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Technical aspects of aortic root sparing surgery: Structural changes occurring during different thawing protocols of cryopreserved human aortic root allografts and the reproducibility of external aortic root annuloplasty using Coroneo ring.

Technické aspekty záchovných operací aortálního kořene: Strukturní změny vzniklé při různých protokolech rozmrazování na lidských kryoprezervovaných allograftech aortálního kořene a reprodukovatelnost externí aortální anuloplastiky za použití prstence Coroneo

PhD Dissertation Theses

Supervisor: MUDr. Jaroslav Hlubocký, Ph.D.

Prague, 2017

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Content

Abbreviations

AV	aortic valve
AVJ	Aorto-ventricular junction
CHARA	cryopreserved human aortic root allografts

Abstract

Aortic valve-sparing procedures treating patients with aortic root aneurysm with or without aortic insufficiency and patients with ascending aortic aneurysm and aortic insufficiency are no longer experimental and unproven procedures. A successful aortic valve-sparing or repair operation aims not only to correct the failing part of the aortic root but also to restore the intro- and the inter-component relationship of the aortic root elements to optimal dimensions and relations. The avoidance of anticoagulation therapy and prosthesis-related complications makes aortic valve repair a tempting procedure. Considering the increasing rate of cusp repair reported in scientific literature, conservative aortic valve surgery seems to be developing into aortic valve repair surgery. This Dissertation Theses are devoted to the study of some specific technical aspects of aortic root sparing surgery, namely to the study of structural changes occurring in cryopreserved human aortic root allografts and the reproducibility of Coroneo ring implantation procedure.

The Introduction of these Dissertation Theses deals with the general review of aortic valve-sparing operations in the light of the historical aspects of used surgical technique, dynamic anatomy and the current situation. One part of the Introduction is devoted to the new non-surgical technical development, i.e. use of fibrin sealants in cardiac surgery and safety aspects of their use, including some information on specific fibrin sealants currently available to the surgeon. The last part of the Introduction discusses some aspects of cryopreservation of allografts for surgery.

The experimental part of these Dissertation Theses was undertaken with the fact in mind that the surgical improvement of the type of surgery described was achieved. The current effort is aimed at optimization of various technical aspects and not on the principal changes of the procedures used. Consequently, this experimental doctoral work was undertaken with the following objectives: 1) To assess morphological changes of the arterial wall that arise during different thawing protocols of a cryopreserved human aortic root allograft (CHARA) arterial wall; 2) to assess morphological changes of CHARA leaflets that arise during different thawing protocols; and 3) to compare the obtained results with the results arising from the comparison of different thawing protocols of CHARA leaflets. Additionally, 4)

reproducibility of Coroneo ring implantation on the aortic annular base under standardized conditions was evaluated.

All parts of this dissertation were performed with keeping up all the relevant and related ethical considerations. Approvals of the Ethical Committees were secured before the work.

For Objectives 1, 2 and 3, two thawing protocols were investigated. For the thawing protocol number 1, CHARA specimens were thawed at a room temperature of 23°C, while when the thawing protocol number 2 was used, CHARAs specimens were placed directly into a water bath at +37°C. After this procedure, microscopic slides for electron microscopy were prepared from all the specimens and evaluated with the use of the scoring system for morphological sample analysis. It was demonstrated that all the samples of CHARAs thawed at the room temperature showed smaller overall structural damage to the arterial wall and no smooth muscle cell contraction in tunica media when compared to the samples thawed in a water bath. Thawing at a room temperature seems to be gentler and does not lead to so severe damage to the CHARAs arterial wall (Objective 1). In the study regarding our objective 2, the performed experimental work following the structural changes occurring during different thawing protocols on cryopreserved AV leaflets showed that different rates of thawing show identical structural changes. Therefore, the rate of thawing does not play a significant role in minimizing structural changes that occur during thawing of cryopreserved AV leaflet. Consequently, it was demonstrated that different types of aortic root tissue (aortic wall versus aortic leaflets react differently when submitted to different thawing protocols, aortic leaflets being less sensitive to the thawing process alteration (Objective 3).

Objective 4 was to determine the reproducibility of external aortic root annuloplasty with the use of Coroneo external aortic annuloplasty ring (Extra-Aortic TM, CORONEO, Inc., Montreal, QC, Canada) in human aortic root allografts (18 human aortic root allografts were used). This part of my Dissertation Theses is important as a dilated AVJ that stays untreated represents a highly significant factor contributing to the failure of aortic valve-sparing operations and this surgical procedure is being used in significant numbers of patients. The procedure of implanting Coroneo ring was performed twice on each aortic annular base and the results were evaluated. Our results on reproducibility of aortic annular base Coroneo ring implantation and re-implantation indicate that the reproducibility of this procedure

is very high and that there is no significant difference between the outcome of the procedures during an implantation and re-implantation of Coroneo ring on the aortic annular base.

Souhrn

Záchovné operace aortálního kořene použité v léčbě pacientů s aneurysmem ascendentní aorty v přítomnosti nebo za absence aortální regurgitace již nepředstavuji experimentální a nedostatečně probádanou proceduru. Úspěšné záchovné operace aortálního kořene nebo opravné operace chlopně jsou uskutečňovány s cílem nejenom korigovat distrofický aortální kořen, ale jde při nich o obnovu funkčnosti jednotlivých komponentů kořene aorty, co se týká jejich rozměrů a propojenosti. Eliminace nutnosti antikoagulační terapie a komplikací, které vznikají při implantaci chlopenních protéz činí záchovné operace aortálního kořene velmi atraktivní pro chirurga i pacienta. Když vezmeme do úvahy údaje z odborné literatury o zvyšujícím se využití plastik chlopní, zdá se, že záchovné operace aortální chlopně a aortálního kořene získávají stále větší význam. Tato Dizertační práce je věnována studiu některých specifický faktorů důležitých pro záchovné operace aortálního kořene, především o stadium strukturních změn vznikajících u kryoprezervovaných alograftů lidských aortálních kořenů a o stadium reprodukovatelnosti procedury implantace (a reimplantace) extraaortálních prstenců Coroneo.

Úvod práce je věnován přehledu situace ve vhodnosti a použití záchovných operací z hlediska vývoje použití jednotlivých chirurgických technik, dynamické anatomie a současné situace. V této části je věnována pozornost také vývoji v oblasti ne-chirurgických technických aspektů záchovných operací, jmenovitě použití ´sealantů´ neboli tmelů na bázi fibrinu v kardiochirurugii a bezpečnosti jejich použití. Tato část úvodu též obsahuje informace o specifických fibrinových sealantech, které jsou v současnosti využívány. Závěr této části je věnován diskuzi některých aspektů kryoprezervace alograftu pro chirurgické použití.

Experimentální část dizertace byla uskutečněna s vědomím, že chirurgická stránka záchovných operací aortálního kořene je na optimální úrovni. V současnosti je pozornost soustředěna na optimalizaci různých technických aspektů, a nikoliv na principialní změny chirurgického zákroku. Tato situace byla východiskem pro experimentální část dizertační práce, která si kladla následující cíle: 1) Zjistit morfologické změny v arteriání stěně, které vznikají při aplikací různých postupů při rozmrazování zmrazených lidských alograftu aortálních kořenů; 2) vyhodnotit

morfologické změny na lidských chlopňových alograftech, které vznikly při různých způsobech rozmrazování; a 3) provést sumární porovnání získanýchvýsledků aplikace různých protokolů rozmrazování. Dalším cílem této práce bylo 4) prověřit reprodukovatelnost výsledků implantace prstence Coroneo.

Všechny části této práce byly provedeny při dodržení relevantních etických podmínek a po schválení patřičnou etickou komisí před provedením experimentů.

Pro dosažení cílů práce 1, 2 a 3 byly zkoumány dva protokoly rozmrazování. Při použití rozmrazovacího protokolu číslo 1 byly zmrazené lidské alografty kořenů aorty rozmrazeny při pokojové teplotě 23°C. Při použití protokolu rozmrazování číslo 2 byly alografty hned umístěny do vodní lázně při +37°C. Po ukončení rozmrazování byly ze všech tkáňových vzorků přípraveny preparáty pro mikroskopické vyšetření změn tkání. Tkáňové změny byly vyhodnoceny s použitím bodového skóre využívaného v analyze morfologických změn. Bylo prokázáno u všech vzorků arteriální stěny, že tkáně rozmrazované při pokojové teplotě byly jako celek méně strukturně poškozeny a neobsahovaly žádné kontrakce hladkých svalů v tunica media, čímž se zásadně lišily of vzorků arteriální stěny rozmrazovaných ve vodní lázni. Rozmrazování při pokojové teplotě se zdá být šetrnější a nevede k výraznému poškození arteriální štěny lidských alograftů (cíl prace 1). Pro dosažení cíle 2 této práce jsem sledoval strukturní změny vznikající při aplikaci různých protokolů rozmrazování pro rozmrazování humánních alograftů lidských chlopní. Získané výsledky prokázaly, že použití obou protokolů rozmrazování způsobilo stejné strukturní změny na chlopních. Rychlost a teplota rozmrazování nejsou v tomto případě tak důležité pro snížení výskytu strukturních změn na chlopních po kryoprezervaci. Získané výsledky prokázaly, že různé tkáně aortálního kořene (tkáně stěn aorty a tkáně chlopní) reagují různě na použití různých protokolů rozmrazování. Aortální chlopně jsou méně citlivé k procesu rozmrazování v porovnání s tkání arteriální stěny (cíl 3).

Cílem číslo 4 této práce bylo prověřní reprodukovatelnosti externí annuloplastiky aortálního kořene při implantaci Coroneo prstence (Extra-Aortic TM, CORONEO, Inc., Montreal, QC, Canada). Pro tuto část práce bylo použito 18 lidských alograftů aortálního kořene. Význam této části práce spočívá v tom, že nestabilizovaný kořen aorty představuje vysoce rizikový faktor, jenž přispívá k selhání záchovné operace aortální chlopně. Tato chirurgická procedura je využita u signifikantního množství pacientů. Procedura implantace Coroneo prstence byla provedena dvakrát na každém kořeni aorty a výsledky implantace a jejího následného opakování byly vyhodnoceny.

Získané výsledky studia reprodukovatelnosti implantace Coroneo prstence prokázaly vysokou reprodukovatelnost této procedury bez významného rozdílu mezi výsledky primární implantace a jejího opakování.

1. Introduction

1.1. Aortic valve sparing operations

Aortic valve-sparing operations were refined in order to preserve the native aortic valve during surgery for the aortic root aneurysm and surgery for the ascending aortic aneurysm with associated aortic insufficiency. The aortic root is an ensemble consisting of distinct entities: the aortic valve leaflets, the leaflet attachments, the sinuses of Valsalva, the interleaflet trigones, the sinotubular junction and aorto-ventricular junction [1-3]. It is a remarkably complex and sophisticated structure [1]. Every single constituent of the aortic root has an optimal macroscopic, microscopic structure and anatomical architecture which contributes to the function of the aortic root: intermittent, unidirectional channelling of large volumes of fluid while maintaining laminar flow, minimal resistance, the least possible tissue stress and damage during varying hemodynamic conditions and demands [4-7]. This synchronized dynamic behaviour of all aortic root components has shown to be of a great importance for a specific flow characteristic, left ventricle function and coronary perfusion [8-10]. When any of the aortic root components fail, it is the recognition of the complexity of the structure that has led to the development and advancements in sparing surgical procedures that respect the fundamental anatomical existence of the individual parts of the aortic root [11-14].

1.2. Historical aspects of used surgical techniques

Aortic root aneurysm aetiology is primarily related to the dystrophic disease. Aortic valve repair is performed in only 1.7% of the cases, versus 69% for mitral valve repair [15,16]. Over the past two decades, there has been a significant paradigm shift towards the aortic valve-sparing procedures over prosthetic valve replacement in suitable candidates [17]. Operations that preserve a patient's native valvular anatomy bring numerous benefits for patients. The most prominent one is the avoidance of life-long anticoagulation therapy [18,19]. The introduction of the composite graft procedure by Bentall in 1966 has become a gold standard for the treatment of aortic root aneurysm and aortic valve regurgitation [20]. Until recently, composite valve and graft replacement was the only standard surgical approach for the aortic root aneurysm [21]. From the early 1990s, valve-sparing procedures have become a feasible alternative, in hope that they will result in improved survival rate and fewer

valve-related complications [18,22]. Two original procedures were initially described as the ‘remodelling' technique proposed by Yacoub: reduction of the sinotubular junction diameter and creation there neo-sinuses of Valsalva with a scalloped Dacron tube graft sutured in the supravalvular position [23]. The second alternative was proposed by David and Feindel: the ‘reimplantation' of the aortic valve within a straight tube, reducing both the annulus and the sinotubular junction diameter while abolishing the sinuses of Valsalva, thus impairing root dynamics [24]. Over the time, modified procedures emerged from the original Yacoub's and David's techniques such as Van Son, Hopkins, Hetzer procedures and others [25-27]. These methods focus on the aortic root reconstruction and the reduction of dilated aortic root diameters in order to restore proper valve function [28,29]. Comparative analysis of early and late results of the aortic root reconstruction with aortic valve-sparing procedures and the composite mechanical valve conduit replacement introduced by Bentall et al. was carried out, showing that the aortic root reconstruction has a low early and late mortality, a high survival free of complications and little need for reoperation. During the late follow-up, the aortic root reconstruction with preservation of the aortic valve showed a lower incidence of bleeding, thromboembolic events and endocarditis [30,31]. Numerous surgical variations have aimed to incorporate preservation of the aortic root dynamics with the treatment of dilated native annulus [32,33]. This multiplicity of aortic valve repair and sparing procedures resulted in a lack of standardization, limiting adoption of such procedures [34]. Additionally, most failures with valve-sparing techniques are due to residual cusp prolapse, either as a primary unrecognized lesion or secondary due to an induced prolapse after root reconstruction [35,36]. Aortic annuloplasty combined with re-suspension of cusp effective height are key steps for a reproducible aortic valve repair. Schäfers et al. proposed to address this issue with a dedicated calliper in order to restore cusp effective height up to 8-10 mm [34,37,38]. Certain controversy remains between external or internal annuloplasty rings [39,40]. Even though subvalvular plane in the right coronary sinus is easier to reach with an internal ring, endovascular placement may interfere with cusp mobility and increase the risk for hemolytic or thromboembolic events [41]. The advantage of the ring placed externally is the avoidance of these complications, as well as limiting placing tension on the device fixation stitches that is caused by the expanding aorta [42].

1.3. Dynamic anatomy

In vitro and in vivo studies have documented that cusp motion and flow patterns across the reconstructed aortic root are more physiologic after the remodelling of the aortic root rather than the reimplantation of the aortic valve, as well as after procedures using a prosthetic conduit fashioned with neo-sinuses of Valsalva than without [41-43].

Dynamic anatomy reports showed that the three-dimensional a sigmoid shape of the aortic annulus could be divided into two two-dimensional planes: one at the base of the aortic annulus (also called the aorto-ventricular junction) and the one at the sinotubular junction [44,45]. Dilatation of both of these diameters is characteristic for lesions of the aortic root aneurysm. These advances in dynamic anatomic knowledge led to the development of different valve-sparing procedures for the treatment of the aortic root aneurysm.

1.4. Current situation

Lansac et al. proposed a standardized approach for aortic valve repair addressing both the aorta and the valve, associating physiological reconstruction of the aortic root according to the remodelling technique with the re-suspension of cusp effective height and an expansible subvalvular ring annuloplasty using expansible aortic ring in order to achieve a complete and calibrated annuloplasty in diastole, while maintaining expansibility of the aortic root (Extra-Aortic ^{TM} ,CORONEO, Inc., Montreal, QC, Canada) [46,47]. This solved a problem in the treatment of aortic root aneurysm and the lack of a geometric annuloplasty ring to facilitate reconstruction of the aortic root that restores physiological annular size and geometry during aortic valve repair. Cusp coaptation height was increased, reducing the stress on the cusps, thus protecting the repair [37]. A multi-centric study analysed preliminary results of this new physiological approach to aortic valve repair with subvalvular aortic ring annuloplasty. In this multi-centric study, unselected patients with aortic root aneurysms were enrolled consecutively, regardless of their aortic insufficiency grade, the presence of bicuspid valve or complex valvular lesion. The addition of a subvalvular aortic ring was systematically performed in all cases to reduce the diameter of the native aortic annular base in diastole. The choice of the aortic ring and the tube graft was standardized, based on the criterion of intraoperative measurement

of a native aortic annular size with the Hegar dilators. The diameter of the prosthetic aortic ring was undersized by one size to restore a normal STJ/annular base ratio of 1.2 [48]. A calibrated expansible aortic ring annuloplasty (Extra-Aortic™, CORONEO, Inc., Montreal, QC, Canada) in different sizes was developed in order to facilitate technical standardization. The result of this multi-centric analysis showed that the aortic function remained stable in most patients. Among the 126 survivors without reoperation, 115 patients had aortic insufficiency of grade < 2 (91.3%) at the end of the follow-up. Freedom of aortic regurgitation of grade II or more was 87.7% at 3 years evaluation (95%, Cl: 80.3-95.1%) [49].

1.5. Non-surgical technical development

As anatomy of the aorta is already highly detailed and elucidated and because surgical aspects of aortic valve-sparing operations are already mastered, the significant improvement is happening in non-surgical technical areas. Two of these are the use of fibrin sealants and cryopreservation. There, new information that is being obtained, may contribute to improved outcomes of aortic valve-sparing operations.

1.5.1. Fibrin sealants

1.5.1.1. Fibrin sealants in cardiac surgery

Technical advancement in cardiac surgery is accompanied not only by the change of used surgical techniques but also by the advancement of other technical aspects contributing towards the success of cardiac interventions. One of such areas is a routine use of fibrin sealants.

A development of sealants occurred during the half of the last century during the war when the need for a fast and reliable method was obvious. The method of sealing battle-related injuries was to stop bleeding occurring even with the use of sutures or to replace the sutures, ligatures or cautery completely. Sealants are modern day auxiliary in surgery [50]. The sealants, including fibrin-based sealants, are dealt with in approximately 200 scientific and clinical reports per year [51].

Presently, sealants are used in in cardiac surgery for several reasons: (a) they should help to control haemostasis through the control of bleeding in the area of surgical intervention (as auxiliary sutures, not as suture replacement); (b) they should

seal openings made by standard sutures; (c) they should be useful in sealing off hollow organs of the body. Ideally, they also should (d) improve wound healing and (e) they may be useful in the delivery of medication to tissues exposed during the surgery. Obviously, the use of sealant in surgery should be simple, safe and well tolerated by patients. The process of disintegration of the sealant should not cause an inflammation, immunological or any other type of unwanted or pathological process. And the cost of the use of sealants in surgery should not be prohibitive. The use of sealants and related products (hemostats, glues, adhesives)

1.5.1.2. Types of sealant in surgery

Sealants in surgery may be classified according to various aspects of their production and structure; i.e. based on biological materials (fibrin) or synthetic substances (cyanoacrylates), a number of substances involved in putting the sealing action into the effect, the tediousness of the use, safety and cost of various types of sealants.

The main types of sealants and glues used in medicine are the following: (a) fibrin sealants; (b) cyanoacrylates; (c) gelatin and thrombin-based products; (d) polyethylene glycol polymers; and (e) albumin and glutaraldehyde-based products.

Fibrin sealants: These are blood-based. They are well absorbed and easy to use. They prevalent use is to control haemostasis in cardiac surgery, liver surgery and after splenic trauma [52,53].

(a) Fibrin sealants: These are blood-based. They are well absorbed and easy to use. They prevalent use is to control haemostasis in cardiac surgery, liver surgery and after splenic trauma [52,53].

(b) Cyanoacrylates: These are synthetic sealants/glues in nature. There are various cyanoacrylates on the market. However, the substances used for medical purposes n-butyl or 2-octyl cyanoacrylate. A bond formed is strong enough makes removal of sutures unnecessary [54,55].

(c) Gelatin and thrombin-based products: In principle, these products may be used in many types of surgery. These products (like all other natural/biological products) are relatively non-toxic [56].

(d) Polyethylene glycol polymers: These are oligomers or polymers of ethylene oxide and are biodegradable within 6 weeks of their use. They are used mainly in neurosurgery [57,58].

(e) Albumin and glutaraldehyde-based products: These mixed-origin (natural albumin and synthetic glutaraldehyde) products have excellent bonding ability in a short time of 2-3 minutes [59]. These products are used in cardiac surgery [60].

1.5.1.3. Fibrin blood clot formation

Fibrin plays an essential role in haemostasis. It is a fibrous protein that plays an important part in the blood clot formation. It is formed through polymerization of fibrinogen (Factor I of the blood coagulation) through an action of the protease enzyme thrombin formed from prothrombin (Factor II). Additionally, thrombin activates other factors of the blood coagulation cascade, such as factor V (proaccelerin), factor VIII (antihemophilic factor) and factor XIII (transglutaminase). Fibrin and platelets (with thrombin receptors) form a hemostatic clot that should close a natural, pathological or surgery-related tissue wound [61].

Fibrin originates from fibrinogen that is a peptide of relatively large molecules (molecular weight 340 kDa). It consists of two tripeptide units connected at their N-terminal regions by disulfide bonds. Aggregation of fibrinogen particles is prevented by charge-charge repulsion. Thrombin cleaves the N-terminal structures making the resulting fibrin molecules capable of aggregation resulting in the formation of the 'soft' clot that is consequently stabilized by fibrin crosslinking [61]. Thus the processes of sealing by fibrin sealants reproduce the final phase of the physiological coagulation, the conversion of fibrinogen into fibrin. This whole process is advantageous for the use of fibrin sealants as it is a process natural to the body.

Europe has the priority in developing the first commercially available fibrin sealants that were approved for clinical use by FDA in 1998 for the use in the United States (Tisseel) [62].

Fibrin as a biological structure is normally well tolerated by patients. In principle, there all the following considerations:

- The risk of immunological reaction to the animal (bovine) or human proteins present in sealants. It was reported that around 2% [63] or even 5% [64] of patients may develop anti-thrombin antibodies.

- The risk of excessive or uncontrolled clotting.
- Potential (currently very small) for the transmission of some diseases, especially the transmission of some viral pathogens, i.e. human parvovirus B19 [65,66]. Additional concerns for hepatitis B, hepatitis C and HIV transmission are justified. However, such cases are not reported in scientific literature currently. What is important is the fact that this aspect of the fibrin sealant use involves not only patients but is potentially risky for the sealant-handling health-care workers.

1.5.1.4. Recent uses of fibrin-based sealants in cardiac surgery – experimental work

Significant scientific work was done in the area of sealants suitability and use in surgery. However, attention is being paid to some aspects the sealant application/use and to clarification of their possible benefits. Some of these works were done not only in a clinical environment but also under experimental conditions during the last 5 years.

Many of such experiments deal with comparing different types of sealants to obtain the information that is not yet available in order to optimize the use of these products. A very important demonstration of not only the platelet-rich fibrin-based glue excellent biocompatibility but additionally of the upregulation of neovascularization was shown in experimental condition using rat model [67]. Additionally, aminomethylbenzoic acid prevents/slow-down the degradation of fibrin glue [67].

Recently, the possibility of using sutureless approach thorough application of a fibrin-based haemostat (TachoComb) was investigated in the experiments including rabbit skin and porcine hearts [68]. It was found that the adhesive strength of the sealant is significantly increased through application of polyglycolic acid sheets and fibrin glue together with the sealant. Thus, combining haemostat with a polyglycolic acid sheet and fibrin glue seems to be a method suitable for difficult clinical situations, such as haemorrhage for the left ventricle. Fibrin glue itself seems to be very suitable for filing needle holes created during cardiac or vascular surgery [69]. As much as this use of a glue in surgery is obvious, not many studies of glue application for improved haemostasis are available. The mentioned report [69] compared different methods of glue application: drip method, a spray method, rub

and spray method and rub and rub method. Comparison of the filling of holes shown that rubbing the fibrin glue onto a hole is the most effective approach. This was confirmed also by a microscopic evaluation that documented effective glugging of the needle holes by rubbing off the glue on [69]. An important finding was documented on the superior effect of fibrin glue compared to cyanoacrylate-based sealant [69] in experiments using rabbit aortic wall. The use of cyanoacrylate-based sealant resulted in thinning of the rabbit aorta while no such thinning was observed with a fibrin-based sealant [69]. Additionally, no apoptotic or necrotic cells were found by histological examination of the aortic tissue.

An interesting study was published [70] devoted to the studying extraction of endoprostheses implanted in the aorta of experimental pigs. The role of fibrin glue was evaluated in forming the interface between the endoprosthesis and tissue. Fibrin glue between the stent graft and the arterial wall increases incorporation of the endoprostheses [70].

1.5.1.5. Recent reports on fibrin-based sealant use in cardio surgery – clinical data

Significantly more reports available in the scientific literature are dealing with sealants/glues used in the clinical situation. We included only reports where fibrin glue or sealant was used and omitted reports, in which the use of non-fibrin glue or sealant were used.

A review summarizing available clinical data from controlled and uncontrolled clinical trial in cardiovascular surgery devoted to using of various sealants appeared in 2013 [71]. However, due to many products available at the market, it deals only with some of them, mainly the product sold under the name TISSEEL. This excellent review [72] did not raise any concerns regarding sealants' safety or tolerability while producing effective haemostasis control in the fields of cardiac and vascular surgery.

The role of bleeding as a predicting factor in morbidity was dealt with in the report on the use of a sealant in composite aortic root replacement in 56 patients [73]. The suture line in these operations was sealed with fibrin glue to prevent possible blood leakage. Only 1 patient required surgical re-exploration for bleeding and no case of operative or hospital death appeared. This was attributed to fibrin sealant application

(spraying) [73]. In another report, fibrin sealant was reported to be successfully used in the case of left ventricular rupture when it was combined with external sutures [74].

A multi-centre, parallel group, randomized, controlled, open-label Phase II/III study was performed in Italy to address the question of safety of fibrin sealant [75]. Two hundred patients were included in this retrospective clinical trial study concentrating on thoracic surgery. Again, no increased risk of any type of adverse effects or surgical complications in the relation to the use of fibrin sealant was observed [75].

An evaluation of the efficacy and also of cost-effectiveness of fibrinogen/ thrombin-coated collagen patch (TachoSil®) use for intraoperative haemostasis in patients (younger than 16 years) with congenital heart disease requiring a reoperation during childhood was performed [76]. The operations of 117 patients took place between 2009 and 2011. The reasons for performing reoperations were the reinforcement of suture lines, lung lesions, epicardial lesions and chest wall lesions. The significant association was observed between the use of fibrinogen/thrombin-coated collagen patch and decreased need for packed red blood cells. This with the elimination of the use of other haemostatic or sealant agents contributed the decreased cost of the operations. This is important, especially because the used patch served as an effective haemostatic agent [77]. The similar results for the same product were reported for patients who developed a lymphatic leakage during operation for congenital heart disease. The use of fibrinogen/thrombin-coated collagen patch was not only safe, but it prevented the development of chylothorax during the period after operation [77].

A scientific study was published that dealt with the success of using platelet and fibrin glue for a desirable non-invasive treatment of non-healing wounds in the sternal region after the operation of coronary artery(ies) bypass [78]. Six patients were treated for serious, life-threatening chronic sternum wounds with multi-drug resistant microbial pathogens. The topical application of platelet and fibrin glue every two days lead to the complete healing of the wound in 5 patients and to significant improvement in one patient without any local or systemic complications or any abnormalities in tissue scarring or another type of tissue formation [78]. However, it was also reported that the use of platelet and fibrin glue sealant may lead to an increased rate of superficial sternal infections [79].

Fibrin glue may also be successfully applied in cases of shot wounds [80]. It was shown that heart lacerations are successful when mattress sutures with felt strips are covered with fibrin glue. In such cases, the use of fibrin glue contributes to an efficient medical care applied in emergencies.

1.5.1.6. Safety aspects in using glues/sealants in cardiac surgery

Fibrin glues or fibrin sealant as such are suitable for the use in surgery because of their biological origin. The only unwanted effect that may really take place is the immunological reaction. There is no report available to us from the period of the last 5 year indicating some fibrin glue-related problems in cardiac surgery [81]. It is essential to note that the adhesive strength of fibrin glue/fibrin sealant is lower compared to the glues based on cyanoacrylate or gelatine–resorcin–formalin mixture. However, no reported toxicity resulting from the use of fibrin sealant or glue in cardiac surgery to repair dissected aorta is obviously a great advantage when compared to other sealant types [81] as some reports indicating issues with non-fibrin glues or sealants are available. For example, there are several reports available on complications related the use of an albumin cross-linked glutaraldehyde glue (BioGlue). This product was implicated in the case report [82] dealing with a patient treated for developed stenosis of the saphenous vein and internal thoracic artery bypass grafts. Occurred fibrotic narrowings were close to the BioGlue use site. The fibrotic reactions were likely associated with a reaction to the glue. Additionally, pulmonary embolism related to the use of BioGlue was reported in the case of type A aortic dissection repair [83]. Additionally, a delayed aorto-pulmonary artery wall disruption with false aneurysm formation after repair of an acute type of aortic dissection with BioGlue was also reported [84]. There are other reports on BioGlue related complications, i.e. on a case of ostial left main coronary artery stenosis possibly related to use of BioGlue [85] and another report on several patients developing late wound healing problems after the use of BioGlue for apical hemostasis during transapical aortic valve implantation [86].

1.5.1.7. Remarks on clinical use of some specific fibrin sealants

Accumulated data on fibrin-based and other sealants created the base for their broad application in practical cardio surgery applications. In today's surgery, any

sealant entering an operating room is of the highest quality and of an approved standard. These sealants are usually approved by FDA (Food and Drug Administration, USA) and by other similar administrations in a particular country. The specific application of some selected sealants is mentioned further for the elucidation of the topic:

A) TachoSil, according to FDA (http://www.fda.gov/BiologicsBloodVaccines/BloodBloodProducts/ApprovedProducts/LicensedProductsBLAs/FractionatedPlasmaProducts/ucm207482.htm), "is a fibrin sealant patch indicated for use with manual compression in adult and pediatric patients as an adjunct to hemostasis in cardiovascular and hepatic surgery, when control of bleeding by standard surgical techniques (such as suture, ligature or cautery) is ineffective or impractical". In other words, TachoSil is used in cardiac surgery in situations where surgical treatment of bleeding is inaccessible due to its anatomical localization or would cause more damage to the anatomical structures. Other examples are TachoSil applications include repair of ventricular rupture, repair of post-infarction repair of the ventricular septal defect, hemostatic support in reoperations. However, it is necessary to underline that TachoSil (and also other sealants) should not be used in place of sutures or other forms of mechanical ligation in the treatment of major arterial or venous bleeding [87-89].

B) BioGlue, according to FDA (http://www.accessdata.fda.gov/cdrh_docs/pdf/p010003b.pdf), is indicated as a supporting method of achieving hemostasis in adult patients in open surgical repair of large vessels. BioGlue has a broad spectrum of use in cardiac surgery such as repair of proximal aortic dissection, aortic root reconstruction procedures, aortic arch reconstruction procedures, ventricular rupture or injury, post-infarction ventricular septal defect repair, valve repair and replacement procedures [90-95]. BioGlue had also shown to be very effective tool in patients with weakened tissue [96].

C) CoSeal, according to FDA (http://www.accessdata.fda.gov/cdrh_docs/pdf/p010022b.pdf), is a hydrogel that works as a vascular sealant. CoSeal is indicated for use in reconstructive surgery to achieve adjunctive hemostasis by mechanically sealing of leakage. Its main use is a

prevention of adhesions in surgery in high risk or young patients where reoperation is expected. Also, CoSeal is used as either a supplement or an alternative to suture repair, obtaining hemostasis both in high-pressure ventricular repair and in the rupture of a friable coronary sinus adjacent to vital structures [97,98].

Based on clinical experience and results of experimental work, the widespread use of fibrin sealants is fully justified as it benefits the patient and also the surgeon through the improved control of hemostasis while not increasing any adverse effects or complication during surgical procedures.

1.5.2. Cryopreservation

Cryopreserved human aortic root allografts (CHARA) have been used extensively in cardiac surgery for their advantages over bio-prosthetic and mechanical valves, such as excellent hemodynamic function, very low thrombotic event rates, and mainly their resistance toward infections [99-101].

The first allograft transplants in cardiac surgery were freshly harvested aortic valves. The first fresh aortic valve allograft transplant was performed by Murray in 1956 [102]. Regardless of the fact that the operation had an imperfect hemodynamic outcome, the allograft performance was outstanding with perfect leaflet function. Other early experimental and clinical trials such as Heimbecker et al., Kerwin et al. and Lam et al. supported the superior properties of fresh aortic valve allografts [103-105]. Nevertheless, the era of allograft transplantation in cardiac surgery began after the first successful aortic valve transplantation performed by Ross in early 1962 based on Brewin experimental work [106,107].

The first allograft transplants in cardiac surgery were freshly harvested aortic valves that underwent minimal treatment with no ABO Blood group matching. Remarkably, these allograft transplants showed outstanding durability and performance, giving the basic foundation for this new type of surgical procedures. Due to do the lack of donors, cardiac centres started to treat allografts with antibiotics in order to prevent disease transmission and cryopreserve them in order to prolong their life span. These techniques of allograft processing and cryopreservation led to significant decrease of allografts durability and their clinical performance between the 1960s and early 1970s leading almost to the abandonment of this type of procedures [108]. This was primarily due to irreversible damage to the cells viability and the loss

of the structural integrity caused by thawing, resulting in the loss of allografts toughness and elastic properties [109-112]. Technical advances in tissue handling had led to the reintroduction of allograft transplants back into use in cardiac surgery [100]. To date, there are no recommended guidelines for cryopreservation and subsequent thawing of cryopreserved allografts that would eliminate damage to the cellular structures in order to obtain allografts of the highest possible quality and durability.

1.6. The lecture learned

Aortic valve-sparing operations treating patients with aortic root aneurysm with or without aortic insufficiency and patients with ascending aortic aneurysm and aortic insufficiency are no longer experimental and unproven procedures. A successful aortic valve-sparing or repair operation aims not only to correct the failing part of the aortic root but also to restore the intro- and the inter-component relationship of the aortic root elements to optimal dimensions and relations. The avoidance of anticoagulation therapy and prosthesis-related complications makes aortic valve repair a tempting procedure [18,19]. Considering the increasing rate of cusp repair reported in scientific literature, conservative aortic valve surgery seems to be developing into aortic valve repair surgery [113-116]. Expansible aortic ring (Extra-Aortic ^{TM}, CORONEO, Inc., Montreal, QC, Canada) was being implanted in an unselected population of patients with aortic root aneurysms enrolled in the prospective multi-centric CAVIAAR trial (CAVIAAR, Conservative Aortic Valve Surgery for Aortic Insufficiency and Aneurysm of the Aortic Root)**.** A standardized management of dystrophic aortic roots towards a physiological approach to valve repair might improve long-term durability of the results. However, the need remains for reliable long-term data comparing valve replacement and valve repair procedures, thus limiting the widespread adoption of this procedure. An international multicentre registry of the aortic valve repair will play a key role to clarify and standardize the place of repair in the aortic valve surgery. AVIATOR (Aortic Valve insufficiency and ascending aorta aneurysm International Registry) is a prospective multicentre registry that shall provide us with the necessary answers in the future. Additionally, as the used procedures became more and more standardized, the importance of individual technical aspects of these surgeries also attract the attention of surgeons.

2. Objectives of the dissertation

As the general situation in the area of our research indicates, as for now, improvement in the type of surgery described relays more on optimization of various technical aspects than on principal changes of the procedures used. Consequently, this experimental doctoral work was undertaken with the following objectives in mind:

1) To assess morphological changes of the arterial wall that arise during different thawing protocols of a cryopreserved human aortic root allograft (CHARA) arterial wall.

2) To assess morphological changes of CHARA leaflets that arise during different thawing protocols.

3) To compare the obtained results with the results arising from the comparison of different thawing protocols of CHARA leaflets.

The hypothesis for the first 3 objectives is that as the complexity of the structures evaluated is significant, the changes in arterial wall structures and in leaflets may be (or may be not) the same regarding the character, complexity and integrity of the tissue. Additionally, the time of thawing (and, consequently, the rate of thawing) may be significant for the outcome of the quality of the tissue that is being prepared for a transplantation.

4) Evaluate reproducibility of Coroneo ring implantation on the aortic annular base under standardized conditions.

This part of the work is meant to clarify whether aortic annular Coroneo ring implantation is reproducible in our conditions and whether standard outcome of the Coroneo ring implantation can be achieved.

3. Methodology

3.1. Ethical statement

All the allografts were harvested in the operation theatre in patients that were organ donors and were pronounced “clinically dead” with compliance to the Czech Republic’s transplants laws.

All 3 clinical departments (2nd Department of Cardiovascular Surgery, General University Hospital, Prague, Czech Republic; Transplant Center & Department of Cardiac Surgery, University Hospital Motol, Prague, Czech Republic; Tissue Bank, Faculty Hospital Hradec Kralove, Charles University - Faculty of Medicine in Hradec Kralove, Hradec Kralove, Czech Republic) have approved regulations dealing with experimental work on cryopreserved human tissues. These regulations were approved by the particular Ethical Committee. Individual consents for the use of tissue are not available as the allografts are not stored under the name of the donor, the individual donor cannot be traced, and the experiments were performed only on allografts that were removed from tissue bank as ‘’unsuitable for patient transplant” (usually when their suitability for transplantation expired after the accepted time or packaging damage).

The study was reviewed and approved by the Ethical Committee of General University Hospital, Prague Czech Republic.

3.2. Allografts harvest and characteristics

Basic allograft characteristics for Thawing Protocol 1 (thawing at a room temperature at 23°C) are summarized in Table 1. Basic allograft characteristics for thawing protocol 2 (thawing in a water bath at +37°C) are summarized in Table 2.

Table 1: Thawing Protocol 1: Basic characteristics of allografts in the experiment.

Gender	Donor's Age	Aorta diameter, mm	ABO, RH Compatibility
Female	55	21	A+
Female	41	21	A+
Male	55	25	AB+
Female	56	24	A+
Male	57	27	B+
Male	59	28	O-

Table 2: Thawing Protocol 2: Basic characteristics of allografts in the experiment.

Gender	Donor's Age	Aorta diameter, mm	ABO, RH Compatibility
Male	34	21	A-
Female	51	24	B+
Male	44	24	B+
Male	44	25	O-
Male	42	27	AB+
Female	37	27	A+

3.3. Allograft processing cryopreservation protocol

All human ARA underwent an initial decontamination according to the standard protocol of the tissue bank. Afterwards, all allografts are stored in an antibiotic cocktail comprising of Cefuroxime 0.2 mg/ml + Piperacilline 0.2 mg/ml + Netilmicin 0.1 mg/ml + Fluconazole 0.1 mg/ml in tissue culture nutrient medium E 199 for 24 hours at + 37 °C. Subsequently, all ARA were moved into the cryoprotectant solution in sterile laminar flow cabinet and packed by double layer technique (sealed in Gambro Hemofreeze bags, NPBI BV, Gambro, The Netherlands). Cryoprotectant used was 10% dimethylsulfoxide in the nutritional source for cell culture E 199. After the packaging was completed, all ARA were

cooled at a controlled rate of -1 °C/min from + 10 °C to -60 °C, then rapidly cooled and stored in a cryocontainers in liquid nitrogen at -196 °C.

3.4. Thawing protocols

Experimental work was based on investigating 12 cryopreserved CHARA specimens. CHARA tissue samples were randomly divided into two groups, each group consisting of six samples. All allografts were thawed in their original packaging (packed by double layer technique and immersed in 10% dimethylsulfidoxide). Two thawing protocols were tested:

- Protocol 1: six CHARA specimens were thawed at a room temperature at 23°C. Thawing times were as follows: minimum 2hr 49 min, maximum 4hr 5 min, with the median of 3hr 19 min.

- Protocol 2: six CHARAs specimens were placed directly into a water bath at +37°C. Thawing times were as follows: minimum 26 minutes, maximum 41minutes, with the median of 32 min.

The time variability in both thawing protocols was given by the different allografts sizes (Tables 1 & 2), as well as different amounts of cryoprotectant used for each allograft during the cryopreservation process. After all the CHARA specimens were thawed, parts of an aortic root arterial wall were dissected from each aortic root and fixated in a 4% formaldehyde solution before they were sent for morphological analysis., fixated in a 4% formaldehyde solution and sent for electron microscope testing. The same procedure was followed for samples of non-coronary AV leaflets of each specimen.

3.5. Microscopic slide preparation

After the thawing protocols were completed, a sample of aortic root arterial wall was carefully dissected from each specimen (the arterial wall of non-coronary sinus was harvested from each allograft). After the samples were collected, they were fixed in Baker's solution. Each sample was divided into 5 -10 mm sub-samples. Samples were mounted on convex polystyrene casts with hedgehog-like spines. All samples

were washed in distilled water for 5 min and dehydrated in a graded ethanol series (70, 85, 95, and 100%) for 5 min at each level. The tissue samples were then immersed in 100% hexamethyldisilazane (CAS No. 999-97-3; Fluka Chemie AG, Buchs, Switzerland) (HMDS) for 10 minutes and air dried in an exhaust hood at room temperature.

Processed samples were mounted on stainless steel stubs, coated with gold, and stored in a desiccator until they were studied and photographed by Electron Microscope on scanning mode operating at 25 kV – BS 301. A special scoring system (from 1 to 6) was introduced in order to analyse morphological changes of the arterial wall of aortic root under the electron microscope: 1. Morphologically intact endothelium – putative physiological changes are not reflected in the superficial morphology of endothelial cells, 2. Confluent endothelium with structural inhomogeneity– irregularities in the form of individual cells and changes of their membranes are detectable, 3. Disruption of intercellular contacts – continuity of endothelial coverage is lost, endotheliocytes shrink while still adhering to basal membrane, 4. Separation of endothelial cells – endotheliocytes separate from the basal lamina. Initially, they protrude by their intercellular edges into the lumen, 5. Complete loss of endothelium – denudation of the endothelial covering with the basal lamina exposed, 6. Damage of subendothelial layers – the valvular surface is covered only by remnants of basal membrane, the fiber structure of the lamina fibrosa and the lamina ventricularis may be dissolved [117] (Table 3 – on the next page).

Table 3: Scoring system for morphological sample analysis.

Score	Morphology
1	Morphologically intact endothelium – putative physiological changes are not reflected in the superficial morphology of endothelial cells.
2	Confluent endothelium with structural inhomogeneity– irregularities in the form of individual cells and changes of their membranes are detectable.
3	Disruption of intercellular contacts – continuity of endothelial coverage is lost, endotheliocytes shrink while still adhering to basal membrane.
4	Separation of endothelial cells – endotheliocytes separate from the basal lamina. Initially, they protrude by their intercellular edges into the lumen.
5	Complete loss of endothelium – denudation of the endothelial covering with the basal lamina exposed.
6	Damage of subendothelial layers – the valvular surface is covered only by remnants of basal membrane, the fiber structure of the lamina fibrosa and the lamina ventricularis may be dissolved.

3.6. Reproducibility of external aortic root annuloplasty

Human aortic root allografts were used for determining the reproducibility of external aortic root annuloplasty with the use of Coroneo external aortic annuloplasty ring (Extra-Aortic ^{TM}, CORONEO, Inc., Montreal, QC, Canada). 18 human aortic root allografts were dissected in a standard manner described by Lansac et al. [46]. This work [46] contains detailed description of the procedure performed.

After the dissection was completed, aortic valve junction (AVJ) was measured with the use of Hegar dilatators. Subsequently, Coroneo ring of the right size was implanted in a standard manner described by Lansac et al. (46). The size of the Coroneo ring was chosen based on standardized sizing of the aortic annulus. Table 4 [46] on the following page shows the sizing of the aortic annulus.

After the implantation was completed, a new AVJ was measured with the use of Hegar dilatators. For each aortic root allograft, the whole procedure of measuring the AVJ, Coroneo ring implantation with subsequently reduced AVJ measuring was repeated twice. The goal of these experiments was to evaluate the reproducibility of this technique and to determine the effect of variable muscular septum thickness on the reduction of AVJ.

Table 4. Criteria for choice of the aortic ring and tube graft diameters [46].

Aortic annular base diameter (Hegar dilators, mm)					
	25-27	**28-30**	**31-33**	**34-39**	**>40**
Tube graft diameter (mm)	26	28	30	32	34
Subvalvular aortic ring diameter (mm)	25	27	29	31	33

3.6.1. Operation technique

The study of the reproducibility of the implantation of the aortic Coroneo ring was performed according to the reference [46] that contains the complete operation technique description. The details of the technique are shown in parts 3.6.1.1. – 3.6.1.3.

3.6.1.1. Dissection of the subvalvular plane

The aneurysmal portion of the ascending aorta is resected and sectioned at the level of the sinotubular junction. External dissection of the aortic root is performed down to the base of the aortic annulus. It begins at the non-coronary sinus. Aortic root is then liberated from the pulmonary artery and infundibulum and from the roof of the left atrium, in order to reach the subvalvular plane. The wall of the aortic sinus is totally removed leaving a fringe of aortic wall of approximately 2 mm. The dissection is completed by freeing the sub-valvular plane and the pulmonary infundibulum. The commissures and coronary arteries are individualized. Choice of expansible ring diameter is based on internal aortic annular base diameter measured with Hegar dilators (Table 4).

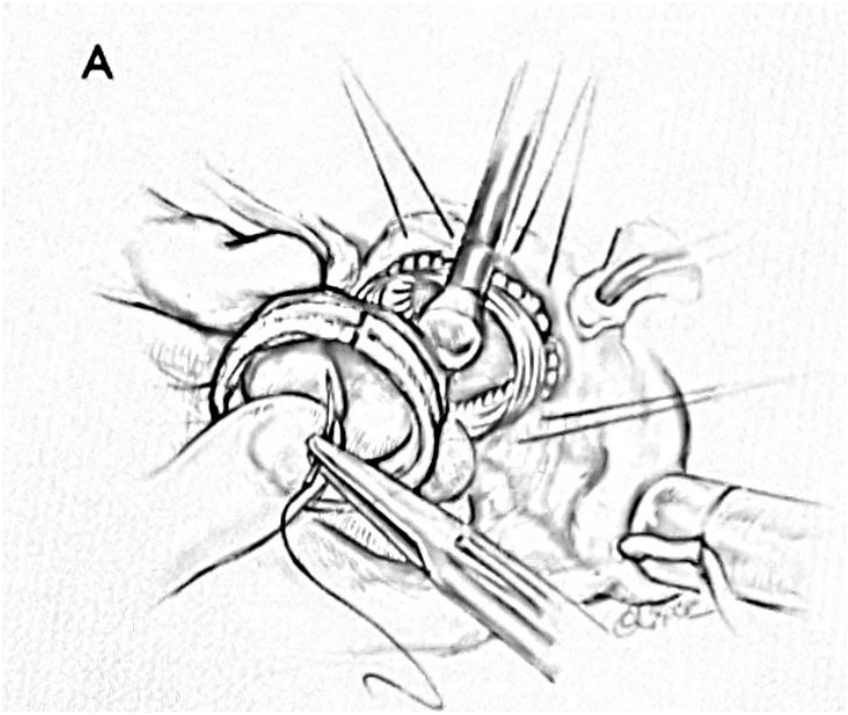
3.6.1.2. Placement of sub-valvular U-anchoring stitches for ring annuloplasty

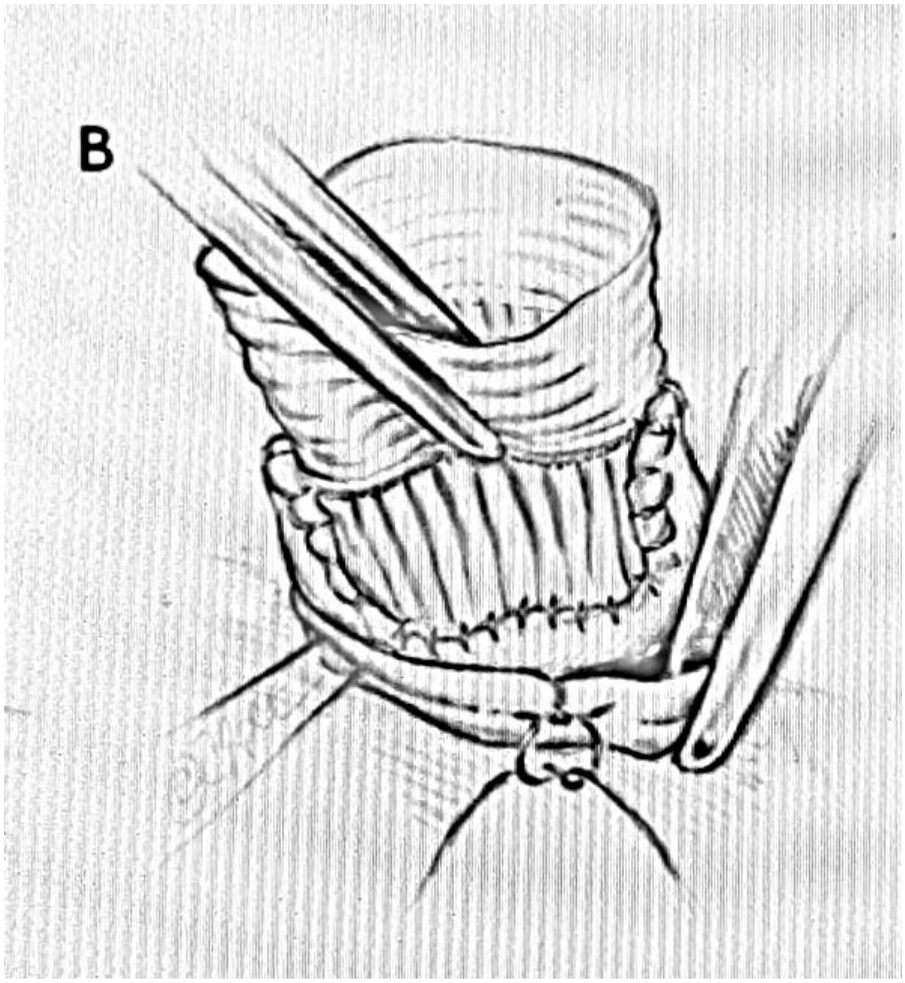
Five threads of 2.0 coated polyester fiber pledgeted are placed from the inside out as “U” stitches (width of 3 mm) circumferentially in the subvalvular plane, clock-wise beginning from the non-coronary sinus. Three sutures are positioned 2 mm below the nadir of each cusp and two are placed at the base of the interleaflet triangles between the non and left coronary sinuses and between the left and right coronary sinuses. A sixth suture is passed from externally at the level of the interleaflet triangle between the right and non-coronary sinus, without pinching inside in order to limit the risk of membranous septum or the bundle of His lesion.

3.6.1.3. Aortic annuloplasty ring placement

The six anchoring “U” stitches are passed through the inner aspect of the prosthetic expansible aortic ring (CORONEO) (Fig. 1A). The ring is then descended around the remodelled aortic root (Fig. 1B) and the “U” stitches are tied to secure the ring in subvalvular position (Fig. 1B). The finalization of the procedure is shown at Fig. 1C.

Fig. 1. Placement of the subvalvular expansible aortic ring (Coroneo) (A,B) and final aspect of the aortic root (C) [46]. – (This and the next page.)

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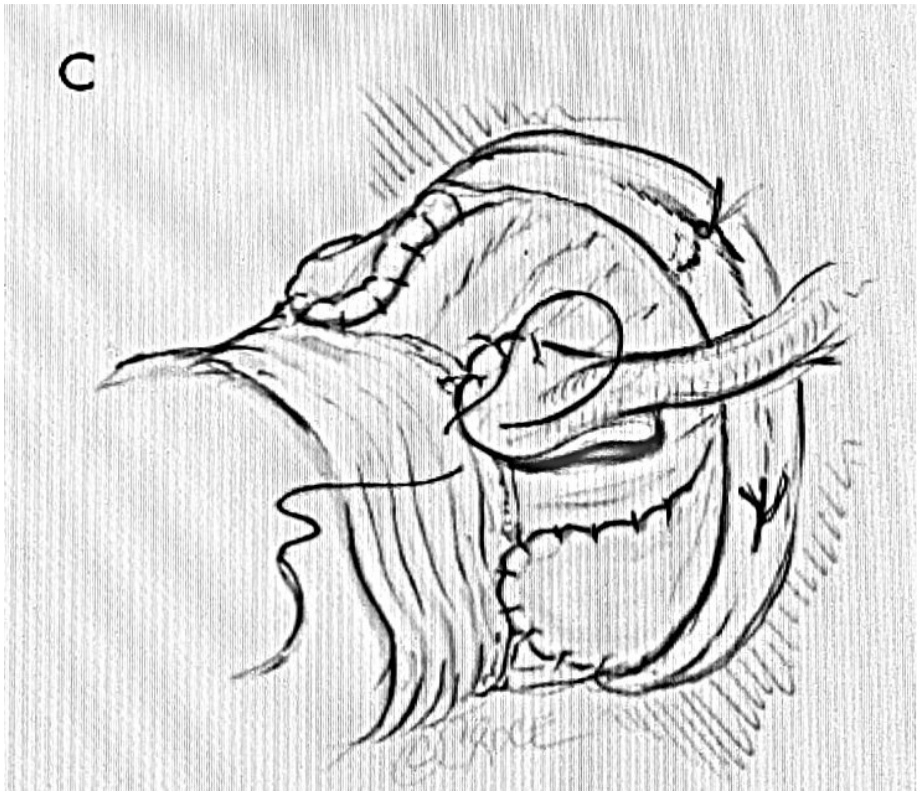

C

4. Results

4.1. Assessment of morphological changes of arterial wall arising from the use of different thawing protocols of CHARA arterial wall specimens

Histological analysis of the aortic root arterial wall was as follows:

- Thawing protocol 1(thawing at a room temperature +23°C): All 6 (100%) samples showed loss of the endothelium exposing the basal lamina, damage to the subendothelial layers with randomly dispersed circular defects and micro-fractures. Arterial wall was not contracted (Fig. 2). Furthermore, 4 (66%) samples showed no damage to the basal lamina (Score 5), 1(17%) sample showed minimal damage to the basal lamina (Score 5), and 1 (17%) sample showed severe damage to the basal lamina (Score 6).

- Thawing protocol 2 (water bath at +37°C): All 6 (100%) samples showed loss of endothelium from the luminal surface, longitudinal corrugations in the direction of blood flow caused by smooth muscle cells contractions in the tunica media with frequent fractures in the subendothelial layer (Fig. 3, Fig. 4). Furthermore, 5 (83%) samples showed severe basal lamina damage (Score 6), and 1(17%) sample showed no basal lamina with a severe damage to the internal elastic lamina (Score 6).

Fig. 2. Aortic root arterial wall (thawing protocol 1): Loss of the endothelium exposing the basal lamina, damage to the subendothelial layers with randomly dispersed circular defects and micro-fractures (magnification 560x).

Fig. 3. Aortic root arterial wall (thawing protocol 2): Loss of the endothelium from the luminal surface, longitudinal corrugations in the direction of blood flow caused by smooth muscle cells contractions in the tunica media (magnification 520x).

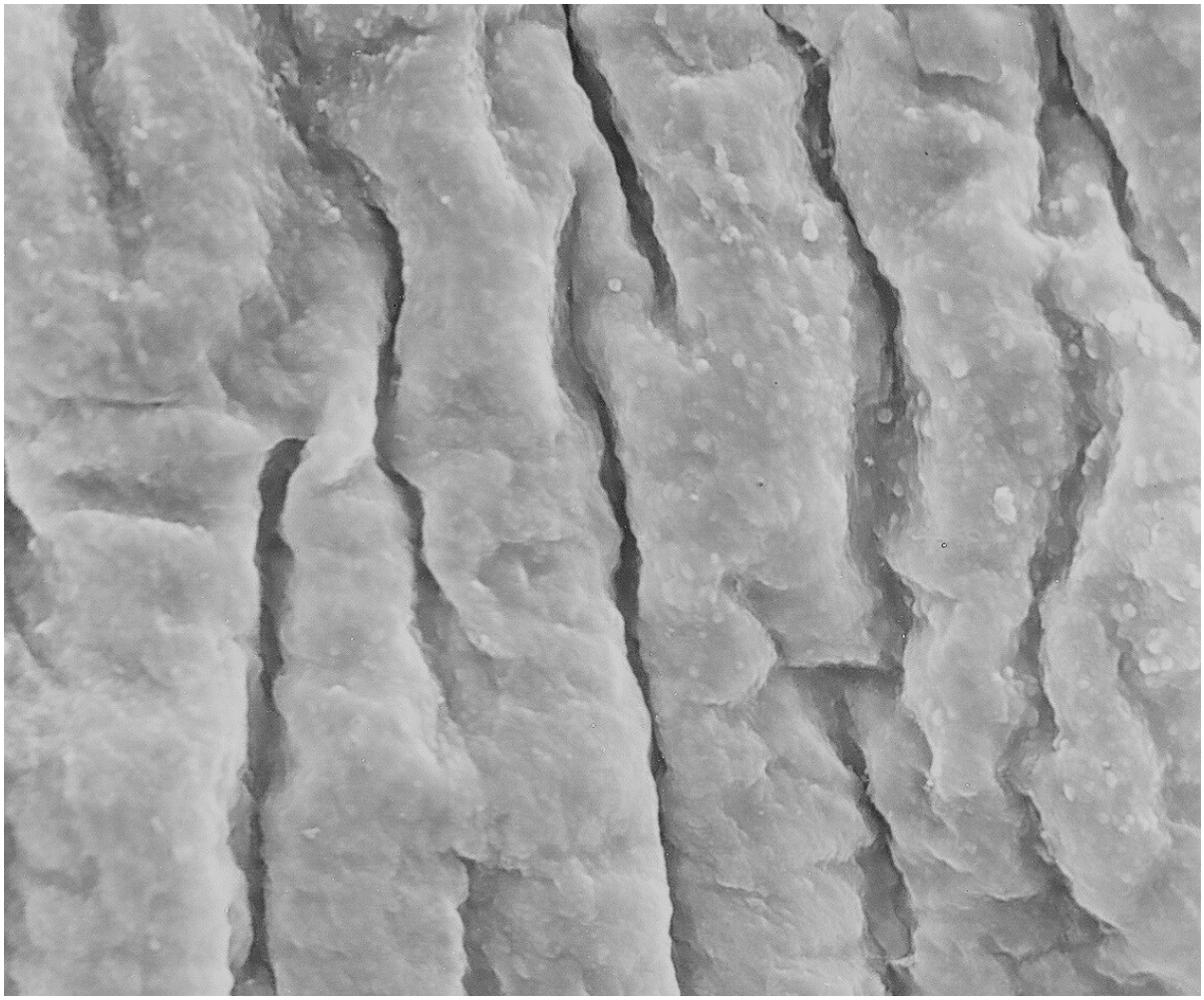
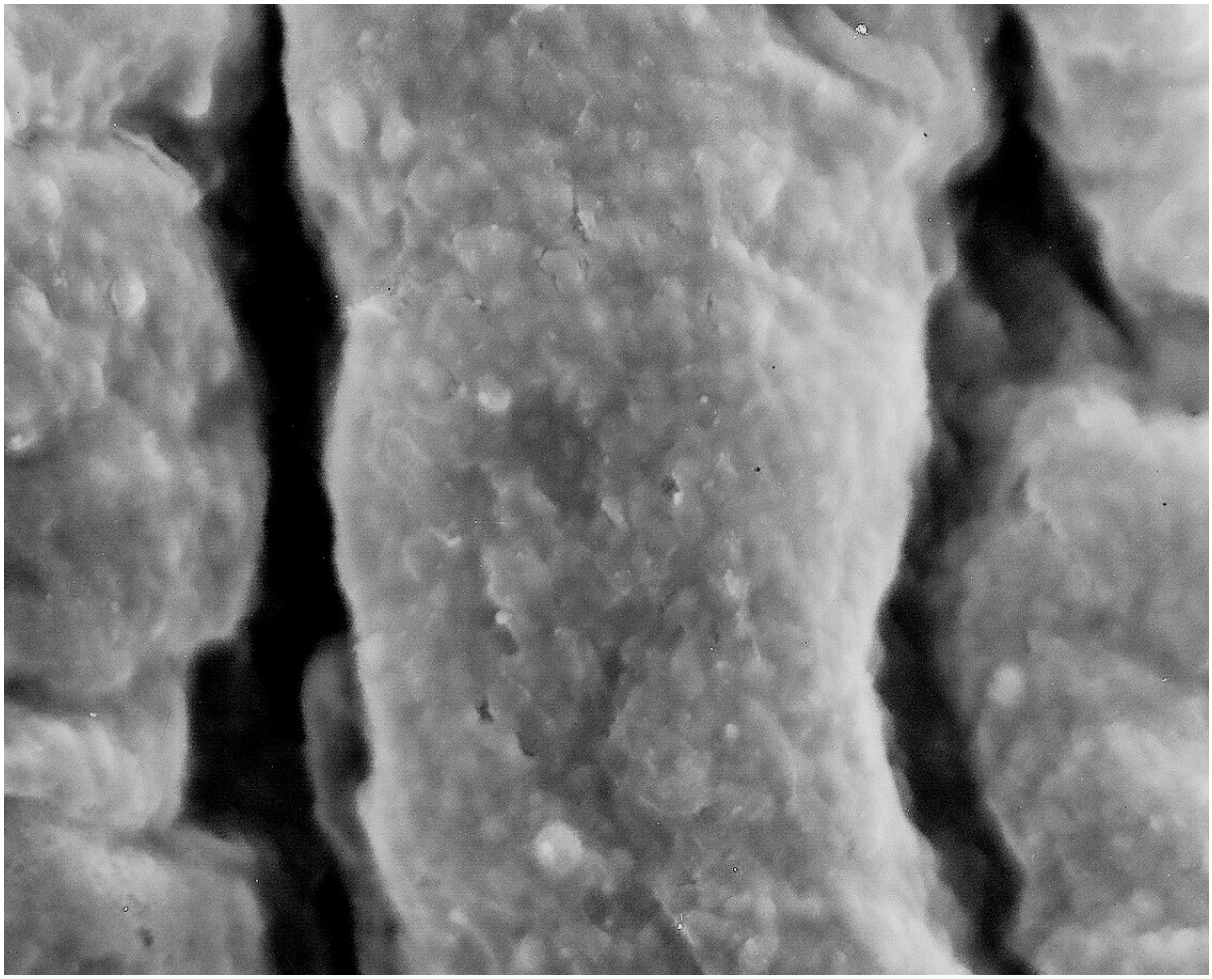


Fig. 4. Aortic root arterial wall (thawing protocol 2): Loss of the endothelium from the luminal surface, longitudinal corrugations in the direction of blood flow caused by smooth muscle cells contractions in the tunica media with frequent fractures in the subendothelial layer (magnification 1700x).



4.2. Assessment of morphological changes of aortic valve leaflets arising from the use of different thawing protocols of CHARA specimens

Histological analysis of the aortic valve leaflets was as follows:

- Thawing protocol 1 (thawing at a room temperature +23°C): 6 (100%) non-coronary AV leaflets showed loos of endothelial cells covering the basal membrane with no damage to the basal lamina (score 5) (Fig. 5).

- Thawing protocol 2 (water bath at +37°C): 5 (83%) non-coronary AV leaflets showed loos of endothelial cells covering the basal membrane with no damage to the basal lamina (score 5), 1 (17%) non-coronary AV leaflets showed significant damage to the basal membrane (score 6). (Fig. 6).

After further investigation of the samples, the severe damage of the non-coronary AV leaflet in thawing protocol 2 was caused by mechanical stresses exerted on the samples during dissection and microscopic sample preparation. One of the examined samples underwent slight stretching during microscopic slide preparation due to its size. This resulted in more severe structural damage compared to the other samples.

Fig. 5. Non-coronary aortic valve leaflet (thawing protocol 1): Loss of endothelial cells covering the basal membrane with no damage to the basal lamina (magnification 520x).

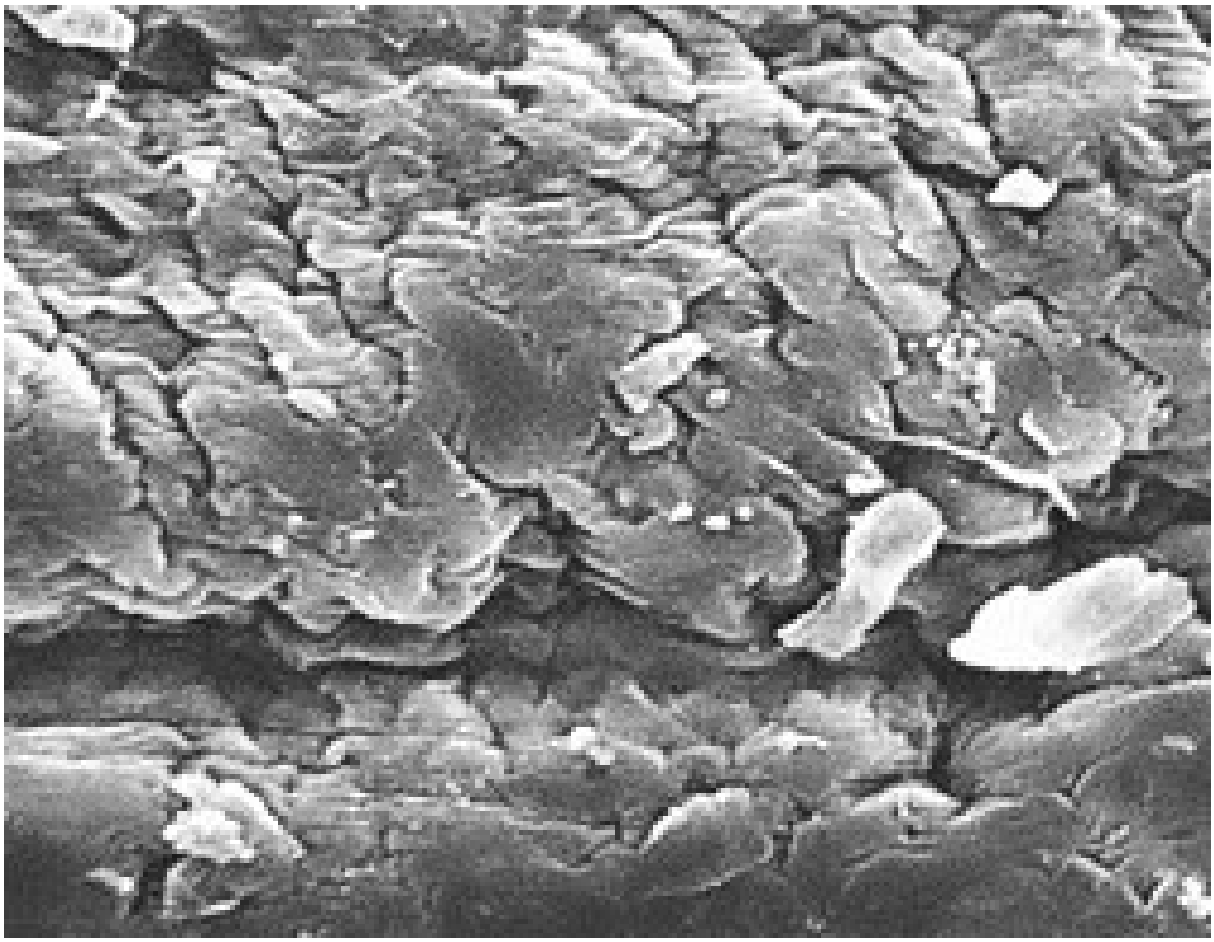
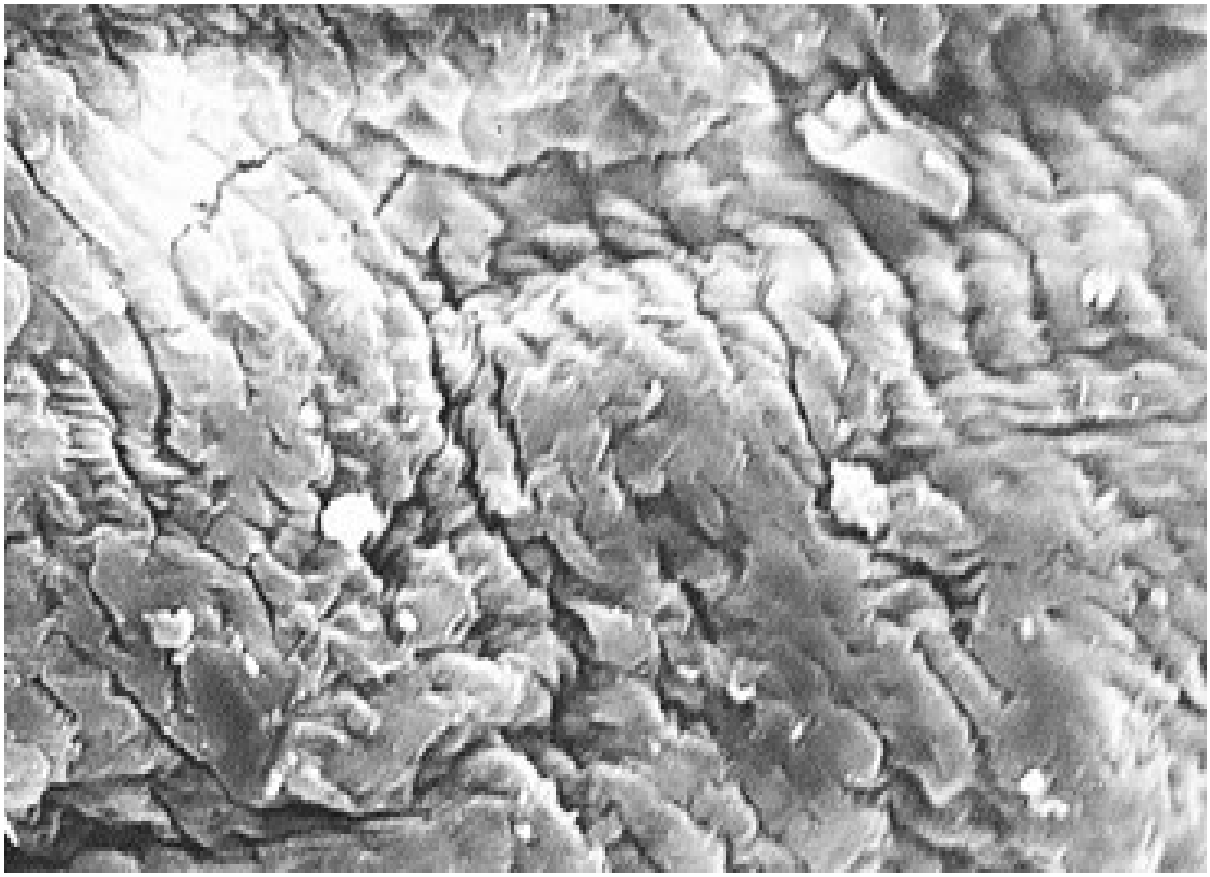


Fig. 6. Non-coronary aortic valve leaflet (thawing protocol 2): Loss of endothelial cells covering the basal membrane and significant damage to the basal membrane (magnification 520x).



4.3. Comparison of the obtained results with different thawing protocols of CHARA tissue samples

Based on our results regarding the Objective 1, we have demonstrated that all the samples of CHARAs thawed at the room temperature showed smaller overall structural damage to the arterial and no smooth muscle cell contraction in tunica media when compared to the samples thawed in a water bath. Thawing at a room temperature seems to be gentler and does not lead to so severe damage of the CHARAs arterial wall. Consequently, based on our histological findings, we can conclude that the samples thawed at a room temperature should be in theory of higher quality compared to samples thaw at water bath, thus they should be more suitable for transplantation. This is despite the fact that the thawing of CHARA allograft specimens take longer when performed at the room temperature (median of 3hr 19 min) compared to the thawing in a water bath at +37°C – median time of thawing of a specimen was only 32 minutes.

Our finding shows the importance of technical aspects even for well-established surgical procedures.

In the study regarding our objective 2, the performed experimental work following the structural changes occurring during different thawing protocols on cryopreserved AV leaflets showed that different rates of thawing show identical structural changes. Therefore, the rate of thawing does not play a significant role in minimizing structural changes that occur during thawing of cryopreserved AV leaflet.

Consequently, it was demonstrated that different types of aortic root tissue (arterial wall versus aortic leaflets react differently when submitted to different thawing protocols, aortic leaflets being less sensitive to the thawing process alteration.

4.4. Reproducibility of external aortic root annuloplasty

As techniques for aortic surgery continue to evolve, the reproducibility of any routinely used surgical procedure becomes more significant. Only techniques that are deemed safe and reproducible, and that are associated with reasonable outcomes and are also superior to similar techniques are employed in surgical practice [118].

A dilated aortic annulus that stays untreated represents a highly significant factor contributing to the failure of aortic valve-sparing operations. Aortic annuloplasty reduces the annulus and leads to an increase of the coaptation height. Consequently, this surgical procedure is being used in significant numbers of patients. The importance of this procedure and the benefits of its use were the reason for us to evaluate the reproducibility of the implantation procedure of Coroneo ring at the aortal annular base.

Eighteen samples of aortic annular bases that were made available for this study by the Transplantation Department & Tissue Bank of the Faculty Hospital Motol in Prague were used for this study. This represents a highly significant number and similar studies with a such high number of aortic roots were not published yet (to my best knowledge).

The procedure of implanting Coroneo ring was performed twice on each aortic annular base and the results were evaluated graphically and by statistical methods.

The characterization of all the used aortic annular bases, including an ID, is at the end of this section as is also the comparison of the first and second Coroneo ring implantations at the aortic annular base outcomes.

Table 5. Reduction in the size of aortic annular base after the first Coroneo ring implantation.

Size of aortic annular base, mm*	**Size of aortic annular base after Coroneo ring implantation 1, mm***	**Decrease in aortic annulus diameter, %**
20	16	20
24	18	25
22	18	18
26	22	15
27	23	15
26	23	12
26	22	15
26	22	15
26	21	19
26	22	15
27	22	19
27	23	15
25	20	20
25	21	16
26	21	19
25	21	16
28	22	21
32	25	22

* Hegar dilators

Fig. 7. Reduction in the size of an aortic annular base after the first Coroneo ring implantation.

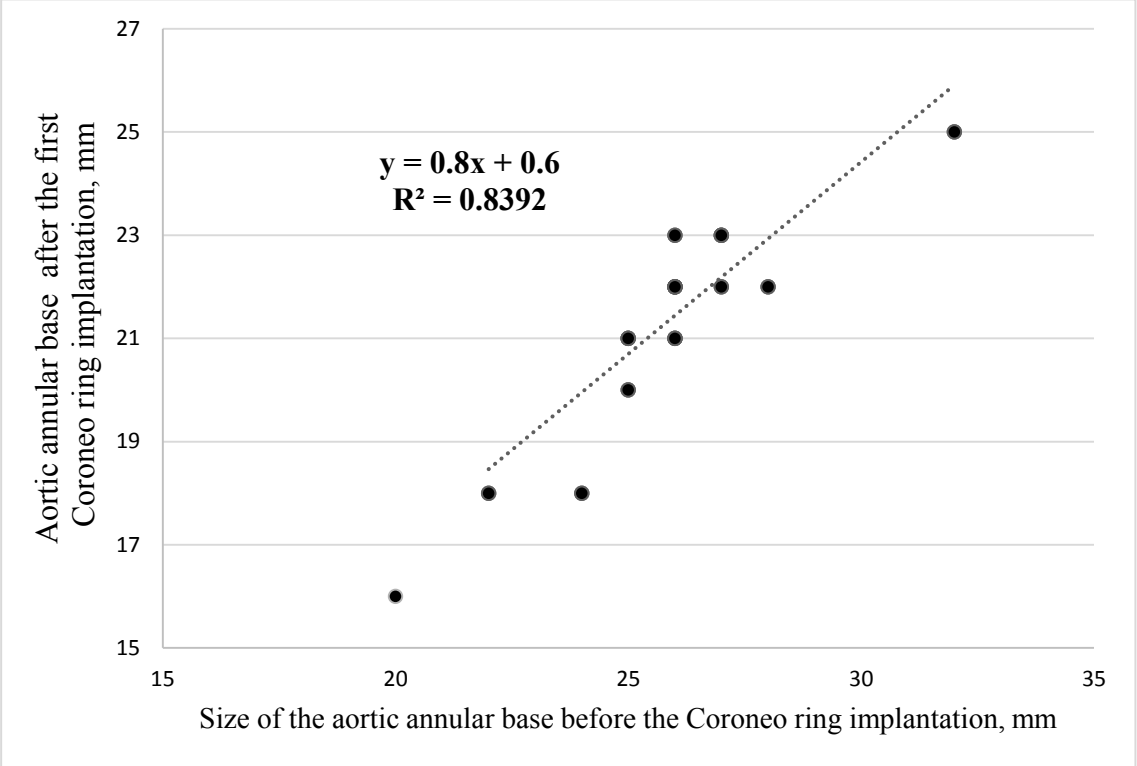


Fig. 8. Percentage reduction in the size of an aortic annular base after the first Coroneo ring implantation.

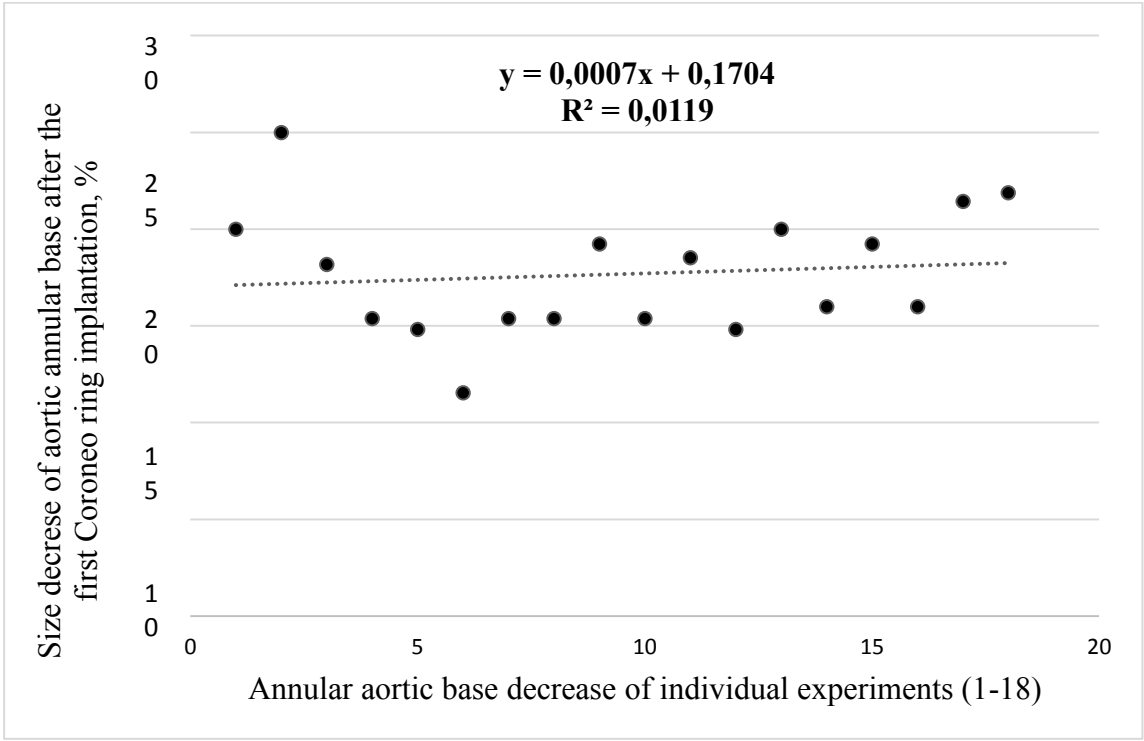


Table 6. Reduction in the size of aortic annular base after the second Coroneo ring implantation.

Size of aortic annular base, mm*	**Size of aortic annular base after Coroneo ring implantation 2, mm***	**Decrease in aortic annulus diameter, %**
20	16	20
24	18	25
22	18	18
26	22	12
27	23	15
26	23	12
26	22	19
26	22	15
26	21	19
26	22	19
27	22	19
27	23	15
25	20	20
25	21	16
26	21	15
25	21	16
28	22	21
32	25	22

* Hegar dilators

Fig. 9. Reduction in the size of an aortic annular base after the second Coroneo ring implantation.

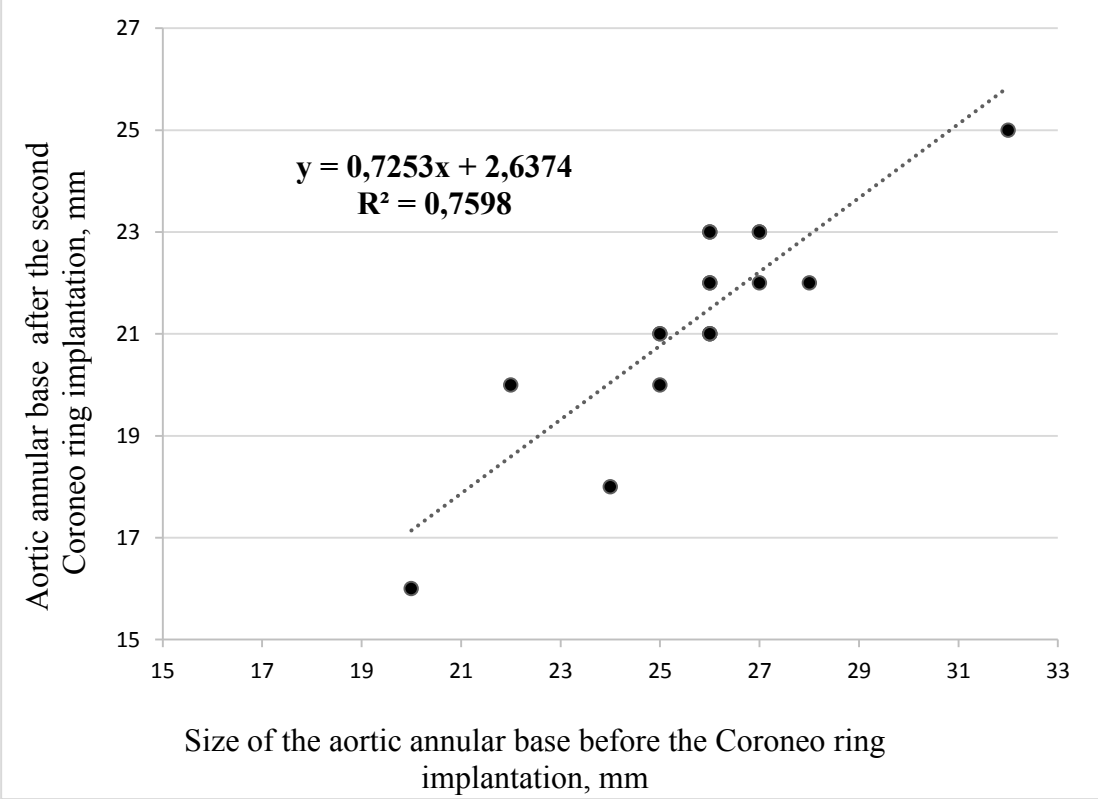


Fig. 10. Percentage reduction in the size of an aortic annular base after the second Coroneo ring implantation.

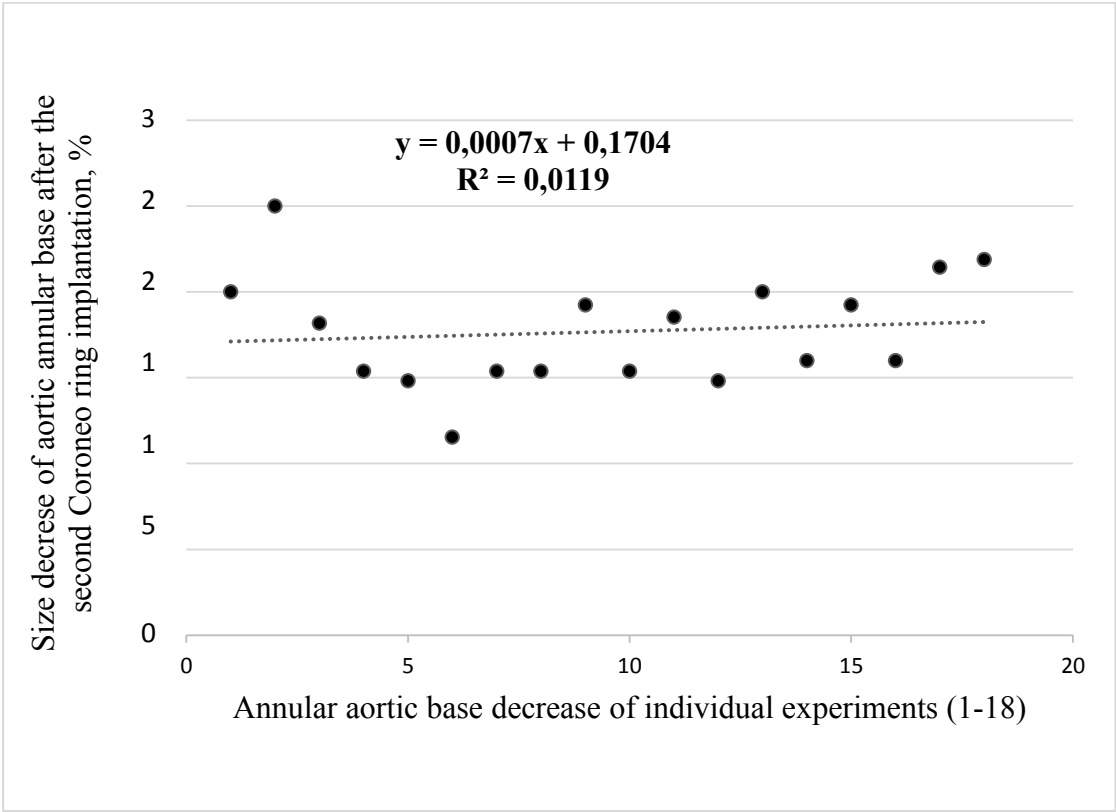


Table 7. Comparison of the size of the aortic annulus base after the first and second Coroneo ring implantations.

Aortic annulus base after the first Coroneo ring implantation, mm	**Aortic annulus base after the second Coroneo ring implantation, mm**	**Difference in size of aortic annulus base after the first and second Coroneo ring implantation, %**
16	16	0
18	18	0
18	20	11
22	23	5
23	23	0
23	23	0
22	21	-5
22	22	0
21	21	0
22	21	-5
22	22	0
23	23	0
20	20	0
21	21	0
21	22	5
21	21	0
22	22	0
25	25	0

Fig. 11. Comparison of the change (%) in the size of the aortic annular base after the first (▨) and second (▮) Coroneo ring implantation.

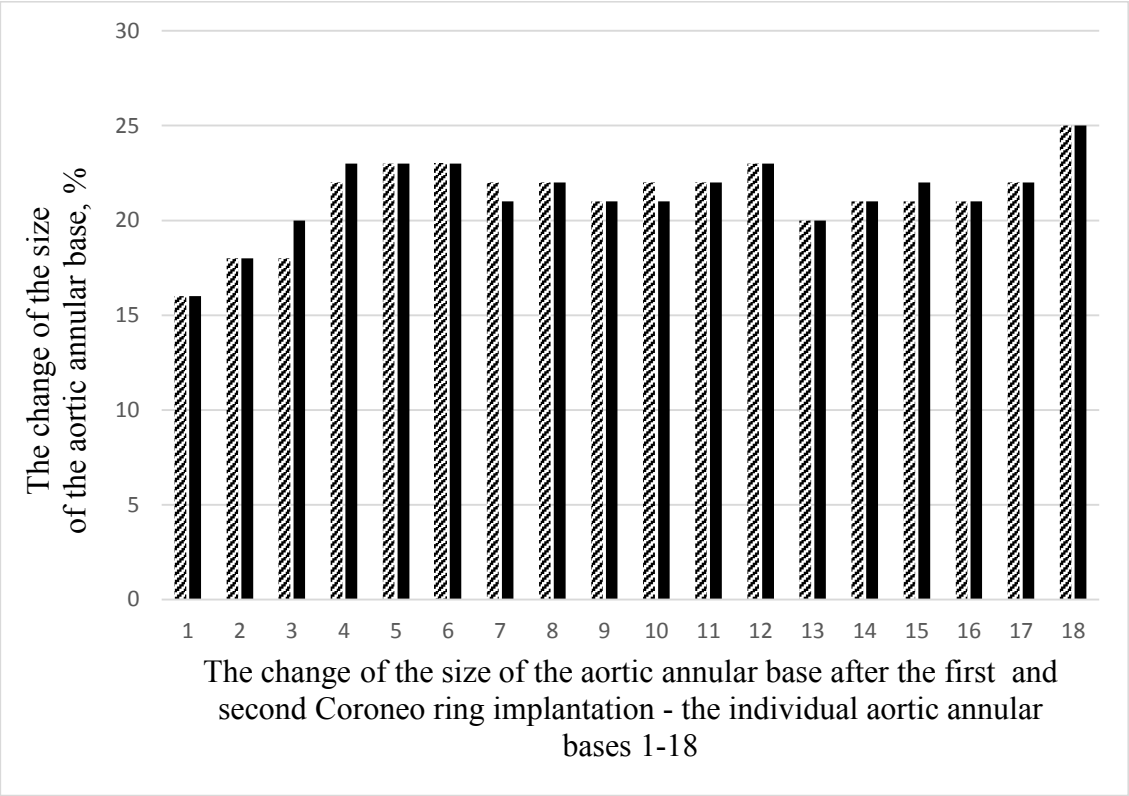


Fig. 12. Comparison of the change (%) in the size of the aortic annular base after the first and second Coroneo ring implantation.

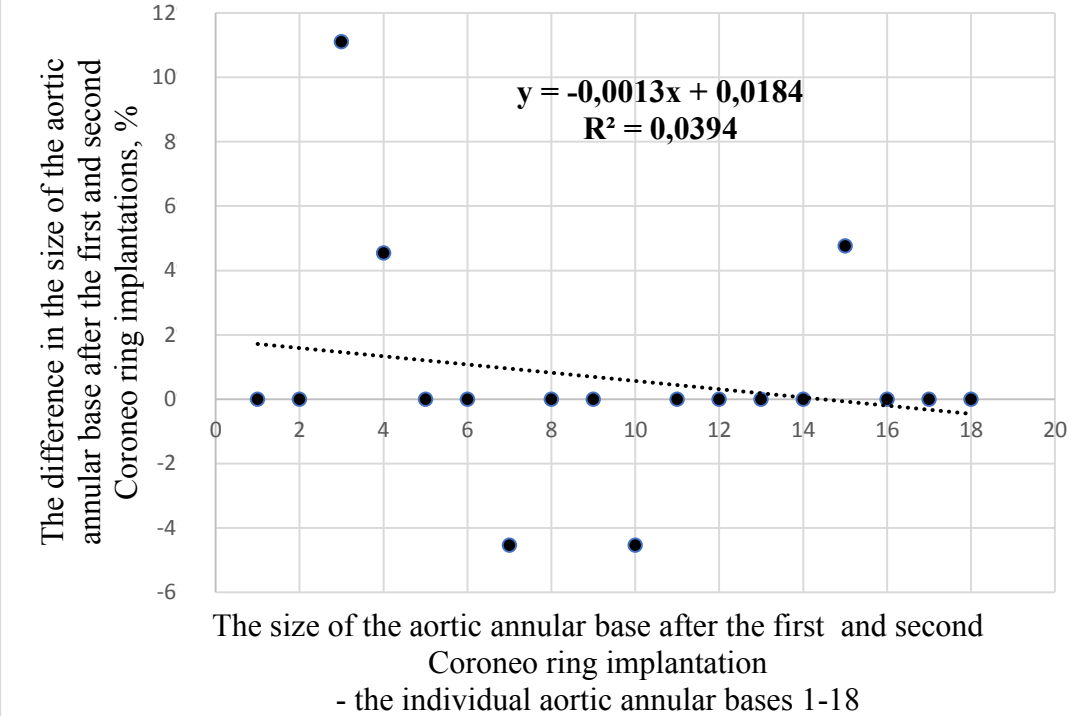


Table 8. The summary of the Coroneo ring implantation results on the size of the aortic annular bases.

No.	ID of the aortic annular base	Aortic annular base, mm	Coroneo ring, mm	Aortic annular base after the implantation of Coroneo ring, mm	
				The first implantation	The second implantation
1	TCAO2009000041A	20	21	16	16
2	TCAO2003000011A	24	23	18	18
3	TCAO2009000020A	22	23	18	20
4	TCAO2009000021A	26	25	22	23
5	03/K057/41	27	25	23	23
6	04/K057/41	26	25	23	23
7	04/K007/41	26	25	22	21
8	03/K042/41	26	25	22	22
9	03/K049/4	26	25	21	21
10	03/K042/42	26	25	22	21
11	TCAO2005000063A	27	25	22	22
12	032/K083	27	25	23	23
13	04/K068/41	25	25	20	20
14	04/K066/41	25	25	21	21
15	05/K027/41	26	25	21	22
16	05/K30/42	25	25	21	21
17	TCAO2004000052A	28	27	22	22
18	03/K044/41	32	29	25	25

Table 9. Comparison of outcomes of the first and second Coroneo ring implantation at the aortic annular base (A) and summary difference between the two implantations performed (B).

A

Parameter	Coroneo ring implantation	
	The first	**The second**
Number of experiments	18	18
Decrease of the size of the aortic annular base:	3 - 7 mm	3 - 7 mm
	12 - 25%	12 - 25%
Average	4.56 mm	4.61 mm
	18%	17%
Median	17%	18.5%

B

Average decrease of the size difference	1%
Median difference of the decrease of the size	1.5%
Average change of the size of the annulus	
between two implantations*	0.60%

*There was no difference between the two implantations outcome in 13 out of 18 experiments.

5. Discussion

5.1. Morphological changes of the arterial wall and aortic root allograft leaflets rising from the use of different thawing protocols

Over the past four decades, cryopreservation of arterial allografts had shown inconsistent results in long-term graft durability. In order to understand the changes that occur during cryopreservation and thawing of allografts, microscopic and immuno-histological techniques are used in order to determine structural and functional changes. One of the most important experimental works performed by M'Bengue-Gaye et al. on a rabbits' carotid arteries clearly showed the effects of cryopreservation on allografts [119]. It had been clearly demonstrated that allograft processing including cryopreservation and thawing are crucial in determining cryopreserved "muscular arteries" allografts durability and clinical performance [120,121].

Immunological reactions caused by cryopreserved arterial allografts are very complex and not fully understood. Arterial allografts are immunogenic as they induce the anti-HLA antibody response in the recipient, thus causing graft rejection. The contribution of anti- HLA class I antibody to the structural allografts degradation is not clear [122]. Cryopreserved aortic root allografts are immunogenic, HLA-ABC and HLA –DR antigen molecules can be identified on the aortic wall and aortic valve leaflet [123]. Rodriguez M et al. proved that slow thawing protocol causes the immune response to subside when compared to fresh arterial allografts [124].

Although cryopreserved aortic root allograft transplants are associated with outstanding hemodynamic, low thromboembolic events and low risk of endocarditis, the biggest concern is their long-term durability and subsequent risk of reoperation based on the allograft structural degradation related to the degradation of valvular leaflets, thus leading to aortic valve insufficiency [125-128]. Progressive allograft atherosclerosis caused by a chronic rejection is one of the major factors that lead to a short-term graft survival with characteristic diffuse intimal thickening, migration and proliferation of smooth muscle cells [110,111]. Allograft vasculopathy is caused by an injury inflicted as a response to the transplantation, causing endothelial dysfunction and intimal hyperplasia development, thus contributing to the development of allograft

atherosclerosis. Experimental work Xu et al. [130] on rat model showed that atherosclerosis – multifactorial complex associated with immunologic and non-immunologic risk factors can be controlled to some extent [119]. Calcification of the aortic root arterial wall is another major preconditioning for aneurysm formation and is the main cofactors in systemic calcific embolization [131]. Another key aspect is the change of toughness and biaxial tensile properties of cryopreserved arterial allografts that lead to early aneurysm formation and ruptures in high-pressure arterial circulation [17].

Additionally, cryopreserved aortic root allografts are widely used in a cardiac surgery as a means of surgical treatment of a prosthesis infection. Based on short and mid-term results, cryopreserved aortic root allografts showed acceptable results with a low risk of reinfection. Patients that receive cryopreserved allografts require a long-term follow-up for both infection and implant durability [122,123]. Despite the advances in understanding morphological and functional changes that are caused by cryopreservation and subsequent thawing, there are still many immunological processes that are not fully understood and are in the need of further investigation [124].

Since the first successful aortic valve allograft transplant performed by Ross in 1962, over 25000 AV allografts have been implanted to-date [106,125]. Over the history of AV allograft transplant use, the procedures of sterilization and storage have evolved immensely; from fresh aseptic harvest with immediate transplantation trough antibiotic sterilization and wet storage at 4°C up to current antibiotic sterilization and cryopreservation [126]. Even though the durability of fresh AV allografts is superior to cryopreserve AV allografts; the lack of donors had forced most of the cardiac centres to focus on allograft cryopreservation. Cryopreservation plays a major role in the degeneration of AV allografts which subsequently leads to progressive calcification and fibrosis affecting up to one-fourth of all implanted AV allografts [127]. Despite the negative impact of cryopreservation on the AV allografts, Fukushimas et al. had shown that cryopreserved AV allografts are durable for over 15 years. He also showed that allograft durability was closely associated and affected by obesity, an age of the recipient and donor. The most important factor was shown to be the surgical technique used during the allograft transplantation [128].

Our experimental results show identical structural changes in both examined thawing protocols. Consequently, the faster rate of thawing does not necessarily mean

that the aortic valve leaflet will be more structurally damaged or structurally compromised. Therefore, they would not/do not require more frequent observation after implantation.

Another aspect that is thought to contribute to the cryopreserved AV allografts failure is gender mismatch. However, the evidence behind this theory is imprecise as gender matching is not done routinely before these transplants. Böll et al. demonstrated that gender-mismatched vs. gender-matched allografts showed no significant difference in regard to death, need for reoperation and allograft function [129].

Experimental work by Brockbank et al. had shown a significantly reduced extracellular matrix damage and well preserved cellular structures in the ice-free leaflets. They also clearly demonstrated that cryopreservation of heart valves transplants at -80°C avoids ice formation, tissue cracking and preserves extracellular matrix [109]. Improvements in modern antibiotic treatment of AV allograft before cryopreservation has a significant impact on infection resistance of AV allografts, as shown in their enhanced bacterial resistance [131].

Cryopreserved allografts represent a gold standard as a treatment in selected indications such as bacterial endocarditis, Ross procedure. However, there is a grooving evidence that decellularized engineered allografts may be superior to cryopreserved allografts [132]. Decellularized aortic valve allografts have shown outstanding mid-term results after their implantation in terms of their stable structural integrity, low rate of calcification and hemodynamic properties [133,134]. Despite the promising short and mid-term results, long-term results are still not known.

Even thou the efforts in minimizing the damage inflicted by cryopreservation on the AV allografts, there are still many factors that need thorough experimental and clinical examination in order ensure allografts of highest possible quality and durability.

5.1.1. Limitations

This part of my dissertation has two obvious limitations:

1) The obvious limitation for these type of experiments is the type of experimental material used – human allografts as the strict ethical regulations are presents and only tissue that is no more suitable for transplantation to patients can be used.

2) The number of tissue samples may always be increased. However, there is no such study involving 12 or even more tissue samples available in the scientific literature (to the best of our knowledge), the numbers in the two groups (6 + 6) were deemed high enough for obtaining relevant results.

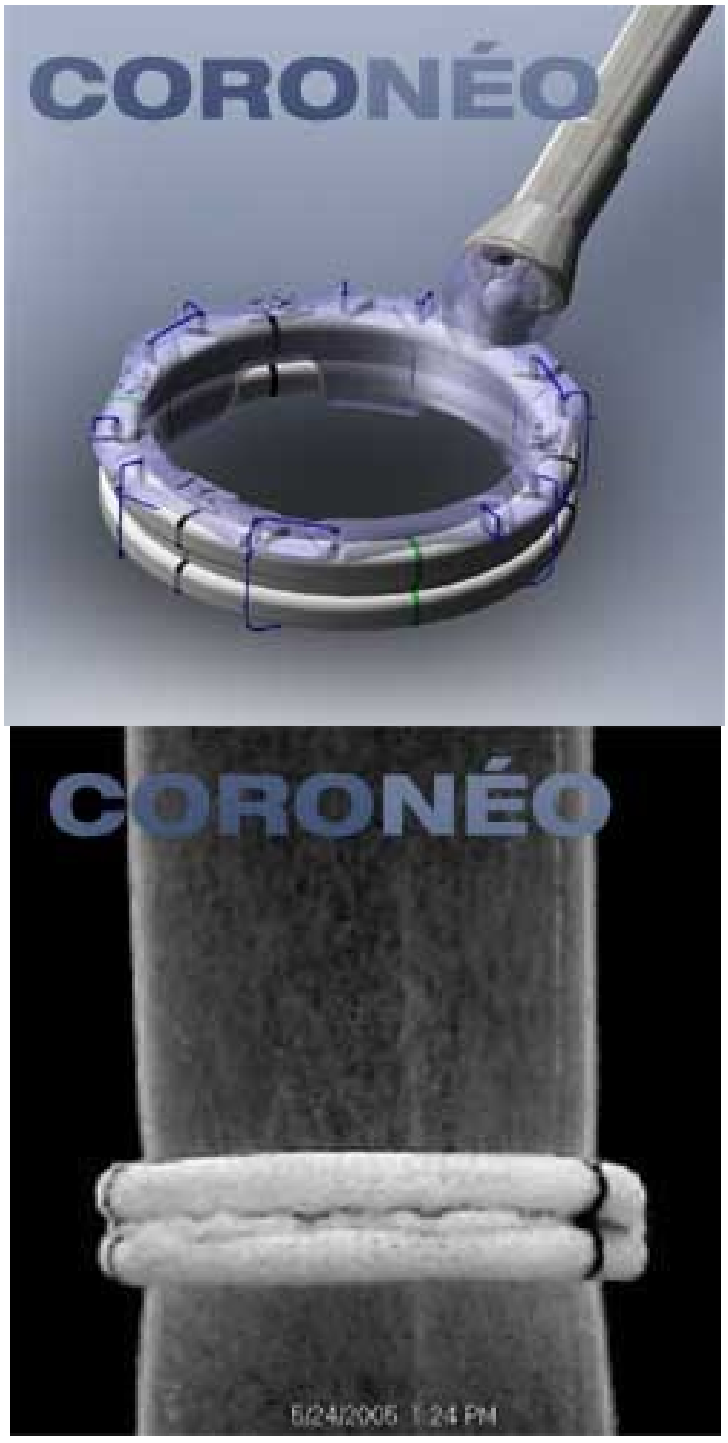
3) The second limitation may be deemed to be based on the fact that only two thawing protocols were investigated - thawing protocol 1 (thawing at a room temperature of +23°C) and thawing protocol 2 (water bath at +37°C). As a slower thawing at the lower room temperature produced tissue of better integrity compared to the thawing in a water bath at +37°C, thawing of the tissue in water bath at temperatures lower than +37°C and higher than the room temperature of +23°C, i.e. +25 or 30 °C may save time during the operation procedure and still produce tissue samples of retained integrity of the structure.

5.2. Reproducibility of Coroneo ring implantation into the aortic root

The aortic annuloplasty ring, Coroneo ring in our study (Fig. 13) [135], is essential for the widespread adoption of aortic valvuloplasty. The introduction of Coroneo ring into everyday surgical practice led to effective standardization through proper selection of patients, assessment of valve performance and prosthetic ring choice. Coroneo ring availability led to improved attention to aortic valve-sparing Techniques for dystrophic disease of aortic root and aortic valve.

Fig. 13. External Coroneo ring.

Coroneo ring as shown in the materials of the Producer (Extra-Aortic™, CORONEO, Inc., Montreal, QC, Canada) [135].



Coroneo ring (Extra-Aortic annuloplasty ring) supports dystrophic tissue of the aorta through its attachment on the outside diameter of the aorta to support the external dilated diameter of the aorta. The elasticity of the ring makes an expansion of the tissues during the diastole and systole possible by 10%. Coroneo ring is available in 6 sizes according to the normal diastolic diameters of the aortic annular base. The sizes available are 23, 25, 27, 29, 31 and 33 mm. The company claims that the rings maintain their elasticity for long time after tissue in-grows into the expansible sheaths around the elastic cores of Coroneo ring [135].

Coroneo ring is attached on the outer wall of the aorta as a hoop supporting the tissue against aortic pressure directly. Consequently, the stress concentrating in the aortic wall is reduced. As the attachment sutures do not have to withstand significant forces, they are applied only in numbers preventing migration of the ring.

The performed study was planned as some related technical aspect of cardiac surgeries are still being investigated, even when it is generally recognized that an untreated dilated aortic annulus represents a major risk for failure of aortic valve-sparing operations or repair and that aortic annuloplasty reduces the annulus and increases the coaptation height. The stabilization of all of the components of the aortic root improves the durability of the valve, and the techniques proposed are reproducible and stable in the medium-term. These operations provide satisfactory long-term results for each ascending aorta phenotype with bicuspid or tricuspid valve. The longer follow-up of the patients is ongoing with the AVIATOR registry [136,137].

Our study concentrated on verification of reproducibility of the Coroneo ring implantation procedure and additionally on the effect of re-implantation of Coroneo ring on the aortic annular base size. The obtained data are presented in Tables 5-9 and Figs. 7-12.

Table 5 summarizes the first set of experiments evaluating the effect of Coroneo ring implantation on the size of aortic annular base. The decrease of the size of the aortic annular base was between 3 and 7 mm with the average decrease of 4.56 mm. The size decrease was between 12 and 25% of the aortic annular base before implantation with the average decrease of 18% and median of 17%.

Table 6 summarizes the second set of experiments of Coroneo ring re-implantation dealing with the effect of the reimplantation on the size of aortic annular base. The decrease of

the size of the aortic annular base was again between 3 and 7 mm with the average decrease of 4.61 mm. The size decrease was between 12 and 25% of the aortic annular base before implantation with the average decrease of 17% and median of 18.5%.

The comparison of the effects of Coroneo ring implantation and second implantation indicates that the differences between these two sets of experiments are small (Table 9A). Additionally, difference between outcomes of Coroneo ring implantation and second-implantation were also minor (Table9B): Average decrease of the size difference between these two sets of experiments was only 1% with median of 1.5%. Average change of the size of the aortic annular base between Coroneo ring implantation and second implantation was only 0.60%. Additionally, there was no difference between the two implantations in 13 out of 18 experiments (72%).

Figs. 7 and 9 illustrate the relationship between reduction in the size of an aortic annular base after the first Coroneo ring implantation (Fig. 7) and after re-implantation/the second implantation (Fig. 9). The distribution of the data is similar and no statistical difference between these two sets of experiments exists (statistical significance determined by applying t-test method for the two sets of experiments is 0.4368 for all sizes of aortic annular base and is only 0.5000 for Coroneo ring size No. 25 experiments).

Figs. 8 and 10 illustrate the reduction of the size of an aortic annular base after the first and after the second Coroneo ring implantation. The outcomes of individual experiments are even and there is no really extreme change in the size of aortic annular base after the implantation.

Fig. 11 illustrates the fact that the size of aortic annular base decreased in similar manner after the first and second Coroneo ring implantations. Fig. 12 demonstrates differences in the change (%) of the size of the aortic annular base after the first and second Coroneo ring implantation. Again, it can be seen that the change in the size of the aortic annular base was the same in 13 out of 18 experimental implantations and re-implantations. The second Coroneo ring implantation (second implantation) resulted in an increased aortic annular base diameter in only 3 experiments (experiments 3, 4 and 15) and in a decreased aortic annular base size in only two experiments (experiments 7 and 10). The size difference was only 1 mm in the four mentioned cases and 2 mm in the experiment No. 3.

5.2.1. Limitations

This part of my dissertation has one obvious limitation:

1) As this part of my dissertation study is technical, the number of tissue samples may be increased. However, based on the data obtained, this would not provide additional information on the reproducibility or improve the data.

6. Conclusion

Our results on assessing morphological changes of the arterial wall and CHARA leaflets arising during different thawing protocols indicate the following:

1) Thawing protocol that allows tissue samples to thaw at the room temperature provides better outcomes regarding the quality of the thawed tissue compared to thawing in a water bath at higher temperature.

2) The changes observed in the tissue samples of the arterial wall and CHARA leaflets included the loss of the endothelium and an exposition of the basal lamina, damage to the subendothelial layers with randomly dispersed circular defects, micro-fractures, and longitudinal corrugations in the direction of blood flow.

3) Cryopreserved AV leaflets are more stable to the use of different thawing protocols compared to the arterial wall tissue.

Our results on reproducibility of aortic annular base Coroneo ring implantation and re-implantation indicate the following:

4) The reproducibility of this procedure is very high.

5) There is no significant difference between the procedures of implantation and second implantation of Coroneo ring on the aortic annular base.

7. Personal Contribution of the Author

I would like to confirm that I performed the experiments and harvesting the thawed allografts personally with the help and participation of several colleagues. The processing of the samples and microscopic slide preparation was performed at the Department of Anatomy, Faculty of Medicine in Hradec Králové by the Head, Assoc. Prof. MUDr. Dáša Slížová, CSc., and MUDr. Otakar Krs, CSc., Vice Head of the same Department. The discussion with these colleagues over the slides was very fruitful.

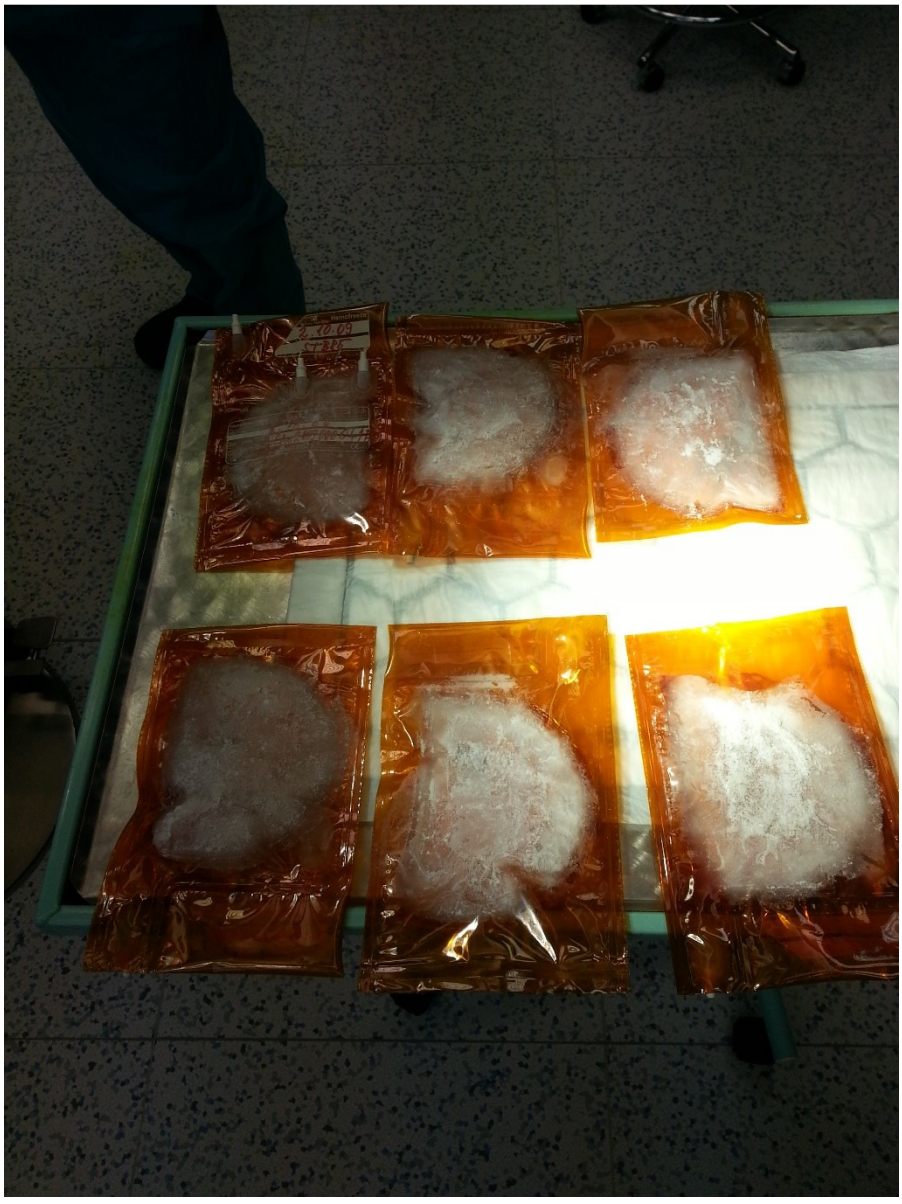
I also personally performed the experiments with Coroneo ring implantation and second implantation, also with help from the colleagues on our team.

The writing-up is my work with some corrections and contribution from my colleagues who were the members of the team and who are mentioned on the publications. I served as a Corresponding Author on these publications (PlosOne, Advances in Clinical and Experimental Medicine 2x).

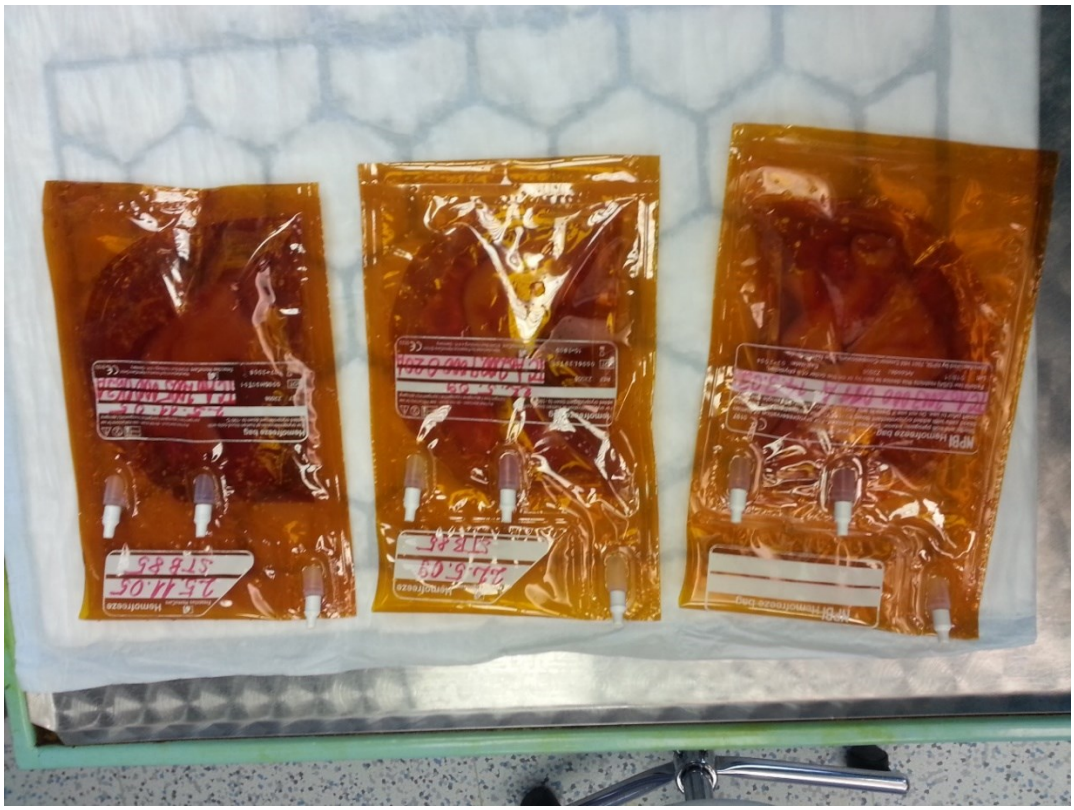
8. Addendum:

Photographic documentation of the experimental work

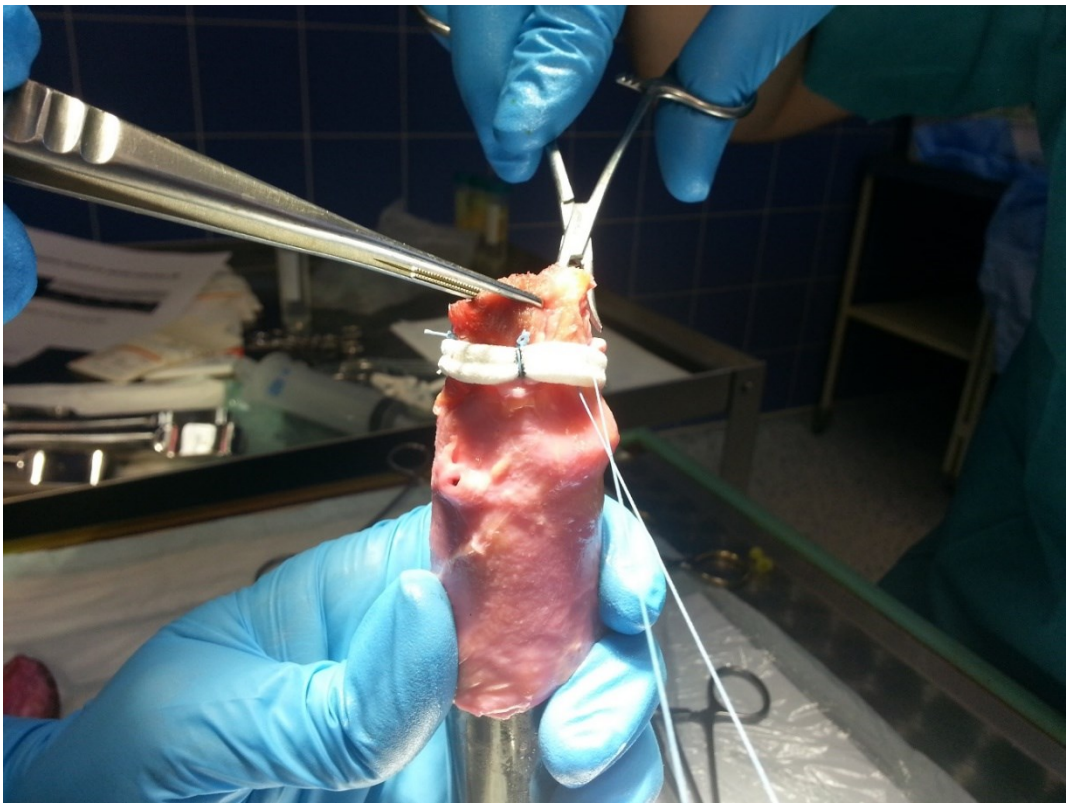
Photography 1. Cryopreserved human aortic roots before thawing.



Photography 2. Thawed human aortic roots.



Photography 3. The process of Coroneo ring implantation on the human aortic root.



10. List of Tables

11. List of Figures

12. References

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