# EFFECTS OF AGE ON THE STRUCTURE OF THE EPIDIDYMIS AND ON THE FERTILITY AND PROGENY OUTCOME IN THE BROWN NORWAY RAT

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## ABSTRACT

The effects of age on the structure and functions of the epididymis were studied using the Brown Norway rat model. Striking quantitative and qualitative changes during aging in the histology of the epididymis were observed. Characteristic features of aging, such as increases in basement membrane thickness and massive accumulation of lipofuscin, were found. In addition, epididymis-specific signs of aging such as polymorphism of lysosomes, presence of giant vacuoles and spermiophagy, and a major increase in the number of halo cells were also found. The precise nature of halo cells was demonstrated by using specific antibodies. In epididymides from young adult animals, halo cells are composed of three types of immune cells: helper T cells, cytotoxic T cells and monocytes. The three immune cell types present increased with age in the epididymal epithelium in a segment specific manner. In contrast their numbers did not increase significantly in the interstitial tissue. Furthermore, the concentration of cytotoxic T cells and monocytes-macrophages was enhanced in the epididymal epithelium of aged rats whose epididymal lumen contained few spermatozoa. The recruitment of immune cells might be triggered by the accumulation of damaged epithelial cells, or by sperm antigens leaking through the blood epididymis barrier. Thus, the epididymal microenvironment that is essential for the maturation of spermatozoa could be altered in old age. The effect of advancing paternal age on pregnancy and progeny outcomes was assessed by mating Brown Norway male rats of

increasing age with young Sprague-Dawley females. The effects of advancing paternal age on the offspring included an increase in preimplantation loss, a decrease in the average fetal weight on day 20 of gestation, and an increase in early neonatal death. These results indicated that the quality of spermatozoa decreases as males age.

# RÉSUMÉ

Les effets de l'age sur la structure et les fonctions de l'épididyme ont été étudiés en utilisant comme modèle le rat Brown Norway. Avec l'age, l'histologie de l'épididyme est énormément modifiée, à la fois qualitativement et quantitativement. Des signes caractéristiques du vieillissement, tels que l'épaississement de la membrane basale et l'accumulation de lysosomes apparaissent. Par ailleurs, des changements spécifiques à l'épididyme, tels que, la polymorphie des lysosomes, la présence de vacuoles géantes, la spermiophagie, et l' augmentation importante du nombre de cellules auréolées, sont observables. L'utilisation d'anticorps spécifiques a permis de démontrer que, chez le jeune adulte, ces cellules auréolées incluent en fait trois types de cellules immunitaires: lymphocytes T effecteurs, lymphocytes T cytotoxiques et monocytes. Ces trois types de cellules immunitaires augmentent avec l'age dans l'épithélium, et chacune, de façon spécifique à chaque segment de l'épididyme. Par contre, en général le nombre de cellules immunitaires présentes dans le tissu interstitiel ne change pas. De plus, dans certains segments, le nombre de lymphocytes T cytotoxiques et de monocytes-macrophages présents dans l'épithélium est supérieur chez les rats avec peu de spermatozoïdes dans la lumière epididymale. Cette migration de cellules immunitaires pourrait être déclenchée par une accumulation de cellules épithéliales endommagées, et par des antigènes, issus de spermatozoïdes ou cellules germinales immatures, capables de traverser la barrière sang-épididyme. Ainsi, le micro-environnement essentiel pour la maturation des spermatozoïdes pourrait

être modifié chez le rat âgé. Des rats mâles Brown Norway d'age croissant, ont été accouplés avec de jeunes femelles Sprague-Dawley afin d'évaluer l'influence de l'age paternel sur la grossesse et sur la descendance. Le vieillissement du père entraîne une augmentation des pertes au stade de pré-implantatoire, une diminution du poids moyen des foetus au jour 20 de gestation et une augmentation des décès peu de temps après la naissance. Ces résultats indiquent que la qualité des spermatozoïdes décroît avec le vieillissement du père.

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# Chapter I

## Introduction

## A. General Introduction: Aging

### 1. Aging of the Population

#### a. Life Expectancy

From the beginning of recorded history until the end of the last century, human life expectancy was increasing rather slowly. Life expectancy was approximately 18 years in 1000 BC, reached 25 years by 100 AD and was 50 at the turn of this century [1, 2]. However, over the past century life expectancy has increased dramatically in developed and developing countries. The average life expectancy was 76.1 years in 1997 [2].

This exponential increase in life expectancy can be explained by recent progress in medicine and agriculture. Improved nutrition, public health, and overall life quality are responsible for a decrease in the number of premature deaths. Old-age mortality has become the norm thus resulting in a major population growth [1, 3]. In contrast to life expectancy, maximum lifespan appears to be fixed and species-dependant; it is about 120 years for humans [2-4]. Hence, the pattern of demographic distribution in industrialized countries is now becoming rectangular as opposed to the pyramidal shape of the early part of this century or that seen for many developing nations [3]. The proportion of the population aged 65 or more will approach 20% of the world population by the year 2030 [1, 2, 5]. An alarming consequence of this increase in life expectancy is the increase of both relative and absolute numbers of elderly among the general population [3, 6].

### b. Health Care Challenge

People from developed nations will live well beyond the age of 65 and death is likely to occur within a small range of years [3, 4]. However, age-related conditions and chronic illnesses may compromise the quality of the latter years of life. Indeed, aging of the population is associated with increased opportunities for age-related conditions, or co-morbidity, such as Alzheimer's disease, osteoarthritis, osteoporosis, obesity, benign prostate hyperplasia and incontinence [7]. Furthermore, cancer, cardiovascular diseases, diabetes and hypercholesterolemia, are responsible for claiming most lives and for a plethora of disabilities [2, 8-10]. Thus, society is facing the challenge of finding new health promotion strategies quickly.

### c. Cost of an Aging Population

> "Living is not the good, but living well.
> The wise man therefore lives as long as he should,
> not as long as he can.
> He will always think of life in terms of quality, not quantity."
>
> Seneca, quoted by Timiras PS [5]

Impaired health and frailty of the elderly population account for an increase in the number of days of restricted activity, and in admittance and length of stays in hospital [11]. Consequently, our society will have to provide sufficient as well as adapted medical facilities and nursing homes [4]. In addition, one has to expect a dramatic increase in the burden of the cost of caring for the elderly, which would

create a threat to the maintenance of government health care systems [1, 3]. In the 1930's, only one out of ten Americans survived to their first retirement paycheck as compared to 75% nowadays [2]. In fact, Laurence Kotlikoff, an economist at Boston University, estimates that "at this rate of growth, programs such as Medicare and Social Security will bankrupt the next generation" [1].

Postponing illness or "compression of morbidity" will be possible if good health can be expanded as close as possible to the end of life [1, 4, 6]. As life expectancy increases in most countries, the next gerontological goal should be decreasing the time spent with disease or disability. Thus, it has become imperative to understand the fundamental mechanisms underlying the process of aging.

## 2. Theories of Aging

> " For when I was a babe, and wept and slept, Time crept;
> when I was a boy and laughed and talked, Time walked;
> Then when the years saw me a man, Time ran;
> but as I older grew, Time flew".

*Whitrow G; The Nature of Time. Holt, Rinehart & Winston, New York 1973 [5].*

Aging can be defined as an accumulation of changes leading to disrupted homeostasis, senescence and death of an organism [7]. Most theories of regulation of aging processes fall into two groups: the genetic or "intrinsic aging" theories, and the environmental exposure or "extrinsic aging" theories. Interactions of genetic and

environmental factors are complex, not mutually exclusive, and result in large inter-individual differences [6, 8, 12]. Effects of aging can be detected at the molecular, cellular, and systemic levels [12-14].

In the past decade, many new observations and hypotheses relating to aging have been put forward. However, the precise mechanisms responsible for aging remain elusive, unproven and subject to considerable speculation. Only the main theories and the recent advances related to the aging process are discussed below. More specific details are provided in the reviews listed at the end of this chapter.

### a. Molecular and Cellular Theories

The pioneering work of Hayflick and Moorhead in the 60's demonstrated that cells in culture have a finite ability to proliferate; this is known as the Genetic Model of Aging [15]. The time of entry into the terminally non-dividing state for normal embryonic fibroblasts is reproducible within narrow limits in vitro [15]. Thus, the limited capacity of cells to divide is a programmed event and a function of the donors' age [15, 16]. This replicative senescence denotes dramatic functional changes within cells and shows clearly that lifespan is under genetic control.

Biogerontology investigates the role of genes in determining lifespan and aging [17]. The yeast and the nematode models have permitted startling advances in our understanding of aging. The yeast model, *Saccharomyces cerevisiae*, has led to the recent discovery that genes are capable of modulating lifespan (RAS-oncogene [18, 19]). Mutations in the genome of the nematode worm model,

*Caenorhabditis elegans*, have been shown to affect biological timing and lifespan thus support the genetic basis of aging (Clk mutant, Daf-2 mutants [20, 21]).

Recently, Harley unveiled a new biomarker of cellular senescence: shortening of telomeres [22, 23]. Telomeres are short repeated DNA sequences capping the end of eucaryotic chromosomes protecting and facilitating their replication [24, 25]. Telomerase is a specialized reverse transcriptase that is accountable for the replication of telomeres and consequently for their length [24, 25]. Telomerase is repressed in somatic cells mostly during development [26]. In contrast, germ cells are telomerase positive; this assures the transmission of full length chromosomes to the next generation [25, 26]. Harley's experiment showed a correlation between the loss of the telomeric repeat array at chromosomal termini and a decreased number of replication [22, 23]. According to the Telomere Hypothesis of Cellular Senescence, shortening of telomeres beyond a critical point signals cell cycle arrest, senescence and death [22, 23]. As predicted, introduction of telomerase increases lifespan [27]. Thus, the limit of lifespan, or the mitotic clock described by Hayflick and Moorhead, is, in part, determined by the critical length of telomeres [15, 27].

The Heterochromatin Loss Model of Aging hypothesizes that there is an age-related loss of heterochromatin-induced gene silencing [28, 29]. This induces, with increasing age, a modest and gradual change in the expression of many genes; these progressive changes lead to the senescent phenotype [29].

The buildup of fateful circles of DNA with age can also act as a timing mechanism. Guarente and Sinclair, using the yeast model, have recently proposed

that the accumulation in the nucleus of extra chromosomal circles of rDNA (ERCs) each time the cell replicates, could be responsible for a cell cycle arrest and aging [30].

According to the Wear and Tear Theory of Aging, the lifespan of a species is limited by a fixed total metabolic potential. An inverse relationship between metabolic rate and life span has been demonstrated. Male houseflies kept at lower temperature or with restricted activity have a longer lifespan [31]. Interestingly, *c elegans* Clk-mutants with increased lifespan, present a hypometabolic phenotype [20].

A strong relationship between caloric restriction and lifespan in mammals has been established [32-34]. These effects have been correlated to a decreased generation of reactive oxygen species (ROS) by lowering oxygen consumption [31, 35]. The cumulative damage generated by toxic by-products of metabolism might contribute to cellular senescence; this implies an important role of the environment and lifestyle in the aging process [36, 37].

One of the most popular theories for the aging process is the Free Radical Theory of Aging that was first presented by Harman [38]. According to this theory, free radicals are by-products of the metabolism process that create membrane damage, accumulation of lipofuscin pigments in lysosomes, and eventually loss of cellular integrity [39]. Reactions leading to the formation of free radicals are ubiquitous in living systems and are accrued during oxidative stress [33]. The Oxidative Stress Theory of Aging proposes that the lack of efficiency of antioxidant

defense lets a small fraction of reactive oxygen species (ROS) inflict extensive oxidative damage in cells [31].

The antioxidant defense system includes free radical scavengers, antioxidant enzymes and proteolytic systems [40, 41]. α-Tocopherol (vitamin E), ascorbic acid (vitamin C) and β-carotene quench reactive oxygen species before they damage cellular constituents [36, 41]. Cellular antioxidant enzymes such as glutathione peroxydases and reductases (GSH Px/ Rx), catalase, and superoxide dismutase (SOD), catalyse the inactivation of oxidant and free radicals [40-42]. Interestingly, Sohal and Orr [31] have shown that co-overexpression of Cu-Zn superoxide dismutase and catalase genes increases the lifespan of *Drosophila melanogaster*. Finally, the proteolytic system (protease and peptidase) may prevent the formation of deleterious oxidized proteins [40, 41].

Alterations to ribonucleic acids (RNA) and deoxyribonucleic acids (DNA) can occur due to oxidative damage or mistake in replication. The Somatic Mutation Theory is based on the age-related increases in the frequency of mutations and lesions to DNA [43]. DNA damage includes strand breaks, sister chromatid exchanges, DNA-DNA and DNA-protein cross-linkages, and base modifications [40]. DNA oxidative damage can be repaired with direct repair mechanisms (methyltransferase) and excision repair mechanisms (endonuclease, exonuclease) [36, 40, 42, 44].

Individuals affected by Werner's syndrome show signs of aging prematurely. The recent discovery that, with this syndrome, the faulty gene encodes a helicase

important for DNA unwinding favours DNA damage as a causal factor of aging [45]. Thus, several lines of evidence suggest that the aging clock is set by genetic factors and modulated by environmental factors. There are convincing indications that the efficiency of the metabolic and repair systems in response to stress or damage is of critical importance in the senescence of an organism. The number of theories of aging proposed is obviously proportional to the complexity of the problem, and a great deal of work remains to be done in determining with certainty the precise relationship between intrinsic and extrinsic factors.

**b. System Level Theories**

The theories reviewed above related to aging at the molecular and cellular levels. However, there is mounting evidence that aging also occurs at a higher level. The System Level Theory proposes that the failure of one system may lead to a cascade of failures of other systems. The main hypotheses belonging to the System Level Theory are the Immune and the Endocrine Theories of Aging.

The Immune Theory of Aging proposes that the age-related impaired functions of the immune cells lead to senescence [46]. The thymus has been referred to as the biological clock for mammalian aging; it reaches its maximum size the first year after birth and then begins to involute [47]. Interestingly, the decrease of the thymic mass is followed by the decline of immunological defences [46, 47]. Consequently, there is a decline in the ability of adapting to environmental stress and

to maintain homeostasis; this is thought to underlie the increased incidence of spontaneous somatic mutations and the increased occurrence of autoimmune diseases [48, 49].

According to the Endocrine Theory, aging is mediated by changes in hormonal levels which consequently disrupt homeostasis and many functions of the body [50]. Endocrine activities of the hypothalamus, pituitary, gonads and adrenals are modified with age in both animals and humans. The circulating levels of numerous hormones change or become irregular [50-52]. However, it remains difficult at the moment to determine whether these complex changes are physiologic or pathologic, if they are related to basic changes in the genome of all cells, and finally, whether they are a cause or a consequence of the aging process.

## 3. Endocrinology of Aging

Endocrinology is the study of glands, tissues, and their hormones. Evidence supporting the endocrine aspect of aging has been brought forward by Brown-Séquard at the end of the nineteenth century; he claimed that self-treatment with soluble extract of dog testes had rejuvenating effects [53]. Intriguingly, a number of traits of normal aging, such as physical frailty induced by osteoporosis, closely resemble features of hormonal deficiency. It is now widely accepted that increasing age is accompanied by a decline in the quantity and/or the activity of hormones in major endocrine systems, such as the pituitary, the adrenal gland and the

reproductive system. Their impaired function or "arrest" leads to somatopause, adrenopause, menopause and andropause [8, 54].

### a. Somatopause

Somatopause is the age-related decline in the production of growth hormone (GH) and insulin growth factor 1 (IGF-1) [8, 55, 56]. Very little is known about the causes and consequences of this deficiency. However, it is postulated that a hormonal replacement therapy in the normal elderly, with GH or growth hormone releasing hormone (GhRH), increases lean body mass and bone turnover [55, 56].

### b. Adrenopause

Adrenopause is the progressive reduction of steroidal products of the adrenal gland that accompanies aging [57]. Serum levels of the main product of the adrenal gland, dehydroepiandrosterone (DHEA), or its sulfate conjugate (DHEAS), are decreased in aged men. Hormonal replacements with DHEA or DHEAS designed to restore serum levels to those of young individuals have been reported to be correlated with higher activity, increased bone mass, longer survival and well-being [58-60]. The value of such replacement therapy remains controversial [60, 61].

### c. Menopause

Aging is accompanied by a decline in reproductive functions. In the female, menopause marks the end of menstrual cyclicity. Early medical writings in the

1840's have described the events following the cessation of menstruations:

> " What she has to expect save gray hairs, wrinkles, the gradual decay of these physical or personal attractions, which heretofore have commanded the flattering image of society... The pearls of the mouth are become tarnished, the hay-like odour of the breath is gone, the rose has vanished from the cheek, and the lily is no longer the vain rival of the forehead or the neck. The dance is preposterous, and the throat no longer emulates the voice of the nightingale..."
>
> *Meigs CD; Females and their Diseases; Philadelphia: Lea and Blanchard 1848 [62].*

The effect of age on the female reproductive system has been extensively studied. The average age of menopause is about 51 years and appears to be genetically determined [8, 63]. It follows a period of progressive lengthening of the menstrual cycle, decreased fertility and increased number of chromosomal aberrations in the offspring [63, 64]. Menopause is defined by an exhaustion of oocytes in the ovary, and major decreases in estradiol and progesterone synthesis and elevated levels of gonadotropins (FSH and LH) [63, 65, 66]. Thus, alterations of both the ovary and the hypothalamus-pituitary axis might be responsible for irregular cycling and transition into menopause [8, 67]. The lack of estrogen during the post-menopausal years dramatically affects numerous other systems and contributes to many age-related conditions such as cardiovascular diseases, osteoporosis and loss

of memory [67, 68]. Early estrogen replacement may protect and improve all the above conditions [69-71].

**d. Andropause**

The first mention of the concept of "male climacterium" was made by Acton in the 19th century.

> " It is usually at the age of fifty or sixty that the generative function becomes weakened. It is at this period that man, elevated to the sacred character of paternity, and proud of his virile power, begins to notice the power decreased, and does so almost with a feeling of indignation. The first step toward feebleness announces to him, unmistakeably, that he is no longer the man he was".
> *William Acton. The Functions and Disorders of the Reproductive Organs 1857 [62].*

The possibility of an effect of age on the male reproductive system has long been denied. Scepticism about the concept of andropause was probably related to the fact that, in contrast to menopause, the effect of age on the male reproductive system had no clear-cut time frame, but rather was progressive. A reemergence of interest was sparked by the clear evidence that sexual dysfunctions, such as impotence, become more frequent with increasing age [72]. In addition, the occurrence of chromosomal disorders is greater in the offspring of older fathers than

Wise PH, eds. Oxford University Press; 1996) [80], and *The Physiology of Reproduction* (Knobil E, Neill J *et al.*, eds. Raven Press;1988) [81, 82, 83].

The hypothalamic-pituitary axis is essential for the maintenance of normal reproductive function. The hypothalamus is located at the base of the diencephalon, below the third ventricle of the brain, and is connected to the pituitary by the median eminence. Gonadotropin-releasing hormone (GnRH) is secreted by the neuroendocrine cells of the basal hypothalamus and is released in a pulsatile manner into the pituitary portal blood. The pituitary gland is located below the hypothalamus, at the end of the infundibular stalk, in the sella turcica. The pituitary is considered to be the "master" endocrine gland; it releases a large number of hormones in response to signals coming from the hypothalamus and the peripheral circulation. GnRH acts on the gonadotrophs of the anterior pituitary, and stimulates the pulsatile secretion of follicle stimulating hormone (FSH), and luteinizing hormone (LH) [84]. Gonadotrophic hormones are released into the blood stream and reach the gonads.

**b. Reproductive Tract**

The testes are encapsulated ovoid organs, suspended in the scrotal sac. The testis is divided into three compartments: the intravascular compartment, the interstitial tissue and the seminiferous tubules. The interstitial tissue contains blood and lymph vessels, nerves, immune cells (mostly macrophages) and Leydig cells [76]. The main function of Leydig cells is the production of testosterone [85].

## 2. Functions of the Male Reproductive Tract

### a. Reproductive Function

The main reproductive function is the production of viable and motile spermatozoa [91, 92]. In the testis, at the base of the seminiferous epithelium, spermatogonia (stem cells) continually divide by mitosis to renew themselves. They have the same DNA content as somatic cells [91]. Some spermatogonia (type B) provide cells for the meiosis process by differentiating into primary spermatocytes. Spermatogenesis is an elaborate process of cell division and differentiation that begins in seminiferous tubules at puberty, as a consequence of stimulation by pituitary gonadotrophic hormones. Primary spermatocytes enter meiosis, which involves two successive cell divisions, to produce spermatids. Each spermatid contains half the DNA content of somatic cells [92, 93].

During spermiogenesis, the spermatid undergoes further modifications including formation of the acrosome, shaping of the nucleus, condensation of chromatin, repression of gene transcription, formation of the tail, reorganisation of cytoplasm and organelles [94]. Following this elaborate remodelling, spermatids undergo spermiation, a process where they lose contact with the Sertoli cell. Spermatozoa are fully differentiated cells that are released into the lumen of the seminiferous tubules [91-94]. They acquire the ability to fertilize an oocyte during their transit through the epididymis, by a process called maturation [87, 95].

Short term pulses of LH act on Leydig cells to stimulate the synthesis of testosterone from cholesterol [103]. Both maintenance and restoration of spermatogenesis are tightly regulated by testosterone [104]. In addition, testosterone and its metabolite 5α-dihydrotestosterone are crucial for the development and maintenance of the male phenotype [105].

## 4. Immunology of the Reproductive Tract.

### a. Overview of the Major Cells in the Immune System

In order to facilitate the understanding of immunology of the reproductive tract a succinct introduction to the fundamental concepts of immunology and a brief description of the main cells of the immune system are presented below. More complete coverage of this highly complex and fast growing field can be found in *Textbook of Immunology* (Bona CA, Bonilla FA, eds. Harwood Academic Publishers; 1996) and *Roitt's Essential Immunology* (Roitt I, ed. Blackwell Science; 1997) [106, 107].

The main function of the immune system is to distinguish self from non-self elements and to defend the body from non-self material. The major cells involved in the immune response are monocytes-macrophages, lymphocytes (T and B), and granulocytes (neutrophils, eosinophils, basophils). Immune cells arise from pluripotent stem cells located in the bone marrow that differentiate into the lymphoid lineage and the myeloid lineage. The former produces lymphocytes and the latter produces monocytes, macrophages and granulocytes.

Monocytes develop in the bone marrow from promonocytes. They are transported to the circulating blood and migrate into different tissues where they can differentiate into macrophages. Macrophages, or "large eaters", are the main phagocytic cells. They have the ability to process and present antigens to specific lymphocytes.

Lymphocytes are produced in the bone marrow. B lymphocytes differentiate in the bone marrow and T lymphocytes mature in the thymus. The T lymphocytes are the main component of cellular immunity and can be divided in two subtypes: the cytotoxic T-cells and the helper T-cells.

CD8 is a marker of cytotoxic T-cells; this adhesion molecule restricts these cells to recognize antigens associated with major histocompatibility complex (MHC) class I. The main function of the cytotoxic T-cells is the selective destruction of cells that could be harmful to the body.

CD4 is a marker of helper T-cells; it confines these cells to recognize antigens associated with MHC class II. The primary role of helper T lymphocytes is to help other cells of the immune system to express effector function. Helper T lymphocytes can be divided into two subsets based on the cytokines they secrete. Helper T lymphocytes type 1 (Th1) can interact with an antigen presented by macrophages or antigen-presenting cells. Helper T lymphocytes type 2 (Th2) can recognize antigens presented by B cells and stimulate B cells.

B lymphocytes are the humoral element of acquired immunity. Activated B lymphocytes can either become memory cells or proliferate and mature into plasma

cells. Plasma cells secrete large amounts of immunoglobulins, also called antibodies. Antibodies can bind and destroy extracellular pathogens and their products.

Neutrophils are phagocytic cells found early in the inflammatory response. Basophils contain granules with histamine and other vasoactive amines; the release of granular content creates an allergic reaction. Eosinophilia is associated with allergy and parasitic infection. It is postulated that eosinophils restrict the inflammatory response by antagonizing the effects of mediators.

**b. Immunocompetent Cells in the Male Reproductive Tract**

Lymphocytes and macrophages are present in the mammalian male reproductive tract (human [108, 109]; mouse [110, 111]; rat [112]). However, there are many divergent results regarding the nature of these cells, and very few studies clearly establish the origin and role played by these immune cells in the male reproductive tract [113]. The fundamental concepts that immune cells have a protective role under normal conditions, that they may become a risk factor for spermatozoa in an abnormal environment, and that they are of considerable importance in the maintenance of fertility will be explored below.

The presence of the blood-testis and the blood-epididymis barriers was demonstrated using intravascular perfusion with the electron opaque dense tracer lanthanum, and with freeze fracture studies [114-116]. Micropuncture studies have shown that the concentration of proteins present in the intraluminal fluid is different

present in the epithelium of the epididymis is the cytotoxic T lymphocyte [108]. Finally, as seen for the testis, vasectomy induces a change in the distribution of immunocompetent cells (mouse [110]; rat [124]).

## C. The Epididymis

A resurgence in interest in the epididymis followed the pioneering work of Orgebin-Crist and Bedford in the late 60's, where it was demonstrated that, upon spermiation, spermatozoa are not fully functional and only acquire their ability to fertilize after passage through the epididymis [95].

### 1. Development of the Epididymis

The epididymis develops during gestation from the upper segment of the mesonephric (Wolffian) duct [77]. The presence of testosterone secreted by the testis is required for the postnatal development of the epididymis [95, 126]. Dramatic changes in the structure and size of the epididymis after birth are correlated with the presence of testicular fluid, and the onset of spermatogenesis and steroidogenesis [77, 127]. In the rat, the development of the epididymis can be divided into three phases: undifferentiated from birth to day 15, differentiation from day 16 to day 44, and expansion from day 44 to 52 [127]. This differentiation of the epididymis progresses from the proximal to the distal segments, and results in

and secondary lysosomes indicates their endocytic properties [129, 132]. In addition, adluminal tight junctions between adjacent principal cells form the blood-epididymis barrier [135]. The zonula occludens is solely responsible for separation of luminal and intercellular spaces [119].

Basal cells are pyramidal-shaped elongated cells, lying on the basement membrane [77, 131]. They are the second most abundant cell type of the epithelium and are found throughout the epididymis [77]. They are characterized by a dome-shaped nucleus and a relatively small amount of cytoplasm. Their exact functions are still unclear. These cells are reactive for gluthathiones S-transferase Yf (GST) (rat [136]) and CuZn-superoxide dismutase (SOD) (human, [137]) and therefore have been proposed to have a protective role against toxic electrophiles. Interestingly, Yeung *et al.* have recently advanced that basal cells could be macrophages and may have a role in immune protection of spermatozoa [111].

Clear cells are typically identified by their lighter appearance compared to their neighbouring principal cells [77]. They are the last epithelial cells to differentiate and are believed to originate from narrow cells (rat [127]). They are found throughout the caput, corpus and cauda epididymidis [77]. They display numerous coated pits, vesicles, endosomes in the apical region, lysosomes in the supranuclear region, and lipids in the basal region [77, 132]. Clear cells play an active role in the endocytosis of luminal components (rat [129, 138]; mouse [139]).

Halo cells are small round cells, found throughout the epididymal epithelium [77]. They display a modest amount of cytoplasm. The nature of these cells is still

### c. Interconnective Tissue, Vascularisation and Innervation

The vascular system of the epididymis consists of two main arteries, spermatic and deferential, and their respective veins. The spermatic artery branches into the superior epididymal artery to supply the initial segment, caput and proximal corpus epididymidis and into the inferior epididymal artery to supply the distal corpus and proximal cauda epididymidis. The deferential artery supplies the distal cauda epididymidis. Lymphatic vessels drain the epididymis and testis and reach the lumbar or para-aortic nodes [76].

Adrenergic and cholinergic nerve fibres are found in the epididymal interstitium of the entire epididymis and are mainly associated with muscles and vascular elements [144]. The cauda epididymis is the more densely innervated region. Denervation alters sperm transport and storage as well as post-fertilization embryo development in the rat [144].

## 3. Functions of the Epididymis

Absorption and secretion by the epididymal epithelium create a specialized intraluminal environment maintained by the blood-epididymis barrier [117, 145, 146]. This microenvironment is segment specific and essential for the maturation and storage of spermatozoa [87]. The absorption and secretion functions are largely dependent on the presence of androgen [143, 147, 148]. The epididymal lumen is also the site of transport of spermatozoa [77].

### a. Absorption and Secretion

#### Fluid and Electrolytes

A large amount of testicular exocrine fluid is absorbed mostly between the rete testis and the caput epididymidis [143, 149]. Thus, there is a major increase in the luminal concentration of spermatozoa by the time they reach the caput epididymidis [141, 143, 150]. This concentration is associated with the phenomena of water reabsorption and electrolyte exchanges by mechanisms involving ion pumps, ion carriers, and ion channels [141, 151]. Anions such as $Cl^-$ and $HCO3^-$ and cation such as $K^+$ and $Na^+$, are secreted or absorbed by the epididymal epithelium [152, 153]. The cystic fibrosis transmembrane conductance regulator (CFTR) is a cAMP-regulated $Cl^-$ channel [154]. Epididymides of cystic fibrosis patients are obstructed, thus suggesting an important role of CFTR in the modulation of secretion of fluid and electrolytes in the epididymis [155]. Adenosine triphosphate ATP activates $K^+$, $Ca^{++}$ and cAMP-$Cl^-$dependent conductances (rat [156, 157]). Fluid and electrolyte transport are dependant on androgens [152]. In addition, Leung and Wong have recently proposed that type-1 angiotensin II receptor (AT1) present in the epididymis may act as a regulator of fluid and electrolyte transport (rat [158]).

#### Luminal Compounds

The epididymal epithelium secretes electrolytes, fluid, and low molecular weight molecules such as carnitine and inositol, as well as proteins [77, 146]. Two-dimension gel electrophoretic analysis has permitted the identification of as many as

146 different proteins in the epididymal lumen of the boar [159]. However their function, in relation to sperm maturation, is largely unknown. The most active region for protein secretion is the caput epididymidis [160]. Interestingly, the fertilizing ability of spermatozoa increases after passage through this region [95].

Proteins secreted and found in the lumen include clusterin (SGP-2) [161], glutathione peroxidase [162], proteins B/C and D/E [163], immobilin [164], N-acetyl - hexosaminidase [165] and lactoferrin [166]. The synthesis and secretion of numerous epididymal proteins are segment-specific and androgen-dependant [163, 167].

Clusterin is secreted into the lumen by principal cells from the caput epididymidis (rat [161, 168]). The secretion of this glycoprotein is segment-specific and androgen-regulated (rat [169]). This glycoprotein is found on epididymal sperm suggesting a role in the maturation process [161, 168, 170]. Glutathione peroxidase 5 (GPX5) is found mainly in the caput and might have a role in the protection of spermatozoa (mouse [162]). Cysteine rich acidic epididymal protein (proteins D/E) is androgen-regulated. This protein is secreted in the lumen and subsequently binds to spermatozoa; it has been postulated to be later involved in gamete recognition (rat [163]). Finally, in human, epididymal protein HE1 might be an epididymal cholesterol carrier, and HE2 might be involved in gamete fusion [171].

The androgen binding protein (ABP) is secreted by Sertoli cells [172]. Large quantities of ABP, important for androgen transport and regulation of 5α-reductase activity, are endocytosed by the proximal segment of the epididymis [173-176]. It

changes in the morphology of the tissue and its response to androgens (mouse [214], rat [215]). For that reason, androgen-supplementation for aging men constitutes a potential risk for triggering the onset or progression of BPH and/or prostate cancer [75, 216, 217].

## 2. Functions of the Male Reproductive System

Aging of the reproductive system in the male is characterized by a decrease in sexual and reproductive functions [218].

### a. Sexual Functions

There is a progressive decline in male sexual activity with advancing age [219-221]. Impotency is defined as "the inability to obtain or sustain an erection" [222]. An increased incidence of impotency, and fewer ejaculations have been reported in aged mammals [222, 223]. In human, impotency starts around 40 years of age and affects more than 50% of individuals over 70-years-old [224, 225].

A low frequency of sexual activity during adulthood, decreased capacity for arousal, and age-related defects in neural, sensory and autonomic systems have been proposed as causal factors [ 72, 225, 226]. One major theory is that this lack of sexual activity is related to alteration of the hypothalamo-pituitary-gonadal axis and the decrease in the level of circulating free testosterone [220, 223, 227, 228]. Although this association is still controversial, preliminary results in the rat suggest a beneficial effect of testosterone supplementation on erectile dysfunction [219, 229].

### b. Reproductive Functions

#### Steroidogenesis

Some reports have shown no age-related changes in serum testosterone in aged men [72, 230]. However, most studies have shown that, with age, there is a gradual decline of serum testosterone levels below the normal range (human [231-233], rat [234]). It has been suggested that this discrepancy is related to the state of health of individuals being studied [230]. In addition, an effect of age is usually found in studies with blood collected in the morning but not in those with blood collected in the afternoon suggesting that the time of blood collection is an important parameter. Age-related changes in the circadian rhythm of testosterone secretion may be responsible for this discrepancy [232, 235].

Testosterone is found in the serum either as "free" testosterone or bound to testosterone estradiol binding globulin (TeBG) [236]. Thus, the large interindividual variation among healthy males may be related to whether it was testosterone or free testosterone that was measured in blood samples. TeBG has been shown to increase gradually with age, and with it, the amount of testosterone bound to TeBG [237]. The testosterone bound to TeBG might not be readily available to the tissues and thus, bioavailable testosterone declines with age [74, 236, 238-240].

Interestingly, the progressive decrease in serum androgen levels is correlated with an increased loss of bone mass and impotency [239]. Thus, there is a potential for hormonal supplementation, however the benefits versus the risks of triggering prostate growth and cardiovascular disease still have to be assessed [75, 240].

In animal models, there is evidence of primary testicular failure during old age characterized by an alteration of Leydig cell functions [241]. In aged rats, Leydig cells present a diminished ability to produce testosterone in vitro after stimulation by exogenous LH [242].

**Spermatogenesis**

An adequate intratesticular concentration of testosterone is essential for spermatogenesis. It is well-established that aging is accompanied by a decline in daily sperm production (human [195, 202, 244], mouse [245], rat [246]). In addition, there is an impairment of sperm motility as a function of age as well as an increase in the percentage of spermatozoa with coiled tails (men [243]). There are very few studies addressing the effect of age on male fertility and progeny outcome. Although the reproductive capacity appears sufficient to allow paternity, a progressive decline in fertility has been reported for aging men and rats (human [247, 248]; rat [249]).

### 3. Regulation of the Male Reproductive System

Aging is accompanied by defects in the hypothalamo-pituitary-testicular axis (human [75, 196, 230, 250]). The effect of age on LH secretion is controversial. Some studies reported an increased serum LH level [230, 233, 237, 251, 252]. Few studies reported no changes or a decrease in bioactive LH [74, 232, 240]. Most recent studies agree on the decreased frequency and amplitude of LH-pulses with age, thus pointing to a hypothalamo-pituitary deficiency (rat [234, 253]). Disruption of LH and T release have also been attributed to alterations in feedback and

comprises many strains each showing unique age-related diseases, such as amyloidosis, osteoporosis, brain atrophy or abnormalities of the immune system.

**b. Klotho Mice**

Klotho mice show, at an early age, characteristic signs of aging such as arteriosclerosis, osteoporosis, skin atrophy, emphysema, and premature death. Recently a defective gene coding for β-glucosidase has been identified in these mice [261]. The characteristics of this model have to be further investigated.

**c. Sprague-Dawley, Lewis and Fisher Rats**

Sprague-Dawley rats (SD) have been widely used for general studies. However, they develop endocrine tumours such as pituitary adenoma, and pancreatic islet cell tumours with age. Furthermore, they become obese as they age; the expansion of tissue and plasma volume make precise hormone measurement difficult to compare with the human [255]. Only 26% of Lewis rats (LEW) reach 36 months; in addition they are susceptible to obesity [262]. The median lifespan of the Fisher rat (F344) is 31 months [262]. However, aged Fisher rats develop testicular interstitial tumours (85%), thyroid carcinoma (22%), mammary tumours (23%) and secrete high levels of progesterone [262, 263]. Thus the Fisher, Sprague-Dawley and Lewis rats strains are inappropriate for studies of aging of the reproductive tract.

### d. Brown Norway Rats

The Brown Norway (BN) rat was proposed for aging studies in the 90's by Zirkin et al [264]. Brown Norway rats have the advantage of remaining in excellent health throughout their long lifespan. Age-related pathologies consist mostly of cardiac lesions (7%) [262, 264]. Once they reach adulthood, the weight of Brown Norway rats increases only slightly [264]. Brown Norway rats do not develop testicular, liver, or pituitary tumours [262, 264]. This permits one to distinguish changes related to aging per se, from changes induced by a specific disease.

Furthermore, recent studies have shown that the age-related changes occurring in the reproductive tract of the Brown Norway rats are very similar to those found in men. In Brown Norway rats as in men, there is, with increasing age, an atrophy of the seminiferous tubules, thickening of the basement membrane, decreased steroidogenesis, changes in secretory patterns of LH and FSH and decreased spermatogenesis [197, 199, 246, 253, 264].

Using the Brown Norway rat model, it was shown that the age-related decrease in serum and testicular testosterone concentrations were due to Leydig cell failure [194, 241, 242]. In addition, gene expression is affected by age in both testis and epididymis [197, 199, 265]. Furthermore, telomerase positive cells have been identified in the seminal vesicles of the Brown Norway rat [266].

Brown Norway rat provides an excellent tool for the study of aging and new exciting data on the mechanism of aging are rapidly emerging.

## F. Formulation of the Project

### 1. Hypothesis

Over the past century life expectancy has increased spectacularly [1, 2]. Furthermore, the proportion of individuals aged 65 and over is now the fastest growing portion of the population in developed and developing countries [1, 2, 5]. Thus, expanding the life expectancy while maintaining good health has become a necessity. This goal will be attainable through a better understanding of the mechanisms of aging.

The reproductive system offers certain clear advantages to studies on aging. Indeed it is a system that can arrest its functions during aging without compromising the survival of the animal. Furthermore, it is one of the first systems to show signs of aging. There have been very few studies on the effect of age on the male reproductive system and as a consequence, the existence of andropause remains controversial.

Couple frequently choose to postpone parenthood. However, little information is available on the effect of paternal age on the offspring. The main functions of the male reproductive system are to produce androgens and spermatozoa of a good quality, thereby a normal progeny. The testis is the site of production of androgen and spermatozoa. The epididymis is the site of transport, maturation and storage of spermatozoa.

Recently, it was shown that the Brown Norway rat is an excellent model to study aging. It has a long lifespan, does not develop age-related pathology and yet, as in humans, remarkable changes occur in the testis with increasing age. In Brown Norway rats, as in men, there is a decrease in the ability to produce testosterone and a decrease in the production of spermatozoa. Thus, since the two major components that enter the epididymis, androgen and spermatozoa, decline with advancing age, we hypothesized that the structure and functions of the epididymis would be affected by age.

**2- Experimental Approach**

To draw parallels between changes in the morphology and/or functions and the presence or absence of spermatozoa, we chose male Brown Norway rats aged 3 months (young adult, full spermatogenesis), 12 months (mature, full spermatogenesis), 18 months (senescent, just before spermatogenesis decreases), and 24 months (aged, spermatogenesis is usually reduced) (Fig. 3).

The first part of the project was to describe the quantitative and qualitative changes in the morphology of epididymis of the Brown Norway rat with age. Brown Norway rats of increasing age were perfused-fixed using glutaraldehyde. The epididymides were then embedded in Epon. We looked at the effect of age on the morphology and distribution of epithelial cells in each segment of the epididymis using light and electron microscopy. We ascertained whether some observed changes were characteristic features of aging tissues.

Very little is known about the type and role of immune cells present in the epididymis. The purpose of the next study was to identify the type of immune cells present in each segment of the epididymis of the young and the old animal. Male Brown Norway rats were perfused and fixed using Bouin's solution then embedded in paraffin. Immunocytochemistry was used to identify cytotoxic T lymphocytes, helper T lymphocytes, B lymphocytes and monocytes-macrophages. Since spermatozoa can be antigenic, we searched for a correlation between the number of immune cells and the content of the lumen of the epididymis. We analysed the number of each type of immune cell as a function of age, epididymal segment, and luminal content.

In the last study, we investigated the effect of paternal age on fertility and progeny outcome by mating Brown Norway rats of increasing age to Sprague-Dawley females of 3 months in proestrus. To assess the effect of paternal age on prenatal progeny outcomes, the females underwent a cesarean-section on day 20 of gestation. The uteri were opened and the number of live fetuses and implantation sites were counted. The ovaries were removed and the number of corpora lutea were counted. This allows for the evaluation of preimplantation and postimplantation losses. To evaluate the effect of paternal age on postnatal progeny outcomes, females were allowed to give birth normally. The pups were examined for external malformations and weighed daily for 2 months.

The results of the studies on the effect of age on the structure of the epididymis are presented in chapter II (effect of age on the morphology of the epididymis) and in chapter III (effect of age on the distribution of immune cells in the

epididymis). The results of the study on the effect of age on the functions of the epididymis are presented in chapter IV (effect of paternal age on prenatal and postnatal outcome). Finally in chapter V, I will discuss the significance of the results from chapter II, III and IV and their implications. Future directions are also proposed.

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**Fig. 1.** Diagrammatic representation showing the testis and the epididymis.

# EPIDIDYMIS

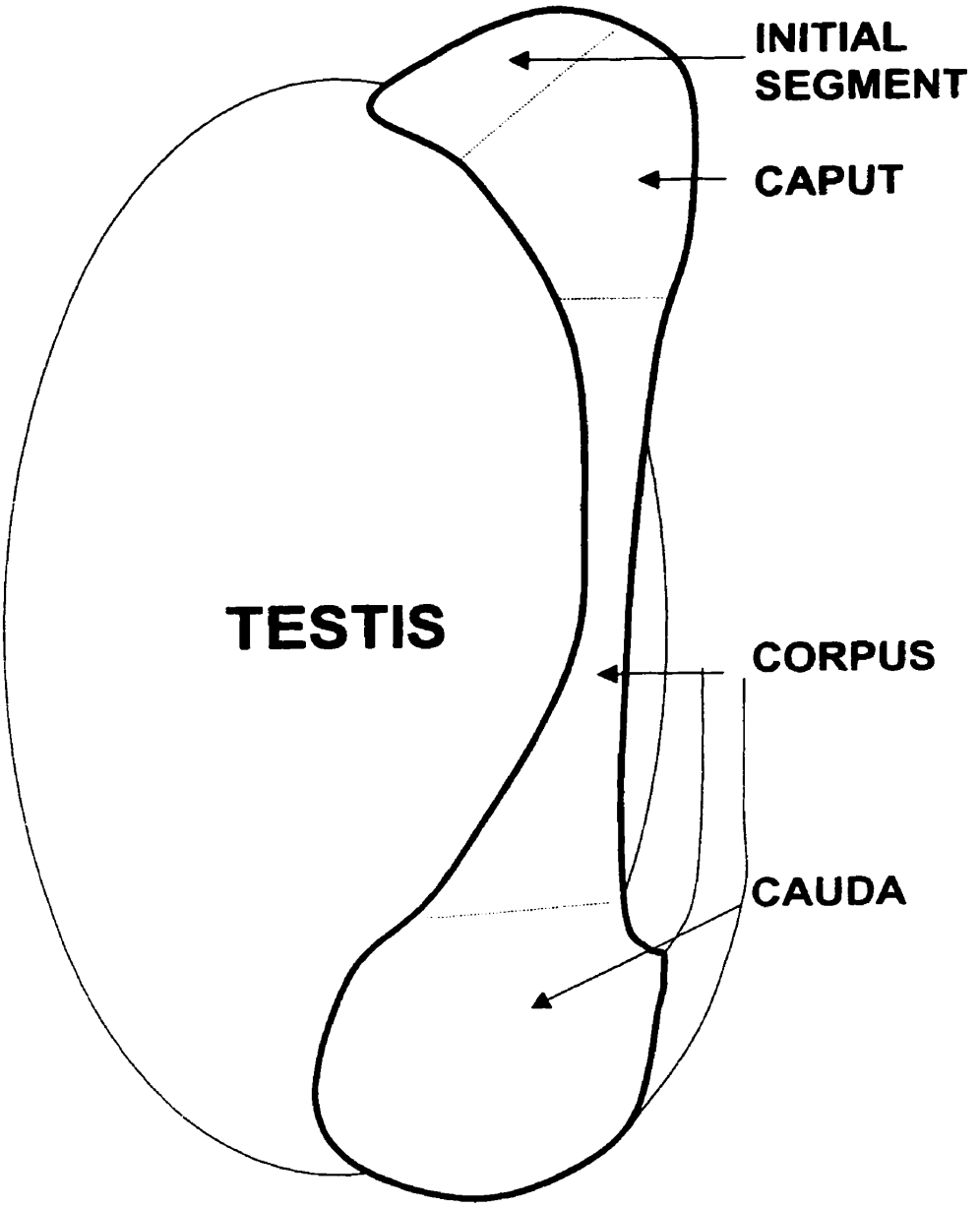


**Fig. 2.** Diagrammatic representation showing a section of the epididymal tubule.

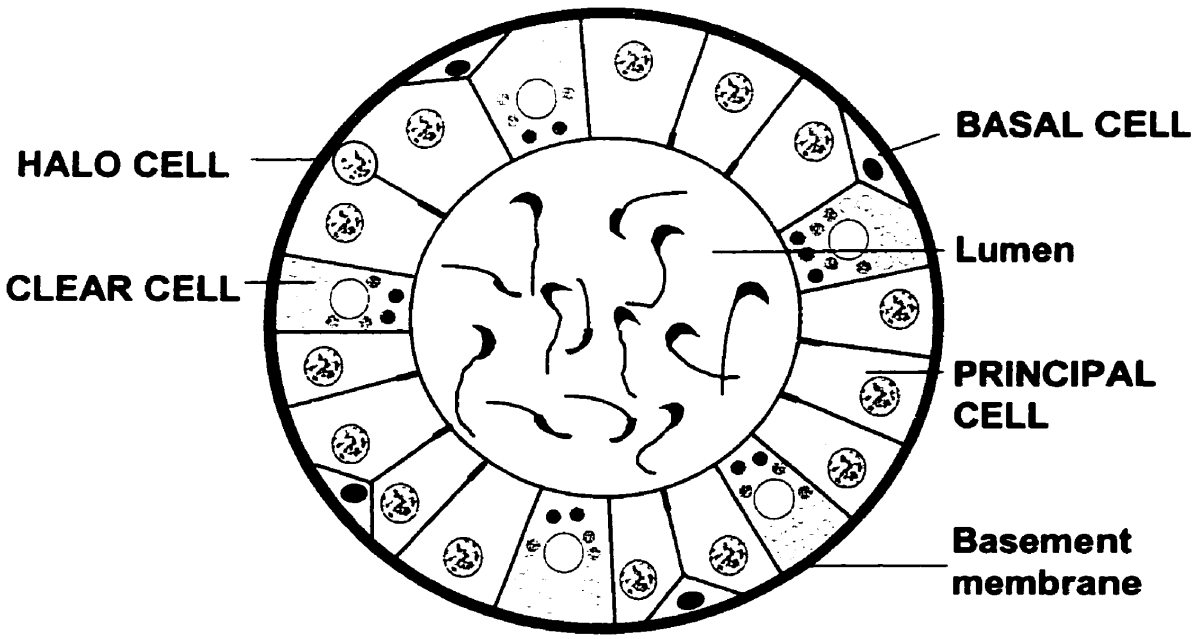
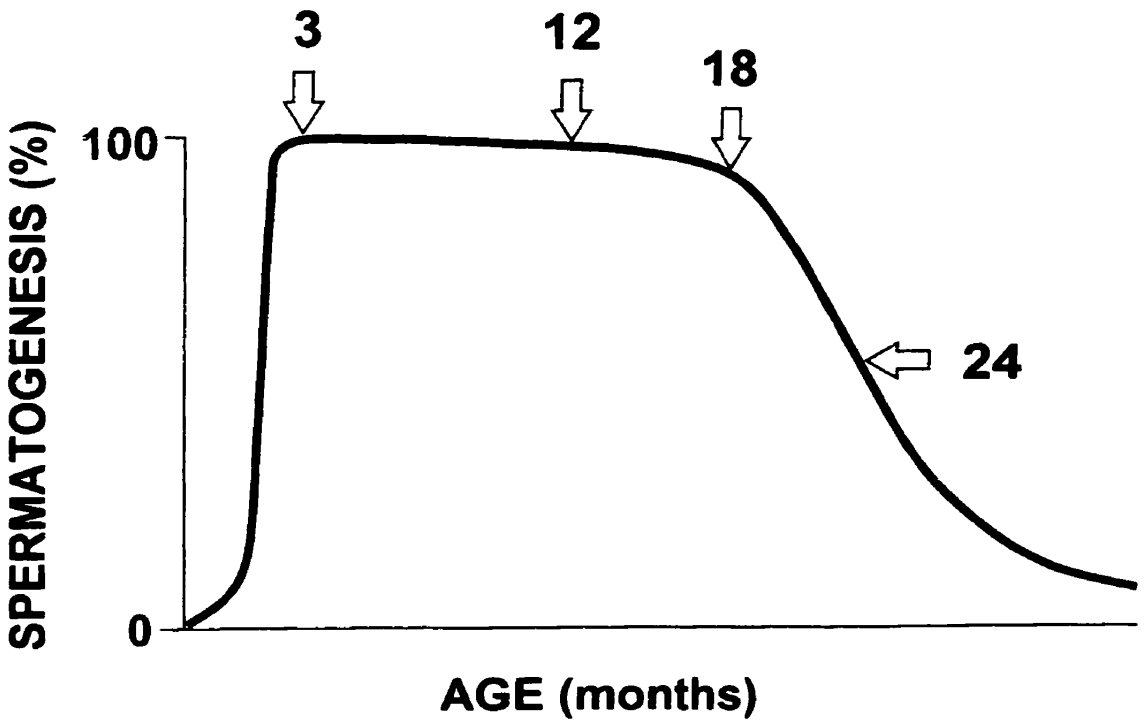

HALO CELL
CLEAR CELL
BASAL CELL
Lumen
PRINCIPAL CELL
Basement membrane

Fig. 3. Diagrammatic representation showing the effect of age on spermatogenesis in the Brown Norway rat.


3
12
18
24
100
0
SPERMATOGENESIS (%)
AGE (months)

## Chapter II

# Segment Specific Morphological Changes in the Aging Brown Norway Rat Epididymis.

**Valérie Serre and Bernard Robaire**

**Abstract**

In aging Brown Norway rats, both spermatogenesis and steroidogenesis decrease. Little is known about changes in the epididymis during aging. However, since the two major components entering the epididymis from the testis change, we hypothesized that epididymal histology would be affected by advancing age. The epididymides of Brown Norway rats ranging in age from 3 to 24 months were prepared for light and electron microscopy. Striking quantitative and qualitative changes were noted. There was an age-dependent increase in the thickness of the basal membrane and in the number of halo cells. There were also major segment-specific changes in the appearance of cells along the epididymis with age. At 12 months, basal cells in the initial segment emitted pseudopods into the basement membrane. By 18 months, in the caput epididymidis, clear cells were filled with lysosomes; these cells frequently showed bulging protrusions into the lumen. In the corpus epididymis, the cytoplasm of principal cells had numerous large lysosomes both below and above the nucleus; apical cells were usually occupied by one giant membranous lysosome. In the proximal cauda, clear cells became filled with dense lysosomes and principal cells presented large clear vacuoles; debris from spermatozoa was found in the larger vacuoles. In summary, aging of the epididymis was accompanied by the emergence of characteristic features of aging and activation of the immune system. Furthermore, there were many cell- and segment-specific changes. Finally, these changes were not related to the presence of spermatozoa, often preceding their disappearance, thus indicating that there may be

an intrinsic mechanism of aging in epididymal epithelial cells.

## Introduction

The epididymis, a highly convoluted tubule that links the testis to the vas deferens, is the site for the maturation and storage of spermatozoa [1, 2]. The epididymal epithelium functions, including absorption, secretion, synthesis and metabolism, create an appropriate luminal environment for the acquisition of fertilizing ability and motility of spermatozoa [3-4]. In addition, tight junctions between epididymal epithelial cells form the blood-epididymis barrier, maintain a specialized and changing luminal micro-environment, and may protect spermatozoa from the immune system [5].

The epithelium contains several cell types: principal, basal, clear, narrow, halo and apical cells. Principal cells are the most abundant cell type and play a major role in secretion and absorption [6]. Basal cells may have a protective role, either by preventing electrophilic attack [7], or by acting like macrophages [8]. Clear cells are not present in the initial segment but are found in the rest of the epididymis. They participate in the uptake of luminal components [9], and the disposal of the contents of cytoplasmic droplets detached from spermatozoa [10]. Narrow cells, only found in the initial segment, may be precursors of clear cells and could be involved in degradation of endocytosed protein [11]. Halo cells are found throughout the epididymis and have been described as lymphocytes [12] or monocytes [13]. They may play a role in the immunological barrier of the male reproductive duct [14].

Apical cells, have been previously described in the initial segment. They have a characteristic apically located spherical nucleus, contain many proteolytic enzymes, and may have a role in protecting the spermatozoa from electrophilic attack [11].

Although many studies have described the histology of the young and adult epididymis, in both humans and experimental animals (rats [6, 15]; human [16, 17]; monkeys [18]), very few have focused on how the epididymis is affected during aging. In 1980, Cran and Jones [19] reported an accumulation of lipofuscin pigment, a marker of cell aging, in the epididymis of the senescent rabbit. Recently, Viger and Robaire, have identified, in the aging Brown Norway rat, region-specific alterations in the expression of certain marker genes specific to epididymal function [20]. The Brown Norway rat is an excellent model to study aging because it has a long life span, it is relatively disease-free, yet remarkable changes occur in the testis when no other disease is apparent. A gradual decrease in the percentage of normal seminiferous tubules [21], total sperm count [22], and the ability of Leydig cells to produce testosterone [23, 24] is found during aging in the Brown Norway rat. Similarly, in humans, aging of the male leads to dramatic changes in the seminiferous epithelium and to decreases in spermatogenesis and steroidogenesis [25, 26].

Maintenance of epididymal structure and function is androgen-dependant [27]. Since spermatozoa and testosterone are major components entering the epididymis from the testis, we determined if there were changes in the architecture of the epididymis. In this study, we examined the morphological changes taking place in

each segment of the epididymis in male Brown Norway rats of increasing age; we assessed the association between observed changes in morphology and the presence or absence of spermatozoa. We found several changes in the epididymis during aging that were highly segment-specific; these changes were not related to the presence of spermatozoa, but rather seemed to be intrinsic to aging.

## Materials and Methods

### *Animals*

Brown Norway rats aged 3, 12, 18 and 24 months were purchased from the National Institute on Aging, Bethesda, MD, and supplied by Charles River Breeding Laboratories (Wilmington, MA). Rats were housed at the Johns Hopkins School of Hygiene and Public Health under controlled light (14 L: 10 D) and temperature (22°C) and with free access to food and water.

### *Tissue Collection*

Rats were anaesthetized with an i.p. injection of sodium pentobarbital (Somnotol; Steris Laboratories Inc., Phoenix, AZ). Right testicular and deferential arteries were ligated. The corresponding testis and epididymis were surgically removed and weighed.

### *Tissue Preparation for Light and Electron Microscopy*

The left epididymis was fixed via perfusion through the abdominal aorta. Retrograde perfusions were used to obtain optimal fixation of the initial segment and caput epididymidis, and prograde perfusions were used to fix the corpus and cauda epididymidis.

Rats were perfused with 2.5% glutaraldehyde buffered in sodium cacodylate (0.1M), pH 7.4. After perfusion, epididymides were sectioned into five segments, i.e., the initial segment, caput, corpus, proximal cauda and distal cauda (Fig. 1). Segments were then cut into 1-$mm^3$ pieces and placed in the same fixative for 2 hrs at 4 °C. The tissues were washed 3 times in a sodium cacodylate buffer (0.1M) containing 3% sucrose, pH 7.4, postfixed in 1% osmium tetroxide and 1.5% potassium ferrocyanide, and embedded in epoxy resin. Semi-thin (2-µm) and thin (75-nm) sections were cut on an ultramicrotome. The semi-thin sections were stained with toluidine blue dye and examined with the light microscope (Leiz Wetzlar, Laborlux D, Montreal, Canada). The thin sections were counterstained with uranyl acetate and lead citrate and examined with a Philips 400 electron microscope (Philips, Eindhoven, The Netherlands).

***Morphological Analysis***

Toluidine blue-stained sections from 3 animals selected at random were examined. For each epididymis, 3 blocks for each segment (initial segment, caput, corpus and proximal cauda epididymidis) were randomly chosen and at least 10 tubule circular cross sections were analysed. A calibrated linear scale was placed in the10X eyepiece of a Wild Leitz microscope. The effect of age on the epididymal tubule morphology, luminal diameter, epithelium height and lamina propria thickness was determined. The effect of age on the relative quantitative cell distribution was assessed by counting the frequency of major cell types (principal, basal, clear,

narrow, and halo cells) in each tubular cross section. Cells located at the base of the epithelium and filled with lysosomes were classified as halo cells.

Thin sections of corpus epididymidis were examined using an electron microscope. Principal cells, which contacted the basement membrane, presented a visible nucleus and reached the lumen were selected at random in different regions of the corpus epididymis. At least 10 entire principal cells for each of 3 rats at each age studied were photographied. Those micrographs allowed quantitation of the numbers, mean areas, and total areas of lysosomes as well as the areas of of principal cells in the corpus region using image analysis (MCID/M4, Imaging Research Inc., Toronto, Canada).

***Statistical Analysis***

One-way analysis of variance followed by Tukey's test was employed to detect significant age effects. The level of significance was taken as $p<0.05$.

## Quantitative Changes in the Epididymis with Age

### *Effect of age on epididymal weight*

The average epididymal weight was not significantly affected by age (3 months - 0.62±0.02g; 12 months - 0.56±0.03g; 18 months - 0.67±0.05g; 24 months - 0.50±0.04g). However, the average testis weight decreased from 3 months (1.68±0.04g) to 24 months (1.27±0.15g). At all ages, the weights of the testes and epididymides were correlated (r=0.87). When the testis was used as a covariable, a significant decrease in weight of the cauda epididymis with age was noted (3 months - 0.217±0.009g; 24 months - 0.172±0.017g).

### *Effect of age on the luminal diameter, epithelial height and thickness of the lamina propria.*

Evaluation of the effect of age on the luminal diameter, epithelial height and thickness of the lamina propria in each segment of the epididymis is shown in Figure 2. In the young adult, there was a progressive increase in the diameter of the lumen, moving from the proximal to the distal segment of the epididymis. As the lumen become wider, the height of the epithelium decreased, while the thickness of the lamina propria remained constant. With increasing age, the luminal diameter tended to narrow. By 24 months, this decrease had become significant in the cauda epididymidis (Fig. 2A). In young animals, and in old ones where the testes were

severely atrophied, a clear relationship was noted between the seminiferous tubule diameter and the diameter of the epididymal lumen; however, in those animals where partial regression of the seminiferous tubules was seen, there was no consistent relationship between the diameter of the semiferous tubules for one testis with the luminal diameter of the adjacent epididymis. There was a marked increase in the height of the epithelium in the corpus region of older rats (Fig. 2B). The height of the epithelium was not affected by age in the three other regions. In contrast, there was a progressive thickening of the lamina propria in each segment of the epididymis with increasing age (Fig. 2C).

***Effect of age on the relative contribution of cells in the epithelium***

A quantitative evaluation of the effect of age on the relative contribution of principal, basal, clear, narrow and halo cells in each segment of the epididymis is shown in Figure 3. Throughout the epididymis, principal cells were the predominant cell type, followed by basal cells. In the young rat, the relative contribution of clear cells increased in the distal segments, contributing to the relative decrease of principal cells; halo cells were rare and located at the base of the epithelium. With increasing age, there was a decrease in the number of principal and basal cells in all segments of the epididymis, of narrow cells in the initial segment, and of clear cells in the corpus epididymidis. This decrease was accompanied by a proportional dramatic increase in the number of halo cells in each segment of the epididymis. (Fig. 3, A and C). Surprisingly, in the caput and proximal cauda epididymidis, the

relative number of clear cells did not change, while the relative number of principal and basal cells decreased significantly (Fig. 3, B and D).

## Morphological Changes Along the Epididymis with Age

### Light microscopy

#### *Initial segment of the epididymis*

Few changes were seen in the epithelial morphology of the initial segment of the epididymis during aging. At 3 months, the luminal diameter was small, and the epithelium was well organized. Principal cells were tall and columnar having a round nucleus located at the base of the cell and long microvilli forming the brush border at the apex. Elongated basal cells were located between principal cells at the base of the epithelium. Narrow cells had a typical dense elongated nucleus located in the upper half of the cell. The few halo cells found had a characteristic clear cytoplasm and were all located at the base of the epithelium. Apical cells were rare and scattered (Fig. 4A).

In older rats there was an increase in the number and size and a change in the location of halo cells (Fig. 4B). A few halo cells spanned the epithelium. Some cells, located at the base of the epithelium, were filled with lysosomes. At 24 months, occasional intra-epithelial cysts were seen (not shown). In these animals, principal cells had lost their columnar appearance and the nuclei were no longer aligned at the base of the epithelium (Fig. 4C).

***Caput epididymidis***

The main effects of age in the caput epididymidis were on the number and size of clear cells and on halo cells. At 3 and 12 months, the epithelium was well organized. Principal cells were columnar, and their nuclei were lobulated. There were a few small lysosomes located apically to the nucleus. Basal cells had a typical elongated aspect. Clear cells could be divided into three distinct regions: endosomes (apical), lysosomes (median), and lipids (basal). Nuclei were round and located in the centre of the clear cells (Fig. 4D).

At 18 months, some clear cells had dramatically swollen; they appeared very active and were bulging into the lumen. Their lysosomal and lipid regions seemed greatly enlarged. (Fig. 4E). At 24 months, these cells retained a similar profile when spermatozoa were found in the lumen. However, in rats in which the lumen was occupied mostly by cell debris, most of the clear cells had lost their bulging protrusions; their nuclei were often irregular and pushed into the apical compartment (Fig. 4F). In this segment, principal and basal cells did not appear to be affected by aging.

***Corpus epididymidis***

Major changes in both principal and halo cells of the corpus epididymidis were seen during aging. At 3 months the epithelium was well organized. Principal and clear cells were cuboidal. Basal cells were elongated and located between principal cells. There were few halo cells (Fig. 5A). At 12 months, there was a minor increase

in the size of lysosomes in a few principal cells (not shown).

By 18 months, there was a major accumulation of lysosomes, both below and above the nucleus (Fig. 5B). This accumulation persisted at 24 months. Arrest of spermatogenesis was accompanied by a severe atrophy of the tubules of the distal corpus epididymidis in the old rats (not shown). The number of halo cells increased progressively with age. Giant cells full of lysosomes were seen on the basement membrane (Fig. 5C). Basal, clear and apical cells did not appear to be affected by age.

*Cauda epididymidis*

The major effect of age in the proximal cauda epididymidis was the emergence of a localized region with large vacuoles. There was no apparent change between 3 and 12 months in the appearance of any cell type. The epithelium was well organized; the height of cells was less than that in the caput epididymidis. The nuclei of principal cells were irregular and were located in the basal region. Basal cells and halo cells were similar to those of the previous segments. Apical cells were not seen. Clear cells had a typical round nucleus and could be divided into three compartments: endosomes, lysosomes and lipid droplets (Fig. 5D).

At 18 months and 24 months, clear cells were larger and filled with dense lysosomes. More distally, there was a region of the epithelium with large clear vacuoles, some of which had the width of several cells (Figs. 5, E and F). Serial sections of the proximal region of the cauda epididymidis in an 18 month old rat

revealed that the vacuoles had an oblong shape and a maximum length of 80 μm. Basal cells did not appear to change. No major change was seen in the epithelium of the distal cauda region with age (not shown).

## Electron Microscopy

### *Initial segment of the epididymis*

At 3 months, basal cells were flat and elongated. They were found at the surface of the basement membrane and had a round or elongated nucleus enclosed by a small amount of cytoplasm; few organelles were noted (Fig. 6A). The basement membrane was narrow (Fig. 6A). As early as 12 months, basal cells emitted pseudopods into the thickening basement membrane. The length of these pseudopods seemed to be proportional to the thickness of the basement membrane (Fig. 6B). Neighboring cells in contact with the basement membrane, such as principal or halo cells, did not show such pseudopods.

The number of halo cells increased progressively from 3 to 24 months. Halo cells had one of two typical appearances. Some had their normal characteristic features, i.e., large nuclei, pale staining cytoplasm and few dense core granules in close approximation to the Golgi apparatus. There was no apparent morphological difference in this type of halo cells between young and old rats (Fig. 7, A and B). They appeared to be recruited to the epithelium from blood vessels located in the interstitial space. Most of these halo cells were at the base of the epithelium. In

older rats, some halo cells were located more apically but they were never seen beyond the tight junctions between principal cells, nor were they found in the lumen. The other type of halo cells was large and was located at the base of the epithelium; these cells were filled with lysosomes whose content was highly heterogenous (Fig. 7C). Such halo cells were not seen at 3 months, but their number increased progressively with age (3 months, 0.15%; 18 months, 2.3 %; 24 months, 3.8%).

***Caput epididymidis***

Clear cells were greatly affected, whereas principal and basal cells did not appear to change with age, in the caput epididymidis. At 3 and 12 months, clear cells from the caput epididymidis were similar; they could be divided into three distinct compartments. The upper region was filled with numerous small pale-stained vesicles, endosomes, multi-vesicular bodies, few scattered cisternae of the rough endoplasmic reticulum, and many mitochondria. The nucleus was pale and round (Fig. 8A). The perinuclear region was occupied by electron dense lysosomes, probably containing lipofuscin. The basal region was filled by numerous lipid droplets. The lipid droplet and lysosome regions were clearly separated (Fig. 8A inset).

At 18 and 24 months, the nuclei of clear cells were irregular and often located in the apical half of the cell. The upper area of these cells contained small vesicles and multi-vesicular bodies. The dense lysosomes and lipid droplet compartments were greatly enlarged and not clearly separated (Fig. 8B). Lysosomes and lipid droplets

were often fused (Fig. 8B inset). Halo cells increased in number and size and could be classified into two groups, as described above for the initial segment (not shown).

***Corpus epididymidis***

At 3 and 12 months, principal cells had lipid droplets in the infranuclear region and few small lysosomes located mostly in the supranuclear region (Fig. 9A). There was a remarkable increase in the size and number of lysosomes at 18 months (Fig. 9B and Fig.10, A and B). Despite an increase in cell height, there was no difference in cell area. Between 3 and 18 months the percentage of the cell surface covered by lysosomes increased 16-fold (Fig. 10C). By 24 months, some principal cells showed just a few, albeit giant, lysosomes (Fig. 9C). Giant lysosomes were usually filled with translucent small vacuoles.

Surprisingly, apical cells were present in the corpus epididymidis of 3- and 12-month-old Brown Norway rats (Fig. 11A). They were funnel-like in shape, with a goblet-shaped nucleus, numerous Golgi stacks and small vesicles close to the trans face, and several mitochondria in the apical cytoplasm (Fig. 11B). By 18 months, all apical cells showed one giant lysosome filled with membranous whirls located apically to the nucleus (Fig. 11, C and D).

As in the proximal segment of the epididymis, the number of halo cells (Fig. 3) increased progressively with age. Halo cells appeared to be recruited from the circulation (Fig. 12). There was no age or region specific difference in the morphology of the classical type of halo cell (Fig. 13A). Mature halo cells number

increased with age (0.1 %, 3 months; 1.7%, 18 months, 3.7%, 24 months); these displayed a large Golgi apparatus, as seen in classical halo cell. However, the lysosomal content of the mature type of halo cell was different from that seen in the proximal region of the epididymis, and appeared to be region-specific. Lysosomes were filled with translucent vesicles, as seen in principal cells of that region (Fig. 13B). Eosinophils were found only in rats aged 18 months and older; these cells were in the interstitial tissue and, occasionally, within the epithelium (Fig. 14, A, B).

***Cauda epididymidis***

At 3 and 12 months, clear cells in the proximal cauda epididymidis had nuclei located at their base. The infranuclear region contained lipid droplets, and the supranuclear region was comprised of a few lysosomes of moderate and dense density, as well as endosomes (Fig.15A). At 18 and 24 months, clear cells located immediately proximal to and within the vacuole region, appeared larger and were filled with dense lysosomes. They had various morphologies (Fig. 15, B and C). Lysosome contents were either translucent vesicles or heterogenous (Fig. 15D); lysosomes of moderate density were rare.

At 18 months, some principal cells had a normal morphology while others contained large vacuoles. Cells that enclosed vacuoles had microvilli on the apical region, flattened cisternae of rough endoplasmic reticulum, and an irregular nucleus (Fig. 16A). Endosomes and lysosomes were frequently seen emptying their contents within large vacuoles (Fig. 16, B and C). Debris from spermatozoa was seen in

endosomes and in a few giant vacuoles at 18 and 24 months. Serial sections of these vacuoles suggested that entire spermatozoa could be found in vacuoles (Fig. 17, A and B). Some vacuoles were in principal cells, which were enclosed by neighbouring cells. Those principal cells had lost their contact with the basement membrane and did not reach the lumen.

## Discussion

The results presented above show that the histology of the epididymis of the Brown Norway rat is radically transformed by aging. Two of the characteristic features of aging, i.e., an increase in basement membrane thickness and a massive accumulation of lipofuscin, were found in the epididymis of the aging Brown Norway rat, further supporting the value of this model in studies of the mechanisms leading to senescence. In addition, four signs of aging found to be specific to the epididymis and described here for the first time are the following: (1) polymorphism of lysosomes, (2) presence of giant vacuoles, (3) spermiophagy, and (4) activation of the immune system.

The first characteristic sign of aging was a thickening of basement membranes, in both the epididymis and the seminiferous tubules of the aging Brown Norway rat. Although many reports have described similar change in the basement membrane thickness in various tissues, including the lung (human [28]), the kidney (rat [29]), capillaries (human [30]) , the retina (rat [31]), and seminiferous tubules (human [30]; rat [32]), the precise mechanisms triggering this increase have remained unresolved.

With advancing age, the basement membrane was found to become progressively thicker while spermatogenesis progressively decreased, as indicated by the narrower luminal diameter in the cauda epididymidal region at 24 months.

Shrinkage of the tissue, by reduction of volume of the luminal fluid, could induce a mechanical expansion of the basement membrane. However, the observation that the luminal diameter was unchanged in the remaining segments at all ages, while the basement membrane increased along the entire epididymis with age, is not consistent with this hypothesis.

Thickening of the basement membrane of the epididymis was measurable using the light microscope as early as 12 months. Ivy et al. [29] have shown similar increases in membrane thickness and the appearance of lysosomes, in young rats treated with a protease inhibitor, and in old rats. Thus, on the basis on these observations, it is inviting to speculate that there might be an age-related decrease in protease efficiency, leading to changes in basement membrane protein turnover and ultimately thickening. An alternative explanation would be an increase in exocytosis of material, emptied from the epithelium into the basement membrane; however the absence of noticeable morphological changes in the epithelium of the epididymis at 12 months suggests that this is not a critical factor.

It is interesting to note that an increase in the basement membrane in the epididymis has been reported previously to follow castration of the prepubertal rat [33]. Although there may be a decrease in the secretion of some substances by the testis as early as 12 months, no change has been reported for spermatogenesis; nevertheless, decreases in testosterone production [24], and possibly dihydrotestosterone [20], are in agreement with a change in endocrine or paracrine environment. In addition, intrinsic changes in the basement membrane cells could

modify susceptibility to this environment and trigger modifications of synthetic and/or degradative pathways.

Thickening of the basement membrane might eventually impair the normal function of the epithelium. The emission of pseudopods by basal cells into the thick basement membrane could possibly be an attempt to maintain epithelial homeostasis. Indeed, by increasing the surface contact, these pseudopods may boost in the efficiency of uptake of paracrine and endocrine products that may have been compromised by the thick basement membrane.

The second characteristic sign of aging we noted was a progressive, marked accumulation of lysosomes in the epithelial cells of the epididymis of the Brown Norway rat. Lysosomes normally contain hydrolytic enzymes that phagocytose either damaged cellular organelles or extracellular products, and can store lipofuscin, the major undigested material. It is now well established that lipofuscin accumulates progressively during aging in post-mitotic cell types in various tissues of many species [29, 31, 34, 35]. Several studies have shown that the process of lipofuscin accumulation is complex and multifactorial; however, there is considerable evidence pointing to a combination of oxidative damage and decline of the degradative pathways as causative factors in aging [29, 35-37].

The buildup of oxidative damage occurs when the antioxidant defence system cannot compensate for oxidative stress. Accumulation of lipofuscin has been reported in epithelial cells after experimental ischemia [38]. Ischemia can be induced by atherosclerotic changes; such as have been reported in the epididymides of older

men [17]. Nevertheless, atherosclerosis is probably not the main factor in lipofuscin accumulation in the aging Brown Norway rat, since no major changes in vascularisation were noticed, except the presence of a highly tangled testicular artery in one of the 24-month-old rats. However, damage could be due to the aftermath of an impaired oxidative shield. Further studies will be needed to determine whether factors involved in protection from oxidative damage are altered during aging [37].

Experimental models in which lysosomal enzyme inhibitors are used lead to an accumulation of lipofuscin-like dense bodies in treated regions in young rats (kidney [29]; brain [39]). Thus, we can speculate that lysosomal proteinases are absent or defective in aged epithelial cells of the epididymis of the Brown Norway rat. The observed accumulation of lysosomes may reflect an age-related defect in intracellular catabolism. The activation of the lysosomal system during normal aging and neurodegenerative conditions such as Alzheimer's disease [40, 41] may appear as contradictory. However, this modification of lysosomal activities could be a response induced by an age-related increase in the numbers of intracellular organelles or in the amount of endocytosed extracellular products susceptible to degradation.

The first of the age-related changes that are specific to the epididymis was the accumulation of lysosomes in specific cells and segments. The reason why one cell type was affected in one particular segment but not in others is still unclear. However, one can speculate that this phenomenon is related to the segment-specific function and regulation of each epithelial cell type [6, 7, 10, 11, 20]. Thus, segment-

specific properties of each cell type entail different susceptibilities to qualitative or quantitative changes in lysosomal substrate. Strikingly, the material present in lysosomes was polymorphic. Interestingly, lysosomes had a similar appearance in principal and mature halo cells in the corpus epididymidis region.

The second specific sign of aging of the epididymis was that, in the 18-month-old Brown Norway rats, giant vacuoles appeared in some principal cells within a localized region of the proximal cauda epididymidis. To our knowledge, only one previous study [19] described the effect of aging on the histology of the epididymis; in that rabbit model, the presence of vacuoles albeit smaller ones, was also noted, in principal cells in the distal region of the epididymis of older rabbits. Because vacuoles could be found whether spermatogenesis was active or not, we suggest that emergence of vacuoles in the mammalian epididymis could be an intrinsic mechanism of aging of principal cells. In addition, the presence of a pale-staining material within the lumen of vacuoles points to the endosomal nature of these vacuoles and indicates a disruption of the endosomal-lysosomal cycling system with age.

Homeostasis of cells depends on the accurate routing of proteins in both biosynthetic and endocytic pathways [42]. We observed, in vacuolated principal cells, an apparently normal morphology of the endoplasmic reticulum and of the Golgi apparatus. However, we noted endosomes and, surprisingly, even dense lysosomes, fusing and emptying their content into the lumen of vacuoles. The relative absence of dense lysosomes in vacuolated principal cells suggests that

mechanisms controlling endosome maturation are impaired as animals age. Furthermore, the active fusion of both endocytic vesicles and dense lysosomes with pre-existing endosomes suggests a dramatic modification of the mechanism controlling the fusion of vesicles.

Vacuoles found in the epididymal epithelium of Brown Norway rats reached up to 80 micrometers; this is wider than the width of several principal cells. Moreover, the number of principal cells in the affected region appeared to be reduced. Usually neighboring cells took on a C-shaped appearance around vacuolated principal cells. Thus, expanding vacuoles may have been pushing away the nuclei of neighboring principal cells. An alternative explanation is that this decrease in cell number involves a mechanism of phagocytosis either by "normal" or by vacuolated principal cells. The presence of vacuoles within cells that neither reach the epithelium nor touch the basement membrane, but rather are enclosed, suggests phagocytosis of the vacuolated principal cells by neighboring cells. However, the paucity of lysosomes in neighboring principal cells favors the hypothesis that vacuolated cells engulf neighboring cells.

The third age-related striking change in the epididymis was the presence of debris from spermatozoa in small endosomes and giant vacuoles, despite the apparent absence of opening of the endosomal vacuoles into the lumen. Spermiophagy has been reported in the spermathecal epithelium, the site of sperm storage of the female salamander [43]. Intraluminal phagocytosis of spermatozoa by macrophages has been reported in the epididymis of monkey and [44] ; in the cauda

epididymidis and vas deferens in rats, mice, hamsters and guinea pigs [45, 46]; as well as in the bat [47]. The occurrence of spermiophagy increases in the rat after surgical or pathological obstruction of the excurrent duct [38, 48, 49]. However, there are very few reports of uptake and phagocytosis of spermatozoa by epithelial cells under normal conditions (human [44]). Thus, we report, for the first time, an active endocytosis and phagocytosis of spermatozoa by principal cells of the proximal cauda epididymidis of nontreated rats.

Finally, the fourth specific change in the epididymis was the apparent activation of the immune system. It is well established that there is a gradual decrease in normal immune function in both men and rodents with advancing age [50, 51]. Two lines of local immune defense have been described in the epididymis [52]; intra-epithelial lymphocytes, mostly subgroup CD8+, lymphocyte T cytotoxic/suppressor cells [53], and intraepithelial macrophages [8,14]. An increased occurrence of autoimmune disorders and focal lymphocytic infiltration has been reported previously in various tissues, such as adrenal cortex [51], kidney [54] and brain [55]; and these have been associated with an alteration of T-cell functions in the elderly.

The age-related immune system activation was characterized by a dramatic increase in the number of characteristic halo cells and the emergence of "mature" halo cells within the epithelium. Neither of these forms of halo cells usually made contact with the basement membrane. The first presented many features of monocytes [6], whereas the latter had many phagocytic inclusions. An age-related

increase in the number of both lymphocytes and macrophages has been reported previously in the human epididymis [14]. It was suggested that these macrophages were derived from blood monocytes [14, 56]. Frequently, we noted a trail of immune cells leaving blood vessels, to reach the epithelium, suggesting an active migration of halo cells (Fig. 12). This observation leads us to suggest that halo cells are monocytes, and that a proportion of the halo cells that migrate into the epididymis in older animals is activated into macrophages.

Intriguingly, the appearance of lysosomes in "mature" halo cells was segment-specific, suggesting that they may have different substrates in the proximal and distal segments of the epididymis. Moreover, all halo cells were located between epithelial cells, stopped by the zonula occludens; they were never seen in the epididymal lumen. This suggests that the lysosome substrate is present in the epithelium and, perhaps, reaches halo cells by transcytosis. However, increases in the number of lymphocytes and macrophages in the epididymis have been reported after vasectomy, at a time when significant degeneration of spermatozoa occurs [56]. Therefore, spermatozoal antigens leaking through the blood-epididymis barrier could be an alternative substrate.

In addition, at 18 and 24 months, some eosinophils were seen in the interstitial tissue near blood vessels and reached the epithelium of the epididymis of the aged rat; they were found only in the corpus and cauda epididymidis whose morphology was dramatically affected. These eosinophils were in direct contact with mature halo cells. Thus, mature halo cells, involved in phagocytosis, may create

sites that contain antigen-antibody complexes, thereby attracting eosinophils. In light of the previous finding, during aging an immune defense mechanism may be activated, possibly to segregate sperm antigens from the circulation.

In summary, our observations on the effect of age on the morphology of the epididymis of the Brown Norway rat show major quantitative and qualitative changes in this tissue. Thickening of the basement membrane was the first sign of aging, possibly caused by changes in the hormonal environment. Endocrine factors might have increasing difficulty in reaching the epididymis, which could eventually impair the homeostasis of the epithelium. Indeed, epithelial cells showed dramatic accumulation of lysosomes. Polymorphism of lysosomes suggested a substrate specificity, and demonstrated the segment-specific nature of epididymal cell regulation. Furthermore, both lysosome accumulation and the emergence of endosomal vacuoles indicated that age greatly affected intracellular traffic. In addition, a spermatophagic function was shown for the first time in principal cells of the proximal cauda epididymidis, and is proposed to be triggered by abnormal or degenerating spermatozoa. Finally, there was an age-related activation of the immune system, as shown by the presence of macrophages and eosinophils. In conclusion we have demonstrated the age-dependant emergence of morphological changes that are epididymal segment-specific, characteristic markers of aging. All changes were seen when spermatogenesis was still active, raising the possibility of an intrinsic mechanism of aging.

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**Fig.1.** Diagrammatic representation showing how the epididymis was sectioned for the studies presented. **1**) initial segment and **2**) caput, **3**) corpus, and **4**) proximal cauda epididymidis.

## EPIDIDYMIS

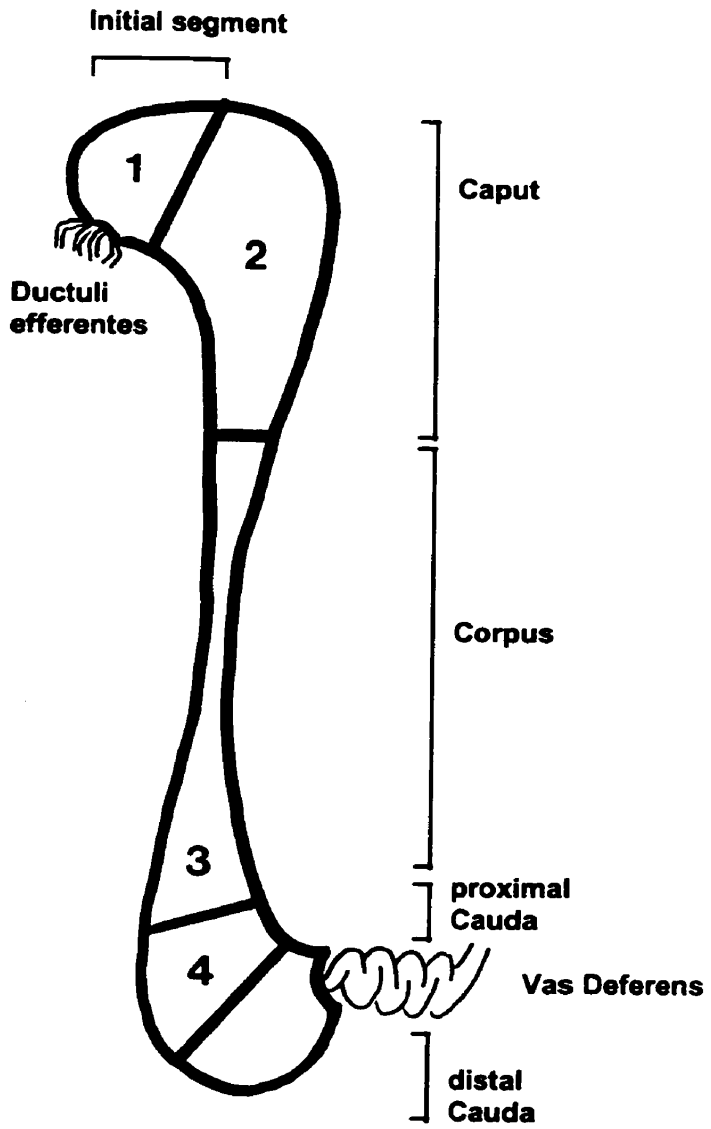
Initial segment
1
2
Ductuli efferentes
Caput
Corpus
3
proximal Cauda
4
Vas Deferens
distal Cauda

**Fig. 2.** Effect of age on the luminal diameter (**A**), epithelium height (**B**), and thickness of lamina propria (**C**), in the four segments of the epididymis shown in Figure 1. Bars represent means ± SEM. Left hatched bar: 3 months; spotted bar: 12 months; right hatched bar: 18 months; crossed-hatched bar: 24 months. n=3. * Values that differ significantly from 3 months, $p < 0.05$.

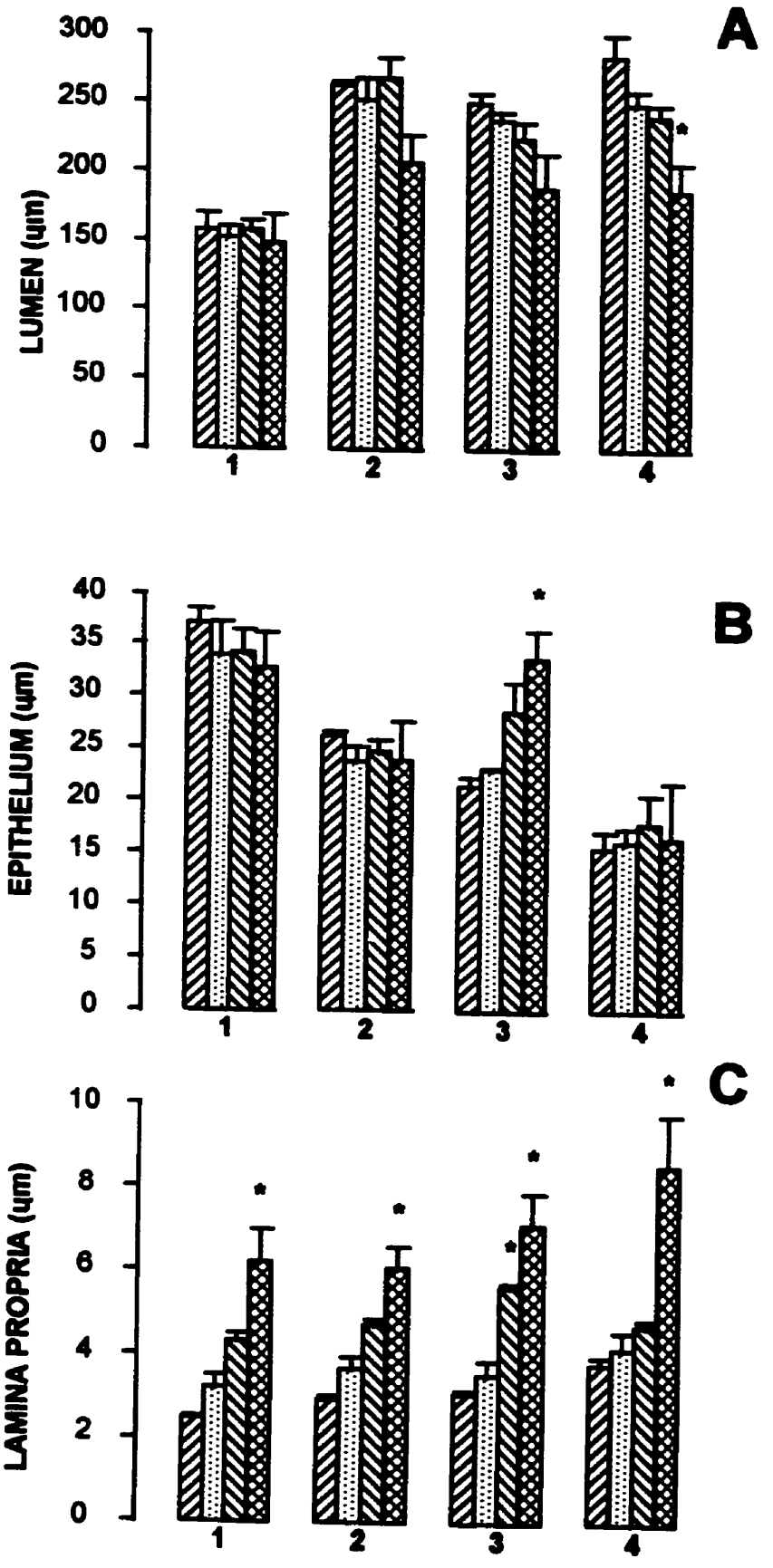
A
LUMEN (µm)
300
250
200
150
100
50
0
1
2
3
4
*
B
EPITHELIUM (µm)
40
35
30
25
20
15
10
5
0
1
2
3
4
*
C
LAMINA PROPRIA (µm)
10
8
6
4
2
0
1
2
3
4
*

**Fig. 3.** Relative cell distributions in four segments (Fig.1) of the epididymis (% of total) at 3, 18, and 24 months in the Brown Norway rat. **A**) Initial segment; **B**) caput; **C**) corpus; **D**) proximal cauda. Bars represent means ± SEM. Right hatched bar: 3 months; right hatched bar: 18 months; crossed-hatched bar: 24 months. n=3. * Values that differ significantly from 3 months, $p < 0.05$.

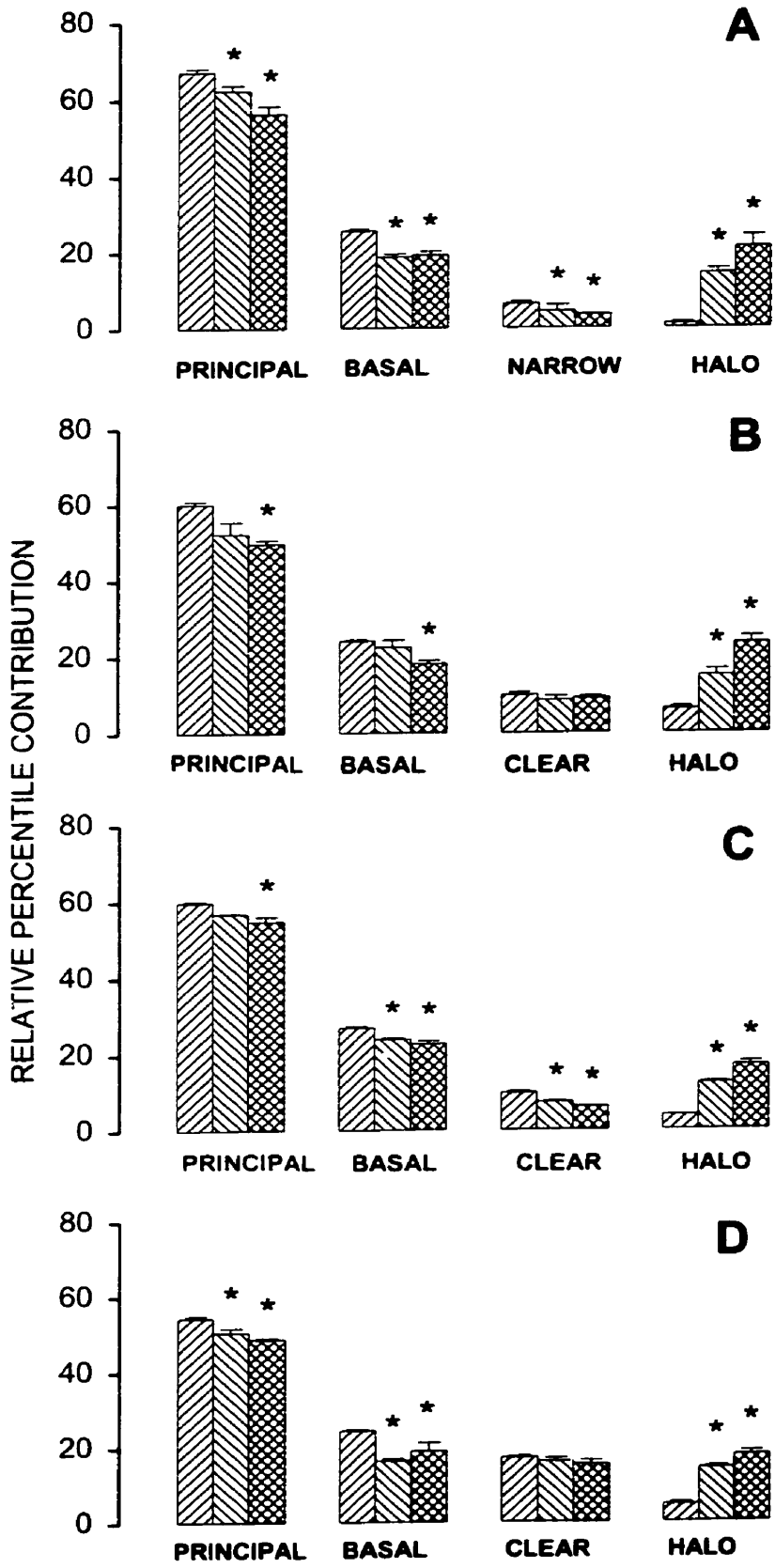
A
B
C
D
RELATIVE PERCENTILE CONTRIBUTION
80
60
40
20
0
PRINCIPAL
BASAL
NARROW
CLEAR
HALO
*

**Fig. 4.** Light micrographs showing the effect of age on the morphology of tubules along the proximal segments of the epididymis. **A-C**) initial segment, and **D-F**) caput epididymidis of the Brown Norway rat. **A**, **D**) 3 months; **B**, **E**) 18 months; **C**, **F**) 24 months. Lu, lumen; P, principal cell; B, basal cell; C, clear cell; N, narrow cell; A, apical cell; H, halo cell; bm, basement membrane; IT, intertubular space. Scale bar, **A-F** = 18 µm.

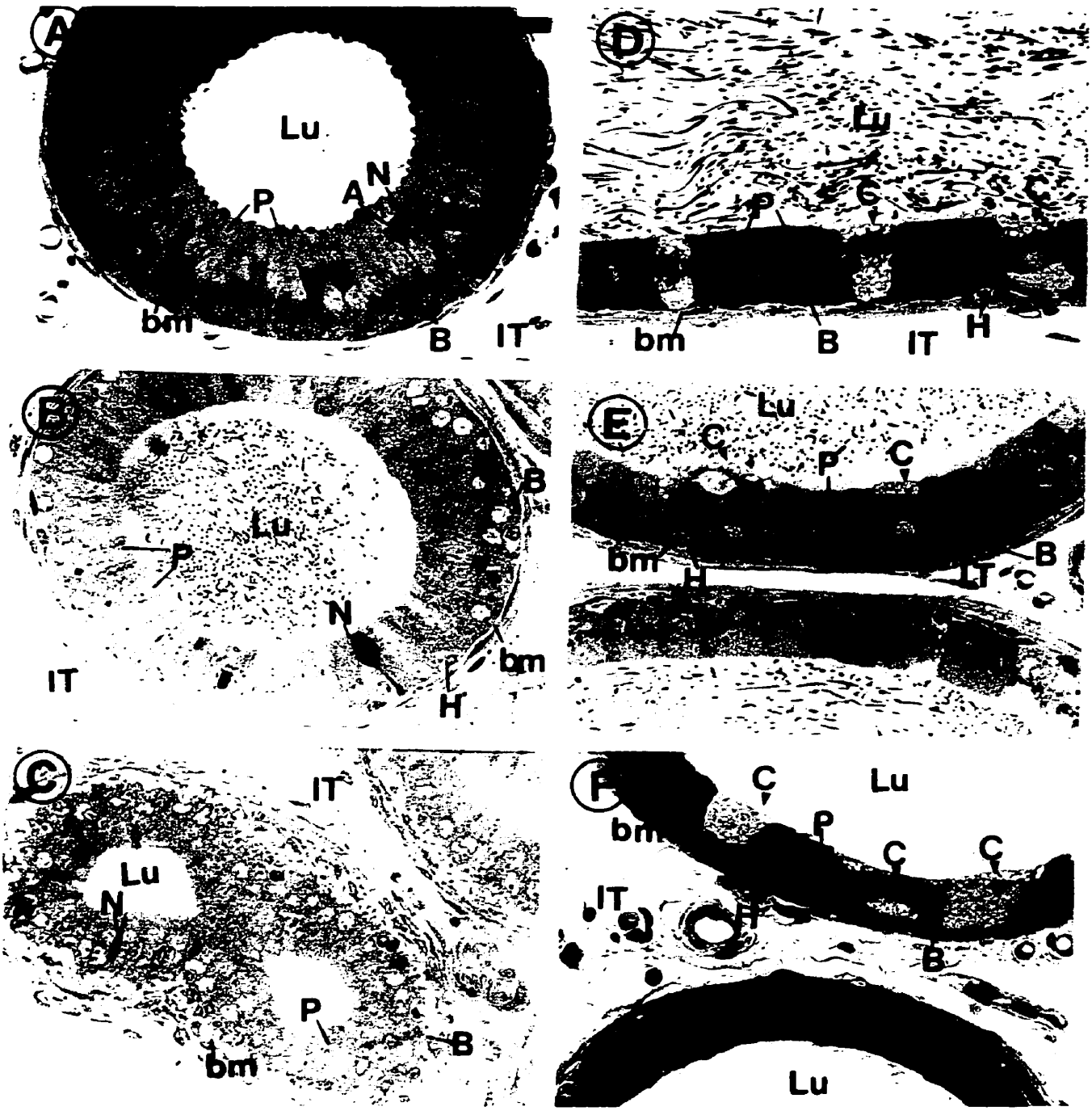
A
Lu
N
A
P
bm
B
IT
D
Lu
P
C
C
bm
B
IT
H
B
Lu
P
N
B
bm
IT
H
E
Lu
C
P
C
bm
B
H
IT
C
C
Lu
IT
Lu
N
P
B
bm
F
C
Lu
bm
P
C
C
IT
H
B
Lu

**Fig. 5.** Light micrographs showing the effect of age on the morphology of tubules along the distal segments of the epididymis. **A-C**) Corpus and **D-F**) proximal cauda epididymidis of the Brown Norway rat. **A**, **D**) 3 months; **B**, **E**) 18 months; **C**, **F**) 24 months. Lu, lumen; P, principal cell; B, basal cell; C, clear cell; A, apical cell; H, halo cell; Vc, vacuole; bm, basement membrane; IT, intertubular space. Scale bar, **A-F**= 18 µm.

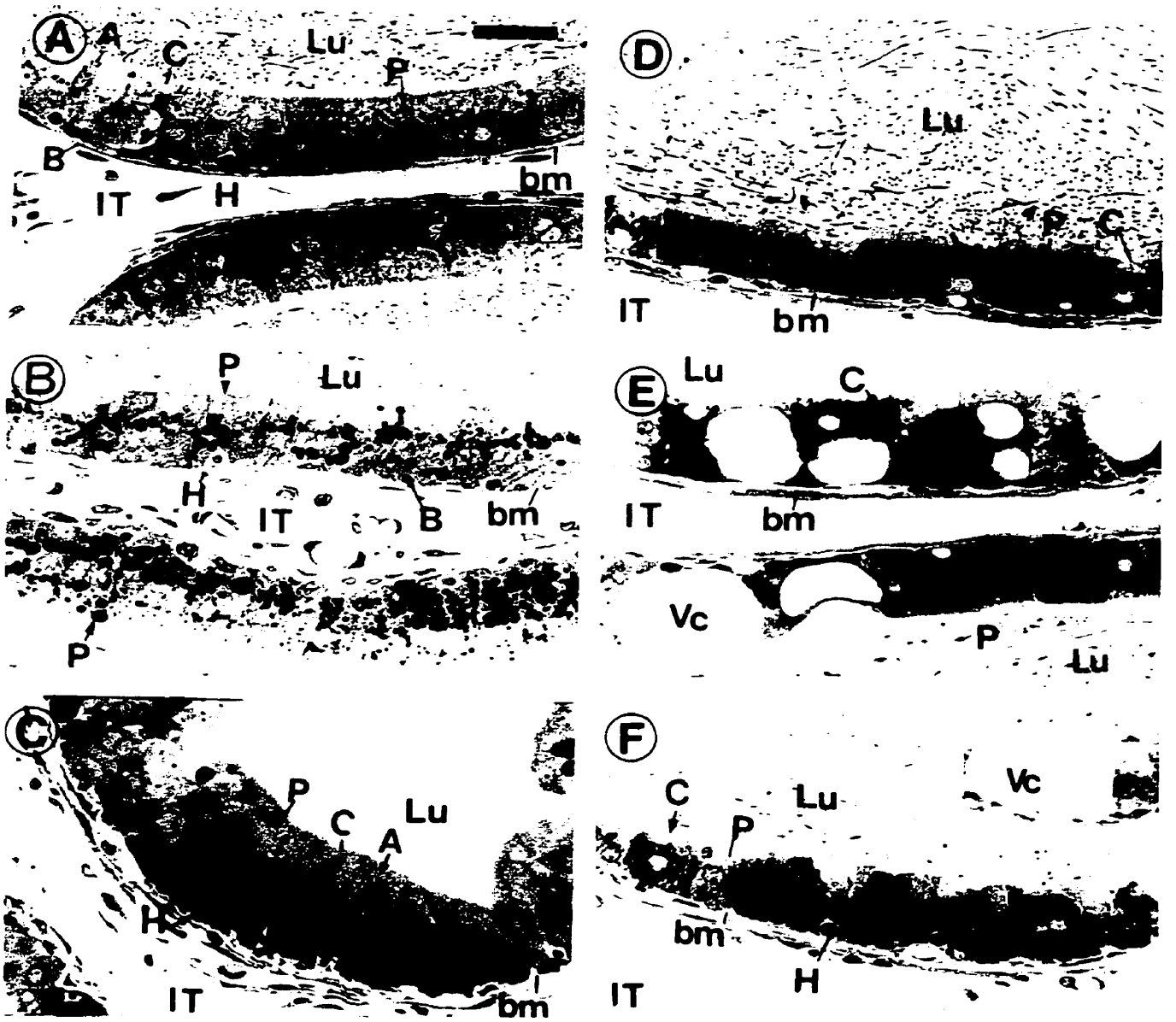
A
A
C
Lu
P
B
IT
H
bm
D
Lu
P
C
IT
bm
B
P
Lu
H
IT
B
bm
P
E
Lu
C
IT
bm
Vc
P
Lu
C
P
C
A
Lu
H
IT
bm
F
C
Lu
Vc
P
bm
H
IT

**Fig. 6.** Electron micrographs showing the effects of age on the basal cells of the initial segment of the epididymis in the Brown Norway rat. **A**) 3 months; **B**) 18 months. P, principal cell; H, halo cell; B, basal cell; N, nucleus; m, mitochondria; ER, rough endoplasmic reticulum; ps, pseudopods; bm, basement membrane; MY, myoid cell; IT, intertubular space. Scale bar, **A**, **B** = 1.2 μm.

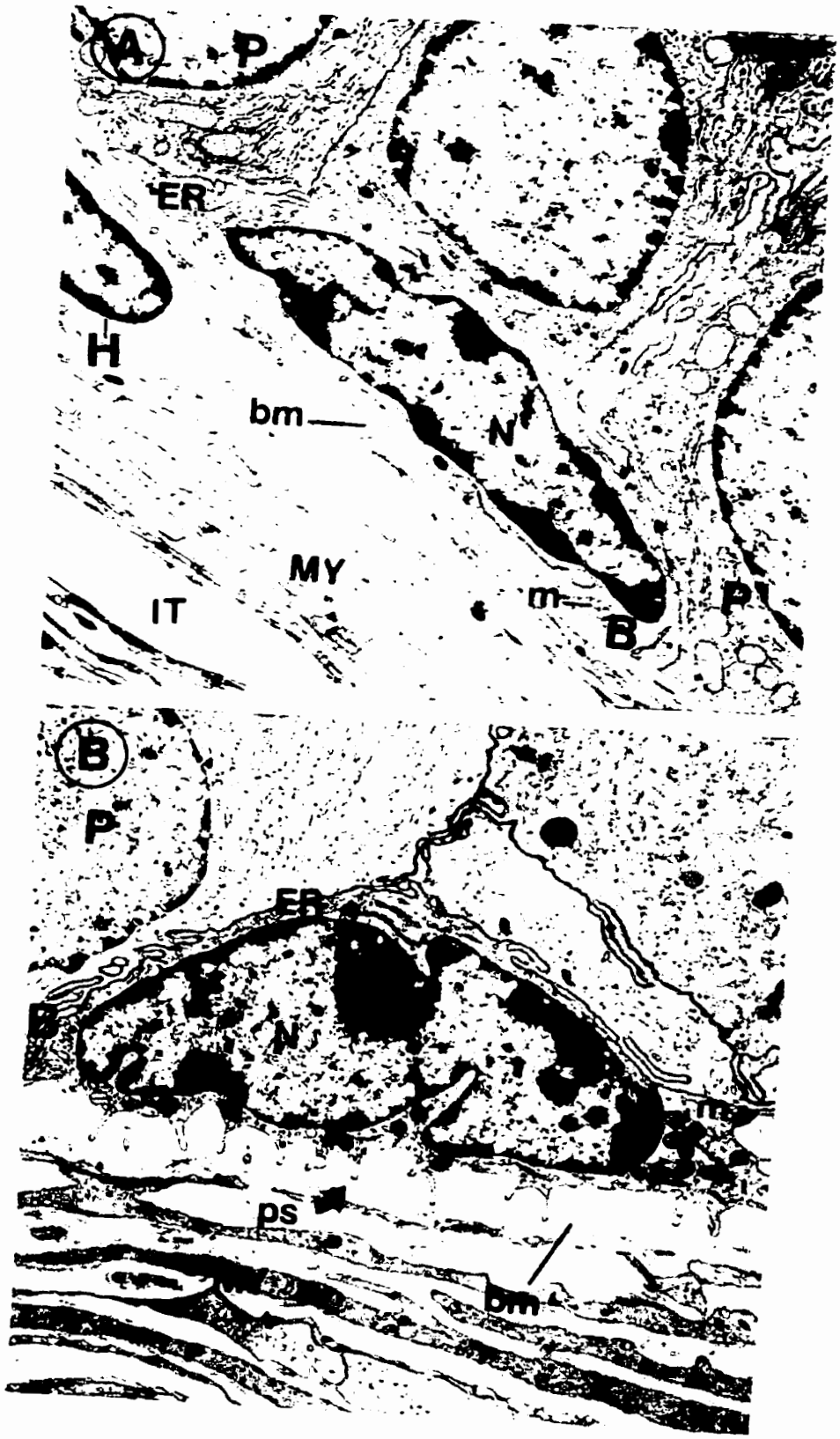
A
P
ER
H
bm
N
MY
IT
m
B
P
B
P
ER
N
ps
bm

**Fig. 7.** Electron micrographs showing the effect of age on the halo cells of the initial segment and caput epididymidis of the Brown Norway rat. Classical halo cell in **A**) a 3-month-old and **B**) 18-month-old rat; **C**) mature halo cell in an 18-month-old rat. P, principal cell; B, basal cell; H, halo cell; Ly, lysosomes; N, nucleus; m, mitochondria; ER, rough endoplasmic reticulum; G, Golgi apparatus; bm, basement membrane; MY, myoid cell. Scale bar, **A-C** = 1.6 µm.

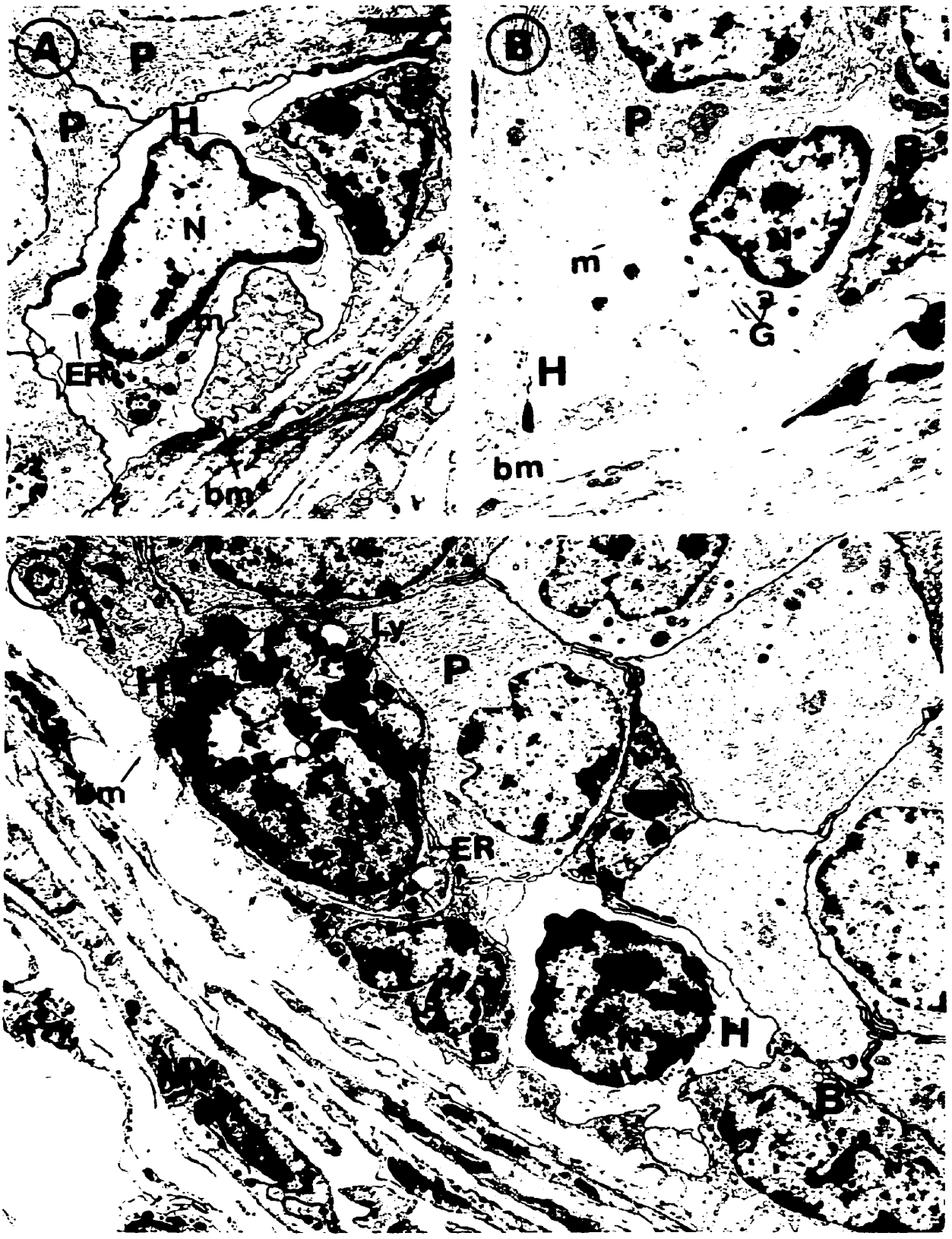
A
P
P
H
N
m
ER
bm
B
P
N
m
G
H
bm
C
Ly
H
P
m
ER
H
B
B

**Fig. 8.** Electron micrographs showing the effects of age on the clear cells of the caput epididymidis in the Brown Norway rat. **A**) 3 months; **B**) 18 months. Lu, lumen; P, principal cell; B, basal cell; H, halo cell; C, clear cell; E, endosomes; Ly, lysosomes; Li, lipid droplets; bm, basement membrane; MY, myoid cell. Scale bar, **A**, **B**= 3.8 µm.

**A**) Inset: High-power electron micrograph of lipid droplets and a lysosome at 3 months. **B**) Inset: High-power electron micrograph of a lipid droplet fused with a lysosome at 18 months. Scale bar (insets, **A**, **B**) = 0.6 µm.

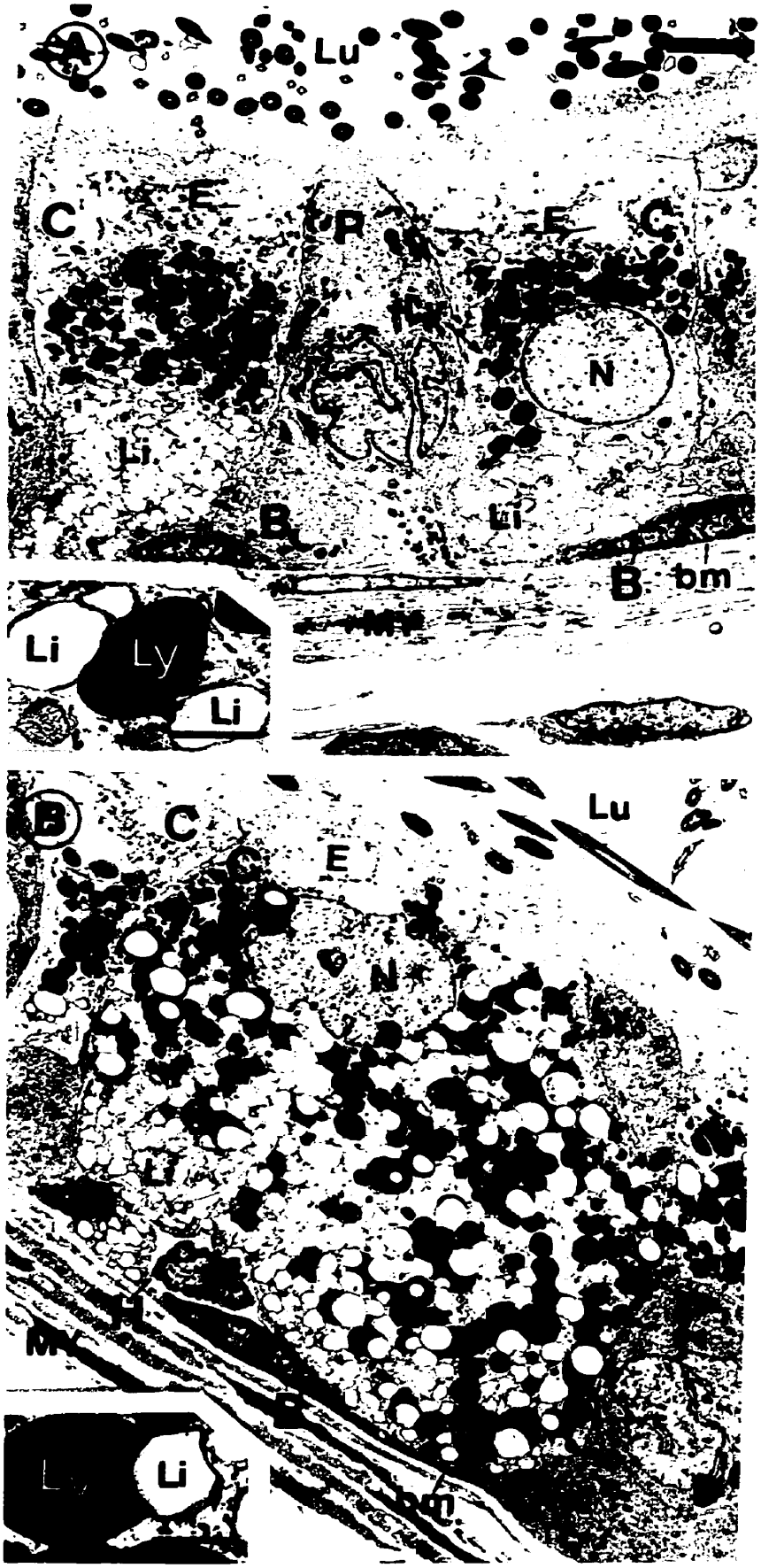
A
Lu
C
E
R
E
C
N
Li
B
Li
B
bm
Li
Ly
Li
B
C
E
Lu
N
Li
Li
Ly
Li

**Fig. 9.** Electron micrographs showing the effects of age on the principal cells of the corpus epididymidis in the Brown Norway rat. **A**) 3 months; **B**) 18 months; **C**) 24 months. Lu, lumen; P, principal cell; B, basal cell; C, clear cell; H, halo cell; A, apical cell; N, nucleus; Ly, lysosomes; ER, rough endoplasmic reticulum; bm, basement membrane; Ca, capillary; MY, myoid cell. Scale bar, **A-C** = 4.3 μm.

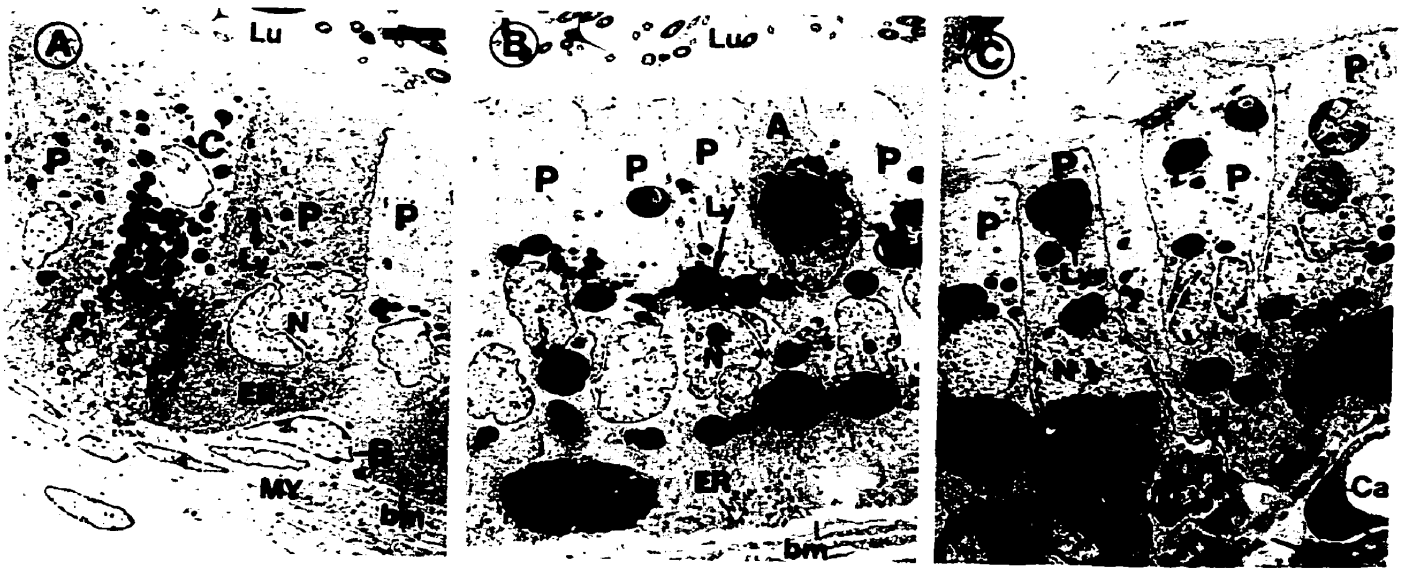
A
Lu
P
C
P
P
L
N
ER
MY
bm
B
Lu
P
P
P
A
P
Ly
N
ER
bm
C
P
P
P
Ly
N
Ca

**Fig. 10.** Effects of age (3 months, 18 months) on the lysosomes in the principal cells of the epididymis in the Brown Norway rat. **A**) Average area of lysosomes; **B**) average number of lysosomes per cell; **B**) percentage of cell surface occupied by lysosomes. Bars represent means ± SEM. n=3, • Values that differ significantly from 3 months, $p < 0.05$.

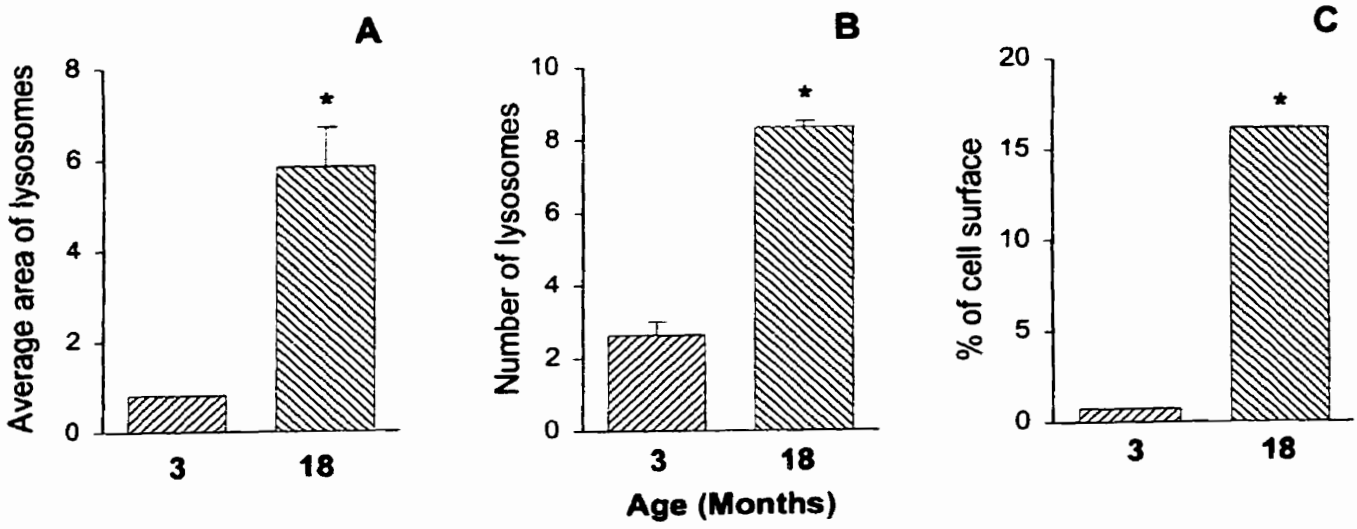
A
B
C
Average area of lysosomes
Number of lysosomes
% of cell surface
0
2
4
6
8
10
5
15
20
*
3
18
Age (Months)

**Fig. 11.** Electron micrographs showing the effects of age on the apical cells of the corpus epididymidis in the Brown Norway rat. Epithelium in the corpus showing apical cells at 3 months (**A**, **B**) and 18 months (**C, D**). **B**, **D**) Magnification of the supranuclear region of the apical cells in **A** and **C**, respectively. Lu, lumen; P, principal cell; B, basal cell; A; apical cell; N, nucleus; G, Golgi apparatus; Ly, lysosome; mw, membranous whirls; m, mitochondria; ER, rough endoplasmic reticulum; Li, lipid droplets; bm, basement membrane. Scale bar : **A**, **C** = 6.5 μm; **B, D** = 1.0 μm.

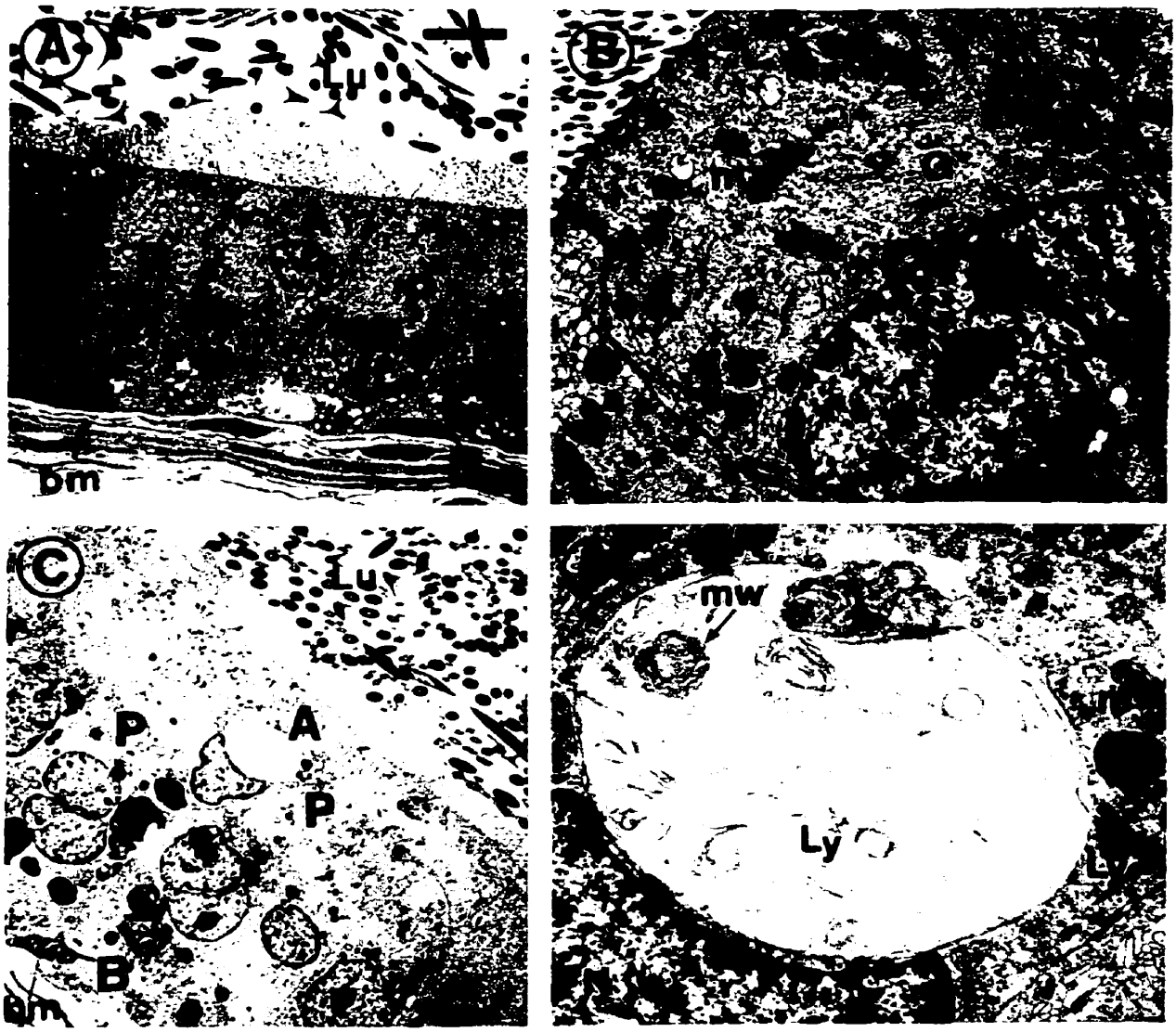
A
Lu
bm
B
n
C
Lu
P
A
P
B
bm
mw
Ly

**Fig. 12.** Electron micrograph showing the apparent migration of immune cells from a capillary into the epithelium in the corpus epididymidis of an 18-month-old Brown Norway rat. P, principal cell; B, basal cell; H, halo cell; MY, myoid cell; bm, basement membrane; Ca, capillary. Scale bar = 2.1 μm.

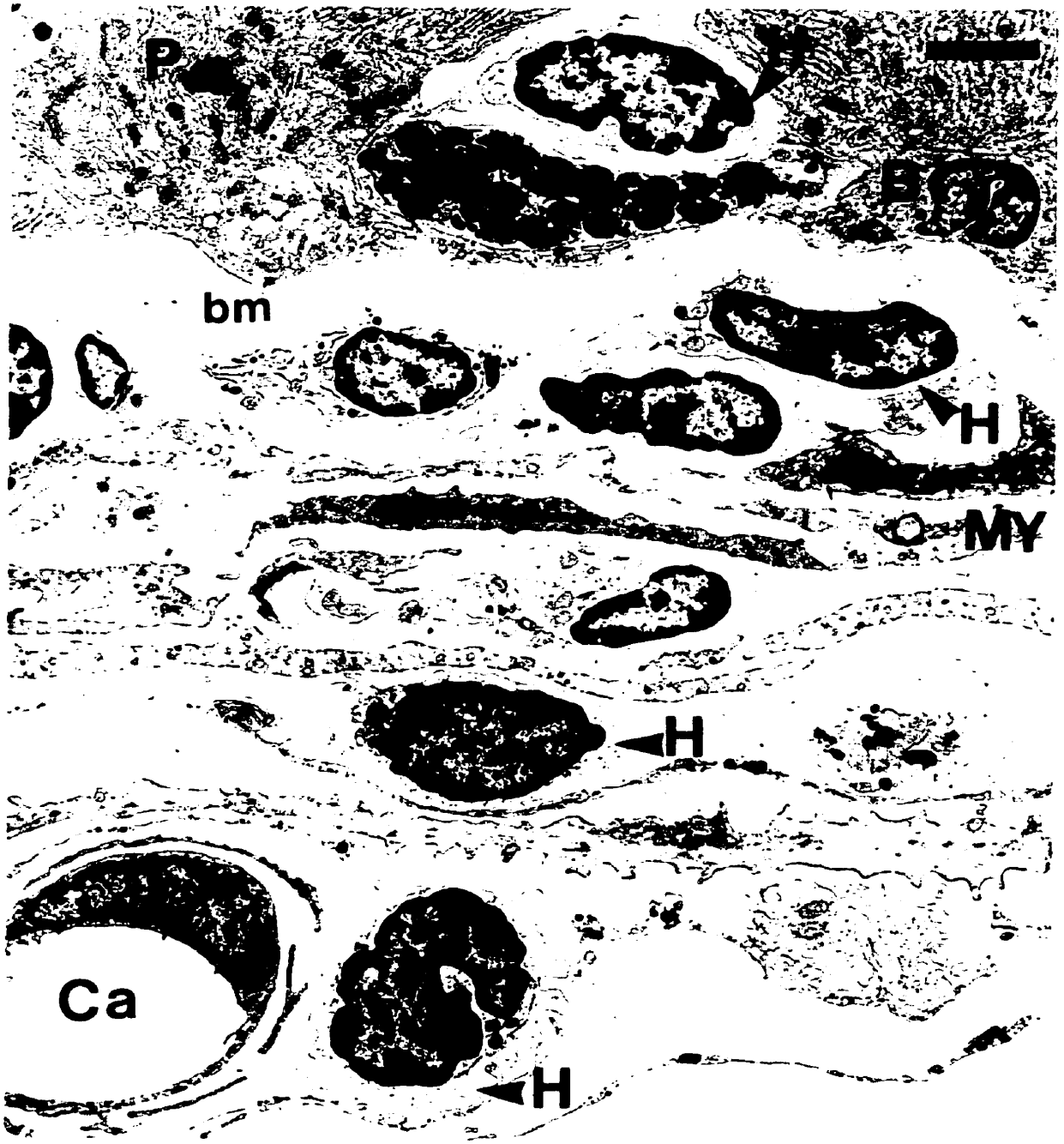
P
bm
H
MY
H
Ca
H

**Fig. 13.** Electron micrographs of the effect of age on the halo cells in the corpus and proximal cauda epididymidis of the Brown Norway rat. Two types of halo cell were present at 18 months: **A**) Classical halo cell, and **B**) mature halo cell. P, principal cell; H, halo cell; Ly, lysosomes; N, nucleus; G, Golgi; ER, rough endoplasmic reticulum; m, mitochondria; bm, basement membrane. Scale bar: **A** = 1.6 μm; **B** = 2.1 μm.

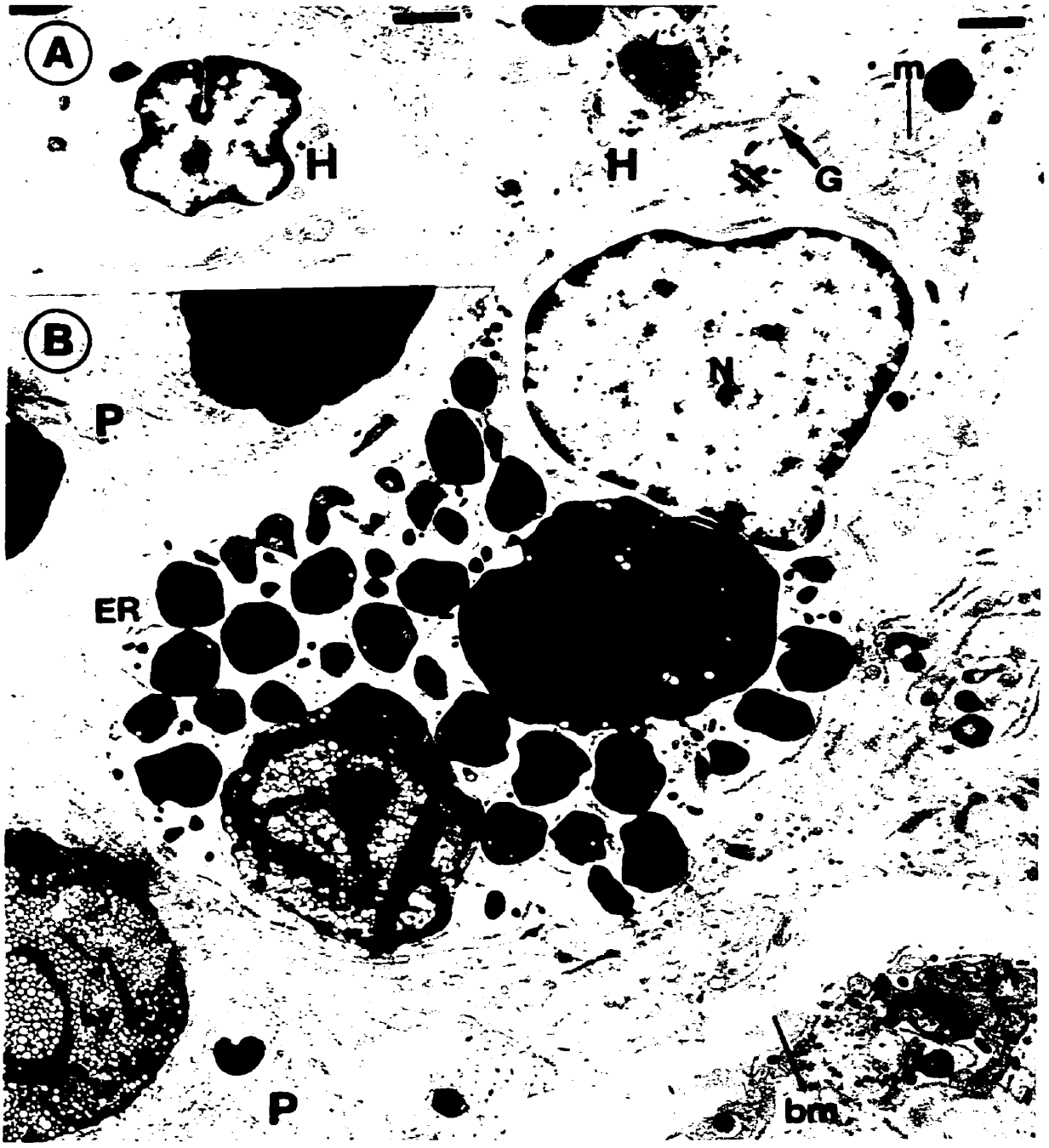
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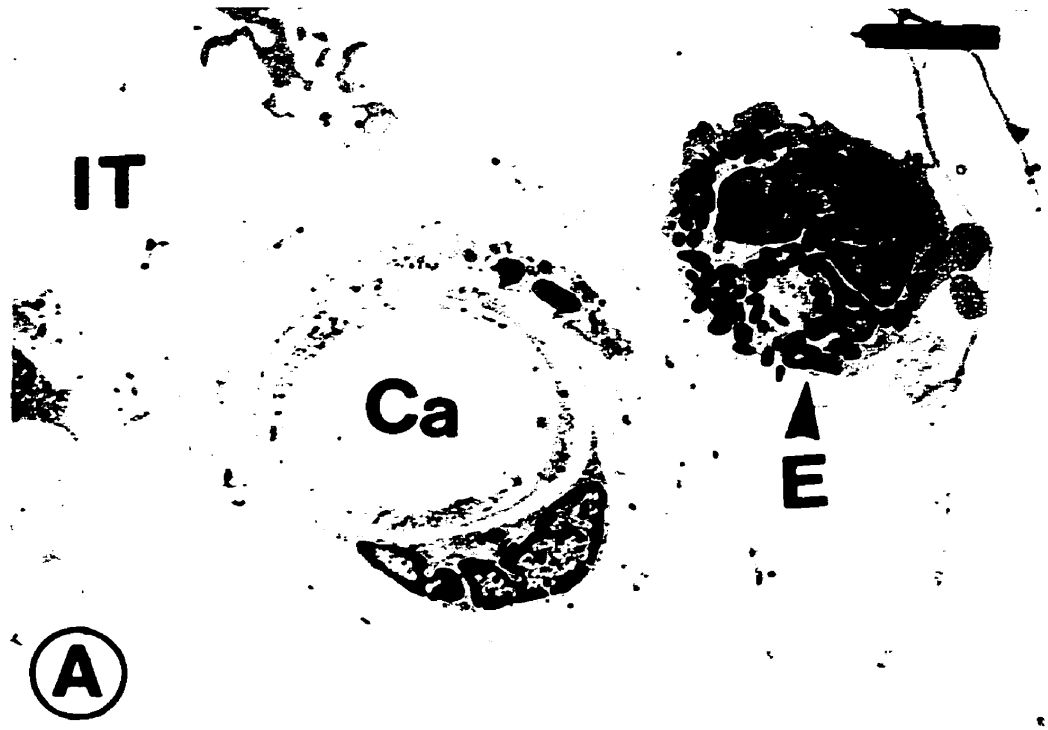
**Fig. 14.** Electron micrographs showing the presence of eosinophils in the corpus epididymidis in an 18-month-old Brown Norway rat. **A**) interstitial tissue; **B**) epithelium. P, principal cell; E, eosinophil; N, nucleus; IT, intertubular space; bm, basement membrane; Ca, capillary. Scale bar: **A** = 2.1 µm; **B**= 2.7 µm. **B)** Inset: High-power electron micrograph of a lysosome with a crystalloid core, Cc. Scale bar (inset, **B**) = 0.3 µm.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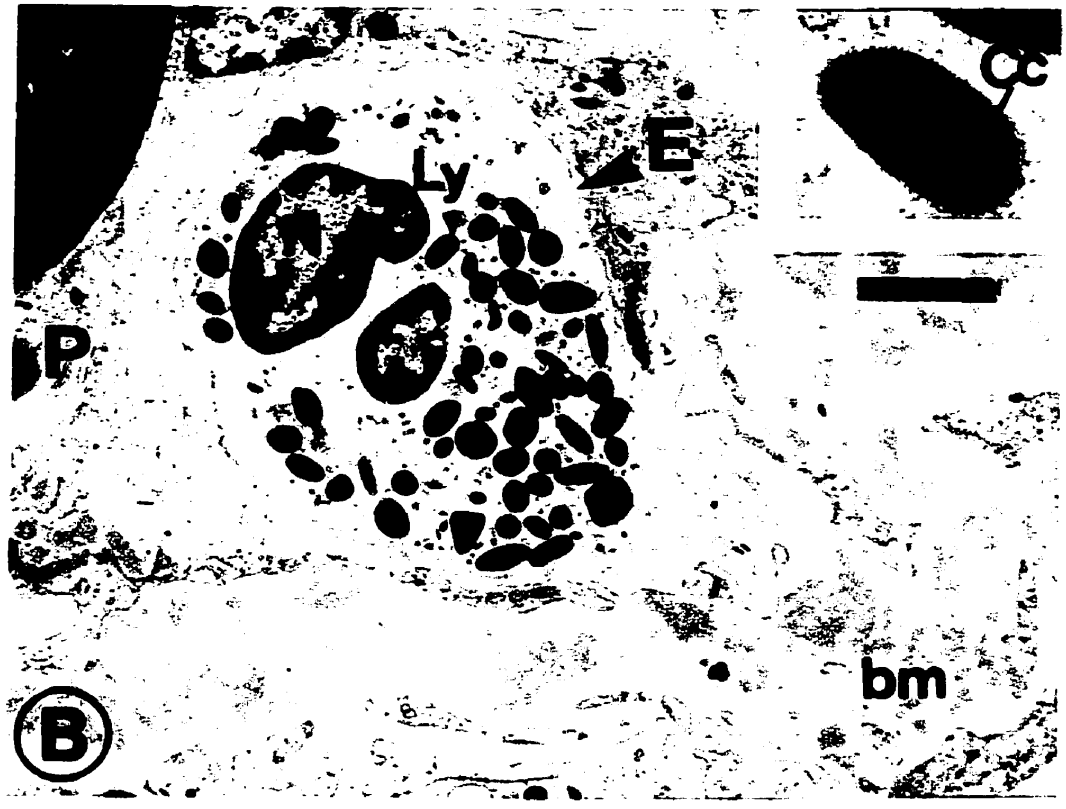
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**Fig. 15.** Electron micrographs showing the effects of age on the clear cells of the proximal cauda epididymidis in the Brown Norway rat. **A**) 3 months; **B**,**C**) 18 months; **D**) high-power magnification of a lysosome from **C**, indicated in the box. Lu, lumen; C, clear cell; P, principal cell; B, basal cell; H, halo cell; m, mitochondria; E, endosomes; Ly, lysosomes; N, nucleus; bm, basement membrane; Ca, capillary. Arrow: electron-dense material enclosed by membranes. Scale bar: **A-C** = 4 μm; **D** = 0.5 μm

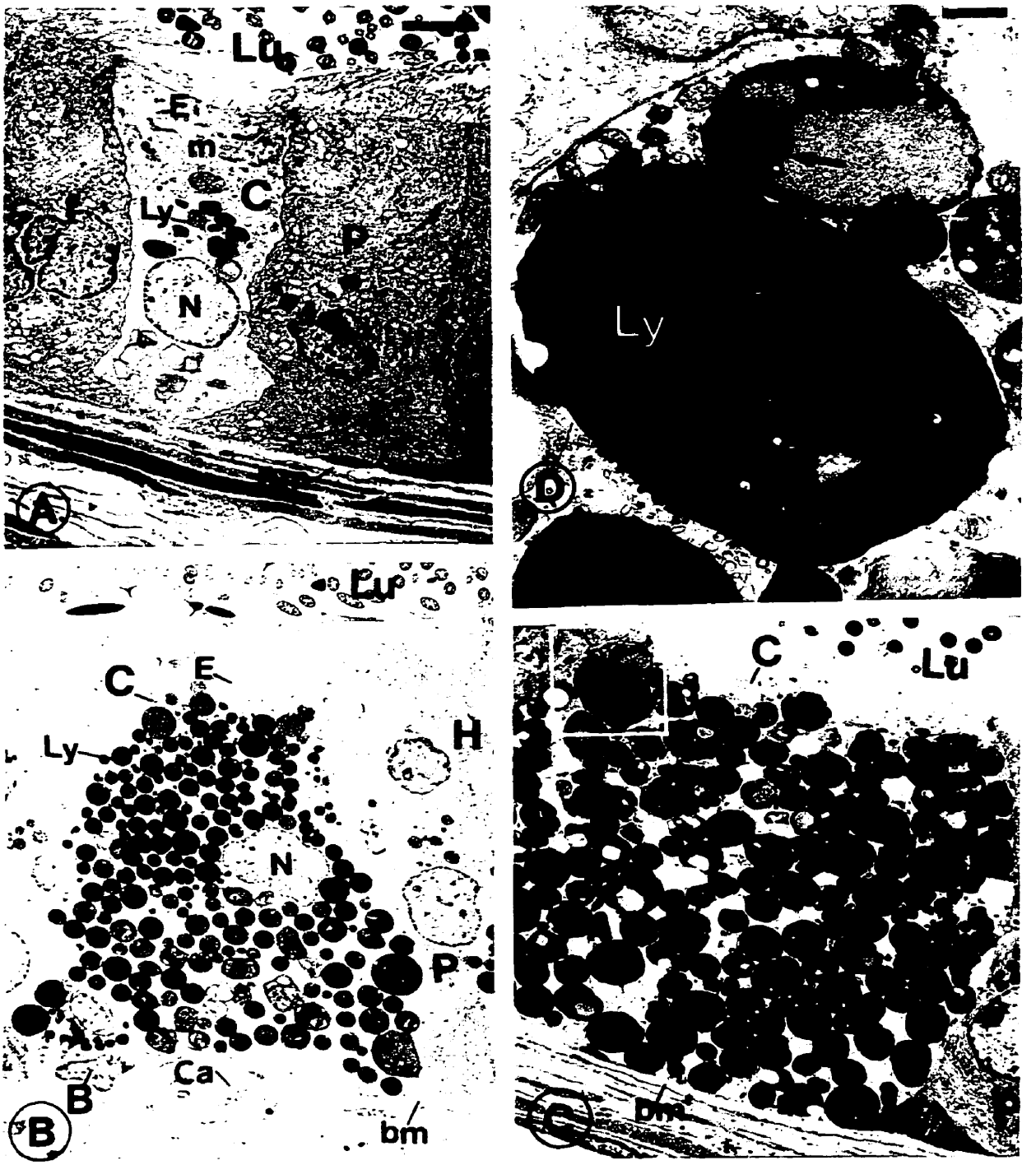
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**Fig. 16.** Electron micrographs showing the origin of vacuoles in epithelial cells of the proximal cauda epididymidis of the 18-month-old Brown Norway rat. **A**) Vacuole in a principal cell; **B**) an endosome fused with a giant vacuole in a principal cell, **C**) high power of a lysosome fused with a giant vacuole in a principal cell. Lu, lumen; Mv, microvilli; P, principal cell; Vc, vacuole; E, endosome; Ly, lysosome; N, nucleus; m, mitochondria; G, Golgi apparatus, • multivesicular bodies; bm, basement membrane; Ca, capillary; MY, myoid cell. Scale bar: **A**, **B** = 2.6 μm; **C** = 0.6 μm.

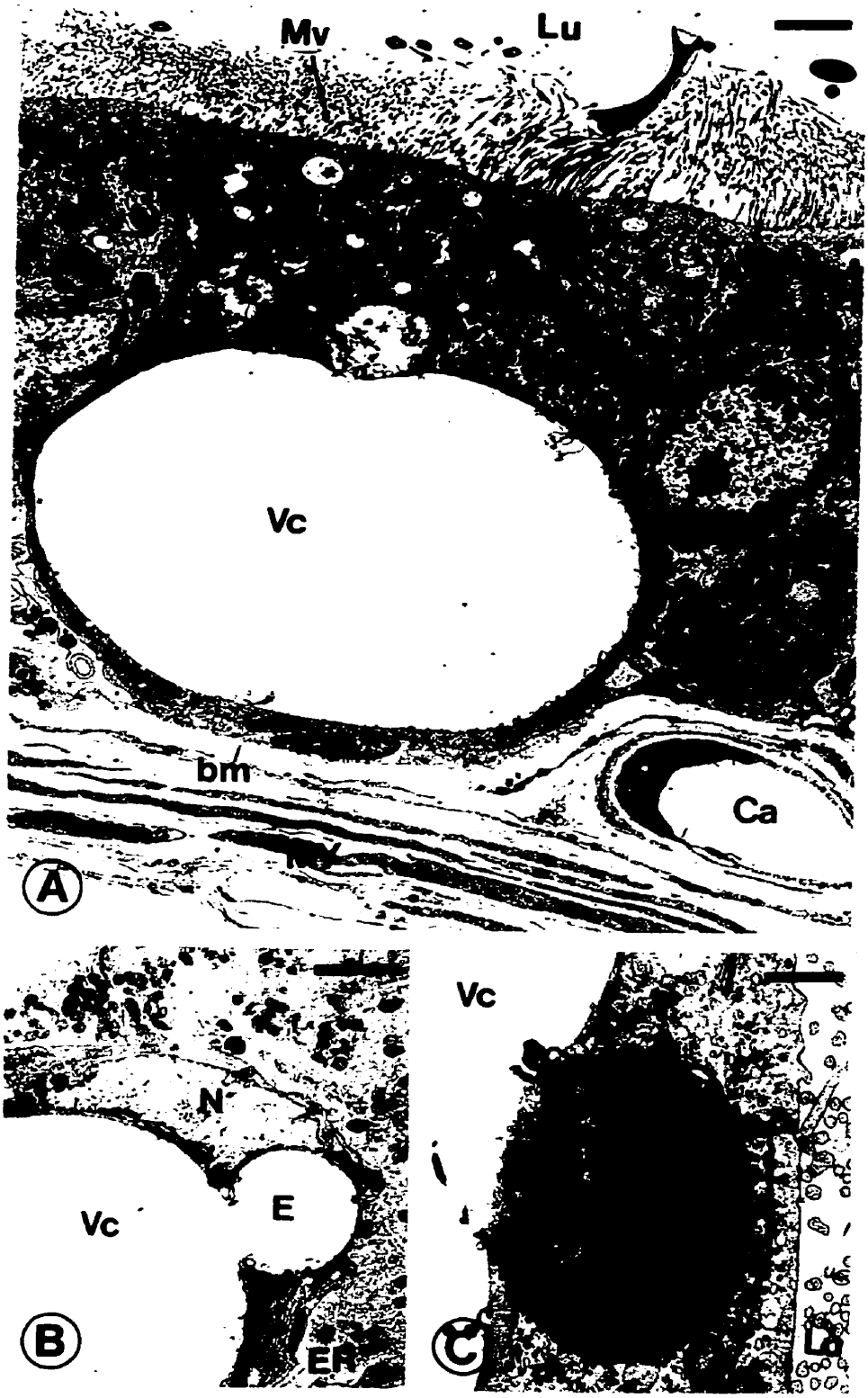
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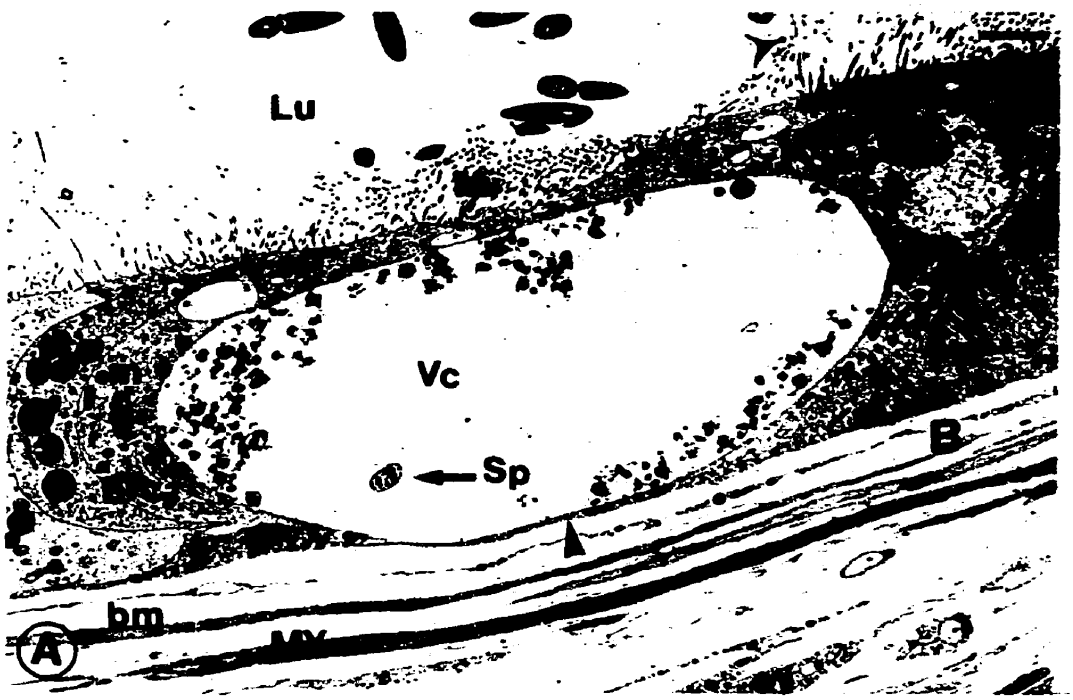
**Fig. 17.** Electron micrographs of two consecutive cuts of a vacuole in the proximal cauda epididymidis of an 18-month-old Brown Norway rat. Spermatozoal debris are visible in the lumen of the vacuole. **A,B)** Serial cut. Lu, lumen; P, principal cell; B, basal cell; C, clear cell; Mv, microvilli; ER, rough endoplasmic reticulum; m, mitochondria; Vc, vacuole; N, nucleus; Sp, spermatozoa; bm, basement membrane, MY, myoid cell. Scale bar, **A**, **B** = 3.4 μm


Lu
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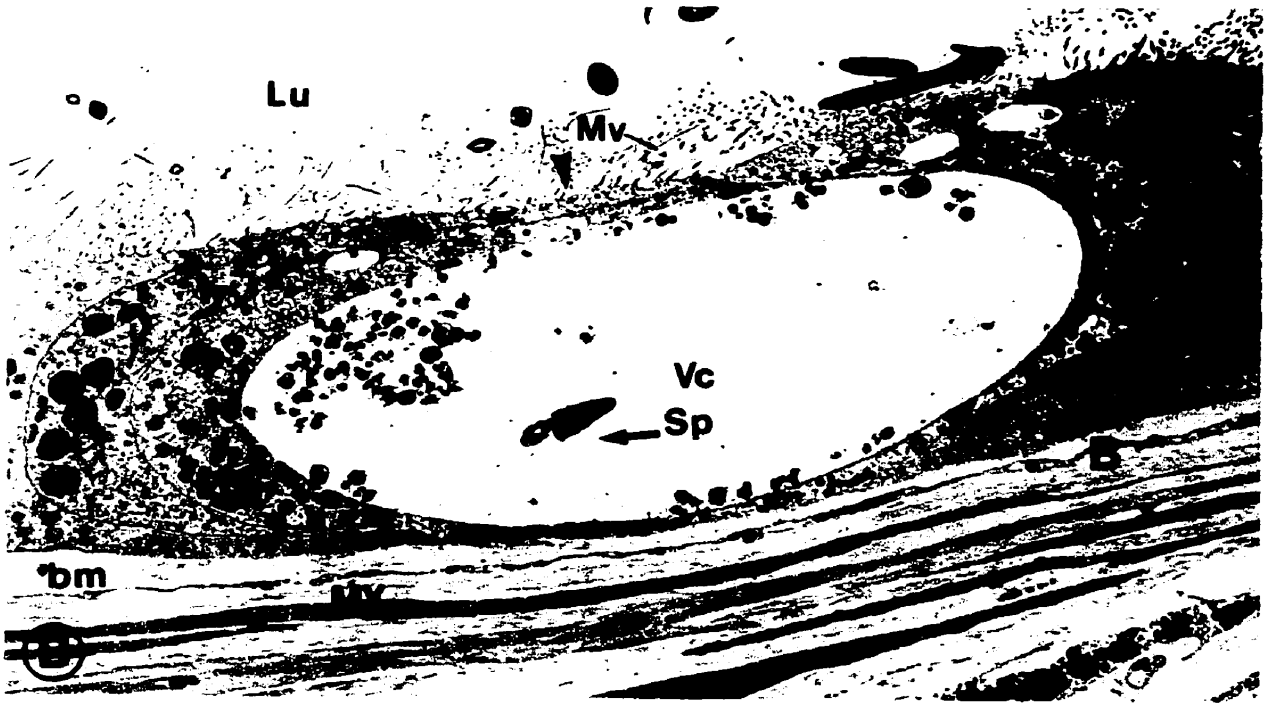
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## Connecting Text- Chapter II to Chapter III

In chapter II, we found that aging of the epididymis of the Brown Norway rat is accompanied by the emergence of characteristic features of aging and a dramatic increase in the number of halo cells. The nature of halo cells is still controversial; they have been postulated to be immunocompetent cells; lymphocytes or monocytes and important for the prevention of autoimmune response against spermatozoa.

Several of the various morphological aspects displayed by halo cells can be related to a specific type of immune cells and/or to a state of activation of these immunocompetent cells. In addition, the presence of numerous trails of immune cells apparently leaving capillaries to reach the epididymal epithelium in the aged rat suggests an active recruitment of these immune cells.

In chapter III, we will determine to which immunocompetent cell type halo cells belong. In addition, we will assess the effect of age and luminal content on the recruitment of immune cells in the epididymal epithelium.

## Chapter III

# The Distribution of Immune Cells in the Epithelium of the Epididymis of the Aging Brown Norway Rat is Segment-Specific and Related to the Luminal Content

Valérie Serre and Bernard Robaire

## ABSTRACT

Remarkable changes occur during aging in the testis and epididymis of the Brown Norway rat. A dramatic increase in the number of halo cells, which are present in the epididymal epithelium and originate from the immune system, is found in animals of increasing age. Halo cells have been postulated to be either lymphocytes or monocytes. We hypothesized that halo cells are a mixture of different immune cells and that their relative composition changes with age. To verify this hypothesis, markers for helper T lymphocytes, cytotoxic T lymphocytes, B lymphocytes and monocytes-macrophages were used to identify the major categories of immune cells in the epididymides of Brown Norway rats ranging in age from 3 to 24 months. The numbers of immunocompetent cells in the epididymis were determined in relation to age, epididymal segment, and luminal content. We found that monocytes, helper T lymphocytes and cytotoxic T lymphocytes belong to the population of halo cells. In addition, a segment specific increase with age in the number of these immune cells was noted. Finally, we report a segment specific recruitment of cytotoxic T lymphocytes and monocytes-macrophages in the epididymal epithelium of aged rats whose epididymal lumen contained few spermatozoa. We postulate that accumulation of damaged epithelial cells and antigens of germ cell origin, leaking through a dysfunctional blood-epididymis barrier, may contribute to the active recruitment of immune cells with age.

## INTRODUCTION

The epididymis, a highly convoluted tubule that connects the testis to the vas deferens, is the site for the maturation and storage of spermatozoa [1, 2]. The epididymal epithelium contains four major cell types: principal, basal, clear and halo cells [1, 3]. Principal cells are the most abundant cells and play a major role in secretion and absorption [1,4]. Tight junctions between principal cells form the blood-epididymis barrier (rat [5,6]). This barrier, in continuation with the blood-testis barrier, is critical for the prevention of autoimmune responses against antigenic post pubertal germs cells [5-7]. Basal cells are flat elongated cells and may have a protective role, either by detoxifying electrophiles [8,9], or by acting like macrophages [10]. Clear cells participate in the uptake of luminal components and the disposal of cytoplasmic droplets detached from spermatozoa [11,12]. Halo cells are found throughout the epididymis. They have been postulated to be lymphocytes or monocytes and are believed to play a role in the immunological barrier of the male reproductive duct [13-16].

The Brown Norway rat is an excellent model to study aging because of its long life span; yet, when no other systemic disease is apparent, remarkable changes occur in the testis [17,18]. The histology of the epididymal epithelium of the Brown Norway rat is also severely affected as animals age [19]. Intriguingly, during aging there is a dramatic increase in the number of halo cells, as well as numerous trails of immune cells, ap parently leaving the blood circulation to reach the tissue epithelium

[19]. These observations suggest a possible age-related activation of the immuno-competent cells in the epididymis.

The major cells involved in immune responses are the monocytes, macrophages and lymphocytes (T and B) [20]. The T lymphocytes can be divided in two subtypes: the cytotoxic T-cells and the helper T-cells. CD8 is a marker of cytotoxic T-cells and CD4 is a marker of helper T-cells [21,22]. Both lymphocytes and macrophages, are present in the mammalian male reproductive tract (human [10,16,23]; rat [14,15,24]; mouse [25]). The immuno-competent cells present in the epididymis have received very little attention. Consequently, the current literature is sparse, and offers divergent, at times contradictory, reports on the predominant cell types present and their distribution in the epididymis.

We hypothesized that halo cells consist of the main types of immune cells and that their distribution in the epididymis changes during aging. To verify this hypothesis, markers for lymphocytes and monocytes-macrophages were used. Our first goal was to clarify the nature and longitudinal distribution of the halo cells in the epididymal epithelium. Our second goal was to assess the effect of age and luminal content on the distribution of these immunocompetent cells in the epithelium and interstitial tissue of each segment of the epididymis of Brown Norway rats.

The present study demonstrates that halo cells consist of helper T lymphocytes, cytotoxic T lymphocytes, and monocytes. Furthermore, we found segment specific increases in the number of each type of immune cell assessed with increasing age. In addition, in the aged rats, we found a dramatic effect of luminal

content on the recruitment of cytotoxic-T lymphocytes and monocytes-macrophages into the epithelium.

## MATERIALS AND METHODS

*Animals*

Male Brown Norway rats aged 3, 12, 18 and 24 months were purchased from the National Institute on Aging (Bethesda, MD) and supplied by Harlan Sprague Dawley Inc. (Indianapolis, IN). Rats were housed at the McIntyre Animal Resources Center, McGill University, under controlled light (14 hours light: 10 hours dark) and temperature (22 °C); animals had free access to food and water.

*Tissue Preparation for Light Microscope Immunocytochemistry*

Rats were anaesthetized with an intra-peritoneal injection of sodium pentobarbital (Somnotol, Steris Laboratories Inc., Phoenix, AZ). The epididymides were fixed with Bouin's solution via perfusion through the abdominal aorta. Retrograde perfusions were done to obtain optimal fixation of the initial segment and caput epididymidis, and prograde perfusions were used to fix the corpus and cauda epididymidis. For the retrograde perfusion, first the common iliac artery on the left side and then the one on the right side were clamped, near the junction of the abdominal aorta; a 18G, 1.5" needle was inserted face up at the point of the junction, pointed in the cranial direction and perfusion with saline was begun. The aorta and

inferior vena cava, cranial to the kidney and below the diaphragm, were clamped immediately. The left renal vein, which should have become inflated, was opened in order to allow efflux of saline. When the effluent is free of blood, the perfusion solution was switched to Bouin's fixative for 10 min. For anterograde perfusions, the common iliac arteries were not clamped and the needle was inserted in the abdominal aorta pointing in the caudal direction, otherwise the same procedure was followed. Following perfusion, epididymides were left 48 hours in Bouin's fixative, dehydrated and embedded in paraffin. Serial longitudinal sections of 5 µm were cut on a microtome and mounted on glass slides.

*Immunostaining*

Sections were deparaffinized with xylene and rehydrated in graded alcohol solution. Endogenous peroxidase activity was neutralized using 70% alcohol containing 1% (vol/vol) hydrogen peroxide. Residual picric acid was inactivated using 70% alcohol containing 1% lithium carbonate. After hydration, free aldehydes were blocked by bathing the sections for 5 minutes in 300nM glycine. Immunohistochemical staining of the sections was done using a Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA). Primary antibodies used for the immunocytochemical staining of cells present in the epididymal epithelium and interstitial tissue were: anti CD4 to label helper T lymphocytes, anti CD8 to label cytotoxic T lymphocytes; anti ED1 to label monocytes and macrophages; anti RLN 9D3 to label B lymphocytes, and antiGSTyf to label basal cells. Goat anti human

CD4 (c-18) (Santa Cruz Biotechnology Inc, Santa Cruz, CA) was used at a dilution 1:50; this polyclonal antibody was raised against an epitope corresponding to an amino acid sequence mapping at the carboxy terminus of the CD4 precursor of human origin which differs from that of the rat's sequences by a single amino acid and cross-reacts with the rat antigen (Santa Cruz technical data sheet). Goat polyclonal anti rat CD8-α (Santa Cruz Biotechnology, Santa Cruz, CA), raised against an epitope corresponding to an amino acid sequence mapping at the carboxy terminus of the rat CD8 alpha chain precursor, was used at a dilution 1:100. Mouse anti rat ED1 (Serotec USA, Washington, DC) was used at a dilution 1:100; this antibody recognizes a cytoplasmic antigen in monocytes and most macrophages. Mouse anti rat RLN-9D3 (Seikagaku America Inc., Rockville, MD) was used at a dilution 1:50, and rabbit anti-rat GST Yf (kindly provided by Dr. J. Hayes; University of Edinburgh, Scotland) at dilution 1:100.

Briefly, the sections were blocked by incubation with diluted normal serum for 30 minutes at room temperature. Sections were incubated for 18 hours at 4°C with primary antiserum diluted in PBS. Sections were then incubated for 30 minutes with appropriate (anti goat, mouse, and rabbit) diluted biotinylated secondary antibody solution and incubated for 30 minutes with Vectastain Elite ABC reagent. Finally, sections were incubated in DAB solution (Vector Laboratories, Burlingame, CA) until stain developed, rinsed in water, counterstained with eosin or methylene blue.

*Cell Counting*

Stained cells were studied in five regions of the epididymis: initial segment, caput, corpus, proximal cauda, and distal cauda; distinctions were based on morphological characteristics [19]. Epididymal sections from 5 to 6 rats per age group were examined by light microscopy at 400X with a green interference filter to enhance contrast (Leitz Wetzlar, Laborlux D, Montreal, Canada). A grid (2.6 $mm^2$) was placed in the center of three cross-sections of the epididymal tubule in each segment of the epididymis. In the distal cauda epididymidis, the area covered by the grid was not large enough to include three cross-sections of tubules, therefore, in this region the grid was placed in the cross sections of two tubules. Immunomarked cells located within the area covered by the grid were counted in both the epithelium and the interstitium [$Ab^+$]. In addition, we counted halo cells that did not stain [$Ab^-$halo] and the non-staining cells [$Ab^-$] located in the interstitium region covered by the grid. The grid was moved randomly and this procedure was repeated 10 times per segment for each animal. Thus the number of cells counted per segment (cell concentration) represents the number of cells per an area equivalent to 0.065$mm^2$ (10 fields of a grid that is 2.6 $mm^2$ viewed at a 400x magnification) .

*Size of ED1 Positive Cells*

The effect of age on the size of ED1 positive cells was determined in 5-6 animals per age-group using light microscopy (400X). A calibrated linear scale was placed in the 10X eyepiece of the light microscope (Leitz Wetzlar, Laborlux D,

Montreal, Canada). The size of monocytes-macrophages are reported to range from 15 to 80 μm [26, 27]. We classified ED1 positive cells as either "small" (less than 20 μm; possibly monocytes), "medium" (20 - 40 μm, likely activated macrophages), or "large" (more than 40 μm, probably mature macrophages) [27]. We classified 100 ED1 positive cells in the epithelium and in the interstitial tissue in each epididymal segment for each animal.

*Statistical Analysis*

To assess the nature of halo cells, statistical evaluation was done using the Student t test (null hypothesis Ho =1). One-way analysis of variance (ANOVA) followed by Tukey's test was employed to detect significant age effects and luminal content effects on the distribution of CD4, CD8, and ED1 positive cells. The level of significance was taken as $p<0.05$.

## RESULTS

*Immunostaining of Immune cells in the Epididymal Epithelium*

Antibodies directed against monocytes-macrophages and lymphocytes, used on sections of the epididymis of Brown Norway rats, revealed the presence of these cells in both the epithelium and interstitial tissue of the epididymis at all ages (Figs. 1-3). Changes with age in the number of these cells in different epididymal segments are shown in Fig. 6.

ED1 positive cells were found throughout the epididymal epithelium and interstitium at all ages and were frequently located in the lower half of the epididymal epithelium close to the basement membrane (Fig. 1). No ED1 positive cells were seen in the lumen at any age. A segment-specific effect of age and luminal content was noted for ED1 positive cells. Results for the initial segment, caput and corpus epididymidis were similar but differed from those for the cauda epididymidis, therefore only representative sections from the corpus (Fig. 1A-C) and distal cauda (Fig. 1D-F) epididymidis are shown.

In the initial segment, caput and corpus epididymidis, the number of ED1 positive cells was increased between 3 months (Fig. 1 A) and 18 months (Fig. 1 B and C). In addition, in these segments, there was a strong immunolabeling of large cells in the epithelium of aged rats that had very few spermatozoa in the lumen (Fig. 1 C). In contrast, neither age nor luminal content appeared to affect the number or the size of

ED1 positive cells in the distal cauda epididymidis (Fig. 1, D-F). It is of interest to note that ED1 positive cells were often in direct contact with non-staining halo cells in the aged Brown Norway rat.

CD4 positive cells were small round cells and were found throughout the epithelium at all ages (Fig. 2; corpus, A-C; distal cauda epididymidis, D-F, as representative sections). These cells were located between epithelial cells, usually close to the basement membrane, but were occasionally found up to the apical point of tight junctions between principal cells. They were never seen in the lumen. A segment specific effect of age was seen in the distribution of CD4 positive cells. There was no change in the relative number of CD4 positive cells in the initial segment, caput, corpus and proximal cauda epididymidis between 3 and 12 months (Fig. 2, corpus A, B). In contrast, an increase in the number of CD4 positive cells was apparent as early as 12 months in the epithelium of the distal cauda epididymidis (Fig 2, D-E). However, by 24 months the number of CD4 positive cells was increased along the entire epididymis (Fig 2, corpus, C and distal cauda, F) except for the caput region.

As was the case for helper T lymphocytes (CD4 positive cells), cytotoxic T lymphocytes (CD8 positive cells) were small round cells, found throughout the epithelium, at all ages (Fig. 3, caput, A-C and distal cauda epididymidis, D-F, as representative sections). These cells were located at various heights between epithelial cells. They also appeared to be stopped at the tight junction level and were never seen in the lumen. A segment-specific effect of age and luminal content

was found in the distribution of CD8 positive cells. The number of CD8 positive cells was increased in the distal cauda epididymidis as early as 12 months (Fig. 3, D and E), whereas it was unchanged in the remaining segments (Fig. 3, A and B; caput). By 24 months, the incidence of CD8 positive cells was higher along the entire epididymis (Fig. 3, caput, C and distal cauda, F).

In contrast, B lymphocytes were very rarely seen in the epididymal epithelium of 3 month old rats and represented less than 1% of immune cells. B lymphocytes were occasionally present at older ages and constituted approximately 5% of immune cells (Fig. 4 initial segment, A; proximal cauda, B, as representative sections). As for T lymphocytes, B lymphocytes were small round cells, often located near the base of the epithelium. They were never seen in the lumen.

*Immunostaining of Epididymal Basal and Halo Cells*

*Basal Cells.*

Previous studies by Yeung et al. (human [10]) have shown intriguing similarities between basal cells and macrophages. To assess whether in the Brown Norway rat, basal cells are immune cells, an antibody directed against the Yf subunit of the glutathione S-transferases (GST), which was previously shown to stain basal cells specifically in the corpus and cauda epididymidis (rat [8]), and antibodies against monocytes-macrophages and lymphocytes were used. The ED1 antibody distinguishes an intracytoplasmic antigen in monocytes, tissue macrophages and free macrophages (rat [28, 29]).

Basal cells from the corpus epididymidis stained with GST Yf, at all ages, as shown by the intense immunolabeling of flat elongated cells located at the base of the epithelium (Fig. 5, corpus, A and B, 3 and 18 months, respectively). In the epithelium of young rats, occasional small cells were stained with the antibody against monocytes-macrophages; however the number and localization of these cells did not coincide with that of basal cells (Fig. 5 C). In aged rats, more ED1 positive cells were present in the epithelium; however, basal cells did not react with ED1 antibody (Fig. 5 D). In addition, basal cells did not stain with antibodies against T lymphocytes or B lymphocytes (data not shown); this is consistent with the size and epithelial localization of these cells shown in Figs. 2, 3 and 4.

*Halo Cells.*

We hypothesized that helper T lymphocytes ($CD4^+$), cytotoxic T lymphocytes ($CD8^+$) and monocytes-macrophages ($ED1^+$) comprise the halo cells population. According to our hypothesis $[CD4^+] + [CD8^+] + [ED1^+] = [\text{total halo}]$. The total number of halo cells present in the epididymal epithelium can therefore be calculated with this formula: $[Ab^+] + [Ab^-\text{halo}] = [\text{total halo}]$. For each animal (N=4 per age group) we counted $[CD4^+]$ and $[CD4^-\text{halo}]$, $[CD8^+]$ and $[CD8^-\text{halo}]$, and $[ED1^+]$ and $[ED1^-\text{halo}]$.

If our hypothesis is correct, then we should expect, for any epididymal segment:

$$\frac{[CD4^+]}{[CD4^+] + [CD4^- \text{halo}]} + \frac{[CD8^+]}{[CD8^+] + [CD8^- \text{halo}]} + \frac{[ED1^+]}{[ED1^+] + [ED1^- \text{halo}]} = 1$$

We found that the average number of halo cells was equivalent to the sum of ED1, CD4, and CD8 positive cells in the epididymis of 3 and 12 months Brown Norway rats (Ho =1; 3 months, 1.04 ± 0.11; 12 months, 1.08 ± 0.07). A small but significant difference was found at older ages (Ho =1; 18 months =1.26 ± 0.04; 24 months = 1.15 ± 0.11). However, ED1 positive cells included "small", "medium" and "large" cells. These "large" cells do not fit the description of halo cells; i.e.: small round cells with a pale rim of cytoplasm. Thus, when we excluded "large" halo cells from the equation, the combined number of ED1, CD4 and CD8 positive cells was then equivalent to the number of halo cells for age 3, 12 and 24 months (Ho =1; 3 months, 1.04 ± 0.11; 12 months, 1.07 ± 0.07; 24 months = 1.05 ± 0.12). A borderline significance was found at 18 months (Ho =1; 18 months, 1.20 ± 0.05; P=0.03).

*Effects of Age on the Number of Immune Cells in the Epithelium*

There was a segment-specific increase with age in the number of monocytes-macrophages (ED1 positive cells), helper T lymphocytes (CD4 positive cells) and cytotoxic T lymphocytes (CD8 positive cells) present in the epididymal epithelium (Fig. 6, A-C). The incidence of monocyte-macrophages was fairly constant along the

epididymal epithelium of 3-month-old Brown Norway rats (Fig. 6 A). In the initial segment, caput and corpus epididymidis, there was no significant change in the number of monocytes-macrophages between 3 and 12 months of age. However, there was a major increase by 18 months in these segments; this increase was sustained at 24 months. In addition, by 24 months, the number of monocytes-macrophages became significantly elevated in the proximal cauda epididymidis, whereas the number of monocytes-macrophages was not affected by age in the distal cauda epididymidis (Fig. 6 A).

The number of helper T cells (CD4+) slowly increased between 3 and 18 months and became significantly higher by 24 months in the initial segment (Fig. 6 B). Although there was a trend toward an increase in the number of helper T lymphocytes with age in the caput epididymidis, no significant effect of age was noted. In the corpus and proximal cauda epididymidis, there was a significant increase in the number of helper T cells at 18 and 24 months, while in the distal cauda epididymidis, this significant increase was seen as early as 12 months and was sustained at 18 and 24 months (Fig. 6 B)

The number of cytotoxic T cells (CD8+) was unchanged between 3 and 12 months but was significantly increased by 18 months in the initial segment (Fig. 6 C). In the caput and corpus epididymidis, the concentration of cytotoxic T cells increased slightly, but not significantly, between 12 and 18 months. However, a dramatic increase of greater than 400% relative to 3 months was found at 24 months. In the proximal cauda epididymidis, the number of cytotoxic T cells was increased

significantly at 18 and 24 months as compared with 3 months. As for helper T lymphocytes, there was a significant increase of cytotoxic T lymphocytes in the distal cauda epididymidis at 12 months; this increase was sustained at 18 and 24 months.

*Effect of Age on the Number of Immune Cells in the Interstitial Tissue*

To develop further insight into the source of immune cells in the epididymal epithelium, the number of immune cells in the interstitial region surrounding the epithelium was assessed. There was no significant effect of age on the number of monocytes-macrophages in the interstitial tissue located in the immediate proximity of the epididymal tubule at all ages. It is noteworthy that there was a trend to a lower concentration at 12 months in the initial segment, caput, corpus, and proximal cauda epididymidis as compared with 3 months, followed by a return at 18 and 24 months to values comparable to those found at 3 months of age [corpus as representative section: 3 months, 30±5; 12 months, 19±3; 24 months 27±8]. In contrast, there was a steady number of monocytes macrophages in the distal cauda epididymis [3 months, 19±5; 24 months, 17±4].

The number of helper T lymphocytes present in the interstitial tissue was not affected by age in any epididymal segment [caput 3 months, 25±1, 24 months 35±12; corpus 3 months, 21±5; 24 months, 30±6, as representative sections]; except for the distal cauda epididymidis [distal cauda 3 months, 9±1; 24 months, 20±4, $p<0.02$].

The number of cytotoxic T lymphocytes present in the intertubular space was not significantly affected by age in any epididymal segment [caput 3 months, 18±3; 24 months, 32±6; distal cauda, 3 months, 16±3; 24 months, 12±2, as representative sections] except for the corpus epididymidis. In that region, the number of cytotoxic T lymphocytes was significantly increased by 24 months [3 months; 23±2; 24 months, 45± 4 $p< 0.001$]. It is of interest that in aged rats, accumulations of immunocompetent cells, mostly cytotoxic T lymphocytes and helper T lymphocytes, were often seen at the periphery of the epididymis, in the immediate proximity of blood or lymph vessels.

*Effect of Luminal Content on the Number of Immune cells in the Epididymal Epithelium and Interstitium.*

In Brown Norway rats of 18 to 24 months of age, the number of monocytes-macrophages present in the epithelium was significantly higher in the initial segment, caput, and corpus epididymidis when few spermatozoa were present in the epididymal lumen as compared to when numerous spermatozoa were found (Fig. 7 A). In contrast, luminal content did not affect the number monocytes-macrophages present in the epithelium of the proximal and distal cauda epididymidis.

The quantity of spermatozoa in the lumen had no effect on the number of helper T lymphocytes (CD4+) present in the epididymal epithelium (Fig. 7 B). Although the number of cytotoxic T lymphocytes (CD8+) was higher in the initial segment and caput epididymidis, this increase was not significant. In the corpus

epididymidis of aged rats, luminal content significantly affected the number of cytotoxic T lymphocytes (Fig. 7 C). In contrast, luminal composition had no effect on the number of cytotoxic T cells in the proximal and distal cauda epididymidis (Fig. 7 C). Luminal composition did not affect the number of monocytes macrophages, helper T lymphocytes and cytotoxic T lymphocytes in the interstitium (data not shown).

*Effect of Age and Lumen Content on the Size of ED1 Positive Cells Present in the Epididymal Epithelium and Interstitium.*

In the epididymal epithelium of 3- and 12-month-old rats, the overwhelming majority of ED1 positive cells was "small" (Fig. 8). In the initial segment and caput epididymidis, there was a significant increase by 18 months in the proportion of "large" ED1 positive cells. This increase was accompanied by an increase in the proportion of "medium" and a relative decrease in the proportion of "small" ED1 positive cells. A similar trend was seen in the corpus by 18 months and proximal cauda epididymidis by 24 months. In contrast, age had no significant effect on the size of monocytes-macrophages in the distal cauda epididymidis.

Furthermore, in aged rats, the proportion of "large" ED1 positive cells was significantly greater in the initial segment and corpus epididymidis when few spermatozoa were present as compared to when the lumen was filled with spermatozoa (Fig. 9). A similar trend was seen for the caput and proximal cauda epididymidis. Luminal composition had no effect on the size of ED1 positive in the

distal corpus epididymidis. In addition, age and luminal composition had no significant effect on the size of ED1 positive cells present in the interstitial tissue (data not shown).

## DISCUSSION

In the present study we used specific markers to identify and quantify immune cells present in the epididymis of Brown Norway rats of increasing age. Monocytes-macrophages, helper T lymphocytes and cytotoxic T lymphocytes were present in the epithelium at all ages. Our counting method allowed us to clarify the nature of halo cells. We confirmed that halo cells are immune cells, and demonstrated that they are comprised of monocytes, helper T lymphocytes and cytotoxic T lymphocytes. Furthermore we found a segment specific increase with age in each of these immune cells in the epididymal epithelium. In addition, the incidence of immune cells was further enhanced in aged animals whose epididymal lumen contain few spermatozoa.

*Identification of Immune cells in the Epididymal Epithelium*

Immune cells were present along the entire epididymal epithelium at all ages. Macrophages and T lymphocytes have been reported in both the epididymal interstitium and the epithelium under normal conditions (human [10, 23]; rat [15, 24]; mouse [25]). However, their precise origin is still unclear.

Yeung et al, proposed that cells positive for an antibody against mature tissue-fixed macrophages were basal cells, based on their similar localization and morphological resemblance (human [10]). In the present study, we demonstrated that in the Brown Norway rat, basal cells identified by an antibody against GST Yf do

not recognize an antibody against monocytes-macrophages (Fig 5). Our results are in agreement with the observation, made by Flickinger et al.[15], that basal cells of the Lewis rat do not stain with an antibody against macrophages. Furthermore, under normal conditions, basal cells are elongated cells whereas ED1 positive cells display various shapes and sizes. In addition, we found that basal cells do not recognize antibodies directed against helper T lymphocytes, cytotoxic T lymphocytes, or B lymphocytes. Together, clearly these observations indicate that basal cells are not immune cells.

The nature of halo cells has been the subject of controversy since their discovery by Reid and Cleland in 1957 [3]. Halo cells are present throughout the epididymal epithelium and are usually defined as small cells with a narrow rim of clear cytoplasm [1]. Lymphocytes and monocytes have similar sizes and their distinction based on nuclear morphology is at best difficult at the light microscope. In the epididymal epithelium of young Brown Norway rats, the number of cells that stained for antibodies against monocytes-macrophages (ED1+), helper T lymphocytes (CD4+) and cytotoxic T lymphocytes (CD8+) was equivalent to the number of halo cells. In young Brown Norway rats, the vast majority of ED1 positive cells was of small or medium size. However, the proportion of large ED1 cells was increased during old age. Interestingly, the combined number of ED1 positive cells is equivalent to the number of halo cells at 24 months, if the large ED1 cells (most probably macrophages) are not entered in the equation. Thus, halo cells are composed of T lymphocytes and monocytes. This suggests that no or very few

additional types of immune cells are resident or migrate into the epididymal epithelium of the Brown Norway rat under normal conditions. In aged rats, eosinophils [19] and B lymphocytes were occasionally seen in the epithelium. Thus, the occurrence of intra-epithelial migration of B lymphocytes, eosinophils and possibly, plasma cells, neutrophils, or basophils, is probably a rare event that occurs only under pathological conditions.

In the epithelium of young Brown Norway rats, T lymphocytes were the predominant immune cells. There appear to be species differences in the main types of immunocompetent cells present in the epididymal epithelium. In murine epididymis, macrophages are the most abundant immuno-competent cells [25]. However, as in Brown Norway rats, human epididymal immune cells are mainly composed of T lymphocytes, with the main lymphocyte sub-population being the cytotoxic T cells [23, 30]. In Lewis and Wistar rats, helper T lymphocytes are present in the greatest relative abundance in the epididymal epithelium [15, 24].

*Effects of Age on the Number of Immune Cells*

An important finding of this study was the demonstration of a dramatic increase with advancing age in the numbers of each type of immune cell present in the epididymis. A higher number of lymphocytes and macrophages was reported in the epididymides of older men [16]. An increase of migratory immune cells with age has been reported in other tissues, such as the kidney (human [31, 32]). The trigger of this age-related immune response is presently unclear.

The increase in immune cells in the epididymis was segment specific. Helper T lymphocytes and cytotoxic T lymphocytes were first recruited in the distal cauda epididymidis as early as 12 months. This recruitment was probably due to subtle modifications of the epithelium since the appearance of the epithelium and lumen of the cauda epididymidis did not seem to differ from that of the 3 months old rats [19]. The proportion of T lymphocytes present in the cauda epididymidis did not change significantly between 12 and 24 months and no major histological changes were reported in that segment at 12 months or at later ages [19]. Furthermore, age had no effect on the incidence of monocytes-macrophages in the distal cauda epididymidis. The main effect of age on immune cells found in the distal cauda epididymidis was a twofold increase in the number of T lymphocytes. The distal cauda epididymidis appeared relatively less affected by aging for reasons that are not clear.

In contrast to the distal cauda epididymidis, age had a major effect on the number of immune cells present in the other epididymal segments. By 18 months, the number of monocytes-macrophages was dramatically increased in the initial segment, caput and corpus epididymidis. Thus, a new substrate or signals for monocytes-macrophage may emerge in these epididymal segments at 18 months.

Despite a significant increase in the total number of monocytes-macrophages, helper T lymphocytes, and cytotoxic T lymphocytes present in the epithelium, the number of immuno-competent cells present in the peritubular interstitium was not markedly affected by age. Although there were few macrophages in the interstitial

tissue at any age, there was a major increase in the proportion of macrophages in the epithelium of Brown Norway rats with age. Together these observations suggest that the substrate and/or signals for the migration of immune cells are not found in the interstitial tissue but rather in the epithelium.

In the epithelium, helper T lymphocytes, cytotoxic T lymphocytes, or monocytes-macrophages were always located between epithelial cells; this is in agreement with other studies [14 - 16]. Lymphocytes were frequently seen in the midst of the epithelium or near the tight junctions at all ages. Unlike helper T lymphocytes or cytotoxic T lymphocytes, monocytes-macrophages were always localized in the lower half of the epididymal epithelium, suggesting that their substrate might be different and located near the basement membrane. It is well established that the interaction between macrophages and lymphocytes is important for antigen uptake and processing [15, 32, 33].

Interestingly, in the epididymal epithelium, monocytes-macrophages and lymphocytes were often found in close proximity, suggesting an active interaction. Overall, a higher concentration of immune cells appeared to be correlated with major alterations of the epithelial structure, such as lysosome accumulation [19], suggesting that modification of the epithelial integrity is an important factor in the recruitment of lymphocytes. In addition, the dramatic accumulation of immune cells seen in old rats may create further damage to the epithelium.

In the young animal, the epithelium may contain enough immune cells to

provide for its efficient protection and the prevention of autoimmune reaction against spermatozoa; the interstitial space may act as a reservoir of immune cells. Although a local proliferation of macrophages is possible [34], mitotic figures were not found in the epithelium or the interstitial space. However, an accumulation of helper T lymphocytes and cytotoxic T lymphocytes was often seen close to blood or lymph vessels located at the periphery of the epididymis. Thus, one may hypothesize that the interstitial reservoir may be insufficient to sustain the age-related increase in the demand for immune cells by the epithelium. Consequently, the interstitial space might serve as a pathway for the active recruitment of immune cells coming from the blood or lymph vessels into the epithelium.

*Effect of Luminal Content on the Number of Immune cells*

In aged rats, there was a segment-specific positive correlation between the presence of an abnormal luminal content and the recruitment of immune cells in the epithelium. An increase in intra-epithelial lymphocytes and macrophages has been reported following vasectomy and sperm degeneration [35, 36]. In contrast, the number of intra-epithelial lymphocytes is lower in sexually immature rats than in the adult rat [14]. Interestingly, the production of spermatozoa begins at puberty, after the formation of the immune system tolerance to self-antigens (rat [6, 7]). Pachytene spermatocytes and later stages of spermatozoa development are identified as foreign components by the immune system (rat [37]). Thus, the breakdown product of spermatozoa or immature germ cells may be an antigenic stimulant [37 - 39].

Macrophages, helper T lymphocytes, and cytotoxic T lymphocytes may cooperate to prevent antigens from reaching the circulation, thereby creating an autoimmune response [15, 23, 30].

The effect of luminal content on the distribution of immune cells was segment specific. The recruitment of cytotoxic T lymphocytes and macrophages was more marked in the initial segment, caput, and corpus epididymidis of aged rats that had few spermatozoa in the lumen. Our results suggest that antigens of spermatozoal origin are present in these epididymal regions and, therefore, that the blood-epididymis barrier is likely to be leaky in aged animals.

In the young Brown Norway rat, a small number of helper T lymphocytes, cytotoxic T lymphocytes, and monocytes-macrophages were present in the epithelium. With increasing age there was a major recruitment of immune cells into the epithelium. This recruitment was segment specific and possibly related to various substrates (abnormal or "foreign" material) that accumulate with increasing age in the epithelium. The synergistic effect of an altered luminal content on the number of immune cells present in the epithelium suggests that some of these chemotactic factors or antigens are spermatozoa and/or the debris of immature germ cells that is leaking through an altered blood-epididymis barrier. The dramatic accumulation of immune cells in aged rats might be damaging to the epithelium and contribute to the senescence of the tissue.

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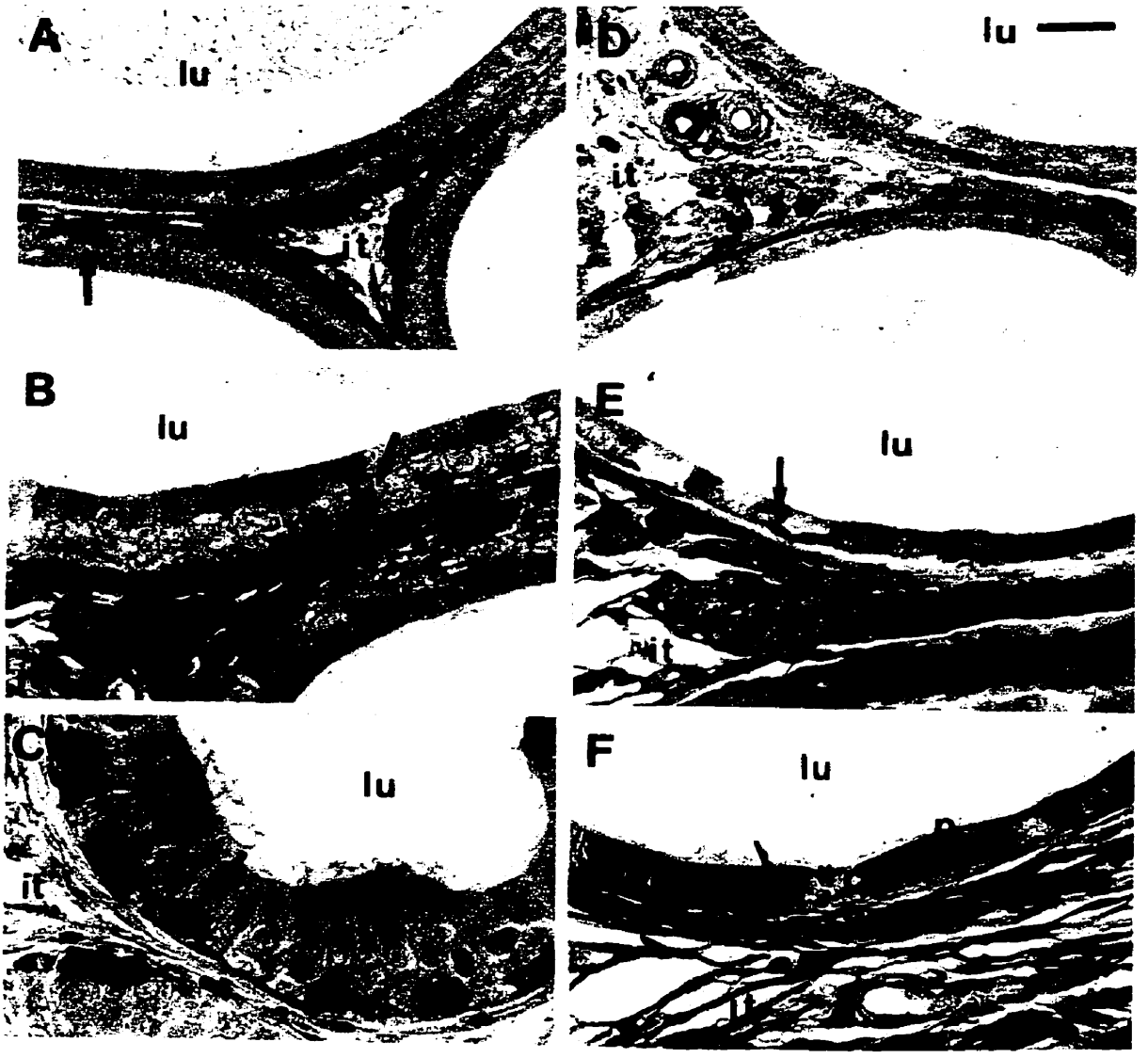
**Fig. 1.** Light micrographs showing sections of the epididymis of the Brown Norway rat stained with an antibody for monocytes-macrophages (ED1). **A-C)** corpus; **D-F)** distal cauda. **A, D)** 3 months; **B, E)** 18 months with numerous spermatozoa in the lumen, **C,F)** 18 months with occasional spermatozoa in the lumen. lu, lumen; it, intertubular space; p, principal cells; b, basal cells; c, clear cells; bm, basement membrane; arrows: ED1 positive cells; asterisks: halo cells. Scale bar, **A-F**= 64 μm


A
lu
it
D
lu
it
B
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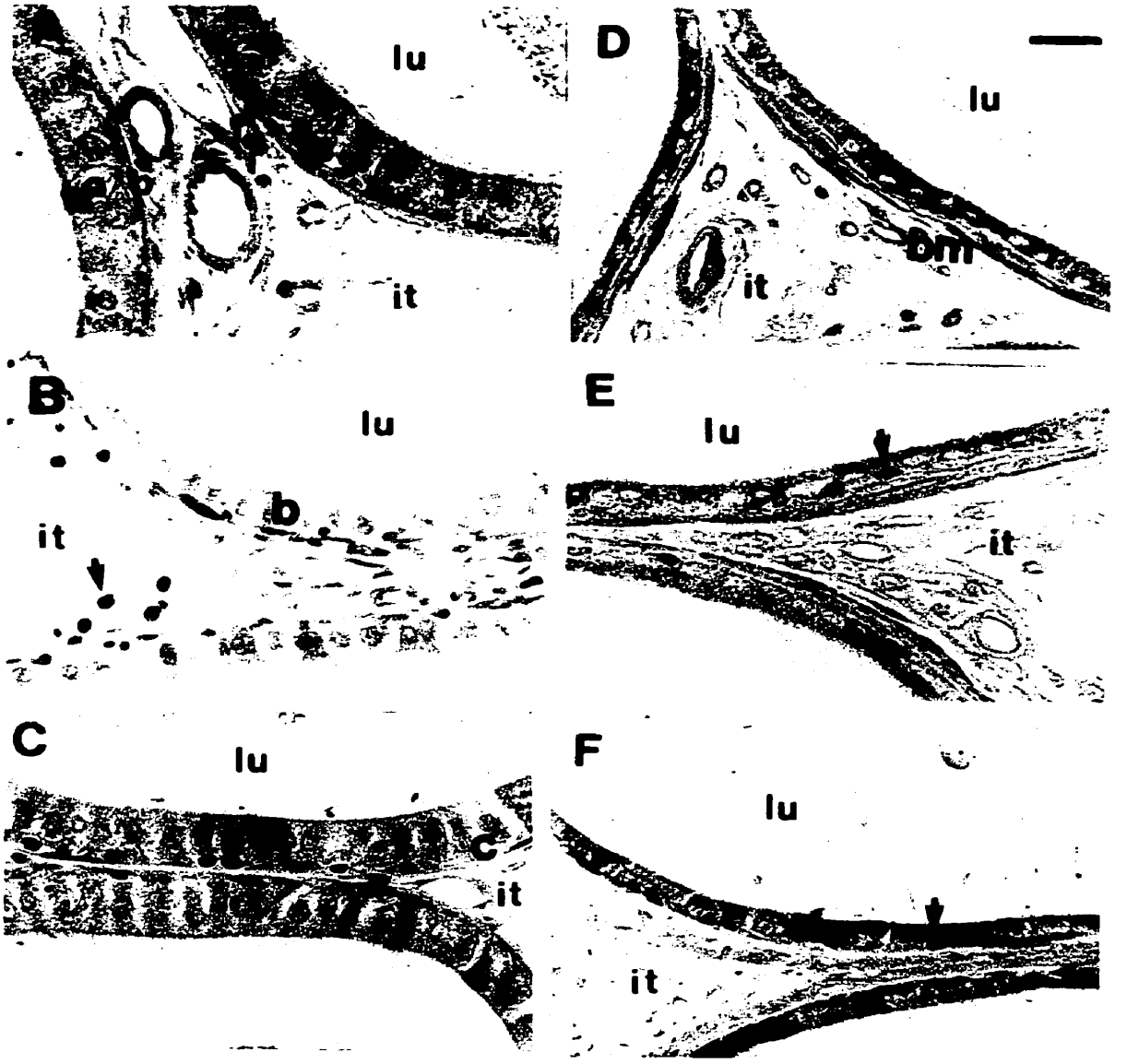
E
lu
it
C
lu
it
F
lu

**Fig. 2.** Light micrographs showing sections of the epididymis of the Brown Norway rat stained with an antibody for helper T lymphocytes (CD4). **A-C)** corpus, **D-F)** distal cauda. **A,D)** 3 months; **B,E)** 12 months **C,F)** 24 months. lu, lumen; it, intertubular space; p, principal cells; b, basal cells; c, clear cells; bm, basement membrane; arrows, CD4 positive cells; asterisks: halo cells. Scale bar, **A-F**= 64 µm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D
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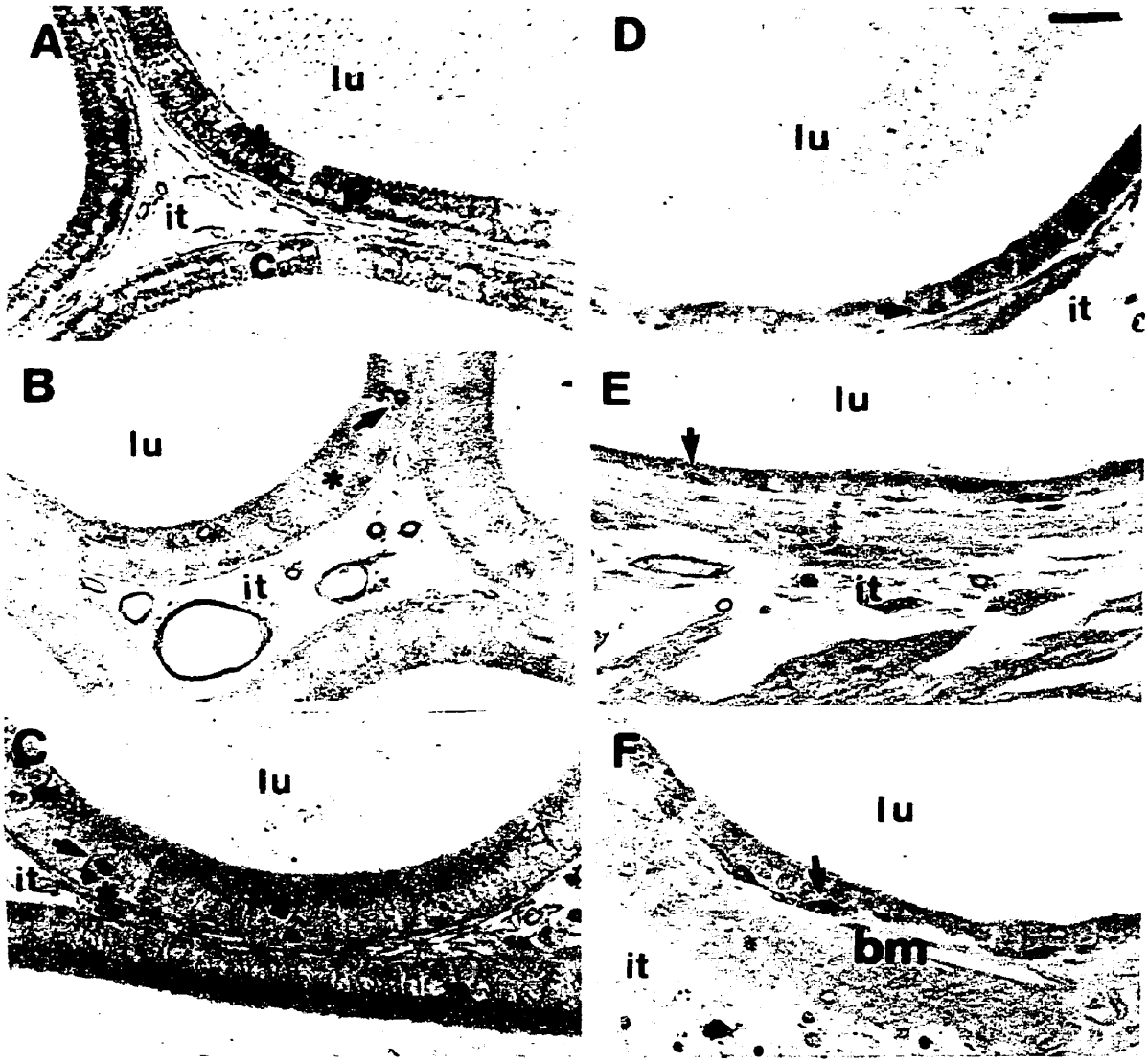
b
it
E
lu
it
C
lu
c
it
F
lu
it

**Fig. 3.** Light micrographs showing sections of the epididymis of the Brown Norway rat stained with an antibody for cytotoxic T lymphocytes (CD8). **A-C)** caput, **D-F)** distal cauda. **A,D)** 3 months; **B,E)** 12 months **C,F)** 24 months. lu, lumen; it, intertubular space, p, principal cells; b, basal cells; c, clear cells; bm, basement membrane; arrows, CD8 positive cells; asterisks: halo cells. Scale bar, **A-F**= 64 µm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it
c
D
lu
it
B
lu
it
E
lu
it
C
lu
it
F
lu
bm
it

**Fig. 4.** Light micrographs showing sections of the epididymis of an 18 month Brown Norway rat stained with an antibody for B lymphocytes (RLN-9D3). **A)** initial segment, **B)** proximal cauda. lu, lumen; it, intertubular space; p, principal cells; b, basal cells; c, clear cells; bm, basement membrane; arrows: B lymphocytes. Scale bar, **A-B**= 64 µm


A
lu
p
bm
it
B
it
lu

**Fig. 5.** Light micrographs showing sections of the corpus of the epididymis of Brown Norway rats. **A, C)** 3 months and **B, D)** 18 months. Section stained with an antibody for **A, B)** GST Yf and **C, D)** ED1. lu, lumen; it, intertubular space; p, principal cells; b, basal cells; c, clear cells; clear arrows: basal cells; dark arrows: ED1 positive cells. Scale bar, **A-D**= 64 µm.

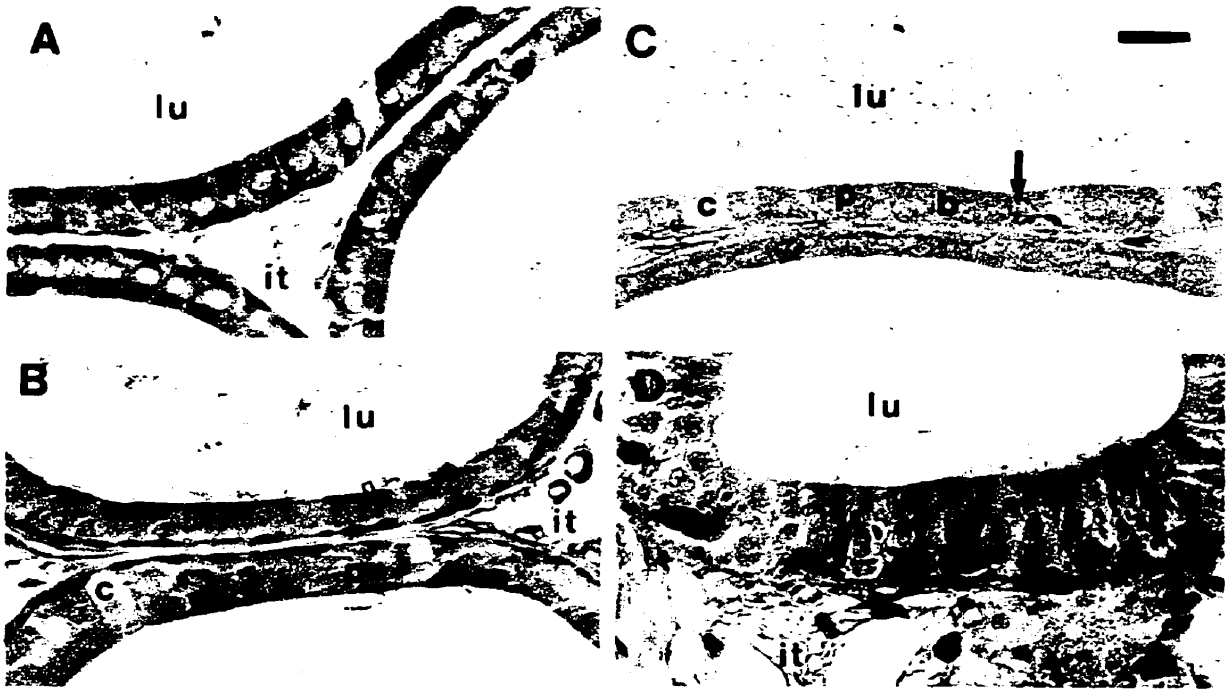

A
lu
it
C
lu
c
B
lu
it
c
D
lu
it

**Fig. 6.** Effect of age on the number of immune cells along the epididymal epithelium of the Brown Norway rat. Average number of immune cells (sum of 10 grids/ rat) in **IS**, initial segment; **CA**, caput; **CO**, corpus; **pCU**, proximal cauda; **dCU,** distal cauda. **A)** Monocytes macrophages (ED1+); **B)** helper T lymphocytes (CD4+), **C)** cytotoxic T lymphocytes (CD8+). Open bar: 3 months; left hatched bar: 12 months; cross hatched bar: 18 months; right hatched bar: 24 months. Bars represent means ± SEM. N= 5-6; asterisks, $p<0.05$ relative to 3 months.

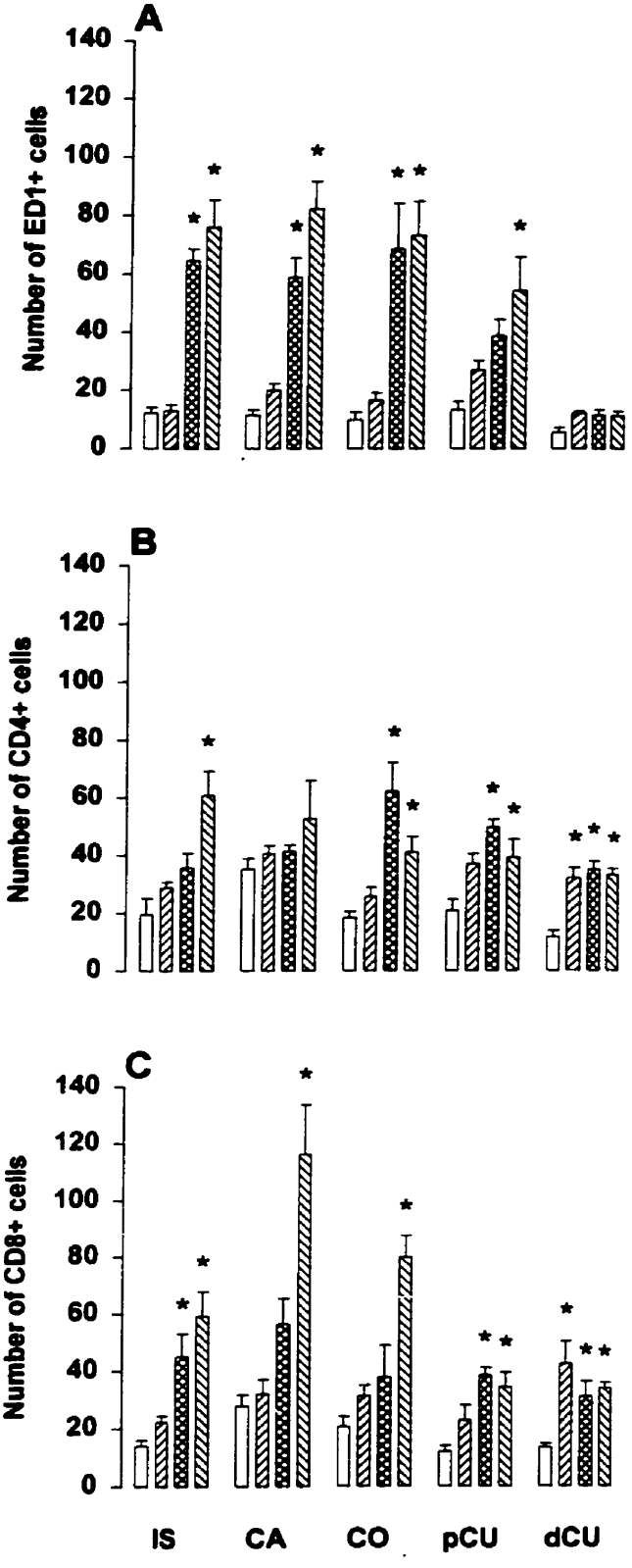

A
Number of ED1+ cells
140
120
100
80
60
40
20
0
B
Number of CD4+ cells
140
120
100
80
60
40
20
0
C
Number of CD8+ cells
140
120
100
80
60
40
20
0
IS
CA
CO
pCU
dCU

**Fig. 7.** Effect of luminal content on the number of immune cells along the epididymal epithelium of the aged Brown Norway rat (18-24 months old). Average number of immune cells (sum of 10 grids/ rat) in **IS**, initial segment; **CA**, caput; **CO**, corpus; **pCU**, proximal cauda; **dCU**, distal cauda. **A)** Monocytes macrophages (ED1+), **B)** helper T lymphocytes (CD4+), **C)** cytotoxic T lymphocytes (CD8+). Open bar: lumen with many spermatozoa; gray bar: lumen with occasional spermatozoa. Bars represent means ± SEM. N= 5-6; asterisks, $p<0.05$.

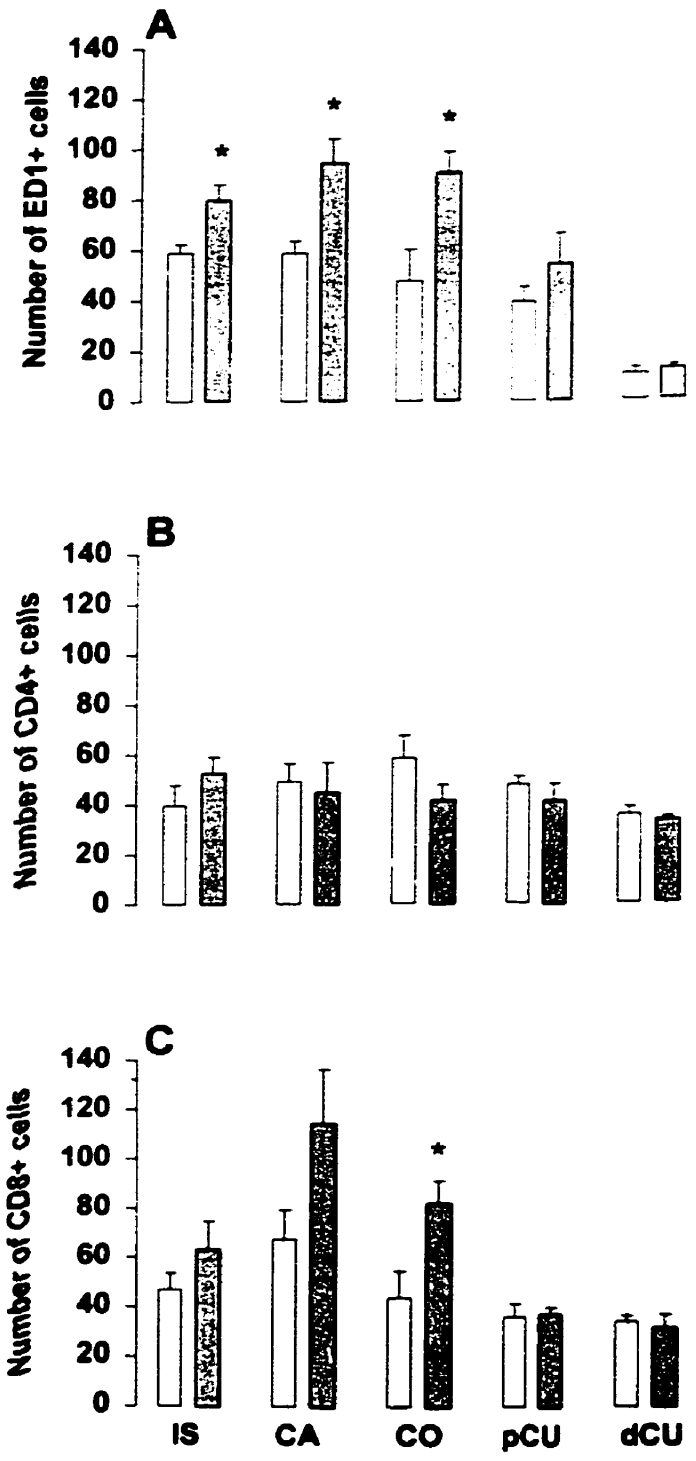

A
Number of ED1+ cells
140
120
100
80
60
40
20
0
*
*
*
B
Number of CD4+ cells
140
120
100
80
60
40
20
0
C
Number of CD8+ cells
140
120
100
80
60
40
20
0
*
IS
CA
CO
pCU
dCU

**Fig. 8.** Effect of age on the size of ED1+ cells along the epididymal epithelium of the Brown Norway rat. Average number of immune cells (sum of 10 grids/ rat) in **IS**, initial segment; **CA**, caput; **CO**, corpus; **pCU**, proximal cauda; **dCU**, distal cauda. Effect of age: **1**, 3 months; **2**, 12 months; **3**, 18 months; **4**, 24 months. Solid black bar: number of "large" ED1+ cells; light gray bar: number of "medium" ED1+ cells; dark gray bar: number of "small" ED1+ cells. Bars represent means ± SEM. N= 5-6; asterisks, $p<0.05$ relative to bar 1 (3 months).

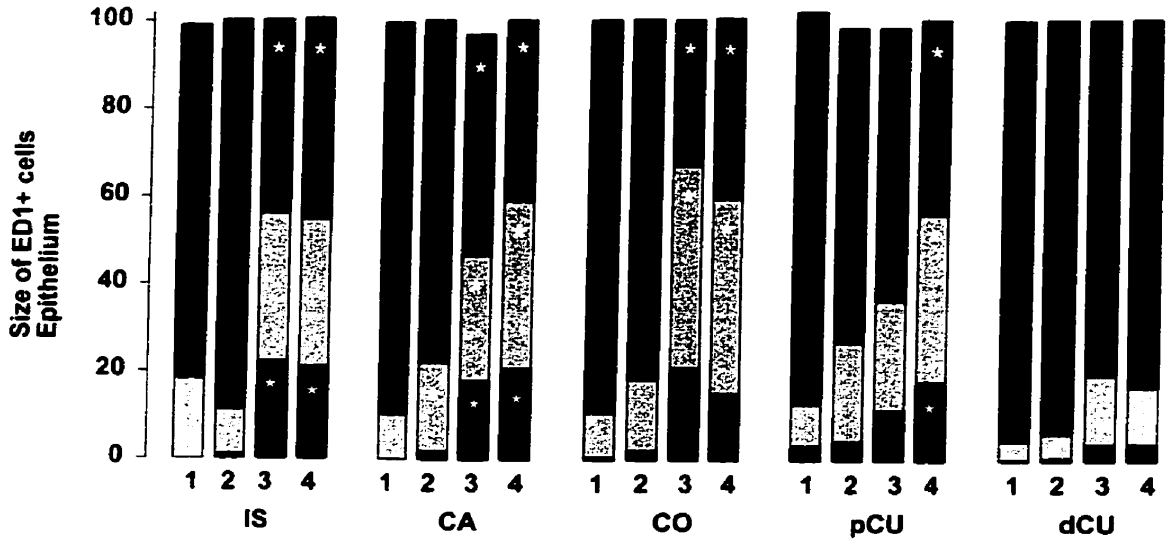
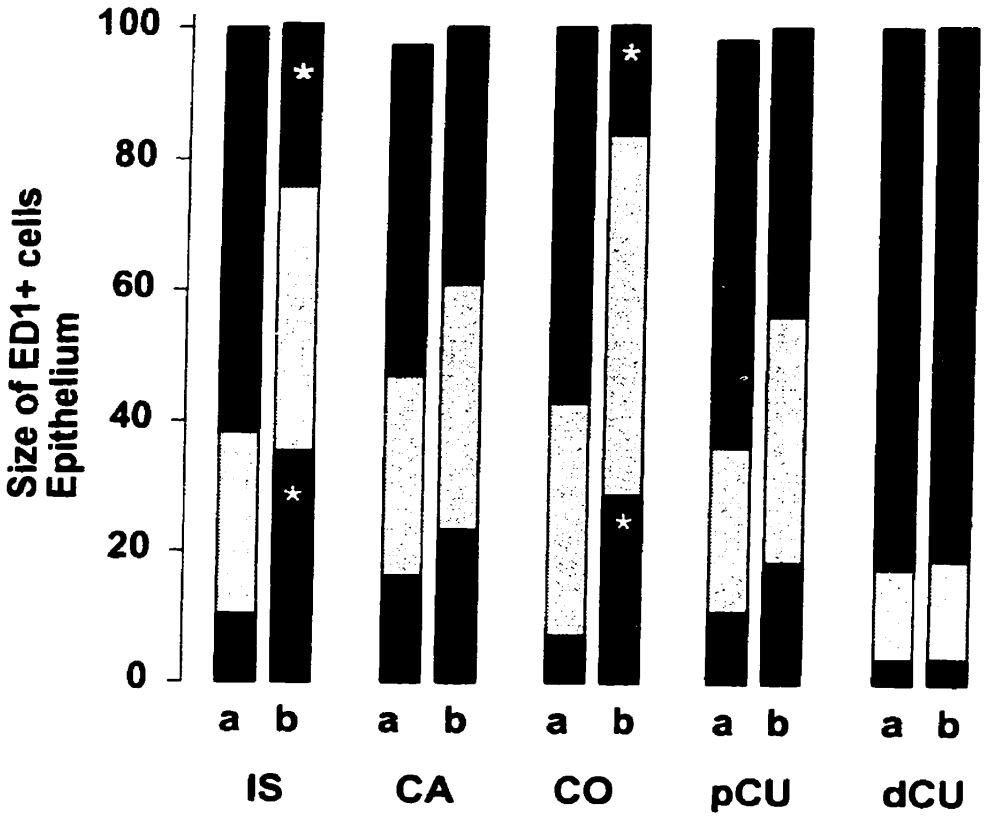

Size of ED1+ cells
Epithelium
100
80
60
40
20
0
1 2 3 4
IS
1 2 3 4
CA
1 2 3 4
CO
1 2 3 4
pCU
1 2 3 4
dCU

**Fig. 9.** Effect of luminal content on the size of ED1+ cells along the epididymal epithelium of the Brown Norway rat. Average number of immune cells (sum of 10 grids/ rat) in **IS**, initial segment; **CA**, caput; **CO**, corpus; **pCU**, proximal cauda; **dCU,** distal cauda. **a,** 18-24 months with numerous spermatozoa; **b,** 18-24 months with occasional spermatozoa. Solid black bar: number of "large" ED1+ cells; light gray bar: number of "medium" ED1+ cells; dark gray bar: number of "small" ED1+ cells. Bars represent means ± SEM. N= 5-6; asterisks, $p<0.05$.


Size of ED1+ cells Epithelium
100
80
60
40
20
0
a b
IS
CA
CO
pCU
dCU

## Connecting Text- Chapter III to Chapter IV

In chapter II, we found dramatic changes in the histology of the epididymis in aged rats. In addition, a spermatophagic function was shown in principal cells of the proximal cauda epididymidis of aged rats; this process was proposed to be triggered by abnormal or degenerating spermatozoa.

In chapter III, we reported a major increase in the number of immunocompetent cells in the epididymal epithelium with age. Furthermore, the number of immune cells was higher when the lumen was filled mostly with immature germ cells and debris. We proposed that the function of immunocompetent cells present in the epididymis was to eliminate either debris found in the lumen and/or spermatozoa leaking from the lumen. Furthermore, we assumed that this major accumulation of immune cells could be detrimental to the structure and functions of the epididymal epithelium.

Aging of the testis is accompanied by a decrease in spermatogenesis and steroidogenesis. In addition, our results from chapters II and III suggest that the epididymal microenvironment, that is essential for spermatozoa to mature normally, may be altered in old age. The effect of advancing maternal age on pregnancy outcome has been studied extensively; in contrast, there is little information about the effect of advancing paternal age on the offspring. In chapter IV, we will investigate whether there is an increased risk of anomalous pregnancy and progeny outcomes with advancing paternal age.

## Chapter IV

# Paternal Age Affects Fertility and Progeny Outcome in the Brown Norway Rat

Valérie Serre and Bernard Robaire

## ABSTRACT

**Objective:** To investigate the effects of paternal age on fertility and progeny outcome using the Brown Norway rat model.

**Design:** Controlled prospective study.

**Setting:** McIntyre Animal Centre, McGill University, Montreal, Canada

**Intervention(s):** Brown Norway male rats of increasing age were mated to young Sprague-Dawley females.

**Main outcome measure(s):** Pregnancy outcome was assessed by counting the numbers of corpora lutea, resorptions and live fetuses on day 20 of gestation. To evaluate progeny outcome, pups were examined for external malformations and weighed daily for 2 months.

**Result(s):** There were no significant changes in the numbers of resorptions, offspring, or in the incidence of external malformations. However, there was an increase in pre-implantation loss (corpora lutea minus implantation sites) in litters fathered by older males. Furthermore, a significant decrease in the average fetal weight was found with increasing paternal age. A significant increase in neonatal deaths for progeny fathered by older males was also found.

**Conclusion(s):** These results indicate that the quality of spermatozoa decreases as males age.

## INTRODUCTION

Life expectancy in man has increased dramatically over the past century. Because couples frequently postpone parenthood, information concerning the effect of age on the success of pregnancy, and on the normal development and health of children is important. The effect of advancing maternal age on pregnancy outcome has been studied extensively. Increased maternal age has adverse effects on progeny outcome; such effects include low birth weight [1,2] and late fetal death [3]. In addition, there are numerous reports of increased occurrences of chromosomal disorders such as Down's syndrome [4] in the progeny of older mothers. As a consequence, genetic counseling of older women wishing to have children has become routine.

There is now a growing concern about the possibility of an adverse effect of advanced paternal age on the offspring. In 1955 Penrose [5] advanced the concept that continuous replication of stem cells in the male during spermatogenesis could increase the probability of a copy error. Recently, it was hypothesized that point mutations may be associated with the replication process and that such mutations may increase with increasing paternal age [6]. Several studies have shown that indeed paternal age can increase the incidence of autosomal and X-linked hereditary diseases [7-9], with mutation rates several times higher in the older age groups [4]. In addition, a recent study reported a correlation between advancing paternal age and impaired development of the progeny [7]. However, there are few well-

controlled studies on the relationship between aging and fertility [10-11]. Consequently, the possibility of an increased rate of adverse effects and mutations in progeny, with advancing paternal age, needs to be further investigated.

The Brown Norway (BN) rat is an excellent model to study aging. These rats have a long life span and are relatively disease-free; they demonstrate remarkable changes in the testis when no other disease is apparent. In the BN rat, as in men, aging of the male leads to dramatic changes in the seminiferous epithelium and to decreases in spermatogenesis and steroidogenesis [rat, 12-14];[man, 15-16]. Histology of the epididymal epithelium is modified dramatically [17]. The basement membrane is thicker, and a major accumulation of lipofuscin is seen in the epithelium.

These results suggest that the environment necessary for the maturation of spermatozoa could be modified with advancing age. Together, these findings implicate paternal aging as having an impact on the offspring. Consequently, we investigated the effects of increasing paternal age on fertility and progeny outcome with use of the Brown Norway rat as a model.

## MATERIALS AND METHODS

We followed the policies and procedures set forth by the facility Animal Care Committees and McGill University as well as those described in the "Guide to the Care and Use of Experimental Animals" prepared by the Canadian Council on Animal Care.

### *Proven Ability to Mate*

Male rats were left for 8 days with two adult Sprague-Dawley female rats (200-250g). Each morning, the tray and the vagina of females were examined for the presence of seminal plugs and vaginal smears were examined for the presence of spermatozoa. For males aged 3, 12 or 18 months, only 8 rats were required to complete each group (n=8), i.e. 100% of males at these ages could give a sperm-positive test. At 24 months, 10 males were required in order to obtain 8 males giving a sperm-positive test. Males who failed to mate during that period were not included in the following studies.

### *Mating Schedules*

Adult virgin female rats were monitored daily to establish their normal estrous cycle. Sexually experienced male rats were housed overnight with two virgin female rats in proestrus. On the next morning the vagina of each female was examined for

the presence of spermatozoa; this was defined as day 0 of gestation for sperm-positive animals.

### *Effect of Rat Strain on Fertility*

To determine whether the previously described low fertility in Brown Norway rats [18] was due to a female or a male factor, we first undertook a study in which male and female Sprague-Dawley (SD) and Brown Norway (BN) rats were mated within and between strains. On day 20 of gestation the females underwent a cesarean section, the ovaries were removed, and the numbers of corpora lutea were counted; the uteri were opened, and the numbers of fetuses were determined.

### *Effect of Paternal Age on Pregnancy Outcome*

To estimate the effect of paternal age on the characteristics of the fetuses, male Brown Norway rats aged 3, 12, 18 and 24 months were used (n=8 per age group). Each male was mated with two virgin female Sprague-Dawley rats (200-250g) [19]. Females underwent a cesarean section on day 20 of gestation. The ovaries were removed, and the numbers of corpora lutea were counted; uteri were opened and the numbers of resorptions and live fetuses were determined. Preimplantation loss was assessed by calculating the difference between the number of corpora lutea and the number of implantation sites (resorptions and live fetuses) for each female. Postimplantation loss was assessed by calculating the difference between the number of implantation sites and the number of live fetuses on day 20 of gestation

[19]. Moles were defined as a dark mass of tissue associated with the placenta. Moles with a weight <100 mg were defined as early resorptions, whereas those with a weight >100 mg were classified as late resorptions [20]. Fetuses were sexed, weighed and examined for external malformations.

### *Effect of Paternal Age on Progeny Outcome*

To assess the effect of paternal age on progeny outcome, females were allowed to give birth normally (day of birth = day 0). Pups were examined for external malformations and weighed; their development was observed daily from day 1 until day 14, and again on days 21 and 60.

### *Statistical Analysis*

To avoid the bias of nutrition advantage, all analyses related to progeny weight were performed with litters of eight or more. The weight of each litter was classified as "large", "normal" or "small for developmental age". Normal litters were defined as litters in which the average weight per litter of the pups or fetuses was within the mean (±2SDs) of the average weight per litter of pups or fetuses fathered by Brown Norway males aged 3 months. "Small" was lower than the mean (-2SDs) [21], while "large" was higher than the mean (+2SDs). The effects of age on pregnancy and progeny outcomes were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test.

For nonparametric data, i.e., percentage of pregnancy per sperm-positive

female, "small" for developmental age, external malformations, and neonatal deaths, analyses were performed with the Kruskal-Wallis $\chi^2$ test. For all analyses, values were considered statistically significantly different at $P < .05$.

## RESULTS

### *Effect of Rat Strain on Fertility*

The influence of rat strain on fertility was assessed with use of crossbreeding of Brown Norway and Sprague-Dawley rats; the results are shown in Figure 1. Matings between male and female Brown Norway rats resulted in a small litter size, compared to male and female Sprague-Dawley rats. To ascertain whether the low number of pups was due to a male or a female factor, the strains of rats were crossbred. Matings between male Sprague-Dawley and female Brown Norway rats resulted in litter sizes similar to those found for matings between male Brown Norway and female Brown Norway rats. However, matings between male Brown Norway and female Sprague-Dawley gave litter sizes similar to those found for matings between male Sprague-Dawley and female Sprague-Dawley rats (Fig. 1). These results showed that the low fertility in the Brown Norway strain was caused by a female factor.

To determine whether the low fecundity associated with female Brown Norway rats was due to a decreased rate of ovulation or to a decreased ability of eggs to be fertilized and/or for fertilized eggs to implant, the numbers of corpora lutea were determined. The Brown Norway females were found to have low numbers of corpora lutea compared with the Sprague-Dawley females (Fig. 1). Thus, the low fecundity of the Brown Norway female was consequent to a decrease in the number of eggs ovulated. Therefore, female Sprague-Dawley rats were used in studies of the effect

of increasing paternal age on pregnancy and progeny outcomes.

### *Effect of Paternal Age on Success of Pregnancy*

Age had no significant effect on mating efficiency among proven breeder (3 months, 69%; 12 months, 59%; 18 months, 44%; 24 months, 59%). There was no significant difference in the percentage of pregnancy per sperm-positive females for all four groups of males (3 months, 88.9%; 12 months, 89.5%; 18 months, 84.6%; 24 months, 83.3%).

### *Effect of Paternal Age on Pregnancy Outcome*

The effects of paternal age on pregnancy outcome as assessed on day 20 of gestation are shown in Figure 2. Preimplantation loss was similar for fathers aged 3, 12, and 18 months. However, there was a significant increase in preimplantation loss with males aged 24 months (Fig. 2). Paternal age had no effect on the percentage of postimplantation loss (Fig. 2). Nonetheless, at ages 3, 12, and 18 months, > 70% of the resorptions were classified as early resorptions. In contrast, no early resorptions were found in litters fathered by rats aged 24 months.

There was no significant difference in litter size at any age (3 months, 14.6 ± 0.4; 12 months, 14.9 ± 0.6; 18 months, 15.0 ± 0.5; 24 months, 12.6 ± 1.7). The lower average litter size at 24 months was a consequence of the increase in preimplantation loss in litters fathered by males of that age; however, this decrease was not significant. The sex ratio of male to female fetuses was similar for the

progeny of males at all ages (3 months, 0.93 ± 0.13; 12 months, 1.06 ± 0.20; 18 months, 0.86 ± 0.10; 24 months, 0.94 ± 0.22).

The effect of paternal age on fetal growth is shown in Figure 3. There was a significant decrease in the weights of fetuses fathered by rats aged 12, 18 and 24 months, when compared to fetuses fathered by rats aged 3 months.

**Effect of paternal age on progeny outcome**

The effect of paternal age on the growth of pups is shown in Figure 3. By day 1 postpartum, there was no significant difference in the average weights of pups/litter with paternal age (Fig. 3). Similarly, no statistically significant differences were found in pup weights with respect to paternal age on day 7 (Fig. 3), day 14, day 21, or day 60 (data not shown). None of the litters sired by males at any age was located in the "large for developmental age" group; on day 20 of gestation <10% of the litters fathered by 3-month-old males were in the "small for developmental age" range. It is of interest that there was a fourfold to eightfold increase in litters fathered by older rats. By day 1 postpartum, none of the litters fathered by the 3-month-old male group were located in the "small for developmental age" range. In contrast, up to 30% of the litters fathered by older rats remained in the "small for developmental age" range.

Although malformations such as short or bent tail and micro-ophthalmia were found in pups fathered by rats aged 12, 18 and 24 months (12 months, 2/125; 18 months, 1/69; 24 months, 1/97), no malformed pups were found in litters fathered by

young rats (3 months, 0/122). However, due to the low incidence of malformations, there were no significant differences among any of the ages.

The effect of paternal age on early neonatal death (between postnatal day 1 and day 14) is shown in figure 4. The average number of deaths per litter was similar for litters fathered by rats aged 3,12, and 18 months. It is surprising that there was a dramatic increase in the number of deaths of pups in litters fathered by 24-month-old rats. Most of those deaths occurred within 4 days, one death was found at day 5, no death occurred between day 6 and 60. Furthermore, most of the pups that died neonatally presented a significantly lower birth weight (5.34 ± 0.27g) when compared to those who survived until day 60 (6.32 ± 0.64g).

## DISCUSSION

Analysis of the effect of paternal age on pregnancy and progeny outcomes was performed in a controlled environment to minimize confounding variables, especially that of the maternal factor. We confirmed the low fertility of the Brown Norway strain [18] and showed that it was due to a female factor. Thus, young adult Sprague-Dawley females were used for all our studies on male aging. In the present study, we report, for the first time, that paternal age affected both pregnancy and progeny outcomes. Three dramatic effects of advancing paternal age on the offspring include an increase in preimplantation loss, a decrease in the average fetal weight on day 20 of gestation, and an increase in early neonatal death.

There was a significant increase in preimplantation loss in litters fathered by aged male rats compared with young rats. To our knowledge, such an increase in preimplantation loss has not been associated previously with increased paternal age; this increase could signify either a decreased ability of spermatozoa to fertilize oocytes or a degeneration of early embryos before implantation.

Studies on the fertilizing ability of spermatozoa from the aged male are few and conflicting [11, 22-27]. Although there are reports of reduced fertility in aged men [11], and of a reduced number of litters per month of breeding by 27 months in Sprague-Dawley male rats and by 30 months in ACI male rats [22], most studies

suggest that reproductive function remained active into extremely old age in both men and rats [23,11]. Furthermore, the age-related decrease in motility and the higher rate of abnormalities of spermatozoa did not appear to affect fertilizing ability [24,25]. Indeed, the capacity of freshly ejaculated sperm from older men to penetrate the ova of zona-pellucida free eggs was comparable to that for younger men [24-26].

Thus, these findings suggested that the increase in preimplantation loss was not related to a decreased fertilizing capacity of spermatozoa during aging, but may be due to an inability of the early embryo to develop and implant.

A decrease in the frequency of ejaculations was reported for aged rats and Rhesus monkeys [27]. In addition, the fertilizing ability of mature spermatozoa from rabbits decreased after experimental retention in the cauda epididymidis [28]. Thus, intra-epididymal retention could "age" spermatozoa in the Brown Norway rat. However, we did not observe any anatomical blockage of the excurrent duct in older animals. All the males used in our experiments mated successfully when presented to females in proestrus as a prerequisite to enter our experimental protocol. Thus, it is unlikely that increased preimplantation loss was a consequence of intraepididymal retention of spermatozoa.

An alternative explanation for the increased preimplantation loss among the progeny of aged males could be a degeneration of the embryo before implantation at

the blastocyst stage. There are no previous reports of failure of implantation with advancing paternal age. However, implantation failure has been reported with increasing maternal age and attributed to an early arrest, possibly related to chromosomal abnormalities [29]. It is of interest that chromosomal aberrations have been found in germ cells of male mice of advanced age [30]. Furthermore, Crow [6] proposed that the increase in the rate of base substitution mutations with paternal age is probably balanced by a quasi-truncation selection. According to his model, harmful mutations are eliminated by "genetic death".

Development, metabolism, and implantation of embryos are facilitated by a complex interaction of many endocrine, paracrine and autocrine factors, such as growth factors and cytokines [31-33]. Among these factors are platelet-activating factor (PAF) and insulin-like growth factors (IGF-2), which have a role as autocrine embryonic factors [31, 33, 34]. Platelet-activating factor is synthesized by two-cell mouse embryos [35]; it stimulates embryo development in vitro and increases viability and the rate of implantations [36, 37]. Insulin-like growth factor-2 is expressed as early as the two cell stage and may have a role in the regulation of embryonic growth during the preimplantation period [38, 39]; thus, it is possible that the level of growth factors, such as PAF or IGF-2, that are secreted by the embryos sired by older fathers is insufficient to sustain growth and implantation.

One of the major findings of the present study was a decrease in fetal weight with advancing paternal age. There are no previous reports of decreased fetal weight with increasing paternal age. However, the obstetrics literature suggests an

association between increasing maternal age and lower birth weight [1, 3]. Despite adjustment for confounding variables, such as complications of pregnancy and smoking, paternal age effect was neglected in these studies [1, 3]. Moreover, long term paternal exposure to alkylating agents such as cyclophosphamide leads to increases in both preimplantation loss and the number of low-weight fetuses [40].

Thus, both increases in preimplantation loss and a decrease in the weights of fetuses may be a sign of germ cell damage. In the Brown Norway rat, intrinsic aging of the germ stem cells, combined with exposure to a modified environment during spermatocytogenesis and spermiogenesis, ultimately may affect the genomic expression of autocrine factors.

Culture of blastocysts for progressively longer periods of time prior to their transfer into carrier females results in decreasing rates of implantation as well as reduced fetal weights [mice; 41]. In addition, decreased endogenous production of the growth factors IGF-1 and IGF-2, during development reduces the weight of the fetuses at day 17 and day 16, respectively [mice; 42, 43]. Together, those results suggest that an inadequate exposure to growth factors in the embryos sired by older males may be responsible for growth retardation on day 20 of gestation.

It is of interest that the gene for IGF-2 has been shown to be paternally imprinted in both mice and humans [mice, 44; men, 45]. Thus, it is tempting to speculate that there may be an alteration in imprinting in spermatozoa during aging. Distinctive methylation patterns emerge during germ cell maturation; the paternal allele for a

number of genes undergoes further modification during embryogenesis [46]. Paternal treatment with 5-azacytidine, a drug that blocks DNA methylation, led to an increase in preimplantation loss; embryos were abnormal at 2 days after fertilization and died before implantation [47]. This raises the possibility of an age-related change in the DNA methylation pattern of imprinted genes such as the IGF-2 gene in the aged Brown Norway rat.

The third major effect of paternal age was an increase in neonatal death. An increase in perinatal mortality has been reported with advancing maternal age [2,48]. Again paternal age was not studied carefully, leaving open the possibility that this effect may have been related also to advancing paternal age. Impaired intrauterine growth acts as a prenatal insult [49-50]. Deficiency in both IGF-1 and IGF-2 leads to reduced weights and increased early postnatal death [42, 43]. It is of interest that in most cases, pups that died early in the postnatal period had lower birth weights than their littermates, and the rate of weight gain was slower. Thus, growth retardation by impaired exposure to a growth factor during development of embryos fathered by old rats may be associated with early postnatal death.

With advancing paternal age, the quality of spermatozoa appears to be affected. Intrinsic aging of germ stem cells an/or exposure to an inadequate environment during maturation may lead to an increase in the rate of mutations and/or changes in the pattern of imprinting. Chromosomal aberrations may result in embryo degeneration and in a greater number of external malformations. Fetal growth

retardation may result from a compromised secretion or response to growth factors, possibly related to changes in DNA methylation.

The ability of most pups to display a normal weight early after birth suggests that the influence of other growth factors predominates postnatally. Nevertheless, the presence of underweight pups at birth suggests that intrauterine damage may have consequences postnatally, such as reduced growth rate and higher postnatal death rate.

The animal studies presented above show the potential for deleterious pregnancy and progeny outcomes as a consequence of increased paternal age. Appropriate clinical studies should be undertaken to establish whether older men want to have children should receive genetic counseling.

**Acknowledgments**

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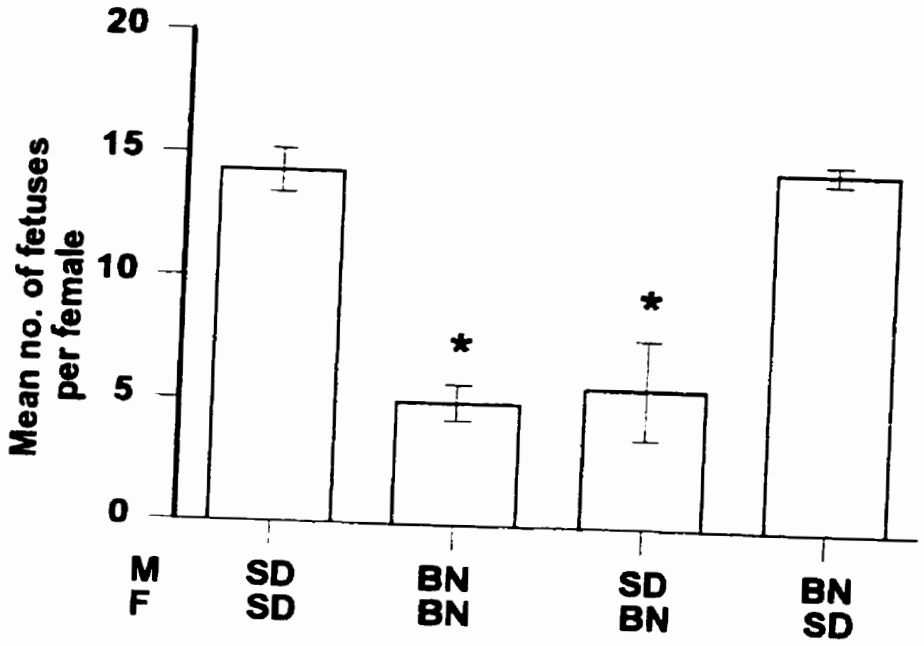
**Fig. 1.** Effect of rat strain on fertility.

***Top:*** Effect of rat strain on litter size (n= 4-8) on day 20 of gestation. Values are the mean number of fetuses per litter per female. ***Bottom:*** Effect of female strain on the numbers of corpora lutea. Values are the mean number of corpora lutea per female. The rat strains are Sprague-Dawley (SD) and Brown Norway (BN). M = male; F = female. Bars represent means ± SEM. *P < 0.05, by ANOVA and Tukey's test.


Mean no. of fetuses per female
20
15
10
5
0
*
*
M
F
SD
SD
BN
BN
SD
BN
BN
SD

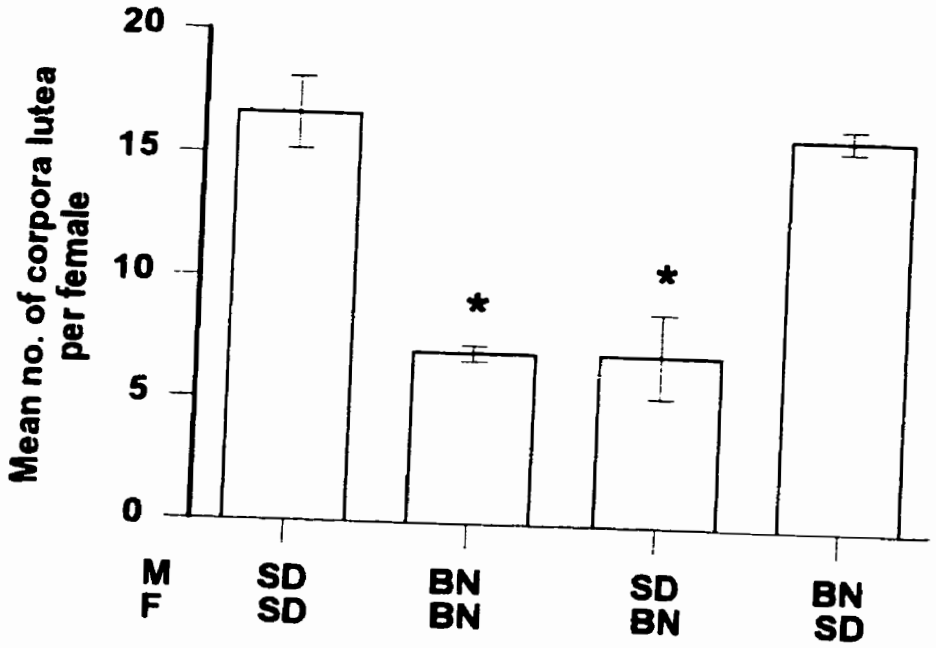

Mean no. of corpora lutea per female
20
15
10
5
0
*
*
M
F
SD
SD
BN
BN
SD
BN
BN
SD

**Fig. 2.** Effect of increasing paternal age on pregnancy outcome.

*Top*: Percentage of preimplantation loss per female rat on day 20 of gestation. *Bottom:* Percentage of postimplantation loss per female rat on day 20 of gestation. Bars represent means ± SEM; n=6-12, *$p < 0.05$, by ANOVA and Tukey's test.

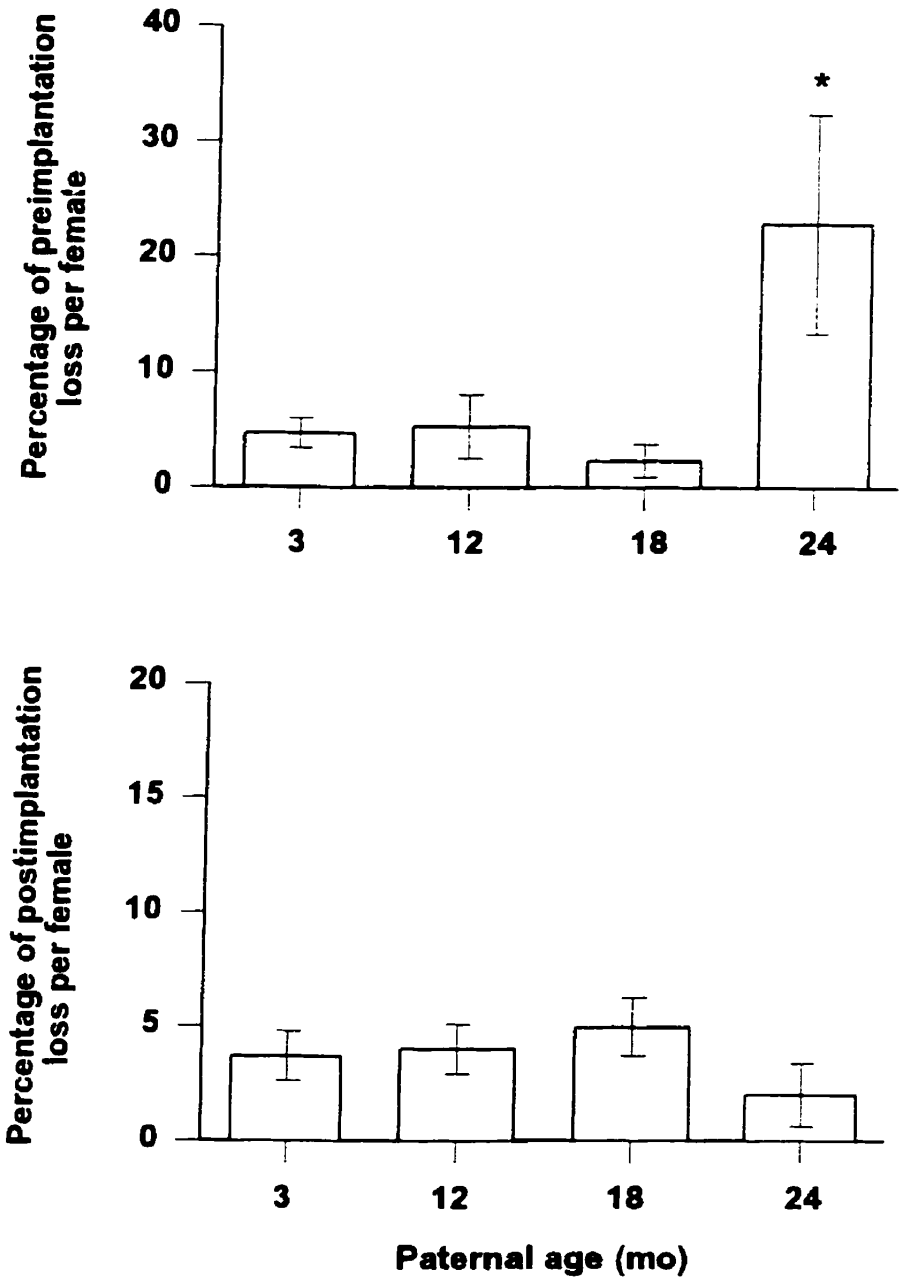
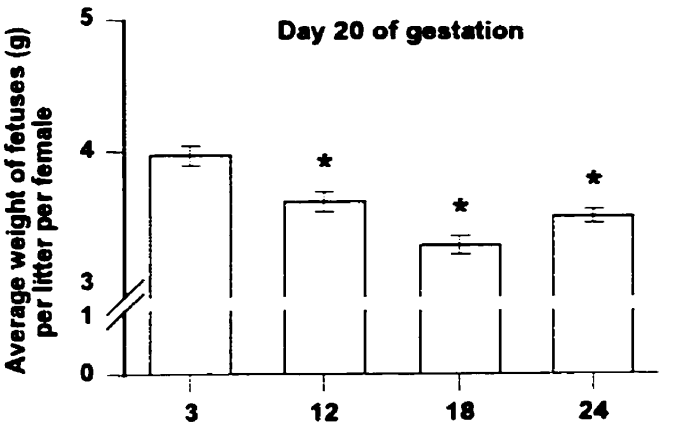
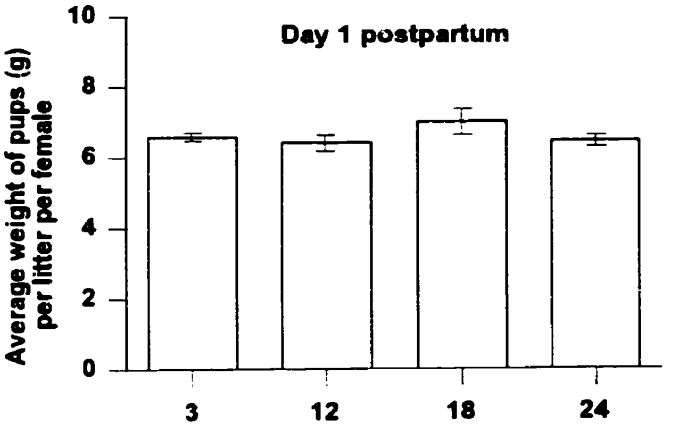

Percentage of preimplantation loss per female
40
30
20
10
0
*
3
12
18
24
Percentage of postimplantation loss per female
20
15
10
5
0
3
12
18
24
Paternal age (mo)

**Fig. 3.** Effect of increasing paternal age on weight of the offspring.

*Top*: Effect of paternal age on the average weights per litter of fetuses (n=6-12) on day 20 of gestation. *Middle:* Effect of paternal age on the average weights per litter of pups (n=6-8) on postnatal day 1. *Bottom*: Effect of paternal age on the average weights of pups (n=6-8) on postnatal day 7. Bars represent means ± SEM. *P < 0.05, by ANOVA and Tukey's test.


Day 20 of gestation
Average weight of fetuses (g) per litter per female
5
4
3
1
0
*
*
*
3
12
18
24


Day 1 postpartum
Average weight of pups (g) per litter per female
10
8
6
4
2
0
3
12
18
24

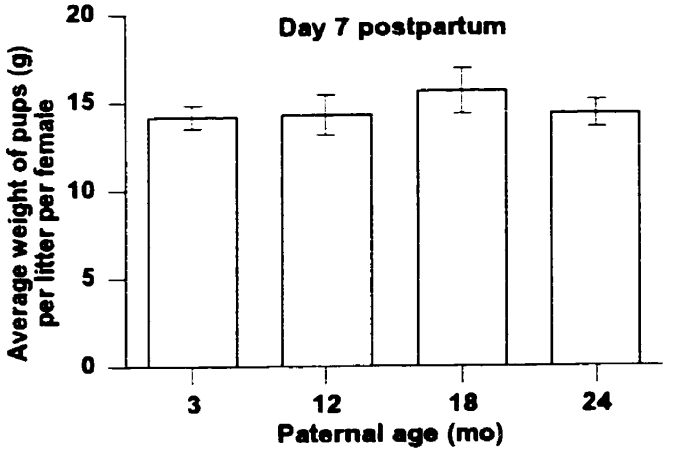

Day 7 postpartum
Average weight of pups (g) per litter per female
20
15
10
5
0
3
12
18
24
Paternal age (mo)

**Fig. 4.** Effect of increasing paternal age on the incidence of neonatal death. Bars represent means ± SEM. N= 6-9. $^{*}P< 0.05$, by Kruskal-Wallis $\chi^{2}$.


Average no. of neonatal deaths per litter
2
1
0
*
3
12
18
24
Paternal age (mo)

# Chapter V

# Discussion

Although considerable information has accumulated regarding the epididymis during its post-natal differentiation phase and in the adult, very few studies have examined how the epididymis is affected by advancing age. The major obstacle to understanding how the male reproductive system is modified by age has been the lack of an appropriate model that would distinguish the effects of age from the effects of disease. Brown Norway rats are remarkably long-lived animals that stay healthy during aging. This strain of rats has proved to be an excellent model for aging studies and has brought some startling advances to our understanding of the mechanisms of aging.

The overall objective of these studies was to determine the effects of aging on the structure and functions of the epididymis. The study presented in chapter II reveals that the histology of the epididymis of the Brown Norway rat is severely altered by aging. The changes in the epididymal epithelium point to a major disturbance of intracellular traffic with age. Modifications of the structure of the epididymis may have considerable implications, such as the recruitment of immune cells described in chapter III. The dramatic infiltration of the epithelium by immune cells is likely to create further damage of the tissue. Alteration of the structure of the epididymal epithelium may impair its secretory and absorptive functions, thereby resulting in a modified intraluminal microenvironment. Interestingly, the study presented in chapter IV showed a decline in male fertility with age which is possibly related to an improper maturation of spermatozoa .

To facilitate the integration of the results collected in chapter II, III and IV, this

chapter is divided into two major sections. In the first, we will briefly review the effects of increasing paternal age on fertility and progeny outcomes, and discuss their significance and implications. In the second, we will focus on the events occurring in the epididymis that may contribute to a decrease in the quality of spermatozoa in aged Brown Norway rats. A hypothesis on the mechanism of aging in the epididymis will be proposed.

## A. Alteration of the Quality of Spermatozoa with Age

A trend in the second half of this century is the postponement of childbearing until a later age. The detrimental effects of maternal age on pregnancy and progeny outcomes are well documented, but there are very few studies that address the influence of paternal age. We showed that, with increasing paternal age, both fertility and progeny outcome are affected in the Brown Norway rat (chapter IV). More specifically, advancing paternal age is associated with increased preimplantation loss, suboptimal fetal growth by day 20 of gestation, and a rise in neonatal deaths.

### 1. Increase in Preimplantation Loss

We report for the first time that there is increased preimplantation loss in litters fathered by aged male rats. This loss can be explained either by a decrease in the ability to fertilize oocytes or by the degeneration of early embryos before

implantation.

### a. Decrease in Fertilizing Ability

#### i. Changes in Motility of Spermatozoa

Fertilizing ability is correlated to sperm motion parameters such as curvilinear velocity [1, 2]. Altered motility patterns are associated with a disruption in the ability of spermatozoa to undergo capacitation and the acrosome reaction [2]. Previous studies report a decrease in sperm motility with advancing age [3-5]. Computer aided sperm analysis (CASA) should be used to analyse the effects of age on spermatozoal motion parameters. Since it takes only a few motile spermatozoa to achieve fertilization [3,6], whether or not changes in these parameters actually translate into an effect on the overall reproductive capacity of an animal needs to be assessed.

#### ii. Morphological Abnormalities of Spermatozoa

The rate of morphological abnormalities in spermatozoa increases with age. For example, many spermatozoa have either axonemal or acrosomal malformations [3,6,7]. Recently, new software for automated sperm morphology analysis (ASTMA) has been developed [8] and should provide new insights into the effects of age on the morphology of spermatozoa. Most authors agree that, in healthy men, increasing

age does not affect the capacity of spermatozoa to penetrate the ova of a zona-pellucida free hamster egg [3,6,7,9,10]. Thus, more experiments should be done to assess the effect of age on the motility, morphological aberrations of spermatozoa and their relation to an altered fertility.

### iii. Nuclear Decondensation

Following penetration of the ova, nuclear decondensation and male pronucleus formation ensue and are critical to subsequent embryonic development. A delay in pronucleus formation, for example may be responsible for alterations in the developing embryo [1,11]. Perreault *et al.* [12] showed that both sperm nuclear decondensation and male pronucleus formation are related to the nuclear disulfides bonds acquired during passage through the epididymis. The nuclear decondensation and the timing of pronuclei formation of aging Brown Norway rat spermatozoa, after penetration of an ova, could provide useful insight into potential damage to the nucleus of spermatozoa during aging.

Nuclear decondensation restores the paternal genome to a replicatively and transcriptionally active conformation [13]. Paternal treatment with cyclophosphamide, an agent known to modify decondensation, results in increases in preimplantation loss and decreases in fetal weight (rat [14]). This suggests that the similar effects observed in progeny sired by aging Brown Norway rats may also be due to defective nuclear decondensation. A method for quantifying the morphometric parameters of sperm decondensation has been developed by Janping

Qiu (laboratory of Dr. B. Robaire) and could be used to determine if sperm nuclear decondensation is affected by age [13].

### b. Embryo Degeneration Prior to Implantation

If fertilization occurs normally with a gamete from an aged rat, then the increased preimplantation loss observed in our studies is likely to be due to a degeneration of the zygote or early embryo. Collecting the eggs at various timepoints post mating would clarify the exact time of embryonic degeneration. In addition, methods that allow study of DNA and mRNA, such as PCR or RT-PCR, could be used to assess any molecular changes occurring at any of these time points.

## I. Chromosomal Aberrations

As a male ages, the number of divisions that his germ stem cells have undergone increases and, as a result, cytogenetic damage is expected to accumulate; this may result in early arrest of embryonic development [15]. Recently, Gavrilov *et al.*, have shown that lifespan decreases in daughters born of aged fathers, thus suggesting that there is a detrimental effect of paternal age on the X chromosome [16]. In addition, chromosomal pathologies and congenital malformations are more frequent with older paternal ages [17,18].

The spermatid micronucleus assay (an antikinetochore antibody) and chromosome painting (in situ hybridisation with a whole chromosome antibody) can

be used to identify chromosomal abnormalities [19]. Lowe *et al.* [19] have used these techniques to show that with age there is an increase in the frequency of chromosomal damage in germ cells (mice [19]). Thus, these methods could be used to determine if early arrest of embryo development is related to chromosomal abnormalities in spermatozoa of aging Brown Norway rats. These methods would also allow us to determine whether or not spermatozoa with abnormal chromosomes are eliminated during their transit though the epididymis.

## 2. Fetal Growth Retardation

### a. Changes in DNA Methylation and Imprinting

The increased chance of fathering growth retarded fetuses is a worrisome consequence of paternal aging. Interestingly, paternal treatment with 5-azacytidine, a compound that blocks DNA methylation, has similar effects on progeny outcomes as increasing paternal age [20]. More specifically, both affect preimplantation loss, but neither litter size nor post-implantation loss are affected [20]. DNA methylation is important in imprinting, a phenomenon whereby different genes are expressed depending on their parental origin [21-23]. DNA methylation is established in the germ line by DNA methyltransferase (DNA MTase) [24,25]. Embryos with a homozygous mutation in the DNA MTase gene have a decreased amount of DNA methylation and their development and survival is compromised [26]. Thus, we can

assume that adequate DNA methylation is critically important to successful embryogenesis. Based on the similarities between these findings and our results, we hypothesize that there are age-related changes in the DNA methylation of male gametes.

### b. Alteration of Imprinting and Growth Retardation

IGF-2 is a paternally imprinted fetal growth factor which, when disrupted, causes growth retardation [27, 28]. Altered imprinting may therefore lead to the growth retardation seen in fetuses fathered by old rats. This interpretation could be confirmed by studying IGF-2 expression in pre- and post-implantation embryos.

## 3. Increase in Early Neonatal Death

We observed that a majority of the growth retarded fetuses had caught up to the normal expected weight by one day post-partum, even though the duration of gestation was identical for every pregnancy, regardless of paternal age. The mechanism of this accelerated growth is obscure. A comparable phenomenon was reported in humans whereby low birth weight infants showed accelerated growth and caught up to their normal counterparts [29]. The accelerated growth may result from the secretion of different growth factors and/or an enhanced response of

underweight fetuses to growth factors.

At birth, some of the fetuses fathered by old Brown Norway rats were severely underweight. These pups lost weight and died within five days post partum. Low weight at birth is correlated to a high mortality rate [30]. Thus, in our results, growth retardation may be directly related to early neonatal death. Careful autopsies should be performed in order to identify the cause(s) of these deaths.

### 4. Causal Factors for a Decrease in the Quality of Spermatozoa with Age

The decrease in the quality of spermatozoa with advancing paternal age may result from intrinsic aging of germ stem cells as well as modified testicular and/or epididymal environment during spermacytogenesis and spermiogenesis.

#### a. Intrinsic Aging of Germ Stem Cells

The precise fate of germ stem cells with age is unclear at the moment. Spermatogenesis is a continuous process of cell division, many authors therefore assume that the quality of spermatozoa is not affected by age. However, spermatogonia (stem cells) continually divide by mitosis to renew themselves [31], thereby increasing the probability of a copy error [32]. More studies are needed to assess whether or not the germ stem cells population structure and functions are affected by age.

### b. Modified Environment

Age affects both the structure and functions of the testis (reviewed in chapter I). It is likely that these changes will compromise the elaborate process of differentiation of germ cells. The epididymis is the site of transport, maturation and storage of spermatozoa [33]. Changes in the structure and functions of the epididymis may also contribute to the decrease in the quality of spermatozoa. This aspect will be developed in the next section.

## B. Changes in the Structure and Functions of the Epididymis with Age

### 1. Loss of Epididymal Epithelial Homeostasis with Age

### a. Changes in the Environment

The structure and functions of the epididymis are dependant on factors coming directly from the testis and indirectly via the blood circulation [33-36]. There are age related changes in the testicular input into the epididymal lumen as shown by decreases in spermatogenesis and steroidogenesis (rat [37-39]; human [40,41]). Furthermore, it is now widely accepted that increasing age is often accompanied by a decline in the quantity of hormones produced by major endocrine systems [42,43]. In addition, a decrease in endocrine and paracrine factors available to the epididymal epithelium might be due to an increased difficulty for these factors to reach the epididymal epithelium. In the aged Brown Norway rat, an increase in the thickness

of the basement membrane was seen as early as 12 months. The basement membrane acts as a filter (Fig 1A). A thicker basement membrane may prevent essential components from entering the epithelium (Fig.1B). A possible consequence of this may be the projection of pseudopods by basal cells in the initial segment to facilitate the uptake of endocrine or paracrine factors (Fig. 1C). Pseudopods may prevent or slow down the age-related loss of epithelial homeostasis.

**b. Destabilisation of the Epithelial Frame**

Basal cells, found throughout the epididymis, lie on the basement and have a cone-like projection that reaches toward the lumen between adjacent principal cells. Although their precise function has not yet been defined [44-47], their unique shape suggests that they may act as a frame to stabilize the epithelium (Fig. 2A). Obviously, a thickening and convolution of the basement membrane, as observed in aging animals (chapter II), will disturb this frame (Fig. 2, B and C). A destabilization of the frame may initially manifest itself as a loss of the columnar aspect of principal cells and then evolve into the disorganisation of the epithelium and an apparent stacking of epithelial cells. Disorganization of the epithelial frame may lead to a loss of cellular homeostasis that is probably of considerable importance regarding the recruitment of immune cells.

### c. Disturbance of the Intracellular Traffic

The morphologic changes seen during aging point to a major disturbance of the intracellular traffic as shown by the dramatic accumulation of dense lysosomes.

## I. Increase in Oxidative Stress

### *Accumulation of Lipofuscin Pigments in Lysosomes*

Lysosomes are composed of two primary components: lipofuscin and hydrolytic enzymes. Lipofuscin is a yellowish brown pigment that accumulates in a variety of mammalian tissues during aging [48-51]. Lipofuscin, when excited with ultraviolet light, is known to emit an intense yellow autofluorescence [52,53]. Principal cells in the corpus epididymidis of aged rats showed a major accumulation of autofluorescent lysosomes (not shown) which is consistent with the presence of lipofuscin. Interestingly, it has been suggested that autofluorescence is linked to oxidative processes and that lipofuscin may accumulate as a product of oxidative stress [52-59]. The buildup of autofluorescent lysosomes in the Brown Norway rat, therefore, may suggest that an increase in oxidative stress might accompany aging and thus lead to the enhanced production of lipofuscin. The increased oxidative stress with age may be the consequence of a decrease in the efficiency of the antioxidant shield and/or an increase in the oxidant exposure.

*Decrease of the Efficiency of the Antioxidant Shield*

The antioxidant shield is primarily composed of enzymes, such as glutathione-S-transferase (GST), glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase which protect the cell against electrophilic attack [56, 60].

Recently, Mueller *et al.* have assessed the expression of various GST isoenzyme subunits (Ya, Yc, $Yb_1$, $Yb_2$, Yo, Yf) in the epididymis of aging Brown Norway rats [61]. Surprisingly, age does not affect the expression of GSTs in the epididymis except in the proximal cauda region. In this region, vacuolated principal cells lose the ability to express GST subunits Yo, Yc, $Yb_1$, and $Yb_2$ yet they retain Ya subunit expression. In contrast, apparently normal, neighbouring principal cells maintain the ability to express subunits Yo, Yc, $Yb_1$ and $Yb_2$ but lose the expression of Ya. These results suggest that altered GST expression renders principal cells more susceptible to oxidative stress and may be responsible for the emergence of large vacuoles [61].

When simultaneously overexpressed, copper-zinc SOD (SOD-1) and catalase, decrease oxidative damage and increase the lifespan of *Drosophila melanogaster* [62]. However, studies in mice show that aging is accompanied by an increase in the activity of SOD1 enzyme and an increase in the ratio of SOD1/Gpx1 activity in organs presenting major peroxidation damage [63]. Furthermore, the levels of lipid peroxidation are higher in the muscles of the murine model for muscular dystrophy; these transgenic mice overexpress SOD1 [64]. It would be interesting therefore to investigate the effect of age on the expression, distribution and activities of SOD,

Gpx, and catalase; we speculate that aging of the epididymal epithelium is accompanied by changes in the balance of these antioxidant enzymes.

### ii. Alteration of the Degradative Pathway

The qualitative and quantitative changes observed in endosomes and lysosomes present in the epididymal epithelium during aging are suggestive of a disturbance in intracellular trafficking. An increase in the load of material to be degraded and/or a decrease in the efficiency of the metabolic machinery could account for this disturbance.

#### *Increase in the Load of Material to be Degraded*

The luminal composition, at least with regard to spermatozoa, changes dramatically in the aged Brown Norway rat (chapter III). The observation that spermatophagy is occurring in the aged epididymal epithelium is significant (chapter II). Spermatophagocytosis by epithelial cells is not normally observed (human, monkey [64]), however it has been reported to occur after excurrent duct obstruction (ram [65], human [66], rat [67]). Uptake and degradation by the epithelium may be stimulated if the amount of immature germ cells, and abnormal and/or degenerating spermatozoa present in the lumen, increases. Experimentally induced degeneration of spermatozoa in the young adult with vitamin A deficient diet [69, 70] induces stasis of spermatozoa. An increase in the load of material to be degraded might cause a disturbance in intracellular traffic. Studies of the effect of age on the internalization

of luminal substrates by epithelial cells would be of interest. Alternatively, since the epididymal epithelium was severely modified with age, an increase in the amount of damaged cellular organelles to be eliminated by intralysosomal degradation may be responsible for the alterations in intracellular trafficking.

### *Decreased Efficiency of the Metabolic Machinery*

Lysosomal enzymes, crucial to the efficiency of the metabolic machinery, are present in the epididymal epithelium. Functional alterations in these enzymes may contribute greatly to the process of aging.

Rat sulfated glycoprotein-1 (SGP-1) is present in the epididymis and is a precursor to lysosomal saposins, which are responsible for activating the hydrolysis of membrane glycosphingolipids by lysosomal hydrolases [71-73]. Since SGP-1 is involved in the degradation of membranes, it would be interesting to study the effects of age on the expression of SGP-1 and on its distribution in the Brown Norway rat. Immunogold labeling studies with anti-SGP-1 antibody could also address lysosomal accumulation in aged animals and the intracellular trafficking defects that may occur.

β-hexosaminidase, along with cathepsins participate in the intracellular degradation of endogenous and exogenous proteins [74, 75]. There are two isoenzymes of the lysosomal beta-hexosaminidase (Hex), Hex A (αβ) and Hex B (ββ). The activity of β-hexosaminidase is high in the testis and epididymis (mouse [76]; rat [77]). A mutation in the α and β subunits of hexosaminidase causes $G_{M2}$ gangliosidosis, such as in Sandhoff disease [78, 79]. In the mouse model for

Sandhoff disease, mice have a mutation in the *hexb* gene coding for the β subunit of beta-hexosaminidase and are Hex A and B deficient (*hexb-/-*) [80, 81]. Interestingly, the epididymides of these mice show striking histological similarities with the aging Brown Norway rat. For example, H*exb-/-* mice also have lysosome accumulation in principal cells and clear cells, clear cell enlargement and interlysosomal fusion [81]. Based on these observations, one would predict that there may be a disruption in the *hexb* subunit in the aged Brown Norway rat.

In rat and human epididymides, lysosomes present in principal cells of the corpus region react with anti-cathepsin D antibody (rat [82], human [83]). Cathepsin expression increases in aging neurons and this protease colocalizes with lipofuscin in lysosomes (rat [84]). The increase in the number of lysosomes present in aged Brown Norway rat principal cells of the corpus epididymidis leads us to anticipate increased expression of cathepsin D. Idgoura [83] has proposed that in the corpus epididymidis, cathepsin D in lysosomes of principal cells may be involved in the degradation of degenerating spermatozoa as well as the degradation of cytoplasmic droplets detached from spermatozoa. In aging Brown Norway rats the proportion of abnormal spermatozoa seems to be increasing. This may result in an increase in intracellular traffic, leading to the accumulation of lysosomes in principal cells.
In contrast, vacuolated principal cells of the proximal cauda epididymidis had few or no dense lysosomes, therefore we would anticipate to see a lack of cathepsin D in these cells. In addition, the presence of spermatozoal debris, and possibly entire spermatozoa, within these vacuoles, which are seen only in older animals, is further

evidence that, in the aged animal, lysosomal degradation is impaired.

The emergence of large vacuoles in the principal cells of the proximal cauda epididymidis of aged animals (chapter II) clearly indicates a disruption in intracellular trafficking, namely the endosomal lysosomal system. These giant vacuoles contained pale material which could indicate either that they contain luminal fluid,or fluid transported in from the interstitium, or that they are endosomal in nature. One way to clarify this issue would be to label the vacuoles with an antibody to a protein known to be found only in the lumen, such as immobilin [85]. We predict that these vacuoles are endosomal, and thus no antibody reactivity is expected.

The observation that dense and pale lysosomes, and multivesicular bodies appeared to empty their content into the large vacuoles is further illustration of a major disruption in endosome-lysosome intracellular traffic. More specifically, it seems that the process of endosomal-lysosomal fusion is disrupted. Study of the effect of age on the intracellular movement of lysosomal glycoproteins found in the lysosomal membrane, such as lysosome associated membrane glycoprotein-1 (Lamp-1) [86], will be of interest. An alteration in the fusion process may result in quantitative, and possibly qualitative, changes in lysosomal enzymes.

## 2. Activation of the Immune System in Response to the Loss of Epididymal Homeostasis

The halo cell population is composed of both T-lymphocytes and monocytes-macrophages; the proportion and distribution of each is age and segment dependent. Macrophages and T lymphocytes have been reported to be present in both the interstitium and the epithelium of the epididymis under normal conditions (human [87, 88]; rat [89, 90]; mouse [91]). Although the reason for their presence is unclear, it has been proposed that they act as an immunological barrier to protect the epithelium [44, 90, 92]. Halo cells are present in 10-day-old rat epididymal epithelia [93], even though there are, as yet, no spermatozoa in the epididymal lumen. Moreover, the fact that tight junctions do not form until day 21 [93, 94] supports the suggestion that one of the main functions of these immune cells is to "watch over" the epididymal epithelium.

### a. Recruitment of Immune Cells and Changes in Intracellular Traffic

#### I. Accumulation of Immune Cells In Damaged Tissue

The primary effect of aging on the immune cell composition of the epididymis was an increase in the number of monocytes-macrophages and lymphocytes (chapter III). However, aging was not usually accompanied by a parallel increase in immune cells in the interstitial space suggesting that there is an active recruitment of

immune cells into the epithelium.

The increase in the number of immune cells present in the epithelium might be related to the emergence of many damaged epithelial cells. With respect to individual cell type populations, the recruitment of immune cells occurs in a segment specific manner. The general trends were that, with age, there were increases primarily in T lymphocytes in the distal cauda epididymidis whereas in the initial segment, caput and corpus and proximal cauda epididymidis both monocyte-macrophage and T-lymphocyte populations increased.

### ii. Impairment of Intracellular Traffic

In the distal cauda epididymidis, the number of T lymphocytes was increased as early as 12 months. At that age, and later ages, there were no major changes in the histology of epithelial cells. There were no further increases between 12 and 24 months in the number of T-cells. Furthermore, the number and size of monocyte-macrophage remained unchanged with increasing age in this region that appeared spared by aging. One striking difference observed between the distal cauda and more proximal regions is that there is a trend for dramatic disturbance of the endosomal lysosomal intracellular trafficking system in the segments where both T lymphocytes and macrophages are actively recruited.

### b. Additional Recruitment of Immune Cells and the Blood Epididymis Barrier

One of the major findings of our study presented in chapter III was that the number of immune cells in the epithelium increases with age and that this increase was further enhanced when the luminal content was abnormal. What is clear from our studies is that changes in luminal composition, at least with regard to spermatozoa, are associated with increased infiltration of immune cells into the proximal epididymis. We noticed that the number of immune cells was the highest in the caput epididymidis of rats with few or no spermatozoa in the lumen. As this region is known to absorb large amounts of testicular exocrine fluid [95, 96], free and soluble antigens from the lumen may cross the epithelium, and come into direct contact with immune cells [90, 92]. Alternatively, the endocytosis of spermatozoa, immature germ cells and/or their respective debris could potentially trigger this phenomena.

#### i. Loss of Cellular Homeostasis and Weaker Blood Epididymis Barrier

A more likely mechanism explaining the immune cell recruitment might be that spermatozoa, post-pubertal germ cells and/or their respective debris are crossing the blood epididymis barrier. To assess whether or not age affects the blood epididymis barrier, aging Brown Norway rats were perfused with lanthanum nitrate, an electron dense tracer that will not reach the lumen if tight junctions are intact. Results show

that the blood epididymis barrier becomes leaky with increasing age [97]. Thus, the loss of cellular homeostasis may result in a decreased efficiency of the blood epididymis barrier.

### ii. Selective Recruitment of Immune Cells and Leaky Blood Epididymis Barrier.

The fact that the number of immune cells is higher in the epithelium of Brown Norway rats whose lumen contained few spermatozoa may appear contradictory. However, one may speculate that more immature germ cells and/or abnormal spermatozoa are present in the epididymal lumen of these animals. These may degenerate in the lumen and increase the antigenic load susceptible to cross the blood epididymis barrier. Gossypol is a contraceptive agent that generates specific defects in late spermatids and epididymal spermatozoa [98]. Interestingly, treatment of rats for six weeks with gossypol does not result in a leaky blood epididymis barrier [98]. Thus, Gossypol treatment could be used in future studies to assess whether an abnormal luminal content in the presence of an intact blood epididymis barrier stimulates the recruitment of immune cells.

## 3. Alteration of the Quality of Spermatozoa Following Loss of Epididymal Homeostasis

### a. Loss of Epididymal Homeostasis and Changes in the Intraluminal Microenvironment

The specialized intraluminal microenvironment essential for the maturation (acquisition of the full capacity for fertilization) and storage of spermatozoa is created by absorption and secretion by the epididymal epithelium, and maintained by the blood epididymis barrier [33, 34, 44].

#### *i. Alteration of the Secretive and Absorptive Functions of the Epididymal Epithelium and Intraluminal Microenvironment*

The secretion of electrolytes, fluid, and low molecular weight molecules and proteins is likely to be greatly modified with aging. Indeed, absorption and secretion by the epididymal epithelium are largely dependant on the presence of androgen [35]. In addition, the alteration of intracellular traffic in epididymal epithelial cells and the disruption of endosomal lysosomal balance presumably impairs absorption and secretion.

### *ii. Alteration of the Blood Epididymis Barrier and Intraluminal Microenvironment*

As previously mentioned, aging is accompanied by a segment specific loss of the blood epididymis barrier integrity in the Brown Norway rat [97]. Consequently, the luminal microenvironment might be modified and the maturation of spermatozoa might be impaired.

## C. Hypothesis on the Mechanism of Aging in the Epididymis

Our studies showed that the structure of the epididymis and the fertility and progeny outcome are dramatically affected by age in the male Brown Norway rat. The histology of the epididymis showed striking qualitative and quantitative changes with age. The thickening of the basement membrane was the among the first sign of aging found. The thickening of the basement membrane may prevent essential paracrine and endocrine factors from entering the epididymis. The accumulation of lysosomes closely follow the changes in the basement membrane and is suggestive of a major age-related disruption of the endosomal lysosomal pathway. An increase in oxidative stress and/or a saturated degradative pathway may contribute to the disruption of the intracellular traffic in the Brown Norway rat. Among possible causal factors are an increase in the amount of load of material to be degraded, possibly damaged intracellular components and spermatozoa and/or immature germ cell debris. The ensuing progressive loss of homeostasis of epididymal epithelial cells

has multiple consequences. The alteration of intracellular traffic will likely compromise the absorptive and secretory functions of the epididymal epithelial cells. More immune cells are migrating into the epithelium, likely to eliminate damaged epithelial cells. Spermatozoa and immature germ cell debris may cross the blood epididymis barrier and create a higher antigenic load that further stimulate the recruitment of immune cells in the epididymal epithelium. Potentially deleterious components may also enter the intraluminal compartment. Together these changes contribute to a dramatic modification of the intraluminal microenvironment composition, thereby resulting in an improper maturation of spermatozoa (Fig. 3). We showed that indeed the quality of spermatozoa decreases as males age. Together, these observations suggest that the mechanisms of aging are multifactorial and interrelated, and likely result from the cumulative effects of intrinsic aging of epididymal epithelial cells and modified environment.

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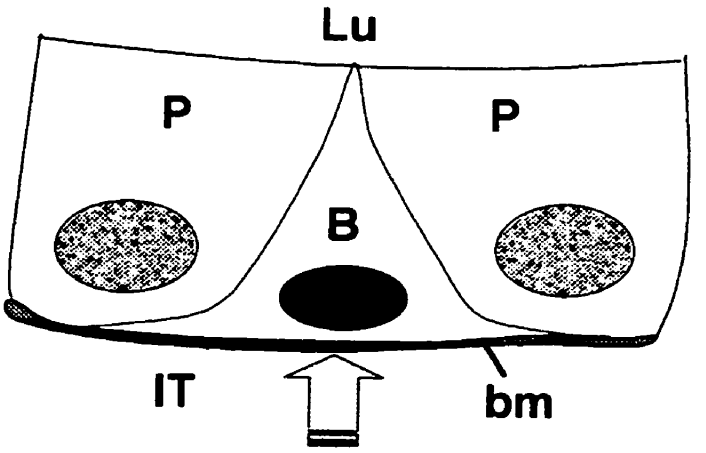
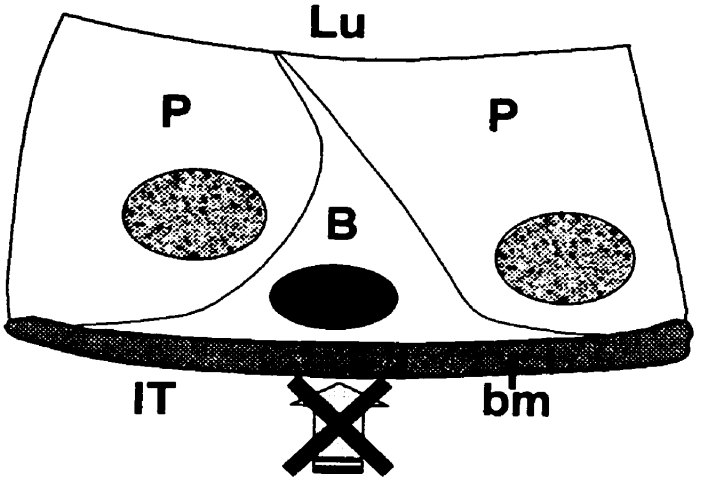
Fig. 1 Diagrammatic representation showing the effect of age on factors entering the epididymal epithelium.

**A**) Young animal, **B**) aged animal (caput, corpus, cauda), **C**) aged animal (initial segment of the epididymis). Lu, lumen; P, principal cell; pp, pseudopodes; B, basal cell; bm, basement membrane; IT, intertubular space. Large Arrow: uptake of products.

A.




B.




C.

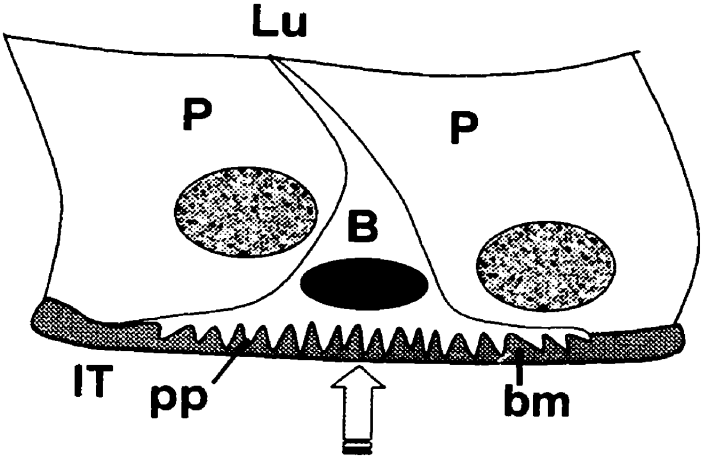
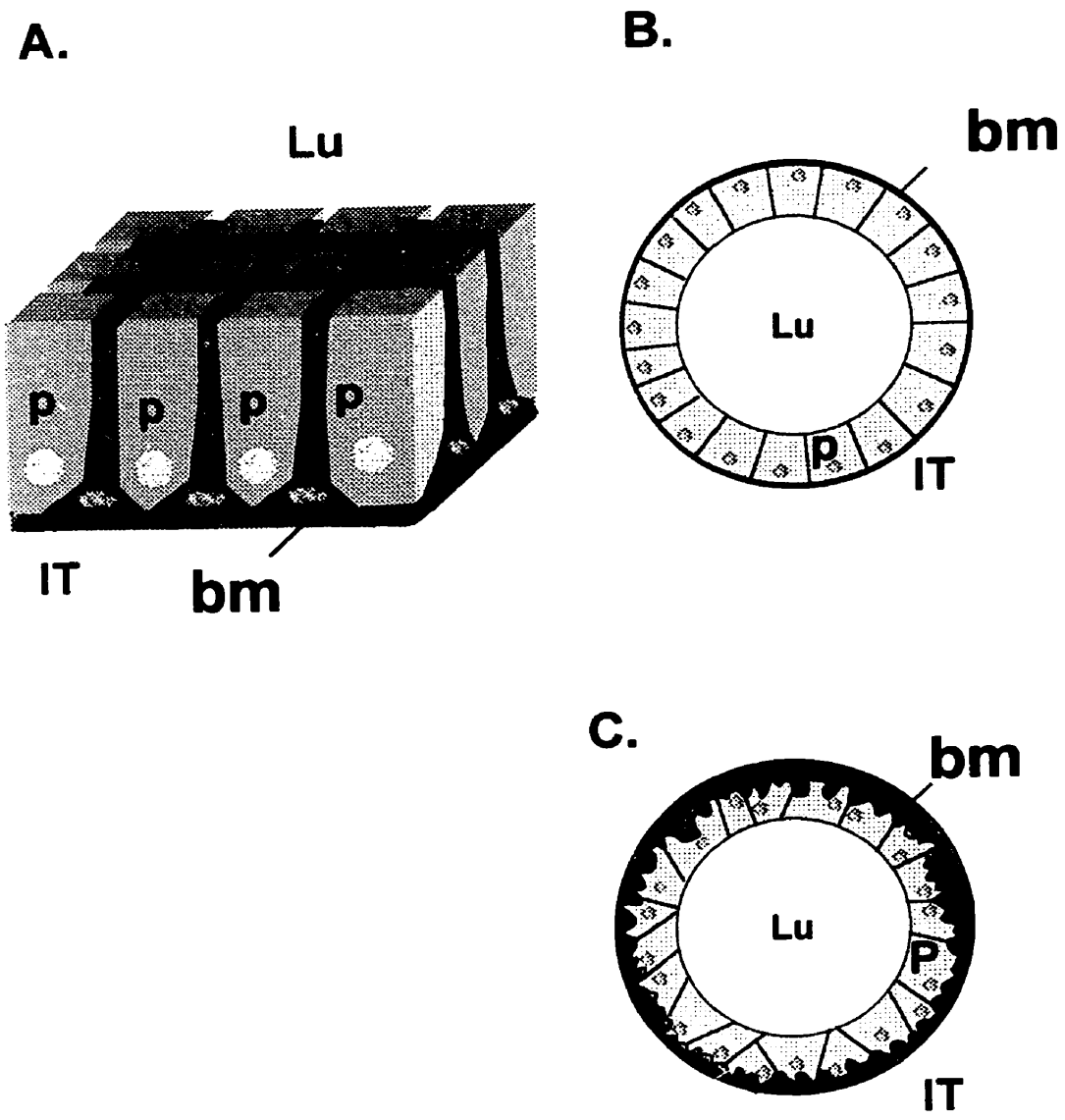


Fig. 2 Diagrammatic representation showing the destabilisation of the epithelial epididymal frame of the Brown Norway rat with age. **A**) Young animal, basal cells act as a frame to stabilise the epithelium (3D) ; **B**) young animal, columnar aspect of epithelial cells (2D), and **C**) aged animal, loss of columnar aspect following thickening of the basement membrane. Lu, lumen; P, principal cell; B, basal cell; bm, basement membrane; IT, intertubular space.


A.
Lu
P
P
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B.
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C.
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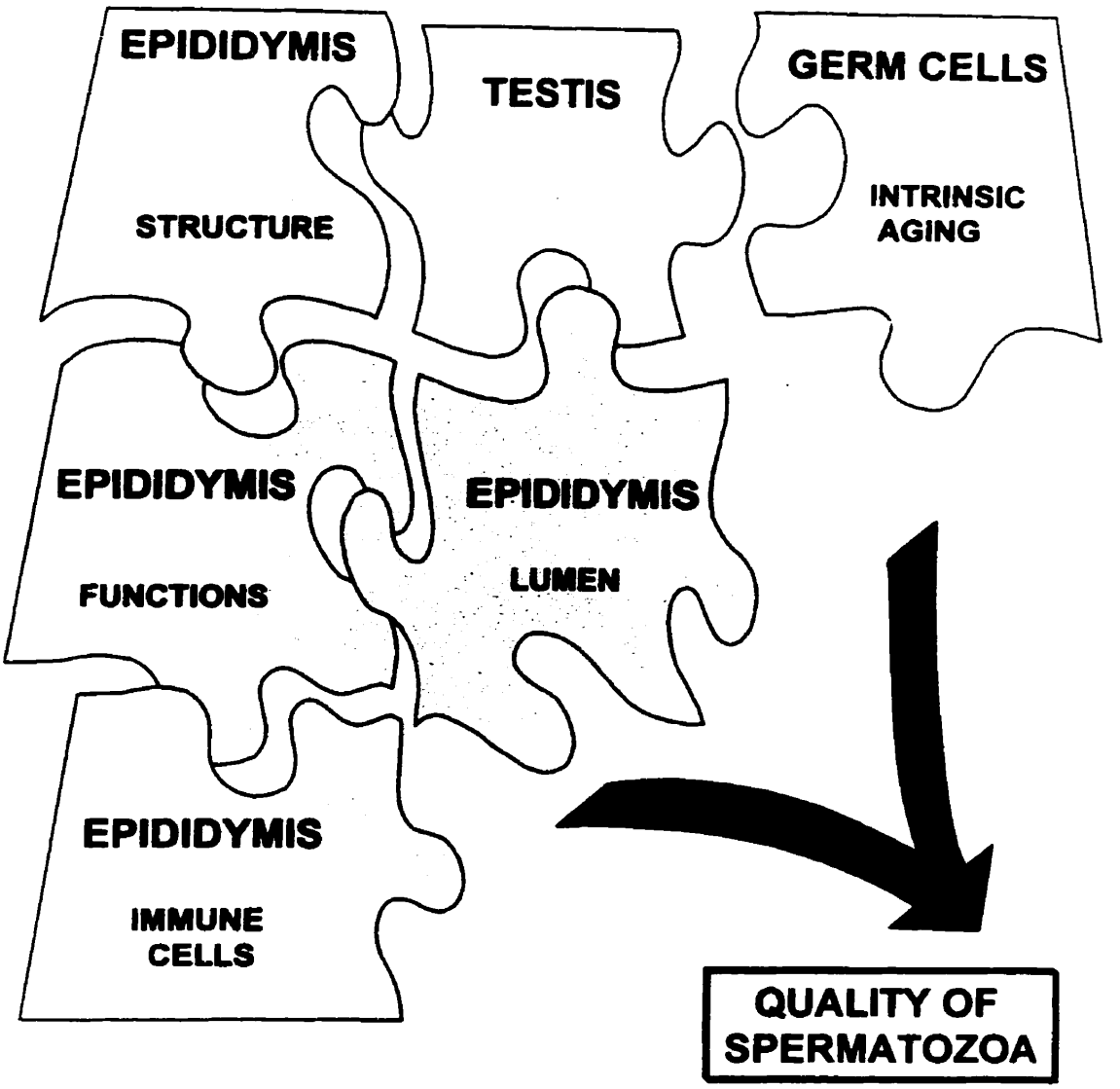
Fig. 3. Diagrammatic representation showing complex interactions of the environment and intrinsic aging on the structure and functions of the epididymis.


EPIDIDYMIS
STRUCTURE
TESTIS
GERM CELLS
INTRINSIC AGING
EPIDIDYMIS
FUNCTIONS
EPIDIDYMIS
LUMEN
EPIDIDYMIS
IMMUNE CELLS
QUALITY OF SPERMATOZOA

## LIST OF ORIGINAL CONTRIBUTIONS

Details of the original contributions are provided in the three manuscripts above. The key findings are:

- There was an age-dependent increase in the thickness of the basement membrane and in the number of halo cells.

- There were also major segment specific changes in the appearance of principal, basal, and clear cells along the epididymis with increasing age.

- Age-dependent changes in the histology of the epididymal epithelium were not related to the presence of spermatozoa, often preceding their disappearance, thus indicating that there may be an intrinsic mechanism of aging in epididymal epithelial cells.

- The demonstration that epididymal halo cells are composed of monocytes, helper T lymphocytes and cytotoxic T lymphocytes belong to the population of halo cells.

- The discovery that there is a segment specific increase with age in the number of immune cells throughout the epididymis.

- The finding that there is a segment specific recruitment of cytotoxic T helper and monocytes-macrophages in the epididymal epithelium of aged rats whose epididymal lumen contained few spermatozoa.

- The hypothesis that accumulation of damaged epithelial cells and antigens of spermatozoal or post pubertal germs cells, leaking through a dysfunctional blood epididymis barrier, may contribute to the active recruitment of immune cells with age.

- The demonstration that increased paternal age is associated with an increase in pre-implantation loss.

- The observation that there is a decreased in average fetal weights on the last day of gestation in litters fathered by older males.

- A significant increase in neonatal deaths in progeny fathered by older males.

**Chapter II. Segment Specific Morphological Changes in the Aging Brown Norway Rat Epididymis**

*Tissue Preparation for Light and Electron Microscopy*

Light microscopy:

- Perfusion: Valérie Serre
- Embedding: Valérie Serre
- Cutting tissues: Valérie Serre

Electron microscopy:

- Perfusion: Valérie Serre , James Lai
- Embedding: Valérie Serre, Marie Ballak
- Cutting tissues: Marie Ballak

*Morphological Analysis:* Valérie Serre

*Statistical Analysis:* Valérie Serre.

**Chapter III. The Distribution of Immune Cells in the Epithelium of the Epididymis of the Aging Brown Norway Rat is Segment-Specific and Related to the Luminal Content.**

Valérie Serre

*Tissue Preparation for Light Microscopy Immunocytochemistry*

*Immunostaining*

*Cell Counting*

*Size of ED1 Positive Cells*

*Statistical Analysis*

**Chapter IV. Paternal Age Affects Fertility and Progeny Outcome in the Brown Norway rat**

Valérie Serre

*Proven Ability to Mate*

*Mating Schedules*

*Effects of Rat Strain on Fertility*

*Effect of Paternal Age on Pregnancy Outcome*

*Effect of Paternal Age on Progeny Outcome*

*Statistical Analysis*

**Development of pictures:** Alan Forster