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Short Communication

Equine synovial fluid protein equalization via combinatorial peptide ligand libraries

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Abstract

To gain further knowledge of the equine synovial fluid (SF) proteome, we propose a protocol based on the equalization of the relative concentrations of its proteins, which leads to the modification of the standard electrophoretic pattern revealing low-abundance proteins that otherwise remain undetected. Fresh SF samples were collected from ten macroscopically normal metacarpophalangeal joints of crossbred horses. The samples were processed using standard procedures as the control and via combinatorial peptide ligand libraries (CPLL) using low ionic forces (NaH2PO4 10 mM) at different pHs (4.0, 7.0, and 9.3) with 10% sodium dodecyl sulfate (SDS) and 25 mM DTT for protein resolubilization. Proteins were then separated by conventional 8% SDS-PAGE and stained with coomassie blue. After separation of the equalized proteins, there was a significant reduction in the albumin (the most abundant protein in the SF) and, at the same time, other protein bands arise that were not visible without CPLL processing. In addition, there was variation in the protein profiles at different pHs. The results suggest that protein equalization of the equine SF by CPLL could be a useful tool to better understand the articular homeostasis and/or for the detection of new biomarkers of joint pathology.

Keywords: Synovial fluid, *Equus caballus*, Equine, Combinatorial peptide ligand library, Biomarker

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Introduction

The performance of sport horses depends, among other factors, on the integrity of the musculoskeletal system, the joints play a key role in this regard (van Weeren, 2014). A full understanding of the mechanisms involved in osteochondral diseases will allow the early recognition of different joint pathologies (Frisbie et al., 2016). Therefore, the study of synovial fluid (SF) biomarkers is important (Chávez et al., 2010). Although still limited, different biomarkers such as cytokines, glycosaminoglycans, hyaluronic acid, osteocalcin, collagen derivatives (Chiaradia et al., 2012), and other proteins related to joint homeostasis (Frisbie et al., 2016) have been identified.

Combinatorial peptide ligand libraries (CPLL) were primarily developed to study the proteome of human biological fluids. This technology allows the equalization of the protein content of a sample, lessening the quantity of high-abundance proteins and increasing the relative concentration of low-abundance proteins (Boschetti and Righetti, 2008). Albumin represents up to 80% of the protein content of SF (Greve et al., 1987) and could mask proteins with similar molecular weight when studied by conventional electrophoresis. These "masked" proteins could be relevant in joint homeostasis and/or pathological conditions, and hence their potential use as biomarkers could be important.

To contribute to the study of equine SF proteins, we implemented a protocol using CPLL technology to analyze these particular fluids by balancing the protein fractions and gaining better access to less abundant proteins.

Materials and Methods

Synovial fluid sampling

Synovial fluid samples, aseptically collected from ten normal metacarpophalangeal joints, were selected for this study. These articulations were obtained from ten adult crossbred horses immediately after being sacrificed at a certified slaughterhouse for human consumption.

To include only non-pathological tissues in this research, all the joints were examined macroscopically

after arthrotomy. Appropriated joints had undegenerated cartilages (i.e., their surface without fibrillation, erosion, or wear lines). Additionally, joints in which the cartilage had a bright pearly color, the synovial membrane had no congestion signs, the SF was clear, and translucent yellowish were considered normal (Adarmes et al., 2003) and eligible for the study.

After collection, the SF samples were kept on ice and then centrifuged at 4,000 xg for 15 minutes (all centrifugation steps were carried out at 5°C). Supernatants were recovered, and their protein concentration was measured using the Lowry method (Lowry et al., 1951). Fluids with 15 mg/mL or higher protein concentrations were discarded to avoid studying samples from joints with an ongoing inflammatory process (Adarmes et al., 2003). The non-discarded SF supernatants were pooled (20 mL), and hyaluronate was removed to avoid potential interference in the interaction between the sample and the resin (Righetti and Boschetti, 2015). The final protein concentration of the pool of SF supernatants was 5.5 mg/mL.

Combinatorial peptide ligand library for equine synovial fluid

The pool of SF supernatants was used to obtain three aliquots of 6.5 mL each. The aliquots were buffered with 13.5 mL of 25 mM acetate pH 4.0; 25 mM phosphate pH 7.0; and 25 mM Tris-HCl pH 9.3, respectively. The total volume of each buffered aliquot was approximately 20 mL, with a total protein content of 35.75 mg. To prepare the combinatorial ligand suspension, 100 mg beads of Proteominer Bulk (Bio-Rad, California) were suspended in 2 mL of 20% ethyl alcohol for 12 hours under constant agitation at room temperature (RT). This suspension was then washed three times with distilled water. One hundred microliters of the suspended beads were buffered with 100 μL pH 4.0, pH 7.0, and pH 9.3 buffers, respectively. Subsequently, 5 mL of each buffered pooled SF supernatants were added to the bead suspensions matching their three respective pHs.

The mixture was then stirred for two hours at RT and centrifuged at 1,000 x g for 5 minutes; the supernatant with unbounded proteins was discarded. This procedure was repeated four times using the same beads. Afterward, 500 μL of 10% SDS and 50 mM dithiothreitol (DTT) were used to recover the proteins bound to the resin. The suspension was centrifuged at 1,000 x g for 5 minutes, and the supernatant was recovered from each tube. This procedure was repeated once more with the same solution to increase the recovery of the resin-bound proteins.

Samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970). Briefly, SDS-PAGE was prepared as follows: stacking gel (5%) containing 0.85 mL acrylamide, 1.25 mL gel buffer (pH 6.8), 2.85 mL deionized water, 5 μL TEMED, 50 μL 10% SDS, 25 mL of 10% ammonium persulfate; and separating gel (8%) containing 2.7 mL acrylamide, 2.2 mL gel buffer (pH 8.8), 4.7 mL deionized water, 5 μL TEMED, 100 μL 10% SDS and 50 μL 10% ammonium persulfate. The samples were heated at 100 ^{o}C for 5 minutes in a loading buffer containing β-mercaptoethanol. Each gel slit was loaded with 15 μL containing $\approx$5 μg/μL of protein. Electrophoresis was carried out at 50 millivolts for 1.5 hours after which the voltage was increased to 100 millivolts for another 1.5 hours. Following electrophoresis, gels were stained with Coomassie blue (Sigma-Aldrich®) overnight at room temperature.

Results and Discussion

The CPLL methodology utilizes solid-phase ligand libraries of immense diversity used for affinity-based binding to the proteins of a given mixture. We combined this approach with additional fractionation methods to further increase the number of detectable species; hence, peptides or undetectable proteins by classical analytical methods became easily accessible as described for other biological fluids by Thulasiraman et al. (2005).

The study of proteins in a particular biological fluid is complicated due to their vast number and concentration range in biological mixtures. Although theoretically, equalization procedures are helpful to solve this problem, they may fail due to nonspecific interactions between trace proteins and the removed high-abundance proteins (Thulasiraman et al., 2005). Overloading the resins with proteins is required to enrich low-abundance protein species (Candiano et al., 2009).

Protein capture depends on both the concentration and affinity constant to the ligand thus the hexapeptide ligand will be saturated as the protein concentration increases (Righetti and Boschetti, 2013). However, Thulasiraman et al. (2005) reported that about 2% of proteins might remain undetectable after CPLL treatment, which indicates that this procedure, which includes protein overloading, does not guarantee total protein capture.

In the current work, CPLL treated synovial samples at different pH values (pH 4.0, 7.0, and 9.3) yielded a total protein content of 4.25, 3.1, and 2.6 mg with a recovery rate of 11.9%, 8.7%, and 7.3%, respectively. Protein separation by SDS-PAGE is shown in Figure 1. The results showed a decline in the concentration of high-abundance proteins and the disclosure of some proteins not seen in untreated SF samples. The electrophoretic pattern showed a significant decrease in the intensity of the albumin band (indicated with letters a, a', and a" in Figure 1) compared with untreated SF (identified with the letter A in Figure 1). As expected, proteins masked due to having a similar molecular weight to albumin in the untreated electrophoretic pattern could be readily recognized. For instance, we could excise from the gel a discrete band that was revealed after the CPLL treatment (indicated with an arrowhead in Figure 1) and then identify it using MALDI TOF MS as an antithrombin-III isoform [*E. caballus*]. The differential protein expression at distinct pH values was also noticeable. For example, bands "b", "c", "d" and "e" illustrated in Figure 1 were more intense at

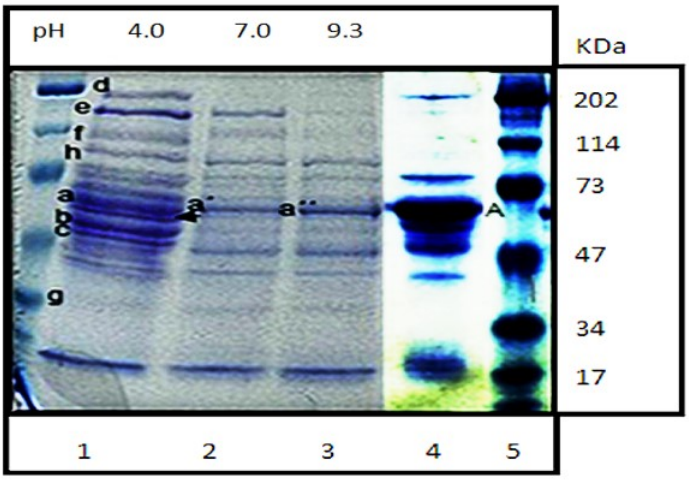

Figure 1: One-dimensional polyacrylamide gel electrophoresis after combinatorial peptide ligand library (CPLL) treatment. Lanes 1 to 3 correspond to synovial fluid (SF) after CPLL treatment and lane 4 is SF without treatment. Molecular weight marker was loaded in lane 5. Letter A (on the right-hand side of lane 4) shows a thick albumin band while the letters a, a', and a" (on the left-hand side of lanes 1, 2 and 3, respectively) show thinner albumin bands, thus revealing the presence of other proteins such as the one in the band of line 1 indicated by a black arrowhead (which was identified as the *Equus caballus* antithrombine-III X isoform). CPLL-treated SF expressed different protein profiles depending on pH as illustrated with the bands indicated by the letters b, c, d, and e, which were more intense at pH 4 (lane 1) than at pH 7 and 9.3 (lanes 2 and 3, respectively). However, the protein band indicated by the letter h was expressed similarly despite pH variation. Finally, some proteins were only visible after CPLL treatment, as shown by the bands indicated by the letters f and g.

pH 4.0 and less perceptible at pH 7.0 and 9.3. On the other hand, band "h" appears to be expressed similarly despite the pH difference, whereas the bands "f" and "g" appear to be unique to the electrophoretic patterns derived from protein equalization by CPLL (Figure 1).

As mentioned above, we used solutions containing low molar concentrations as the capture medium to avoid possible interference of the binding phase with the resins and proteins. It is important to consider that electrostatic bonds represent the main method of bonding between hexapeptide ligands and proteins. The ionic forces are a major factor in the capture efficiency because they modulate the peptide-protein interaction. Guerrier et al. (2006) achieved better protein recovery when using low ionic force solutions, which is consistent with the results of Di Girolamo et al. (2011), who found that protein recovery was three times more efficient when NaCl was removed from the phosphate-buffered saline solution (PBS). However, there is also evidence that some protein species are better captured when high ionic forces are used (Boschetti and Righetti, 2013), so two-dimension capture methods have been developed to capture the entire protein spectrum (Santucci et al., 2013). Furthermore, it is known that hexapeptides and proteins can be ionized depending on the pH of the medium, so pH variations modulate the ability and strength of electrostatic bonds between them (Boschetti and Righetti, 2013). When buffered at pH 7.0, equalized human serum shows a protein loss between 15% (Fasoli et al., 2010) and 20% (Santucci et al., 2013), but when samples were treated at other pHs (4.0 and 9.3), these losses were considerably reduced.

As described above, low molar concentration solutions without NaCl and at different pH were used in this SF study. In addition, proteins were recovered from the resin using 10% SDS and 50 mM DTT solution to optimize the results, as previously described (Di Girolamo et al., 2011; Boschetti and Righetti, 2013). The differences in the electrophoretic patterns at different pH values can be attributed to the predominance of acidic protein species (about 2/3) within the SF proteome (Candiano et al., 2012) and the hydrophilicity caused by the carboxyl group predominance. Fasoli et al. (2010) described that some protein species are better or exclusively seen under certain pH conditions. Additionally, it has been reported that acid protein species are best captured under low ionic forces (Santucci et al., 2013).

Conventional CPLL has been used to characterize the protein content of SF in healthy and osteoarthritic (OA) equine joints, identifying a set of proteins and neopeptides that may act as potential biomarkers to distinguish between normal and OA articulations (Peffers et al., 2015). Nevertheless, the total SF volume (1 mL) and total protein quantity (5 mg) used in the aforementioned pioneering study were relatively low, and these conditions, according to Thulasiraman et al. (2005), may reduce the efficiency of the protein equalization. This may be the reason why only 33% of

additional species were found in that study, which is more similar to the yield found when immunodepletion (25–30%) is used than the one expected to be obtained after CPPL treatments. In this respect, studies in human samples have shown a higher increase in the number of protein species using the CPLL technique: 335% in serum (Beseme et al., 2010), 490% in urine (Righetti et al., 2006), and 628% in erythrocytes (Righetti et al., 2010).

In our study, the electrophoretic profile showed a lack of bands at pH 7.0 and 9.3 under the albumin band location (between 50 and 60 kDa), which is different to the pattern at pH 4.0 and to the untreated SF. The largest number of new protein species has been found within this range in various human biological fluids (Righetti et al., 2006). Thereby, our results using CPLL demonstrated that a differential electrophoretic profile occurs under different pH conditions, expanding the range of protein detection, which in turn could be useful for identifying the SF proteins and their association with physiological or pathological phenomena of equine joints.

We conclude that the implemented protocol, that takes advantage of CPLL technology, allowed equine SF protein equalization in which the concentration of high-abundance proteins decreased while enrichment of the concentration of low-abundance proteins was apparent. It is important to emphasize that protein overloading of the hexapeptide ligands is necessary for the proper outcome of this technology. The differential capture of proteins from the SF at different pH values is a significant tool to complement the SF proteome study.

Finally, although we developed a procedure using CPLL that proved useful for revealing a wider range of protein content within the SF, additional research is necessary to optimize this technology to find new biomarkers of horse joint diseases and gain a better understanding of joint homeostasis.

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Conflict of Interest.The authors declare no conflict of interest.

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