VILNIUS UNIVERSITY

Saulius Raugelė

COMPARISON OF HEART VALVE BIOPROSTHESIS ANTICALCIFICATION TREATMENT METHOD'S EFFICACY IN EXPERIMENTAL MODELS

Summary of Doctoral Dissertation

Biomedical Sciences, Medicine (06B)

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VILNIAUS UNIVERSITETAS

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BIOLOGINIŲ VOŽTUVŲ TRANSPLANTATŲ APRUOŠIMO METODŲ EFEKTYVUMO PALYGINIMAS EKSPERIMENTE

(eksperimentinis darbas)

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Abbreviations:

Ala – L-Alanine
Ao – aorta
CHAPS - 3- [(3-cholamidopropil) dimethylamonio] -1 –propanosulfonate
CMC-critical micele concentration
CPB – cardiopulmonary bypass
DEO – Sodium deoxycholic acid
DNA – deoxyribonucleic acid
ECM – extracellular matrix
EDC – 1-ethyl-3-(3-dimethylaminopropil) carbodiimide
EDTA – ethylendiamintetracetic acid
GAG - glikozaminoglicanes
Glu – glutaraldehyde
NHS – N-hydroksisukciimide
Norv – L-Norvaline
PA – pulmonary artery
RNA – ribonucleic acid
SDS – Sodium dodecyl sulfate

1. Introduction

Every year a lot of people suffering from congenital or acquired heart valve diseases overcome heart valve surgery. More than 300 000 patients yearly had heart valve surgery worldwide. Numbers of heart valve surgery cases are increasing every year, therefore heart valve prosthesis industry is also developing new models, and many trials are taking place to investigate new chemical agents and technologies.

At present there are two basic types of heart valve substitutes: mechanical and biological. Mechanical ones are durable and functioning lifelong, however, there is a need for continuous uptake of anticoagulants, which may cause bleeding or thrombembolic complications since it made from foreign materials. Biological prostheses are modern and anticoagulants are used only for several months, besides, they are similar to native valves in terms of anatomical and physiological characteristics. Nevertheless, these substitutes also have disadvantages; recipient's immune cells recognize these valves as antigens (since they are harvested from animals) thus calcification and degeneration occurs impairing function and longevity of implanted valves. The younger the patient the faster these processes begin. Chemical treatment may prolong prosthesis longevity, but only up to a certain limit. According to some trials, present bioprostheses available in the market are to function up to 15-20 years. However, the problem is still unsolved, since more and more patients are juvenescent and the usage of bioprostheses due to their longevity is therefore limited. Tissue engineering methods could be a solution to existing limitations.

Tissue engineering is described as actions and manipulations with cells, and cell cultures creating new integrated structures or tissues that could perform intended function - be able to adapt to metabolic changes and perform role of functioning substitute for native tissues or organs. This kind of valve prosthesis could be ideal choice for every patient for the whole life. It would be immunologically non-reactive, living structure, capable to remodel and renew.

There are many reports how to make heart valve scaffolds or whole valved conduit using different tissue engineering methods. However, only decellularization methods are applied not only in experimental works, but also in clinical practise. Acellular or even recellularized valves are used for implantations. Different scientist groups declare different results; sometimes animal experiments show better results than clinical data when used to patients. Therefore discussion about safety and compatibility with human tissues is still open and united approach is non-existent.

Approximately 500 heart valve surgery cases are performed in Lithuania yearly, most of them due to aortic valve diseases. Like in other countries distribution of patients is similar, the majority of them being under 60-65 years of age. According to guidelines, this is a limit for bioprosthesis usage, with some exceptions.

Considering newest achievements in tissue engineering we decided to investigate existing methods and upgrade them using new combinations of substances and compounds, creating unique tissue treatment method efficient both in vitro and in vivo experiments.

2. The aim of the study, objectives, scientific novelty, practical application

2.1 The aim of the study

To investigate different tissue treatment methods intended to lower or eliminate tissue calcification after implantation and to evaluate their efficacy in experimental models.

2.2 The objectives of the study

1. Selection of chemical agents for aortic and pulmonary valved conduit treatment and development of treatment protocols.
2. To evaluate efficacy of chemical treatment in experimental model without direct contact to hemodynamic system (experiments with small animals).
3. To evaluate efficacy of chemical treatment in experimental model with direct contact to hemodynamic system (experiments with large animals).
4. Selection of detergents and other substances, and development of treatment protocols for decellularization of aortic and pulmonary valved conduits in vitro.
5. To evaluate efficacy of decellularized valved conduits in vivo.
6. To compare efficacy of all investigated models.

2.3 Scientific novelty and originality of the study

Restricted longevity is the main disadvantage and reason for limited use of bioprosthesis. From the very beginning of their usage, different treatment methods were applied in order to prolong their functioning. Many substances were investigated, changing their concentrations, other compounds and detoxication. Parallel to that the rise of a new method - tissue engineering, took place. It is designed to manipulate with cells and cellular structures where calcification first begins.

In this research we investigate efficacy of new, previously not used substances (Alanine, Norvaline), and upgrading former treatment protocols (Carbodiimide). In vitro and in vivo experiments show good short-term results by means of bioprosthesis function. This confirms the idea of further upgrading and investigation of successful treatment protocol.

This investigation was also designed to create and evaluate new protocols for tissue decellularization. We found out that two of eight probative protocols were successful. One of these two protocols showed good short-term result in experiments in vivo in terms of calcification mitigation and excellent bioprosthesis function.

2.4 Practical application

First part of investigation showed good results when using Carbodiimide mixture with other chemical agents. We also had good results using original decellularization protocol when detergents were used with other substances. All experiments showed good short-term prosthesis function and anatomy.

All these protocols are original, and could be patented. There is a need for further investigation for long-term results. In case of success, new methods could be applied to treat bioprosthesis used in conventional surgery or minimal invasive technologies.

3. Materials and methods

The research started with obtaining permissions of local Bioethics committee (2007 07 12 No 0159 and 2009 10 14 No 0193). All anaesthetic and surgical procedures were performed and complied with the Principles of Laboratory Care and the Guidelines for the Care and Use of Laboratory Animals.

Research models were managed in special order to perform experiments in series in vitro and later on in vivo and step by step expanded in extent. Porcine aortic and pulmonary artery valved conduits were chosen as the most similar to human ones in terms of anatomy and function.

Chemical agents used for tissue treatment were chosen as original ones or already used somewhere, but innovated by means of concentration, exposure time or other compounds.

Stages and steps of experiments:

3.1 Porcine aortic valved conduit treatment with chemical agents in vitro in order to reduce calcification possibility after implantation.
3.2 Implantation of treated tissue samples subcutaneously in Wistar rats (only aortic).
3.3 Implantation of treated valved conduits in pig's abdominal aorta (only aortic).
3.4 Decellularization of valved conduits – in vitro.
3.5 Implantation of decellularized valved conduits in ovine heart.

3.1 Selection of chemical agents for aortic and pulmonary valved conduit treatment and development of treatment protocols.

During this stage we used male pigs 20-30 kg in weight, sedation induced with Diazepam 5 mg/kg, heparinisation (200vv/kg) and euthanasia were performed shortly afterwards. Under sterile conditions porcine hearts were harvested and valved conduits were prepared leaving a short rim of muscles and about 2-3 cm of conduit's wall. All fat tissue and adventitia were removed. Conduits were kept in cold saline while transferred to treatment solutions.

Chemical agents used for tissue treatment (four groups and control – without treatment):

- *Glutaraldehyde 0,1% (Glu);*
- *L-Alanine (Ala) 100 mM pH 7,6;*
- *L-Norvaline (Norv) 100 mM pH 7,6;*
- *Carbodiimide (EDC) 0,3 M with N-hydroxisuciimide 0,1 M (NHS) and poli(propilen glikol)bis 2-(aminopropil) ether 0,1M (Jeffamin).*

Tissue samples were fixed to special rotating (50 – 70 times per minute) membrane and kept in room temperature in all cases in order to ensure flotation of conduit's valve leaflets.

Exposure with Glu lasted 24 hours; thorough rinse in cold saline (4ºC) was

performed afterwards. In groups were tissues were treated with Alanine and Norvaline, initial exposure to Glu also lasted 24 hours, afterwards rinsing in cold saline additional 24 hours treatment with Ala or Norv was performed. The last group initial treatment with EDC with NHS lasted 24 hours, after thorough rinse in cold saline 3 hours tissue samples were kept in Jeffamine and finalizing additional 24 hours in Ala 100 mM pH 7,6. In all cases care was taken to ensure free flotation of tissue samples and valve leaflets. After main treatment part was done, samples were placed into cold saline until implantation. It lasted up to 24 hours.

3.2 To evaluate efficacy of chemical treatment in experimental model without direct contact to hemodynamic system (experiments with small animals)(N=120)

During this stage we used male 150-200 g weight Wistar rats. 12 hours to surgery animals starved, but were given water. Anaesthesia was induced with Ketamine (Bremer Pharma GmbH, Germany), antibiotics were given afterwards 0,1 g Reflin (Cephazolin, Ranbaxy Ireland Limited). Dorsal surface was prepared for surgery by means of cutting hairs and washing with Cutasept. Three incisions bilateral spine were performed and subcutaneous pockets were prepared by blunt dissection. Then treated tissue samples of approximately 1 cm^2 were placed inside and incisions were sutured with adaptive sutures using 4/0 Prolene. Tissue samples from every group were prepared before surgery approximately 1 cm^2 sample was cut from each anatomical field (wall and leaflet).

After surgery animals were observed in vivarium for 60 days. Then according to Principles of Laboratory Care and the Guidelines for the Care and Use of Laboratory Animals euthanasia was performed and samples were explanted. Explants were placed in dry cold conditions.

Just before evaluation of residual calcium level, samples were kept for 24 hours in dry cold conditions; later Hitachi 175-50 (Japan) atomical absorbtional spectrograph was used. All adjustments were made according to manufacture's recommendations. Residual calcium level was measured by mg/g of dry tissue mass.

Results were processed with SPSS, means were compared using Student's t-test and one way ANOVA, when $p<0,05$ means were kept as statistically different.

3.3 To evaluate efficacy of chemical treatment in experimental model with direct contact to hemodynamic system (experiments with large animals) (N=20) (only aortic).

In this series porcine valved aortic conduits treated as previously described were used. Just before implantation thorough rinse with saline and antibiotic solutions was performed.

Male pigs, in weight approximately 20-30 kg were used as recipients. Diazepam 5 mg/kg and Ketamine 10 mg/kg were given for sedation. Cannulation of ear artery and vein for drug injections and arterial pressure monitoring were done. Using invasive probes three main leads of ECG was obtained. Fentanil 0,005mg/kg, Propofol 2,5mg/kg, Tracrium were given for anaesthesia induction. As soon as intubation was performed with 7, 7,5 or 8 sized tubes, ventilation with air was established maintaining blood gas parameters normal. We used Isoflurane (Forane), Fentanil, Propofol for maintenance of anaesthesia. Cephazoline

1g injection before, during and after surgery were used as prophylaxis against infection.

Operative field was shaved off, washed with Cutasept, surrounding was covered with sterile drapes. 10 cm length skin incision was done just below costal arch in the left side. Subcutaneous tissues were dissected with electrocautery and peritoneum separated by blunt dissection in order not to enter peritoneal cavity. As the retroperitoneal space was reached aorta, left renal artery, inferior vena cava were recognized. Abdominal aorta was dissected from veins and surrounding tissues, several lumbar branches were ligated, heparinisation took place afterwards (200vv/kg). Aorta was cross-clamped just below renal artery and several centimetres caudal from first clamp, and cross-sected. Valved conduit sutured end to end with two continuous 4/0 Prolene sutures, clamps released, almost in all cases there were no signs of bleeding, no drains were left and all tissues were sutured according to layers. Animals were observed in operating room until spontaneous breathing and chewing movements appeared, then extubated. Further on animals were observed in vivarium for 60 days, painkillers were given during first days after surgery.

Repeated surgery was performed after intended period. Preparation to surgery and further anaesthetic and surgical steps were done as mentioned previously, periaortic adhesions were divided sharply when retroperitoneum space was reached.

Heparinisation was followed by direct pressure measurement in aorta cranialy to conduit and caudaly, mean pressure gradient was calculated. Euthanasia and explantation of conduit performed afterwards. Adherent tissues were removed from explanted sample, conduit was cut longitudinally, and pictures of internal surface were taken. Tissue samples from every anatomical field were cut; one half of every sample was designated for residual calcium level evaluation by histological examination, the other for evaluation of residual calcium level by atomical absorbtional spectrography. Tissues for histological examination were put into 10% Formaldehyde (Lach-Nez, s.r.o., Czech) solution for several days, after the microtome incisions were made, samples were stained with von Kossa, and calcium deposits were seen as black. Calcium level estimated by spectrography was described before. All results were processed with SPSS, Student's t-test and one way ANOVA were used to compare means, when $p<0,05$ the difference was statistically significant.

3.4 Selection of detergents and other substances, and development of treatment protocols for decellularization of aortic and pulmonary valved conduits in vitro.

Male pigs, weighted approximately 30-35 kg (N=24) were used as donors for this step. We used valved pulmonary and aortic conduits from 3 different pigs to test every decelularization protocol. Diazepam 5 mg/kg and Ketamine 10 mg/kg were given for sedation and premedication, anaesthesia was maintained with Thiopental and Fentanyl, heparinisation took place as usual (200vv/kg), and animals were euthaniased afterwards. Hearts were harvested under sterile conditions and preparation of valved conduits was made removing all adipous tissue and adventitia, leaving small rim of muscles, taking care not to injure leaflets. Conduits were kept in cold saline until placed in treatment solution. Just before that they were fixed on special rotating membrane so that when membrane rotated 60 times per minute valve leaflets were closed or almost closed and slightly floating. Membrane with conduits was placed in special flask with treatment solution. According to

protocol, flasks could be placed either in refrigerator or in thermostat.

We used different agents to achieve decellularization: detergents (sodium deoxycholic acid, sodium dodecyl sulfate, Triton-X-100, CHAPS), enzymes and nucleases (Trypsyn, DNA-ase ir RNA-ase), other compounds and buffers.

Initial treatment with detergents was always done in thermostat at 37°C, 5% CO_2 for certain period, washing with cold saline was done afterwards. In order to remove all remnants of nuclei, DNA, RNA, cellular structures and membranes, additional treatment with endonucleases was performed. This step of treatment is very important by means of porcine retroviruses removal, which could infect recipient after implantation. Conduits were rinsed afterwards and immersed into L-15 medium (Leibovitz) (Gibco, UK) with L-glutamine, antibiotics (peniciline/streptomicine) were added to this medium, where tissues could be kept 7 days at 4°C before implantation. At present step there were no need to use medium, since just after decellularization, efficacy was evaluated.

We used 8 decellularization protocols, some of them were described by other authors (such as with Trypsine), others were upgraded by means of concentrations or additives, and some were our own newly chosen. All possible effective agents were included in these protocols under different mixture.

Some additives were not disclosed because of possible commercial and future patent secret.

Decellularisation protocols:

1. *0,1% sodium deoxycholic acid, 0,1% sodium dodecyl sulfate.*
2. *0,1% sodium deoxycholic acid, 0.25% tert-octylphenyl-polyoxiethylene (Triton X-100).*
3. *0.5% trypsin, 0.2% ethylenediaminetetraacetic acid (EDTA).*
4. *0.05% trypsin, 0.02% ethylenediaminetetraacetic acid (EDTA).*
5. *0,45% hypotonic solution, 0,5% sodium deoxycholic acid, 0,1% sodium dodecyl sulfate, 0.25% tert-octylphenyl-polyoxiethylene (Triton X-100).*
6. *0,45% hypotonic solution, 0,1% sodium deoxycholic acid, 0.25% tert-octylphenyl-polyoxiethylene (Triton X-100) su 0.2% EDTA.*
7. *0,45% hypotonic solution, 0,1% sodium deoxycholic acid, 0,1% sodium dodecyl sulfate, 0.2% EDTA.*
8. *1% CHAPS, 0.25% tert-octylphenyl-polyoxiethylene (Triton X-100).*

Efficacy of decellularisation was assessed by visual evaluation, tissue quality, elasticity, valve competence and histological examination to confirm absence of cellular structures and quality of elastic fibers. Tissue samples were immersed in 10 % Formaline for several days, Hematoxiline/Eosine staining used for basic evaluation of nuclei and cellular structures presence, van Gieson staining used to evaluate elastic fibers.

3.5 To evaluate efficacy of decellularized valved conduits in vivo (N=9).

Valved conduits treated as previously mentioned were stored in cold saline (4°C) with antibiotics solutions for 7 days until implantation. We have chosen conduits treated by two protocols where decellularization was confirmed.

Male sheep, in weight approximately 20-25 kg, were used as recipients for porcine conduits as xenotransplants, this model was designated as it imitates xenotransplant

implantation in human heart, but ovine immune system is much more active.

Ear vein was cannulated Diazepam 5 mg/kg and Ketamine 10 mg/kg were injected to sedate animals. Fentanyl 0,005mg/kg, Propofol 2,5mg/kg were used for anaesthesia induction, Tracrium injected afterwards and intubation took place with 7, 7,5 or 8 sized endotracheal tubes. Mixture of oxygen and Isoflurane was used for ventilation; blood gases were monitored and kept in normal range. Jugular vein was cannulated and infusions of Fentanyl and Propofol were started to maintain anaesthesia. Cephazoline 1g was given before, during and after surgery for infection prophylaxis. Ear of leg artery cannulated for arterial pressure monitoring and temperature probe placed in esophagus, ECG basic leads established using invasive electrodes.

Operative field was shaved off, washed with Cutasept, surrounding was covered with sterile drapes. Left thoracotomy in 3rd intercostal space was made. Subcutaneous tissues ant muscles were cut using electrocautery. Left lung was pulled aside; stay sutures after pericardiotomy were placed. Ascending aorta, aortic arch were prepared and two purse-string 5/0 Prolene sutures placed in arch for arterial cannulation. 14 Fr arterial cannula inserted after heparinisation (200vv/kg). Single 3/0 Prolene purse-string suture placed on right atrium, two stage venous cannula inserted, all cannulas connected to pediatric membrane oxygenator (Dideco D905 EOS, Mirandola, Italy) in cardiopulmory bypass mashine (Gambro) and bypass was started (flow rate 2 – 2,5 l/min/m^2). All procedures performed in ovine normothermia (37 – 37,5ºC) on beating heart.

Native ovine pulmonary artery was prepared, separated from aorta, taking care not to injure coronary arteries. It was transsected 2 cm distally to pulmonary artery valve annulus to remove valve leaflets and remnants of vessel wall. Continuous 4/0 Prolene sutures were used to make proximal and distal anastomoses. Just before starting to wean from cardiopulmonary bypass lungs were insufflated, infusions of Adrenaline and Dexamethasone started to lower pulmonary resistance. After weaning from cardiopulmonary bypass, protamine sulfate was given and additional haemostasis applied if needed. Drain left in left pleura until spontaneous breathing appeared, then removed. All tissues sutured by layers. Animals observed in operative room first postoperative day, pain killers were given if needed, in second postoperative day animals were transported to vivarium. 45 to 60 days sheep were kept in vivarium before repeated surgery. All anaesthetic and surgical steps were the same as during first operation, adherent tissues were divided either by blunt or sharp way. After heparinisation epicardial echoscopy was performed and exsanguination took place.

Conduit explanted and cut longitudinally, pictures of internal surface were taken. Tissue samples from every anatomical field were cut; one half of every sample was designated for residual calcium level evaluation by histological examination, the other for evaluation of residual calcium level by atomical absorbtional spectrography. These procedures were described before.

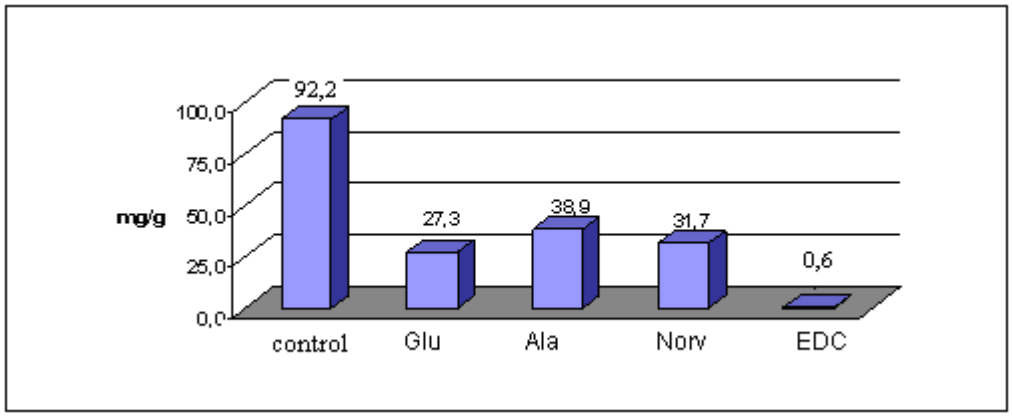
4. Results

4.1 Selection of chemical agents for aortic and pulmonary valved conduit treatment and development of treatment protocols..

Tissues were treated with established protocols, prepared to implantation procedures. Any histological examination was not performed, tissues were assessed visually, all parts were changed in colour, more rigid than native ones. Valve leaflets were normal, closure was competent.

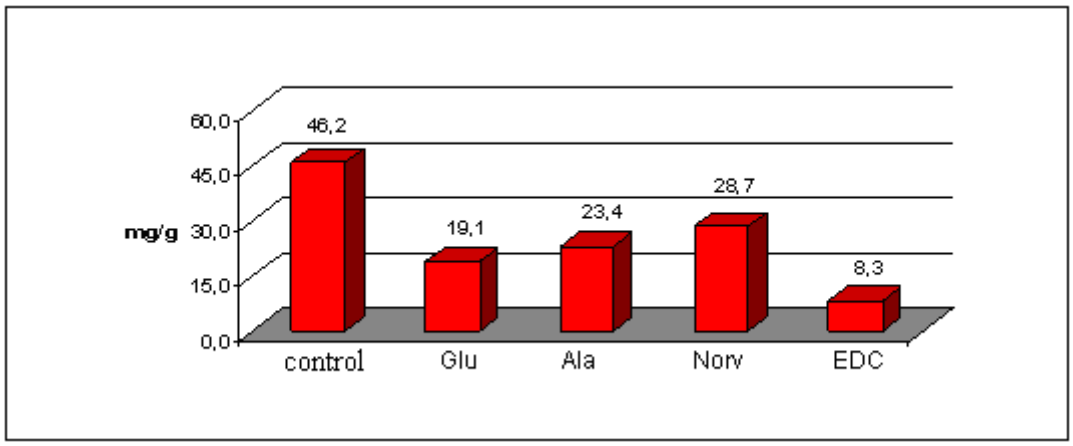
4.2 To evaluate efficacy of chemical treatment in experimental model without direct contact to hemodynamic system (experiments with small animals) (only aortic).

120 experiments were made. Each group of investigative chemical agent had 60 implants of each anatomical structure (aortic wall or aortic valve). All rats survived without any complications. Analysis of residual calcium level was calculated, statistical evaluation was made and data presented in tables and figures.



Picture 1. Residual calcium level in aortic valve
Glu – glutaraldehyde, Ala – L-Alanine, Norv – L-Norvaline, EDC – carbodiimide, Control – control group, without treatment.

Evaluation of residual calcium level in aortic valve is shown in picture 1, where data expressed by means. The most expressed calcification was found in control group, where tissues were without any treatment. Results show that Glu lowers calcification significantly, but in next two series, where Ala or Norv were added to Glu, calcium amount was even slightly higher comparing to treatment with single Glu. The lowest calcium amount found in the last group, where EDC was used, number dropped to 0,6 mg/g. It is more than a hundredfold lower than when untreated. All data differ statistically significantly except between groups Ala and Norv, where p>0,05.



Picture 2. Residual calcium level in aortic wall
Glu – glutaraldehyde, Ala – L-Alanine, Norv – L-Norvaline, EDC – carbodiimide, Control – control group, without treatment.

Calcification was less expressed in subcutaneous aortic wall implants in the same experimental model comparing to aortic valve (pic 2). As expected, the biggest calcium level was found in untreated tissue samples. Other series showed similar results as in aortic valve series, Glu lowered calcification significantly; additives such as Ala and Norv did not improve calcification mitigation. The best result showed treatment with EDC, calcium amount falls to 8,3 mg/g from 46,2 mg/g when untreated. All results are statistically different, except Ala and Norv groups, where p>0,05.

	Control group (N=120)	*Glu (N=60)*	*Glu+Ala (N=60)*	*Glu+Norv (N=60)*	*EDC+NHS+ Jeffamin+Ala (N=60)*
AoV	*92,2±6,1*	*27,3±3,1*	*38,9±2,1 **	*31,7±1,4 ●*	*0,6±0,1*
AoS	*46,2±5,3*	*19,1±0,9*	*23,4±1,2 **	*28,7±1,8 ●*	*8,3±1*

Table 1. Residual calcium levels, comparison between aortic tissues.
AoS – aortic wall, AoV – aortic valve, Glu – glutaraldehyde, Ala – L-Alanine, Norv – L-Norvaline, EDC – carbodiimide, Control – control group, without treatment.
*NHS - N-hydroxisukcinimide, * and ●- p>0,05*

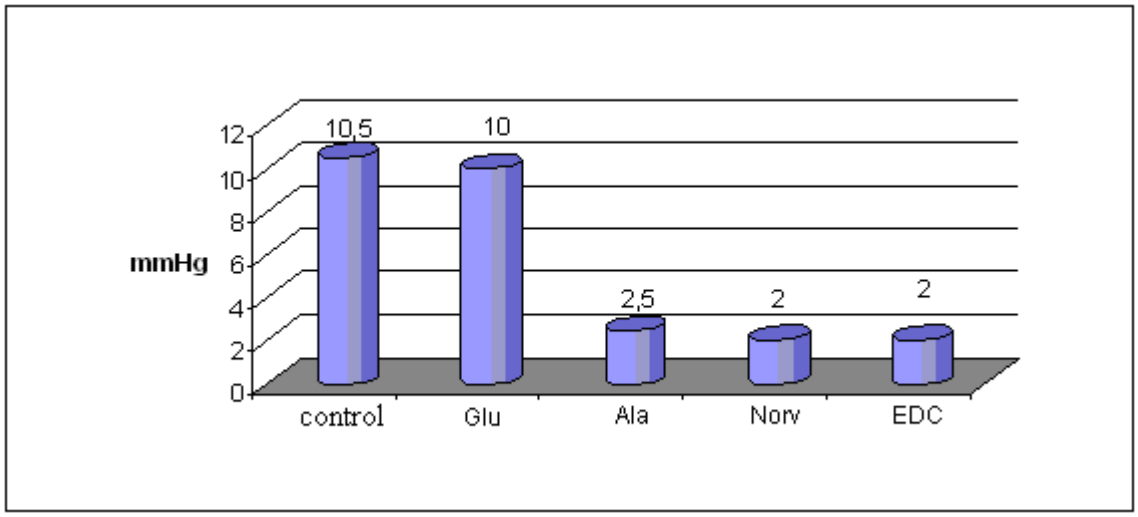
As mentioned before there are some similarities in calcification level comparing different tissues, as could be seen in summary table 1. Residual calcium amount variation was more or less repeated, but in different amounts. More extensive lowering was observed in EDC group, where aortic valve calcium numbers differ more than tenfold comparing to aortic wall. Comparing means of both groups, there is a statistical difference, except for Ala and Norv series.

Speaking generally about this experimental model, it is clear that treatment with EDC gave the most impact on calcification mitigation. Other substances were not so effective except Glu, which lowered calcification level significantly lower comparing to other substances. Additional treatment with amine acids did not made

improvement in these series.

4.3 To evaluate efficacy of chemical treatment in experimental model with direct contact to hemodynamic system (experiments with large animals) (only aortic).

In total there were 20 surgery experiments, all animals survived, one pig had small retroperitoneal abscess far from implant, which was detected during repeated surgery. 4 valved conduits were treated in the same way (there were 5 groups of treatment) and implanted. After certain time, during repeated surgery we had 4 explants, from each of them two pieces from the same anatomical field (2 aortic valve leaflets, two pieces of aortic wall) were cut and sent for evaluation of residual calcium level and remaining one leaflet and piece of wall was sent for histology. Thus every series had 8 pieces to calculate calcium. Pressure gradient was assessed, pictures of internal surface were taken, all data presented in summary table and pictures.



Picture 3. Mean pressure gradient in different series.
Glu – glutaraldehyde, Ala – L-Alanine, Norv – L-Norvaline, EDC – carbodiimide, Control – control group, without treatment.

The biggest pressure gradients were detected in two groups, were tissues were untreated or treated with Glu, 10.5 mmHg and 10 mmHg respectively (pic 3). Severe calcifications and narrowing of lumen caused these changes. In the rest of groups numbers were similar (2-2.5mmHg). Slight dilatation could happen after implantation or valve leaflets were pressed to inner wall and stayed there even when calcified not creating high-pressure gradient. No statistical evaluation was possible due to small number of samples.

4.3.1 Macroscopic and histological examination results (only aortic).

All tissue samples were stained van Kossa and imagination 40 times used to determine residual calcium which is seen as black dots.

4.3.1.1 Control group: pictures of internal surface show severe calcifications in all implant surfaces. Aortic wall changed in colour, damaged, calcified and restricted leaflets seen which seems to be rigid and not able to move. Histological examination showed well-structured aortic wall and valve, however, full of calcium deposits. All leaflet and wall tissue layers (endothelial, media and adventitia or ventricular in leaflet sides) are in black dots, in some places black colour in confluent, especially in places of internal surface side. This is a sign of severe calcification.

4.3.1.2 Glu group: photo of inner surface seems very similar to control group. Aortic wall looks changed in colour; small calcifications could be recognized on surface. Valve leaflets are mobile but thickened, they also look unnatural, changed in colour and slightly restricted. Histological pictures also show well-structured wall and leaflets, but with plenty of black dots – calcium deposits. Comparing to previous group, calcification there is less expressed, aortic wall is more damaged in both surface sides (endothelial and adventitia), but the media has only few black marks. Whereas leaflet looks opposite, many deposits could be seen in medial layers, ands less of these dots are in both surface sides. According to the findings, calcification is still severe, but efficacy of treatment is obvious.

4.3.1.3 Glu and Ala group: picture of explant completely explains why gradients were so low in this group. Valve leaflets are damaged with well seen calcifications, all three leaflets are restricted, rigid and shortened, and seems like they hide in sinuses. Aortic wall is very thick, but surface looks smooth without any visible calcium particles. Histological findings show less expressed calcification. Aortic wall endothelial surface has few calcium deposit marking dots and subendothelial layer is severely filled with calcium deposits, media and other places have black markings but they are not very concentrated. Valve leaflet has very similar view as described in Glu group. Both surface sides are free from deposits, but starting from subendothelial layer black colour is dominating, meaning that amount of residual calcium deposits is high. Despite very bad macro view, histological data suggest this treatment to have some impact on calcification.

4.3.1.4 Glu and Norv group: macro pictures of this group on the first sight look better than all mentioned before. Aortic wall is damaged, changed in colour, but mobile, smooth, without visible calcifications. Leaflets also severe damaged, restricted to the sinuses, shortened, but asymmetrically, some are still more or less mobile. Histological examination confirms this macro view. Aortic endothelial and subendothelial layers are infiltrated with calcium and going more far to media and to adventitia side there are less and less visible black dots. Valve leaflet stays damaged in medial layer severely, deposit dots are confluent, but both surface sides are more or less normal. This is why aortic wall is more natural, and nevertheless treatment had impact on calcification.

4.3.1.5 EDC+NHS+Jeffamin+Ala group: the best results were observed in the last group. Picture of internal surface looks normal. Aortic wall in normal colour, mobile, smooth, sinuses are well seen, no sign of calcification. Leaflets mobile, pliable,

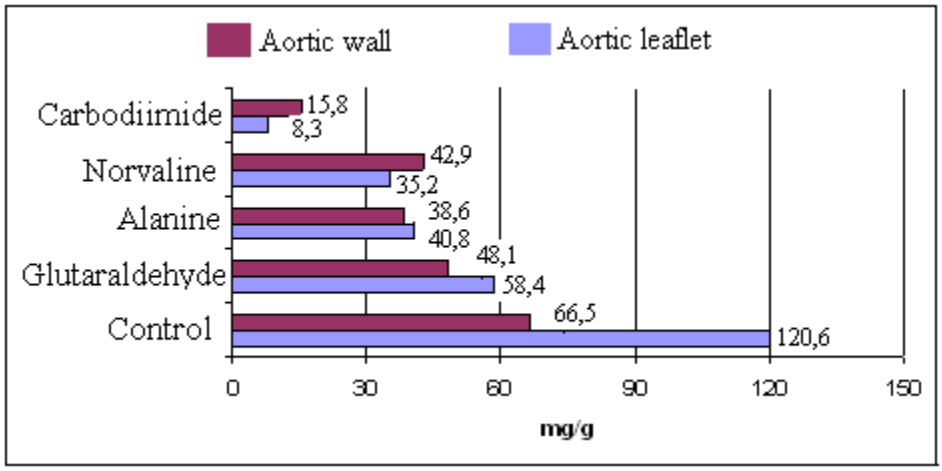
transparent, there are no vegetations or thrombus. However, histological pictures show some minor injuries. Wall internal layers, to the media are slightly infiltrated with calcium deposits. Leaflet both endothelial surfaces are covered with deposits showing dots. Other medial layers are untouched. These findings totally correspond to macro view and it seems this treatment has made the greatest impact preventing calcification.

4.3.2 Quantitative evaluation of residual calcium amount (only aortic).

It is performed using atomical absorbtional spectrometry. Data are shown in summary table 2 and figure 4.

	Control group (N=8)	*Glu (N=8)*	*Glu+Ala (N=8)*	*Glu+Norv (N=8)*	*EDC+NHS+ Jeffamin+Ala (N=8)*
AoV	*120,6±9,2*	*58,4±4,2*	*40,8±2,6 **	*35,2±1,9 ●*	*8,3±0,5*
AoS	*66,5±4,8*	*48,1±5,4*	*38,6±4,1 **	*42,9±2,4 ●*	*15,8±0,8*

Table 2. Residual calcium levels, summary table.
AoS – aortic wall, AoV – aortic valve, Glu – glutaraldehyde, Ala – L-Alanine, Norv – L-Norvaline, EDC – carbodiimide, Control – control group, without treatment.
*NHS - N-hydroxisukcinimide, * ir ●- p>0,05*



Picture 4. Comparison of residual calcium levels between different tissues.

Spectrometric results are correspondent to examinations made before. Control group has the most calcium in tissues; this was the cause of high gradient, and changes seen in histological and internal surface pictures. Comparing two tissues, difference is almost twofold and it is statistically significant (picture 4 and table 2).

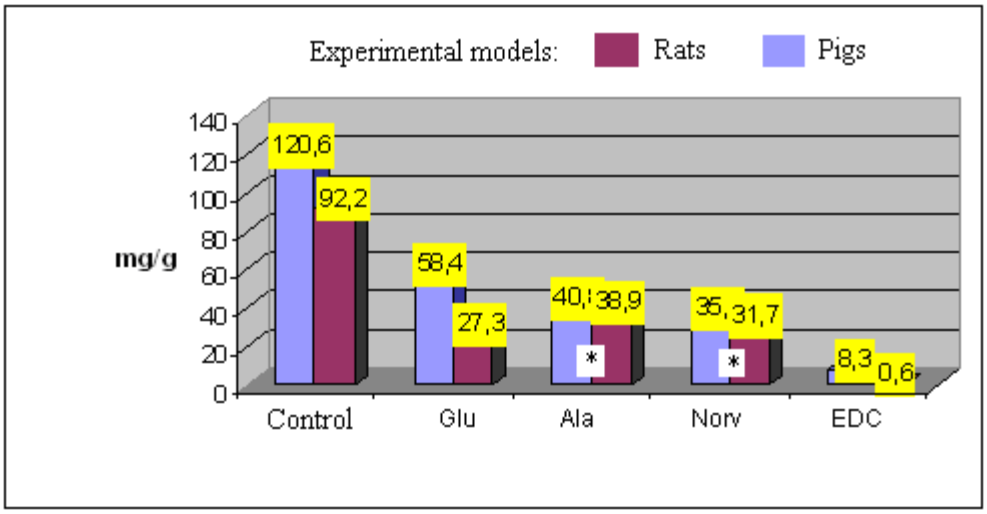
Treatment with Glu changed situation, and there was lowering in residual calcium amount. Nevertheless, calcification is still severe, valved conduits are too damaged. Difference between results is statistically significant. Like in control group, valve leaflets are heavier calcified than wall, thus making valve non-functioning.

Heavy calcification is also present in the next groups. Despite of lower residual calcium amount comparing to groups before, Ala and Norv treatment does not mitigate calcification enough. Data are statistically different when comparing to other groups, but when comparing between tissues in these groups, difference is not statistically significant, p>0,05. (picture 4 and table 2).

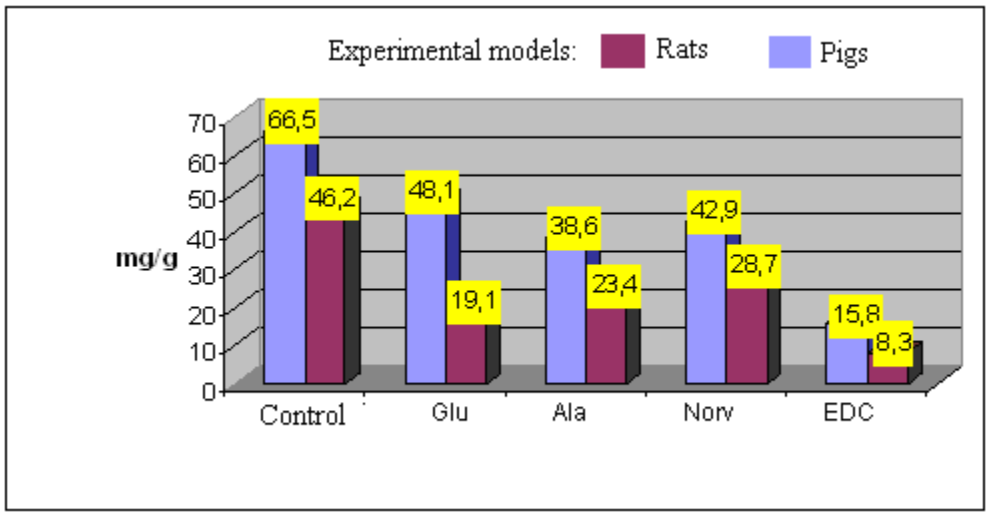
Estimating all gathered data it is clear, that EDC had the best impact on postoperative calcification. In this group residual calcium level was the lowest, comparing to other groups, and the difference is statistically significant, tissues were well structured and functional.

4.3.3 Comparison of treatment efficacy in different experimental models (only aortic).

It is interesting to review results, how the treatment with the same agent is effective comparing different experimental models. In all series explants from pigs had more calcium, but in some series these differences are not so significant. Picture 5 shows two models (porcine and rat) and five series of treating aortic valve. The calcification is most expressed in groups where there was no treatment at all or treatment with Glu, but in rats Glu treatment made substantial lowering in calcium level, next two series were almost in the same range and as described before EDC had the best impact on calcium level. All differences in data while comparing between animals are statistically significant, except Ala and Norv groups.



*Picture 5. Comparison of calcium amount in aortic valve between two experimental models (rats – red colour, pig – blue colour) Glu – glutaraldehyde, Ala – L-Alanine, Norv – L-Norvaline, EDC – carbodiimide, Control – control group, without treatment, * - p>0,05.*



Picture 6. Comparison of calcium amount in aortic wall between two experimental models (rats – red colour, pig – blue colour). Glu – glutaraldehyde, Ala – L-Alanine, Norv – L-Norvaline, EDC – carbodiimide, Control – control group, without treatment. In all series p<0,05.

Differences while comparing treatment efficacy in aortic wall are more apparent. In all series calcium amount is higher in porcine model, these differences are statistically significant (picture 6).

Summarizing these data it is evident, that tissues in porcine model were more exposed to immunological system, were directly approaching hemodynamic system, which caused more extensive calcification.

4.4 Selection of detergents and other substances, and development of treatment protocols for decellularization of aortic and pulmonary valved conduits in vitro.

During this stage pulmonary and aortic valved conduits were harvested from porcine hearts and treated in 8 different protocols. 6 of them were original and 2 were reported before, but we repeated them in order to verify efficacy. We treated 3 aortic and 3 pulmonary valved conduits by the same protocols, later histological examination staining with Hematoxilin/Eosin for decellularization efficacy was done. In case of effective decellularization elastin fibers were examined with staining van Gieson. This was also done in control case where decellularization was not full. All data are shown in summary table 3.

4.4.1 Basic histological examination

I protocol: 0,1% DEO + 0,1% SDS: *two ionic detergents are included in this original treatment protocol. Concentrations are low and similar. Two agents with similar features had synergetic effect on tissues. Histological examination confirms absence of nuclei in pulmonary artery, valve and in aortic valve. However, there are signs of nuclei (blue dots) in aortic wall starting in media towards adventitial side. In both conduits endothelial layer*

is also absent.

II protocol: 0,1% DEO + 0,25% Triton-100: *combination of ionic and non-ionic detergents was used in second original protocol. In both histograms endothelial cells or basal membrane could be seen, also there are plenty of nuclei (blue dots) in all layers. Thus decellularization failed in this series.*

III protocol: 0,5% Trypsin + 0,2% EDTA: *treatment with enzymes and chelates is well described, therefore we tried to verify efficacy. We used standard concentrations which should be sufficient for membrane disruption, but, unfortunately, histological pictures showed plenty of nuclei, cellular structures also could be seen, presence of endothelial cells remnants could by suspected and only somewhere one could find small islands of acellular ECM. Besides, tissues were in poor condition by means of strength and shape, showed failure of decellularization in both conduit tissues.*

IV protocol: 0,05% Trypsin + 0,02% EDTA: *the fourth protocol modified the third one by means of lowering concentrations of compounds ten times. As in previous series, decellularization failed, in some places we can see quite normal tissue structure. Endothelial cells are present, blue coloured nuclei are present everywhere in all layers. As in previous series, ECM fragmentation began, tissues are in poor quality.*

V protocol: Hypotonic s. + 0,5% DEO + 0,1% SDS + 0,25% Triton-100: *many compounds are included into this original protocol. Ionic, non-ionic detergents, hypotonic solution. Basically, it is an upgraded second protocol with SDS, hypotonic solution and using higher DEO concentration. This combination ensures all types of action to membranes and cellular structures. Histological examination shows removal of cellular structures in all layers and endothelial cells only in pulmonary artery wall. Nuclei are removed partly from aortic wall, media and adventitial side is well structured and full of nuclei. Both conduits' valves are also with nuclei in all layers. In general terms, decellularization failed.*

VI protocol: Hypotonic s. + 0,1% DEO + 0,25% Triton-100*: is created upgrading second and fifth protocols by removing SDS in order to decrease chance to damage. It was expected to have mild but also effective interaction with tissues. Nevertheless, this combination failed to decellularize tissues. Efficacy is very superficial, where structural elements are absent, but otherwise all tissue layers both in pulmonary and aortic conduits, such as media and adventitial side, are full of cellular structures. Even endothelial cells are present somewhere.*

VII protocol: Hypotonic s. + 0,1% DEO + 0,1% SDS: *this original method was created by upgrading the first protocol with hypotonic solution and some additives . It is the most effective protocol. Aortic and pulmonary tissues are acellular. Endothelial cell layers are removed effectively, cellular structures also destroyed and removed as well as nuclei from tissues. Histological pictures show completely acellular ECM.*

VIII protocol: 1% CHAPS + 0,25% Triton-100: *the last original protocol which includes zwiterionic and non-ionic detergents. These two detergents have mild effect on tissues, which is also well seen on histograms. Tissues are well structured, almost untouched. All layers are present in pulmonary and aortic tissues with plenty of nuclei. Efficacy is poor; there was nearly no effect on tissues, decellularization failed.*

Summarising results we have two protocols (first and seventh) with completed decellularization. One of them shows complete acellular ECM. This is achieved by using ionic detergents and other additives, and even in low concentrations these agents act well. Probably having higher concentration of these substances, exposure time will shorten. It is important to lower damage to ECM as much as possible. All protocols are summarised in table 3, red marked cells mean complete acellularity of ECM. One tendency is also obvious, pulmonary artery tissues could be treated more effective than aortic ones, and this could be caused by anatomical features.

No	*Treatment protocol*	*Ao wall*	*Ao valve*	*PA wall*	*PA valve*
1	*0,1% DEO + 0,1% SDS*	-	+	+	+
2	*0,1% DEO + 0,25% Triton 100*	-	-	-	-
3	*0,5% Trypsin + 0,2% EDTA*	-	-	-	-
4	*0,05% Trypsin + 0,02% EDTA*	-	-	-	-
5	*0,45% Hypotonic s. + 0,5% DEO + 0,1% SDS + 0,25% Triton-100*	-	-	+	-
6	*0,45% Hypotonic s.+ 0,1% DEO + 0,25% Triton-100 + 0,2% EDTA*	-	-	-	-
7	*0,45% Hypotonic s.+ 0,1% DEO + 0,1% SDS + 0,2% EDTA*	+	+ /-	+	+
8	*1% CHAPS + 0,25% Triton-100*	-	-	-	-

Table 3 Summary table of decellularization protocols.
Ao – aortic, PA – pulmonary artery. + complete decellularization; +/- incomplete decellularization (few structural elements); - failed decellularization (full of structural elements)

4.4.2 Histological examination of elastic fibers

All tissues, especially acellular, should undergo assessment of elastic fibers. The assessment

of collagen is also important, but in case there is no possibility to perform electronic microscopy, elastic fibers could be evaluated with light microscopy, staining with van Gieson.

Elastic fibers were assessed in the seventh protocol, where acellular ECM was detected. Histogram shows normal, solid, well-lined fibers in all tissues whereas in the second protocol, where decellularization failed, elastic fibers are fragmented in all tissue depth. Thus it is important to create such mixture of compounds, to protect ECM from structural damage during procedure. This is a direct reason to early postoperative failure of conduit.

4.5 To evaluate efficacy of decellularized valved conduits in vivo.

Preparation of valved conduits for implantation was made according to decellularization results in vitro. All conduits were treated by the seventh protocol and EDC, which also showed good results in porcine model.

Nine successful experimental operations were performed, 3 valved conduits from the same anatomical origin (3 PA, 3 Ao and 3 more Ao treated EDC) were used for implantation. During surgery we used cardiopulmonary bypass (CPB). Mean CPB time 42,8±7,7 minute. Animals, which did not survive surgery because of gastrointestinal disorders or severe resistant pulmonary hypertention, were excluded. Observation time was 45 days, repeated surgery was done afterwards, intraoperative epicardial echo performed, pictures of internal surface, histological examination and assessment of residual calcium level was done.

	Residual calcium level (mg/g)	*Mean pressure gradient (mmHg)*
Aortic valved conduit treated with EDC (N=3)	*12,05±0,2*	*2±0,2*
Decellularised pulmonary artery valved conduit (N=3)	*0,27±0,03*	*1,3±0,12*
Decellularised aortic valved conduit (N=3)	*0,45±0,025*	*1,6±0,2*

Table 4. Comparison of impact of different treatment methods on tissues.

As mentioned before, epicardial echo took place, and valve leaflet function, gradient and velosity through the valve were assessed. All three series had normal gradient and velosity. Slightly lower numbers are in decellularized conduit groups (table 4). A little different situation is evaluating residual calcium level, where aortic conduit treated with EDC had much higher numbers comparing to decellularized ones. Theoretically this amount could be normal in implanted valve, since functionally and anatomically it looks well structured. Valve leaflets are thin, pliable, and transparent with no signs of vegetations or thrombus. Aortic wall looks normal in colour, smooth, elastic, valve function normal, competent. The same features have decellularized valved aortic and pulmonary conduits, but their residual calcium level

is much lower.

5. Discussion

Heart valve bioprostheses are considered to be tissues having plenty of collagen, thus these xenotransplant polymers with many antigenic structures after implantation may induce acute or chronic rejection reaction in recipient organism. In order to mitigate or exclude possibility of these reactions tissue are treated with various substances which lower antigenicity by stabilising polymers. This could be agent having several features useful for stabilisation. It could bind between collagen polymers with its amine or/and carboxy groups. But during this kind of interaction always zero length links are made. This is a link between two radicals of the same polymer, excluding stabilisation with adjacent polymer. Zero length links changes tissue features by loss of elastisity, becoming rigid, not pliable. In order to enable possibility to form there links special agents are used to disrupt formation of zero length links. They block and become end capping mollecules for amine or carboxy groups.

Glutaraldehyde is the main substance used in tissue treatment, this agent has mixed features, but also may form zero-length links. Mollecule of glutaraldehyde forms links with two collagen polymers by its carboxy groups.

In our series we used low concentration glutaraldehyde (0,1%). This was done expecting for better tissue preservation and better stabilising effect at the same time. Since there are reports where it is declared that high concentrations (>0,625%) of glutaraldehyde even enhances calcification.

Our results show that even low concentration of glutaraldehyde causes severe calcification after implantation in different experimental models. Comparing to literature one can find different results. Some scientists declare that residual calcium amount is high, especially in young patients. This happened because implants are implanted in very fast metabolism host organism, where immune system is fast has severe reaction to xenotransplants. Other authors report opposite results, despite of possible adverse action glutaraldehyde lowers immune reaction by stabilizing antigenic structures and enables valved conduit to grow. Nevertheless there are clear recommendations of usage bioprosthesis, they should be implanted to patients older than 65 years.

Treatment with glutaraldehyde is wide used in bioprosthesis making in market. But scientists are looking for new chemicals with better features and longer function of treated tissues. This caused usage additional fixative agent to prevent and block radicals which could form of zero length links. There are many combinations of different substances, one of the most popular and also used in market is alpha amine oleic acid. This is monononsaturated fatty omega 9 acid, derived from oleic acid and takes part in metabolism processes. The latter easily bonds to carboxy groups of collagen by covalet links and disables calcium metabolism to tissues. The best result is observed in valve leaflets. There are articles where experimental data confirm even better results when it is combined with more additives. We did not have purpose to repeate patented technology, this made us to

change mixture compounds with original chemicals never used before, but with similar features.

Alanine and Norvaline, are chort chain amine acids which like alpha amine oleic acid block carboxy groups. This should make tissues more pliable and possible to regenerate. We have choosen L kind of isomers, because these are more chemically active and have stable, inactive –COO$^-$ carboxy radical if looses hydrogen atom.

However after explants were analysed, we had not so good result as expected. Both these substances did not stopped calcification in tissues. Residual calcium level was lower than untreated or treated with glutaraldehyde. There is no comparison in literature according to these substances, but it is said diamines could make impact on tissues mitigating calcification. Other substance used in mixtures is – 1-ethyl-3-(3-dimethylaminopropil) carbodiimide. Carbodiimide is water soluble substance, which crosslinks with collagen carboxy and amine groups (from aspartate and glutamate acids). This agent is also able to form zero length links, like glutaraldehyde. Thus to prevent it we used with N-hydroksisukci-imide (NHS), which stabilises cross-links, converts O-acyliurea, a chemical group which formed during chemical interaction, to carboxy group which can bond with amine goup. Another mediator blocking zero-length links is Jeffamin. Both two substances are not nowel, used previously in many experiments and are well known. But we added more additives creating treatment protocols and results were excellent. Calcification level was the lowest. Similar results are described in literature. We had slightly better results, we think because of one more additive – L-Alanine. It is clear that about 50% of zero length links are persistent and not disrupted despite of how good treatment was. Usage of Alanine as end capping molecule possilbly helped.

In next series we used detergents for valved conduit treatment. Basically there are three types of detergents: ionic (sodium deoxycholic acid, sodium dodecyl sulfate), non ionic (Triton-X-100), zwiterionic (CHAPS). These groups are formed by its features. One of the most important feature is CMC-critical micele concentration point, it is a certain concentration of detergent when it forms active micele. This concentration is different for each type, different circumstances may influence CMC. Ionic are dependent on solution strength, non ionic – on temperature.

If CMC is lower than needed there are too few or inactive micele, if CMC is too high, miceles are insoluble and do not affect membranes. In our series we used various mixtures and low concentrations of detergents. One of eight protocols was successful, decellularization was full in both aortic and pulmonary valved conduits. Calcium amount was lower comparing to treatment with other chemical agents. It is well confirmed by histological examination. Also these results are similar to reported in the literature. Using this method in experiments where implants were exposed to real haemodynamic surrounding, early posoperative results were good. Supposingly decellularization removed all cellular structures adn antigenic structures as well, thus made low calcium amount postoperatively. Very important is to evaluate valve function – we did it during repeated surgery and all parameters were in normal range. There were no stenotic flow or incompetence. And comparing two methods with the best results we saw tendency of better results when decellularisation was performed. It is described by authors, having good

results made prototypes of these experimental bioprostheses and used them in clinical practise for restoration of right ventricle outflow tract. But despite of good experimental results one faces really bad results in humans. This is the reason why we are going to resume with more close research of early and late results with decellularization.

6. Conclusions

1. Xenotransplant treatment with chemical agents showed the best impact on tissues when used Carbodiimide in experimental models with small or large experimental animals.

2. Decellularisation of xenotransplants was achieved using mixture of detergent and other agents (0,5% hypotonic s. with 0,1% DEO, 0,1% SDS and 0,2% EDTA). The rest of treatment protocols did not resulted in full decellularisation effect.

3. Tissues treated by decellularisation had less residual calcium amount comparing to other methods.

SANTRAUKA

Sutrumpinimai
Ala – L-alaninas
Ao – aorta
AoS – aortos sienelė
AoV – aortos vožtuvas
CHAPS – 3-[(3-cholamidopropil) dimetilamonio] -1 –propanosulfonatas
CMC – kritinė micelių koncentracija (critical micele concentration – angl.)
DEO – natrio deoksicholinė rūgštis
DKA – dirbtinė kraujo apytaka
DNR – dezoksiribonukleino rūgštis
ECM – ekstraląstelinis matriksas
EDC – 1-etil-3-(3-dimetilaminopropil) karbodiimidas
EDTA – etilendiamintetracto rūgštis
GAG – glikozaminoglikanai
Glu – glutaraldehidas
HE – hematoksilinas ir eozinas
NHS – N-hidroksisukciimidas
Norv – L-norvalinas
PA – plaučių arterija
PAS – plaučių arterijos sienelė
PAV – plaučių arterijos vožtuvas
RNR – ribonukleino rūgštis
SDS – natrio dodecilsulfatas

1. Įvadas

Kasmet daugeliui žmonių prireikia širdies operacijos dėl įgytos ar įgimtos vožtuvų ydos. Per metus daugiau nei 300 000 pacientų protezuojami širdies vožtuvai. Esant tokiam poreikiui, vystosi dirbtinių vožtuvų pramonė, kuriami nauji modeliai, gamybai ir apdorojimui naudojamos naujos medžiagos.

Šiuo metu naudojami du pagrindiniai protezų tipai: mechaniniai ir biologiniai. Mechaniniai protezai ilgalaikiai, tačiau reikalaujantys antikoaguliantų terapijos visą likusį gyvenimą ir išlieka trombembolinių ar kraujavimo komplikacijų rizika. Biologiniai protezai šiuo požiūriu modernesni, nereikia kraujo krešumą mažinančių vaistų, paties vožtuvo funkcija artima natyviniam. Tačiau pacientų aktyvi imuninė sistema greitai paveikia bioprotezą, ir tai labai apriboja (kalkėja, suyra) jo funkciją. Tai ypač greitai nutinka jauno amžiaus pacientams. Apdorojant cheminėmis medžiagomis pailginti protezų funkcionavimo trukmės nepasisekė, galiojimo laikotarpis siekia 15–20 metų. Todėl mokslininkai, kurdami širdies vožtuvus, pradėjo taikyti audinių inžineriją.

Audinių inžinerija širdies vožtuvų chirurgijoje apibrėžiama kaip veiksmų ir

manipuliacijų visuma, kai naudojant ląstelių kultūras sukuriamos naujos struktūros, galinčios tinkamai atlikti numatytas funkcijas bei prisitaikyti prie metabolinių pokyčių. Tokie sukurti vožtuvai būtų idealus pasirinkimas pacientams. Protezas taptų imunologiškai nereaktyvus, tai būtų gyva, visą likusį paciento gyvenimą atsinaujinanti struktūra.

Taikant įvairius audinių inžinerijos būdus, sukurta daugybė įmanomų karkasų vožtuvams gaminti ir pačių biologinių vožtuvų. Tačiau tik deceliulizacijos sritis pritaikyta praktikoje, atliktos operacijos implantuojant aceliulinius ir naujai ląstelėmis apaugintus protezus. Skirtingų mokslininkų pateikiami rezultatai labai įvairūs, eksperimentuose gauti geri artimi rezultatai ne visada pasitvirtina praktikoje. Nėra vieningos nuomonės, ar šis metodas saugus.

Lietuvoje kasmet apie 500 pacientų atliekamos vožtuvų implantacijos operacijos, daugiausia dėl aortos vožtuvo ligų. Kaip ir kitose šalyse, nemažą dalį sudaro pacientai iki 65 metų. O tai reiškia, kad šiai amžiaus grupei, vadovaujantis šių dienų širdies chirurgijos gairėmis, nerekomenduojama implantuoti biologinių protezų. Pasitelkdami pastarųjų metų mokslo laimėjimus, norime ištirti naujausių technologijų galimybes, esant galimybei patobulinti jas, surasti geresnes medžiagas audinių apdorojimui bei išbandyti eksperimentiniuose modeliuose.

2. Darbo tikslas, uždaviniai, naujumas, praktinė reikšmė

Darbo tikslas:

Ištirti širdies biologinių vožtuvų apdorojimo būdus, skirtus sumažinti ar panaikinti audinių kalkėjimą po implantacijos, bei patikrinti efektyvumą eksperimentiniuose modeliuose.

Uždaviniai:

1. *Cheminių medžiagų parinkimas ir aortos vožtuvo transplantatų apdorojimo jomis protokolų sukūrimas.*
2. *Cheminio apdorojimo efektyvumo patikrinimas eksperimentiniame modelyje be sąlyčio su kraujotakos sistema (su smulkiaisiais eksperimentiniais gyvūnais).*
3. *Cheminio apdorojimo efektyvumo patikrinimas eksperimentiniame modelyje esant tiesioginiam sąlyčiui su kraujotakos sistema (su stambiaisiais eksperimentiniais gyvūnais).*
4. *Aortos ir plaučių arterijos vožtuvinių transplantatų apdorojimo detergentais ir kitomis medžiagomis protokolų sukūrimas ir taikymas in vitro, pašalinant ląstelines struktūras (deceliulizacija).*
5. *Nuląstelintų (deceliulizuotų) vožtuvinių transplantatų patikrinimas eksperimentiniame modelyje in vivo.*
6. *Skirtingų apdorojimo metodų rezultatų palyginimas.*

Naujumas ir originalumas:

Biologinių protezų trumpaamžiškumas – pagrindinė kliūtis jų platesniam naudojimui, o jų apdorojimas įvairiomis medžiagomis siekiant pailginti funkcionavimo trukmę – tema, gvildenama nuo pat biologinių vožtuvų atsiradimo. Tobulinami įvairūs cheminio

apdorojimo būdai, keičiant cheminių medžiagų koncentraciją, pritaikant detoksikacijos metodus po apdorojimo; lygiagrečiai atsirado nauja mokslo kryptis – audinių inžinerija. Šios krypties taikinyje ląstelinės audinių struktūros, kurios tampa ankstyvo vožtuvų suirimo priežastimi.

Šiame moksliniame darbe plėtojama audinių apdorojimo cheminėmis medžiagomis tyrimo kryptis, taikant visiškai naujas medžiagas: alaniną, norvaliną. Buvo patobulintas karbodiimido naudojimo apdorojimui protokolas. Tyrimo metu in vitro ir in vivo eksperimentiniuose modeliuose gauti geri artimi naujo apdorojimo būdo rezultatai.

Eksperimentiniame darbe pavyko patobulinti ir audinių inžinerijos būdu apruošiamus vožtuvus. Tyrimo metu atradome, kad net 2 iš mūsų naujai pasiūlytų apdorojimo protokolų efektyviai sumažina kalkėjimą. Įvertinę visus duomenis, galime teigti, kad vienas iš pastarųjų dviejų gerai apsaugo biologinių protezų audinius artimu laikotarpiu.

Praktinė reikšmė:

Mokslinio eksperimentinio darbo metu įrodytas karbodiimido ir kitų cheminių medžiagų kombinacijos efektyvumas. Taip pat geri artimi rezultatai gauti atlikus deceliulizaciją originaliais metodais.

Jau šiuo metu sukurti metodai patentuotini, nes yra originalūs. Esant teigiamiems ikiklinikiniams tyrimams, tikėtina naujoves įdiegti, kuriant vožtuvus įprastinėms vožtuvų protezavimo operacijoms ar perkateterinėms technologijoms.

3. Tyrimų metodikos

Moksliniam eksperimentiniam darbui gauti Bioetikos komiteto leidimai (2007-07-12 Nr. 0159 ir 2009-10-14 Nr. 0193).

Eksperimentiniai modeliai sukurti taip, kad tiriamos medžiagos būtų išbandomos tiek in vitro, tiek in vivo, nuosekliai, eksperimentai suplanuoti ir atlikti nuosekliai didinant eksperimento apimtį.

1. Cheminių medžiagų parinkimas ir aortos vožtuvo transplantatų apdorojimo jomis protokolų sukūrimas.

Eksperimento metu naudotos vienos lyties 20–30 kg kiaulės, kurios slopintos ir užmigdytos naudojant benzodiazepinus, po to sukelta analgezija ir anestezija, atlikta heparinizacija (200vv/kg) bei eutanazija. Steriliomis sąlygomis išpreparuoti aortos ir plaučių arterijos vožtuviniai transplantatai. Apdorojimui pasirinktos cheminės medžiagos: glutaraldehidas, L-alaninas (Ala), L-norvalinas (Norv), karbodiimidas (EDC) su priedais.

Apdorojimas su Glu truko 24 valandas, po ko audiniai buvo skalaujami šaltame fiziologiniame tirpale. Alanino ir norvalino grupėse pradinis audinių apdorojimas su Glu truko 24 valandas, po skalavimo šaltame fiziologiniame tirpale papildomai 24 valandas apdorota atitinkamai Ala ir Norv medžiagomis nuolatinio judėjimo sąlygomis. Paskutinėje grupėje pradinis apdorojimas EDC su NHS truko 24 valandas, taip pat naudotas skalavimas šaltu fiziologiniu skysčiu, po to 3 valandas skalauta jeffamine ir dar 24 valandas su Ala 100 mM pH 7,6 su fosfatiniu buferiu. Apdorojus cheminėmis medžiagomis audinius, pastarieji buvo laikomi šaltai 4ºC fiziologiniame

tirpale iki 24 valandų prieš implantavimą.

2. Cheminio apdorojimo efektyvumo patikrinimas eksperimentiniame modelyje be sąlyčio su kraujotakos sistema (su smulkiaisiais eksperimentiniais gyvūnais). (tik aortos vožtuvinio transplantato).

Eksperimentui naudotos Wistar veislės žiurkės vienos lyties, 150–200 g svorio. Nejautra sukeliama į pilvo ertmę suleidžiant ketamino ir 0,1 g reflino infekcijos profilaktikai. Įvertinus nejautrą, nukirpti nugaros plaukai ir odos paviršius apdorotas antiseptiku. Atlikta po 3 odos pjūvius abipus stuburo, buku būdu išpreparuoti paodžio audiniai, paruošiant apie 1 cm^2 guolį. Anksčiau apdoroti audiniai (kaip aprašyta anksčiau) išimti iš laikymo tirpalų bei praplauti fiziologiniu skysčiu. Iš kiekvienos anatominės srities (vožtuvo, kraujagyslės sienelės) iškirptas 1 cm^2 gabalėlis, kuris įdėtas į paruoštą guolį. Oda ir paodis užsiūti pavienėmis adaptacinėmis 4/0 proleno siūlėmis.

Po 60 dienų atlikta pakartotinė operacija, kurios metu sukelta nejautra anksčiau minėtu būdu, po to atlikta eutanazija, išimti anksčiau implantuoti audinių gabalėliai.

Prieš atliekant liekamojo kalcio kiekio nustatymą, audinių gabalėliai laikyti sausai 24 valandas, tyrimui naudotas Hitachi 175-50 (Japonija) spektrografas. Kalcio kiekis matuotas mg/g sausos audinio masės.

Gauti rezultatai apdoroti statistikos programa SPSS, palyginti gautieji vidurkiai, naudojant Stjudento t-metodą ir ANOVA, kai $p<0,05$, manoma, kad vidurkiai skiriasi statistiškai patikimai.

3. Cheminio apdorojimo efektyvumo patikrinimas eksperimentiniame modelyje esant tiesioginiam sąlyčiui su kraujotakos sistema (su stambiaisiais eksperimentiniais gyvūnais (tik aortos vožtuvinio transplantato).

Eksperimente naudoti vožtuviniai transplantatai, apdoroti cheminėmis medžiagomis, kaip aprašyta anksčiau.

Eksperimente naudoti paršeliai 20–30 kg. Premedikacijai suleista diazepamo 5 mg/kg ir ketamino 10 mg/kg. Išpunktuotos, kaniuliuotos ausies vena ir arterija, stebėtas arterinis kraujo spaudimas, elektrokardiograma. Anestezijos indukcijai suleistas fentanilio 0,005mg/kg, propofolio 2,5mg/kg, tracrium. Atlikta endotrachėjinė intubacija, pradėta dirbtinė plaučių ventiliacija oru, palaikant normalius kraujo dujų rodiklius. Anestezija palaikyta isofluranu, fentaniliu, propofoliu. Infekcijos profilaktikai skirtas cefazolinas 1g operacijos pradžioje ir pooperaciniu periodu.

Operacinis plotas paruoštas su antiseptiku, padengtas steriliais apklotais. Žemiau šonkaulių lanko iš kairės pusės atliktas apie 10 cm odos pjūvis, išpreparuoti paodis ir raumenys iki pilvaplėvės. Pastaroji buku būdu atskirta nuo retroperitoninio tarpo, kur išpreparuota aorta žemiau a. renalis sin., heparinizuota (200 vv/kg), aorta perspausta aortiniais spaustukais žemiau inkstų arterijų ir perkirpta. Dviem ištisinėmis 4/0 proleno siūlėmis įsiūtas vožtuvinis transplantatas, audiniai susiūti pasluoksniui. Gyvuliukai stebėti, ekstubuoti ir pervežti į vivariumą. Tolimesnis gydymas ir stebėjimas tęstas vivariume 60 dienų. Po numatyto laiko gyvuliukams atlikta pakartotinė operacija. Išpreparuota aorta ties vožtuvinio transplantato įsiuvimo vieta, heparinizuota (200 vv/kg) (6 pav.).

Matuotas tiesioginis spaudimas iki transplantato ir už jo, apskaičiuotas spaudimu

gradientas. Atlikta eutanazija ir iškirptas implantatas. Audiniai iškirpti pagal anatomines sritis ir padalinti pusiau. Vienoje pusėje nustatinėtas liekamojo kalcio kiekis, kita audinių pusė, skirta histologiniam ištyrimui. Liekamojo kalcio kiekio vizualizavimui dažyta von Kossa būdu, kur kalcis matomas juodų taškelių pavidalu. Gauti rezultatai apdoroti statistikos programa SPSS, palyginti gautieji vidurkiai, naudojant Stjudento t-metodą ir ANOVA, kai p<0,05, manoma, kad vidurkiai skiriasi statistiškai patikimai.

4. Aortos ir plaučių arterijos vožtuvinių transplantatų apdorojimo detergentais ir kitomis medžiagomis protokolų sukūrimas ir taikymas in vitro, pašalinant ląstelines struktūras (deceliulizacija)

Eksperimentui naudotos 30–35 kg kiaulės, kiekvienam protokolui išbandyti naudoti 3 skirtingų kiaulių aortos ir plaučių arterijos vožtuviniai konduitai. Gyvunėliai užmigdyti ir atlikta eutanazija. Steriliomis sąlygomis išpreparuoti aortos ir plaučių arterijos vožtuviniai transplantatai. Tiriami transplantatai prifiksuojami prie specialios membranos, kuri sukasi 60 k/min greičiu pagal laikrodžio rodyklę taip, kad vožtuvo burės laisvai juda. Indas pagal poreikį dedamas į termostatą arba į šaldytuvą.

Deceliulizacijos efektui pasiekti naudojamos medžiagos: detergentai (Na deoksicholinė rūgštis, Na dodecyl sulfatas, triton-X-100, CHAPS), fermentai (tripsinas, DNRaze ir RNRaze), įvairios koncentracijos tirpalai ir buferinės terpės.

Deceliulizacijos efektui gauti mūsų tyrime naudoti 8 protokolai, vieni iš jų anksčiau aprašyti kitų autorių, šiek tiek patobulinti, kiti originalūs. Deceliulizacijos protokolai:

1. *0,1% natrio deoksiholinė rūgštis, 0,1% natrio dodecilsulfatas;*
2. *0,1% natrio deoksiholinė rūgštis, 0.25% tert-octylphenyl-polyoksietilenas (triton X-100);*
3. *0.5% tripsinas, 0.2% etilendiamintetracto rūgštis (EDTA);*
4. *0.05% tripsinas, 0.02% etilendiamintetracto rūgštis (EDTA);*
5. *0,45% hipotoninis tirpalas, 0,5% natrio deoksiholinė rūgštis, 0,1% natrio dodecilsulfatas, 0.25% tert-octylphenyl-polyoksietilenas (triton X-100);*
6. *0,45% hipotoninis tirpalas, 0,1% natrio deoksiholinė rūgštis, 0.25% tert-octylphenyl-polyoksietilenas (triton X-100) su 0.2% EDTA;*
7. *0,45% hipotoninis tirpalas, 0,1% natrio deoksiholinė rūgštis, 0,1% natrio dodecilsulfatas, 0.2% EDTA;*
8. *1% CHAPS, 0.25% tert-octylphenyl-polyoksietilenas (triton X-100).*

In vitro įvertinime transplantatai nelaikyti mitybinėje terpėje, po apdorojimo vizualiai įvertinta audinių kokybė, elastingumas, vožtuvo sandarumas, po to nusiųsti histologiniam tyrimui, ląstelinių struktūrų pašalinimo efektyvumui bei elastinių skaidulų būklei įvertinti.

Vertinant bendrą audinio būklę, branduolių buvimą audiniuose, dažyta hematoksilinu/eozinu, papildomai vertinant elastines skaidulas dažyta van Gieson būdu.

5 Nuląstelintų (deceliulizuotų) vožtuvinių transplantatų patikrinimas eksperimentiniame modelyje in vivo.

Eksperimentui pasirinktas nuląstelinimo būdas, su pilnu deceliulizacijos efektu.

Imituoti ksenotransplantato įsiuvimo modelį recipientais pasirinktos avys, kaip kitos rūšies gyvūnai, 20–25 kg. Išpunktuota ausies vena ir suleidus diazepamo 5 mg/kg ir ketamino 10 mg/kg sukeltas slopinimas. Anestezijos indukcijai suleista fentanilio 0,005 mg/kg, propofolio 2,5 mg/kg, tracriumo, atlikta intubacija pradėta dirbtinė plaučių

ventiliacija deguonies ir isoflurano mišiniu. Išpunktuota v. jugularis, tolimesnė nejautra palaikyta fentaniliu, propofoliu. Punktuota ausies arba kojos arterija, invaziniam spaudimui sekti prijungti elektrokardiogramos elektrodai bei įvestas į nosiaryklę temperatūros matavimo zondas.

Operacinis plotas nuskustas bei paruoštas su antiseptiku, padengtas steriliais apklotais. 3-ame tarpšonkauliniame tarpe kairėje pusėje atlikta torakotomija, audiniai išpreparuoti naudojant elektrokoaguliaciją. Išpreparuotas aortos lankas, krūtininė nusileidžiančioji aorta su diafragmos nervais. Heparinizuota (200 vv/kg) aortos lanke įsiuvus dvi tabokinės siūles 5/0 prolenu, įkišta tiesi 14Fr arterinė kaniulė, prijungta prie dirbtinio kraujo apytakos (DKA) aparato. Dešinysis prieširdis apsiūtas 3/0 prolenu tabokinės siūle, įkišta dviejų spindžių veninė kaniulė, prijungus prie DKA, pradėta dirbtinė apytaka. Plaučių arterijos operacijos atliktos normotermijoje (37–37,5ºC), perfuzijos greitis 2–2,5 l/min/m^2.

Pplaučių arterija perkirpta skersai apie 2 cm distaliau PA vožtuvo žiedo, pašalintas natyvinis vožtuvas , ištisine 4/0 proleno siūle suformuota proksimalinė anastomozė, po to sutrumpinus ir pritaikius kraujagyslių galus, ištisine 4/0 proleno siūle suformuota distalinė anastomozė. Sustabdžius DKA, suleistas protamino sulfatas. Įsitikinus, kad nėra kraujavimo, drenuota kairė pleuros ertmė, audiniai užsiūti pasluoksniui. Gyvuliukai stebėti, pašalintas drenas. Pirmąsias paras laikytas intensyvaus sekimo kambaryje užtikrinant nuskausminimą, stebint ir skatinant diurezę, arterinį spaudimą. Tolimesnis gydymas ir stebėjimas tęstas vivariume 45–60 dienų.

Po numatyto laiko gyvuliukams atlikta pakartotinė operacija. Išpreparuota širdis, plaučių arterija ties vožtuvinio transplantato įsiuvimo vieta, atlikta epikardinė echoskopija, įvertinta kraujotaka per vožtuvą, spaudimų gradientas. Atlikta eutanazija ir iškirptas implantatas. Audiniai iškirpti pagal anatomines sritis ir, kaip aprašyta anksčiau, tirti histologiškai bei nustatinėtas liekamojo kalcio kiekis.

4. Rezultatai

1. Cheminių medžiagų parinkimas ir aortos vožtuvo transplantatų apdorojimo jomis protokolų sukūrimas.

Tiriami audiniai apdoroti chemikalais pagal numatytus protokolus, paruošti naudojimui. Histologinis tyrimas nedarytas nesitikint jokių struktūrinių pakitimų. Vizualiai audiniai pakitę, pakeitę spalvą, truputį rigidiški. Apžiūrint vožtuvą, pakitimų nepastebėta.

2. Cheminio apdorojimo efektyvumo patikrinimas eksperimentiniame modelyje be sąlyčio su kraujotakos sistema (su smulkiaisiais eksperimentiniais gyvūnais). (tik aortos vožtuvinio transplantato)

Iš viso atlikta 120 eksperimentų. Kiekvienoje chemikalų grupėje buvo atlikta 20 eksperimentų, kiekvieno metu implantuojant po 2 tos pačios tiriamosios medžiagos gabaliukus, taigi implantuota po 60 gabalėlių audinio, apdoroto tokiu pat būdu, išskyrus kontrolinę, kur buvo papildomai 60 implantų. Duomenys suskirstyti pagal poveikį AoV ir AoS audiniams bei jie palyginti tarpusavyje skirtingų chemikalų grupėse.

Didžiausia kalcifikacija, kaip ir tikėtasi, yra kontrolinėje grupėje be jokio specialaus apdorojimo. Glu žymiai sumažina kalcio kiekį, tačiau apdorojant audinius tolimesniuose etapuose papildomai pridedant amino rūgščių, kalcifikavimas netgi padidėjo. Mažiausias liekamojo kalcio kiekis rastas EDC grupėje – 0,6 mg/g. Visi duomenys skiriasi statistiškai patikimai, išskyrus vidurkių skirtumą tarp amino rūgščių, kur p>0,05.

Tame pačiame eksperimentiniame modelyje, implantavus aortos sienelę į paodį, pastebėtas kiek mažesnis liekamojo kalcio kiekis. Ryškiausia kalcifikacija stebėta kontrolinėje grupėje, kur transplantatai neapdoroti chemikalais. Glu žymiai sumažina kalcio kiekį, panašiai kaip ir aortos vožtuvo atveju, taip ir čia, Ala ir Norv papildomai pridėjus apdorojant audinius, rezultatas gautas blogesnis. Vienintelis ir geriausias apdorojimo būdas su EDC turėjo mažiausiai kalcio audiniuose – 8,3 mg/g. Visų grupių vidurkiai skyrėsi statistiškai patikimai, išskyrus amino rūgščių grupes, kur p>0,05.

Lyginant liekamojo kalcio kiekį tarp aortos vožtuvo ir sienelės skirtingose cheminių medžiagų grupėse, matome dėsningumus. Abiejuose mėginiuose kalcifikavimas kito panašiai. Tačiau žymiai mažesnis pakitimų lygmuo matomas aortos sienos audiniuose, išskyrus EDC grupę, kur vožtuvo kalcifikavimo lygmuo daugiau nei 10 kartų mažesnis už aortos sienos. Lyginant vidurkius tarp audinių grupėse, matome, kad tik amino rūgščių rodikliai nesiskyrė statistiškai patikimai.

3. Cheminio apdorojimo efektyvumo patikrinimas eksperimentiniame modelyje esant tiesioginiam sąlyčiui su kraujotakos sistema (su stambiaisiais eksperimentiniais gyvūnais (tik aortos vožtuvinio transplantato).

Atlikta 20 eksperimentų. Kiekvienoje cheminės medžiagos grupėje po 4 implantus, iš kiekvieno konduito paimti po 2 sienelės ir burių mėginiai kalcio kiekiui nustatyti, vienas – histologiniam tyrimui. Išpreparavus audinius, heparinizavus pirmiausia atliktas tiesioginis vidutinių spaudimų matavimas iki ir už transplantato įsiuvimo vietos, apskaičiuotas spaudimų skirtumas – vidutinis gradientas. Aortos diametras šioje vietoje apie 1,5 cm.

Tiek kontrolinėje, tiek Glu grupėse susidarė didžiausi vidutiniai gradientai, tai leidžia daryti prielaidą apie ryškiausią kalcifikaciją ir aortos spindžio siaurėjimą. Likusiose grupėse gauti spaudimų skirtumai beveik lygūs, tačiau tai nėra tiesioginis įrodymas, jog nėra spindžio stenozės.

Kitame etape, atlikus eutanaziją, iškirpti transplantatai, išilgai perpjauti ir išskleisti detalesniam apžiūrėjimui. Audiniai iškirpti pagal anatomines sritis, padalinti pusiau. Viena pusė skirta histologiniam, kita – liekamojo kalcio kiekio tyrimui. Histologinio tyrimo metu taikytas von Kossa dažymas, kuris parodo kalcio intarpus kaip juodus taškus. Mikroskopuota šviesos mikroskopu 40 kartų didinimu.

<u>Kontrolinė grupė:</u> Matoma labai pakitusią transplantato sienelę, pritrauktas, suragėjusias, nejudrias bures, kurios kalcinuodamos traukiasi uždarydamos spindį. Histologinio tyrimo vaizduose matyti kalcio intarpų (juodi taškeliai) pilna ir AoS, ir AoV visame plotyje: tiek paviršiuje, tiek viduriniame sluoksnyje. Abu vaizdai patvirtina didelės apimties kalcifikaciją.

<u>Glutaraldehido grupė :</u> vožtuvinio transplantato sienelės pakitusios, šiurkščios, rigidiškos, burės vos judančios, sustorėjusios. Histologiškai kalcio intarpų mažiau, AoV kalcinatai

susikaupę labiau viduriniame sluoksnyje, kai tuo tarpu AoS -– atvirkščiai. Kalkėjimas labai žymus paviršiniuose sluoksniuose, giliai media išlieka normali galbūt dėl ribotos rezorbcijos po apdorojimo.

Glutaraldehido ir L-alanino grupė: preparato burės visiškai patrauktos ir užsilenkusios į vidų, neointima užaugusi iš abiejų pusių, uždengusi ir bures. Histologiškai burės išorinis sluoksnis kalcio intarpų neturi, tuo tarpu vidurinis sluoksnis gausiai sukaupęs kalcio. Galima preliminariai teigti, kad šis apdorojimo tirpalas bures veikia labiau paviršutiniškai, o AoS priešingai – vidinėje dalyje kalcio nėra, tik paviršiuje.

Glutaraldehido ir L-norvalino grupė: makro- ir mikrovaizdai panašūs į Ala grupę. Makroskopiškai labai pakitusi sienelė, burės pritrauktos, suirusios, nefunkciuonuoja, mikroskopiškai – kalcis susikaupė burės vidiniame sluoksnyje, o AoS – išoriniame.

Karbodiimido, N-hidroksisukciimido, poli(propileno glikol)bis 2-(aminopropil) eterio ir L-alanino grupė: audiniai nepakitę, lankstūs, elastingi, burės permatomos, judrios, vientisos. Mikroskopijoje nedideli kalcio kiekiai paviršiniuose sluoksniuose tiek AoV, tiek AoS.

Lyginant liekamojo kalcio kiekį transplantato burėse su sienoje, skirtumas beveik dvigubas, burės turėjo tiesioginį kraujotakos smūginį poveikį, didesnis stresas audiniams.

Liekamojo kalcio kiekis Glu grupėje žymiai mažesnis, tačiau pakitimai taip pat ryškūs ir koreliuojantys tarpusavyje. Sukaupto kalcio kiekis atspindi transplantato disfunkciją. Lyginant AoV su AoS, burėse vėlgi kalcifikavimas didesnis.

Ala ir Norv grupėse, kaip ir iki tol darytuose kitų eksperimentų tyrimuose, vyrauja kalcifikavimas. Kalcio sankaupos labai pažeidė struktūras, galima teigti, kad Ao spindžio stenozavimas buvo abiem atvejais reikšmingas. Taip pat abiejose grupėse vidiniai sienelių sluoksniai buvo geriau apsaugoti nei burių.

Susumavus visus duomenis, šiame eksperimentiniame modelyje EDC apdorojimo cheminės medžiagos labiausiai apsaugojo implantatą nuo kalcinavimo, suirimo. Audiniai išlaikė natyvinę struktūrą ir funkciją ir mažiausiai sukaupė kalcio.

Vertinant liekamojo kalcio kiekį tarp audinių skirtingų chemikalų grupėse, statistiškai patikimai nesiskyrė tik amino rūgščių rezultatai, kur $p>0,05$.

Liekamojo kalcio kiekio palyginimas tarp skirtingų eksperimentinių modelių

Aortos vožtuvas labiau kalkėjo kiaulės eksperimentiniame modelyje. Tai matyti visose chemikalų grupėse. Taip pat pastebimas proporcingas kalcio kiekio mažėjimas keičiantis apdorojimo protokolui. Blogiausi rezultatai išlieka kontrolinėje ir Glu apdorojimo grupėse, mažiausiai kalcio intarpų sukaupė EDC.

Vertinant skirtumus aortos sienoje, pastebimos tos pačios tendencijos, tačiau kalcifikavimo lygmuo beveik per pusę mažesnis kai kuriose grupėse. Žiurkių eksperimentiniame modelyje kalcio kiekiai reikšmingai mažesni vertinant abu audinių mėginius.

4. Aortos ir plaučių arterijos vožtuvinių transplantatų apdorojimo detergentais ir kitomis medžiagomis protokolų sukūrimas ir taikymas in vitro, pašalinant ląstelines struktūras (deceliulizacija).

Eksperimentai atlikti tiriant 8 skirtingus deceliulizacijos protokolus. Iš jų 6 originalūs, 2 atkartoti, norint įsitikinti fermentų efektyvumu. Kiekvienam protokolui naudoti

3 aortos ir 3 plaučių arterijos vožtuviniai konduitai. Tiriamoji medžiaga buvo paimta iš skirtingų kiaulių. Trumpai apžvelgsime kiekvieno protokolo rezultatus vadovaujantis histologinių tyrimų duomenimis.

I protokolas: **0,1% DEO + 0,1% SDS:** *naudoti du joniniai detergentai, kurių koncentracijos vienodos. Tai originalus metodas. Gauti vienas kitą papildantys poveikiai, kada efektyviai suardomos visos struktūros. Audinių pavyzdžiai rodo efektyvią deceliulizaciją beveik visuose audiniuose, išskyrus aortos sieną. Aortos sienoje deceliulizacijos pėdsakai matomi iki vidurinio sluoksnio vidurio, toliau einant išorės link matomi branduoliai (melsvi). Pakankamai storas aortos sienos audinys turėtų būti apdorojamas arba ilgiau, arba agresyviau.*

II protokolas: **0,1% DEO + 0,25% Triton 100:** *naudoti detergentai iš skirtingų grupių (joninis ir nejoninis), deceliulizacija neefektyvi. Medžiagų koncentracijos nedidelės ir nejoninis triton-100 turėjo ilgiau veikiant švelniai papildyti prieš tai agresyviai ardžiusį DEO. Visuose pavyzdžiuose matomi branduoliai (mėlyni), tikėtina, kad ir kitos ląstelinės struktūros nevisai pašalintos.*

III protokolas: **0,5% Trypsin + 0,2% EDTA:** *kombinacijoje naudojamas chelatas ir fermentas. Tripsinas naudojamas nedidelės koncentracijos, tačiau per ilgą laiko tarpą suardo audinio baltymines jungtis, visai nedaug pakeisdamas ląstelių struktūrų sudėtį. Deceliulizacija neįvyko nė viename pavyzdyje, o audiniai pakenkti stipriai, gana gerai matyti fragmentavimasis. Šio proceso metu, kaip ir kituose protokoluose, naudotas ištisinis plovimas, kuris šiuo atveju nepašalino ląstelių, o tikriausiai priešingai – sulaikė fermentą audiniuose. Todėl poveikis labai paviršutinis, tik pačiuose vidiniuose endotelio sluoksniuose sienelėse, o vožtuvuose – visai nėra poveikio.*

IV protokolas: **0,05% Trypsin + 0,02% EDTA:** *protokolas analogiškas prieš tai buvusiajam, tik 10 kartų mažesnės medžiagų koncentracijos, o pati procedūra lygiai tokia pati. Paviršutinis poveikis į plaučių arterijos sienelę, kur audinių sluoksniai plonesni, tuo tarpu kiti pavyzdžiai lieka nepaveikti – deceliulizacija neįvyko. Audiniai, kaip ir prieš tai, pakenkti, fragmentuojasi. Vadovaujantis histogramomis, galima teigti, kad fermentinis apdorojimas su chelatais norimo efekto neduoda, o tik labiau žaloja pačius audinius.*

V protokolas: **Hipoton.+0,5%DEO+0,1%SDS+0,25%Triton-100:** *originalus apdorojimo protokolas pasižymi įvairių medžiagų gausa, kurios skiriamos nedidelėmis koncentracijomis ir veikia pakaitomis. Iš principo, tai antrojo protokolo patobulinimas, didinant DEO koncentraciją, naudojant hipotoninį tirpalą ir triton-100 su SDS. Sudaromos sąlygos ardyti ląstelių membranas, taip palengvinant visapusišką detergentų poveikį. Audinių histogramose matomas poveikis, visiška deceliulizacija įvyko tik plaučių arterijos sienelėje, vožtuvų burėse. Nors ląstelių struktūriniai elementai smarkiai paveikti, tačiau nepakankamai gerai išplauti iš audinio gilumos. Aortos storesnė sienelė efektyviausiai paveikta tik iki vidurio, toliau matomi nesužaloti branduoliai. Šis metodas efektą davė, tačiau nepakankamą, tai galėtų būti arba dėl per mažos koncentracijos, arba dėl per trumpos apdorojimo trukmės.*

VI protokolas: **Hipoton.+0,1% DEO+0,25% Triton:** *originalus protokolas – tai antrojo ir penktojo patobulinimas, nuo paskutinio pašalinant SDS. Tai atlikta siekiant sušvelninti žalojantį detergentų poveikį organoidams. Tačiau rezultatas neigiamas, visuose audinių pavyzdžiuose gausu nepakenktų branduolių, deceliulizacija tik paviršinė. Tikintis*

geresnio efekto, reikalinga ilgesnė ekspozicija detergentais ir didesnė jų koncentracija.

VII protokolas: **Hipoton+0,1% DEO + 0,1% SDS:** *originalus audinių deceliulizacijos protokolas – tai pirmojo patobulinimas, įvedant hipotoninį tirpalą, chelatą. SDS ir DEO lieka tomis pačiomis koncentracijomis. Tai efektyviausias iš visų protokolas, visuose pavyzdžiuose visiška deceliulizacija, branduolių nematyti, gal kiek blogiau išplauti organoidai iš aortos vožtuvo, tačiau tendencija teigiama. Teorinis tokio efekto pagrindas – subalansuotas poveikis audiniams, iš pradžių ardant išorines membranas, o toliau ir organoidus. Darome prielaidą, kad papildomai veikiant DNR-azei ir RNR-azei, visiškai suardyta tiek branduolinė, tiek nebranduolinė DNR ir RNR. Tai labai svarbu atokiam rezultatui, kalcifikacijos prevencijai.*

VIII protokolas: **1% CHAPS + 0,25% Triton 100:** *originalus, neatkartojantis nė vieno iki tol buvusio. Naudojamas mišrus detergentas su švelnaus poveikio nejoniniu. Deja, norimas efektas nepasiektas, deceliulizacija nė viename audinio pavyzdyje neįvyko. Matomi mėlyni branduoliai. Manome, kad tai lemia per švelnus ir tausojantis poveikis audiniams.*

Įvertinę visus audinių apdorojimo protokolus, matome, kad efektyviausiai veikia joninių detergentų ir kitų medžiagų kombinacijos. Deceliulizaciją geriausiai pavyksta plaučių arterijos audiniuose.

Septintojo protokolo audiniai, nepaisant visiškos deceliulizacijos, turi būti vertinami skaidulų elastingumo atžvilgiu. Neturėdami galimybės tirti kolageno skaidulas, kaip ekvivalentą tyrėme elastines skaidulas. 7-o protokolo histogramose vientisos liniuotos elastines skaidulas, beveik visos išsidėsčiusios viena kryptimi. Tuo tarpu lygindami su 2-u protokolu, kur neįvyko deceliulizacija, elastines skaidulas pakenktos, fragmentacija.

5 Nuląstelintų (deceliulizuotų) vožtuvinių transplantatų patikrinimas eksperimentiniame modelyje in vivo.

Remiantis anksčiau gautais cheminio apdorojimo ir deceliulizacijos rezultatais, toliau atlikti eksperimentai in vivo, naudojant vožtuvinius konduitus, apdorotus EDC ir 7-u protokolu.

Atlikti 9 eksperimentai, po 3 implantacijas kiekvienoje grupėje, EDC apdoroti tik aortos vožtuviniai konduitai. Eksperimento metu naudota DKA, vidutinis DKA laikas 42,8±7,7 minutės. Eksperimentiniai gyvūnai, kurie turėjo refrakterinį širdies silpnumą ar plautinę hipertenziją ir dėl to eksperimento metu ar po jo nugaišo – neįtraukti.

45 dienos po implantacijos įvertintas liekamojo kalcio kiekis, echoskopiškai įvertinta kraujotaka per transplantatą, makroskopinis ir mikroskopinis vaizdai.

Operacijų metu prieš eksplantaciją buvo atliekamos epikardinės echoskopijos, vertinama vožtuvo funkcija, vidutinis spaudimo gradientas per vožtuvą. Rezultatai parodė, jog visų trijų grupių vožtuviniai implantai turėjo nedidelį, fiziologiškai priimtiną gradientą. Kiek mažesni skaičiai buvo deceliulizuotų vožtuvų. Tačiau vertinant liekamojo kalcio kiekį, skirtumai daug didesni, deceliulizuotų vožtuvų liekamojo kalcio kiekis mažesnis net 30 ar 60 kartų nei apdoroto EDC. Vertinant morfologiškai, deceliulizuotas vožtuvas po implantacijos niekuo nesiskiria nuo natyvinio. Burės plonos, permatomos, be kalcifikavimo ar irimo židinių. Sienelė elastinga, normalios spalvos, be kalcinatų. Histogramose, ieškant liekamojo kalcio

intarpų, nematyti jokių tamsių dėmių, kaip tai buvo cheminio apdorojimo metu, kur net EDC grupėje liekamojo kalcio žymių buvo.

Eksperimentiniame in vivo modelyje lyginant skirtingus apdorojimo metodus, geresnius rezultatus pavyko gauti deceliulizavus vožtuvinius konduitus. O lyginant deceliulizuotus aortos ir plaučių arterijos implantus, geresni rezultatai plaučių arterijos konduitų.

5. Išvados

1. *Atlikus ksenotransplantatų apdorojimą cheminėmis medžiagomis, mažiausias kalkėjimas gautas apdorojant karbodiimidu eksperimentuose ir su mažais, ir su dideliais gyvūnais.*

2. *Ksenotransplantatų deceliulizacija gauta naudojant detergentų ir kitų medžiagų kombinaciją (0,45% hipotoninis NaCl tirpalas su 0,1% DEO, 0,1% SDS ir 0,2% EDTA). Visi kiti apruošimo protokolai davė nepakankamą deceliulizaciją arba buvo neefektingi.*

3. *Deceliulizacijos būdu apdoroti vožtuviniai transplantatai kalcį kaupia mažiau nei kitais būdais paruošti.*

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