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Original Article

Differential Surface Thrombogenicity of Heparin Coated Bovine Pericardium: Implication in Bioprosthetic Valve Fabrication

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Sub-acute valve thrombosis limits the full potential of bovine pericardial valves. In this study, the effect of heparin coating and the influence of rough versus smooth pericardial surface on thrombogenicity was evaluated by *in vitro* methods. This was followed by mitral implantation of bioprosthetic valves with different inflow surfaces in sheep. The presence of conjugated heparin was demonstrated through O-toluidine blue staining and micro Raman spectroscopy. Heparin was estimated by a colorimetric assay for depletion of O-toluidine blue stain following exposure to heparin modified tissue. Functionality of surface bound heparin was demonstrated by platelet adhesion and protein adsorption studies. Difference in surface thrombogenicity of heparin coated rough and smooth surface was evaluated by modified whole blood kinetic clotting time method. Bioprosthetic valves with either rough or smooth surface of heparin coated pericardium forming the inflow surface were implanted in the mitral position of sheep and observed for device thrombosis upto 6 months. Heparin coating rendered the pericardial surface hydrophilic. Adsorbed protein on heparin coated pericardium was less compared to non-coated one. SEM images for platelet adhesion showed absence of platelets on heparin-coated tissue. Surface thrombogenicity evaluation showed difference in thromboresistance for heparin coated and uncoated pericardial surfaces and between heparin coated rough/smooth surfaces. Sheep implanted with valve having heparin coated rough surface as valve inflow thrombosed in 53 days. In comparison, the valve having heparin coated smooth surface of pericardium forming the valve inflow surface completed the observation period of 6 and 12 months without any valve thrombosis.

Introduction

Biological heart valves can be homograft or allograft when it is of human origin [1-5] or it can be made from chemically processed animal tissues [6,7]. Bioprosthetic heart valves (BHV) are made from glutaraldehyde processed animal tissues such as porcine aortic valve or bovine pericardium with supporting fabric covered stents or without it. Glutaraldehyde cross-linked bovine pericardia is widely used in the fabrication of BHV since 1970s [8] and have evolved through various pericardial post processing techniques to make it more conducive for clinical application. One of the major benefits of BHV over mechanical valve is the decreased dependency on anticoagulant therapy [9]. However, unlike its mechanical counterpart, both transcatheter and surgical BHV have been reported to fail on account of calcific structural degradation and thrombosis [10-12] thus limiting its durability [1]. For any blood-contacting device, clot formation can result in dysfunction leading to device failure, or alternatively it may release emboli into the systemic circulation causing fatal thromboembolism of distal organs [13]. Occurrence of subclinical leaflet thrombosis in transcatheter heart valves (THVs) is found to range between 7 and 35% [5]. Although, the long established glutaraldehyde process is known to mask most of the cellular antigens present on the collagenous tissue, its residual immunogenicity and consequent chronic inflammation is still found to be associated with long term failure including valve thrombosis [14,15]. Glutaraldehyde treated pericardial surface is known to be hydrophobic and vulnerable to surface fibrinogen binding [16]. In conclusion, in *vivo* calcification and valve thrombosis has since remained elusive factors limiting the potential durability of glutaraldehyde processed bovine pericardial valve [17-19]. Surface immobilization of heparin is known to induce thromboresistance and even temporary immobilization of heparin to biomaterial surfaces has previously

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shown to reduce thrombus formation on implanted blood contacting devices [20-22].

The present study demonstrates thromboresistance induced by surface immobilized heparin on glutaraldehyde processed bovine pericardium by way of resistance to platelet adhesion and reduced surface protein adsorption. Furthermore, this study highlights the difference in thromboresistance between rough/fibrous surface and smooth/ parietal surface of heparin coated pericardium by *in vitro* and more importantly it's effects on bioprosthetic valve thrombosis in sheep mitral implantation model.

Materials and Methods

Conjugation of heparin onto glutaraldehyde processed bovine pericardium

A proprietary method was used for glutaraldehyde processing of bovine pericardium and single step heparin conjugation onto to it. In brief, bovine pericardia of veterinary certified cattle slaughtered for food purpose at Meat Products of India Ltd., Ernakulam Dist., Kerala was manually defatted and cleaned with several changes of clean water to remove blood and tissue debris. This was again cleaned and packed in sterile normal saline with antibiotic-antimycotic (AA) solution (HiMedia) and transported to the laboratory within 24 hours at room temperature. This pericardium was subjected to low concentration detergent treatment in deionized distilled water for overnight at RT followed by low concentration glutaraldehyde cross-linking for over three weeks. This glutaraldehyde processed bovine pericardium was thoroughly washed in normal saline and immersed in 0.05 Molar HCl solution containing 0.4g heparin for 48 hours at room temperature. This was followed by washing in normal saline solution. Processed tissues were sterilized and stored in 70% ethanol based liquid chemical sterilant until further use. Glutaraldehyde processed bovine pericardium prepared by the same method except for heparin surface conjugation was treated as control.

Demonstration of bound heparin

Heparin specific O-Toluidine blue staining

The presence of surface conjugated heparin post conjugation was demonstrated through O-toluidine blue staining. 1cm x 1cm pieces of heparin coated bovine pericardia were placed in 1ml of PBS (in triplicates and at pH 7.4) and incubated for 60 minutes under constant agitation. Another set of heparin coated samples were incubated in PBS for 7 days under agitation at 37ºC. Heparin remaining bound at the end of 60 minutes and 7 days were demonstrated by staining with O-toluidine blue stain (20µg in 10ml deionised distilled water, which is made up to 100ml with deionized distilled water) for 30 minutes. The samples were observed for metachromatic purple staining. This was compared with uncoated sample.

Micro Raman Spectroscopy

The presence of surface conjugated heparin was further confirmed using the highly sensitive Raman spectroscopy technique. 1cm x 1cm pieces of heparin coated and uncoated processed bovine pericardium samples were completely air dried to remove any moisture and was analyzed for Raman spectra using Confocal Raman Microscope (alpha 300A, Witec Inc. Germany); following the method adopted from Witec Alpha 300FR Confocal Raman Microscope User Manual. Instrument was pre calibrated using silicon wafer calibration standard (accepted peak at 520 cm^{-1}). All samples were observed under 20x objective and excited using 785nm Laser with integration time of 5s and a grating of 300 lines/mm. The resultant spectra were compared to identify laser induced fluorescence peaks for heparin.

Static water contact angle measurement:

Static water contact angle using sessile drop method of the Heparin coated and Heparin uncoated glutaraldehyde processed tissues were measured and compared to that of native tissue to identify the hydrophilic/hydrophobic nature of the material surface. 2cm x 2cm patches in triplicates were used from the native, heparin modified and glutaraldehyde processed pericardia. The angle of contact of a small sessile drop of distilled water placed on the material surface was measured with six measurements on each sample using Goniometer GII, Kerno Instruments Inc, USA.

Estimation of Bound Heparin

The heparin content on the modified bovine pericardium was estimated indirectly by measuring the depletion of the metachromatic dye O-Toluidine blue in the immersion liquid following tissue immersion for set periods [23,24,25]. 500mg (blotted dry weight) heparin coated tissues were placed in 2.5mL of 40mg% O-toluidine blue solution followed by the addition of 2.5mL of normal saline. Tissues were incubated for 15min and to this 5mL hexane was added and vortexed. OD values for the supernatants were read at 631nm. A calibration curve was plotted using known concentrations of heparin and a polynomial equation was derived showing the relationship between OD values and the concentration of heparin by curve fitting. The unknown concentration of heparin was derived from the above mentioned equation.

Demonstration of functional activity of bound heparin

Platelet adhesion study by Environmental Scanning Electron Microscopy (ESEM)

Morphology and platelet adhesion on Heparin coated (test) and Heparin uncoated tissues (control) were observed using environmental scanning electron microscopy. For platelet adhesion, the pericardial samples (both test and control) were prepared by washing the samples in normal saline in three changes and were exposed to whole blood containing ACP for 30 minutes at 37ºC followed by several washes with PBS. Fixation of samples were done using 2% Glutaraldehyde and kept at -20ºC overnight and were repeatedly washed in sterile PBS. Samples were then treated in graded concentrations of 30%, 50%, 70%, 90% and 100% alcohol for 15 minutes each and observed using ESEM (SEM, Hitachi S-2400 Japan) using high vacuum secondary electron detector.

Estimation of adsorbed protein

Total protein adsorbed onto heparin coated tissue was evaluated by Lowry's method of protein estimation. Briefly, heparin coated and heparin uncoated processed bovine pericardia were rinsed thoroughly with normal saline for three times. The samples were then exposed to whole blood containing ACP for duration of 30 minutes at 37ºC followed by several washes in phosphate buffered saline solution. To this tissue was added 0.1 N sodium hydroxide and was incubated overnight at room temperature to cleave all the proteins adsorbed to the surface. Samples were then centrifuged at 12000 rpm for 20 minutes at 4ºC and the supernatant thus obtained was used to measure the adsorbed protein content on the tissue surface by following Lowry's method of protein estimation.

In vitro surface thrombogenicity evaluation

Heparin coated (test) and heparin uncoated (control) bovine pericardia were evaluated for the thrombogenicity of both their

rough and smooth surfaces using a modified procedure of the whole blood kinetic clotting time method [26,27]. Briefly, rough and smooth surfaces of the two treatment samples (n=6) having a 2 cm x 2 cm dimension were placed with their test surface facing upward in 6-well tissue culture plates. 10% (v/v) of 0.1 M $CaCl_2$ was added to citrated whole blood to activate the clotting reaction and a 100µL volume of the activated blood was placed on top of the pericardia immediately after activation. Samples were incubated at 37ºC for 15 min followed by addition of 2.5 ml of distilled water and incubated for 5 min. This step caused hemolysis of free RBCs not entrapped in the clot on material surface thereby releasing hemoglobin into the supernatant, which is an indirect indication of clot size generated on the test material surface. Each well was sampled from the supernatant, in triplicates (200 µL each) and transferred to a 96-well plate. The concentration of hemoglobin in the supernatant solution was assessed by measuring the absorbance at 540 nm using a 96-well plate reader which is an indirect indicator of the size of clot formed on the tissue surface.

Sheep mitral implantation study

Animal protocol was approved by Institutional Animal Ethics Committee and CPCSEA, Government of India. Four healthy adult male sheep as confirmed by clinical and laboratory examination were implanted with bioprosthetic heart valve in the mitral position under general anesthesia and cardiopulmonary bypass. Atropine sulphate at 0.1mg/Kg was given subcutaneously as premedication, followed by intramuscular Xylazine hydrochloride 0.3mg/Kg and Ketamine hydrochloride 10mg/Kg. General anesthesia was induced using Thiopentone sodium at 5mg/Kg administered intravenously. Anesthesia was maintained with inhalant anesthetic Isofluorane at 1.5 to 2% and propofol 50 mg intravenous bolus administered as and when required. Sheep was positioned on right lateral recumbence and thoracotomy was done through left 4th intercostal space. Lung was retracted, azygous vein was ligated and divided. Pericardium was opened and tented. Under systemic heparinisation at 3mg/Kg, descending aorta and right atrium was cannulated using 18 size aortic cannula 31size DLP cannula respectively to institute CPB. Under cardioplegic heart arrest by delivery through aortic root, bioprosthetic valve was implanted using 15 to 18 3/0 pledgeted coated polyester sutures in horizontal mattress fashion taken at the mitral annulus retaining the native valve and chordae tendinae. Mitral annulus was approached through left atrium via a diagonal incision. Valve opening and closing was checked and left atrial incision was closed with two layers of 5/0 prolene sutures. Aortic clamp was removed and heart resumed rhythm spontaneously in most of the time. In some cases external defibrillation was required. Heart was de-aired, bypass was terminated and heart decannulated in stages with dopamine support. Chest was closed in layers with a pleuro-pericardial drain. The sheep were given 100mg Dipyridamol once daily for up to one-month post operatively as antiplatelet. Apart from this intramuscular Tramadol 50mg SID, Dexamethasone 4mg SID for 3 days, Ceftriaxone-Sulbactum 1.5 g SID for 7 days, Paracetamol 300mg and Meloxicam 5mg SID for 3 days were given along with oral probiotics post-operatively. Chest tube was removed on the next day and sutures were removed on 10th day.

Test valve fabrication

Test valves were fabricated using heparin conjugated bovine pericardium which had completed ISO 10993 tests and *in vivo* calcification study in juvenile rat subcutaneous implantation model. The first sheep was implanted with a bioprosthetic valve which is having heparin coated rough (fibrous) surface as the inflow surface, and the rest three sheep with the bioprosthetic valve with the heparin coated smooth surface (parietal surface) as the inflow surface. The planned observation period was 6 months. One sheep was retained up to 12 months.

Statistical analysis

Statistical analysis was performed using Microsoft Excel software. F test was performed to study the variance and based on its significance, Student's t- test (for equal or un-equal variance) was used for further comparison between test and control groups. The results were considered significant at p-values less than 0.05 and presented as mean ± standard deviation (SD) along with p-value.

Results

Demonstration of bound heparin

Qualitative study: O-Toluidine blue staining

The presence of the conjugated heparin remaining on the sample surface after 60 minutes and 7 days of incubation under agitation was demonstrated by ortho toluidine blue staining reaction. The intense blue color of the metachromatic dye in comparison to light blue color of control samples indicated presence of surface bound heparin on heparin coated processed pericardium even after incubation in PBS (pH 7.4) for 7 days under agitation (figure 1).

Micro Raman Spectroscopy

Analysis of the Raman spectra of heparin coated and uncoated glutaraldehyde processed pericardia showed high level of background fluorescence in both groups on account of glutaraldehyde treatment as observed elsewhere [29]. Compared to the control group, spectrum for heparin coated glutaraldehyde processed bovine pericardium showed a distinct peak of low intensity at 1085 cm^{-1} and an increase in intensity of the peak in the range 1250-1260 cm-1 (figure 2), which can be assigned to the absorption by the primary and secondary sulfate groups of the heparin molecule as reported earlier [30].

Static water contact angle measurement

Water contact angle for both the rough and smooth surfaces of the heparin coated sample as well as the native bovine pericardium were below 90º and hence it is assumed to be hydrophilic. Moreover, a decrease in contact angle for the smooth surface compared to that of the rough surface as indicated in figure 3 was observed in both the groups irrespective of having heparin coating. Following heparin coating the contact angle decreased more for rough surface than for smooth surface which was statistically significant. Measured contact angle decreased from 80.45º ± 4.37º to 68.11º ± 6.87º for the rough

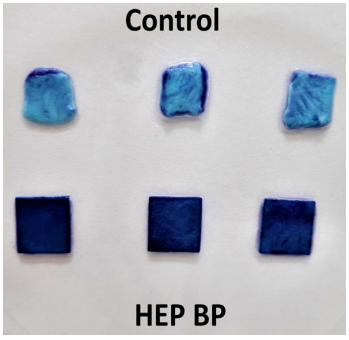


Figure 1: Photograph showing O-toluidine adsorption by heparin coated bovine pericardium as compared to native tissue even after seven days of incubation in PBS (pH 7.4)

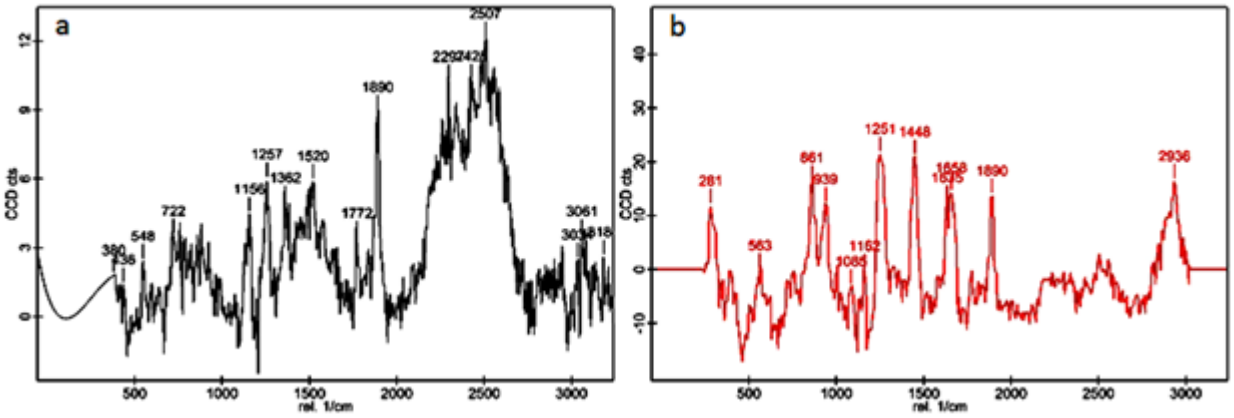


Figure 2: Micro Raman spectra of (a) Heparin uncoated glutaraldehyde processed bovine pericardium control group and (b) Heparin conjugated glutaraldehyde processed bovine pericardium. Figure (b) shows a distinct peak of low intensity at 1085cm $^{-1}$ and an increase in intensity of the peak in the range 1250-1260cm^{-1}

surface (p=0.001) and from 74.54º ± 5.32º to 66.9º ± 6.57º for the smooth surface (p=0.02), implying that following heparin coating, the pericardial surfaces have become more hydrophilic, with the effect of coating more prominent in the case of rough surface. The contact angle for glutaraldehyde processed heparin uncoated tissue could not be measured as sessile drop could not be formed on its surface and is reported as highly hydrophobic.

Estimation of bound heparin

Colorimetric assay determined the residual O-toluidine dye in the immersion liquid by measuring the spectrophotometric absorbance at 631nm after the set incubation periods. The depletion in absorbance was used as an indirect measure of bound heparin concentration on processed tissue surface. Following the assay and substituting the observed absorbance value in the polynomial equation, the heparin content was measured to be 0.717 ± 0.019 mg/500mg (wet weight) processed tissue following heparin conjugation.

Functional evaluation of bound heparin

Platelet adhesion study

When compared to uncoated surface, heparin coating reduced platelet adhesion after incubation in anti-coagulated whole blood for 30 minutes at physiologic pH and temperature as observed from the SEM image given as figure 4. Hence it is understood that the covalent immobilization has not compromised the physiological anticoagulant activity of heparin and the processed bovine pericardium surface is thus rendered less susceptible to platelet adhesion.

Protein adsorption study

Protein adsorption studies done through estimation of eluted protein from the whole blood exposed surfaces revealed that following heparin coating the adsorbed protein content on the heparin coated surface significantly reduced from 0.402 ± 0.019

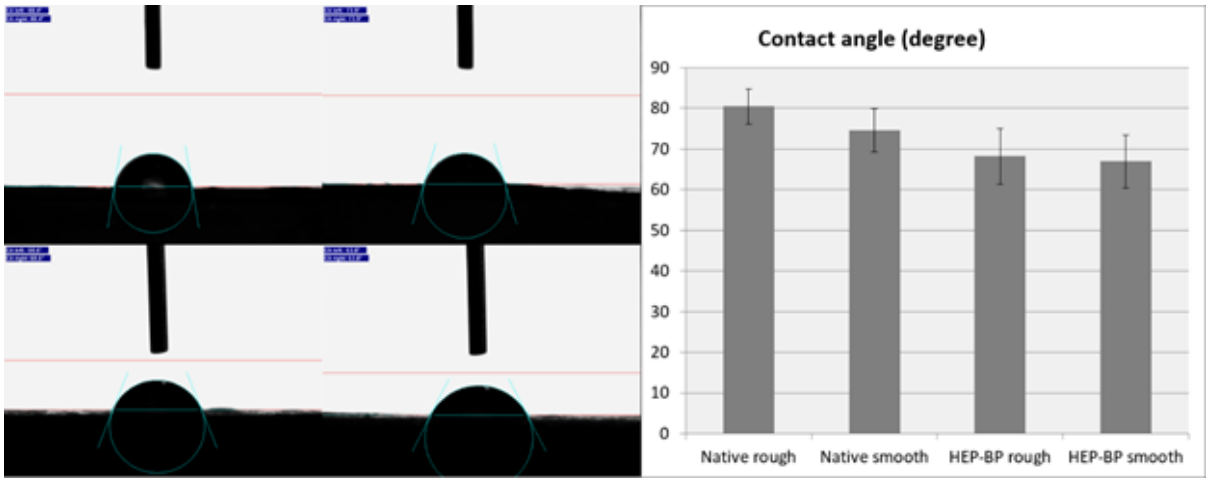


Figure 3: Chart showing water contact angle measurement of A) Native bovine pericardium rough surface, B) Native bovine pericardium smooth surface C) Heparin coated bovine pericardium rough surface and D) Heparin coated bovine pericardium smooth surface

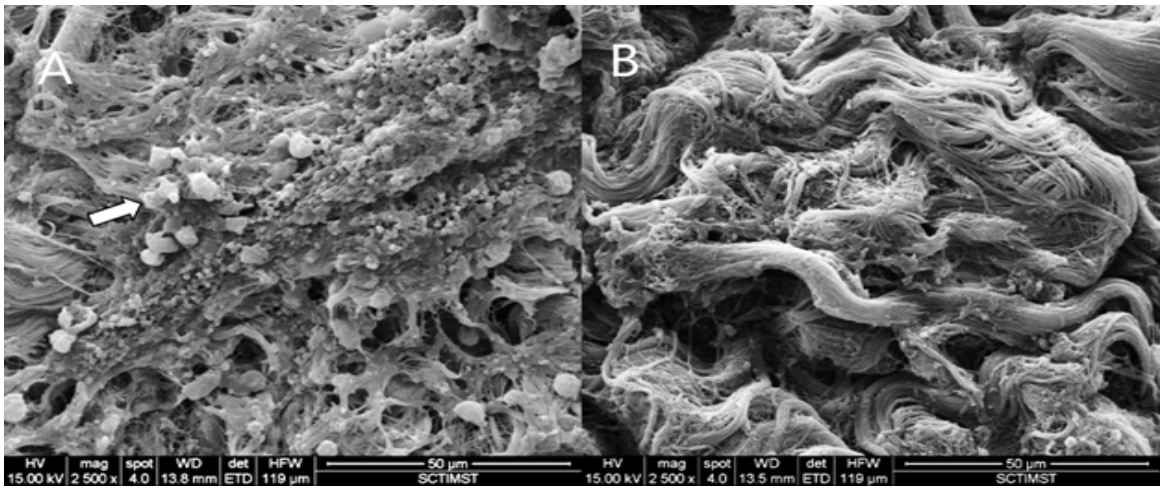


Figure 4: ESEM picture showing the **presence of platelet adhesion on heparin unbound BP surface (A-bold arrow) in comparison to its absence in heparin coated BP surface (B)**

mg/100mg tissue to 0.227 ± 0.0519 mg/100 mg tissue (p=0.01). This indicated the possibility for reduced blood coagulation on heparin coated pericardial surface (figure 5). This observation correlates well with the knowledge that when attached to a surface, covalently immobilized heparin has the capacity to reduce the thrombogenicity of artificial materials by way of reducing pro-coagulant protein adsorption on to surface [32], the effectiveness of which is dependent on the extent to which the catalytic activity of heparin is preserved after immobilization process, specifically on the AT uptake capacity of the heparin coated surface [33].

In vitro surface thrombogenicity evaluation

Thrombogenicity of the heparin coated tissue surface was analyzed in comparison to that of uncoated tissue following whole blood kinetic clotting time method with standardized time interval of 15 minutes. The contribution of rough and smooth pericardial surface to its surface thrombogenicity was also studied. The concentration of free hemoglobin measured by reading the absorbance at 540nm is as an indirect indication of the size of clot formed on the tissue surface by tissue-blood interaction. It is assumed that only the RBCs outside of the clot are lysed by the addition of distilled water resulting in the release of hemoglobin into the medium. Therefore, absorbance value observed is inversely proportional to the size of clot, and hence to the surface thrombogenicity thereby indicating that higher hemoglobin value corresponds to lesser surface thrombogenicity (figure 6). As per this test, heparin coated smooth surface of the pericardium showed least thrombogenicity followed by heparin coated rough surface, heparin un-coated smooth surface and finally heparin uncoated rough surface which showed the least thromboresistance among the samples (figure 7). It was observed that, following heparin coating, the thromboresistance of smooth surface of glutaraldehyde processed pericardium significantly improved (p=0.0001) compared to that of rough surface (p=0.0005). It was also observed that the rough and smooth surfaces of heparin coated tissue also differed significantly (p=0.006) in their thromboresistance, whereas in the case of heparin uncoated tissue, this difference, though present, was not statistically significant (p=0.067).

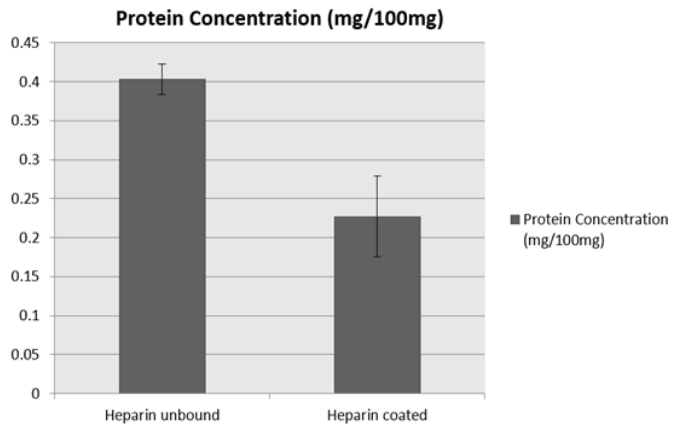


Figure 5: Chart showing eluted protein content in heparin uncoated and coated processed bovine pericardium respectively

Bioprosthetic valve implantation in sheep

Sheep implanted with the bioprosthetic valve having rough surface of pericardium forming the inflow surface showed bilateral jugular pulse at the base of the neck by 46th post-operative day and this ascended upward distally. Concomitantly, animal started showing tachypnea and respiratory distress which was managed with bronchodilators and diuretics. However, on 53rd day this sheep was found dead. Autopsy of this animal showed thrombus formation on the inflow surface of the valve almost occluding the valve affecting the forward blood flow (figure 7).

The other two sheep implanted with the bioprosthetic valve having smooth pericardial surface forming the inflow surface, completed the scheduled observation period of 6 months. Autopsy at the end of this observation period revealed thrombus free inflow surface (figure 8).

Discussion

The present study aimed at producing a thromboresistant surface

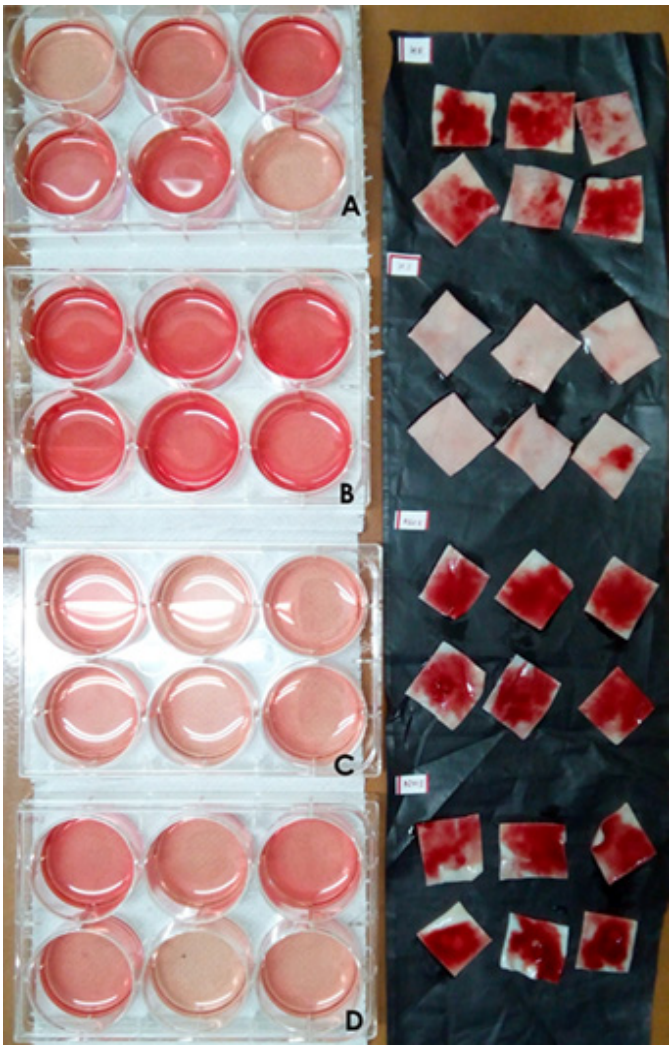


Figure 6: Photograph showing difference in clot formation in treatment groups A: Heparin coated rough surface, B: Heparin coated smooth surface C: Non-heparin coated rough surface D: Non-heparin coated smooth surface. Note the color intensity of **pericardial wash in petri** dish correlates with clot **formation on the pericardial surface as maximum clot formation in sample D and vice versa in sample B**

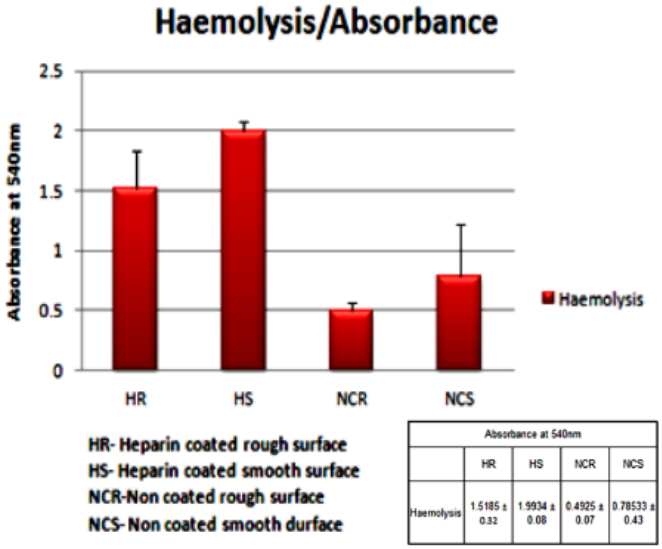
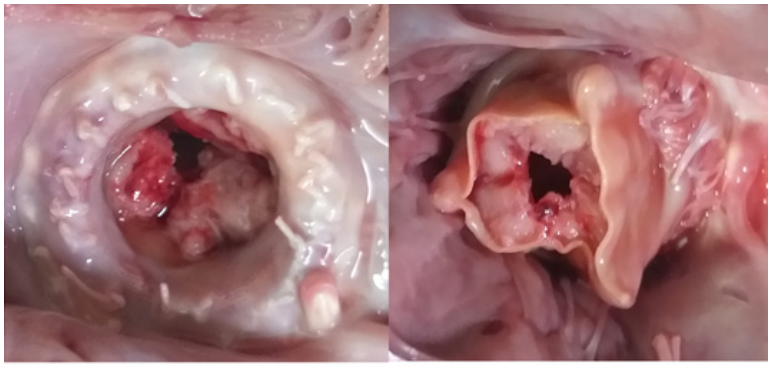


Figure 7: Chart showing the absorbance values at 540nm for the rough and smooth surfaces of Heparin coated and uncoated processed pericardia. Graph indicates absorbance as a measure of hemolysis. The greater the absorbance value, lesser the size of clot formed

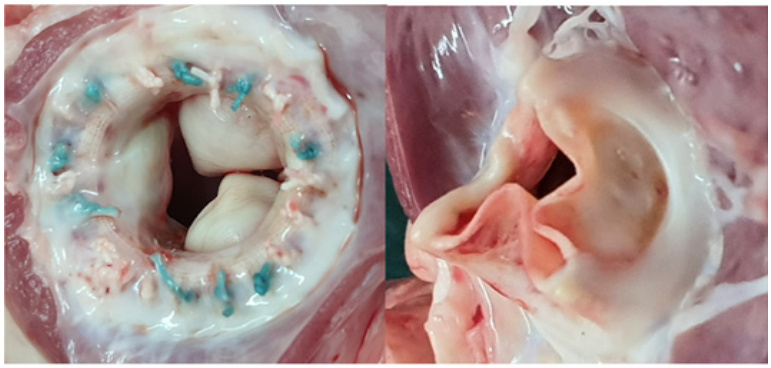
on glutaraldehyde processed bovine pericardium using a simple single step method and demonstrated the difference in thrombogenicity of rough (fibrous) and smooth (parietal) surface of heparin coated pericardium. It was carried out by heparin conjugation to a glutaraldehyde processed bovine pericardial surface, by hemi-acetal and acetal formation between aldehyde groups of the glutaraldehyde treated pericardium and OH groups of the heparin under acidic pH, without affecting sulphate groups responsible for the anticoagulation property of the heparin. This simple single-step reaction possibly has led to the formation of direct covalent linkage between heparin and glutaraldehyde treated pericardium resulting in a stable, functionally active heparin coated tissue surface. Literature reveal that the activity of surface bound heparin is largely dependent on the type of surface interaction between heparin and the material [34-36]. Therefore, surface binding of heparin does not always guarantee effective anti-thrombotic activity. Through this study it was demonstrated that this method of heparin conjugation on glutaraldehyde processed bovine pericardium did not compromise the anticoagulant activity of heparin. The successful immobilization of heparin to the processed pericardium tissue was visualized qualitatively through O-toluidine blue staining of heparin coated tissue in comparison to native tissue as control and quantitatively through the estimation of bound heparin.

The presence of heparin on glutaraldehyde processed bovine pericardium surface was confirmed by micro Raman spectroscopy. For glutaraldehyde cross linked pericardium, a weak peak in the range between 1150-1060 cm^{-1} corresponds to the asymmetric stretch C-O-C vibration [41] which can be attributed to an ether-type bond formation between the collagen fibers and glutaraldehyde [42]. In the present study, the presence and intensity of the peak in the range 1150-1060 cm^{-1} in comparison with the control tissue may assumed to be contributed additionally by the acetal bond formation between the aldehyde groups of glutaraldehyde and hydroxyl groups of heparin. Studies have previously shown that glutaraldehyde cross-linking in pericardium results in very high fluorescence background, which interferes with the Raman spectra [29]. This may be factored into consideration for the very low intensity of the peaks formed and the presence of large number of peaks that could possibly skew the distribution of the significant peaks for heparin.

Results of protein adsorption and platelet adhesion studies showed an overall decrease in the amount of coagulation effectors on the heparin bound pericardium which may be attributed to its anticoagulant activity observed in the heparin coated samples. Heparin, through the interaction of its high affinity pentasaccharide sequence with antithrombin [37], inhibits the downstream coagulation cascade through inactivation of coagulation enzymes such as thrombin factor (IIa), factors Xa, IXa, XIa, and XIIa [38] Also, it is known that heparin is a highly hydrophilic molecule and studies have previously shown decreased adsorption of plasma fibrinogen and subsequent thrombin formation on hydrophilic surfaces due to specific surface adsorption through áC-domain of



Thrombus on the inflow region formed by rough pericardial surface at 53 days



Thrombus free inflow region formed by smooth pericardial surface at 6 months

Figure 8: Photograph showing thrombus formation on valve inflow in the first row (heparin coated rough pericardial surface) compared to thrombus free inflow in the second row (heparin coated smooth pericardial surface)

fibrinogen [39]. The results of static water contact angle for the heparin coated tissue indicate that this surface modification rendered the surface even more hydrophilic than the native pericardium surface. Heparin itself is a hydrophilic molecule with various functional groups that contribute to its hydrophilic nature [40]. Direct conjugation of heparin to the glutaraldehyde processed bovine pericardium is thus expected to lower the contact angle for the otherwise hydrophobic modified surface, which could have potentially lowered both protein adsorption and subsequent platelet adhesion by the altered surface adhesion of plasma proteins. The reduced protein adsorption through the above mentioned mechanisms may be attributed to the overall decrease in platelet adhesion on the heparin coated glutaraldehyde processed tissue as visualized in the SEM.

The difference in thromboresistance of heparin coated rough and smooth pericardial surfaces were demonstrated by whole blood kinetic clotting time method as this method allowed specific study of coated surfaces. Results indicated that the surface thrombogenicity for the rough and smooth surfaces of heparin coated tissue varied significantly with increased thromboresistance for the smooth surface. Interestingly, for the heparin uncoated tissue, the difference in surface thrombogenicity identified was not statistically significant. Pericardial valves are typically fabricated with the rough side toward the inflow, to keep this surface well washed and thereby minimize the possibility of thrombosis [44]. However, the first bioprosthetic valve fabricated in this fashion with the heparin coated rough surface forming the inflow surface, thrombosed in 53 days in spite of the proven anti-platelet therapy given to this sheep [45]. Interestingly, the rest two sheep implanted with the bioprosthetic valve having heparin coated smooth surface forming the inflow, completed the planned observation period of six months without valve thrombosis. This raises an interesting question, that is 'does the hydrophilic heparin coating make the rough surface of pericardium vulnerable to valve thrombosis even when it is significantly more thromboresistant than hydrophobic heparin uncoated pericardium?. A conclusive answer to this question will be relevant in the manufacture of bioprosthetic heart valve using pericardium coated with hydrophilic thromboresistant coating such as heparin.

Conclusion

Through the *in vitro* experiments it is successfully verified that the key events in thrombus formation such as protein adsorption and platelet activation which are critical in determining the success of bioprosthetic valve with respect to subclinical valve thrombosis, are considerably reduced in heparin modified glutaraldehyde processed bovine pericardium in contrast to heparin unmodified tissue. And, differential thromboresistance were observed in the smooth (parietal) and rough (fibrous) surface of the pericardium with maximum thromboresistance observed in heparin coated smooth surface and the least thromboresistant was non-heparin coated rough surface of pericardium. However, contrary to the established knowledge, rough surface of pericardium caused valve thrombosis when it is heparin coated and hydrophilic.

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