Determining the presence of Osteopontin in fluid and cells from follicles of various sizes in bovine species

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Abstract: The objective of this study was to identify Osteopontin (OPN) in fluid and cells from follicles of various sizes using one dimensional poly-acrylamide gel electrophoresis (1D-PAGE), and western blot analysis. One dimensional poly-acrylamide gel electrophoresis was used to separate the various sized proteins in each sample using a 7.5% - 12.5% acrylamide gradient. For the western blot analysis, anti OPN, goat anti rabbit strep aviden horse radish peroxidase, and an electrochemoluminesence were used to localize and identify OPN from all other follicular proteins. OPN was found at molecular weights ranging from 27.0 kDa to 95.7 kDa in the small, medium, and large follicles.

Introduction:

Osteopontin (OPN) is a glycosylated phosphoprotein that was originally isolated from bone matrix [1], OPN is produced by osteoblasts, osteoclasts, and macrophages [2], and OPN levels increase in active sites of bone metabolism. OPN is also found in bodily fluids such as milk, blood, urine, and tissues such as smooth muscle cells, kidney, leukocytes, some tumors, and has been shown to promote migration of breast cancer cells [3-7]. OPN has been shown to promote cell adhesion, signaling, and migration in the immune cells [8]. OPN is also produced in the reproductive tissues, such as the uterus, placenta, seminal plasma, and has been positively correlated with bull fertility.

Folliculogenesis is the development of follicles from the primordial stage through a series of stages morphologically defined as: primary, preatral, antral, and culminating in the Graffian or preovulatory follicle stage. After ovulation the follicle undergoes transformation to the corpus luteum in cattle. There are waves of follicular growth that occur during the estrous cycle [10]. Each wave is characterized by the development of a number of follicles, where one follicle develops into the dominant follicle. The dominant follicle does not have to become an ovulatory follicle it can regress like the other smaller follicles in the wave, allowing another wave of follicles to develop. There can be as many as four to five follicular waves, but only one follicle will become the ovulatory follicle. There are suggestions that follicular development is continuous, and are independent of any particular stage of the estrous cycle. Sirois and Fortune et al, through real–time ultrasonography, measured and counted follicles during a normal estous cycle, early pregnancy, growth, and ovulation or regression of individual follicles during the late luteal and the follicular phase of the estous cycle [11].

Killian et al detected OPN in seminal plasma and the relative amount was positively correlated with bull fertility [7]. The observation of a correlation between the concentration of OPN in higher fertility bulls and the presence of OPN in follicular development led to the speculation that OPN in follicular fluid may play a role in ovum fertility. As initial efforts to better understand the role of OPN in the developing follicle,
this study was to identify OPN in follicular fluid and follicular cells of various sized follicles.

Methods and Materials:

Collection of Follicular fluid and cells

Cow ovaries were obtained from the Taylor Packing slaughter house in Pennsylvania and washed with 7x cleaner solution and 2% Chlorohexadine and allowed to stand for 10 minutes to disinfect the surface tissue. Then the ovaries were washed with PBS. The follicles were extracted from the ovaries and separated into labeled containers to get a specific OPN reading to determine whether there are differences in the OPN amounts in various size follicles. When emptying the syringes into the labeled containers, we used the 10x heparinized saline solutions to prevent clotting of the tissues. The small follicles ranged from 1mm to 5mm in diameter, medium 6mm to 10mm, and large follicles ranged 10mm and above. The Samples were then centrifuged at 3,000 rpm for 10 minutes at 4° C. The supernatant was then removed and re-centrifuge at 10,000 x gravity for one hour at 4° C, while the cells remained chilled on ice. The centrifugation of the samples was done to isolate the follicular fluid from follicular cells. The follicular fluid was aspirated off and re – centrifuged to get particulate materials out of the follicular fluid. The supernatant was then removed from the pellet giving pure follicular fluid. Then sample were stored in the freezer at –80°C.

Extraction Buffer

An extraction buffer was prepared containing 200 micro liters (µl) of 10 millimolars (mM) Tris at pH 7, a buffer to stabilize the pH; 40µl of 1mM EDTA, 1µl of 1mM Dithiothreitol, which reduces disulfide bonds; 20µl of 20mM Phenolmetholsulphonyl fluoride, and 95µl of Aprotonin were used as protease inhibitors and then raised to 20mL with distilled water in a 50ml conical tube. 5ml of extraction buffer were added to each cell sample, vortexed and titrated. One µl of 0.2M PMSF was added to each sample of cells. One ml of each sample was then added to the micro centrifuge tubes, vacuum pumped for 2hrs at 45°C. Then the samples were put through a Lowry’s reagent test to calculate the unknown concentrations, so that the protein concentrations are not to concentrated or diluted when the one dimensional poly acrylamide gel is run. If the proteins concentrations are too concentrated or diluted then the western blot will show the two extremes and the proteins will not be distinguishable from each other, or they will not show the proteins.

Western Blot Analysis

The one dimensional poly acrylamide gel was placed onto one sheet of 19 x 15 nitrocellulose paper inside a Pharmacia Nova Blot for one hour. We put 200mL of
PBS/Tween 20 inside a container and added our primary antibody, BSA and heat inactivated normal goat serum (HINGS) to bind to all of the proteins on the nitrocellulose paper except the OPN proteins. The secondary antibody used was anti OPN, to bind the OPN proteins. The tertiary antibody was the goat anti rabbit strep aviden horse radish peroxidase antibody (SA-HRP), which is used to bind to the anti OPN and is a receptor for the electrochemoluminescence to emit a fluorescence to expose the kodak photography paper. Then the nitrocellulose paper was then died with India ink to show the proteins transferred from the poly acrylamide gel.

Results:

Table 1:

<table>
<thead>
<tr>
<th></th>
<th>Unknown sample</th>
<th>SDS treatment Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small cells</td>
<td>3.0µl</td>
<td>96.7µl</td>
</tr>
<tr>
<td>Medium cells</td>
<td>6.3µl</td>
<td>93.7µl</td>
</tr>
<tr>
<td>Large cells</td>
<td>.62µl</td>
<td>99.38µl</td>
</tr>
<tr>
<td>Small F. fluid</td>
<td>.13µl</td>
<td>99.87µl</td>
</tr>
<tr>
<td>Medium F. fluid</td>
<td>.12µl</td>
<td>99.88µl</td>
</tr>
<tr>
<td>Large F. fluid</td>
<td>.14µl</td>
<td>99.86µl</td>
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</tbody>
</table>

The standard curve of protein concentration is used to determine the unknown concentration present in the follicular samples.

Figure 1 Standard Curve

In 1-D electrophoresis all proteins in follicular fluid or cells were separated by their molecular weights.
In Western Blot we transferred OPN on to nitrocellulose paper and visualized OPN at two different molecular weights.

**Discussion:**

This study was done during a restricted eight-week research program, not leaving time for an in-depth study. OPN was viewed at the correct molecular weights where osteopontin can be found. Also using the correct antibody receptors, along with precise laboratory technique, there has been a conclusion that OPN is present within follicular cells and fluid. Throughout the study there were several inconclusive findings of two of the three samples due to improper applications of the antibodies to the nitrocellulose paper, improper 1D electrophoresis set up, and the application of unknown protein concentrations to the 7.5% - 12.5% poly acrylamide gel. Our third unknown sample was rendered useless because of the failure to apply the SDS treatment buffer to the samples before setting them in boiling water, therefore the unknown sample three solidified. The
solidification occurred because the sulfur bonds were not broken by the SDS TRT buffer. This may mean that there is a positive correlation with fertile cows and fertile bulls.

**Conclusion:**

These studies determined that similar molecular weight forms of OPN are present in all sized follicles, suggesting that the egg is exposed to OPN throughout follicular development. These studies also may suggest in future studies a positive correlation between high fertility bulls and highly fertile cows.

**References:**

10. Rajakoski E. The Orarian follicular system in sexually mature heifers with special reference to seasonal, cyclical and left-right variations. Acta Endocrinol (suppl.) 52; 1-68.