A non-invasive method for measuring mammary apoptosis in dairy animals

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What determines milk production?

Milk yield is the result of three major biological processes:

1) Mammary cell production and differentiation
2) Milk secretion rate per cell
3) Cell death rate
Background

- Apoptosis of secretory cells is one of the key drivers of milk yield throughout lactation.
- Past methods to measure apoptosis during lactation have been problematic.
- Microparticles are released at times of cell activation and apoptosis.
- Measuring microparticles in milk may help us understand one of the key determinants of milk yield – cell death rate by apoptosis.
Microparticles

- Microparticles (MP) are membrane-bound vesicles of less than 1 μm diameter released from many different cell types.
- Microparticles are formed by blebbing of the parent cell membrane.
- During cell membrane blebbing and MP formation, phospholipids become exposed on the outer leaflet of the plasma membrane and the outer surface of the microparticle.
- It is the presence of these normally hidden molecules that allow for the detection of microparticles by binding to specific markers.
Milk samples

- Monthly milk samples collected from 12 cows over a 5 month period
- Farms records on milk production were taken from routine monthly recording on the farm
- 60 whole milk samples were frozen and transferred to the laboratory for MP analysis
- Microparticle density estimated using flow cytometry
Laboratory method

- The phospholipid phosphatidylserine specifically binds to annexin V (AV), a calcium-dependent phospholipid-binding protein.
- The negatively charged lipophilic dye merocyanine (MC) 540 used to detect the presence of disordered phospholipids on the membranes of MP.
- MP with attached markers used to count cell numbers using flow cytometry.
Data analysis

- Milk yield parameters plus four MP densities analysed
  - AV+ = Annexin-V positive MP density
  - MC+ = MC540 positive MP density
  - Both+ = MPs +ve for both Annexin-V and MC540 density
  - Total = all MP density
- MP = μ + Cow + b(DIM:Cow) + error
- Pearson correlation coefficients between parameters
## Results - data collected from 12 cows on 5 monthly recording days (n=57)

<table>
<thead>
<tr>
<th></th>
<th>Original data</th>
<th>Log&lt;sub&gt;10&lt;/sub&gt; transformed data</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>Days in milk (d)</td>
<td>201</td>
<td>68</td>
</tr>
<tr>
<td>Milk yield (kg/d)</td>
<td>25.7</td>
<td>7.6</td>
</tr>
<tr>
<td>Total (mp/μl)</td>
<td>334,705</td>
<td>198,669</td>
</tr>
<tr>
<td>Both+ (mp/μl)</td>
<td>5,207</td>
<td>8,753</td>
</tr>
<tr>
<td>AV+ (mp/μl)</td>
<td>120,473</td>
<td>104,898</td>
</tr>
<tr>
<td>MC+ (mp/μl)</td>
<td>108,686</td>
<td>70,374</td>
</tr>
</tbody>
</table>

AV+ = Annexin-V positive microparticles; MC+ = MC540 positive microparticles; Both+ = microparticles positive for both Annexin-V and MC540; Total = all microparticles.
## Results - ANOVA summary fitting effects of cow (n = 12) and days-in-milk (n = 5) within cow as a linear function

<table>
<thead>
<tr>
<th></th>
<th>DMY (kg/d)</th>
<th>Total (Log no/µl)</th>
<th>Both⁺ (Log no/µl)</th>
<th>AV⁺ (Log no/µl)</th>
<th>MC⁺ (Log no/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cow</strong></td>
<td>***1</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td><strong>DIM² within cow</strong></td>
<td>***</td>
<td>0.051</td>
<td>*</td>
<td>***</td>
<td>**</td>
</tr>
<tr>
<td><strong>Residual</strong></td>
<td>5.59</td>
<td>0.038</td>
<td>0.249</td>
<td>0.070</td>
<td>0.037</td>
</tr>
<tr>
<td><strong>Mean cow intercept</strong></td>
<td>44.9</td>
<td>4.9</td>
<td>2.7</td>
<td>4.2</td>
<td>4.3</td>
</tr>
<tr>
<td><strong>SE</strong></td>
<td>± 2.90</td>
<td>± 0.080</td>
<td>± 0.46</td>
<td>± 0.027</td>
<td>± 0.11</td>
</tr>
<tr>
<td><strong>Mean slope (/d)</strong></td>
<td>-0.097</td>
<td>0.003</td>
<td>0.004</td>
<td>0.004</td>
<td>0.003</td>
</tr>
<tr>
<td><strong>SE</strong></td>
<td>± 0.010</td>
<td>± 0.0004</td>
<td>± 0.0024</td>
<td>± 0.0014</td>
<td>± 0.0005</td>
</tr>
</tbody>
</table>

1Probability values or *** P < 0.001; ** P < 0.01; * P < 0.05.
2DIM = days in milk; DMY = daily milk yield.
AV⁺ = Annexin-V positive microparticles; MC⁺ = MC540 positive microparticles; Both⁺ = microparticles positive for both Annexin-V and MC540; Total refers to all microparticles
**Results** - Correlations between persistency and the regression slope of the 4 microparticle densities on DIM for each cow (n = 12)

<table>
<thead>
<tr>
<th></th>
<th>Persistency</th>
<th>Total</th>
<th>Both(^+)</th>
<th>AV(^+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>-0.65</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Both(^+)</td>
<td>-0.32</td>
<td>0.37</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AV(^+)</td>
<td>-0.50</td>
<td>0.69</td>
<td>0.85</td>
<td></td>
</tr>
<tr>
<td>MC(^+)</td>
<td>-0.49</td>
<td>0.76</td>
<td>0.71</td>
<td>0.74</td>
</tr>
</tbody>
</table>

Correlations which were > 2 SE shown in bold
SE of correlations: correlations of 0 to 0.4 - SE of ~0.27; 0.5 to 0.7 - SE of ~0.19; > 0.7 - SE of ~0.11.
AV\(^+\) = Annexin-V positive microparticles; MC\(^+\) = MC540 positive microparticles;
Both\(^+\) = microparticles positive for both Annexin-V and MC540; Total = all microparticles.
Implications

- Extraction of microparticles from milk is viable
- From a limited dataset we have shown that changes in microparticle density are related to the decline in milk yield in late lactation
- This is likely to be linked through apoptosis
- We have found a useful non-invasive method for monitoring apoptosis of mammary cells
Further work

- Compare pregnant and non-pregnant cows
- Differentiate between MP from apoptosis and other cell activities
- Study MP density throughout the milking process
- Study MP production throughout more, complete lactations
- Study factors affecting MP production
Acknowledgements

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