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POOR NOCICEPTIVE INNERVATION of NECK SKIN in THREE DOMESTIC RUMINANTS

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***Abstract**:*
Background: Skin innervation in ruminants has received less attention than other animal skins. The objective was to compare the tissue layers of the skin and detect the expression of nociceptive nerve endings and axons within the neck region of these animals. Methods: Sections of neck (cervical) region of three domestic ruminants involved sheep, goat and bull slaughtered at Kerkuk Slaughter House were studied for both histological, using routine stain, hematoxylene and eosin (H&E) and immune-histochemically using substance-P primary antibody. Results: No conspicuous fundamental differences in the skin layers between these three animals do exist except the bull skin was much thicker than that of sheep and goat, respectively; while more adipose tissue was deposited in sheep in comparison with goat and bull with abundant loose hypodermis in all. Immunohistochemical examinations showed scanty expression of substance-P nerve endings in the epidermis and nerve fibers in the submucosa and hypodermis of all three animals except a few at hair follicles were detectable. Conclusion: The neck skin is charcterised either scanty nerve endings and fibers supplied or run perpendicular to skin surface which could be advantageous for such commercially used meat productive animals to minimize pain during the Islamic manner of slaughtering used to eliminate pain during slaughtering in comparison with other painful methods used in Western society

Keywords: Immunohistochemistry; neck skin; nociceptive; ruminants; substance-P.

INTRODUCTION

Ruminants are important in their traditional role in both agricultural researches and teaching but are also extensively used for studies in molecular biology, genetic engineering, and biotechnology for basic science, agricultural and clinical applications **(Underwood et al. 2015).** Ruminants have also been useful subjects for reproductive research such as embryo transfer, artificial insemination (AI), and control of the reproductive cycle **(Wall et al. 1997).** Several important milestones in gene transfer, cloning, and genetic engineering techniques have been developed or demonstrated using these species **(Cibelli, et al. 1998a; Cibelli, et al. 1998b).** Healthy sheep and goats are often used for antibody production. Genome mapping has developed rapidly since 1998 in ruminants and other domestic species **(Elsik et al. 2009).** Despite all these various usages their skins have occupied more interste in histologcsl research than other topics. The important role of the skin generates from the fact that it services the body in many aspects. It consists of the four main types of tissues arranged in a special way to perform various functions i.e. protection, thermoregulation, external sensory, awareness, immunological defense, wound healing, perception, excretion and as an effective barrier to prevent desccation of electrolytes and macromolecules from the body. The skin is the multilayered organ comprising epidermis, dermis, and hypodermis and its layers get

modified depending upon the species habitat and body region of the animal. Skin cover the surface of the body and consist of two main layers, the surface epithilume or epidermis generate from ectoderm and a subjacent deeper connective tissue layer or the dermis from mesoderm.

Thickness of skin meassurs less than one millimeter to few millimeter and it functions as important sensory organ which has tactile thermal and painful nerve ending. Each component of the skin plays a role in its daily function, therefore every component is a source of vital information that can be captured and assessed with a skin biopsy. Epidermis, the next layer under the stratum corneum, functions to protect the body contains sensory nerves specifically small diameter sensitive temperature fibers. It is these sensory nerves that are helpful when evaluating a skin biopsy following immunostained **(Proksch et al. 2008).** The sensory nerves in the epidermis serve to sense and transmit heat, pain, and other noxious sensations. When these nerves are not functioning properly they can produce sensations such as numbness, pins-and- needles, pain, tingling, or burning **(Stücker et al. 2002).** When evaluated, characteristics of the nerve such as total number, contiguity, diameter, branching, swelling, and overall health are taken into consideration **(Cranenburgh 2019).** An antibody raised for nociceptive nerve ending called substance-P may represent the best tool to diagnose and locate these nerve endings in the tissue.

Dermis, the next layer underneath epidermis is characterized by loose, ribbon-like cells that hold dermal structures in place and serves to contain fluids and other compenents i.e. arrector pili muscle a tiny muscle attaches to the base of a hair follicle at one end and to dermal tissue on the other end. The arrector pili muscle is a source of information when evaluating a skin biopsy since it is well-innervated with autonomic nerves that control when the muscle contracts. These autonomic nerves are also visible when the skin biopsy is immunostained **(Hughes 1960).** Basket cells, the structures surround the base of hair follicles and serve as pressure sensors, are a source of valuable information when assessing overall nerve health and condition **(Colin 1952).** Nerve endings in the skin represent the distal end of the axon of a nerve fiber terminates, called sometimes, a free nerve endings (FNE) are unspecialized, afferent nerve fiber sending its signal to a sensory neuron which bring information from the body's periphery towards the brain. They function as cutaneous nociceptors and are essentially used by vertebrates to detect pain.

For most scientists the study of nerve terminations in mammalian skin has been an exercise in descriptive morphology. A single histological technique was usually considered adequate and presumably impeccable, meanwhile the possibility of artefacts was not considered **(Weddle et al. 1955).** Published immunohistochemical researches of ruminants on neck skin are so scanty. The intention of this humble work has therefore been designed to study the expression of nociceptive nerve endings using antibodies raised against Substance-P nerve endings within the neck skin layers.

MATERIALS AND METHODS

Skin samples were collected for histological and immunohistological examination by a skin biopsy on the site of the *Kerkuk Sloughter House*. Only three different ruminants were available for test i.e. Goat, sheep and Bull. The histological and immunhistochemical (IHC) experiments were carried out **(Lor 2018).** The skin blocks cut out smaller than 1 $cm,^3$ were immersed in the 10% formalin and kept for 24 hours. Dehydration was carried out using ascending series of ethanol alcohol started from 50-90% for 10 minutes each ended up at 100% for 1 hour at room temperature. Tissue blocks were then immersed in 100% xylenex

for 1 hour (2 chnages) followed by mounting in fresh paraffin wax. Skin sections were cut using Reichart misrotome at thickness of 5μm. Sections were streached over a water bath and picked up on ulbuminised clean slides. For histological examinations these sections were stained using heamatoxylene and eosin stains **(Lor et al. 2019)**.

Immunohistochemical staining was performed for substance-P using VIP (Vector/Novocastra, Burlingame, CA), (Biogenesis, Brentwood, NH), and GAP-43 (Vector/Novocastra, Burlingame, CA). Paraffin sections (4-5 μm) were collected on Superfrost slides (Fisher, Pittsburgh, PA) from each of the paraffin-embedded skin specimens of goat, sheep and bull. Slides were incubated for 16 hours at 55^0C and then deparaffinated by a 30-minute immersion in xylene followed by rehydration through a graded alcohol series to deionized water over 10 minutes. To enhance antigen retrieval, the slides were immersed in Antigen Unmasking Solution (Vector Labs, Burlingame, CA; Cat. # H-3000) and microwaved for 3 minutes. Slides were rinsed in distilled water and PBS (0.1 mol/L phosphate buffer, *p*H 7.4). Blocking for 30 minutes at room temperature was performed using nonimmune serum from the species in which the secondary antibody was raised (1:10 dilution) in PBS, followed by a 16-hour incubation with the specific primary antibody with a dilution SP, 1:200. The slides then were washed with PBS followed by a second blocking step with methanol containing 0.6% hydrogen peroxide (H_2O_2) for 30 minutes at room temperature. Slides then were rinsed with deionized water, then PBS, followed by incubation with species-specific biotinylated IgG secondary antibody (1:200 dilution) for 30 minutes at room temperature. The slides were washed with PBS for 10 minutes and avidin-biotin complex (ABC) added for 30 minutes at room temperature. The slides then were rinsed well in PBS, and developed with chromagen 3,3-diaminobenzidine for 2 minutes at room temperature. Counterstaining was performed using HARRIS hematoxylin for 20 seconds. The slides then were washed well in distilled water, dehydrated in alcohol, clarified in xylene, and mounted with permount. Slides were then examined using Olympus binuclear microscope and pictures were taken.

RESULTS AND DISCUSSION

The skin layers in the remnants, generally, looked very similar, however, the skin of bull was much thicker than both goats and sheep's skins. This is not a surprising issue as the bigger size the animal is the thicker the skin would be. Simultaneously, the extra adipose tissue accumulated in the hyopdermis of sheep in comparison with goat might be attributed to fact that goats are more active than the sheep. However, the similarities were restricted in the configuration of the layers in the three skins being studies. While the difference in the thickness was confined in all layers of the skins the hypodermis, particularly, showed loose connective tissue in the three animals. Such looseness might provide the animal more fixability in moving the neck region in various direction i.e. twisting and tying the animals from. The most conspicuous characteristic of the skins was the abundant hair within the neck region of both bull and sheep but lesser in goat. The latter, inevetibly, caused some difficulties in sectioning the skin blockes.

Recently, the use of new techniques has shown that it is characteristic of all nerves entering mammalian skin to terminate in an arborization manner of almost <1μm in diameter, naked, axoplasmic filaments with a probabity of free ends. Ensheathed stem fibres give rise to unencapsulated nerve endings in all skin strata, and terminals from neighbouring stem fibres overlap and interdigitate extensively. Axoplasmic filaments terminate in the cellular layers of the epidermis and in the dermis but in no specific relation to the capillaries **(Weiss & Wardrop 2010).** They are found in relation to the myoepithelial and gland cells of sweat glands, and in relation to the adventitia and media of blood vessels. Axons of <1 *u*m is too

difficult to be visualized by light microscopy while capsulated nerve endings or nerve cells might be much esasier. The letter may create a hurdle in visualizing the nerve axons running in the skin strata.

The skin sections of these ruminants showed very rare substance-P expressed nerve components i.e. cell bodies or nerve fibers. Scanty Substance-P cells may indicate to either a lack or poor supply nociceptive nerve cells in the neck regions of these three ruminats which function to transmit pain during the sloughering processes. Such a method involves also cutting the cervical blood vessles which would stop supply the head with blood too **(Radostits et al. 2007).** The latter will minimize the pain as a very little or no blood will reach the brain to feel the external stimuli. This method represents additional merciful way to minimize pain generated from slaughtering process in comparison with others applied in the non-musdlim countries.

In hairy skin there are, in addition to the unencapsulated nerve endings, nerves which end specifically in relation to hair follicles. Ensheathed myelinated stem axons give rise to two distinct and separate series of arborizations of fine, naked, axoplasmic filaments which lie at right angles one within the other. The outer series encircle the hair lying among the cells of the middle layer of the dermal coat. The inner series lie among the cells of the outer root sheath parallel to the hair shaft. No encapsulated nerve endings are seen in hairy skin **(Brunjeni et al. 2010).** Another interpretation may also be postulated that is the direction of these afferent nerve fibers might be running perpendicular or at the right angle to the skin. The latter would minimize the transmission of pain when the neck skin is cut by butcher's knives used Islamic manner in scarifying these animals.

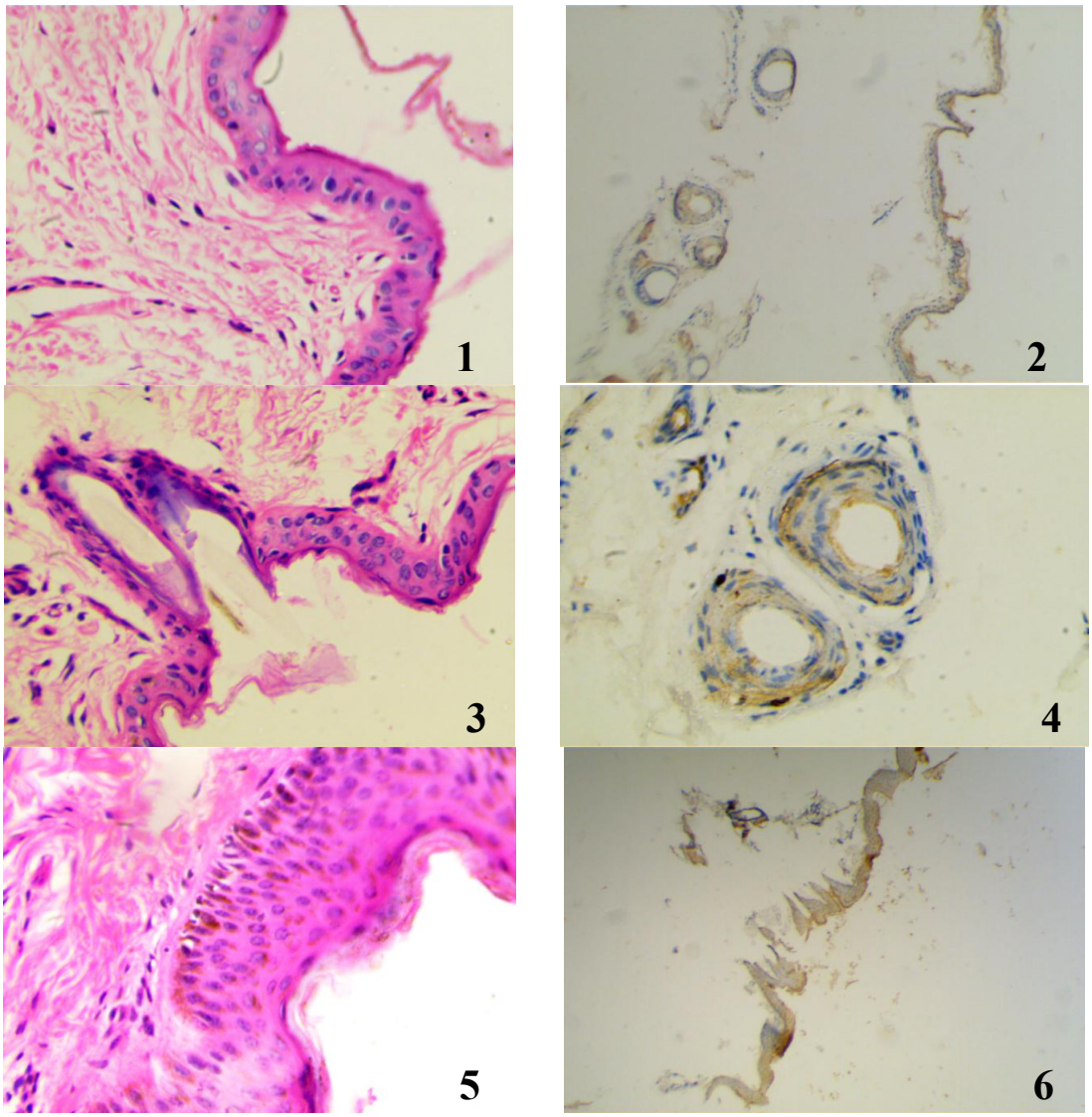
The hairy necks might add another reason for invisibility of neve fibers as well as encapsulated nerve endings. They are of different sizes and shapes, and the ensheathed myelinated stem fibre(s) enter the capsule pursue more or less tortuous course **(Ma et al. 2004).** They then give rise in all cases to an arborization of fine, naked, axoplasmic filaments which end freely among the capsular cells in planes roughly parallel to the surface layer of cells **(Radostits et al. 2007).** It is therefore, unsurprised to conclude presence of scanty nerve ending in the neck skin as a source of nociceptive pain generating from the neck skin during the slaughtering.

In the light of above information a re-analysis of the observations force the scientists to interprete that the facts reported by various authors have more in common than would have been supposed from a perusal of their summaries and conclusions. Hence, the innervation of skin using different techniques, i.e. hyaluronidase, it is now possible to re-interpret the observations in the old literatures **(Weddle & Pallie 1954a; Weddle & Pallie. 1954b)**. The technique of immunohistochemistry might be an excellent tools to explore further details of the skin cells in all other animals **(Weddle et al. 1954).** If the literature is re-interpreted in this way, the points concerning the innervation of mammalian skin via improved neurohistological techniques may correspond both to those described by a few authors whose work is usually ignored or discounted **(Smith 2009; Smith & Sherman 2009).**

A very few positive substance-P cells were detectable in the follicular cells at the bottom of the follicles represent the nociceptive pain transmitter for the hair shafts. This is not surprising as snatching a hair may generate some pain via these nerve endings. Some melanocytes in the germinating layers of the sheep had picked up the substance-P might indicate to the reaction of Diaminobenzidin (DAB) stain used to stain the substrate of the

complex conjugated with the melanocytes. This phenomenum needs further confirmation in our future studies.

There is not and has never been a convincing histological evidence for the commonly accepted statement that morphologically specific nerve endings subserve each of the primary modalities of cutaneous sensibility. According, the skin of these animals have been under-exploerd in comparison with other tissues. Further antibodies are required to explore other types of nerve supply and endings in the skin of these animals. Needs are due to erect theories of cutaneous sensibility based on the existence of four primary modalities, touch, warmth, cold and pain, operating within the 'law of specific nervous energies'.



Sections through the skin of Goat: A thin epidermis (3-4 layers) with loose subcutaneous connective tissue consisted of irregular dense connective tissue with less hair folloicle than those of sheep and bull [Fig. 1, H&E x350 & Fig. 2, IHC x100]; Sections through skin of sheep with a thin skin a thin epidermis of (4-6 layers) [Fig.3;(H&E x350]. Many hair follicles are embedded within the hypodermis consists of loose irregular connective tissue [Fig.4, IHC x350). A thicker bull skin (12-15) layers epidermis covered with a keratin layer Fig. 5, H&E x350). Some Malpigean cells germinating layers cell appeared filled with melanocytes [Fig. 6, IHC x100].

CONCLUSION

Using immunohistochemistry, the nociceptive nerve endings in the epidermis of the neck skin were either scanty or at the right angle to the axis of the neck which serve to minimize pain during the slaughtering process. The morphology and distribution of nerve terminals in mammalian skin should be further studied from other aspects i.e. functions as transducers of stimuli (mechanical, thermal or chemical) into propagated action potentials rather than taxonomically.

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