MORPHO-HISTOLOGICAL STRUCTURE STUDY OF EQUINE HOOF CORONARY DERMAL PAPILLAE

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ABSTRACT

This study investigated the features of this important histological papilla structure. The frequent renewal of the hoof wall occurs at the coronary dermal papillae. The importance of hoof material synthesis from the papillae, in the pathology of laminitis has been underlined in different species. Sixteen equine hooves were collected and tissue samples were harvested from the coronary region. The results revealed that the average number of dermal papillae was found to be 21±0.67 mm^2 across 15 horses which is more than reported in cattle. There was a strong correlation between the ratio of collagen fibres in dermal papillae and deep dermal tissue. In addition, this study indicates that the significant difference of papillae epithelial thickness at the heel region in comparison to dorsal and quarter, suggested that these difference may be have an influence on the production and reduction of horny material.

Key words: dermal papillae, hoof wall, coronary region, epidermal cells.

INTRODUCTION

The dermal papillae constitute the middle hoof layer that is one of the biological materials recognised as being the most resistant to fractures (Kasapi and Gosline, 1997 ; Wang, 2016). The equine hoof dermal papillae exhibit some particular individualities which are relatively different from even-toed cattle or pigs and mostly different from those in the other homologous in digital end organs for example the nail and claw (Bragulla, 2003). The ultimate forms of the hoof wall are mainly defined by the conformation of the interface between dermal and epidermal tissue, which is represented in hoof wall by dermal papillae (Hamrick, 2001 ; Davies *et al.*, 2007). Additionally, the thickness of the hoof capsule is related to the proportion of keratinization, which occurs in layers from the papillae and along the abaxial axis of the hoof and develops along the proximo-distal axis (Bragulla, 2003). The presence of a constant amount of collagen fibres in the dermal lawyer is essential in protecting the architecture of hoof shape (Kuwano *et al.*, 2005). These fibres are organised and extended over the reticular lawyer to formative primary dermal laminae, which attach with the

secondary laminae that interface with the epidermal laminae. Consequently, any defect of these fibres can cause a detachment between the dermal and epidermal connection and then separation of the distal phalanx. It is now well recognised from a variety of studies, that the bulk of the keratinised hoof wall (stratum medium) is generated by the stratum basale which covers and lining the dermal papillae (Daradka and Pollitt, 2004 ; Eurell and Frappier, 2013). Because knowledge of the hoof wall structure is a requirement to understanding its growth and function, the investigation of the coronet, in particular coronary dermal papillae will provide new evidence on the morphological structure of equine hoof capsule. This study set out with the aims that (i) the numbers of dermal papillae govern the proportion of keratinization (ii) the amount of the proportion of epithelial thickness at the coronary region differs between different hoof regions; (iii) the amount of collagen fibres at the dermal epidermal junction determines the integrity of dermal epidermal attachment.

MATERIALS AND METHODS

Equine hooves were collected from Swindon slaughterhouse, United Kingdom. Ethical permission was given by The School of Veterinary Medicine and Science, University of Nottingham ethical committee. Sixteen hooves (Cross breed horse) were utilized to obtain tissue samples from the dorsal coronet as well as from the quarter and heel regions and then fixed in 4% para formaldehyde (PFA) for 24 hours at 4 °C (Pollitt, 1996). Tissue sections were stained with (i) H and E stain. This stain enabled to measure the epithelial thickness at the coronary tissue and also the number and length of dermal papillae from dorsal, quarters and heels hoof regions. Measurements were calculated using calibrated a Fiji J software. These measurements were recorded as mean±SEM and calculated for each sample using Excel software and GraphPad software (Prism). (ii) Periodic Acid Schiff (PAS) was used to assess the integrity of basement membrane; five photomicrographs were taken from each section. (iii) Masson's trichrome with light green staining was used to assess the presence and proportion of collagen fibres in coronary hoof tissues. Collagen vs non collagen percentage coverage was assessed for each photomicrograph and the percentage mean±SEM were recorded and calculated for each sample using Excel software. The manufacturer's protocol was followed for all stains.

RESULTS AND DISCUSSION

Examination of tissue samples stained with H&E revealed the occurrence of coronary tissue (stratum medium) on the coronate region (Figure 1). This

stratum formed the bulk of the hoof wall and consisted of tubules arranged parallel and vertically oriented from dermo-epidermal junction at the coronary region as expected from previous investigations (Dellmann and Eurell, 1998; Stewart, 2013). Additionally, at the coronary region numerous perioplic and coronary dermis were invaginated and papillated under the epidermis in a precise orderly way, which seemed to have a finger like projection extended and protruded deeply in epidermal tissue (termed dermal papillae) (Bragulla, 2003) and were visible in longitudinal tissue sections (Figure 1).

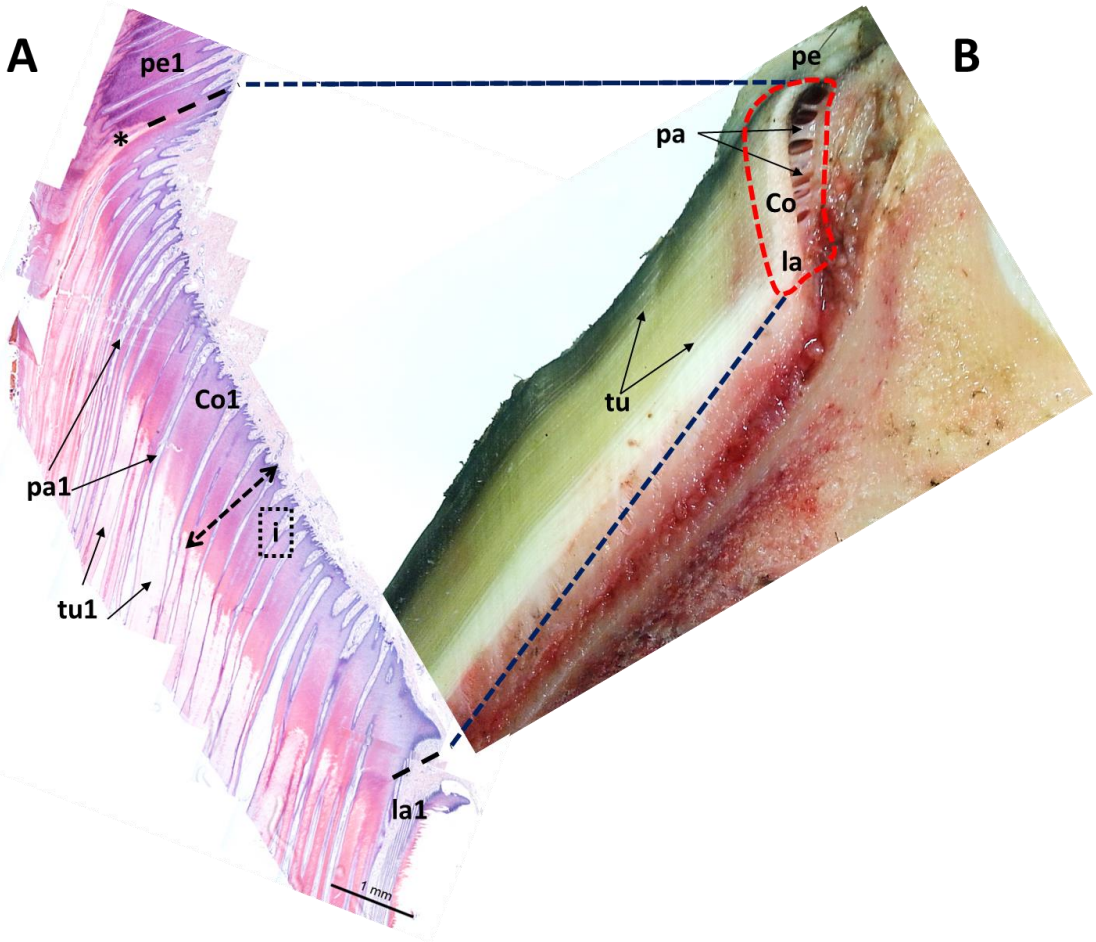

Figure 1. Section of coronary hoof tissue

A) Shows coronary region which is appeared clearly in stitched photomicrographs, while (B) represents the hoof sagittal section marked with red line showing the site of coronary region, whereas (*) the asterisk mark illustrates the area (pe, pe1) periople and (co, co1) the coronary region, while (la, la1) represents the lamellae, in addition (i) signifies the interdigitation, while (pa, pa1) shows the dermal papillae, and (tu, tu1) represents the tubular horn that originated from papillae, Scale bar represents 1mm. (H and E stain)

The numbers of dermal papillae were counted on each stitched image for 15 equine feet at quarter and heel regions. This results revealed that there were no significant difference between the numbers of papillae in each hoof region

(dorsal; n=7, 19.93±0.86 (mm^{-2})), (quarter; n=15, 22.15±0.69 (mm^{-2})) and (heel; n=15, 20.38±1.11 (mm^{-2})) (Figure 2). In terms of the number of papillae at the coronary region, no one reported the number of papillae at the coronary region; most of the research reported the density of tubular horn at the middle hoof region (Reilly *et al.*, 1996 ; Reilly *et al.*, 1998 ; Pollitt, 2004 ; Lancaster *et al.*, 2013). One of these studies reported, counts of tubules on four zones at the stratum medium with average 16 tubules mm^{-2} (Reilly *et al.*, 1998). However, these previous studies have not counted the number of papillae at the coronary region. The average number of dermal papillae in the current study was found to be 21±0.67 mm^{-2} across 15 horses is more than reported in cattle, which showed 13.4±0.927 mm^{-2} (Singh *et al.*, 1992). These findings are likely to be related to the nature and mechanism of hoof growth and raise the possibility that the dermal papillae and dermal lamellae adaptations of the dermis, which are understood as an endeavour to increase the surface attachment with inner, hoof (Davies *et al.*, 2007). A possible explanation has been stated by Budras *et al.*, (1996) that the papillary region at the coronet is highly vascularised and has a high rate of horny production when comparing to the poorly vascularised lamellae. This could be a similar rationale behind the differing amounts of horny material produced in the horse and cattle. As a consequence of this reaction, there is an impact on the hoof growth resulting in changes in shape and thickness of the hoof capsule (Budras *et al.*, 1996 ; Hirschberg *et al.*, 2001 ; Davies *et al.*, 2007).

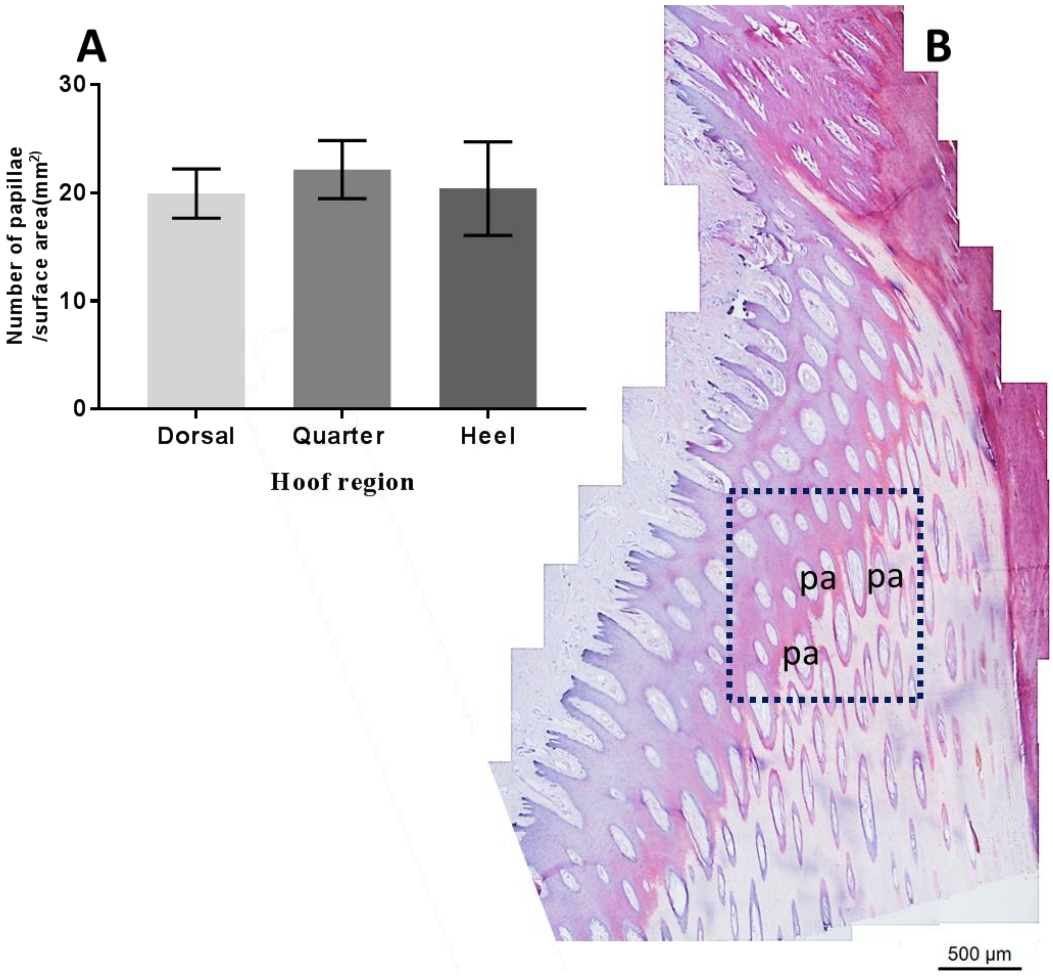

Figure 2. Graph and photomicrograph stitched showing the number of papillae

A) No significant difference were found between different hoof regions (dorsal quarter and heel). B) Shows black square represents sites of accounting papillae (pa). Statistical comparisons between groups were analysed using One Way ANOVA, Scale bar represents 500µm. N= 15 for quarter and heel and 7 for dorsal. (H and E stain)

Analysis for measuring entire length of dermal papillae revealed that the average length of each papilla was 3.96±1.49 mm; two of these papillae are shown in sagittal section in figure 3. The diameter of central core of each papilla was similar to that of the distance between the core and the surrounding dermal tissue and in the most distal region of dermal papilla it became gradually constricted until the end of papilla (Figure 3). As the dermal tissue become further away from the coronary border, the amount of vasculature also diminished (Bragulla, 2003) (Figure 3, yellow arrows). The cells that line the dermal papillae and rest on the basement membrane constituent the mature hoof wall tissue (Leach, 1980 ; Pollitt, 2004). The method which maintains a constant number of basal epidermal cells in the coronet participating in renewing hoof tissue (Pollitt, 1998 ; Daradka and Pollitt, 2000). These are delivered through a physiological process called tissue homeostasis involving progenitor stem cells

possessing the capability for self-renewing and to differentiate into different cell lineages when specifically stimulated (Blanpain and Fuchs, 2009).

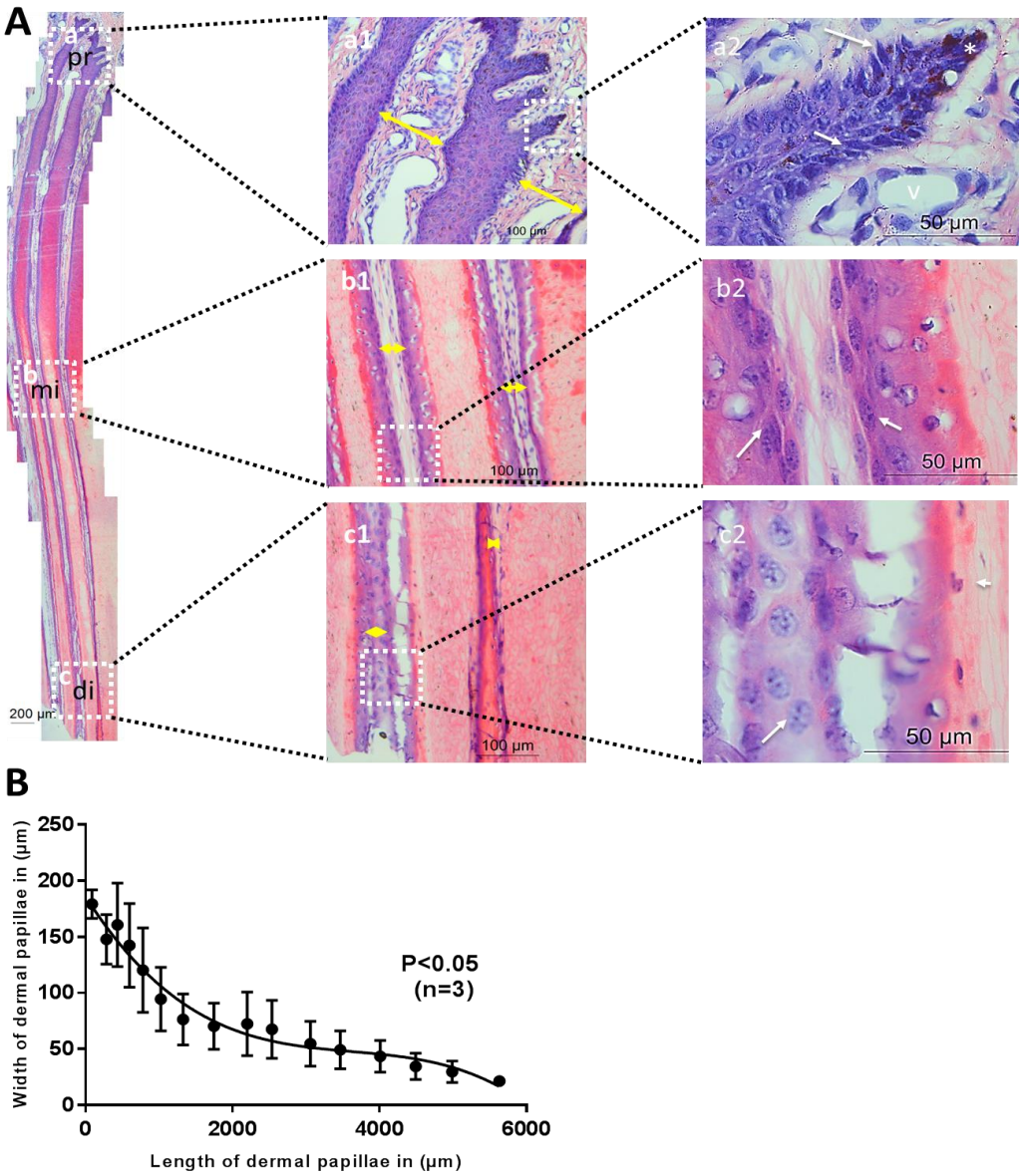

Figure 3. Photomicrograph stitched images and graph show dermal papillae diameter A) Represents stitched images of dermal papillae showing three portions, (a), (pr) the proximal region while, (b), (mi) represents the middle region and finally (c) the distal end of papilla (di). B) Shows a nonlinear regression was plotted on the papillae diameter against papillae length. Image (A) scale bar represents 200µm and images (a1), (b1) and (c1) 100µm, and (a2), (b2) and (c2) 50 µm. (H and E stain)

The epidermal layers, constituted from stratified squamous keratinised epithelium were continuous with skin from the leg and seemed divided clearly into the three expected layers: The thin deepest layer of basal cells (stratum basal), the second layer (stratum spinosium) and further distally, as the cells become more flattened toward the external layer (stratum corneum) (Figure 4).

This sequential development of epidermal cells and cornification are analogous to that of most structural hard keratin, for example hairs, nails, wool, quills, hooves, horns and claws (Lynch *et al.*, 1986; Marshall *et al.*, 1991; Rouse and Van Dyke, 2010).

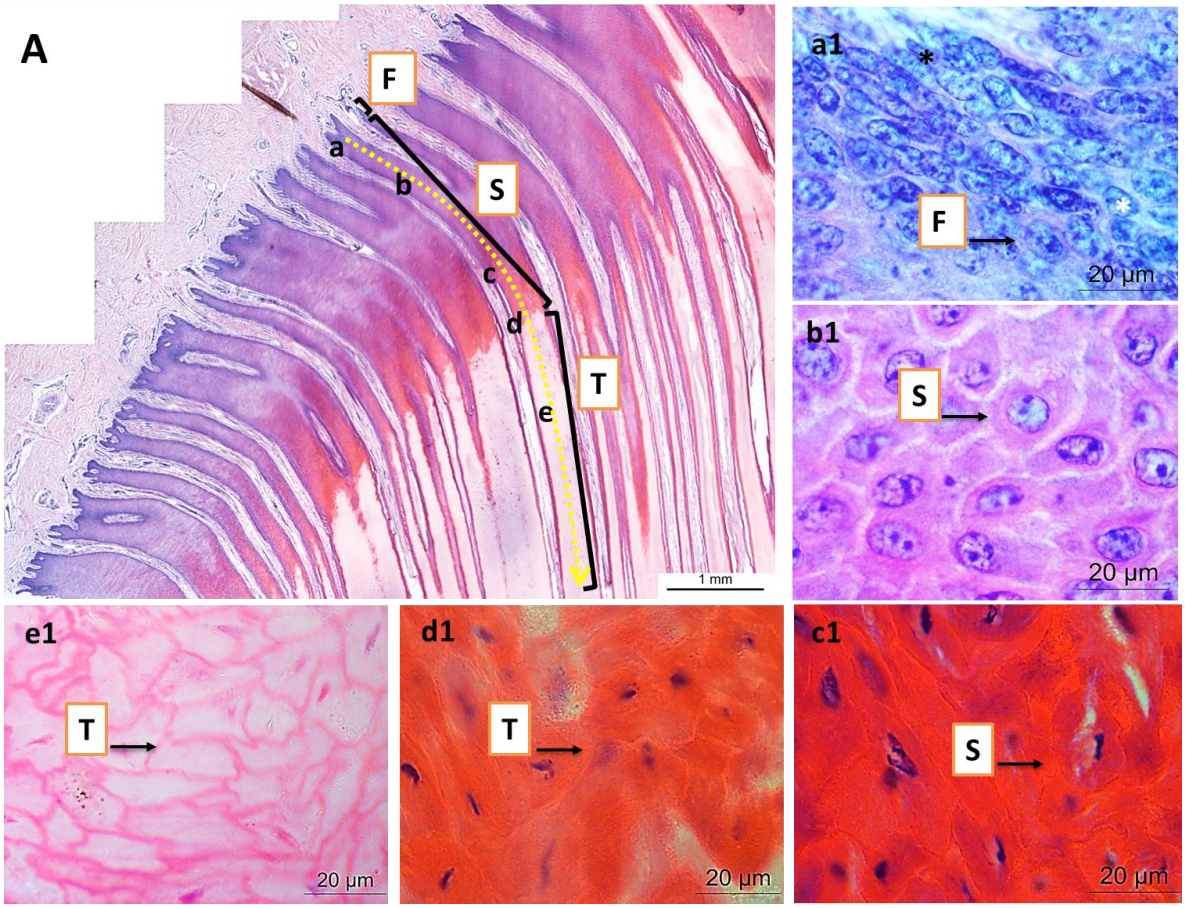

Figure 4. Photomicrographs illustrate intertubular epidermal layers

A) First layer (stratum basale) (F), second layer (stratum spinosium) (S) and third layer (stratum corneum) (T), while (a, a1) shows the basal and supra basal layers while in (b, b1) the second layer (S) formed from polyhedral cells move as it is appeared in (c, c1) the keratinised cells (d, d1) in third layers (T) with a fully keratinized appeared in (e, e1). Image (A), scale bar represents 1mm and images (a1) (b1) (c1) (d1) and (e1) 20µm. (H&E stain)

The results obtained from the analysis of epithelial thickness between the basement membrane and the junction between stratum spinosium and stratum corneum revealed there was a significant difference between hoof regions at the heels region ($p<0.05$; n=16, 1.34±0.14 mm) in comparison with both the dorsal region (n=16, 0.97±0.05 mm) and quarters region (n=16, 0.81±0.05 mm). The average epidermal thickness at the heel was increased by 41% in the heels region in comparison to the dorsal region and 65% in comparison to the quarter region. This analysis also indicated a significant difference between hoof regions at the heels region ($p<0.05$; n=16, 0.9±0.13 mm) in comparison with dorsal

(n=16, 0.54±0.06 mm) and quarters regions (n=16, 0.7±0.06 mm). The average stratum corneum thickness was increased by 67% in the heels region in comparison to the dorsal region and by 28% in comparison to the quarter region. The results of this study indicate that the significant difference of epithelial thickness at the heel region in comparison to dorsal and quarter (Figure 5), suggested that these difference may be have an influence on the production and reduction of horny material. It seems possible that these results are due to influenced by nutrition (Ott and Johnson, 2001) environment (Hampson, 2011), genetics (Ducro *et al.*, 2009); age (Frackowiak and Komosa, 2006). It is therefore probable that such differences have an impact on hoof wall growth, along with foot lameness (Dyson *et al.*, 2011).

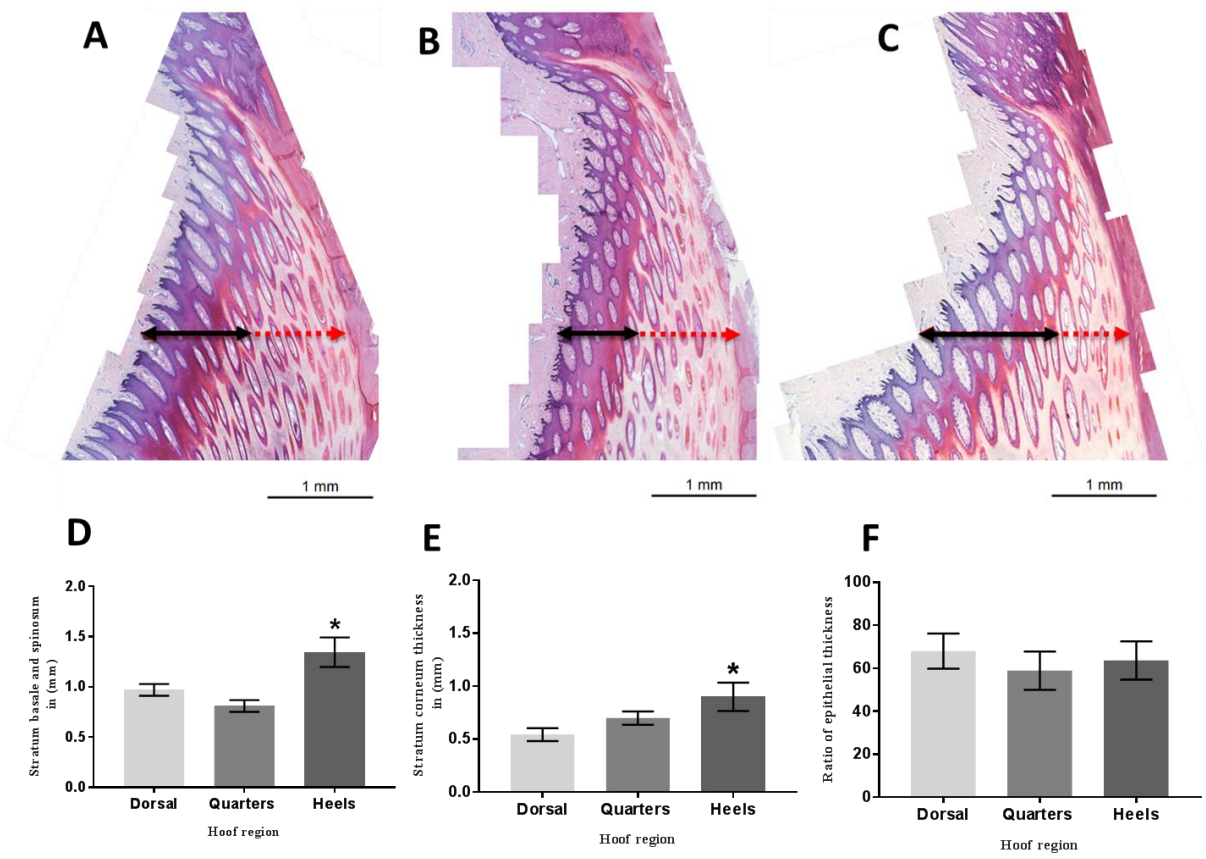

Figure 5. Epithelial coronary thickness

A) dorsal, B) quarters and C) heels, whereas black arrows represent the stratum basale and corneum, while red arrows show the stratum corneum. Statistical comparison shows a significant difference in graph (D) and (E) for stratum basale and spinosum, and corneum respectively at heels region, while (F) no significant difference was found to quarters region. Scale bar represents 1mm. (* indicates P<0.05, heel vs dorsal and quarter regions). Statistical comparisons between groups were analysed using One Way ANOVA SPSS. N= 16 per group. (H and E stain)

The results of Periodic Acid-Schiff (PAS) staining indicated that the basement membrane BM at the end of dermal papillae was folded into several ridges corresponding with the long alliance of the papilla. These results corroborate the ideas of Davies *et al.*, (2007), who stated that these folds act as guides for the orientation of tubular horns in a properly oriented proximodistal direction. In addition, there are equivalents to the secondary epidermal lamellae, which might play a significant role in increasing the attachment between the dermal and epidermal tissue to the periosteal surface of the distal phalanx. The integrity and borderline nature of the BM is important in indicating lesions of the epidermal tissue (Pollitt, 1996 ; Pollitt, 2004). This finding supports the evidence found that laminitis could be initiated by changes in the BM which have been suggested to indicate the beginning of laminar failure (Pollitt, 1996; Visser and Pollitt, 2011).

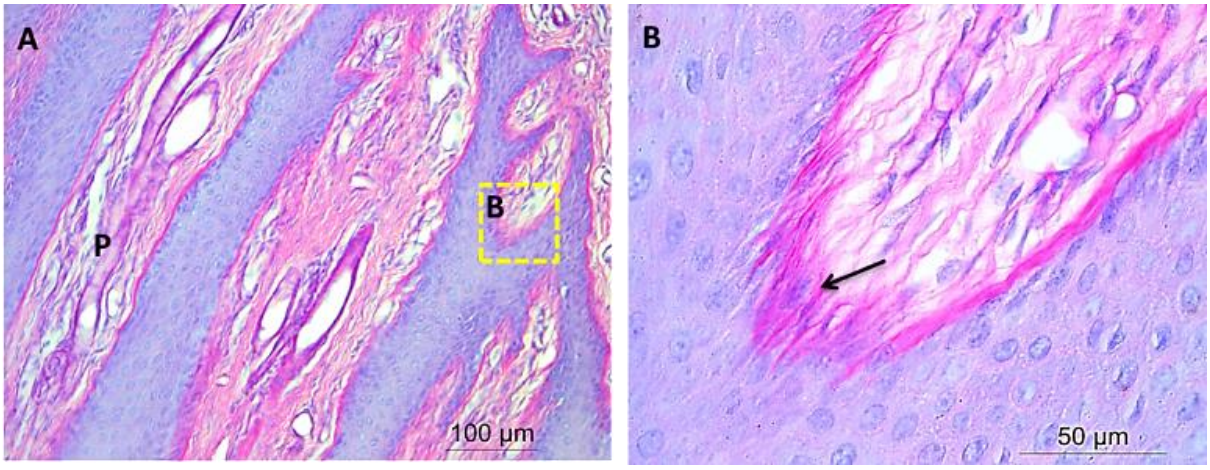

Figure 6. PAS stained photomicrograph of dermal papillae
A) Shows dermal papillae (p). B) Shows twisting (black arrow). Scale bar represents 50µm. (PAS stain)

The proportions of collagen versus other connective tissues, particularly muscle were assessed using Masson's trichrome stain. This method has been shown to be appropriate in investigating abnormal lesions of collagen fibres (White *et al.*, 2004). The preliminary results showed the normal distribution of the green-coloured collagen fibres in the dermal tissue of equine foot (Figure 7), and red-coloured collagen bundles were also observed in some of the stained sections (Figure 7). Previous studies have found that the red colour is due to a normal tension in collagen fibres before fixation (Flint *et al.*, 1975 ; Flint and Merrilees, 1977). However, the current study was in agreement with a later study conducted by Pollitt and Collins (2016) that showed the importance of collagen fibres in the structure of the suspensory apparatus of distal phalanx of

equine hoof. They used Masson's trichrome to demonstrate the connection of the parietal surface of the distal phalanx to the lamellar hoof wall. As is it, the chronic condition of this apparatus will consequently leads to disorders in capsular growth rate (Collins *et al.*, 2010 ; Pollitt, 2010). This proposes that the amount of collagen fibres inside the dermal papillae was originated and extended from the under attachment deep dermal tissue and this might be explain if any abnormalities or degeneration appeared in deep dermis, consequently it will affect severely on both collagen fibres inside dermal papillae and the attachment with epidermal tissue (Pollitt, 2010). The results of Masson's stained sections indicated that the collagen fibres of dermal tissue were arranged in long organised bundles with green and some of them had red colour, no abnormalities in collagen fibre arrangement were observed in the samples investigated (Figure 7). There was no significant different between the amount of collagen fibres at dorsal (n=12, 35±3.68%) and quarters regions (n=12, 33±1.64%). However, there was a strong correlation between the ratio of collagen fibres in dermal papillae and the ratio of collagen fibres inside dermal tissue (Figure 7). Overall, the current data measured and assessed collagen fibres which did not show any abnormalities in collagen fibres either when they were attached inside dermal papillae or when they were in the deep dermis at the coronary region.

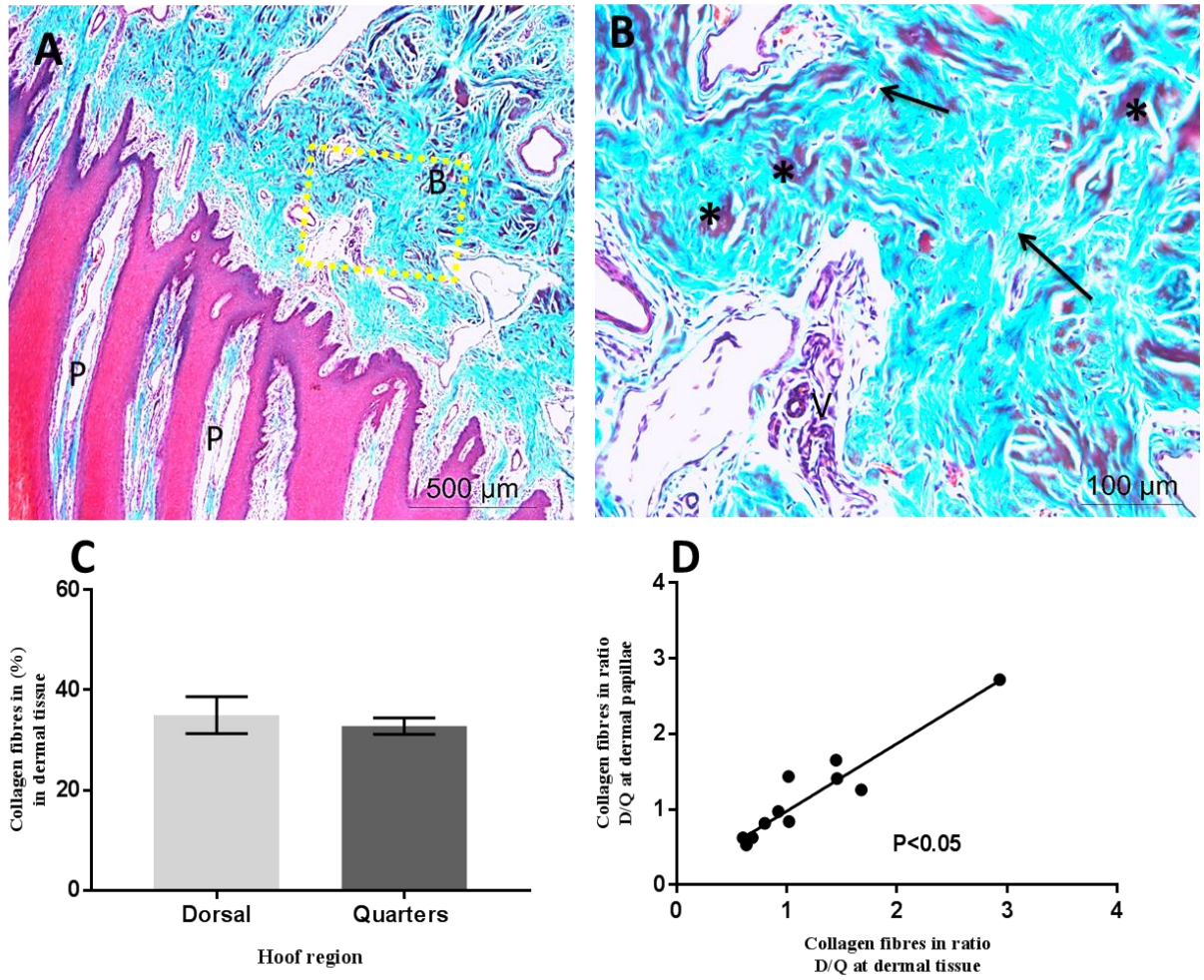

Figure 7. Photomicroscope images illustrating the collagen bundles at dermal tissues

A) Shows the papillae (p) had collagen bundles lay close to the epidermal tissue. B) Shows a deeper tissue where collagen appeared in longitudinal sections. C) Graph shows no statistical difference between the amount of collagen fibres. However, a clear correlation coefficient was observed in (D) between the ratio of both papillae and deeper collagen fibres. Statistical analysis was carried out using T-test. Image (A) scale bar represents 500 µm and (B) 100µm. N= 12 per group. (Masson's trichrome stain)

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دراسة التركيب الشكلي النسيجي لحليمات الادمة في المنطقة التاجية لحافر الخيول

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المستخلص

بحثت هذه الدراسة ملامح هذه البنية الحليمة النسيجية الهامة. يحدث التجدد المتكرر لنمو الحافر من الحليمات الجلدية التاجية، وقد تم التأكيد على أهمية تكون الحافر من الحليمات التاجية، ودورها الامراضي في التهاب الصفيحة في أنواع مختلفة. جمعت ست عشرة عينة من حوافر الخيول ومن ثم جمعت العينات النسيجية من المنطقة التاجية للحافر. أوضحت النتائج أن متوسط عدد الحليمات الجلدية بلغ 21 ± 0.67 مم$^{-2}$ لمجموعة 15 خيل وان عدد هذه الحليمات هو أكثر مما هو مذكور في حافر الأبقار، وبينت الدراسة وجود ارتباط قوي بين نسبة ألياف الكولاجين في الحليمات الجلدية ونسيج الجلد العميق، فضلاً عن ذلك، تشير هذه الدراسة إلى الاختلاف الكبير في سمك الظهارة لحليمات الحافر في منطقة الكعب مقارنةً بالجزء الامامي وجانبي الحافر، ان تشير هذه النتائج إلى أن هذا الاختلاف قد يكون له تأثير في إنتاج وتقليل المواد القرنية.

الكلمات المفتاحية: الحليمات الجلدية، جدار الحافر، المنطقة التاجية، خلايا البشرة.