THE RABBIT OLFACTORY MUCOSA: POSTNATAL MORPHOLOGY AND THE EFFECTS OF ANTICANCER DRUGS VINBLASTINE AND DOCETAXEL

by

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DECLARATION

I, Boniface M. Kavoi, hereby declare that the data presented in this thesis is my original work and has not been submitted for a degree in any other University

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DEDICATION

This work is dedicated to my father, PAUL KAVOI and mother, ESTHER KAVOI

for their incalculable contribution towards my education,

and to MY FAMILY with love

"Knowledge is a vast ocean that no single person can claim to have circumnavigated"

(Yusuf Dawood, 2011)

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PUBLICATIONS

Part of this work was, before the compilation of this thesis, published in the following articles:

1) **Boniface M. Kavoi**, Andrew N. Makanya, Plendl Johanna and Stephen G. Kiama (2012) Morphofunctional adaptations of the olfactory mucosa in postnatally developing rabbits. *Anatomical Record* **295**: 1352-1363

2) **Boniface M. Kavoi**, Andrew N. Makanya and Stephen G. Kiama (2012) Anticancer drug vinblastine sulphate induces transient morphological changes on the olfactory mucosa of the rabbit. *Anatomia Histologia Embryologia* **41**: 374-387

ABSTRACT

The olfactory mucosa is a chemoreceptor structure located within the vertebrate nasal cavity and functions in detecting and discriminating between odors of different substances. Unlike in most parts of the nervous system where neural formation is confined to intra-uterine life, neurogenesis in the olfactory mucosa is a lifelong process. Rabbits are reputed for their heavy reliance on the olfactory cue owing to their being born blind and deaf and the exceptionally little maternal care that they receive during the suckling period. The preponderant use of this species as a model for toxicological research has also been widely reported. In conventional toxicity studies, substances administered by inhalation have been shown to cause injury to the olfactory mucosa. However, when chemicals are administered systemically, the effort to evaluate the mucosa for lesions has been minimal. Cancer chemotherapy, which is mostly antiproliferative, is associated with various forms of olfactory dysfunction of which the accompanying structural lesions remain largely unknown. In the present work, light and electron microscopy, immunohistochemistry and morphometry were used to analyze the extent and the pattern of structural refinement of the rabbit olfactory mucosa at neonatal (0-1 days), suckling (2 weeks), weanling (4 weeks) and adult (6-8 months) stages of postnatal development. Further, the impact of anticancer drugs vinblastine and docetaxel on olfactory mucosal structure, and on olfactory function (using the buried food recovery test) were investigated in adult rabbits. In all the postnatal ages, the basic components of the olfactory mucosa were present. However, Ki-67 immunostaining revealed an age-related decrease in the proliferative rates of cells of the olfactory

epithelium. Between birth and adulthood, cross-sectional diameters of axon bundles, packing densities of olfactory cells and cilia numbers per olfactory cell knob increased by 5.5, 2.1 and 2.6-fold respectively. Volume fraction for the bundles increased by 5.3% from birth to suckling age and by 7.4% from weaning to adulthood, and the bundle cores were infiltrated with blood capillaries in all age groups except in the adults. Single vinblastine and docetaxel injections to the adults at respective doses of 0.31 and 6.26 mg/kg resulted in marked differences in regard to the degree and duration of lesions that the drugs induced on the olfactory mucosa. On days 3 and 5 in the vinblastine-treated rabbits, there was disarrangement of the normal layering of nuclei of the mucosal epithelia, degeneration of axon bundles, occurrence of blood vessels within the bundles, localized death of cells of Bowman's glands and glandular degeneration. In the docetaxel-exposed animals, the above changes were evident on postexposure days 5 and 10. Relative to control values, bundle diameters, olfactory cell densities and cilia numbers in vinblastine-treated animals decreased to as low as 54.4%, 75.2% and 70.8%, respectively, on day 5 whereas in the docetaxel-exposed rabbits, the aforementioned parameters were lowest on day 10, with their respective values being 49.3%, 63.4% and 50.0%. In the control animals, volume fractions for the bundles and glands were 26.3% and 41.5% respectively. Respective volume fractions for these structures dropped to a low of 16.8% and 38.4% in the vinblastine-treated rabbits at day 5 and 13.4% and 34.9% in the docetaxel-exposed animals at day 10. In contrast, the volume fraction for the blood vessels in the controls was 19.9%, a value which was significantly lower than that of vinblastine-treated animals at day 3 (34.3%) or day 5 (31.5%) and of docetaxel-

exposed rabbits at day 5 (26.6%) or day 10 (28.5%). Fifteen days after treatment with the two anticancer drugs, all the above changes were resolved and the mucosa was morphologically indistinguishable from that of the controls. In the vinblastine-treated rabbits, the buried food test demonstrated a progressive increase in food-finding time in the first three days of exposure (latency score increased from the control value of 44 ± 9 to 179 ± 14 sec on postexposure day 3). On day 5, the animals failed to find the piece of cookie (latency 341 ± 18 sec). This was followed by recovery from the olfactory deficit with the food-finding time decreasing to 83 ± 12 sec on day 7 and subsequently to 48 ±8 sec on day 15. With docetaxel treatment, the food-finding time increased progressively in the first seven days (reached 169 ± 15 sec on day 7). Inability to find the cookie was noted on day 10 (latency 329 ± 21 sec), after which recovery from the olfactory deficit was observed by day 15 (latency 115 ± 14 sec). Results of this study show that the structural and functional changes imparted by single parenteral doses of vinblastine or docetaxel on the olfactory mucosa are transient and that regenerative recovery, which restores the normal structure of the mucosa and olfactory function, is relatively more delayed during treatment with docetaxel as compared with vinblastine. Further, the progressive modifications in olfactory mucosal structure during postnatal development may be attributed to the high olfactory functional demands documented in this species. While the findings on the normal postnatal morphology of the rabbit olfactory mucosa may form an important basis for further work involving more ages and species, the data on the impact of the anticancer drugs on the olfactory mucosa may be useful in the design and development of better management strategies for patients with cancer.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

The olfactory mucosa is a peripheral sensory tissue located in the vertebrate nasal cavity whose function is to detect and distinguish a wide variety of odors at low concentrations (Lancet *et al.*, 1993; Liberles & Buck, 2006). As evidenced by the number of published articles, there is increased interest in olfactory neural research. After Richard Axel and Linda Buck were awarded the Nobel Prize for Medicine and Physiology in 2004 for their successful studies on olfactory sensation mechanisms, relatively more detailed reports on the olfactory system have been published. In animals that use the lung for respiration, the olfactory mucosa is a vital remote chemical captor (Sturkie, 1982), indispensable to sensing and reacting to chemical stimulus. Many animals therefore use the faculty of olfaction in a number of life-sustaining activities including food acquisition (Estes and Goddard, 1967), predator avoidance and reproduction (Estes and Goddard, 1967; Shillito-Walser and Alexander, 1980; Wright, 1994; Rawson and Gomez, 2002; Terrazas *et al.*, 1999; Graham *et al.*, 2000; Restrepo *et al.*, 2004; Trinh and Storm, 2004). In rabbits where the newborns are functionally blind and deaf (Coureaud *et al.*, 2008) and receive unusually limited maternal assistance during suckling, olfaction is particularly critical in aspects of mother-neonate interaction (Hudson and Distel, 1983, 1984; Schaal *et al.*, 2003; Hudson *et al.*, 2008).

The olfactory system is a highly plastic region of the nervous system (Plendl and Sinowatz, 1998). Primary olfactory neurons in the olfactory mucosa replicate

throughout the life of an animal, a feature that is unique in regard to these neurons (Farbman, 1994; Calof et *al.*, 1998; Barnett & Riddell, 2004). Thus, the olfactory mucosa provides an excellent model for studying neurogenesis. Owing to the peripheral location of the olfactory mucosa, the olfactory cells are highly vulnerable to the injurious effects of noxious agents contained in inhaled air (Schiffman, 1983). In conventional toxicity studies, substances administered by inhalation are reported to cause damage to the lining of the nasal cavity, including the olfactory mucosa (Appelman *et al.*, 1982; Jiang *et al.*, 1983; Kerns *et al.*, 1983; Buckley *et al.*, 1985; Gaskell *et al.*, 1988; Thomas and Morgan, 1988; Hastings *et al.*, 1991). However, data on how systemically administered substances impact on the structure of the olfactory mucosa are largely unavailable.

Microtubule-disrupting drugs, also referred to as antimicrotubule, antimitotic or microtubule targeted drugs, constitute a group of parenterally administered agents which exert their effect on highly mitotic cells, including cancer cells, by interfering with spindle microtubule dynamics (Bender, 1987; Don *et al.*, 2004). The integrity of microtubules is critical in several cellular processes including maintenance of cell shape, cell migration, arrangement of organelles within the cell (Rodriguez, 2003) and in the establishment of epithelial cell surface polarity (Yap & Manley, 2001). Previously, the effects of microtubule targeted drugs were studied on highly proliferative tissues including lymphohematopoietic, gastrointestinal (Todd *et al.*, 1979; Kanter *et al.*, 1994), testicular (Todd *et al.*, 1976) and cutaneous tissues (Dorr and Alberts, 1985; Lishner *et al.*, 1999) with limited focus on the olfactory mucosa. Furthermore, the few studies available on the impact of antimicrotubule agents on

olfactory mucosal structure have revealed remarkable differences in susceptibility to lesions in regard to the drug type (Kai *et al.*, 2004), the dose level (Kai *et al.*, 2005) and the species of the animal (Kai *et al.*, 2006). In clinical studies on antimicrotubule anticancer drugs (Leopold, 1995; Seiden, 1997), data on various forms of olfactory dysfunctions have been presented. However, very little effort has been made in detailing the nature and the extent of olfactory lesions that are associated with such smelling disorders. Therefore, this study strives to provide a detailed analysis of the impact of single parenteral doses of antimicrotubule drugs vinblastine or docetaxel on olfactory mucosal structure and olfactory function in the rabbit. Additionally in this study, the morphofunctional status of the rabbit olfactory mucosa is investigated during postnatal development.

1.2 LOCATION OF THE OLFACTORY MUCOSA

In vertebrates, the olfactory mucosa is located in the nasal cavity (Williams, 1995). The nose is a very complex organ with multiple functions that include not only olfaction, but also conditioning of inhaled air (Harkema *et al.*, 2006). From a comparative viewpoint, humans are micromastic i.e. they have relatively simple noses with breathing as the primary function, while other mammals have more complex noses with olfaction being their primary role (i.e. are macrosmatic) (Witt and Hummel, 2006; Harkema *et al.*, 2006). Inside the nasal cavity, the olfactory mucosa is positioned in two major regions: the posterodorsal region of the cavity (*regio olfactoria*) as the main olfactory mucosa (olfactory mucosa proper) and within the vomeronasal organ as the vomeronasal or accessory olfactory mucosa (Adams, 1992; Farbman, 1992; Kjell and Didier, 1998; Wakabayashi *et al.*, 2002).

In some mammals, a small island of olfactory tissue located bilaterally at the ventral base of the nasal septum, referred to as septal organ of Masera (Masera's organ), is considered to be a third olfactory organ (Breipohl *et al.*, 1989; Taniguchi *et al.*, 1993; Ma *et al.*, 2003; Kociánová *et al.*, 2006).

1.2.1 The main olfactory mucosa

The main olfactory mucosa primarily lines the ethmoturbinates (Adams, 1992; Kumar *et al.*, 2000; Kocianova *et al.*, 2001; Restrepo *et al.*, 2004) and spreads to cover the posterior regions of the dorsal and middle nasal turbinates as well as the nasal septum (Breipohl *et al.*, 1989; Dyce *et al.*, 1996). In humans, the mucosa extends from the posterior upper part of the lateral nasal wall to also cover the spheno-ethmoidal recess, the upper part of the perpendicular plate of ethmoid and the roof of the nose arching between the septum and the lateral wall including the underside of the cribriform plate (William, 1995). The percentage of the nasal airway that is covered by this type of mucosa varies among animal species. In the nasal cavity of F344 rats, the olfactory mucosa covers an area of approximately 50% (Gross *et al.*, 1982) whereas in humans, the mucosa covers an area of about 500 mm^2, which is only ~ 3% of the total surface area of the nasal cavity (Sorokin, 1988).

1.2.2 The vomeronasal olfactory mucosa

The vomeronasal olfactory mucosa covers the medial wall of the lumen of the vomeronasal organ (the lateral side is lined by a respiratory mucosa) (Adams, 1992; Booth and Katz, 2000). The vomeronasal organ, also referred to as the Jacobson's

organ (named after Jacobson Ludwig who provided the first systematic observations of this organ in several species of animals), is a pair of cartilage-supported ducts situated on the anterior floor of the nasal cavity on either side of the nasal septum (Krazing, 1971; Taniguchi and Mikami, 1985; Adams, 1992; Kjell and Didier, 1998; Wakabayashi *et al.*, 2002). Depending on species, the ducts of the vomeronasal organ open directly into either nasal or buccal cavities or both (Meredith and O' Connell, 1979; Adams, 1992). The vomeronasal organ and therefore the accessory olfactory mucosa is present as a complete and independent chemosensory organ in most reptiles, amphibians, and mammals (Witt and Woz´niak, 2006) but is lacking in marine mammals, birds (Doving *et al.*, 1993; Witt and Woz´niak, 2006), Old World monkeys (Dennis *et al.*, 2004), crocodiles and some species of bats (Eisthen, 1997), and is vestigial in human adults and some apes (Dennis *et al.*, 2004; Witt and Woz´niak, 2006).

1.2.3 The septal organ of Masera

The Masera's organ was first observed in newborn mice by Broman in 1921 and was named 'Riechepithelinsel' (island of olfactory epithelium). Subsequently, this structure was described by Rodolfo Masera (1943) after whom it was named. On the ventral region of the nasal septum, the organ of Masera occurs as an isolated patch of olfactory tissue separated from the principal olfactory mucosa by a region of modified respiratory mucosa (Pedersen and Benson, 1986; Farbman, 1992; Giannetti *et al.*, 1995; Weiler and Farbman, 2003). Though documeted to be a separate accessory olfactory structure, the septal organ is believed to play roles similar to those of the vomeronasal olfactory mucosa (Weiler and Farbman, 2003).

1.3 STRUCTURE OF THE OLFACTORY MUCOSA

The olfactory mucosa comprises an epithelium having olfactory, supporting and basal cells as its principal cell types, and a lamina propria that accommodates Bowman's glands, bundles of olfactory cell axons and vascular elements (Mendoza, 1993; Menco and Morrisson, 2003; Kavoi *et al.*, 2010). The structural organization of the olfactory mucosa is demonstrated in Figure 1.

1.3.1 The olfactory epithelium

The olfactory epithelium is an atypical epithelium which is constituted by a sheet of heterogeneous cells namely, basal cells, supporting (sustentacular) cells and olfactory receptor neurons (Mendoza, 1993; Kavoi *et al.*, 2010). The olfactory epithelium sits on a basement membrane, whose thickness is almost twice that of the respiratory epithelium (Kumar *et al.*, 2000). In the nasal cavity, the area covered by the epithelium varies from a few square centimeters in man or frog to more than a hundred square centimeters in the dog (Lancet, 1986). In histological sections, the epithelium presents three distinct zones named from the apical surface as the free zone, the non-nuclear (cytoplasmic) zone and the nuclear zone (Menco, 1984; Breipohl *et al.*, 1989, Kumar *et al.*, 2000; Kavoi, 2008).

The free zone is constituted by the microvilli of the supporting cells, which form a dense meshwork over the olfactory knobs and their cilia, whereas the non-nuclear zone represents the supranuclear parts of the supporting cells (Loo, 1977; Burkitt *et al.*, 1993; Kumar *et al.*, 2000). Located between the free and the non-nuclear zones

of the epithelium is the terminal bar, which is believed to represent occluding junctions that connect the apical borders of olfactory dendrites to the supporting cells (Bukitt *et al.*, 1993). The nuclear zone comprises of three layers or strata: an uppermost stratum of elongated nuclei of supporting cells, a middle layer of rounded nuclei of olfactory cells and a lower row of basal cells (Farbman, 1986; Breipohl *et al.*, 1989; Burkitt *et al.*, 1993; Kumar *et al.*, 2000). While the basal stratum occurs as a single row of nuclei just above the basement membrane, the upper and the middle strata form several staggered rows of nuclei (Breipohl *et al.*, 1989; Suzuki *et al.*, 2000).

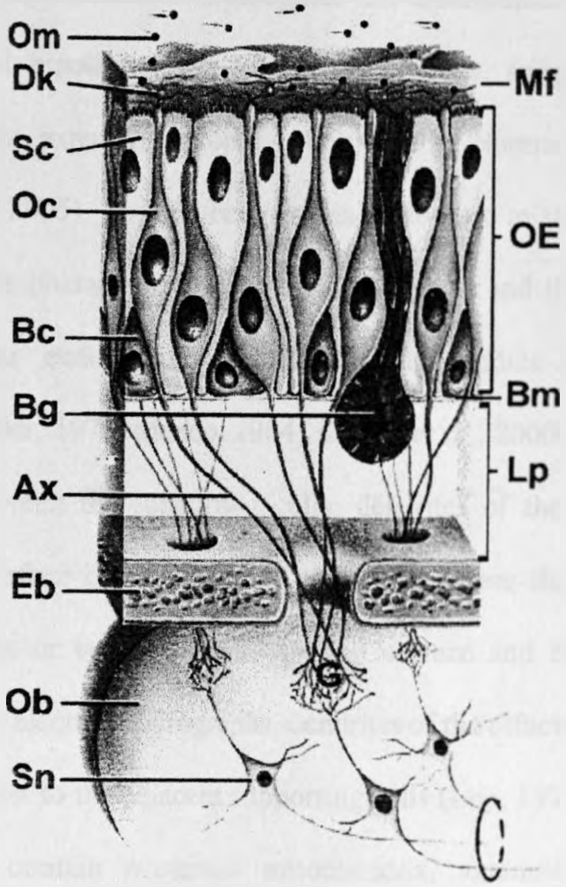

Figure 1. A schematic representation of the components of the olfactory mucosa. The mucosa comprises an epithelium (OE) and a lamina propria (LP). The epithelium is pseudostratified columnar consisting of olfactory (Oc), supporting (Sc) and basal cells (Bc). The olfactory cell is a bipolar neuron with an apically directed dendrite that terminates by forming a cilia-bearing knob (Dk) and a basally extending axon (Ax) that pierces the basement membrane (Bm) to enter the lamina propria. Within the propria, axons of several olfactory neurons converge to form large axon bundles, which pass through the apertures of the cribriform plate of ethmoid bone (Eb) to establish synapses with second order neurons (Sn) within the glomerulus (G) of the olfactory bulb (Ob). Also located in the lamina propria are Bowman's glands (Bg), whose secretions are delivered through vertically oriented ducts (D) to the surface of the epithelium where the secretions contribute to the formation of the mucus film (Mf) which serves to dissolve odor molecules (Om).

1.3.1.1 The olfactory cells

The olfactory cell, whose function is to recognize and discriminate numerous odor molecules, is a typical bipolar neuron having a cell body, an apically directed dendrite and a basally extending axon (Anholt, 1993; Nomura *et al.*, 2004; Stuelpnagel and Reiss, 2005). The cell body (soma) is located in the central region of the epithelium and is characterized by a rounded nucleus and the abundance of granular and agranular endoplasmic reticulum, Golgi bodies and lysosomes (Kerjaschki and Horander, 1976; Menco, 1984; Kumar *et al.*, 2000; Nomura *et al.*, 2004). By passing between the supporting cells, dendrites of the olfactory cells project to the apical surface of the olfactory epithelium where they terminate by forming club-like knobs or vesicles (Lenz, 1977; Costanzo and Morrison, 1989; Nomura *et al.*, 2004). In electron micrographs, dendrites of the olfactory cells appear electron-lucent in contrast to the adjacent supporting cells (Loo, 1977; Kavoi *et al.*, 2010). The dendrites contain numerous mitochondria, agranular endoplasmic reticulum, smooth and coated vesicles, microtubules and some ribosomes (Menco *et al.*, 1976; Krause, 1992; Willams, 1995).

Projecting from the knob of the olfactory cell dendrite are the olfactory cilia, whose cross-sectional diameter is approximately 0.25 microns (Lancet, 1986). The average number of cilia per dendritic knob varies with species being 7 in the sheep (Kavoi *et al.*, 2010), 17 in the bovine (Menco, 1978) and 18 in the dog (Kavoi *et al.*, 2010). The pattern of projection of the cilia from the dendritic knobs varies with species. In humans (Lenz, 1977), horses (Kumar *et al.*, 2000) and dogs (Kavoi *et al.*, 2010), the cilia arise from the bases of the olfactory cell knobs in a radial (stellar) fashion

whereas in the ox (Menco, 1978), goat (Kumar *et al.*, 1999) and sheep (Kavoi *et al.*, 2010), the cilia project in parallel from the tips of the knobs in form of a tuft. In the vomeronasal neuroepithelium, the olfactory receptor cells possess microvilli rather than cilia on their free endings (Barber and Raisman, 1978; Naguro and Breipohl, 1982; Yoshida-Matsuoka *et al.*, 1999; Hofer *et al.*, 2000; Menco *et al.*, 2001).

Structurally, the olfactory cilia are distinct in their extended length (30-200 µm long) and in tapering to a 0.15 µm diameter with loss of microtubuli along their distal length (Reese and Brightman, 1970; Lenz, 1977; Lancet, 1986; Bukitt *et al.*, 1993). Similar to cilia from other tissues, olfactory cilia have basal bodies and a tubulin containing 9 x 2 + 2 microtubular core (axoneme), and are ensheathed in a lipid bilayer (Lacet, 1986; Morrison and Constanzo, 1990; Williams, 1995, Kociánová *et al.*, 2001). In mammals, the peripheral doublets of the microtubules lack dynein arms (the ciliary energy-transducing ATPase) and are therefore non-motile and atypical (Lidow & Menco 1984; Ross and Reith, 1985). The plasmallema of the cilia is studded with numerous intramembraneous particles, which represent the putative sites for odor binding (Menco, 1984; Buck and Axel, 1991).

In the olfactory epithelium, the basally directed axons of the olfactory cells run close to each other forming small intraepithelial fascicles which, on piercing the basement membrane, become entrapped by processes of olfactory ensheathing cells (Williams, 1995; Field *et al.*, 2003; Nomura *et al.*, 2004; Barraud *et al.*, 2010). In the lamina propria of the mucosa, the fascicles progressively group into large axon bundles (Lidow and Menco, 1984; Costanzo and Morrison, 1989; Williams, 1995; Field *et*

al., 2003). Inside each bundle, the axon-containing fascicles are aligned, packaged and compacted by sheet-like processes of the olfactory ensheathing cells (Field *et al.*, 2003). The sizes of the axon bundles, which have been shown to vary with species and age (Kavoi *et al.*, 2010), directly relate to the packing densities of the olfactory cells (Meisami, 1989; Yamagishi *et al.*, 1989).

The olfactory ensheathing cells are a unique class of vertebrate glial cells that envelope axons of olfactory nerves, both peripherally in the olfactory mucosa and within the olfactory nerve layer of the olfactory bulb (Field *et al.*, 2003; Raisman and Li, 2007). These cells are similar in many ways, including in their morphological and antigenic characteristics, to Schwann cells (Boyd *et al.*, 2005; Barraud *et al.*, 2010). Although evidence supporting the capacity for the olfactory ensheathing glial cells to myelinate axons *in vitro* has remained controversial, it is well known that these cells interact with the Schwann cells to create a 3-dimensional matrix that provides a permissive microenvironment for successful axon regeneration in the adult mammalian central nervous system (CNS) (Boyd *et al.*, 2005).

1.3.1.2 The supporting cells

The supporting or sustentacular cells are tightly packed and act as glia to the olfactory epithelium besides serving other functions which includes K^+ transport, maintenance of water/salt balance in the neuroepithelium mucus layer (Menco *et al.*, 1998; Rochelle *et al.*, 2000; Vogalis *et al.*, 2005) and phagocytosis of dendritic fragments (Rafols & Getchell 1983, Suzuki *et al.*, 1996). They are columnar and

span the full length of the olfactory epithelium with their upper one third being broader than the lower two thirds (Farbman, 1986; Mendoza, 1993). The broad apical part of these cells carries a vertically elongated nucleus (Menco, 1984; Breipohl *et al.*, 1989, Meiasami *et al.*, 1990; Kavoi, 2008). These cells contain many mitochondria, Golgi apparatus, smooth and rough endoplasmic reticuli and electron-luscent lysosomal structures in their supranuclear cytoplasm (Reese and Brightman, 1970).

The thin basal parts of the supporting cells form foot-like processes that spread on the basement membrane to create tunnel-like spaces for guiding the olfactory cell axons into the nerve bundles (Costanzo and Morrison, 1989; Mendoza, 1993; Nomura *et al.*, 2004). Remains of secondary lysosomes (residual bodies) in the end feet of the supporting cells take the form of lamellated dense bodies, which have been associated with the characteristic yellowish-brown color of the olfactory mucosa (Reese and Brightman, 1970). At the apical region of the epithelium, the lateral borders of the supporting cells contact adjacent olfactory cell dendrites by means of tight junctions (Loo, 1977; Krause, 1992; Burkitt *et al.*, 1993; Kociánová *et al.*, 2001; Herrera *et al.*, 2005).

1.3.1.3 The basal cells

The basal cells, which occupy the basal region of the olfactory epithelium, serve to replace olfactory and other epithelial cells lost during normal turn over or injury (Moran *et al.*, 1982; Chen *et al.*, 2004; Jang *et al.*, 2007). Based on morphology and location, basal cells are categorized as horizontal and globose (Crew and Hunter,

1994). The horizontal cells, also called basal cells proper, are somewhat flat or angular with a small heterochromatic nuclei and lie close the basement membrane (Breipohl *et al.*, 1989; Suzuki and Takeda, 1991). The globose or blastemal cells are, on the other hand, located more superficially and are rounded to ellipsoid in shape with a larger euchromatic nucleus (Graziadei and Monti Graziadei, 1979; Yamagishi *et al*, 1989; Suzuki and Takeda, 1993; Herrera *et al.*, 2005).

In developing and mature olfactory epithelium, stem cells are in a state of constant mitotic division (Plendl and Sinowatz, 1998; Viktoroy *et al.*, 2006). Daughter cells forming as a result of asymmetrical division pass through a succession of several stages during migration and differentiation into mature olfactory neurons (Viktoroy *et al.*, 2006). Thus, the olfactory epithelium contains cytokeratin-positive horizontal and cytokeratin-negative globose stem cells, primary progenitor cells expressing proneuronal Mash 1 (mammalian achaete scute homolog 1) gene and secondary progenitor cells expressing Neurogenin (Ngn 1) proneuronal gene (Calof *et al.*, 1998; 2002; Beites *et al.*, 2005; Viktorov *et al.*, 2006). Cell culture studies by Carter *et al.*, (2004) showed the horizontal stem cells to form a heterogenous population including two subpopulations: globose stem cell precursors and glial olfactory ensheathing cell precursors. The globose stem cells have been shown, through *In vivo* experiments, to serve as the immediate precursors of olfactory receptor neurons (Mackay-Sim and Kittel, 1991) and of supporting cells of the olfactory epithelium (Beites *et al.*, 2005).

1.3.2 The lamina propria of the olfactory mucosa

The lamina propria comprises the region of the olfactory mucosa between the basement membrane and the bony or cartilaginous core of the ethmoturbinate (Herrera *et al.*, 2005; Kavoi *et al.*, 2008). The propria consists of loose irregular connective tissue that supports Bowman's glands, olfactory nerve fiber bundles and blood vessels (Ross and Reith, 1985; Kumar *et al.*, 2000).

1.3.2.1 The Bowman's glands

The Bowman's glands are branched tubular structures lying within the lamina propria of the olfactory mucosa (Williams, 1995). These glands are present in the olfactory mucosa of all vertebrates except fish (Getchell and Getchell, 1992). The glands consist of a secretory portion which contains serous or mucus cells or both, and a duct that delivers the glandular secretions to the olfactory epithelial surface (Getchell and Getchell, 1992; Kumar *et al.*, 1994, 2000). On the epithelial surface, the secretions form a thin layer of mucus where odorants dissolve prior to their interaction with the ciliary membrane (Lancet, 1986). In a number of mammalian species including horses (Kumar *et al.*, 2000), hamsters (Costanzo and Morrison, 1989), rats (Nomura *et al.*, 2004), dogs and sheep (Kavoi *et al.*, 2010), these glands are tubulo-acinar with pyramid-shaped secretory cells. At the basal part of the epithelium in rats, ducts of these glands occasionally form globular swellings or bulges (Nomura *et al.*, 2004).

In air breathing vertebrates, secretions of the Bowman's glands dissolve odorants present in air thereby allowing their diffusion to the sensory receptor sites on the

surface of the olfactory cell cilia (Costanzo and Morrison, 1989; Greer, 1991; Getchell & Getchell, 1992; Kumar *et al.*, 1994; Williams, 1995; Nomura *et al.*, 2004). Continuous flow of the Bowman's gland secretions cleans the apical portion of the olfactory cells to ensure that compounds that stimulate the sense of smell are constantly being removed, thus keeping the receptors in a state of readiness to respond to new stimuli (Hart and Haugen, 1971). Species and age-related differences have been demonstrated in regard to the distribution of the Bowman's glands. In the adult of the sheep (Kavoi *et al.*, 2010) and in the horse (Kumar *et al.*, 2000), the glands mainly occur in the superficial part of the lamina propria whereas in the lamb, puppy and adult dog (Kavoi *et al.*, 2010), the glands are distributed throughout the propria.

1.3.2.2 The axon bundles

The axon bundles represent the proximal segments of the olfactory nerve fibers that travel in groups in the lamina propria of the olfactory mucosa (Williams, 1995; Field *et al.*, 2003). Olfactory axons contained in these bundles are accompanied by olfactory ensheathing glial cells, which most authors refer to as "Schwann cells" following the terminology applied to other peripheral nerve glia (Gasser, 1956; De Lorenzo, 1957; Graziadei, 1971; Chuah and Au, 1991). The ensheathing cells are unique in that they do not envelope individual axons but extend tongues of cytoplasm which encircle and compact such groups of axons into longitudinally aligned parallel fibers called fasciculi (De Lorenzo, 1957; Field *et al.*, 2003). Eventual merging of several of these fascicles results in the formation of the axon bundles (Field et al., 2003; Herrera *et al.*, 2005; Kavoi *et al.*, 2010). In fact, the

simplest fascicle consists of a group of axons (50 or more) ensheathed by a single olfactory ensheathing cell (Williams, 1995; Field *et al.*, 2003). Externally, the axon bundles are surrounded by a fibroblastic sheath (Costanzo and Morrison, 1989; Williams, 1995; Field *et al.*, 2003; Nomura *et al.*, 2004; Herrera *et al.*, 2005) are separated from each other by a collagen-packed extracellular space (Costanzo and Morrison, 1989; Williams, 1995; Field *et al.*, 2003; Nomura *et al.*, 2004; Herrera *et al.*, 2005).

The number of axons encircled by one olfactory ensheathing cell varies greatly and increases with progressive depth in the lamina propria, reaching more than 500 axons per fascicle (Herrera *et al.*, 2005). Individual olfactory axons never branch and are remarkably thin and uniform in diameter (0.2-0.4 μm) and contain 5-10 microtubules (Field *et al.*, 2003). Lack of myelination of the olfactory cell axons has been attributed to their slender nature (small caliber) (Williams, 1995; Field *et al.* 2003; Gordon *et al.*, 2005; Viktorov *et al.*, 2006). Internally, the olfactory axons contain neurofilaments (Williams, 1995). To reach the olfactory bulb, the axon bundles pass through the apertures of the cribriform plate of ethmoid bone (Farbman and Margolis, 1980; Plendl and Schmahl, 1988; Kavoi and Hassanali, 2011). On reaching the bulb, the bundles lose their fibroblastic envelope and the sheath of olfactory glial cells binding their fascicles open out to allow axons from different fascicles to establish connection with dendritic tufts of secondary sensory neurons in the olfactory bulb glomeruli (Graziadei and Monti Graziadei, 1978; Meisami, 1989; Williams, 1995; Kasowski *et al.*, 1999; Field *et al.*, 2003; Herrera *et al.*, 2005).

The axon bundles together with the conglomerates of Bowman's glands constitute the largest proportion of the lamina propria (Herrera *et al.*, 2005). In many species including humans, guinea pigs (Yamagishi *et al.*, 1989), dogs and sheep (Kavoi, 2008; Kavoi *et al.*, 2010), the axon bundles are confined to the deep part of the propria. In the horse, however, the bundles spread to occupy the superficial region of the propria as well (Kumar *et al.*, 2000). In a recent study (Kavoi *et al.*, 2010), cross-sectional diameters of the axon bundles were shown to increase with age and to vary across taxa. A unique finding in the axon bundles of the dog was the presence of blood capillaries within the bundle cores, which was demonstrated in both puppies and adults (Kavoi, 2008; Kavoi *et al.*, 2010). Larger sizes of the axon bundles are reflective of a higher packing density of olfactory cells and vice versa (Meisami *et al.*, 1989; Kavoi *et al.*, 2010). Furthermore, the thickness of an axon bundle directly correlates to the ratio of convergence between its constituent axons and second-order neurons in the olfactory bulb (Van Drongelen *et al.*, 1978; Meisami, 1989).

1.4 OLFACTORY PATHWAYS

Olfactory pathways are sets of nerve fibers that convey chemical stimuli from the external environment to higher centers and include the main olfactory (cortical) pathway and the vomeronasal (subcortical) pathway (Allison, 1953; Powell *et al.*, 1965; Heimer, 1972; Raisman, 1972; Broadwell, 1975; Scalia and Winans, 1975; Giachetti and MacLeod 1975, 1977; Keverne, 1978; Takagi, 1979, 1980, 1981; Witt and Woz´niak, 2006). The main olfactory pathway starts from the main olfactory mucosa in the nasal cavity, passes through the olfactory bulb, the prepyriform cortex, the mediodorsal nucleus of the thalamus and ends in the orbitofrontal cortex

(Takagi 1980, 1981). The vomeronasal pathway, on the other hand, begins from the neuroepithelium of the vomeronasal organ and enters the accessory olfactory bulb, the corticomedial nuclei of the amygdala and terminates in limbic areas including the the preoptic and the septal nuclei of the hypothalamus (Takagi, 1980, 1981; Witt and Wozniak, 2006).

A third olfactory pathway, which has been associated with the septal organ of Masera (Witt and Woz´niak, 2006) and demonstrated in rats (Giachetti and MacLeod, 1975, 1977) and rabbits (Imarura et al.,1980), goes to the neocortical taste area through the ventroposteromedial nucleus of the thalamus (Takagi, 1981). In a study in the monkey (Yarita *et al.*, 1980), the olfactory pathways were found to be very different from those in lower mammals.

1.5 DEVELOPMENT OF THE OLFACTORY MUCOSA

1.5.1 Prenatal development

The prenatal development of the olfactory mucosa is characterized by several distinct processes, some of which occur sequentially and others simultaneously. In the mice, Cuschieri and Bannister (1975) divided these processes, somewhat arbitrarily, into: (1) an initial stage of stem cell proliferation occurring chiefly during the formation of the olfactory placode, (2) the differentiation of receptor cells marked by the outgrowth of their axons, (3) the formation of receptor dendrites and terminal swellings, (4) the final steps in olfactory receptor cell maturation and (5) the differentiation of non-nervous elements including supporting cells and Bowman's glands.

1.5.1.1 Placode stage

The first sign of olfactory organ development in mammalian embryos, as well as other vertebrates, is the appearance of bilateral patches of thickened ectoderm referred to as olfactory placodes and which are actually embryologically parts of the CNS (Couly and Le Douarin, 1985; Farbman, 1986; Whitlock, 2004; Bhattacharyya *et al.*, 2004; Taniguchi and Taniguchi, 2007; Katoh et al., 2011). These thickenings are oval-shaped and are positioned on either side of the rostral end of the embryo, medially connected to the neuroectoderm (Hamilton *et al.*, 1962; Arey 1965; Taniguchi and Taniguchi, 2007). As growth continues, each side of the placode acquires a shallow depression, which ultimately deepens into two nasal pits (Farbman, 1986). The deep part of the nasal pits becomes lined by the primitive olfactory epithelium, which consists mainly of undifferentiated cuboidal cells (Farbman, 1977, 1986; Williams, 1995; Taniguchi and Taniguchi, 2007). Even at the placode stage, the presumptive receptor cells are elongated and many of them display a bulbous surface process that will become the dendritic terminal (Cuschieri and Bannister, 1975; Waterman and Meller, 1973; Farbman, 1977; Menco and Farbman, 1985). At this stage, the pattern of mitotic activity in the epithelium closely resembles that of the developing neural tube, with divisional stages being present only in the superficial marginal zones of the placode (Smart 1971; Cuschieri and Bannister, 1974).

Later with ongoing development, replication of the cells appears deeper in the epithelium and finally becomes confined to the basal layers, where division persists

throughout life (Smart 1971; Cuschieri and Bannister, 1975). From the invaginating olfactory placode arises the following cell types: (1) olfactory sensory neurons that grow towards the apical part of the corresponding cerebral hemispheres to form the olfactory tract (2) olfactory ensheathing cells that provide essential growth and guidance for the olfactory neurons and (3) gonadotropin-releasing hormone (GnRH)-secreting neurons that migrate caudally to enter into the future hypothalamic region of the brain (Verwoerd and Van Oostrom, 1979; Schwanzel-Fukada and Pfaff, 1989; Wray *et al.*, 1989; Schwanzel-Fukada *et al.*, 1996; Su and He, 2010). More recently, fate-mapping and genetic Cre-lox lineage tracing experiments (Barraud *et al.*, 2010; Forni *et al.*, 2011) have revealed that ectodermal cells actually intermix with neural crest cells (a multipotent population of migratory cells that delaminate from the neuroectoderm to lie at the border between neural and non-neural ectoderms on the lateral edge of the neural plate) in the nasal placode to give rise to the aforementioned cell types. These studies served to correct the earlier misconception (Chuah and Au, 1991) that olfactory ensheathing cells originate from precursors residing in the olfactory epithelium. Failure of the GnRH-secreting neurons to migrate to the future hypothalamic region results in a condition referred to as Kallmann syndrome in which patients lack GnRH-secreting neurons and therefore develop small genitalia and sterile gonads (Stout and Gradziadi, 1980; Schwanzel-Fukada *et al.*, 1996; Chan *et al.*, 2009; Wray, 2010).

1.5.1.2 Differentiation of olfactory receptor cells

The differentiation of the olfactory receptor cells has earlier been investigated with a reasonable documentation of the structural sequence of events in a number of

vertebrates including rats, mice (Cuschieri and Bannister, 1975) and zebra fish (Whitlock and Westerfield, 1998). In the mouse by embryonic day 10 (gestation period = 21 days), multiplication of the stem cells is presumably already over, since the olfactory placode is fully formed and differentiation of the olfactory receptor cells has commenced (Cuschieri and Bannister, 1975). At this stage, cells of the epithelium can be grouped into pale and dark types, with the pale type being the differentiating olfactory receptor cells while the dark are the original stem cells of the early placode (Cuschieri and Bannister, 1975). In the zebra fish (Whitlock and Westerfield, 1998), the original stem cells, also called pioneer neurons, were observed to initiate the first connection between the olfactory placode and the developing olfactory bulb. These neurons are unique in that they establish a pathway and then undergo apoptosis once the adult axons, which follow them, have made connections with the target site (McConnell *et al.*, 1994; Whitlock and Westerfield, 1998). In the zebra fish also, the pioneer neurons for the olfactory sensory system appear 20 hours post-fertilization as large cell bodies in the basal part of the olfactory placode juxtaposed to the telencephalon (Whitlock and Westerfield, 1998).

1.5.1.2.1 Axon growth

An early event in olfactory cell development, as in other neurons (Jacobson, 1978), is the genesis of the axon. In the rat (gestation period 22 days), this occurs between embryonic day 13 and 14, a time when the olfactory dendrites are yet to mature and are therefore without cilia (Farbman and Squinto, 1985). In the mouse, small axons are seen as early as gestational day 10 (Cuschieri and Bannister, 1975). Within the epithelium, the cell body of the olfactory neuron grows a single axon which begins

to assemble with other axons to form small fascicles. These fascicles break through the basal lamina and grow through the connective tissue towards their synaptic targets in the olfactory bulb (Farbman, 1986).

In the rat, the olfactory cell axons first reach the olfactory bulb on prenatal day 15-16 while in the mouse, this takes place on gestational day 11-12 (Hinds, 1972; Cuschieri and Bannister, 1975). In prenatal mouse (Hinds, 1972) and rats (Farbman and Squinto, 1985), the establishment of synapses between the olfactory axons and the bulb cells do not occur until after two days following the arrival of the axons in the bulb. Ultrastructural and light microscopic observations of cells found straddling between the olfactory epithelium and lamina propria have led most researchers to believe that the olfactory axons are enveloped by olfactory ensheathing cells as they grow towards the olfactory bulb (Van Campenhout, 1937; Wesolowski, 1970; Cuschieri and Bannister, 1975; Mendoza *et al.*, 1982; Farbman and Squinto, 1985).

1.5.1.2.2 Dendrite development

From the differentiating olfactory receptor cell perikaryon extends the dendrite, which begins to make its way to the epithelial surface (Hinds and Hinds 1972; Costanzo and Graziadei, 1983; Farbman 1994; Williams, 1995). On reaching the epithelial surface, the dendrites form rounded terminal expansions into which centrioles migrate from the olfactory cell soma to become the bases of the olfactory cilia, which grow from them distally (Williams, 1995). In the rat, dendritic terminals start to appear on the surface of the olfactory epithelium as early as embryonic day 12-13, a stage at which the olfactory cell can be distinguished from a supporting cell

(Farbman, 1986). At this stage also, the dendrite and its knob contains ribosomes, microtubules and a single primary cilium (Menco and Farbman, 1985).

Microtubules are characteristic features of dendrites in both developing and mature neurons (Tennyson, 1965; Lyser, 1968) and in the olfactory neuronal cells, their formation coincides with the acquisition of the typical bipolar shape of the neurons (Cuschieri and Bannister, 1975). In the rat, the primary cilia, which are found in many types of cells during differentiation (at the G1 phase of cell cycle) (Sorokin, 1968; Tucker and Pardee, 1982; Menco and Farbman, 1985), do occur on some olfactory dendritic knobs as late as gestational day 22 (Menco and Farbman, 1985). While still below the surface of the epithelium, the membrane of dendritic ends of the growing neurons possesses discontinuous strands, which are the earliest indication of the membrane specialization that will constitute the primitive tight-junction belts that fuse with similar junctions of the supporting cells (Kachar and Pinto da Silva, 1981; Farbman, 1986).

In the rat fetus, the characteristic pattern of organization and distribution of olfactory receptor cells begins to become apparent on intrauterine day 14 (Menco and Farbman, 1985), a stage which coincides with the presence of several centrioles in the dendritic knobs (Mulvaney and Heist, 1971; Farbman and Squinto, 1985). Olfactory cells have multiple cilia on gestational day 13 in the mouse (Noda and Harada, 1981), gestational day 79 in the sheep (Kociánová *et al.*, 2003) and prenatal week 9 in humans (Pyatkina, 1982). In the rat (Menco and Farbman, 1985), the cilia are present on prenatal day 16, which, it will be recalled, is approximately when

axons first contact their olfactory bulb targets. It has been suggested that the timing of these two developmental events (i.e. ciliogenesis and axons reaching their targets) is consistent with the interpretation that the contact of the axons with their olfactory bulb targets does indeed influence aspects of olfactory receptor cell differentiation, including ciliogenesis (Cuschieri and Bannister, 1975).

In prenatal mice at day 17, the olfactory receptor cells undergo the final stages of maturation, which mainly include gradual increase in the number of cilia and change in shape of the terminal swellings (Cuschieri and Bannister, 1975). In the perikarya, granular and agranular endoplasmic reticula proliferate, Golgi complexes enlarge, lysosomes become abundant and the cytoplasm comes to resemble that of mature postnatal cells (Frisch, 1967). Few microtubules occur in the dendrites and mitochondria no longer occupy the terminal swellings (Cuschieri and Bannister, 1975). Another interesting finding at this stage of olfactory cell maturation is that the olfactory cell bodies and dendrites, which were initially isolated from each other by the usual 20 nanometer intercellular gaps, became more and more separated by supporting cells as is the case in the postnatal animals (Graziadei, 1971; Cuschieri and Bannister, 1975). Results of morphometric studies on ciliogenesis in the fetal rat (Menco and Farbman, 1985) show that the average number of cilia per dendritic ending increases linearly from a value of < 1 (because some knobs have no cilia) at prenatal day 16 to about 7 cilia at postnatal day 22. Moreover, at gestational day 18-19, an increasing number of growing cilia in the rat fetuses begin to taper as is the case in adult cilia (Menco and Farbman, 1985).

1.5.1.3 Differentiation of non-nervous elements of the olfactory mucosa

The non-nervous components of the olfactory mucosa which include supporting cells and Bowman's glands arise from the olfactory placode (Cuschieri and Bannister, 1975; Williams, 1995; Mendoza *et al*, 1982; Klein and Graziadei, 1983; Couly and Le Douarin, 1985). In the mouse fetus, an abrupt onset of differentiation of these components occurs on gestational day 17, a time when olfactory receptor cells have already attained maturity (Cuschieri and Bannister, 1975). In the swine (gestation period 114 days) supporting cells are identifiable from the olfactory epithelium at day 41 of gestation (Holubcova *et al.*, 1997). In the rat fetus (Farbman and Squinto, 1985), the formation of the Bowman's glands occurs at around day 18-20.

The supporting cells are formed directly from stem cells, whose nuclei are situated in the most superficial layers of the epithelium where they are identifiable by the conspicuous amounts of rough and smooth endoplasmic reticula, mitochondria, pinocytotic vesiscles and numerous straight and branched microvilli (Cuschieri and Bannister, 1975). In rat fetuses at day 18-19, a growth spurt of the microvilli of the supporting cells accompanies the massive growth of olfactory cilia (Menco and Farbman, 1985). In the Syrian hamster (gestation period 23 days), the free surfaces of the supporting cells are densely covered by microvilli by the 15th day of gestation (Taniguchi and Taniguchi, 2007). At day 79 of fetal development in the sheep (gestation period 150 days), the irregular-shaped microvilli are present on the apical surface of the supporting cells and a continuous strip of dark fine granular material

occurs in the cytoplasm immediately beneath the apical surfaces of these cells (Kociánová *et al.*, 2003). This granular material is believed to be identical with the terminal tissue of microtubules and filaments found in fully differentiated cells (Moulton and Beidler, 1967; Kocianova *et al.*, 2003).

The Bowman's glands are, in contrast, formed from stem cells whose nuclei are situated at the basal region of the olfactory epithelium (Cuschieri and Bannister, 1975). The late differentiation of the non-nervous elements of the olfactory mucosa is reminiscent of the central nervous system in that there is a predetermined 'programming' of differentiation related to the number of divisions of a particular stem cell (Fujita, 1967; Angevine, 1970; Cuschieri and Bannister, 1975). In the developing Bowman's gland, a sequence of cellular changes occurs before the mature structure is achieved. During prenatal development, the Bowman's glands are formed from two cells types: dark cells which are the stem cells of the gland (identified as such by their lack of secretory apparatus and their similarity to the stem cells of the main epithelium) and pale cells which are the secretory cells (Cuschieri and Bannister, 1975). In postnatal life, the position is apparently reversed since the secretory cells then become 'dark' because of the proliferation of agranular endoplasmic reticula, whereas the seemingly non-secretory cells are now 'pale' (Frisch, 1967).

At day 16 of gestation in the mouse, occasional capillaries occur within the developing olfactory epithelium (Cuschieri and Bannister, 1974, 1975). Intraepithelial capillaries have also been demonstrated in the fetal olfactory

epithelium of the same species by Herken *et al.*, (1989) and also in rats (Farbman and Squinto, 1985), humans (Sangari *et al.*, 1992, 2000) and guinea pigs (Sangari *et al.*, 2002). Within the epithelium, each blood vessel consists of the usual endothelial lining surrounded by a thin basement membrane with no mesenchyme between the vessels and the surrounding epithelial cells (Cuschieri and Bannister, 1975).

1.5.1.4 Development of the olfactory nerve fasciculi

In the mouse at gestational day 10, processes similar or identical to developing axons are visible in the mesenchyme adjacent to the olfactory epithelium (Cuschieri and Bannister, 1975). These processes occur in small groups bounded at least in part by neighboring cells with the characteristics of developing Schwann cells (Tennyson, 1965; Cuschieri and Bannister, 1975). At this stage, the axons contain microfilaments, microtubules, mitochondria and small vesicles, and their diameters vary widely between 0.5- 2.0 μm with a single axon showing one or more dilatations in a single longitudinal section (Cuschieri and Bannister, 1975). According to Cuschieri and Bannister (1975), three stages or processes are evident during the prenatal development of the axonal bundles: (1) the number of axons within the confines of the Schwann cell sheaths gradually increase, (2) the axons decrease in diameter and reach a more uniform size and (3) various changes take place in the arrangement of the axons so that the axon bundle as a whole decreases in size. As opposed to early stages of fetal development where bundle diameters are widely varied due to the presence of numerous growth cones and other dilatations of the sprouting axons, the later stages are characterized by the sprouting of smaller proportions of the axons (some with diameters as low as 0.05 μm) thereby resulting

in gradual reduction in the bundle diameters (Cuschieri and Bannister, 1975). In the well-formed and rounded axon bundles seen in mouse fetuses at day 11, finger-like extensions of the olfactory ensheathing cells later penetrate them to separate the axons of the bundles into smaller fascicles (Cuschieri and Bannister, 1975). The change in the number of axons within an axonal fasciculus correlates with the changing mechanical and possibly nutritional interactions between the olfactory ensheathing cells and the axons (Cuschieri and Bannister, 1975).

1.5.2 Postnatal development

The formation of olfactory receptor cells continues in postnatal life (Moulton *et al.*, 1970; Graziadei, 1973; Calof *et al.*, 1998; Barnett and Riddell, 2004; Lazarini and Lledo, 2011) and therefore many of the events which occur during prenatal development also occur after birth, although in a less clear sequence (Cuschieri and Bannister, 1975). In the early postnatal period in the mouse, cells of the olfactory epithelium are at their final steps of structural maturation (Cuschieri and Bannister, 1975). In the pig (Holubcova *et al.*, 1997), apical structures of supporting and sensory cells form by postnatal day 55 and bands of axon bundles and Bowman's glands are visible by the 94th day of postnatal life. In postnatal day 1 in the Syrian hamster (Taniguchi and Taniguchi, 2007), the main olfactory epithelium is almost completely differentiated while vomeronasal olfactory epithelium differentiates slowly to retain some immature properties for a while even after postnatal day 10. In the sheep, complete morphological development of structures of the vomeronasal olfactory mucosa takes place by gestational day 98 (Salazar *et al.*, 2003). In a study detailing the morphology of the olfactory bulb neurons in 1 to 21-day-old rabbits

(Yilmazer-Hanke *et al.*, 2000), the following changes were observed (1) formation of knob-like growth cones and a few collateral branches in neuronal axons of the olfactory nerve layer (2) increase in axon thickness and number of axon collaterals and formation of rather complex and irregular growth cones in the region close to glomeruli and (3) formation of terminal branches and boutons within the glomerulus coupled with the removal of the extraglomerular branches once the axons had entered the glomerulus.

In the postnatal period, particularly during juvenile development, a number of morphometric parameters of the olfactory mucosa have been documented. Between birth and weaning (postnatal day 30) in the rabbits (Meisami *et al.*, 1990), the area covered by the olfactory epithelium increases by 3-fold and the thickness of the epithelium increases from 65 to 90 μm. In rats between birth and postnatal day 10, the height of the epithelium increases from 94 to 98 μm (Sakashita *et al.*, 1995). In the rabbit (Meisami *et al.*, 1990), packing densities of olfactory and supporting cells increases by 5.5 and 2.5-fold, respectively, between birth and postnatal day 30. In the dog between suckling (3-4 weeks) and adult ages (12-15 months) (Kavoi *et al.*, 2010), packing densities of the olfactory and supporting cells increase by 22.5% and 12.6% respectively. Proliferative activity of the basal cell population in the rat olfactory epithelium (Weiler and Farbman, 1998) was shown to decrease with age with the BrdU-labeling index falling from 30% at birth to 5% at postnatal day 181. In the dog between suckling and adult ages (Kavoi, 2008; Kavoi *et al.*, 2010), the number of cilia per olfactory cell knob, the diameters of axonal bundles and the sizes of the Bowman's glands increase by 5.6%, 7.5% and 39.5% respectively.

1.5.3 Olfactory neurogenesis

The term neurogenesis refers to a process in which neuronal progenitor cells proliferate and differentiate into functional neurons (Shou *et al.*, 1999; Ming and Song, 2011). Throughout most of the 20th century, a 'central dogma of neurobiology' held that neurogenesis takes place only during prenatal development and that the postnatal CNS was static, lacking any capacity for regeneration (Colucci-D'Amato *et al.*, 2006). In the brain, neurogenesis continues throughout life in two areas namely, the dentate gyrus of the hippocampal formation and the subventricular zone of the lateral ventricles (Gould, 2007; Whitman and Greer, 2009; Kriegstein and Alvarez-Buylla; 2009; Gage, 2000; Lazarini and Lledo, 2011). In the latter zone, neuroblasts move along a well-delineated pathway, the rostral migratory stream, *en route* to the olfactory bulb where they mature into inter-neurons (Kornack and Rakic, 2001; Gould, 2007; Whitman and Greer, 2009; Kelsch *et al.*, 2010, Lazarini and Lledo; 2011). Thus in the olfactory system, there is continuous remodeling of olfactory circuitry throughout life as neurons are added and replaced (Lledo *et al.*, 2006).

At the periphery within the olfactory mucosa, studies using nuclear labeling methods have shown that there is a steady loss and replacement of olfactory neuronal cells throughout life, with the stem cells situated near the base of the olfactory epithelium giving rise to immature olfactory neurons (Moulton *et al.*, 1970; Graziadei 1973; Graziadei and Monti Graziadei, 1979; Moran *et al.* 1982; Williams, 1995). The replacement of the olfactory receptor cells occurs during normal turnover, which

lasts 4 to 8 months in man, or following mucosal injury (Bear *et al.*, 1996). Neurogenesis in the olfactory mucosa entails a host of events including olfactory neuronal proliferation, migration, differentiation, axonal path finding, dendritic formation and degeneration (Hsu, 2005; Yoshihara *et al.*, 2005). Many studies have shown that continuous regeneration of the olfactory neurons is influenced by abundant components of the olfactory extra-cellular matrix called glycoconjugates which include laminin, fibronectin, glycoproteins and glycosaminoglycans (Pfenninger *et al.*, 1984; Silverman and Kruger, 1990; Snyder *et al.*, 1991; Takami *et al.*, 1994; Nishizuka and Arai, 1996; Okabe *et al.*, 1996; Raabe *et al.*, 1997; Sharon, 1998; Plendl and Sinowatz, 1998). For purposes of maintaining an equilibrium between basal cell mitosis, neuronal cell differentiation, basal and neuronal cell death and survival in the olfactory epithelium, olfactory neurogenesis is highly regulated by peptide growth factors in the families of nerve growth factor, epidermal growth factor, vascular endothelial growth factor, transforming growth factor, fibroblast growth factor and platelet-derived growth factor (Hsu, 2005; Chae *et al.*, 2012). A study by Enwere *et al.*, (2004) showed that the reduction in epidermal growth factor receptor signaling during aging results in diminished olfactory neurogenesis and deficits in fine olfactory discrimination.

1.6 FUNCTION OF THE OLFACTORY MUCOSA

The role played by the receptor cells of the olfactory mucosa is that of olfaction, also referred to by the layman as "smelling". With regard to function, some authors argue that the main olfactory mucosa is specialized in the detection of small volatile odor molecules whereas the vomeronasal sensory mucosa is responsible for detecting

non-volatile chemicals (Kosel *et al.*, 1981; Halpern, 1987; Wright, 1994; Booth and Katz, 2000; Hagino-Yamagishi *et al.*, 2001). This controversy has been resolved by later workers (Gelez and Fabre-nys, 2004; Restrepo *et al.* (2004) and it is now generally agreed that the two systems play overlapping roles in the detection of biologically relevant chemical signals.

1.6.1 Mechanism of olfaction

The mechanism by which olfactory receptor cells detect odorants and pass the appropriate information to the CNS, `the so called odor transduction, can be divided into three steps: (1) in the nasal olfactory epithelium, the olfactory cells receive stimulus of diverse odor, generating corresponding active potentials (2) the olfactory nerves transmit these active potentials to the olfactory bulb and (3) the active potentials are transmitted, by nerve cells and fibers, to more superior olfactory centers generating olfactory sensation (Buck and Axel 1991; Breer and Boekhoff, 1992; Williams, 1995; Yang, 2002).

In mammals, chemical stimuli are transduced into spike trains by olfactory sensory neurons which express olfactory receptor proteins (Buck and Axel, 1991; Lazarini and Lledo, 2011). These sensory receptors release glutamate onto the distal dendrites of mitral and tufted cells, the two relay neurons of the olfactory bulb circuit (Lazarini and Lledo, 2011). This circuit actively process and refines sensory information using two classes of local inhibitory inter-neurons: periglomerular cells and granule cells (Wilson, 2008). Odor discrimination, learning and memory are thought to depend on the synaptic interplay between inter-neurons and mitral cells

(Yokoi *et al.*, 1995; Laurent, 2002; Lledo and Lagier, 2006). After being processed and refined in the olfactory bulb, odor information is transmitted to higher-order brain structures in the primary and accessory olfactory cortex (Shipley and Ennis, 1996).

In air breathing vertebrates, odor molecules dissolve in a film of mucus on the surface of the olfactory epithelium before they reach the receptor sites on the surface of the olfactory cell cilia (Buck and axel, 1991; Ohloff, 1994; Bear *et al.*, 1996). At the receptor sites, the odorants recognize and bind to specific receptor proteins on the membranes of the cilia (Pace *et al.*, 1985). The interaction of the odorant and the receptor causes the activation of one or more second messenger systems, which entrain the opening of ion channels in the cilia membrane thereby initiating a transduction process (Pace *et al*, 1985; Menco *et al.* 1992, 1994). Olfactory recognition is mediated by a large ensemble of sensory cells, each conveying a fraction of the information that signifies the nature of the odorant and its concentration (Lancet, 1986). Olfactory receptor cells may fail to transmit a signal after a while if they are not cleared off the material bound to them, a phenomenon referred to as "olfactory fatigue" (Hart and Haugen, 1971).

1.6.2 Olfaction ability

Behavioral data generated from several studies (Alberts and May, 1980; Slotnick and Shoonover, 1984; Van Toller *et al.*, 1985; Raimund *et al.*, 1991) show that maximum olfaction ability is attained when an animal reaches adulthood. The increase in odor detection ability as an animal approaches maturity is associated with

the increase in the number (density) of olfactory cells as well as the length and number of cilia of the olfactory cells (Hinds and McNelly, 1981; Farbman and Menco, 1986; Meisami and Najafi, 1986; Rehn *et al.*, 1986; Breipohl *et al.*, 1989; Stahl *et al.*, 1990; Raimund *et al.*, 1991; Wailer and Farbman, 1997).

The packing density of olfactory cells has also been found to directly correlate with the ratio of convergence between axons of olfactory cells and mitral cells in the olfactory bulb of the brain, a factor that Van Drongelen *et al.* (1978) associate with the ontogenic and phylogenic variations in odor sensitivity. In studies detailing changes in the olfactory system of aging animals (Van Toller *et al.* 1985; Hirai *et al.*, 1996; Rosli *et al.*, 1999), diminished olfaction ability in the older animals was associated with the atrophic changes that lead to the reduction in the number of sensory neurons and the loss of cilia in the existing neuronal cells.

1.7 INJURY TO THE OLFACTORY MUCOSA

The peripheral olfactory system is able to recover after injury i.e. the olfactory epithelium reconstitutes, the olfactory nerve regenerates, and the olfactory bulb is reinnervated (Schwob, 2002). The ability to regenerate primary sensory neurons, which is unique in the olfactory system, is a phenomenon of much neurobiological interest (Williams, 1995). As a consequence of their relatively unprotected position in the nasal cavity, cells of the olfactory mucosa can easily be damaged by exposure to toxins, infectious agents and trauma (Monath *et al.*, 1983; Williams, 1995; Schwob, 2002). Throughout life, the olfactory epithelium retains a population of proliferating progenitor cells in its basal region, whose daughters can be "chased"

apical ward with the passage of time into the neuronal compartment of the epithelium (Graziadei and Graziadei, 1979; Schwob, 2002). Indeed, death of both non-neuronal and neuronal cells directs multipotent globose basal cell progenitors to give rise individually to sustentacular cells and horizontal basal cells as well as neurons (Schwob, 2002). Because of their relative accessibility compared to intra-cranially located neuronal stem cells, olfactory epithelial stem cells make attractive candidates for autologous cell-based therapy (Ducray *et al.*, 2002; Viktorov *et al.*, 2006).

To many people, the ability to sense thousands of different odors is something that they may take for granted. However, to a minority, this natural skill is lost due to a dysfunction of the olfactory system (Leopold, 1995; Seiden, 1997). The loss of the sense of smell can have profound psychological and somatic consequences (Van Toller, 1999). Loss of smell often leads to a loss of taste and although often discounted and overlooked in the basic clinical examination, deficiencies in these chemosensory cues can cause anxiety, depression and even nutritional deficiencies due to decreased enjoyment of food (Leopold, 1995). Olfactory dysfunction is associated with a wide range of conditions which include sino-nasal disease (Smith and Seiden, 1991; Cullen and Leopold, 1999; Wolfensberger and Hummel, 2002), head trauma (Smith and Seiden, 1991; Duncan and Smith, 1995) and toxicity of systemic or inhaled drugs (Leopold, 1995). Some abnormalities in smell can signal the existence of several diseases of the nervous system such as Alzheimer's (Doty, 1997; Seiden, 1997; Thompson *et al.*, 1998) and Parkinson's (Li *et al.*, 1995). The

sense of smell is also impaired with aging, with a noticeable average decline in function during the 7th decade of life in humans (Leopold, 1995).

Smell and taste changes is a major side effect subsequent to cancer chemotherapy (Bernhardson *et al.*, 2008; Steinbach *et al.*, 2009) and loss of olfactory/ gustatory function can lead to malnutrition, weight loss, prolonged morbidity of chemotherapy-induced adverse effects, decreased quality of life and decreased therapy response (Steinbach *et al.*, 2009). According to Seiden (1997), smell dysfunction come in four major forms: complete loss of smell referred to as anosmia, partial loss of smell called hyposmia, enhanced smell sensitivity termed hyperosmia and distortion in odor perception named dysosmia. It has been suggested that dysosmia is a sign of regeneration and eventual recovery and is more common in patients with post-traumatic hyposmia (Smith *et al.* 1987; Seiden, 1997).

1.8 THE CELL CYTOSKELETON

The internal organization, shape, motility and life cycle of eukaryotic cells are all controlled by a complex network of polymeric filaments called cytoskeleton (Janke and Bulinski, 2011). These filaments provide, within every cell, a supporting framework that maintains the structural stability of the cell (Burkitt *et al.*, 1993). The cytoskeleton is dynamic and strong, ever ready to adapt to demands on the cell and is constituted by three main kinds of cytoskeletal filaments: actin filaments (also called microfilaments), microtubules, and intermediate filaments (Amos *et al.*, 2004; Harold, 2007).

1.8.1 Actin filaments

Actin filaments are extremely fine strands (about 5 nm in diameter) of a protein known as actin which consists of two strings of bead-like subunits twisted together like a rope (Burkitt *et al.*, 1993). These subunits are stabilized by Ca^{2+} and are associated with ATP molecules which provide energy for contractile properties (Burkitt *et al.*, 1993; Williams, 1995). At the cell membrane, microfilament assembly protrudes the membrane in actively moving cells (Bray, 2000; Ridley *et al.*, 2003; Pollard and Borisy, 2003; Pollard, 2003). Actin filaments can also play a passive structural role by providing the internal stiffening rods in microvilli, maintaining cell shape and anchoring cytoskeletal proteins (Mooseker *et al.*, 1980; Gartzke and Lange, 2002). Infact, decreased actin turnover and rigidity of cytoskeletal structures have been associated with aging and cell death (Kronenberg *et al.*, 2010). The ERM (ezrin, radixin and moesin) protein family crosslink actin filaments with plasma membranes (Tsukita *et al.*, 1997; Yonemura *et al.*, 1998) and have together been documented to play role in epithelial cell organization and functions (Fiévet *et al.*, 2007). Using immunohistochemistry, confocal microscopy and Western blotting, Persson *et al.*, (2010) demonstrated the presence of the ERM protein radixin in neuroblasts of the adult rostral migratory stream (RMS), cerebral cortex, striatum, cerebellum, thalamus, hippocampus as well as the granular and periglomerular layers of the olfactory bulb, where the protein plays role in neuronal migration and differentiation. Myosin VIIa, a putative actin-based mechanoenzyme, has been documented as common component of cilia of olfactory neurons, cochlear hair cells and cells of kidney distal tubules and of lung bronchi (Wolfrum *et al.*, 1998).

1.8.2 Intermediate filaments

Intermediate filaments were first identified in the developing skeletal muscle in the Howard Holtzer's laboratory (Ishikawa *et al*, 1968) as polymers differing from microfilaments and microtubules in that they are intermediate in size between microtubules and actin filaments. Intermediate filaments are the most resilient component of the cytoskeleton that helps individual cells to get integrated into tissues thereby contributing to a precise cell-cell organization (Song *et al.*, 2009). Intermediate filaments, whose diameter is 10-12 nm, have a stable fibrous structure made up of a variety of different irregular molecular strands, which appear to be specific to particular cells. In epithelial cells, intermediate filaments constitute structures called tonofilaments, which form a tough supporting meshwork within the cytoplasm and are anchored to the plasma membrane at strong intracellular junctions within the adjacent epithelial cells (Burkitt *et al.*, 1993). In CNS neurons, the primary components of the intermediate filaments are α-internexin and neurofilament triplet proteins (Liem and Messing, 2009) while in peripheral nervous system, peripherin is expressed along with the neurofilament triplet proteins (Ching and Liem, 2006). In astrocytes, the key component of the intermediate filaments is glial fibrillary acidic protein, although there are lower levels of other intermediate filaments including vimentin, nestin, and synemin (Liem and Messing, 2009).

1.8.3 Microtubules

Microtubules are cytoskeletal components having a diameter of 25 nm and are constituted by globular protein subunits designated alpha (α) and beta (β) tubulins,

which are readily assembled and disassembled to provide for alteration in cell shape and position of organelles (Burkitt *et al.*, 1993). In most cells, cytosolic microtubules are involved in intracellular transport, organelle positioning, change in shape of the cell and motility (Rodriguez *et al.*, 2003; Tran *et al.*, 2007) whereas more complex microtubule structures form the core components of centrosomes, the centrioles, and the core structures of cilia and flagella, which are called axonemes (Rodriguez *et al.*, 2003; Janke and Bulinski, 2011). Microtubules have been implicated in the establishment of epithelial cell surface polarity and in controlling differentiative processes (Yap and Manley, 2001; Janke and Bulinski, 2011). Moreover, epithelial surface specilializations such as cilia are dominated by microtubules (Wolfrum *et al.*, 1998).

Neurons are striking examples of cells in which microtubules are essential for achieving a high degree of morphological and functional complexity (Janke and Bulinski, 2011). In fact, the biochemical similarities in structure between the filaments of the mitotic spindle and those of the neuronal axons have been used to justify the vulnerability of neurons to lesions by microtubule disrupting agents (Pace *et al.*, 1996; Topp *et al.*, 2000). Despite their functional diversity, all microtubules are assembled from heterodimers of α and β tubulins, which are two highly similar proteins that are conserved among all eukaryotic species (Janke and Bulinski, 2011). Soluble α-tubulin–β-tubulin dimers polymerize into microtubules in the presence of guanosine-5'-triphosphate (GTP) at physiological temperatures (Vallee, 1986). Both *in vivo* and *in vitro*, microtubules undergo cycles of polymerization and rapid depolymerization, a property that was first described and referred to by Mitchison

and Kirschner (1984) as "dynamic instability" and which is believed to be a crucial feature to many microtubule functions. During metaphase stage of mitosis, the microtubules polymerize to form hollow tubules which grow, by addition of the tubulin subunits, between a pair of centrioles and serve to hold and segregate chromosomes located at the equatorial plate of the cell (Burkitt *et al.*, 1993).

Many, if not all, functions of microtubules are mediated by a highly complex and diverse set of microtubule-interacting proteins, which include two major families of microtubule motors; kinesins and dyneins (Bustamante *et al.*, 2000; Vallee *et al.*, 2004; Verhey and Hammond, 2009). Upon interaction with microtubules, motor proteins generate force and these forces are used for various intracellular functions, most obviously intracellular transport (Janke and Bulinski, 2011). In neuronal cells for example, cargoes need to be transported over long distances (in some cases, more than one meter) from the cell body to the synapses (Sheetz *et al.*, 1989). Besides their transport functions, motor proteins generate forces for ciliary beating and for the self-organization of microtubule arrays, such as the mitotic spindle (Surrey *et al.*, 2001; Lindemann and Lesich, 2010). Moreover, other kinesin motors catalyze the depolymerization of microtubules thereby participating in the complex network of interacting proteins that regulate microtubule dynamics (Howard and Hyman, 2007). The heterogeneous group of non-motor microtubule associated proteins comprises not only many proteins that stabilize microtubules (for example, the neuronal proteins tau, motor associated protein 1 and motor associated protein 2) but also severing proteins, such as spastin and katanin, which destabilize the microtubule lattice (Roll-Mecak and McNally, 2010).

Another group of intensively studied microtubule associated proteins is that of the microtubule plus end-tracking proteins (+TIPs), which help to control microtubule dynamics and interactions with other cellular organelles and subcellular domains (Akhmanova and Steinmetz, 2008). Studies have also shown that the assembly and dynamics of axonemal microtubules are regulated by the TTLL3 (Tubulin tyrosine ligase-like family, member 3) protein/ gene, whose deletion results in (1) shortening and increased resistance of the axonemes to paclitaxel-mediated microtubule stabilization and (2) shortening or loss of cilia in several organs, including the Kupffer's vesicle and olfactory placode (Wloga *et al.*, 2009). In a study focusing on the olfactory epithelium (Kulaga *et al.*, 2004), deletions of the BBS (Bardet-Biedl syndrome) gene, which is responsible for proper functioning of basal bodies and cilia, resulted in severe reduction of the ciliated border of the epithelium, disorganization of the dendritic microtubule network, trapping of olfactory ciliary proteins in the dendrites and partial or complete anosmia.

1.8.4 Role of microtubules in cell replication

1.8.4.1 Cell replication

The division and duplication (replication) of a eukaryotic cell is achieved when a cell undergoes a series of events which constitute the cell-division cycle or the cell cycle (Morgan, 2007). The cell cycle is divided in two main phases: (1) a non dividing phase called interphase in which the cell grows and duplicates its DNA and (2) a relatively short mitotic or M phase in which two events occur, namely, mitosis and cytokinesis (Lilly and Duronio, 2005; Morgan, 2007). With the development of

radio-isotopes, the interphase stage was shown to have four discrete periods which include (i) G_1 phase in which the cell increase in size and prepares for DNA synthesis (ii) S- phase during which DNA is replicated/ synthesized (iii) G_2 phase in which the cell continues to grow and prepare to enter the M-phase and (iv) G_0 phase which is a resting phase where the cell has left the cycle and has stopped dividing (Elledge, 1996; Morgan, 2007; Slavov and Botstein, 2011). While facultative dividers enter the G_0 phase but retain the capacity to re-enter the cell cycle when suitably stimulated, some other types of cells progress continuously through the cell cycle to accommodate tissue growth or cell turnover (Burkitt *et al.*, 1993).

During mitosis, the eukaryotic cell separates the chromosomes in its nucleus into two identical sets (Lloyd and Chan, 2006). This is followed immediately by cytokinesis, which divides the nuclei, cytoplasm, organelles and cell membrane into two cells containing roughly equal shares of these cellular components (Nanninga, 2001; De Souza and Osmani, 2007). The process of mitosis is fast and highly complex with the sequence of events being divided into prophase, prometaphase, metaphase, anaphase and telophase (Lloyd and Chan, 2006; De Souza and Osmani, 2007). During these stages, the mitotic spindle, whose fundamental machinery are the spindle microtubules, plays a critical role in the migration of chromosomes to the opposite ends of the cell (Walczak and Heald, 2008).

1.8.4.2 Spindle microtubules

The mitotic spindle is a complex macromolecular structure responsible for the movement of chromosomes during mitosis and has three principal components

microtubules, centrosomes and kinetochores (O'Connell and Khodjakov, 2007). The spindle forms as chromosomes become connected to the two centrosomes (spindle poles) by microtubule bundles that link each pole to a specialized macromolecular assembly on the chromosome body termed kinetochore (Hayden *et al.*, 1990; Rieder and Alexander, 1990; O'Connell and Khodjakov, 2007). Capture of kinetochores by dynamic astral microtubules was originally proposed as the basis of spindle formation (Hayden *et al.*, 1990; Rieder and Alexander, 1990; Skibbens *et al.*, 1993). However, mounting evidence later indicated that there are multiple microtubule nucleation and capture sites throughout the spindle (Carazo-Salas *et al.*, 2001, 2005; Luders and Stearns, 2007; O'Connell and Khodjakov, 2007).

A number of chemical substances are known to interfere with cell replication by disrupting spindle structures. These include antimicrotubule drugs, which activate multiple response pathways to arrest cells division at the M-phase (Jordan *et al.*, 1991; Gidding *et al.*, 1999). In higher eukaryotic cells, chromatin-binding proteins, including many bromodomain proteins, play a critical role in preserving a properly acetylated chromatin status (Muchardt *et al.*, 1996; Segil *et al.*, 1996). Indeed, mitotic arrest resulting from antimicrotubule drugs has been associated with the rapid release of the mammalian bromodomain protein Brd4 from the cell chromosomes (Nishiyama *et al.*, 2006). Upon withdrawal of the antimicrotubule drug, this protein is reloaded onto chromosomes thereby allowing the cells to proceed to complete cell division (Nishiyama *et al.*, 2006).

1.9 CANCER AND ITS MANAGEMENT

1.9.1 CANCER

Cancer is the uncontrolled growth of cells anywhere in the body and the abnormal cells are termed cancer or tumor cells (Gupta and Massague, 2006; Wang, 2010; Hussein and Komarova, 2011; Putre, 2011). Except for the benign type which is localized (Goswami *et al.*, 2012; Girish *et al.*, 2012; Mannarini *et al.*, 2012), most cancers are of the malignant type i.e. metastasise and invade other tissues or organs (Leong *et al.*, 2012). Cancer is a health problem not only in humans but also in animals, where it is a major cause of death particularly in pets (Withrow, 2007; Rabinowitz *et al.*, 2009). Many forms of cancer are thought to be caused by the interaction between genetic susceptibility and environmental toxins (Vogelstein and Kinzler, 1998; Lichtenstein *et al.*, 2000; Brennan, 2002; Blessmann *et al.*, 2012). The many cancers and the abnormal cells that compose the cancer tissue are further identified by the name of the tissue that the abnormal cells originated from e.g. breast cancer, cervical cancer, testicular cancer, prostate cancer, lung cancer, colon cancer. Based on the Global Burden of Cancer (GLOBOCAN) estimates, about 12.7 million cancer cases and 7.6 million cancer deaths are estimated to have occurred in the year 2008; of these, 56% of the cases and 64% of the deaths occurred in the economically developing world (Jemal *et al.*, 2011).

According to the National Cancer Institute, there are over 200 types of cancers majority of which fit into the following categories (Muir and Percy, 1991): (1) carcinomas which begin in the skin or in tissues that line or cover internal organs, (2) sarcomas which start in bone, cartilage, muscle, blood vessels, or other

connective or supportive tissue, (3) leukemias which begin in blood-forming tissue such as the bone marrow and cause large numbers of abnormal blood cells to be produced and to enter the blood, (4) lymphomas and myelomas which begin in the cells of the immune system, and (5) central nervous system cancers which begin in the tissues of the brain and spinal cord.

1.9.2 MANAGEMENT OF CANCER

Cancer can be managed in a number of ways. The most common management strategies include surgery, radiation therapy, hormonal therapy, use of angiogenesis inhibitors, targeted therapy and chemotherapy (Nemoto *et al.*, 1980; Skeel, 2003; Chabner and Longo, 2005; Camphausen and Lawrence, 2008; Takimoto and Calvo, 2008; Zhukov and Tjulandin, 2008).

1.9.2.1 Surgery

In theory, non-hematological cancers can be cured if entirely removed by surgery. However, when the cancer has metastasized to other sites in the body prior to surgery, complete surgical excision is usually impossible (Lacroix, 2006). In the Halstedian model of cancer progression, tumors grow locally, spread to the lymph nodes, then to the rest of the body (Xie *et al.*, 2011). Although local-only treatments such as surgery are best suited for small cancers, the excision of the primary tumor, particularly in the breast, has been associated with increased systemic metastatic burden (Hussein and Komarova, 2011; Hussein *et al.*, 2011, 2012). Examples of surgical procedures for cancer include mastectomy for breast cancer (Nemoto *et al.*, 1980; Hussein *et al.*, 2011), hysterectomy for uterine or cervical cancer (Steren *et*

al., 1993; Cantuaria *et al.*, 1999; Brandsborg, 2012), prostatectomy for prostate cancer (Merglen *et al.*, 2007; Welz *et al.*, 2008) and orchidectomy (castration) for testicular cancer (Scher *et al.*, 2004; Labrie, 2011).

1.9.2.2 Radiation therapy

Radiation therapy, also called radiotherapy, X-ray therapy or irradiation, is the use of ionizing radiation to kill cancer cells and shrink tumors (Shaw *et al.*, 1989; Formenti and Demaria, 2008; Camphausen and Lawrence, 2008). Radiation therapy injures or destroys cells in the area being treated by damaging their genetic material, making it impossible for these cells to continue to grow and divide (DeVita *et al.*, 2008; Camphausen and Lawrence, 2008). Dewey *et al.*, (1977) recommend the combination of hyperthermia with X-irradiation in cancer therapy because cycling tumor cells in S-phase are most likely to survive an X-ray dose. Although radiation damages both cancer cells and normal cells, most normal cells can recover from the effects of radiation and function properly (Emami *et al.*, 1991). Radiation therapy is used to treat almost every type of solid tumor with the radiation dose to each site depending on a number of factors, including the radiosensitivity of the cancer type and whether or not there are tissues and organs nearby that may be damaged by radiation (Emami *et al.*, 1991; Nieder *et al.*, 2000). Proton beam therapy, the latest advancement in the treatment of various types of cancers (Slater *et al.*, 2004; St. Clair *et al.*, 2004; Levin *et al.*, 2005; Dinesh, 2011), is a precise form of radiotherapy, which uses a beam of protons to target the cancer cells and destroys them. Because healthy tissues are largely spared in this type of therapy, oncologists

can, in theory, deliver much higher doses of radiation, while improving local control and reducing the risk for recurrence and morbidities (Dinesh, 2011).

1.9.2.3 Hormonal therapy

Hormonal therapy involves the manipulation of the endocrine system through exogenous administration of specific hormones, particularly steroid hormones, or drugs which inhibit the production or activity of such hormones (Stetler-Stevenson and Kleiner, 2001; Takimoto and Calvo, 2008). Because steroid hormones are powerful drivers of gene expression in certain cancer cells, changing the levels or activity of certain hormones can cause certain cancer cells to cease growing, or even undergo death (Bernstein and Ross, 1993; Risch, 1998; Choi *et al.*, 2002; Leung and Choi, 2007; Armaiz-Pena *et al.*, 2009). Hormonal therapy is used for several types of cancers derived from hormonally responsive tissues, including the breast, prostate, endometrium, and adrenal cortex (Lippman *et al.*, 1976; Stetler-Stevenson and Kleiner, 2001; Brunton *et al*, 2006; Labrie 2011). Perhaps the most familiar example of hormonal therapy in oncology is the use of the *selective estrogen-response modulator* tamoxifen, a drug which acts as antagonists of the estrogen receptor and is used as the first-line treatment for nearly all pre-menopausal women with hormone receptor-positive breast cancer (Stetler-Stevenson and Kleiner, 2001; Jordan, 2008; Gjerde *et al.*, 2010).

1.9.2.4 Use of angiogenesis inhibitors

When solid cancers are small, they are supplied with nutrients by diffusion from nearby blood vessels. In order to grow larger, they need their own blood vessels,

which they create by angiogenesis promoters such as vascular endothelial growth factor (VEGF) (Hicklin and Ellis, 2005). VEGF regulates both vascular proliferation and permeability, and functions as an anti-apoptotic factor for newly formed blood vessels (Haspel *et al.*, 2002; Rosen, 2002). Angiogenesis inhibitors such as the anti-VEGF monoclonal antibodies (e.g. bevacizumab) prevent the extensive growth of blood vessels that tumors require to survive (Rosen, 2002; Diaz-Rubio, 2006). One of the main problems with anti-angiogenesis drugs is that when one angiogenesis promoter is blocked, cancers eventually grow blood vessels using another angiogenesis promoter (Bergers and Hanahan, 2008; Hwang and Heath, 2010). Other problems include route of administration, maintenance of stability and activity and targeting at the tumor vasculature (Kleinman and Liau, 2001).

1.9.2.5 Targeted therapies

Targeted therapy is a type of medication that blocks the growth of cancer cells by interfering with specific targeted molecules needed for carcinogenesis and tumor growth (Zhukov and Tjulandin, 2008). This kind of therapy, which became available in the late 1990s, has had a significant impact in the treatment of some types of cancers. Small molecule targeted therapy drugs (e.g. Bcl-2 antagonist and tyrosine kinase inhibitors imatinib, gefitinib and erlotinib) are generally inhibitors of enzymatic domains on mutated, overexpressed, or otherwise critical proteins within the cancer cell (Warr and Shore, 2008; Katzel *et al.*, 2009). Monoclonal antibody therapy is another version of targeted therapy in which the therapeutic agent is an antibody (e.g. anti-HER2/neu antibody trastuzumab and anti-CD20 antibody

rituximab) which specifically binds to a protein on the surface of the cancer cells (Drebin *et al.*, 1986).

1.9.2.6 Chemotherapy

Chemotherapy is the treatment of cancer with drugs that can destroy cancer cells (Skeel, 2003). In current usage, the term "chemotherapy" usually refers to cytotoxic drugs, which affect rapidly dividing cells in general (Takimoto and Calvo, 2008), in contrast with targeted therapy which interferes with specific targeted molecules needed for tumor growth (Zhukov and Tjulandin, 2008). All drugs used in cancer chemotherapy affect cell division or DNA synthesis (Takimoto and Calvo, 2008). Most forms of chemotherapy target all rapidly dividing cells and are not specific to cancer cells, although some degree of specificity may come from the inability of many cancer cells to repair DNA damage, while normal cells generally can (Rantala *et al.*, 2010). Hence, chemotherapy has the potential to harm healthy tissues, especially those that have a high replacement rate (Chabner and Longo, 2005). Since some drugs work better together than alone, two or more drugs are often given at the same time, the so called combination chemotherapy (Figgitt and Wiseman, 2000)

The majority of chemotherapeutic drugs can be divided into alkylating agents, platinums, cytotoxic antibiotics, antimetabolites, alkaloids and terpenoids (Takimoto and Calvo, 2008). An alkylating agent attaches an alkyl group to the guanine base of DNA, at the number 7 nitrogen atom (N7) of the purine ring thereby damaging cancer cell (Scott, 1970; Takimoto and Calvo, 2008). Classical alkylating agents include melphalan, chlorambucil, ifosfamide, carmustine, lomustine, streptozocin,

busulfan (McClean *et al.*, 1999) while the non-classical include procarbazine and altretamine (Armand *et al.*, 2007; Yasko *et al.*, 1998).

Platinum-based chemotherapeutic drugs permanently coordinate to DNA by binding at N7 of guanine to interfere with DNA repair and are therefore described as "alkylating-like" (Pizzo and Poplack, 2006; Cruet-Hennequart *et al.*, 2008). They include cisplatin, carboplatin, nedaplatin, oxaliplatin, satraplatin and triplatin tetranitrate (Pizzo and Poplack, 2006; Takimoto and Calvo, 2008; Vermorken *et al.*, 2008).

Cytotoxic antibiotics are a group of antibiotics that are used for the treatment of cancer owing to their ability to interfere with DNA replication and protein synthesis (Takimoto and Calvo, 2008). These antibiotics include actinomycins and anthracyclines (e.g. doxorubicin, daunorubicin, valrubicin, idarubicin and epirubicin) (Takimoto and Calvo, 2008). The actinomycins, of which the most significant is actinomycin D, was the first antibiotic shown to have anti-cancer activity (Waksman and Woodruff, 1940; Turan *et al.*, 2006). The anthracyclines are effective against more types of cancers (Weiss, 1992; Minotti *et al.*, 2004).

Antimetabolites used in cancer therapy interfere with DNA production, halt cell division and hence the growth of the tumor (Smith, 1997; Peters *et al.*, 2000). Antimetabolites prevent purines and pyrimidines from becoming incorporated into DNA during the S-phase thereby stopping normal cell development and division (Silverman, 2004). Examples of these agents are purine analogues (e.g. azathioprine,

mercaptopurine, thioguanine and fludarabine) and pyrimidine analogues (e.g. 5-fluorouracil, floxuridine and cytosine arabinoside) (Takimoto and Calvo, 2008).

Alkaloids and terpenoids are plant derivatives widely used in cancer chemotherapy and their activity is targeted on microtubules (Zhou and Rahmani, 1992; Pazdur *et al.*, 1993; McGrogan *et al.*, 2008). For targeting microtubules, these drugs have been referred by names such as microtubule-targeted drugs, microtubule inhibitors, antimicrotubule or antimitotic drugs. Alkaloids and terpenoids act on highly mitotic cells, including cancer cells, by interfering with spindle microtubules dynamics and the main examples of these drugs are vinca alkaloids and taxanes (Bender, 1987; Don *et al.*, 2004).

1.9.2.6.1 Vinca alkaloids

Vinca alkaloids are a group of anti-microtubule drugs that are widely used in cancer therapy (Zhou and Rahmani, 1992). These drugs are derived from the Madagascar periwinkle plant *Catharanthus roseus* (formerly known as *Vinca rosea*) (Bender *et al.*, 1987). They are a class of cell-cycle-specific drugs that bind to specific sites on tubulin thereby inhibiting the assembly of the tubulin into microtubules to arrest cell division at M-phase (Jordan *et al.*, 1991; Gidding *et al.*, 1999). The synthetic forms of this group of drugs include vinblastine, vincristine, vindesine, vinorelbine and vinflunine (Bender, 1987; Grindey, 1989; Zhou and Rahmani, 1992; Gidding *et al.*, 1999; Takimoto and Calvo, 2008; Kruczynski and Hill, 2001). Vinblastine sulphate, which is administered intravenously to human patients at weekly doses of 3.7 mg/m^2 (0.10 mg/kg), is among the widely used antimicrotubule drug, particularly for being

the drugs of choice in the management of Kaposi's sarcoma, whose incidence has increased dramatically since the advent of the HIV pandemic (Zidan *et al.*, 2001; Onyango and Njiru, 2004, Ferdinado *et al.*, 2009; Phipps *et. al*, 2010).

1.9.2.6.2 Taxanes

Taxanes represent a novel class of antineoplastic drugs. These drugs inhibit mitotic cell division by stabilizing guanosine diphosphate (GDP)-bound tubulin in the microtubule to block microtubule disassembly (Pazdur *et al.*, 1993; McGrogan *et al.*, 2008). The prototype taxane is the natural product paclitaxel, originally known as taxol, which is derived from the bark of the Pacific Yew tree *(Taxus baccata)* (Bender *et al.*, 1987). The toxoid paclitaxel and its semi-synthetic analogue docetaxel (taxotere) are the widely known representatives of this group of drugs (Pazdur *et al.*, 1993; Fumoleau *et al.*, 1995). Other examples are larotaxel, ortataxel and tesetaxel (Don *et al.*, 2004; McGrogan *et al.*, 2008).

Although docetaxel and paclitaxel are often considered similar in activity and tolerability, these agents are indeed different in regard to their benefits and adverse effects (Michaud *et al.*, 2000). In managing breast cancer, docetaxel shows excellent efficacy with approximately 50% responses in tumors resistant to other antineoplastic drugs (Capri *et al.*, 1996). In human patients, docetaxel is administered at doses of 60-100 mg/m^2 (1.62- 2.70 mg/kg) but controversy remains regarding dose-benefit relationship and scheduling differences (i.e. weekly versus every 3 weeks) (Michaud *et al.*, 2000). Promising results have, however, been

achieved in phase I/ II trials of a weekly regimen of docetaxel (Figgitt and Wiseman, 2000).

1.10 EFFECTS OF ANTIMICROTUBULE DRUGS ON TISSUES

By disrupting spindle structures, antimicrotubule drugs exert their effects not only on cancer cells but also on normal tissues that are characterized by highly mitotic numbers which includes gastrointestinal, lymphohematopoietic, testicular (Todd *et al.*, 1976) and cutaneous tissues (Dorr and Alberts, 1985; Lishner *et al.*, 1999). In the use of vincristine to manage cancer, gastrointestinal and hematologic toxicity are the dose-limiting factors with the associated lesions being necrosis and hemorrhage of the gastrointestinal (GIT) mucosa, bone marrow atrophy and necrosis and atrophy of the lymphoproliferative tissues (Kanter *et al.*, 1994). In a study comparing the hematologic toxicity of various antimicrotubule agents in different species of animals, vincristine was found to produce severe leucopenia in chickens, cats and monkeys (Todd *et al.*, 1979). In rats, administration of vincristine and vindesine results in depressed blood cell counts and extramedullary hematopoiesis while in dogs, leucopenia and erythropenia are the main findings (Todd *et al.*, 1976). In the use of paclitaxel and docetaxel in cancer patients, neutropenia is the dose limiting toxicity (Pazdur *et al.*, 1993; Fumoleau *et al.*, 1995). In both rats and dogs, exposure of the testicular tissue to vincristine and videsine results in inhibition of spermatogenesis (Todd *et al.*, 1976). The clinical use of docetaxel has been associated with unpredictable and severe skin toxicity, as well as edema and effusions due to a capillary-leak syndrome (Capri *et al.*, 1996). While alopecia is commonly reported in cancer patients undergoing combined chemotherapy (Lishner

et al., 1999), experimental exposure of vincristine to mice induces ulcerative lesions on the skin (Dorr and Alberts, 1985).

In rats, systemic administration of vincristine results in the degeneration of axons of myelinated and large diameter peripheral nerves (Topp *et al.* 2000). In chemotherapeutic management of cancer using vinorelbine in humans, sensory-motor axonal neuropathy has been reported (Pace *et al.*, 1996). In mice administered with vincristine, vinblastine, videsine and paclitaxel, axonopathy and myelin fragmentation of the trigeminal nerve was observed (Kai *et al.*, 2004). Peripheral neuropathy has also been reported in the clinical use of docetaxel and paclitaxel in humans (Pazdur *et al.*, 1993; Capri *et al.*, 1996). Systemic exposure of the mouse olfactory mucosa to antimicrotubule agents resulted in varied histopathological changes including atrophy, hemorrhage and single cell death in the epithelium (Kai *et al.*, 2004, 2005), axonal degeneration (Kai *et al.*, 2005) and presence of cell debris in the lamina propria (Kai et al., 2002).

1.11 RATIONALE AND OBJECTIVES

1.11.1 RATIONALE

Rabbits pups are born blind and deaf and physically weak and their young ones receive unusually limited maternal care. In this species therefore, the olfactory cue plays an important role in aspects of mother-neonate interaction, particularly in the release and guidance of suckling behavior. As part of this work, changes in structure of the various components of the olfactory mucosa are analyzed at different

postnatal ages to ascertain whether such changes may be attributed to the high olfactory functional demand associated with juvenile development in this species.

In toxicity studies, chemicals administered by inhalation have been shown to alter the normal functioning and structure of the olfactory mucosa. However, when the chemicals are administered systemically, reports on the lesions imparted on the olfactory mucosa are largely lacking. Microtubule-disrupting drugs are a group of parenterally administered anticancer drugs, which impart their effects on highly proliferative tissue cells. The olfactory mucosa is one such type of tissue. In chemotherapeutic management of cancer using these drugs, data on smell dysfunction have widely been documented with hardly any reports on the accompanying olfactory lesions. Thus, as a second part of this study, the olfactory mucosa was analyzed for both structural and functional alterations following administration of antimicrotubule anticancer drugs vinblastine or docetaxel in the rabbit, a widely used animal model in toxicological studies (Amann, 1982; Morton, 1988; Williams *et al.*, 1990; Frame *et al.*, 1994; Simunek *et al.*, 2004; Che *et al.*, 2011).

1.11.2 OBJECTIVES

1.11.2.1 Overall objective

This principal aim of the present work is to establish the structural changes that take place in the rabbit olfactory mucosa during postnatal development and to find out to what extent the anticancer drugs vinblastine and docetaxel interfere with the integrity of the olfactory mucosa in the adult rabbit.

1.11.2.2 Specific objectives

1. To describe the morphological changes associated with postnatal development of the olfactory mucosa in the rabbit.
2. To determine the morphometric changes associated with postnatal development of the olfactory mucosa in the rabbit.
3. To analyze the effects of microtubule-disrupting drugs vinblastine and docetaxel on the structure of adult rabbit olfactory mucosa.
4. To evaluate the effects of vinblastine and docetaxel administration on olfactory function in the adult rabbit.

1.11.3 Hypotheses

The present study seeks to elucidate the hypotheses that (1) the structural refinement of the olfactory mucosa during postnatal development provides some implications on its functional status in the rabbit (2) the structural alterations imparted on the adult rabbit olfactory mucosa vary among the anticancer drugs vinblastine and docetaxel and (3) the administration of vinblastine or docetaxel produces varied level olfactory impairment in the adult rabbit.

CHAPTER TWO

MATERIALS AND METHODS

2.1 EXPERIMENTAL ANIMALS

New Zealand White rabbits acquired from commercial breeders in Njoro, Rift Valley Province, Kenya, were used in this study. The rabbits were kept in the Departmental of Veterinary Anatomy and Physiology animal housing facility in individual wire mesh cages measuring 52 cm long x 42 cm wide x 41 cm high (Chirino *et al.*, 2007) and under natural lighting and temperature conditions (12-hour light/ dark cycle, average temperature 23°C and relative humidity 55 ± 15%). They were fed on standard rabbit pellets (Unga feeds Ltd, Nairobi) and tap water was available *ad libitum.* All protocols for the experimentation of the animals were approved by the Animal Use and Care Committee of the Faculty of Veterinary Medicine, University of Nairobi.

Details of how the animals were distributed to the various experimental groups are given in Table 1. For the study on the postnatal morphology of the olfactory mucosa, the animals were divided into four groups of ten animals each, comprising of adult males aged 6-8 months, weanlings 4 weeks, sucklings 2 weeks and neonates/ newborns 0-1 days. In order to study the effect of microtubule disrupting drugs vinblastine and docetaxel on olfactory mucosal structure and on olfactory function, a total of 115 animals were used. These animals were distributed into the various groups as shown in Table 1. For the study on the impact of the antimicrotubule drugs on mucosal structure, the animals were injected with the test drugs at the same time but harvesting was done at different time points.

Table 1. Summary of distribution of animals to the various experimental groups

		No. groups	*No. animals/ group*	*Total no. animals used*
Study on development	Neonates	1	10	40
	Sucklings	1	10	40
	Weanlings	1	10	40
	Adults	1	10	40
Structural test: VBS	Exp groups	4	10	40
	Control	1	10	10
Structural test: DCT	Exp groups	4	10	40
	Controls	1	10	10
Functional test: BFT	VBS	1	5	5
	DCT	1	5	5
	Controls	1	5	5

Abbreviations VBS, DCT, BFT and 'Exp' stands for vinblasine, docetaxel, buried food test and 'experimental' respectively.

2.2 ADMINISTRATION OF TEST DRUGS

Lyophilized formulations of vinblastine sulphate and docetaxel (Surgipharm Ltd, Nairobi) were dissolved in physiological saline and administered to the adult rabbits as single intravenous doses of 0.31mg/kg and 6.26 mg/kg respectively, via the ear vein. These dosage values were equivalents to those used for cancer chemotherapy in human patients (Reagan-Shaw *et al.*, 2007). In humans, vinblastine is administered at a dose rate of 3.7 mg/m^2 (0.10 mg/kg) (Hochadel, 2011) whereas docetaxel is given at 75 mg/m^2 (2.03 mg/kg) (Bang *et al.*, 2002) and therefore the doses given to the rabbits were worked out using the formula

Animal dose= Human dose X (Human K_m factor / Animal K_m factor)

[Where: K_m factor, body weight (kg) divided by body surface area (m^2), is 37 for humans and 12 for rabbits (Reagan-Shaw *et al.*, 2007)]

Control animals were injected with physiological saline also through the ear vein. Representative groups of animals administered with the test drugs (vinblastine or docetaxel) were euthanized with lethal doses of pentobarbital sodium (200 mg/kg, given intraperitonially) on postexposure days 3, 5, 10 and 15.

2.3 TISSUE FIXATION

After euthanizing the animals, perfusion fixation of the olfactory mucosa was done intracardially through the left ventricle at a pressure head of 150 mmHg (Hayat,

2000). Animals intended for light microscopic studies (n= 5 animals per group) were perfused with saline followed by 10% formaldehyde whereas those for electron microscopy (n= 5 animals per age group) were perfused with 0.1M phosphate buffer followed by 2.5% phosphate buffered glutaraldehyde (pH 7.4).

2.4 TISSUE HARVESTING AND SAMPLING

A mid-sagital cut was made through the skulls of the heads, using a bone saw, to open up the nasal cavities. Using a sharp blade, the nasal septum was dissected out to expose the ethmoid turbinates or concha, on which the olfactory mucosa is located. The turbinates were then detached from the ethmoid bone and sampled as illustrated in Figure 2. After separating the turbinates from each other, they were transected perpendicularly to their long axes to obtain segments representing their posterior, middle and anterior portions. By systematic random sampling, tissues for microscopy were selected from sub-segments obtained from each of the three levels.

2.5 DECALCIFICATION OF BONE

Conchal pieces for microscopy were decalcified to remove/ dissolve the thin bone onto which the olfactory mucosa lies. After washing the tissue with distilled water, decalcification was done using 5% ethylenediaminetetraacetic acid (EDTA) in phosphate buffer (pH 7.4) for a period of two to three weeks and at a temperature of 4°C with weekly renewal of the EDTA (Alers *et al.* 1999; Kavoi *et al.*, 2010).

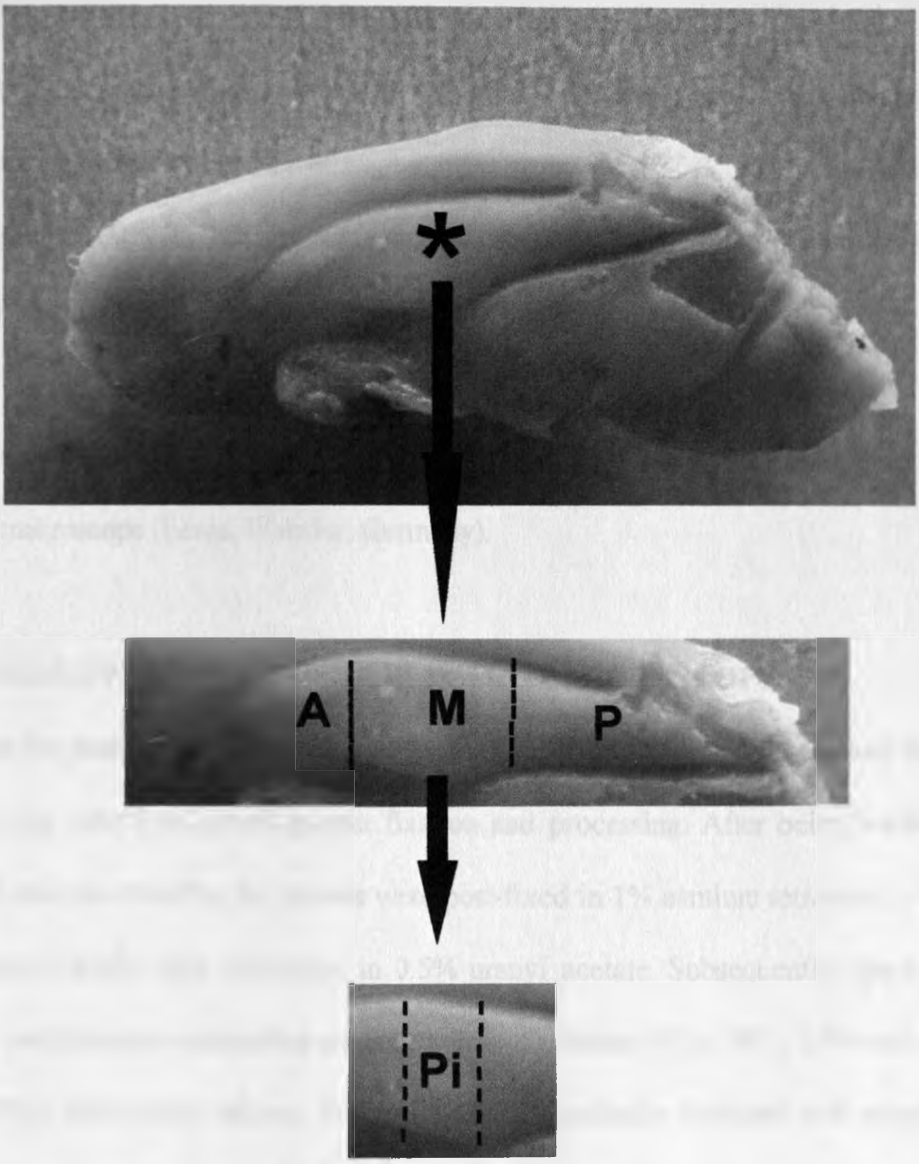

Figure 2. Macrographs of the ethmoturbinates of the rabbit outlining the steps followed in tissue sampling. Tissue blocks of the turbinate were cut from posterior (A), middle (M) and anterior portions (P) using a sharp blade. Each of these was further transected into smaller pieces (Pi). By systematic random sampling, tissue pieces obtained from each of these levels were selected for microscopic analysis.

2.6 TISSUE PROCESSING FOR LIGHT MICROSCOPY

Decalcified tissues were thoroughly rinsed in distilled water and dehydrated in increasing concentrations of ethanol starting from 70%, 80%, 95% and finally two changes in 100%. The tissues were cleared using methyl benzoate and infiltrated and embedded in paraffin. The embedded tissues were sectioned on the transverse plane at 5 µm using a rotary microtome (Leitz Wetzlar, Germany), stained with hematoxylin and eosin (H&E) or Masson's trichrome and observed on a Leica DMR light microscope (Leica, Wetzlar, Germany).

2.7 TISSUE PROCESSING FOR ELECTRON MICROSCOPY

Tissue for transmission electron microscopy (TEM) were trimmed into small blocks (of about $1mm^3$) to permit proper fixation and processing. After being washed in 0.1M phosphate buffer, the tissues were post-fixed in 1% osmium tetroxide in 0.1M phosphate buffer and contrasted in 0.5% uranyl acetate. Subsequently, the tissues were dehydrated in increasing concentrations of ethanol (70%, 80%, 95% and twice in 100%) after which ethanol in the tissues was gradually replaced with propylene oxide before infiltrating and embedding the tissues in epoxy resin. From the resin blocks, semi-thin and ultra-thin sections were cut with glass knives using a Sorvall® ultramicrotome. The semi-thin sections were picked on glass slides and stained with 0.5% toluidine blue for light microscopy. The ultra-thin sections were collected on 200-mesh copper grids, stained with lead citrate and observed with a Hitachi H 7100 or Philips 201 C TEM under an accelerating voltage of 60 kV.

After dehydration, some sections were selected for scanning electron microscopy (SEM), critical-point dried in liquid carbon dioxide and mounted on aluminum stub using double stick carbon tape. The samples were then coated with a thin film of gold-palladium complex before being examined on a Leo 1530 or Jeol 330 SEM at 17 kV.

2.8 IMMUNOHISTOCHEMICAL DETECTION OF Ki-67 IN THE OLFACTORY MUCOSA

Ki-67 immunohistochemistry was done on tissue sections cut at 3-4 μm from representative paraffin blocks of the olfactory mucosa. The nuclear antigen Ki-67 (also designated MK167), which is associated with and is necessary for cellular proliferation is expressed during the active phases of the cell cycle (G1, S, G2, and mitosis) but not in resting cells (Go) (Urruticoechea *et al.*, 2005). The Ki-67 immunostaining is therefore used to estimate the level of cell proliferation in a given tissue (Scholzen and Gerdes, 2000; Urruticoechea *et al.*, 2005). Following deparaffinization and rehydration, the olfactory tissues were boiled for 15 min at 600 watts in 10mM citrate buffer (pH 6.0) in a microwave followed by cooling at room temperature. After washing in Tris-buffered saline (TBS) (50 Mm Tris base, 150 Mm NaCl and 0.002% Triton X-100, pH 7.6) the tissues were quenched for endogenous peroxidases by incubating the slides in 3% H_2O_2 in methanol for 30 min. The sections were then incubated for 60 min at room temperature with primary antibody mouse monoclonal anti-human Ki-67 antigen clone MIB-1 (Immunotech S.A., Marseille, France) (1:25 and 1:50, dilutions in TBS) (Note: this step was omitted for the negative controls). Thereafter, the sections were washed in 0.17M

NaCl and incubated with biotinylated anti-mouse IgG (1:500, in antibody diluent, DAKO) for 45 min at room temperature after which they were washed in the NaCl and further incubated with avidin-biotin complex solution (Vectastain) for 45 min at room temperature. After a second wash in the NaCl, immunostaining was visualized by the use of diamino-benzidine (DAB) (mix 7.5µl H_2O_2 to 1ml of 1% DAB) stained for 10 min followed by counterstaining with Meyer's hematoxylin. Assessment was carried out on the entire epithelium following distinct nuclear staining of the dividing cells.

2.9 TERMINAL DEOXYRIBONUCLEOTIDYL TRANSFERASE MEDIATED dUTP-DIGOXIGENIN NICK-END LABELLING (TUNEL) ASSAY

TUNEL assay was carried out on 5-6 µm sections following the standard procedure (Mainwaring *et al.*, 1998). This assay detects DNA degradation in apoptotic cells because one of the hallmarks of late stage apoptosis is the fragmentation of nuclear chromatin, which results in a multitude of 3'-hydroxyl termini of DNA ends (Gavrieli *et al.*, 1992). The assay therefore relies on the presence of nicks (the exposed 3'-hydroxyl ends) in the DNA, which can be identified by terminal deoxynucleotidyl transferase (TdT), an enzyme that will catalyze the addition of dUTPs that are secondarily labeled with a marker (Gavrieli *et al.*, 1992; Stefanis *et al.*, 1997). For this study, endogenous peroxidase activity in the tissue sections was inactivated for 10 min with 1% H_2O_2 in phosphate-buffered saline (PBS) (pH 7.4). Nuclei of tissue sections were stripped of proteins by incubation with 0.5% pepsin (pH 2.0) (Sigma Chemical Co, Poole, Dorset, UK) for 30 min at 37°C. The sections were washed five times in distilled water to remove all traces of pepsin. Each section

undergoing the TUNEL protocol was incubated for 5 min in Tris buffer (pH 7.6) and then for 1 hr at 37°C in 100 µl of reaction mixture consisting of 15 units TdT FPLC pure (Pharmacia, Windsor, Berkshire, UK), 0.5 nmol biotin-16-dUTP (Boehringer Mannheim, Mannheim, Germany), 5 mM cobalt chloride, 0.2 M sodium cacodylate, 25 mM Tris HCl (pH 6.6), and 0.25 mg/ml bovine serum albumin (BSA) dissolved in distilled water. After extensive washing in distilled water, the sections were incubated for 30 min at room temperature in 1:400 dilution of horseradish peroxidase conjugated to streptavidin (Dako UK Ltd.) in PBS supplemented with 1% BSA and 0.5% Tween 20. Color was developed for 10 min using 0.05% diaminobenzidine plus 0.07% imidazole plus 0.1% H_2O_2 and further intensified in 0.5% $CuSO_4$ with 0.9% NaCl for 1 min. The sections were counterstained in Mayers haematoxylin, dehydrated, cleared in xylene and mounted on slides using DPX mounting medium.

2.10 MORPHOMETRIC ANALYSIS

In anatomical studies, morphometry reveals details which would go undetected by qualitative observations (Maina, 1987). Quantitative data were analyzed at light and electron microscopy following a sampling protocol previously applied for the olfactory mucosa in the dog and sheep (Kavoi *et al.*, 2010). For each animal group, tissue samples were randomly selected from ten animals (five for light microscopy and five for electron microscopy). For each tissue block, 10-15 light micrographs and 8-10 electron micrographs were prepared. Quantitative parameters were analyzed on 30-35 test fields generated from randomly selected micrographs. At light microscopy, the following parameters were analyzed: (1) thicknesses of the

olfactory epithelia, (2) diameters of axonal bundles, (3) volume fraction of the axon bundles, Bowman's glands and blood vessels and (4) Ki-67 labeling index of the olfactory epithelial cells. At SEM, packing densities of olfactory cells and cilia numbers per olfactory cell knob were also estimated.

2.10.1 Estimation of thickness of olfactory epithelium

The thickness of the olfactory epithelium was measured from the basal lamina to the apical surface (apical end of the tangled mat) on images selected/sampled on a Leica DMR light microscope and projected on a computer monitor where multiple measurements were made using a digital ruler.

2.10.2 Estimation of diameters of axon bundles

Mean linear intercept lengths (Karlsson and Gokhale, 1997) were adopted in the estimation of cross-sectional diameters of the axon bundles. For this purpose, a set of test lines were overlaid with a uniform random position on a micrograph and the set of lines intercepting the bundle profiles generated linear intercepts within the profiles whose mean total length ($\bar{X}TL$) and number ($\bar{X}TN$) were used to calculate the mean diameter of the bundle profiles ($\bar{X}Di$) as follows:

$$\bar{X}Di = \bar{X}TL / \bar{X}TN$$

2.10.3 Estimation of volume fractions of lamina propria components

According to Weibel (1979), the volume fraction (also called volume density) of a tissue component can be estimated by point counting using an overlay of coherent test system of points. For this study, volume fraction estimation of the axon bundles,

Bowman's glands and blood vessels was carried out as described for components of the pectin oculi (Kiama *et al.*, 2001) and the marsupial lung (Makanya *et al.*, 2007). To this end, a transparent test grid bearing a square lattice of points was overlaid with random position on histological fields of the lamina propria projected on a computer screen and the total number of test points hitting the components of interest (bundles, glands or vessels) and those falling on the projected field were counted. The volume fraction of the component of interest Vv(c) was then worked out as the ratio of the total number of points falling on the component of interest (ΣNPc) to the total sum of points falling on the entire field of the propria (ΣNP) and expressed as a percentage as follows:

$$Vv\,(c) = (\Sigma NPc / \Sigma NP) \times 100$$

2.10.4 Estimation of packing densities of olfactory cells

At SEM level, estimation of packing densities of the olfactory cells was done by counting, on the epithelial surface, the number of projecting olfactory cell knobs per square area (Apfelbach *et al.*, 1991). To avoid bias while counting the olfactory cell knobs within the square area, the forbidden line rule and the counting frame of Gundersen (1977) were applied. To this end, the dendritic knobs that fell within or touched the two dotted inclusion lines of the counting frame were counted whereas those that that fell outside or touched the bolded exclusion lines were not counted.

2.10.5 Estimation of Ki-67 labelling index of olfactory epithelial cells

In the olfactory epithelium, the number of Ki-67 labeled cells was estimated using the direct manual counting technique previously applied in measuring the mitotic rates in the endometrium of primates (Brenner *et al.*, 2003). To this end, Ki-67 positive cells were counted on randomly chosen areas of a section on a light microscope at a magnification of x400 with non-overlapping fields being selected with the help of an ocular grid. In each area of the section, negative (unstained) cells were also counted. Four trained laboratory technicians counted the same sections and the counts made by each observer was summed and averaged. Where KiT denotes the total number of cells counted (positive and negative for Ki67) and Ki+ stands for the total number of Ki-67 positive cells counted on the same area, the Ki67 labeling index, also called proliferative index (PI) was calculated and expressed as a percentage using the formula

$$PI = (Ki+ / KiT) \times 100$$

2.10.6 Estimation of number of cilia per olfactory cell knob

Estimation of the number of cilia per olfactory cell knob was done following the method of Menco (1978, 1980), which considers that 25% of the cilia remain obscured behind visible structures during observation. Thus, where Nk is the total number of cilia per olfactory cell knob and Nc is the number of cilia observed and counted on each knob, then $Nc = \frac{3}{4} Nk$ and therefore

$$Nk = 4/3 \cdot Nc$$

2.11 ASSESSMENT OF OLFACTORY FUNCTION USING THE BURIED FOOD TEST

The buried food recovery experiment was performed to investigate the effects of vinblastine and docetaxel administration on olfactory function in the rabbit. This test was first described in the early 1970s (Alberts and Galef, 1971; Edward *et al.*, 1972) and since then, various versions of this test have been described under names such as "hidden cookie test", "food exploration test", "food localization test" (Dawson *et al.*, 2005; Quiroz-Padilla *et al.*, 2006) or "buried food pellet recovery test" (Nathan *et al.*, 2004). Cereals, chocolate chips, various kinds of cookies, and food pellets have been successfully used. In contrast to the sand-buried food test which measures an animal's ability to associate an odorant with a food reward (Wong *et al.*, 2000; Trinh and Storm, 2003), the buried food test measures the animal's natural tendency to use olfactory cues for foraging and therefore its general ability to smell. In the buried food test, the main parameter is the latency to uncover a small piece of palatable food hidden beneath a layer of cage bedding (Yang and Crawley, 2009). This test is more sensitive when the animals are given an overnight fast rather than moderate food deprivation (Dawson *et al.*, 2005) and works under the assumption that the food-restricted animals which fail to use odor cues to locate the food within a given period ($\geq$ 5 minutes for the rabbits) are likely to have deficits in olfactory abilities.

For the current study, the test was performed stepwise as follows:

Step 1. Odor familiarization: A piece of dry cookie scented with a drop of lavender oil was put in the subject's cage for 2-3 consecutive days before the test. It was confirmed the following morning if the cookie had been consumed.

Step 2. Food deprivation: 18-24 hrs before the test, all food was removed from the home cage including any scattered pellet fragments within the cage. The subject had free access to water.

Step 3. Scoring the latency to find the cookie:

(i) The subject rabbit was placed in a standard cage measuring 52 cm long x 42 cm wide x 41 cm high (Chirino *et al.*, 2007) and containing clean bedding. The subject was allowed to acclimate to the cage for 5-10 min.

(ii) In each trial, the subject was transferred to an open wooden box of similar dimension as the standard cage containing clean bedding underneath which was buried a 5 g piece of cookie (approximately 6 cm beneath the surface) scented with 1 drop of lavender oil (see set up in Figure 3).

(iii) After placing the subject in the box, the experimenter (who was blinded to the test information in all trials) retreated to the observation station (located about 2 meters away from the cage) where a video camera was fixed. The video was started, and then stopped when the subject rabbit uncovered the cookie. The latency time was recorded and defined as the time between placement of the rabbit in the box and grasping the cookie with its teeth. If the subject failed to find the cookie after 300 sec had elapsed, the test was stopped and the subject was considered to have a deficit in olfactory ability.

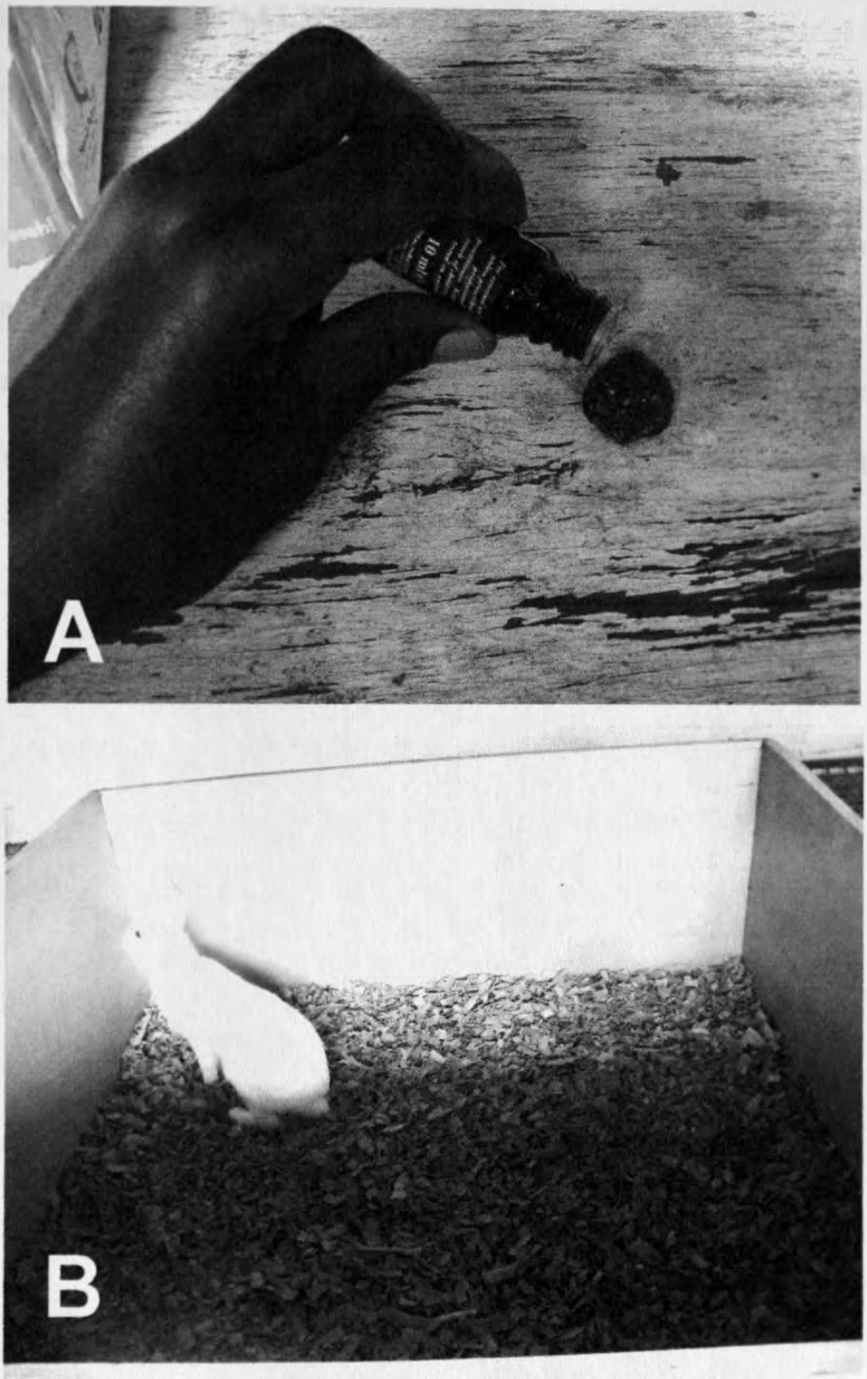

Figure 3. A photograph of the set up used in the buried food test. A: a piece of cookie is scented with one drop of lavender oil B: the cookie is buried about 6cm beneath the surface of clean wooden shavings contained in an open wooden box after which the subject is introduced into the box and allowed time to locate and uncover the cookie.

2.1.2 STATISTICAL ANALYSIS

Morphometric differences in various parameters of the olfactory mucosa were analyzed using the Student's *t*-test. Such parameters included thicknesses of the olfactory epithelia, diameters of axonal bundles, packing densities of olfactory cells, cilia numbers per olfactory cell knob and volume fraction of axonal bundles, Bowman's glands and blood vessels. In all cases, statistical significance was set at $p < 0.05$ and mean values were presented together with their standard deviations (SD).

CHAPTER THREE

RESULTS

3.1 GENERAL OBSERVATIONS

3.1.1 Rabbits during postnatal development

At birth, the rabbits were furless and unable to use their eyes since the eyelids remained closed (Fig. 4) until after postnatal day 10-12. The olfactory mucosa was characteristically yellowish-brown in color and covered the caudal roof of the nasal cavity (*fundus nasi*) where it spread over the ethmoturbinates, the posterior portions of the dorsal and middle nasal turbinates and the nasal septum (Fig. 5A). In this species, the endoturbinates, which are the rostral projections of the ethmoid bone, were four in number (Fig. 5B). The largest and the most dorsal was endoturbinate I, which provides the bony support for the dorsal nasal turbinate. This was followed by endoturbinate II (EII), which forms the bony basis for the middle nasal turbinate (Fig. 5B). The remaining were endoturbinates III and IV, which form the bony core for the scroll-like ethmoturbinates (Fig. 5B). Additionally in this species, the ventral nasal turbinate was characterized by prominent folds of lamellae (Fig. 5B).

Table 2 shows the values for the greatest lengths of the ethmoturbinates and body weights of the rabbits at the various stages of postnatal development. The length of the ethmoturbinates increased progressively with age being 3.4 times longer in the adult as compared to the neonate (Table 2). The increment in length was greater between suckling and weanling ages (1.8-fold; 0.8 to1.4 cm) as compared to the stage between birth and suckling (1.6- fold; 0.5 to 0.8cm) and between weaning and adulthood (1.2- fold; 1.4 to 1.7 cm) (Table 2). Body weight gain in the rabbits was

56.3-fold between birth and adulthood. Between birth and weaning, the weight increased 7.6 times (from 48.7 to 369.9 g). The weight gain was greatest between weaning and adulthood (7.4- fold; 369.9 to 2742.4 g) as compared to period between birth and suckling (4.8-fold; 48.7 to 233.6 g) and between suckling and weaning (1.6-fold; 233.6 to 369.9 g) (Table 2).

Figure 4. A photograph showing the external body features of the rabbit at birth. Notice the absence of fur on the skin and also the closed eyelids in the newborn of this species.

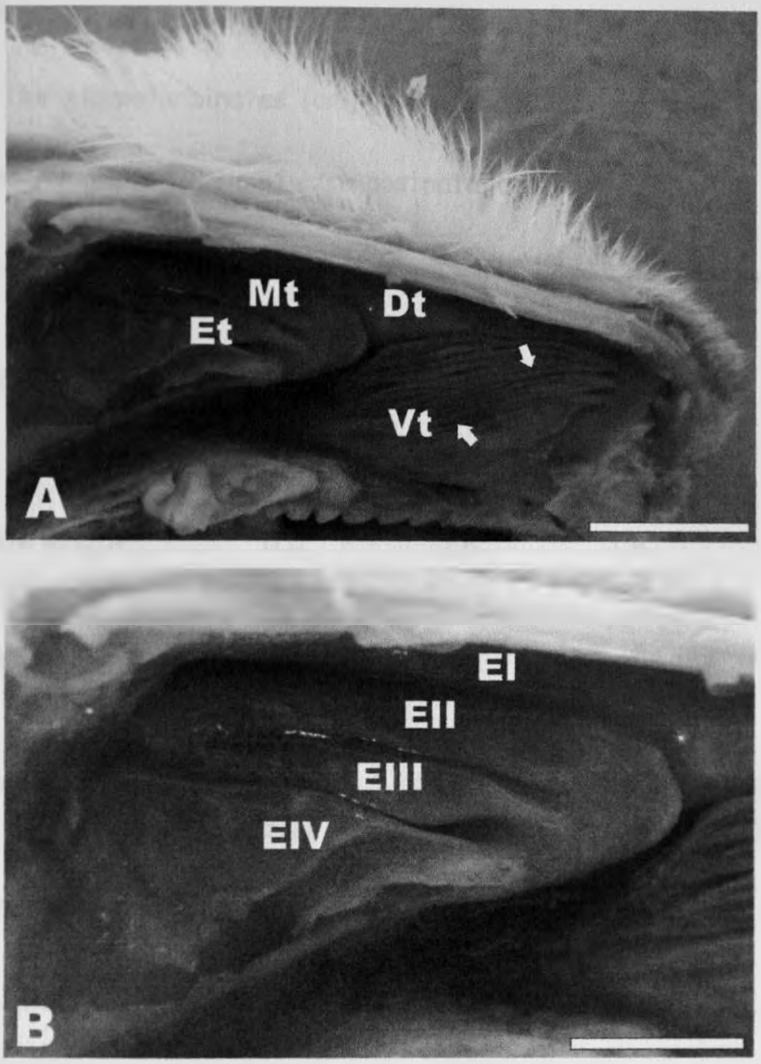

Figure 5. A macrograph of the left side of the split head of a rabbit showing the location of the olfactory mucosa and the positioning of the endoturbinates in the nasal cavity. A: at the posterior roof of the nasal cavity, the olfactory mucosa lies on the ethmoid turbinates (Et) and the caudal parts of the dorsal (Dt) and middle nasal turbinates (Mt). Notice the presence of numerous lamellae folds (arrows) on the surface of the ventral nasal turbinate (Vt). B: the endoturbinates, which are projections of the ethmoid bone, are four: endoturbinate I (EI) which is the largest and most dorsal and forms the bony basis for the dorsal nasal turbinate, endoturbinate II (EII) which supports the middle nasal turbinate and endoturbinates III (EIII) and IV (EIV), which constitute the scroll-like ethmoturbinates. Bar = 2 cm in A & 0.5 cm in B.

Table 2. Mean values (± SD given in parentheses) for the body weight (g) and length of the ethmoturbinates (cm) in newborn, suckling, weanling and adult rabbits, and the growth ratio at each postnatal age.

Parameter	*Newborn*	*Suckling*	*Weanling*	*Adult*
Turbinate length	0.5 (0.2)	0.8 (0.2)	1.4 (0.3)	1.7 (0.3)
Growth ratio		1.6	2.8	3.4
Body weight	48.7 (8.5)	233.6 (39.7)	369.9 (43.4)	2742.4 (200.3)
Growth ratio		4.8	7.6	56.3

Growth ratio= mean weight of a given age group/ mean weight at birth (Meisami *et al.*, 1990).

3.1.2 Rabbits treated with antimicrotubule drugs vinblastine and docetaxel

3.1.2.1 Effects of the drugs on body weight

Figure 6 shows the mean body weights of controls and of rabbits treated with vinblastine or docetaxel and weighed on days 3, 5, 10 and 15. Relative to body weight value in controls (2950 ± 44g), there was a progressive decrease in body weight in the first 5 days after vinblastine-exposure (i.e. 2830 ± 38 and 2695 ± 42 g at days 3 and 5 respectively). Thereafter, the body weights for the rabbits increased to reach control values by day 10 (2860 ± 35g) or 15 (3080 ± 47g) (Fig. 6). In the docetaxel-treated animals, the body weights were significantly lower at day 10 (2706 ± 46g) as compared to controls. No significant differences in body weights were noted between controls and docetaxel-treated animals at day 3 (2958 ± 48g), day 5 (2905 ± 39g) or day 15 (3056 ± 51g) (Fig. 6).

3.1.2.2 Effects of the drugs on food intake

Data on food consumption (rabbit pellets in g/ day) in controls and in animals treated with vinblastine or docetaxel are shown in Figure 7. In the controls, food intake was 17.1 ± 2.6 g per day. Following treatment with vinblastine, the food consumption decreased progressively in the first 5 days (9.3 ± 1.9 g on day 3 and 6.4 ± 1.2 g on day 5) (Fig. 7). Thereafter, the intake increased progressively attaining control value by day 10 (14.5 ± 2.1 g) or day 15 (20.6 ± 2.4 g). In the docetaxel-treated animals, suppression of food intake was higher on day 5 (11.2 ± 1.8 g) and day 10 (7.6 ± 1.4 g). No significant differences in food intake was noted between controls and docetaxel-exposed animals at day 3 (15.7 ± 2.3 g) or day 15 (18.9 ± 2.8 g) (Fig. 7).

3.1.2.3 Effects of the drugs on skin condition

Administration with vinblastine or docetaxel resulted in a number of body surface lesions on the rabbits as demonstrated in Figure 8. These lesions were clearly noticeable 3-5 and 5-10 days following administration of vinblstine and docetaxel respectively. The lesions included alopecia, which was more pronounced on the loose skin of the dorsal neck region, necrotic lesions involving the thin skin of the eyelids and of the inner side of the pinna, and diarrhea, which lasted one to two days, leaving the animal with a soiled tail and hind limbs (Fig. 8).

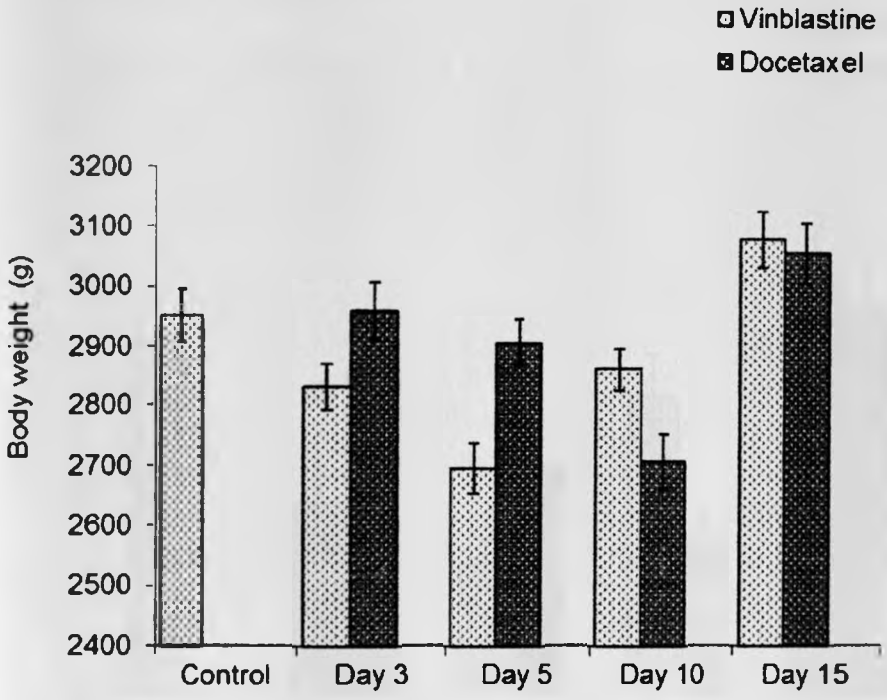

Figure 6. Bar graphs showing mean body weights (g) of controls and of rabbits injected with vinblastine or docetaxel and weighed after days 3, 5, 10 and 15. With vinblastine treatment, the body weights decrease progressively in the first 5 days. Thereafter, the weights increase progressively reaching control values on day 10 or 15. Compared to controls, body weights in docetaxel-treated animals are remarkably lower at day 10 but not significantly different at day 3, 5 or 15, $p < 0.05$.

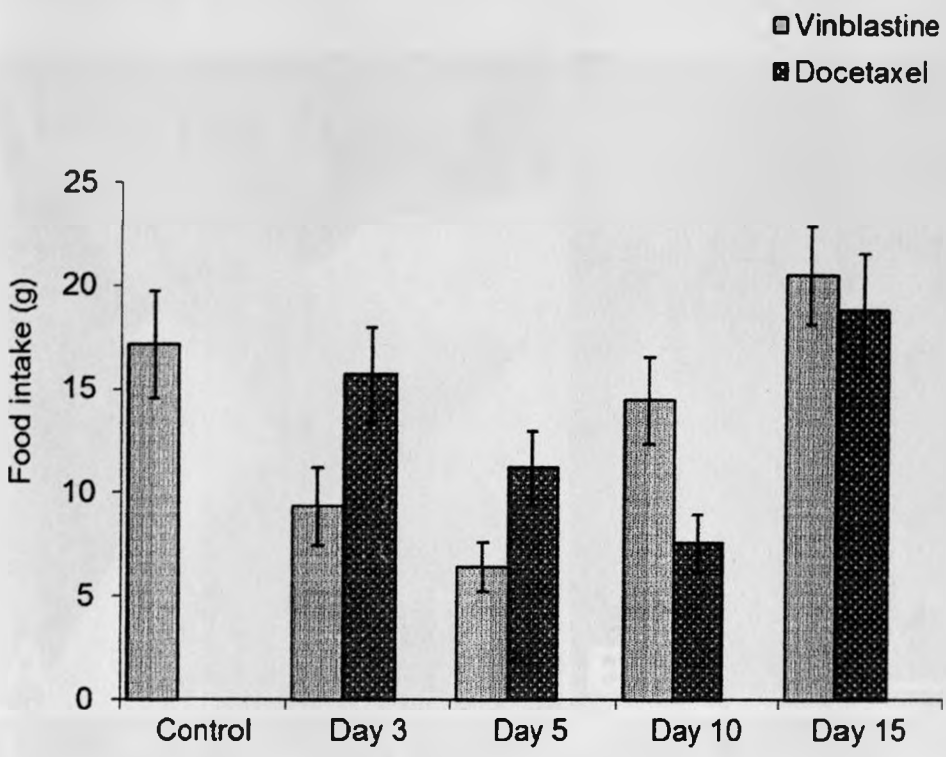

Figure 7. Graphical representation of mean food intake (g/ day) in controls and in vinblastine or docetaxel-treated animals at days 3, 5, 10 and 15. In animals treated with vinblastine, food intake reduces progressively in the first 5 days, after which it increases to control levels on day 10 or 15. In the docetaxel-exposed rabbits, suppression of food intake is only significant on days 5 and 10, $p < 0.05$.

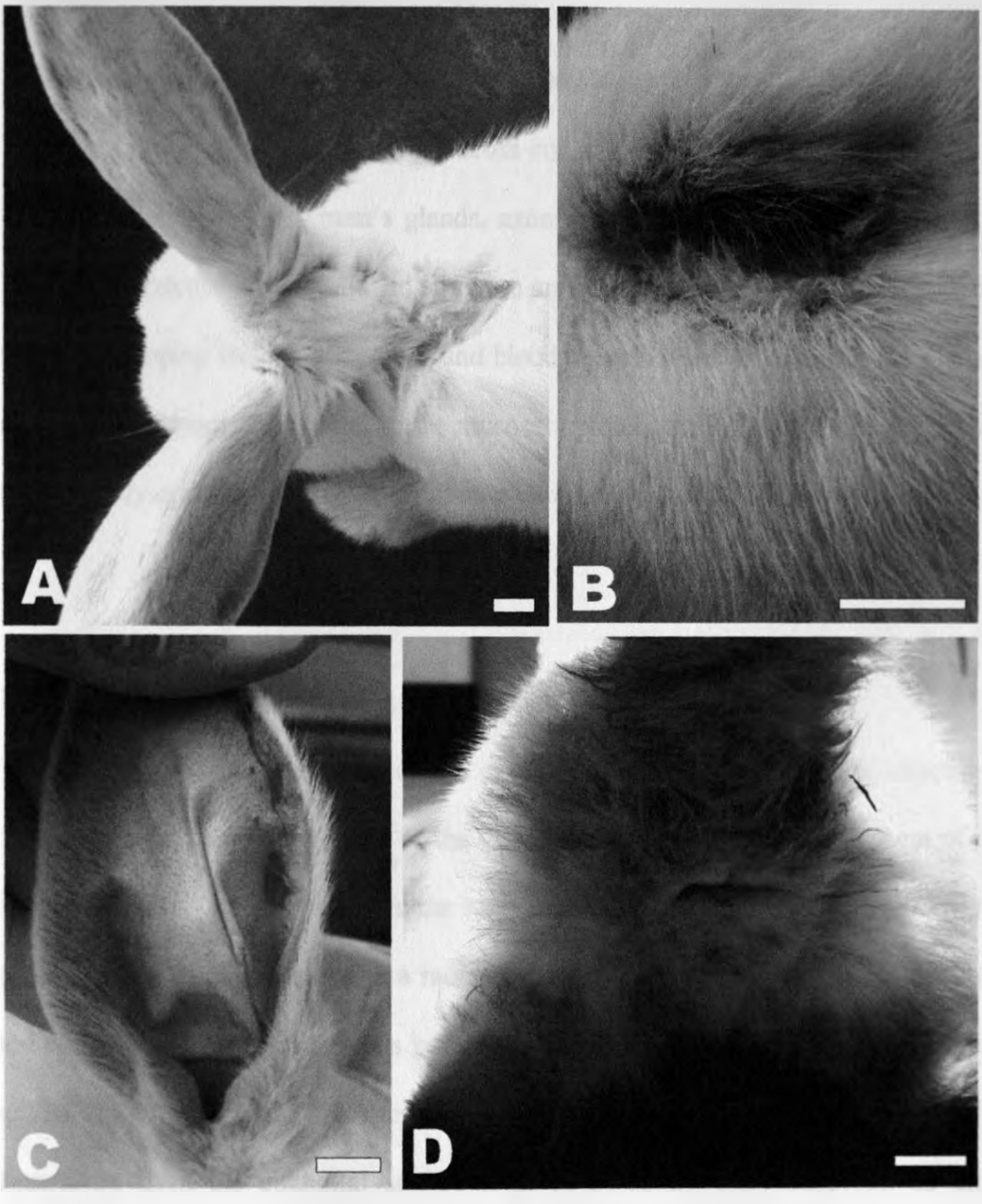

Figure 8. Photographs showing the lesions imparted by vinblastine or docetaxel on the body of adult rabbit. These drugs induce alopecia, which is more conspicuous on the dorsal neck (A), necrotic skin lesions involving the eyelids (B) and the inner side of the pinna (C), and diarrhea, which results in soiled tail and hind limbs (D). Bar = 1 cm in A-D.

3.2 POSTNATAL STRUCTURE OF RABBIT OLFACTORY MUCOSA

3.2.1 MORPHOLOGIC OBSERVATIONS

3.2.1.1 Mucosal components and their distribution

In all the age groups, the olfactory mucosa consisted of an epithelium sitting on a basement membrane, Bowman's glands, axon bundles and blood vessels (Fig. 9). These components were similar in structure and distribution except in the neonates where developing Bowman's glands and blood vessels were observed in the region of the lamina propria subjacent to the mucosal epithelium (Fig. 9 A). The height of the olfactory epithelium was observed to progressively increase with age (Fig. 9 A-D).

3.2.1.2 Surface features of olfactory epithelium

On the surface of the olfactory epithelia of newborn and suckling animals, olfactory cell cilia projected from the tips of the dendric knobs to run parallel in form of a bundle (Fig. 10 A & B) whereas in the weanlings and adults, the cilia emerged from around the bases of the knobs in a radial pattern (Fig. 10 C & D). In the weanling and adult rabbits, supporting cells projected their apical parts on the surface of the epithelium whereas in the neonates and sucklings, the apices of these cells were not discernible from the epithelial surface (Fig. 10 A & B). Also observed on the epithelial surface were tunnel-like openings, which were more predominant in the adult, weanling and suckling animals (Fig. 10 B-D).

3.2.1.3 Proliferative activity of olfactory epithelial cells

As defined by Ki-67 immunostaining, proliferative activity of cells of olfactory epithelium appeared to decrease progressively with age. Thus, the Ki-67 positive cells were more prevalent in the neonates followed by sucklings and weanlings and least in the adults (Fig. 11). In all cases, the Ki-67 reactivity was confined to a population of cells localized in the basal region of the olfactory epithelium (Fig. 11).

3.2.1.4 Morphology of axon bundles

In all age groups, axon bundles were surrounded by a fibroblastic sheath whereas individual fascicles within the bundles were encircled by sheet-like processes of olfactory ensheathing cells, whose nuclei appeared superimposed on sections of the bundles (Fig. 12). In the neonate, suckling and weanling animals, cores of the axon bundles contained blood capillaries, which were lacking from the bundle cores of the adult animals (Fig. 12). On cross-sections of the axon bundles, relative sizes of the constituent fascicles were observed to increase with age (Fig. 13). These fascicles were separated by distinct gaps, which became smaller and smaller as the animals matured (Fig. 13 A-D). Ultrastructurally, cross-sectional profiles of the bundle fascicles in all the age groups demonstrated the presence of the unmyelinated axons of the olfactory nerves, which contained numerous neurofibrils (Fig. 14 A-D). In the neonates and to a small extend the sucklings, extensive processes of olfactory ensheathing cells were seen to entrap and compact newly forming axons (Fig. 14 A& B). In the weanlings and adults, the axons were relatively more closely packed (well compacted) and were without such entrapments (Fig. 14 C&D).

3.2.1.5 Morphology of Bowman's glands

Age-related variations were noted with regard to the structural forms (types) of the Bowman's glands whereby in the neonates, sucklings and weanlings, the glands were of the acinar type (Fig. 15 A-C) whereas in the adults, the glands were predominantly tubular (Fig. 15 D).

3.2.2 MORPHOMETRY

Morphometric values related to the thickness of the olfactory epithelium, cross-sectional diameters of axon bundles, packing densities of olfactory cells, cilia counts per olfactory cell knob and Ki-67 labeling indices of olfactory epithelial cells are provided in Table 3. Relative to the value at birth (55.5 ± 2.7 μm), the height of the olfactory epithelium increased by 1.3, 1.5, and 1.7- fold in the sucklings, weanlings and adults respectively (Table 3). The diameter of the axon bundles, which was 27.4 ± 4.3 μm at birth, increased 2.0, 2.3 and 5.5 times at suckling, weaning and adult ages respectively (Table 3). The packing density (mm^{-2} x 10^3) for the olfactory cells was 42.3 ± 3.5 at birth, a value which increased by 1.3, 1.8 and 2.1-fold at suckling, weanling and adult ages respectively (Table 3). At birth, the number of cilia per olfactory knob was estimated at 9 ± 4 and this value increased 1.4, 1.9 and 2.6 times at suckling, weanling and adult stages respectively (Table 3). The Ki-67 labeling index (%) was estimated in the newborn as 31.2 ± 6.7, a value which decreased to 28.8 ± 6.0 in the suckling animals, 18.6 ± 5.5 in the weanlings and 11.3 ± 4.6 in the adults (Table 3).

Volume fractions (%) of lamina propria components, which included Bowman's glands, axon bundles and blood vessels were estimated as presented in Figure 16. In all age groups, volume fraction values for the glands remained higher compared to those for the bundles and vessels. The volume fraction for the glands, which was 32.1 ± 3.4% in neonates, 31.3 ± 2.9% in sucklings, 40.4 ± 3.2% in weanlings and 44.3 ± 3.7% in adults, was significantly different only between the suckling and weanling ages (Fig. 16). Volume fraction for the axonal bundles increased with progressing age, with differences in these values being significantly different between newborn and suckling ages (9.3 ± 2.1% to 14.6 ± 2.5%) and between weaning and adulthood (16.7 ± 2.8% to 24.1 ± 3.3%) (Fig.16). Volume fraction for the blood vessels did not show any significant differences between the various ages (13.0 ± 1.5% in neonates, 11.5 ± 1.4% in sucklings, 13.7 ± 1.6% in weanlings and 12.5 ± 1.5% in adults) (Fig. 16).

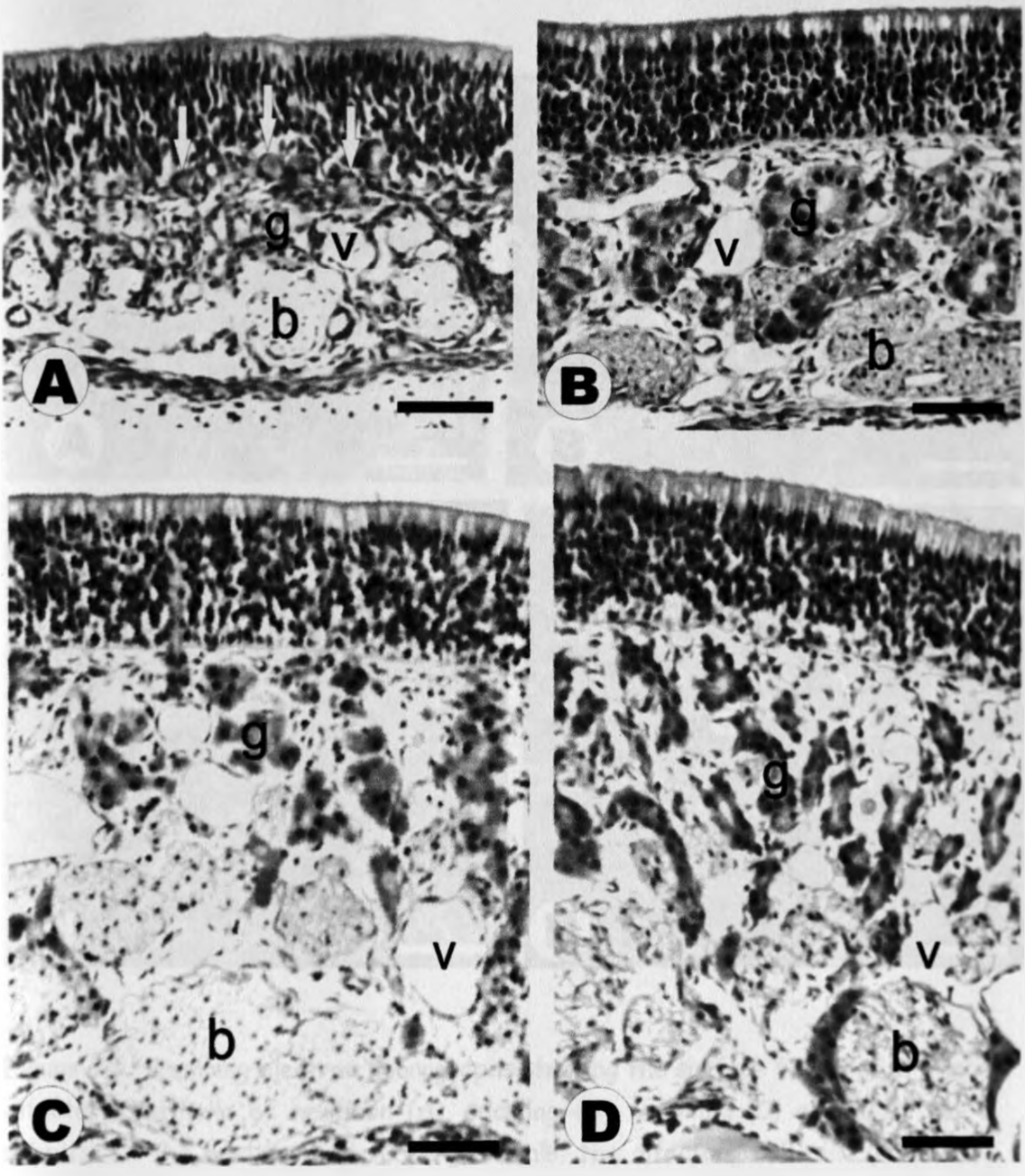

Figure 9. Histological sections of olfactory mucosa of newborn (A), suckling (B), weanling (C) and adult (D) rabbits. All mucosal components including Bowman's glands (g), axon bundles (b) and blood vessels (v) are present in all the age groups. In the neonate, developing Bowman's glands and blood vessels (arrows) are observed in the region of the lamina propria subjacent to the mucosal epithelium. Notice also the age-related increase in the height of the epithelium. H&E stain, Bar = 50 μm in A-D.

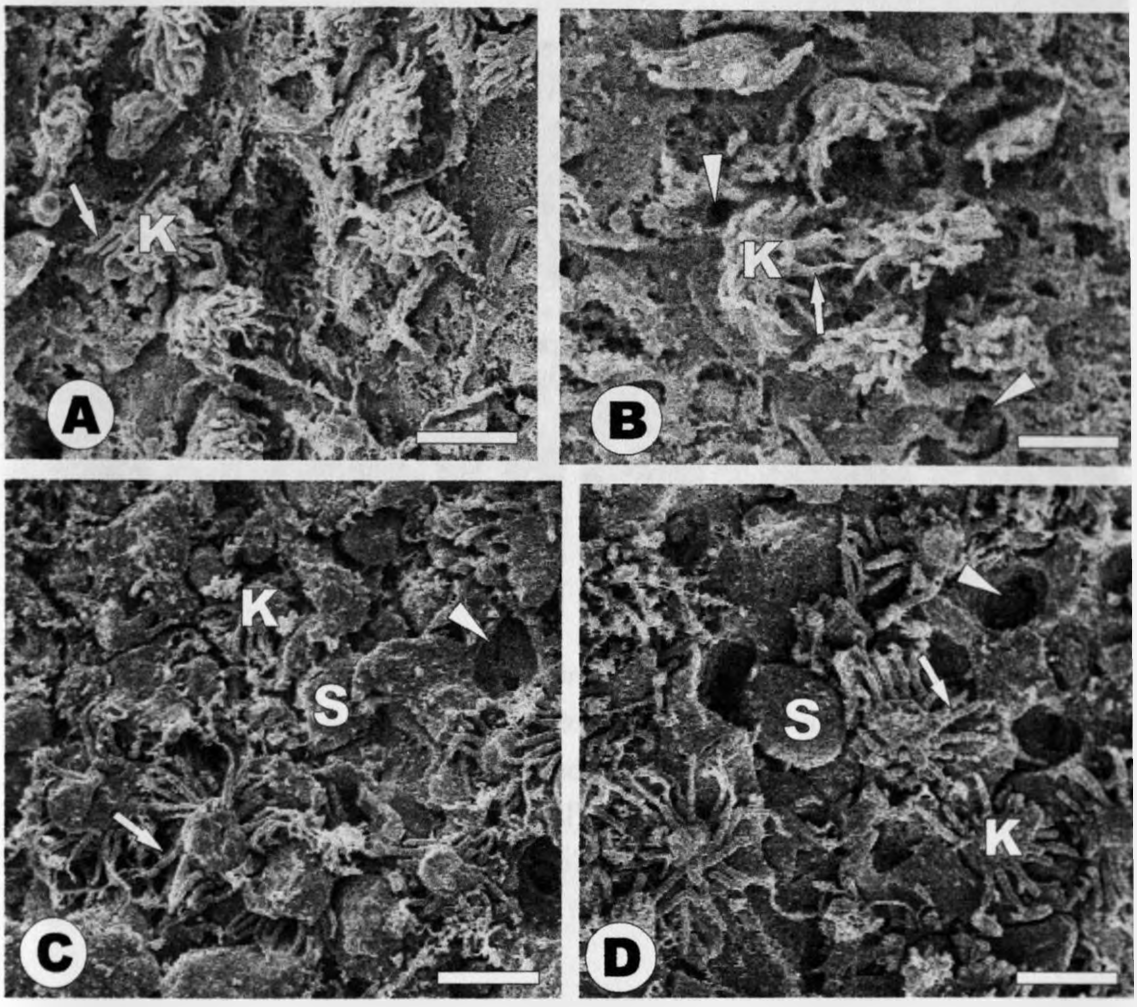

Figure 10. Scanning electron micrographs showing the surface features of the olfactory epithelia of newborn (A), suckling (B), weanling (C) and adult (D) rabbits. In the newborn and suckling animals, the olfactory cell cilia (arrows) project as a bundle from the tips of the dendritic knobs (k) in contrast to the weanlings and adults where the cilia radiate from around the bases of the knobs. Projecting apices of supporting cells (s) are present from the epithelial surfaces of adults and weanlings but are absent in neonates and sucklings. Notice also the predominance of tunnel-like openings (arrow heads) on the epithelial surfaces of adults, weanlings and sucklings. Bar = 2 μm in A-D.

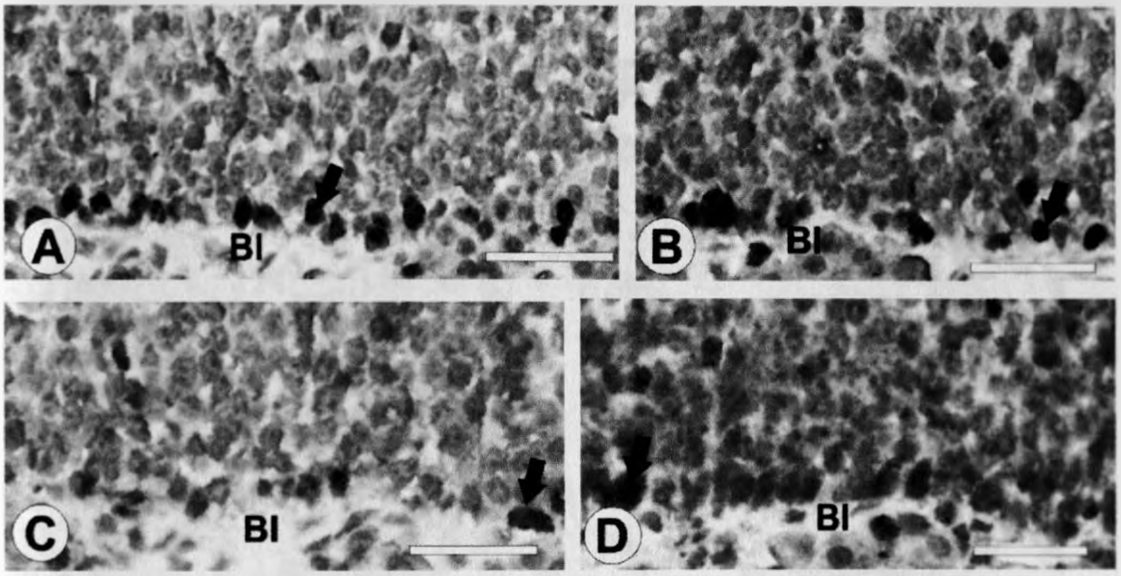

Figure 11. Light micrographs demonstrating Ki-67 immunostaining of the olfactory epithelia of newborn (A), suckling (B), weanling (C) and adult (D) rabbits. The Ki-67 reactivity is restricted to the basal part of the epithelium just above the basement membrane (Bl) and the proliferative activity, as evidenced by the prevalence of the Ki-67 positive cells (arrows), appears to decrease with advancing age. Bar = 30 μm in A-D.

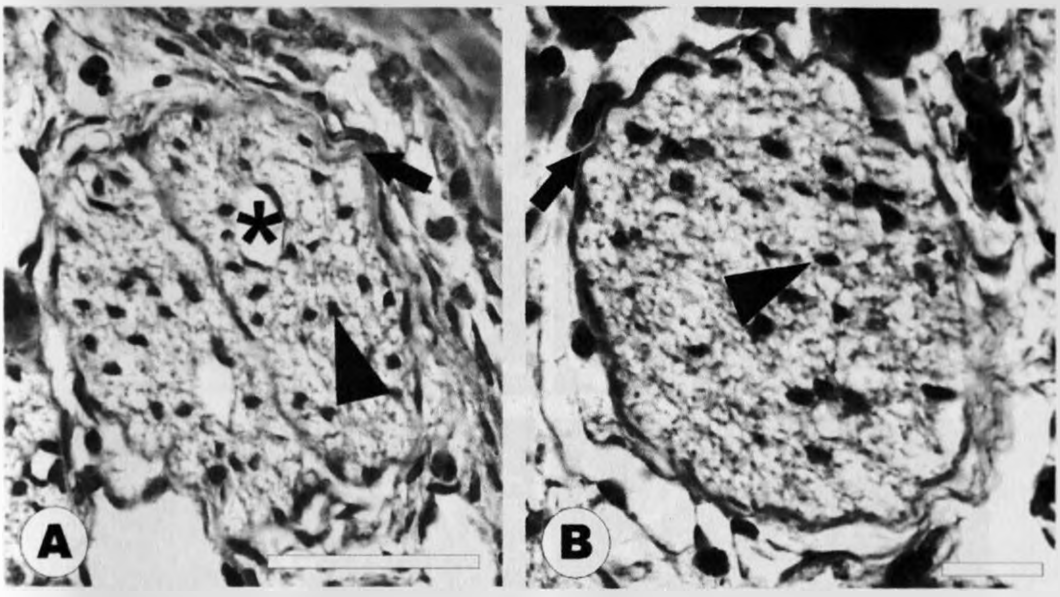

Figure 12. Histomicrographs of axon bundles taken from suckling (A) and adult (B) rabbits to illustrate features of their cross-sectional profiles. In these age groups and also in the neonates and sucklings, the bundles are encircled by a fibroblastic sheath (arrows) and their cores are infiltrated with blood capillaries (asterisks) except in the adults where such vessels are lacking. Within the bundles, individual fascicles are enveloped by processes of olfactory ensheathing cells, whose nuclei (arrow heads) appear superimposed on sections of the bundles. Masson's trichrome stain, Bar = 25 μm in A & B.

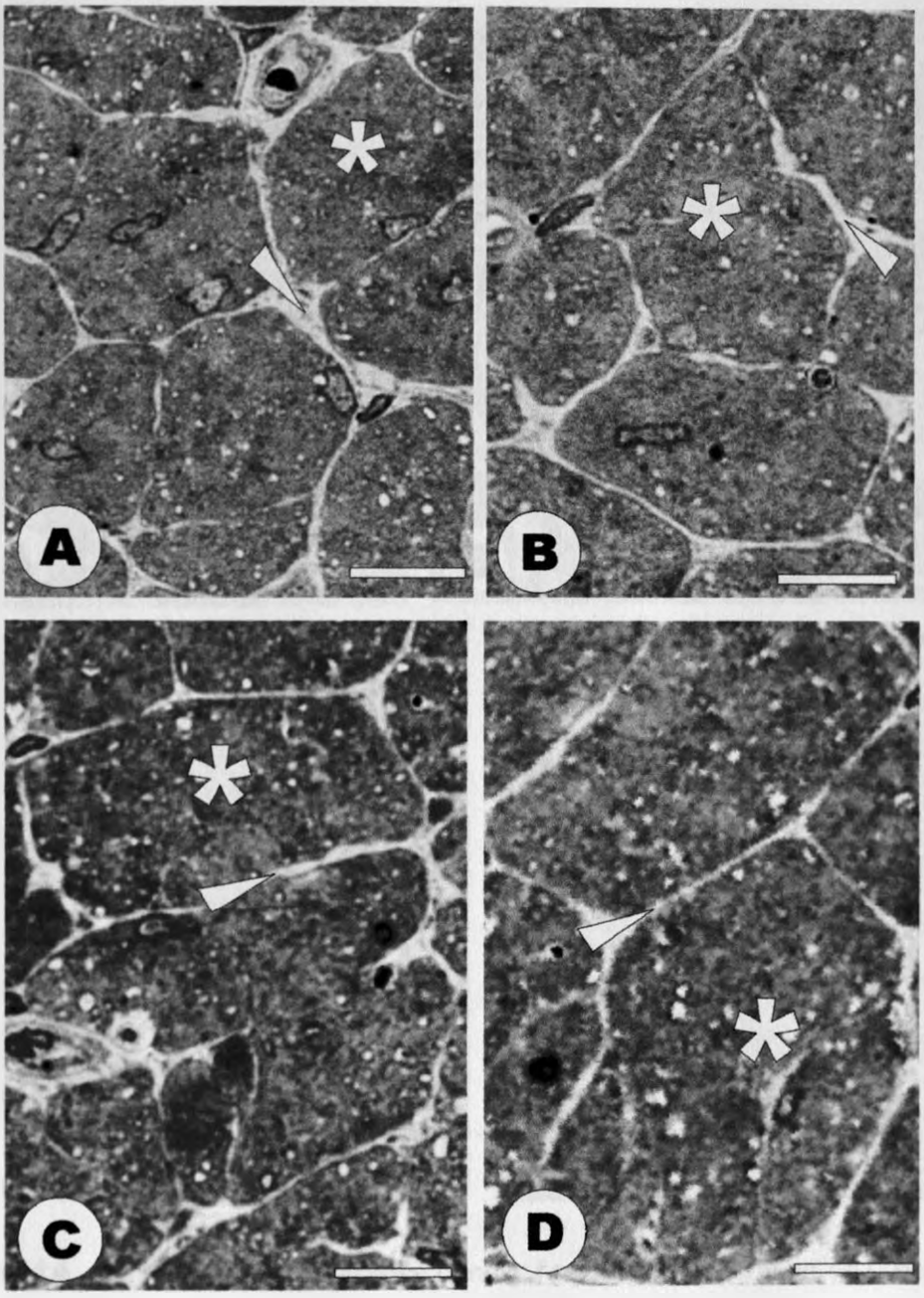

Figure 13. Semi-thin sections across axon bundles demonstrating the structural features of their fascicles in newborn (A), suckling (B), weanling (C) and adult (D) rabbits. With progressing age, relative sizes of the fascicles (asterisks) increase while gaps between the fascicles (arrow heads) become narrower and narrower. Toluidine blue stain, Bar = 20 μm in A-D.

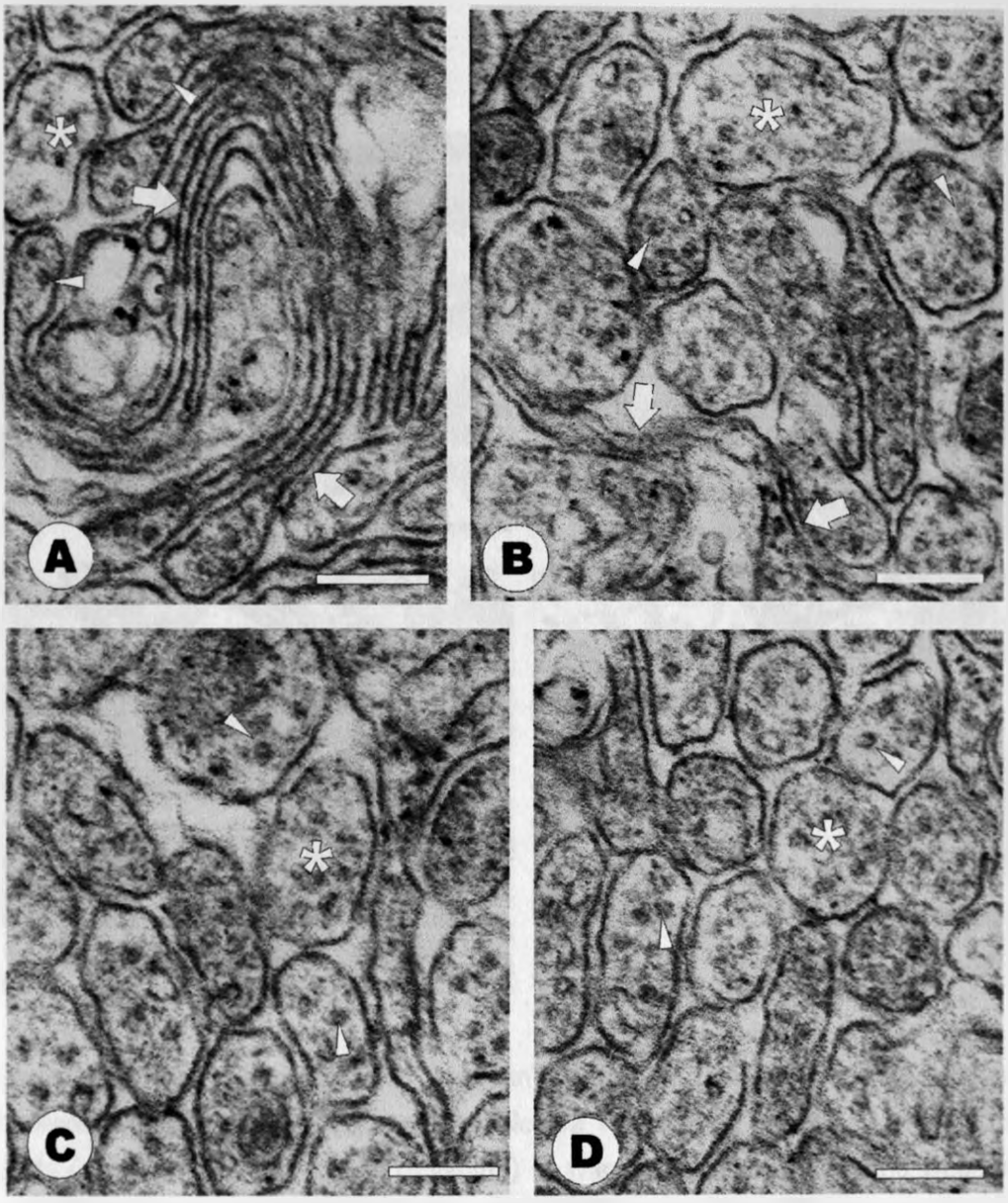

Figure 14. Ultrathin section across bundle fascicles of newborn (A), suckling (B), weanling (C) and adult (D) rabbits. Notice the unmyelinated axons (asterisks) of olfactory nerves, which are packed with numerous neurofibrils (arrow heads). In the newborn and suckling animals, extensive sheaths of Schwann cell processes (arrows) entrap and compact groups of newly forming axons. Bar = 0.1 μm in A-D.

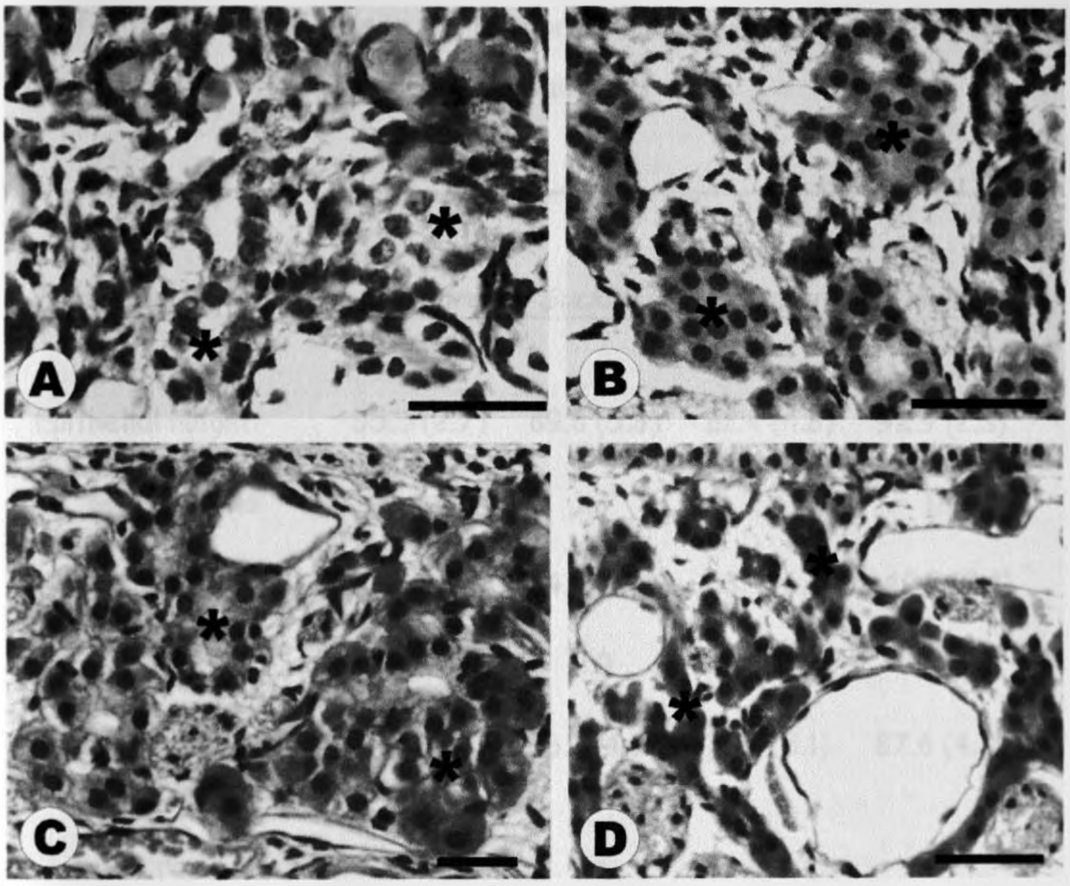

Figure 15. Light micrographs of Bowman's glands of the newborn (A), suckling (B), weanling (C) and adult (D) rabbits. Notice the change in type (form) of the glands (asterisks) from acinar (alveolar) in the neonate, suckling and weanling to being predominantly tubular in the adult. H&E stain, Bar = 50μm in A-D.

Table 3. Mean values (±SD given in parentheses) for olfactory epithelial height (μm), axon bundle diameters (μm), olfactory cell densities ($mm^{-2} \times 10^3$), cilia counts per olfactory cell knob and proliferative (Ki67 labelling) index (%) of olfactory epithelial cells in the newborn, suckling, weanling and adult rabbits, and the growth ratio of each of the parameter.

Parameter	*Newborn*	*Suckling*	*Weanling*	*Adult*
Epithelial height	55.5 (2.7)	69.8 (5.3)	82.4 (4.8)	92.9 (2.3)
Growth ratio		1.3	1.5	1.7
Bundle diameter	27.4 (4.3)	54.6 (8.3)	63.4 (6.1)	149.5 (9.4)
Growth ratio		2.0	2.3	5.5
Olfactory cell density	42.3 (3.5)	56.2 (4.3)	74.6 (5.1)	87.6 (4.8)
Growth ratio		1.3	1.8	2.1
Cilia number	9 (4)	13 (3)	17 (3)	23 (4)
Growth ratio		1.4	1.9	2.6
Ki67 labelling index	31.2 (6.7)	28.8 (6.0)	18.6 (5.5)	11.3 (4.6)

Growth ratio = Mean of a given age group/ Mean of newborn (Meisami et al., 1990)

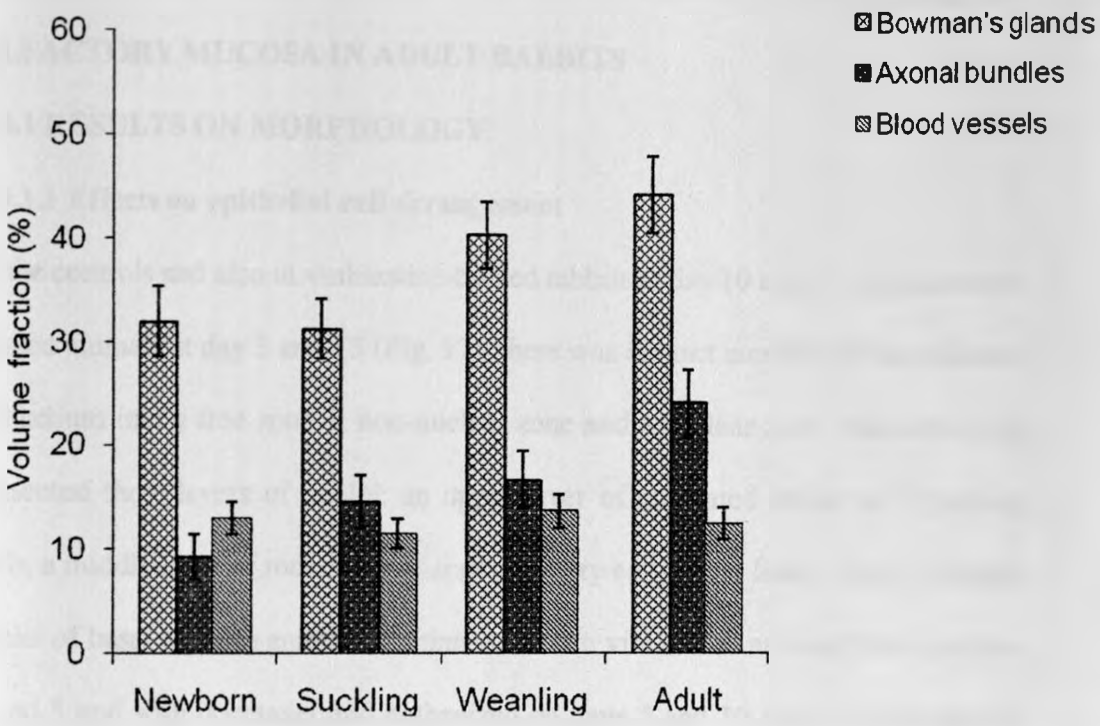

Figure 16. Bar graphs showing mean volume fraction (%) for Bowman's glands, axon bundles and blood vessels in newborn, suckling, weanling and adult rabbits. Volume fraction values for the glands are generally higher than for the other structures and the increase is age-related, being significantly different only between suckling and weanling ages. Volume fraction for the bundles increase with age, with significant differences occurring between birth and suckling age and between weanling and adult ages. No significant differences are noted with regard to the volume fraction for the blood vessels between the various ages, $p < 0.05$.

3.3 EFFECTS OF VINBLASTINE AND DOCETAXEL ON STRUCTURE OF OLFACTORY MUCOSA IN ADULT RABBITS

3.3.1 RESULTS ON MORPHOLOGY

3.3.1.1 Effects on epithelial cell arrangement

In the controls and also in vinblastine-treated rabbits at day 10 and 15 and docetaxel-treated animals at day 3 and 15 (Fig. 17), there was distinct zonation of the olfactory epithelium into a free zone, a non-nuclear zone and a nuclear zone. The latter zone presented three layers of nuclei: an upper layer of elongated nuclei of supporting cells, a middle layer of rounded nuclei of olfactory cells and a lower row of flattened nuclei of basal cells. In animals administered with vinblastine and sacrificed on days 3 and 5 and with docetaxel and euthanized on days 5 and 10 (Fig. 17), the normal layering of the nuclei of the epithelia was disrupted so that the nuclei appeared disarranged.

3.3.1.2 Effects on epithelial surface structures

In the control and also in vinblastine-treated rabbits at days 10 and 15 and docetaxel-treated animals at days 3 and 15 (Fig. 18), knobs of olfactory cells and apices of supporting cells projected above the general surface of the epithelium. In these animal groups, the cilia projected from around the bases of the knobs in a radial pattern. In animals treated with vinblastine and euthanized on days 3 and 5 and with docetaxel and sacrificed on days 5 and 10 (Fig. 18), apices of the supporting cells were not observable from the surface of the epithelium but the knobs of the olfactory cells were readily visible, with their cilia projecting from the tips of the knobs to run parallel in form of a bundle.

3.3.1.3 Effects on epithelial cell proliferation

Proliferative activity of cells of the olfactory epithelium was shown by Ki-67 immunostaining to vary among the different animal groups, with Ki-67-positive cells being most prevalent in vinblastine-treated rabbits at day 5 followed by docetaxel-treated animals at day 10 (Fig. 19). The Ki-67 labeling was relatively less prevalent in the controls and in vinblastine-treated rabbits at days 3, 10 and 15 and docetaxel-exposed animals at day 15 (Fig. 19). In docetaxel treated animals at days 3 and 5, cells of the epithelium were negative for the Ki-67 immunostaining (Fig. 19). In all animal groups showing Ki-67 immunoreactivity, the labeling was confined to a population of cells localized in the basal region of the epithelia (Fig. 19).

3.3.1.4 Effects on axon bundles

In controls and also in vinblastine-treated rabbits at days 10 and 15 and docetaxel-exposed animals at day 15, the bundles were of normal structure, with their cross-sectional profiles showing a complete and intact fibroblastic ensheathment (Fig. 20). In vinblastine-treated rabbits at days 3 and 5 and docetaxel-treated animals at days 3, 5 and 10, the bundles were degenerate, with the loss of the cells beginning from periphery towards the inside (Fig. 20). More significantly, cores of the bundles of vinblastine-treated rabbits at days 3 and 5 and of docetaxel exposed animals at days 5 and 10 were infiltrated with blood capillaries (Fig. 20).

3.3.1.5 Effects on Bowman's glands

Figure 21 shows the structure of the Bowman's glands in controls and in animals injected with vinblastine or docetaxel. In controls and in the rabbits treated with vinblastine and sacrificed on days 10 and 15 and with docetaxel and euthanized on days 3 and 15, the glands were of normal structure, with their acini having intact spherical shaped secretory cells (Fig. 21). Glands of vinblastine-treated rabbits sacrificed on day 3 and of docetaxel-exposed animals euthanized on days 5 and 10 showed localized cell death. Glands of animals administered with vinblastine and sacrificed on day 5 were characterized by abnormally large cells and degeneration (Fig. 21).

3.3.1.6 Effects on mucosal cell survival

Terminal deoxyribonucleotidyl transferase-mediated dUTP-digoxigenin nick-end labeling (TUNEL) assay performed on the olfactory mucosa of the rabbits confirmed apoptotic death of cells of the olfactory epithelia, olfactory axon bundles and Bowman's gland of vinblasine-treated rabbits at days 3 and 5 and of docetaxel-exposed animals at days 5 and 10. Apoptotic death of the cells was confirmed by the presence of TUNEL-positive cells within these structures (Fig. 22). In the olfactory epithelium, reactivity of the cells to the TUNEL assay was relatively more evident in the middle and basal regions.

3.3.2 RESULTS ON MORPHOMETRY

Data on the impact of vinblastine and docetaxel on the thickness of the olfactory epithelium, diameters of axon bundles, packing densities of olfactory cells and cilia

numbers per olfactory cell knob provided in Table 4. Following treatment with vinblastine, the height of the olfactory epithelium decreased from the control value (98.4 ± 3.1 μm) by 39.1%, 34.2%, 7.3% and 1.3 % on days 3, 5, 10 and 15, respectively. With docetaxel treatment, the decrease in epithelial height was 7.3% on day 3, 25.1% on day 5, 36.5% on day 10 and 1.6% on day 15 (Table 4). In animals administered with vinblastine, cross-sectional diameters of axon bundles decreased from the control value (121.2 ± 10.6 μm) by 39.0%, 54.4%, 21.7% and 3.5% on days 3, 5, 10 and 15, respectively (Table 4). In docetaxel-treated animals, the bundle diameters decreased by 5.6%, 26.1%, 49.3% and 2.2% on days 3, 5, 10 and 15 respectively (Table 4). The packing density (mm^{-2} x 10^3) of olfactory cells in controls was 89.6 ± 4.6, a value which decreased after vinblastine administration by 65.3% on day 3, 75.2% on day 5,10.3% on day 10 and 1.5% on day 15 (Table 4). Injection with docetaxel resulted in a density decrease of 6.4%, 45.5%, 63.4% and 2.2% on post-exposure days 3, 5, 10 and 15, respectively. In vinblastine treated animals at days 3, 5, 10 and 15, the number of cilia reduced from the control value (24 ± 2 per knob) by 62.5%, 70.8%, 16.7% and 4.2%, respectively, whereas in the docetaxel-exposed animals, the cilia counts decreased by 20.8% on day 3, 33.3% on day 5, 50.0% on day 10 and 12.5% on day 15 (Table 4).

Figures 23-25 show volume fractions (%) of the components in lamina propria (axonal bundles, Bowman's glands and blood vessels) of the olfactory mucosa. Relative to the control value (26.3 ± 2.1%), the volume fraction for the axon bundles (Fig. 23) decreased significantly in the first five days (17.2 ± 1.3% on day 3 and 16.8 ± 1.2% on day 5). Thereafter, the volume fraction for the bundles increased to reach

control values by day 10 (25.2 ± 2.0%) or day 15 (25.9 ± 2.1%). In the docetaxel-treated animals, volume fraction for the bundles decreases progressively in the first ten days of exposure (i.e. 20.4 ± 1.2% on day 3, 17.7 ± 1.4% on day 5 and 13.4 ± 1.3% on day 10), after which the volume fraction increases to attain the control value on day 15 (24.8 ± 1.7%) (Fig. 23). Compared to the control value (41.5±1.8%), the volume fraction for the Bowman's glands did not significantly vary from that of the animals administered with either vinblastine (i.e. 38.6 ± 1.6% on day 3, 38.4% ± 1.6% on day 5, 39.7 ± 1.7% on day 10 and 41.0 ± 2% on day 15) or docetaxel (36.1 ± 1.3% on day 3, 35.6 ± 1.7% on day 5, 34.9 ± 1.4% on day 10 and 37.2 ± 1.6% on day 15) (Fig. 24). Volume fractions for the blood vessels (Fig. 25) were remarkably lower in the controls (19.9 ± 1.4%) than in vinblastine-treated rabbits at day 3 (34.3 ± 1.8%) and day 5 (31.5 ± 2%) but not significantly different from that vinblastine rabbits at day 10 (16.3 ± 1.7%) and day 15 (16.5 ± 1.6%). In docetaxel-exposed animals at day 5 and 10, volume fraction values for the vessels, which were estimated at 26.6 ± 1.2 and 28.5 ± 1.5, respectively, was significantly higher than of controls and of docetaxel rabbits at day 3 (15.3 ± 1.6%) and day 15 (15.8 ± 1.8%) (Fig. 25).

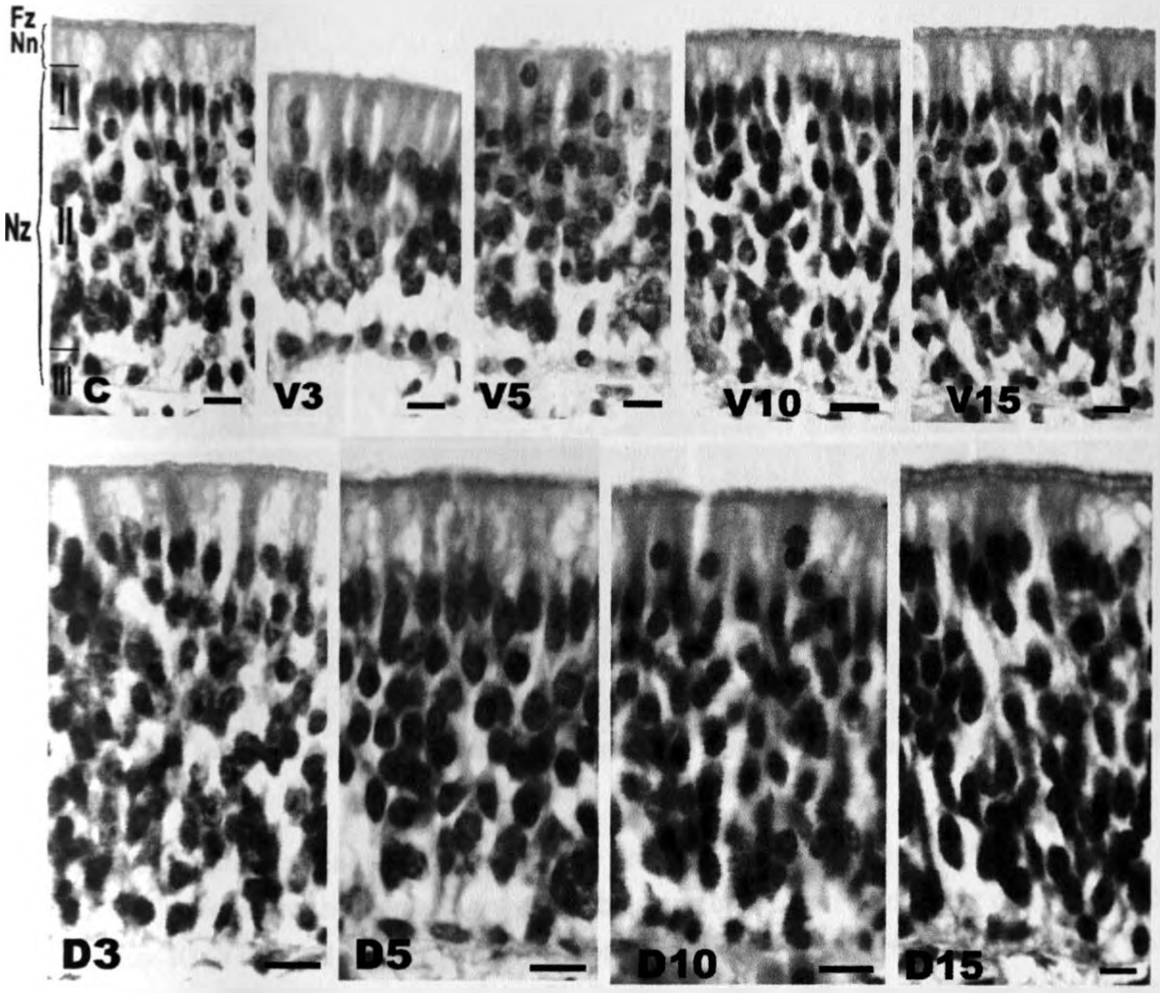

Figure 17. Histological sections of olfactory epithelia of controls (C), and of rabbits administered with vinblastine and sacrificed on day 3 (V3), day 5 (V5), day 10 (V10) and day 15 (V15), and with docetaxel and euthanized on day 3 (D3), day 5 (D5), day 10 (D10) and day 15 (D15). In the controls and also in the vinblastine-treated rabbits at day 10 and 15 and docetaxel-treated animals at day 3 and 15, the epithelium is organized into a free zone (Fz), a non-nuclear zone (Nn) and a nuclear zone (Nz), with the latter zone being constituted by an upper layer of elongated supporting cell nuclei (I), a middle layer of rounded olfactory cell nuclei (II) and a lower row of flattened basal cell nuclei (III). In vinblastine-treated rabbits at days 3 and 5 and docetaxel rabbits at days 5 and 10, the normal stratification is disrupted so that the nuclei appear disarranged. H&E stain, Bar = 5 μm (in all sections).

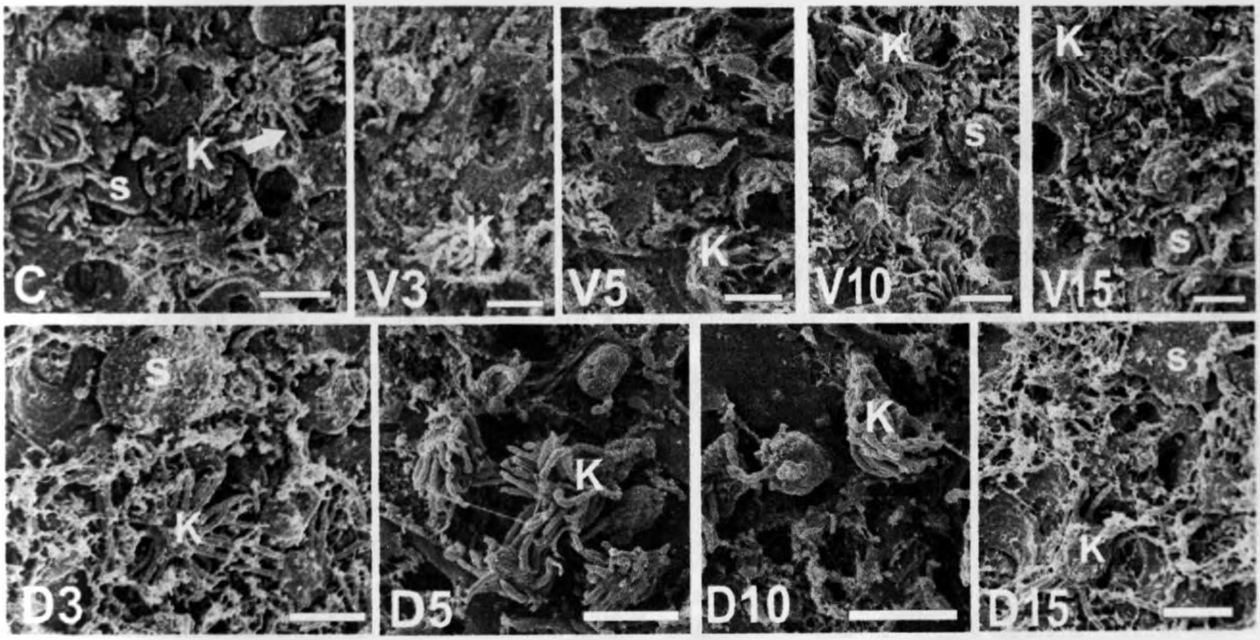

Figure 18. Scanning electron micrographs showing the surfaces of the epithelia of controls (C), and of rabbits treated with vinblastine and sacrificed after day 3 (V3), day 5 (V5), day 10 (V10) and day 15 (V15), and with docetaxel and euthanized on day 3 (D3), day 5 (D5), day 10 (D10) and day 15 (D15). In the control and also in vinblastine-treated rabbits at days 10 and 15 and docetaxel-treated animals at days 3 and 15, the apical ends of supporting cell (s) and knobs of olfactory cells (k) are discernible from the surface of the epithelium. In these animals, the cilia (arrow) project in a radial fashion from around the bases of the knobs. In vinblastine-treated rabbits at days 3 and 5 and in docetaxel-administered animals at days 5 and 10, apices of the supporting cells are not visible from the surface of the epithelium but knobs of the olfactory cells are readily seen, with the cilia arising from the apex of the knobs to run in parallel in form of a tuft. Bar = 2 μm.

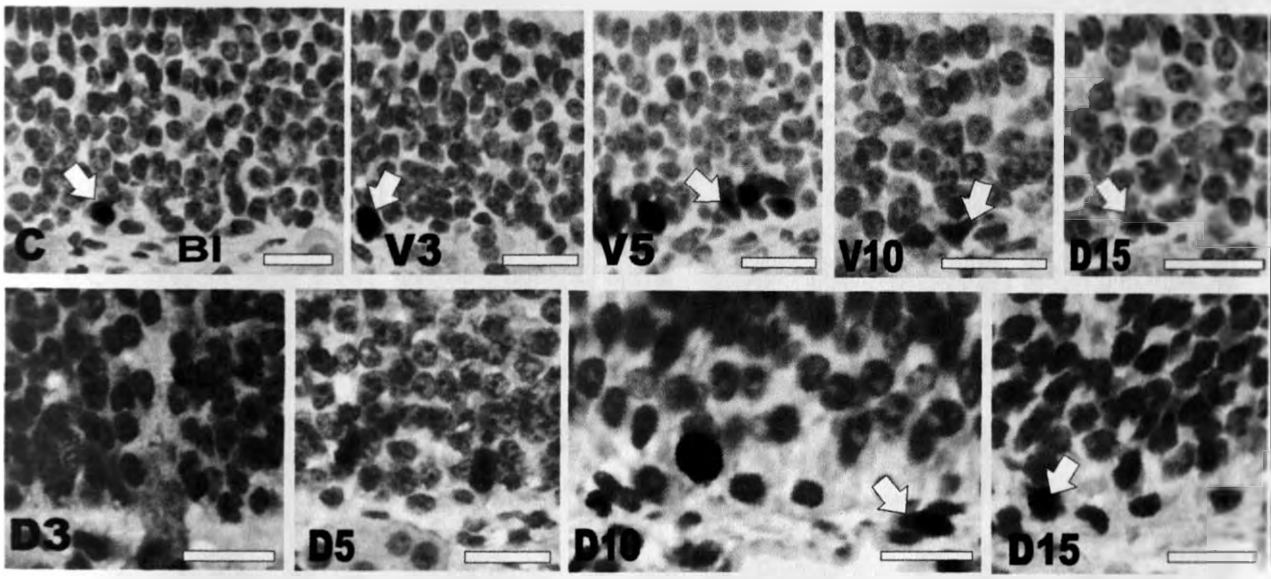

Figure 19. Light micrographs showing Ki-67 immunostaining of the olfactory epithelia of controls (*C*), and of rabbits administered with vinblastine and sacrificed on day 3 (V3), day 5 (V5), day 10 (V10) and day 15 (V15), and with docetaxel and euthanized on day 3 (D3), day 5 (D5), day 10 (D10) and day 15 (D15). In all positive cases, Ki-67 reactivity is restricted to the basal part of the epithelium just above the basement membrane (Bl). Ki-67 positive cells (arrows) show the highest prevalence in vinblastine-treated rabbits at day 5 and docetaxel-treated animals at day 10. The Ki-67 cells are relatively less prevalent in the control animals and in vinblastine-treated rabbits at days 3, 10 and 15 and in docetaxel animals at day 15. The Ki-67 labeled cells are absent in the epithelia of docetaxel-treated animals at day 3 and 5. Bar = 30 μm.

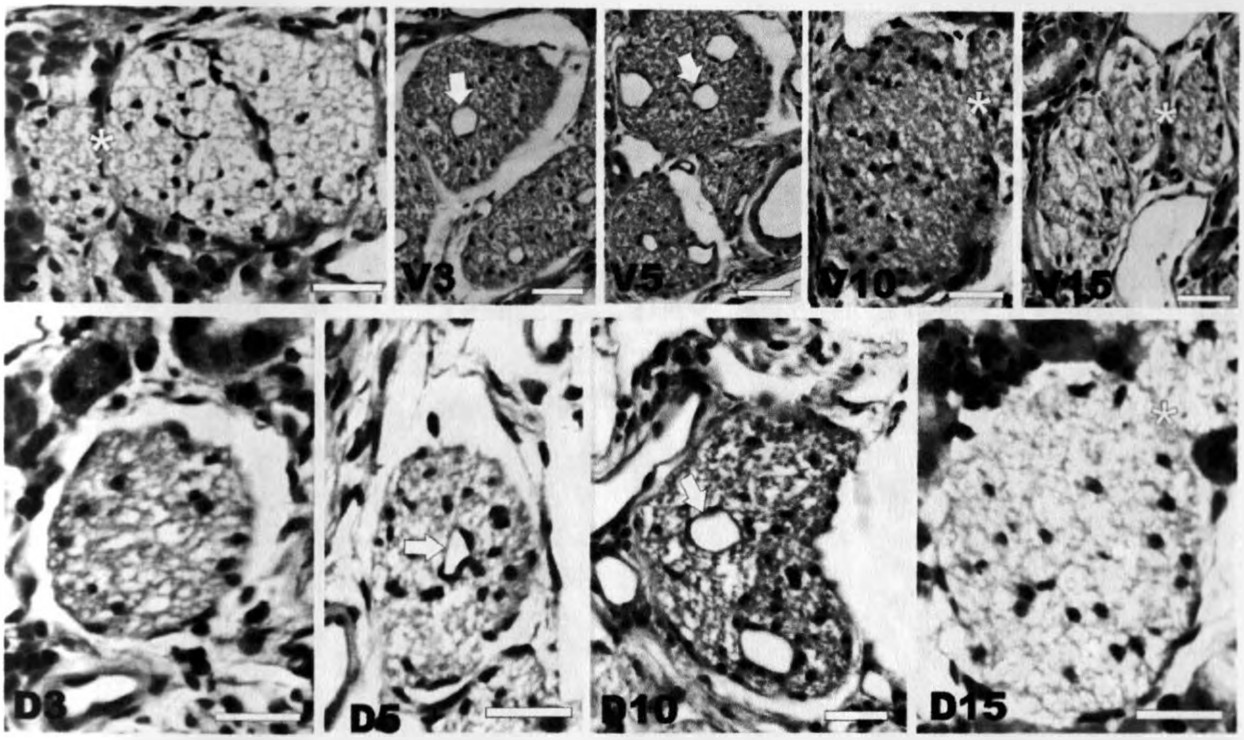

Figure 20. Histological sections across axon bundles of controls (C), and of animals injected with vinblastine and euthanized on day 3 (V3), day 5 (V5), day 10 (V10) and day 15 (V15), and with docetaxel and sacrificed on day 3 (D3), day 5 (D5), day 10 (D10) and day 15 (D15). In the controls as well as in vinblastine-treated rabbits at days 10 and 15 and docetaxel-treated animals at day 15, the bundles appear structurally normal, with an intact fibroblastic envelope (asterisks). In rabbits treated with vinblastine at days 3 and 5 and with docetaxel at days 3, 5 and 10, the bundles undergo degeneration, with the loss of cells beginning from outside the bundle. Cores of axonal bundles of vinblastine-treated rabbits at days 3 and 5 and of docetaxel-treated animals at days 5 and 10 contained blood capillaries (arrows). H&E stain, Bar = 20 μm.

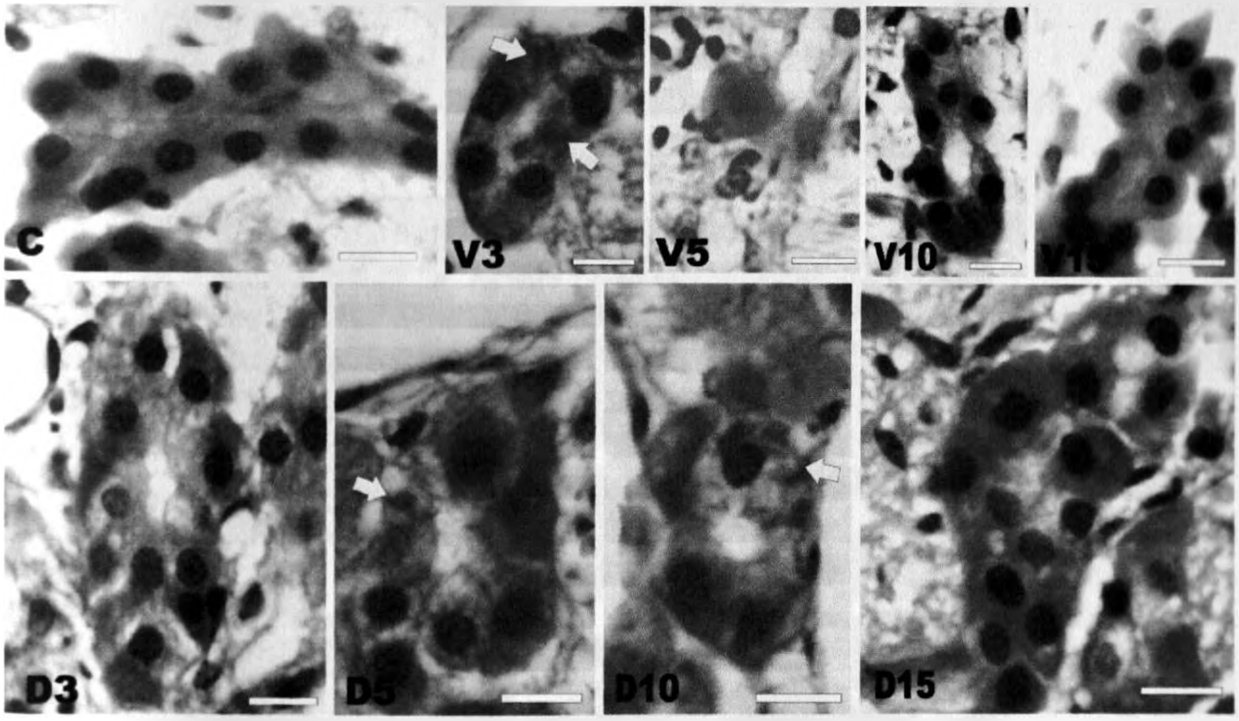

Figure 21. Light micrographs illustrating the structure of the Bowman's glands in controls (C), and in rabbits treated with vinblastine and sacrificed after day 3 (V3), day 5 (V5), day 10 (V10) and day 15 (V15) and with docetaxel and euthanized on day 3 (D3), day 5 (D5), day 10 (D10) and day 15 (D15). In controls and also in vinblastine-treated rabbits at day 10 and 15 and docetaxel-treated animals at days 3 and 15, the secretory parts of the glands are tubular and with intact spherical secretory cells. In vinblastine-treated rabbits at day 3 and docetaxel-treated animals at days 5 and 10, there is loss involving the glandular cells (arrows) while in vinblastine-treated animals at day 5, cytomegaly and cell loss are the key features observed. H&E stain, Bar = 10 µm.

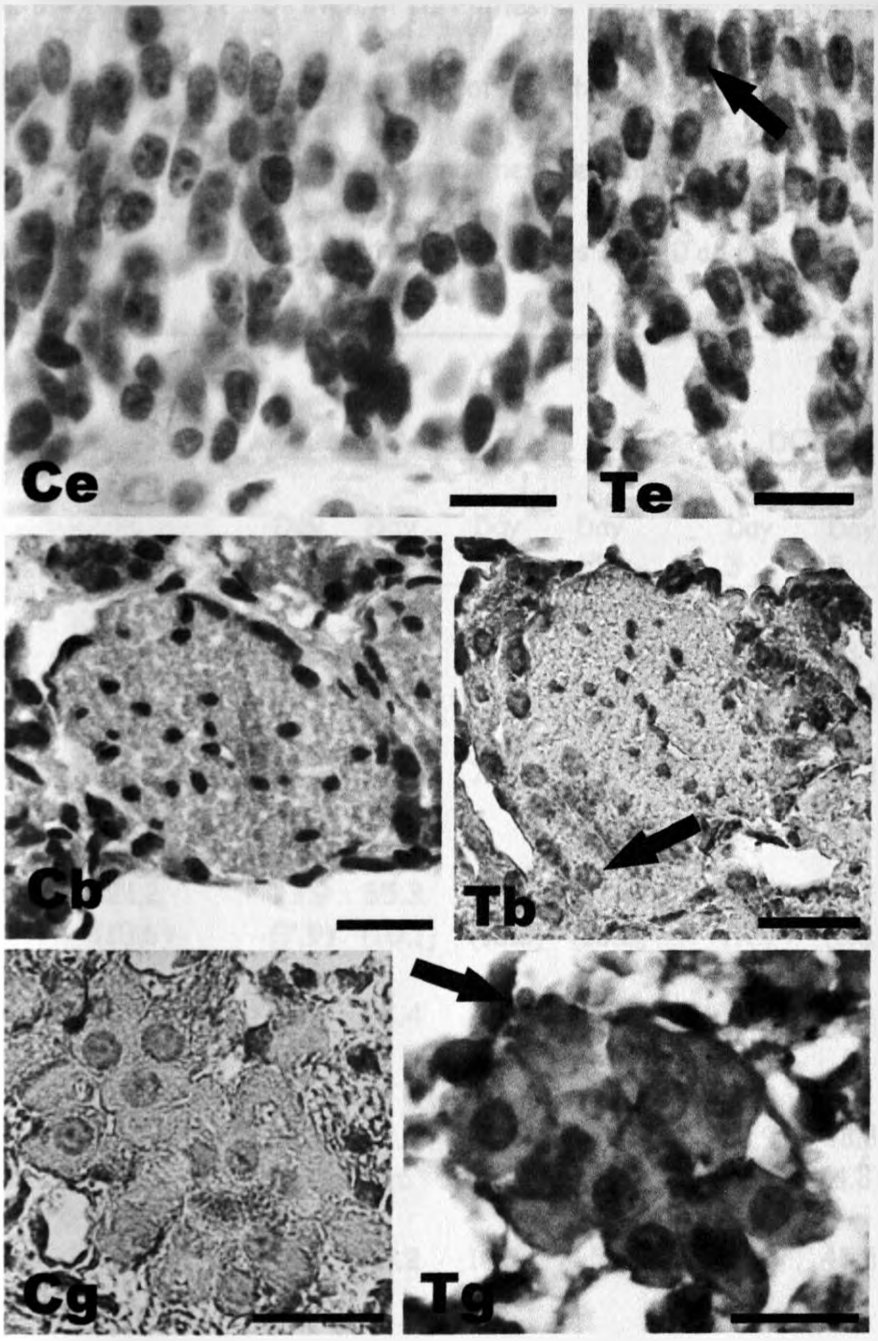

Figure 22. Terminal Transferase dUTP Nick End Labeling (TUNEL) assay of olfactory epithelia, axon bundles and Bowman's glands of controls (Ce, Cb and Cg respectively) and of vinblastine or docetaxel treated animals (Te, Tb and Tg respectively) at day 5. Notice the presence of TUNEL-positive cells (arrows) in the epithelia, bundles and glands of the treated animals. In the epithelium, the TUNEL-positive cells occur in the middle and basal regions of the epithelium. Bar = 20 μm.

Table 4. Mean values (± SD given in parenthesis) for olfactory epithelial height, axon bundle diameters (in μm), packing density of olfactory cells ($mm^{-2} \times 10^3$) and cilia count (per olfactory cell knob) in the rabbit and their percentage decrease after vinblastine and docetaxel administration and euthanasia at days 3, 5,10 and 15.

		VINBLASTINE				DOCETAXEL			
	Control	Day 3	Day 5	Day 10	Day 15	Day 3	Day 5	Day 10	Day 15
Epithelial height	98.4 (3.1)	59.9 (3.2)	64.7 (2.9)	91.2 (2.9)	97.1 (3.6)	91.2 (4.2)	73.7 (3.4)	62.5 (4.3)	96.8 (3.7)
% decrease		39.1	34.2	7.3	1.3	7.3	25.1	36.5	1.6
Diameter of bundle	121.2 (10.6)	73.9 (7.9)	55.3 (10.1)	94.9 (12.6)	117.0 (10.3)	114.4 (11.7)	89.6 (8.3)	61.4 (8.9)	118.5 (9.7)
% decrease		39.0	54.4	21.7	3.5	5.6	26.1	49.3	2.2
Olfactory cell density	89.6 (4.6)	31.1 (4.1)	22.2 (2.6)	80.4 (4.2)	88.3 (5.4)	83.9 (4.6)	48.8 (4.8)	32.8 (3.3)	87.6 (4.2)
% decrease		65.3	75.2	10.3	1.5	6.4	45.5	63.4	2.2
Cilia count/ knob	24 (2)	9 (1)	7 (2)	20 (2)	23 (2)	19 (2)	16 (2)	12 (3)	21 (2)
% decrease		62.5	70.8	16.7	4.2	20.8	33.3	50.0	12.5

% decrease = (Control value - Value after drug exposure/ Control value) x 100

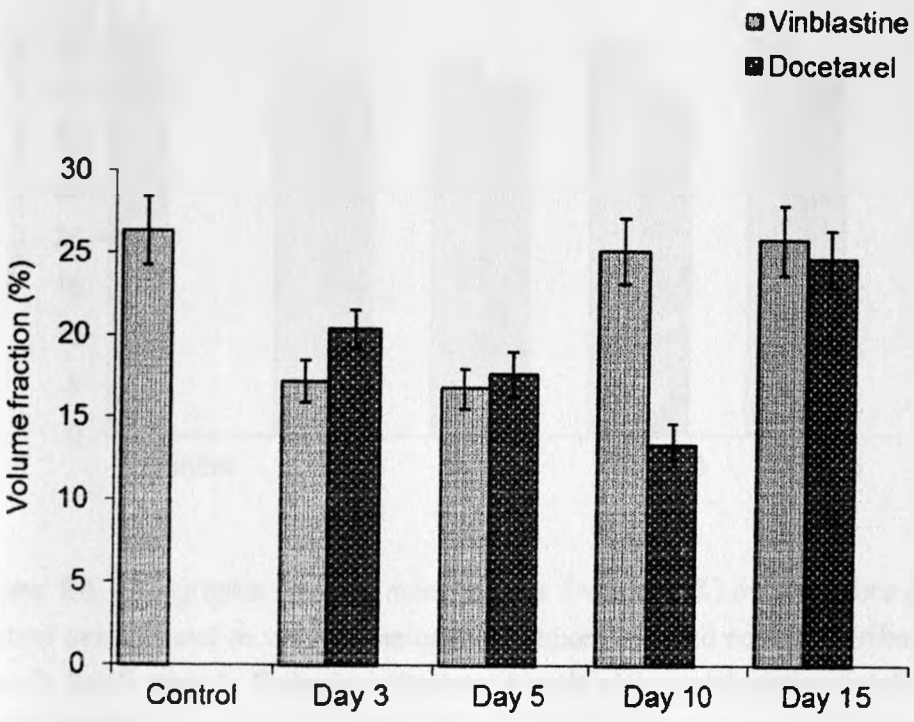

Figure 23. Graphical illustration of mean volume fraction (%) of axon bundles in control animals and in vinblastine and docetaxel-treated rabbits euthanized on days 3, 5, 10 and 15. Following vinblastine exposure, the volume fraction for the bundles decreases significantly in the first 3 to 5 days. Thereafter, the volume fraction increases to reach control values on day 10 or 15. For the docetaxel-treated animals, the volume fraction for the bundles decreases progressively in the first 10 days, after which the volume fraction increases to control values on day 15, $P < 0.05$.

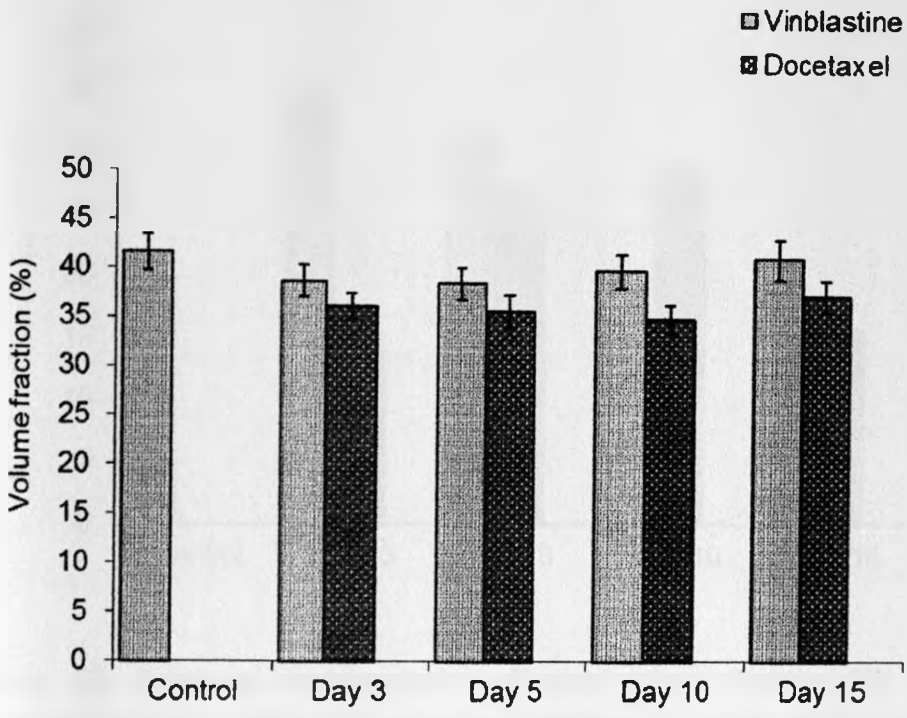

Figure 24. Bar graphs showing mean volume fraction (%) of Bowman's glands in control animals and in vinblastine and docetaxel-treated rabbits euthanized on days 3, 5, 10 and 15. Following treatment with either vinblastine or docetaxel, the volume fraction for the glands does not significantly vary from that of the controls, $P < 0.05$.

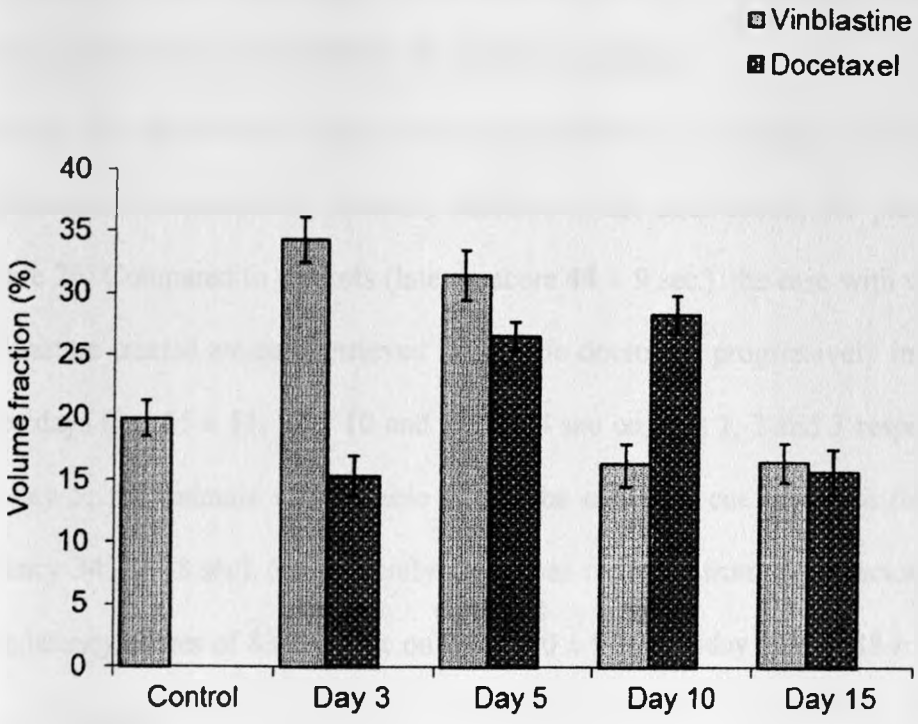

Figure 25. Graphical representation of mean volume fraction (%) of blood vessels in controls and in vinblastine and docetaxel-treated rabbits euthanized on days 3, 5, 10 and 15. The volume fraction for the vessels is remarkably lower in the controls than in vinblastine-treated rabbits at day 3 and 5 but insignificantly different from that for the animals at days 10 and 15. Compared to the control, the volume fraction for the vessels is lower than for the docetaxel-exposed rabbits at days 5 and 10 but not significantly different from that of the animals at day 3 and 15, $P < 0.05$.

3.4 EFFECTS OF VINBLASTINE AND DOCETAXEL ADMINISTRATION ON OLFACTORY FUNCTION IN ADULT RABBITS

Data for the buried food test, which was performed to investigate the effects of vinblastine or docetaxel on olfactory function in the adult rabbit, are presented in Figure 26. Compared to controls (latency score 44 ± 9 sec.), the ease with which the vinblastine-treated animals retrieved the cookie decreased progressively in the first three days (i.e. 65 ± 11, 81 ± 10 and 179 ± 14 sec on days 1, 2 and 3 respectively). On day 5, the animals were unable to use the olfactory cue to locate the cookie (latency 341 ± 18 sec). Subsequently, there was recovery from the olfactory deficit with latency scores of 83 ± 12 sec on day 7, 60 ± 9 sec on day 10 and 48 ± 8 sec on day 15 (Fig. 26).

In animals administered with docetaxel, the ease with which they retrieved the cookie decreased progressively in the first 7 days (i.e. 56 ± 7, 68 ± 9, 123 ± 13 and 169 ± 15 sec on days 1, 2, 5 and 7 respectively) (Fig. 26). On day 10, the animals failed to uncover the cookie (latency 329 ± 21 sec). Thereafter, there was recovery from the olfactory deficit with a latency score of 115 ± 14 sec being recorded on day 15 (Fig. 26).

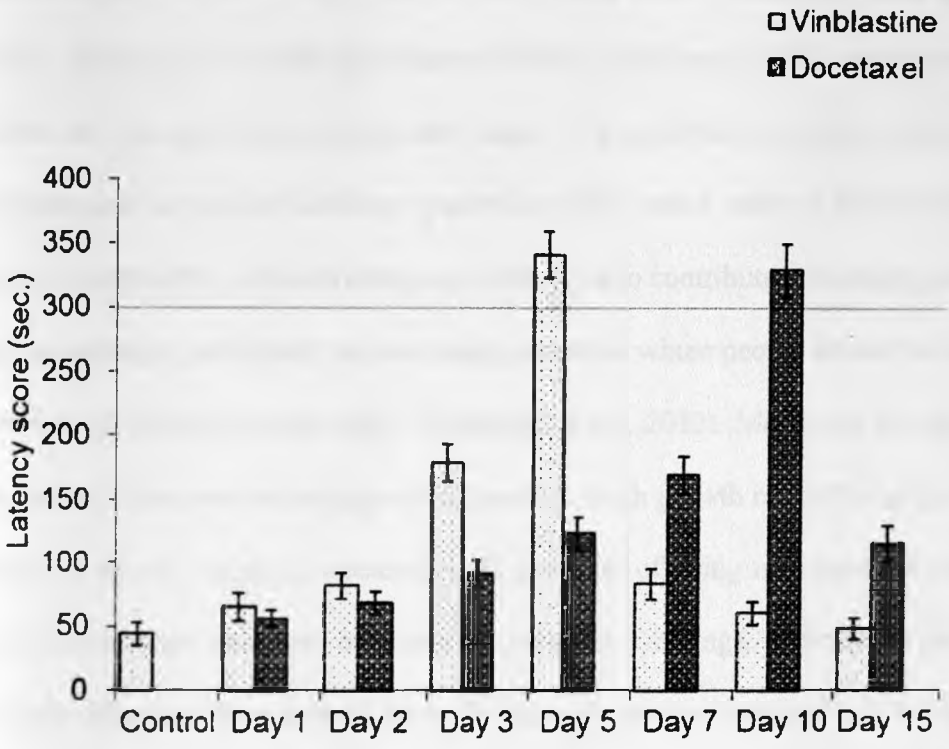

Figure 26. Bar graphs showing mean latency scores of controls and of rabbits injected with vinblastine or docetaxel and tested for olfaction ability after days 1, 2, 3, 5, 7, 10 and 15. Following treatment with vinblastine, latency scores increase progressively till day 5, when the animals are unable to locate the cookie. Thereafter, recovery from the olfactory deficit occurs on day 7 and progressively to day 10 and day 15. In docetaxel treated animals, the ability to uncover the cookie decrease progressive till day 10, when the animals fail to uncover the cookie. Afterwards on day 15, the animals recover from the olfactory deficit, $P < 0.05$.

CHAPTER FOUR

DISCUSSION AND CONCLUSIONS

4.1 GENERAL DISCUSSION

This study uses the rabbit as a model to study the remodeling structure of the olfactory tissue during postnatal development. Coincidentally, the study touches on aspects of growth and development as they relate to the olfactory system in this species. Being reflex or induced ovulators (Bakker and Baum, 2000), developmental studies in this species are rather easy since it is possible to initiate mating and predetermine the time of kindling (parturition). The rabbit, with its high fecundity and non-competitive diet with humans, is envisaged to contribute to elevating animal protein shortage, particularly in developing countries where people subsist on cereal diets low in protein (Owen, 1981; El Rahman *et al.*, 2012). Moreover, this species has several other overt advantages which include: high growth rate (attaining market weight of about 2 kg at 12 weeks of age), potential of being in a constant state of reproduction (can mate and conceive 24 hrs after kindling), capacity to produce multiple offspring (litter size of up to 7), meat of excellent quality (rich in protein and low in fat and cholesterol) and high suitability to both small scale and large-scale commercial production (El-Amin, 1978; King, 1978; Lawrie, 1979; El Rahman *et al.*, 2012). Accordingly, research in this species has for the last few decades been confined to aspects of development with an aim of improving its productivity (Dinh Van Binh, 2002, 2008).

Rabbit pups are born altricial, i.e. with glabrous skin, devoid of vision and audition, and with limited motor abilities (Caba and González-Mariscal, 2009). The eyes of

rabbits remain closed until around postnatal day 10-13 (Ripisardi *et al.*, 1975; Langenbach *et al.*, 2001) whereas the ears remain sealed until day 7-8 after birth (Gottlieb, 1971; Coureaud *et al.*, 2008). Thus, in the early part of postnatal life in this species, the interaction between the young and the dam (doe) for purposes of feeding (suckling) is highly dependent on olfaction. Indeed, specific odors on the mother's ventrum associated with the substance 2-methylbut-2-enal in the doe's milk have been shown to guide the rabbit newborns to achieve the behavioral sequence necessary for obtaining milk: contacting the female's abdomen, locating a nipple by searching movements, and orally contacting it to suck (Schaal *et al.*, 2003).

In this study and in an earlier one (Broekhuizen *et al.*, 1986), the furless young are observed to remain in their nest under the warm cover of the hair that the doe obtains from her own body shortly before parturition, a behavior that may be necessitated by the fact that the doe nurses it's young only for a few minutes (5 or less) once each day (Hudson and Distel, 1983, 1984; Coureaud *et al.*, 2008; Hudson *et al.*, 2008; Caba and González-Mariscal, 2009). In the growing rabbits, body weight gain varies between the various stages. Between birth and weaning, for example, body weights for the rabbits increase from 48.7 to 369.9 g. In a study comparing birth and weaning weights in different breeds of rabbits (El Rahman *et al.*, 2012), respective values of 42.6-62.7 g and 383.6-415.4 g were obtained. A study on growth performance in rabbits (Anous, 1999) showed birth and weaning weights to correlate with feed conversion ratio in the doe.

The nasal cavity of the rabbit carries a total of four endoturbinates. A similar number of endoturbinates occurs in the dog whilst in the sheep, the turbinates are five in number (Kavoi, 2008; Kavoi *et al.*, 2010). On the turbinates of freshly euthanized rabbits, the olfactory mucosa is identified by its yellowish-brown color. This coloration is thought to result from the deposition of remains of secondary lysosomes (residual bodies) in the end-feet of the supporting cells following phagocytosis of particulates by these cells (Reese and Brightman, 1970; Williams, 1995). In the adult rabbit, measurement of the greatest length of the ethmoturbinates gave a mean value of 1.7 cm. In the dog and sheep, respective lengths of the ethmoturbinates were estimated at 4.3 and 3.4 cm (Kavoi *et al.*, 2010). Similar to an earlier observation in the sheep (Kavoi *et al.*, 2010), the ethmoturbinates of the rabbit possess smooth surfaces. This contrasts with the situation in the dog where the turbinate surfaces are characterized by prominent folds of lamellae (Kavoi *et al.*, 2010). Such folds provide a larger surface for the receptor cells and their presence is believed to be a key morphological indicator of better olfaction ability (Pihlstrom *et al.* 2005, Kajiura *et al.* 2005; Schluessel *et al.* 2008, 2010).

In the rabbits, administration of vinblastine or docetaxel results in body weight loss, which was most significant on day 5 and 10 following exposure to vinblastine and docetaxel respectively. Previously, loss in body weight was demonstrated following systemic administration of several other anticancer drugs including cisplatin, cyclophosphamide, actinomycin D, 5-fluorouracil, vincristine (Yamamoto *et al.*, 2007) and doxorubicin (Che *et al.*, 2011). This study and several others (Sakata *et al.*, 1998; Sava and Cocchietto, 2000; Nelson *et al.*, 2002; Rademaker-Lakhai *et al.*,

2004) documents gastrointestinal problems including diarrhea, emesis, inappetence and reduced food intake as key adverse reactions associated with administration of most anticancer drugs. In the current investigation, the reduction in food intake is a likely reason for the body weight loss observed when the rabbits were injected with the anticancer drugs. In studies on the emetogenic levels of various anticancer drugs, the potential and the duration taken to induce emesis was shown to vary with the type of the anticancer drug (Hesketh *et al.*, 1997; Schnell, 2003; Grunberg *et al.*, 2005). Similarly, differences in the ability of vinblastine and docetaxel to induce anorexia may explain why rabbits treated with docetaxel take longer than those treated with vinblastine to regain appetite and body weight. Clinical reports on anticancer drugs suggest that gastrointestinal reactions such as anorexia and emesis are as a result of gastric motor dysfunction (Nelson *et al.*, 2002). Furthermore, appetite is governed by peripheral hormones and central neurotransmitters that act on the arcuate nucleus of the hypothalamus and nucleus tactus solitarius of the brainstem and whose functions may be interrupted by the anticancer drugs (Davis *et al.*, 2004).

Diarrhea, alopecia and skin necrosis are other major adverse reactions associated with vinblastine and docetaxel administration in the rabbits. Abnormal gastric emptying has been documented as the cause of gastrointestinal problems associated with the clinical use of most anticancer drugs (Nelson *et al.*, 2002). By disrupting spindle structures, antimicrotubule drugs exert their effects on rapidly proliferating tissues (Cleton, 1995). The gastrointestinal tract and the skin belong to this group of tissues (Todd *et al.*, 1976; Dorr and Alberts, 1985; Cleton, 1995; Lishner *et al.*,

1999). Ulcerative lesions of the skin were previously demonstrated in the mouse following exposure to vincristine (Dorr and Alberts, 1985) whereas alopecia is common in cancer patients undergoing combined chemotherapy (Lishner *et al.*, 1999). In the rabbits, necrotic lesions are more conspicuous on the skin around the eyes and the ears. Susceptibility of these skin areas to necrotic lesions may be attributed to the thin nature of the skin and the presence of numerous and delicate blood vessels that are superficially placed. Furthermore, such skin areas are more prone to phlebitis, edema and effusions (due to a capillary-leak syndrome), all of which have been associated with the use of anticancer drugs (Capri *et al.*, 1996; Rademaker-Lakhai *et al.*, 2004).

4.2 STRUCTURE OF OLFACTORY MUCOSA IN POSTNATALLY DEVELOPING RABBITS

The basic structure of the adult rabbit olfactory system has extensively been studied (Allisson and Wawick, 1949; Allison, 1953; Le Gros Clark, 1951, 1956; Mulvaney and Heist, 1970; Yamamoto, 1976; Mori *et al.*, 1985; Onoda and Fujita, 1988, Harkema *et al.*, 2006) and investigations on olfactory function are also well documented (Shepherd, 1971; Freeman and Schneider, 1982; Hudson and Distel, 1983; Distel and Hudson, 1984; Chaput and Holley, 1985; Hudson and Distel, 1987; Imamura *et al.*, 1992; Schaal *et al.*, 2003; Hudson *et al.*, 2008). In view of the unique nature of the rabbit olfactory mucosa with respect to functional challenges during postnatal life (Hudson and Distel, 1983, 1984; Hudson *et al.*, 2008), it is surprising that the postnatal morphology of the mucosa has been so little studied. In a study by Meisami *et al.*, (1990) in which the structure of the nasal olfactory tissue

was compared in newborn and weanling rabbits, key mucosal components including axon bundles, Bowman's glands and blood vessels were not investigated. Moreover, histology was the only analytical method used. Hence, in the present work, several other analytical techniques including scanning and transmission electron microscopy and Ki-67 immunostaining are employed to investigate the morphological changes of the rabbit olfactory mucosa from birth through weaning, suckling and adult stages.

Despite the considerably mature cytoarchitecture of the rabbit olfactory epithelium at the time of birth, results of this study show a progressive modification in the qualitative and quantitative structure of the epithelium from birth to adulthood. In rats (Meisami, 1989), a dramatic refinement involving an increase in the surface area of the olfactory receptor sheet and the number of primary sensory afferent units was observed in the first few postnatal weeks. Epithelial thickness has been cited as a key developmental index of the olfactory epithelium (Sakashita *et al.*, 1995). From the time of birth in the rabbit, the olfactory epithelium, whose mean height is 55.5 microns, increases progressively to reach 92.9 microns at adult age. This is in contrast to the situation in the rat (Sakashita *et al.*, 1995) where the average height of the epithelium has been shown to increase only minimally from 94 to 98 microns between birth and postnatal day 10 after which it declines to a mean of 57 microns at adult age.

Barber and Boyde, (1968) recognized the use of SEM as a key breakthrough in the analysis of surface structures of biological specimens. In the current study, SEM is

used in the estimation of packing densities of olfactory cells and in describing the structure of olfactory nerve endings. In newborn and weanling rabbits, Meisami *et al.*, (1990) determined the densities of olfactory cells based on cell shape and staining characteristics with no regard to the state of their maturation. It is generally accepted that only those olfactory neurons whose apical dendrites terminate in a ciliated knob can be considered functional (Le Gross Clark, 1956; Graziadei 1971, Monti Graziadei *et al.*, 1980). Accordingly, the data by Meisami *et al.*, (1990) in which the packing densities of the olfactory cells were determined on histological sections, may be less reliable in relating aspects of mucosal morphology with olfactory function. In the adult rabbits, Meisami *et al.*, (1990) estimated the average packing density at 100,000 mm^{-2} while counts using SEM in this study give a mean value of 87,600 mm^{-2}. The discrepancy in these values may have resulted from errors in counting procedures and / or differences in the degree of shrinkage where, at SEM, the shrinkage is minimal since it is compensated by the obligatory metal coating (Menco, 1978).

On the surface of the rabbit olfactory epithelium, the number of cilia per olfactory cell knob increases with progressing age, reaching a mean of 24 cilia per knob in the adult. In earlier studies, mean values for the cilia number per knob were 18 in the dog, 7 in the sheep (Kavoi *et al.*, 2010) and 17 in the bovine (Menco, 1978). Since the receptors for odor binding are mainly located on the cilia of olfactory neurons (Getchell, 1986; Buck and Axel, 1991; Kinnamon and Getchell, 1991; Menco *et al.*, 1992, 1997; Lowe and Gold, 1993; Liberles and Buck, 2006), it can be argued that

the number of cilia per olfactory cell knob is reminiscent of the olfactory functional capability of a particular species.

In the rabbit olfactory epithelium, the prevalence of Ki-67 positive cells decreases progressively with increasing age, with the labeling index being 11.3% in adults as compared to 31.2% in the neonates, and Ki-67 reactivity is restricted to the population of cells positioned at the base of the epithelium. Mitotic activity of olfactory epithelial cells during postnatal development have been demonstrated in other species including guinea pigs (Nakamura *et al.*, 1998; Higuchi *et al.*, 2005), mice (Ohta and Ichimura, 2000) and rats (Weiler and Farbman, 1997, 1998) with a similar finding that the rate of cell proliferation decreases postnatally. In the rat olfactory epithelium (Weiler and Farbman, 1998), 5-bromo-2'-deoxyuridine (BrdU)-labeling index for the basal cell population was shown to decrease from a high of 30% at postnatal day 1 to a low of 5% at postnatal day 181. The age-related decrease in the number of labeled olfactory progenitor cells have been attributed to a number of factors, which include: age-related decrease in the concentration of growth associated factors (Weiler and Farbman; 1998), longer cell cycle time in older animals (Weiler and Farbman, 1997, 1998) and greater rate of expansion of the olfactory area in the younger than in the older animals (Weiler and Farbman, 1997).

In the basal region of the olfactory epithelium, multipotent basal cell progenitors give rise to immature neuronal and supporting cells which migrate with the passage of time to take defined positions in the apical region of the epithelium (Graziadei and Graziadei, 1979; Williams, 1995; Schwob, 2002). This apical migration of

maturing neuronal cells has actually made it possible to determine the neuronal age by position (Graziadei and Monti-Graziadei, 1979; Farbman and Margolis, 1980). In the current investigation, it is suggested that the early part of postnatal life in the rabbits is mainly devoted to basal cell replication, which slows down in the later stages when an adequate number of mature epithelial cells has been attained. This offers a likely explaination to the finding of a relatively higher Ki-67 reactivity in the basal region of the olfactory epithelium of neonates and sucklings as compared to weanling and adult animals.

At neonatal stage, axon bundles in the rabbit olfactory mucosa appear as fully formed structures while Bowman's glands and blood vessels continue to develop in the area of the lamina propria subjacent to the mucosal epithelium. Similar variations in the level of development of these structures have been reported in prenatally developing animals where, for example, in the Syrian hamster (Taniguchi and Taniguchi, 2007) the primitive axonal bundles were found at the base of the epithelium at mid gestation while in the mouse (Cuschieri and Bannister, 1975), small outgrowths representing the future Bowman's glands appeared in the same location at late gestation. Developing vasculature has also been demonstrated in the olfactory neuroepithelium of prenatal humans (Sangari *et al.*, 2000) and mice (Herken *et al.*, 1989).

The current study documents an age-related variation in the structural form of the Bowman's glands where the glands are of acinar type in the neonates, sucklings and weanlings and are mainly tubular in the adults. In the histogenesis of the olfactory

mucosa in humans (Sangari *et al.*, 1992), the Bowman's glands develop as buds of epithelial tissue in the lamina propria during fetal development. In the postnatal rabbits, the age-associated variation in the structural forms of the Bowman's glands may probably be linked to the anatomical transformation that takes place as the glands develop from primitive to definitive forms. Between birth and adulthood, diameters of axon bundles increase over 5 times and the volume fraction for the bundles increases by over 2.5-fold in comparison to the Bowman's glands whose volume fraction increment is merely 1.3-fold. According to studies by Van Drongelen *et al.* (1978) and Meisami (1989), large sized axon bundles are required for a functionally effective olfactory mucosa since the size of a bundle is directly related to the ratio of convergence between its axons and those of secondary neurons in the olfactory bulb. Moreover, olfactory receptor proteins located on the axonal processes are believed to act as molecule sensors for odorants as well as cell recognition molecules guiding the axons to their appropriate glomerulus in the olfactory bulb (Menco *et al.*, 1994; Strotmann *et al.*, 2004).

In the neonate, suckling and weanling animals, blood vessels occur within the cores of the axonal bundles. Previously, similar vessels demonstrated in axon bundles of dogs were associated with the great thicknesses of the bundles and hence the great diffusion distances that oxygen and nutrients must cross to supply ensheathing cells located deep within the bundle cores (Kavoi *et al.*, 2010). In the neonate, suckling and weanling rabbits, the bundle sizes are not so large to limit oxygen diffusion and thus the presence of vasculature in the bundle cores in these age groups is likely to be a feature of development. Moreover, vascularization of developing olfactory

mucosal structures such as the olfactory epithelium (Sangari *et al.*, 1992) has been attributed to the increased metabolic demand of the replicating cells towards the completion of their maturation.

In the rabbits during postnatal development, relative sizes of the bundle fascicles increase while the gaps separating these fascicles narrow with age. The increase in the fascicle sizes and the decrease in the inter-fascicular gaps with age may be associated with the age-related increase in olfactory neuronal densities, the increased number of axons arising from the newly forming neurons and consequently the increased rate of packaging of the neuronal axons within individual fascicles (as evidenced by TEM in neonates and sucklings). Furthermore, the increase in the number of primary olfactory cells and hence the higher convergence upon the central relay neurons enhances the physiological capacities of the olfactory afferent pathways by increasing the opportunity for spatial summation and facilitation thereby resulting in improved olfactory sensitivity with development (Meisami, 1989).

At the time of birth in the rabbit, dendritic endings of the olfactory cells possess fully developed cilia. In mice (Cuschieri & Bannister, 1975) and Syrian hamsters (Taniguchi and Taniguchi, 2007), growth of cilia on dendritic terminals occurs 3-4 days before term. In prenatally developing rats, densities of odor binding proteins, which take the form of freeze-fracture intramembranous particles in the ciliary membrane, increase with development (Menco, 1987) whereas immunoreactivity for antibodies to the olfactory signal-transduction proteins parallel cilium development

(Menco *et al.*, 1994). These findings, coupled with the fact that the cilia are the principal sites for the initial events of olfactory transduction (Getchell, 1986; Buck and Axel, 1991; Kinnamon and Getchell, 1991; Lowe and Gold, 1993; Menco *et al.*, 1997; Jenkins *et al.*, 2009) imply that the full development of the cilia in the rabbit neonates is reflective of an early functional maturation of the olfactory mucosa in this species.

Regarding the projection of the cilia from the olfactory cell knobs, the change in pattern from parallel in the newborns and sucklings to radial in the weanling and adults is not clear. However, the radial pattern, which has also been demonstrated in dogs (Kavoi *et al.*, 2010), horses (Kumar *et al.*, 2000) and humans (Lenz, 1977), seems to be associated with higher cilia numbers when compared to the parallel pattern commonly reported in bovids (Menco, 1978; Kavoi *et al.*, 2010). At the early stages of development of the olfactory mucosa, olfactory cells proliferate in the superficial layer of the epithelium and send extensions towards the free surface while on the contrary, proliferation of the supporting cells remains confined in the middle or basal layers of the epithelium (Taniguchi and Taniguchi, 2007). This may therefore explain why, in the neonatal and suckling stages, the supporting cells had not projected their apices on the surface of the olfactory epithelium. In a previous study by Barber and Boyde (1968), the tunnel-like openings seen on SEM micrographs of the rabbit olfactory epithelium were identified as the exit points for the Bowman's gland ducts on the surface of the olfactory epithelium.

Studies conducted in several mammals have provided a more dynamic view of the role of olfaction in the regulation of maternal care (Levy *et al.*, 2004). In the sheep, for example, parturient ewes were shown to be more attracted to a model lamb smeared with amniotic fluid than to one without amniotic fluid (Vince *et al.*, 1985), an indication that olfactory cues (provided by amniotic fluid) are necessary to ensure appropriate maternal behavior at parturition. Similar results were reported in rabbits (Melo and Gonzalez-Mariscal, 2003). In the rat, however, olfaction plays no crucial role in the initiation of maternal behavior at parturition. In this species, following prepartum destruction of the olfactory mucosa by zinc sulfate application, normal onset of maternal behavior was found in primiparous parturient females (Benuck and Rowe, 1975; Jirik-Babb *et al.*, 1984; Kolunie and Stern, 1995). Functional studies in the rabbit (Distel and Hudson, 1984) revealed that, between birth and postnatal day 5, the median time taken by the pups to attach to nipples decreases from 11.8 to 3.2 seconds. This improvement suggests an age-related increase in the ability to react to odors and may reflect a likely contribution of the aforementioned modifications in olfactory mucosal structure to the enhancement of olfactory sensitivity with development.

In contrast to earlier work on the postnatal morphology of the nasal olfactory tissue in the rabbit (Meisami *et al.*, 1990), the present study provides quantitative data on the proliferating rates of cells of olfactory epithelium and volume fractions of axon bundles, Bowman's glands and blood vessels. This study also demonstrates, for the first time, the presence of vasculature within the cores of the axon bundles in early postnatal rabbits. In conformity with findings in other species, values for neuronal

densities (Apfelbach *et al.*, 1991; Weiler and Farbman, 1997; 1998); cross-sectional diameters of axon bundles and cilia numbers per olfactory cell knob (Kavoi *et al.*, 2010) increase postnatally. However, for corresponding postnatal ages, the values for these parameters show great interspecies differences. This observation suggests that the principles of development and morphology are similar across taxa but that the quantitative and temporal variations are maintained.

4.3 EFFECTS OF VINBLASTINE AND DOCETAXEL ON OLFACTORY MUCOSAL STRUCTURE

A wide variety of chemical substances has been reported to induce morphological changes in various components of the nervous tissue (Rennie, 1993; Dangata and Kaufman, 1997; Zul Izhar and Kuldip, 2007; Giari *et al.*, 2011). In a few studies carried out on the olfactory epithelium using antimicrotubule drugs, susceptibility to lesions were shown to vary with the type of drug (Kai *et al.*, 2004), the dose level (Kai *et al.*, 2005) and the species of the animal (Kai *et al.*, 2006). In the present study, the rabbit is the preferred species for the reason that it has for long served as a useful model in toxicological investigations (Amann, 1982; Morton, 1988; Hasting, 1990; Williams *et al.*, 1990; Frame *et al.*, 1994).

Results of this study show that the morphological changes imparted on the olfactory mucosa by single injections of antincer drugs vinblastine and docetaxel are transient. Further, regenerative recovery, which restores the normal structure of the mucosa, is relatively more delayed during treatment with docetaxel as compared with vinblastine. In both cases, however, full recovery from olfactory lesions occurs 15

days post-exposure. In an investigation in which rats were exposed to methyl bromide fumes for 2 weeks at 200 p.p.m for 4 h/day for 4 days, olfactory lesions were retained for a period of 71 days (Hastings *et al.*, 1991). In mice (Kai *et al.*, 2004), studies on vinblastine, paclitaxel and videsine showed that the recovery from lesions occurred by day 5 except in the paclitaxel where the recovery was delayed until day 15. Differences in the duration of tissue recovery from toxic effects of drugs have been associated with the concentration of the drug that is distributed and retained within the tissue (Kai *et al.*, 2004, 2005). Accordingly, it seems probable that the variations in the rates of regenerative recovery of the antimicrotubule drugs are due to their differences in the mode of action and degree of retention within the olfactory mucosa.

In the normal rabbit olfactory mucosa (Meisami *et al.*, 1990), the olfactory epithelium is organized into a free zone, a non-nuclear zone and a nuclear zone, with the latter being organized into an upper, middle and a lower nuclear layer of supporting, olfactory and basal cells, respectively. The present study demonstrates a similar organization in the control and in vinblastine-treated rabbits at days 10 and 15 and docetaxel-treated animals at days 3 and 15 but a disruption of the nuclear stratification in vinblastine-exposed rabbits at days 3 and 5 and docetaxel-treatred animals at days 5 and 10. In the olfactory epithelium of the rats (Kai *et al.*, 2004), a similar disorganization of nuclear stratum was noted following administration with LD10 of paclitaxel. To occupy defined positions within the olfactory epithelium, immature cells arising from progenitor cells must migrate from the base of the epithelium (Newman *et al.*, 2000; Bock *et al.*, 2009), a processes that is facilitated

by microtubules (Rodriguez *et al.*, 2003). Therefore, disruption of microtubules by vinblastine and docetaxel may be the cause of the cell disarrangement observed in this study. Furthermore, the role of microtubules in epithelial polarization and morphogenesis and in cell movement is well document (Yap and Manley, 2001; Rodriguez *et al.*, 2003).

Degeneration of axonal bundles, which involve the apoptotic death of peripherally located cells, characterizes the olfactory mucosa of vinblastine-administered rabbits at days 3 and 5 and of docetaxel-exposed animals at days 3, 5 and 10 (both drugs were administered at human equivalent doses). This finding concurs with that of Kai *et al.*, (2004) in which a number of tubulin-targeted drugs were shown to induce apoptosis on olfactory epithelial cells of mice. While LD10 of paclitaxel in mice induces apoptosis on olfactory epithelium resulting in the atrophy of the epithelium 5-15 days post-exposure (Kai *et al.*, 2004), an equal dose of vincristine causes no morphological changes on the axon bundles (Kai *et al.*, 2002, 2005). This implies that the impact of microtubule-targeted drugs on the nasal olfactory mucosa may vary with the specific drug as well as the species of the animal. Furthermore, the presence of numerous microtubules in the axons of olfactory neurons renders the neurons highly vulnerable to toxic lesions by the microtubule-targeted agents (Schwartz *et al.*, 1991; Huard and Schwob, 1995).

Axonal bundles of vinblastine-treated rabbits at day 3 and 5 and of docetaxel-treated animals at days 5 and 10 were infiltrated with blood capillaries. This stands out as a unique finding with regard to studies involving antimicrotubule drugs. In a recent

study on the normal olfactory mucosa (Kavoi *et al.*, 2010), similar vessels were demonstrated in axon bundles of the dog. Such vessels were thought to facilitate the delivery of oxygen and nutrients to the olfactory ensheathing Schwann cells lying deep within the typically thickened bundles of the dog. Accordingly, it may be possible that the immediate need for axonal repair, regrowth and ensheathment following insult by vinblastine or decetaxel may call for an increased demand for oxygen and nutrient, which therefore results in the infiltration of the bundles by blood capillaries.

Bowman's glands of vinblastine-treated rabbits at day 3 and of docetaxel-exposed animals at days 5 and 10 show focal cell death. On day 5 following treatment of the animals with vinblastine, the glands undergo degeneration. The death of the glandular cells, as evidenced by the TUNEL assay, is by apoptosis. In mice administered with LD10 of vincristine (Kai *et al.*, 2002), the Bowman's glands remained intact, while in hamsters exposed to furfural vapours for 13 weeks, abnormal enlargement of the gland cells was observed (Feron *et al.*, 1979). This suggests that the degree and nature of lesions caused by drugs on the Bowman's glands may not only depend on the drug type but also on the duration of exposure and the route of administration. Furthermore, the capacity of an antimicrotubule drug to induce apoptosis on the nasal olfactory tissue is dependent on other factors like the route of excretion (El Dareer *et al.*, 1977) and the half-life of the drug (Krishna *et al.*, 2001).

Apoptotic cell death is also noted in the middle and basal regions of the olfactory epithelium of vinblastine-treated rabbits at days 3 and 5 and of docetaxel-exposed animals at days 5 and 10. In a study in which olfactory lesions were studied in the mouse using several categories of antincancer drugs, which included antimicrotubule agents, antibiotics, fluoropyrimidines and platinums (Kai *et al.*, 2004), only the antimicrotubule group of drugs were shown to induce apoptotic cell death in the olfactory epithelium. In conformity to the finding in the rabbit, apoptotic cell death in the mouse was restricted to the middle and basal regions of the epithelium (Kai *et al.*, 2004, 2006). The induction of apoptosis by the antimicrotubule drugs result from interference with mitotic spindle microtubules and intra-axonal transportation (Gidding *et al.*, 1999; Kai *et al.*, 2004, 2006). The confinement of the apoptotic cells in the middle and basal parts of the epithelium has been associated with the presence, in these regions, of immature sensory cells and globose basal cells (Kai *et al.*, 2004), which are known to divide with the most rapid mitotic cycle (Schwartz *et al.*, 1991; Suzuki and Takeda, 1991).

Normally, the nasal epithelium remains in a steady state of equilibrium between cell loss and cell replacement (Fabrikant & Cherry, 1970). Exposure to noxious substances may disrupt this fine balance, leading to modification in the normal architecture or function of the cells lining the various structures in the nasal cavity (Boorman *et al.*, 1990). Data obtained in the present study indicated a substantial increase in cell proliferation (DNA synthesis) occurring in the rabbit olfactory epithelium on the 5^{th} and 10^{th} day following administration with vinblastine and docetaxel respectively. These data correlate with the histological evaluations and

may be considered reflective of the regenerative tissue repair mechanisms following exposure to the antimicritubule drugs.

In previous studies involving antimicrotubule drugs, the surface features of the olfactory epithelium have not been examined at SEM level. In this study, it was found that the pattern of projection of the cilia from the olfactory cell knobs varies with the duration of exposure of the olfactory mucosa to the antimicrotubule drugs. In the control and in rabbits treated with vinblastine and sacrificed on days 10 and 15 and with docetaxel and euthanized on days 3 and 15, the knobs projected their cilia from around their bases in a radial pattern. In contrast, vinblastine-treated rabbits at days 3 and 5 and docetaxel-exposed animals at day 5 and 10 had the cilia projecting from the tip of the knobs to run in parallel in the form of tuft. The normal disposition of the cilia in the rabbit is similar to that of humans (Lenz, 1977), horse (Kumar *et al.*, 2000) and dogs (Kavoi *et al.*, 2010). The change in the pattern of cilia projection from radial to parallel a few days after exposure to the antimicrotubule drugs and back to radial several days post-exposure may be due to the disruption of the cilia microtubules by these drugs.

Relative to control values, the packing density of the olfactory cells and the number of cilia per olfactory cell knob are significantly lower in vinblastine and docetaxel-treated rabbits on days 3, 5, and 10. In rats exposed to methyl bromide, repair of the olfactory epithelium from the injurious effects of this substance was characterized by the development of abnormally large olfactory cell knobs with reduced number of cilia (Hastings *et al.*, 1991). A recent study by Giari *et al.* (2011) reports a similar

loss involving kinocilia in neuromasts of fish exposed to cisplatin. While the reduction in the packing density of the olfactory cells may be a direct consequence of the mitotic inhibitory effects (reduced formation of olfactory neurons) by the antimicrobule drugs vinblastine and docetaxel, the loss of cilia on individual nerve cells may portray the disruptive effects of these drugs on microtubules. Besides, microtubules have been cited as a key structural component of the cilia (Burkitt *et al.*, 1993).

Treatment of the rabbits with vinblastine or docetaxel results in a decrease in the thickness of the olfactory epithelium, with return to normal thickness by day 15. Death of supporting and olfactory cells is a key feature following topical application of benzalkonium chloride on the olfactory mucosa of rabbits (Cureoglu *et al.*, 2002). In this species also, loss of mature olfactory neurons because of antenatal hypoxia-ischaemia causes a significant reduction in the height of the olfactory epithelium (Drobyshevsky *et al.*, 2006). In the rat (Apfelbach *et al.*, 1991), a direct correlation between the number of neuronal cells and the thickness of the epithelium has been demonstrated. In this study therefore, the observed decrease in epithelial thickness may have resulted from death and/or decrease in the formation of the olfactory cells following exposure to the antimicrotubule drugs.

Diameters of axon bundles decrease by close to 50% in vinblastine-treated animals at day 5 and in docetaxel-exposed animals at day 10. Thereafter, the bundle diameters progressively increase to control sizes. Death of olfactory sensory cells, which results in atrophy of the axon bundles and therefore a reduction in the

thickness of the bundles, has been reported following inhalation exposure of the olfactory mucosa to acetaldehyde (Appelman *et al.*, 1982), formaldehyde (Kerns *et al.*, 1983) and dimethylamine (Buckley *et al.*, 1985). In toxicity studies in mice (Kai *et al.*, 2004), degenerative changes involving axon bundles were associated with the disruption of axonal transport, a process that has also been implicated in the induction of neuropathy. Thus, degeneration of the axon bundles, which occurs from the periphery towards the inside, may be the cause of the decreased bundle diameters values noted after treatment of the rabbits with the antimicrotubule drugs.

Volume fractions for the axonal bundles decrease significantly in vinblastine-treated rabbits at days 3 and 5 and in docetaxel-exposed animals at days 3, 5 and 10. For the blood vessels, the volume fraction in the controls is lower than in vinblastine-treated animals at day 3 or 5 and in docetaxel-exposed animals at day 5 or 10. While the decrease in volume fraction for the bundles may be associated with their degeneration following exposure to the antimicrotubule drugs, the post-exposure increase in volume fraction of the vasculature may reflect an increased demand for oxygen and nutrients during the process of mucosal repair. The insignificant change in the volume fraction noted in regard to glands in the treated (either with vinblastine or docetaxel) may imply that the glands were less susceptible to insult by the drugs as compared with the bundles.

Cancer chemotherapy has a significant effect on olfactory and gustatory function, possibly causing nausea, reduced appetite, low energy intake and weight loss (Bernhardson *et al.*, 2008; Steinbach *et al.*, 2009). Additional spices and flavouring

may compensate for this diminished chemosensory function, thereby enhancing patient compliance and quality of life (Steinbach *et al.*, 2009). In human cancer therapy, vinblastine and docetaxel are given at weekly doses of approximately 0.10 mg/kg and 2.03 mg/kg respectively (Tucker and Winkelmann, 1976; Klein *et al.*, 1980; Solan *et al.*, 1981). These, according to Reagan-Shaw *et al.*, (2007), translate to respective dosage value of 0.31 mg/kg and 6.26 mg/kg in the rabbit. In the current study, the drugs vinblastine or docetaxel were administered as single doses, and their effects on olfactory mucosal morphology examined over a period of approximately two weeks, with the finding that the mucosa acquires normal morphology and function within this period. Unlike in previous studies on antimicrotubule drugs in which analysis for changes in structure was mainly confined to the epithelium of the olfactory, the current investigation incorporated other key mucosal components including axonal bundles, Bowman's glands and vasculature. Moreover, animals for this study were sacrificed at fairly short durations following exposure to the antimicrotubule drugs.

4.4 EFFECT OF VINBLASTINE AND DOCETAXEL ADMINISTRATION ON OLFACTORY FUNCTION

In the present work, analysis of olfaction following treatment of the rabbits with antimicrotubule drugs vinblastine and docetaxel was performed using the buried food recovery test. This behavioral test, which is used to gauge olfactory function, tasks the subjects with relying on the sense of smell to locate food (Duncan-Lewis *et al.*, 2011). Olfactory function has also been studied using other techniques including olfaction maze test (Lu *et al.*, 2008), odor discrimination test (Gheusi *et al.*, 2000;

Enswere *et al.*, 2004) and the 2-nozzle drink test (Lien *et al.*, 1999; Schaefer *et al.*, 2000). However, none of these methods have hitherto been applied to study olfaction in lagomorphs. Compared to the buried food test, the odor discrimination and the 2-nozzle drink tests are less accurate methods in that they require learning and memory inputs, and the 2-nozzle drink test examines the sense of taste and its associated pathways (facial and glossopharyngeal nerves) in addition to the sense of smell (Lu *et al.*, 2008). In toxicity studies using microtubule disrupting drugs, susceptibility to olfactory lesions has been shown to vary among species (Kai *et al.*, 2006). This notwithstanding, the use of the buried food test to assess drug-induced and other types of anosmia has for over the last four decades been confined to animals in the order rodentia (Alberts and Galef, 1971; Edwards *et al.*, 1972; Hurtt *et al.*, 1988; Genter *et al.*, 1996; Luo *et al.*, 2002; Nathan *et al.*, 2004; Dawson *et al.*, 2005; Getchell *et al.*, 2006; Lu *et al.*, 2008; Yang and Crawley, 2009; Duncan-Lewis *et al.*, 2011).

Results of the buried food test in vinblastine-treated rabbits show a progressive increase in food-recovery time in the first 3 days of exposure. Five days after, the animals are unable to use the olfactory cue to find the cookie. This is followed by recovery from the olfactory deficit and therefore a subsequent decrease in the food-finding time on day 7 and progressively to day 15. With docetaxel treatment, the food-finding time decreases progressively but with the olfaction loss being delayed until post-exposure day 10, after which the animals drastically recover from the olfactory deficit. In toxicity studies, olfactory impairment has been linked to lesions produced on the olfactory mucosa by several substances including zinc sulfate

(Alberts and Galef, 1971; Slotnick and Gutman, 1977), zinc gluconate (Duncan-Lewis *et al.*, 2011), methyl bromide (Hurtt *et al.*, 1988; Hasting, 1990; Hasting *et al.*, 1991), 3-methylindole (Turk *et al.*, 1986, 1987; Peele *et al.*, 1991), 3,3'-iminodipropionitrile (IDPN), dichlobenil and methimazole (Genter *et al.*, 1996). With vinblastine or docetaxel treatment, normal olfactory function is observed even in the presence of continuing morphological evidence of damage. These findings agree with those of inhalation studies by Hurtt *et al.*, (1988) and Hasting (1990) who demonstrated that recovery from methyl-bromide-induced olfactory deficits precedes complete histological recovery.

Previously, the buried food test has been used to study some interesting phenomenon related to olfaction. In the study by Lu *et al.*, (2008), this test was used to compare olfaction of normal mice with those lacking aquaporin-4, a water-selective transport protein whose deficiency in glial cells results in altered neuroexcitation. The authors report that the latency times in the normal mice were lower compared to aquaporin-4 deficient mice. In a recent paper (Duncan-Lewis *et al.*, 2011), the buried food test was used in the mice to investigate the effects of zinc gluconate, an intranasally administered drug used to treat common cold and whose safety was in doubt, with the finding that the mean latency time in the treated animals was significantly higher than of the controls. In a study comparing the effects of IDPN, dichlobenil and methimazole (Genter *et al.*, 1996) on olfactory function, the magnitude and duration of olfactory impairment were shown to differ among the three chemicals. While the IDPN and dichlobenil caused transient deficits in olfactory function, the methimazole produced extensive mucosal damage resulting in prolonged loss of

olfactory function. Going by these reports, it can be inferred that the degree of damage of olfactory tissue varies in different drugs probably due to variations in their mode of action. Since vinblastine and docetaxel act differently on microtubules, they are also most likely to vary in their rates and levels of olfactory tissue damage and therefore olfaction impairment.

Drugs used in the present study are antiproliferative. Consequently, olfactory deficits produced in the rabbits by these drugs are likely due to failure of continous formation and replacement of degenerating neuronal cells. In the assessment of olfactory function using the buried food experiment, Genter *et al.*, (1996) attributes several other factors to the increased latency following toxicant administration. These include (1) the presence of debri in the airways, resulting from the sloughing of the damaged epithelium, impairing of air flow and therefore the interaction of the inhaled odorants with the odorant receptors and (2) the feeling of general malaise associated with administration of most toxicants. Necrotic lesions involving the delicate skin around the eyes and ears are observed in the present study. In the clinical use of vincristine, necrotic lesions have also been documented in other organ systems including the gastrointestinal tract (Kanter *et al.*, 1994).

It is noteworthy that the data generated from this test need to be interpreted with caution. This is because the drugs, unlike those used in the aforementioned studies, were administered systemically thereby imparting their effects not only on the olfactory mucosa but also on other nervous tissue components including olfactory bulb, hippocampus, amygdala, orbital frontal cortex, anterior olfactory nucleus and

entorhinal cortex, all of which are important for processing olfactory information (higher-level odor tasks) (Eichenbaum *et al.*, 1983; Boyles *et al.*, 1985; Nordin and Murphy, 1996; Grehan *et al.*, 2001; Nathan *et al.*, 2004). Hence, the lesions imparted by the drugs in these other nervous tissue areas may contribute to some of the observed olfactory deficits.

4.5 CONCLUSION AND FUTURE DIRECTION

4.5.1 CONCLUSION

Results of this study show that the structure of the rabbit olfactory mucosa is progressively refined during postnatal development and that such modifications may be ascribed to the unusually high olfactory functional demands reported in the juveniles of this species. It is further concluded that single injections of anticancer drugs vinblastine and docetaxel produce reversible alterations in the structutre of the olfactory mucosa and in olfactory function in the adult rabbit. Regenerative recovery, which restores the normal structure of the olfactory mucosa and olfactory function, is relatively more delayed during treatment with docetaxel as compared with vinblastine.

4.5.2 FUTURE DIRECTION

Future studies should compare the developmental patterns of the olfactory mucosa with those of other components of the olfactory system, including the olfactory bulb, preferably using a wider range of age groups and species. Additionally, the long-term effects of repeated doses of various anticancer agents on the olfactory mucosa and on other olfactory structures should be studied. Besides, data on the molecular

mechanisms explaining the lag phase between vinblastine and docetaxel treatment and on the effects of chemical, microbial and/ or physical perturbations of the various olfactory pathways are warranted.

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