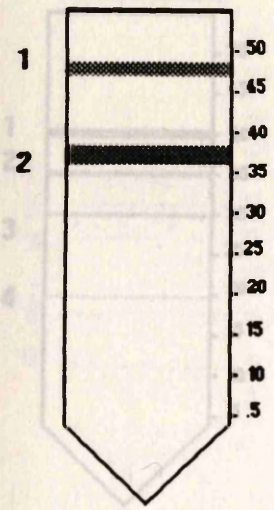
2.- Leydig cells, very clean, the best band.

3.- Very few cells, some Leydig cells, large amounts of tissue debris, a few spermatozoa, crenated cells.

Method 2 is a variation of method 1. Notice the difference in cell band formation, the 1st and 2nd bands being very thin. The third band is very wide and contained some Leydig cells as well, but there were many damaged cells in this band.

Fig. 43

Formula 3



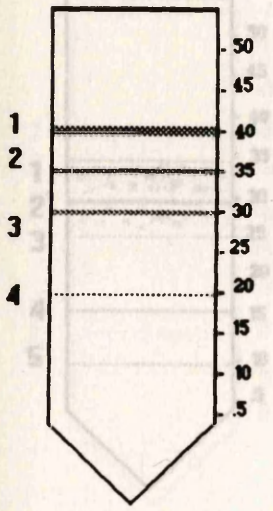
1.- Germ and Leydig cells mixed up, not much tissue debris.

2.- Few cells, many erythrocytes.

Formula 3 is another version of the first method. Only two bands were formed, but the germ and Leydig cells were found in the same band. Even the erythrocytes were not clearly separated as their band included tissue debris as well.

Fig. 44

Formula 4.



1.- Germ and Leydig cells in this band mixed with clumps of debris.

2.- Germ and Leydig cells in clumps also clumps of debris.

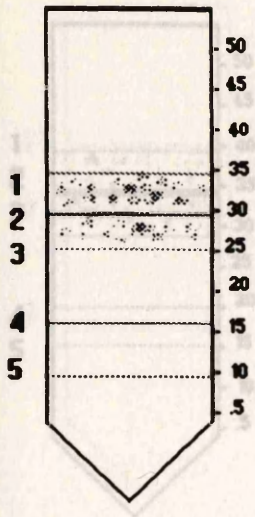
3.- Larger clumps of cells and debris.

4.- Erythrocytes.

Formula 4 is also a modification to the first method. In this gradient, all the contents of the cell suspension separated in bands but in clumps of both tissue debris and cells.

Fig. 45

Formula 4
Using one day old refrigerated tissue or frozen tissue.



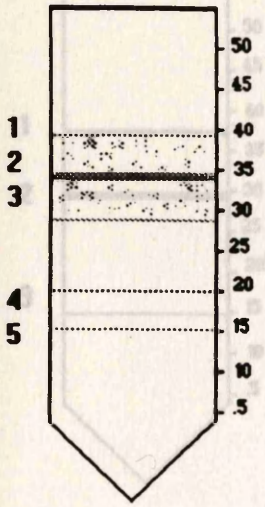
1, 2, 3 Bands very few cells of all kinds, all dead, large amounts of debris.

4, 5 Narrow bands, once collected no cells were recovered.

This is again formula 4, but using refrigerated one day old tissue, or frozen tissue. Notice that the first bands contained mostly debris and that the few cells which were found were dead. Bands 4 and 5 were very narrow. Once Percoll was rinsed off, no cells were recovered from the last two bands.

Fig. 46

First method, using a cleaner cell suspension.



1.- Cloudy band, very few cells, some tissue debris.

2.-Thick band, many Leydig cells, clean of debris.

3.- Few spermatozoa and some debris.

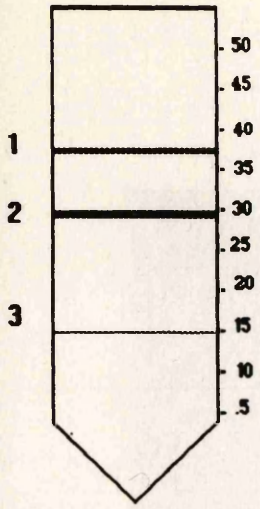
4 & 5 Very thin bands.

This is the first method using a cleaner cell suspension. The Leydig cell harvest was very good and the extra cell bands were very thin. Much of the contamination by tissue debris and spermatozoa has been eliminated. Once Percoll was rinsed off bands 4 and 5, no cells could be recovered.

Fig. 47

First formula.

Using a cleaner and more diluted cell suspension.



1.- Leydig cells mixed with small amounts of tissue debris.

2.- More Leydig cells, almost no debris

3.- Erythrocytes.

This figure shows the results of using a cleaner and more diluted cell suspension. Most tissue debris, spermatozoa and erythrocytes have been eliminated. Notice that there are no more cloudy bands and that the bands present are very clearly defined.

Figure 48. This is the actual photograph of a discontinuous gradient as shown in figure 47. Notice the width of the first band, in which most of the three sizes of Leydig cells were found, with smaller numbers of the same cells in the second band. Erythrocytes were found in the third.

Figure 49. This figure shows the production of testosterone in 3 culture dishes, each containing 100 ul of dog cell suspension after incubation for 24 hours. The Leydig cells were still not identified at this stage. For this reason the amount of testosterone produced is per 100 ul of cell suspension and not per 1000 cells. Notice that although the three dishes have the same amount of cell suspension, the testosterone production is not the same, suggesting that they may not contain the same numbers of cells.

Fig. 49. Testosterone produced by dog cell cultures after incubation for 24 hours.

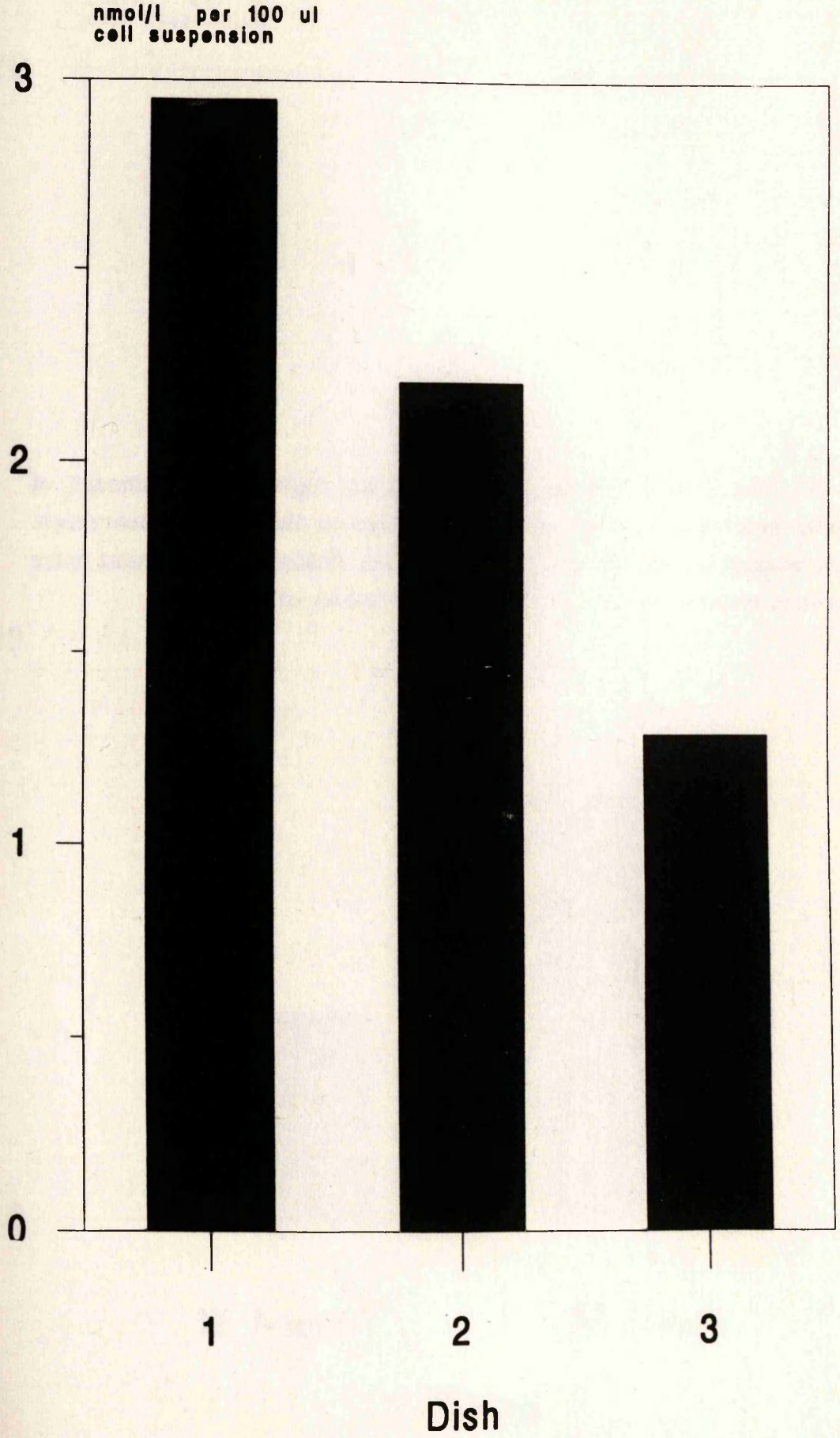


Figure 50. The results presented in this figure represent the amount of testosterone produced in two different culture dishes incubated at different times. Again the amount of testosterone is per 100 ul/dish. Notice there is almost twice the amount of testosterone after 24 hour incubation than after 12 hours.

Fig. 50. Testosterone produced by initial untreated dog cell cultures incubated for 12 and 24 hours.

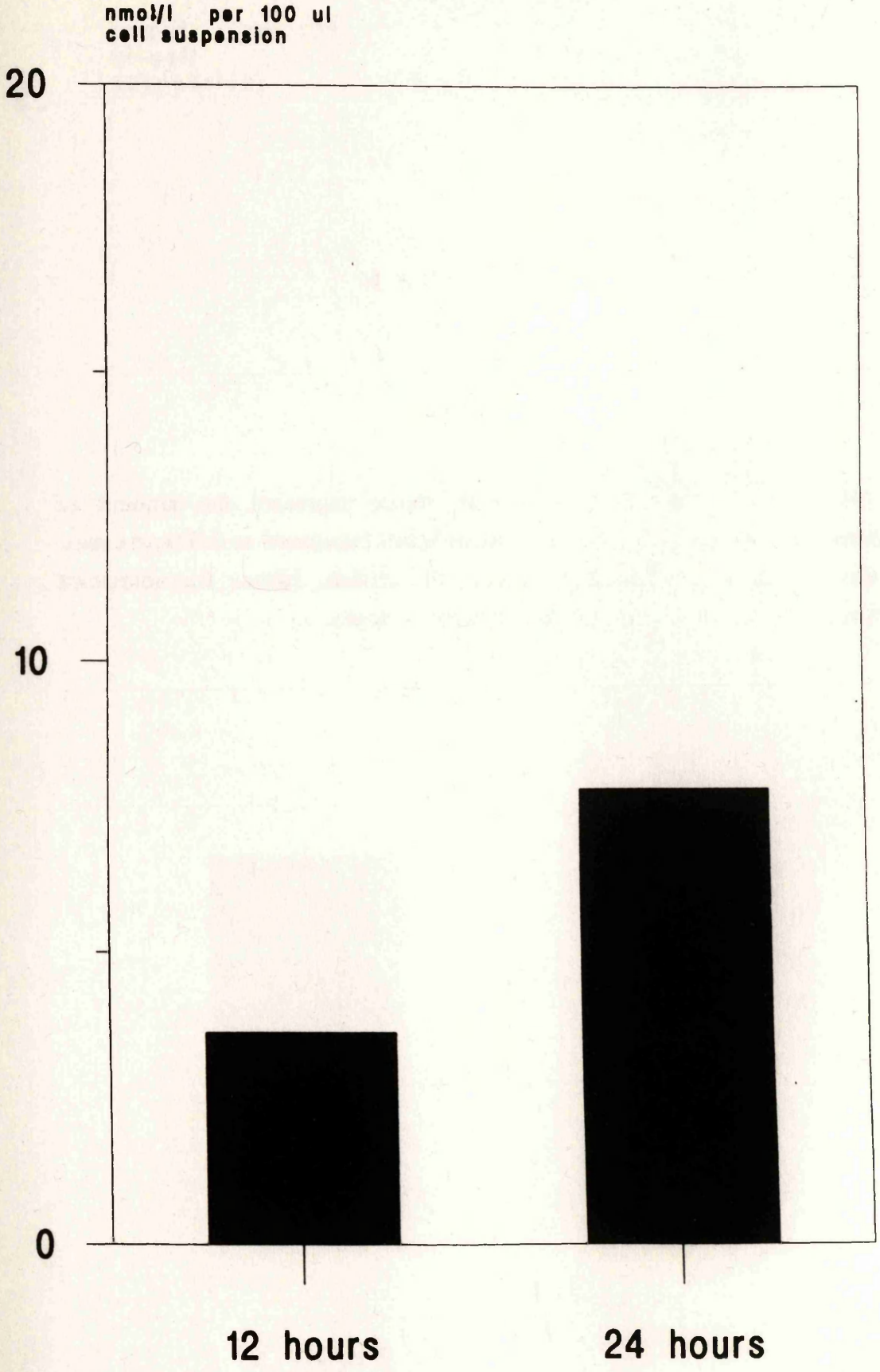
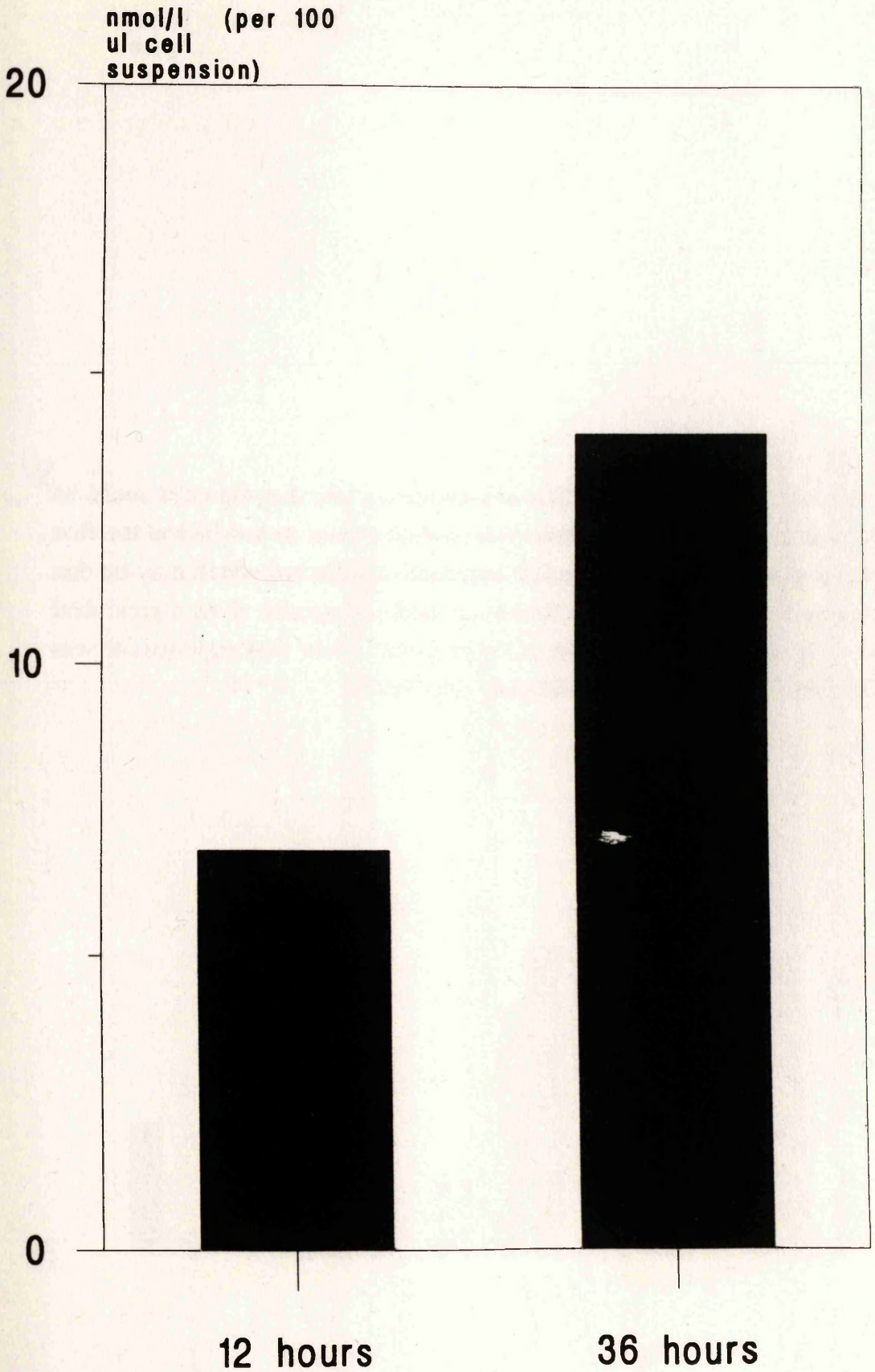


Figure 51. The results presented in this figure represent the amount of testosterone produced in two different culture dishes incubated at different times. Again the amount of testosterone is per 100 ul/dish. Notice the increased testosterone after 36 hours incubation than after 12 hours.

Fig. 51. Testosterone produced by untreated dog cell cultures incubated for 12 and 36 hours.



2 Dishes.

Figure 52. Represents one of the first cell cultures where Leydig cells could be counted. Notice that although the cells were counted prior to incubation the first supernatants are not uniform in the first supernatant collected,which may be due to the presence of cell clumps. The following supernatants also show a great deal of variation in their response to the hCG treatment. The first supernatant was collected after 8 hours incubation without treatment.

Fig. 52 Testosterone produced by dog Leydig cell culture during 2 days of treatment with 0.15 iu HCG (Chorulon)

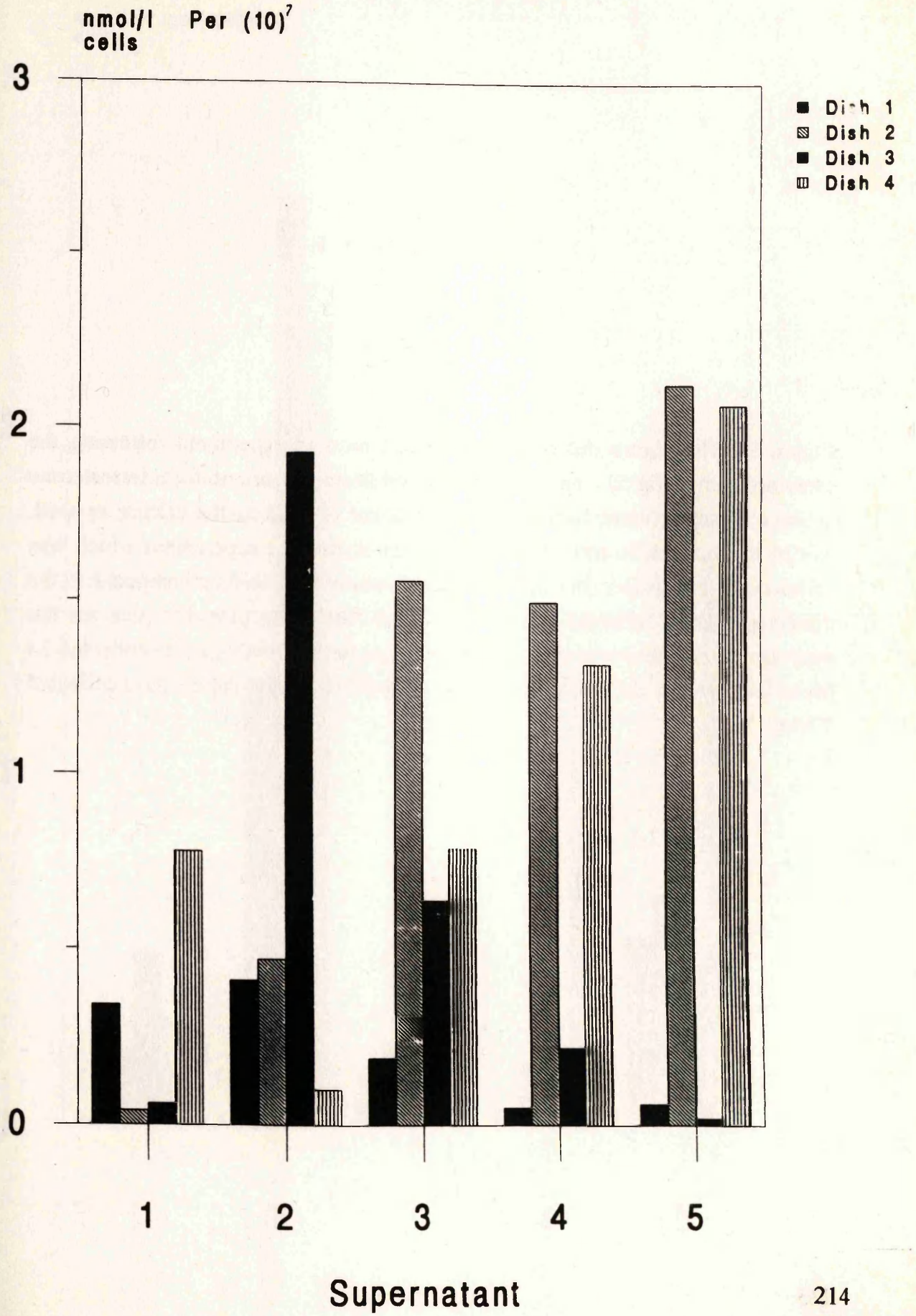


Figure 53. This figure presents the results of another experiment following the same protocol as fig 52. The first supernatant shows a more uniform testosterone amount reflecting more similarity in the amount of cells in the culture as well. Notice the increase in testosterone production in the 2nd supernatant which was collected 24 hours later In comparison there was a decreased cell response in the third supernatant collected 3 hours later. Two interesting points to note are the decrease in testosterone production in the 4th supernatant which was collected 24 hours later and the cell's response to the treatment in the 5th supernatant collected 3 hours later.

Fig.53. Testosterone produced by Leydig cell culture during 3 days of treatment with 0.15 iu HCG (Chorulon

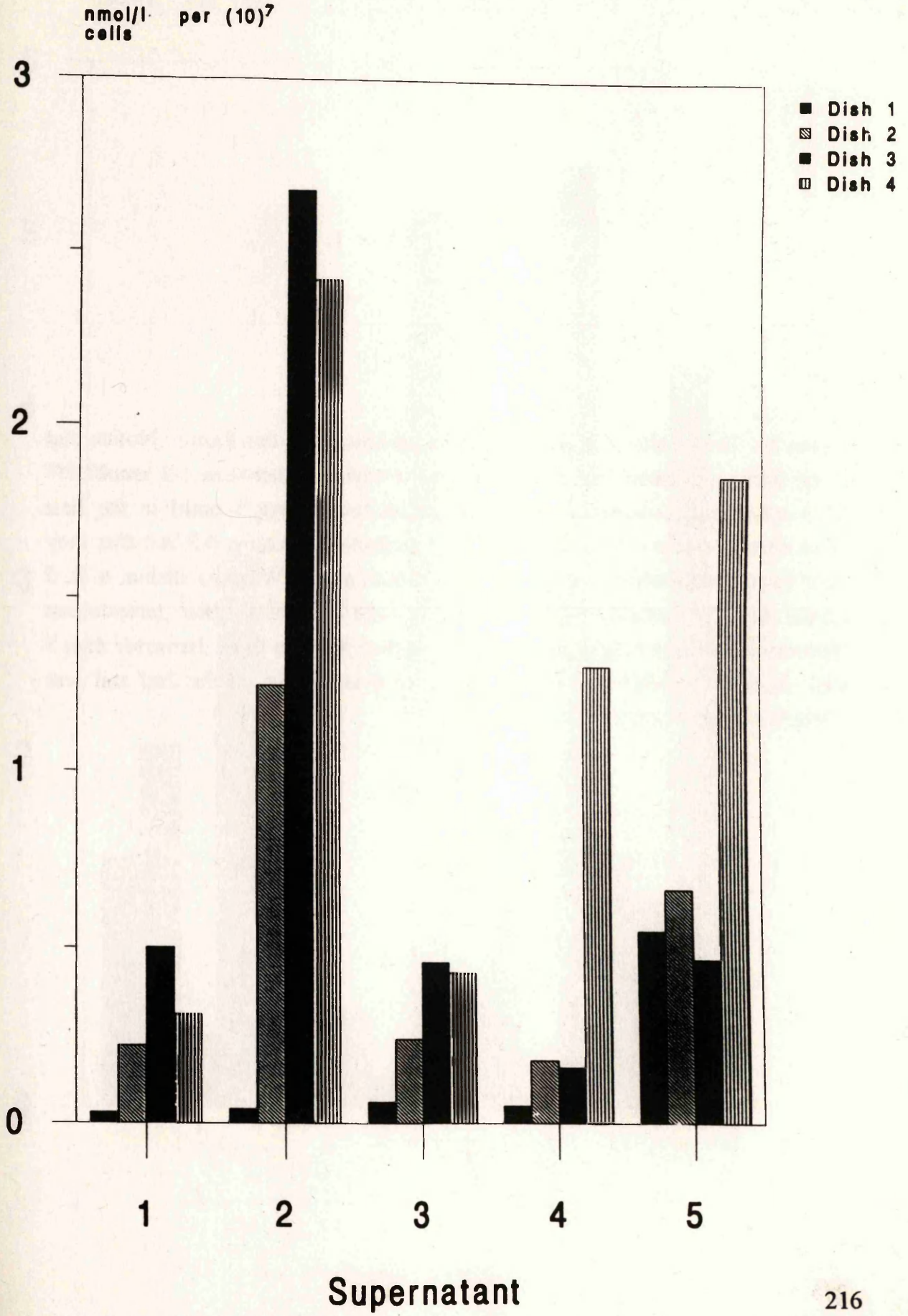


Figure 54. The results of a 2 day culture are presented in this figure. Notice that in the first supernatant the production of testosterone is between 1-3 nmol/l per 1000 cells. Notice dishes 1 and 2 which had levels over 1 nmol in the first supernatant, 24 hours later decreased their production to below 0.5 but after they were treated they improved on the third supernatant. Whereas dishes 4 & 5 which had the highest levels initially with increased their testosterone production in the 2nd supernatant and maintained it in the third. However dish 3 with the lowest initial supernatant, increased dramatically on the 2nd and was above the initial levels by the third collection.

Fig. 54. Testosterone produced by dog Leydig cell culture during 2 day incubation and treatment with 0.15 iu HCG (Chorulon)

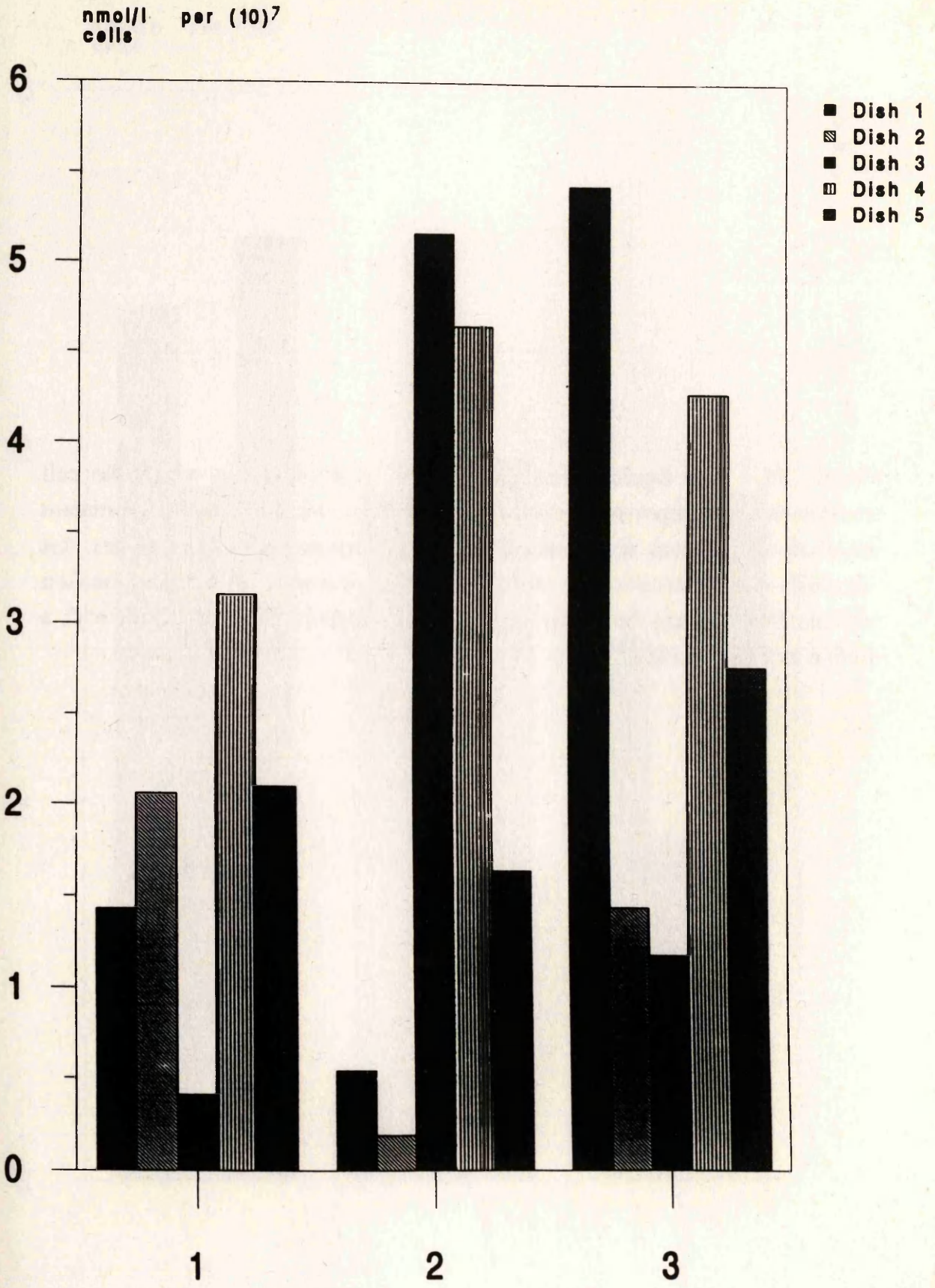


Figure 55. This figure presents the results of a culture in which the cell resuspension was improved to eliminate cell clumps. Notice the first supernatant values in the 3 dishes show more uniform testosterone levels. The values that were high in the first collection, dishes 1 and 3, were low in the second collection whereas dish 2 where the values were lower are high in the second, but with a decline in the third supernatant.

Fig.55 Testosterone produced by Leydig cell culture, with improved cell suspension, during 2 days of treatment with 0.15 iu HCG.

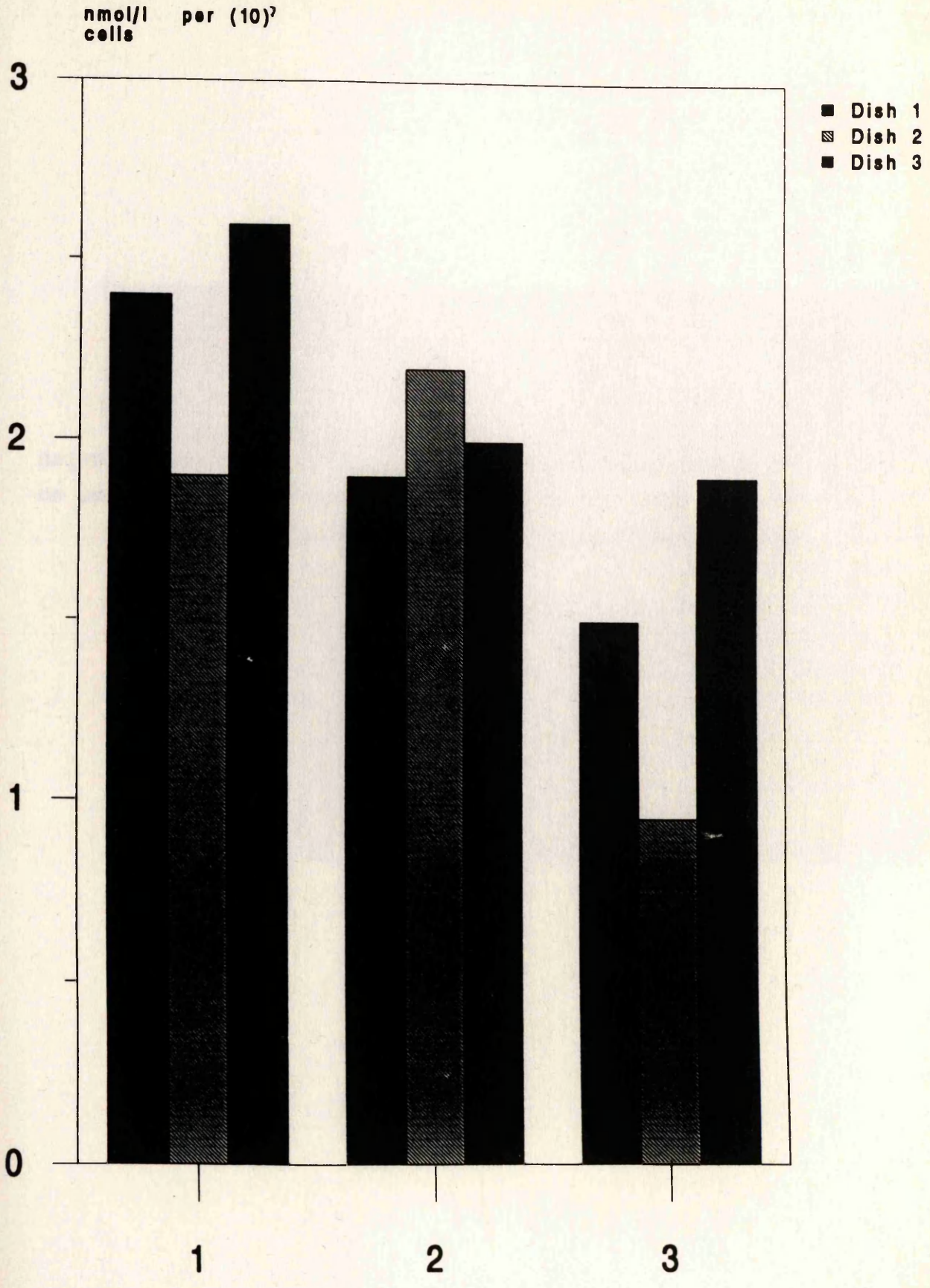


Figure 56. This is a photo of dead purified Leydig cells stained blue with trypan blue. Note how clean this cell suspension is, there is no tissue debris, no spermatozoa. This is a result of the improved Percoll gradient method 1.

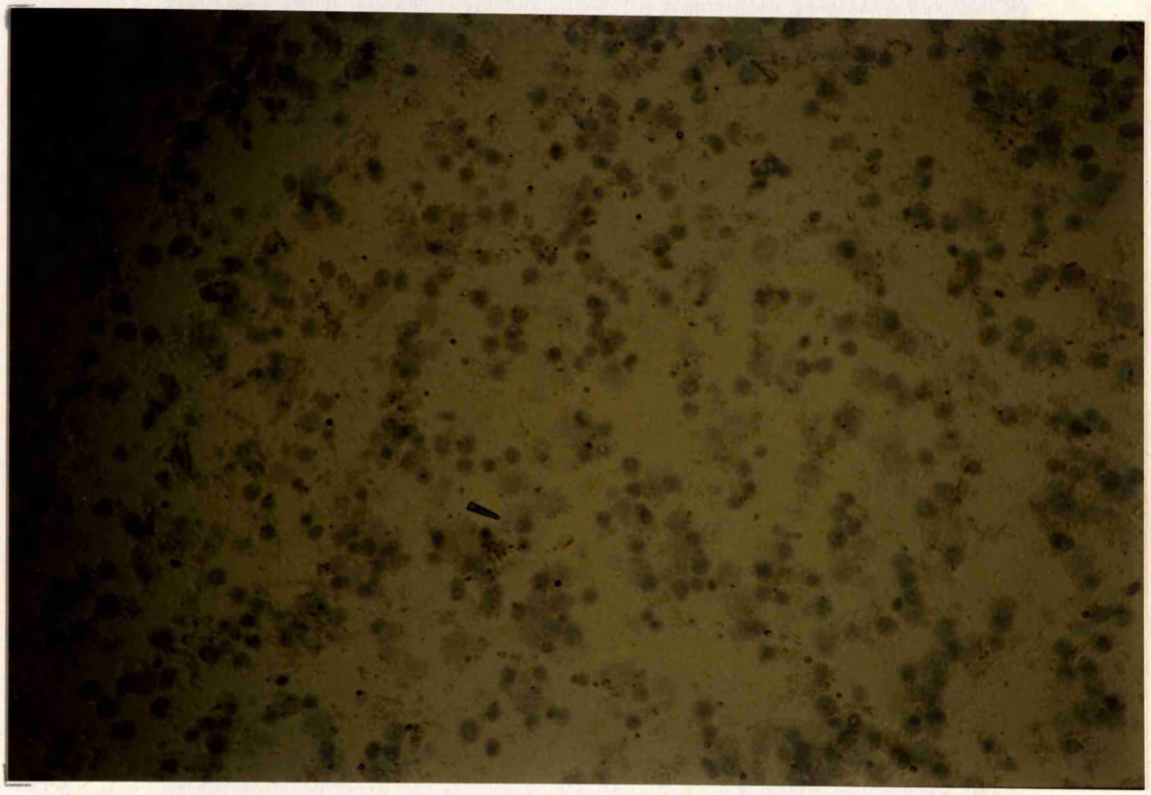


Figure 57. Materials used for filtering the dog testicular tissue; a) plastic strainer b) glass funnel with the attached millipore filter, c) syringe and needle used for setting up gradients, as well as for cell collection from the gradients.

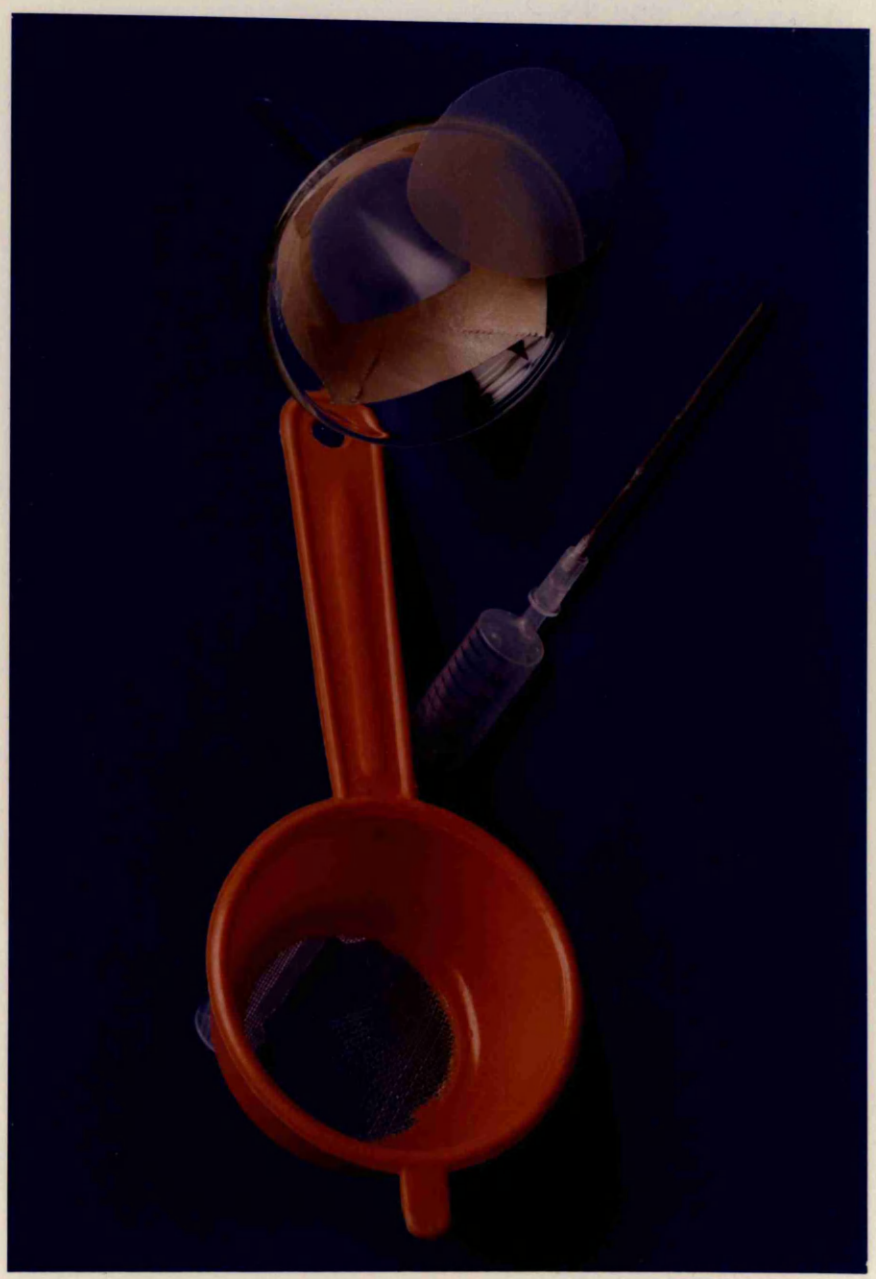
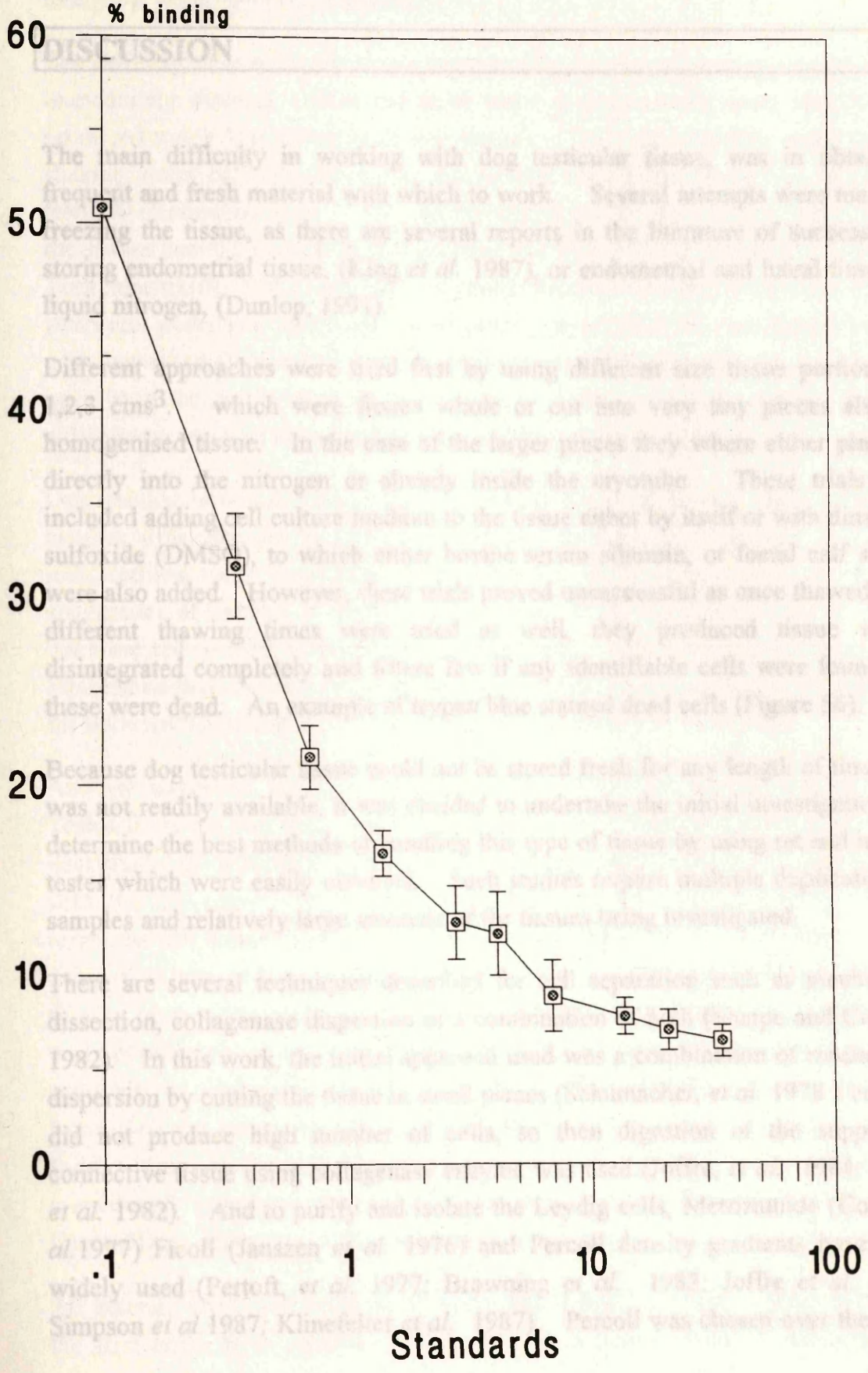


Figure 58. Presents the standard curve for repeatability of testosterone evaluation in cell cultures.

Fig. 58 Repeatability of standard curve for testosterone in Leydig cell culture.



DISCUSSION

The main difficulty in working with dog testicular tissue, was in obtaining frequent and fresh material with which to work. Several attempts were made at freezing the tissue, as there are several reports in the literature of successfully storing endometrial tissue, (King *et al.* 1987), or endometrial and luteal tissue in liquid nitrogen, (Dunlop, 1991).

Different approaches were tried first by using different size tissue portions of 1,2,3 cms^3. which were frozen whole or cut into very tiny pieces also as homogenised tissue. In the case of the larger pieces they where either plunged directly into the nitrogen or already inside the cryotube. These trials also included adding cell culture medium to the tissue either by itself or with dimethyl sulfoxide (DMSO), to which either bovine serum albumin, or foetal calf serum were also added. However, these trials proved unsuccessful as once thawed, and different thawing times were tried as well, they produced tissue which disintegrated completely and where few if any identifiable cells were found but these were dead. An example of trypan blue stained dead cells (Figure 56).

Because dog testicular tissue could not be stored fresh for any length of time and was not readily available, it was decided to undertake the initial investigations to determine the best methods of handling this type of tissue by using rat and mouse testes which were easily obtained. Such studies require multiple duplication of samples and relatively large amounts of the tissues being investigated.

There are several techniques described for cell separation such as mechanical dissection, collagenase dispersion or a combination of both (Sharpe and Cooper, 1982). In this work, the initial approach used was a combination of mechanical dispersion by cutting the tissue in small pieces (Schumacher, *et al.* 1978) but this did not produce high number of cells, so then digestion of the supportive connective tissue using collagenase enzyme was used (Joffre, *et al.* 1984; Gale, *et al.* 1982). And to purify and isolate the Leydig cells, Metrizamide (Conn, *et al.*1977) Ficoll (Janszen *et al.* 1976) and Percoll density gradients have been widely used (Pertoft, *et al.* 1977; Browning *et al.* 1983; Joffre *et al.* 1984; Simpson *et al.*1987; Klinefelter *et al.* 1987). Percoll was chosen over the other

products for the present studies, because being the latest developed product it was thought it would give better results.

The differences in testicular tissue between dog and the laboratory animals were immediately obvious. Rat and mice testis disperse much more readily and produced much less debris than dog tissue. The recommended speed of the shaking water bath incubator used for rat and mice tissue had to be increased from 80 (Rommerts *et al.* 1982), to 100 cycles for the dog as dog tissue left at the slower speed became like cooked meat and little cellular separation occurred. Testicular tissue from older dogs was much tougher than that from dogs of two years and under and took longer to disperse. In addition, the number of Leydig cells harvested from testes from older dogs was markedly reduced. This may have been the result of the animal's age or to the extended exposure time to collagenase required to break down the tougher connective tissue.

Determining how much time was required for dispersal to take place presented another difficulty, as the published reports on this aspect in rats and mice indicated that tissue breakdown ceases when the seminiferous tubules present the appearance of clumps of threads. These were easily identified in the rat tissue but were not so clear when dog tissue was used. With more experience, it was established that material from young dogs required to be exposed to collagenase for between 15-20 minutes and for about 30-35 minutes in older dogs.

Another important factor recognised was the freshness of the dog material. Testes from rats and mice were processed within 5-10 minutes of their removal. Sacrificing of laboratory animals can be programmed and all the necessary solutions and equipment prepared in advance. This was not possible with dog testes, as this tissue became available at unexpected times. Thus there was a delay of at least an hour in processing after their removal from the animal, to give time to prepare the necessary solutions. Initially, a small amount of dog tissue was not preserved in any way immediately after castration as theatre staff was not available to undertake these procedures. Immediately on arrival at the laboratory, the testes were placed in a plastic container to avoid further loss of fluid. Later in the study the removed testis were kept in 36^{o}C saline solution immediately upon their removal from the dog. Efforts were made to keep the interval from castration to processing to a minimum.

All external blood was removed from the intact dog testes with a paper towel, as the first attempts to separate Leydig cells on a Percoll gradient indicated that

blood cells interfered with this purification stage. Although the entire capsule was removed from the rat and mice testes, decapsulating the whole dog testes was considered unnecessary and time consuming, as the whole testes was not used.

In the rat, the very small amounts of tissue debris present at the end of collagenase digestion, was easily eliminated by filtering the resulting homogenate through an 80 um Millipore filter. However, such large quantities of debris were present in the dog tissue at this stage that they clogged the filter. Therefore, a plastic strainer was used prior to the homogenate being passed through the Millipore filter (Figure 55). These extra procedures were bound to have an effect on the numbers of viable cells ultimately collected.

Initially a continuous Percoll gradient was used to purify Leydig cells. However, preparation of such a gradient required about 1 hour and time was critical since as mentioned before, fresher tissue produced better results therefore they had to be processed as quickly as possible. Another disadvantage observed in this gradient was that it did not produced well defined bands, where cells were not separated from the contaminating spermatozoa or debris (Figure 39). However, as mentioned below better results would have been possible by using a cleaner more diluted cell suspension.

In their work in the rat, Simpson *et al.* (1987) used a more practical discontinuous gradient that could be quickly prepared as the different solutions required could be prepared in advance and kept refrigerated. This reduced the time factor and when used for the separation of the rat and mice Leydig cells produced reasonable results and thus this was the method adopted for the dog tissue and with some experience also produced acceptable results.

However, initially when using this gradient the same problems that had occurred with the previous discontinuous one arose, such as undefined cell bands, with cloudy layers of cells located before and after the 2nd band where most of the viable cells could be found (Figure 40). At this point some modifications were made to Simpson's original formula gradients (Figures 41, 42, 43, 44) in an attempt to improve the separation of bands since it was difficult to harvest the bands without aspirating cells from other bands as well.

The modifications to the first formula were done by using different amounts of Percoll and medium therefore formula 2, 3, and 4 originated. The first thing that

became apparent was that, in the original formula, it was possible to visually appreciate the different concentrations of the gradient by placing the gradient tube against the light and seeing the change in light refraction. However, this was not the case in the later methods tried and the results of these modified formulas did not show any improvement on the band separation. (Figures 41, 42, 43) for they did not eliminate spermatozoa or debris but did increase the number of dead cells.

While trying out these new gradient modifications, work was also going on, in an attempt to improve the first original method, which finally did happened by using a cleaner and more diluted cell suspension (Figures 45, 46, 47).

The cleaner cell suspension was the result of making such changes as whipping the blood off the dog testicles, before slicing off the testicular capsule thus markedly reducing the amount of erythrocytes. Most of the tissue debris was eliminated by filtering once through a plastic mesh and then through an 80 um filter (Figure 56). The spermatozoa were mostly eliminated by rinsing the pellet with medium, centrifuging and resuspending the resulting pellet with more medium

Considering that the cell suspension to be purified is placed in top of the gradient, then by reducing the presence of unwanted material, it becomes easier for the Leydig cells to go down through the different densities and settle down in cleaner bands. If not purified they could become trapped in the topmost layer and also because there was a tendency for the Leydig cells to stick together in the gradient bands forming clumps that were difficult to separate. As this occurred when very concentrated cell suspensions were used, the problem was reduced by increasing the dilution. It is easy to see, that one reason why the erythrocytes separate better, always going to the bottom of the gradient, might very well be because they are the smallest cell found in the cell suspension and they do not stick to each other, so they can even go through thick cell suspensions. .

It is important to realise that all the changes introduced to the discontinuous Percoll gradient were necessary only for dog tissue, but not for the mice or rat, since in these later species the amount of contaminating tissue was very small. In the rat, it was actually possible to eliminate most of the blood contamination by pulling off all the major blood vessels from the decapsulated testis. This meant that from the beginning, the discontinuous Percoll gradient produced very good results, as the bands were very clear and well defined.

As a general comment regarding Percoll gradients, it would seem that the number of bands obtained by this method as the four bands reported by Simpson *et al.* (1987) in the rat might not be so much species related, but due to a more heterogeneous cell suspension used for purification. Therefore this was the method that was used for the remainder of the experiments and no more modifications were made.

It was actually while using formula 4 (Figure 44) to separate dog tissue from an animal which had been castrated the day before, that it became apparent that the tissue had to be fresher as the resulting bands were full of debris and the few cells found were dead. Similar findings resulted when frozen tissue was used.

The identification of the isolated Leydig cells presented difficulties. Initial attempts at identification were made by observing a fresh cell preparation stained either with Diff Quick (Fig. 35a, b, c) with trypan blue (Figures 36a and b) and observed under a light microscope. However, it was not possible at that stage to do a proper identification.

Another method for identifying these cells was to demonstrate the presence of the 3βHSD enzyme in isolated Leydig cells. This enzyme is contained in steroid producing cells and this method was attempted but the results were also disappointing (Figures 37, a, b, c, d) because the cell preparation either did not stain at all, or although with different degrees of intensity stained everything blue.

Since identification of the Leydig cells was proving difficult, some cell suspensions were cultured to determine testosterone concentrations in the cell culture supernatant, as this would be an indication of the presence of Leydig cells.It was encouraging to determine that there was indeed testosterone indicating the presence of Leydig cells in these initial crude preparations (Figure 48). Furthermore the longer the cells remained undisturbed, the greater testosterone production (Figures 49 and 50).

However, the attempts at devising a reliable identification method continued. Schumaker *et al.* (1978), Joffre *et al.* (1984), Gale *et al* .(1982) and Hunter *et al.* (1982) reported that using phase contrast microscopy, rat Leydig cells had a distinctive bright yellow halo around them. Initial attempts at using this method in the dog were unsuccessful.

Eventually, by using a more sophisticated contrast phase microscope, it was possible to detect the yellow halo in the Leydig cells, (Figures 38 a and b). This was the identification method that was subsequently used in all the cell culture experiments.

Once identification of cells was possible, three different sizes of Leydig cells were seen in the preparations from all three species, namely mice, rats and dogs (Figure 46).The most common were medium sized fewer small and even fewer large ones. Unfortunately it was not possible to correlate these findings with differences such as age of the animal not only because of the small amount of dog tissue available, but because of the very diverse population of rats, mice and dogs.

The heterogeneity of the Leydig cell population has been reported by other researchers. Qureshi & Sharpe. (1993) found in man that Leydig cells can be light or dark. Aquilano and Dufau, (1984) that when using centrifugal eleutration and Metrizamide gradients Leydig cells settled into different sedimentation velocities while Molenaar *et al.* (1983) found differences in the amounts of testosterone produced when stimulating with hCG, Laws *et al.* (1985) also reported finding at least two cell bands in the Percoll gradient with the cells in one of the bands actually containing a smaller number of hCG receptors. A similar finding was also reported by Bhalla *et al.* 1987.

A different approach to identification of the Leydig cells was tried simultaneously with o the development of a better Percoll gradient. Based on work by Molenaar *et al.* (1983), Hunter *et al.* (1981), Rommerts *et al.* (1982), Themmen *et al.* (1987).Viable Leydig cells attach to culture dishes, whereas the erythrocytes and germ cells float to the surface of the dish. It was thought that by trying this method it would be easier to obtain and identify viable Leydig cells.

However, these initial cell culture attempts failed due to lack of experience in this area, for example until distilled water was added in the empty wells of the culture dishes, the cell medium evaporated, leaving dead dried cells in the bottom of the dish. Also a problem with the original incubator went undetected, and the temperature was set at 37°C and considering that Leydig cells are part of the testis which is maintained at a lower temperature, this could have accounted for the high dead cell rate observed in the first cultures, the temperature was changed to 32°C. The over frequent observation of the dishes probably

contaminated the cultures. To avoid this contamination, the multiwell dishes were eliminated in favour of individual dishes that made examination easier.All material used in the entire process was sterilised the solutions, gradients and cultures were prepared in a positive pressure sterile hood

However there were very small probabilities of identifying one particular cell type in the initial heterogeneous cell suspensions which contained everything from spermatozoa, erythrocytes, interstitial tissue to Sertoli and germ cells . The germ cells would be contaminating the suspension because as mentioned before it was not possible to eliminate the dog seminiferous tubules before they digested thus adding to the confusion of detecting Leydig cells. Specially because in the beginning the impression was that Leydig cells were all the same size, as later it was learned that this was not so plus the fact that the improved cell suspensions increased the number of viable cells attaching to the dishes, identification became easier.

Considering the possibility that dog cells were not attaching to the culture dishes because they required longer incubation periods, different times were tried from 3-7 hours up to 24 and 36 (Figures 49 and 50,). However, these initial figures present results on two different dishes which were left untouched until the end of the incubation period when the supernatant was collected and the bottom of the dish examined. Once the supernatant was removed, trypan blue was added , and the dishes showed varying numbers of cells still attached to the bottom of the dishes.(Figures 38 a, b, c), this particular find just proved that Leydig cells were present in the culture and that the longer they were left untouched they were able to continue producing testosterone.

As mentioned before, a characteristic that was noticed in Leydig cells was that after Percoll centrifugation the cells in the pellets proved difficult to disperse as the cells tended to form clumps. This problem became apparent in the initial cell cultures that showed a very uneven testosterone production in the first supernatant collected after 7 hours of plating (Figures 51 and 52) . Although the concentration of the cell suspension had indeed been evaluated prior to culture, it was obvious that the culture dishes did not contain the same amount of cells to begin with because they still contained some cell clumps which however small could still account for increased testosterone amounts produced in a particular culture dish.

A better resuspension was achieved when instead of using glass Pasteur pipettes, disposable plastic pipettes (Merck Ltd. Lutterworth, UK) were used and it was noted that the cells tended to stick less to these than to glass. By adding .5 ml of medium to the pellet and softly but repeatedly passing the cell pellet through the plastic pipette, it was possible to produce a more homogeneous suspension that was then diluted further until an appropriate suspension was obtained. By agitating the cell preparation container repeatedly while pipetting into the culture dishes, the problem of the cells settling to the bottom was reduced. An example of this improvement can be seen in Figures 53 and 54, as it shows more homogeneous testosterone production levels in the first supernatant collected from all the dishes.

However, the problem of the variable numbers of cells attaching to the dishes continued as demonstrated in the very varied testosterone found in the next supernatants. It was not always possible to relate the increase or decrease in testosterone contents in the subsequent supernatants to the initial values, for it was not possible to be sure whether the lack of testosterone production was due to a problem in the cells themselves or just due to a decrease in testosterone producing cell numbers.

Although the problem of cell attachment was not entirely satisfactory, at this point it was decided that the initial work was producing viable cells and that it was time to try a more advanced approach to cell culture that would provide more information regarding Leydig cell function in the dog.

CHAPTER 4.

LEYDIG CELL FUNCTION STUDIES

1. INTRODUCTION

The results thus far had shown that the Nafarelin acetate treatment was suppressing the production of LH and testosterone in the dog, and that the degree of inhibition was related to length of treatment. At the same time, the isolation and purification techniques used so far were producing viable dog Leydig cells. Therefore a more advanced study of the Leydig cells mechanisms appeared feasible. The aims were to monitor the injection effect over an extended period of time, in an attempt to find out if the suppression of LH and testosterone was through desensitisation of the pituitary as in most species, or also directly on the gonad as in the case of the rat. In this species GnRH-like receptors have also been identified in the gonads (Hsueh & Erickson, 1979; Sharpe and Fraser, 1980; Hsueh and Jones, 1981; Popkin, *et a*l. 1985; Huhtaniemi, *et al.* 1986). It was also intended to study the effects of LH and testosterone suppression in the Leydig cells and therefore with the purpose of obtaining these cells, two more dogs were treated with Nafarelin acetate, one for 30 days and a second for 60 days and their Leydig cells were used for the study.

2. MATERIALS AND METHODS

2.1. ANIMALS USED

a) Testes from untreated animals: Dogs, mice, rats.

b) Testes from 2 dogs treated with daily subcutaneous injections of Nafarelin acetate.

c) Testes from 16 mice: 8 Treated with daily subcutaneous injection of Nafarelin acetate for 30 days, 8 mice were kept as control.

Identification	Breed	Age, years	Weight kgs.	Body condition	Treatment length
Kenny	Beagle	7	21.2	good	60 days
Dennis	Beagle	7	21.2	good	30 days

2.2. DOG'S MAINTENANCE.

As discussed in chapter 2, section A.

2.3. TESTICULAR MEASUREMENTS

As discussed in chapter 2, section A.

2.4. SCANNING.

As discussed in chapter 2, section A.

2.5. LIBIDO EVALUATION

As discussed in chapter 2, section A.

2.6. SEMEN EVALUATION

As discussed in chapter 2, section A.

2.7. ALKALINE PHOSPHATASE

As discussed in chapter 2, section A.

2.8. BLOOD SAMPLE COLLECTION AND PROCESSING.

As discussed in chapter 2, section A.

2.9. MEDICATION USED.

The two dogs and the 8 treated mice were given Nafarelin acetate, kindly supplied by Syntex Labs. Palo Alto, California administered at a dose of 62 ug/day by subcutaneous injections.

2.10. TISSUE COLLECTION

As discussed in chapter 3.

2.11. TISSUE DISSOCIATION

Using the third method discussed in chapter 3.

2.12. CELL COUNTS, VIABILITY AND IDENTIFICATION

As discussed in chapter 3.

2.13. CELL PURIFICATION

As discussed in chapter 3

2.14. CELL CULTURE METHODS

The method used was as described by O'Shaughnessy et al (1991).

Material used.

1. 20 ml poly propylene vials. (Bibby Sterilin Ltd. Tilling Dr., Stone, Staff., England ST 150SA)

2. 2 ml Eppendorf tubes with perforated caps(Merck Ltd. Lutterworth, Leics. England).

3. Disposable pipettes (Merck Ltd., Lutterworth, Leics., England).

4. Boiling water

5. Aluminium foil

Method number 1 for the treatment of isolated Leydig cells, to determine cell viability by their testosterone production response.

Cell experimental design			
Tube No.	Cell medium (µl)	Treatment suspended in (µl)	Cell suspension (µl)
1-2	900	no treatment 0	100
3-4	890	hCG-(200 m IU) 10	100
5-6	890	dbcAMP (2mM) 10	100
7-8	892	22 ROHC (25 uM) 8	100

In this method the following extra treatments were added to the above protocol.

Additional treatments.			
Tube	Cell medium (µl)	Treatment suspended in (µl)	Cell suspension ((µl)
9-10	890	Nafarelin acetate (8mM) 10	100
11-12	890	Nafarelin acetate (4 mM) 10	100
13-14	890	Nafarelin acetate (2 mM) 10	100
15-16	890	Nafarelin acetate (1 mM) 10	100
17-18	890	Nafarelin acetate (0.00058 ug/ml) 10	100

2.15. INCUBATION

The 20 ml culture tubes were incubated in a ICN 160 model automatic CO_2 incubator (ICN Flow Laboratories, Herts, England) at 32°C and 5% CO_2 for 3 hours.

Cell culture was collected after incubation

1. The cell culture medium was transferred from the incubating tubes into the Eppendorf tubes with perforated caps.

2. The Eppendorf tubes were inserted in the aluminium foil circle which was then placed over a container full of boiling water and allowed to boil for 2 minutes. This was done with the purpose of bursting the cells open thus releasing all remaining testosterone into the medium.

3. The contents of the perforated tubes were transferred into Eppendorf tubes with intact caps.

4. The tubes were then centrifuged in an Eppendorf centrifuge 5415 (Eppendorf Geratebau, Hamburg Germany) at 14000 rpm/min for 1 minute.

5. The tubes were decanted and the supernatant was transferred to another identified set of Eppendorf tubes with intact caps and stored frozen for future analysis.

HORMONE ANALYSIS.

As discussed in chapter 3.

Histochemistry 3BHSD staining.

Method 1

As described in chapter 3.

Method 2.

This method was the one described by Payne, Downing and Wong (1980).

Material required for method 2.	
100 ml	Phosphate buffered saline
0.001 gr	Bovine serum albumin. (No. A.9647)
99.5 mg	Nicotinamide adenine dinucleotide (No. 7004)
20.4 mg	Nitro blue tetrazolium (No. 6639)
5.8 mg	Ethiocholan (No. E5252)

All reagents were purchased from Sigma (Chemical Co. Ltd., Poole, Dorset, England).

Preparation procedure. This solution is prepared by dissolving all the reagents in the 100 ml of phosphate buffered saline, the ethiocholan having been dissolved in 1 ml of methanol. This solution is prepared fresh just prior to use as it loses efficacy with storage.

Staining of slides. 100 ul samples of cell suspension were placed in a glass slide and dried at room temperature. Immediately after drying, the cells were covered with the solution.

GnRH RECEPTORS

Labelling of GnRH The Chloramine-T method was used as described by Jeffcoate et al. (1978).

Material used:

Material used
1. GnRH Code no.L-7134. (Sigma Chemical, Co. Ltd., Dorset, England)
2. 1 ml polystyrene vials with rubber stoppers (Luckhaus LP3)
3. Phosphate buffer.

Phosphate buffer made up as follows

A.-6.9 g Na H_2 PO_4	dissolved in 100 ml distilled water
B.-7.1 g Na 2 HPO_4	dissolved in 100 ml distilled water
Add A to B	to make up the pH 7.5 solution.

RADIO IODINATION OF GnRH.

1	Place 2.5 ug GnRH (2.4 µl) in the 1 ml vials.
2	Add 25 ul of 0.5 phosphate buffer (pH 7.5).
3	Add 1 mCi 125 I (20 µl).
4	Count to determine accuracy of iodine addition.
5	Stopper reaction vial and add 40 µl Chloramine-T (20 µg).
6	Shake gently for 15 sec.
7	Add 100 µg (50 µl) of sodium metabisulfite (2mg/ml).
8	Add 100 µl of 1% BSA-PBS and shake gently.

9	Transfer reactive mixture to surface of 11 ml. column of Sephadex G-25 (fine). Column pre-saturated with 10 ml.
10	Elude column with 0.05m (PBS + BSA, 25%).
11	Collect 1 ml fraction.
12	Count 10 or 20 µl fractions to determine peptide peak.
13	100 µl aliquots were made and stored frozen for further use.

PREPARATION OF HOMOGENATED TISSUE.

Material used	
1	Round bottom polystyrene tubes 130 mm x 17mm diameter (Sarstedt Ltd. Leicester England).
2	Ultra-turrax-T25 homogeniser (Janke & Kunkel IKA Labortechnik, Germany).
3	Tris buffer.
4	14 ml. centrifuge tubes (Greiner Labortechnik Ltd., Dursley, England).

Homogenising procedure

1 500 gram pieces of tissue sample plus 1 ml of tris buffer were placed in the bottom of the round polystyrene tube.

2 The tissue was homogenised in a Ultra- turrax-T25 at 20,500 rpm.

3 The homogeniser's probe was rinsed with an additional 1 ml of buffer by adding it a little at a time as the procedure had to stop every few seconds to avoid overheating of the tissue.

4 The tube containing the tissue was placed in crushed ice during these short resting periods, this being continued until the tissue was completely homogenised.

5 The homogenate was then transferred into 14 ml centrifuge tubes (Greiner Labortechnik Ltd. Dursley) and resuspended into an additional 6 ml of buffer.

6 The mixture was thoroughly mixed by turning the tube by hand before placing in the centrifuge and spun at 3000 g for 5 minutes.

7 The tubes were decanted and the pellet was resuspended in 2 ml. This preparation was then set up for incubation as follows.

Material used for incubation	
1	Labelled GnRH.
2	Incubation tubes. Rohren 3.5 ml, 55 x 12 mm dia. (Sarsted, Leiscester England).
3	Tris buffer.
4	Crushed ice.

Protocol for determination of GnRH receptors in the Leydig cells.

Tube no.	Labelled μl GnRH	Unlabelled Nafarelin μl acetate	Tissue
1,2	100		
3,4,5,6,	100		100
7,8,9,10	100	100	100
11,12	100	50	100
13,14	100	20	100
15,16	100	10	100

3. RESULTS

Table 17 Protocol for the Nafarelin acetate treatment of an adult male beagle (Kenny) treated for 60 days. (Day of 1st. treatment= day 1).

Days	Procedure
-7	Semen collection
-7, 1 and then weekly	Animal weighed
-17 to -14	Daily samples taken .
-13	8 Hourly samples.
1, 2, 3, 4, 5 and then weekly	1 Blood sample before injection 3 Hourly samples taken after injection
-3 +10 and then weekly	One blood sample
1 to 60	Daily injection and body temperature taken
7 and then weekly	Semen collection, libido evaluation, scanning.
59	8 Hourly samples.

Table 18. This table presents the protocol details of the treatment period of a male beagle dog (Kenny) that was treated for 2 months with daily subcutaneous injections of Nafarelin acetate. Note that when 4 blood samples were obtained, the first one was taken prior to the daily injection, whilst the other 3 were collected at hourly intervals following the injection. The well being of the animal was observed daily. Temperature and body weight were monitored on regular basis. There was no indication of the treatment having an effect on either of these two parameters.

Table 18. Bleeding and treatment protocol of a male beagle dog (Kenny) treated with Nafarelin acetate

Date	Nafarelin acetate in ug	Bleeding	Temp.	Weight in kgs
14/03/93			102	
15/03/93			102	21.8
16/03/93			;02	
17/03/93			101.8	
18/03/93			102	
19/03/93		B	101	21.7
20/03/93		B	101.2	
21/03/93		B	102	
22/03/93		B	101.8	21.1
23/03/93		Window	;01.4	
24/03/93		B	101.8	
25/03/93			101.8	
26/03/93		B	101.6	
29/03/93		B	101.8	20.2
30/03/93		B	101.2	
31/03/93		B	101	
01/04/93			101	
02/04/93			101.8	
03/04/93			101.2	
04/04/93			101.2	
05/04/93	62	4 samples	101.2	21
06/04/93	"	4 samples	101.2	
07/04/93	"	4 samples	101.2	
08/04/93	"	4 samples	101.2	
09/04/93	"	4 samples	101.2	
12/04/93	"	4 samples	101	20.2
13/04/93	"		100.8	
14/04/93	"	B	100.7	
15/04/93	"		101.2	
16/04/93	"	B	101.2	
19/04/93	"	4 samples	102	20.2
21/04/93	"	B	101.8	
22/04/93	"		101	
23/04/93	"		100.8	
24/04/93	"	B	101	
26/04/93	"	4 samples	101	20.1
27/04/93	"		101.2	
28/04/93	"	B	101	
29/04/93	"		101	
02/05/93	"		102	
03/05/93	"		101.4	19.9
04/05/93	"	4 samples	101	
05/05/93	"	B	100.6	
06/05/93	"	B	1001	
07/05/93	"	B	101	
10/05/93	"	4 samples	100.3	19.7
11/05/93	"		101.8	
12/05/93	"	B	101	
13/05/93	"		100.9	
14/05/93	"	B	101.1	
17/05/93	"	4 samples	101	20.2
18/05/93	"		102	
19/05/93	"	B	102	
20/05/93	"		102	
21/05/93	"	B	101.9	
24/05/93	"	4 samples	101.4	19.6
26/05/93	"		101.3	
28/05/93	"	B	101.2	
29/05/93	"		101.3	
31/05/93	"		101.2	19.8
02/06/93	"	window 8 hr	101	20.4
	"			

Table 19. The evaluation of external genitalia in this animal (Kenny) starts showing a decrease in testicular size, as well as a change in consistency that can be appreciated on the 30th of April, i.e. 25th day of treatment until the end of the 60 day treatment period. Libido in this animal was low and the numerous collection attempts both prior to and during treatment proved unsuccessful with just one exception on the second day of treatment. A second positive response was observed 25 days after treatment in the presence of an oestrous teaser as he developed an erection and attempted to mount but an ejaculate was not produced.

Table 19. Evaluation of external genitalia of an adult male beagle (Kenny) treated with Nafarelin acetate.

	Testicular measurements							Clinical evaluation							
												Abnormalities			
Date	Left Width (cms)	Left Length (cms)	Right Width (cms)	Right Length (cms)	Testicular consistency	Epididymis	Libido	Volume ml		Progressive motility %	Concentration x (10)6/ml	Live:dead	Head %	Acrosome %	Tail %	Total %
31/03/93	8.5	8	8	8	firm	normal	2									
06/04/93					firm	normal	3	1.5	cloudy	65	153	95:5	1	normal	7	8
21/04/93	8.5	8	8	8	firm	normal	1									
30/04/93	8	8	7	7.5	firm	normal	1									
14/05/93	7	7	7	6.5	soft	small	2									
26/05/93	5.5	5	5.5	5	soft	small										
28/05/93	5	4	5	4	soft	small	0									
01/06/93	4	3	4	3.5	soft	small	0									

Figure 59. This figure presents the results of daily bleeding in Kenny both prior to and during treatment. It shows the results of daily blood samples. On the days when 4 samples were taken only the first pre-injection sample is included. Note the testosterone levels on the first sample and the dramatic decrease of testosterone 0.01-16.99 nmol/l to almost undetectable (by our assay) levels by the 4th day post injection this low levels were maintained until the end of treatment. However, LH continued to be synthesised in some instances to levels that were even higher than in the pre-treatment period H 0.05-7.2 ng/ml.

Fig. 59 Circulating plasma LH and testosterone in daily samples taken prior to and during treatment with Nafarelin acetate (Kenny)

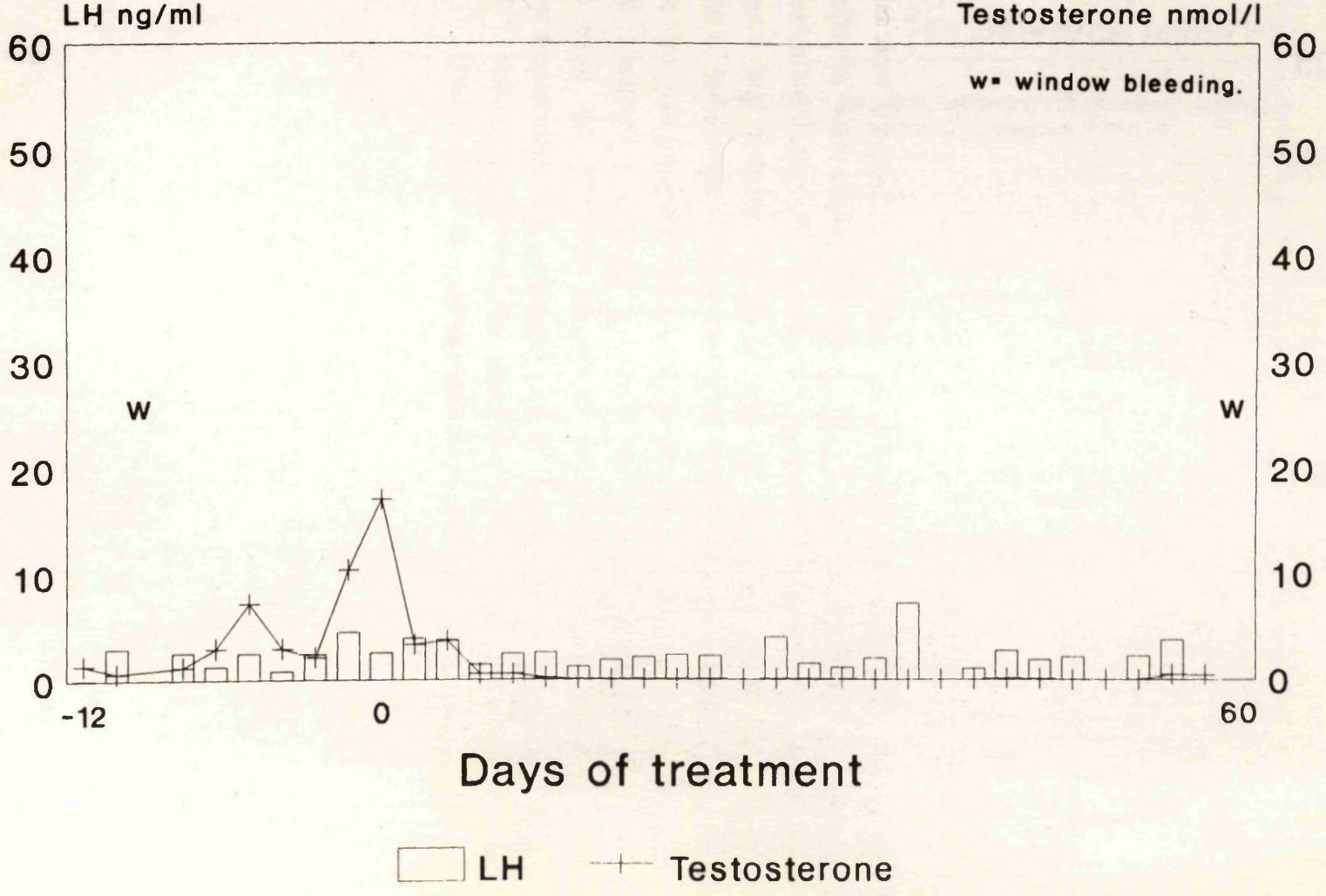


Figure 60. This figure includes only the days when 4 samples were taken. The first sample was collected prior to the application of the daily injection and the next 3 samples at hourly intervals post injection. The first 5 of these sampling periods took place daily for the first week of treatment and every 7 days thereafter. Notice the stimulatory effect that can be appreciated in the 3 hourly samples collected after the first injection. By the following day this stimulation effect has decreased and by the third day there is a dramatic increase in testosterone production which by the fourth and fifth day have diminished considerably. The suppression of both hormones but testosterone in particular was maintained throughout the remainder of the treatment period The ranges were for LH 0.0-25.3 ng/ml and for testosterone 0.0-33.06 nmol/l.

Fig. 60 Circulating plasma LH & testosterone concentrations in multiple samples taken during treatment with Nafarelin acetate in Kenny.

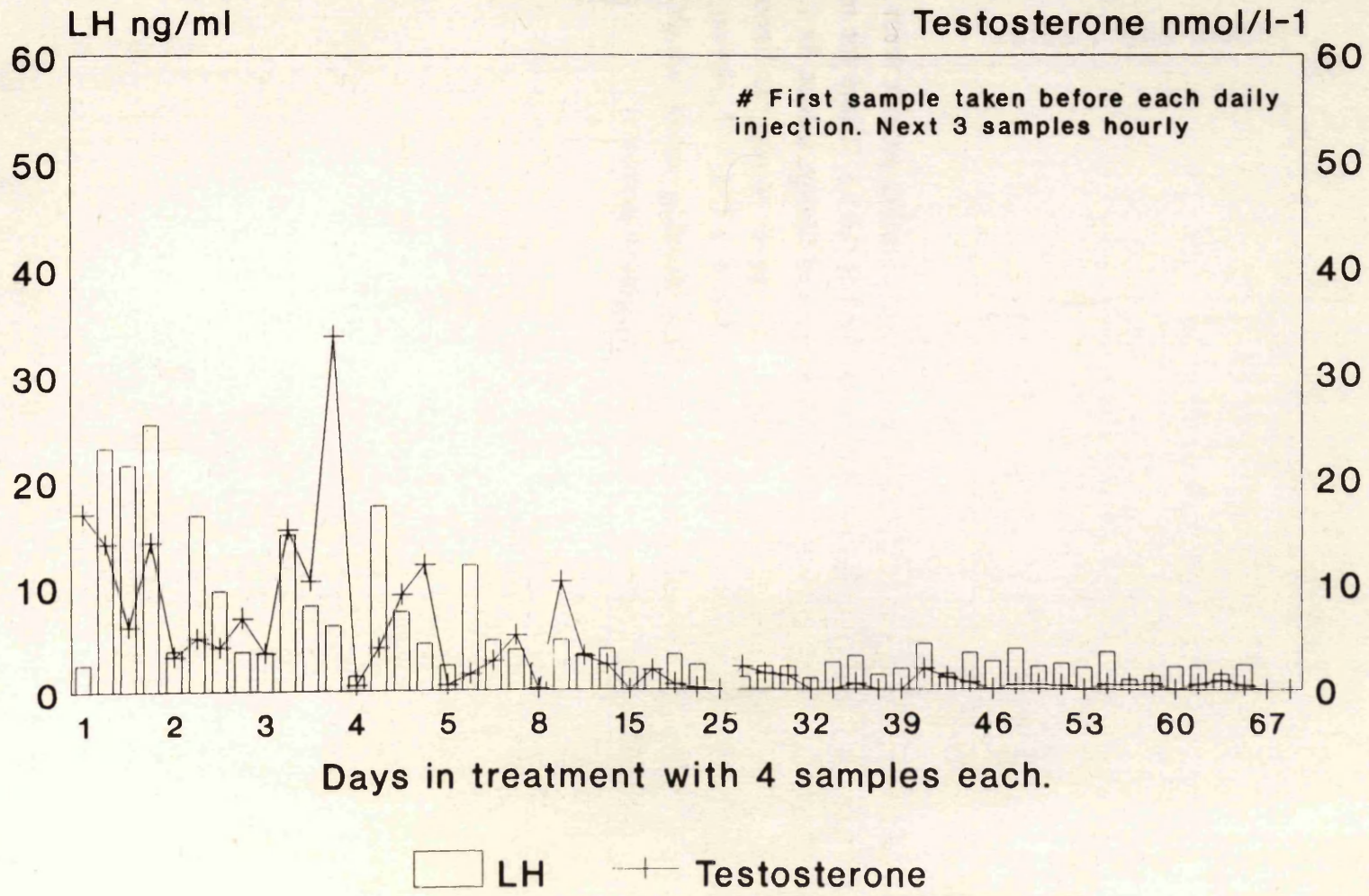


Figure 61. In this animal (Kenny) two hourly bleeding periods were taken, one prior to initiation of treatment (The range for LH 0.01-3.71 and for testosterone 1.16-5.28 nmol/l), and the second towards the end (Range values for LH 1.5-3.7 and for testosterone 0.11-0.58 nmol/l) No major hourly oscillations can be appreciated in either hormones, but particularly i. e LH. However, there is a clear suppression of testosterone in the second bleeding period, which apparently did not occur in LH, with levels of this being almost normal.

Fig. 61 Circulating plasma LH & testosterone in samples taken every hour for 7 hours. Before and after treatment (Kenny)

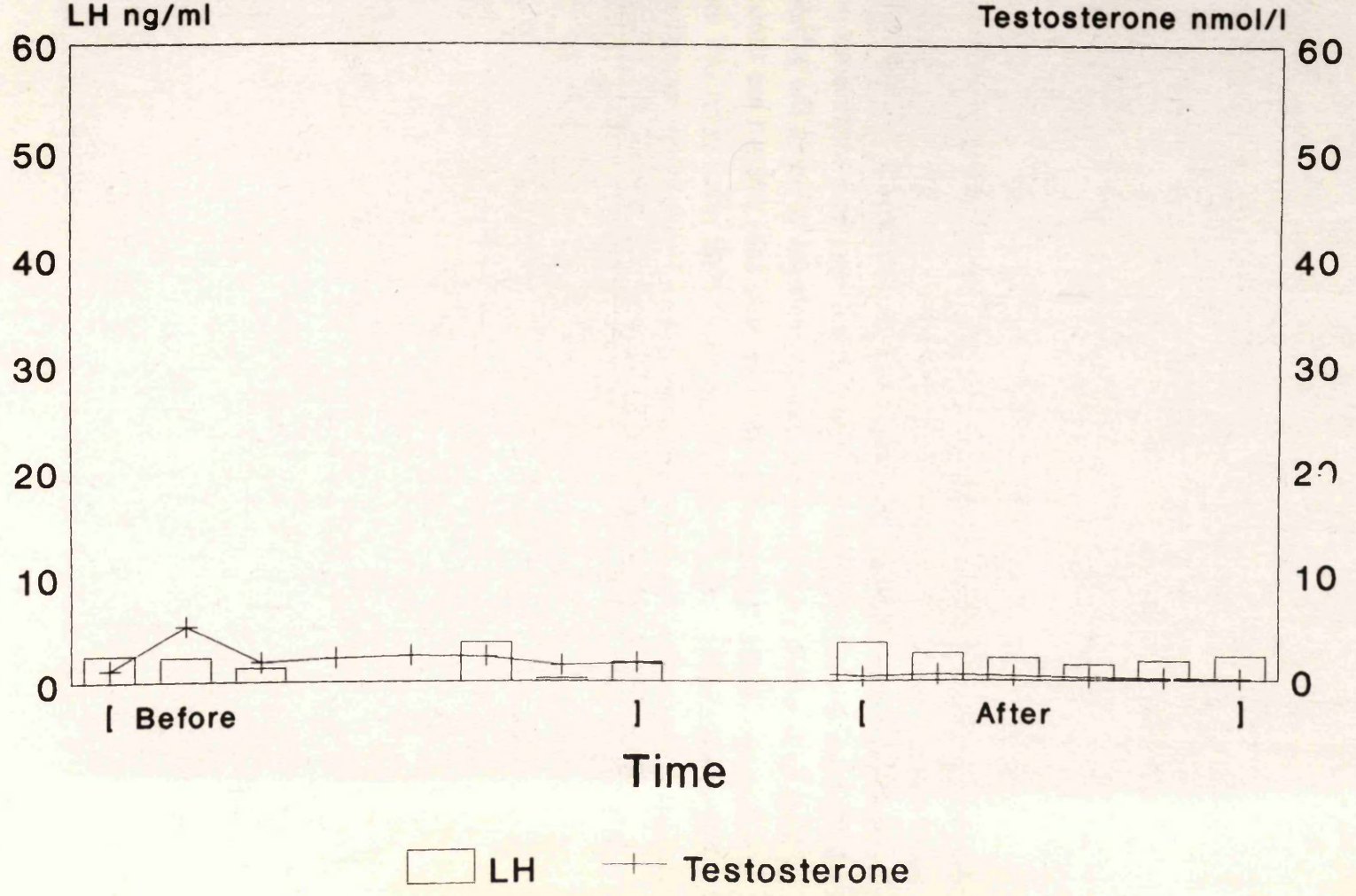
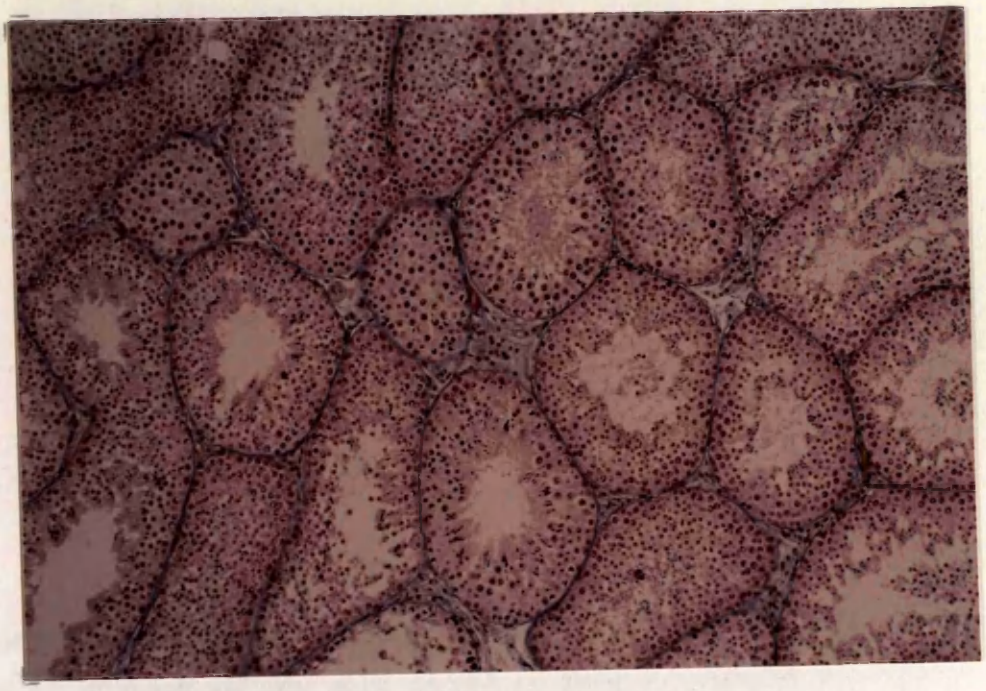


Figure 62. These are the histological sections of testes from a) normal dog, b) an adult beagle dog (Kenny) treated with Nafarelin acetate by daily subcutaneous injections for two months. Notice that in the normal dog the seminiferous tubules have a complete germ cell complement seen as a continuous progression of germ cells which start from the basal membrane towards the tubule's lumen. In comparison in the treated dog testis, the only cells present are those closer to the basal membrane. Note the disappearance of all other germ cell associations as well as the tubular lumen. Magnification 10 x. Both slides stained with MSB. A blue microscope filter was used for the photograph of the normal animal.

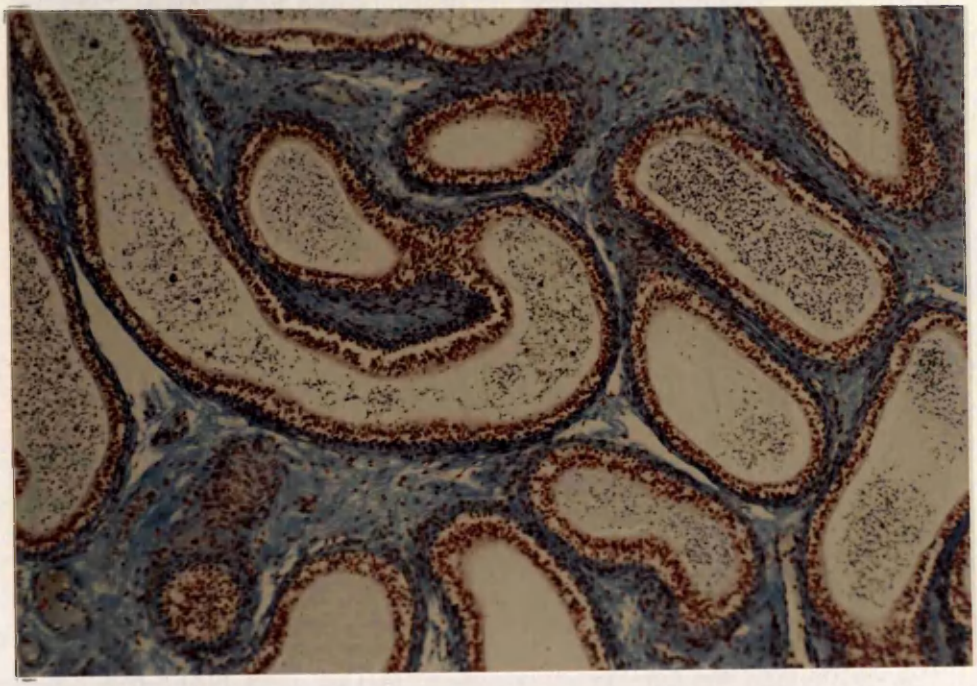
a



b

Figure 63. Histological sections of epididymal body a) from a normal dog b) from a beagle dog (Kenny). Note in the normal dog the uniform cellular arrangement indicated by the position of the nuclei which are almost all placed at the same distance from the basal membrane. In the nuclei from the treated dog there is a somewhat disarranged appearance with the placement of the nucleus at different distances from the basal membrane. The presence of spermatozoa in this section of the epididymis is not as abundant as in the head, but in the normal dog they can be seen in one of the tubules. However none are apparent in the section from the treated dog. Magnification 10 x. Both slides stained with MSB.

a



b

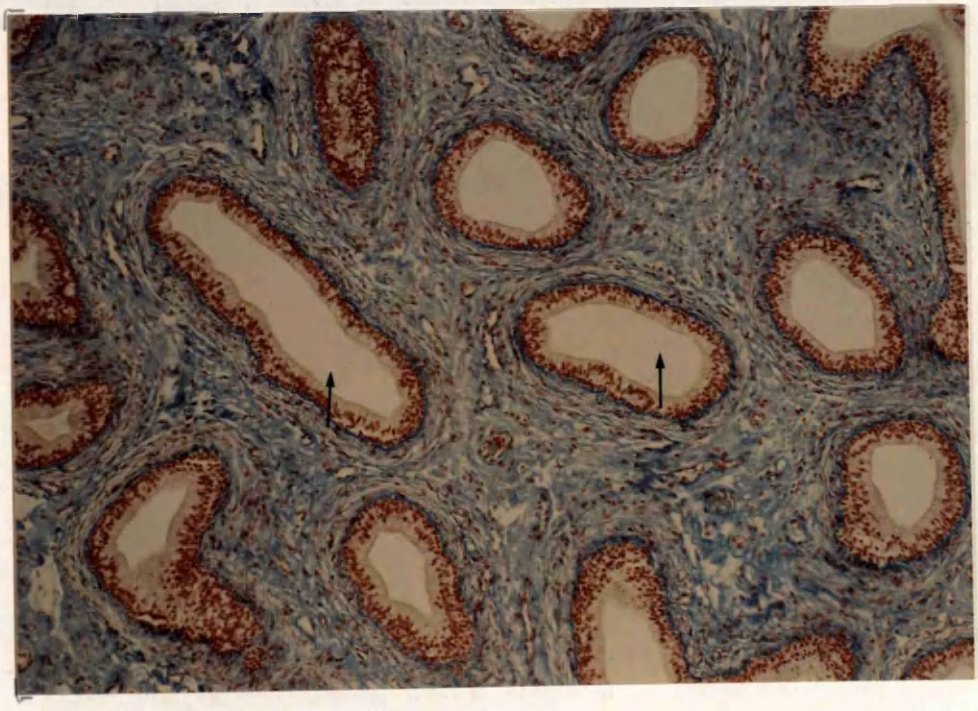
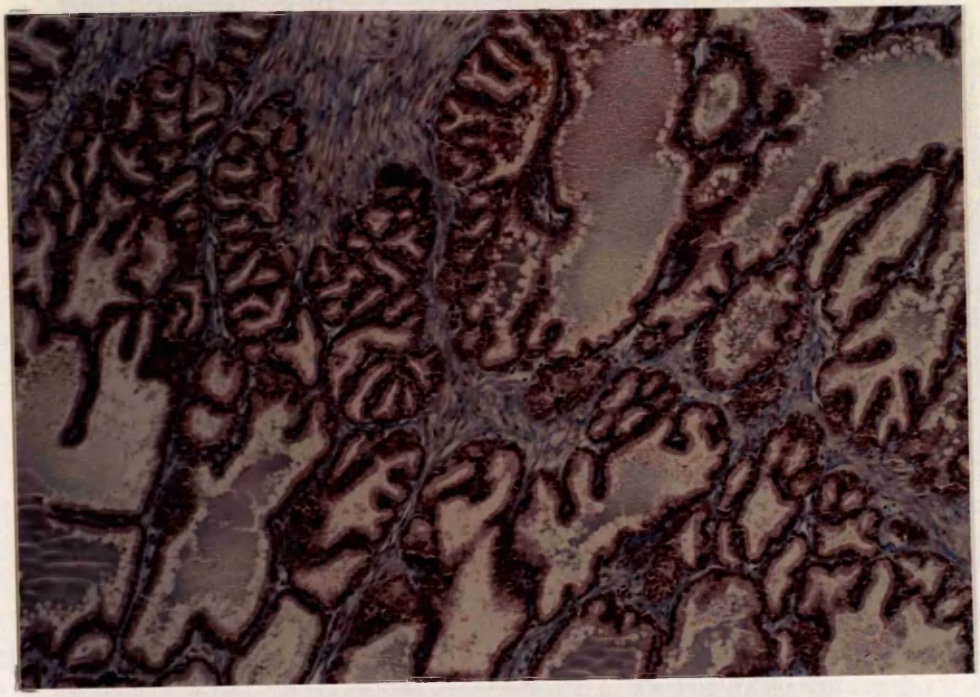


Figure 64. This figure presents histological sections taken from the prostate of a) a normal dog b) from a beagle dog (Kenny) treated with Nafarelin acetate. In the treated animal the prostatic alveoli have a shrunken appearance compared to those in the normal dog. This also shows secretion in the alveoli lumen that is not present in the treated animal's sections which show an obvious increase in connective tissue as an indication of the gland's atrophy. a) Photograph magnification 10x .A blue microscope filter was used for this photograph b) photograph 54 x . Both slides stained with MSB

a



b

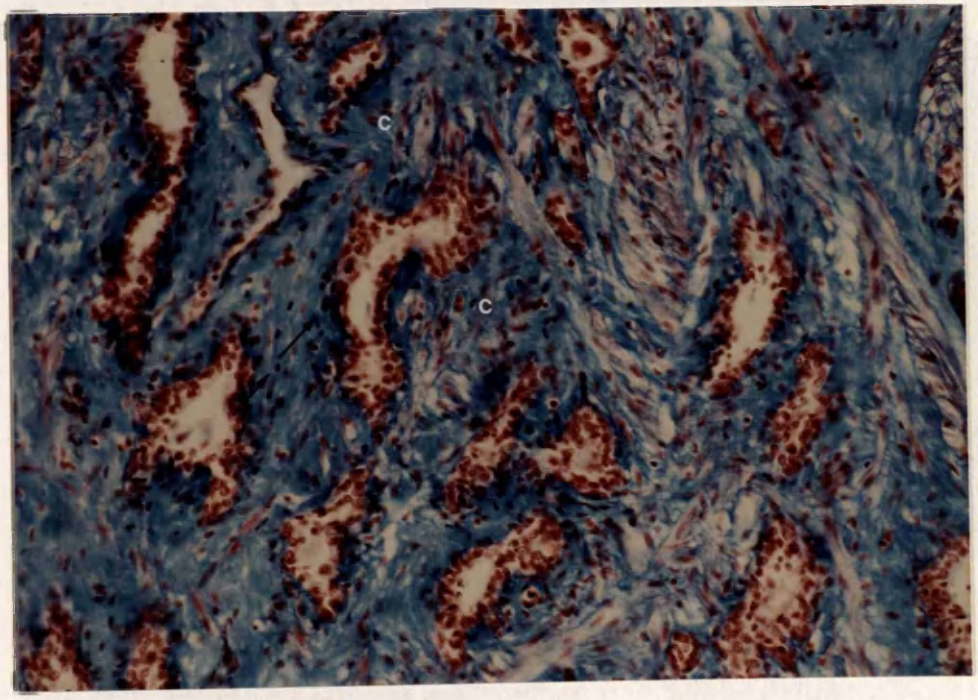
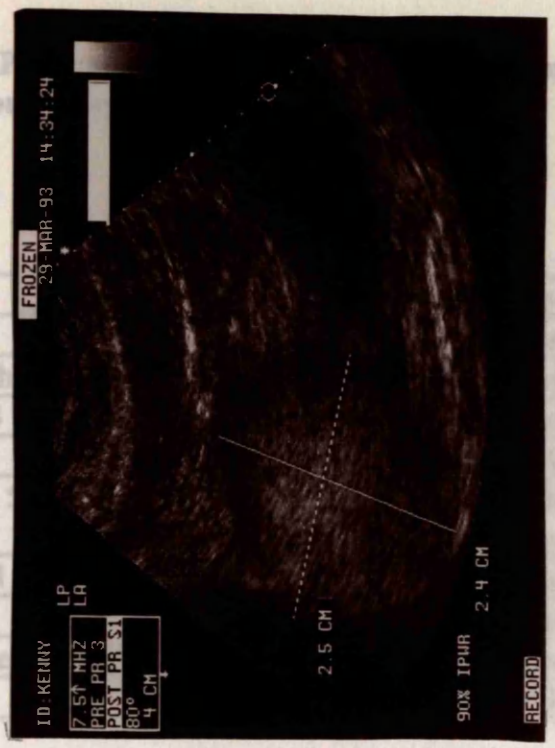
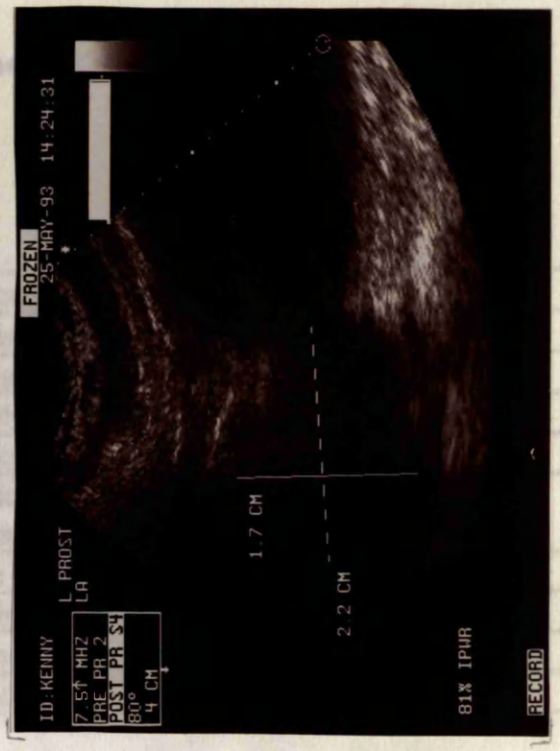
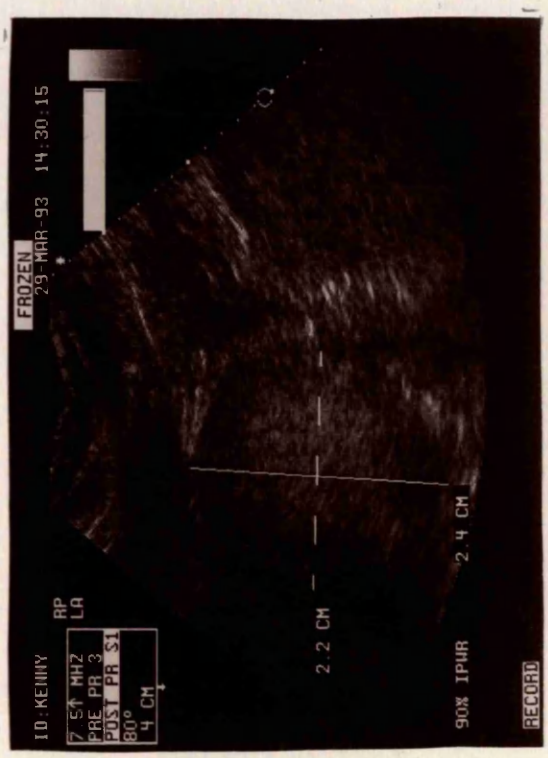


Figure 65. Ultrasonographs of the prostate show the difference in sizes between those taken prior to treatment on March 29 (1) , and the ones taken 56 days later (2). The longitudinal axis of the left prostate is shown in photos A-1 and A-2 and the right side shown B-1 and B-2. Notice that the greatest difference is in the width with a decrease of 0.7 cm from the initial diameter and 0.2 cm in the length, both left and right.


FROZEN
29-MAR-93 14:34:24
ID:KENNY
LP
LA
7.5↑ MHZ
PRE PR 3
POST PR S1
80°
4 CM
2.5 CM
90% IPWR
2.4 CM
RECORD


FROZEN
25-MAY-93 14:24:31
ID:KENNY
L PROST
LA
7.5↑ MHZ
PRE PR 2
POST PR S4
80°
4 CM
1.7 CM
2.2 CM
81% IPWR
RECORD


FROZEN
29-MAR-93 14:30:15
ID:KENNY
RP
LA
7.5↑ MHZ
PRE PR 3
POST PR S1
80°
4 CM
2.2 CM
90% IPWR
2.4 CM
RECORD

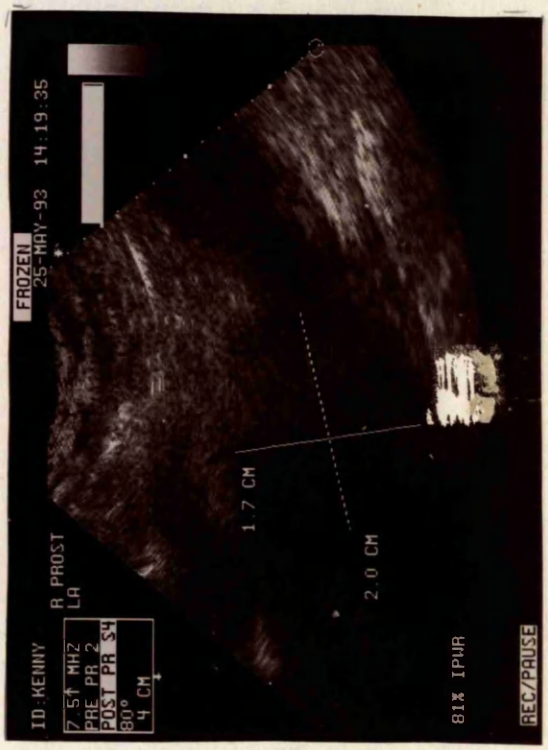

FROZEN
25-MAY-93 14:19:35
ID:KENNY
R PROST
LA
7.5↑ MHZ
PRE PR 2
POST PR S4
80°
4 CM
1.7 CM
2.0 CM
81% IPWR
REC/PAUSE

Table 20 Protocol for the Nafarelin acetate treatment of an adult male beagle (Dennis) treated for 30 days.

Days	Procedure
-7	Semen collection
-7,1 and then weekly	Weight taken
-17 to -14	Daily blood samples
-13	8 Hourly samples
1 ,2, 3, 4, 5 and then weekly	1 Blood sample before injection 3 hourly samples after injection
-3, 10 and then weekly	One blood sample
1 to 30	Daily injection and body temperature taken
+7 and then weekly	Semen collection, libido evaluation, scanning.
30	8 Hourly samples.

Table 21. This table presents the protocol details of the treatment period of a male beagle dog (Dennis) that was treated for 1 month with daily subcutaneous injections of Nafarelin acetate. Note that when 4 blood samples were obtained, the first one was taken prior to the daily injection administration, whilst the other 3 were collected at hourly intervals following the injection. The well being of the animal was observed daily and temperature and body weight were monitored on a regular basis and there is no indication of the treatment having an effect on either of these two parameters.

Table 21. Bleeding and treatment protocol of a male beagle dog (Dennis) treated with Nafarelin acetate

Date	Nafarelin acetate in ug	Bleeding	Temp. o F	Weight in kgs
14/03/93			102	
15/03/93			101.4	
17/03/93			101.8	
18/03/93			102	
19/03/93		B	101	21.7
20/03/93		B	101.4	
21/03/93		B	102	
22/03/93		B	101.8	21.1
23/03/93		7 hour bleeding window	102	
24/03/93		B	101.8	
25/03/93			101.8	
26/03/93		B	101.6	
29/03/93		B	101.8	20.2
30/03/93		B	101.2	
31/03/93		B	101.1	
01/04/93			101	
02/04/93			101.6	
03/04/93			101.6	
04/04/93			101.4	
05/04/93	62	4 samples	101.2	21
06/04/93	"	4 samples	101.2	
07/04/93	"	4 samples	101.2	
08/04/93	"	4 samples	101.2	
09/04/93	"	4 samples	101.2	
12/04/93	"	4 samples	101.2	20.2
13/04/93	"		100.8	
14/04/93	"	B	100.7	
15/04/93	"		101.2	
16/04/93	"	B	101.2	
19/04/93	"	4 samples	102	20.2
21/04/93	"	B	101.8	
22/04/93	"		101	
23/04/93	"		100.8	
24/04/93	"	B	102	
26/04/93	"	4 samples	101	20.1
27/04/93	"		101.2	
28/04/93	"	B	101	
29/04/93	"		101	
02/05/93	"		101.1	
03/05/93	"		101	19.9
04/05/93	"	7 hour bleeding window	101.3	
05/05/93	"	B	100.8	
06/05/93	"	B	101	

Table 22 As this table indicates, the libido in this animal (Dennis) was poor (0 and 1) even on those occasions when an oestrus bitch was used to collect semen. Note that the testicular measurements started decreasing in size by the 23th day of treatment, but no obvious changes were appreciated in the epididymis.

Table 22. Evaluation of external genitalia and libido of a male dog (Dennis) treated with Nafarelin acetate

Date	Libido	Testicular measurements in cms				Testicular consistency	Epididymis	Comments
		Left Length	Left Length	Right Width	Right Lenght			
31/03/93	o	7	8.5	7	9	firm	normal	
21/04/93	o	7	8.5	7	9	firm	normal	
05/04/93								treatment started
28/04/93	1					firm	normal	
29/04/93	1					firm	normal	
30/04/93	1	6.5	8	6.5	8	firm	normal	
02/05/93	1					firm	normal	
03/05/93	1					firm	normal	
05/05/93	1	6.5	7	6.5	6.5	firm	normal	

Figure 66. This figure presents the results of daily bleeding in Dennis both prior to and during treatment. It shows the results of daily blood samples and for those days when 4 samples were taken, only the first pre injection sample is included. Note the testosterone levels on the sample taken one day before the treatment started and the decrease of testosterone to almost undetectable (by our assay) levels by the 5th day, which were maintained until the end of treatment. However, LH continued to be synthesised in some instances to levels that were even higher than in the pre treatment period. The range of both hormones was (LH 1.87-8.69 ng/ml; testosterone 0.01-30.99 nmol/l).

Fig. 66 Circulating plasma LH & testosterone in daily samples taken prior to and during treatment with Nafarelin acetate (Dennis)

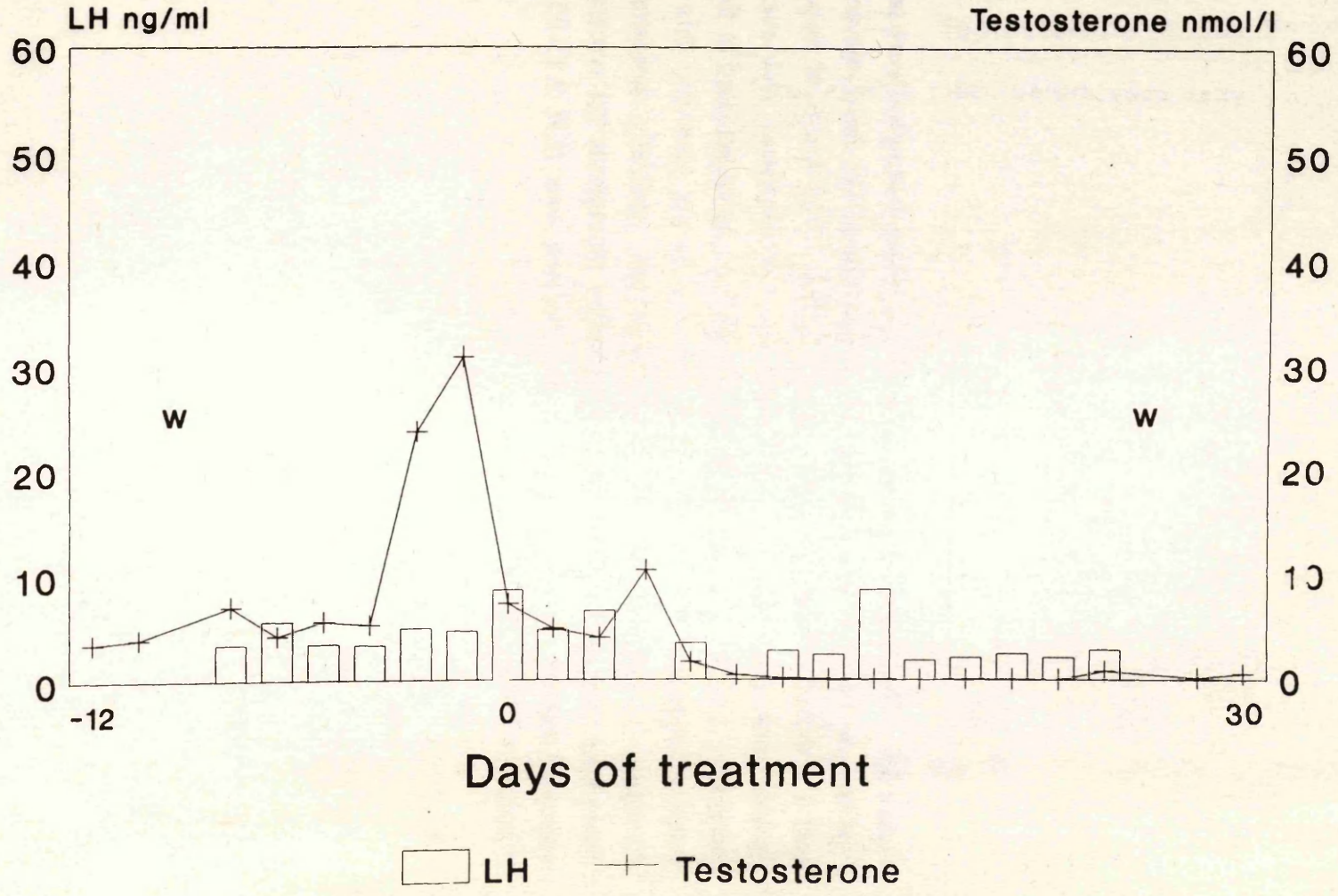


Figure 67. This figure includes only the days when 4 samples were taken. The first sample was collected prior to the application of the daily injection and the next 3 samples at hourly intervals post injection. The first 5 of these sampling periods took place daily for the first week of treatment and every 7 days thereafter. Notice the stimulatory effect that can be appreciated in the 3 hourly samples collected after the first injection, but by the next day this effect has decreased. By the fifth day both hormones, specially testosterone, have diminished considerably and this is maintained throughout the remainder of the treatment period. The range of both hormones was (LH 2.13-35.67 ng/ml; testosterone 0.06-54.07 nmol/l).

Fig. 67 Circulating plasma LH & testosterone in samples taken during treatment with Nafarelin acetate (Dennis)

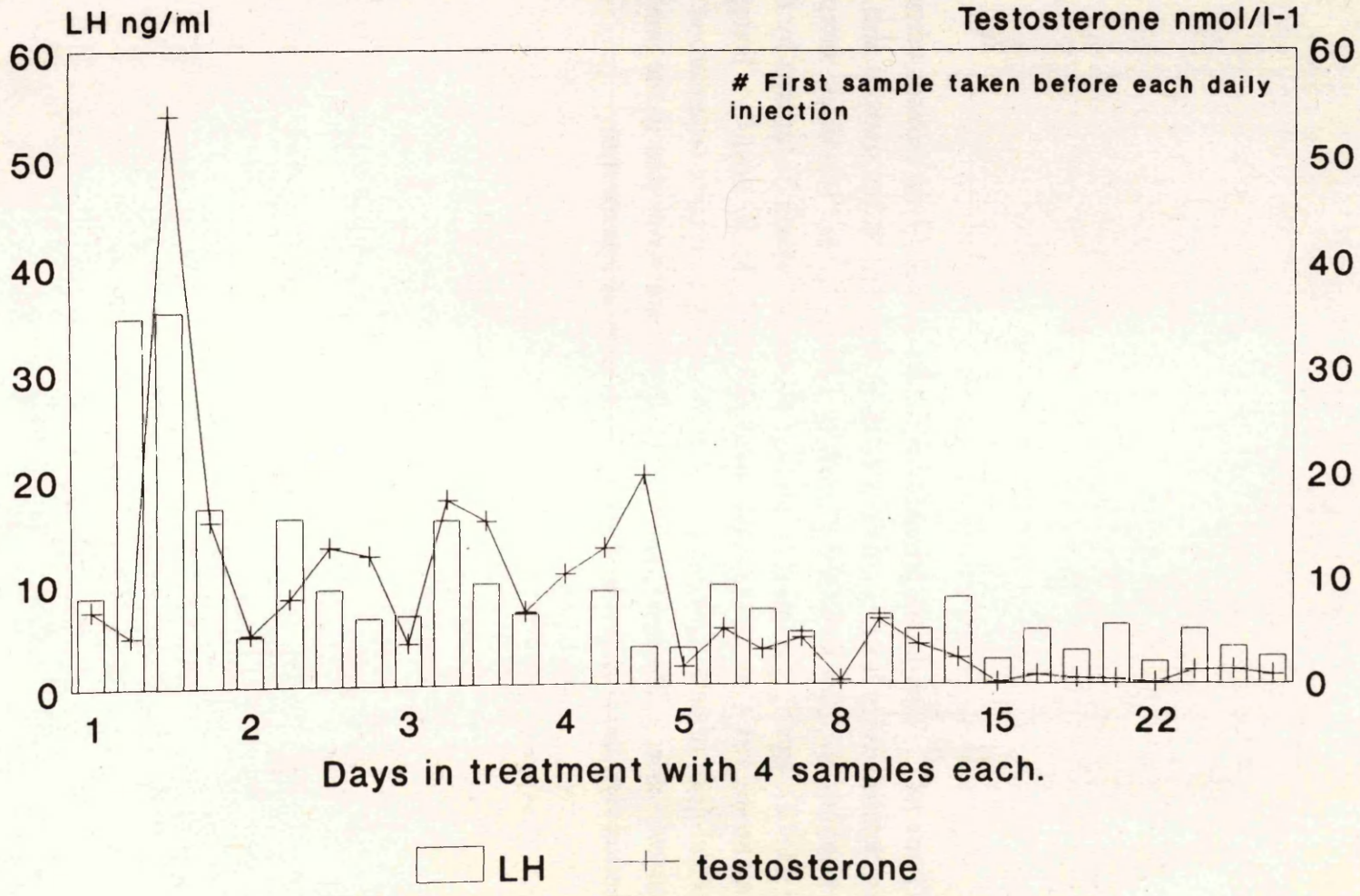


Figure 68. This figure presents the results of two 7 hour periods when samples were taken every hour. As can be appreciated both testosterone and LH show variations in their oscillatory pattern during the pre treatment sampling (LH 3.21-7.31 ng/ml; testosterone 2.03-15.55 nmol/l) which disappears by the end of treatment(LH 1.91-7.01 ng/ml; testosterone 0.3-23.56 nmol/l). Except for the initial peak of both hormones in all the remaining samples testosterone is almost undetectable. However, although LH levels are lower than in the pre treatment period they never show the marked suppression of testosterone.

Fig. 68 Circulating plasma LH & testosterone in samples taken every hour for 7 hours before and at end of treatment (Dennis)

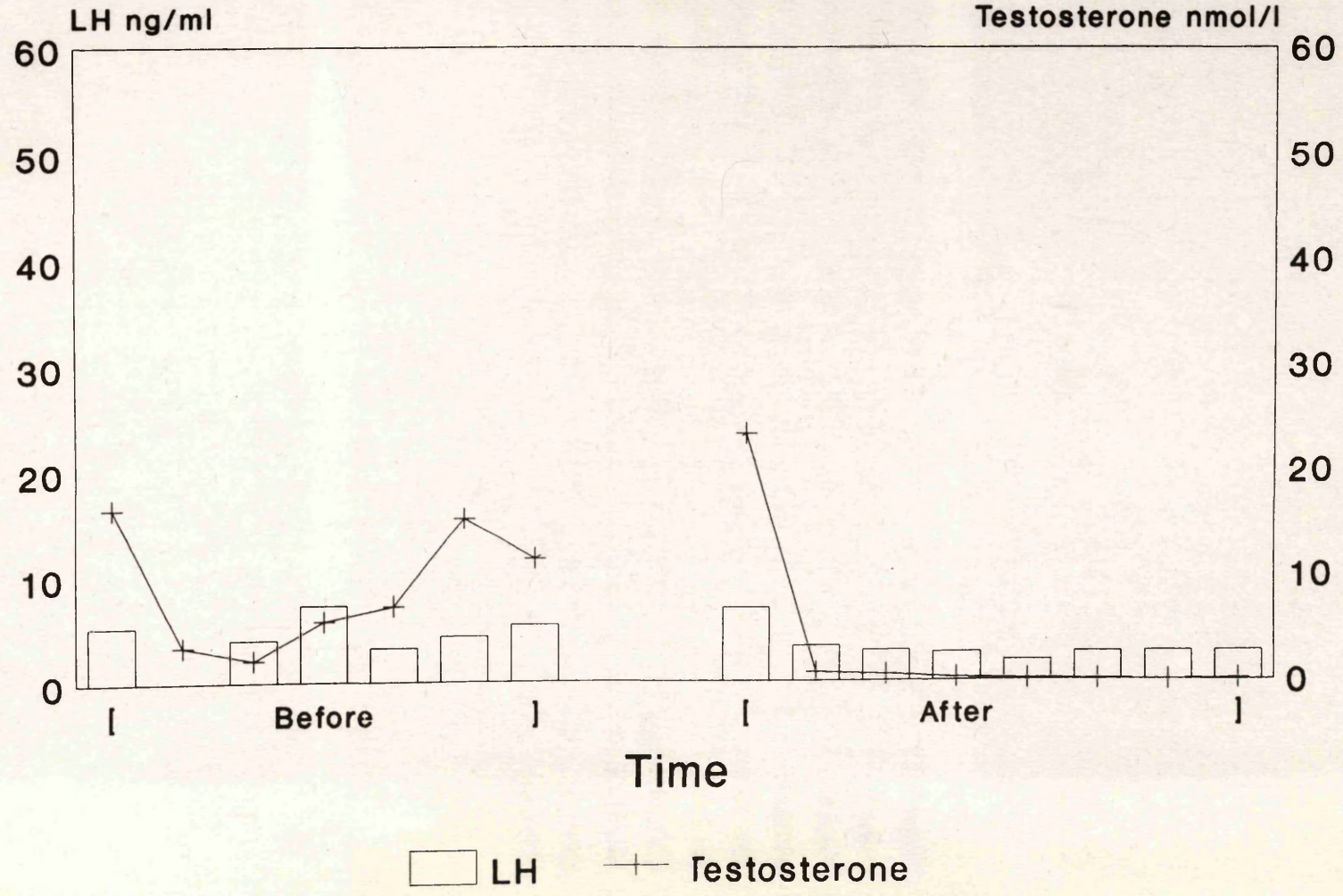
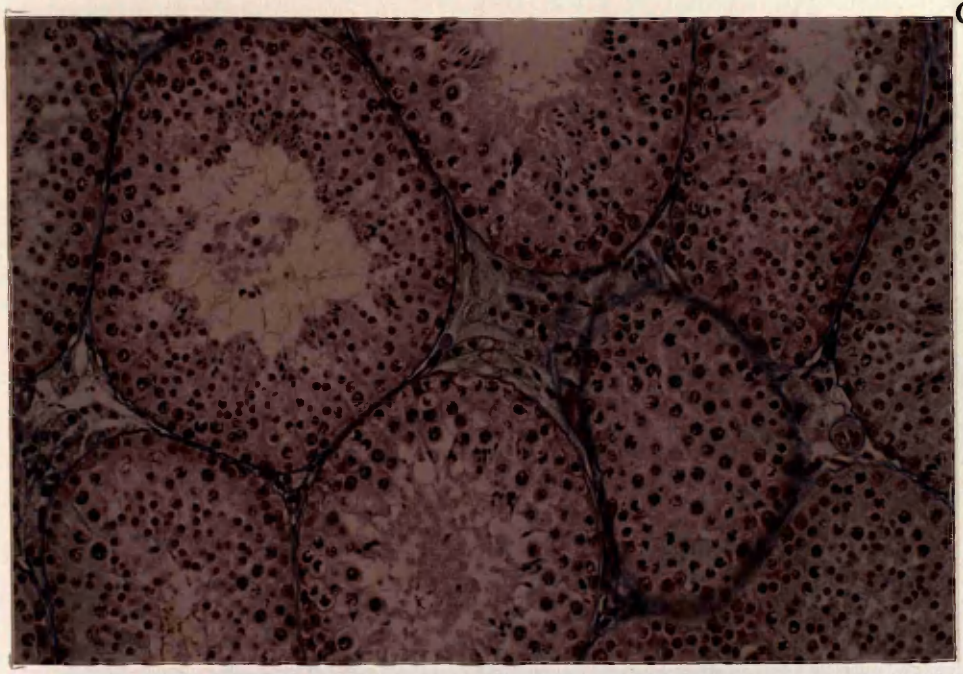
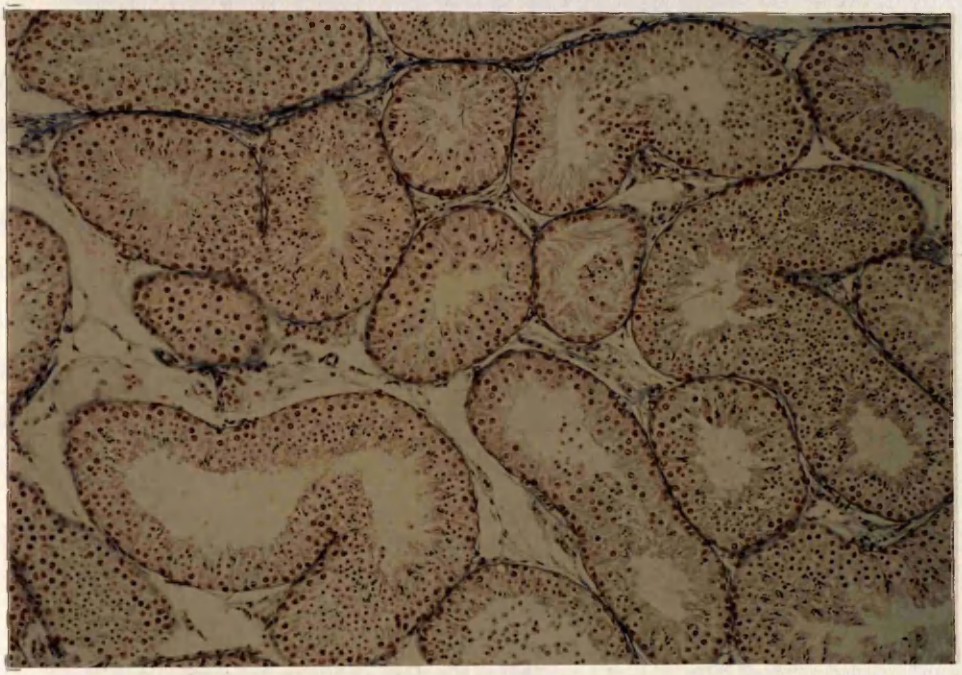


Figure 69. Histological sections of testes from a) normal dog, b) and c) male beagle (Dennis) treated with Nafarelin acetate by daily subcutaneous injections. Notice in the normal dog, the presence of a full germ cell complement which occupies the space between the basal membrane and the lumen of the tubule. The cell's nuclei in the normal dog appear almost all at the same distance from the basal membrane, whereas in the treated cells their nuclei appear to be at different levels, giving the impression of a disorganised epithelium. Magnification of (a) and (c) photographs 54 x, and (b) photograph is 10 x . The three slides were stained with MSB. A blue microscope filter was used for photograph (a).

a



b



c

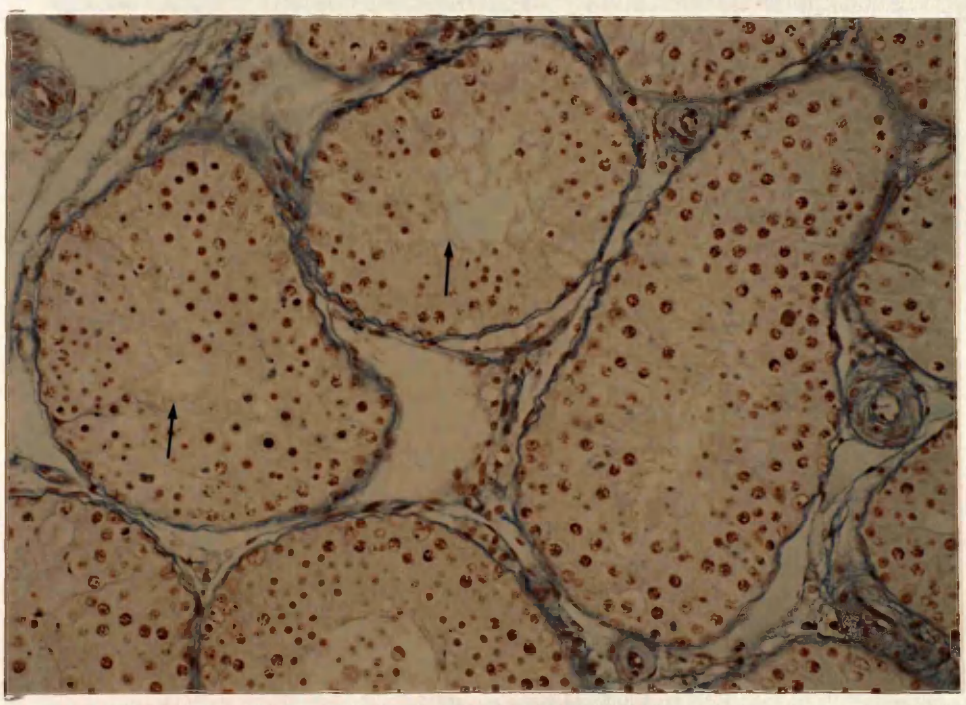
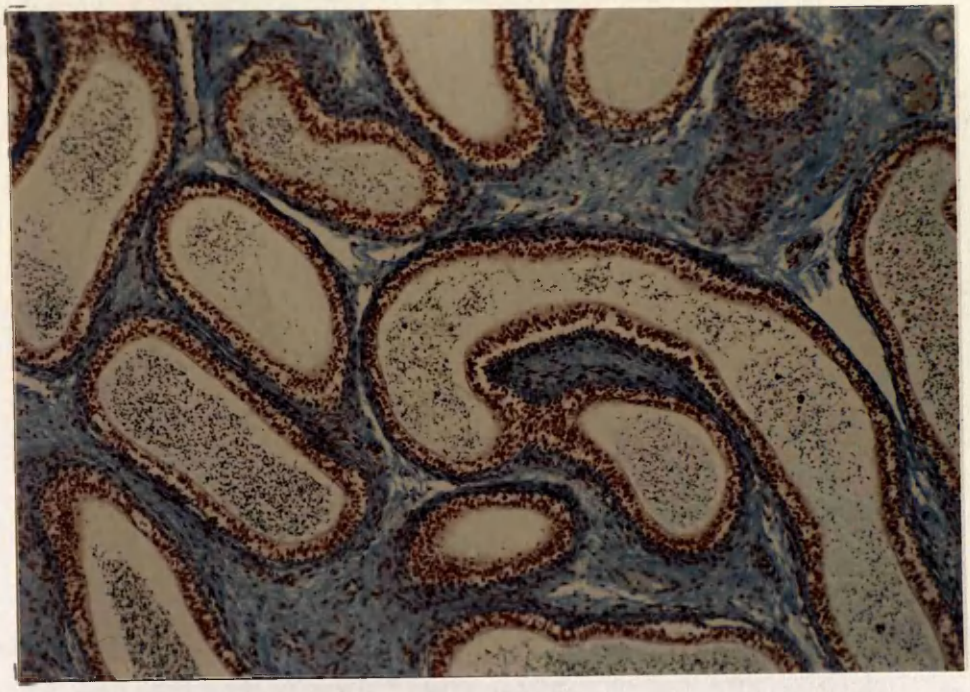


Figure 70. Histological sections of epididymal body a) from a normal dog b) from a beagle dog (Dennis). Note the uniform cellular arrangement indicated by the position of the nucleus which are almost all placed at the same distance from the basal membrane, whereas in the treated nucleus there is a somewhat disarranged appearance with the placement of the nucleus at different distances from the basal membrane. The presence of spermatozoa in this section of the epididymis is not as abundant as in the head but in the normal dog they can be seen in one of the tubules, while none are apparent in the section from the treated dog. Magnification for photograph (a) 10 x and for (b) 54 x. Both slides stained with MSB.

a



b

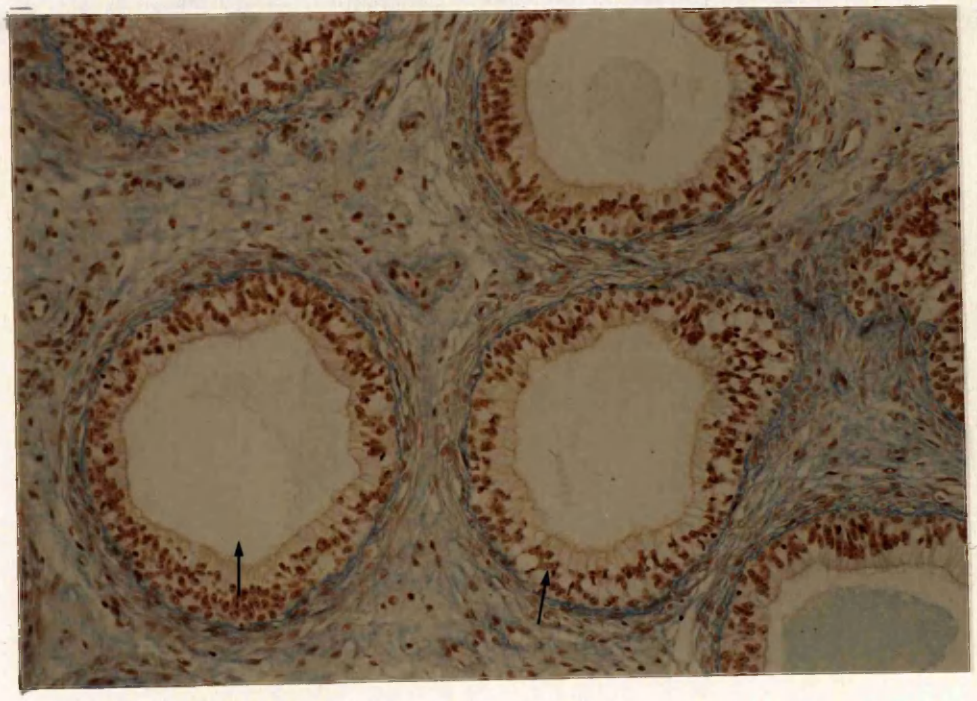
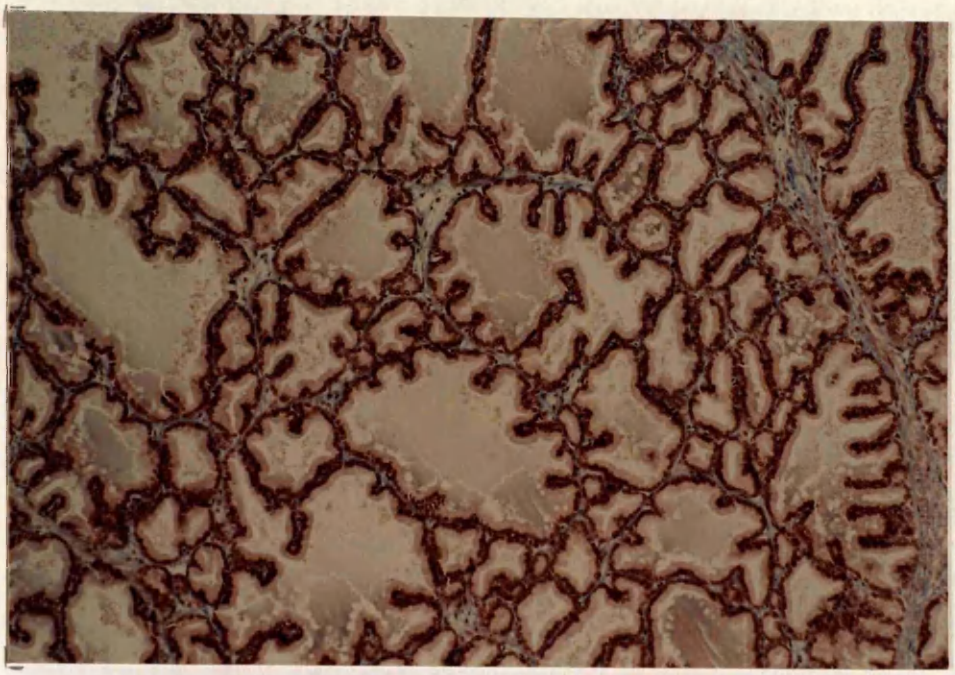


Figure 71. This figure presents histological sections taken from the prostate of a) a normal dog b) from a beagle dog (Dennis) treated with Nafarelin acetate. In the treated animal the prostatic alveoli have a shrunken appearance compared to those in the normal dog. The latter also shows secretion in the alveoli lumen that is not present in the treated animal's sections, but where an obvious increase in connective tissue can be appreciated as an indication of the gland's atrophy. Magnification of (a) photograph 10x, and of (b) is 54x . Both slides stained with MSB.

a



b

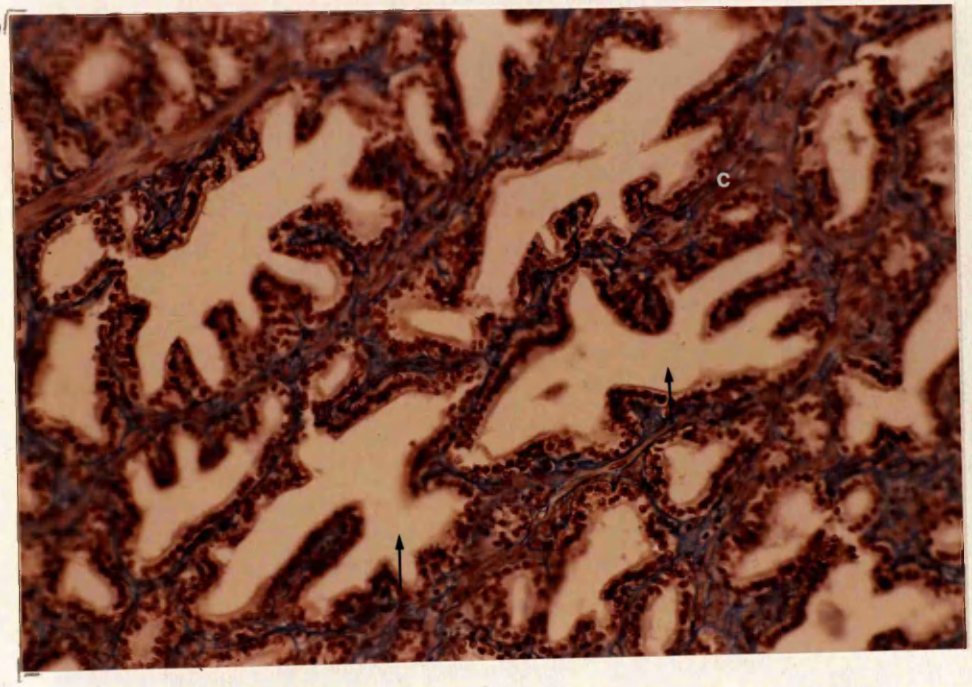
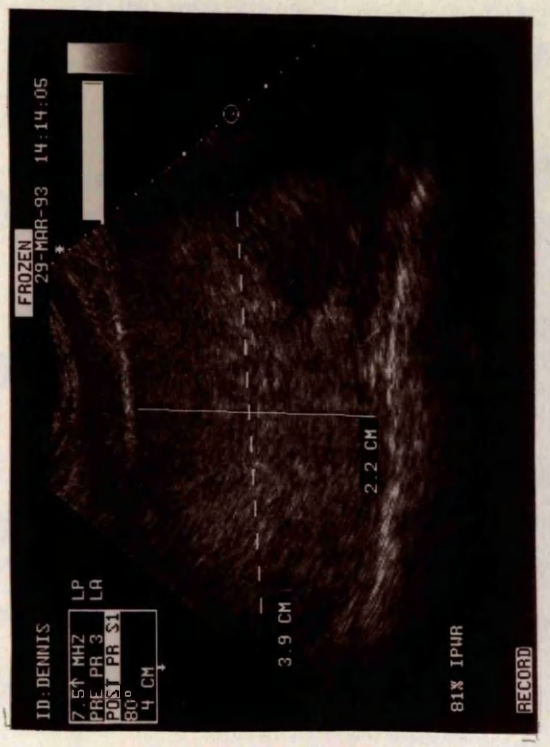
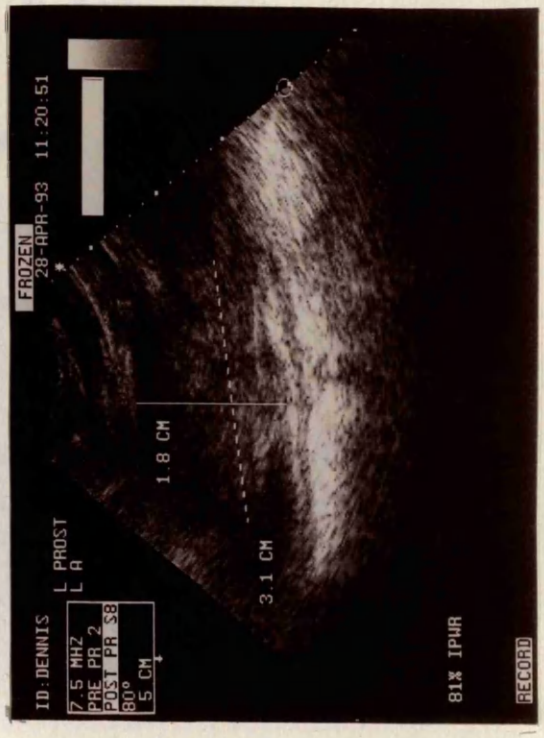
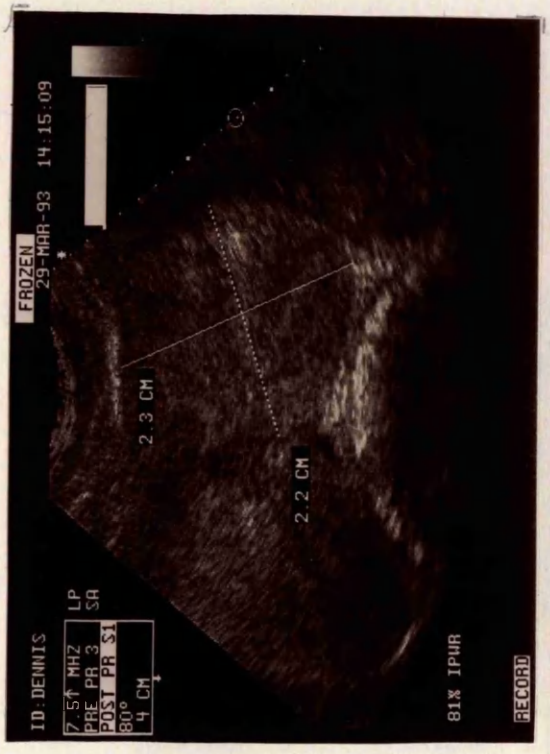


Figure 72. Ultrasonograms photos of the prostate of Dennis. This series shows the differences in sizes between the ones taken prior to treatment on March 29 (1), and those taken on April 28 (2). Longitudinal axis of left prostate A-1 and A-2 width difference of 0.4 length 0.8 cm. In the sagittal axis photos B-1 Vs B-2 , the difference in width is 0.3 cm and length 0.4 cm.


ID:DENNIS
FROZEN
29-MAR-93 14:14:05
7.5↑ MHZ
PRE PR 3
POST PR S1
80°
4 CM
LP
LA
3.9 CM
2.2 CM
81% IPWR
RECORD


ID:DENNIS
FROZEN
28-APR-93 11:20:51
7.5 MHZ
PRE PR 2
POST PR S8
80°
5 CM
L PROST
L A
1.8 CM
3.1 CM
81% IPWR
RECORD


ID:DENNIS
FROZEN
29-MAR-93 14:15:09
7.5↑ MHZ
PRE PR 3
POST PR S1
80°
4 CM
LP
SA
2.3 CM
2.2 CM
81% IPWR
RECORD

ID:DENNIS
FROZEN
28-APR-93 11:22:00
7.5 MHZ
PRE PR 2
POST PR S8
80°
5 CM
L PROST
S A
2.0 CM
1.8 CM
81% IPWR
RECORD

Experiment 1.

Figures. 73-1 and 73-2

To evaluate technical aspects of Leydig cell isolation.

Figure 73-1. Cells were isolated from 6 groups of mice (36 mice total) and cultured for 3 hours: a) with no additives; b) hCG (200 mIU ml); c) dbcAMP (2mM); d) 22 R-hydroxycholesterol.(25 μ M). The results seen in this graph indicate that the cell isolation and culture procedure produced cells capable of responding to exogenous stimulation.

Further analysis of the results using the interaction as the error term showed that there was a significance ($P<0.001$) difference between treatments.

$$C2=\frac{31.18}{3.15}=9.8=P<0.001$$

$$C1=\frac{21.03}{3.15}=0.7=P<0.01$$

Fig. 73-1 Testosterone produced by pooled mouse Leydi cells cultured for 3 hours with HCG, dbc AMP and 22ROHC (6 groups 36 mice total)

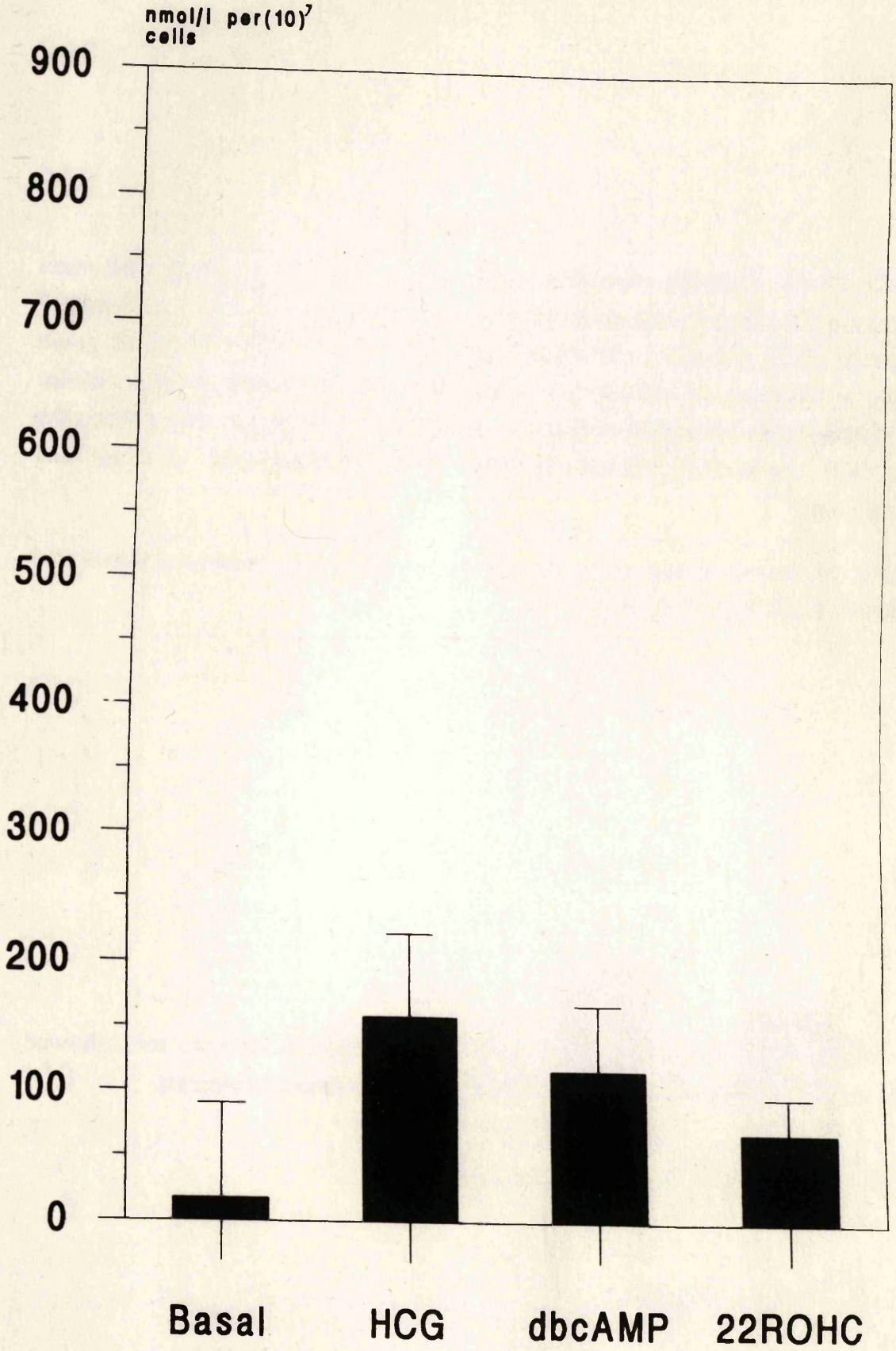


Figure 73-2. Leydig cells from control dogs (n=11). These cells were incubated for 3 hours: with a) no additives; b) hCG (200 mIU ml); c) dbcAMP (2mM); d) 22 R-hydroxycholesterol (25 μM). The results seen in this graph show an increase in testosterone production with the additives of a similar magnitude to that observed with the mouse cells. The results indicate that the procedure produced functional dog cells capable of responding to exogenous stimulation.

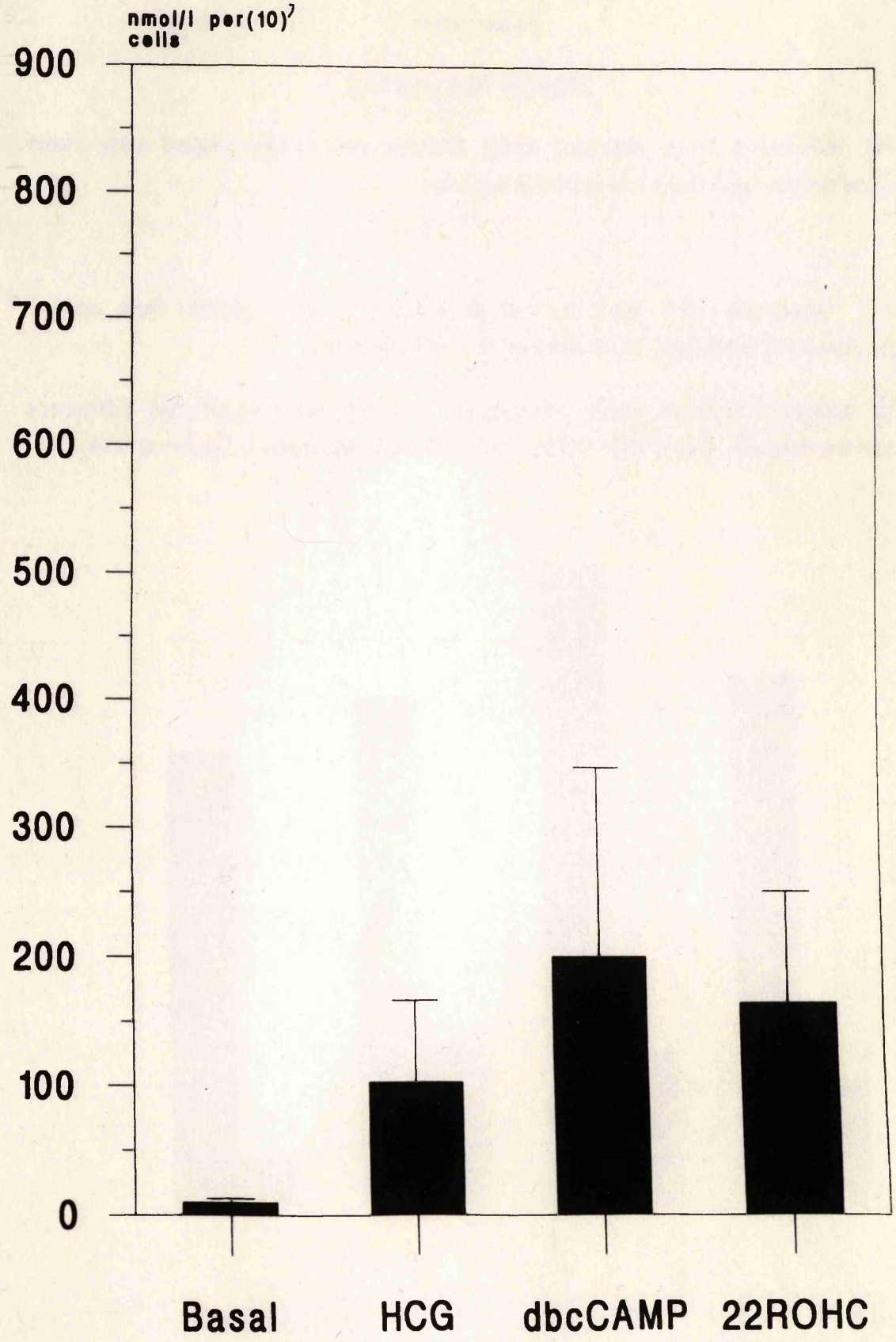
Statistical analysis shows there was significant interaction between animals and treatments (C2*C2= P<0.026)

And further analysis of the results using the interaction as the error term showed that there was a significance (P<0.001) difference between treatments.

$$C2= \frac{28.78}{1.89} = 15.2 \frac{19.91}{1.89} = 10.5 == P<0.001$$

$$C1= \frac{19.91}{1.89} = 10.5 = P<0.001$$

Fig. 73-2 Testosterone produced by dog Leydig cells cultured for 3 hours with HCG and dbcAMP, 22ROHC (11 dogs)



Experiment 2

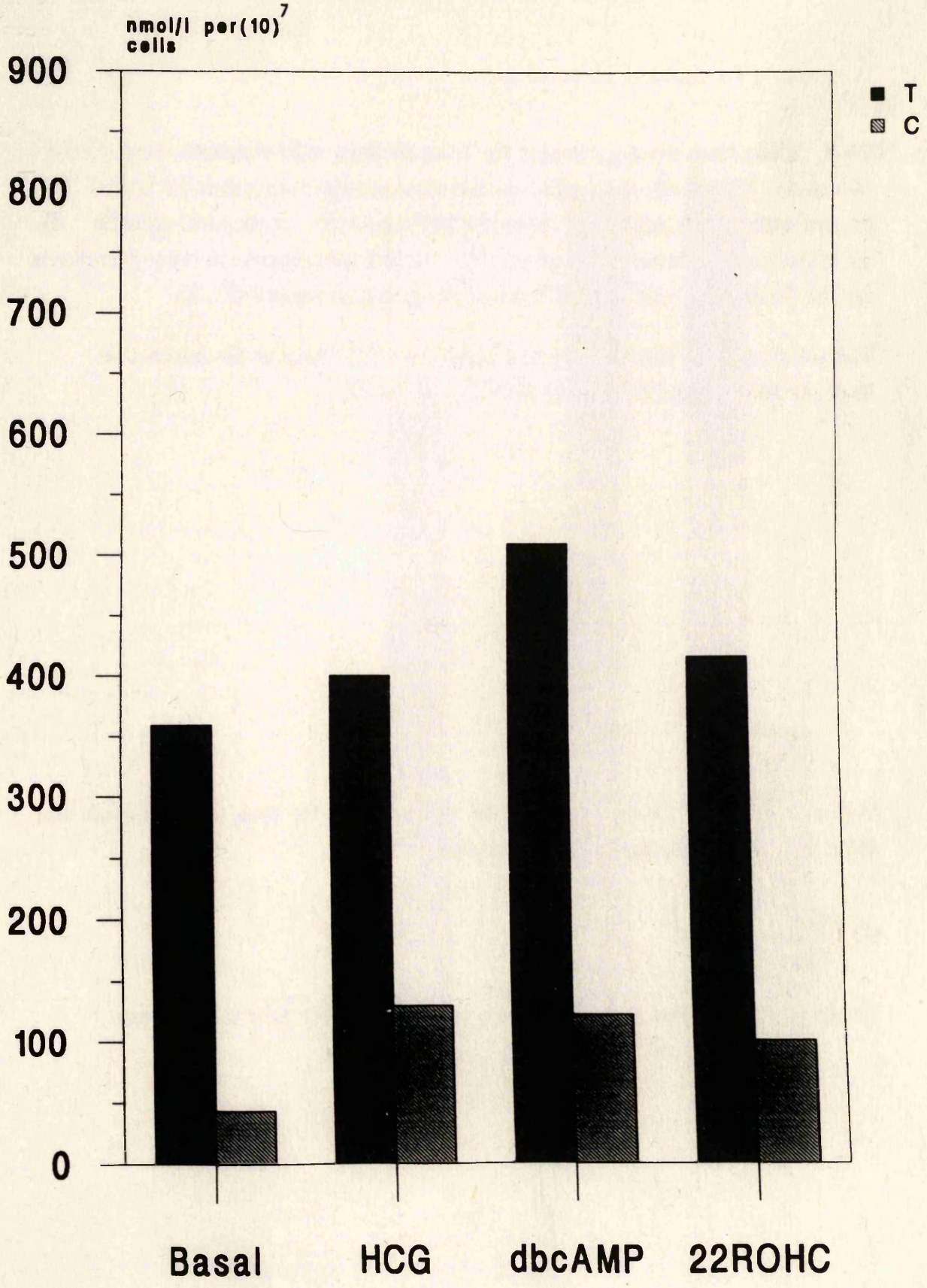
Figures 74-1 and 74-2

The cells used were obtained from animals previously treated with daily subcutaneous injections of Nafarelin acetate.

74-1. Treatment of 8 mice for 30 days resulted in a greater than control stimulation of testosterone production for all treatments.

The statistical analysis shows no interaction effect but a significant difference between animals (C1= P=<0.0001). And between treatments (C2=P=<0.039)

Fig. 74-1. Testosterone produced by Leydig cells from 8 mice treated for 30 days with daily injections of Nafarelin acetate compared with 8 control mice



74-2. Cells from the dogs treated for 30 or 60 days with Nafarelin were cultured. These cells showed an increase in testosterone production when treated with : a) no additives; b) hCG (200 mIU ml); c) dbcAMP (2mM); d) 22 R Hydroxycholesterol (25 microM). In fact the response to these stimulants for the 60 day dog was several hundred times that of control (71-2).

Statistical analysis shows there was significant difference in the interaction between animals and treatments (C2*C2= P<0.003)

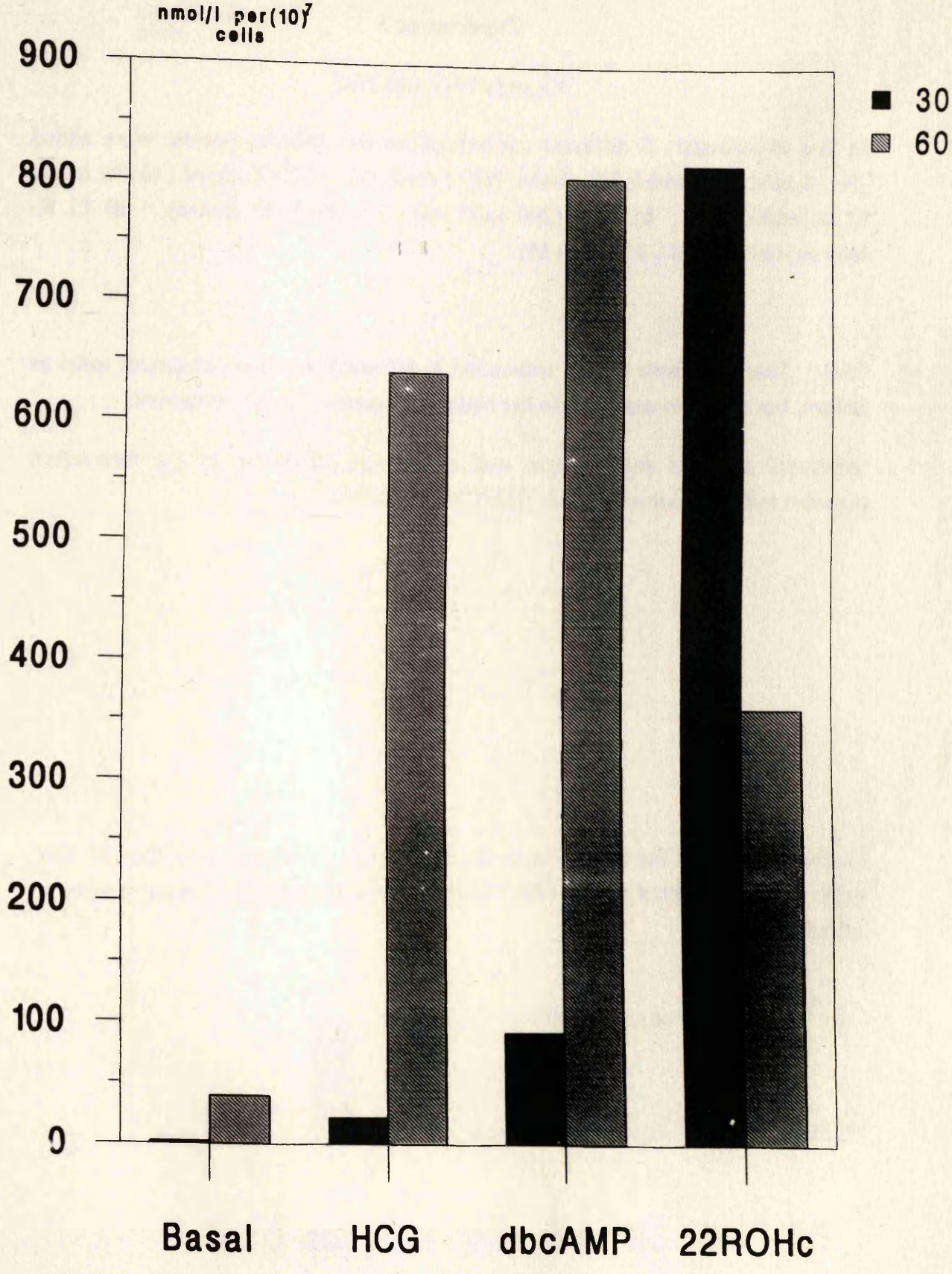
Further analysis of the results using the interaction as the error term showed that there was no significance between treatments

$$C2=\frac{52.6}{11.57}=4.54 \text{ NS}$$

The interaction shows that the animals responded differently to treatment

(P=< 0.003)

Fig. 74-2. Testosterone produced by Leydig cells from dogs treated with Nafarelin acetate for 30 and 60 days and then cultured for 3 hours with no additives, HCG, dbcAMP and 22ROHC



Experiment 3

Figures 75-1 and 75-2

In this experiment 5 different concentrations of Nafarelin acetate were added (N1, 8 mM; N2, 4mM; N3, 2mM; N4, 1 mM; N5, 0.00058 ug/ml.) to the initial a) no additives; b) hCG (200 mIU ml); c) dbcAMP (2mM); d) 22 R-hydroxycholesterol (25 micro M).

75-1. The cells from 4 dogs responded to the initial hormone treatment same as before, but show no response to the Nafarelin acetate (N1-N5) treatment.

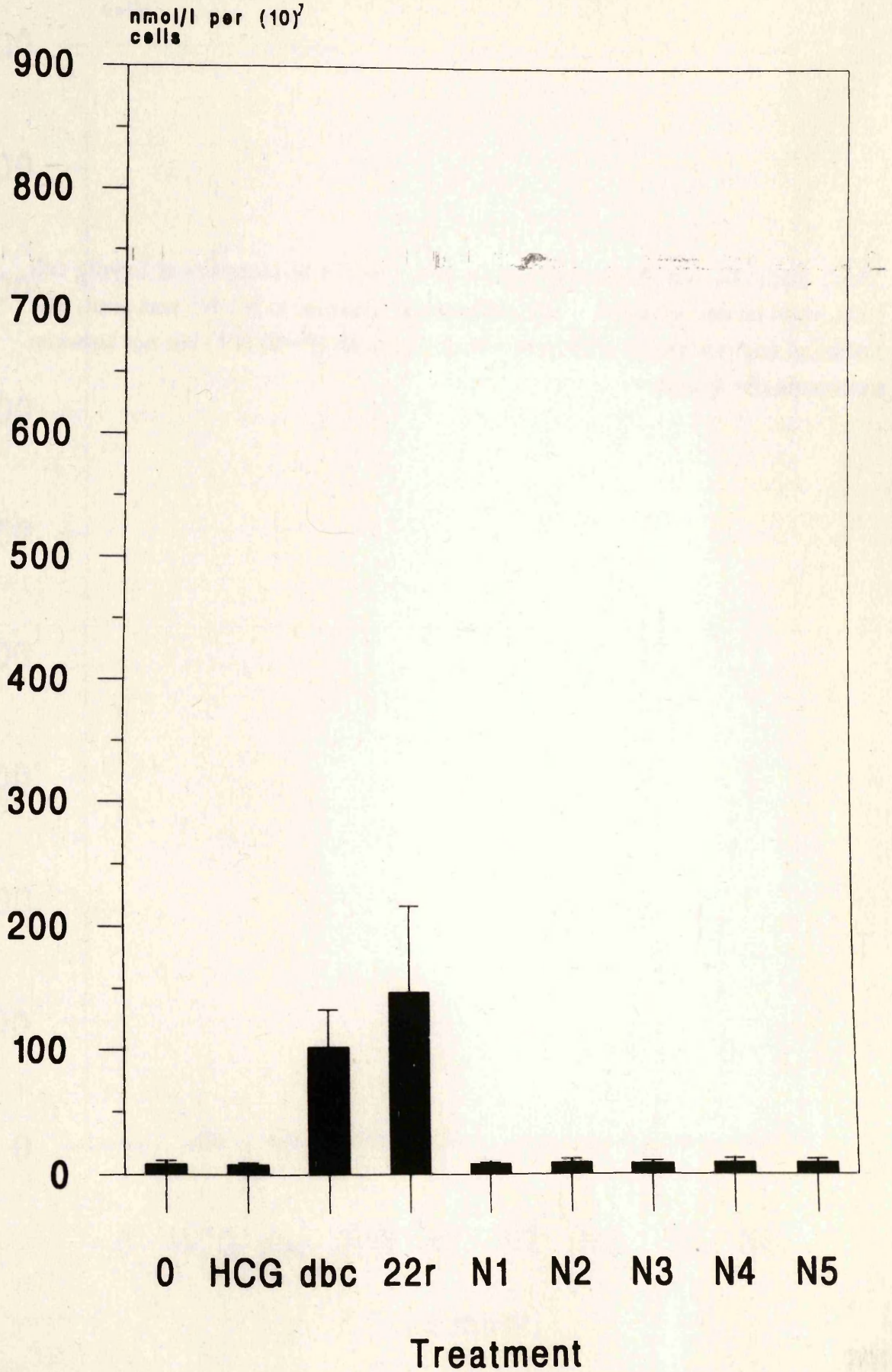
Statistical analysis shows there was significant difference in the interaction between animals and treatments (C2*C2=P<0.0001)

Further analysis of the results using the interaction as the error term showed that there was a significant (P<0.01) difference between treatments, but not between animals (P= 2.67)

$$C1=\frac{47.05}{17.6}=2.67 \text{ not significant}$$

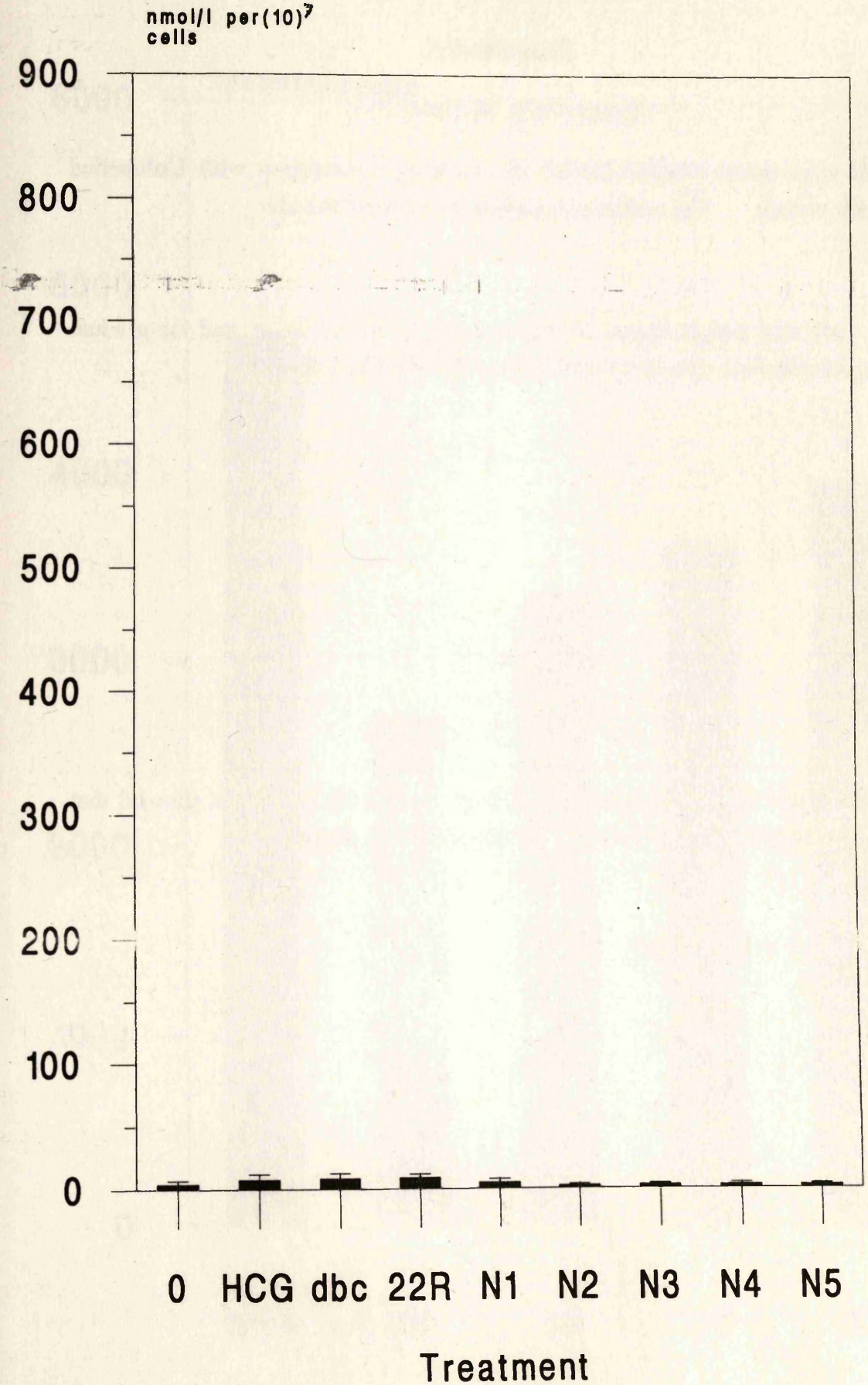
$$C2=\frac{66.5}{17.6}=3.78=P<0.01$$

Fig. 75-1 Testosterone produced by dog Leydig cells cultured for 3 hours with: No additives, HCG, dbcAMP and 22ROHC, and 5 concentrations of Nafarelin acetate (N1-N5). (4 dogs)



75-2. Rat cells, which are supposed to have GnRH-like receptors at Leydig cell level, were treated as above. No testosterone response to N1-N5 was seen. The statistical analysis shows difference between animals (P=<0.001) but not between treatments (P= 0.307).

Fig. 75-2 Testosterone produced by rat Leydig cells cultured for 3 hours with no additives, HCG, dbcAMP and 22ROHC and 5 concentrations of Nafarelin acetate (N1-N5) (9 rats)



Experiment 4

Figures 76-1, 76-2 and 76-3

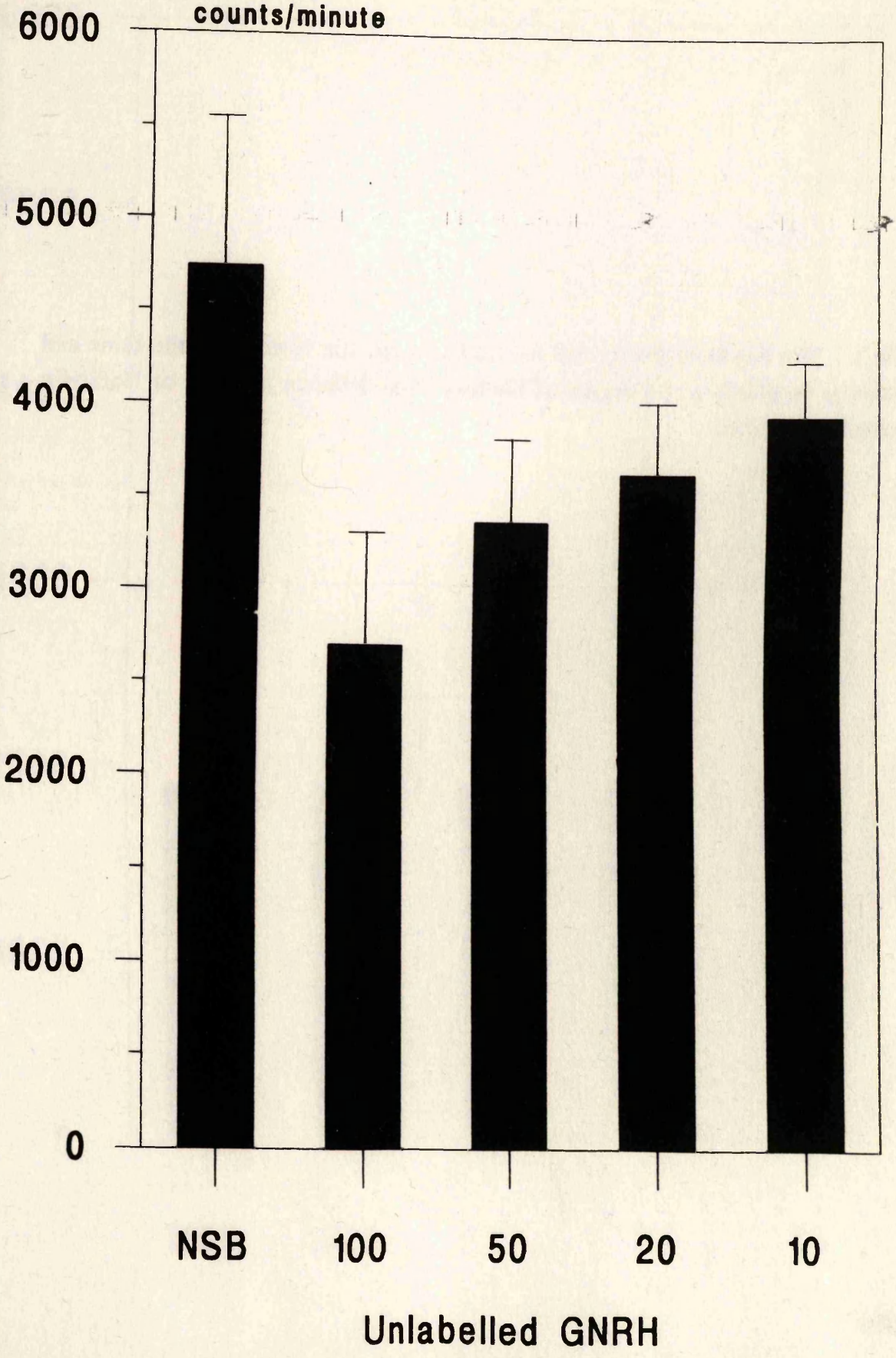
For this experiment labelled GnRH was used in combination with Unlabelled Nafarelin acetate. The results are expressed in counts/minute

76-1. Indicated a high degree of total binding, in the rat tissue, and the gradual binding interference caused by the Unlabelled Nafarelin acetate.

Further analysis of the results using the interaction as the error term showed that there was a significance ($P<0.001$) difference between treatments.

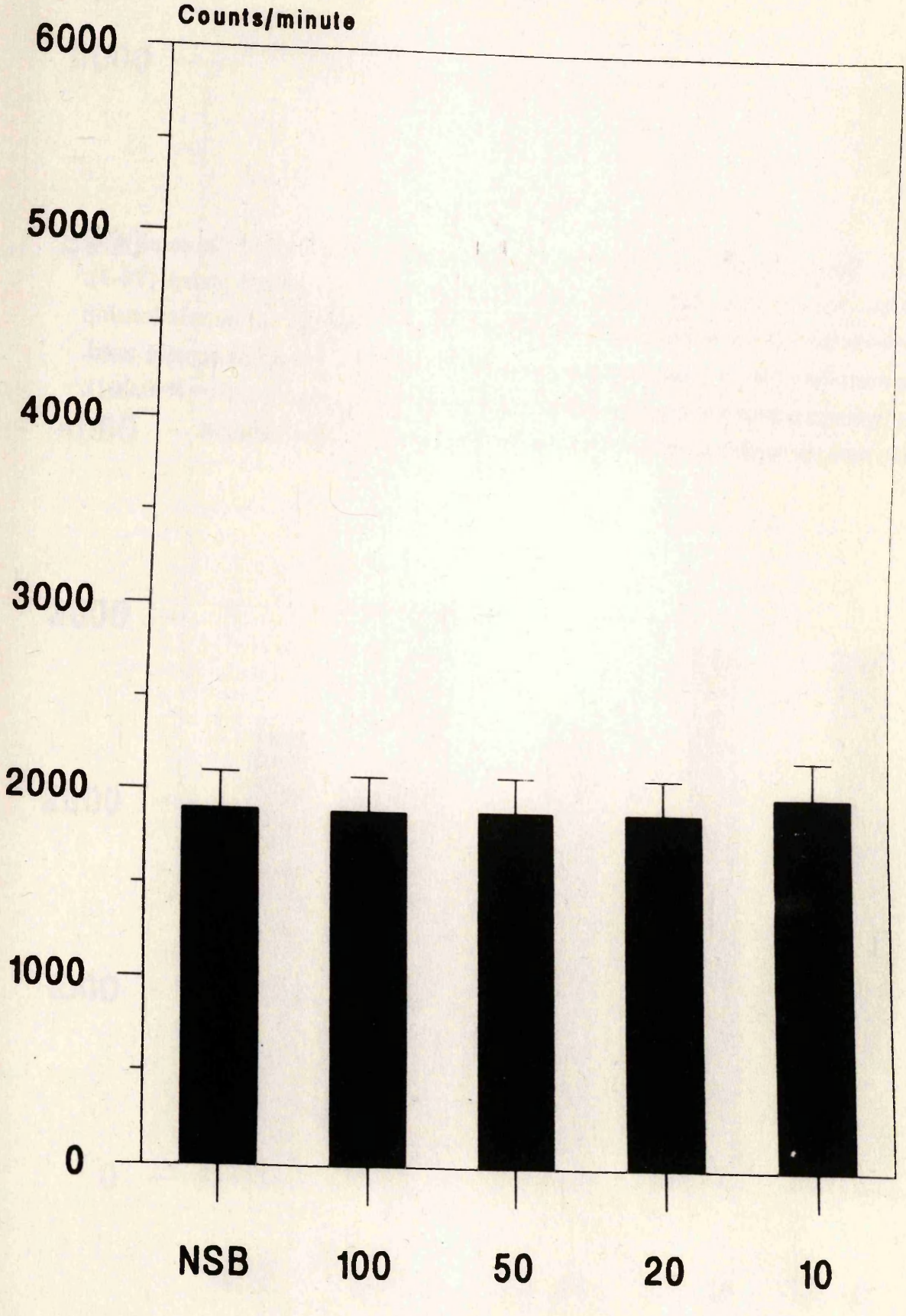
$$C2= \frac{34.12}{3.74} = 9.12 = P < 0.001$$

Fig.76-1. Rat testicular homogenated tissue, treated with labeled GNRH to determine GNRH receptors in Leydig cells (9 rats)



76-2. The dog homogenate and the total non specific binding are the same and there is no effect in the degree of binding when different amounts of Nafarelin acetate are added.

Fig. 76-2. Dog testicular homogenated tissue, treated with labeled GNRH to determine GNRH receptors in Leydig cells (11 dogs).



76-3. The testicular tissue from the two treated dogs, was treated the same way and the 30 day dog shows even lower binding than the control group (74-2). However the 60 day animal shows a higher degree of binding, but no relationship was seen between binding and the amount of unlabelled Nafarelin acetate used. The statistical analysis shows a difference between animals only (C1= $P<0.001$). There was no significant difference between animals and/or treatment.

Fig. 76-3. Dog testicular homogenated tissue from 2 dogs treated for 30 and 60 days with Nafarelin acetate Treated with labelled GNRH to determine GNRH receptors in the Leydig cells.

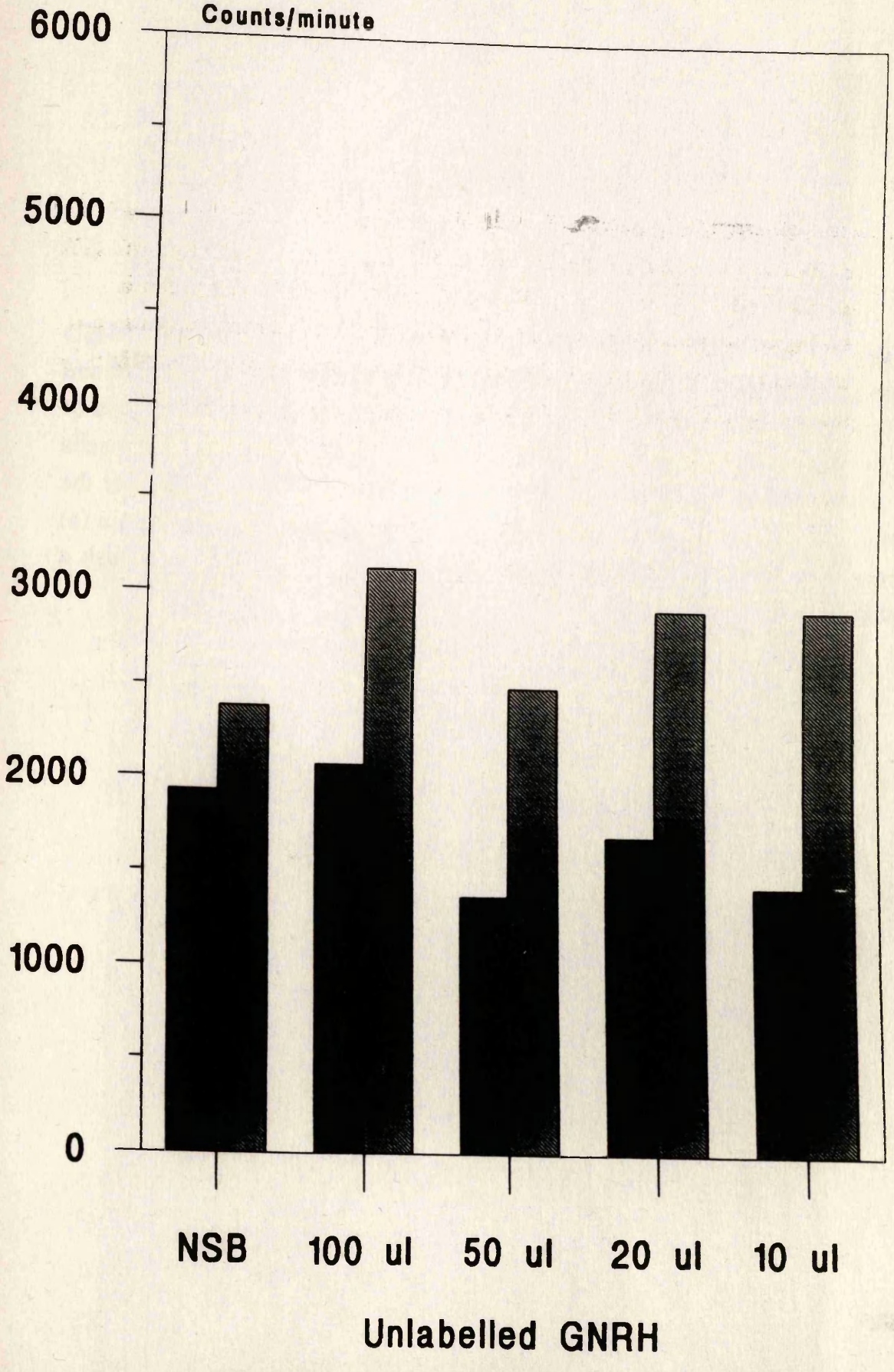
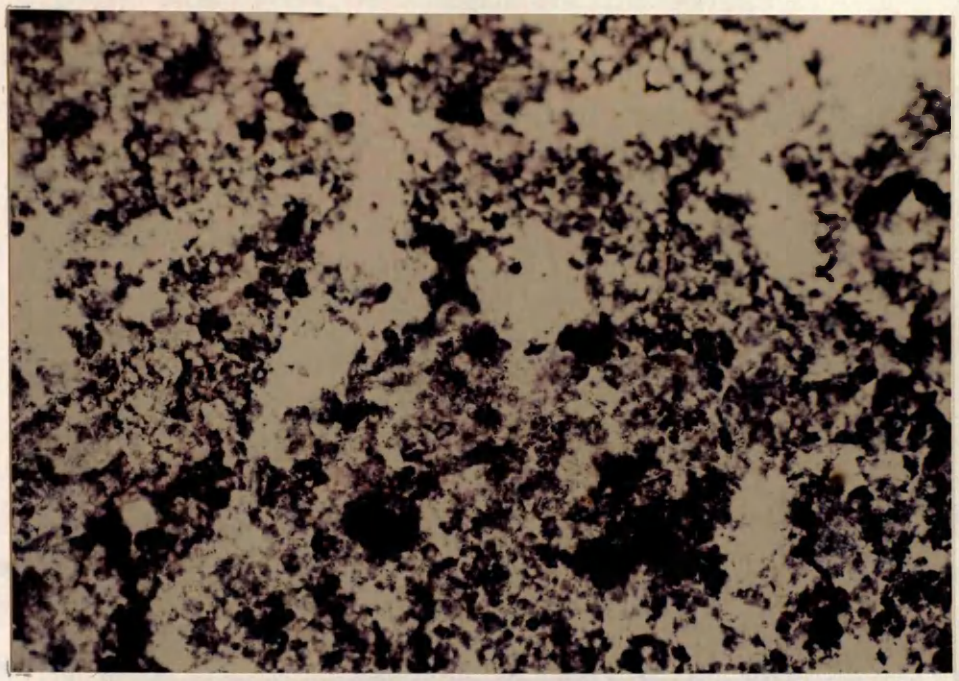
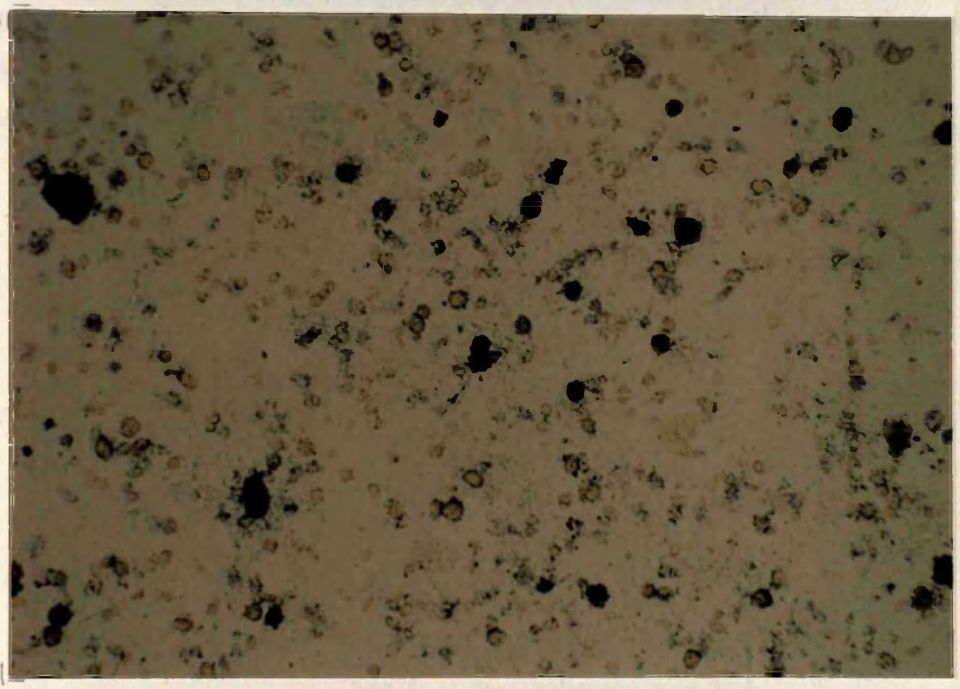


Figure 77. This figure presents two methods for the detection of the 3BHSD enzyme in isolated Leydig cells. First (a) examples of the method of Lobel and Levy (1968) which was used in the previous chapter. Secondly the method of Wang et al. (1980) (b) and (c). With the second method, only the Leydig cells stain blue whereas the remaining cells remain transparent. Note also that the cell suspension is very clean and there is hardly any tissue debris as in figure (a) where everything stains blue and purple and it is not possible to distinguish a particular type of cell.

a



b



c

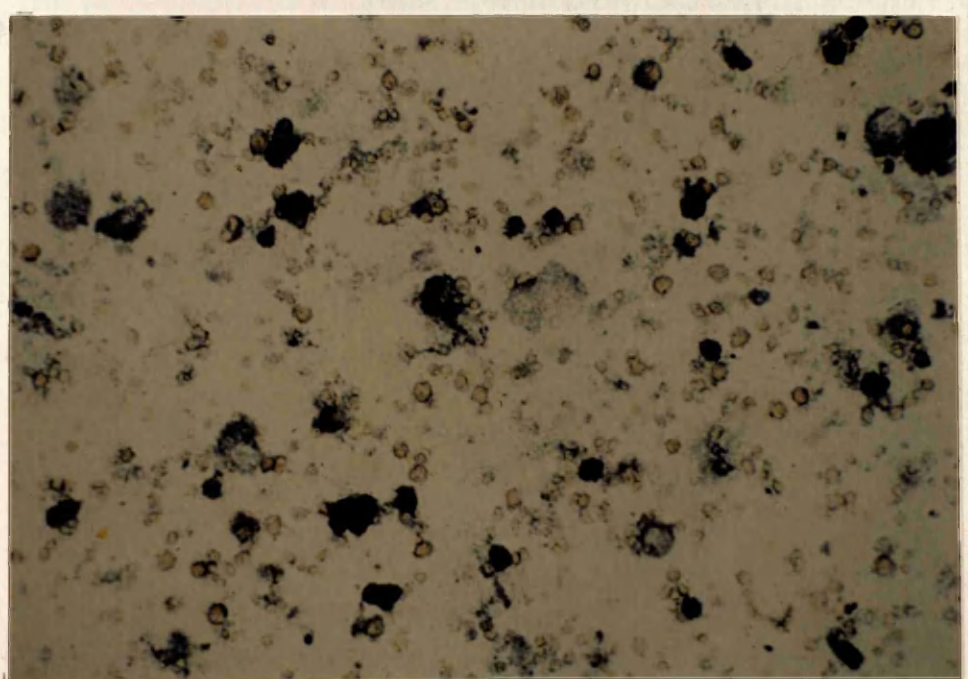


Table 23. This table presents the testosterone concentrations found in circulation and testicular vein blood plasma samples. Notice that the mean of the untreated dogs is about 5 times that of the circulation blood levels. The mean found in testicular veins is 47.13 42 ± 18.55nmol , means that the 0.3 nmol/ l levels found in both treated dogs, is well below the lowest limit of 28.58 nmol/l. And the jugular vein testosterone levels of 8.88±1.75 nmol l means that the 0.66 and 0.3 nmol/l levels found in both treated dogs is also below the lowest limit of 7.13 nmol/l

4. DISCUSSION

4. DISCUSSION

The original daily bleeding protocol that was followed in the previous dogs, namely Adam, Baxter and Dexter, was used for the two dogs used in this section, Dennis and Kenny. However, as indicated before, it was modified to allow for a closer observation of the effect of the daily injection (Tables 17, 18, 20, 21). The results of the daily bleeding showed a decline in both hormones by days 4 and 5 in both Kenny and Dennis (Figures 59 and 66) respectively . This was similar to that in Adam's, which was discussed in chapter 2-B and which agree with Vickery *et al.* (1985) who found depressed hormone levels also by the 5th day.

However, as a result of the modifications to the protocol it was possible to observe the hormone increase one hour post injection in both animals, an increase that was maintained for the next three hours (Figures 60 and 67). As no further samples were taken it must be assumed that the effect of the injection is of at least 3 hours. This same type of response although more subdued continued throughout the treatment period.

An interesting observation is again the difference in individual hormone levels, a finding which has been observed all throughout the experiments beginning with the MPA treated dogs and which was seen again in Kenny (Figure 60) who has the lowest hormone profile with levels in the range of 0.0-25.3 ng/ml of LH and 0.0-33.06 nmol/l of testosterone. In contrast, Dennis (Figure 67) had the highest plasma concentrations of both hormones 2.13-35.67 ng/ml of LH and 0.06-54.07 nmol/l of testosterone. The difference in normal pre-treatment levels can also be appreciated in the two window bleedings taken prior to treatment (Figures 61 and 68) where again Dennis showed a higher hormone profile than Kenny. However, in the window taken at the end of treatment, the suppression effect is almost the same in both dogs.

A general observation regarding the hormone levels in all the dogs treated with either Nafarelin derivative or Nafarelin acetate, is that LH does not show the degree of suppression as testosterone. In several instances, the levels are almost the same pre treatment as at the end. It might be that the daily injection effect allows LH to recover to almost normal levels which does not occur in the case of

testosterone, which may be affected by just a temporary alteration to the LH stimulus to the Leydig cell.

The histological examination of the testes of both dogs, clearly shows the time-related effect of the treatment. Kenny who was treated longer (60 days), showed seminiferous tubules depleted of most germ cells with only Sertoli cells and those spermatogonia found closer to the basal membrane being seen (Figure 62). In Dennis, who was only treated for 30 days, there appears to be a gradual disappearance of cells (Figure 69) in that only one or two tubules show the same severe germ cell depletion seen in Kenny.

The examination of the body of the epididymis in both dogs (Figures 63 and 70) did not present the vacuoles that had been found in other dogs. The only change appeared to be the absence of spermatozoa from the lumen of the tubules, otherwise the epithelium was apparently normal.

The prostate sections (Figures 64 and 71) show an increase in connective tissue around the alveoli which is more apparent in Kenny where the alveoli look smaller than in Dennis, again probably as an effect of the more prolonged treatment period. The decrease in the gland's size can also be appreciated in the ultrasonographs photos taken of both animals, before and at the end of treatment (Figures 65 and 72). This particular effect on the prostate has already been discussed and the same arguments would apply here.

The testosterone concentrations from jugular and testicular vein blood were evaluated in these two animals and a larger number of control dogs than shown in Table 15 was used. The testicular blood showed a five fold increase over the jugular blood concentrations. In contrast in the treated animals the same values were obtained in both jugular and testicular blood in Kenny and in Dennis, the jugular blood values are slightly higher. These differences could very well reflect again the time related effect of the treatment which here shows the effect directly in the testis. This find has been discussed before in chapter 2-B.

The testicular tissue from both dogs was processed as described in the previous chapter in order to isolate the Leydig cells. The only change introduced in the process was that the identification of Leydig cells was carried out using the presence of 3βHSD by the method of Payne *et al.* (1980). This proved to be a very practical method as it did not take long to prepare and it finally produced positive results (Figure 77). Although correlations were made between the

fresh and the 3βHSD stained cells, the work so far had depended on counting fresh preparations of cells and identification of the Leydig cells continued to be made mainly by the presence of the yellow halo as described before.

The approach used to treat these Leydig cells, which had been isolated from tissue in which germ cells had been either partially or totally eliminated, was similar to that used by Abayasekara *et al.* (1990) in the rat and O'Shaughnessy *et al.* (1991) in sex reversed mice. In these studies germ cells were also absent and it seemed an appropriate treatment to use in this study. The Leydig cells capacity for androgen synthesis was evaluated by stimulating the cell at different levels with hCG, dibutyryl cyclic AMP and 22-R-hydroxycholesterol.

Therefore, the same method was tested first in mouse cells to compare to O'Shaughnessy's results and develop a working routine that would evaluate the technical aspects of Leydig cell isolation and culture. These initial results of experiment 1 (Figures 73-1 and 73-2) proved that the cell preparation techniques that were being used in the present work did indeed produce viable cells capable of responding to exogenous stimulation. However, when compared to O'Shaughnessy's work, the present results in the mouse experiment (Figure 73-1) showed a 5 fold increase in the amount of testosterone produced by the mouse cells. This difference could be explained by the fact that in the present work the mice which were available were not always the same strain. There seems to be some evidence to the effect that there is a strain-related difference in testosterone production (Stalvey and Payne, 1983). Again comparing the present results, the basal, the hCG and dbcAMP stimulated cell responses are similar. Except in those cells where 22 ROHC was used, which show lower levels of testosterone in comparison to what O'Shaughnessy reported in his experiments.

Experiment 1 using dog cells (Figure 73-2) produced the lowest response at membrane level with the hCG stimulation. The highest effect was at postmembrane level with the other two additives, dbc AMP and 22ROHC. As mentioned in the previous chapter, dog tissue required a longer collagenase dispersion phase, which could well have caused damage at membrane level especially damaging LH-hCG receptors which might have interfered with the cell's capacity of response. A similar occurrence appears to take place in isolated human Leydig cells, where a tenfold decrease in the sensitivity of these cells in comparison to rat cells has been observed (Sharpe, 1990). It appears, However, that the cells capacity to respond to the postmembrane treatments were not affected by the membrane damage and that both dbcAMP and 22 ROH were able

to produce the desired stimulation at 2nd messenger and mitochondrial level respectively.

In the next study, experiment 2, the Leydig cells were treated with the same additives as above, with the difference that these cells were from animals that had been previously treated *in vivo* with the same Nafarelin acetate dose. Once again mice cells were used and it was most interesting to see in this experiment the clear stimulatory effect that the treatment had in these animals (Figure 74-1). In the treated mice the response was several hundred times higher than the control group which responded in a similar way to that seen in the first experiment (Figure 73-2). It is reported that in mice the inhibitory effect only occurs when high doses of the agonist are used. Bex *et al.* (1982) used 100 ug/mouse/day for 14 days which had no inhibitory effect on testicular weight and this dose is 7500 times higher than that required in rats to reduce reproductive organ weight. A more recent report indicated that very high doses, 1000 ug/kg of Buserelin, were required to produce the decrease in testis weight and sperm production supression (Kher and Kalla, 1993).

The dog cells (Figure 74-2), however, behaved very differently from the mice cells, but they also show completely opposite results between the 30 and 60 day treatment than what was expected, as the results in the 30 day treated dog (Dennis) show lower basal, hCG and dbcAMP responses than experiment 1 (Figure 73-2). As far as the 22ROHC response is concerned it was several hundred times higher. It was expected at the time that an animal that had been treated for twice as long as Dennis would have an even more depressed response. This was not so in Kenny's case as all the cells responded with an increased production of testosterone, except for the cells treated with 22ROHC where the lowest stimulatory response was produced.

At this point, one could speculate on the reasons for this contradictory finding. The basal response was low because the *in vivo* treatment had inhibited the cells normal testosterone production and as they were incubated with no additives at all there was no stimulation. In Dennis, the *in vivo* GnRH agonist treatment would have increased the production of LH in the first days, therefore occupying all available LH membrane receptors, so that his Leydig cells would have been refractory to the effects of hCG in the culture. This same effect might have also affected the 2nd messenger level response which the dbcAMP additive was supposed to activate. Nevertheless the cells were able to respond to the 22ROHC stimulus because they did not go through the membrane but directly to

the mitochondria and therefore the membrane interference would not affect the results of this treatment.

In Kenny the results give the impression of cells, which once released from the inhibition, being able to respond with more than twice the amount of testosterone production to each of the additives as if it had been holding back reserves. There is evidence that in some cases Leydig cells accumulate progesterone when exogenous oestrogens are administered which produce a lesion in the steroidogenic pathway in the 17-20 desmolase level which does not allow progesterone to undergo further transformations (Risbridger, *et al.* 1981; Risbridger, 1990). However, the additives used in the present work produce the stimulation way before this particular step in the steroidogenic pathway occurs, so that this explanation would not apply in this particular case. But this does not discard the possibility that something similar might have occurred in Kenny's cells.

Another interesting finding is that when comparing the testosterone produced by the cell cultures to the circulating levels in the live animal, the highest testosterone cell production came from Kenny who had the lowest circulating blood levels initially. On the other hand, Dennis who had the highest circulation hormone profile, showed the lowest cell stimulation.

This difference between *in vivo* and *in vitro* testosterone responses might be better explained by an observation made in the gonads of both animals, which was that not only the size was reduced (Tables 19 and 22) but during *post-mortem* examination they were paler both outside and inside compared with, the normal testis. There was very little bleeding when they were removed from the dog and what appeared as a reduction in the size of the blood vessels became apparent when collecting the blood samples from the surface testicular blood vessels, as it proved more difficult than in the untreated dogs. This was probably due to a reduction in blood flow to the smaller testis. Similar information has been provided by Damber *et al.* (1985) in unilaterally cryptorchid adult rats stimulated with hCG, which shows that less secretion of testosterone is produced by the smaller abdominal testis. However, after hCG treatment it had increased therefore proving that it was the reduction of blood flow to the testis and not a disability of the Leydig cells to produce less testosterone suggesting also that LH-hCG control the blood flow to the testis. Similar information arises from an experiment with irradiated rat testes which caused a disruption in spermatogenesis, where again they found that the capacity of the testes was limited by a decrease in testicular blood flow, but not by the

ability of the Leydig cells to produce testosterone (Wang *et al.* 1983. Setchell, 1990a, 1990b). This explanation would explain the ability of the cells in the present work to respond to additives in cell culture, but it would still leave open the question about the difference between dogs.

The results from these two dogs also suggest that the Leydig cell's capacity to produce testosterone might not necessarily be reflected in the actual testosterone levels found in circulating plasma. As they have more LH-hCG receptors than they need to maintain the continuous production of testosterone, referred to as "spare receptors" by Catt and Dufau (1973). This could help explain Kenny's increased cell testosterone production suggesting that perhaps his Leydig cells had more receptors than Dennis's, but that the full capacity of his cells was not reflected in his circulating plasma levels of testosterone, because they were not required for the dog to function normally. This experiment does indicate that once the cells were removed from an LH deprived environment and placed in the stimulating cell culture environment, they recovered their testosterone production capabilities, which suggests that the absence of LH did not apparently cause irreversible changes.

One aspect of the Nafarelin acetate treatment that required further investigation was to determine if it had produced the suppression of LH and testosterone by acting at pituitary level alone, or if it had been through the previously mentioned gonadal GnRH-like receptors as is the case in the rat.

Therefore in experiment 3, the cells were not only incubated with the usual additives, but with different concentrations of Nafarelin acetate. The results in the dog cells (Figure 75-1) were similar to that of experiment 1 in the control animals with the original additives, but there were no indications of stimulation to the cells of the rat (Figure 75-2). There were no indications of any type of stimulation to the Nafarelin acetate additive in either group. The lack of stimulation in the rat cells was surprising in the light of information provided by Browning *et al.* (1983) in their studies with the GnRH agonist HOE 766, where they stimulated rat Leydig cells with even smaller concentrations than those used in the present work. It is not clear why the rat cells were not stimulated either by the initial additives. However, since the main interest in the present work was to study the dog, no further attempts to study this mode of treatment in the rat was tried.

As the direct treatment with Nafarelin acetate had not proved successful, another approach was attempted which became experiment 4, using homogenated

testicular tissue from dogs and rats and measuring the displacement binding of ^{125}I GnRH as no ^{125}I Nafarelin acetate was available, a similar method has been used for pituitary GnRH detection (Clayton *et al.* 1979. The results of this experiment in rat tissue (Figure 76-1) shows a gradual dose related interference of the Nafarelin acetate binding. This part of the experiment indicated that the treatment procedure was indeed working in detecting the rat's GnRH-like receptors mentioned by others (Hsueh and Erickson, 1979; Hsueh and Jones 1981; Sharpe and Fraser, 1980; Perrin *et al.* 1980. Bourne *et al.* 1980. Bourne *et al.* 1982. Bambino *et al.* 1980. Huhtaniemi, *et al* 1986). It also showed that it would be an effective way of assessing the presence of such receptors in the dog Leydig cells.

However, this experiment in 11 control dogs (Figure 76-2) show no evidence of an interference by the Nafarelin acetate with the ^{125}I GnRH, as all the counts are below 2000 per minute. When the homogenates from the two *in vivo* treated dogs, Dennis and Kenny (Figure 76-3) were treated in the same manner, there was a statistically significant difference between dogs, but no relationship between binding and the amount of unlabelled Nafarelin acetate used.

These last results suggest that with the methods used in the present work it was not possible to demonstrate the presence of GnRH-like receptors in dog Leydig cells.

In view of the extremely complicated interactions present in the testes it would be most unwise to believe that the results from only two treated dogs can be conclusive, but it points the need to attempt to answer some of the many questions that have arisen so far, by repeating the experiments with a larger population of *in vivo* treated animals.

CHAPTER 5

GENERAL DISCUSSION

This work was undertaken against the background of a need for a reliable means of chemical castration of the dog. The experimental approach selected was to attempt suppression of LH and testosterone production and monitor the effect of this interference on the testes. A degree of suppression was achieved using a GnRH agonist. Subsequent studies focussed on determining the mechanism of action of the agonist at the level of the Leydig cell.

Treatment with Medroxyprogesterone acetate failed to produce the desired suppression of LH or testosterone but produced a very interesting response in the epididymis. Spermatozoa recovered from the area showed severe acrosomal damage which would be likely to interfere with fertilisation. This offers the possibility of using Medroxyprogesterone acetate as a contraceptive in the male dog. Moreover, the effect occured after just one injection and one month of treatment. Further investigation is warranted to determine whether sperm damage occured through the direct action of the progestogen on the spermatozoa or as result of a change in the epididymal environment. It would be necessary to ascertain how quickly these changes occur and whether the changes are reversible. It may be possible to find a better means of drug administration than subcutaneous injection, such as an implant or slow-release device releasing a known amount of progestagen. An electron microscopy study of the epididymis and of the spermatozoa located in this area might contribute to a better understanding of the cause of sperm damage.

The results of the GnRH agonists studies showed that daily subcutaneous injections did not totally suppress LH release into the circulation. It is evident that LH always maintained detectable levels. Circulating testosterone levels were apparently more susceptible to interference by the treatments. The resistance of LH to suppression suggests that probably a longer, more continuous presence of the treatment would be required, such as the osmotic pumps could provide. The problems which arose with the pumps would be overcome with more experience in their use.

It was interesting to observe the capacity of the gonad to protect its function from exogenous influences. Regardless of the length of exposure to GnRH treatment, the Sertoli cells and the stem spermatogonia always remained in the seminiferous tubules, thus safeguarding the gonads capability of recovering normal function.

As difficult as it was to work with Leydig cell cultures it proved to be a very interesting area. It was disappointing to have only two treated dogs to study and, tempting as it is to speculate about the results, the fact remains that a larger experimental dog group is required, however this was not possible during the present work due to the high cost of each experimental dog. It is well recognized that cell culture systems only provide some answers regarding how cells really function. Once isolated from interactions with other substances in their normal environment it is difficult to assess the physiological importance of responses to stimuli. LH and testosterone blood plasma concentrations varied markedly between animals. Similar variation was observed in the cell cultures, pointing to regulatory mechanisms in the Leydig cell which determine its capacity to produce testosterone. Alternatively this could be a reflection of the local interaction occurring at interstitial-seminiferous tubule level.

It would have been very helpful to study the Leydig cells from the treated testes using electron microscopy and this is an area worthy of further investigation. The existence of large and small luteal cells with different physiological functions has long been acknowledged. The observation of three sizes of Leydig cells in the dog is worthy of further investigation to determine whether functional differences exist between morphological types. Other types of differences have been acknowledge by other researchers. A possible reason for these differences could be due to the Leydig cells adaptation to the different stages of the spermatogenic cycle taking place within the seminiferous tubule.

A difference in the testosterone production by Leydig cell from untreated and Naferelin treated mice suggested that in this species the GnRH agonist acted at the level of the pituitary and directly at the gonad. Gonadal GnRH-like receptors have been identified in the rat by other workers and the finding confirmed in this study. However, using the same methodology, no evidence of gonadal GnRH receptors was found in the dog. It must be concluded that there are species differences in Leydig cell function. The evidence from this study suggests that the GnRH agonists used produced an inhibitory effect on LH and testosterone by down-regulating the pituitary and not by working directly in the gonad. This observation might prove useful in future research

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