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Optimization of Pulsation Rate of the Milking System for the Mammary Gland Remodeling during Involution in Thai Crossbred Holstein Cows

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ABSTRACT

This study aimed to investigate the effects of pulsation rate of the milking machine on the proteinous components and gelatinase activity in the mammary secretion for optimal tissue remodeling during the dry period in tropical dairy cows. Nine healthy primiparous Thai crossbred Holstein cows (75%HF) were milked with various pulsation rates (50, 60, and 70 cycles/min) one week after calving. The total protein contents of the secretions increased along the time course in 50 and 60 cycles/min groups but not in 70 cycles/min group and were not different among the 3 groups along the time course. Lactoferrin and BSA abundance of the 3 groups as well as γ-globulin in 50 and 70 cycles/min groups also increased in a time-dependent manner, in which only γ-globulin abundance in 60 cycles/min group 14 days before drying off and lactoferrin in 50 cycles/min group 14 days after drying off were significantly higher than those of the other 2 groups. In tissue remodeling by gelatinase activity analysis, 50 cycles/min group showed dramatic increases of Matrix metalloproteinase-9 (MMP-9) and MMP-2 activities after drying off, whereas 60 and 70 cycles/min groups had a significant but a smaller change ($p<0.05$) along the time course. Cows with 60 cycles/min exhibited dramatic increases of MMP-9 and MMP-2 activities than the other groups before drying off ($p<0.05$). The findings suggested that milking pulsation rate at 60 cycles/min resulted in higher activity of remodeling during mammary involution and thus may benefit the renewal and health of the udder in the long run.

Keywords: pulsation rate; extracellular matrix; mammary gland; drying off; Thai crossbred dairy cows

INTRODUCTION

The milking pulsation can affect milk yield and also the health of the udders. Any adverse effects on either of these factors would negatively influence profits. An optimal pulsation rate is able to control congestion and the onset of edema in the teat tissue and further reduce the rate of new intramammary infection rate (IMI) (Mein, 2012). Several studies have investigated the effects of pulsation rates on the health of the udders (Ferneborg & Svennersten-Sjaunja, 2015; Romero *et al*., 2020), as well as the teat end conditions (Besier & Bruckmaier, 2016). An increase of new infection rate was reported in pulsation failure, such as the absence of pulsation (Besier *et al*., 2016), and insufficient duration of liner closure (Penry *et al*., 2018) or of d-phase pulsation (Upton *et al*., 2016). Past studies showed a worsening status of the udder health with pulsation rates of 90 and 120 cycles per min (Atigui *et al*., 2015) or lower than 55 cycles per min (Osteras *et al*., 1995).

Drying off in dairy cows results in changes in the composition and functionality of the secretion retained in the udder cisterns. In the initial stages after drying off, blood proteins are transferred into the alveolar lumen, by which the concentrations of bovine serum albumin as well as the milk immunoglobulins are significantly enhanced (Hurley & Theil, 2011; Tiantong *et al*., 2015b). These alterations are believed to arise as a consequence of transport facilitation or due to the loss of integrity at the junctions connecting mammary epithelial cells (Stelwagen & Singh, 2014). Besides, there is a spontaneous change in the relative abundance of the main milk proteins (Tiantong & Mwabena, 2019). Although such observations have been made, the effects of pulsation rate of milking machine on the alterations of milk components during the involution of the mammary gland have not been well studied.

The extracellular matrix (ECM) forms a milieu surrounding cells that reciprocally influences cellular function (Hynes, 2009). Matrix metalloproteinases (MMP) function as selective enzymes to cleave proteinous components, notably ECM or chemokine peptides (Khokha & Werb, 2011), and thus are tightly related to the process of remodeling, especially when the damaged

tissues undergo repairing processes in the case such as inflammation. The activity of MMP is tightly controlled on various levels, including the transcription, the secretion, and the post-secretion activation (Li *et al*., 2016).

When the mammary glands become inflamed, especially at early phase of the dry period of the dairy cattle, the neutrophil levels in somatic cells can rise long before any increase in the other cell types (Pezeshki *et al*., 2010; Yu *et al*., 2012). *In vitro* studies demonstrated a dramatic but transient increase of MMP-9 due to the neutrophils infiltration in the mammary secretions of freshly dry dairy cattle (Yu *et al*., 2011). Therefore, it can be inferred that the high levels of MMP-9 found in the early dry secretions of dairy cattle can reflect the procession and physiological significance during milk stasis (Gifre-Renom *et al*., 2020).

The study aimed to examine the effect of three pulsation rates, 50, 60, and 70 cycles/min, that are frequently used by the smallholder dairy farmers in Thailand, on the abundance of protein components and ECM degradation of the mammary secretions in respect to gelatinase activity for remodeling progression in Thai crossbred Holstein cows.

MATERIALS AND METHODS

Animals and Experimental Design

Nine healthy primiparous Thai crossbred Holstein cows (n=9; 75% HF) were chosen from the herd at the Silpakorn University Phetchaburi IT Campus dairy farm in Phetchaburi Province, Thailand. These cows were randomly divided into three groups with different milking pulsation rates; 50, 60, and 70 cycles per min in a pulsation ratio 60:40, throughout the lactation period (280 ± 30 d in milk) with negative CMT (California mastitis test) results and no signs of intramammary infection. One week after calving, cows were milked twice daily at 0600 and 1630 h with milking machines at different pulsation rates. During the lactation period, cows were fed on a total mix ration (TMR); 3,300 kcal/kg metabolizable energy, and 20% crude protein. When milk yields were less than 5 kg/d with more than 280 ± 30 days in milking (DIM), cows were moved to milk-stasis stalls and fed twice per day with a 2500 kcal/kg metabolizable energy, 14% crude protein, and rice straw and water were provided ad libitum throughout the period of sample collection within two weeks. The experimental design and sampling protocols were approved by the Animal Use and Care Committee of the Faculty of Animal Sciences and Agricultural Technology, Silpakorn University under approval number Asat.SU 031/2562, based on the Ethics of Animal Experimentation of the National Research Council of Thailand.

Sampling Processes and Preparation of the Samples

Milk samples (~25 mL) were manually collected from individual quarters of each cow 14 days (d -14) and 7 days (d -7) prior to the expected drying-off and the day before dry-cow therapy (d 0), and 3 days (d 3), 7 days (d 7), and 14 days (d 14) after dry-off. Collected milk was skimmed by 400 x *g* at 4°C for 20 minutes in order to collect the clear supernatant. The supernatant, free of fat and cells, was then placed in aliquots under -20°C until further analyses within a two-months (Tiantong *et al*., 2015a).

The protein concentration of skimmed supernatants was determined using a dry-binding reagent (Bio-Rad Laboratories, Hercules, CA, USA) in a microplate format (SPECTROStar Nano, BMG Labtech GmbH, Ortenberg, Germany) with BSA (Sigma-Aldrich) as a standard. A standard curve was plotted for quantification of SDS-PAGE analysis and gelatin zymography.

Gel Electrophoresis (SDS-PAGE)

The proteinous components of milk were identified by 9% of resolving gel in SDS-PAGE system of Laemmli (1970). Briefly, aliquots of the skimmed milk supernatants equivalent to 10 μg protein content were treated with 2X Native Sample Buffer (161-0738, Bio-Rad Laboratories, Hercules, CA, USA) before loading. Afterward, gels were stained for 1 h with the Coomassie Brilliant Blue R-250 staining solution (161-0436, Bio-Rad Laboratories, Hercules, CA, USA) and washed with distilled water until clear visualization. The respective band image was captured with Epson Stylus TX130 (Seiko Epson Corporation, Nagano, Japan) and then measured by Image J software (v. 1.50; National Institutes of Health, Bethesda, MA, USA). Adjustment of the integrated band zone then took place to take total protein loading into consideration.

Gelatin Zymography

Gelatin zymography was performed under native SDS-PAGE conditions, whereby the resolving gel made use of gelatin to serve as the substrate. MMP-2 and -9 were identified on the molecular size of gelatinolytic band at 37 °C as described previously (Piamya *et al*., 2015). Briefly, the skimmed milk supernatants of 20 μg total proteins were first mixed with a zymogram sample buffer (161-0764, Bio-Rad Laboratories, Hercules, CA, USA), and then was subjected to 7.5% native SDS-PAGE, with 0.1% gelatin obtained from bovine skin (Sigma-Aldrich) within the resolving gel. The gels then underwent further incubation using a renaturing solution of 2.5% (v/v) Triton-X100 (Sigma-Aldrich) for 30 minutes at room temperature while completely dripped using distilled water. Additional incubation then took place at a temperature of 37°C for 19 h in zymogram development buffer (161-0766, Bio-Rad Laboratories, Hercules, CA, USA) for gelatinolytic activity. Subsequently, the gels underwent staining with 0.5% coomassie brilliant blue R-250 (161-0400, Bio-Rad Laboratories, Hercules, CA, USA) for 30 minutes, and were then destained for 30 min in the destaining solution containing 30% methanol, 7.5% glacial acetic acid, and 62.5% distilled water twice, prior to final preservation in the form of clear bands which were visible against the blue background. The MMP-2 and -9 levels were presented as the area of the band, which was captured by the Epson Stylus TX130 (Seiko Epson Corporation, Nagono, Japan) before integration with Image J software, version 1.50 (National Institutes of Health, Bethesda, MA, USA).

Statistical Analyses

Data of total protein concentration, proteinous components, and MMP-proteolytic capacities were analyzed using the GLM procedure and Duncan's new multiple range tests (R Core Team, 2018) with sampling time (14 days and 7 days before drying off, and on the day of drying off and 3, 7, or 14 days after drying off) and the different pulsation rates (50, 60, or 70 cycles per min) as the major factors. Interaction between sampling time and the different pulsation rates were also assessed. All results were presented as mean ± SE. Statistical significance was considered at $p<0.05$.

RESULTS

Total Protein Concentration

Cows with pulsation rates at 50 and 60 cycles/min, but not at 70 cycles/min, showed a time-dependent increase of total protein concentrations, particularly on 7 days and 14 days after drying-off. However, the total protein concentrations were not significantly different among cows with different pulsation rates (Figure 1).

Protein Composition

Typical images of the semi-quantitative SDS-PAGE in the case of skimmed supernatants from mammary secretions can be observed in Figure 2 (A). The SDS-PAGE image of supernatant of mammary secretion samples showed a characteristic increase of band intensity corresponding to of γ-globulin, lactoferrin, and BSA from 14 days before milk stasis until 14 days after drying off period, while the band intensity of caseins increased from 14 days before milk stasis until the day of drying off then slightly decreased until 14 days after drying off therapy.

It was found that the mean values along with standard error (SE) for the quantity of γ-globulin in the supernatant of the mammary secretions were significantly ($p<0.05$) greater 14 days before milk stasis of cows with pulsation rate at 60 cycles/min than that of cows with pulsation rates at 50 and 70 cycles/min (Figure 2B). However, the abundance of γ-globulin of cows with pulsation rates at 50 and 70 cycles/min increased significantly along the time course, but not in cows with 60 cycles/min (Figure 2B).

The mean of the abundance of lactoferrin was significantly ($p<0.05$) reduced in those cows which had pulsation rates of 60 cycles per minute compared to those at 50 cycles/min 14 days after drying off period. Also, the abundance of lactoferrin in the 3 groups increased along the time course with top levels 14 days after drying off and the lowest levels 14 days before drying off period.

The mean of the abundance of BSA in mammary supernatant was not significantly different among the 3 groups. Similar to lactoferrin, BSA abundance in the 3 groups increased along the time course with a top 14 days after drying off and the nadir 14 days before drying off. For cows with 50 cycles/min pulsation rate, the abundance of caseins increased to 3 days after drying off period and then decline, while the casein content of cows with 60 cycles/min pulsation rate remained constant until a quick drop 14 days after drying off period and cows with 70 cycles/min showed an increase to the day of dry off period and then a constant decreased level on 14 days after drying off period. The proteins which are typical for the dry period, γ-globulin, lactoferrins, and BSA, showed the most significant changes from 3 days to 14 days in milk stasis.

Matrix Metalloproteinase-2 and -9 (MMP-2, -9)

Figure 3 presents the gelatin zymograms of the supernatants of skimmed mammary secretions. It was possible to detect both MMP-2 and MMP-9 in the secretions of cows with a pulsation rate at 50, 60, and 70 cycles/min during the dry period. The means of MMP-9 activity in cows at 60 cycles/min had a top level on 14

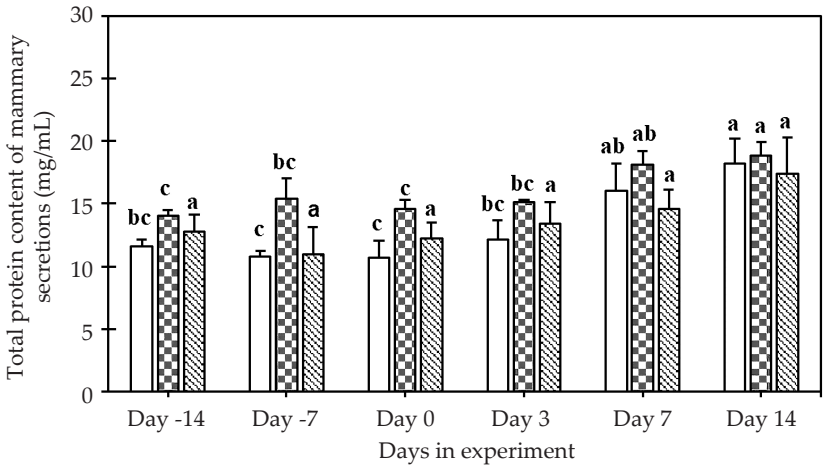

Figure 1. Total protein concentration of mammary secretion supernatant of the experimental Thai crossbred Holstein cows during the dry period with the differential pulsation rates, including 50 cycles/min; 60 cycles/min, and 70 cycles/min. [a-c]Values with different superscripts differ significantly ($p<0.05$) among days in experiment within the same animal.
□= 50 cycles/min; ▩= 60 cycles/min; ▨= 70 cycles/min.

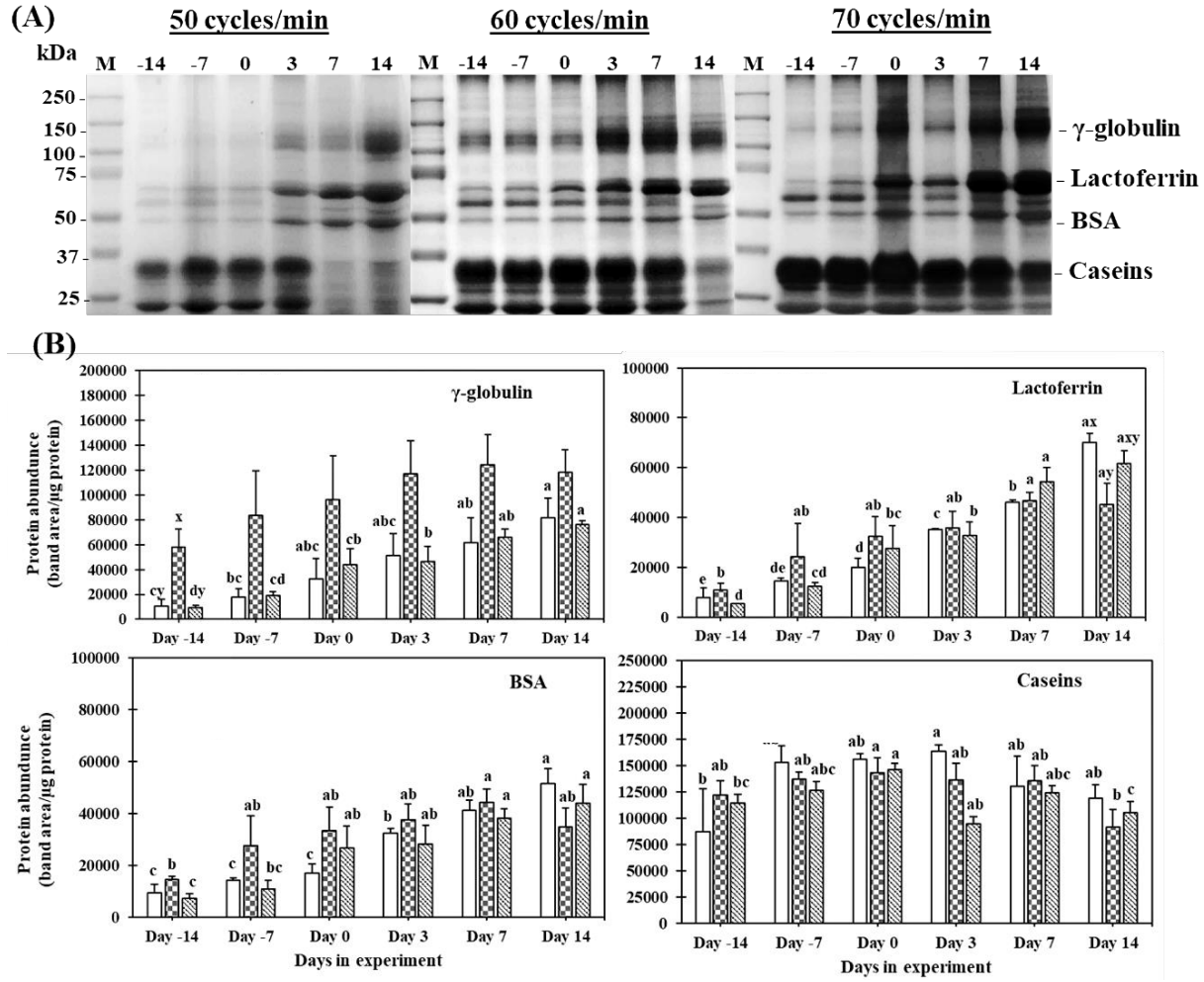

Figure 2. (A) SDS-PAGE images and (B) the abundance of protein composition of the mammary secretion supernatant of the experimental Thai crossbred Holstein cows during the dry period with the differential pulsation rates, including 50 cycles/min; 60 cycles/min, and 70 cycles/min. [a-c]Values with different superscripts differ significantly ($p<0.05$) among days in experiment within the same animal. [x-z]Values with different superscripts differ significantly ($p<0.05$) in various levels of pulsation rate within the same day in experiment.
□= 50 cycles/min; ▩= 60 cycles/min; ▨= 70 cycles/min.

days before milk stasis and then declined on the day of dry off, then increased 3 days again after drying off period and finally reach a nadir 14 days after drying off period ($p<0.05$). In cow with 50 or 70 cycles/min, MMP-9 activity increased along the time course to reach a top 7 days after drying off period and then declined slightly 14 days after drying off period ($p<0.05$), in which MMP-9 levels during the milk stasis stage (3 days and 14 days after drying off period) were significantly higher than those before drying off period ($p<0.05$).

Before milk stasis (14 and 7 days before and at the day of drying off period), MMP-9 activities were significantly higher ($p<0.05$) in the case of cows which had pulsation rates of 60 cycles per minute compared to those with 50 and 70 cycles/min. After milk stasis, the MMP-9 level was ranked as 70 >60>50 cycles/min 3 days after drying off period, and 50=70>60 cycles/min 7 days and 14 days after drying off period ($p<0.05$).

The means of MMP-2 activity of cows with pulsation rate at 50 cycles/min increased with time course to reach a top 3 days after drying off period and then declined 14 days after drying off period, in which MMP-2 levels after the milk stasis stage (3 days to 14 days after drying off period) were significantly higher than those before dry-off ($p<0.05$). The MMP-2 level in cows with 60 cycles/min decreased on the days of milk stasis, then increased 3 days after drying off period, and finally declined to the nadir 14 days after the drying off period, whereas cows with 70 cycles/min exhibited a constant MMP-2 activity 3 days before milk stasis and then declined to the nadir 14 days after the drying off period ($p<0.05$). The MMP-2 activity was ranked as 50<60=70 cycles/min 14 days and 7 days before drying off period, 50<60<70 cycles/min on the day of milk stasis, 50>70>60 cycles/min 3 days after drying off period, 60>50=70 cycles/min 7 days after drying off period, and 60<50=70 cycles/min 14 days after drying off period ($p<0.05$).

DISCUSSION

Based on the protein components and MMP activity in the milk secretions, it appears that milking with

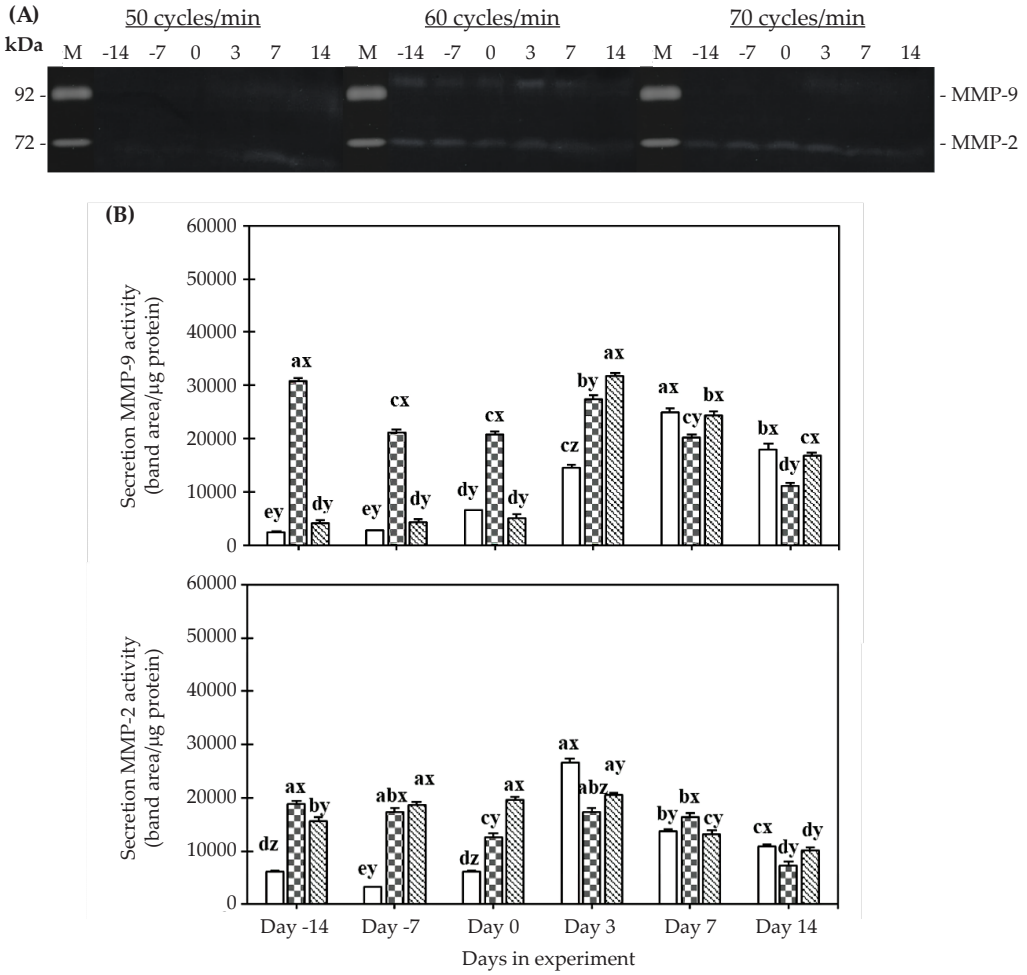

Figure 3. (A) Gelatin zymography images and (B) the gelatinolytic activity abundance of the mammary secretion supernatant of the experimental Thai crossbred Holstein cows during the dry period with the differential pulsation rates, including 50 cycles/min; 60 cycles/min, and 70 cycles/min. [a-c]Values with different superscripts differ significantly ($p<0.05$) among days in experiment within the same animal. [x-z]Values with different superscripts differ significantly ($p<0.05$) in various levels of pulsation rate within the same day in experiment.
□= 50 cycles/min; ▩= 60 cycles/min; ▨= 70 cycles/min.

pulsation rate at 60 cycles/min can optimize the mammary gland remodeling during involution. Pulsation is defined as the cyclic opening and closing of the teat cup liner, which is an important factor in dairy cattle farm productivity. In Thailand, it is common to use alternating pulsation at a rate of 60 cycles/min for which the ratio is 60:40 for the front to rear cups on the teats. This arrangement is widely used by dairy farmers because they believe it is a good compromise by the system to provide both speed milking and better udder health. As milk yields per cow continue to increase and, therefore, milking times per cow become longer, an optimal combination of pulsator setting might change to a rate of 55 cycles/min in a ratio of 65:35. Besides, some farmers in Thailand also recommend that the pulsation rate can be increased up to 75 cycles/min to increase milk harvest and reduce the milking time without the new IMI. However, current quantitative recommendations for milking machine installation for Thai crossbred Holstein cows showed no specification for the pulsation characteristics, given the absence of any studies regarding to these features with infection risk and mammary gland remodeling during the dry period. In this study, three different pulsation rates (50, 60, and 70 cycles/min) as widely used in Thailand were tested to optimize the pulsation rate for the smallholder dairy farms. The present results showed no differences in total protein concentration of milk by the 3 pulsation rates. These results are consistent with the previous works by Tromas *et al*. (1991), who showed no differences in milk composition such as fat and protein percentage, or somatic cell count due to pulsation rate at the same level. Meanwhile, Ferneborg & Svennersten-Sjaunja (2015) reported that the pulsation rate of 60 cycles/min with the 60:40 ratio increased milking efficiency in automatic milking system and had no negative effects on teat condition or milk somatic cell counts.

Besides, we found that the total protein concentration steadily increased until 14 days after drying off. According to our period works (Tiantong *et al*., 2015b; Tiantong & Mwabena, 2019), this increase is primarily attributed to the increased concentrations

of immunoglobulins, serum albumin, and lactoferrin. Also, this increase can be attributed to the decreased mammary gland functionality in the first two weeks of the dry period, with alterations in mammary secretion components due to the decreased synthesis of casein (Raimondo *et al*., 2013), and changes in cell junctions and vascular permeability by the increased protein concentration from blood serum (Boutinaud *et al*., 2003).

Examination of relative abundance of the main proteins in the mammary secretions was achieved through the biochemical methodology SDS-PAGE, but this was not the case for absolute concentration. In drying off mammary secretions, there is an almost reciprocal change in the ratios of γ-globulin, lactoferrins, and BSA (Chen *et al*., 2007; Ho *et al*., 2010). There are two possible reasons for the lowering of the casein proportion. These are acceleration of caseinolysis (Nielsen, 2002; Ferranti *et al*., 2004) and the attenuation of the synthesis of casein (Miller *et al*., 2006; Sorensen *et al*., 2006). In contrast, a rise in the BSA fraction might be partially linked to the heightened permeability of the blood-mammary gland barrier (Wenz *et al*., 2010). One early sign of mastitis is high levels of BSA in milk (Wenz *et al*., 2010). The situation for milk γ-globulin and lactoferrins differs from BSA due to endogenous origins; they can be considered as marker proteins which indicate mammary gland involution, since they usually appear in high proportions following the drying off period (Ho *et al*., 2010).

The normal kind of reciprocal shift in the prevalence of casein-like bands and lactoferrin-like bands were observed in the SDS-PAGE, in which cows with pulsation rate at 60 cycles/min showed an earlier occurrence than those with 50 and 70 cycles/min (Figure 2), which is indicative of a rapid decline in the synthetic capacity of the milk.

The turnover of mammary epithelial cells accelerate the early dry period in dairy cattle (Khokha & Werb, 2011), although the extent of the breakdown of the mammary architecture was so trivial as to be invisible via morphological or histological technology (Sorensen *et al*., 2006). MMP has been employed for organ remodeling in the case of diseases such as Osteoarthritis as a result of its potential for ECM degradation (Maldonado & Nam, 2013). It has also been effective for cow mastitis (Li *et al*., 2016) and also in dairy cattle which are in drying-off period (De Vries *et al*., 2010). The particular units of MMP-9 in the milk rise as lactation progresses in dairy cattle, or after milk stasis (Chen *et al*., 2007; Weng *et al*., 2008; Yu *et al*., 2012). In this work, the increase in MMP-9-specific units was observed at an earlier stage in the milk secretions of the cows which had a pulsation rate at 60 cycles/min quarters than those with pulsation rate at 50 and 70 cycles/min quarters (Figure 3). However, specific units of MMP-2 which were almost constant could be seen in those cows with both 60 and 70 cycles/min quarters throughout the experiment period (Figure 3).

Our observations of MMP-2-specific units found in the secretions of quarters at various pulsation rates along the drying off progression can solidify the endogenous aspects of mammary tissue, which confirms the earlier results for MMP-9 results. It can therefore be inferred that the ECM for those quarters which involved pulsation rates of 60 cycles/min would show a greater degradation as a consequence of the enhanced PMN chemotaxis as well as the related release of MMP-9.

CONCLUSION

The milking machine with pulsation rate at 50, 60, or 70 cycles/min did not affect total milk protein, whereas the pulsation rate at 60 cycles/min may promote mammary gland remodeling process during early involution, and thus may reduce the risk of new IMI. Accordingly, the rate of pulsation of 60 cycles/min is recommended to increase milking efficiency in automatic milking systems without damaging the mammary tissues.

CONFLICT OF INTEREST

The authors hereby declare that no conflict of interest exists of a personal or financial nature with any other person or organization, in the context of the contents of the manuscript presented.

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