Mammary Cell Turnover under High Temperature during the Dry Period in Dairy Cows

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ABSTRACT : The influence of high temperature on mammary cell turnover during the dry period is still unclear. The objective of this study was to investigate mammary cell turnover and p53 protein expression in the mammary tissue under high temperature conditions. Mammary gland biopsy samples from 8 dairy cows were obtained at 7, 25, 40, and 53 d during the dry period in summer or spring (n = 4, each season). Cell cycle, cell turnover, and p53 protein expression were analyzed by flow cytometry. During the dry period in summer, the percentage of mammary epithelial cells in the G0/G1 phase was the highest, but those in the S and G2/M phases were lower. However, the proportion of cells in the different stages of the cell cycle was not significantly different among the different biopsy time points, except in the G2/M phase. Under different temperature conditions, the cells were significantly different in their apoptotic rate and proliferation index; moreover, the tendencies of these indicators to change significantly differed. In general, the samples under high temperature conditions showed significantly lower apoptotic rates and proliferation indices. Under high temperature conditions, the apoptotic rate and proliferation index were the lowest (2.17% and 3.26%, respectively) at day 40, and the highest at day 53 (3.67% and 4.61%, respectively). However, under normal temperature conditions, the values of these indicators were the lowest (7.60% and 5.54%, respectively) at day 7, and almost the highest at day 25 (12.85% and 6.47%, respectively). Moreover, p53 protein expression was significantly higher under high temperature conditions than under normal temperature conditions, except at day 25. The level of p53 protein was the lowest (13.10%) under high temperature conditions at day 25, but was the highest (26.07%) under normal temperature conditions. Our findings suggest that high temperature delayed the G2/M phase of the cell cycle and the cell turnover rate, but remarkably increased p53 protein expression. Thus, the results indicate that high temperature extends the recovery period of mammary epithelial cells. (Key Words : High Temperature, Dry Period, Cell Cycle, Apoptosis, Proliferation, Dairy Cow)

INTRODUCTION

Dairy cows require a non-lactating period before parturition to maintain normal development of the fetus and to repair the mammary gland for optimized milk production in the subsequent lactation. The non-lactating period, or dry period, lasts for approximately 2 months (Leidy et al., 1989). Although cell turnover in the mammary tissue during the lactation and dry periods in dairy cows has been studied (Sorensen et al., 2006), mammary cell proliferation and apoptosis of the mammary gland under high temperature conditions is still unclear. The optimal temperature range for dairy cows is between 10°C to 20°C. Dairy cows reduce their daylight eating time and have an endocrine imbalance when the maximum daily ambient temperature exceeds 25°C, resulting in a reduced milk yield (West et al., 2003). Previous studies have shown that high temperature blocks mammary gland formation and regeneration (Cavestany et al., 1985; Mu et al., 1989). When cows were subjected to heat stress, calf birth weight and milk yield of the subsequent lactation was reduced (Collier et al., 1982). Thus, we hypothesized that high temperature would influence the cell turnover rate during the dry period. Previous studies have demonstrated that p53 regulates expression of several genes that are related to DNA repair, cell cycle arrest and apoptosis induction (Oren, 1999). However, the role of p53 in heat-stress induced apoptosis of mammary epithelial cells in cows is still unclear. Previous studies in vitro may not reflect the actual situation in vivo, thus a study in vivo is important to understand mammary cell turnover under high temperature conditions during the
dry period.

In the present study, mammary biopsy samples were obtained during the dry period in the spring and summer. We determined the cell proliferation rate, apoptotic rate, and p53 protein expression level in the mammary gland to understand the cell turnover rate during the dry period under high temperature conditions.

MATERIALS AND METHODS

Animals and diets
Eight healthy Holstein cows in the first to fourth parity were provided by Beijing Jinxiu Dadi dairy farm (Beijing, China). The animals were housed in tie-stalls and handled according to standard procedures. The cows were milked daily at approximately 0800 and 2000 h, and fed a ration based on grass silage and concentrate. Approximately 60 days before parturition was expected, the cows were dried off and their intake was restricted, with free access to water during the dry period. The dry period of 4 cows (body weight = 549.6 kg; milk yield = 28.4 kg/d) was in April and May (daily ambient temperature, <25°C) and that of the other 4 cows (body weight = 557.5 kg; milk yield = 22.6 kg/d) was in July and August (daily ambient temperature, >35°C). The experiments followed the guidelines of the regional Animal Ethics Committee.

Sampling
Biopsy samples were collected from the rear part of the mammary gland using semi-automatic biopsy needles (Precisa, Hospital Service, Rome, Italy). The biopsy samples were obtained at days 7, 25, 40, and 53 after the beginning of the dry period. From the mammary gland of each cow, 3 samples were obtained. Approximately 20 mg of tissue was harvested for each biopsy.

For cell suspensions, the tissue samples were minced and then digested in medium 199 supplemented with collagenase (1 mg/ml; Elixigen, Huntington Beach, CA) and hyaluronidase (1 mg/ml; Sigma, St. Louis, MO), as previously described (McGrath, 1987). The digested tissues were passed through a 300-μm stainless steel mesh (Toyobo, Tokyo, Japan). The cell suspension was washed twice by centrifugation, and the cell number was adjusted to 1 x 10^6 cells/ml. The characteristics of mammary epithelial cells were observed under a light microscope (data not shown).

Flow cytometric analysis
For determination of total DNA content, cells (1 x 10^6) were stained with a solution containing 50 g/ml propidium iodide (PI) and 100 g/ml RNase 1 in phosphate buffered saline (PBS) for 30 min at 37°C. The cells were filtered through a nylon mesh to remove cell clumps and then analyzed for cell cycle distribution with a FACScan flow cytometer (Becton Dickinson, San José, CA). Apoptosis was measured by staining the cells with a combination of fluoresceinated (FITC) annexin V and PI using an apoptosis kit (Multisciences, Hangzhou, China) according to the manufacturer’s instructions. For the detection of p53 protein expression level, the cell suspensions were isolated from the mammary gland samples obtained at 7, 25, 40, and 53 d during the dry period. The cells were permeabilized with 0.5% Triton X-100 for 10 min and blocked with 3% bovine serum albumin (Sigma, St. Louis, MO) for 30 min at room temperature. Then, the cells were incubated with phycoerythrin (PE)-labeled p53 monoclonal antibody (Cat# ab27697; Abcam, Cambridge, UK) and control PE-labeled mouse IgG2a (Cat# ab18456; Abcam) for 15 min at room temperature in the dark. The cells were washed 2 times with PBS and resuspended in 1% paraformaldehyde. A minimum of 10,000 cells were included in each sample. Data were analyzed using CellQuest software (Becton Dickinson), and the percentage of p53+ cells was determined. Proliferation index (PI) was expressed as follows: PI = ((S+G2/M)/(G0/G1+S+G2/M))×100%.

Statistical analysis
A statistical package for social sciences (SPSS) 13.0 (SPSS incorporated, Chicago) was used for all analyses. All experiments were performed for a minimum of 4 times. All values were expressed as mean±standard deviation (SD). Statistical evaluation of the raw data was performed using one-way analysis of variance (ANOVA) and the least significant difference (LSD) test. p<0.05 was considered statistically significant.

RESULTS

Mammary cell cycle progression during the dry period in the different seasons
As shown in Figure 1, most of the cells were in the G0/G1 phase at each biopsy time point during the 2 seasons. The percentage of mammary epithelial cells in different phases of the cell cycle during the different dry periods was consistent. Under the normal temperature condition, the proportion of cells in the G0/G1 phase was highest at 7 d (94.46±1.51%) and decreased slightly at 25 (93.53±0.15%) and 40 d (93.20±2.46%). The proportion of cells in the S phase increased through the dry period, peaking at day 53 (3.06±1.12%), while the proportion of cells in the G2/M phase decreased, with the lowest value observed on day 53 (2.64±0.44%). Under the high temperature condition, the proportion of cells in the G0/G1 phase at days 7 and 40 was relatively high, accounting for 96.58±0.14% and 96.74±0.17%, respectively. The proportion of cells in the S and G2/M phases was highest at day 53 (2.69±0.12% and 1.92±0.24%, respectively) and was lowest at days 7

(1.58±0.12% and 1.85±0.12%, respectively) and 40 (1.77±0.09% and 1.50±0.17%, respectively). However, the proportions of cells in the G0/G1 and S phases at each biopsy time point in the different seasons did not differ significantly (p>0.05). The proportions of cells in the G2/M phase were significantly lower (p<0.01) under high temperature conditions than under normal temperature conditions.

**Mammary cell turnover during the dry periods in the different seasons**

As shown in Figure 2, the proliferation index and apoptotic rate of mammary cells (Figure 3) were lower under high temperature conditions than those under normal temperature conditions, and their changing tendencies were different between the high and normal temperatures. Under the normal temperature condition, the apoptotic rate of mammary epithelial cells was higher (p<0.01) at day 25 (12.85±0.78%) than at days 7 (7.60±1.13%), 40 (8.70±1.27%), and 53 (7.65±0.35%). The proliferation index at the different biopsy time points gradually increased at first, and then decreased, with the highest value at day 40 (6.80±2.46%) and lowest value at day 7 (5.54±1.51%). However, these differences were not significant (p>0.05). Under the high temperature condition, the changes in the apoptotic rate and proliferation index were similar during the dry period: lowest at day 40 (2.17±0.67% and 3.26±0.17%, respectively) and highest at day 53 (3.67±0.75% and 4.61±0.32%, respectively). The apoptotic rate at day 40 was significantly lower (p<0.01) than that at days 25 (3.27±0.32%), 40 (3.60±0.50%), and 53 (3.67±0.75%). The proliferation index at day 40 was significantly lower (p<0.01) than that at days 25 (4.31±0.29%) and 53 (4.61±0.32%).

**Expression of p53 protein in the mammary cells during the dry period in the different seasons**

Protein expression of p53 was significantly higher under the high temperature conditions than that under normal temperature conditions. In addition, the changing tendency differed between the 2 conditions. Under the normal temperature condition, the changes in the apoptotic rate and p53 protein expression were similar during the dry period. P53 protein expression was the highest at day 25 (26.07±3.49%). This was significantly higher (p<0.01) than at days 7, 40, and 53. Under the high temperature condition, p53 protein level in the early dry period at day 7 was 59.83±6.00%, which was higher (p<0.01) than at 25, 40, and 53; thereafter, the level decreased sharply to 13.10±2.27%, which was lower (p>0.05) than at 40 and 53 d. The levels of p53 protein were significantly different (p<0.05) at 40 and 53 d, and the levels were higher when cells were under high temperature condition (except on day 25) than when cells were under a normal temperature condition (Figure 4).
DISCUSSION

Turnover of mammary epithelial cells

Adequate apoptosis and proliferation of mammary secretory epithelium during the dry period of dairy cows appear to be essential for maximal milk production during lactation (Oliver et al., 1989). Proliferation and apoptosis of mammary epithelial cells were two alternately continuous processes during the complete dry period of dairy cows, and their changing trends were similar. Mammary glands progressed through 3 functional states during the dry period: 3-week active recovery, 2-week steady rehabilitation, and 3-week cellular growth and differentiation (Smith et al., 1982; Oliver et al., 1989; Capuco et al., 2001). A large number of mammary cells underwent natural death or apoptosis during the early dry period (7 d), and the apoptotic rate increased. During 25-35 days of the dry period, cells were in a steady recovery phase. Cells were in the transition phase from steady recovery to growth and differentiation at 35 d, and cell turnover rate decreased; thereafter, the original cells in the mammary gland formed new lactating cells, which differentiated into functional lactating cells. The proliferation index of cells gradually increased until pre-partum. An increase in the cell apoptotic rate was probably caused by excessive mammary epithelial cell differentiation (Li et al., 2005), or a secondary effect that the cows suffered as a result of the high temperature (Neill et al., 1998; Breen et al., 1999).

This period has been acknowledged by researchers. The relevant indicators have already been detected under the normal temperature condition. However, the influence of high temperature on mammary cell turnover in vivo during the dry period remains unclear. Therefore, in our research, indicators of the mammary cell cycle, such as cell turnover and P53 protein, were monitored systematically under the high temperature condition.

Our results indicate that under the high temperature condition (>35°C), the cows were obviously stressed. In addition, the results reflect mainly on the reduction of cell turnover rate and the high level of p53 protein expression. Compared to normal temperatures, the cell cycle, including the G0/G1 and S phases, did not show any significant

Figure 2. The proliferation index and apoptotic rate of mammary epithelial cells under normal and high temperatures. Data were obtained from FACS assay, (mean±SD of 4 independent experiments). A-B: significant differences between different biopsy days (p<0.01).
Figure 3. Representative dot plot diagrams obtained by flow cytometry of Annexin V/PI double-stained mammary epithelial cells.
changes at high temperatures. However, the high temperature inhibited the G2/M phase in differentiating mammary cells. These results are consistent with those of previous studies. Jin et al. (2004) reported that the increased occurrence of G2/M arrest in H1299 cells was statistically significant at high temperatures. Narita et al. (1998) and Tadashi et al. (2004) also reported significant high temperature-induced cell cycle disorder and G2/M arrest. The difference in the points of cell cycle arrest that are induced by high temperatures may be due to the difference in cell types and high temperature strengths, because various cell types have different sensitivities and tolerances to heat (Lee et al., 1994; Lim et al., 2006). In our study, we found that temperatures >35°C in the summer were not high enough to cause serious damage to the cell cycle (only the G2/M phase), and the cows still performed normally in the summer.

The proliferation index and apoptotic rate of mammary cells were significantly lower under high temperature than under the normal temperature conditions. This indicates that the high temperature inhibited both the apoptosis and proliferation of mammary cells. The changing tendencies of the proliferation index and apoptotic rate of mammary cells were also different between the high and normal temperature conditions. Under the normal temperature condition, the proliferation index and apoptotic rate of mammary cells were almost the highest at day 25, because the mammary cells were in an extremely active phase. At day 40, the mammary cells were in a steady recovery phase and the apoptotic rate decreased sharply. At day 53, cellular growth and differentiation of mammary cells were almost complete, so the proliferation index and apoptotic rate were relatively lower. From the above results under the normal temperature condition, the changing tendencies of the proliferation index and apoptotic rate were consistent with the recovery progression of mammary cells. Under the high temperature condition, the proliferation index and apoptotic rate of mammary cells were lower, and this might extend the recovery period of mammary cells. Interestingly, under the high temperature condition the proliferation index and apoptotic rate were the lowest at day 40. On the basis of the results of previous studies, mammary cells might have the lowest cell turnover rate when in the transition phase from steady recovery to growth and differentiation. We inferred that under the high temperature condition, mammary cells might start entering this transition phase at day 40, but under the normal temperature conditions this might occur at day 35. Consistent with this hypothesis, under the high temperature condition the proliferation index increased again at day 53, indicating that the mammary cells were still actively growing and differentiating. Overall, the high temperature suppressed the turnover rate of mammary cells, thereby extending the recovery period.

Various stress stimuli can result in cell apoptosis. Cells adapt to stress by improving their endurance against the lethal effects of stress. High temperature induces delayed and gradual cell apoptosis. Du et al. (2006) found that the apoptotic rate of mammary cells was significantly higher under high temperature conditions, and, using an Annexin V-FITC/PI staining method and observing the cell ultrastructure with transmission electron microscopy, they showed that the cell apoptotic rate was highest during the late dry period. Immune suppression was probably induced in cows following the high temperature-induced apoptosis of mammary cells. On the other hand, cows maintained a steady number of mammary cells through the continuous proliferation of mammary cells to alleviate the heat-stress reaction. In addition, the decline in mammary cell number by apoptosis was accompanied by a degree of cell renewal (Capuco et al., 2001). Thus, the proliferation index of mammary cells also increased to a maximum.

![Figure 4](image-url)
p53-mediated cell turnover under high temperature condition

p53 plays a significant role in cellular monitoring by integrating and reacting with various intracellular and extracellular stress signals. Wildtype p53 in normal cells primarily exists in an inactive form that can be degraded rapidly by proteolysis and is maintained at relatively low levels. When cells are exposed to high temperature, such as DNA damage, hypoxia, nucleotide pool imbalance, peroxidation injury and oncogene product imbalance, the absolute p53 protein content increases and converts from an inactive to the active state, which enhances its ability to combine with specific DNA sequences and increases its stability (not easily degradable). One significant role of activated p53 is to arrest the cell cycle to allow for damage repair, thus avoiding the accumulation of damaged DNA or afferent daughter cells. In general, a relatively small amount of DNA damage causes cell cycle arrest, whereas larger DNA damage ultimately induces cell death.

Expression of p53 protein was significantly higher under the high temperature conditions than under the normal temperature conditions. Under the normal temperature conditions, changes in the tendencies of the apoptotic rate, proliferation index, and p53 protein expression were similar. Thus, the results indicate that the low level of p53 protein expression did not strongly influence the process of cell turnover. Under high temperature conditions, the increasing level of p53 protein expression reduced cell turnover. In addition, the changing tendencies were opposite between p53 protein expression and the apoptotic rate or proliferation index. Interestingly, under the high temperature condition, p53 protein expression decreased sharply at day 25. We supposed that, at day 25, a dynamic balance was present between the apoptotic rate and proliferation index. As the body recognized this state of balance, p53 protein expression decreased. This balance was disrupted with the new functional recovery phase; thus, p53 protein expression increased again at day 40.

CONCLUSIONS

During the dry period, changes in the cell cycle were not significantly different among each biopsy time point in the different seasons; however, the apoptotic rate and proliferation index of the mammary epithelial cells in the summer were lower than those in the spring. p53 protein levels were higher when cells were under heat stress than when cells were under a normal temperature condition. These findings suggest that high temperature retards the G2/M phase of the cell cycle and the cell turnover rate, but induces a high level of p53 protein expression. We inferred that the recovery period of mammary epithelial cells was extended because of high temperature.

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