Surface architectural anatomy of the penile and preputial epithelium of bulls[*]

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Abstract

Microscopic examinations of epithelial tissues are valuable methods to evaluate surface and histologic architecture. The objectives of this study were to determine: 1) the changes in the thickness of the surface epithelium of the penis and prepuce, 2) the size of epithelial folds, 3) the location and distribution of epithelial folds in the surface epithelium, and 4) examination for the presence of crypts on the penis and prepuce of two groups of beef bulls (*Bos taurus* and *Bos indicus*). Bulls two years of age and bulls $\geq$5 years of age were selected. Neither the area encompassed by epithelial folds ($p < 0.05$) nor the number of epithelial folds ($p < 0.05$) differed between age groups based upon Image J analysis of penile and preputial epithelium.

Keywords: Penile epithelium, preputial epithelium, crypt, epithelial fold, beef bulls

Introduction

It has long been assumed that as bulls age the folds or crypts on the surface of the penis and prepuce become deeper. The presence of deeper crypts purportedly facilitates the chronic carrier state of bovine venereal disease by providing a protected environment suitable for long term maintenance of infection. No published reports were found to support this assumption and this study was conducted to characterize the surface architectural anatomy of the epithelium and epithelial crypts in younger and older bulls.

The gross anatomy of the bovine penis and prepuce has been described and the intricate mechanisms leading to erection and ejaculation defined.[1] Compounded wrinkles and folds of the preputial epithelium and creases on the surface of the glans penis are present and thought to allow for the mechanics of penile extension during erection.[‡] The surface architecture of the genital epithelium has been speculatively discussed for decades but quantitative studies of the microscopic anatomy and the effects of ageing are lacking. It has been suggested that that the epithelial microstructure of the penis and prepuce undergoes significant age-associated changes and that these changes are important in establishment of persistent infection with *Tritrichomonas foetus* (*T. foetus*) or *Campylobacter fetus* subsp. *venerealis* (*C. fetus*).[2] Persistence of infection with these organisms has been speculated to be related to deeper folds or crypts in the preputial epithelium of mature bulls.

A crypt is defined as a blind pit or tube-like structure on a free surface.[3] Specific structures such as the crypts of Liberkühn are well-defined as a lumen of intestinal glands on the surface of the intestinal mucus membrane that serve to secrete or are absorptive in nature.[4] However, epithelial crypts are poorly defined on the penis and prepuce of the bull.

Penile anatomy of the bull

The surface of the penis and the prepuce within the preputial cavity are among the very few non-haired areas of skin present on the bovine. The free portion of the non-erect penis distal to the attachment of the prepuce is approximately 12 cm long and lies within the caudal portion of the preputial cavity when the penis is non-erect. The free portion is capped by a small cushion of asymmetrical, ventrally directed, slightly spiraled tissue which comprises the glans penis.[5]

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The prepuce of the bull is 35 to 40 cm long and approximately 4 cm in diameter with wide variations among breeds.[6] The prepuce is composed of an external, parietal, and visceral layer. The parietal and visceral layers of the prepuce are covered with stratified squamous epithelium.

The free portion of the penis is covered with stratified squamous epithelium which is very tightly adhered over the apex. This epithelium transitions caudally to become more loosely attached at the junction of the free portion and prepuce, allowing the epithelium to change its orientation when the penis is extended or withdrawn.[5] The distal portion of the penis is encapsulated by the smooth cap-like glans penis, while the remainder of the glans has a rough uneven epithelial surface. The epithelium of this area is characterized by a network of fine epithelial folds which vary from slightly irregular depressions to relatively deep, closely-arranged crevices that produce a papillate appearance.[7] It has been alleged that epithelial folds or crypts become deeper as a bull matures and that folding of the epithelium provides a protected environment suitable for the development of a chronic carrier state of venereal diseases.[8]

Materials and methods

Animals

Healthy sexually mature bulls were placed into two groups of six (Table). Group 1 (N=6) consisted of bulls two years of age (± 3 mo.). Group 2 (N=6) consisted of bulls five years old or older (five to 12 yrs.). These age groups were selected because most bulls enter the breeding herd at two years of age and most are removed from the herd at five years of age. It was hypothesized that age-related penile and preputial epithelial differences would be apparent by five years of age. All tissue samples were collected from bulls presented to the John Thomas Vaughan Large Animal Teaching Hospital, College of Veterinary Medicine, Auburn University for routine breeding soundness examinations. Written consent was obtained from owners prior to tissue collection. Each bull was sampled for presence of *T. foetus* and *C. fetus.* The Auburn University Institutional Animal Care and Use Committee approved all protocols.

Biopsy technique

Three locations were chosen for epithelial biopsy: 1) the distal penis 1 cm proximal to the glans, 2) the proximal portion of the free end of the penis, 1 cm distal to the attachment of the prepuce, and 3) the proximal prepuce 6 cm distal to the attachment of the prepuce and the sheath. Each bull was restrained in a livestock chute and the penis was manually extended, held by sterile surgical gauze, and cleaned with water prior to aseptic surgical preparation. The dorsal penile nerves were blocked with 2% lidocaine hydrochloride (5 ml) and a #22 scalpel blade was used to excise a 1cm diameter sample of surface tissue from each target area. Each excision area was closed with 0 chromic gut in a cruciate pattern. Collection of all samples was conducted by the same investigator to minimize differences of collection methods. Tissue samples were pressed onto sections of tongue depressors to reduce artifact contracture and folding during fixation. Each tissue sample was immersion fixed and stored in a mixture of 4% paraformalin, 1% glutaraldehyde (Electron Microscopy Services, Hatfield, PA), and 150 mM phosphate buffered saline (Sigma Chemical, St Louis, MO) prior to processing.

Light microscopy

All steps of tissue preparation were performed under a ventilated hood in compliance with OSHA guidelines. Tissues for light microscopy were prepared by rinsing in phosphate buffered saline (PBS) in three separate rinses of 20 min each to remove fixative agents. Fixative-free tissues were dehydrated with ethyl alcohol (ETOH) in graded strengths to displace water from the tissues. Fixation was performed in the following manner: 1) each sample was placed in 30% ETOH and agitated at room temperature for 30 min, 2) the samples were placed in a series of ascending concentrations of ETOH (50%, 70%, 85%, 100%) for 30 min each, 3) samples were washed two times in hexamethyldisilazane (HMDS; Electron Microscopy Services) for 30 min each to clear residual ETOH, and 4) tissue samples were placed under a fume hood overnight to allow remaining chemicals to be removed.

Tissue samples were placed in molds for embedding in paraffin. Samples were processed in a Tissue-Tek VIP E300™ (Ames Co., Inc., Elkhart, IN) for 2-3 hrs. Each sample was cut into 7 micrometer sections using a Reichert-Jung 2040 Autocut Microtome™ (Germany). The sections were mounted on glass microscope slides with resin and allowed to dry for 20 minutes, then stained with hematoxylin and eosin (Sigma Chemical).

Each biopsy sample was examined at 40 X magnification to evaluate the morphology of the penile and preputial surface epithelium. Evaluation was performed by a veterinary pathologist blinded as to the group and location from which the biopsy specimen was collected. Image J software (NIH.gov) was used to determine epithelial surface area, the area of epithelial folds, and the number of epithelial folds per section. Measurements of each parameter from each location were compared within and between groups.

Scanning electron microscopy

Tissues for scanning electron microscopy (SEM) were collected and stored in a mixture of 4% paraformalin, 1% glutaraldehyde (Electron Microscopy Service), and phosphate buffered saline (PBS; Sigma Chemical) at 4°C. The tissues were rinsed in PBS for 20 min with agitation. Tissues were fixed in 4% osmium tetroxide/PBS (Electron Microscopy Services) for two hours, rinsed with PBS for three changes of 20 min each, and rinsed with distilled water for 20 min.

Tissues were dehydrated as previously described for light microscopy. All samples were stored under a vacuum to ensure complete dehydration. The samples were mounted on an aluminum specimen stud (Electron Microscopy Services) and sputter coated with colloidal gold (Electron Microscopy Services) prior to viewing with an EVO 50 SEM™ (Zeiss XVP, USA). Images were captured with the EVO 50™ (Zeiss XVP, USA) computer and stored on removable file.

Similar to light microscopy samples, surface epithelium of each sample was evaluated by a veterinary pathologist blinded to group and location from which biopsy specimens were collected. Differences in the surface epithelium at each anatomical location were compared within and between groups by subjective evaluation.

Biopsy analysis

The following parameters were measured for each biopsy (3.256 mm): 1) area of the epithelium between the basement membrane and the luminal surface, 2) area of epithelial folds under a line drawn from the pinnacles of the surface epithelium at the edges of the epithelial fold, 3) the number of epithelial folds, and 4) presence of crypts.[3,4,9] An epithelial fold was defined as a deviation encompassing an area greater than 200 square microns, the area that would accommodate a *Tritrichomonas foetus* organism (20x10 microns). These parameters could be consistently measured with Image J software.

Area of epithelium

Each sample of epithelium was labeled according to the anatomical location from which it was collected. From each specimen color images of 4080 x 3072 pixel resolution (40X magnification) were acquired with a light microscope (Olympus ® BH-2) and digital camera (Olympus® DP-71). Epithelial borders were traced and epithelial areas were determined using Image J analysis software.

Area of epithelial folds

For each sample of epithelium, the borders of the epithelial folds were outlined, the area of the epithelial fold determined and the number of folds recorded using Image J software.

Examination for crypts

Each biopsy was examined with scanning electron microscopy for presence of structures that met the criteria for crypts as described.[3,4,9] The surface epithelium of each biopsy from each bull was evaluated and compared within and between age groups.

Statistical analysis

Area of epithelium, area of epithelial folds, and total number of epithelial folds per biopsy were compared within and between age groups by ANOVA using the SAS analytical system (SAS software, SAS Institute, Cary, NC). A value of $p < 0.05$ was considered significant.

Results

Area of epithelium

The area of the epithelium did not differ within ($p = 0.35$) or between ($p = 0.77$) groups (Figure 1). The mean ± SD epithelial areas were: Group 1: distal 0.8 x 10^6 ± 0.3 x 10^6 µM^2, middle 0.7 x 10^6 ± 0.2 x 10^6 µM^2, and proximal 1.0 x 10^6 ± 0.3 x 10^6 µM^2; Group 2: distal 0.8 x 10^6 ± 0.3 x 10^6 µM^2, middle 0.7 x 10^6 ± 0.3 x 10^6 µM^2, and proximal 0.8 x10^6 ± 0.3 x 10^6 µM^2.

Area within epithelial folds

The area of epithelial folds did not differ within ($p = 0.07$) and between groups ($p = 0.15$) (Figure 2). The areas of epithelial folds (mean ± SD) for group 1 were: distal 0.1 x 10^6 ± 0.1 x 10^6µM^2, middle 0.1 x 10^6 ± 0.03 x 10^6 µM^2, and proximal 0.2 x 10^6 ± 0.1 x 10^6 µM^2. The areas of epithelial folds of group 2 were: distal 0.1 x 10^6 ± 0.09 x 10^6 µM^2, middle 0.07 x 10^6 ± 0.05 x 10^6 µM^2, and proximal 0.1 x 10^6 ± 0.1 x 10^6 µM^2, (Figure 2).

Number of folds

The number of epithelial folds did not differ between biopsy sites within ($p = 0.56$) and between ($p = 0.41$) age groups. The number of epithelial folds (mean ± SD) per site was: Group 1 distal 12.3 ± 3.8, middle 9.2 ± 3.4, and proximal 12.7 ± 5.8; Group 2 distal 12.3 ± 6.7, middle 10.6 ± 5.3, and proximal 14.1 ± 11.4 (Figure 3).

Electron microscopic evaluation

Tissues were examined by electron microscopy for presence of epithelial crypts at 31–170X magnification. Folds of epithelium were present on all sections examined but no structures comparable to intestinal crypts were identified.[9]

Discussion

In 1941, shortly after the initial description of *T. foetus*, Abelein noted that there were numerous infoldings present on the surface of the penis. The author suggested that *T. foetus* established long-term infection in these crevices.[10] It has been stated that presence of deeper crypts in older bulls allows the development of chronic infection with *C. fetus* subsp. *venerealis*.[2] For decades older bulls have been assumed to have deeper crypts than younger bulls, but little documentation exists to support this conclusion. This study was undertaken to evaluate the differences in the penile and preputial epithelium between different aged bulls at three different anatomical locations.

The first objective of this study was to determine the thickness of the epithelial layer of the penis and prepuce as the bull matures. The area of the surface of the epithelium did not differ within or between groups.

A second objective of this study was to determine the area encompassed by the epithelial folds and to compare differences within and between groups. Area within the epithelial folds did not differ within or between groups. This was unexpected and contrary to the widely held belief that older bulls would have increased areas of the epithelial folds. In each age group the area within the epithelial folds was greater at the proximal and distal sites than the middle.

The final objective was to determine the total number of epithelial folds in each age groups. The total number of folds did not differ within or between the groups. Also, based upon examination by SEM, there is an absence of structures that can be classified as crypts. Due to the absence of the crypt or crypt-like structures, the authors propose that epithelial folds be used as the preferred descriptive term.

Architectural characterization and electron microscopic evaluation of penile and preputial epithelium may enhance future studies of the pathophysiology and immunology of venereal infections in bulls.

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Group 1	Group 2
Angus 25 mo.	Angus 6 yrs.
Angus 26 mo.	Angus 5 yrs.
Angus 25 mo.	Angus 12 yrs.
Brahman 24 mo.	Angus 5 yrs.
Angus 27 mo.	Angus 7 yrs.
Angus 26 mo.	Angus 6 yrs.

Table. Age and breed of bulls.

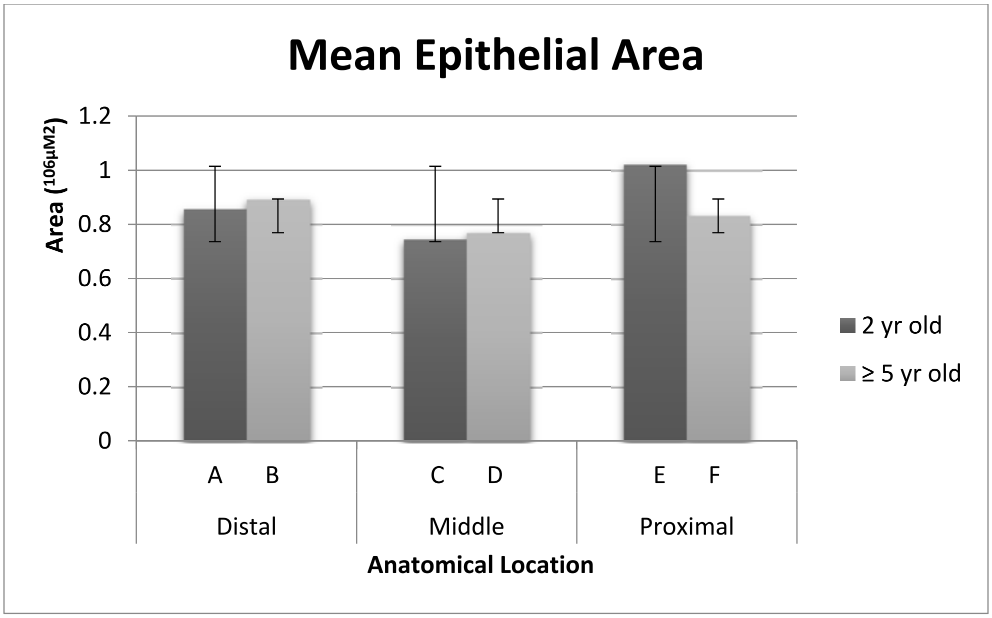

Figure 1. Mean epithelial area and standard deviation (A-F) per site. Area between groups is not different (p= 0.77) A) SD ± 0.3 x 10^6 B) SD ± 0.3 x 10^6 , C) SD ± 0.2 x 10^6 , D) SD ± 0.3 x 10^6 , E) SD ± 0.3 x 10^6 , F) SD ± 0.3 x 10^6.

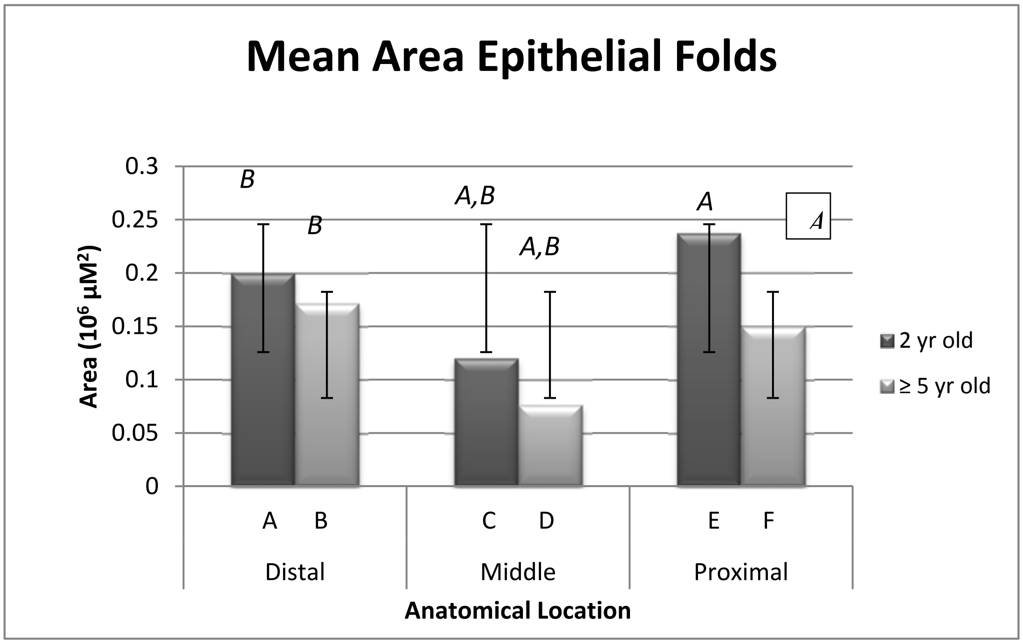

Figure 2. Mean area of infoldings and standard deviations (A-F). Area of infoldings between age groups is not different (p = 0.15) A) SD ± 0.1 x 10^6 B) SD ± 0.09 x 10^6 , C) SD ± 0.03 x 10^6 , D) SD ± 0.05 x 10^6 , E) SD ± 0.1 x 10^6 , F) SD ± 0.1 x 10^6
A) Mean area of infoldings within each group was greater in proximal sites than in middle sites (p = 0.025).
B) Mean area of infoldings within each group was greater in distal sites than in middle sites (p = 0.028).

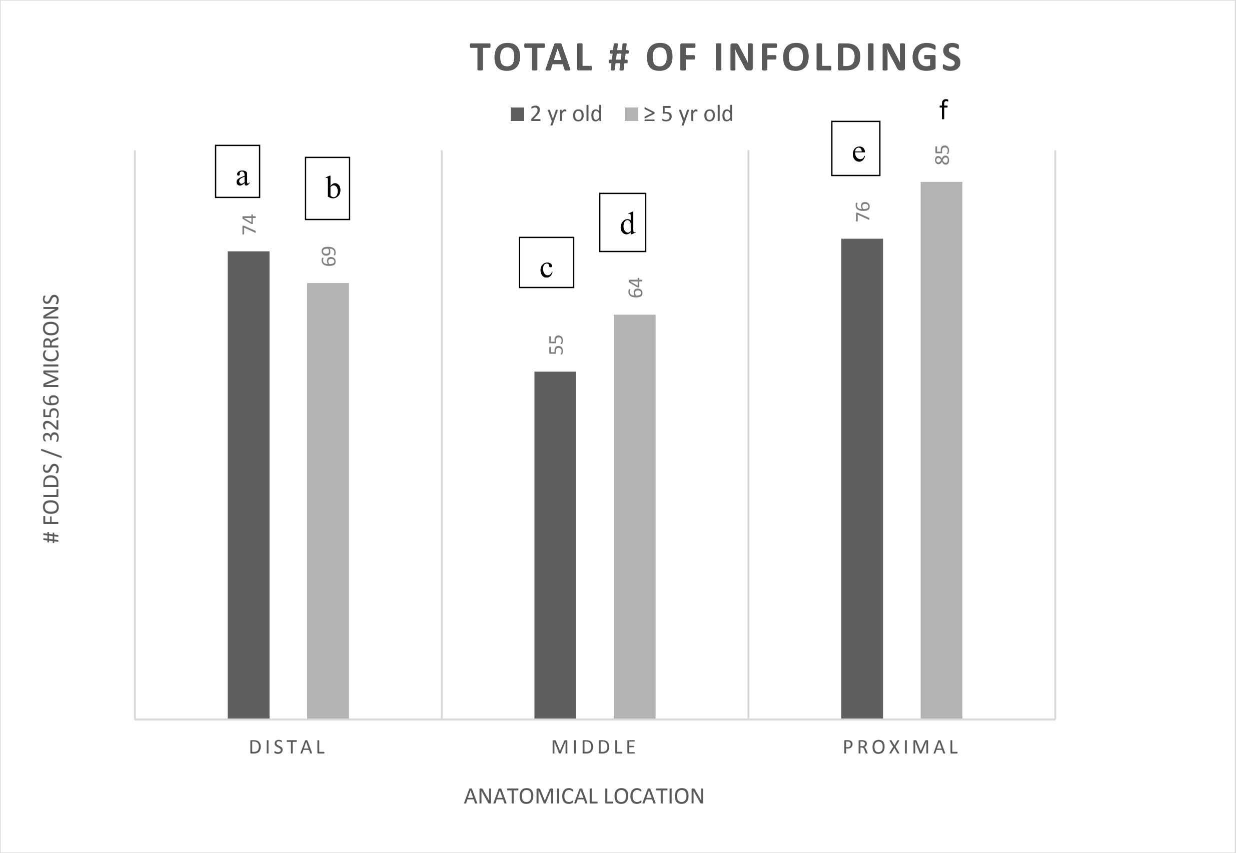

Figure 3. Total number of folds/3256 µM and standard deviations (a-f).
a) Mean 12.3 SD ± 3.8, b) Mean 12.3 SD ± 6.7, c) Mean 9.2 SD ± 3.4, d) Mean 10.6 SD ± 5.3, e) Mean 12.6 SD ± 5.8 f) Mean 14.2 SD ± 11.4.