Quantification of pericaudal adipocyte diameter in dairy cattle: a complementary method to study energetic status using conventional histology.

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Key words: adipocyte, dairy cow, peripartum.

Abstract.

The energetic status of high yielding Holstein-Friesian dairy cattle was studied under field conditions using Body Condition Score (BCS), glycaemia, seric ß–hydroxi-buthyrate and adipose tissue cellularity. This last method was tested as a complementary tool for energetic status assessment. Biopsies of pericaudal subcutaneous adipose tissue were obtained from twenty five multiparous animals at 28 days before and 21 days after parturition. Samples were routinely processed for histological examination and stained with hematoxylin and eosin. The Mean Diameter of Adipocytes (MDA) was measured with the aid of a digital image processor. During the same period, animals were blooded weekly for metabolite determinations. Differences between MDA 28 d pre-partum and 21 d post-partum were significant at $p < 0.1$ (72.11 vs. 66.18 µm respectively, $p= 0.055$ *), as also BCS (3.32 vs. 3.19 respectively, $p = 0.068$ *). At prepartum, the BCS was positively correlated with MDA (Pearson's $r = 0.521$, $p =0.016$ *), and with glycaemia (Pearson's $r = 0.404$, $p = 0.056$ *). No significant correlation with ß–hydroxi-buthyrate was observed. Our results suggest that routine histological preparations of biopsies from subcutaneous adipose tissue could be included as an easy and valuable tool to evaluate metabolic adaptation of dairy cows to peripartum. This complementary method associated with BCS and/or metabolic parameters could be used to evaluate the energy status of commercial herds.

Introduction

Lactation is essential for the survival of neonate mammals and it involves a substantial transfer of energy from mother to offspring. This transfer is facilitated by numerous endocrine and cellular adaptations that enhance lipid mobilization from white adipose tissue. This physiological response of adipose tissue during peripartum of dairy cows is critical for milk production, feed intake, reproduction, health status and, in turn for the dairy industry (Bauman & Currie, 1980; Waltner et al., 1994; Blok et al., 2001). The inability to quickly adjust lipid metabolism to meet the increased energetic needs frequently results in acute and subclinical metabolic disorders of dairy cattle (Bauman & Currie, 1980; Reist et al., 2002; Dann et al. 2005; Hachenberg et al., 2007).

Dairy cows experience major changes in energy metabolism during the peripartum period, especially those with high yielding performance. The sudden onset of lactation highly increases the metabolic activity of the mammary glands which raises total energy requirements by approximately fourfold (Block et al., 2001). A pronounced energy deficit develops because voluntary feed intake do not suffice to meet this increased energy expenditure, which leads to an important lipidic catabolism in white adipose tissue (Block et al., 2001).

Previous studies of subcutaneous adipose tissue cellularity in man and domestic animals, including cattle, have demonstrated that total fat tissue mass and adipocyte mean cell size are fairly well related, and this relationship could be used to indirectly assess total fat mass in living animals (Hirsch & Gallian, 1968; Hood & Allen, 1973; Etherton et al., 1977; Robelin, 1981; Alzón et al., 2007; Faulconier et al, 2007). As fat deposition in cattle has important economic and medical interests, the increase in adipocyte diameter can be used as an

indicator of lipogenic activity under different metabolic challenging situations - i. e., during growth, undernourishment, or breeding - (Alzón et al., 2007; Faulconier et al, 2007). In the case of the lactating dairy cow, subcutaneous adipose depots have crucial importance for energy metabolism during lactation, and its amount varies according to lactation stage (Smith & McNamara 1990). Besides genetic merit, milk production rate and energy intake, adipose cellularity also varies with changes in body mass and BCS - Body Condition Score - (Garnsworthy & Topps, 1982; Smith & McNamara 1990; Waltner et al, 1994, Domecq et al, 1995). Therefore, adipocyte diameter was used to estimate the amount of body fat deposition during bovine lactation (Pike & Roberts, 1980; Waltner et al, 1994). Currently, computer image analysis can be used to quantify MDA -Mean Diameter of Adipocytes- based on isolated cellular preparations (Etherton et al., 1977), as an indicator of lipogenic activity in adipose tissue (Schoonmaker et al., 2004; Alzón et al., 2007). However, data concerning the lipolytic activity of adipose tissue in the lactating dairy cow is complex to interpret due to the intense lipolytic activity that usually develops to overcome the Negative Energy Balance (NEB) faced during early lactation (Chilliard et al., 1999).

Body Condition Score is the most practical and widely extended method for the monitoring of fatness in dairy cows of commercial farms, because it is a non-invasive, quick, and non-expensive technique (Edmonson et al, 1989). The BCS although subjective, is valuable when used consistently (Edmonson et al, 1989). Unfortunately, little information is available on how BCS and the total amount of body fat are related in the dairy cow (Waltner et al, 1994). Also, there is no information about concurrent changes in BCS and adipocyte size using routine histology during peripartum in commercial dairy herds.

In dairy farms, seric concentrations of glucose and ketone bodies (among other metabolites) during peripartum were frequently used associated with BCS to evaluate adaptation to NEB (Kunz et al. 1985; McNamara et al, 1995; Rukkwamsuk et al. 1998; Kokkonen et al, 2005). The decreased availability of glucose precursors in the liver during early lactation increases ketone body production because of the high rate of gluconeogenesis, both enhanced by a limited food intake (Kunz et al., 1985; Chilliard 1999). As a result of this, significant lipid mobilization from subcutaneous adipose tissue leads to a progressive loss of body mass which decreases BCS (Chilliard, 1999).

A practical quantitative method for measuring adipose tissue mobilization in live animals associated to other metabolic parameters would be desirable, but particularly limited information is available for dairy cattle commercial herds, especially during peripartum (Pike & Roberts 1980; Smith & McNamara, 1990).

The aim of the present work was to quantify changes in MDA during peripartum of dairy cows, by biopsy sampling subcutaneous pericaudal white adipose tissue using conventional histology. We investigated the association between MDA and BCS with glycaemia (GLY) and seric levels of ß–hydroxi-buthyrate (BHBA) during the adaptation to the NEB that occurs during early lactation.

Materials and Methods.

Animals and adipose tissue sampling.

Experimental protocols were approved by the Institutional Animal Care and Use Committee of Universidad de Santiago de Compostela.

Twenty five multiparous high-yielding Holstein-Friesian cows housed in a stall of a commercial dairy farm of Lugo Province (Sociedad Agraria de Transformación Vilamaior de Negral, Guntín, Spain) were studied. Cows were fed with grass and corn silage offered *ad libitum* and supplemented with concentrate. The average milk yield of the preceding lactation was about 29 liters/cow/day, within 310 d of lactation. Two subcutaneous fat samples were obtained from each animal, the first at 28 d before partum and the second 21 d after calving. Biopsies were taken from alternate sides of the tailhead, after injection of a 2% procaine hydrochloride solution as anesthetic. The biopsy area was shaved and cleaned with an iodine scrub and disinfectant, an incision of 2-3 cm length was made between the tailhead and the tuber ischii, and a sample of approximately 1 cm^3 of subcutaneous white adipose tissue was removed. Incisions were sutured and treated with topical antibiotics agents. Biopsy procedure was done according Smith & McNamara (1990) and Lemor et al. (2009). Tissue samples were cut into 0.5 cm^3 pieces and fixed in 10% formalin until analysis.

Body Condition Scoring (BCS) and Blood samples processing.

BCS was evaluated in a 1 to 5 scale (Edmonson et al., 1989). Blood samples were taken weekly by caudal venipuncture from 28 days before to 21 days after parturition (between 9:00 to 11:00 a. m.) to measure GLY by standard colorimetric methods and BHBA by spectrophotometric method. Weekly data collection is summarized in table 1.

Adipose tissue processing and cell size determinations.

Tissue samples were embedded in paraffin and sliced taking no simultaneous slides (3 per biopsy) to stain with hematoxylin-eosin (H&E). For each slide, 15 different microscopic

fields were analyzed at 200x magnification with an Olympus Provis® microscope. Every adipocyte in each field was measured using an image processor Olympus U-MCB® with analysis software Olympus Micro Image 4.0®. Measurements were smallest and largest diameters, used to calculate mean diameter.

Statistical analysis

Analyses were performed with statistical software package SPSS 11.5 (SPSS Inc., Chicago, IL, USA). Mean comparisons and relationships are based on -28 d and +21 d data. Summaries are expressed as mean ± SEM. We used Student's t test for dependent samples to compare means, and Pearson's correlation coefficient besides the test of null correlation to examine relationships. Critical significance levels (p-values) are shown when $p<0.10$..

Results.

Body Condition Scoring and Blood metabolites.

The decrease in BCS of dairy cows between pre-partum (3.32 ± 0.08) and post-partum (3.19 ± 0.06) under usual production management was significant ($t = 1.908$, $p = 0.068$,).

At prepartum, results shown that there is a positive correlation between BCA and MDA ($r=0.521$, $p=0.016$) and between BCA and GLY ($r=0.404$, $p=0.056$), but no relationship can be observed between BCA and BHBA.

The observed variation in BCS, GLY and BHBA agrees with expected normal values for dairy cows during peripartum (Kunz et al., 1985; Chilliard, 1999; Dann et al., 2005; Hachenberg et al., 2007).

153

154 Table 1 (here)

155

156 **Adipose tissue processing and cell size determinations.**

157 The panoramic microscopic images obtained from adipose tissue biopsy of each individual
158 cow before and after parturition, showed marked adipocyte shrinkage after calving (figure
159 1). Mean postpartum MDA was lower than at prepartum (66.18 ± 2.915 vs. 72.41 ± 3.212
160 µm, t = 2,071, p = 0.055, *). MDA summaries of adipocytes (n = 3748) during pre- and
161 postpartum was 70.02 ± 27.39 µm.

162

163

164 Figure 1 (here)

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166

167 **Discussion**

168 Image analysis of conventional H&E histological preparations of adipose tissue biopsies in
169 the present work was a sensitive method for the study of cellularity changes during
170 peripartum. We observed that adipocytes suffered a significant shrinkage post-calving.
171 Decreased MDA observed in our conditions at 28 d prepartum against 21 d postpartum (8.6%
172 decrement) were similar to previous reports in dairy cows at 4 wk pre- vs. 4 wk post-partum
173 using adipocyte cell suspensions (about 11% decrement, Pike & Roberts, 1980; Smith &
174 McNamara, 1990). Similar but not significant shrinkage was observed by Pike & Roberts

175 (1980) in adipocyte suspensions, in which it is not possible to evaluate *in situ* adipocyte
176 shrinkage.
177 The whole MDA values (70.02 μm) under field conditions were similar to results previously
178 obtained with osmium tetroxide-fixed adipocyte suspensions (Smith & McNamara, 1990).
179 However, other authors obtained higher MDA values in adipocyte suspensions (Pike &
180 Roberts, 1980; Waltner et al., 1994; McNamara et al., 1995; Faulconier et al., 2007). These
181 differences could be due to the variability of adipose tissue from different anatomical regions
182 (Hood & Allen, 1973), adipocyte dissociation method (Pike & Roberts, 1980; Smith &
183 McNamara 1990; Waltner et al., 1994; McNamara et al., 1995; Faulconier et al., 2007) and
184 because adipocytes were counted and sized only when cell diameter was higher than certain
185 thresholds (McNamara et al., 1995, 20 μm; Faulconier et al., 2007, 12.5 μm). We sized
186 adipocyte subpopulation with MDA lower than 20 μm, and it constituted about 2.5% of total
187 adipocyte population, with no effect on the total decrease in MDA (data not shown). In our
188 case, formalin fixed samples preserved connective tissue matrix, which had probably
189 determined differences observed with latter mentioned work.
190 Smith & Mc Namara (1990) proposed the use of mean cell volume (MCV) instead of MDA,
191 a cubic function of MDA that would be a more accurate indicator of changes in adipocyte
192 lipid storage, but this may holds true only for cell suspensions. This method estimates
193 adipocyte size from spherical dissociated cells, and then the usage of image analysis
194 processor could be a good predictor of mean cell volume. In our case, this method would bias
195 MCV because adipocytes in conventional biopsy slides appear like irregular polygons in
196 different depth planes instead of sphere-shaped particles (Etherton et al., 1977).

Traditional quantification of cellularity in adipose tissue samples was certainly not easy to implement using conventional histology, probably due to difficulties derived from the sizing of a polygonal two dimensional adipocyte image obtained in slides, instead of a rounded "three-dimensional" one of isolated adipocytes obtained by other methods (Etherton et al., 1977).

The BCS is a widespread indirect measure of fat storage in dairy cows (Edmonson et al., 1989), sometimes used associated to the measurement of adipocytes from subcutaneous adipose tissue biopsies (Smith & McNamara, 1990; Waltner et al., 1994), to fat depth measured by ultrasonography (Garnsworthy & Topps, 1982; Domecq et al., 1995), or to body weight (McNamara et al., 1995). We used fat cell diameter to have a picture of the metabolic status of lactating dairy cows, given the significant correlation observed between MDA and BCS at prepartum. Conventional light microscopy associated to simple image analysis allowed an easy and safe method to study fat biopsies in a cost-effective manner. This routine could be incorporated as a complementary method in experimental designs for the study of metabolic disorders of the high-yielding dairy cow, like in protocols that evaluate different nutritional status during peripartum. This primary study of fat tissue cellularity during peripartum in commercial conditions suggests that cell shrinkage could be better evaluated using conventional histology.

Acknowledgements. This work was supported by project N° XUGA PGIDT 00AGR26101PR, Xunta de Galicia, Spain, and Spanish Agency of International Cooperation (AECI, Spain). We thank Claudio Borteiro (Facultad de Veterinaria, UdelaR, Uruguay) and

Luis Alberto Ramil Novo (Departamento de Estadística e Investigación Operativa, Universidad de Santiago de Compostela, Spain) for their constructive criticism and manuscript proofreading.

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294

295 Table 1. Changes in Body Condition Score (BCS, scale 1 to 5), glycaemia (GLY, mg/dl) and
296 hydroxibuthyrate (BHBA, mmol/l) during peripartum in dairy cattle (n = 25). Values are
297 mean ± SEM.

Days relative to parturition									
	-28d	-21d	-14d	-7d	partum	7d	14d	21d	Total period
BCS	3.47 ± 0.09	3.41 ± 0.06	3.36 ± 0.07	3.26 ± 0.07	3.06 ± 0.09	3.21 ± 0.05	3.21 ± 0.06	3.15 ± 0.07	3.25 ± 0.03
GLY	62.67 ± 1.78	64.35 ± 1.28	64.80 ± 1.42	62.08 ± 1.45	61.90 ± 2.26	61.81 ± 1.95	59.05 ± 2.00	57.38 ± 2.33	61.7 ± 0.70
BHBA	0.72 ± 0.07	0.71 ± 0.04	0.60 ± 0.05	0.70 ± 0.07	0.55 ± 0.04	0.61 ± 0.04	0.62 ± 0.07	0.73 ± 0.05	0.65 ± 0.02

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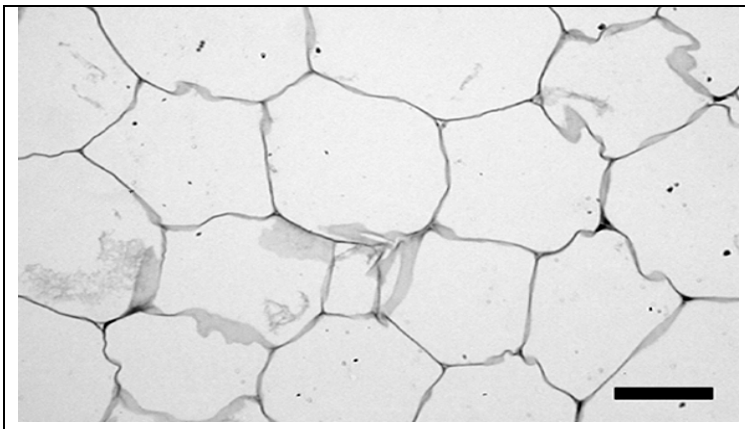

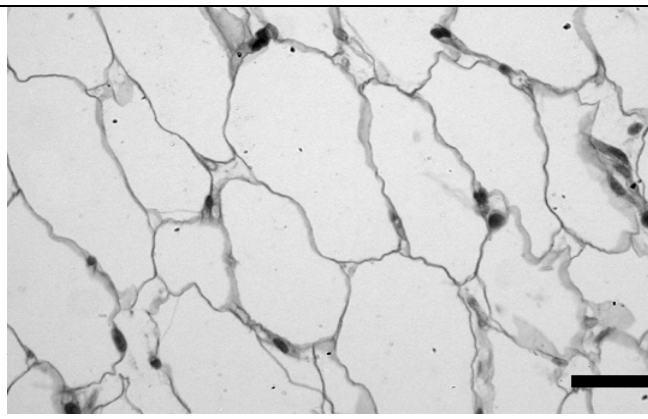

Figure 1. Representative photomicrographs of pericaudal subcutaneous adipose tissue biopsies taken from the animal 28 days before (left) and 21 days after parturition (right). Note shrinking of cell shape in the latter. x200 magnification, scale bars = 50µm).

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