White rhinoceros *Ceratotherium simum* horn development and structure: a deceptive optical illusion

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Abstract

The alleged traditional medicinal properties of rhinoceros horn resulted in a dramatic escalation in rhinoceros poaching in South Africa. Despite the listing of all species of rhinoceros in the International Union for Conservation of Nature Red List of threatened animals, their numbers are still declining rapidly. Based on the assumption that rhinoceros' horn consists of a collection of hollow tubules and intertubular hollow spaces, which allow internal fluid distribution, a horn devaluation procedure through infusion of chemicals and dyes was recently introduced. This procedure is costly and has a mortality risk. This study provides the first detailed description of the development and resultant structure of the rhinoceros horn. The unique solid structure which consists of a large number of tightly packed filaments is the result of the cellular orientation of squamous epithelium corneocytes. What was previously thought to be microtubules is an optical illusion created by the orientation of the corneocytes in the solid filaments. We contest the scientific basis for infusing chemicals into the rhinoceros horn as a deterrent for human use.

Keywords: White rhinoceros; horn structure; onychokeratinization; keratin; dermal papillae

Introduction

South Africa is home to more than 80% of Africa's rhinoceros population and more than 70% of all wild rhinoceros worldwide. The continued use of rhinoceros horn for its alleged traditional medicinal properties has resulted in a dramatic escalation in poaching and illegal horn trade in Africa (Daffue, 2014). Rhinoceros appear on the International Union for Conservation of Nature Red List of threatened species, with three out of five species classified as critically endangered (IUCN 2014). The general macroscopic features of African species of rhinoceros horns are well described (Kock & Atkinson, 1993). Although varying

opinions and terminology on the microscopic structure of rhinoceros horn exist, no descriptive study relating to the actual development of rhinoceros horn is available in the literature. The lack of knowledge on the developmental biology of the horn and its resultant structure leave wildlife conservationists with no scientific basis to consider proactive devaluation procedures aimed at saving the rapidly diminishing rhinoceros populations from extinction.

The infusion of horns with a combination of indelible dye and ectoparasiticides (Rhino Rescue Project, 2013) is based on the assumption that the horn consists of a collection of hollow tubules or canals (Boas, 1931; Makinson, 1954), hollow keratin fibres (Penny, 1987), cracks (Van Orden & Daniel, 1993) or that it has gas spaces (Ryder, 1962) which could be filled with fluid. This venture, aimed at preventing end-of-line usage carries a mortality risk and is now adding another threat to the future of these animals (Ferreira *et al*., 2014). The aim of this study was to provide the first detailed description of rhinoceros horn development in order to explain its unique microscopic structure and establish a scientific basis for programmes aimed at discouraging end use of the product.

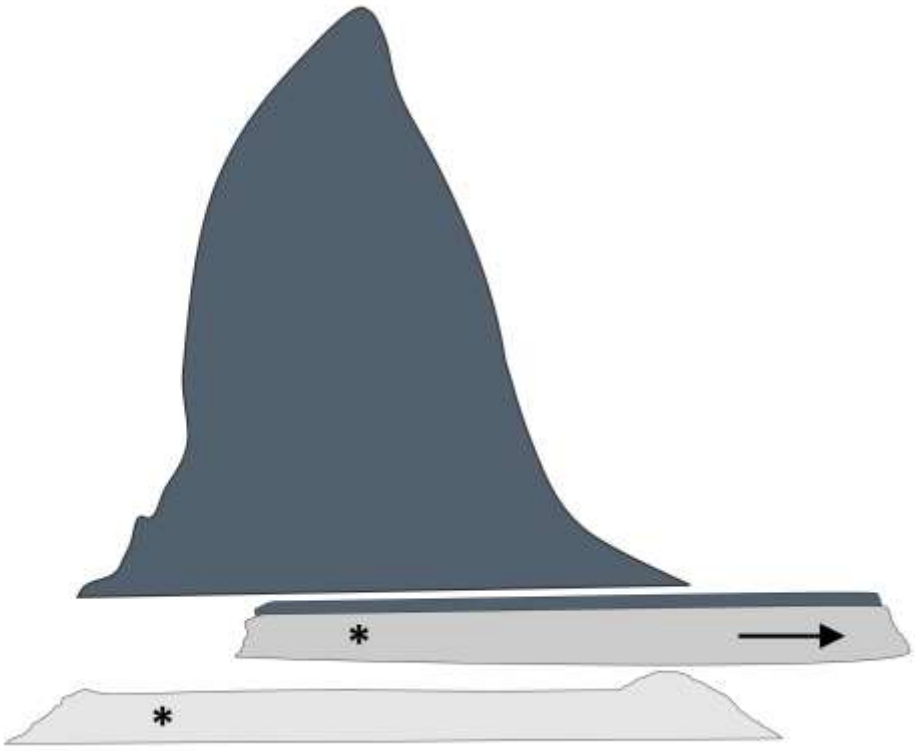


Figure 1. The diagram demonstrates the horn base area as defined in this study. The dark grey represents the horn structure proper and the more proximally situated light grey area in the schematic drawing (asterisk) represents the soft tissue that attaches the horn to the underlying nasal bridge. The horn base in this study is therefore the most proximal aspect of the rhinoceros horn and the connective tissue that attaches it to the nasal bridge of the animal.

Materials and methods

The horn bases (Fig. 1) of six white rhinoceros *Ceratotherium simum* were studied macroscopically and by light, digital and stereomicroscopy. For the purpose of this study, the horn base was defined as the most proximal aspect of the horn and the connective tissue directly underneath it (Fig. 1). The sample contained a mixture of fragments from both frontal

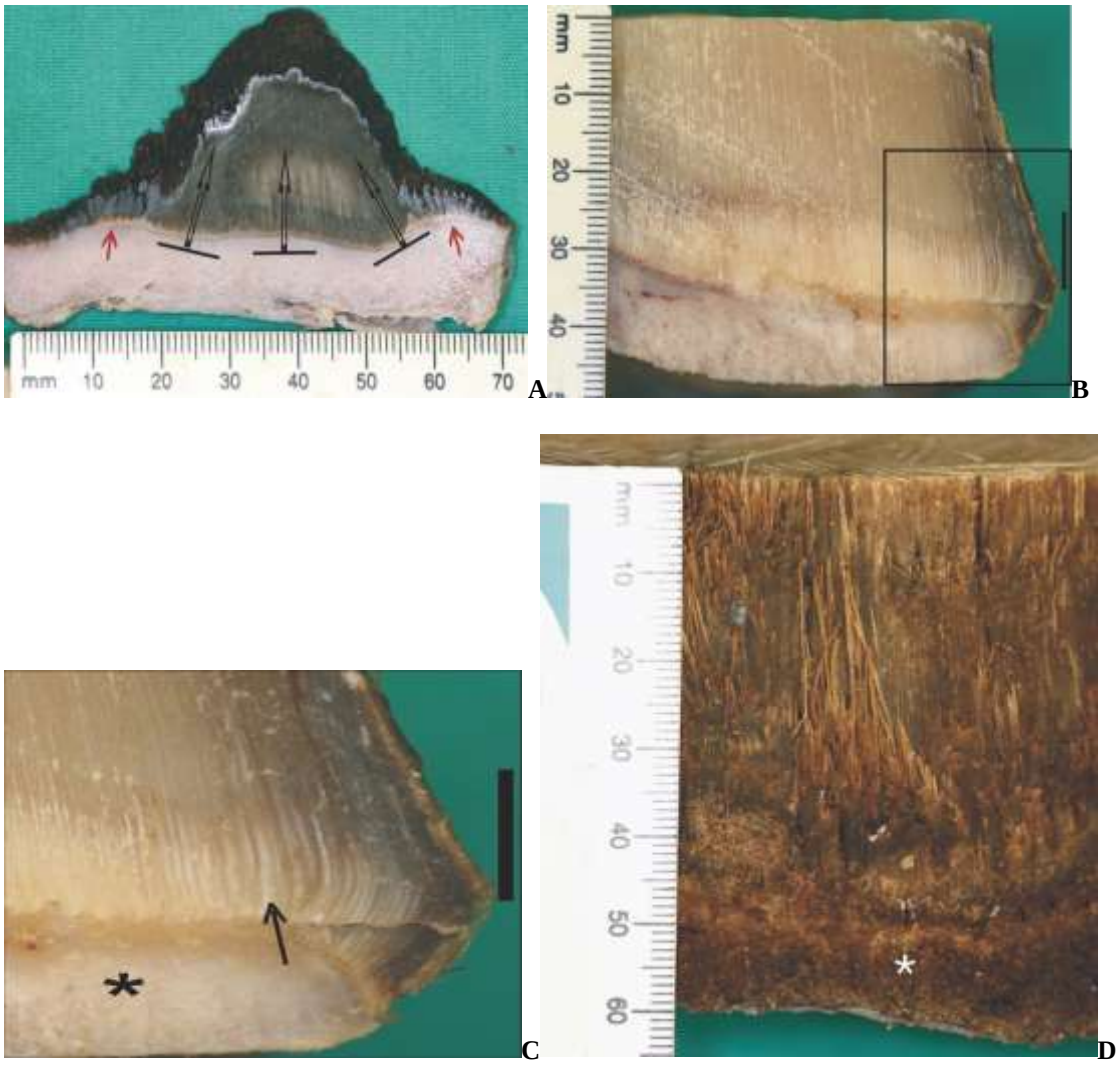


Figure 2. Macroscopic images of sagittal sections through (a) an early developing calve horn demonstrates how the epidermal basal membrane dips into the underlying connective tissue. The slanting of the filamentous structures towards the central aspect of the horn is demonstrated with long arrows. All elongating filaments adhere to each other as a solid mass extending dorsally. Addition of more filaments on the peripheral aspect of the elongating horn is demonstrated on the photo (short arrows). Sagittal sections through the adult horn base (b) demonstrate the individual filamentous structures which originate from the corneum and extend dorsally forming the bulk of the horn. (c) Higher magnification of the marked area in (b). The arrow points to the individual filaments and the asterisk shows the connective tissue of the horn base. (d) Dry and tethered filaments extending from the skin (asterisk) as seen on the outer surface of an adult horn. (Scale bar in b and c = 10 mm).

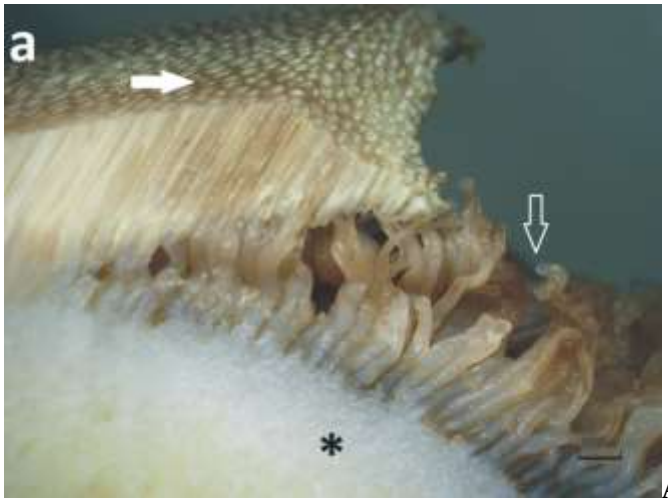
and nasal horn bases. The animals were four adult bulls (one animal 15 years and the other three 5 years of age) and two calves (9 months old and one new born). The animals were presented for postmortem examinations at the Onderstepoort Veterinary Faculty of the University of Pretoria for reasons unrelated to this study. For the purpose of this study, the skin area where the horn develops in the calves will be referred to as the horn placode. The horn bases and placodes were sectioned in mid-sagittal and mid-coronal planes to visualize the skin-to-horn transition as well as the attachment of the horn base to underlying connective tissue (Fig. 2 plate). Blocks for microscopic examination were obtained from the entire width of the horn bases and placodes extending from the adjacent skin through the central aspects of the horns. All specimens were fixed in 10% buffered formalin and processed overnight. Sections, 3–4-μm thick were cut from the paraffin embedded tissue blocks and stained with haematoxylin and eosin according to standard histology laboratory protocol. The microscopic slides were examined by two experienced senior histopathologists. Scientific and ethics committees of the South African National Parks (SANParks) and University of Pretoria, South Africa approved the study.

Results

Macroscopic examination: adult horn base

Macroscopically, a transverse section through the skin adjacent to the horn bases confirmed the epidermis to gradually thicken as it approached the horn (Fig. 2a). Closely packed filamentous structures became visible in the skin closer to the horn gradually increasing in length to eventually form the horn proper as it extend dorsally through the entire length of the horn structure examined (Fig. 2a–c). Distinctly evident in the sections through the young calf horn was the epidermal basal membrane which changed its orientation to dip into the underlying connective tissue (Fig. 2a). This resulted in the associated filamentous structures to slant slightly towards the central aspect of the young horn (Fig. 2a). These filamentous structures on the outer surface of the adult horns presented as tethered dry structures originating from the underlying skin area continuing dorsally parallel to each other (Fig. 2d). Further examination of the basal/connective tissue aspects of the horn using a digital microscope (Nikon ShuttlePix P-MFSC system; Nikon Corporation, Tokyo, Japan) revealed slender dermal papillae which originated from the underlying connective tissue (Fig. 3a). The papillae could focally be separated from a harder sheath-like structure surrounding it (Fig. 3a). A stereomicroscopic surface view of a transverse section made 28 mm dorsal to the underlying connective tissue base confirmed the soft dermal papillae to be absent at this distance. Instead, a solid mass of clearly delineated structures was seen (Fig. 3b). The calf's horns were mostly black in colour with the pigmentation extending from the soft dermal papillae into the overlying horn tissue. The black pigmentation of the dermal papillae and its

associated hard tissue was, however, only present towards the central area of the adult horn with the peripheral aspects in a lighter sandstone colour (Fig. 2).



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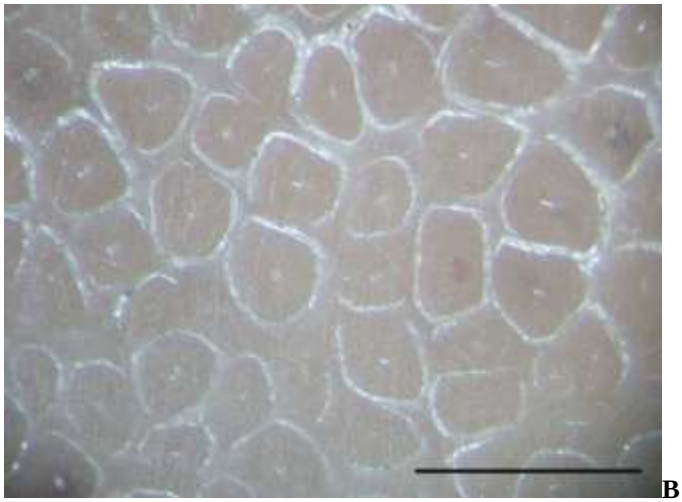
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Figure 3. A sagittal section through the central core of an adult horn base demonstrates (a) the dermal papillae (open arrow) extending from the underlying connective tissue base (asterisk). The white structure seen on the left (solid arrow) represents a sheath-like structure from which the papillae were pulled when cut. (b) Stereomicroscopic visualization of the tissue depicted in (a) in a coronal plane shows the solid nature of the horn structure with no empty holes 28 mm dorsal from the underlying connective tissue. (Scale bar = 1 mm).

Hair and its associated follicles were still visible between the filamentous structures in the adjacent skin but disappeared in the horn base area. Beneath the epidermis, three different zones were clearly discernible within the underlying connective tissue. The first, situated immediately under the epithelium, exhibited a more delicate and almost translucent appearance compared with the second, thicker zone where the white connective tissue fibres appeared coarser (Fig. 4). The third zone could be seen in some specimens and represented the connective tissue of the underlying periosteum.

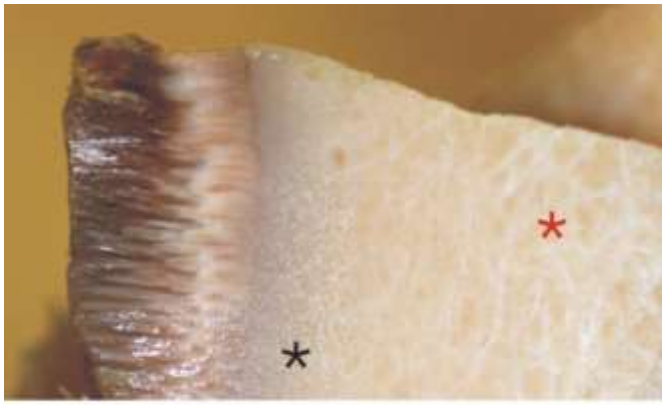


Figure 4. The macroscopic image shows two of the three connective tissue zones described. The thinner and more translucent connective tissue zone (asterisk at the bottom) is clearly delineated from the deeper zone with visibly coarser connective tissue arrangement (asterisk at the middle right side).

Macroscopic examination: calves' horn bases and placodes

The skin overlying the horn placodes had a smoother appearance than the surrounding skin (Fig. 5). A slight indentation in the skin delineated the placode area from the skin adjacent to

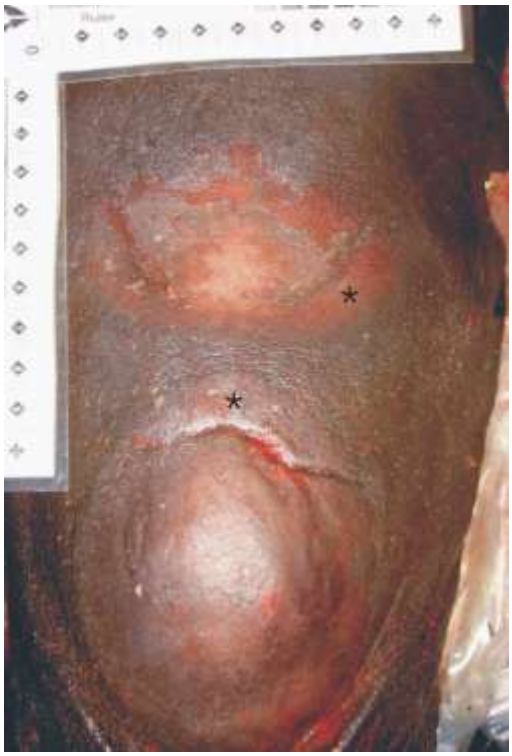


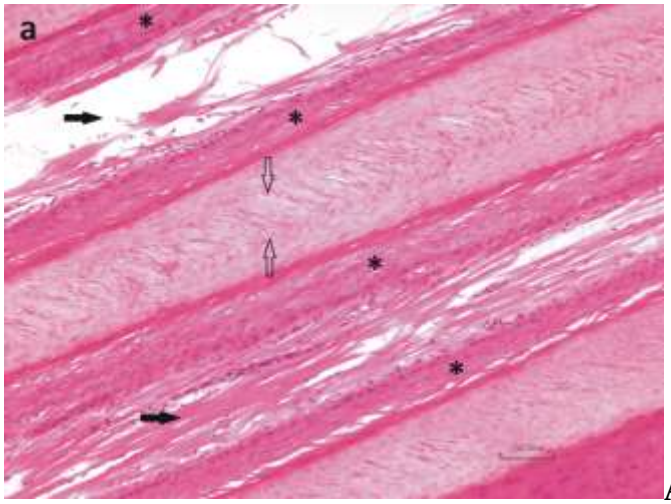
Figure 5. A clinical photograph of the developing frontal and nasal horns of a rhinoceros calf demonstrates the change in the appearance of the skin in the transition zone from skin to horn placode area (asterisks).

it. Sections through the thin placodes and calves' horn bases demonstrated similar features to those of the adult including the slanting of the shorter filamentous structures and different

connective tissue zones. Prominent blood-filled spaces were apparent between the filaments. The connective tissue zones, similar in appearance and proportions to the adult horn, were thinner measuring less than 1 cm in thickness in the newborn calve horn placode. Hair follicles were completely absent in the smooth skin of the horn placode.

Microscopic examination: adult horn base

Microscopic examination of the epidermis demonstrated a gradual transition from orthokeratinization to onychokeratinization commencing more than 10 mm away from the horn. A gradual loss of keratohyaline granules in the superficial epithelial layers with abrupt keratinization characterized this zone. The epidermal thickening towards the horn was partly because of the increased keratinization but mostly the result of prominently increased interdigitation between epithelial rete ridges and its elongating dermal papillae (Fig. 6a). The dermal papillae increased in length, each covered on all its surfaces by squamous epithelium organized in three distinct zones. The first represented a basal or germinative layer of epithelial cells with basophilic nuclei, the second consisted of 5–8 layers of the stratum spinosum and the third of 3–4 layers of flattened keratinocytes with brightly eosinophilic cytoplasm, some with and others without nuclei (Fig. 6a,b). The third layer of the epithelium was covered by pale staining degenerating corneocytes characterized by a clear cytoplasm, conspicuous thin cell membranes and scattered retained small eosinophilic nuclei (Fig. 6b). A distinct long-axis arrangement characterized the pale corneocytes, orientated parallel to the surface epithelium it originated from. Those originating from the tips of the dermal papillae and from the valleys between two adjacent dermal papillae were orientated with an almost right angle to those originating from the epithelial cells covering the long walls of the dermal papillae. The columns of differently orientated corneocytes resulted in the long filamentous structures seen throughout the entire rhinoceros horn. Vascular channels were demonstrated in the entire length of the dermal papillae which extended approximately 13 mm into the horn (Fig. 6a,b). Papillae in the central aspects of the horns were extensively pigmented because of the intracytoplasmic melanin in most of the epithelial cells covering it. Slides prepared from the tethered filaments on the outer surfaces of some of the adult horn bases displayed sheets of corneocytes with similar features but without dermal papillae. Neurovascular bundles of varying sizes and some large muscular arteries were found focally in the sub-horn connective tissue. Skin adnexal structures identified within the dermis of the adjacent skin included hair follicles with normal but relatively small sebaceous units and apocrine-type glands with its associated excretory ducts opening on the epithelial surface. None of these structures were found in the connective tissue beneath the horn base. Instead, large neural structures reminiscent of tactile corpuscles were found in this area. It consisted of a collection of neural bundles, several hundred micrometres in diameter and distributed evenly in the dense connective tissue.



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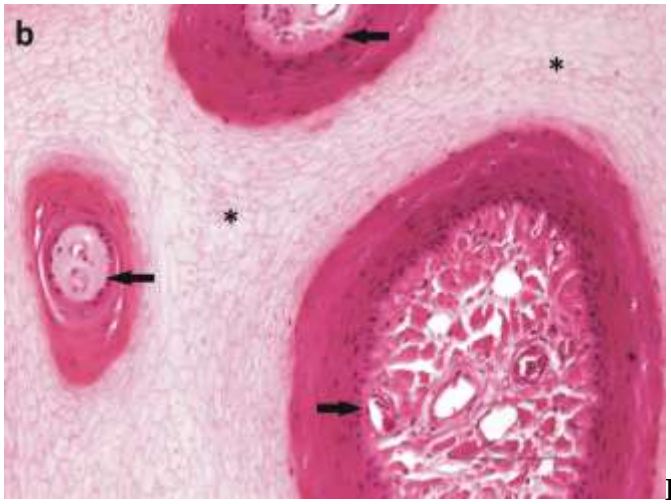

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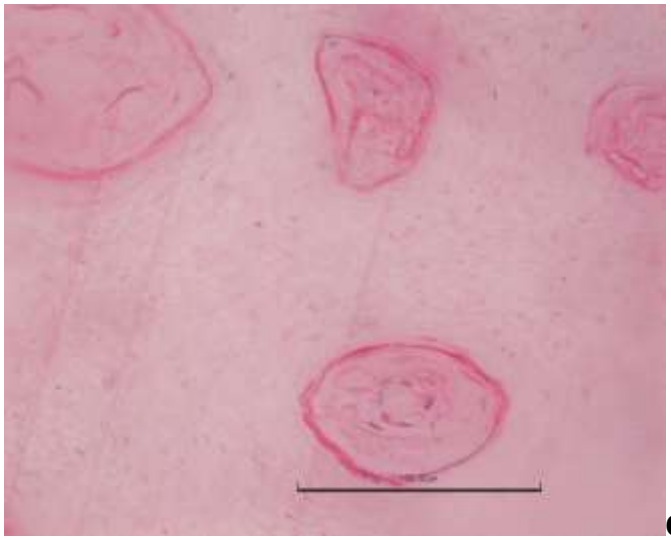

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Figure 6. The microscopic image of (a) a longitudinal section through the dermal papillae as seen in Fig. 3a demonstrates the connective tissue (closed arrows) covered on its lateral walls (asterisks) with differently orientated onychokeratinizing epithelium maturing towards the outer aspects (open arrows). (b) High magnification of a cross-section of the same area depicted in Fig. 3a demonstrates onychokeratinization (asterisks) on the lateral walls of three adjacent dermal papillae (arrows). (c) A cross-section of the tip of the calves' horn in Fig. 2a shows differently orientated corneocytes with no dermal papillae visible. No tubules or other open spaces are visible between the columns of corneocytes (Scale Bar = 0.100 mm).

Microscopic examination: calves' horn base and placode

The calves' horn base and placode showed features identical to those of the adult horns with difference only in the shorter and more vascular onychokeratinizing dermal papillae and inflammatory cell infiltrates within the papillae and superficial keratin layers. The tightly packed columns of corneocytes and their cellular orientations in relation to one another were clearly visible in all sections examined. Slides prepared from the most distal aspect of the calves' horns displayed sheets of differently orientated corneocytes without dermal papillae (Fig. 6c).

Discussion

The literature is incongruous on the structure and composition of rhinoceros horn. Work dating back to the 1930s described it to be composed of tubules, canals (Boas, 1931; Makinson, 1954), matted hair and keratin fibres cemented together (Montagna, 1959). Results obtained from studies on other cornified papillary epidermal appendages, such as ungulate hoof walls, bovid artiodactyl horns, baleen plates and cockatoo bills, are frequently extrapolated to the micro-anatomical aspects of rhinoceros horn (Homberger, 2001) causing further confusion. In his seminal article published in Nature in 1962, Ryder indicated his preference for the term filament rather than tubule (which suggests a hollow structure) or fibre (which suggests equivalence to hair). He, however, completed his manuscript by stating that there is no doubt about the evolutionary association between hair and rhinoceros horn structure and concluded the horn to be composed of closely packed filaments of concentric laminae around a solid medulla with interfilamentous material in the interstices (Ryder, 1962). Describing rhinoceros horn as a collection of hollow keratin fibres or to have structural similarities to hair, however, persisted into the new millennium (Lynch, Robinson & Anderson, 1973; Van Orden & Daniel, 1993; Hieronymus & Witmer, 2004; Hieronymus, Witmer & Ridgely, 2006). Rhinoceros horn structure has been investigated by light microscopy (Ryder, 1962; Van Orden & Daniel, 1993; Hieronymus *et al*., 2006), scanning electron microscopy (Van Orden & Daniel, 1993), energy dispersive X-ray spectroscopy and X-ray diffraction analysis (Van Orden & Daniel, 1993) and most recently X-ray computed tomography (Hieronymus *et al*., 2006). Although recent authors mention the horn to develop from a generative layer of epidermis covering a dermal papilla (Hieronymus *et al*., 2006), schematic representations depict the horn tubules to have a medullary cavity surrounded by a cortex both set in an amorphous intertubular matrix. It is this image that has resulted in the development of the currently highly debated anti-poaching technique of chemical infusion believed to be effective as a result of the distribution of chemicals throughout the horn via these empty tubules.

The results from this detailed micro-anatomical study confirmed rhinoceros horn to consist of multiple columns of tightly packed corneocytes originating from elongated dermal papillae covered by onychokeratinizing epithelium. This distinct but well-known type of keratinization is similar to that seen in human nail development and growth (Martin, 2013). The nail grows as a single, flat and broad structure whereas rhinoceros horn filaments are numerous, tubular and thin. Both are the result of the epithelial covered dermal structures they originate from. The nail develops from the proximal nail bed matrix which is situated between two epithelial covered dermal structures namely the ventral portion of the proximal nail fold dermis and nail bed dermis. These two epithelial covered dermal structures resemble the lateral surfaces of two adjacent rhino horn dermal papillae while the proximal attachment between the ventral and proximal nail fold epithelium correlates with one of the valleys between two horn papillae (Fig. 7). The human nail does not have an anatomical equivalent to the tips of the horns' dermal papillae. In contrast to the single nail bed for each finger or toe, approximately eight dermal papillae originate per square millimeter connective tissue patch on the nasal bridge of the rhinoceros. Through onychokeratinization of the tips, valleys and lateral surfaces of each of these dermal papillae, a conical mass of corneocyte columns develop. Our study failed to identify intertubular matrix, empty medullary cavities or spaces between the corneocyte columns as previously suggested (Lynch *et al*., 1973; Van Orden & Daniel, 1993; Hieronymus *et al*., 2006). The previously described small ‘holes’ visible in the horn structure on cross-sections can be explained in two ways. Within the first 10–20 mm of the horn connective tissue, they are the result of the dermal papillae surrounded by keratinocytes and corneocytes (Fig. 6a). Those more distally is an optical illusion created by the differently orientated columns of corneocytes (Fig. 7). The empty-appearing cavities in fact represent corneocytes visualized in cross-section through their long axis and the so-called intertubular matrix between the corneocytes visualized in their long axis. No empty cavities other than small artefactual tears in the thin slides were found in any of the transverse sections evaluated on all levels of the horns examined (Fig. 6c).

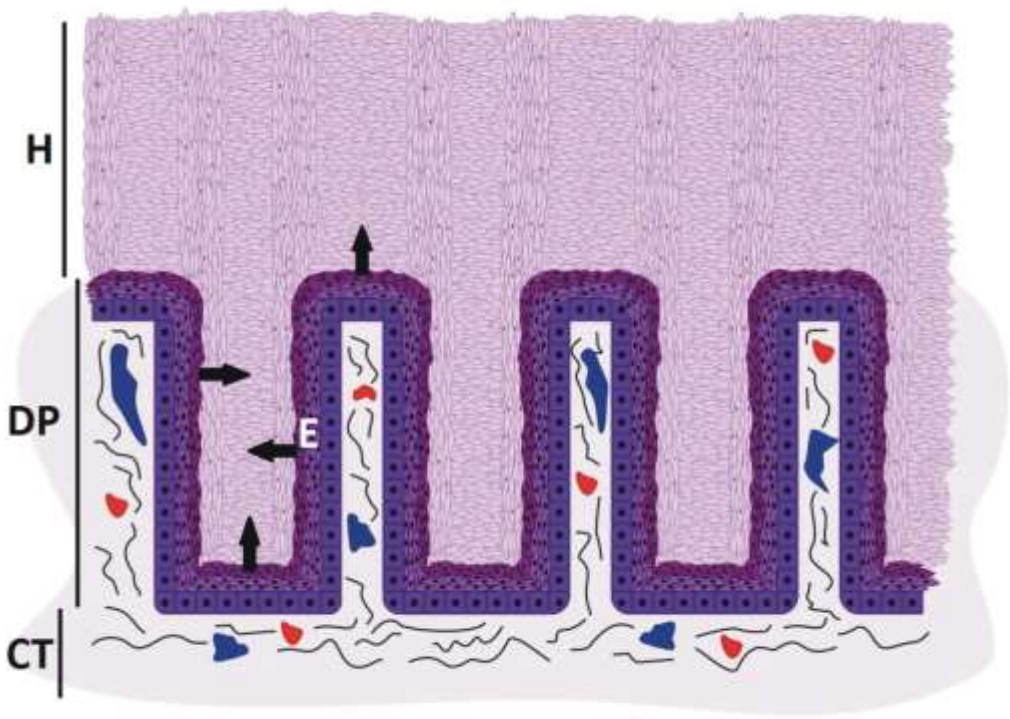

Figure 7. The diagram demonstrates the microscopic image of an approximately 3 cm slice taken from the horn base area as defined by this study. It includes some underlying dermal connective tissue (CT), the dermal papillae (DP) that measures about 15 mm in length and about 13 mm of horn structure (H). The DP is covered by onychokeratinizing epithelium (E) which gives origin to the corneocyte columns responsible for the entire horn structure. The different long-axis orientations of the corneocytes originating from the different epithelial areas are shown with arrows. The corneocyte columns are tightly packed with no hollow tubules or cavities.

Various reasons to explain the conical morphology of the rhinoceros horn has been proposed. Suggestions included higher concentration of melanin and calcium salts in the centre of the horn (Hieronymus *et al*., 2006) and differential wear. The current study adds another possibility to explain this phenomenon. In the sections through the calf horn, the epidermal basal membrane dipped into the underlying connective tissue (Fig. 2a). This results in the associated dermal papillae to slant slightly towards the central aspect of the horn (Fig. 2a). The corneocyte columns associated with each dermal papilla elongate individually and fuse with the central part of the horn. With advancing age, the horn base increases in diameter adding more dermal papillae at the periphery of the lengthening mass of corneocyte columns (Fig. 2a). The new peripheral columns fuse with the central part and are shorter because of their more recent development. As the horn increases in length, the most distal columns undergo dehydration and shrinking, further enhancing the conical shape of the horn. The oblique positioning of the basal membrane and its dermal papillae was not observed in the adult horns. It is postulated that the slanting seen in the early developing calf's horn dictate and initiate the conical form of the rhinoceros horn.

No theory that relates the structure of the horn with its mechanical function is proposed in the literature. This might be because of the lack of information on the actual role of the horn besides it being used in self-defence or to protect their young (Boeyens & van der Ryst, 2014). For this function, however, it needs to be strong and able to withstand forces from different directions, therefore flexible to a certain extent without being brittle. It is postulated that like in human nails (Farren, Shayler & Ennos, 2004), the different orientations of keratinocytes within the horn structure provides this multidirectional yet flexible strength comparable with the human nail. The large tactile-like neural corpuscles in the sub-horn connective tissue most probably act as specialized mechanoreceptors with a sensory function for touch and pressure. It is postulated that these corpuscles also act as protective mechanism to prevent the animal from exerting excessive pressure on its horn during the use thereof. Further studies are, however, required to establish their morphology and function.

The information added to the literature by this study provides proof that both the microscopic and macroscopic structures of the rhinoceros horn are the direct result of its development and should form the basis for improved scientific and clinical research relating to rhinoceros conservation.

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