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Glutaraldehyde-fixed biological tissue calcification: effectiveness of mitigation by dimethylsulphoxide

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The conditions defining the extent of dimethylsulphoxide (DMSO) effectiveness in mitigating calcification of glutaraldehyde (GA)-fixed tissue have been evaluated. Exposure of GA-fixed tissue porcine aortic valve cusps to low concentrations of DMSO does not impart calcification inhibitory activity. Mitigation in calcification becomes evident only as the DMSO component nears 100% and is best when neat DMSO is used. In all instances, regardless of the DMSO concentration, exposure to DMSO resulted in an increase in the tissue shrinkage temperature, attributed to further cross-linking in the tissue. Histological examination of samples before implantation indicate some deleterious effects to the tissue, the degree dependent on concentration, time and temperature of DMSO exposure. The results of this study suggest that treatment of GA-fixed tissue with high concentrations of DMSO for a short duration at a lowered temperature could give a bioprosthesis that has good mitigating calcification properties with retention of tissue integrity.

1. Introduction

The bioprosthetic heart valve fabricated from glutaraldehyde (GA fixed) porcine aortic valves remains the most successful example of a heterograft-based implant device [1]. However, the continuing problems of GA fixation, poor long-term durability and *in vivo* calcification continue to limit their wider use [2]. Alternative methods to replace GA fixation, such as the promising dye-mediated photo-oxidation of bovine pericardium, have yet to be demonstrated for long-term clinical viability [3]. Therefore, the removal of undesirable effects of GA fixation such as the control of calcification remains, at least for now, the preferred option in any product improvement for the continued commercial viability of bioprosthetic heart valves.

Many proposals for the control of calcification have been presented. Each has been shown to be effective in the small-animal stage but usually has not moved on to advance trials or has produced inconclusive results [4, 5]. Previously this laboratory reported the surprising discovery that treatment of GA-fixed tissue with dimethylsulphoxide (DMSO) endowed the tissue with anticalcification properties [6]. The method that we used was to immerse tissue in neat DMSO for 24 h. While the result that we obtained was encouraging, the extent of DMSO effectiveness in calcification mitigation was not evaluated. This is important because, although DMSO is a relatively non-toxic chemical and has been used as a cryopreservative for tissue, it is best to minimize DMSO exposure if possible [7, 8]. Therefore, the influence of DMSO exposure parameters such as the effective concentration range, the time and temperature and its influence on the integrity of tissue and anticalcification needs to be resolved. In this report, these important considerations are addressed.

2. Materials and methods

Aortic valve cusps were excised from freshly harvested porcine aortic valves, rinsed with 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) buffered saline and fixed with 0.45% GA solution in 0.05 M HEPES buffer for more than 24 h at 4 °C. The cusps were subsequently exposed to various combinations of DMSO (20–100% v/v) with co-media of distilled water, 0.05 M HEPES-buffered saline or 0.05 M HEPES-buffered 0.45% GA solution at 25 or 37 °C for 1 or 6 h. At 100% DMSO concentration, further fixation time periods up to 24 h were imposed. At the end of each treatment, cusps were blot dried, rinsed with HEPES-buffered saline and stored in 0.45% GA solution in 0.05 M HEPES buffer at 4 °C until utilized for analysis or implantation.

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2.1. Shrinkage temperature

A small piece of cusp (about 2.5 mm × 2.5 mm) was cut, rinsed with distilled water and blot dried. The cut piece was placed in between a fluorophosphate-coated pan and cover and hermetically sealed. The shrinkage temperature was obtained by differential scanning calorimetry (DSC) under a nitrogen atmosphere at a heating rate of 5 °C min^{-1} from room temperature to 100 °C according to the method of Loke and Khor [9]. The sample size was 5 or more.

2.2. Implantation

The variously treated cusps (15 or more 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 days (and 120 days for the final study), the animals were sacrificed and the samples retrieved and rinsed thoroughly with distilled water. Samples were subsequently dried at 110 °C for 24 h, weighed and hydrolysed in 2 ml of 6 N HCl for 24 h at 85 °C. The hydrolysates were analysed for calcium content by atomic absorption spectroscopy [10].

2.3. Histology

Treated cusps or retrieved implants were washed with buffered saline followed by distilled water and fixed in 10% buffered formalin and processed by routine histological preparative methods. The samples were sectioned at 5 μm thickness and stained with haematoxylin and eosin and von Kossa for calcium. The sections were examined blind. The integrity of the tissue and the presence of dystrophic calcification were evaluated.

3. Results

3.1. Visual observations

Upon removal from DMSO solutions, samples were generally found to have shrunk and appeared darker in colour compared with GA-fixed tissue. The extent of shrinkage and colouration was concentration dependent (the higher the concentration of DMSO, the larger the shrinkage), and it was more prominent at 37 °C and following longer exposure times. The shrinkage was found to be reversible. For example, the length of the cusps prior to DMSO exposure and after were determined by caliper measurements and found to recover up to 75% of their original length almost immediately upon transfer back to buffered GA solution (Table I). The amount of recovery was DMSO concentration dependent (the higher the DMSO concentration, the less the recovery to original length).

3.2. Shrinkage temperature evaluation

3.2.1. Dimethylsulphoxide-exposed non-implanted samples

The shrinkage temperatures for GA-fixed porcine aortic valve cusps (PAVCs) after exposure to DMSO was

TABLE I Sampling of length measurements for tissue after exposure to 100% DMSO ($n = 10$; mean ±SD; number in parentheses indicate percentages of original length)

Treatment	Original length (mm)	Length immediately after exposure (mm)	Length after soaking in buffer (10 min) (mm)
100% DMSO; 6 h at room temperature	29.99 ± 3.18	15.43 ± 1.82 (51.45%)	22.01 ± 1.92 (73.39%)
100% DMSO; 6 h at 37 °C	29.08 ± 4.23	14.24 ± 2.05 (48.97%)	20.40 ± 2.54 (70.15%)

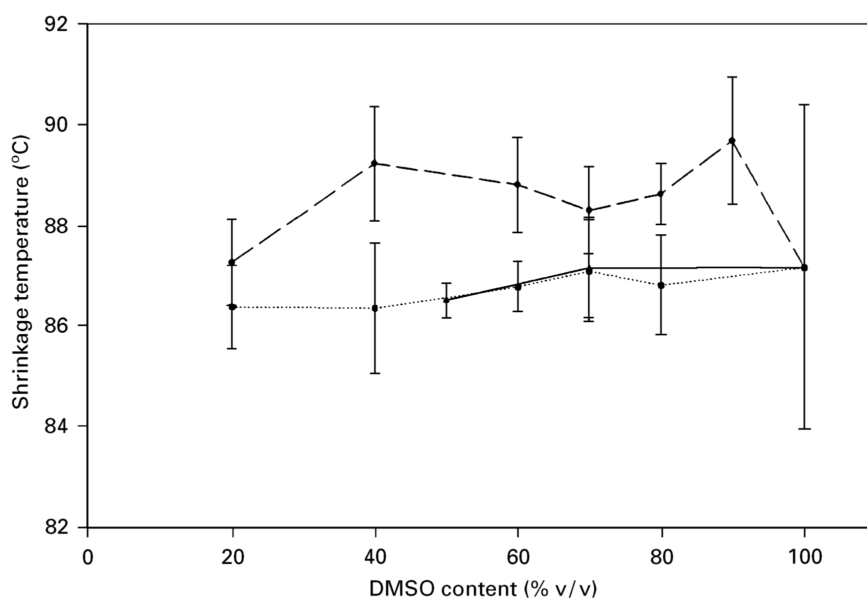

Figure 1 Shrinkage temperature plots of DMSO-exposed GA-fixed PAVCs. (– –), DMSO–saline; (···), distilled water; (——), DMSO–GA.

TABLE II Shrinkage temperature of PAVCs exposed to DMSO–saline under various conditions (GA-fixed PAVC has a shrinkage temperature of 85.79 ±0.52 °C (from [6])

DMSO concentration (%)	Shrinkage temperature (°C) at the following times and temperatures of exposure			
	1 h		6 h	
	Ambient	37 °C	Ambient	37 °C
20	88.78 ± 0.84	89.01 ± 0.50	87.28 ± 0.86	87.43 ± 0.63
60	88.85 ± 0.94	88.61 ± 0.60	88.82 ± 0.94	89.17 ± 0.62
70	89.29 ± 0.38	88.51 ± 0.98	88.32 ± 0.86	88.78 ± 0.44
80	89.05 ± 0.38	88.79 ± 0.47	87.28 ± 0.86	88.43 ± 0.75
90	89.71 ± 0.95	89.77 ± 1.30	89.70 ± 1.26	89.81 ± 1.76
100	87.79 ± 1.64	80.62 ± 4.82	87.20 ± 3.22	88.64 ± 1.82

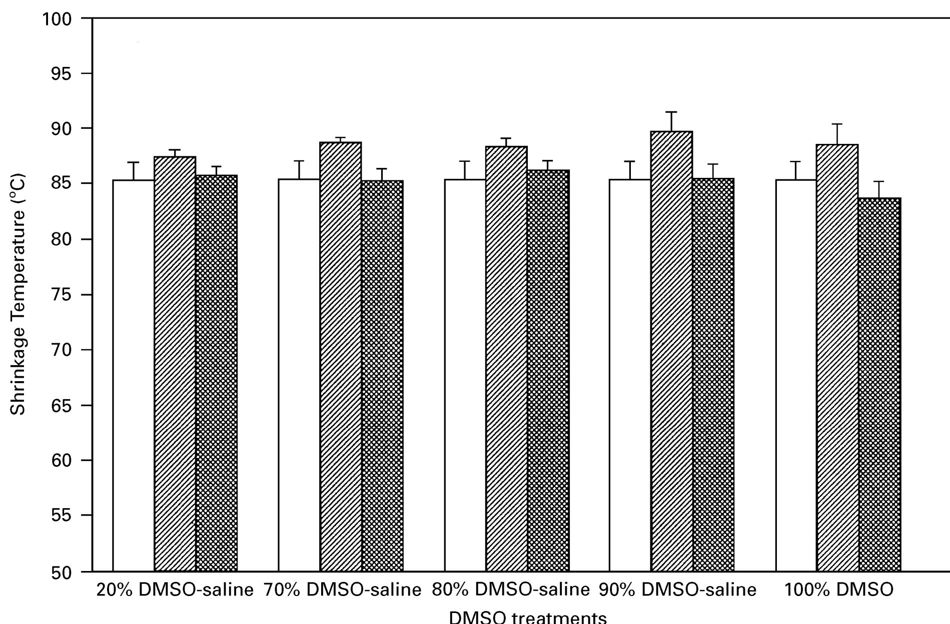

Figure 2 Shrinkage temperature profiles for DMSO-exposed GA-fixed PAVCs. (□), GA control; (▨), non-implanted; (▩), post-implanted.

obtained for three variations of co-media, two temperatures and two times of exposure. In all instances, the shrinkage temperature increased by 1 °C to 3 °C after DMSO exposure. A comparison of the performance of the three co-media is shown in Fig. 1. In general, the co-medium of saline gave the most consistent results and the highest increase in shrinkage temperatures and was selected for all subsequent studies.

Table II summarizes the shrinkage temperatures determined for a series of samples of various DMSO concentrations with saline for the two temperatures and the two times of exposure. The data indicate that the two time periods and the two temperatures of exposure to DMSO apparently do not signifcantly influence the integrity of the tissue as evaluated by shrinkage temperature determinations, although variations within a particular treatment group were found to be higher as the DMSO concentration increased (note the standard deviation (SD)). Generally, 3–6 h was found to be sufficient to increase the shrinkage temperature compared with the GA-fixed control.

3.2.2. Post-implantation

Fig. 2 shows the distribution of shrinkage temperature for the GA-fixed PAVCs after exposure to DMSO–saline for 6 h in the concentration range from 20% to 100% after 21 days residence in the rat subdermal mode. In general, the shrinkage temperatures were comparable with the GA control after the same period of implantation but reduced compared with pre-implant samples.

3.3. Histology

3.3.1. Dimethylsulphoxide exposed non-implanted samples

The effects on tissue morphology imparted by DMSO exposure appear to vary and to be time and temperature dependent. Fig. 3 exemplifies the category of features that can be found from a histology study of DMSO-exposed non-implanted samples using the 100% DMSO-exposed (37 °C) GA-fixed tissue.

Fig. 3a shows an area of a sample exposed for 3 h displaying the normal cellular details with intact collagen and myxoid stroma. Fig. 3b shows another area of the same sample as in Fig. 3a, displaying mild shrinkage manifested as the appearance of artefactual changes in the myxoid areas of the connective tissue. When the time of exposure is increased to 18 h, the degree of shrinkage increases with separation of the stromal connective tissue (Fig. 3c). Finally, when

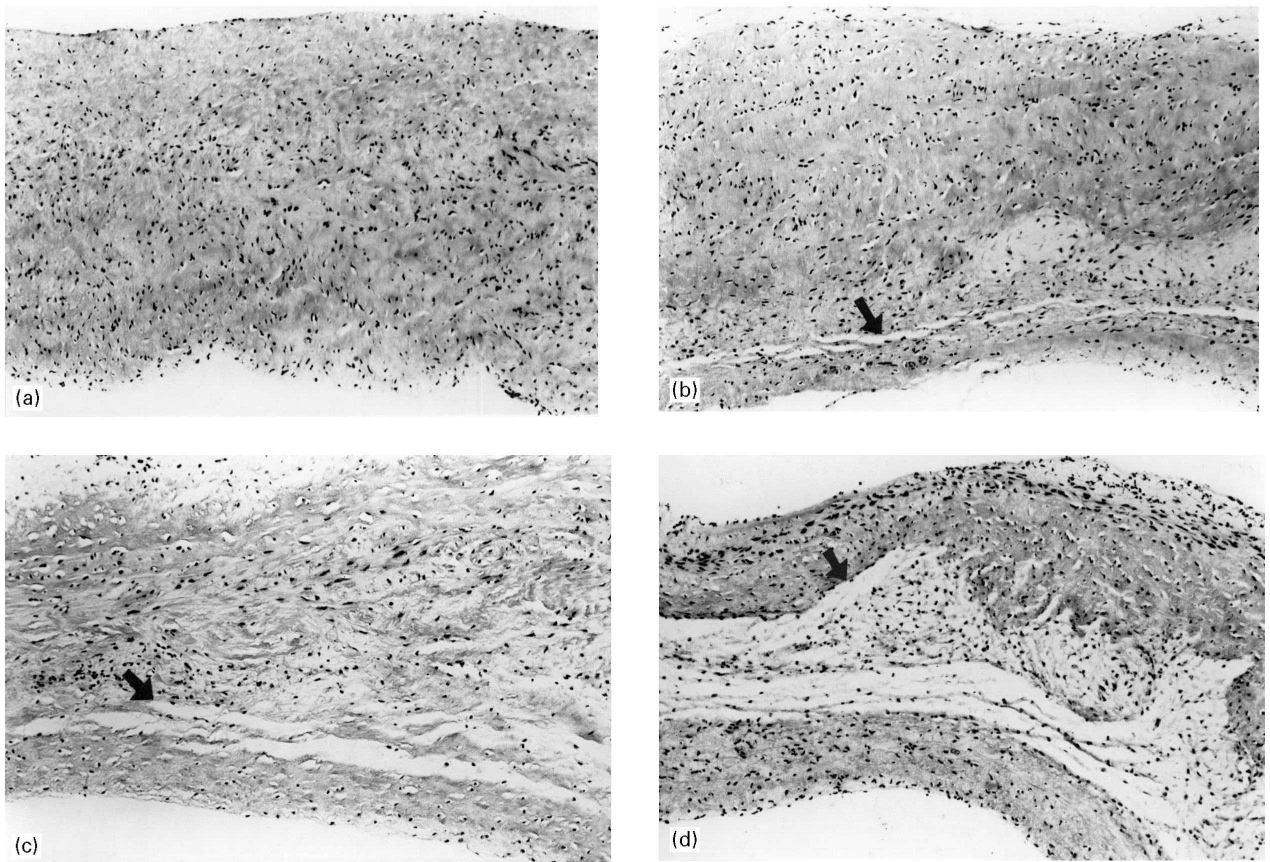

Figure 3 Representative photomicrographs displaying the various effects that DMSO exposure has on tissue morphology (DMSO–saline, 37 °C; H&E stain; magnification, 65 ×): (a) an area showing normal cellular details with no evidence of shrinkage (grading, o); (b) an area showing mild shrinkage artefactual changes (arrow) in the myxoid areas of the connective tissue (grading, +); (c) an area showing moderate degree of shrinkage with separation of the stromal connective tissue (grading, + +); (d) tissue showing marked shrinkage artefacts with obvious separation of the stromal connective tissue (grading, + + +).

TABLE III Sampling of unfavourable tissue morphology after exposure to DMSO–saline solutions (o, none; +, mild; + +, moderate; + + +, obvious; ND, not determined)

DMSO concentration (%)	Sampling for the following times and temperatures of exposure			
	1 h		6 h	
	Ambient	37 °C	Ambient	37 °C
20	o	+	+ +	+ +
60	+	+	+ +	+ +
70	+	+ +	+ +	+
80	+ +	+	+	+
100	+	+	+ +	+ +
1:1 DMSO:GA	+ + +	+ + +	ND	ND

the time of exposure is 24 h, marked shrinkage artefacts with obvious separation of the stromal connective tissue was found (Fig. 3d). These features were found in most samples under various conditions of concentrations of DMSO, times and temperatures of exposure. In general, lower concentrations of DMSO, lower temperatures and shorter times of exposure gave the most normal tissue features. A summary of these effects for various concentrations and times and temperatures of exposure for DMSO–saline samples are provided in Table III.

3.3.2. Post-implantation

Fig. 4 shows the photomicrographs of histology sections from retrieved PAVCs implantation for 21 days (von Kossa; x150). The GA-fixed PAVC post-fixed with 100% DMSO for 24 h samples (Fig. 4a) is representative of all 100% DMSO-exposed samples where no calcification was found; the black marks are the nucleus with a regular size (arrow). In contrast, the sample GA fixed and post-treated with 80% DMSO–saline for 6 h at 37 °C (Fig. 4b) shows band-like dystrophic calcification surrounding the nucleus, evident by the black patchy stains (arrow). The GA-fixed control (Fig. 4c) also shows extensive calcification throughout the explant (arrow).

3.4. Calcium levels in explants

Fig. 5 shows the calcium profiles of 2 days explants obtained for samples exposed to a range of DMSO concentrations for 6 h. The level of calcium detected in the explants decreased as the concentration of DMSO increased and was the least at 100% DMSO. Therefore, the 100% DMSO-exposed samples were selected for a further time of exposure to DMSO at room temperature. The results in Fig. 6 support the mitigating effects on calcification of this treatment, evidenced by the very low calcium levels obtained for all time periods. Finally, the 6 h, 100% DMSO treatment was

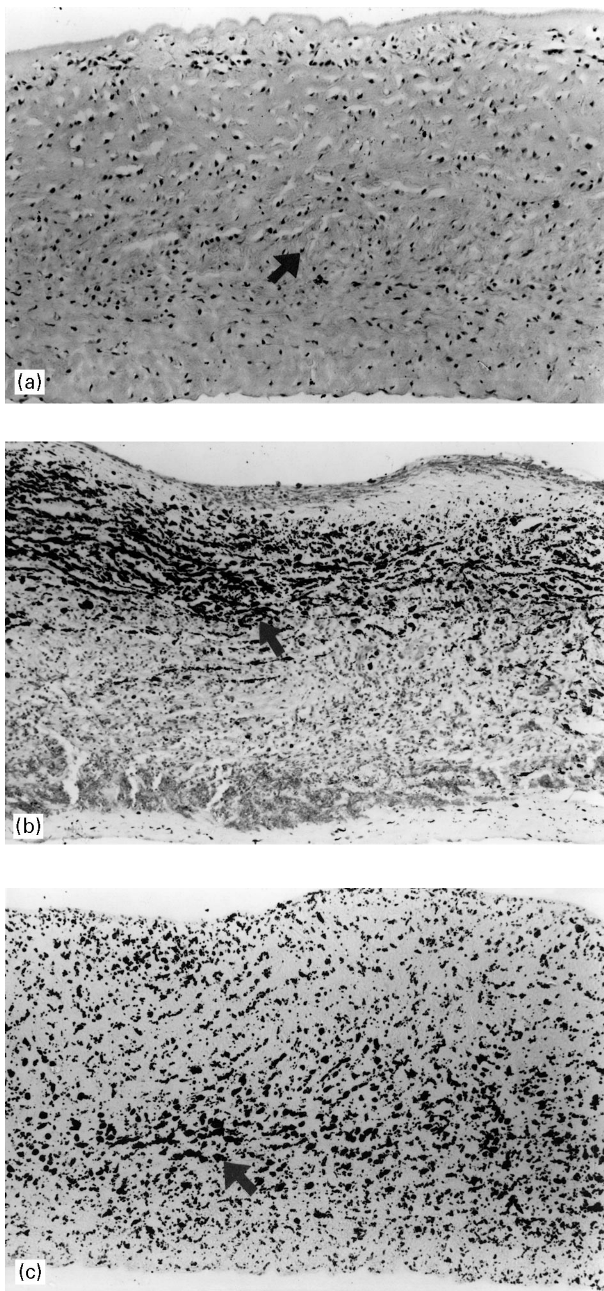

Figure 4 Photomicrographs of histology sections from retrieved porcine aortic valve cusps after 21 days implantation (von Kossa; magnification, 97.5 ×). (a) GA-fixed and post-fixed with 100% DMSO for 24 h. No calcification is observed. The black dots are the normal nuclei of the stromal cells (arrow). (b) GA-fixed and post-fixed with 80% DMSO–saline for 6 h at 37 °C. Band-like dystrophic calcification is observed. Calcification surrounding the nucleus is evidenced by the black patches (arrow). (c) GA-fixed. Extensive calcification throughout the explant (arrow).

selected for an extended-term study. The calcium levels in explants retrieved after 120 days in the animals for these treated samples were 26.24($\pm$31.16 SD)mg (g tissue)$^{-1}$ ($n = 14$), much lower than the GA-fixed controls of 235.19($\pm$55.9 SD)mg (g tissue)$^{-1}$ ($n = 16$), demonstrating that the effect is long lived.

4. Discussion

The general observations of shrinkage and darker colour upon exposure to DMSO is not surprising. DMSO is readily miscible with water and, when placed in the presence of tissue, can replace water molecules surrounding the collagen molecules. In effect, dehydration takes place, resulting in tissue shrinkage [11]. The greater the amount of DMSO, the more water molecules are displaced, with the process being accelerated at a higher temperature. The reversibility of the dehydration implies that the three-dimensional structure of collagen was not irreparably damaged after exposure to DMSO, which is desirable. The recovery of the length up to 75% is not ideal but suggests that improved methods of exposure to DMSO could further mitigate this undesirable side effect. All in all, the results imply that controlled exposure of tissue to organic solvents with minimal detrimental effects may be possible and could be the basis for future tissue reactions with organic reagents including water-sensitive polymers.

4.1. Shrinkage temperature evaluation

4.1.1. Dimethylsulphoxide exposed non-implanted samples

The increase in shrinkage temperature after DMSO exposure is again not surprising. The process of dehydration causes collagen chains to come closer with the accompanying opportunity for secondary covalent bonding between adjacent chains to occur, such as disulphide linkages [12, 13]. Furthermore, DMSO has been found to stabilize proteoglycans (mucopolysaccharides) present in connective tissue *in vitro* [14]. The net result is an increase in shrinkage temperature by 1 °C to 3 °C overall.

The influence of the co-solvent is interesting. Saline gives the highest increase while the GA solution and distilled water appear to have neutralizing effects, giving more modest increase in shrinkage temperatures compared with the controls. It is known that DMSO can extract neutral-salt-soluble collagen from tissue [14]. This could cause other chains to come closer, increasing the opportunity for cross-linking which gives rise to the higher shrinkage temperatures observed.

In general, the time or temperature of exposure does not appear to influence the shrinkage temperature results although, within the 100% DMSO set, deleterious effects were found at long exposure times (see Section 4.2) [13]. This can be rationalized by referring to the GA fixation of tissue, where an initial increase in shrinkage temperature was found which quickly reaches a plateau [15]. With increasing time, only negligible increases were obtained. The same explanation is used here, i.e., after the preliminary reaction time of causing additional cross-linking, the system quickly equilibrates. Temperature only accelerates the process.

4.1.2. Post-implantation

The slight lowering of shrinkage temperature after implantation is within acceptable limits and similar to the behaviour displayed by the GA control.

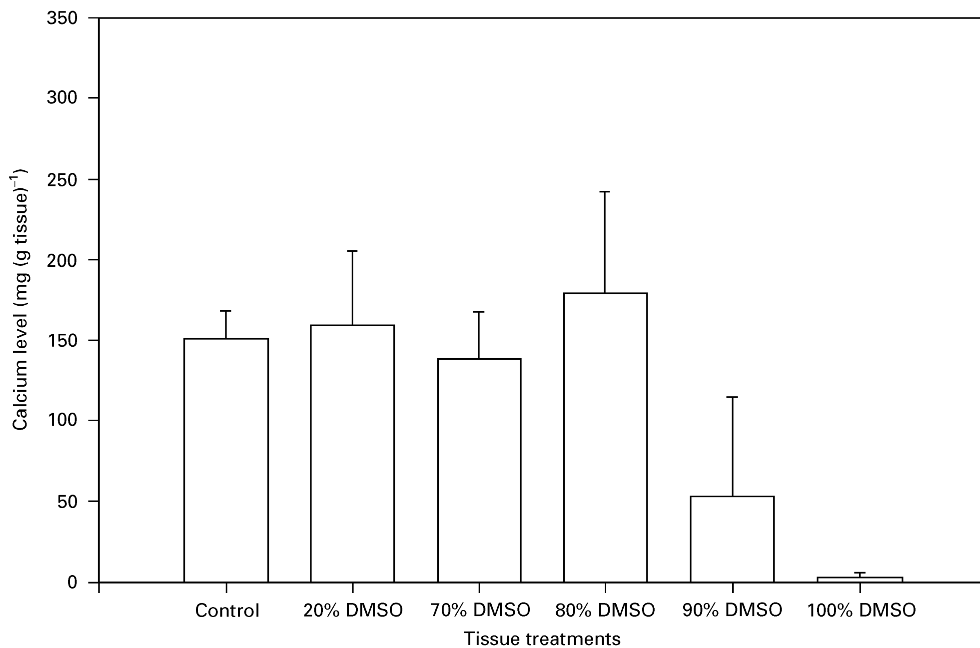

Figure 5 Calcium level in retrieved implants after 21 days for GA-fixed PAVC samples exposed to various concentrations of DMSO.

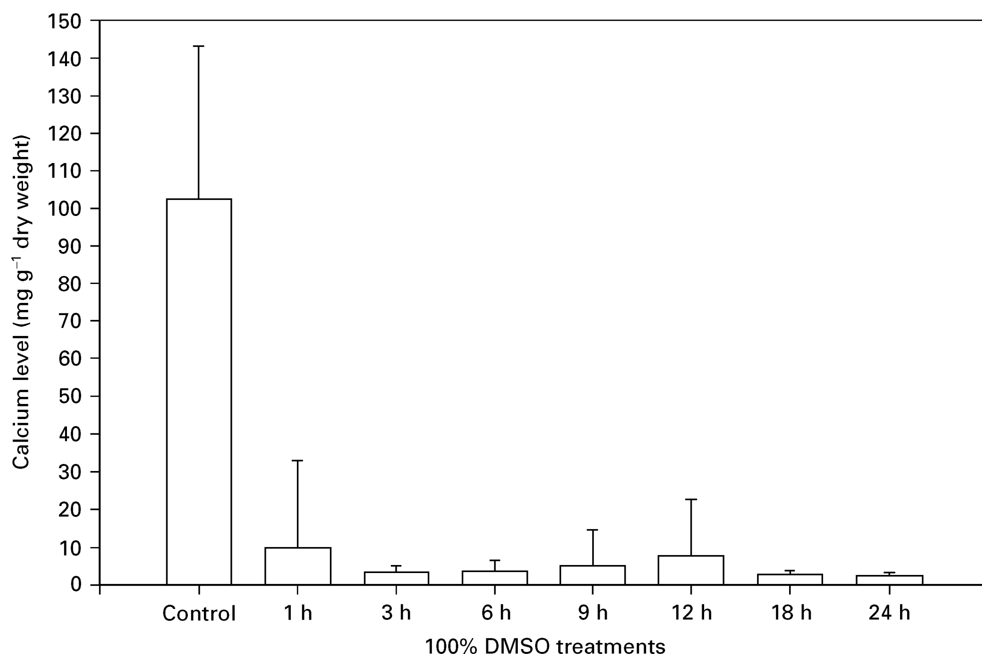

Figure 6 Calcium level in retrieved implants after 21 days for GA-fixed PAVC samples exposed to 100% DMSO for various time periods.

4.2. Histology

4.2.1. Dimethylsulphoxide exposed non-implanted samples

The varied features found in this histology study are interesting and point to some deleterious effects that DMSO can have on tissue [16]. Most previous work on collagenous tissue exposure to DMSO limits the concentration to about 20% with a typical high end limit of 70%, while this study has spanned the whole range up to 100%. Gries [14] has reported that rabbit-skin-based collagen after exposure to 100% DMSO for 24 h has its neutral salt-soluble collagen reduced and attributes this to the solvent effect of DMSO. Nevertheless, our results show that, even at 100% DMSO, good tissue morphology can be obtained by limiting the time and temperature of exposure if pre-fixed with GA. It was not an aim of this project at this stage to determine the best DMSO exposure conditions for good tissue morphology but this study has shown that this issue is of utmost importance, apart from the reduction in calcium levels.

4.2.2. Post-implantation

The post-implantation histology results are similar to our previous results [6]. The lack of calcification in the 100% sample is strong support that 100% DMSO must be used in preference to lower concentrations, exemplified by the 80% DMSO–saline solution.

4.3. Calcium levels in explants

The calcium levels reinforce the conclusion that 100% DMSO must be used. The results of the time-of-exposure study indicates that shorter exposure time to DMSO is just as good in inhibiting calcium uptake and therefore, in the light of the possible damage to tissue morphology, perhaps a 1 h exposure time is sufficient. The result for 120 days suggests that little degradation to the sample occurred and that the DMSO calcium mitigation effect is long lived.

4.4. Implications of the mitigating effects of dimethylsulphoxide on calcification

DMSO has long been used as a cryoprotectant in the preservation of tissue, cells, etc. [8]. Typically DMSO is used at low concentrations usually below 5%. DMSO has been reported to impart both good and adverse effects. The hydrophilic nature of the reagent permits the replacement of water by DMSO in the tissue structure. Therefore, the structural integrity of the biological system is retained. At higher concentrations and temperatures, the removal of coordinated water promotes bonding in the hydrophobic regions of collagen, giving rise to the increased shrinkage temperatures. The implications of this work clearly suggest that undiluted DMSO must be used in order to benefit from the calcification mitigation effects of DMSO on GA-fixed tissue. It is known that 100% DMSO does not necessarily impair the proper function of biological entities after the solvent is removed [17]. Therefore, treatment of tissue with pure DMSO is not as absurd an idea as at first confronted. The advantageous pre-fixation with GA is another factor in favour for using neat DMSO for bioprostheses. In order to minimize the adverse effects that DMSO can have on tissue, which includes the rupture of existing cross-links in collagen at higher temperatures and concentrations and localized areas of denaturation, care must be exercised, such as the use of a lower temperature. A minimum time of exposure is necessary, to permit the diffusion and equilibration of tissue to DMSO.

Finally, the results of this work have important implications for bioprosthesis calcification. The finding that exposure to low concentrations does not prevent calcification suggests that calcium deposition and nucleation is collagen related. At higher concentrations of DMSO, some degree of tissue denaturation is expected and was found. However, in this instance, prior GA fixation retains the overall network of collagen, essential for tissue integrity. Therefore, it is proposed that the reduction in calcification imparted by DMSO is the nullifying of epitactic sites that can attract Ca^{2+} ions. This can be visualized for example as two pendant -COO^- groups of amino acids oriented to attract Ca^{2+}. Pure DMSO changes the secondary three-dimensional structure in tissue, blocking or at the very east minimizing the oriented Ca^{2+}-attracting sites in tissue. Combined with the inherent tissue shrinkage caused by DMSO exposure, sites along the fibrils that are nucleation sites for calcium crystal formation as proposed by Glimcher [18] are reduced. This explanation combined with the hypothesis that GA can also be nucleating sites for Ca^{2+} probably accounts for the complete picture of *in vivo* dystrophic calcification of bioprosthetic tissue.

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