Comparison of vehicles to collect dentinal fluid for molecular analysis

Zehnder, Matthias ; Rechenberg, Dan-Krister ; Bostanci, Nagihan ; Sisman, Filiz ; Attin, Thomas

DOI: https://doi.org/10.1016/j.jdent.2014.01.014

Comparison of vehicles to collect dentinal fluid for molecular analysis

Matthias Zehnder [a]*, Dan-Krister Rechenberg [a], Nagihan Bostanci [b], Filiz Sisman [a], Thomas Attin [a]

[a] Clinic for Preventive Dentistry, Periodontology, and Cariology
[b] Oral Translational Research, Institute of Oral Biology
University of Zürich, Center of Dental Medicine, Plattenstrasse 11, CH-8032 Zürich

Keywords:
Dentinal fluid
Diagnostics
MMP-2

**Corresponding author:* Tel.: +41 44 634 3438, fax: +41 44 634 4308
E-mail address: matthias.zehnder@zzm.uzh.ch

ABSTRACT

Objectives: To test the hypothesis that a material with higher water absorption than polyvinylidene fluoride (PVDF) could increase the yield of target molecules from exposed dentin.

Methods: In a series of standard tests, different cellulose membranes were compared to a PVDF counterpart for their ability to absorb water and release protein. In a subsequent randomized clinical trial, the cellulose material with the most favorable values was compared to PVDF regarding the levels of MMP-2 that could be collected from exposed dentin of healthy human teeth during filling replacement. MMP-2 levels were determined by enzyme-linked immunosorbent assay (ELISA). Data from the laboratory experiments were compared between materials using the appropriate parametric tests. The frequency of cases yielding quantifiable levels of MMP-2 was compared between materials by Fisher's exact test. The level of significance was set at 5%.

Results: The cellulose membrane with the largest pore size (12 – 15 μm) absorbed significantly ($P < 0.05$) more water than PVDF. It showed a protein release that was similar to that of PVDF, while the cellulose membranes with smaller pore size retained significantly more protein ($P < 0.05$). Using the large-pore cellulose membrane, MMP-2 could be collected at a quantifiable level from the dentin of healthy teeth in 9 of 13 cases, compared to 1 of 13 with the PVDF membrane ($P < 0.05$).

Conclusions: Under the current conditions, a large-pore cellulose membrane yielded more of a molecule of diagnostic value compared to a standard PVDF membrane.

Clinical significance: Molecular diagnostics of dentinal fluid are hampered by low yields. In the current study, it was shown that cellulose membranes are more useful to collect MMP-2 from dentinal fluid than PVDF membranes.

1. Introduction

A recent systematic review identified sparse evidence regarding the accuracy of current pulp tests.[1] When a tooth contains an allegedly vital pulp before it is restored, the dentist has but few means to decide whether to perform a root canal treatment prior to restoration or not. This may explain why pulp necrosis is the most common biological complication in abutment teeth.[2, 3]

A novel experimental approach to non-invasively identify the state of the pulp is molecular diagnostics.[4] It is based on the collection and analysis of dentinal fluid.[5, 6] However, the collection of dentinal fluid is not unproblematic. The fluid and protein flux across the pulpodentin complex is influenced by multiple factors.[5] In addition, a simple and clinically useful tool for dentinal fluid collection is currently not available. In the first trial on the levels of matrix metalloproteinase-9 (MMP-9), an enzyme associated with pulpal breakdown,[7] a folded polyvinylidene fluoride (PVDF) filter membrane was applied to exposed dentin for dentinal fluid collection. This was based on the excellent protein release from PVDF.[8] However, the first clinical study using this approach showed that the target protein was below the detection limit in the majority of the samples, also those that should contain it. It was concluded that better vehicles for dentinal fluid collection must be identified to increase the yield of target molecules before further studies can be performed along this line.

It was the aim of the current investigation to test the hypothesis that a material with higher water absorption than PVDF could increase the yield of target molecules from exposed dentin. In standard laboratory tests, cellulose filter membranes of similar thickness and different pore size were compared to the PVDF membrane used in the first clinical trial[4] regarding their water absorption and protein release. Subsequently, the cellulose membrane with the most favorable values was compared to the PVDF counterpart in a randomized clinical trial. The outcome measure was the yield of a metalloproteinase from exposed dentin of clinically healthy teeth in need of restoration replacement. The hypothesis tested here was that cellulose membranes absorb more water than PVDF counterparts, and thus could increase the yield of diagnostically relevant molecules.

2. Materials and methods

2.1 Filter membranes under investigation

Three types of cellulose membranes were compared. They were Sartorius 1288, 1289, and 1290 (Sartorius Stedim Biotech GmbH, Göttingen, Germany). These filter membranes were available as sheets, which were required for the water absorption test (below). They had a standard thickness of 0.21 mm and a pore size of 12 – 15 µm (1288), 8-12 µm (1289), and 3 – 5 µm (1290). The PVDF membrane under investigation (Merck Millipore, Billerica, MA, USA) had a thickness of 0.125 mm and a pore size of 0.45 µm.

2.2 Water absorption

For this test, the method according to Klemm was used (DIN 53 106). In brief, the filter membranes were cut to a width of 1.5 cm and a length of 10 cm. One end of these strips was attached to a custom-made holder. For measurements, the holder was placed over a glass container filled with deionized water, so that the membrane strips were immersed 2 mm below the water surface. The height of the water rise was marked with a sharp pencil on the membranes after 30 sec, 60 sec, and 120 sec of immersion. This test was performed three times per material.

2.3 Protein release

For this test and the subsequent trial, round filter membranes with a diameter of 8 mm were used. Using a micro precision pipette (Gilson, Middleton, UK), 5 µL of a 2 mg/mL bovine serum albumin (BSA) solution (Pierce, Rockford, USA) was blotted on the membranes. Subsequently, membranes were transferred to microcentrifuge tubes (Eppendorf, Hamburg, Germany) containing 1 mL of sterile distilled water. The tubes were vortexed for 5 sec. Subsequently, each membrane was transferred to a new microcentrifuge tube and again washed in 1 mL of distilled water as described. The procedure was repeated once more for a total of three washing steps. Protein recovery was measured using a commercially available protein assay (BCA Protein Assay Kit, Pierce) against a BSA standard curve. The absorbance at 562 nm was measured using a microplate reader (Epoch, BioTek, Luzern, Switzerland). Measurements were corrected for blank reading (mere membrane without protein processed as described).

2.4 Clinical trial

This trial was approved by the institutional review board. The patients participating did so by giving informed written consent.

Sartorius 1288 filter membranes were compared to Millipore PVDF counterparts for their capacity to collect a metalloproteinase from exposed dentin of clinically healthy teeth in need of filling replacement. MMP-2 was chosen as the target molecule, because this enzyme is involved in normal tissue turnover and thus present in healthy pulp tissue.[7] The membranes were folded to form a cone as described.[4] They were kept in micro centrifuge tubes with low protein binding (Life Technologies, Carlsbad, CA, USA). The membranes and tubes were sterilized in ethylene oxide (3M Sterigas, Two Harbors, MN, USA).

Thirteen lateral teeth from 8 patients (6 males and 2 females, aged 29 to 56) were selected for this study. These teeth reacted normal to cold test, and showed no signs of caries or inflammation. They required replacement of a filling for prosthetic or esthetic reasons. Teeth were treated under local anesthesia and rubber dam isolation. The occlusal aspect of the filling reaching into the dentin was removed first, i.e. the inter-proximal aspects of the fillings were left in place, to exclude contamination with crevicular fluid. A figure depicting this procedure can be found in an earlier publication.[4] Diamond-coated burs were used in a high-speed hand piece under water-cooling to remove the old filling. When the dentin was exposed, a coin was flipped to determine which material to use first. The cavity was dried with compressed air. Thereafter, the first folded membrane was applied to the exposed dentin using sterile cotton pliers. The membrane was kept in place for 30 sec. Subsequently, and without any intermediate procedure, the second membrane of the alternative material (as defined by the randomization process) was applied for 30 sec. Membranes were immediately transferred to their microcentrifuge tubes, which were kept on ice. These tubes were then transferred to a freezer and kept at -20°C until further analysis.

On the day of analysis, the samples were eluted in 330 µL of sterile phosphate buffered saline (PBS, pH 7.2) by centrifuging at 2000xg for 30 min at 4˚C. The levels of MMP-2 in these samples were measured by a commercially available specific enzyme-linked immunosorbent assay (ELISA) kit, according to the manufacturer's instructions (Duoset, R&D Systems, Abingdon, UK). The absorbance at 450 nm was assessed using a microplate reader (Epoch), with a wavelength correction set at 570

nm to subtract background. A standard curve was generated using a four-parameter logistic curve fit for each set of samples assayed. The concentrations of MMP-2 were expressed as ng/mL/30 sec of collection. The sensitivity of this assay, defined as the lowest detectable protein concentration that could be differentiated from zero, was 1.25 ng/mL.

2.5 Statistics

Values relating to water absorption were analyzed using two-way repeated measures ANOVA to consider the impact of time and membrane type on water rise. Water rise at each time point and protein release were compared between materials using one-way ANOVA followed by Tukey's HSD test. The frequency of detection of MMP-2 in the clinical trial between materials was compared using Fisher's exact test. MMP-2 data from the clinical trial was skewed (Shapiro-Wilk test) and thus compared between groups using Wilcoxon Signed Rank test. The alpha-type error was set at 5% ($P < 0.05$).

3. Results

In the analysis of the values generated in the water absorption test according to Klemm, two-way ANOVA showed that both time and membrane material had a significant ($P < 0.05$) impact on water rise. Cellulose membranes took up water faster and in larger quantity than the PVDF material under investigation (Table 1). The cellulose materials with the larger pore size showed higher water absorption than the filter membrane with the small pore size.

Protein release was statistically similar ($P > 0.05$) between the cellulose materials with the large pore size (Sartorius 1288 and 1289) and the PVDF membrane. After 3 wash steps, 1288 released 93 ± 9% of the BSA, 1289 released 96 ± 3%, and the PVDF membrane 101 ± 4%. The cellulose material with the small pore size (1290) released significantly ($P < 0.05$) less protein, 81 ± 4%.

In the clinical trial, the cellulose material that performed most favorably in the laboratory screening (1288) was compared to the PVDF filter membrane regarding the detection of MMP-2 from exposed dentin of clinically healthy teeth with vital pulps. Round membranes of 8 mm diameter were folded to form tapered cones. The tip of the cone was pressed against the exposed dentin using cotton pliers. The materials were applied to the exposed dentin in random sequence during the trial, 1 membrane per material and tooth. With cellulose, MMP-2 could be detected in 9 of 13 cases, compared to 1 of 13 with PVDF ($P < 0.05$). Consequently, the levels of MMP-2 were significantly higher ($P < 0.05$) when a cellulose membrane was used to collect dentinal fluid (Fig. 1). MMP-levels were statistically similar ($P > 0.05$) between first and second collections, indicating no influence of whether a membrane material was used first or second on a specific tooth (Fig. 2).

4. Discussion

The current study showed that cellulose of large pore size showed clear advantages over PVDF in dentinal fluid collection. This was attributed to the better water absorption of cellulose compared to PVDF.

The clinical part of this study was limited by the fact that only clinically healthy teeth were included. This was done to assess the possibility to consistently collect steady-state molecules present in pulpal interstitial fluid. While the cellulose membrane under investigation performed clearly better than the PVDF membrane that was used in a first trial,[4] the target molecule was still not detected in 30% of the cases. This could be related to differences in dentin microstructure between the teeth under investigation.[9] It may be so that dentinal fluid collection is simply not possible in all teeth, especially those with complete sclerosis of dentin.[10] However, the repeated collection with the 2 different materials yielded similar amounts of the target molecule (Fig. 2). This would suggest that in future trials, at least 2 sampling procedures should be performed per tooth in order to increase fluid yield. Alternatively, the membrane used for collection could also be left in place for longer periods of time. As suggested by the *in vitro* part of the current communication, exposing a filter membrane to water for 1 min instead of 30 sec may lead to better water uptake. A further limitation of this study was the low number of teeth under investigation. However, we merely compared 2 methods of dentinal fluid collection in healthy teeth. Based on the in vitro results, a difference between the 2 methods of more than 50% in absolute MMP-2 levels was expected. As indicated by the highly significant difference between the 2 methods, this assumption was correct, and the number of samples was adequate.

Absorbency of the vehicles used for dentinal fluid collection may play a role because the natural flow of fluid through dentin is rather low.[5] It may be so that the cellulose membrane actively soaked more fluid from the dentin wound than the PVDF membrane did. Dentinal fluid flow is related to pulpal pressure.[11] This pressure might vary based on the inflammatory state of the underlying pulp.[12] However, even necrotic teeth contain dentinal fluid.[13] Using the cellulose membrane technique described here, we were able to collect MMPs from teeth with necrotic teeth also (data not shown). Consequently, absorption of fluid through dentin by cellulose may be necessary to get maximum yields regardless of the condition of the pulp. This, however, needs to be investigated in future trials.

In the current study, a simple ELISA assay was used. In the first trial on MMP-9 levels in the dentinal fluid of healthy and inflamed pulps, a more sensitive, yet also more complex assay was employed, which detects the target molecule indirectly via substrate turnover.[4] That assay detected MMP-9, a molecule related to pulpal tissue breakdown, in the dentinal fluid of 7 out of 16 teeth diagnosed with irreversible pulpitis, compared to 0 of the 12 healthy control teeth. That first study should now be repeated to investigate whether the use of a cellulose material for dentinal fluid collection would allow for sample analysis with straightforward ELISA kits, or whether more sensitive assays are still required. However, even though the kit used here is easy to use, the current approach to analyze dentinal fluid would still be far away from chair-side practicability in a dental office setting.

As the field of molecular diagnostics of the pulp is relatively new, basic studies such as the current investigation are necessary to identify ideal and simple tools for the collection of dentinal fluid. Dentinal fluid could, for a long time, only be collected from extracted teeth, e.g. by heating the dentin or by centrifugation.[14, 15] In the original study on dentinal fluid analysis of vital human teeth, phosphate-buffered saline was placed in the cavity over the exposed dentin for 15 min and then collected using micropipettes.[6] Similar approaches were also used to study dentinal fluid flux in dog teeth.[5, 11] These collection methods, however, are impracticable in the clinical situation. The current research aimed towards the development a chair-side assay that could reflect the health of pulp before a tooth is restored. Whether this goal can ever be achieved is not yet clear. Nevertheless, current knowledge on pulp biology and sensitive molecular assays could prove to be useful in this context. It is beyond doubt that pulpal breakdown is triggered by bacterial invasion of dentin,[16] yet propagated by enzymes secreted by polymorphonuclear leukocytes (PMNs).[17] The idea to use PMN-associated enzymes as markers for pulpal health was formulated, yet not tested, more than 20 years ago.[5] In this context, the MMP system of enzymes is interesting. Some of these enzymes are produced by resident cells.[18] They are related to normal tissue turnover, and are thus not elevated or even less active in inflamed pulps compared to normal counterparts.[7] On the other hand, PMN-related enzymes such as MMP-9 (also called neutrophil gelatinase), are strongly correlated to pulpal inflammation and breakdown.[7] It may thus be suggested to use the ratio between MMP-9 and MMP-2 in future clinical trials to relate dentinal fluid composition to the state of the pulp. It must be cautioned, however, that MMPs are also contained

in the dentin itself, and can be released by caries-related acids.[19, 20] Furthermore, proteins of varying size and structure might be retained differently during their passage through the pulp-dentin complex.[5] It may thus be wise to investigate the levels of different proteins and their relation to different conditions of the pulp to identify the most robust markers. In summary, much work lays ahead to further develop dentinal fluid-based diagnostics.

5. Conclusion

The identification and development of better vehicles for dentinal fluid analysis is essential for the propagation of non-invasive molecular diagnostics of the dental pulp. This research can be seen as a first step into that direction. Future clinical trials should now reveal whether a cellulose membrane is a suitable collection tool to consistently identify marker molecules related to pulpal health and disease.

Conflict of interest

The authors declare that they have no conflict of interest

REFERENCES

1. Mejare IA, Axelsson S, Davidson T *et al.* Diagnosis of the condition of the dental pulp: a systematic review. *Int Endod J* 2012;**45:**597-613.

2. Tan K, Pjetursson BE, Lang NP, Chan ES. A systematic review of the survival and complication rates of fixed partial dentures (FPDs) after an observation period of at least 5 years. *Clin Oral Implants Res* 2004;**15:**654-66.

3. Pjetursson BE, Sailer I, Zwahlen M, Hämmerle CH. A systematic review of the survival and complication rates of all-ceramic and metal-ceramic reconstructions after an observation period of at least 3 years. Part I: Single crowns. *Clin Oral Implants Res* 2007;**18 Suppl 3:**73-85.

4. Zehnder M, Wegehaupt FJ, Attin T. A first study on the usefulness of matrix metalloproteinase 9 from dentinal fluid to indicate pulp inflammation. *J Endod* 2011;**37:**17-20.

5. Maita E, Simpson MD, Tao L, Pashley DH. Fluid and protein flux across the pulpodentine complex of the dog in vivo. *Arch Oral Biol* 1991;**36:**103-10.

6. Knutsson G, Jontell M, Bergenholtz G. Determination of plasma proteins in dentinal fluid from cavities prepared in healthy young human teeth. *Arch Oral Biol* 1994;**39:**185-90.

7. Gusman H, Santana RB, Zehnder M. Matrix metalloproteinase levels and gelatinolytic activity in clinically healthy and inflamed human dental pulps. *Eur J Oral Sci* 2002;**110:**353-7.

8. Yao Y, Grogan J, Zehnder M *et al.* Compositional analysis of human acquired enamel pellicle by mass spectrometry. *Arch Oral Biol* 2001;**46:**293-303.

9. Bergenholtz G. Pathogenic mechanisms in pulpal disease. *J Endod* 1990;**16:**98-101.

10. Vasiliadis L, Darling AI, Levers BG. The amount and distribution of sclerotic human root dentine. *Arch Oral Biol* 1983;**28:**645-9.

11. Pashley DH, Nelson R, Pashley EL. In-vivo fluid movement across dentine in the dog. *Arch Oral Biol* 1981;**26:**707-10.

12. Heyeraas KJ, Kvinnsland I. Tissue pressure and blood flow in pulpal inflammation. *Proc Finn Dent Soc* 1992;**88 Suppl 1:**393-401.

13. Papa J, Cain C, Messer HH. Moisture content of vital vs endodontically treated teeth. *Endod Dent Traumatol* 1994;**10:**91-3.

14. Spreter von Kreudenstein T, Stüben J. Dentinstoffwechselstudien. III. Die thermische Methode zum Nachweis des Dentinliquors. *Dtsch Zahnarztl Z* 1955;**10:**1178-82.

15. Coffey CT, Ingram MJ, Björndal AM. Analysis of human dentinal fluid. *Oral Surg Oral Med Oral Pathol* 1970;**30:**835-7.

16. Reeves R, Stanley HR. The relationship of bacterial penetration and pulpal pathosis in carious teeth. *Oral Surg Oral Med Oral Pathol* 1966;**22:**59-65.

17. Rauschenberger CR, McClanahan SB, Pederson ED, Turner DW, Kaminski EJ. Comparison of human polymorphonuclear neutrophil elastase, polymorphonuclear neutrophil cathepsin-G, and alpha 2-macroglobulin levels in healthy and inflamed dental pulps. *J Endod* 1994;**20:**546-50.

18. Reynolds JJ, Hembry RM, Meikle MC. Connective tissue degradation in health and periodontal disease and the roles of matrix metalloproteinases and their natural inhibitors. *Adv Dent Res* 1994;**8:**312-9.

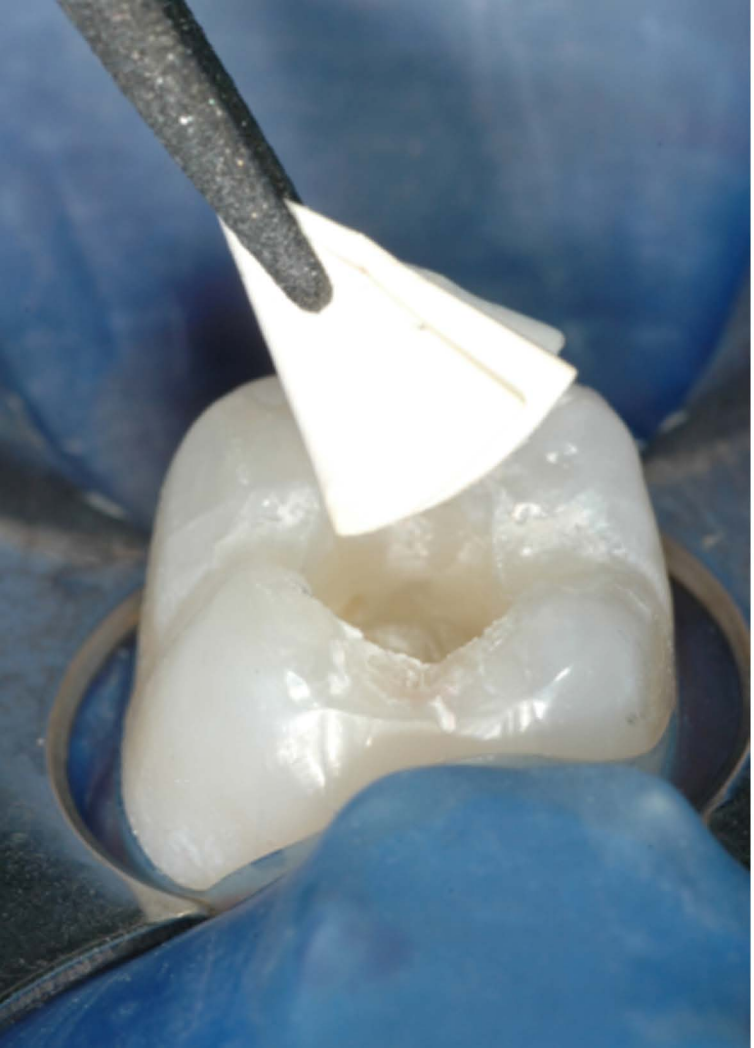
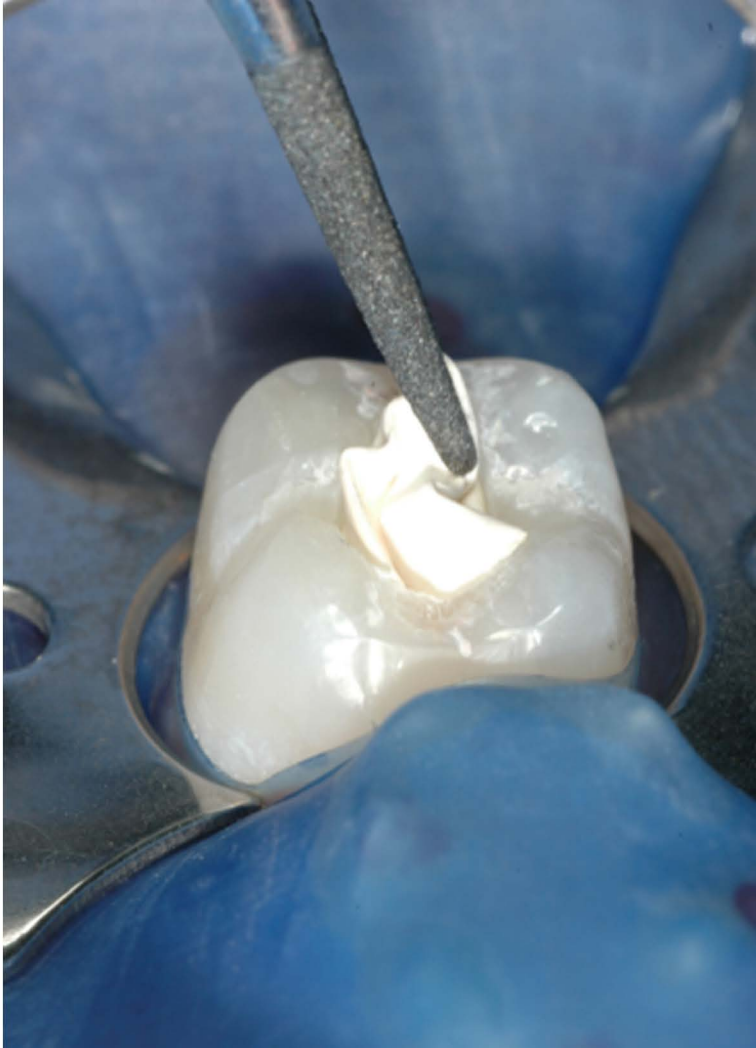
19. Tjäderhane L, Larjava H, Sorsa T, Uitto VJ, Larmas M, Salo T. The activation and function of host matrix metalloproteinases in dentin matrix breakdown in caries lesions. *J Dent Res* 1998;**77:**1622-9.

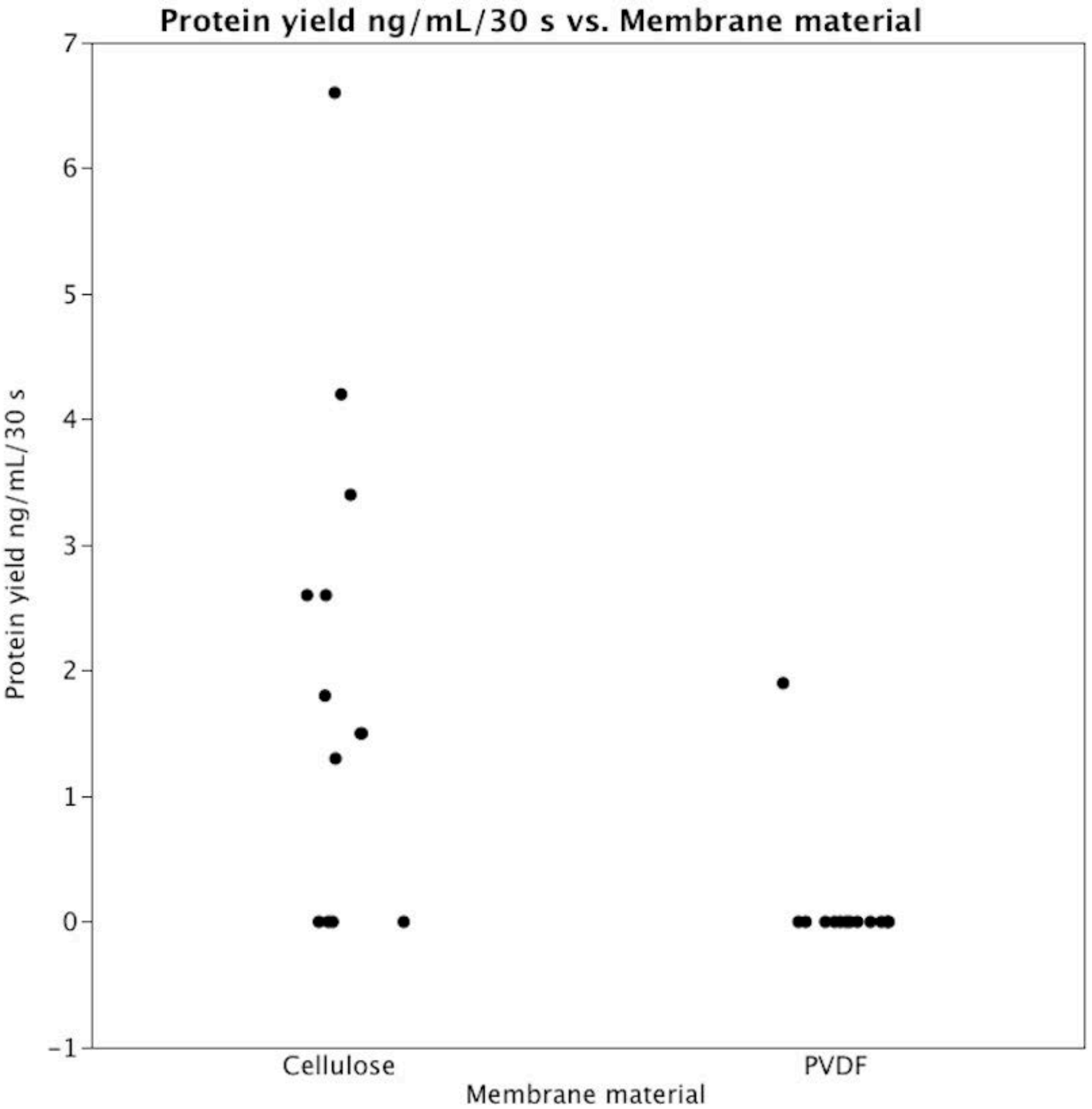
20. Chaussain-Miller C, Fioretti F, Goldberg M, Menashi S. The role of matrix metalloproteinases (MMPs) in human caries. *J Dent Res* 2006;**85:**22-32.

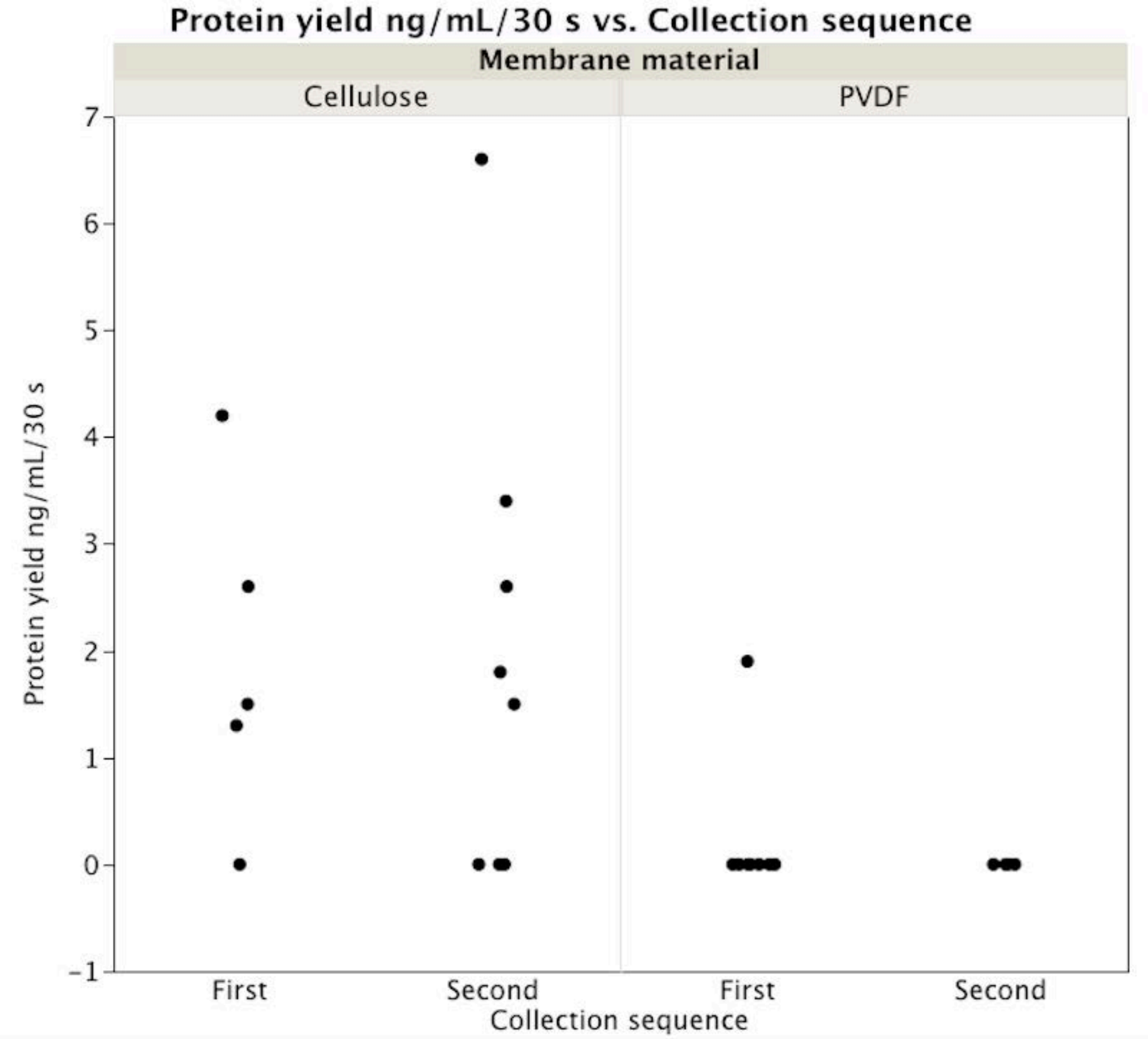
Figure Captions

Fig.1 – Dot plot depicting the MMP-2 yields from the two different filter membranes under investigation. Materials were applied to exposed dentin for 30 sec. There was a significant difference in MMP-2 yields between the two materials under investigation (Wilcoxon Signed Rank test, *P* = 0.0023).

Fig. 2 – Dot plot relating MMP-2 yields to the sequence the filter membranes were applied in the clinical trial. There was no difference in yield whether a membrane was applied first or second (Wilcoxon Signed Rank test, *P* > 0.05).


Protein yield ng/mL/30 s vs. Membrane material
Protein yield ng/mL/30 s
7
6
5
4
3
2
1
0
-1
Cellulose
PVDF
Membrane material


Protein yield ng/mL/30 s vs. Collection sequence
Membrane material
Cellulose
PVDF
Protein yield ng/mL/30 s
7
6
5
4
3
2
1
0
-1
First
Second
First
Second
Collection sequence

Height of water rise at different times in mm (Klemm method)			
Membrane	30 s	60 s	120 s
Sartorius 1288	21.7 ± 1.2 [A]	31.0 ± 1.0 [A]	37.7 ± 0.6 [A]
Sartorius 1289	20.0 ± 1.0 [A]	31.0 ± 4.4 [A]	37.7 ± 0.6 [A]
Sartorius 1290	14.0 ± 1.7 [B]	17.7 ± 2.1 [B]	20.0 ± 0.0 [B]
Millipore PVDF	9.0 ± 0.0 [C]	13.0 ± 0.0 [C]	20.0 ± 0.0 [B]

Values indicate means and standard deviations of triplicates. Two-way repeated measures ANOVA showed that both time and membrane had a significant ($P < 0.05$) impact on water rise. Data sets sharing a superscript letter did not differ significantly at a given point in time (one-way ANOVA, Tukey HSD)