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Rīgas Stradiņa universitāte

Mārtiņš Kalējs

OPTIMAL MECHANICAL PARAMETERS FOR STRUCTURAL COMPONENTS OF HEART VALVE BIOPROSTHESES AND SELECTION OF A MATCHING SUBSTITUTE MATERIAL

Summary of the Doctoral Thesis
for obtaining the degree of a Doctor of Medicine
Speciality – medical biomechanics, cardiac surgery

Riga, 2014

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The study was carried out from 2007 to 2013 at: Pauls Stradins Clinical University Hospital, Department of Cardiac Surgery; Rīga Stradiņš University, Laboratory of Biomechanics; Rīga Stradiņš University, August Kirchenstein Institute of Microbiology and Virology; Centre Hospitalier Universitaire Vaudois (CHUV), Lausanne, Switzerland.

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Doctoral Thesis is available at the library of Rīga Stradiņš University and on the home page: www.rsu.lv

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Secretary of the Promotional Council:
Dr. habil. med. Professor **Andrejs Skaģers**

CONTENTS

1. INTRODUCTION

Heart valve diseases are widespread and potentially life-threatening affecting ~2.5% of the general population in economically developed countries. It is more common among the elderly, reaching 11.7% prevalence in those older than 75 years of age. Abundance of valve diseases and especially of aortic stenosis is linked with advanced age. Aortic valve stenosis nowadays is the most common heart valve disease in developed countries [8, 21].

Search for a better substitute for the severely diseased human aortic valve is still an urgent issue in the whole world. Currently biological and mechanical heart valve prostheses are used in the absolute majority of valve replacement cases. Valve bioprostheses made either from specially treated porcine aortic valve leaflets or bovine pericardium play an especially important role in aortic valve replacement in the elderly patients. The biggest drawback of bioprostheses is their limited longevity caused mainly by calcifying and non-calcifying degeneration after implantation [32]. For this reason there is an ongoing search for a perfect material for production of long-lasting bioprostheses.

Calcification of biomaterials currently used in biopostheses in part is determined by the very process of chemically fixating them with glutaraldehyde which promotes deposition of calcium salts [32]. For this reason during the production of modern bioprostheses different, very often secret, post processing anti-calcification techniques are used. Glutaraldehyde treatment also changes the mechanical properties of the biomaterials [2, 25], tissue become more rigid causing changes in stress distribution which in turn may promote mechanical damage and subsequent calcification [41].

There is no publicly available data comparing the mechanical properties of currently used biomaterials. These data are necessary to evaluate which of

the treatment methods and conditions allow preserving the mechanical properties of the biomaterials as similar to the native human aortic valve as possible. Several authors have raised concerns that not only calcification alone but also mechanical properties different from the native valve and different stress distribution in valve leaflets can lead to bioprosthetic valve dysfunction [41].

Stress distribution in leaflets of bioprostheses is even more influenced by mechanical properties of the fixating apparatus or stent. This claim has been proven with mathematical modelling of stress distribution in bioprosthetic valve leaflets [3]. A rigid stent significantly increases stress on the valve leaflets especially on the comissure region where leaflets are fixed to the fixative stent. At the current moment this study is of great importance, because aortic stent-prostheses for transcatheter delivery are rapidly spreading in clinical praxis. There are prostheses with very little or no radial deformability and with unknown long-term results. The results of this study potentially could allow to make prognosis on the possible longevity of these valves as well as would give guidance on what mechanical properties should next generation stent-prostheses conform to.

Nevermind the drawbacks, heart valve bioprostheses are one of the most successful medical devices created from biological tissue. Created from non-living, chemically treated tissue, heart valve bioprostheses have proven themselves superior in terms of biocompatibility when compared to mechanical prostheses, with good short and mid-term results (up to 10–15 years). They are immunologically inert because of the decellularization process in preparation of the tissue which removes almost all cellular structures as well as due to similar mechanical properties to the native human valve because of the interspecies similarity of collagen molecules. On the other hand absence of living cells in bioprostheses leads to several negative effects: 1) inability to regenerate

components of extracellular matrix, including collagen, 2) inability to repair damage caused by tissue fatigue, 3) inability to actively resist infection, 4) inability to resist calcification, 5) inability to grow and remodel [34]. Although these drawbacks are known for decades, bioprostheses have an important role in the treatment of heart valve diseases because of the predictability of their durability and clinical results. Nevertheless it doesn't stop scientists from searching for better ways to create a perfect heart valve substitute, a substitute free of the imperfections of bioprostheses. In recent years tissue engineering has come to the forefront as a method possibly capable of giving a solution to this problem. One of the unanswered problems in aortic valve tissue engineering is the selection of an appropriate matrix for this purpose. Its mechanical properties should match those of the native heart valve, it has to be biocompatible, as well as it should not biodegrade at a rate that is faster than the rate of creation of a new extracellular matrix by valve interstitial cells.

1.1. Objective

To determine the optimal mechanical parameters of heart valve prostheses leaflets and their fixative stents based on the studies of mechanical properties of native porcine aortic valves, native aortic roots and various bioprostheses.

Additional objective

To find a polymer nanofiber material to be used as a matrix for heart valve tissue engineering, which would mimic the mechanical properties of a native valve as close as possible.

1.2. Tasks of the study

The study can be thematically divided into three parts with the following tasks:

- Mechanical studies of aortic valve and bioprosthetic valve leaflets
    1. To commit studies of mechanical properties of native porcine aortic valve leaflets and leaflets from several often used bioprostheses in a uniaxial test system.
    2. To determine the limits of mechanical properties which leaflets of aortic valve bioprostheses must confirm to.
- Mechanical studies of fixative stents and the native aortic root
    1. To study the radial deformability of native porcine aortic root and fixative stents of several bioprostheses with means of sonomicrometry in a pseudostatic pressure system.
    2. To determine the limits of mechanical properties fixative stents of bioprostheses must confirm to after summarizing data on the native root and tested bioprostheses.
- Assessment of application potential of polymeric nanofiber materials as a matrix for valve tissue engineering.
    1. To study mechanical properties of different electrospun nanofiber materials in a uniaxial test system.
    2. To select the nanofiber material which by mechanical properties suits best for the aortic valve tissue engineering purpose according to previously determined criteria.
    3. To test biocompatibility of the selected material with COS-7 cells by assessing the proportion of viable cells after 6 weeks cultivation on the selected material.

4. To test resistance to biodegradation *in* vitro of the selected materials by testing their mechanical properties after 6 weeks cultivation with COS-7 cells.

1.3. Hypotheses

1. Mechanical parameters of leaflets and fixative stents of commercial bioprostheses differ significantly from those of the leaflets of native aortic valve and those of native aortic root.

2. It is possible to select a polymer nanofiber material with mechanical properties comparable to the leaflets of the native aortic valve and which is biocompatible and resistant to premature biodegradation at the same time.

1.4. Novelty

The main idea of this study is to define limits of mechanical properties of bioprosthetic valve structural components – leflets and fixative stents based on analysis of several current bioprostheses with known good clinical results and „the gold standard" – native aortic valve. There have been relatively many studies on the mechanical properties of native valve leaflets, less on bioprostheses but none has intended to define the limits of their optimal mechanical properties. In this work I define for the first time the mechanical properties which should be followed when assessing new biomaterials and treatment methods for creation of leaflets of bioprostheses.

To my best knowledge there are no studies on the radial deformability of fixative stents of bioprostheses. Nevertheless in many publications there is special stress on the importance of pliability of bioprostheses fixative stents for their long term functionality. This work for the first time defines the minimal

and optimal values of radial deformability of fixative stents which standard and transcatheter valve bioprostheses should conform to.

One of the unsolved questions in aortic valve tissue engineering is selection of an appropriate matrix for this purpose. This work suggests an original solution – to create a biocompatible, slowly biodegrading composite material with mechanical properties matching those of the native aortic valve.

1.5. Practical application

The defined limits of mechanical properties of bioprosthetic valve leaflets can be used for assessment of new biomaterials and treatment methods.

The defined limits of radial deformability can be used while creating new classical and transcatheter stented aortic valve bioprostheses. These data have special importance for creation of transcatheter prostheses because their stents must be with very high radial force to fix them at the level of the aortic fibrous ring.

The new nanofiber material design can be potentially used for tissue engineering of aortic valve leaflets as a matrix.

1.6. Structure

Doctoral Thesis has been written in Latvian and is composed of 14 sections. It consists of 114 pages including 34 figures, 7 tables and 3 annexes. List of literature consists of 192 references.

2. MATERIAL AND METHODS

Experimental studies were performed with permission from the ethics committee of Pauls Stradins Clinical University Hospitals Development society.

Experimental studies were perfomed at:

- Pauls Stradins Clinical University Hospital , Department of Cardiac Surgery;
- Rīga Stradiņš University, Laboratory of Biomechanics;
- Rīga Stradiņš University, August Kirchenstein Institute of Microbiology and Virology;
- Centre hospitalier universitaire vaudois – CHUV , Lausanne, Switzerland.

2.1. Mechanical studies of aortic valve and bioprosthetic valve leaflets

According to previous studies performed on native and chemically treated porcine and human aortic valves done by Mavrlias and Missirlis, and our group [10, 16] using uniaxial tests, the mechanical properties of porcine and human aortic valves are comparable. Native porcine valve material is comparably only a little more deformable and less rigid than human aortic valve. In my study porcine aortic valve material was preferred due to this similarity and most importantly due to its accessibility when compared to pathologically unchanged human valve material. There was no comparison with previously attained data on human aortic valve attempted due to different technical equipment used in this study and previous ones while this fact would introduce a significant error in such a comparison.

Mechanical studies were done with aortic valve leaflets from 10 porcine hearts which were obtained from a slaughterhouse no later than 24 hours after

death of the animal. Valve leaflets were excised along their basis and kept in isotonic salt solution up to 12 hours before performing the tests. At least 15 mm long and exactly 3.5 mm wide specimens were prepared from the excised leaflets – 14 in circumferential and 13 in radial direction as shown in figure 2.1.

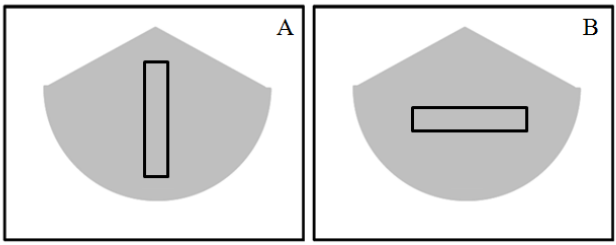

Figure 2.1. Directions in which specimens from aortic valve leaflets were cut: A) radial and B) circumferential

Three different xenoaortic bioprostheses were compared with the native valve material, testing 3 prostheses (altogether 9 leaflet samples) from each type: 3 EPIC prostheses – fig. 2.2. A (St. Jude Medical, Minnesota, USA), 3 Hancock II bioprostheses – fig. 2.2. B (Medtronic inc., Minnesota, USA), 3 Freestyle stentless bioprostheses – fig. 2.2. C (Medtronic inc., Minnesota, USA). To test how much differs a bioprosthesis made from another material, I tested 3 Sorin Soprano – fig. 2.2. D (SORIN S.p.A., Milano, Italy) bioprostheses made from chemically treated bovine pericardium. The bioprostheses I used for testing were just after their expiry date (up to 4 weeks post expiry date). Specimens were prepared in the same fashion as from the native valves, as shown in fig. 2.1.

The methodology of testing both for native valves and bioprostheses is described in detail in section 2.3.1.

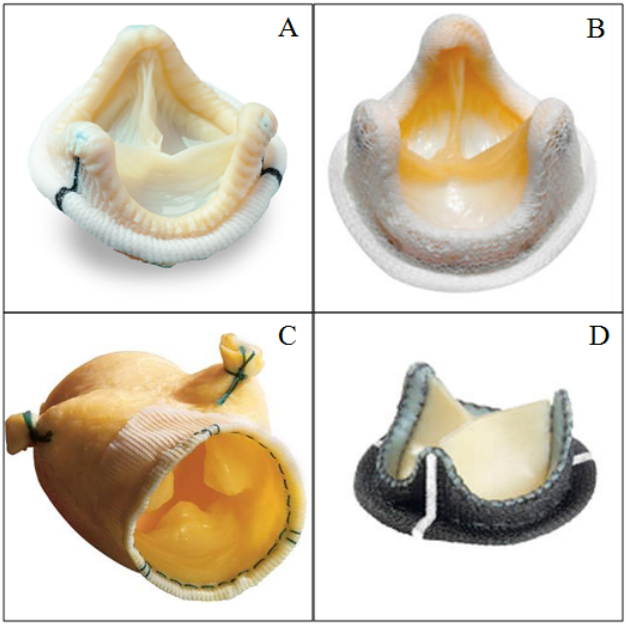

Figure 2.2. Bioprostheses used in the study:
A) St. Jude Medical Epic, B) Medtronic Hancock II,
C) Medtronic Freestyle, D) Sorin Soprano

Image source: A) http://cdn.medgadget.com/img/54355sjm2.jpg; B) http://img.medicalexpo.com/images_me/photo-g/aortic-valve-bioprosthesis-porcine-70691-3020049.jpg; C) http://www.ctsnet.org/vendor_graphics/products/401.jpg; D) http://img.medicalexpo.com/images_me/photo-mg/aortic-valve-bioprosthesis-70922-96641.jpg

2.2. Mechanical studies of fixative stents and the native aortic root

Following bioprostheses were used for fixative stent deformability testing: Medtronic Intact (Medtronic Blood Systems, Irvine, CA, USA) produced in 1988 and Carpentier Edwards Standard (American Edwards Laboratories, Irvine, CA, USA) made in 1987. Both bioprostheses were kept at

room temperature in their original packaging. They were compared to 3 different modern traditional stented bioprostheses with known good long-term clinical results [20, 39, 43]: St. Jude Epic (St. Jude Medical Inc., St. Paul, MN, USA), Medtronic Hancock II (Medtronic Inc., Minneapolis, MN, USA), Sorin Soprano (Sorin S.p.A., Milano, Italy) and ATS (ATS Medical Inc., Minneapolis, MN, USA) stentprosthesis – Enable with a nitinol stent intended for sutureless implantation in the aortic position during open surgery.

All of the prostheses were tested at 3 different transvalvular pressure gradients: 0–10 mmHg, 70–90 mmHg and 120–140 mmHg, using 3 TRX 2mm sonomicrometry probes (Sonometrics Corp., London, Ontario, Canada), fixed at the level of comissures – fig. 2.3.

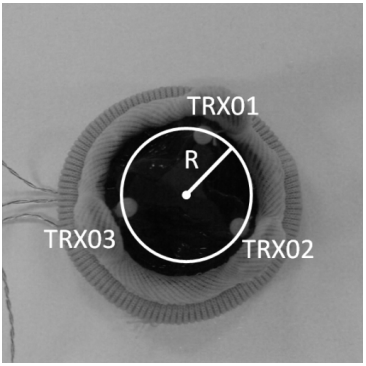

Figure 2.3. Positions of sonomicrometry probes and the circle circumscribed around them

The experiment was performed *in vitro* in a specially constructed test system (see fig. 2.4.). To analyze the mechanical properties of fixative stents of different bioprostheses, changes in radius of a circle circumscribed around the 3 sonomicrometry probes at different transvalvular pressure gradients were compared.

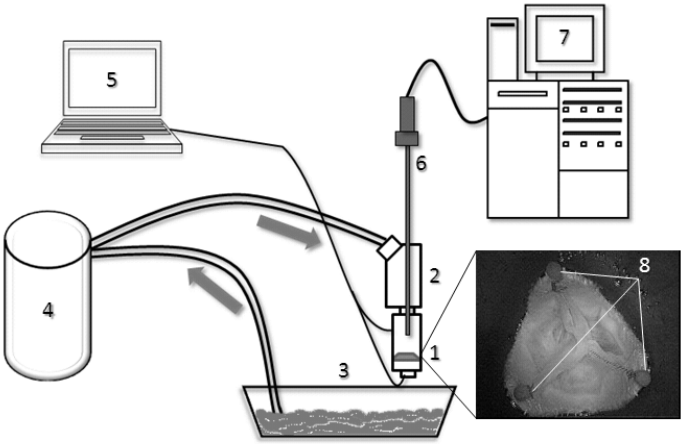

Figure 2.4. A schematic representation of the test setup used in this experiment

1 – valve-mounting chamber;
2 – chamber with two ports;
3 – water bath with 0,9% NaCl solution at room temperature;
4 – roller pump;
5 – system for real-time pressure recording;
6 – endoscope;
7 – endoscope "tower";
8 – three Sonometrics probes attached at the inner sides of stent posts.

Measurements were acquired at a frequency of 626 Hz. Mean values from 2400 data points from 3 measurements of the interprobe distances were used to calculate the changes in the radius of the circle circumscribed around the 3 piezoelectric probes. We used the formula R = abc/4S in our calculations, were a, b and c are the respective interprobe distances and S – area of triangle formed by the three probes which was calculated using Heron's formula.

The deformability of the bioprosthetic valves was compared with circumferential compliance at the level of commissures of 3 fresh porcine (animal weight 51± 2 kg) aortic roots, where the sonomicrometry probes were

fixed at the inner commissure regions. Otherwise the testing methodology was similar and the aortic roots were mounted in the same test system which is schematically depicted in fig. 2.4.

For bioprostheses the changes in radius of the circle around the stent posts were compared at 70–90 mmHg (transvalvular pressure gradient during diastole) and at 0–10 mmHg (transvalvular pressure gradient during systole). To assess radial deformability of the native aortic root the respective changes in radius values were compared at pressures 70–90 mmHg (corresponding to pressure during diastole) and 120–140 (corresponding to pressure during systole). Deformability was defined as the relative increase of radius during the transition from systole to diastole in percent.

2.3. Assessment of application potential of polymeric nanofiber materials for valve tissue engineering

Electrospun nanofiber materials were custom made by Czech company Elmarco (Elmarco s.r.o., Liberec, Czech Republic) from several polymer materials including gelatin, polyurethane (PUR), polilactic acid (PLA) and polycaprolactone (PCL). These materials were produced with different fiber diameters and different area density: PUR 6.2 and 10.4 g/m^2, PCL 3.86 and 12.0, and 15.7 g/m^2, PLA 5.2 and 11.0 g/m^2, gelatin 5.7 g/m^2. Materials were custom made on individual order and Elmarco granted scanning electron microscopy images of each material. Thickness of all materials was measured on site using cathetometer MK-6 (LOMO, Saint Petersburg, Russia) with ± 0.01 mm precision. For mechanical testing materials were cut into 3.5 mm wide and 20 mm long specimens. Five specimens were prepared from each of the tested materials.

2.3.1. Uniaxial testing of the synthetic and biological material

Universal testing machine Zwick/Roell BDO-FB0.5TS equipped with a 50 N tensile force module was used for mechanical properties testing. It was used with a computer equiped with testXpert software for device control and data processing. Specimens for tensile testing were cut from the given material 3.5 mm wide and ~15 to 30 mm long (specimens were shorter in the radial direction) fig. 2.1. Thickness of all native valve and bioprosthetic valve leaflets as well as nanomaterials was measured with cathetometer MK-6 (LOMO, Saint Petersburg, Russia) with ± 0.01 mm precision prior to mechanical testing. During uniaxial tensile testing specimens were deformed with a speed of 5 mm/min until rupture. Maximum (ultimate) strain (ε_{max}) and maximum (ultimate) stress (σ_{max}) were determined for each specimen. Stiffness of specimens was defined as a tangensial modulus of elasticity (E) at the linear part of the stress/strain curve and calculated as a tangent from the angle it forms with the strain axis (angle β in fig. 2.5.).

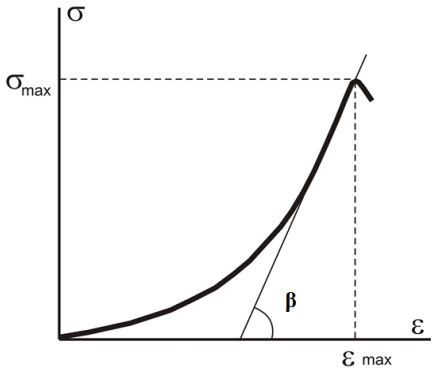

Figure 2.5. An example of a stress/strain curve and the main mechanical parameters

Due to their small sizes specimens were fixed in the testing machine by means of a specially constructed fixator which can be seen in fig. 2.6.

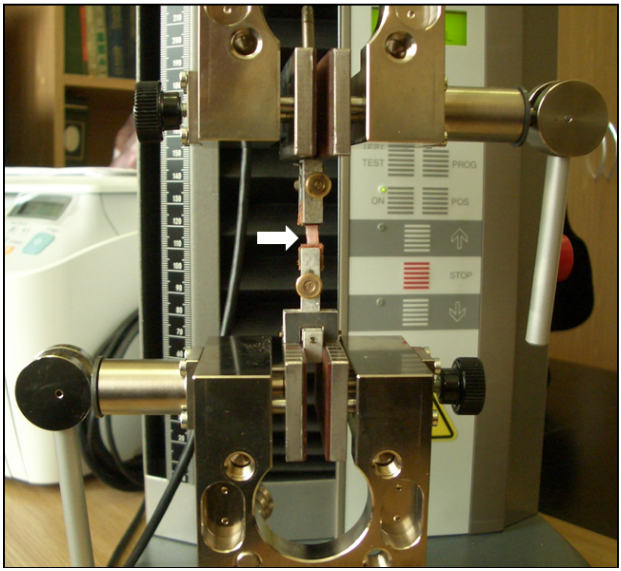

Figure 2.6. Uniaxial testing system Zwick/Roell BDO-FB0.5TS, specially constructed specimen fixator can be seen. Arrow marks the specimen.

Data obtained from uniaxial tensile testing were further analyzed using testXpert software. Ultimate stress and strain as well as tangential modulus of elasticity at the linear part of stress/strain curve were determined using this software.

2.3.2. Cell culture on the polymer nanofiber material

For thorough analysis three materials were selected: gelatin with area density 5.7 g/m^2, PCL – 15.7 g/m^2 and PUR – 6.2 g/m^2. Specimens from these materials were inserted into standard 8-well culture plates and cultured for

6 weeks with COS-7 cells, which initially are derived from an African Green monkey kidney fibroblast cell line (ATCC 1651 CRL). Cells were maintained in *Dulbecco's Modified Eagle Medium* (DMEM) with 5% fetal calf serum supplement at 37 ºC in humidified 5% CO_2 atmosphere. Medium was changed twice a week. After 6 weeks in culture, viability of cells was assessed by acridine orange and ethidium bromide staining which colors living cells green and dead cells orange in fluorescent light. For quantification cells were counted in microscopic fields of view – 200 cells for each specimen, from which the proportion of living cells was calculated.

2.4. Data analysis

Experimental data were processed using SPSS for Windows 16.0 (SPSS Inc., Chicago, IL, USA) and Microsoft Excel (Microsoft, Redmond, WA, USA). Descriptive and analytical statistical methods were used for analysis. Mean values and standard deviations were calculated. Mean values of two separate sample groups were compared using the Student's t-test. Statistically different pairs were defined as having $P < 0.05$. Mean values among three or more separate groups were compared using single factor ANOVA and appropriate *post hoc* tests.

3. RESULTS

3.1. Mechanical properties of leaflets of aortic valve and bioprostheses

Altogether 14 samples in circumferential and 13 in radial direction were tested from 10 porcine aortic heart valves. In circumferential direction the mean modulus of elasticity was 9.7 ± 1.3 MPa, ultimate stress – 2.3 ± 0.6 MPa and ultimate strain is 44.8 ± 5.9 %. The respective parameters of the native valve in radial direction are: modulus of elasticity 1.0 ± 0.2 MPa, ultimate stress 0.5 ± 0.2 MPa and ultimate strain was 95.6 ± 31.4%. Stress/strain curves from selected experiments in both tested directions can be seen in fig. 3.1. From these curves one can clearly appreciate the distinct differences in mechanical properties between both tested directions.

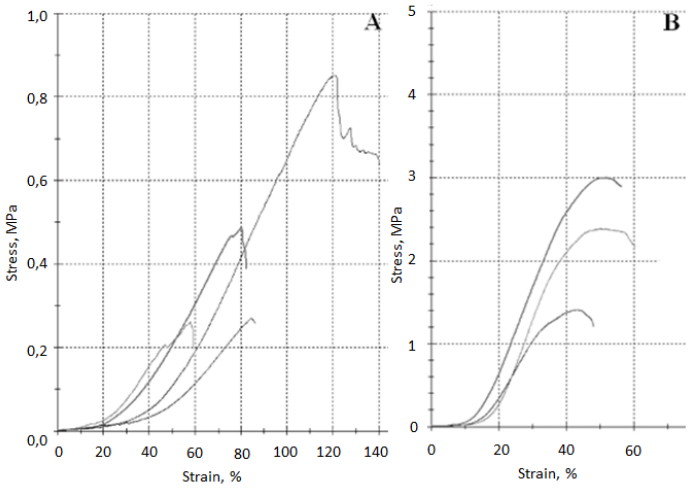

Figure 3.1. Stress/strain curves of porcine valve leaflets from selected experiments: A) radial and B) circumferential direction

Mechanical properties of all of the tested bioprostheses are summarized in table 3.1. which includes modulus of elasticity (E), ultimate stress (σ_{max}) and ultimate strain (ε_{max}) mean values ± standard deviation for the tested leaflets both in radial and circumferential direction. The table includes also data on native porcine aortic valve.

Table 3.1.

Summary of mechanical properties of native porcine aortic valve and bioprostheses

	E (Mpa)	σ_{max} (Mpa)	ε_{max} (%)
Native porcine valve, circumferential	9.7 ± 1.3	2.3 ± 0.6	44.8 ± 5.9
Native porcine valve, radial	1.0 ± 0.2	0.5 ± 0.2	95.6 ± 31.4
Epic, circumferential	19.7 ± 3.9	3.8 ± 0.7	28.7 ± 6.3
Epic, radial	2.0 ± 0.3	0.8 ± 0.2	53.0 ± 19.8
Freestyle, circumferential	9.0 ± 3.0	2.8 ± 0.5	64.9 ± 10.3
Freestyle, radial	0.8 ± 0.3	0.4 ± 0.1	89.0 ± 13.9
Hancock II, circumferential	22.5 ± 2.2	4.7 ± 0.2	36.8 ± 9.1
Hancock II, radial	2.5 ± 0.2	1.1 ± 0.0	58.3 ± 13.2
Soprano, circumferential	29.5±6.0	8.9 ± 1.2	56.9 ± 9.3
Soprano, radial	15.8 ± 5.4	5.2 ± 0.9	65.2 ± 10.4

The tested bioprostheses have a non-linear stress/strain response similar to that of the native aortic valve. But bioprostheses have more rigid leaflet material, as judged by modulus of elasticity values: Epic and HancockII being two times of that of the native valve and Soprano even three times as high. The leaflets of Soprano bioprosthesis are also least anisotropic and least deformable

and far more rigid when compared to other xenoaortic bioprostheses. For example, leaflets of HancockII and Epic bioprostheses are 2,5 and 2 times more rigid in radial direction than the native valve ($p < 0.05$), but the treated bovine pericardium used in Soprano valve is 15 times more rigid ($p < 0.001$). Mechanical properties of Freestyle bioprosthesis match those of the native valve best, see table 3.1. These differences between various bioprostheses are not only due to the material used, because all of them except Soprano are xenoaortic, but also because of the chemical treatment and conditions used for their fixation. Freestyle is the only one of these prostheses being fixed at near zero pressure; while the others are fixated at Medium high pressures (exact pressure of fixation is not known). At higher pressures connective tissue fibers are fixed in an already partially distended state which limits their further deformability [2, 25]. This question will be further addressed in the discussion section of this work. When summarizing the mechanical properties of native valves and various bioprostheses, it is possible to compile non–exhaustive recommended limits of mechanical parameters for bioprosthetic valve leaflets which can be found in the "Practical recommendations" section of this work.

3.2. Mechanical properties of the aortic root and fixative stents of bioprostheses

The results are summarized in table 3.2. Diastolic radius of aortic root and bioprostheses was measured at pressure 70–90 mmHg, systolic radius – for the aortic root at 120–140 mmHg (equals the pressure during systole) but for the prostheses at 0–10 mmHg (transvalvular pressure gradient during systole). Deformability is defined as relative increase of radius in percent during transition from diastolic to systolic pressures.

Table 3.2.

Mechanical properties of aortic root and fixative stents of bioprostheses

	Radius of prosthesis at the level of comissures, mm			Increase in radius (%) diastole - systole
Transvalvular pressure gradient, mmHg	0-10 (systole)	70-90 (diastole)	120-140	
Edwards CES (1987.)	7.99	7.35	7.20	8.70
Medtronic Intact (1988.)	7.24	6.97	6.85	3.87
Medtronic Hancock II	10.24	9.96	9.78	2.85
Sorin Soprano	10.37	10.07	9.95	2.99
St. Jude Epic	8.76	8.66	8.65	1.18
ATS 3F Enable	11.66	11.32	11.13	3.00
Transvalvular pressure gradient, mmHg	0-10	70-90 (diastole)	120-140 (systole)	
Native porcine aortic root	11.64 ± 1.70	14.38 ± 3.10	15.40 ± 2.80	7.09 ± 2.20

Deformability of traditional bioprostheses varies between 1.18% for St. Jude Epic and 8.7% for Carpentier Edwards Standard, pliability of which even exceeds that of the native porcine aortic root. Mean deformability in the group of all traditional bioprostheses with good long–term results is 3.9 ± 2.8%. Deformability of Medtronic Intact, one of the old bioprostheses is 3.87% and almost matches this mean value. Deformability of the only tested stentprosthesis for implantation during open surgery – ATS 3F Enable is

exactly 3.0%. Mean deformability of older bioprostheses is 6.3 ± 3.4%, significantly higher ($p < 0.05$) than that of modern bioprostheses which is 2.3 ± 1.0%.

3.3. Assessment of application potential of polymeric nanofiber materials for valve tissue engineering

Summary of mechanical properties of different nanofiber materials before culture with COS-7 cells can be found in table 3.3. For convenience table 3.3. includes data also on native aortic valve leaflets.

Table 3.3.

Mechanical properties of non-treated polymer nanofiber materials

	E (MPa)	σ_{max} (Mpa)	ε_{max} (%)
Porcine aortic valve, circumferential	9.70 ± 1.30	2.30 ± 0.60	44.80 ± 5.90
Porcine aortic valve, radial	1.00 ± 0.20	0.50 ± 0.20	95.60 ± 31.40
PCL 3.86 g/m^2	0.88 ± 0.25	0.2 ± 0.03	42.8 ± 5.08
PCL 12 g/m^2	0.48 ± 0.12	0.09 ± 0.02	20.79 ± 3.31
PCL 15.7 g/m^2	1.30 ± 0.20	0.30 ± 0.05	37.90 ± 2.46
PLA 5.2 g/m^2	3.35 ± 0.48	0.55 ± 0.03	41.94 ± 3.67
PLA 11 g/m^2	0.23 ± 0.03	0.14 ± 0.01	54.94 ± 8.15
Gelatin 5.7 g/m^2	0.64 ± 0.14	0.38 ± 0.05	82.53 ± 10.20
PUR 6.2 g/m^2	3.90 ± 0.50	5.30 ± 1.68	141.80 ± 43.90
PUR 10.4 g/m^2	4.55±0.78	7.65 ± 1.65	181.06 ± 39.83

From the tested materials, PUR 6.2 g/m^2 matched the mechanical properties of native aortic valve in circumferential direction best, with a modulus of elasticity (E) 3.9 ± 0.5 Mpa, ultimate stress and strain 5.3 ± 1.68 MPa and 141.8 ± 43.9 % respectively. Stress/strain curves of PUR 6.2 g/m^2 in comparison with the native valve in circumferential direction can be seen in figure 3.2. Gelatin 5.7 g/m^2 fits best to the properties of the native valve in radial direction with a modulus of elasticity (E) 0.64 ± 0.14 MPa, ultimate stress 0.38 ± 0.05 MPa and ultimate strain 82.53 ± 10.20 %. Stress/strain curves for gelatin 5.7 g/m^2 specimens in comparison with native porcine aortic valve leaflets in radial direction can be seen in fig. 3.3.

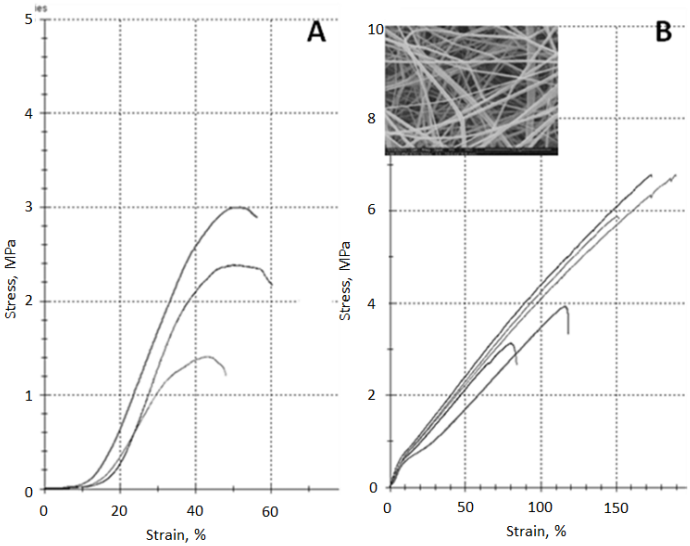

Figure 3.2. Stress/strain curves: A) porcine aortic valve circumferential direction and B) PUR 6.2 g/m^2. Insert – a scanning electron microscope image of PUR nanofiber material

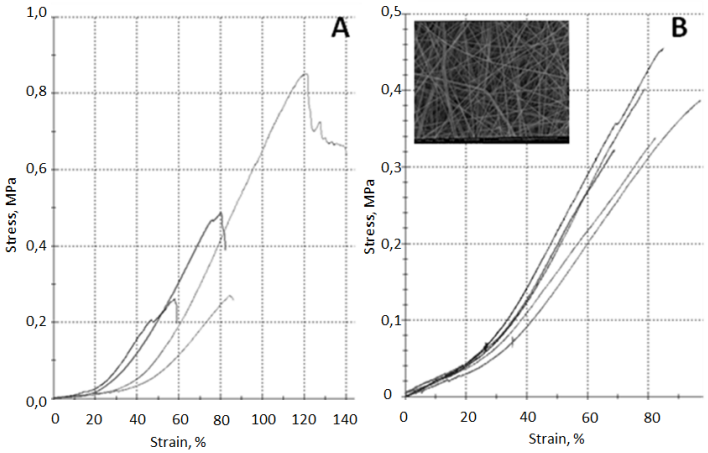

Figure 3.3. Stress/strain curves: A) porcine aortic valve circumferential direction and B) gelatin 5.7 g/m^2. Insert – a scanning electron microscope image of gelatin nanofiber material

3.3.1. Biocompatibility of nanomaterials with COS-7 cells *in vitro*

Biocompatibility was tested for three nanofiber materials: PUR with area density 6.2 g/m^2 and gelatin because their mechanical properties fitted best native porcine aortic valve in circumferential and radial direction respectively. Also PCL 15.7 g/m^2 was tested because of the long-standing experience with this polymer as a surgical material and because its mechanical parameters fit those of the circumferential direction of native valve reasonably good. In tested conditions COS-7 cells were growing very good, forming a uniform monolayer of cells, figure 3.4. Proportion of living cells after 6 weeks in culture was PUR – 90.2% ± 7.9%; PCL – 92.6 ± 5.4%; gelatin – 88.1 ± 5.7% with no significant differences among materials and samples.

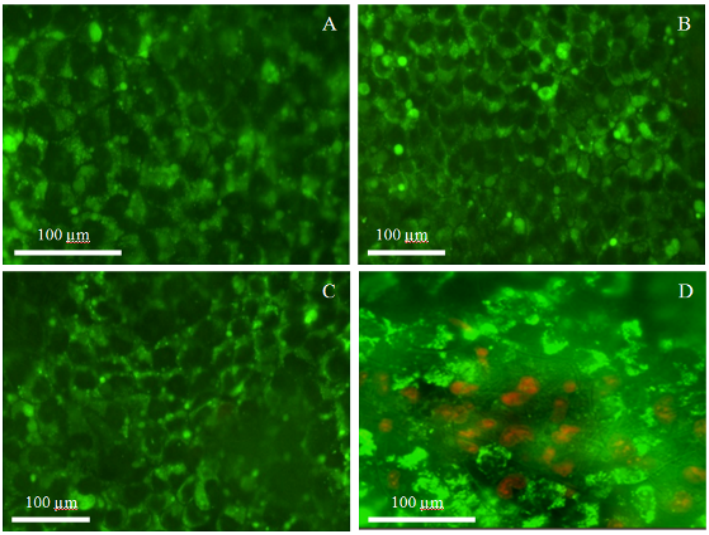

Figure 3.4. Fluorescence microscopy images of COS-7 culture grown on: A) PCL 15.7 g/m^2; B) gelatin 5.7 g/m^2, C) PUR 6.2 g/m^2, D) PUR 6.2 g/m^2 (with more dead cells) samples for 6 weeks, acridine orange and ethidium bromide staining

3.3.2. Mechanical properties of nanofiber materials after 6 weeks *in vitro* culture with COS-7 cells

Data on mechanical properties of nanofiber materials after cultivation with COS-7 cells can be found in table 3.4. The ultimate stress values of PCL did decrease significantly from 0.49 ± 0.01MPa to 0.37 ± 0.03MPa ($p < 0.05$), see fig. 3.5. A. meanwhile ultimate strain of gelatin increased from 87.2 ± 13.8% to 157.1 ± 15.2% ($p < 0.05$), see fig. 3.5. B. There were no significant changes in mechanical properties of PUR material when comparing it before and after cell culture. It has to be mentioned that none of the materials changed its modulus of elasticity as a result of 6 week cell culture, table 3.4.

Table 3.4.

Mechanical properties of nanofiber materials prior to and after 6 weeks culture with COS-7 cells

	E (MPa)	σ M (Mpa)	σ X (%)
PCL 15.7 g/m^2	2.10 ± 0.10	0.49 ± 0.01	34.70 ± 4.00
PCL 15.7 g/m^2 (COS-7)	2.20 ± 0.10	0.37 ± 0.03	28.50 ± 1.00
Gelatin 5.7 g/m^2	0.73 ± 0.17	0.43 ± 0.09	87.20 ± 13.80
Gelatin 5.7 g/m^2 (COS-7)	0.62 ± 0.16	0.45 ± 0.08	157.10 ± 15.20
PUR 6.2 g/m^2	3.90 ± 0.50	5.30 ± 1.70	141.80 ± 43.90
PUR 6.2 g/m^2 (COS-7)	4.20 ± 0.60	4.70 ± 2.10	153.60 ± 51.00

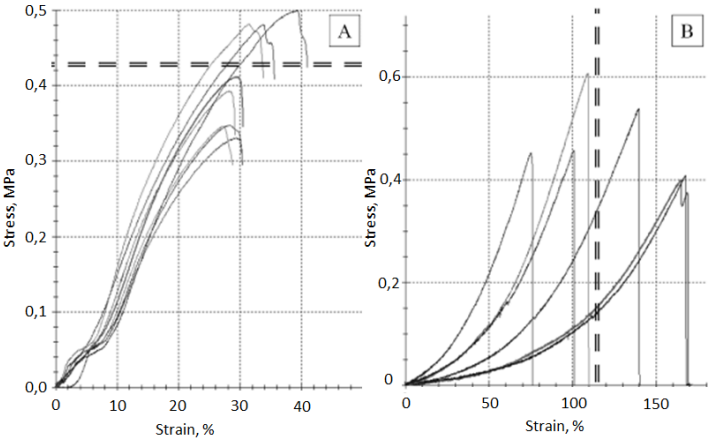

Figure 3.5. Stress/strain curves from a single experiment with nanofiber materials PCL 15.7 g/m^2 and gelatin: A) PCL – the curves extending above the double dotted line correspond to samples before culture, curves bellow it - after culture, B) gelatin – the curves on the left side of the double dotted line correspond to samples before cell culture, the curves located to the right – after culture.

4. DISCUSSION

4.1. Potential influence of mechanical properties of bioprosthetic valve leaflets on their longevity

Experimental results show that the mechanical properties of aortic valve leaflets are different in radial and circumferential directions, which is in agreement with previously published data on the anisotropy of this biomaterial [11, 23, 29, 35]. It is determined by the ultrastructural peculiarities of valve leaflets, more precisely, orientation of connective tissue fibers in valve leaflets. Several authors have managed to demonstrate that elastic fibers are mostly orientated evenly in all directions, but collagen fibers are mainly orientated in circumferential direction [28, 35]. In this study it is demonstrated that ultimate stress of native porcine valves is 4.6 times higher in circumferential direction than in radial, which can be explained by concentration of collagen fibers in this direction. Similarly modulus of elasticity of native valves is even 9.7 times higher in circumferential direction, similar results have been obtained by Sauren and Missirlis [18, 29] – they have reported on modulus of elasticity even 21 times higher in circumferential direction.

As demonstrated in earlier works [2, 25], mechanical properties of the valve tissue are determined by chemically fixing it in a loaded or unloaded state. Fixation in a loaded state causes a shift to the left of the stress–strain curve closer to the stress axis but fixation in an unloaded state to the right. This happens because in a loaded state collagen fibers become fixed in an already partially extended position which limits their further deformability.

Taking into account results from previous studies and theoretical considerations Mano Thubrikar and colleagues came to a conclusion that for the valve leaflets to function properly they require mechanical duality of the material with high deformability at low pressure levels and material strength

and rigidity at high mechanical stress situations [7, 36]. Mechanical strength especially is required during diastole to prevent leaflet bulging and prolapse. On the other hand during the early phases of systole high deformability and elasticity is essential for the valve to open as quickly and as effective as possible, and it is dependent on the flexion rigidity of the valve leaflets. Flexion rigidity on the other hand is directly proportional to modulus of elasticity and tissue thickness [37]. Accordingly, more rigid valve leaflets, due to fixation, are exposed to greater flexion stresses and it can cause accelerated mechanical damage formation in comparison with materials which mechanical properties are at the level of a non-treated native aortic valve. In a recent study, Mirnajafi and colleagues have shown that the region most exposed to flexion deformity is around comissures – the leaflets during opening undergo a rotation of ~65° at this region [17]. Hence this region is exposed to great stress because of flexion deformation and because of pressure caused by the retrograde blood flow during diastole. It is supported by observations that bioprostheses are very often damaged exactly in this region [36]. Flexion fatigue is thought to be one of the main causes of leaflet rupture [36, 42]. Arcidiacono and colleague [1] with means of computer modeling have shown that even the slightest differences in rigidity of valve leaflets have an impact on the dynamics of valve opening and closure, stressing the importance of leaflet mechanical properties homogeneity within one valve. All these previous studies point out that increased leaflet rigidity characterized by higher modulus of elasticity values may have significant impact on bioprosthesis longevity and hemodynamic properties.

Data on the failure of bioprostheses to copy the mechanical properties of the native human heart valves are in accordance with a large number of publications about non-calcifying valve leaflet degeneration [26, 41]. Although it is most likely that these processes – gradual structural deterioration and calcification work hand in hand. It is supported by many studies which demonstrate that calcification often begins in the regions of increased stress and

deformation [13, 31, 37]. Further on calcification causes a loss of elasticity and an increase in flexion stress which accelerates further tissue degeneration. Although, when talking about the current generation of bioprostheses calcification is not the most actual problem – there is an increasing tendency to highlight damage caused by mechanical factors [41].

4.2. Potential influence of mechanical properties of bioprosthetic valve stents on their longevity

Our results demonstrate that the older 2nd generation bioprostheses were created with more pliable stents than the modern traditional bioprostheses. Nevertheless it must be noted that the deformability of the two older bioprostheses differ significantly: 3.9% for Medtronic Intact and 8.7% for CE Standard ($P < 0.001$). Also the materials used as a base for stents of these two valves are very different – the Medtronic valve has a stent with a polymer core while the stent of CE Standard valve is made of a metal alloy called Elgiloy. Nevertheless the long-term results of both valves are equally good [5, 6, 9], which allows an assumption that 3.87% is an acceptable deformability. The fixative stent of CE in fact even exceeds in radial deformability the native aortic root. All the newer generation valves have more rigid stents when compared to the mean values of older traditional bioprosthetic valves and native aortic valve. Still the long-term results of these valve prostheses are good [20, 39, 43]. Hence it becomes even more difficult to answer the question about minimum required deformability of valve stents. From a technological point of view maybe this question is not so important when creating traditional bioprostheses, because it does not create an engineering challenge to make bioprostheses with a little bit more or less pliable stent posts. To the contrary, when designing new transcatheter valve prosthesis it becomes a challenge, because these prostheses are kept in their position only by the means of the radial force of their stents and thus rigid stents with high radial force would be

preferential. For this reason stents of transcatheter valves have to be rigid enough to keep their position and to have pliability just enough not to damage valve leaflet tissue. In this study ATS 3F Enable aortic valve prosthesis was tested which has recently become available and is used for sutureless implantation during conventional open surgery, it is made of nitinol and it shares many design features with self-expanding transcatheter valves. Its radial deformability is 3.0 ± 0.0%, which is even more than the mean of modern standard bioprostheses, but currently only early results of this valve are known [15, 27]. It has to be noted that the field of transcatheter heart valves is evolving rapidly [38], new prostheses enter the market with stents made from various materials, including stainless steel. Many of these stents give a subjective impression of being very rigid, because they must maintain the shape and position of the valve prosthesis after implantation often in very calcified and rigid surrounding tissue. Correspondingly some concerns arise about the longevity of these prostheses, which could face problems similar to the first generation of bioprostheses. It is these bioprostheses, after acquiring data on their long-term results, which could give us the answer about the clinical significance of the deformability of bioprosthetic valve fixative stents.

4.3. Nanomaterials matching mechanical properties of aortic valve

Polymer scaffolds have been used for tissue engineering purposes with varying success for many years already [30]. Various polymers – natural and synthetic, their combinations and modified molecules have been tested for different tissue engineering tasks [4, 14, 33]. Many polymer scaffold types have been used previously for cardiac and cardiac valve tissue engineering [19, 24, 30]. The materials tested in this work are widely studied before and well known: one of them – polycaprolactone (PCL) has been used as a material in

surgery for decades with mechanical and biological properties, including biodegradation well described [22, 44]. Still, to my best knowledge, there is limited published data on changes in mechanical properties of PCL, PUR and gelatin after prolonged cultivation with fibroblast cells.

PCL is a very good candidate for a tissue engineering scaffold for load bearing components, like cartilage and bone as well as heart valve tissue due to its slow degradation rate *in vivo* [44]. Although experimental results of this study show significant reduction of maximum stress of PCL matrices after 6 weeks of culture with COS-7 cells, this reduction of 25% is rather technically non-significant, because in the physiological conditions loading of the valve would never reach those tested and it has to be stressed that modulus of elasticity remained unchanged for the samples before and after culture.

Gelatin, although commercially widely used, still remains a rather poorly understood substance manufactured from native collagen. Still it is rather very popular in the modern biomedical field, being exploited as a drug and cell carrier/scaffold [12, 14]. Its mechanical and biological properties have been extensively studied [14, 40]. In my study gelatin after 6 weeks in culture didn't lose its mechanical strength, but gained more deformability, which cannot be explained without ultrastructural studies. This in praxis could lead to problems in practical application of heart valve tissue engineered constructs with a scaffold made only from gelatin, because it could result in excess bulging and even prolapse of valve leaflets.

Taking into account the importance of the anisotropy of aortic valve leaflets for their optimal functioning as discussed in section 4.1., none of the tested materials is optimal as a substitute. Although PUR matches the circumferential direction with an emphasis on material strength and rigidity very good, it lacks the deformability required for the radial direction. On the other hand gelatin is deformable enough, but it lacks mechanical strength. As a potential solution a composite electrospun nanofiber material made from

gelatin and PUR with their fibers predominantly arranged in perpendicular directions can be suggested. Fibers of PUR would be aligned mostly in circumferential direction, while gelatin in radial direction.

4.4. Study limitations

The main limitation of our study is that it has been carried out in an *in vitro* setting – namely data from a cell culture with COS-7 cells cannot be extrapolated to an *in vivo* situation with its highly dynamic environment in the circulatory system and exposure to high dynamic pressures, and other mechanical and biological factors. Similarly, although cultivation with COS-7 cells allows us to make judgments on biocompatibility with these cells, general conclusions about biocompatibility *in* vivo or about biocompatibility with other cell lines cannot be drawn from it.

It has to be noted that only a single valve per bioprosthesis type has been tested for stent deformability, albeit several times, due to limited availability. This precludes the evaluation of material deformability among the various copies of a specific prosthesis model. Although fixative stents are the part of bioprosthesis production of which can be standartized most precisely, a chance still exists that by testing several copies of the same prosthesis type significant variability could be found. The number of bioprostheses available for the study was dictated not only by financial considerations but also by the fact that the two older bioprostheses were available only in a single copy and ATS 3F Enable was also kindly provided by its producer only in a single copy. A similar problem was encountered with bioprosthetic valve leaflet material studies, for them only 3 from each prosthesis type were available, which gives 9 valve leaflets from each bioprosthesis type. It was sufficient in this study to obtain statistically significant results, mainly due to relatively small variability in results among tested prostheses leaflets of one type.

5. CONCLUSIONS

Conclusions I: Mechanical properties of leaflets of aortic valve and bioprostheses

Mechanical properties of the leaflets of aortic valve bioprostheses differ significantly from native porcine aortic valve leaflets. Their mechanical properties are different in accordance with their different treatment, treatment conditions and biomaterial used.

All tested bioprostheses have a non-linear stress-strain relationship and anisotropy similar to the native valve tissue.

Conclusions II: Mechanical properties of the aortic root and fixative stents of bioprosthese

The tested older bioprostheses have more pliable stents when compared to the modern bioprostheses.

The minimal deformability of a bioprosthetic valve fixative stent is 1.2% for a xenoaortic and 3.0% for a xenopericardial prosthesis.

Conclusions III: Assessment of application potential of polymeric nanofiber materials for valve tissue engineering

Gelatin is best to mimic radial direction and polyurethane – circumferential direction of native valve tissue. To model the mechanical properties of native valve tissue as precise as possible, a material made from both polyurethane and gelatin with their fibers predominantly orientated in a perpendicular fashion is suggested.

The tested nanofiber materials were proven to be biocompatible in a prolonged culture with COS-7 cells. They can be used in heart valve tissue engineering because of their biocompatibility and slow biodegradation.

6. PRACTICAL RECOMMENDATIONS

A. Limits of the recommended mechanical properties for aortic valve leaflet substitute materials are given in table 6.1. Values which are given as optimal correspond to the results obtained from native valve material.

It has to be noted that it cannot be postulated that bioprostheses not conforming to these parameters would be prone to accelerated degradation, because it was not studied. It is possible only to suggest that bioprostheses which do conform to these recommended properties will have a satisfactory lifespan at least as much as it depends on the leaflet material.

Table 6.1.

Recommended mechanical properties of aortic valve leaflet material

Modulus of elasticity, Mpa			
	Min	Optimal	Max
Circumferential	9.0	9.7	29.5
Radial	0.8	1.0	15.8
Ultimate stress, Mpa			
	Min	Optimal	Max
Circumferential	2.3	2.3	8.9
Radial	0.4	0.5	5.2
Ultimate strain, %			
	Min	Optimal	Max
Circumferential	28.7	44.8	64.9
Radial	53.0	95.6	95.6

B. Recommended mechanical properties of fixative stents of bioprosthetic valves based on studies on native aortic root and commercial bioprostheses can be found in table 6.2. Values which are given as optimal correspond to the results obtained from native aortic root material.

Table 6.2.

Recommended mechanical properties of fixative stents of bioprostheses

Radial deformability, %		
Min	Optimal	Max
1.2*	7.1	8.7
3.0#		

* for xenoaortic bioprostheses
# for xenopericardial bioprostheses

C. To model as precisely as possible the mechanical properties of native aortic valve it is suggested to use a combined material from gelatin and PUR with their fibers predominantly orientated in perpendicular directions. Gelatin would grant deformability important for radial direction and polyurethane would give additional strength to the circumferential direction.

7. PERSPECTIVE FOR FUTURE STUDIES

Development of new heart valve bioprostheses currently is a booming field of applied research experiencing many innovations in regard to development of new models of prostheses, new routs of delivery and new materials. Results of this study stimulate further research in the field of new prostheses and their structural components. Development of an autologous tissue engineered heart valve substitute should be mentioned as the ultimate goal for future studies. This work already sets ground for such future studies by defining mechanical properties of an ideal material for tissue engineering of aortic valve leaflets. Future research should be centered on creation of a composite material as described in this work, in depth studies of its mechanical properties, biocompatibility and cell infiltration capabilities etc. Certainly a very perspective field is also studies of other newly created polymer nanofiber materials, cultivation of cells on them, evaluation of cell infiltrative capabilities depending on the porosity and fiber diameter of the tested material because these are the aspects success of tissue engineering is most dependent on.

8. SCIENTIFIC PUBLICATIONS

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9. CONFERENCE THESES

There have been altogether 24 presentations in international conferences on the topics addressed in this study, list of conference theses can be found in the full text of this doctoral thesis.

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