Invited Review: Formation of Keratins in the Bovine Claw: Roles of Hormones, Minerals, and Vitamins in Functional Claw Integrity

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ABSTRACT

Keratins are the characteristic structural proteins of the highly cornified epidermis of the skin, feathers, and hoof. Keratin proteins provide the structural basis for the unique properties of the biomaterial horn and its protective function against a wide range of environmental factors. Hoof horn is produced through a complex process of differentiation (keratinization) of epidermal cells. Formation and biochemical binding of keratin proteins and synthesis and exocytosis of intercellular cementing substance (ICS) are the hallmarks of keratinization. It is finalized by the programmed death of the living epidermal cells, i.e., cornification, that turns the living epidermal cells into dead horn cells. The latter become connected by the intercellular cementing substance. The functional integrity of hoof horn essentially depends on a proper differentiation, i.e., keratinization of hoof epidermal cells. Keratinization of hoof epidermis is controlled and modulated by a variety of bioactive molecules and hormones. This process is dependent on an appropriate supply of nutrients, including vitamins, minerals, and trace elements. Regulation and control of differentiation and nutrient flow to the epidermal cells play a central role in determining the quality and, consequently, the functional integrity of hoof horn. Decreasing nutrient supply to keratinizing epidermal cells leads to horn production of inferior quality and increased susceptibility to chemical, physical, or microbial damage from the environment. A growing body of evidence suggests that hormones, vitamins, minerals, and trace elements play critical roles in the normal development of claw horn and correct keratin formation.

(Key words: keratin protein, laminitis, bovine hoof, nutrition)

Abbreviation key: EGF = epidermal growth factor, ICS = intercellular cementing substance, IFAP = intermediate filament-associated protein, SOD = superoxide dismutase, TG = transglutaminase.

INTRODUCTION

Keratin proteins comprise a major portion of the protective matrix of the skin, hair, horn, beak, and feathers of mammals and fowl (Fraser and MacRae, 1980). Formation of keratin proteins is part of a systematic process of cellular differentiation that transforms living, highly functional epidermal cells into cornified, i.e., dead, structurally stable cells with no metabolic activity (Mülling, 2000a). The dermal layer of the skin and its ability to provide much needed nutrients, along with hormonal exposure, modulates and controls cell differentiation in the epidermis, including keratin formation. It is this process of keratinization and the programmed cell death (cornification) of epidermal cells that dairy cattle rely on for formation of healthy skin, hair, and horn (Fraser and MacRae, 1980). When nutrient supply to keratin-forming cells is compromised or completely interrupted, inferior keratinized tissue, i.e., horn, is produced, which may lead to increased susceptibility to claw disorders and ultimately to lameness (Mülling et al., 1999). Trace minerals and vitamins play important roles in production and maintenance of healthy keratinized tissues (Mülling et al., 1999). Increasing the bioavailability of trace minerals improves their utilization and thus can help improve the integrity of keratinized tissues (Ballantine et al., 2002).

Lameness is a major health concern of the dairy industry. There may be 30% or more dairy herds experiencing some degree of laminitic insults, accompanied by the economic losses associated with lameness (Hendry et al., 1997). In addition to the cost in animal suffering, lameness is accompanied by loss of production on a per hundredweight basis comparable to mastitis (Warnick et al., 2001).

Lameness is the ultimate manifestation of a variety of conditions that may have distinctly different origins.

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Figure 1. Diagram of the structure of mammalian (bovine) skin. Micrograph of a section of bovine skin about 2 cm above the hoof. Hematoxylin and eosin stain. The dermis is covered by the densely arranged cells of the stratum basale of the epidermis. This is the layer where proliferation takes place. Cells are pushed into the next layer and enter the process of differentiation. These cells build up the stratum spinosum. Towards the end of differentiation, basophilic dense keratohyaline granules accumulate in the cells, which are therefore described as stratum granulosum. It is this layer that forms the border of cornification in which cells die, i.e. cornify and turn into horn cells which establish the stratum corneum.

However, the majority of these conditions are associated, to varying degrees, with lesions within the horny tissues of the hoof (Kempson and Logue, 1993) and, of these, one of the most common afflictions (especially in lactating dairy cows) is laminitis (Pododermatitis aseptica diffusa) (Greenough, 1991; Vermunt, 1994). Laminitis is the generic term for conditions in which the sensitive dermal structures between the pedal bone and the epidermal claw capsule are damaged (Hendry et al., 1997; Mülling and Lischer, 2002). Laminitis leads to poor quality horn. It is thought to be associated with impaired, generally decreased, or a complete lack of the synthesis or disturbed chemical binding of keratins, the structural proteins of the hoof or claw, with resultant deterioration of the macromolecular organization that gives the horn mechanical strength (Hendry et al., 1997).

The objective of this paper is to summarize the processes involved in the formation of keratin proteins in claw horn. Special emphasis is placed on the nutritional and hormonal factors that affect claw keratin formation during the periparturient period and their potential role in the production of inferior horn tissue, resulting in increased lameness.

Keratin Proteins

Keratin is a fibrous (keratin filaments) or amorphous (intermediate filament associated protein, [IFAP]), proteinaceous material that is produced by epidermal cells in the integument (epidermal layer) or outer covering of the body (Steinert and Idler, 1975; Calhoun and Stinson, 1981). The primary role of keratin is to

make the skin, hair, and horn a pliable, insoluble, and unreactive barrier against the natural environment. The fibrous structural proteins of the epidermis are many and varied and are collectively termed keratin proteins. Keratin is often misunderstood as a single substance even though it is composed of a complex mixture of proteins.

Keratin formation. To the casual observer, the keratinization process is purely a degenerative process, i.e., keratin simply arises through drying of degenerated and dead epithelial cells. Histological, biochemical, and molecular biological investigations, however, have clearly shown that keratin is formed through highly specific cell processes that aim to produce proteins with certain chemical and physical properties (Steinert et al., 1984; Dale et al., 1993; Franke and Kartenbeck, 1993; Parry and Steinert, 1995). The presence of the following substances in keratinizing cells serves as a positive indicator of intense cellular activity: ribonucleic and deoxyribonucleic acid, ascorbic acid, free aldehyde groups, alkaline phosphatase, lipids, glycogen, and glutathione (Fraser and MacRae, 1980; Hendry et al., 1997).

The dermis, located just beneath the epidermis, also referred to as corium is termed the “real” living layer of the skin. It forms the supportive connective tissue layer for the epidermis, containing blood vessels and nerves. The principal fibrous proteins of the dermis are collagen and elastin. From this layer, nutrients and hormones are provided to the stratum basal, or germinal layer, for the production of epidermal cells (Figure 1). The germinal layer is located on the laminar or papillary surface of the dermis and is the site of mitotic cell division (Calhoun and Stinson, 1981). All distal layers of the epidermis are derived from these cells by a process of proliferation and differentiation.

It is the dermal layer that directs the differentiation processes within the epidermis. Early works by Larson et al. (1956) demonstrated that in the early stages of acute laminitis in the equine hoof, histopathological changes occur only in the epidermis, whereas the capillaries and the connective tissue remained intact during the initial phase. Hendry et al. (1997) made histological examinations of laminitic dairy cows and found marked changes in the microvasculature of the dermal laminae. Mülling and Lischer (2002) reported that changes in the dermal vascular system during an acute laminitic insult may lead to disruption in cell differentiation and result in the production of inferior horn.

The epidermis consists of 4 distinguishable layers of cells—the stratum basal, the stratum spinosum, the stratum granulosum exclusively in regions where soft horn is produced, and the stratum corneum (Figure 1). Horny tissue is generated by keratinization and corni-

Figure 2. Micrograph of the heel region of the bovine hoof, Periodic acid Schiff reaction (PAS) with hematoxylin counterstain. The dermis contains a dense vascular system (arrows) with smaller vessels entering the dermal papillae (P). The dermal papillae are covered by the stratum basale of the epidermis, where cell division takes place. In the stratum spinosum cellular differentiation takes place characterized by formation of keratin proteins subsequently accumulating in the cells. Synthesis of intercellular cementing takes place in the lower third of stratum spinosum, exocytosis starts between middle and upper third (level indicated by line) and as a result PAS-positive intercellular material becomes visible in the upper third. Programmed cell death occurs at the level of cornification indicated by a sudden change in appearance, i.e., color and shape of the cells. Dead horn cells connected by PAS positive intercellular cementing substance build up the stratum corneum. Box indicates the tissue area which is shown enlarged in Figure 4.

fication of cells generated by cell division in the basal layer of the epidermal tissue. The cytoplasm of cells in the stratum basal contains fine filaments with a low density. These filaments frequently occur in bundles or fibrils. The filaments are often referred to as tonofilaments and the fibrils as tonofibrils (Parry and Steinert,

1995). The stratum spinosum is several cells thick (in hoof up to 120 layers of cells), and it is in the inner spiny layers that keratin synthesis appears to proceed at a very rapid rate. During this process, the columnar basal cells reorient to lie perpendicular to the basal cell layer and become enlarged, flattened, and polygonal in

Figure 3. Micrographs of middle stratum spinosum from the epidermis of the sole. Left, Periodic acid Schiff reaction (PAS)/hematoxyline stain. Right, polarized light. Cells are filled with keratin filaments. The cell boundaries are well contrasted by the PAS positive intercellular cement connecting individual cells.

shape as the cells progress toward the stratum corneum (Mülling 2000a) (Figures 2 and 3).

Keratin is formed intracellularly, and the epidermal cells responsible for keratin synthesis are therefore termed keratinocytes (Dale et al., 1993). Remnants of mitochondria and ribosomes can still be seen in the cytoplasm of immature keratinocytes. There is a dramatic increase in the cytoplasmic content of fibrils as the cells become more dense (Fraser et al., 1972). As cells proceed outward toward the stratum granulosum, the presence of very dense basophilic bodies within the cytoplasm become apparent, called keratohyalin granules (Fraser et al., 1972). On the electron microscopic level, the keratohyalin granules are electron-dense, irregular-shaped granules occurring in the cells of the stratum granulosum. During cornification, they dissolve and merge with the filamentous keratin proteins to a homogenous mass of middle-electron density filling the body of the horn cells.

As the cells move outward, there is an abrupt transition to the stratum corneum cell-type involving the complete filling of the cytoplasm with keratin and disappearance of the nucleus and virtually all of the cytoplasmic organelles and contents (Figure 2). This is the terminal stage of epidermal differentiation and keratin filament aggregation when the intermolecular cross linkages are formed and the keratinocyte dies (Figure 3).

Keratinization involves the continuous and progressive replacement of most of the cell contents by keratin proteins (Figure 4), their macromolecular organization into tonofilaments, and subsequent incorporation into the cell cytoskeleton by intermediate filament-binding proteins (Budras et al., 1989; Grosenbaugh and Hood, 1992; Kempson and Logue, 1993). The keratin filaments are aligned parallel to the long axis of the squame, with disulfide cross linkages formed by an interaction with IFAP and cell envelope proteins. This process gives rigidity to the cell thus giving it mechanical strength to withstand the impact of the forces of locomotion (Mülling and Budras, 1998). Each squame is tightly bound to its neighbors by desmosomal intracellular junctions and an intercellular cementing substance (ICS). This material is synthesized during keratinization and extruded into the intercellular space toward the end of keratinization in the upper third of the stratum spinosum (Budras et al., 1989; Leach, 1993; Mülling and Budras, 1998). The final step in the keratinization/cornification process is the secretion of a lipid-rich extracellular matrix, the so-called ICS, by which mature keratinocytes become glued together (Grosenbaugh and Hood, 1993; Mülling, 1998; Mülling et al., 1999). These keratinization processes can be summarized as keratin

Figure 4. Schematic of mammalian epidermal cells. Left, mammalian basal cells have little rough endoplasmic reticulum (RER). Membrane coating granules (MCG) appear and discharge their contents into the intercellular spaces. Bundles of filaments (F) develop in the cytoplasm and in the more distal cell layers keratohyalin granules (KH) are evident. In the final stages of cellular differentiation the plasma membranes of the cells thicken (TPM) and the cytoplasmic components disintegrate except for the fibrils which consolidate into the filament-matrix complex of keratin. Also illustrated are the dermis (D), basal lamina (BL) and anchor fibrils (AF). Right, micrograph of upper third of stratum spinosum and lower stratum corneum (enlarged area from Figure 2, indicated there by a box). Periodic acid Schiff reaction (PAS)/hematoxyline stain. With progressive differentiation the cells in the stratum spinosum change their shape, their nuclei show peripheral condensation and fragmentation of chromatin. The intercellular material is exocytosed and fills the intercellular space as an intensive PAS-positive line. With cornification a sudden flattening of cells occurs. The nuclei disappear and the cells become filled by homogenous intensively stained keratin masses.

protein synthesis, aggregation and stabilization, and synthesis and exocytosis of ICS (Figure 4).

Some (Grosenbaugh and Hood, 1993; Mülling, 1998; Mülling et al., 1999) have likened this process to building a brick and mortar wall. The keratinocyte "bricks" are formed as they move down the hoof wall and are "fired" to hardness during cornification. Three major protein groups are instrumental in establishing integrity and rigidity in the keratinocyte. Keratins function as the internal scaffolding, intermediate filament-associated proteins enable the proper alignment of the keratin filaments, and cell envelope proteins align themselves along the inner cell wall to give the outer shell of the mature keratinocyte its rigidity (Grosenbaugh

Figure 5. Electron micrograph of horn cells in upper stratum spinosum undergoing the terminal stage of differentiation. Keratin filament bundles are cross-linked by disulfide bonds to a homogenous “keratin mass” filling the body of cornified cells in the stratum corneum. (right micrograph). The individual horn squames (H) are connected by intercellular cementing substance (arrows).

and Hood, 1993; Mülling et al., 1999). The structural building blocks, keratinocytes, are attached to one another by desmosomal attachments. After extrusion of the ICS toward the end of differentiation, the keratinocytes are embedded in and connected by a lipid-rich intercellular matrix (i.e., mortar) (Figure 4). Any defects in the cornification process may lead to loss of structural integrity and possibly functional loss.

Classification of Keratins

Keratins are normally differentiated as “soft” or “hard,” corresponding to the products of 2 apparently

different modes of biosynthesis (Giroud and Leblond, 1951). Soft keratins occur in the stratum corneum, corns, callouses, and the eponychium (coronary band) around the hoof (Figure 1), whereas hard keratins are found in hair and horn (Figure 2). The terms “soft” and “hard” are descriptive of tactile sensation, but there are marked differences in histological development and composition (Mercer, 1961; Fraser and Macrae, 1980).

Typically, the keratin proteins (filaments and IFAP) in soft horn have a low degree of consolidation and are thus a key component in desquamating tissues (those that shed cells, such as skin). Chemically, skin keratin is distinguished by a sulfur content of about 1% (dry weight) and a lipid content of about 4%. The sulfur is rather evenly distributed between cysteine and methionine (both combined to form protein). The sulfur and tonofibrils are concentrated near the cell boundaries. The fatty material includes fatty acids, phospholipids, and sterol.

Hard keratin or hard horn differs from soft keratin, as the IFAP is produced in the hard type of keratinization and is higher in sulfur/cysteine (high sulfur proteins, according to Parry and Steinert, 1995) having up to 5% mainly in the form of combined cysteine (Ward and Lundgren, 1954; Grosenbaugh and Hood, 1992). Presence of nonprotein constituents is very low. For example, appreciable amounts of lipid and glycogen are not present. The “hard” keratins form more coherent structures with higher tensile strength. The transition from the germinal layer to the fully hardened product occurs more gradually over a much wider region in which the sulfur content is increased markedly (Ward and Lundgren, 1954). The structures forming “hard” keratin are more highly complex and specialized (Figures 5 and 6).

Figure 6. High-power electron micrographs of keratin filaments in stratum spinosum cells. Left, bundle of aggregated keratin filaments in a cell in middle stratum spinosum. Right, magnification of center of bundle shown left, electron dense (dark) filaments are connected by material of lower density, i.e., intermediate filament associated protein (IFAP).

Architecture of Horn

The architecture of hoof horn is determined by the surface formation of the underlying dermis (Mülling et al., 1999). The dermal papillary body not only provides mechanical support and supplies nutrients and oxygen but also determines the cellular composition of the claw horn. Corresponding to the horn tubules and horn laminae are two structural formations present in the papillary body, dermal papillae that are present in all regions of the claw, and dermal laminae present exclusively in the wall region. In areas with dermal papillae, the epidermis forms tubular horn; in the laminar region of the wall, horn lamellae are formed (Budras et al., 1989, 1996). The tubular horn consists of horn tubules built by the epidermis around the dermal papillae and above their tips. These tubules are connected by the intertubular horn between them. Each horn tubule consists of an outer cortex originating from the living epidermis located around the dermal papilla and an inner medulla originating from the epidermis over the tip of the papilla. The diameter and density of tubules, as well as the ratio between cortex and medulla, determine the quality of hoof horn. The medullar cells develop at the tip of the dermal papilla due to continuous proliferation in the basal layer of the keratinizing cells and are rapidly moved away from the nourishing underlying dermal blood vessels. These locations can be seen in the micrograph in Figure 2 and the micrograph of sagittal section of bovine claw (hoof) in Figure 6.

The mechanical strength and quality of hoof horn is directly related to the dimensions of tubules, i.e., the diameter and proportion of medulla and cortex and, therefore, the arrangement and spatial relationship of tubular, intertubular, and laminar horn cells (Mülling et al., 1999). In the white line (the junction of wall and sole horn), large horn tubules with a wide medulla establish sites of predisposition for bacterial invasion. Once the medullar horn has fallen out of the tubules, microorganisms may invade the tubule, start horn cell destruction, and ascend within the tubule toward the inner living tissue layers. This results in the development of white line disease (Kempson and Logue, 1993). In complicated cases, once the bacterial invasion reaches the dermis, white line abscesses and wall separation can develop. Many of these claw abnormalities occur in early lactation (Green et al., 2002) and may be the result of nutritional deficiencies or hormonal changes occurring in dairy cattle at this stage of lactation.

Dermo-Epidermal Interactions

Many physiological changes occur in late gestation and into early lactation of the dairy cow that affect

nutrient uptake and flow. During the process of keratinization, epidermal cells rely upon the dermal layers for the supply of nutrients, macro and trace minerals, and vitamins. This supply must be provided entirely via diffusion from blood vessels in the underlying dermis because the epidermis is an avascular tissue (Mülling et al., 1999). Hendry et al. (1999) reported that little is known about the control mechanisms for nutrient flow and rate of hoof keratinization.

Hormonal control of horn growth. An interesting area of developing research relates to the hormonal control of horn-protein production and how changes at calving may affect the potential for future lameness. In research to investigate keratinization control, Hendry et al. (1999) demonstrated that insulin binding was detected in both the epidermal and the dermal layers of explanted bovine hoof tissue. In the suprabasal epidermal layers, insulin binding was located at the periphery of the keratinocytes and also in the nucleus. Little binding was detected in the horn. Protein and DNA synthesis in bovine hoof tissue explants was stimulated by culture for 24 h in the presence of insulin.

In the early lactating dairy cow, there is a decrease in insulin sensitivity (Cowie et al., 1980) and an inverse relationship between circulating insulin and animal productivity (Hart et al., 1978). Therefore, the decrease in insulin sensitivity, and or concentration, in early lactation could compromise production of claw-horn keratin due to depressed uptake of glucose and amino acids (Hendry et al., 1999). It is conceivable that this could be exacerbated if horn tissue shares the postreceptor insulin resistance shown by other tissues during the periparturient period (Vernon, 1988). Vermunt and Greenough (1994) suggested that overfeeding during the dry period, which gives rise to hyperinsulinemia and hyperglycemia (2 classic signs of insulin resistance) in early lactation, appeared to predispose cows to laminitis. Green et al. (2002) reported the incidence of first lameness was highest 3 mo after calving, suggesting that factors affecting horn growth during the dry period and in early lactation result in production of inferior horn and lameness in early lactation.

Epidermal growth factor. Hendry et al. (1999) reported that epidermal growth factor (EGF) may impact keratin formation and result in formation of inferior horn production. They reported that EGF, with its potent mitogenic and anti-differentiative effects in other epithelial tissues (alimentary and uterine tracts), was bound more locally than insulin, being found only in the differentiating epidermal layer (Hendry et al., 1999). This differed from findings by Grosenbaugh et al. (1991), where EGF was found predominantly in the basal layer in equine tissue, and little EGF was detected in the horn or the dermis. EGF stimulated protein syn-

thesis in bovine hoof-tissue explants (Hendry et al., 1999), whereas EGF was reported to decrease keratin expression in healthy equine tissue (Grosenbaugh et al., 1991). Hendry et al. (1999) reported that the EGF response increased with time and was maximal at physiological concentrations of the growth factor in the bovine.

The effect of EGF on hoof-protein synthesis indicates that endocrine control of keratinization is modulated by local (autocrine or paracrine) signaling within the tissue (Hendry et al., 1999). It is likely that, as in other tissues, local growth-factor control not only modulates but is also modulated by changes in systemic hormone concentrations. For example, steroid hormones elevated in pregnancy down-regulate local production of EGF in a number of tissues (Plaut, 1993). If this also occurs in the claw, the result would be an inhibition of keratin synthesis (Hendry et al., 1999).

Prolactin. Another hormone of particular interest during the periparturient period is prolactin. The major lactogenic hormone prolactin may also influence EGF-dependent keratin deposition (Cowie et al., 1980). Hendry et al. (1999) found that hoof-explant culture stimulation of protein synthesis by EGF was antagonized to a modest degree by prolactin. Although prolactin itself did not influence hoof protein synthesis, its ability to decrease EGF-stimulated protein synthesis in hoof tissue cultures may be another factor in reducing keratin synthesis during lactation (Hendry et al., 1999).

Glucocorticoids. Goff and Horst (1997) reported that periparturient dairy cows are often subjected to stress, with a subsequent increase in cortisol. Glucocorticoids are thought to have an impact on maturation of keratinocytes through regulation of protein synthesis as cortisol affects the metabolism of glucose, protein, and fats (Goff and Horst, 1997). Hendry et al. (1999) found that hydrocortisone inhibited keratin protein synthesis in bovine hoof-tissue explants. Epidemiologists have yet to identify a causative relationship between systemic glucocorticoid concentration and laminitis in dairy cows. Yet, it is notable that highly productive herds, which have a greater incidence of laminitis (Nocek, 1997), also have higher glucocorticoid levels (Johnson and Vanjonack, 1976). Milne (1985) reported that steroid treatment of horses exacerbates laminitis. Stress and subsequent elevation of cortisol during the periparturient period and during lactation (Goff and Horst, 1997) may predispose dairy cows to claw disorders resulting from production of inferior claw horn.

Required Nutrients for Keratinization

Amino acids. The amino acids Cys, His, and Met play key roles in establishing the structural integrity

of the keratinocyte (Ekfalck, 1990; Ekfalck et al., 1990). Fraser and MacRae (1980) reported that the formation of disulfide bonds between Cys residues was an integral step in the final stage of keratinization and in cornification and establishment of the cellular envelope, providing cell-wall rigidity and high resistance against a variety of proteolytic enzymes (Elias, 1981). Grosenbaugh and Hood (1993) reported that cultured explants preferentially incorporated ^{35}S -Cys into partially keratinized epidermal lamina as opposed to the uptake of ^{35}S -Met, thus supporting the requirement for Cys in formation of the keratin-rich cornified hoof wall.

Amino acid requirements of dairy cattle are not known with much certainty (NRC, 2001). However, the NRC (2001) does suggest that high-producing dairy cows may not be able to produce adequate quantities of metabolizable protein to meet the demands of milk production, especially in early lactation when DMI is depressed (Marquardt et al., 1977). This lack of metabolizable protein in early lactation could contribute to insufficient protein synthesis by developing keratinocytes and thus result in production of inferior horn and predispose the dairy cow to lameness.

Minerals. The onset of lactation places such a large demand on mechanisms of calcium homeostasis that most cows develop some degree of hypocalcemia at calving (Goff and Horst, 1997). This is important in that calcium plays an integral role in the keratinization and cornification process. Calcium is needed for activation of epidermal transglutaminase (TG), which is active in cross-linkage of the cell envelope keratin fibers and, in addition, is involved in the initiation and regulation of the terminal differentiation of the epidermal cells. This enzyme helps activate the final step in the production of the mature squame (i.e., fully differentiated keratinocyte) by linking cell envelope proteins on the cytoplasmic side of the cell wall via glutamyl-lysine bonds to form a ridged cell wall (Mülling et al., 1999).

Insufficient Ca provided to the maturing keratinocyte due to inadequate vascular supply (Nocek, 1997) or Ca availability due to hypocalcemia may lead to depressed TG activity and formation of dyskeratotic horn. Mülling et al. (1999) reported that differentiating epidermal cells were very sensitive to changes in plasma Ca levels. They suggested that inconsistent levels of Ca around parturition, in particular with the onset of lactation, would certainly influence the metabolism in differentiating epidermal cells. This may provide an explanation for the horn rings consistently observed associated with pregnancy in cows. Horst (1986) reported that between 5 and 10% of all milking cows suffer with hypocalcemia during, or shortly after, calving. There is an apparent increase in incidence with increasing parity from the second to sixth lactation (Curtis et al., 1984). Nocek

(1997) reported that the incidence of laminitic insults in dairy cows increases with age. Therefore, it may be probable that some of the laminitic insults seen in high-producing dairy cows (typically moderately hypocalcemic) and those that have suffered from hypocalcemia may be in part related to impaired TG activity and its impact on terminal differentiation control and formation of the cellular envelope.

Zinc. Zinc has been identified as a key mineral in the processes of keratinization (Smart and Cymbaluk, 1997; Mülling et al., 1999; Mülling, 2000b). The ubiquitous distribution of Zn among cells, coupled with Zn being the most abundant intracellular trace element, points to very basic functions. Whereas Zn is a component of over 200 enzyme systems, it has a role in 3 key functions in the keratinization process—catalytic, structural, and regulatory (Cousins, 1996). Catalytic roles are found in enzymes such as RNA nucleotide transferases, RNA polymerase, alkaline phosphatase, carboxypeptidase, alcohol dehydrogenase, and the carbonic anhydrases (Cousins, 1996; NRC, 2001). As indicated earlier, the presence of ribonucleic and deoxyribonucleic acid, ascorbic acid, free aldehyde groups, and alkaline phosphatase in keratinizing cells serves as a positive indicator of intense cellular activity (Frazer and MacRae, 1980; Hendry et al., 1997). These catalytic enzymes are Zn metalloenzymes and, as such, are dependent upon Zn as an activator, and thus an integral component in the differentiation of keratinocytes.

Zinc also plays a key role in the formation of the structural proteins during the keratinization process. Zinc-finger proteins are involved in functions requiring protein-to-protein interactions, most of which are thought to affect cellular differentiation or proliferation (Cousins, 1996). Two examples are the transcription factors of retinoic acid and calcitriol (1,25-dihydroxycholecalciferol) receptors (Cousins, 1996). Interestingly enough, Zn-finger proteins are thought to have the following general structure: -C-X₂-C-X_n-C-X₂-C-, where C designates Cys and X designates other amino acids. Fraser and MacRae (1980) reported that the favored pentapeptide sequence Cys-Gln-Pro-(Ser, Thr)-Cys was identified in the α -helix chain of hard mammalian keratins. They postulated that the Cys-favored positions may form the β -bend conformation, which is stabilized by a disulfide linkage between Cys residues. Therefore, it is postulated that insufficient Zn status may decrease the formation of Zn-finger proteins and thus the formation of keratin filaments needed in the developing keratinocyte.

The third key role of Zn in differentiating cells, including differentiating keratinocytes, is regulatory. Zinc regulates calmodulin, protein kinase C, thyroid hormone binding, and inositol phosphate synthesis

(NRC, 2001). Calmodulin is responsible for binding Ca²⁺ and carrying it into the cytosol of the cell when activated. This is important in the final step of the developing keratinocyte because, as noted earlier, calcium activates epidermal transglutaminase. Protein kinase C (which is also calcium dependent) is responsible for phosphorylation of proteins, thus adding available energy to the differentiation process. Thyroid hormone acts to regulate the action of calmodulin and protein kinase C. Inositol phosphate acts to increase Ca²⁺ by mobilizing the ion from intracellular stores, primarily from the endoplasmic reticulum.

Zinc is also required for activation of the cytosolic enzyme Cu/Zn superoxide dismutase (SOD). In Cu/Zn SOD, Cu functions at the catalytic site, whereas Zn has a role in the 3-D structure of the enzyme (Cousins, 1996). The Cu/Zn SOD is responsible for prevention of lipid peroxidation. Protection of the ICS is critical in the maintenance of the structural integrity and biological function of the claw (horn) (Mülling et al., 1998, 1999). Mülling (2000b) reported that organic Zn has an important role in the activation and regulation of keratin protein production by horn tissue explants.

British workers (Baggott et al., 1988) reported findings of lower Zn concentration in claws of lame cows than those with no history of lameness. Claws of lame cows were also softer than the nonlame. This suggests an insufficiency of Zn or lack of adequate vascular supply to the developing keratinocytes. At dairies having a high incidence of foot problems, cows fed 2 to 3 g/d of ZnSO₄ for 70 d had fewer claw problems than cows not receiving supplemental Zn (Demertizis, 1973). In contrast, sheep fed rations supplemented with ZnSO₄ for up to 6 mo did not show a reduction in claw problems (Cross and Parker, 1981). Inconsistent responses to feeding Zn in the form of ZnSO₄ can be attributed to antagonists present in the diet affecting the bioavailability of the Zn (NRC, 2001). Organic sources of Zn, such as zinc methionine, have proven to be more bioavailable than Zn from inorganic sources (Wedekind et al., 1992).

Several studies have shown that complexed Zn improves claw integrity. In a year-long study conducted at Illinois State University, cows fed an additional 200 mg/d of Zn from Zn Met had fewer cases of foot rot, heel cracks, interdigital dermatitis, and laminitis than cows not fed Zn Met (Moore et al., 1989). Observations on ulcers and white line disease (indications of dyskeratotic, structurally altered horn tissue) trended toward improvement. Of beef cattle receiving 216 mg/d of Zn from complexed Zn, 2.45% had foot rot, whereas 5.38% of cattle not receiving complexed Zn had foot rot (Brazle, 1993). These studies indicate that feeding organic Zn complexed to a single amino acid has a beneficial influ-

ence on keratinizing tissues, thus improving hoof horn and skin integrity, resulting in improved animal well-being and performance. Zinc requirements for dairy cows vary by stage of lactation (NRC, 2001). Milk production creates a significant drain on zinc stores, thus zinc requirements are highest in early lactation (NRC, 2001). Insufficient supplies of bioavailable zinc during the periparturient period and during lactation may predispose cows to production of inferior horn tissue, with a concomitant increase in lameness.

Copper. Much like Zn, Cu is instrumental in the activation of enzymes. Copper is needed for activation of the cytochrome oxidase enzyme involved in aerobic respiration, lysyl and thiol oxidases for structural integrity of cells, ceruloplasmin, which is essential for absorption and transport of iron for hemoglobin synthesis, and superoxide dismutase, which works with Zn in reducing the toxic effects of oxygen metabolites (NRC, 2001). Of greatest importance in the keratinizing horn cell is the activity of thiol oxidase (O'Dell, 1990). Copper activates thiol oxidase enzyme, which is responsible for formation of the disulfide bonds between Cys residues of keratin filaments (O'Dell, 1990). This process is essential for structural strength on the cellular level, giving rigidity to the keratinized cell matrix.

Cattle suffering from a subclinical Cu deficiency are more susceptible to heel cracks, foot rot, and sole abscesses (Puls, 1984). This response may be the result of insufficient cytochrome-c oxidase activity, resulting in reduced respiration and oxidative phosphorylation and thus deficient energy supplies for differentiating keratinocytes (Linder, 1996). Heel cracks and abscesses may also be the result of insufficient Cu availability for activation of Cu/Zn SOD. Reduced activity of the Cu/Zn SOD is expected to enhance the fragility of cell membranes because unsaturated lipids in the cell periphery are particularly vulnerable to oxidative damage (Linder, 1996). The intercellular lipids are an integral part of the cementing substance responsible for cell-to-cell adhesion (Mülling and Budras, 1998). Therefore, any nutrient deficiency that leads to the production of inferior ICS or predisposes it to excessive oxidative damage may lead to production of dyskeratotic horn tissue, with increased susceptibility to cracking and wear.

Selenium. Selenium is a constituent of the enzyme glutathione peroxidase. Glutathione peroxidase is responsible for reduction of H_2O_2 and free O_2 to H_2O (NRC, 2001). Thus, by acting much like Cu/Zn SOD, glutathione peroxidase plays a role in protecting both the intra- and extra-cellular lipid membranes against oxidative damage. This way Se may contribute to the protection and maintenance of physiological function of the lipid-rich ICS.

Excessive supplementation of Se may be damaging to developing keratinocytes. Selenium in the form of selenomethionine (SeMet) is readily absorbed by the same mechanism as Met (Combs, 2000). Inorganic Se absorption does not appear to be regulated and is quite high (>50%) (NRC, 2001). Bodily storage of inorganic Se from selenite or selenate occurs as selenoamino acids SeCys and SeMet. Combs (2000) indicated that the most biologically efficacious of these is the SeMet form, yet SeCys is also very active. Combs (2000) reported that the Se and/or selenoamino acids may be preferentially incorporated into sulfur requiring AA sites during protein production and thus change the integrity of the protein structure.

Larson et al. (1980) reported that dairy cows supplemented with 50 mg of injectable Se (over $6.6 \times$ NRC requirement) during the dry period suffered severe claw problems in the postpartum period. They indicated that between 48 and 69% of cows receiving the supplemental Se injection had increased lameness, sore feet, deformed claws, and loss of hair from the tail versus 28 to 30% claw problems in nonsupplemented cows. It is very likely that the excessive Se supplement was incorporated into keratin fibers of the maturing keratinocytes with the key Cys and Met sites replaced by SeCys or SeMet. Therefore, critical disulfide bridge formation was reduced or inhibited during the cornification process, creating inferior hoof horn lacking structural rigidity with poor integrity. The recommended level of Se in dairy diets is 0.3 mg/kg DM and should be closely monitored to ensure over supplementation does not occur, especially during the dry and early lactating periods (NRC, 2001).

Manganese. Manganese plays an indirect role in the keratinization process. Manganese helps minimize feet problems by maintaining proper leg formation (Miller et al., 1988). Manganese is needed for activation of galactotransferase and glycosyltransferase enzymes, which are needed for the synthesis of chondroitin-sulfate side chains of proteoglycan molecules (Keen and Zidenberg-Cherr, 1996; NRC, 2001). Proteoglycans are essential building blocks in the formation of normal cartilage and bone. Animals suffering from a Mn deficiency will exhibit skeletal abnormalities, crooked legs, and shortening of tendons, as noted by knuckling over of feet (NRC, 2001).

Manganese also plays a role in the activation of other critical enzyme systems, such as pyruvate carboxylase, an enzyme that catalyzes the first step of carbohydrate synthesis. This process is responsible for gluconeogenesis and the production of cellular energy, an essential component in the production of quality horn tissue (Keen and Zidenberg-Cherr, 1996). Similar to Cu/Zn SOD, Mn plays a role in the activation of Mn superoxide

dismutase (Mn SOD) and the removal of superoxide-free radicals. Therefore, Mn SOD may play a protective role for the lipids involved in cementing together mature keratinocytes.

Combinations of trace minerals. There are significant interactions between trace minerals, and hence it is imperative that nutritionists formulate rations to maintain an appropriate balance of trace minerals in order to maximize animal performance. Research has demonstrated that supplying a combination of complexed trace minerals is more beneficial to claw integrity than supplying a sole complexed trace mineral because of synergistic effects. A 2-yr study conducted on 5 commercial dairy herds in Central New York indicated that cows fed 360 mg of complexed Zn, 200 mg of complexed Mn, 125 mg of complexed Cu, and 25 mg of complexed Co resulted in better claw integrity than cows fed only 360 mg of complexed Zn or no complexed trace minerals (Nocek et al., 2000). Supplementation of the diet with a combination of complexed trace minerals reduced the incidence of double soles, white line separation, digital dermatitis, sole hemorrhages, and ultimately, sole ulcerations (Nocek et al., 2000). In addition, 300 cows on a large commercial dairy in Florida were fed a combination of complexed Zn, Mn, Cu, and Co to evaluate claw health (Ballantine et al., 2002). Cows fed complexed trace minerals tended to have fewer incidents ($P < 0.15$) of claw disorders than cows fed inorganic trace minerals at 75 d postpartum (23.6 vs. 34.1%) and numerically lower incidence at 250 d postpartum (10.0 vs. 17.7%). Feeding complexed trace minerals reduced incidents ($P < 0.05$) of white line disease at 75 (9.5 vs. 14.6%) and 250 d postpartum (4.9 vs. 8.8%). Feeding complexed trace minerals during the late dry period and during early lactation tended to improve claw lesion scores and thus was associated with improved claw health and integrity.

Role of Vitamins in Horn Growth

Vitamin A. Vitamins also play an integral role in developing the structure and quality of keratinized horn tissue. Vitamin A is needed for cell differentiation (Olson, 1996). Differentiating cells have specific binding sites for vitamin A and, once bound, can both stimulate or inhibit gene expression. Vitamin A is needed for normal growth and development and for maintenance of skeletal and epithelial tissues (NRC, 2001). The role of vitamin A in keratinizing cells is tied to its action in gene expression (NRC, 2001).

Vitamin D. One of the most important biological regulators of calcium metabolism is vitamin D (synonym calciferol) (NRC, 2001). Derived from cholesterol, an Mn-dependent process, vitamin D is responsible for

minute-by-minute calcium and mineral homeostasis. In its biologically active form $1,25(\text{OH})_2\text{D}_3$, vitamin D is required for control of Ca^{2+} reabsorption, absorption, and mobilization/accretion from bones (Norman, 1996). Because the body can endogenously produce vitamin D_3 and because it is retained for long periods of time in vertebrate tissues, it is not likely that dairy animals would be deficient in vitamin D. However, with increased confinement and reduced exposure to direct sunlight, dairy animals lacking sufficient supplementation could succumb to minor vitamin D deficiencies. Therefore, any lack of vitamin D will certainly impact calcium metabolism and thus affect the keratinization process.

Vitamin E. The best understood role of vitamin E is as a lipid-soluble cellular antioxidant (NRC, 2001). Via this function and possibly others, vitamin E is involved in the maintenance of cellular membranes. This function is important to the integrity of keratinized tissues, as the ICS is composed of lipid rich material (Mülling et al., 1999). A deficiency of vitamin E at the cellular level is generally accompanied by an increase in lipid peroxidation of cellular membranes (Sokol, 1996). This may lead to decreased energy production by mitochondria, oxidation, and mutation of DNA, and alteration of normal transport processes of the plasma membrane (Sokol, 1996). Therefore, cells exposed to oxidative stress (i.e., a laminitic insult) will show more rapid injury and necrosis when rendered vitamin E deficient (Sokol, 1996). This may also help explain why transition dairy cows fed low levels of vitamin E and subjected to undue stress at parturition incur higher than normal levels of lameness and production of poor horn tissue (Nocek, 1997).

Biotin. A water-soluble "B" vitamin, biotin is possibly the vitamin of greatest importance to the keratinization process. Biotin is essential for the formation and integrity of the keratinized tissues (skin, hair, claws, and footpads) in mammals and birds (Maynard et al., 1979). Biotin is a cofactor for enzymes used in a diverse array of metabolic pathways. Amino-acid metabolism, cellular respiration, gluconeogenesis, and lipogenesis involve enzymes that require biotin (Mock, 1996). There are 4 biotin-containing enzymes found in mammalian cells—acetyl-CoA carboxylase, B-methylcrotonyl-CoA carboxylase, propionyl-CoA carboxylase, and pyruvate carboxylase. All 4 enzymes require biotin to become activated (Weiss and Zimmerly, 2000).

With regard to keratin formation, biotin-dependent enzymes are directly involved in the synthesis of lipids and glucose, with particular importance placed on synthesis of long-chain fatty acids (Meyer et al., 1998; Weiss and Zimmerly, 2000). Mülling et al. (1999) demonstrated that biotin was essential for the formation of

complex lipid molecules in the ICS. They also demonstrated, in biotin-deficient calves, that biotin deficiency affected keratinizing epidermal cells, as well as the composition of the intercellular cement (Mülling et al., 1997). Research in pigs and horses has shown that biotin positively influenced the integrity of the hoof horn (Geyer, 1998).

Functioning ruminants are able to produce biotin in the rumen. However, high grain (>50% DM) rations reduce ruminal synthesis of biotin *in vitro* (DaCosta-Gomez et al., 1998). This response may be due to an insufficient conversion of lactate to pyruvate. Mock (1996) reported that biotin deficiency was tied to insufficient pyruvate-carboxylase activity resulting in cellular lactic acidosis. It may be possible that ruminants receiving proportionately high-grain diets lack sufficient biotin in their rumen to convert lactic acid to pyruvate and then oxaloacetate, thus predisposing them to lactic acidosis. Nocek (1997) reported lactic acidosis as one of the possible contributing factors in lameness of dairy cows. Studies by Fitzgerald et al. (2000), Weis and Zimmerly (2000), and Hedges et al. (2001) indicate that dairy cows respond favorably (improved claw integrity and reduced lameness) when provided supplemental biotin (20 mg/cow per day) for a period of greater than 6 mo. In a study of 5 dairies with a total of 900 cattle, Pötzsch et al. (2003) reported biotin supplemented at 20 mg/d for longer than 6 mo reduced white line disease in multiparous cows by 45% in 8.5 cases per 100 cow years. However, the effect of biotin in primiparous cows was not significant. These studies indicate that biotin reduced the incidence of white line abnormalities in particular and other claw diseases, such as sole hemorrhage, sole ulcers, digital dermatitis, and heel erosion.

Mülling et al. (1999) proposed the analogy of building a brick wall to the effect of supplements such as Zn and biotin on hoof keratin formation. Zinc is needed for activation of the enzyme systems needed for formation of sound cellular structure (bricks), whereas biotin is needed for production of the ICS (mortar). Together, they allow the mason (keratinizing squamous cells) to generate a stronger wall with greater integrity that will better withstand environmental stresses. It is this ability to withstand environmental stress that ultimately determines the productivity and potential profitability of the animal.

CONCLUSIONS

Formation of keratin proteins is an essential/crucial part of a systematic process of cellular changes that transform living, highly functional epidermal cells into fibrous, structural horn cells with no metabolic activity.

This differentiation of epidermal cells is very complex and very sensitive to hormonal controls, nutrient flow, and environment. It is the process of nutrient flow, as impacted by hormonal controls that plays a major role in determining the quality and integrity of keratinized tissues of the horn. When nutrient supply to keratin-forming cells is compromised or completely altered, inferior keratinized tissue is produced. Inferior tissue increases the susceptibility to development of claw disease and may ultimately lead to lameness. Calcium, Zn, Cu, Mn, vitamins A, D, and E, and biotin all play important roles in the production and maintenance of healthy keratinized tissues. Increasing the bioavailability of trace minerals, especially Zn, Cu, and Mn, improves their utilization and thus contributes to an improved integrity of keratinized tissues, such as skin and claw. Integrity of claw horn is one prerequisite for claw health, which in turn is the prerequisite for overall animal well being, productivity, and potential profitability.

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