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ABSTRACT

QUANTITATIVE ESTIMATION OF HEPARIN CONTENT ON BIOLOGICAL PERICARDIUM TISSUE USING IN VITRO ANTI-FACTOR Xa ASSAY

by
Jean-Claude Simeon

Shelhigh Inc. (Millburn, NJ) has developed a new heparin binding technique to biological tissues. The technique seems to bind heparin to tissues in a more permanent fashion. Shelhigh's heparinized biological tissues are intended for use in long term implants. This investigation was undertaken to assess whether heparin leaching is prevalent when tissues are quarantine for long shelflife at room temperature, whether tissues maintained thromboresistance properties when in contact with plasma and to estimate heparin content of the tissues.

The study used anti-factor Xa assaying methods: clotting time and chromogenic factor Xa sensitive substrate in order to fulfill the aforementioned objectives. Anti-factor Xa assay was chosen because of its specificity. It characterized the role of heparin in the inactivation of factor Xa. Storage solutions containing heparinized pericardium tissues were mixed with equal volume of plasma in order to assess for heparin leaching. The average clotting time for these solutions was 22+/-.50 seconds. This result was in the range of clotting time of zero unit heparin in plasma. When Shelhigh's heparinized pericardium tissues were exposed to plasma at $37^{\circ}C$, neutralization of clotting factor Xa was accelerated as measured by the clotting time and chromogenic substrate method, and this was attributed to trace amounts of heparin released from the pericardium tissues.

This demonstrated that Shelhigh's pericardium tissues have thromboresistance properties when in contact with plasma.

To estimate the heparin content on the pericardial tissues, conventional methods used to estimate heparin content on material surfaces was not applied. In this investigation, heparin is not immobilized on the tissue surfaces. Rather, the heparin is attached onto the matrix of the tissues. Therefore, to estimate heparin content on heparinized pericardium tissues, the tissues had to be dried, ground and incubated in plasma at $37^{\circ}C$. The estimated heparin content measured on tissue samples according to the clotting time and chromogenic substrate was observed to be varying, although all of the tissues were heparinized using standard manufacturing procedures.

The contribution of heparin released from Shelhigh's heparinized biological pericardium tissues stored in storage solution at room temperature was found to be negligible. When the tissues were in contact with plasma, neutralization of clotting factor Xa increased dramatically. Therefore, it can be concluded that heparin from Shelhigh's pericardium tissues markedly improves thromboresistance properties.

QUANTITATIVE ESTIMATION OF HEPARIN CONTENT ON BIOLOGICAL PERICARDIUM TISSUE USING IN VITRO ANTI-FACTOR Xa ASSAY

by
Jean-Claude Simeon

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May 1998

APPROVAL PAGE

QUANTITATIVE ESTIMATION OF HEPARIN CONTENT ON BIOLOGICAL PERICARDIUM TISSUE USING IN VITRO ANTI-FACTOR Xa ASSAY

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Dedicated
to my family,
mom, sister, brothers, nieces and nephews

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CHAPTER 1

INTRODUCTION

1.1 Background Information

Heparin is a sulfated glycoaminoglycan mixture, composed of repeating monosaccharide units of L-iduronic and glucosamine (Figure 1) (1). Due to its structure and surface charge, heparin is able of interacting with several blood clotting factors in a specific manner (9). Heparin is widely used as an anticoagulant that aids in the circulation of blood in patients. In addition, it has been used in extracorporeal devices (i.e. hemodialysis, surgical procedures and/or heart-lung machines) and for biological tissues other than the natural endothelial lining wall to procure thromboresistance properties. Experiments have shown that the blood compatibility properties of bound heparin behave in similar manner to that of the fluid phase. That is, like heparin in solution, bound heparin accelerated the inactivation of thrombin and factor Xa by antithrombin-dependent mechanism (2-7).

The finding that bound heparin exerts similar anticoagulant activity to that in solution has led to several hypothetical mechanisms about the fates of bound heparin (Table I). For example, when heparin is released from a surface, there exist a heparin microenvironment at the material/blood interface to impart thromboresistance properties (4).

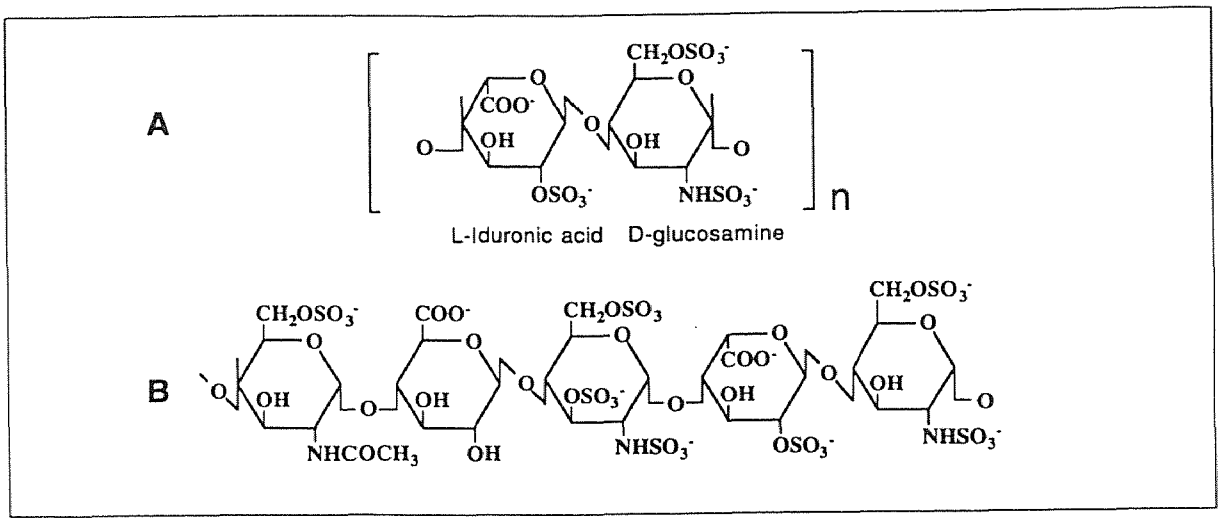

Figure 1. Heparin Structure

Heparin is a linear polysaccharides mixture composed of repeating units of L-Iduronic acid and D-glucosamine. [A] is the most common disaccharide unit, representing 90 % of the beef-lung structure, and up to 70% pig-mucosa heparin. [B] is a unique pentasaccharide binding site for antithrombin.

Taken from (1, p.4) by Chromogenix Monograph Series

Table I. Conceivable Fates of Surface-Bound Heparin

- Bound heparin is released creating a heparin microenvironment at the material/blood interface.
- Bound heparin remains attached to the material, but becomes saturated with inactive complex.
- Heparin acts catalytically and does not become saturated, but a high production of inactive complex causes a systemic hypocoagulability.
- Heparin does not become saturated, and inactive complex formation is maintained to within tolerable levels.

Taken from (4, p.149) by M.F.A Goosen and M.V. Sefton

The anticoagulant activity of heparin is characterized by its interaction with antithrombin III, the primary inhibitor in the blood coagulation system. Antithrombin III inactivates thrombin by forming an irreversible-antithrombin III complex. In addition, antithrombin III inactivates several serine proteases clotting factors, namely factors XIIa, XIa, IXa, and Xa. However, of all the blood coagulation proteases, only the antithrombin III dependent-mechanism of thrombin and factor Xa is effectively inhibit (2,3,7). The inhibitory capacity of antithrombin III is enhanced in the presence of heparin. Heparin accelerated the rate of formation of irreversible complexes between antithrombin III and thrombin and factor Xa. Heparin inactivates thrombin and factor Xa by antithrombin III bound to end-point attachment mechanism (Figure 2) (1,9).

The accelerating function of heparin depends on a highly specific antithrombin-binding pentasaccharide sequence of the heparin molecule. Binding to this pentasaccharide sequence causes a conformational change in antithrombin III that increases the anti-thrombin and anti-factor Xa activity several folds. In addition, the effect of heparin on the reaction between antithrombin III and thrombin require the heparin chain to be of a certain length (> 18 monosaccharides). The inactivation of factor Xa, on the other hand, can be achieved by very small heparin chain length (>5 monosaccharides) (1,9). Despite the facts mentioned above, the elucidated mechanism between heparin and antithrombin III is open to debate. There exist two prevalent opinions about the mode of heparin action with antithrombin III. First, heparin (Table II) is thought to react with thrombin producing a conformational change in thrombin causing a rapid complex formation with antithrombin III (Figure 2). The alternative mechanism, proposed that heparin binds to antithrombin III, inducing a conformational change that convert antithrombin III from a slow inhibitor to a very fast inhibitor (1-7). The heparin-antithrombin III complex then inhibits several coagulation proteases, which included factors IXa, Xa, XIa, XIIa, thrombin and as well as plasmin and kallikrein, supporting the heparin to antithrombin III mechanism (Figure 3). However, there are numerous experiments or results that favored the thrombin-heparin mechanism (2,13).

Table II. Mechanisms for Inactivation of an Enzyme by Inhibitor in the Presence of Activator

- Heparin binds to enzyme (Thrombin or Factor Xa) to make it more susceptible to antithrombin.
- Heparin reacts with the enzyme-inhibitor complex and accelerates the formation of a covalent bond between enzyme and inhibitor.
- Heparin activates antithrombin to make it a more potent/ faster inhibitor

Taken from [13, p.838] by J. Sturzebecker and F. Markwardt

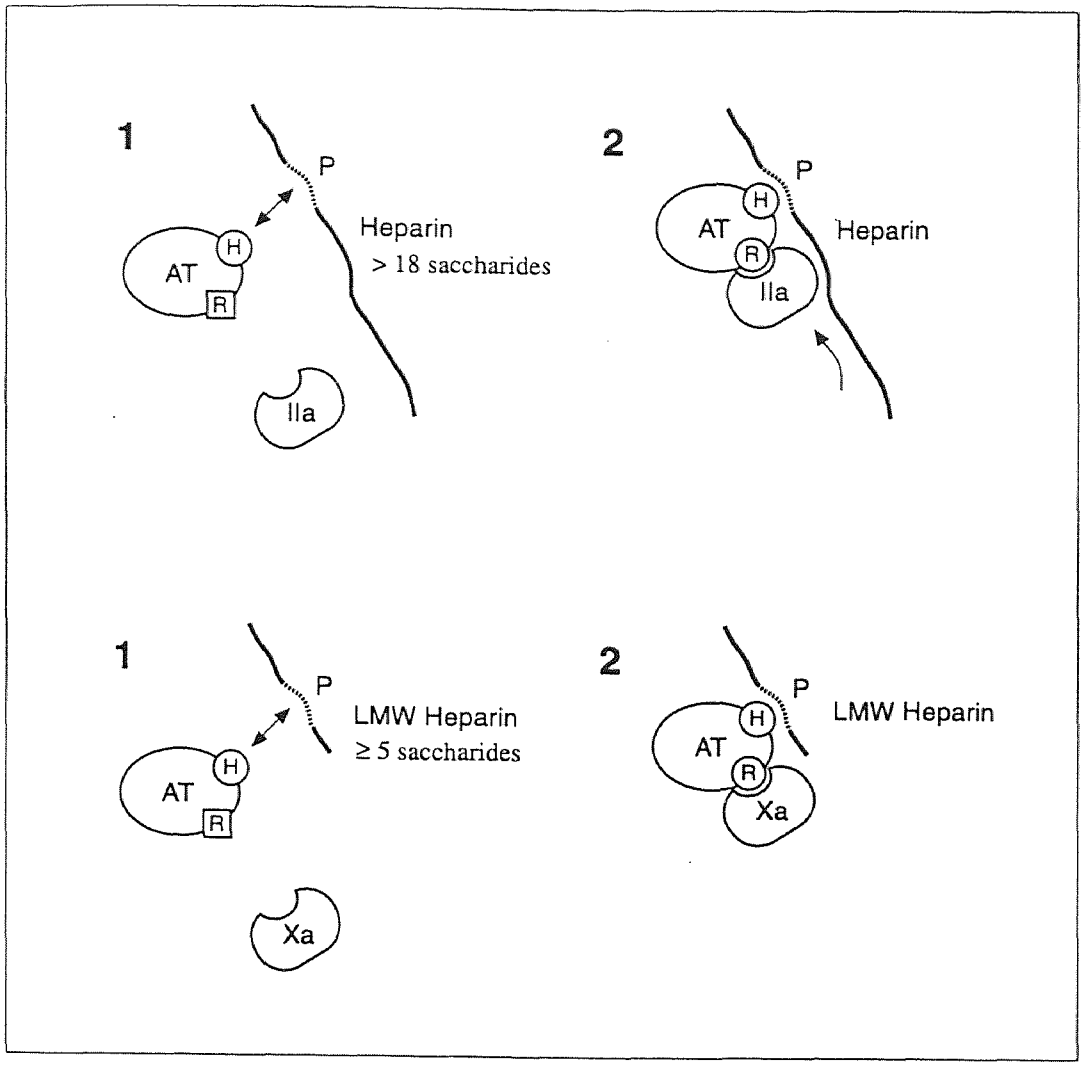

Figure 2. Model Describing How Heparin Catalyzes the Antithrombin-Protease Reaction [H] represents the heparin binding site and [R] is the reactive site in the antithrombin and [P] is the unique antithrombin sequence binding sequence of heparin,. Binding to this sequence causes a conformational change in antithrombin, which enhanced reaction with it proteases target. **Top:** Demonstrated the effect of heparin on the reaction between antithrombin (IIa), the heparin which must be of certain length (> 18 monosaccharides) reacts with enzyme-inhibitor complex to accelerate the formation of covalent bond between enzyme and inhibitor. Thrombin binds nonspecifically and slides along until encounter the bound antithrombin. **Bottom:** Inactivation of factor Xa does not require ternary complex formation and is achieved solely through antithrombin binds to heparin. *Taken from (1, pp.7) by Chromogenix Monograph Series*

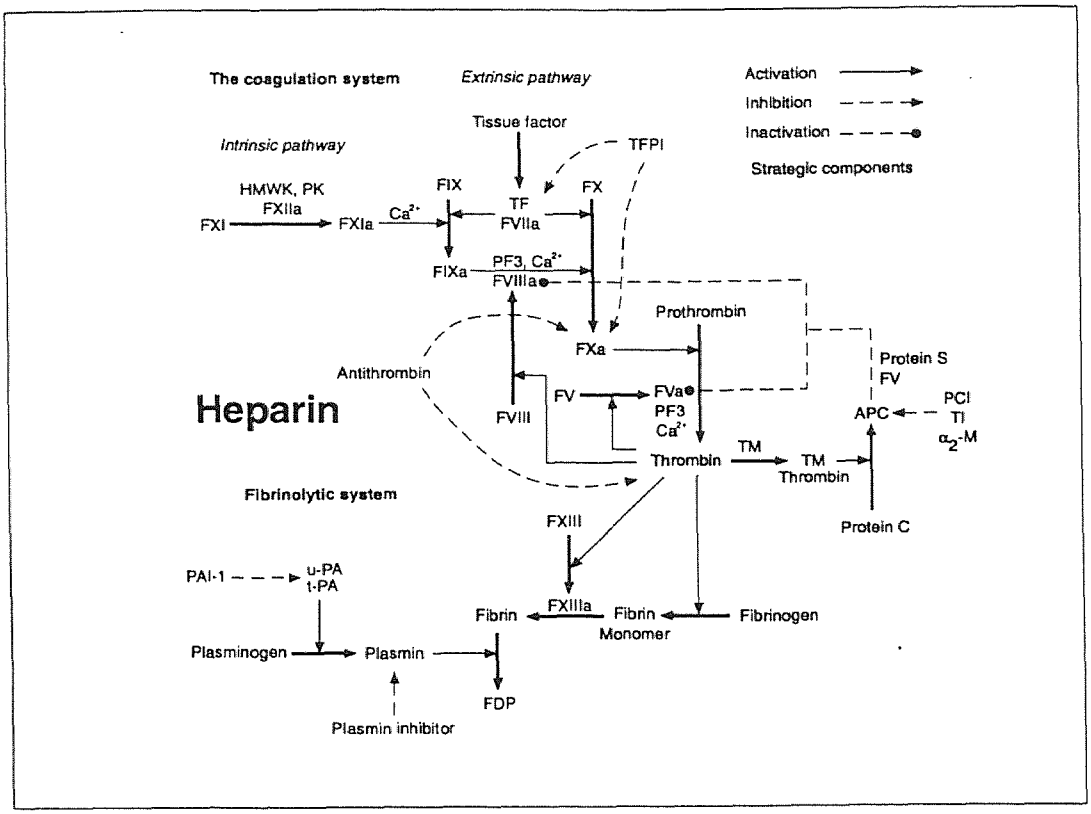

Figure 3. Coagulation Cascade. Blood coagulation is an enzymatic event initiated in response to tissue damage and introduction of foreign materials other than the natural endothelial lining. Heparin, an anticoagulant agent inhibits blood from clotting primarily by its binding to antithrombin, thereby accelerating the inhibition of Xa and thrombin in plasma. *Taken from (1, pg. 4) by Chromogenix Monograph Series.*

The finding that bound heparin exerts anticoagulant effect similar to that of fluid phase has led to numerous studies to alter implantable artificial material (i.e. polymers and biological tissues) in order to deem them blood compatible properties. Researchers typically take two approaches in modifying foreign biomaterials: modification of foreign biomaterials surface, in which preexisting materials are altered or new materials are produced with properties that procure thromboresistance, or the pharmaceutical approach, in which anticoagulant agents (e.g. heparin) are applied along with the materials (9,12).

Heparinization of foreign biomaterials has been achieved by two well-established methods: covalent and ionic bonding. Covalent end-point attachment of nitrite degraded heparin on artificial biomaterials procures good thromboresistance properties to foreign biomaterials, without systemic anticoagulant treatments (2,3,5). Ionic bonding involves formation of quaternary amine groups onto biomaterial surface into which negatively charged heparin can be attach. Heparinization treatment of foreign biomaterials prepared by ionic coupling also procured good blood compatibility properties to biomaterial surfaces. However, due to heparin's rapid removal rate from the biomaterials, this approach is rendered to be unsuitable for long-term implants (2,3). Shelhigh Inc. (Millburn, NJ) has developed a new heparin binding technique to biological tissues. The technique seems to bind heparin to tissues in a more permanent fashion. Shelhigh's heparinized biological tissues were able to prolong in vitro clotting times, and extend the blood biological patency when in vivo. This study was intended to evaluate the biological activity of heparin bound to biological pericardium tissues.

1.2 Objectives

In light of the facts aforementioned, the following objectives for the present investigation were set:

1. To assess whether heparin is released from heparinized biological pericardium tissues into storage solution (2 % benzyl alcohol) during long shelf life.
2. To assess whether heparinized biological pericardium tissues have anticoagulant properties when in contact with plasma.
3. To quantitatively estimate heparin content in heparinized biological pericardium tissues.

This study used anti-factor Xa assays: clotting time assay introduced by Yin et al. (20) and a chromogenic factor Xa sensitive substrate assay introduced by Teien et al. (18). The anti-factor Xa assay was chosen because of its specificity. It characterized the ability of heparin-accelerated antithrombin to inactivate clotting factor Xa. The clotting time assay introduced by Yin et al. is based on heparin catalyst activity to accelerate the neutralization of factor Xa. Then, a clotting time technique (i.e. Fibrometer) is used to measure the residual factor Xa. The chromogenic factor Xa sensitive substrate assay is a photometry version of anti-factor Xa. The assay used a synthetic factor Xa sensitive substrate to measure the residual factor Xa, which correspond to a heparin concentration present in a testing sample.

This study used the aforementioned assays to determine whether heparin is leached from Shelhigh's biological pericardium tissues when quarantined for a long shelflife; whether heparin from biological pericardium tissues is biologically active when in contact with plasma; and to quantitatively estimate the heparin content in biological pericardium tissues. This investigation was performed at Shelhigh Inc. (Millburn, NJ) facilities.

CHAPTER 2

MATERIALS AND EXPERIMENTAL METHODS

2.1 Materials

Saline 0.9 gram sodium chloride dissolved in 100 ml distilled water to make a 0.9 (w/v) % saline solution. Used to dilute concentrated heparin.

Heparin. A highly sulfated polysaccharide derived from porcine intestines (Elkins Sinn, Inc., Cherry Hill, NJ, lot no. 106080), 5000 Units/ml diluted in 0.9 (w/v)% saline solution to 100 Units/ml. Heparin is anticoagulant when combines with antithrombin III increases the effectiveness of antithrombin III from a hundred-to-a thousandfold in neutralizing thrombin and factor Xa.

Acetic acid 3 M or 20 % acetic acid. Used to stop reaction in the chromogenic factor Xa sensitive substrate assay (end-point method).

Fresh Frozen Plasma. Collected and processed by the Blood Bank Center of New Jersey Inc., E. Orange, NJ. Obtained from the University of Medicine and Dentistry of New Jersey (Newark, NJ) blood bank.

Biological pericardium tissues. Commercial pericardium tissues (Shelhigh Inc., Millburn, NJ)

2.2 Anti-factor Xa Clotting Time Assay Reagents

Bovine Factor Xa. Catalog No. F 3649 (SIGMA DIAGNOSTICS)

Lyophilized bovine Factor Xa reconstituted with 2 ml distilled water according to the manufacturer instructions contains bovine serum albumin, sodium chloride, PEG and tris-maleate pH 7.5. The factor Xa is neutralized when incubate in heparinized plasma. The residual factor Xa is measured using a Fibrometer to measure the time it takes for plasma to clot. The amount of factor Xa neutralized is proportional to the heparin concentration present in the coagulation reaction mixture.

RECALMIX, Catalog No. R0887 (SIGMA DIAGNOSTICS)

Lyophilized rabbit brain cephalin with buffer reconstituted with 2 ml distilled water according to the manufacturer instructions, contains calcium chloride and bovine plasma fraction rich in fibrinogen and factor V, pH 7.5. This mixture contained maximum concentration of calcium chloride and brain cephalin in bovine plasma fraction, used to recalcify a reaction mixture after a predetermined incubation period.

2.3 Anti-factor Xa Chromogenic Assay Reagents

Bovine Factor Xa. Catalog No. B 4031 (SIGMA DIAGNOSTICS)

Lyophilized bovine Factor Xa contains approximately 20 nKat/vial with 0.1% sodium azide as preservative, reconstituted with 5 ml distilled water according to the manufacturer instructions. The neutralization of factor Xa in the chromogenic assay is based on the same principle as described above. However, the residual factor Xa is measured using a synthetic factor Xa chromogenic substrate.

Human Antithrombin III. Catalog No. F3524 (SIGMA DIAGNOSTICS)

Lyophilized preparation, when reconstituted with 5 ml distilled water contains human antithrombin III, 0.5 units/vial, tris-HCl, 0.05 M, sodium chloride, 0.175 M, EDTA, 7.5 mM, pH 8.4 and 0.1 % sodium azide as preservative. The addition of antithrombin II helped to reduce the effect of varying antithrombin II concentrations in the assay.

Factor Xa Substrate MeO-CO-D-CHG-Gly-Arg-pNA. Catalog No. F 3524 (SIGMA DIAGNOSTICS)

Lyophilized MeO-CO-D-CHG-Gly-Arg-pNA, reconstituted with 5 ml-distilled water according to the manufacturer instructions. The factor Xa substrate is used to measure the residual factor Xa. When incubated with factor Xa, the paranitroaniline (pNA) attached to the end is cleaved and a yellow color is observed. The yellow color is inversely proportional to the heparin concentration present in the reaction mixture.

2.4 Instruments

Spectrophotometer 405 nm, Shimadzu Scientific Instruments, Inc. (7120 Riverwood Drive, Columbia, Maryland, 21046 USA).

Fibrometer BBL Fibrometer, Precision Coagulation Timer (Becton Dickinson Microbiology System, 250 Schilling Circle, Cockeysville, Maryland 21030 USA).

Heating device Precision, Molecular Biology Waterbath Model 180

Grinder Braun Grinder, KSM2, Model 4041

2.5 Experimental Methods

2.5.1 Preparation of Standards

2.5.1.1 Anti-factor Xa Clotting Time Assay: This method is based on the principle of incubation activated factor Xa with plasma at $37^{\bullet}C$. A RECALMIX solution, which contained brain cephalin, calcium chloride and bovine plasma fraction rich in fibrinogen and factor V, pH 7.5 is then added to the reaction mixture. The time it takes (i.e. seconds) for the plasma mixture to clot is recorded using a Fibrometer and converted to units heparin per ml plasma using the prepared standard curve. The presence of heparin in the reaction mixture prolonged the clotting time by neutralizing the factor Xa in the incubation mixture. The neutralization of clotting factor Xa is directly proportional to the heparin content present in testing plasma (17, 20).

The assay in detailed is composed of pre-warming the RECALMIX mixture for at least 5 minutes at $37^{\bullet}C$. Then, $100\mu L$ of test plasma or standard plasma is added to a pre-warm coagulation reaction cuvet and incubated for 2 minutes at $37^{\bullet}C$. After the incubation, $100\mu L$ factor Xa is added to the coagulation reaction cuvet and mixed thoroughly. After exactly 2 minutes, the mixture is transferred to a warm coagulation fibrotube cup containing $100\mu L$ RECALMIX mixture and clotting time is recorded using the BBL Fibrometer, Precision Coagulation Timer (Becton Dickinson Microbiology Systems, 250 Schilling Circle, Cockeysville, Maryland 21030 USA). Clotting time (seconds) is recorded and converted to units heparin per ml plasma using the established heparin standard curve. Tests were performed in duplicates and the results averaged.

When clotting time for a run was more than 10% higher than the shorter clotting time (or vice-versa), a third clotting time trial was performed. Test samples, that gave clotting times outside normal range of the standard curve, were diluted with plasma in 1 in 2, 1 in 3, 1 in 5, or 1 in 10, or with respect to the anticipatory heparin concentration (20).

In order to obtain accurate and reproducible results using the Fibrometer system, the following recommendations were considered: (1) Prewarmed the RECALMIX for exactly five minutes according to the manufacturer instructions. (2) Incubate test plasma or standard plasma and factor Xa together for exactly two minutes. (3) Prewarmed the coagulation reaction cuvet. (4) Clean the Fibrometer probe between run.

2.5.1.2 Anti-Factor Xa Chromogenic Substrate Assay: The present assay analyzed heparin as a complex of heparin-antithrombin III (Table III). The assay involved incubating test plasma or standard plasma with excess factor Xa. Some of the factor Xa is neutralized by the Heparin-ATIII complex in relative proportion to the concentration of heparin present (1). The remaining factor Xa cleaves paranitroaniline (pNA) from a factor Xa specific chromogenic substrate (MeO-CO-D-CHG-Gly-Arg-pNA).

The assay in details involved mixing $25\mu L$ test plasma or standard plasma with $200\mu L$ human antithrombin III. The mixture is then incubated for 2 minutes at $37^{\bullet}C$. After the incubation, $200\mu L$ bovine factor Xa is added to the reaction vessel, solution is mixed and incubated for exactly 60 seconds at $37^{\bullet}C$. After exactly 60 seconds, $200\mu L$ factor Xa substrate was pippeted to the reaction vessel and thoroughly mixed.

The reaction mixture is then incubated for exactly 5 minutes at $37^{\circ}C$. After the incubation, $200\mu L$ of glacial acetic acid is then added into the reaction vessel to stop the reaction (end-point method). The absorbance of the mixture is read spectrophometrically at 405 nm against a blank reagent sample. The absorbance measured decreased linearly with increasing heparin concentration in the samples. The absorbance of a test sample was compared to the appropriate prepared standard curve to yield a corresponding units of heparin per ml plasma. Absorbance was recorded using Shimadzu UV 160U, UV-VIS Recording Spectrophotometer (Shimadzu Scientific Instruments, Inc., 7102 Riverwood Drive, Columbia, Maryland 21046 USA). To obtain accurate and reproducible results the following recommendations were taken into considerations: (1) Used proper pipetting technique and devices. (2) Adhered to incubation times and incubation temperature (i.e. $37^{\circ}C$).

Plasma heparin concentrations for the preparation of the standard curves were prepared by diluting 5000 Units/ml heparin to 100 Units/mL in 0.9% saline solution. $900\mu L$ normal human plasma was mixed with $100\mu L$ of 100 U/mL heparin to give 10 U/ml solution. $100\mu L$ of the 10 U/mL solution was mixed with $900\mu L$ normal human plasma to give a 1 Units/mL concentration. Standard curves were produced for each in vitro assay for solution heparin concentrations of 0.0, 0.125, 0.250, 0.500 units per mL plasma. The heparin used to prepare the standard curves came from a single lot.

Table III. The Measurement Principle of the Chromogenic Substrate Assay

Heparin + ATIII (excess)→ [Heparin*ATIII]
[Heparin*ATIII] + FXa (excess)→[Heparin*ATIII*FXa] + FXa (residual)
FXa Substrate + FXa (residual) → Peptide + pNA

Taken from [1, p. 17] by Chromogenix Monograph Series

2.5.2 Stability of Heparin in Biological Pericardium Tissues

Four vials each contained 20-mL saline solution and 5x5 heparinized tissues were stored under sterile conditions at room temperature. $400\mu L$ of the storage solution from each vial was withdrawn every other week and tested for the amount of heparin released from the tissues. After three weeks of storage, the 5x5 heparinized tissue from each vial was withdrawn from the saline solution and washed with PBS (Phosphate-buffered saline) pH 7.4. Two pieces were then dried in an oven overnight at $60^{\bullet}C$ and later homogenized with normal human plasma. The other pieces were placed in 20-mL normal human plasma and incubated for 8 days in a water bath at $37^{\bullet}C$. $400\mu L$ of the incubated plasma was withdrawn every day and tested for heparin released using the standard methods.

Samples of storage solution (2 % benzyl alcohol) containing heparinized biological pericardium tissues were also tested. These samples solution were subjected to standard assays to quantify heparin leakage during long shelflife. The solutions were taken from different year of manufacturing period. Some of these samples have been quarantined or remained in shelf for more than three years. To quantify heparin leakage from heparinized biological pericardium tissues in storage solution during shelflife, $400\mu L$ of the storage solution was withdrawn and mixed with equal volume of normal human plasma.

2.5.3 Estimation of Heparin Content in Biological Pericardium Tissues

Heparinized and non-heparinized biological (served as control) tissues were removed from storage solution (2 % benzyl alcohol) and washed with PBS, pH 7.4. The tissues were then placed into an oven to dry overnight at $60^{\circ}C$ for 2-3 days. After the tissues were dried, they were ground in a grinder (Braun Grinder, KSM2, Model 4041) for twenty minutes or until tissues are finely ground. The ground tissues were weighed and placed into a test tube containing 4-mL normal human plasma. The mixture was mixed and allowed to incubate in water bath at $37^{\circ}C$ for a 4 hours period, with frequent stirring to enhance the diffusion of heparin from the tissues. After the incubation, plasma was pippetted into a cuvet, which were subsequently tested for heparin using the in vitro chromogenic factor Xa substrate and clotting time assays as described above.

In some cases, a small amount of grinding tissue was weighed and homogenized with $400\mu L$ normal human plasma. The mixture was then allowed to incubate for 4 hours period, with frequent stirring at $37^{\circ}C$. After the incubation, $400\mu L$ factor Xa was added and incubated for an additional 2 minutes according to standards preparation. $200\mu L$ of the mixture was withdrawn and mixed with $100\mu L$ RECALMIX and recorded clotting time.

Similarly, a small amount of ground tissue was weighed and incubated as above. After the incubation, human antithrombin III, bovine factor Xa, factor Xa substrate and acetic acid were added in the same time period as in the preparation of standards.

CHAPTER 3

RESULTS AND DISCUSSIONS

3.1 Standard Curves for the Anti-factor Xa Assays

Heparin was added in vitro to plasma in order to prepare standard curves. In the anti-factor Xa clotting time method, clotting time increased linearly with increasing heparin concentration in the standards (see Figure 4). That is, the amount of factor Xa neutralized is proportional to heparin concentration in standards or test plasma. For the chromogenic factor Xa substrate method, there is a linear relation between absorbance and heparin concentration in the standards (see Figure 5). Absorbance is inversely proportional to the heparin concentration in standards or test plasma.

The minimum detectable heparin concentration for the clotting time and chromogenic factor Xa substrate method was 2.302 E-2 and 2.9157 E-2 units heparin per mL plasma, respectively. The clotting time method was set to provide a zero heparin clotting time of 18 +/-2.0 seconds.

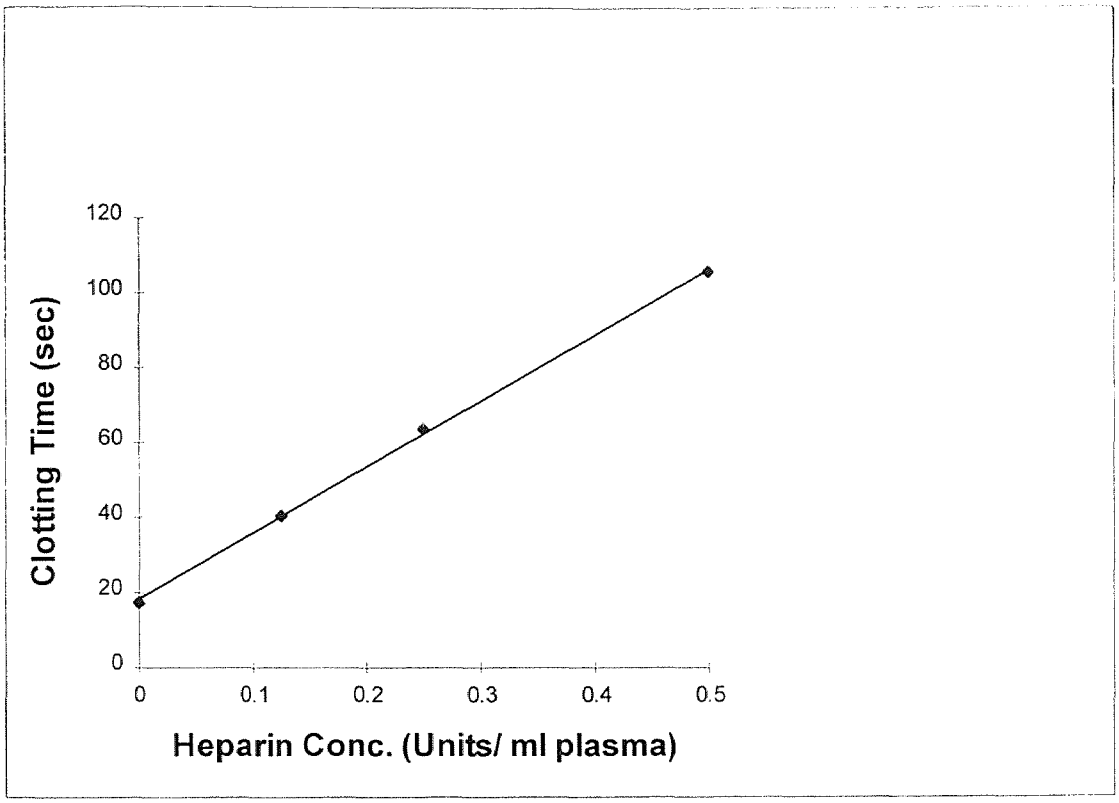

Figure 4: Standard Curve for Anti-factor Xa Clotting Time Assay

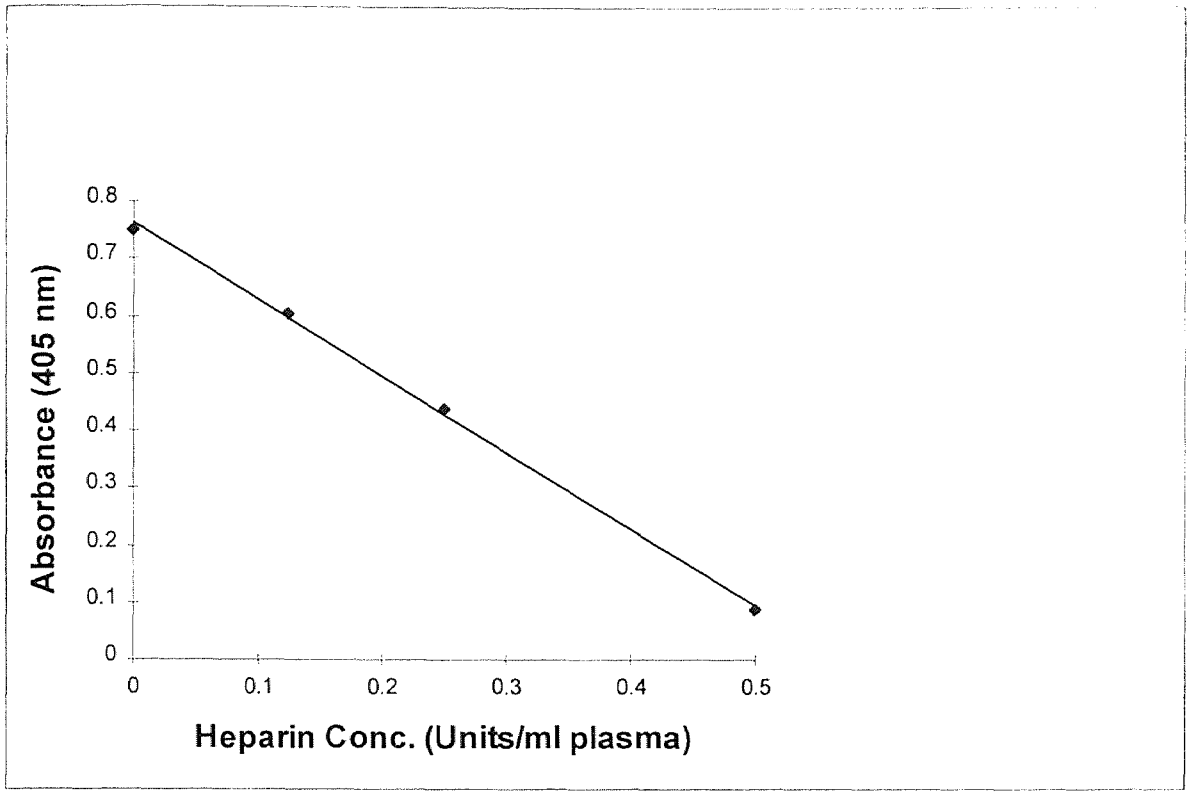

Figure 5: Standard Curve for the Chromogenic Factor Xa Substrate Assay

3.2 Stability of Heparin in Biological Pericardium Tissues

The released of bound heparin from pericardium tissues in saline solution during the three weeks storage is negligible so as to be of no concern. Heparin concentration measured in saline solution is so low that it can be assumed to be zero. Furthermore, heparin concentration remained fairly constant with time as indicated by Figure 6. The average clotting time measured for the overall three weeks storage was 20.0+/-1.0 seconds. The approximate heparin content measured in the solution for the storage period allowed using the clotting time and chromogenic substrate was 2.898E-3+/-0.0009 and 3.2961E-3+/-0.0005 units heparin per mL saline solution (n=21). Thus, it was concluded that heparin from biological pericardium tissues leached very little or not at all into the storage solution.

Storage solutions that contained heparinized biological pericardium tissues for more than three years shelflife were also tested to determine whether heparin is released from the biological tissues. The average heparin concentrations measured for the solutions using the clotting time and chromogenic factor Xa substrate method was 1.536E-2+/-0.00 and 1.526E-2+/-0.00 units heparin per mL storage solution . The average clotting time measured for these solutions 22.0+/-0.52 seconds (n=25). This result confirmed that the heparin content measured in solutions stored for the three weeks at room temperature. In addition, it showed that heparin does not leached from Shelhigh's heparinized biological pericardium tissues when quarantined for long shelflife. Thus, Shelhigh's heparinized pericardium tissues when implanted should have extended blood compatible efficacy.

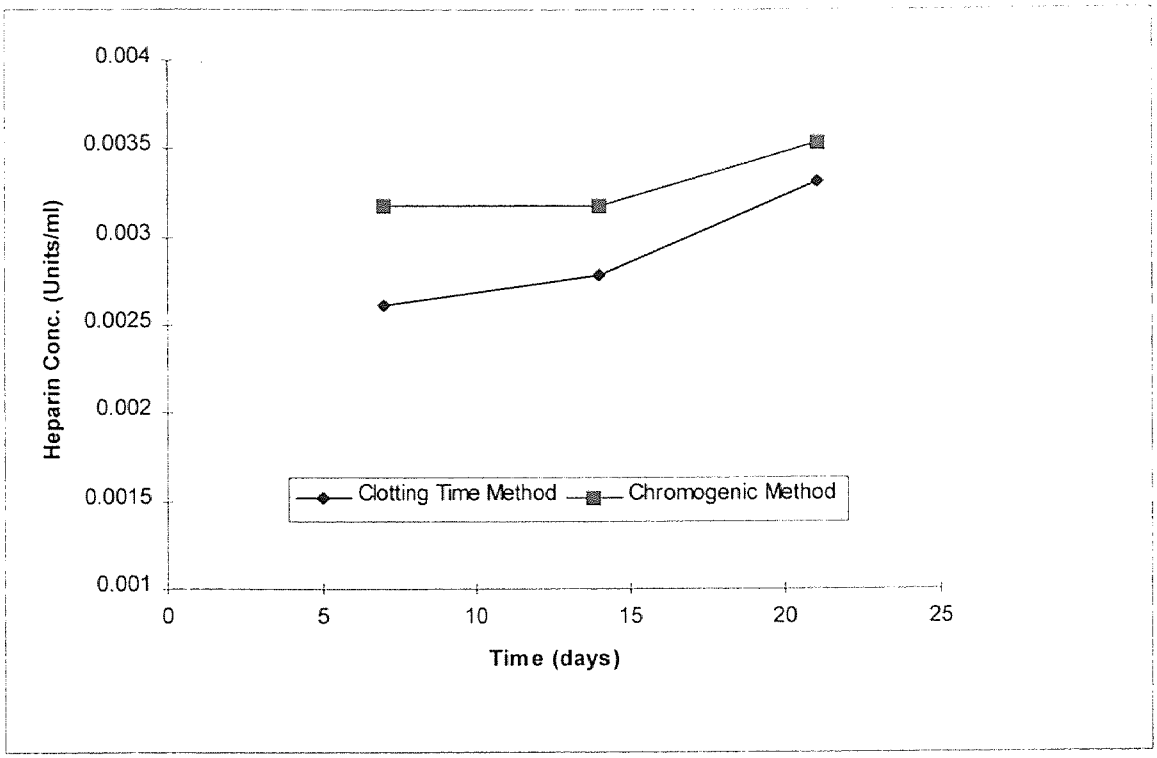

Figure 6: Heparin Released from Pericardium Tissues Stored at Room Temperature for 21 Days

The amount of heparin released from the heparinized biological pericardium tissues when incubated in plasma at $37^{\circ}C$ is higher in comparison to tissues incubated in storage solution at room temperature (Figure 7). This result is consistent with expectation, when heparinized biological pericardium tissues are in contact with plasma at $37^{\circ}C$ heparin should be released from the tissues creating a heparin microenvironment, thus prevent the plasma from clotting.

The incubated plasma containing the heparinized pericardium tissues neutralized factor Xa according to the clotting time and chromogenic factor Xa substrate assay. This indicated that heparin was indeed released from the pericardium tissues into the plasma. Furthermore, it indicated that the heparinized pericardium tissues have anticoagulant properties when in contact with plasma. Heparin concentration in plasma during the incubation period increased rapidly then appeared to stabilize (Figure 7). In time, it is expected for heparin to be released slowly from the pericardium tissues to create a heparin microenvironment at the material/plasma interface to impart thromboresistance characteristics. The percentage change of heparin concentration for the incubation period allowed was measured for the clotting time and chromogenic substrate factor Xa assay to be 10.6% and 9.97% respectively. The approximate heparin concentration measured in plasma using the clotting time and chromogenic substrate assay was.1415+/-.007 and .1479 +/-.0365 units heparin per ml plasma (n=6) respectively.

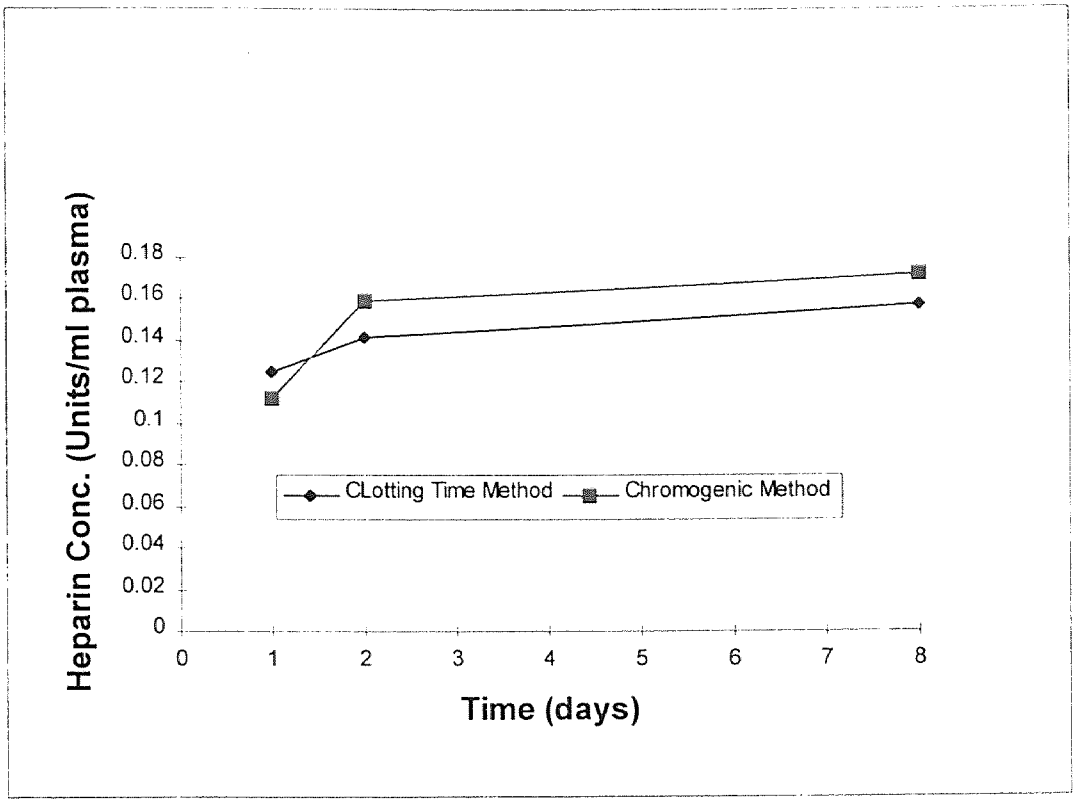

Figure 7: Heparin Released From Pericardium Tissues Incubated in Plasma at $37^{\bullet}C$ for 8 Days

3.3 Estimation of Heparin Content in Biological Pericardium Tissues

The prolongation of clotting time caused by the addition of ground heparinized pericardium tissues to plasma is in contrast to plasma to which non-heparinized ground tissues have been added. The present investigation demonstrated that factor Xa is neutralized by heparin from the ground heparinized pericardium tissues for the incubation period allowed. In the estimation of heparin content on heparinized biological pericardium tissues, the loss of factor Xa was observed to increase when the pericardium tissues are finely ground, and this was observed to vary from tissue to tissue, although all of the tissues used for the study were pericardium. The average clotting time measured for plasma with non-heparinized pericardium tissues was 26+/-2.0 seconds. This clotting time value gave heparin concentration in plasma that is in close approximity to the minimum detectable heparin concentration in a testing plasma. Therefore, incubating ground non-heparinized pericardium tissues did not resulted in a loss of factor Xa activity as measured by clotting time or chromogenic factor Xa substrate assay. The neutralization of factor Xa occurred exceedingly only in plasma containing ground heparinized pericardium tissues. According to the clotting method, clotting time for ground heparinized pericardium tissues was excessively higher in comparison with plasma containing non-heparinized tissues.

The estimated heparin content (Units heparin per gram dried tissues) in pericardium tissues according to the clotting time and chromogenic factor Xa substrate assay showed that heparin content varied in Shelhigh's pericardium tissues (Table IV). The results obtained were intriguing, since heparinization of the pericardium tissues was performed, using standard manufacturing procedures. However, there exist several reasons to explain the lack of heparin content consistency in the pericardium tissues. First, the degree of how fine each tissue was ground varied from tissue to tissue as mentioned before. Second, each pericardium tissue may have different heparin content on its matrix or different heparin diffusion rate. This implied that Shelhigh's heparinization process or binding of heparin onto tissues matrix may not be conforming to homogeneity. Therefore, the process might need to improve to ensure the amount of heparin immobilized on tissue matrix remains the same for all of the tissues and for the diffusivity of heparin to be also the same for all the tissues.

Table IV. Estimated Heparin Content in Biological Pericardium Tissues

Lot. No.	Dried tissue mass (gm)	Clotting Time Method: (Units/gm dried tissues)	Chromogenic Substrate Method: (Units/ gm dried tissues)
961016	.2258	3.404	3.245
970917	.1472	1.809	1.918
960203	.2745	2.143	2.640
970909	.2017	5.929	2.944
970925	.1431	1.936	1.958
970814	.1008	2.428	2.055
961211	.1252	2.221	1.819
960401	.2842	2.139	1.871
960401	.2581	6.726	6.393
960401	.2064	8.481	7.831

Table IV. (Cont.)

970811	.2083	7.403	7.153
941213	.2251	10.314	9.605
931206	.2786	4.212	3.482
950222	.2101	25.586	25.507
970929 (16)	.179	24.203	20.581
970929 (15)	.2185	12.985	11.731
970124	.2011	2.325	1.989
940901	.1988	8.6076	7.736
960215	.3446	96.143	93.407
Control 1	.1711	.4425	-----
Control 2	.2084	.5783	.6948
Control 3	.1816	.9104	.8877
Control 4	.192	.8611	.8396

3.4 Discussions

The aims of this study were to evaluate whether heparin leached from Shelhigh's heparinized pericardium tissues when quarantined for a long shelflife; whether these tissue have thromboresistance properties when in contact with plasma and to estimate heparin content in the tissues. From the results obtained, it can be concluded that heparin leaching from Shelhigh's pericardium tissues is negligible and these tissues have thromboresistance characteristics when in contact with plasma. Accordingly, solutions of quarantined pericardium tissues at room temperature did not have enough heparin to accelerate the inactivation of factor Xa, thus prolonging plasma clotting time.

In the work present here, when heparinized pericardium tissues were removed from storage solution, washed with PBS pH 7.4 and incubated at $37^{\circ}C$. Heparin was released from the heparinized biological tissues to accelerate the neutralization of factor Xa, therefore, prolonging the plasma clotting time. Factor Xa inactivation occurred in the presence of heparinized pericardium tissues but not in the presence of non-heparinized tissues. Therefore, when Shelhigh's heparinized pericardium tissues are implanted, the tissues should have blood compatibility properties. The heparin from the heparinized biological pericardium tissues is slow released, but the amount of heparin is sufficient in creating a heparin microenvironment to procure thromboresistance properties.

Since heparin in the biological pericardium tissues is not surface-bound as in the immobilization of heparin on polymer surfaces. To estimate heparin content on biological pericardium tissues, the tissues had to be dried, ground and homogenized with

plasma. Diffusion of heparin and lack of homogeneity in the tissues ground are presumed to be major reason as to why heparin content estimated on the pericardium tissues varied (Table IV). Nonetheless, when ground heparinized tissues were homogenized with plasma and incubated at $37^{\circ}C$, the apparent loss of factor Xa activity as measured by the clotting time and chromogenic factor Xa method was attributed by the trace amounts of heparin found in plasma. The inactivation of the clotting factor Xa was proportional to the amount of heparin present in the plasma. Furthermore, the inactivation of factor Xa increased even more when the tissues were ground to fine powder, mixed thoroughly and allowed to soak overnight in the plasma before incubating at the incubation temperature (i.e. $37^{\circ}C$). Therefore, the heparin content estimated for a tissue depends on the length of incubation period allowed and how fine a tissue was ground. Of these two factors, only the length of incubation period was more apt to control. The degree of how fine a tissue may be ground was observed to vary from tissue to tissue, although all of the tissues were pericardium, dried and ground under similar conditions. Consequently of this observation, it was concluded that the estimation of heparin content in heparinized pericardium tissue may not be very reproducible. To resolve this problem, alternative attempts were made in order to make the estimation of heparin content in pericardium tissues more reproducible. One such attempt was using a meat grinder to grind the tissues while wet, instead of drying the tissues in an oven. However, such attempts failed because the tissues may have undergone before, during or after the tissues are heparinized.

Therefore, the meat grinder was not able to cut the tissues into small fine pieces. As a result, it was concluded the best method for the present investigation was to dry large of pericardium tissues in an oven at $60^{\circ}C$ for approximately 2-3 days. Then, ground the tissue to very fine powder, and this was observed to vary from tissue to tissue.

The method of toluidine blue (10) was also used to estimate heparin content in homogenized solution. However, it was observed when the homogenized solution was added to an equal volume of an aqueous toluidine blue solution, the tint of the dye solution did not change color (i.e. from blue to red violet for high heparin concentration or intermediate shades of purple for low heparin concentration) as prescribed by the protocol. APTT test was performed on similar homogenized solution after diluted with equal volume of normal human plasma. It was observed, that clotting time for the plasma increased dramatically compared with the plasma without heparin added. Although the APTT is a global test, it does not specifically measure heparin ability to accelerate the inactivation of clotting factors such as factor Xa or thrombin, the prolonged clotting time of the plasma, when mixed with the homogenized solution, indicated the presence of heparin in the homogenized samples.

To test whether errors were made in the part of the experimenter or whether there existed some experimental flaws in the toluidine blue protocol, different heparin concentration in 0.9% saline solution was prepared by two members in the laboratory. The heparin concentration in the samples was not revealed to the experimenter, and the solutions were tested using the toluidine blue and APTT methods.

The results obtained for the heparin concentration in the 0.9 % saline solution using the toluidine blue and APTT method, agreed within the limits of experimental error. Thus, in the homogenized solution, it was concluded there existed an unknown interference(s) that prevents the reaction between the heparin and the aqueous toluidine dye. Soluble proteins or elements released from the ground heparinized pericardium tissues might have been the culprit that inhibits reaction between heparin in homogenized solution with toluidine blue dye or to a significant degree inhibits the formation of the heparin-dye complex. However, further studies are required in order to confirm such claims.

CHAPTER 4

CONCLUSIONS AND SUGGESTIONS

The work presented here in this thesis serves as a basis for estimating heparin content in biological tissues. Although it was observed that the method used to estimate heparin content in biological pericardium tissues was not very reproducible, the objectives of the authors for this investigation were fulfilled. It was shown that heparin from heparinized pericardium tissues when quarantined for long shelflife leached very little or not at all into storage solution. Also shown in this study was, when Shelhigh's biological pericardium tissues were in contact with plasma, loss of factor Xa activity was observed. Thus, Shelhigh's pericardium tissues have thromboresistance characteristics. In addition, the investigation revealed heparin content on pericardium tissue varied from tissue to tissue, although the tissues were heparinized under similar manufacturing procedures and all of the heparinized pericardium tissues used for the study were dried, ground and incubated under similar conditions. The heparin content estimated on different pericardium tissues showed that the diffusitivity of heparin from the tissues may vary from tissue to tissue and each tissue may have different heparin content immobilized on its matrix despite the fact all of the tissues were heparinized under standard manufacturing procedures.

Therefore, future research will require one to study the diffusion rate of heparin from pericardium tissues incubated in plasma. In addition, since heparin is not bound onto the surface of biological pericardium tissues as in heparin immobilization on polymer surfaces, future research will also oblige one to find a way to extract immobilized heparin from tissues matrix after the tissues undergone fixation procedures. Currently, there are no known available methods to extract heparin from heparinized biomaterials. However, there is a method to extract or remove heparin from solution through the use of heparinase. But the method inactivates heparin in the solution, thus making heparin less susceptible to be measured using established methods.

Nonetheless, the apparent loss of factor Xa activity as measured by the clotting time and the chromogenic factor Xa substrate assay demonstrated that Shelhigh's pericardium tissues have thromboresistance characteristics when in contact with plasma. Therefore, heparin activities on Shelhigh's pericardium tissues is sufficient in accelerating the neutralization of thrombin and factor Xa, thus able to reduce or prevent thrombus formation on the pericardium tissue surface when implanted. Explanted pericardium tissues are now being tested to determine whether explanted tissues from patients still retain blood compatibility in vitro after long implantation. Furthermore, more studies will be performed at Shelhigh Inc. to find ways to reduce variation in heparin content on tissues and to make the estimation of heparin content more reproducible and accurate.

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