Evaluation of the effect of routine histologic processing on the size of skin samples obtained from dogs

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Objective—To determine the effects that routine histologic processing has on the dimensions of samples of normal skin of dogs and assess whether the inclusion of a muscle or fascial layer in such samples alters those effects.

Sample Population—Skin samples obtained from 6 medium-sized adult dogs with grossly normal skin.

Procedure—From each dog, skin samples (with or without underlying fascia or muscle) were obtained from 3 sites bilaterally (6 samples/dog) and processed routinely for histologic evaluation; their dimensions were measured at intervals during the experiment.

Results—As a result of processing, skin samples decreased in size (combined percentage change in length and width) and increased in thickness, compared with their original dimensions. Samples without fascia or muscle decreased in size by 21.1% to 32.0% and increased in thickness by 45.1% to 75.8%. The site of sample origin influenced processing-associated changes in sample size but did not affect the change in thickness. Decreases in dimensions did not vary with inclusion of fascia but did vary with inclusion of muscle. The change in thickness did not vary with inclusion of a layer of fascia or muscle.

Conclusions and Clinical Relevance—Processing of skin samples obtained from dogs for histologic evaluation can cause changes in sample dimensions; samples may decrease in length and width by as much as 32% and increase in thickness by 75.8%, compared with their original dimensions. The presence of muscle in canine skin samples can restrict the amount of shrinkage in length or width associated with processing. (*Am J Vet Res* 2005;66:500–505)

Neoplasia is a very important health issue in companion animals. Surgical resection remains a cornerstone of treatment for many neoplastic diseases. In fact, complete surgical removal of nonhematologic solid tumors provides a cure in more human patients than any other single treatment modality.[1] In human and veterinary medicine, whether the tumor has been removed in its entirety is largely determined by the appearance of the tumor margins at the light microscopic level. The completeness of excision (ie, complete vs incomplete excision) is an important prognostic factor in predicting local recurrence for many neoplasms. To determine whether the margins are clean or contaminated, excised tissues are usually examined histologically for the absence or presence of neoplastic cells along the surgical edges[2-4]; however, pathologists must make this determination on the basis of representative samples, as they cannot examine every margin of the tissues submitted. Neoplasia may extend to within a very short distance (within 1 or several millimeters) of the surgical margins. In these situations, the margins are sometimes referred to as "clean but close" by pathologists. The quantification of clean margins by pathologists, especially when tumor cells are located close to the surgical margins, is becoming more commonplace. There is much debate regarding the most prudent course of action to take for patients in whom the tumor has been excised with narrow margins.[2,5–7] At present, these debates center predominately on personal bias and beliefs with very few scientific facts available to support a dogmatic response.

To understand the clinical importance of margins that are determined to be only a few millimeters wide after excision and processing of the tumor tissues, knowledge of the biological behavior of the tumor in question and the extent of the margin in situ before the tissue was excised, manipulated, and processed is essential. Without doubt, tissue undergoes many gross and microscopic alterations during excision and processing for histologic evaluation. Tissue shrinkage is a well-recognized consequence of excision and processing.[2,3,7–18] Quantification of the alterations in size that canine skin samples undergo during excision and routine histologic processing would be useful in the understanding of the clinical importance of margins that are designated as clean but close. In time, such information would help veterinarians make scientifically reasoned recommendations regarding whether additional treatment is necessary in a given situation.

In human medicine, the need to understand morphologic alterations during tissue handling has been recognized. Many studies[8,9,12,13,15-17] have been performed on various tissues to determine shrinkage patterns and size alterations during various processing techniques; these studies have included investigation of many different types of neoplasia including cancer of the breast,

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prostate, colon, lung, brain, liver, and kidney.[7,10,14-16,18] In humans, the ability to accurately predict tissue shrinkage that occurs between surgical excision and histologic assessment has allowed surgeons to more effectively attain adequate margins at the time of the primary surgery. For example, for excision of colonic adenocarcinoma, a histologic margin of at least 2 cm is recommended to achieve local control of the tumor, and the excised colonic tissue is expected to shrink by 60% after excision and processing. Therefore, the surgeon knows that excision of the tumor must include 5-cm margins in situ.[10] This information has proven to be very useful in the surgical treatment of humans with tumors.[2,7,10,15]

At present, little is known about the effect of processing (with regard to size alterations) on tissue samples obtained from dogs.[8,11,13] Masses within the skin and subcutaneous tissues are the most commonly diagnosed neoplasms in dogs.[19] To the authors' knowledge, no quantification of skin shrinkage following collection and processing of canine skin samples has been performed. This would be very useful information and could provide a basic stepping-stone for recommendations regarding adjuvant care in dogs with tumors, as well as a basis on which to plan the first surgical resection in affected dogs.

Inclusion of a fascial or muscular layer is commonly performed when removing cutaneous or subcutaneous tumors in an effort to obtain a deep margin that is free from neoplastic disease.[20] The inclusion of a fascial or muscular layer may alter degree of change that occurs as a result of tissue processing. It is also possible that the degree of processing-associated change depends on the anatomic site of excision. The purpose of the study reported here was to determine the effects that routine histologic processing has on the dimensions of samples of normal skin of dogs and assess whether the inclusion of a muscle or fascial layer in such samples alters those effects.

Materials and Methods

Six medium-sized adult dogs with a body score of 4 to 6 (on a scale of 1 to 9)[21] were included in the study. These 6 dogs were part of a terminal-use teaching program unrelated to this study and were to be euthanatized following their use. The dogs weighed 12 to 30 kg; on physical examination, they were free of any grossly apparent dermatologic disorders. The experimental protocol used in this study was approved by the University of California-Davis Animal Care and Use Committee. Each dog was anesthetized; anesthesia was maintained by use of inhaled isoflurane for approximately 8 hours before the skin samples were collected. This time frame was necessary to allow procedures unrelated to this study to be performed. During this period, the dogs received lactated Ringer's crystalloid solution IV at a rate of 10 mL/kg/h. During anesthesia, dogs were monitored by a combination of systolic blood pressure measurements obtained via Doppler signal and cardiac auscultation via an esophageal stethoscope. On the basis of these variables, as well as eye position, jaw tone, and pulse quality, the inhaled anesthetic concentration was adjusted to ensure an adequate plane of anesthesia throughout the procedures.

To collect the skin samples, the dogs were placed in ventral recumbency and 6 areas were routinely clipped and prepared for a surgical procedure by use of a microbicidal surgical scrub followed by a rinse with isopropyl alcohol. For each dog, a skin sample was obtained from 3 sites bilaterally (ie, 6 samples/dog; **Figure 1**). All of these samples were elliptical in shape. A 4 × 2-cm ellipse was surgically removed from the head at a site centered between the rostral extent of the pinna and midline (site 1). The longer axis was oriented in a medial to lateral direction, parallel to the line of skin tension in this region.[22] The right and left site 1 samples were randomly assigned to excision of either skin and subcutis alone (sample 1a) or skin and subcutis with inclusion of the underlying superficial fascia of the temporalis muscle (sample 1b). Site 2 was the lateral aspect of the thorax immediately caudal to the forelimb and triceps musculature. An 8 × 4-cm ellipse of skin was removed with the long axis of the ellipse oriented in the dorsal-ventral direction; this orientation provides a sample that is parallel to the lines of skin tension in this region.[22] Each side of the dog was randomized for excision of either skin and subcutaneous tissue to a level immediately deep to the cutaneous trunci muscle (sample 2a) or skin and subcutaneous tissue with inclusion of underlying latissimus dorsi muscle (sample 2b). The final site (site 3) was the lateral aspect of the hind limb, immediately distal to the stifle joint. The depth of the skin sample was as deep as but not including the superficial fascia of the cranial tibial muscle on both the left and right sides (sample 3a1 and sample 3a2, respectively). The elliptical incision was 4 × 2 cm with the longer axis oriented in the proximal-distal direction. The orientation of this sample was perpendicular to the lines of skin tension in this region.[22] With our sample designation system, all samples designated by use of a lowercase letter *a* in the sample description were free from any major underlying muscle or fascia and included only skin and subcutaneous tissue.

After preparation for the biopsy procedure, the ellipse was drawn over the selected areas by use of a standard surgical skin marker.[a] The marker included a sterile ruler, which was used to measure the elliptical boundaries. The initial skin incision was made by use of a No. 10 scalpel blade routinely mounted on a No. 3 scalpel handle. This initial incision was made through only the most superficial portion of the epidermis to prevent skin contracture before the initial measurements were taken. One drop of tissue dye[b] was then placed in each of 4 locations on the surface of the skin. These locations represented the approximated extremities of the incision in the cranial (or rostral), caudal, medial (or dorsal or proximal), and lateral (or ventral or distal) directions and served as the points from which the measurements were obtained. The color selected for each region was standardized to all specimens to prevent loss of orientation during subsequent measurements and manipulations of the tissues. All specimen measurements were made by use of a digital precision caliper[c] and were repeated 3 times at each measurement. The mean of these 3 measurements was selected to minimize laboratory error.

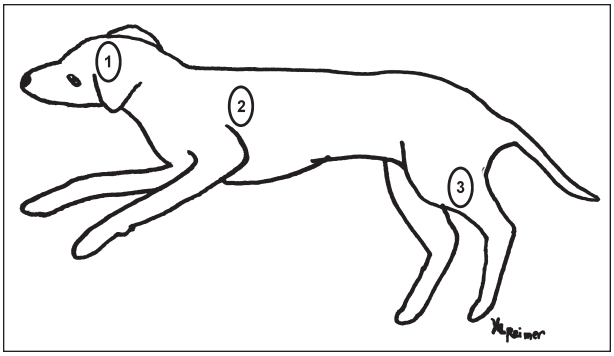

Figure 1—Illustration of the sites (1, 2, and 3) of skin sample acquisition performed bilaterally in 6 dogs (6 samples/dog).

The first set of measurements was taken immediately after the superficial incision was made into the epidermis (time point, T1). The measurements included the rostral (cranial) to caudal distance and the medial (or dorsal or proximal, depending on the site) to lateral (or ventral or distal, depending on the site) distance. For simplification, these measurements are referred to as width and length, respectively. The tissue was then undermined at the appropriate depth and removed from the body of the dog. The tissue dye was used to mark the 4 different thicknesses at the same location that had been previously marked by the small drops of tissue dye on the skin surface. The same color was selected to correspond to the surface dye, and a fine line of dye was applied to the tissue from the superficial to the deep limitation of the excised tissue in the same 4 locations that had just been measured for length and width. The second set of measurements that were made (time point, T2) included the previously outlined distances as well as the thicknesses of the tissue at all 4 marked marginal locations.

Ten minutes were allotted to allow the dye to air-dry. The 6 measurements were then repeated (length, width, and 4 thickness measurements; time point, T3). The tissue was placed in an amount of neutral-buffered 10% formalin that was approximately 10 times its volume for optimal tissue preservation. The tissue remained in the formalin for 36 hours. Another set of the 6 individual measurements was obtained at that time (ie, postfixation; time point, T4). Finally, a veterinary pathologist (HED) cut the tissues (including the 6 regions of interest) for standard paraffin embedding and histologic preparation. For each tissue sample, one 5-µm-thick section was prepared and stained with H&E. The 6 dimensions of interest were measured (time point, T5) with a ruler that has a scale to 0.5-mm accuracy. From each tissue sample, the transverse margin was reembedded in the opposite orientation for measurement of the dimension that needed to be added to the longitudinal section for accuracy. This maneuver was performed to compensate for the temporary inability to include this tissue in the dimension of the orthogonal measurement. Again, 5-µm-thick sections were obtained, stained with H&E, and measured, as described.

Data collected included 3 separate measurements at each of 6 anatomic sites at each of the 5 defined time points during processing (T1 through T5). Measurements were taken of the width (D1), length (D2), and each of 4 thicknesses (D3 through D6). Thicknesses could be assessed only at T2 through T5 because at T1, only a partial-thickness incision had been made through the epidermis and although D1 and D2 were measured, thickness measurement was not possible. The percentage changes in D1 and D2 from the original values were combined (percentage total surface change) to assess the change in size of samples during processing. These data were analyzed by use of repeated measures of variance to evaluate the main effects of site, time, and dimension, as well as their respective interactions. The effect of location was determined via comparisons among the samples from site 1 without a fascial layer (sample 1a), site 2 without the latissimus dorsi (sample 2a), and site 3 (left and right sides; samples 3a1 and 3a2). The effect of including a fascial layer was determined via comparison of samples from site 1 with a fascial layer (sample 1b) and samples from site 1 without a fascial layer (sample 1a). The effect of including a muscle layer was determined via comparison of samples from site 2 with latissimus dorsi (sample 2b) and samples from site 2 without muscle (sample 2a). Values of $P < 0.05$ were considered significant.

Results

Effect of location—With respect to width and length (D1 and D2, respectively), the processed skin samples from the head, lateral aspect of the thorax, and right and left sides overlying the tibia did change in size with time (ie, T1 through T5; $P < 0.001$). On assessment of the percentage total surface change that occurred from T1 through T5, the size of samples 1a, 2a, 3a1, and 3a2 decreased by 21.1%, 26.6%, 32%, and 31.8%, respectively, compared with their original sizes. This change in size with processing time (from T1 through T5) did vary ($P < 0.001$) by location but did not vary ($P = 0.31$) by dimension being measured (D1 vs D2). As expected, changes detected in samples 3a1 and 3a2 were not significantly ($P = 0.24$) different.

With regard to their thickness (D3 to D6), the samples became thicker ($P < 0.001$) during processing (from T2 through T5). The thickness of samples 1a, 2a, 3a1, and 3a2 increased by 75.8%, 45.1%, 75.2%, and 72.9%, respectively, compared with their original thicknesses. This change in thickness with processing time did not vary by location ($P = 0.094$) nor by dimension being measured (D3 to D6; $P = 0.45$).

Effect of inclusion of a fascial layer—With respect to width and length, the processed skin samples obtained from the heads of the dogs (with and without inclusion of the fascial layer) did change in size with time (ie, T1 through T5; $P < 0.001$). On assessment of the percentage total surface change from their original sizes that occurred from T1 through T5, the samples without the fascial layer (samples 1a) decreased in size overall by 21.1% whereas the samples with the fascial layer (samples 1b) decreased by 20.7%. This change in size with processing time did not vary by the inclusion or exclusion of a fascial layer ($P = 0.75$) but did vary by dimension (D1 vs D2; $P < 0.001$). Compared with their original lengths and widths, both samples decreased more in length (25%) than width (11.8%).

With regard to their thickness (D3 to D6), the samples became thicker ($P < 0.001$) during processing (from T2 to T5). The thickness of samples 1a and 1b increased by 75.8% and 56.2%, respectively, compared with their original thicknesses. This change in thickness with processing time did not vary by the inclusion or exclusion of a fascial layer ($P = 0.15$) nor by the dimension being measured (D3 to D6; $P = 0.57$).

Effect of inclusion of a muscle layer—With respect to width and length, the processed samples did change in size over time (ie, T1 through T5; $P < 0.001$). On assessment of the percentage total surface change from their original sizes that occurred from T1 through T5, the samples without a muscle layer (samples 2a) decreased in size overall by 26.6% whereas the samples with a muscle layer (samples 2b) decreased by 17.6%. This change in size with processing time did vary ($P = 0.038$) by inclusion or exclusion of a muscle layer but did not vary by the dimension being measured (D1 vs D2; $P = 0.67$).

With regard to their thickness (D3 to D6), the samples became thicker ($P < 0.001$) during processing (from T2 to T5). The thickness of samples 2a and 2b increased by 45.1% and 36.2%, respectively, compared with their original thicknesses. This change in thickness with processing time did not vary by inclusion or

exclusion of a muscle layer (P = 0.16) nor by the dimension (D3 to D6; P = 0.36).

Discussion

The results of our study reaffirm the fact that routine histologic processing causes considerable morphologic alterations of excised tissues. In the interval between in vivo collection and microscopic assessment, all canine skin samples used in the present study underwent a change in size as a result of manipulation and processing. Samples decreased in length and width and increased in thickness, compared with their original dimensions. The underlying causes of this phenomenon include contraction of underlying muscular supportive structures in the tissue samples or dehydration associated with immersion in hypertonic fixatives.[11,23]

The assessment of the percentage total change in surface dimensions (width and length) revealed that the size of the samples decreased during processing, compared with their original dimensions, which was expected. However, the overall increase in thickness of samples during processing detected in our study was unexpected because other investigators have reported a decrease in sample thickness with routine processing of tissues obtained from the oral cavity of dogs.[11] In 1 study[9] of human cervical tissue, routine histologic processing of tissue samples resulted in no shrinkage in an anterior to posterior direction, but there was shrinkage in the other 2 directions. To the authors' knowledge, no net increase in dimension has been reported to occur in excised tissue samples as a result of histologic processing. Experimentally, sample thickness proved to be logistically the most difficult dimension to measure, which alerted us to the possibility that this finding was a result of laboratory error. However, the consistency of this finding among the different biopsy specimens examined, regardless of the observer, makes laboratory error unlikely. Subjectively, the tissues appeared grossly thicker at each measurement stage as well. If the decrease of the surface dimensions is caused by contraction of myoepithelial cells within the tissue,[11] then the increase in thickness of the sample may be a consequence of the presence of thicker, contracted myoepithelial cells underlying the contracted skin surface. However, among the biopsy specimens processed in our study, one would expect that the samples that underwent the greatest shrinkage in the surface dimensions would have the greatest increase in thickness, but this was not necessarily what we observed. Although significant differences in the change in surface dimensions were detected among samples from different locations on the body or between samples from a given location with and without inclusion of a muscle layer, there was no significant difference in the increases in thickness among samples from different locations on the body or between samples from a given location with and without inclusion of a muscle layer.

A significant difference in the extent of surface shrinkage was detected among the samples from different sites that contained only skin and subcutaneous tissue (ie, samples 1a, 2a, 3a1, and 3a2). Of these samples, those obtained from the skin overlying the tibia had the greatest decrease in surface dimensions, whereas those obtained from the skin of the head had the least amount of shrinkage. The skin samples from the thoracic area were intermediate in their degree of shrinkage. An explanation for this observation may be found in the inherent properties of the subepithelial tissues in these regions. It is possible that better organization of myoepithelial structures in skin may impart a greater capacity for extensive contraction following surgical transection; this may be a feature of the skin overlying the proximal portions of the tibiae of the dogs used in our study. Another explanation may be that, independent of the myoepithelial structures, certain anatomic locations on the body such as limbs are associated with more skin tension than others, which would lead to a greater degree of shrinkage of the skin after incision. In this manner, skin may be considered analogous to an elastic band; the more stretched an elastic band is before it is cut, the more it will shrink when cut. However, the factors that influence the extent to which skin in different anatomic locations shrinks on incision are currently unknown.

With exception of the samples of skin overlying the tibia, the long axis of each sample obtained from the study dogs was oriented parallel to the lines of tension of the skin, as previously reported.[22] This was performed to mimic clinical situations in which it is considered optimal to attempt skin closure parallel to these lines of tension except over the extremities, where incisions are commonly made parallel to the long axis of the limb (ie, perpendicular to the lines of tension).[24] Situations will arise in the clinical setting where this orientation is not attainable because of the extent and location of the disease process that requires resection. In the present study, we measured only 2 surface dimensions (length and width) in the skin samples, and excision of skin samples in other tangential orientations may result in an alteration of overall shrinkage.

Although not significant, there were differences in thickness among the samples collected from different locations on the body; the thickness of the skin samples from the thoracic site increased by 45.1%, compared with increases in thickness of 75.8%, 72.9%, and 75.2% for the samples from the other 3 locations. It is possible that a type II error occurred (ie, the null hypothesis was not rejected when there was truly a difference) and that had samples been collected from more dogs, a difference would have been detected leading to a rejection of the null hypothesis.

In our study, no difference in the extent of shrinkage during processing between skin samples with and without fascia was detected. Although inclusion of this structure during resection of a cutaneous tumor is widely viewed as an important physiologic barrier to neoplastic extension,[20] our results suggest that the presence of a superficial fascial plane in a skin sample has no effect on the dimensional alterations of that sample caused by histologic processing. The reason why inclusion of a fascial layer would allow the length of a skin sample to decrease significantly more than the width (25% vs 11.8%, respectively) during processing

is unknown. The suggestion that the orientation of the samples with respect to the lines of skin tension plays a role is appealing; if this were true, whichever dimension of the skin sample was parallel to the line of skin tension would be expected to shrink more than others. However, the same result should be expected from all the samples consisting of skin only, which was not evident in our study. It may be possible that this result is specific to the head location and that if other anatomic locations on the body had been investigated for the effect of inclusion of a fascial layer in skin samples, the length and width of samples would have been altered by the same degree.

As for a fascial layer, the inclusion of an underlying muscular plane with an en bloc resection of a cutaneous tumor is viewed as an appropriate surgical principle to apply in an attempt to control local neoplastic disease.[20] The results of the present study suggest that the inclusion of a relatively large muscle segment (eg, the latissimus dorsi muscle) in a skin sample has an effect on the degree of alteration of the sample's surface dimensions caused by routine histologic processing. It has been suggested that muscle sustains relatively less shrinkage during processing when compared to other tissue types.[17] This protective effect was evident in the skin samples evaluated in our study.

The present study has some limitations. We only had a small number of dogs from which the samples were collected (6 samples from each of 6 dogs; 36 samples in total). Attempts to achieve exact orientation for all measurements of each sample at each time were time-consumptive yet critical. This makes inclusion of large numbers of samples in this type of study logistically impossible. All dogs were of medium size and fairly standard conformation. It would be conceptually easy to imagine that samples of skin obtained from dogs of breeds in which skin is not stretched taut before transection (eg, Shar Peis) would sustain less shrinkage during processing, compared with those obtained from dogs of breeds in which skin is stretched much more taut (ie, Greyhounds). Therefore, in assessment of dimensional alterations in skin samples during histologic processing, breed and individual differences almost certainly exist; patient age and underlying condition (benign vs malignant disease process) have also been shown to affect the degree of tissue shrinkage.[25,26]

In the skin samples used in our study, the surface dimensions were fairly simple to measure at each stage; however, samples were more difficult to orient in a standard manner for measurement of thickness at each time stage as different tissue planes of samples tended to slide past each other or fold on excision. Without doubt, on occasions during the series of evaluations, the perfect orientation of samples for measurement of thickness was not achieved. The fact that there was a consistent increase in the thickness measurement over time lends weight to our overall findings that sample thickness does actually increase over time.

All the dogs in our study were free from dermatologic disease and had grossly normal skin, which would respond in a physiologically normal manner to surgical resection. Clinical application of these findings must be made with some caution because the behavior of skin affected with pathologic conditions may be decidedly different, compared with that of normal skin. It is likely that the tumor tissue within a sample would not undergo the same degree of morphologic changes because of inherent differences in tissue composition between different types of tumors as well as differences between tumors and normal tissues. However, because the margins of normal tissue around an excised tumor are of interest in clinical practice, the results of the present study may be helpful in understanding pathologists' reports that include a quantitative description of the margins. Also, in our study, the duration of formalin fixation of skin samples was 36 hours because this coincides most appropriately with our clinical setting; other clinical situations may necessitate longer or shorter fixation times, which presumably may alter the overall dimensional changes in skin samples.

Our results have suggested that cutaneous samples processed for histologic evaluation in a routine manner undergo alteration of their length, width, and thickness; in skin samples obtained from dogs processed according to the conditions of our study, these alterations resulted in a decrease in their surface dimensions, but an increase in their thickness over time. In dogs, clinical investigation of different cutaneous tumors needs to be performed to determine the optimal margin of pre-excisional tissue needed to obtain local control of neoplastic diseases.

a. Securline, Precision Dynamics Corp, San Fernando, Calif.
b. The Davidson Marking System, Bradley Products Inc, Bloomington, Minn.
c. Mitutoyo Corp, Aurora, Ill.

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