Magnesium Presence Prevents Removal of Nuclear-Associated Protein Antigens from Bovine Pericardium for Heart Valve Engineering

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BACKGROUND

Current heart valve prostheses are associated with significant complications including aggressive immune response, limited valve life expectancy, and inability to grow in juvenile patients.1 Animal derived “tissue” valves undergo glutaraldehyde fixation to mask tissue antigenicity, however chronic immunological responses and associated calcification still commonly occur.2 A heart valve formed from an unfixed bovine pericardium (BP) extracellular matrix (ECM) scaffold, in which antigenic burden has been eliminated or significantly reduced, has potential to overcome deficiencies of current prostheses.3,4 Our group recently developed a novel antigen removal (AR) paradigm which utilizes protein chemistry principles of sequential differential solubilization to promote protein solubility and reduce residual BP antigenicity.5

Due to the diversity in amino acid composition of tissue proteins, antigen removal solutions must be optimized in order to maximize antigenic protein solubilization and thus removal from xenogeneic tissue.6 The reported AR solution contained magnesium (a heavy metal chelator) which is known to result in precipitation of DNA and promote both inter- and intra-molecular disulphide bond formation. We wanted to understand whether the removal of magnesium from AR solutions would enhance protein solubility, specifically of nuclear-associated proteins, and thereby further reduce overall residual antigenicity.

HYPOTHESIS

We hypothesized that removal of magnesium from AR solutions will enhance protein solubility, resulting in increased removal of previously precipitated antigens, particularly those of nuclear origin, thus decreasing residual antigenicity of BP following AR (BP-AR).

METHODS

Magnesium (2 mM magnesium chloride hexahydrate) was removed from either both AR solutions (OM), only the lipophilic AR solution (ARM), or neither AR solution (AR). Each group was subjected to the hydrophilic AR solution (10 mM Tris HCl pH 8.0, 1% antibiotic antimycotic solution, 0.5 mM pefabloc, 100 mM dithiothreitol, 100 mM KCl (48 h)), then lipophilic AR solution (hydrophilic solution with addition of 1% amidosulfobetaine-14 (ASB-14)) (48 h), followed by nuclease digestion (24 h) and washout (48 h).

Manually minced BP-AR scaffolds were subjected to extraction of residual proteins using sequential 0.1% (hydrophilic extract) followed by 1% SDS (lipophilic extract) in Tris HCl (pH 8.0), 1 mM dithiothreitol, 2 mM magnesium chloride hexahydrate, 10 mM potassium chloride and 0.5 mM pefabloc. Residual hydrophilic and lipophilic extracts (n = 12 per group) were assessed for residual antigenicity using one-dimensional gel electrophoresis and western blotting, probed with anti-native BP serum and assessed for IgG positivity, quantified via densitometry, as previously reported.

Biopsy punches of BP-AR scaffolds were taken and subjected to DNA content analysis using Invitrogen PicoGreen Assay Kit and for immunohistochemistry to assess ECM morphology and nuclei counts using hematoxylin and eosin (H&E) staining. Lastly, concentration and DNA content of protein removed from BP scaffolds during each step of antigen removal was quantified using Bio-Rad DC Colormetric assay and Invitrogen PicoGreen Assay Kit respectively. Equal volumes of proteins removed into the AR buffer during each step of the AR process were also analyzed via mass spectrometry.

Means were analyzed using Student’s T-Test between AR and ARM or AR and OM, with significance defined at p<0.05.

RESULTS

Removal of magnesium from either step of the AR process had no significant effect on removal of water-soluble antigens (Fig. 1a). However, absence of magnesium from either the hydrophilic AR step (ARM) or both the lipophilic and hydrophilic steps (OM) resulted in increased removal of lipid-soluble antigens P = 0.004 (Fig. 1a). Assessment of antigen content of the nuclease digestion supernatant demonstrated that significantly more antigens were removed in the absence of magnesium P = 0.04 (Fig. 1b).

CONCLUSIONS AND FUTURE WORK

In order to improve upon the reduction of residual antigenicity achieved using our previously reported method, we considered the removal of magnesium from AR solutions, given its propensity to precipitate DNA and cause inter- and intra-molecular disulphide bonds.5

In the absence of magnesium, there is a statistically significant reduction in lipid soluble antigenicity, as well as an overall increase in DNA content washed out from BP-AR scaffolds. We also determined that more histones are removed in the absence of magnesium than in the presence. We therefore conclude that in the absence of magnesium, DNA precipitation is avoided and therefore more nuclear-associated proteins are soluble and available for removal during subsequent washout steps (Fig. 4).

Future investigations are required to confirm these findings, including DNA content analysis and mass spectrometry results for BP scaffolds. However, our results suggest that DNA removal is approaching 100% with the reported method. Additionally, future studies are necessary to determine the effect of magnesium removal on scaffold ECM structure/function properties.

REFERENCES


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