Histology of collagen in Merino sheep skin and its association with skin wrinkle formation and follicle curvature

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1 Introduction

Wrinkle formation in Australian Merino sheep skin is a phenomenon with serious economic and political consequences. It has long been known (Seddon, Belschner, and Mulhearn (1931) [22]) that wrinkled sheep are more susceptible to blowfly strike. The use of the *mulesing* operation to control flystrike in Merino sheep has recently been the subject of intense animal ethics scrutiny. No effective alternative management option has appeared. The most effective long term solution would seem to be to breed the wrinkle out of Merino sheep. This approach has at times met with resistance from some Australian Merino breeders who feel that the extra skin surface area of wrinkled sheep is necessary to achieve high levels of wool production. Breeding plans which include some culling on wrinkle usually do not lead to its complete elimination (for example Turner Dolling and Kennedy (1968) [26]).

This study is an attempt to go back to the basic biology of wrinkle formation, to see whether we can understand the tissue structure of a wrinkle, and to see if that suggests a better approach breeding of wrinkle-free sheep, without lowering productivity or adversely affecting wool quality.

There have been very few attempts to define what a wrinkle actually is. The early work of Carter(1943) [3] went as far as describing and naming all the folds on the neck, body, and breech, and developed a set of photographic scores for degree of wrinkle. Carter used the terms *fold* and *wrinkle* interchangeably, but he distinguished the small *pin wrinkles* present in all Merinos, from the larger folds which develop to varying degrees as the sheep matures. From this early start, there is, somewhat surprisingly, nothing on the biology of wrinkles, until the study of Mitchell et al(1984) [16].

The Mitchell et al(1984) [16] paper defines five tissue layers in sheep skin.

Layer1 epidermis is mainly keratinised protein

Layer2 contains wool follicles and accessory glands, and is part of the dermis. Sometimes called *papillary layer*.

Layer3 layers 2 and 3 together called 'dermis' . Contains fibrous proteins, collagen, and elastin. Sometimes called *reticular layer* although the structure is not always reticular, but may be interwoven.

Layer4 contains voluntary muscle, collagen and elastin

Layer5 adipose tissue

These are illustrated in Figure 1

Only the first 3 layers curve upward in a folded section of skin, layers 4 and 5 remain straight. This can be seen in Figure 1. Mitchell et al note that Layer2 is much weaker than Layer 3 (collagen not as hard). When wrinkles or folds

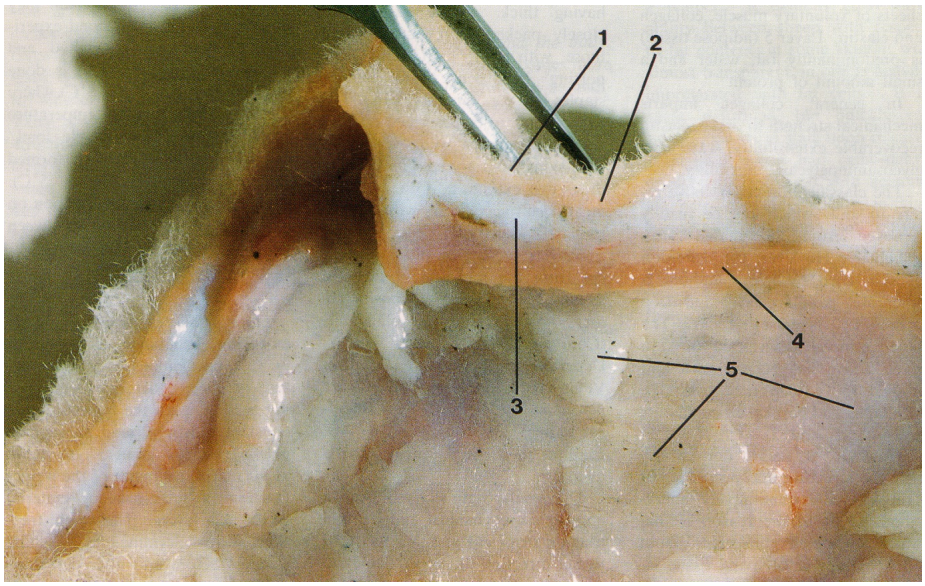

Figure 1: Merino sheep skin showing layers. 1. epidermis with wool fibres; 2. papillary layer of dermis; 3. reticular layer of dermis; 4. areolar tissue and muscle; and 5. adipose tissue. Two wrinkles are present; one alongside each side of the forceps (from Mitchell et al (1984) [16])

occur in the skin, Layers 1,2, and 3 buckle up into a fold, while Layers 4-5 are straight. It appears as if wrinkles are formed either by an overgrowth of Layers 1-3, or by a shrinkage or tightening of Layer 4. Mitchell has demonstrated this by showing that if Layer4 (and Layer 5) are dissected away from a skin specimen with wrinkles, the folds in Layers 1-3 flatten out. So in a wrinkled sheep, Layer 4 is holding the skin under some tension, which relaxes when Layer 4 is removed.

Even less is known about wrinkle development. Merino lambs are born with visible wrinkles. A somewhat obsure reference (Bogolyubsky (1940) [1]) asserts that wrinkles were observed forming in foetal skin of Karakul and Merino lambs at around 100 days of gestation. That is about the time at which the secondary derived follicles initiate. Carter(1943) [3] presents a photograph of the skin surface of a 10 day old Merino lamb (Plate 13 Figure 1) which clearly shows small *pin wrinkles*. There are no other studies of foetal wrinkle development, but there is a considerable literature on follicle development (see Fraser and Short(1960) [4] and Maddocks and Jackson(1988) [14] and Ryder and Stevenson(1968) [21] for reviews). There is some literature on collagen development in sheep skin, and we will look at that below.

What is to be investigated in this study is that the amount and type (and maybe timing and arrangement in the skin) of collagen development might be a factor involved with both wrinkle development and follicle development. So what is known about collagen? Well, it is already present in the dermis

(layers 2 and 3) of foetal skin at the time follicles develop (Knight et al (1993) [13]). These authors distinguish two collagen types (Type III or 'soft' collagen, and Type I or 'hard' collagen) and note that Type III is highest at 75 days of gestation, and falls progressively as the foetus develops, while Type I is low at day 75 and rises to over 50 percent by birth. Collagen fibres are formed from cells called *fibroblasts*. At 75-80 days the fibroblasts appear as plump, immature cells surrounded by reticular collagen fibres which are composed of Type III collagen. By birth the fibroblasts have matured and the collagen fibres may be intermeshed to varying degrees. If the fine reticular fibre pattern remains, it is soft collagen, if the fibres intermesh the collagen tissue is hardened to various degrees.

Collagen development, secondary follicle development and wrinkle formation all seem to commence at the same time of around 100 days of foetal age. Follicle development ceases at around birth (150 days) but development of collagen and wrinkles continues into the adult sheep. In this study we look at the end points of development - that is we study collagen and follicles in adult sheep with and without wrinkles. That will not reveal the details of development, but it should make clear any obvious associations between collagen, wrinkles, and follicles.

2 Materials and Methods

The experimental design was to choose, by visual inspection, individual sheep with wrinkle-free skin and wrinkly skin from each of a number of Australian Merino flocks. The flocks available for this study were mostly flocks which were undergoing breeding towards the SRS ^{TM} Merino type. Consequently most of the sheep chosen as examples of wrinkle-free sheep would have the loose and supple skin which is characteristic of SRS ^{TM} Merinos. There is another sort of wrinkle-free sheep which has low follicle density and tight skin and this type is probably not well represented in the present study.

Two trials were conducted

Trial 1 Two sheep were chosen from each of five Merino flocks, one wrinkle-free and one wrinkles. This is a randomized block design without replication . The blocks are the five flocks, and the treatment is the presence or absence of wrinkle.

Trial 2 Eighteen sheep were chosen from each of two flocks, nine wrinkle-free and nine with wrinkles. This is a randomised block design with replication. The second of these two flocks was more wrinkled and was not breeding towards the SRS ^{TM} Merino type.

2.1 Skin samples

In Trial 1 a biopsy sample was taken from the midside position on each sheep and the specimens were trimmed in the normal manner before processing, so

that only Layer 1 (epidermis) and Layer 2 (papillary dermis) were present for histological observation.

In Trial 2 , for the sheep with wrinkly skins, skin biopsies were collected from on the wrinkles as well as between the wrinkles. For the wrinkle-free sheep only one biopsy sample was collected. These specimens included Layers 1 to 4, ie only the adipose tissue was trimmed.

Midside skin samples were collected using a 10 millimetre circular trephine (Acu Punch skin biopsy punches, Acuderm, Inc.) and fixed in 10% formol saline solution.

2.2 Macroscopic skin observations

Skin samples were washed in several changes of water, the wool stubble trimmed and then examined under a magnifying lamp (x 3 magnification). Scores for suppleness (1 = hardened to 5 = supple) of the papillary layer and reticular layer were made. Each skin sample was examined to determine if layers 2 and 3, and layers 3 and 4, were free or fixed and whether localized hardening and folding of the skin had occurred.

The thicknesses of the papillary dermis and the reticular dermis were measured using a ruler graduated in one millimetre divisions. A Mitutoyo ballpoint gauge (model no. 2046S) was then used to measure the compressed thickness at four sites for each skin sample.

2.3 Histological skin processing and observations

2.3.1 Collagen observations

Skin samples used for haematoxylin and eosin staining (H-E) and picrosirius red (PSR),were fixed in 10% neutral buffered formalin for 24 hours before being processed to wax in an automated tissue processing platform (Shandon Excelsior, Thermo Scientific, USA), and then embedded in paraffin wax. Four micron sections were cut and placed onto slides for H-E staining for tissue morphology. Serial section was also employed on a separate slide for PSR staining to highlight collagen content. Staining was performed manually.

Sections were then reviewed microscopically (BX53 Olympus, Australia)), and images taken on 3 CCD camera (DP72, Olympus, Australia) under both bright field and polarized conditions for PSR staining.

For PSR collagen analysis, the 40x objective was employed at a fixed exposure to take high power images of 5 random deep dermal fields of view for computational analysis.

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[ Sanaz it seems to me that these 5 random fields would have been
chosen within the red stained areas with collagen present.
I think we should say so]
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The images for each sample were then uploaded for quantitative analysis via the ImagePro Plus (Media Cybernetics, USA) 7.1 software in which thresholds

were set to count all pixels comprising of the red staining fibres in the PSR stained specimen against the total pixels. A mean was calculated for each of the specimens' 5 images and graphed.

Polarised light was employed in order to try and determine the type of collagen present within each of the samples.

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[Sanaz, you made a comment about this on Jim's last draft.

The yellow and green reflectances are likely to indicate soft (Type III)
 collagen (Sanaz, please check this statement).
 Need to be careful here Jim, as no one has been able to definitively
 prove the birefringences of PSR staining with collagen fibres,
 and some of the literature contradicts itself.
 I can pull a few papers to reference as a guide to the reviewers?

and this

Birefringence measurements of PSR stained skin sections indicate that
 nearly all  (. %) of the collagen sheets in the subfollicular
 layer of the papillary dermis have the deep red light reflectance
indicative of hard (type I) collagen. (Sanaz, please check this statement).
 Again Jim, we have to tread carefully here making definitive
 statements based on colour birefringence. We can certainly point
 out that the thicker fibres were red, and the thinner fibres more
 green, with some yellowish-orange colours in between.

Can you make some statement that is either definitive or indicative
 and give a reference please?

I think it belongs here in the Methods, not mixed up with Results where Jim had it.
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2.3.2 Vertical skin sections

Vertical skin sections, approximately 0.3 millimetres wide, were cut freehand with a sharp razor blade on a freezing stage and stained with 0.25 % Nile blue sulphate, as described by Nay (1973). The sections were cut parallel with the angle of emergence of the fibres to avoid cutting through follicles. Mean follicle curvature was scored from 1 = straight follicles to 7 = tangled follicles by reference to a set of standard drawings used by Nay and Johnson (1973). Follicle depth was measured as both the perpendicular and angular distances (in millimetres) between the skin surface and the lower ends of the follicle bulbs, along with follicle bending, as described by Maddocks and Jackson (1988).

2.3.3 Horizontal skin sections

Horizontal skin sections were also prepared as described by Maddocks and Jackson (1988) using the frozen section technique and measurement procedures of

Nay (1973). The sections were used to measure follicle density, secondary follicle to primary follicle ratio (S/P ratio), primary fibre diameter and secondary fibre diameter of the sheep.

JW to describe measurement of orientation of follicle groups and measurements made of collagen sheets in subfollicular layer of papillary dermis.

2.4 Summary of measurements

2.5 Statistical Methods

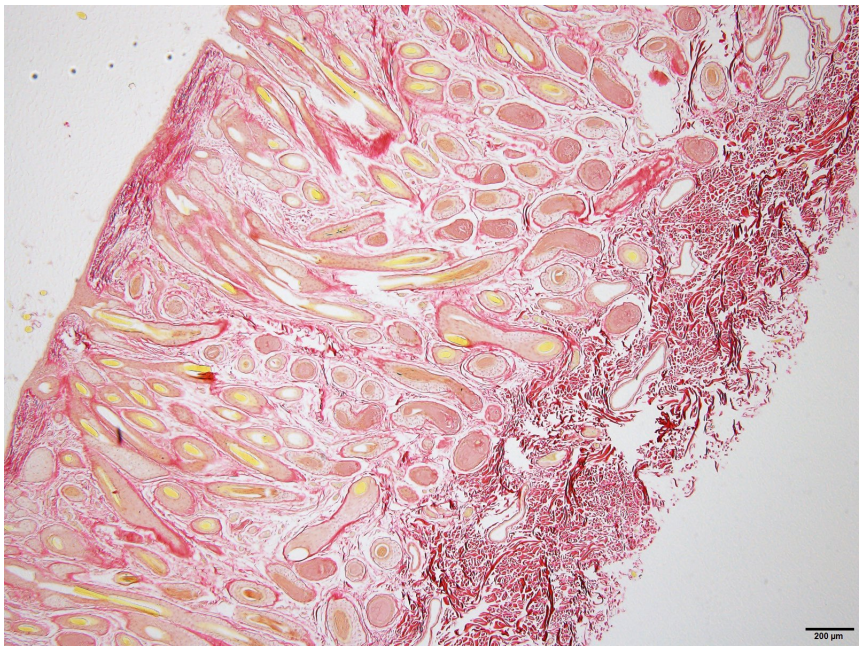
Data were imported into the R statistical program [20] and analysed using the *aov()* function for analysis of variance.

3 Results

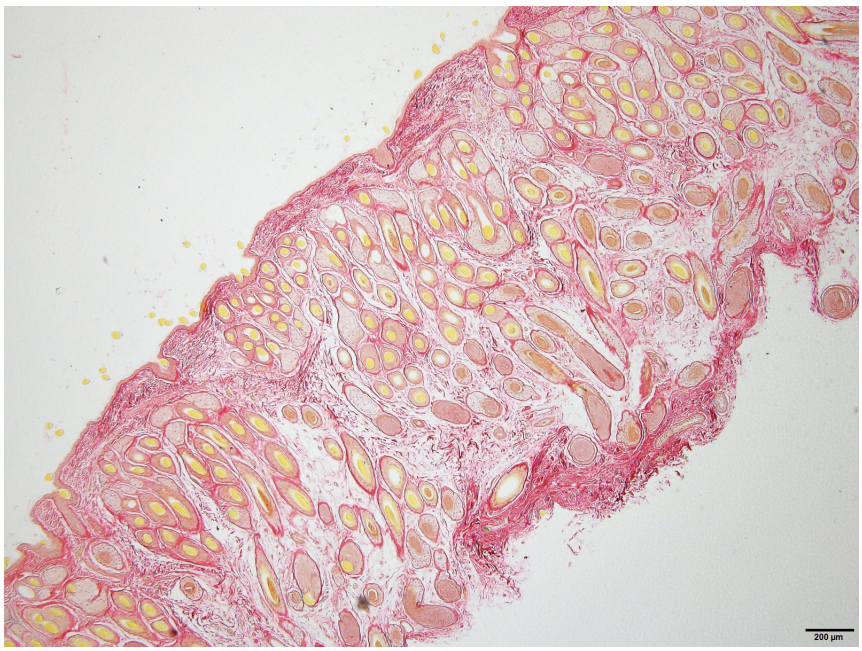
We follow the path of looking first at overall morphology of skin specimens, then at the details of collagen structure, and finally at other related measurements

3.1 Skin tissue Morphology

The pairs of wrinkle free and wrinkled sheep from each flock in Trial 1 showed consistent visual differences in their tissue structure. Figure 2 shows vertical sections stained with H-E from the wrinkled and wrinkle-free pair of sheep from flock 1.



(a) Sheep w479-2 Wrinkled



(b) Sheep x490-2 Wrinkle-free

Figure 2: Vertical sections from a wrinkled (a) and a wrinkle-free (b) sheep from Trial 1 flock 3 stained

4 Discussion

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