Short communication: Temporal changes in the skin morphology of dairy cows during the periparturient period

Banri Suzuki,* Toshie Sugiyama,* Chikako Yoshida,^{†1} and Toshihiko Nakao[‡]

*Faculty of Agriculture, Niigata University, 2-8050 Ikarashi, Niigata, Japan

[†]Field Center for Sustainable Agriculture and Forestry, Niigata University, 6934 Ishizone, Gosen, Niigata, Japan

[‡]Faculty of Veterinary Medicine, Rakuno Gakuen University, 582 Bunkyo-daimidori-cho, Ebetsu, Hokkaido, Japan

ABSTRACT

Management of dairy cow productivity requires monitoring of their nutritional status by visual observation. It has been suggested that changes in hair coat appearance are among the indicators of nutritional state in dairy cows. Temporal changes in the skin morphology in cows, however, have not been reported. In this study, we examined the changes in the skin of dairy cows that occur during the peripartum period. Seven pluriparous cows were used. Skin samples were collected at 28 d before the due date and 28 d and 56 d after calving for morphological examination. Hair follicle width was $108.8 \pm 5.9 \mu\text{m}$ ($\pm\text{SD}$) in the dry period, $95.5 \pm 5.5 \mu\text{m}$ at 28 d after calving, and $104.2 \pm 5.3 \mu\text{m}$ at 56 d postpartum. The percentages of anagen hair follicles during these 3 periods were 41.4 ± 3.4 , 18.5 ± 3.4 , and $32.3 \pm 3.3\%$, respectively. The corresponding sebaceous gland sizes were $8,362.0 \pm 707.6$, $7,800.0 \pm 831.4$, and $9,186.8 \pm 962.6 \mu\text{m}^2$, respectively. Hair follicle width was positively correlated with percentage of anagen hair follicles. The thickness of epidermal and proliferation rate of epidermal cell were also correlated. However, the hair follicle width, sebaceous gland size and cell proliferation rate, and thickness and proliferation rate of epidermal cells did not show any marked changes.

Key words: dairy cow, skin, morphology, periparturient

Short Communication

Skin of malnourished animals and humans shows signs of atrophy (Mathur and Doe, 1976; Thavaraj and Sesikeran, 1989). Malnourished beef cattle show follicular atrophy and decreased epidermal thickness (Uesaka and Yahata, 1955; Haaland et al., 1977). The nutritional state of hair follicles determines hair growth in sheep (Ryder, 1973), and it has also been suggested

that hair thickness is influenced by the nutritional state of the hair follicles in humans and rats (Sims, 1968; Salas et al., 1995). The sebaceous glands of humans respond to prolonged caloric deprivation with diminished sebum secretion and altered sebum composition (Pochi et al., 1970). Undernourished animals have a poor hair coat (Miyamoto et al., 1941; Nielsen et al., 1975; Watson, 1998; Kirby et al., 2009); thus, changes in the hair coat may indicate an animal's nutritional status. Hair growth is a cyclical process that varies during the different follicular phases; hair is produced during the anagen phase and hair growth stops during the telogen phase. Sebum, which gives hair its luster, is secreted by the sebaceous glands. Identification of early changes to the hair coat requires histological examination. Changes in the hair coat may reflect underlying changes in the skin (Landaeta-Hernández et al., 2011). Modern dairy cows produce a large quantity of milk with inadequate energy intake during early lactation, and thus are in negative energy balance during the postpartum period (Berglund and Danell, 1987; Butler, 2003). It is hypothesized that hair follicle size and cycle phase, sebaceous gland size and cell proliferation, and epidermal thickness and cell proliferation in cows show temporal changes during the periparturient period. The aims of this study were, therefore, to identify changes in the skin morphology of dairy cows during the peripartum period.

The study protocol and all procedures were conducted in accordance with the guidelines of the Ethics Committee for the Care and Use of Laboratory Animals for Research of Niigata University, Japan. This experiment was conducted at the Field Center for Sustainable Agriculture and Forestry of Niigata University. Seven multiparous Holstein cows that calved between June and December 2014 were used. The mean $\pm$ standard deviation of lactation number was 3.4 ± 1.3 . The average milk yield per cow was 8,700 kg/305 d. The herd was managed in a tiestall shed and fed concentrate and forage separately. In the dry period, cows were given 2.0 kg/d of concentrate, 1.0 kg/d of beat pulp pellets, and haylage ad libitum. After parturition, the amount

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¹Corresponding author: cyoshida@agr.niigata-u.ac.jp

of concentrate given was increased by 1 kg every week up to an appropriate volume for each cow depending on its milk yield and BW. The study period consisted of the close-up period (3 wk before parturition) to early lactation (3 wk after parturition), during which time the energy balance decreased drastically and then recovered (Chagas et al., 2007; Bicalho et al., 2017). Over the experimental period, skin and blood samples were collected 3 times, at 28 d before the expected calving date, and 28 and 56 d after parturition. To indicate the energy metabolism of cows, the serum concentrations of fatty acids were measured using a discrete-type clinical chemistry automated analyzer (TBA-120FR, Toshiba Medical Systems, Tochigi, Japan) with NEFA-HR (Wako Pure Chemical Industries, Osaka, Japan). Body condition scores were measured at the same time. One diclofenac tablet containing 25 mg of diclofenac sodium (Bolabomine 25 mg, Tsuruhara Pharmaceutical, Osaka, Japan) was administered per 100 kg of BW 30 min before skin sample collection. For ease of collection, the shoulder is the best position because it is easy to immobilize and take a piece of skin. Two skin biopsies were collected from the left shoulder of each cow using an 8-mm dermatome (Biopsy Punches, Kai Industries, Tokyo, Japan); these biopsies were later used for histological and immunohistochemical analyses.

Skin samples were fixed in a 10% buffered-formalin solution. After fixation with 10% buffer-formalin solution, skin samples were divided pieces 3 mm × 4 mm × 2 mm of size and embedded in paraffin wax or frozen in optimal cutting temperature compound (Sakura Finetek Japan, Tokyo, Japan). Fifteen 5-μm-thick paraffin-wax-embedded sections were stained with hematoxylin and eosin (HE). Five 10-μm-thick cryosections from 15 frozen optimal cutting temperature samples were stained with Oil Red O to allow observation of the location of lipids within the tissue and to analyze its size. After general light microscopy analysis, the morphometry of each skin sample was analyzed as described below.

Immunohistochemical detection of proliferating cell nuclear antigen (PCNA), a marker of cell proliferation (Mokřý and Nĕmecek, 1995), was performed using a Vectastain ABC-AP standard kit (Vector Laboratories, Burlingame, CA), in accordance with the manufacturer's protocol. In brief, the paraffin sections described above were reacted with a primary mouse anti-PCNA antibody (Abcam, Cambridge, UK) and a secondary biotinylated goat anti-mouse IgG antibody (Vector Laboratories) at dilutions of 1/16,000 and 1/200, respectively. The reaction was visualized using ImmPACT Vector Red Alkaline Phosphatase Substrate (Vector Laboratories). Proliferating cells showed red staining in their nuclei, and the cell proliferation was

represented as the ratio (%) of PCNA-positive cells to total cells counted.

The hair follicle width and activity (percentage of follicles in the anagen phase), sebaceous gland size and activity (cell proliferation rate), and epidermal thickness and activity (epidermal cell proliferation rate) were examined as skin morphological parameters. Three to 5 skin sections were chosen randomly from each paraffin block. To assess hair follicle width, hair follicle phase, and epidermal thickness, skin sections stained with HE were used. To measure sebaceous gland size, Oil Red O-stained sections were used. Longitudinal (vertical) sections were used for analyzing hair follicle width, sebaceous gland size and activity, and epidermal thickness and activity, whereas transverse (horizontal) sections were used for investigating hair follicle phase. Five sections were selected randomly and 3 images (magnification × 100) were selected randomly for each section and the skin morphological parameters were assessed using image analysis software (Nikon NIS-Elements Documentation Ver. 3.13, Nikon Corporation, Tokyo, Japan). The numbers of hair follicles in the anagen and telogen phases of the hair cycle were counted and the anagen:telogen ratio was calculated. Exogen follicles were considered as those with features of both anagen and telogen phases. Hair cycle stages were identified using the method described by Innerå et al. (2013) and Kim et al. (2014). Hair follicles in the anagen phase were defined as having a well-defined inner root sheath (Figure 1a). Hair follicles in the telogen phase were defined as having an inner root sheath that appeared as a red amorphous keratin mass (Figure 1b). Hair follicles with characteristics of both anagen and telogen phases were observed (Figure 1c); these follicles were considered to be in the exogen phase, the phase during which a new hair shaft grows before the resting shaft has been shed (Stenn et al., 1998). Epidermal thickness was defined as the distance of the stratum basale and the stratum granulosum. The thickness of each skin section was calculated as the mean of 30 distances randomly measured. Stratum corneum, rete pegs, and hair follicles were excluded from the measurements. To measure the proliferation rate of sebaceous gland cells, 3 randomly selected, paraffin-embedded skin sections per cow were used. The sebocytes in the sebaceous glands were counted. All sebaceous glands on each slide were observed. In addition, to evaluate the epidermal cell proliferation rate, 5 randomly selected skin sections were used. For each skin section, between 200 and 300 epidermal keratinocytes were counted.

Data were saved in Microsoft Excel 2010 (Microsoft Corp., Redmond, WA). Statistical analyses were conducted using JMP (Japanese version 4.4, SAS Institute Japan, Tokyo, Japan). The significance of differences in

Figure 1. Classification of hair cycle stages in dairy cows using transverse hair follicle sections ($\times 400$). Bars = 10 μm . (a) An anagen hair follicle characterized by a well-defined inner root sheath (arrow). (b) A telogen hair follicle characterized by an inner root sheath that appears as an amorphous keratin mass (arrow). (c) An exogen hair follicle (Stenn et al., 1998), which has characteristics of both anagen (black arrow) and telogen (white arrow) phases. Color version available online.

the skin sample parameters between sampling periods was assessed by multivariate ANOVA. The correlations between certain pairs of skin parameters were assessed by regression analysis. Significance was designated at $P < 0.05$.

Generally, blood fatty acid lower than 200 $\mu\text{Eq/L}$ indicates positive energy balance. The critical threshold of fatty acid is considered more than 300 $\mu\text{Eq/L}$ (Ospina et al., 2010). The cows in this study were in an appropriate nutritional state at 28 d before the due date. Three of 7 cows showed more than 300 $\mu\text{Eq/L}$ of fatty acid at 28 d after calving; they were considered still remaining in low nutritional condition. Their condition recovered at 56 d. Experimental cows showed BCS of 2.75 to 3.5 before parturition, reduced after calving to that of 2.5 to 3.0 at 28 d, and recovered to 2.75 or more at 56 d. Over the experimental period, all cows were in the ideal BCS range (Chagas et al., 2007; Roche et al., 2009).

Figure 2a shows a representative image of an HE-stained section of skin showing the hair follicles and epidermis. Functional sebaceous glands were identified by their lipid content using Oil Red O-stained sections (Figure 2b). Sebocytes and epidermal cells expressing PCNA protein were identified by immunohistochemistry (Figure 2c).

Table 1 shows skin changes during the peripartum period. The percentage of anagen follicles was decreased at 28 d after parturition relative to their preparturient values, but had recovered by 56 d postpartum. Hair follicle width and sebaceous gland size, sebaceous gland cell proliferation rate, epidermal thickness, and epidermal cell proliferation rate were similar throughout the study period.

The correlations between certain pairs of skin parameters were analyzed: hair follicle width and the percentage of anagen follicles, sebaceous gland size and cell proliferation rate, and epidermal thickness and cell proliferation rate. Significant correlations were observed between hair follicle width and the percentage of anagen follicles ($n = 21$, $r = 0.37$, $P < 0.01$) and between epidermal thickness and cell proliferation rate ($n = 21$, $r = 0.33$, $P < 0.01$). Although the changes in sebaceous gland size and sebaceous gland cell proliferation rate were similar, no significant correlation was observed between these parameters ($n = 21$, $r = 0.06$, $P = 0.3$).

Malnutrition causes atrophy of the hair follicles, sebaceous glands, and epidermis in animals and humans (Uesaka and Yahata, 1955; Mathur and Doe, 1976; Haaland et al., 1977; Thavaraj and Sesikeran, 1989). In this study, hair follicle width, sebaceous gland size, and sebaceous gland cell proliferation decreased after calving; however, epidermal thickness and cell proliferation did not change during the same period. The hair

Figure 2. Longitudinal skin section used for skin morphology analysis ($\times 100$). Bars = 100 μm . (a-1 to a-3) Hair follicles (Hf) and epidermis (Ep) are shown by hematoxylin and eosin staining. (a-1) At $-d$ 28, (a-2) at $+d$ 28, and (a-3) at $+d$ 56. (b-1 to b-3) Lipid in sebaceous glands is shown by Oil Red O staining (arrowheads). (b-1) At $-d$ 28, b-2) at $+d$ 28, and (b-3) at $+d$ 56. (c-1, c-2) Immunostaining revealed proliferating cell nuclear antigen (PCNA) protein localization in sebaceous glands (arrows): positive (black arrow), negative (white arrow). Counterstained with hematoxylin. Color version available online.

cycle of cattle is also dependent on season (Dowling and Nay, 1960; Haaland et al., 1977); therefore, we collected samples over multiple seasons, from summer to winter, to avoid seasonal bias. They were kept in a cowshed throughout the experiment. The range of changes in each skin parameter differed among the individuals. No seasonal effects could be found due to the small number of animals.

Regression analysis revealed a significant ($P < 0.05$) positive correlation between hair follicle width and the

percentage of hair follicles in the anagen phase. The reduction in hair follicle width might be caused by the decreased percentage of anagen hair follicles because hair follicles in the telogen phase are in a regressed state (Ryder, 1973; Stenn et al., 1998; Ross and Pawlina, 2006), and are thus probably smaller than during the anagen phase of the hair cycle. Estrogen also influences the skin of humans and rodents (Conrad and Paus, 2004; Ohnemus et al., 2006). Estrogen administration decreases sebaceous gland size and epidermal thickness

Table 1. Skin changes in dairy cows during the peripartum period¹

Item	Days relative to parturition		
	28 d before	28 d after	56 d after
HF width (μm)	108.8 $\pm$ 5.9	95.5 $\pm$ 5.5	104.2 $\pm$ 5.3
Anagen HF rate (%)	41.4 $\pm$ 3.4 ^a	18.5 $\pm$ 3.4 ^b	32.3 $\pm$ 3.3 ^{ab}
Sebaceous gland size (μm^2)	8,362.0 $\pm$ 707.6	7,800.0 $\pm$ 831.4	9,186.8 $\pm$ 962.6
SGC proliferation rate (%)	30.9 $\pm$ 4.3	24.6 $\pm$ 2.7	32.6 $\pm$ 3.4
Epidermal thickness (μm)	30.4 $\pm$ 1.6	29.4 $\pm$ 1.5	28.8 $\pm$ 1.8
EpC proliferation rate (%)	39.1 $\pm$ 6.6	37.2 $\pm$ 3.5	34.0 $\pm$ 3.0

^{a,b}Different superscripts indicate significant differences ($P < 0.01$).

¹HF = hair follicle; SGC = sebaceous gland cell; EpC = epidermal cell. Values are means $\pm$ SE (n = 7).

in rodents (Hooker and Pfeiffer, 1943; Oh and Smart, 1996) and induces a premature catagen phase in the hair follicles of mice (Oh and Smart, 1996; Hu et al., 2012). Conversely, in humans, estrogen prolongs the anagen phase, resulting in an increased number of follicles in the anagen phase of the hair cycle during pregnancy (Conrad and Paus, 2004; Gizlenti and Ekmekci, 2014). In cows, blood estrogen levels increase during pregnancy because of placental estrogen secretion (Domeki, 1998a,b). Estrogen levels in the late gestation period are high, with estradiol-17 β over 0.29 ng/mL (Shah et al., 2006). After parturition, blood estradiol levels decrease to lower than 6.1 $\pm$ 1.7 pg/mL (Rettmer et al., 1992; Sumiyoshi et al., 2014). The life cycle of hair follicles during the periparturient period has not been determined, and remarkable differences in blood estrogen concentrations before and after calving may have considerable effects on hair follicles.

Fatty acids may influence gene or protein expression in human hair follicles (Schmidt and Horsley, 2012), and a reduction in intradermal adipocytes delays the follicular transition to anagen (Maklad et al., 2009; Sugawara et al., 2010). The composition of fatty acids, particularly the oleic and palmitic acid concentrations, has been shown to regulate gene transcription in adipocytes (Dennis et al., 1991; Distel et al., 1992; Clarke and Jump, 1996). Specific fatty acids can also influence the sebaceous glands. Linoleic acid stimulates, and palmitic acid inhibits, human sebocyte proliferation in a dose-dependent manner in vitro (Akai et al., 1994). However, in this study, neither blood estrogen levels nor specific fatty acid components were determined.

In addition, a significant and consistent correlation was present between epidermal thickness and the epidermal cell proliferation rate during the peripartum period. This suggests that epidermal thickness is determined by the proliferative activity of the epidermal cells. However, despite the similarity in their changes during the peripartum period, no correlation was observed between sebaceous gland size and sebaceous gland cell proliferation rate. This suggests that, in cows, changes

in sebaceous gland size during the peripartum period may not depend on the number of sebocytes resulting from cell proliferation but more likely depend on the size of the individual sebocytes, which is determined by their stage of maturation.

In conclusion, the skin morphology showed temporal changes in terms of the percentage of anagen hair follicles in dairy cows during the periparturient period. The hair follicle width, size of the sebaceous gland, and thickness of epidermal cells did not show any marked changes.

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