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

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Abstract:

Introduction

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

Methods

Strips of AM were incubated in sealed tubes with hydrogen peroxide (H_2O_2). After being sonicated, uptake of reactive oxygen species (ROS) was measured by the Amplex Red Hydrogen Peroxide/Peroxidase assay. 1630kDA HA was used for comparison.

Results

There was uptake of ROS by all AMs samples, which decreased with increasing concentrations of H_2O_2. Mean ROS uptake for 5 different AMs at 1 hour was significantly greater for 50uM (83%; SD 11.7, SEM 5.23) compared to 100uM (67%; SD 20.48, SEM 9.16; p=0.028, 95% CI (2.8,29.2)). The HA comparison group showed similar uptake and trend.

Conclusion

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

Word Count: 195

Introduction

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

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

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

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

vitro pilot study AM was exposed to various concentrations of hydrogen peroxide (as an exogenous source of free radicals). HA was used as a control group to compare the magnitude of any uptake of H_2O_2.

Methods:

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

Prior to use, AM samples which had been stored at -80^{o}C were defrosted and washed 4 times with phosphate buffered saline (PBS). 1cm by 0.5cm strips of AM were cut and used throughout. The AM strips were incubated in the dark at 37^{o}C with 300uL of H_2O_2. The tissue was incubated in sealed tubes to prevent evaporation. Incubation times were for 1 hour at 15 minute intervals. The H_2O_2 solutions were freshly prepared from a 30% stock solution (Sigma).

Control tubes with no AM were included at each concentration of H_2O_2 for every assay. There was no change in the control values during the experiment indicating that there had been no degradation or evaporation of the peroxide. This value was considered to be the 'initial concentration' in the subsequent calculations of uptake.

After incubation, the tissues were sonicated in an MSE sonicator for 1 minute in 300uL PBS/0.5% Triton X and spun in a microfuge for 10 minutes at 4000rpm. The supernatant was evaluated for Reactive oxygen species (ROS) by the Amplex Red Hydrogen Peroxide/Peroxidase method (Invitrogen, Paisley, UK). This procedure was done in duplicate for all tested measurements. The uptake was calculated by subtracting the concentration of H_2O_2 left in the supernatant from the initial concentration.

The Amplex Red Hydrogen Peroxide/Peroxidase Assay is a sensitive, one-step assay that uses the Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine) to detect hydrogen peroxide or peroxidase activity. It has been used to detect H_2O_2 released from biological samples, enzyme-coupled reactions and is ultrasensitive even when H_2O_2 is in excess. In the presence of peroxidase, the Amplex Red reagent reacts with H_2O_2 in a 1:1 stoichiometry to produce the red-fluorescent oxidation product, resorufin. This reaction has been used to detect as little as 10 picomoles of H_2O_2 in a 100 μL volume. [For more information, see www.invitrogen.com]

The differences in uptake at different concentrations of H_2O_2 were tested statistically using a paired t-test in Minitab (version 15).

We wanted to work with small volumes of AM and H_2O_2 to make it clinically relevant to the ocular tissues. In light of this, we chose to standardise our samples to 1cm by 0.5cm strips. By weighing several pieces of AM on standard laboratory scales, we found that our small samples had an average weight of 4.5mg. We then wished to estimate the concentration of hyaluronic acid in our small strips. Published studies have quoted a concentration of 0.45mg of hyaluronic acid per gram on wet AM tissue.[18] We were then able to calculate that the hyaluronic acid content was approximately 2ug per strip. Hyaluronic acid Streptococcus equi. 1630 kDa (Sigma Cat. No. 53747) was chosen as the comparative equivalent as AM predominantly contains high molecular weight long chain hyaluronic acid. Using the conditions described above, 2ug hyaluronic acid was incubated with various concentrations of H_2O_2 and resultant uptake was calculated. In the same assay 3 different AM samples replaced hyaluronic acid so that comparison of any uptake could be made.

Results:

There was uptake of ROS by the AM for all tested H_2O_2 concentrations within the first hour. The level of uptake decreased with increasing concentrations of H_2O_2. To illustrate this effect, samples from one individual AM consistently had 70% removal of ROS for 50uM H_2O_2, 45% removal for 100uM and 18% removal for 200uM after incubation for 1 hour.

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

Figure 2 illustrates the percentage uptake in two individual AM samples within the first hour of exposure. For 50uM H_2O_2 there was an average 55% uptake at 15 minutes, 80% uptake at 30 minutes and 91% uptake at 60 minutes. For 100uM H_2O_2 uptake at 15 minutes was 65%, at 30 minutes uptake was 87% and at 60 minutes it was 86%. These results provide further confirmation of uptake, but also demonstrate biological variability even within the same AM.

In the hyaluronic acid comparison group, the uptake of H_2O_2 at 1 hour for the 50uM and 100uM concentrations was in the same range as for 3 different AMs sampled. Average uptake at 50uM H_2O_2 showed a trend towards higher uptake than at 100uM H_2O_2 (53% uptake vs 50.9% uptake). This was lower than the uptake measured in the AMs (53% vs 69.9% for 50uM H_2O_2 and 50.9% vs 68.3% for 100uM H_2O_2). The sample size (n=3) was too small to perform meaningful statistical analysis, but did act as an appropriate comparison group for the magnitude of the uptake. [Table 1]

The accuracy of all these results was confirmed by duplication of testing and also by testing sealed controls which contained equivalent H_2O_2 without presence of AM. There was no reduction of H_2O_2 in these controls.

Discussion:

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

We have demonstrated uptake of peroxide by all samples of AM, which increased over time, but appeared to plateau at 1 hour. The uptake was less at the greater concentrations, and was statistically significant between the 50uM and 100uM at one hour for 5 different AMs. This could point to a saturation effect, where the tissue's ability to absorb the peroxide is overwhelmed by the higher concentrations of free radicals. If the antioxidant capacity of the AM was depleted by injurious agents, then the resultant oxidative stress would continue in the clinical setting, leading to further cellular damage. This overwhelming saturation would render the AM ineffective, and could contribute in part to the reported variability and failure of AM transplantation.[13,16,17] The uptake in the HA comparison group was of a similar magnitude to the AM group. However, the average results suggest that AM had a greater potency for removing H_2O_2 than HA alone.

Free radicals and the cornea

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

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

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

Limitations:

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

Conclusion:

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

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Legend:

Figure 1:

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

Figure 2:

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

Table 1:

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