JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE 7 (1996) 691–693

Dimethyl sulfoxide as an anticalcification agent for glutaraldehyde-fixed biological tissue

E. KHOR, A. WEE*, W. K. LOKE, B. L. TAN‡
Departments of Chemistry and Pathology, National University of Singapore Kent Ridge, Singapore 0511*

Dimethylsulfoxide has been found to mitigate the calcification of glutaraldehyde-fixed biological tissue in the rat subdermal model. This effect is achieved with neat dimethylsulfoxide either prior to or after glutaraldehyde fixation of the biological tissue. The calcium levels for the 21 days post-implant for both methods are over 20 times less than the controls. However, fixation of the tissue with glutaraldehyde before dimethylsulfoxide treatment appears to better control calcification over the longer term. Histological examination of samples after exposure before or after implantation show no deleterious effects to the tissue due to dimethylsulfoxide.

1. Introduction

Pathologic calcification is acknowledged as the primary cause of failure in the present generation of bioprosthetic heart valves fabricated from glutaraldehyde-fixed bovine pericardium or porcine aortic valves [1, 2]. Glutaraldehyde fixation is believed to cause degenerative changes in these biological tissues, predisposing them to calcification [3]. To date, however, glutaraldehyde-fixation remains the most successful method available to crosslink these collagenous tissues. Therefore, the control of calcification in glutaraldehyde-fixed biological tissue remains the preferred option in any product improvement for the continued commercial viability of xenograft bioprosthetic heart valves.

Many potential agents have been shown to prevent or at least mitigate calcification in various *in vivo* animal models, primarily the rat subdermal model. Extensive work has focused on small organic molecules such as sodium dodecyl sulfate and 2-amino-oleic acid, synthetic analogs of naturally occurring calcium mediating compounds collectively known as the diphosphonates, chloroform/methanol mixture, metal ions such as Fe^{3+} and Al^{3+} and most recently, detoxification using amino acids [4–9]. Presumably, these compounds interrupt various points of the mineralization events that can lead to calcification, such as extraction of phospholipids (which can attract calcium ions) from the biological tissues or as in the case of metal ions, repel the similarly charged calcium ions, or act as competitors of nucleation sites of calcium phosphates.

Our laboratory has also been actively investigating the development of anticalcification strategies. We have found, surprisingly, that the treatment of glutaraldehyde-fixed biological tissue with the organic reagent dimethylsulfoxide (DMSO) can render the tissue resistant to calcification as determined with the rat subdermal model. This serendipitous discovery arose from an evaluation of preliminary biocompatibility studies on work related to using polyurethane as a potential crosslinking agent to replace glutaraldehyde, where dimethylsulfoxide was the reaction solvent [10, 11]. Histology of retrieved tissue sections showed no calcification in samples fixed with our polyurethane process. Careful deduction of the data led us to conclude that dimethylsulfoxide was the agent responsible for the calcification prevention. We have confirmed this deduction and present our preliminary report of this finding.

2. Materials and methods

Dimethylsulfoxide (HPLC grade) was obtained from Aldrich Chemical Company.

Freshly harvested porcine aortic valve cusps were excised, rinsed with sterile HEPES-buffered saline and treated in one of the following ways:

1. Immersion in DMSO for 24 h at ambient temperature (25 °C). At the end of this period, the cusps were washed thoroughly in sterile buffered saline and fixed in 0.45% glutaraldehyde solution in 0.05 M HEPES buffer at 4 °C for longer than 24 h.
2. The cusps were fixed in 0.45% glutaraldehyde solution in 0.05 M HEPES buffer for over 24 h at 4 °C and subsequently immersed in DMSO for 24 h at 25 °C. Finally, the cusps were washed in sterile buffered saline and replaced in similar glutaraldehyde solution for storage.

‡Visiting research technologist, permanent address: St. Vincents Meditech Pte. Ltd., 51A The Faraday, Science Park, Singapore 0511

0957–4530

The variously treated cusps (≥ 15 per treatment randomly distributed) were implanted subcutaneously in the abdominal region of 3-week-old male Sprague Dawley rats. Each animal received four implants. At 21, 60, 90 and 120 days, the animals were sacrificed and samples retrieved and rinsed thoroughly with distilled water. Samples were subsequently dried at 110 °C for 24 h, weighed and hydrolyzed in 2 ml of 6N HCl for 24 h at 85 °C. The hydrolysates were analyzed for calcium content by atomic absorption spectroscopy.

Histology was performed on retrieved samples washed in buffered saline and distilled water, fixed in 10% buffered formalin and routinely processed. Paraffin-embedded histological sections were cut at 5 μm thickness and stained with hematoxylin and eosin (H&E).

3. Results

Fig. 1 shows graphically the calcium analyses results. For all time periods, the calcium levels were significantly lower than the glutaraldehyde-fixed controls. It is interesting to note further that up to the 90 day period, the two methods' anticalcification behaviours are similar. However, at 120 days, samples that were first fixed in glutaraldehyde followed by DMSO treatment fared better.

Fig. 2 shows photomicrographs of the histological examination of representative samples. The control (Fig. 2a) shows the usual dystrophic calcification that develops in these sample types. In contrast, the glutaraldehyde-fixed followed by DMSO treatment samples (Fig. 2b) shows no evidence of calcification. Furthermore, no deleterious effects on the DMSO treated biological tissue were found since the normal wavy features of collagen are still observed for all samples and for all time periods.

4. Discussion

Most of the present strategies for anticalcification rely on the prevention of calcium ion penetration into the tissue. This is a reasonable approach since the intake of calcium ions should be the first step in any calcification process. Previous work from this laboratory using metal ions showed that this strategy would be deficient over the longer term because of the leaching out of metal ions [12]. This is due to the lack of chemical "anchoring" of the metal ions to the biological tissue. Although site-specific drug delivery may be an answer to this situation, new problems such as drug replenishment arise [13].

Our discovery that dimethylsulfoxide can delay the onset of calcification in glutaraldehyde-fixed biological tissue is unprecedented. This is because solvent-mediated crosslinking reactions on biological tissue are rare if at all, in view of the potential for protein denaturation. The favourable low levels of calcium detected in the tissues compared to the controls over the entire implant period of 120 days are promising. However, the unexpected higher level of calcium at 120 days for the DMSO followed by glutaraldehyde-fixation method suggests that the order of DMSO exposure of the tissue may be important. Further indication of this may be concluded from the sample retrieval number (Table I). At 21 and 60 days post-implant, essentially all samples were retrieved. Beyond this point the sample retrieval number becomes less consistent. Allowing for a small number (1 or 2) being lost by natural attrition, the nearly 50% loss suggests

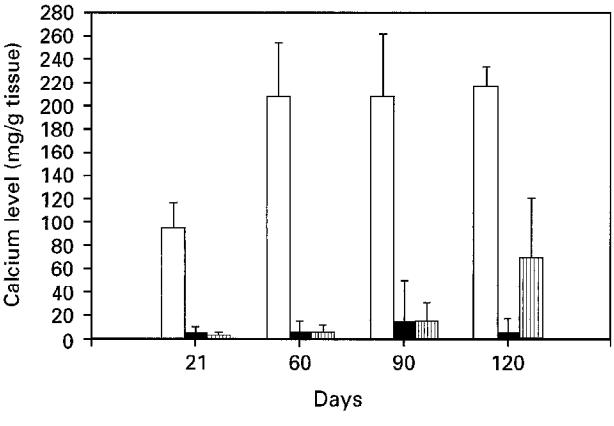

Figure 1 Calcium levels in retrieved porcine pericardium: glutaraldehyde fixed □; glutaraldehyde fixed and post-fixed with DMSO ■; pre-treated with DMSO followed by glutaraldehyde-fixation ▥ (error bars indicate standard deviation based on number of samples retrieved).

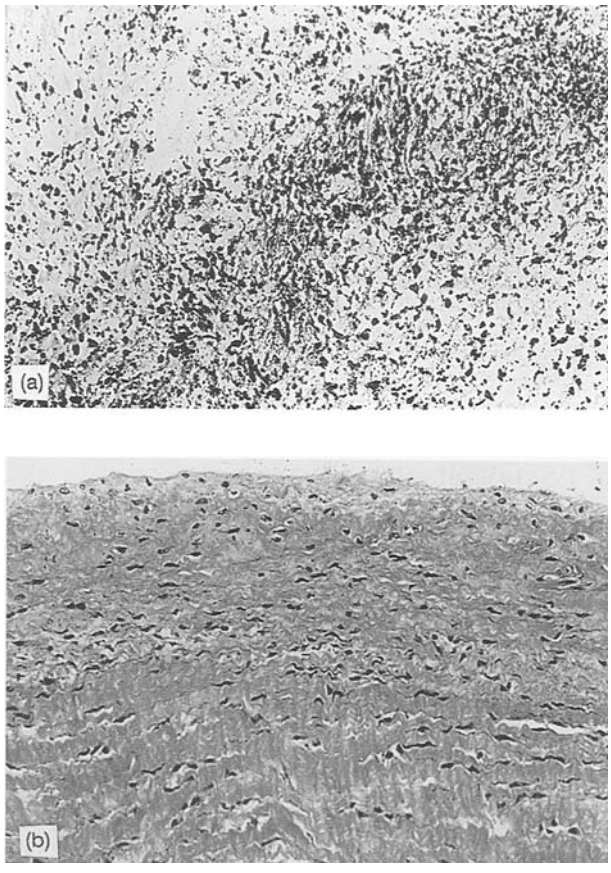

Figure 2 Photomicrograph of (a) glutaraldehyde-fixed implant after 120 days of implantation showing band-like dystrophic calcification in the fibrous layer (H & E, ×100). (b) Glutaraldehyde fixed implant followed by treatment with DMSO after 120 days of implantation showing intact collagenous tissue with no evidence of calcification (H & E, ×200).

TABLE I Sample retrieval number

Time period (days)	Glutaraldehyde-fixed controls	Glutaraldehyde-fixed-DMSO	DMSO-Glutaraldehyde-fixed
21	13/13	23/23	23/23
60	11/11	15/15	15/15
90	9/11	12/15	13/15
120	5/11	12/15	8/15

that the treatment of fresh tissue with DMSO may predispose the tissue to less efficient crosslinking in the subsequent glutaraldehyde fixation. *In vivo*, the tissue would tend to be more prone to degrade, hence the poorer recovery of samples. This would be less likely if glutaraldehyde fixation was first performed. However, the situation is further compounded by the loss of over half the control samples. Webb *et al.* have studied the anticalcification of Al^{3+} up to 120 days [14]. Although, no retrieval number was reported, the data suggests that no excessive lost was encountered. Therefore further investigation is warranted to resolve this ambiguity.

The exact reason why DMSO has anticalcification properties is not known, but may include the extraction of phospholipids in the fresh biological tissue, in a similar manner to other organic reagents such as sodium dodecyl sulfate and chloroform/methanol. Dimethylsulfoxide is known to be relatively non-toxic to biological systems and has been found useful as a cryopreservant for tissues [15]. Therefore, this work may have further implications beyond the application to glutaraldehyde-fixed xenograft based bioprostheses. It may be that cryopreserved homografts may possess anticalcification properties by virtue of their exposure to dimethylsulfoxide [16]. Further conclusions would be premature pending a complete study to determine the conditions and limits the anticalcification effects are displayed by dimethylsulfoxide.

5. Conclusions

Dimethylsulfoxide has been demonstrated to reduce significantly, the calcification of glutaraldehyde-fixed biological tissue. Further work is ongoing to define the threshold as well as to elucidate the mechanism responsible for the observed calcification mitigation effect.

Acknowledgements

The authors are grateful to the National Science and Technology Board, Singapore for financial sponsorship (NSTB 16/5/10) and St Vincents Meditech Pte Ltd for the supply of porcine aortic valve cusps. TBL acknowledges the National Science and Technology Board, Singapore, for a Research EXchange award.

References

1. F. J. SCHOEN, R. J. LEVY and G. GOLOMB, *CRC Crit. Rev. Biocompat.* **2** (1986) 147.
2. F. J. SCHOEN, H. HARASKI, K. M. KIM, C. ANDERSON and R. J. LEVY, *J. Biomed. Mater. Res.* **22A1** (1988) 11.
3. R. J. LEVY, F. J. SCHOEN, H. C. ANDERSON, H. HARASAKI, T. H. KOCH, W. BROWN, J. B. LIAN, R. CUMMING and J. B. GAVIN, *Biomaterials* **12** (1991) 707.
4. F. J. SCHOEN, R. J. LEVY, S. C. HILBERT and R. W. BIANCO, *J. Thorac. Cardiovasc. Surg.* **104** (1992) 1285.
5. J. P. GOTT, P. CHIH, L. M. A. DORSEY, J. L. JAY, G. K. JETT, F. J. SCHOEN, and R. A. GIRARDOT, *Ann. Thorac. Surg.* **53** (1992) 207.
6. E. JORGE-HERRERO, P. FERNANDEZ, M. GUTIERREZ and J. L. CASTILLO-OLIVARES, *Biomaterials* **12** (1991) 683.
7. M. T. BALDWIN, B. L. CIESLIGA, L. D. BARKASI and C. L. WEBB, *ASAIO Trans.* **37** (1991) M170.
8. C. L. WEBB, W. E. FLOWERS, C. HORTON, F. J. SCHOEN and R. J. LEVY, *Amer. J. Path.* **138** (1991) 971.
9. E. PASQUINO, S. PASCALE, M. ANDREON and S. RINALDI, *J. Mater. Sci. Mater. Med.* **5** (1994) 850.
10. E. KHOR, W. K. LOKE, SH TEOH, A. WEE, and K. S. CHIAN, Proceedings of the 4th Annual Conference of the Australian Society for Biomaterials (1994) p. 69.
11. W. K. LOKE, MSc thesis, National University of Singapore (1995) in preparation.
12. W. M. TAN, W. K. LOKE, B. L. TAN, A. WEE, E. KHOR and K. S. GOH, *Biomaterials*, **14** (1993) 1003.
13. R. J. LEVY, T. P. JOHNSTON, A. SINTOV and G. GOLOMB, *J. Control. Release* **11** (1990) 245.
14. C. L. WEBB, W. E. FLOWERS, C. HORTON, F. J. SCHOEN and R. J. LEVY, *ASAIO Trans.* **36** (1990) M 408.
15. B. TEASDALE, V. K. SIEBER, D. J. RICHES and J. NANCALAL, *Burns* **19** (1993) 406.
16. J. K. KIRKLIN, D. SMITH, W. NOVICK, D. C. NAFTEL, J. W. KIRKLIN, A. D. PACIFICO, A. C. NANDA, F. R. HELMCKE, and R. C. BOURGE, *J. Thorac. Cardiovasc. Surg.* **106** (1993) 154.

Received 5 January
and accepted 21 September 1995