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Comparing Perilymph Proteomes Across Species

Jonathan C. Palmer, ME; Megan S. Lord, PhD; Jeremy L. Pinyon, PhD; Andrew K. Wise, PhD; Nigel H. Lovell, PhD; Paul M. Carter, PhD; Ya Lang Enke, BSc; Gary D. Housley, PhD; Rylie A. Green, PhD

Objectives/Hypothesis: Biological components of perilymph affect the electrical performance of cochlear implants. Understanding the perilymph composition of common animal models will improve the understanding of this impact and improve the interpretation of results from animal studies and how it relates to humans.

Study Design: Analysis and comparison of the proteomes of human, guinea pig, and cat perilymph.

Methods: Multiple perilymph samples from both guinea pigs and cats were analysed via liquid chromatography with tandem mass spectrometry. Proteins were identified using the Mascot database. Human data were obtained from a published dataset. Proteins identified were refined to form a proteome for each species.

Results: Over 200 different proteins were found per species. There were 81, 39, and 64 proteins in the final human, guinea pig, and cat proteomes, respectively. Twenty-one proteins were common to all three species. Fifty-two percent of the cat proteome was found in the human proteome, and 31% of the guinea pig was common to human. The cat proteome had similar complexity to the human proteome in three protein classes, whereas the guinea pig had a similar complexity in two. The presence of albumin was significantly higher in human perilymph than in the other two species. Immunoglobulins were more abundant in the human than in the cat proteome.

Conclusions: Perilymph proteomes were compared across three species. The degree of crossover of proteins of both guinea pig and cat with human indicate that these animals suitable models for the human cochlea, albeit the cat perilymph is a closer match.

Key Words: Perilymph proteome, human, guinea pig, cat, cochlear implant, impedance.

Level of Evidence: NA

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INTRODUCTION

The electrode array of a cochlear implant (CI) is surgically implanted into the scala tympani of the cochlea that contains fluid called perilymph. Along with endolymph, this comprises the fluid environment of the inner ear. Although these fluids are related, they are not identical.[1] Perilymph is a complex biological fluid that contains a variety of proteins, amino acids, and salt ions.[2] Proteins are produced by local cells along with proteins intrinsic to the cerebrospinal fluid (CSF) that connects with perilymph via connections such as the cochlear aqueduct.[3] Although CIs can contact cells lining the scala tympani, the majority of functional components, such recessed platinum electrodes, are bathed in perilymph. For this reason, it is important to understand the composition of perilymph, as biological fluids have a significant impact on impedance and dissolution properties of electrodes. The impact of the biological environment on device performance is the underlying area of interest that motivated the current study. Protein adsorption is the first interaction that occurs after introduction of a synthetic material, such as a CI, to a biological environment. These initial interactions dictate ongoing responses of the body to the material or implant, for example inflammation, cell adhesion, and tissue growth. This will also affect impedance.[4] For CIs and other bionic devices, the amount and type of protein present in a biological fluid will impact on the electrical properties and performance of the implant.

The efficiency and efficacy of auditory nerve activation is key to the long-term performance of a CI. Protein adsorption to an electrode increases the impedance of the electrode surface. This change reduces the performance of an implant as the capacity to pass charge

Additional Supporting Information may be found in the online version of this article.

From the Graduate School of Biomedical Engineering (J.C.P., M.S.L., N.H.L., R.A.G.), the Translational Neuroscience Facility and Department of Physiology, School of Medical Sciences (J.L.P., G.D.H.), University of New South Wales Sydney, Sydney, New South Wales, Australia; Bionics Institute (A.K.W.), East Melbourne, Victoria, Australia; Cochlear Ltd (P.M.C., Y.L.E.), Macquarie University, Sydney, New South Wales, Australia; Department of Bioengineering (R.A.G.), Imperial College London, London, United Kingdom.

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Send correspondence to Jonathan Palmer, Graduate School of Biomedical Engineering UNSW Australia, Sydney, NSW, 2052, Australia. Email: jonathan.palmer@unsw.edu.au

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efficiently into the body is diminished. A study by Newbold et al.[5] found adsorption of bovine serum albumin (BSA) to the surface of gold electrodes increased polarization impedance by 60%. The types of protein adsorbed to the surface will have different effects on the electrode properties. It was found in a study by Zhang et al.[6] that BSA adsorption to a surface saw a change in capacitance 50% greater than when fibrinogen was adsorbed to the same surface. These protein absorption effects can alter the rate of dissolution (loss of material from electrodes). It was identified by Robblee et al.[7] that protein absorption reduced stimulating electrode dissolution by up to 90%. Despite these findings, there is very little understanding of the mechanism by which proteins protect electrodes.

One of the major limitations in these in vitro studies is that perilymph cannot be adequately represented by a single protein such as BSA. Two or more proteins in solution create competitive and time-dependent adsorption effects.[8] An understanding of the composition of perilymph, and in particular the relationship between human data and animal models used to study CI performance, is necessary to enable improved understanding of in vivo CI properties.

A proteome (protein expression of a genome), coined by Wilkins et al.,[9] is the full expression of proteins by a particular set of genes. Recent developments in mass spectrometry (MS) enabled the identification of large numbers of proteins in a relatively short time. Liquid chromatography with tandem MS (LC-MS/MS) is the most common configuration used due to its relatively high accuracy and resolution from small sample sizes. In 2011, while demonstrating a diagnostic technique, Lysaght et al.[10] used LC-MS/MS to obtain the first broad proteome of human perilymph. This was a substantial improvement on an earlier study by Palva and Raunio,[11] where albumin was identified as the major component of human perilymph, and Arrer et al.[12] discovered the presence of transferrin and immunoglobulins. Lysaght et al.[10] identified almost 300 unique proteins across four samples of human perilymph, of which 71 were common to all and determined to be the principal components of the human perilymph proteome. A study in 2017 by Schmitt et al.[13] used an alternate extraction technique to identify 878 proteins across 41 samples of human perilymph, with 73 proteins found in all samples. Although these studies provide a basis for relating CI performance to human perilymph, showing which proteins are present to cause an effect, this is not immediately useful for in vitro studies probing protein electrode interactions or translational studies using animal models.

The majority of research studies that investigate CI performance are conducted using animal models. Animal models are critical to design and regulatory studies that demonstrate safety and efficacy of implant devices. Animals provide a more accessible system for analyzing implant performance and allow implant retrieval for analysis at the conclusion of the study. Guinea pigs and cats have been used extensively for CI studies.[14,15] However, few studies have sought to examine the perilymph proteome and establish a comparison between the animal models and the human environment. Swan et al.[16] established a proteome for mouse perilymph, but as this is currently not a commonly used model, it has minimal relevance to CI performance. The guinea pig and cat models are so well established that CI companies produce specifically designed devices for these species.[14,15] An early profile of guinea pig perilymph, reported by Thalmann et al.,[17] identified some proteins common to CSF; however, as a high-resolution technique was not available, the published proteome was limited. To address this research gap and start to understand the impact of the biological environment, the current study used contemporary LC-MS/MS techniques to analyze perilymph of guinea pigs and cats. Results were used to demonstrate the degree of similarity and critical differences to the published human proteome.[10] This has a potential relationship to device performance and drug therapies.

MATERIALS AND METHODS

Perilymph Collection

Due to the restrictive nature of acquiring human perilymph and availability of preexisting high-quality data, the human perilymph proteome was obtained from the published dataset available from Lysaght et al.[10] Animal perilymph was obtained using protocols approved by the University of New South Wales Sydney Animal Care and Ethics Committee (guinea pigs) and Bionics Institute Animal Research Ethics Committee (cats). Eight perilymph samples were collected from the left and right ears of four guinea pigs. For extraction, the skull was bisected; the entire cochleae were removed from the skull and cleaned of contaminants using saline. Perilymph was obtained by inserting a needle into the round window of the cochlea and gently extracting with a syringe. A total of six specimens of cat perilymph were extracted. In this case, cochleae were exposed externally due to the relative toughness of the skull. The exposed area was cleaned and perilymph was extracted using a syringe. Maximum volume extracted for both species was 10 μL. Perilymph was stored at −80 °C until used. Samples were not refrozen so as to minimize protein degradation.

Mass Spectrometry Analysis of Perilymph

Perilymph samples were diluted to 10 μl with 25 mM ammonium bicarbonate (NH_4HCO_3). Specimens were reduced with 10 mM dithiothreitol for 10 minutes at 95 °C and alkylated with 25 mM iodoacetamide for 20 minutes at 25 °C. Solutions were digested for 16 hours at 37 °C with 0.4 μg/μL trypsin in 50 mM NH_4HCO_3. Final solutions were centrifuged, and the supernatant was diluted 10 × in buffer (1% formic acid and 0.05% heptafluorobutyric acid in water). Liquid chromatography was performed using an Ultimate 3000 HPLC and autosampler system (Dionex, Amsterdam, the Netherlands). The column was filled with C18 silica (1.9 μm, 120 Å ReproSil-Pur 120 C18-AQ, Dr. Maisch GmbH, Ammerbuch, Germany). MS analysis was performed by a LTQ Orbitrap Velos mass spectrometer (Thermo Electron, Bremen, Germany). Mass/charge ratios were scanned between 350 and 1,750 and specific ratios dynamically excluded for 35 seconds. Peptides were identified by searching the Mascot database (Matrix Science Inc., Boston, MA) to find parent proteins.

The following exclusion criteria were applied: MOWSE (molecular weight search) score based on the frequency of hits

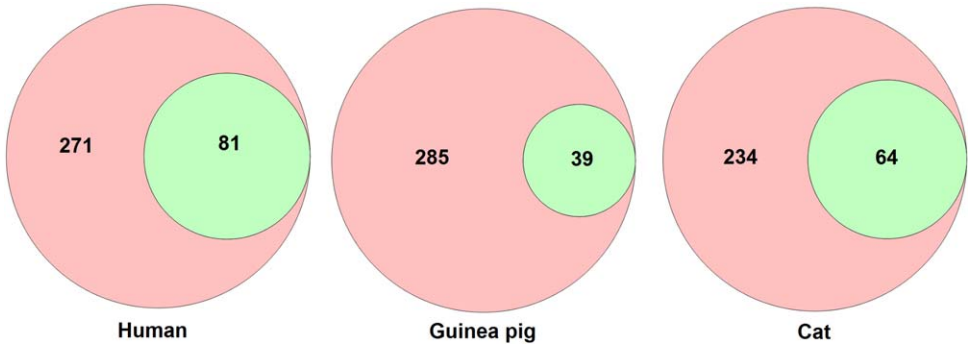

Fig. 1. The number of proteins in the perilymph proteome of each species (smaller green) based on analysis of the total number of protein matches (larger red) found by liquid chromatography with tandem mass spectrometry for that species. [Color figure can be viewed in the online issue, which is available at www.laryngoscope.com.]

for an identified protein was used.[18] Proteins with a score less than 50 were removed due to low confidence in identification. Proteins that were unknown or identified as hypothetical were also excluded. Any proteins identified as being from a species not closely associated to the one tested were removed. Finally, extracted perilymph can become contaminated by known impurities such as keratin from skin and hemoglobin from blood. These were removed from the proteome.

All proteins were grouped by functional classes: albumins, apolipoproteins, complement factors, enzymes, glycoproteins, immunoglobulins, protease inhibitors, and a section termed "ungrouped" for the few proteins that fell outside the previous classes. The proteome for each species was formed by identifying proteins present in 75% of samples for each species. This figure was chosen due to the large variance across MS analyses.[10] Isoforms and alternate names for a protein were counted as a single entry. Relative compositions were divided into protein classes, identified by tallying spectral counts and MOWSE scores.

Statistical Analysis

A one-way analysis of variance (ANOVA) with $\alpha = .05$ was used to compare the number of proteins in sample. A two-way ANOVA ($\alpha = .05$), with Tukey multiple comparisons, was performed to identify any significant mean difference in classes between proteomes.

RESULTS

The full proteome for each species is found in the Supporting Information. The number of proteins found in each animal species is shown in Figure 1. The overall number of proteins identified in each species is of similar magnitude for all species. The greatest number found was the guinea pig and was 22% higher than the cat's. Human perilymph had the most proteins when restricted to the perilymph proteome definition.

Figure 2 shows overlap in the refined proteome datasets. A total of 21 proteins were common to all three proteomes, which accounted for 25.9% of the human proteome. An additional four proteins were found in common between the guinea pig and human, which increased the overlap to 30.9%. Forty-two proteins were found in common between human and cat perilymph, which accounted for 51.8% of the human proteome.

Proteins were sorted into classes according to fit by the following criteria. Albumins are globular proteins that bind solutes such as fatty acids and assist in maintaining oncotic pressure.[19] Apolipoproteins bind to lipids (forming lipoproteins) to allow transport.[20] Complement components trigger the innate immune system,[21] and enzymes catalyze a wide range of reactions.[22] Glycoproteins are proteins with a covalently bound carbohydrate side chain that are branched and irregular. Total content varies significantly depending on the type of protein.[23] Immunoglobulins, or antibodies, bind to antigens as part of the immune system response.[24] Protease inhibitors prevent or limit the action of enzymes.[25] Ungrouped proteins did not fit into any of these classes. When each species proteome is divided into these broad classes, the areas of difference in composition become apparent, as shown in Table I. On average, the greatest variety was found in the protease inhibitors and the least in the albumins. For all three proteomes, the range of proteins in these two classes was comparable across all three proteomes.

Proportional composition was calculated by breaking samples down by protein class and tallying each class by spectral count/MOWSE score. The average for all samples within a species is shown in Figure 3. The statistical analysis for this section was a two-way ANOVA with an α of .05. The highest level of albumin

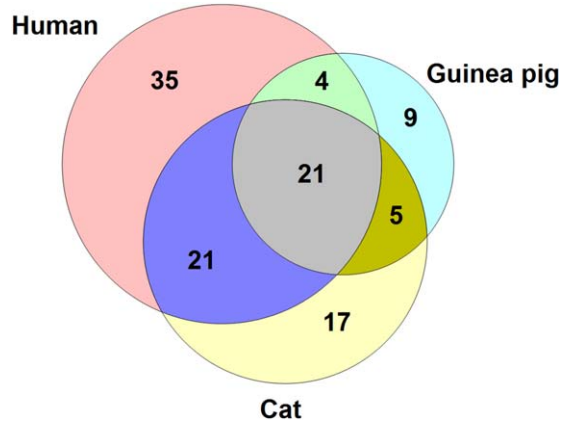

Fig. 2. Overlap of proteins identified in the proteomes of human, guinea pig, and cat perilymph. [Color figure can be viewed in the online issue, which is available at www.laryngoscope.com.]

TABLE I.
The Number of Proteins by Type Within Each Class of the Proteomes

Class	Human	Guinea Pig	Cat
Albumins	3	2	4
Apolipoproteins	8	3	9
Complements	9	4	6
Enzymes	13	5	9
Glycoproteins	14	6	11
Immunoglobulins	10	3	5
Protease inhibitors	13	13	15
Ungrouped	11	3	5
Total	81	39	64

was found in the human proteome, accounting for 40% of the composition. The albumin content of the cat proteome was significantly lower at 26.3%, and the guinea pig's was the lowest at 20.6%. Levels of protease inhibitors in the human proteome were significantly lower than the guinea pig or cat proteomes. The amount of immunoglobulins in the human proteome was significantly greater than in the cat proteome. For each of the remaining classes, to a 95% confidence interval, there was no significant difference between each proteome.

DISCUSSION

Any combination of proteins within a confined volume will have competitive interactions based on polarity, net charge, reactive regions, and size. These properties impact on protein adsorption to an implant.[8] As such, when looking for an appropriate animal model for human cochlea fluid, one with a similar perilymph proteome will have greater likelihood of demonstrating relevant behaviors. The overall number of proteins found in each perilymph indicates fundamental similarity between all three species. The proportion of proteins included in the proteome compared to the total number of proteins found was very similar for the human and the cat. The guinea pig had a much smaller proteome than the human, which may imply a reduced complexity in this model.

There was substantial variation in the number of proteins found across different samples. The numbers presented here for the human proteome are different to those originally presented by Lysaght et al.[10] due to reanalysis in line with the current study. Although the initial human proteome was defined as proteins that were found in all samples, here it was chosen to use those found in three of four samples (75%) to allow for variance and equal analysis with animal perilymph. Despite controlled extraction and identical analysis within a species, each animal had a varied perilymph composition. Extracted volume varied from 1 μL in humans[10] to between 2 and 10 μL for cat and guinea pig. Volume of perilymph in humans is as much as five times higher than a guinea pig[26] and at least two times higher than a cat.[27] Variation in samples may cause low-abundance proteins to fall below detection limits.[10] Although attempts were made to match extraction procedures, human and cat samples were obtained intraoperatively and guinea pigs were postmortem. It was likely that the intraoperative samples would have faced some contamination from CSF flow. The CSF flow rate is variable between animals, but the flow is generally low (~0.6 μL/min[28]. Given the fast extraction of samples, the level of CSF contamination would have been minimal. Some degeneration of fluid occurs postmortem, including changes in CSF flow. However, this was minimized by taking samples immediately after euthanasia. Samples taken in this manner reduce contamination from blood flow.[11]

Analysis of human LC-MS/MS results was performed using the Paragon algorithm, whereas cat and guinea pig samples were analyzed using Mascot. Mascot performs identification by matching peptide fragments to all possible results until the most likely protein is found. Paragon performs a selection process to limit the number of possible proteins it searches against.[29] These differences may have introduced variation; however,

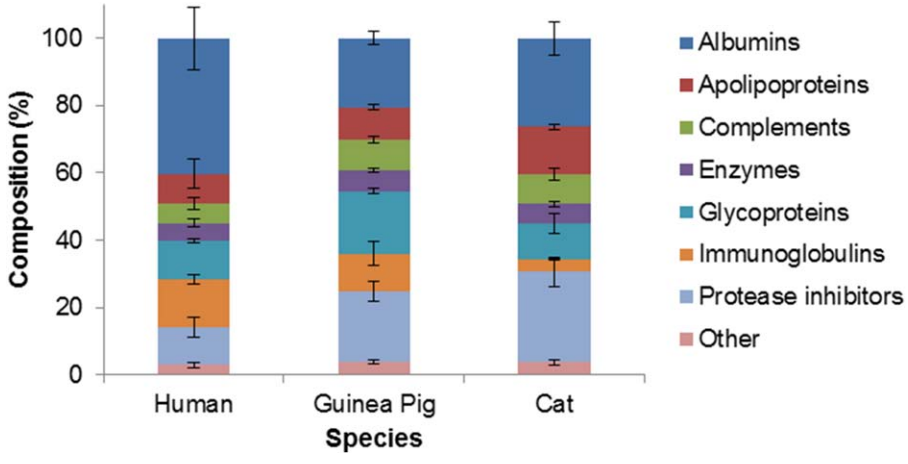

Fig. 3. Composition of human, guinea pig, and cat perilymph proteomes by protein classes. Error bars are standard error of the mean. [Color figure can be viewed in the online issue, which is available at www.laryngoscope.com.]

cochlin, a known biomarker for perilymph, was present in the proteome of all three species.[13] Large variation in protein number was also found by Schmitt et al.[13] Variation in protein content was dependent on the age of the patient and the type of surgery. Lysaght et al.[10] obtained perilymph from patients undergoing vestibular schwannoma removal or CI surgery. These disease conditions may affect the perilymph composition by changing protein production. Surgery itself may also cause a change in protein content by inflammation and other effects. The surgical approach used may have also had an effect on the protein expression, depending on whether implantation was through the round window or a cochleostomy.[30] It is possible that the higher proportion of immunoglobulins present in the human proteome is related to the disease state of these sample providers. Although cat perilymph was taken intraoperatively and guinea perilymph was postmortem, both were from otherwise healthy animals. Thus, the animal models would not have experienced the same disease state effects as the human subjects, and may ultimately impact on the proteome definition.

Some protein classes will have a greater effect than others on interactions with materials. It is unlikely that albumins will have any long-term effect, as they do not specifically bind to surfaces and will not be dominant at the surface of a chronic implant.[19] Apolipoproteins do not bind strongly with platinum,[20] making their relatively high content in animal models a minor consideration. Glycoproteins have oligosaccharide side chains that affect their biological functions, including binding properties.[23] Guinea pig perilymph proteome had fewer glycoprotein types but a proportionally larger volume. This changes the type and amount of carbohydrates present, potentially affecting binding behavior and impedance performance of an electrode.[31] Complement proteins and immunoglobulins are expected to be present at an interface where implant damage and disease are dominant, inciting inflammation and other immune responses.[24,32] Complements are part of the innate immune system and can recruit and promote a cascade of immune reactions. A greater amount of complements was found in guinea pig and cat perilymph in comparison to human, but with less variety in the types of complement proteins present. In contrast, immunoglobulins interact with the adaptive immune system. They attach to the surface of antigens and are known to interact with metals.[24,33,34] The levels and types of immunoglobulins were much less in the guinea pig and cat proteome than in the human proteome, suggesting a key area of difference and consideration when using these models. Propofol, which was used to anesthetize the cats, has an immunosuppressive effect[35] and may have resulted in reduced presence of immune-related molecules.

CONCLUSION

Perilymph proteomes were defined for the guinea pig and cat. The perilymph of all three species had relatively similar numbers of proteins, with 21 proteins identified as common to all three species, and comparable overall compositions based on protein class. This indicated that both guinea pigs and cats make acceptable models in terms of biological content of the perilymph and hence potential protein interactions with CI devices. The cat and human proteomes are closer to each other, in terms of numbers of proteins present, than the guinea pig and human proteomes, and as such is considered the better model. Future studies will seek to develop an artificial perilymph that can be used to conduct in vitro studies for predicting implant performance and lifetime.

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