

1 **Antioxidant properties of amniotic membrane: novel observations from a pilot**  
2 **study**

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18

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22

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26 **Antioxidant properties of amniotic membrane: novel observations from a pilot**  
27 **study**

28 **Abstract:**

29 **Introduction**

30 Amniotic membrane (AM) is used to manage various debilitated ocular surface  
31 conditions. The impact of oxidative stress and free radicals on the ocular surface is  
32 increasingly being recognised. Hyaluronic acid (HA) has anti-inflammatory  
33 properties and is abundantly present in AM. In this *in vitro* pilot study we investigated  
34 AM's potential for intrinsic free radical scavenging properties.

35

36 **Methods**

37 Strips of AM were incubated in sealed tubes with hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). After  
38 being sonicated, uptake of reactive oxygen species (ROS) was measured by the  
39 Amplex Red Hydrogen Peroxide/Peroxidase assay. 1630kDA HA was used for  
40 comparison.

41

42 **Results**

43 There was uptake of ROS by all AMs samples, which decreased with increasing  
44 concentrations of H<sub>2</sub>O<sub>2</sub>. Mean ROS uptake for 5 different AMs at 1 hour was  
45 significantly greater for 50uM (83%; SD 11.7, SEM 5.23) compared to 100uM (67%;  
46 SD 20.48, SEM 9.16; p=0.028, 95% CI (2.8,29.2)). The HA comparison group  
47 showed similar uptake and trend.

48

49 **Conclusion**

50 This pilot study demonstrates that AM is able to remove ROS from its' environment.  
51 Demonstrating total antioxidant capacity in AM provides evidence for use as a free  
52 radical scavenger. The antioxidant properties of AM and the contribution from HA  
53 require more research.

54

55 Word Count: 195

56

57 **Introduction**

58 Oxidative stress is increasingly being recognised as the common inflammatory  
59 cellular pathway in ocular surface disease.<sup>1,2</sup> It is the result of the imbalance between  
60 total antioxidant capacity and reactive oxygen species.<sup>3</sup> The healthy eye has a variety  
61 of protective antioxidant defences, including the constituents of the normal tear film.<sup>1</sup>  
62 It follows that any chronic ocular surface injury can exhaust these protective defences  
63 and cause local free radical damage. For example, glutathione has been shown to be  
64 depleted in the tear film of patients with keratoconus.<sup>4</sup> Oxidative stress can happen at  
65 both an exogenous and endogenous level to the cornea, and has recently been  
66 described in pterygia, corneal dystrophies, dry eyes, trauma, a host of inflammatory  
67 conditions and chemical injuries.<sup>2,5,6</sup>

68

69 Amniotic membrane (AM) is often used to reconstruct the debilitated ocular surface,  
70 including after chemical injury.<sup>7-9</sup> It has been shown to facilitate epithelial healing and  
71 analgesia, when used either as a patch dressing, or in extract form, as a suspension or  
72 drops.<sup>10-12</sup> However, the reported benefits of AM in chemical injury have not always  
73 being replicated in other studies.<sup>13-17</sup>

74

75 Hyaluronic acid (HA), a multifunctional glycosaminoglycan and component of extra  
76 cellular matrices, has been shown to be abundantly present in AM, and a recent study  
77 demonstrated a covalent complex of heavy chain-hyaluronic acid (HC-HA) as the  
78 active component responsible, in part, for anti-inflammatory and anti-scarring  
79 actions.<sup>18</sup> HA in the AM stroma has been shown to play a role in entrapping  
80 inflammatory cells, so reducing further damage to ocular tissue.<sup>19</sup> Studies have shown  
81 that high and low molecular weight HA have different biological effects. For  
82 example, high molecular weight HA has been shown to be anti-inflammatory, and can  
83 protect the cornea from oxidative stress associated with preservatives in ophthalmic  
84 preparations (such as BAK and EDTA) and UV-related free radical damage.<sup>20-27</sup>  
85 Alternatively, low molecular weight HA has been shown to be generated by oxidative  
86 fragmentation (such as due to peroxide) and to accumulate with inflammation.<sup>28-30</sup>

87

88 Total antioxidant capacity has previously been described in amniotic fluid.<sup>31</sup> We  
89 wondered if the same was true of AM and hypothesised that some of the benefits of  
90 AM may be due to intrinsic free radical scavenging antioxidant properties. In this *in*

91 *vitro* pilot study AM was exposed to various concentrations of hydrogen peroxide (as  
92 an exogenous source of free radicals). HA was used as a control group to compare the  
93 magnitude of any uptake of H<sub>2</sub>O<sub>2</sub>.  
94

95 **Methods:**

96 This study was approved as part of a non-substantial amendment to utilise surplus  
97 AM tissue from a previous project by the West of Scotland Ethics Committee and  
98 Research and Development Office. [See R&D Ref: WN08OP219; Ethics Ref:  
99 08/S0709/98] Briefly, AM was collected from human placentas delivered after  
100 caesarean section following written informed consent. The placentas were thoroughly  
101 rinsed with balanced salt solution containing streptomycin, penicillin, neomycin and  
102 amphotericin. The amnion was separated from the chorion by blunt dissection, cut  
103 into one square inch pieces and stored at -80°C in a 50/50 mixture of glycerol and  
104 Roswell Park Memorial Institute medium supplemented with 10% FCS, penicillin-  
105 streptomycin and L-glutamine (Invitrogen, Paisley, UK).

106

107 Prior to use, AM samples which had been stored at -80°C were defrosted and washed  
108 4 times with phosphate buffered saline (PBS). 1cm by 0.5cm strips of AM were cut  
109 and used throughout. The AM strips were incubated in the dark at 37°C with 300uL of  
110 H<sub>2</sub>O<sub>2</sub>. The tissue was incubated in sealed tubes to prevent evaporation. Incubation  
111 times were for 1 hour at 15 minute intervals. The H<sub>2</sub>O<sub>2</sub> solutions were freshly  
112 prepared from a 30% stock solution (Sigma).

113

114 Control tubes with no AM were included at each concentration of H<sub>2</sub>O<sub>2</sub> for every  
115 assay. There was no change in the control values during the experiment indicating that  
116 there had been no degradation or evaporation of the peroxide. This value was  
117 considered to be the 'initial concentration' in the subsequent calculations of uptake.

118

119 After incubation, the tissues were sonicated in an MSE sonicator for 1 minute in  
120 300uL PBS/0.5% Triton X and spun in a microfuge for 10 minutes at 4000rpm. The  
121 supernatant was evaluated for Reactive oxygen species (ROS) by the Amplex Red  
122 Hydrogen Peroxide/Peroxidase method (Invitrogen, Paisley, UK). This procedure was  
123 done in duplicate for all tested measurements. The uptake was calculated by  
124 subtracting the concentration of H<sub>2</sub>O<sub>2</sub> left in the supernatant from the initial  
125 concentration.

126

127

128 The Amplex Red Hydrogen Peroxide/Peroxidase Assay is a sensitive, one-step assay  
129 that uses the Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine) to detect  
130 hydrogen peroxide or peroxidase activity. It has been used to detect H<sub>2</sub>O<sub>2</sub> released  
131 from biological samples, enzyme-coupled reactions and is ultrasensitive even when  
132 H<sub>2</sub>O<sub>2</sub> is in excess. In the presence of peroxidase, the Amplex Red reagent reacts with  
133 H<sub>2</sub>O<sub>2</sub> in a 1:1 stoichiometry to produce the red-fluorescent oxidation product,  
134 resorufin. This reaction has been used to detect as little as 10 picomoles of H<sub>2</sub>O<sub>2</sub> in a  
135 100 µL volume. [For more information, see [www.invitrogen.com](http://www.invitrogen.com)]

136

137 The differences in uptake at different concentrations of H<sub>2</sub>O<sub>2</sub> were tested statistically  
138 using a paired t-test in Minitab (version 15).

139

140 We wanted to work with small volumes of AM and H<sub>2</sub>O<sub>2</sub> to make it clinically relevant  
141 to the ocular tissues. In light of this, we chose to standardise our samples to 1cm by  
142 0.5cm strips. By weighing several pieces of AM on standard laboratory scales, we  
143 found that our small samples had an average weight of 4.5mg. We then wished to  
144 estimate the concentration of hyaluronic acid in our small strips. Published studies  
145 have quoted a concentration of 0.45mg of hyaluronic acid per gram on wet AM  
146 tissue.<sup>18</sup> We were then able to calculate that the hyaluronic acid content was  
147 approximately 2µg per strip. Hyaluronic acid Streptococcus equi. 1630 kDa (Sigma  
148 Cat. No. 53747) was chosen as the comparative equivalent as AM predominantly  
149 contains high molecular weight long chain hyaluronic acid. Using the conditions  
150 described above, 2µg hyaluronic acid was incubated with various concentrations of  
151 H<sub>2</sub>O<sub>2</sub> and resultant uptake was calculated. In the same assay 3 different AM samples  
152 replaced hyaluronic acid so that comparison of any uptake could be made.

153

154

155 **Results:**

156 There was uptake of ROS by the AM for all tested H<sub>2</sub>O<sub>2</sub> concentrations within the  
157 first hour. The level of uptake decreased with increasing concentrations of H<sub>2</sub>O<sub>2</sub>. To  
158 illustrate this effect, samples from one individual AM consistently had 70% removal  
159 of ROS for 50uM H<sub>2</sub>O<sub>2</sub>, 45% removal for 100uM and 18% removal for 200uM after  
160 incubation for 1 hour.

161

162 One hour analysis of 7 AM samples at 50uM had a mean uptake of 82.6% (minimum  
163 70%, median 82%, maximum 100%, SD 9.8). One hour analysis of 5 AM samples at  
164 100uM had a mean uptake of 67% (minimum 45%, median 67%, maximum 95%, SD  
165 20.48). When we compared matched samples from 5 different AMs, mean ROS  
166 uptake at 1 hour was significantly greater at 83% (SD 11.7, SEM 5.23) for 50uM  
167 compared to 67% (SD 20.48, SEM 9.16) for 100uM (p=0.028, 95% CI (2.8,29.2)).  
168 The mean difference was 16% (based on the 5 paired data values for analysis).

169 [Figure 1]

170

171 Figure 2 illustrates the percentage uptake in two individual AM samples within the  
172 first hour of exposure. For 50uM H<sub>2</sub>O<sub>2</sub> there was an average 55% uptake at 15  
173 minutes, 80% uptake at 30 minutes and 91% uptake at 60 minutes. For 100uM H<sub>2</sub>O<sub>2</sub>  
174 uptake at 15 minutes was 65%, at 30 minutes uptake was 87% and at 60 minutes it  
175 was 86%. These results provide further confirmation of uptake, but also demonstrate  
176 biological variability even within the same AM.

177

178 In the hyaluronic acid comparison group, the uptake of H<sub>2</sub>O<sub>2</sub> at 1 hour for the 50uM  
179 and 100uM concentrations was in the same range as for 3 different AMs sampled.  
180 Average uptake at 50uM H<sub>2</sub>O<sub>2</sub> showed a trend towards higher uptake than at 100uM  
181 H<sub>2</sub>O<sub>2</sub> (53% uptake vs 50.9% uptake). This was lower than the uptake measured in the  
182 AMs (53% vs 69.9% for 50uM H<sub>2</sub>O<sub>2</sub> and 50.9% vs 68.3% for 100uM H<sub>2</sub>O<sub>2</sub>). The  
183 sample size (n=3) was too small to perform meaningful statistical analysis, but did act  
184 as an appropriate comparison group for the magnitude of the uptake. [Table 1]

185

186 The accuracy of all these results was confirmed by duplication of testing and also by  
187 testing sealed controls which contained equivalent H<sub>2</sub>O<sub>2</sub> without presence of AM.

188 There was no reduction of H<sub>2</sub>O<sub>2</sub> in these controls.

189 **Discussion:**

190 Studies have demonstrated that AM suppresses myofibroblastic differentiation,  
191 suppresses matrix metalloproteinase expression in the stroma, and can modulate the  
192 immune response by absorbing live inflammatory and immune cells into its' stroma  
193 and render them into apoptosis.<sup>32-35</sup> Rabbit model studies have evaluated the use of an  
194 immediate AM patch following an alkali wound and have demonstrated promotion of  
195 wound healing by inhibiting both proteinase activity and polymorphonuclear  
196 leucocyte infiltration.<sup>36</sup>

197

198 We have demonstrated uptake of peroxide by all samples of AM, which increased  
199 over time, but appeared to plateau at 1 hour. The uptake was less at the greater  
200 concentrations, and was statistically significant between the 50uM and 100uM at one  
201 hour for 5 different AMs. This could point to a saturation effect, where the tissue's  
202 ability to absorb the peroxide is overwhelmed by the higher concentrations of free  
203 radicals. If the antioxidant capacity of the AM was depleted by injurious agents, then  
204 the resultant oxidative stress would continue in the clinical setting, leading to further  
205 cellular damage. This overwhelming saturation would render the AM ineffective, and  
206 could contribute in part to the reported variability and failure of AM  
207 transplantation.<sup>13,16,17</sup> The uptake in the HA comparison group was of a similar  
208 magnitude to the AM group. However, the average results suggest that AM had a  
209 greater potency for removing H<sub>2</sub>O<sub>2</sub> than HA alone.

210

211 **Free radicals and the cornea**

212 Our pilot study suggests that AM is able to scavenge reactive oxygen species. This  
213 total antioxidant capacity has been previously described in the evaluation of amniotic  
214 fluid.<sup>31</sup> The antioxidant capacity of AM may be an additional mode of action for the  
215 surgeon to utilise as they seek to reconstruct the debilitated ocular surface. Free  
216 radical damage is increasingly being identified as a cellular component of corneal  
217 disease.<sup>37</sup> Exposure to exogenous free radicals has been shown to cause mitochondrial  
218 DNA damage in corneal epithelial cells.<sup>38</sup> Corneal fibroblasts have been shown to  
219 decline with age in response to oxidative stress. Through measuring antioxidant  
220 enzymes in primary cultured corneal fibroblasts from patients and healthy subjects,  
221 recent research has implicated oxidative damage induced by decreased catalase  
222 expression as a causative factor in the pathogenesis of corneal dystrophies.<sup>39</sup>



223 Oxidative stress has also been shown to keep Pax6 in a chronic wound state, and the  
224 effect on subcellular localisation, signalling and gene dosage effect contributes to  
225 aniridia-related keratopathy.<sup>40</sup> Oxidative stress can also be exogenous, and the source  
226 could be due to external factors such as surface toxicity from multiple medications.  
227 Intrinsic free radical presence has been reported in topical and intracameral  
228 ophthalmic preparations, independent of preservatives or pH.<sup>41-43</sup> Low grade chronic  
229 oxidative stress could explain residual inflammation in vulnerable ocular surfaces  
230 even when using long term unpreserved medications.

231

232 An increased awareness of the role of free radicals in corneal disease may lead to  
233 future treatment strategies using antioxidant agents. For example, HA has been shown  
234 to decrease oxidative DNA damage induced by EDTA and BAK in human corneal  
235 epithelial cells.<sup>20,21</sup> Antioxidant capacity could be an additional benefit to those  
236 described with early intervention with AM in the acute stages of an ocular chemical  
237 injury.<sup>44,45</sup> Topical and oral vitamin C is already used for its' antioxidant properties in  
238 this scenario. This relationship between oxidative stress and antioxidant protection is  
239 already being actively explored in anterior segment diseases such as glaucoma,  
240 cataract and posterior segment disease such as age-related macular degeneration.<sup>46-48</sup>

241

#### 242 **Limitations:**

243 We acknowledge that this was a pilot study with small numbers which could affect  
244 the statistical analysis of our results. However, our original premise was to perform a  
245 proof of principle study to evaluate if AM had antioxidant capacity. Variability in our  
246 results may have been due to effect of storage and processing of the samples. This  
247 clinical concern has been raised previously in the literature, and is the motivation for  
248 the development of a reproducible biosynthetic amniotic membrane which retains the  
249 properties of the human tissue.<sup>49</sup> Variability in AM has been suggested as the reason  
250 for failure of treatment in ocular surface reconstruction. We did not measure the  
251 breakdown products of HA, and so our study does not allow direct comparison of the  
252 uptake of peroxide between the AM and the HA. However, it does act as a reasonable  
253 control regarding the magnitude of the uptake, and does provide a basis for future  
254 research.

255

256

257 **Conclusion:**

258 This pilot study demonstrates that amniotic membrane is able to remove ROS from  
259 its' environment. Demonstrating total antioxidant capacity in amniotic membrane  
260 provides evidence for use as a free radical scavenger. An increased awareness of the  
261 role of free radicals in corneal disease may lead to treatment strategies utilising  
262 antioxidant agents derived from hyaluronic acid or amniotic membrane. The role of  
263 hyaluronic acid and the antioxidant properties of amniotic membrane require further  
264 research.

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414

415 **Legend:**

416

417 **Figure 1:**

418 Graph showing spread of percentage uptake of hydrogen peroxide by 5 different  
419 amniotic membranes at 1 hour, consistently demonstrating decreased uptake with  
420 stronger concentrations. Mean ROS uptake for 5 AMs at 1 hour was significantly  
421 greater for 50uM (83%) compared to 100uM (67%,  $p=0.028$ ).

422

423 **Figure 2:**

424 Graph showing average percentage uptake of hydrogen peroxide by 2 different  
425 amniotic membranes at 15 minute intervals in the first hour of exposure,  
426 demonstrating variability within individual membranes, and a plateau effect by 1  
427 hour.

428

429 **Table 1:**

430 Comparison table of hyaluronic acid group results demonstrating similar, but  
431 increased uptake of ROS with different 3 AMs compared to hyaluronic acid (HA) at 1  
432 hour.