SEZNAM PŘÍLOH

PŘÍLOHA 1 a-h

Přehled hodnot Spearmanova korelačního koeficientu určujícího vztah mezi délkou kryoprezervace a mechanickými a strukturálními vlastnostmi pro jednotlivé vzorky aortálních a pulmonálních alograftů srdečních chlopní (AASCH).

PŘÍLOHA 2

Fulltext článku:

Fiala R., Kochová P., Kubíková T., Cimrman R., Tonar Z., Špatenka J., Fabián O., Burkert J. (2019) **Mechanical and structural properties of human aortic and pulmonary allografts do not deteriorate in the first 10 years of cryopreservation and storage in nitrogen**, *Cell Tissue Bank*. 2019 Mar 22. doi: 10.1007/s10561-019-09762-x. [Epub ahead of print]

IF 1,527 / 2019

PŘÍLOHA 3

Fulltext článku:

Novotný R., Slížová D., Hlubocký J., Krs O., Špatenka J., Burkert J., Fiala R., Mitáš P., Měřička P., Špaček M., Hlubocká Z., Lindner J. (2018) **Structural changes arising from different thawing protocols on cryopreserved human allograft's aortic valve leaflets**, *Advances in Clinical and Experimental Medicine*, 2018, vol. 27(8), s. 1033-1036.

IF 1,262 / 2018

PŘÍLOHA 4

Fulltext článku:

Kubíková T., Kochová P., Fiala R., Špatenka J., Burkert J., Králíčková M., Tonar Z. (2016) **Histological Composition and Mechanical Properties of Cryopreserved Samples of Aortic and Pulmonary Valves**. *Solid State Phenomena,* 2016, 258**,** 341-344.

PŘÍLOHA 1a

Spearmanův korelační koeficient určující vztah mezi délkou kryoprezervace a mechanickými a strukturálními vlastnostmi vzorků stěny aortálních alograftů srdečních chlopní (AASCH). E_0 a E_1 – Youngův modul elasticity v malých, resp. velkých deformacích; MT – tloušťka stěny, A_A – plošný podíl v referenční ploše vzorku. Barevně označeny významné korelace (p<0,05).

AASCH stěna	věk (roky)	pohlaví	kryo (roky)	E_0 (MPa)	E_1 (MPa)	mezní deformace	mezní napětí	MT (µm)	A_A (elastin)	A_A (kolagen I)
věk (roky)	1.000000	-0.040671	-0.099272	-0.029375	-0.054305	**-0.415899**	-0.087869	**-0.353632**	**-0.316216**	0.264709
pohlaví	-0.040671	1.000000	0.055301	0.179880	0.240807	0.133460	0.220498	0.034919	0.187905	-0.094796
kryo (roky)	-0.099272	0.055301	1.000000	**-0.477699**	**-0.295945**	0.015765	**-0.312017**	0.172407	-0.047311	-0.102773
E_0 (MPa)	-0.029375	0.179880	**-0.477699**	1.000000	**0.765612**	0.119898	**0.708367**	-0.043848	0.008079	-0.083701
E_1 (MPa)	-0.054305	0.240807	**-0.295945**	**0.765612**	1.000000	0.197959	**0.851735**	-0.142152	0.016528	0.033986
mezní deformace	**-0.415899**	0.133460	0.015765	0.119898	0.197959	1.000000	**0.529490**	0.041998	0.106016	0.073770
mezní napětí	-0.087869	0.220498	**-0.312017**	**0.708367**	**0.851735**	**0.529490**	1.000000	-0.205550	0.017022	0.043485
MT (µm)	**-0.353632**	0.034919	0.172407	-0.043848	-0.142152	0.041998	-0.205550	1.000000	0.209812	**-0.430779**
A_A (elastin)	**-0.316216**	0.187905	-0.047311	0.008079	0.016528	0.106016	0.017022	0.209812	1.000000	**-0.439798**
A_A (kolagen I)	0.264709	-0.094796	-0.102773	-0.083701	0.033986	0.073770	0.043485	**-0.430779**	**-0.439798**	1.000000

PŘÍLOHA 1b

Spearmanův korelační koeficient určující vztah mezi délkou kryoprezervace a mechanickými a strukturálními vlastnostmi vzorků cípů aortálních alograftů srdečních chlopní (AASCH)., E_0 a E_1 – Youngův modul elasticity v malých, resp. velkých deformacích; MT – tloušťka stěny, A_A – plošný podíl v referenční ploše vzorku. Barevně označeny významné korelace (p<0,05).

AASCH cíp	věk (roky)	pohlaví	kryo (roky)	E_0 (MPa)	E_1 (MPa)	mezní deformace	mezní napětí	MT (µm)	A_A (elastin)	A_A (kolagen I)
věk (roky)	1.000000	0.140319	-0.242532	-0.142143	**-0.332011**	-0.114550	**-0.425053**	-0.030556	-0.097910	**-0.264418**
pohlaví	0.140319	1.000000	0.021155	**0.259357**	0.061477	0.069162	0.138323	-0.163299	0.167141	0.026896
kryo (roky)	-0.242532	0.021155	1.000000	**-0.295474**	**-0.265908**	0.204288	-0.133695	0.024113	0.157729	**0.276099**
E_0 (MPa)	-0.142143	**0.259357**	**-0.295474**	1.000000	**0.531782**	**-0.294659**	**0.494870**	-0.036594	0.163035	-0.026494
E_1 (MPa)	**-0.332011**	0.061477	**-0.265908**	**0.531782**	1.000000	-0.247118	**0.805553**	-0.097039	-0.066790	-0.092173
mezní deformace	-0.114550	0.069162	0.204288	**-0.294659**	-0.247118	1.000000	**0.270915**	0.107192	**0.293760**	**0.479429**
mezní napětí	**-0.425053**	0.138323	-0.133695	**0.494870**	**0.805553**	**0.270915**	1.000000	0.059545	0.190534	**0.273083**
MT (µm)	-0.030556	-0.163299	0.024113	-0.036594	-0.097039	0.107192	0.059545	1.000000	0.031994	0.215918
A_A (elastin)	-0.097910	0.167141	0.157729	0.163035	-0.066790	**0.293760**	0.190534	0.031994	1.000000	**0.313749**
A_A (kolagen I)	**-0.264418**	0.026896	**0.276099**	-0.026494	-0.092173	**0.479429**	**0.273083**	0.215918	**0.313749**	1.000000

PŘÍLOHA 1c

Spearmanův korelační koeficient určující vztah mezi délkou kryoprezervace a mechanickými a strukturálními vlastnostmi vzorků ventrikulo-arteriální (V-A) junkce aortálních alograftů srdečních chlopní (AASCH)., E_0 a E_1 – Youngův modul elasticity v malých, resp. velkých deformacích; MT – tloušťka stěny, A_A – plošný podíl v referenční ploše vzorku. Barevně označeny významné korelace (p<0,05).

AASCH V-A junkce	věk (roky)	pohlaví	kryo (roky)	E_0 (MPa)	E_1 (MPa)	mezní deformace	mezní napětí	MT (µm)	A_A (elastin)	A_A (kolagen I)
věk (roky)	1.000000	0.040728	-0.245738	-0.021575	-0.084686	-0.184091	-0.109487	-0.310379	**-0.446546**	0.035154
pohlaví	0.040728	1.000000	0.014818	0.185109	0.111065	-0.125874	0.029617	-0.019979	0.203788	0.139854
kryo (roky)	-0.245738	0.014818	1.000000	**-0.530563**	-0.221102	0.217470	-0.067783	-0.097061	0.210819	0.170080
E_0 (MPa)	-0.021575	0.185109	**-0.530563**	1.000000	**0.530645**	**-0.575000**	0.045968	0.030033	-0.059844	-0.231146
E_1 (MPa)	-0.084686	0.111065	-0.221102	**0.530645**	1.000000	-0.320161	**0.453226**	0.024249	-0.068743	-0.146607
mezní deformace	-0.184091	-0.125874	0.217470	**-0.575000**	-0.320161	1.000000	**0.537903**	0.169744	0.066518	0.244494
mezní napětí	-0.109487	0.029617	-0.067783	0.045968	**0.453226**	**0.537903**	1.000000	0.094994	0.020690	0.308565
MT (µm)	-0.310379	-0.019979	-0.097061	0.030033	0.024249	0.169744	0.094994	1.000000	0.181758	-0.217353
AA (elastin)	**-0.446546**	0.203788	0.210819	-0.059844	-0.068743	0.066518	0.020690	0.181758	1.000000	0.038487
AA (kolagen I)	0.035154	0.139854	0.170080	-0.231146	-0.146607	0.244494	0.308565	-0.217353	0.038487	1.000000

PŘÍLOHA 1d

Spearmanův korelační koeficient určující vztah mezi délkou kryoprezervace a mechanickými a strukturálními vlastnostmi vzorků ringů aortálních alograftů srdečních chlopní (AASCH)., E_0 a E_1 – Youngův modul elasticity v malých, resp. velkých deformacích; MT – tloušťka stěny, A_A – plošný podíl v referenční ploše vzorku. Barevně označeny významné korelace (p<0,05).

AASCH ring	věk (roky)	pohlaví	kryo (roky)	E_0 (MPa)	E_1 (MPa)	mezní deformace	mezní napětí	MT (μm)	A_A (elastin)	A_A (kolagen I)
věk (roky)	1.000000	0.139463	-0.234036	0.232685	**0.453272**	**-0.591189**	-0.046577	-0.125416	-0.124004	0.291448
pohlaví	0.139463	1.000000	0.067881	0.143218	0.331662	-0.316587	0.226134	0.195982	-0.052764	0.346843
kryo (roky)	-0.234036	0.067881	1.000000	0.019972	-0.314908	0.288078	0.006859	0.056082	**0.357071**	0.154273
E_0 (MPa)	0.232685	0.143218	0.019972	1.000000	**0.522581**	**-0.472177**	0.278226	**-0.427419**	**0.520565**	**0.483412**
E_1 (MPa)	**0.453272**	0.331662	-0.314908	**0.522581**	1.000000	**-0.813306**	**0.426613**	-0.218548	0.141129	**0.457598**
mezní deformace	**-0.591189**	-0.316587	0.288078	**-0.472177**	**-0.813306**	1.000000	0.079839	0.317339	-0.094355	**-0.419078**
mezní napětí	-0.046577	0.226134	0.006859	0.278226	**0.426613**	0.079839	1.000000	0.171774	-0.000403	0.212564
MT (μm)	-0.125416	0.195982	0.056082	**-0.427419**	-0.218548	0.317339	0.171774	1.000000	**-0.411694**	-0.227690
AA (elastin)	-0.124004	-0.052764	**0.357071**	**0.520565**	0.141129	-0.094355	-0.000403	**-0.411694**	1.000000	**0.361601**
AA (kolagen I)	0.291448	0.346843	0.154273	**0.483412**	**0.457598**	**-0.419078**	0.212564	-0.227690	**0.361601**	1.000000

PŘÍLOHA 1e

Spearmanův korelační koeficient určující vztah mezi délkou kryoprezervace a mechanickými a strukturálními vlastnostmi vzorků stěny pulmonálních alograftů srdečních chlopní (PASCH). E_0 a E_1 – Youngův modul elasticity v malých, resp. velkých deformacích; MT – tloušťka stěny, A_A – plošný podíl v referenční ploše vzorku. Barevně označeny významné korelace (p<0,05).

PASCH stěna	věk (roky)	pohlaví	kryo (roky)	E_0 (MPa)	E_1 (MPa)	mezní deformace	mezní napětí	MT (µm)	A_A (elastin)	A_A (kolagen I)
věk (roky)	1.000000	**0.320459**	-0.153302	0.257312	0.067735	**-0.380332**	-0.097573	**0.401348**	**0.344141**	**-0.282498**
pohlaví	**0.320459**	1.000000	0.117804	**0.525754**	**0.290899**	-0.072058	**0.282893**	0.118953	0.198754	0.192941
kryo (roky)	-0.153302	0.117804	1.000000	0.119522	0.164190	-0.266190	0.104361	-0.060062	**0.310087**	-0.103178
E_0 (MPa)	0.257312	**0.525754**	0.119522	1.000000	**0.418733**	**-0.516923**	0.107783	0.163571	0.241906	-0.086329
E_1 (MPa)	0.067735	**0.290899**	0.164190	**0.418733**	1.000000	**-0.278371**	**0.665611**	0.126122	0.080823	-0.060767
mezní deformace	**-0.380332**	-0.072058	-0.266190	**-0.516923**	**-0.278371**	1.000000	**0.338371**	-0.081020	**-0.337067**	**0.602464**
mezní napětí	-0.097573	**0.282893**	0.104361	0.107783	**0.665611**	**0.338371**	1.000000	0.207755	0.047963	**0.392816**
MT (µm)	**0.401348**	0.118953	-0.060062	0.163571	0.126122	-0.081020	0.207755	1.000000	0.214557	0.031327
AA (elastin)	**0.344141**	0.198754	**0.310087**	0.241906	0.080823	**-0.337067**	0.047963	0.214557	1.000000	-0.049189
AA (kolagen I)	**-0.282498**	0.192941	-0.103178	-0.086329	-0.060767	**0.602464**	**0.392816**	0.031327	-0.049189	1.000000

PŘÍLOHA 1f

Spearmanův korelační koeficient určující vztah mezi délkou kryoprezervace a mechanickými a strukturálními vlastnostmi vzorků cípů pulmonálních alograftů srdečních chlopní (PASCH). E_0 a E_1 – Youngův modul elasticity v malých, resp. velkých deformacích; MT – tloušťka stěny, A_A – plošný podíl v referenční ploše vzorku. Barevně označeny významné korelace (p<0,05).

PASCH cíp	věk (roky)	pohlaví	kryo (roky)	E_0 (MPa)	E_1 (MPa)	mezní deformace	mezní napětí	MT (µm)	A_A (elastin)	A_A (kolagen I)
věk (kryo)	1.000000	**0.343921**	-0.211089	0.110723	-0.040529	**-0.315921**	-0.207783	-0.257917	-0.119093	0.014094
pohlaví	**0.343921**	1.000000	-0.009303	0.178600	0.104269	-0.029939	0.114593	-0.184797	0.250865	**0.399526**
kryo (roky)	-0.211089	-0.009303	1.000000	0.030090	0.109243	0.147063	0.231236	-0.054853	0.109243	0.087437
E_0 (MPa)	0.110723	0.178600	0.030090	1.000000	**0.738472**	**-0.656710**	**0.521979**	-0.153683	-0.203236	-0.114461
E_1 (MPa)	-0.040529	0.104269	0.109243	**0.738472**	1.000000	**-0.606509**	**0.778400**	-0.120738	**-0.288382**	-0.210065
mezní deformace	**-0.315921**	-0.029939	0.147063	**-0.656710**	**-0.606509**	1.000000	-0.060383	**0.393251**	**0.458119**	**0.444400**
mezní napětí	-0.207783	0.114593	0.231236	**0.521979**	**0.778400**	-0.060383	1.000000	0.135718	-0.031468	0.076809
MT (µm)	-0.257917	-0.184797	-0.054853	-0.153683	-0.120738	**0.393251**	0.135718	1.000000	-0.059215	0.087485
AA (elastin)	-0.119093	0.250865	0.109243	-0.203236	**-0.288382**	**0.458119**	-0.031468	-0.059215	1.000000	**0.547818**
AA (kolagen I)	0.014094	**0.399526**	0.087437	-0.114461	-0.210065	**0.444400**	0.076809	0.087485	**0.547818**	1.000000

PŘÍLOHA 1g

Spearmanův korelační koeficient určující vztah mezi délkou kryoprezervace a mechanickými a strukturálními vlastnostmi vzorků ventrikulo-arteriální (V-A) junkce pulmonálních alograftů srdečních chlopní (PASCH). E_0 a E_1 – Youngův modul elasticity v malých, resp. velkých deformacích; MT – tloušťka stěny, A_A – plošný podíl v referenční ploše vzorku. Barevně označeny významné korelace (p<0,05).

PASCH V-A junkce	věk (roky)	pohlaví	kryo (roky)	E_0 (MPa)	E_1 (MPa)	mezní deformace	mezní napětí	MT (µm)	A_A (elastin)	A_A (kolagen I)
věk (roky)	1.000000	**0.388438**	-0.176785	0.090575	0.053142	-0.274733	-0.027072	-0.012463	0.166422	-0.069648
pohlaví	**0.388438**	1.000000	0.009560	0.241978	0.280185	-0.331127	0.050943	-0.105127	-0.064433	0.308599
kryo (roky)	-0.176785	0.009560	1.000000	-0.087473	-0.201706	0.203546	-0.018899	0.295542	0.308567	-0.058888
E_0 (MPa)	0.090575	0.241978	-0.087473	1.000000	**0.898730**	**-0.649398**	**0.699198**	-0.012830	-0.286657	-0.315616
E_1 (MPa)	0.053142	0.280185	-0.201706	**0.898730**	1.000000	**-0.625000**	**0.788102**	-0.175220	-0.300220	-0.183651
mezní deformace	-0.274733	-0.331127	0.203546	**-0.649398**	**-0.625000**	1.000000	-0.095254	0.125367	0.240103	**0.444282**
mezní napětí	-0.027072	0.050943	-0.018899	**0.699198**	**0.788102**	-0.095254	1.000000	-0.068548	-0.222507	-0.004399
MT (µm)	-0.012463	-0.105127	0.295542	-0.012830	-0.175220	0.125367	-0.068548	1.000000	0.188783	-0.201246
AA (elastin)	0.166422	-0.064433	0.308567	-0.286657	-0.300220	0.240103	-0.222507	0.188783	1.000000	-0.070381
AA (kolagen I)	-0.069648	0.308599	-0.058888	-0.315616	-0.183651	**0.444282**	-0.004399	-0.201246	-0.070381	1.000000

PŘÍLOHA 1h

Spearmanův korelační koeficient určující vztah mezi délkou kryoprezervace a mechanickými a strukturálními vlastnostmi vzorků ringů pulmonálních alograftů srdečních chlopní (PASCH). E_0 a E_1 – Youngův modul elasticity v malých, resp. velkých deformacích; MT – tloušťka stěny, A_A – plošný podíl v referenční ploše vzorku. Barevně označeny významné korelace (p<0,05).

PASCH ring	věk (roky)	pohlaví	kryo (roky)	E_0 (MPa)	E_1 (MPa)	mezní deformace	mezní napětí	MT (µm)	A_A (elastin)	A_A (kolagen I)
věk (roky)	1.000000	**0.356075**	-0.108054	0.174487	0.037023	-0.075513	0.212977	-0.086510	0.278486	-0.227294
pohlaví	**0.356075**	1.000000	0.061098	0.105127	0.077997	-0.301816	0.064433	-0.145821	0.040706	-0.027132
kryo (roky)	-0.108054	0.061098	1.000000	0.086406	**-0.412402**	0.092277	-0.213172	0.053201	0.004312	0.016421
E_0 (MPa)	0.174487	0.105127	0.086406	1.000000	0.063416	-0.174487	0.188416	-0.179985	0.136584	0.189350
E_1 (MPa)	0.037023	0.077997	**-0.412402**	0.063416	1.000000	0.156891	**0.846041**	-0.212610	-0.282152	0.315828
mezní deformace	-0.075513	-0.301816	0.092277	-0.174487	0.156891	1.000000	**0.455645**	-0.214076	0.234852	0.098066
mezní napětí	0.212977	0.064433	-0.213172	0.188416	**0.846041**	**0.455645**	1.000000	-0.263930	-0.105784	0.296765
MT (µm)	-0.086510	-0.145821	0.053201	-0.179985	-0.212610	-0.214076	-0.263930	1.000000	-0.009900	-0.120246
AA (elastin)	0.278486	0.040706	0.004312	0.136584	-0.282152	0.234852	-0.105784	-0.009900	1.000000	-0.098093
AA (kolagen I)	-0.227294	-0.027132	0.016421	0.189350	0.315828	0.098066	0.296765	-0.120246	-0.098093	1.000000

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Mechanical and structural properties of human aortic and pulmonary allografts do not deteriorate in the first 10 years of cryopreservation and storage in nitrogen

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Abstract The aortic and pulmonary allograft heart valves (AHV) are used in the cardiac surgery for replacing the impaired semilunar valves. They are harvested from donor hearts and cryostored in tissue banks. The expiration period was set to 5 years arbitrarily. We hypothesized that their mechanical and structural properties do not deteriorate after this period. A total of 64 human AHV (31 aortic and 33 pulmonary) of different length of cryopreservation (fresh, 0–5, 5–10, over 10 years) were sampled to different tissue strips (artery, leaflet, ventriculo-arterial junction) and tested by tensile test with loading velocity 10 mm/min until tissue rupture. Neighbouring regions of tissue were processed histologically and evaluated for elastin and collagen area fraction. The results were evaluated statistically. In aortic AHV, the physical deformation response of wall samples to stress did not changed significantly neither during the process of cryopreservation nor during the first 10 years of storage. In pulmonary AHV, the ultimate strain dropped after 5 years of cryopreservation

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indicating that pulmonary artery was significantly less deformable at the time of rupture. On the other hand, the ultimate stress was equal during the first 10 years of cryostorage. The changes in collagen and elastin amount in the tissue samples were not associated with mechanical impairment. Neither elasticity, stiffness and solidity nor morphology of aortic and pulmonary AHV did not change reasonably with cryopreservation and in the first 10 years of cryostorage. This evidence suggests that the expiration period might be extended in the future.

Keywords Heart valve allograft · Homograft · Cryopreservation · Tissue banking · Mechanical characteristics · Structural changes

Introduction

Despite the undisputable progress in vascular and heart valve prosthesis research and development, the ideal substitute remains an unmet clinical need. Among wide range and designs of manufactured mechanical and tissue (biological) heart valve prosthesis exists a special group of human donor valves—allografts. The use of allograft heart valves (AHV) was developed by Duran and Gunning on animal model in Oxford, UK. They introduced the subcoronary aortic allograft valve transplantation into the subcoronary position (Duran and Gunning 1962). The history of clinical introduction of the method was fascinating. Three surgeons, in three continents replaced the diseased aortic valve by aortic AHV transplantation independently: Raymond Heimbecker in Toronto, Canada (Heimbecker et al. 1962) Donald Ross in London, UK (Ross 1962) and Sir Brian Barratt-Boyes, Aucland, New Zealand (Barrat-Boyes 1964) from March to August 1962. Pulmonary valve replacement with AHV was introduced subsequently by Donald Ross (Ross 1967; Ross and Somerville 1966). Nevertheless, clinical use of AHV remains controversial up to now. Nowadays, in many cardiac surgery centres of excellence aortic and pulmonary AHV are routinely used. AHV were proved to be extremely useful in congenital heart defects surgery. In heart valve surgery of adolescents and adults they became popular for repair of particular acquired pulmonary and aortic valve disease. Most often for infective endocarditis and for aortic valve replacement by means of pulmonary autograft (Ross 1967) in young and middle-aged adults and in women of childbearing age. AHV offer an excellent hemodynamic performance by restoring nearly normal anatomy, have higher resistance to infection compared to all other valves, do not require anticoagulation, but their use is limited by long-term durability issues (Barron et al. 2010; Da Costa et al. 2006; Stoliński et al. 2006). The most important drawback of the use of allografts remains their availability (Antunes 2018).

The AHV are harvested from cadaveric donor hearts not suitable for transplantation (for any reason). The morphological suitability of AHV strongly depends on the age of donors. The age limit for donor is usually set to 65 years (Jashari et al. 2004). Some facilities have raised the age limit to 70 years following the findings that especially pulmonary valves are not affected by age-related tissue changes (Grosse et al. 2008). The further utilization depends on the time of transplantation. Previously used "homovital" AHV were harvested in the regime of organ transplant from the brain-dead organ donors or cardiac transplant recipients, stored at 4 °C in tissue culture medium and kept unprocessed until their implantation within 48 h (Yacoub et al. 1995). Present legislation has led to abandoning this method in many countries.

The "fresh" AHV can be harvested either at the time of standard multiorgan harvesting or from "non-heart-beating donors" with a warm ischemia time of less than 6 h. The hearts are processed under sterile conditions in a laminar flow cabinet. Fresh AHV are sterilized by a solution containing a combination of antibiotics in which they remain at 4 °C for 7 days. Thorough quality and safety testing (microbiological and histological) take place within this period. Fresh AHV have to be transplanted within 6 weeks after the decontamination period (Anastasiadis et al. 2004) and therefore do not allow longer storage and wider availability.

Current method of choice is antibiotic decontamination and subsequent cryopreservation. The AHV are harvested and processed in the same way as fresh and thereafter frozen (programme cooled) in liquid nitrogen and stored in tissue banks. The main advantage of cryopreservation and the existence of a tissue bank is the on-call availability for cardiac surgery centre. However, there can be an imbalance between supply and demand. Shelf life of cryopreserved aortic

and pulmonary allografts was arbitrarily set on 5 years in most cardiovascular tissue banks around the world. Expired heart valves are discarded. Hence the number of available AHV can be limited (Spatenka and Burkert 2018).

Aortic and pulmonary (semilunar) AHV structure

Semilunar AHV consist of the arterial root, valve leaflets and a corresponding artery. Despite their similar function, aortic and pulmonary roots differ in their gross anatomy and histology, reflecting the different hemodynamic condition they have to act in. Aortic root exposed to the systemic pressure is thicker, stouter and contains more fibro-elastic tissue than the pulmonary one (Muresian 2018). The base of the aortic heart valve that supports the valve leaflets (annulus) is formed by dense collagenous tissue. According to Muresian's work, the pulmonary valve leaflets has no annulus in the sense of a fibrous ring. They take off directly from the muscles of the right ventricular infundibulum (Muresian 2016). Both aortic and pulmonary valves have 3 leaflets of semilunar shape. The leaflets of aortic valve are commonly named by their relationship to the coronary arteries—left coronary, right coronary and non-coronary. The pulmonary valve leaflets are classified as left, right and anterior according to usual nomenclature (Nomina anatomica), although their real-life positions are rather posterior, right anterior and left anterior (Muresian 2016). Human valve leaflets are normally pliable and thinner than 1 mm. Aortic leaflets are slightly thicker than the pulmonary ones as they belong to left-side of the heart maintaining significantly higher pressures than the right side (Hinton and Yutzey 2011).

The structure of valve leaflets consists of three layers—the fibrosa, spongiosa and ventricularis. Their histological structure is composed by endothelium, connective tissue cells and extracellular matrix. The main constituents of the connective tissue are collagen fibrils, elastic fibres and proteoglycans (Hopkins 2005). Collagen is the major extracellular matrix (ECM) component of the valves forming approximately 50% of the total valve by dry weight, elastin comprises 13% of the ECM (Bashey et al. 1967; Kubíková et al. 2017). Elastic arteries (ascending aorta, pulmonary artery) consists of three layers as follows: the tunica intima (endothelium and subendothelial layer), the tunica media (elastic membranes and smooth muscle cells) and the tunica adventitia (elastic and collagenous fibres, connective tissue cells, blood vessels, nerve fibres) (Kubíková et al. 2016).

Aortic and pulmonary valve mechanics

The heart valve's function is to maintain unimpeded unidirectional blood flow. While the heart ventricles propel the blood into aorta and pulmonary artery in systole, the semilunar valves placed in the left and right ventricular outlets prevent reversal blood flow to the ventricles in diastole. Therefore, their biomechanical properties are very important, essential to life. The dynamics anatomy of the aortic root is very complex (Vojacek et al. 2018). In a simplified interpretation the leaflets are passive elements that open and close rapidly to blood flow. They are pushed to open as soon as the pressure in the ventricle exceeds the pressure in the artery and closed by the backflow of the blood when the pressure in the ventricle drops at the end of systole. The valve leaflets are fully loaded in diastole (when the valve is closed) and relaxed in systole. It is believed that biomechanical behaviour is dominated by collagen and elastin under high tensile loading (Eckert et al. 2013). Collagen and elastin are curved fibres that change their shape according to the valve movement—crimping when the tissue is relaxed and flattening when the tissue is under stress. The collagen provides strength to the leaflets, while the purpose of the elastin is to maintain a specific collagen fibre configuration and return the fibres to this "resting" state between loading cycles (Vesely 1998). The single layers of valve leaflets are bonded by transverse collagen fibres and work as one unit (Buchanan and Sacks 2014).

Study aims

The aim of our study is to assess the mechanical and structural properties of aortic (AAHV) and pulmonary (PAHV) allograft heart valves according to the duration of cryopreservation and compare them to the fresh (not cryopreserved) grafts. We hypothesize that mechanical and structural attributes of AHV do not deteriorate after 5 years of cryopreservation period reasonable. We wonder if there are any signs of mechanical and/or structural deterioration detectable after cryostorage longer than 5 years and whether the mechanical and structural parameters of

AAHV and PAHV differ between fresh and less than 10 years cryopreserved ones.

Materials and methods

Specimens

A total of 64 AHV (31 aortic and 33 pulmonary) were studied. The control group (group 0) consisted of fresh AHV. The rest of AHV of different duration of cryopreservation were divided into three groups as follows:

Group I: cryopreservation period 0.1–4.9 years.
Group II: cryopreservation period 5–9.9 years.
Group III: cryopreservation period more than 10 years.

The group demographics and cryostorage characteristics are summarized in Table 1.

All samples of fresh and cryopreserved AHV were obtained from the National AHV Bank of the Department of Transplantations and Tissue Bank, Motol University Hospital, Prague, Czech Republic. They were harvested from the pool of heart beating multi-organ donors in case when the donor heart was not used for transplantation as an organ for any reason. All AHV were dissected from the donor hearts and in the time of the quality control assessed as suitable for clinical use. They were decontaminated by an antibiotic cocktail according to the established protocol of the tissue bank (Spatenka et al. 1997). Subsequently, all AHV were moved into the cryoprotectant solution—10% dimethylsulfoxide (DMSO)—and packed by double layer technique (sealed in Gambro Hemofreeze bags, NPBI BV, Gambro, Netherlands). Packed AHV were programme cooled (at a rate of − 1 °C/min) and stored in cryocontainer in the liquid phase of liquid nitrogen at − 196 °C in compliance with the Czech legislation (Czech Law on Quality and Safety of Human Tissue and Cells for Application in Humans 296/2008 of Czech Legal Code).

Randomly selected samples in their original packaging were transported in the cryocontainers (CXR 500, Wharton-Tylor International LLC, USA, with a digital thermometer and datalogger) to the research

Table 1 Demographics and cryostorage characteristics

Variable	Group 0 (fresh)	Group I (cryo 0.1–4.9 years)	Group II (cryo 5–9.9 years)	Group III (cryo > 10 years)
Aortic AHV				
n	3	9	11	8
Gender				
Male	2	4	2	3
Female	1	5	9	5
Donor age (years) (median, interquartile range)	51.35 (33.07–58.14)	49.08 (40.73–51.91)	45.64 (40.59–50.33)	44.36 (35.43–47.37)
Cryopreservation (years) (median, interquartile range)	N/A	3.89 (0.94–4.48)	5.26 (5.10–5.32)	12.74 (10.51–14.45)
Pulmonary AHV				
n	4	12	8	9
Gender				
Male	3	5	3	5
Female	1	7	5	4
Donor age (years) (median, interquartile range)	54.74 (37.64–62.93)	49.99 (45.23–54.96)	53.36 (51.28–56.69)	46.66 (38.91–53.79)
Cryopreservation (years) (median, interquartile range)	N/A	2.98 (1.11–4.54)	7.48 (6.80–8.85)	15.21 (11.81–17.28)

AHV allograft heart valve

facility. After removal they were left at room temperature for at least 15 min to equilibrate. Packages were then immersed directly into a water bath at + 37 °C for another 15 min at least. This thawing protocol was selected based on previous own unpublished experimental work (protocol validation) and is still routinely used in our tissue establishment.

After completing the thawing protocol, the allografts were unpacked and processed. AHV was opened longitudinally by incision between the leaflets. Custom made devices with two longitudinally placed razor blades in the distance of 5 and 10 millimetres were then used for tissue strips cutting to ensure the samples homogeneity. From each allograft we cut one specimen of the ventriculo-arterial junction (10 mm in width), prepared two leaflet specimens (5 mm in width) and 1–3 (depending on the AHV size) artery specimens (10 mm in width) as shown in Fig. 1. The ventriculo-arterial junction was selected as the composite specimen containing both arterial wall and leaflet tissue. The number of specimens is summarized in Table 2. Neighbouring region of each sample was used for histological assessment. All samples were washed in saline solution (0.9% solution of sodium chloride) before further evaluation. The mechanical testing was performed on the day of sampling. Tissue samples for histological analysis were fixed with 4% buffered formalin and underwent standard histological processing.

Table 2 Number of tissue samples in different groups according to their duration of cryopreservation

	Group 0	Group I	Group II	Group III
Aortic AHV	3	9	11	8
Artery	6	11	26	6
Leaflet	6	18	21	16
Junction	3	9	11	8
Pulmonary AHV	4	12	8	9
Artery	9	21	10	11
Leaflet	7	21	16	14
Junction	4	12	8	9

AHV, allograft heart valve; group 0, fresh; group I, cryopreserved for 0.1–4.9 years; group II, cryopreserved for 5–9.9 years; group III, cryopreserved for more than 10 years

Mechanical loading

Mechanical properties, namely Young's moduli of elasticity (E), ultimate stresses and strains, of all AHV samples were assessed by tensile test. Tissue strips were tested using Zwick/Roel Z50 traction machine (Zwick GmbH & Co, Germany) equipped with a 1 kN load cell. Geometrical measurements of sample thickness were obtained using a calliper. The specimens were clamped into the jaws of measurement device at the initial length of 10 mm (Fig. 2a). The specimens were first preconditioned using 20 loading and unloading cycles up to 10% of the initial specimen's length with the velocity 10 mm/min. After preconditioning the specimens were loaded by strain velocity 10 mm/min until the tissue rupture (Fig. 2b). All specimens were tested at room temperature and held moistened by saline solution during the experiment. The mechanical measurement and evaluation protocol were described previously in our own studies (Kubíková et al. 2016, 2017).

Fig. 1 **a** Scheme for the preparation of tissue specimens from the allograft heart valve (AHV) under study: Two 5 mm wide transversal cuts in the middle of the leaflets and one 10 mm wide longitudinal cut through the ventriculo-arterial junction (V-A junction) with adjacent part of the leaflet were made in each AHV. The 10 mm wide samples of arterial wall were cut longitudinally out of the ascending aorta. **b** Pulmonary AHV opened by longitudinal incision between leaflets. **c** Aortic AHV sampling was performed in coronary leaflets, V-A junction in non-coronary leaflet

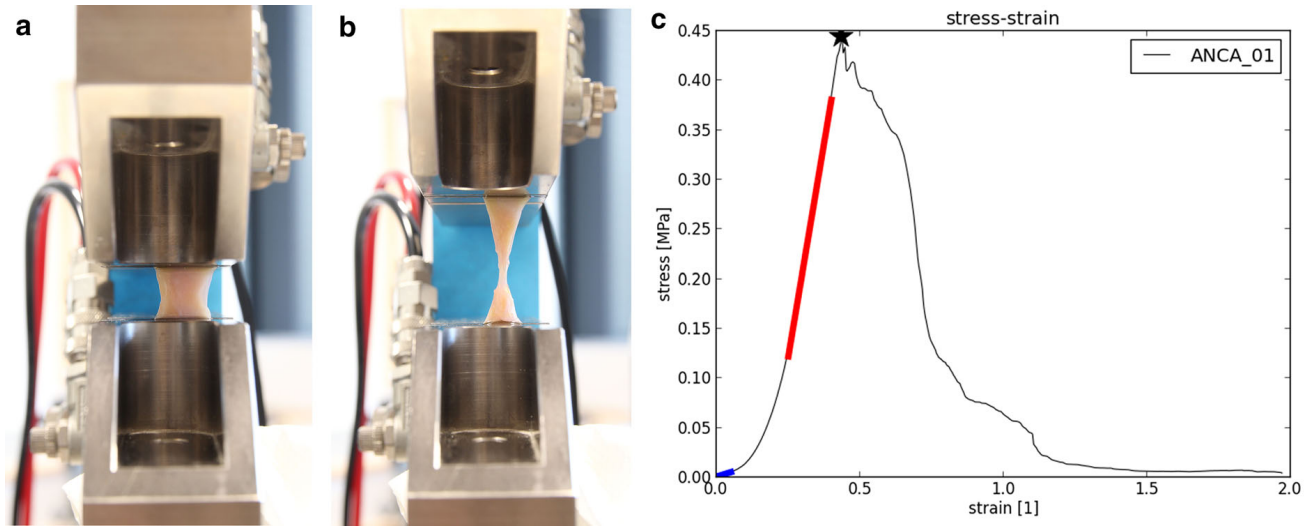

Fig. 2 Mechanical measurement: **a** The aortas wall specimen in the jaws of the measurement device Zwick/Roell Z050. **b** A rupture of the aortas wall specimen. **c** An example of a nonlinear stress–strain curve of the aortic wall. The small deformation region (blue line) with a low stiffness and the linear region of large deformations (red line) with a higher stiffness. The star marks the beginning of the rupture of the tissue characterized by ultimate stress and strain. (Color figure online)

The result of mechanical measurement was the relation between loading and deformation: the stress–strain curve (Fig. 2c). The strain was defined as the actual specimen elongation divided by initial length of the specimen. The stress was defined as the actual force divided by the initial cross-sectional area. The Young's moduli of elasticity in the small deformation region (E_0) as well as in the large deformation region (E_1) were determined using linear regression of the stress–strain curve. The strains intervals of the small and large deformation regions were approximately 0–4% and 20–40% for the elastic arteries, 0–2% and 10–20% for the leaflets, 0–2% and 10–20% for the ventriculo-arterial junctions, respectively, depending on the shape of the curve. The ultimate stresses and the ultimate strains were determined at the start of the rupture. Note: the Young's modulus of elasticity characterizes physiological stiffness of the tissue. The in-house software enabling semiautomatic evaluation of mechanical measurements (Elfpy) was used for the evaluation (Elfpy2018, http://docs.sfepy.org/elfpy/doc-devel/index.html#).

Histological processing and assessment

The 5-µm-thick histological sections obtained from each formalin-fixed, paraffin-embedded tissue block were used for assessment. Two sections were stained with haematoxylin–eosin and two sections were stained with Verhoeff's haematoxylin and green trichrome to visualize the connective tissue. Two sections were stained with orcein to visualize the elastic fibres, two sections were stained with picrosirius red to visualize the type I collagen using circularly polarized light. In total, 849 slides and 5094 micrographs were examined histologically (Table 3, Fig. 3).

The area fraction (A_A) of elastin and collagen were evaluated by stereological morphometry using a stereological grid point test system based on the Cavalieri's method (Kochová et al. 2012; Kubíková et al. 2017; Mouton 2002; Tonar et al. 2015).

Statistics

The data were processed using Statistica Base 10 (StatSoft, Inc., Tulsa, OK, USA). Normality of mechanical and structural parameters was tested by the Shapiro–Wilk's W test, for the differences in parameters between groups the Kruskal–Wallis ANOVA and Man-Whitney U tests were used. The Spearman's rank correlation test was used to find relation between duration of cryopreservation and mechanical and structural parameters. The significance level was set at the p value of 0.05 or less. The resultant parameters are shown as median (interquartile range).

Table 3 Sampling of micrographs for measuring the structural parameters of the artery, leaflet and ventriculo-arterial junction

Evaluated parameter	Staining	Number of microphotographs per specimen	Used objective in microscope
Whole wall thickness (MT)	Verhoeff's haematoxylin and green trichrome	2	4×
Area fraction of elastin within wall [A_A(elastin)]	Orcein	8	40×
Area fraction of collagen within wall [A_A(collagen)]	Picrosirius red	8	20×

A_A area fraction, *MT* medium thickness

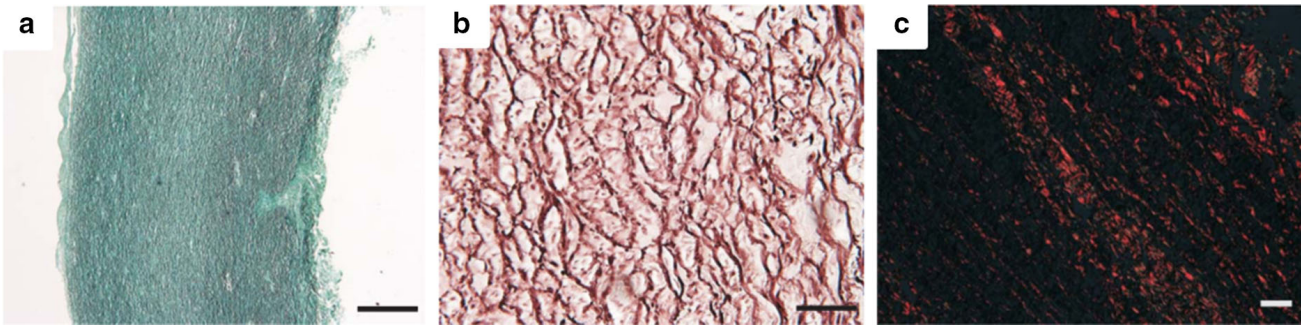

Fig. 3 An example of histological staining for the aortic wall (group II): **a** Verhoeff's haematoxylin and green trichrome to visualize the connective tissue (collagen I—green, elastin—black). Scale bar = 500 µm. **b** Visualization of the red–brown elastic fibres with orcein. Scale bar = 50 µm. **c** Visualization of the red collagen I fibres with picrosirius red in polarized light. Scale bar = 50 µm. (Color figure online)

Results

Mechanical and structural properties of aortic AHV (AAHV)

The mechanical and structural properties of AAHV are summarized in Table 4 and Figs. 4, 5, 6. The complete primary data from the analyses are provided in Supplement 1.

Mechanical and structural properties of pulmonary AHV (PAHV)

The mechanical and structural properties of PAHV are summarized in Table 5 and Figs. 7, 8, 9. The complete primary data from the analyses are provided in Supplement 1.

Comparison of mechanical and structural properties of aortic and pulmonary AHV according to the duration of cryopreservation

Differences between the groups 0–III (Kruscal-Wallis ANOVA) were found in the Young's moduli of elasticity in the small deformation region, ultimate strain and collagen amount for the PAHV arterial wall samples; in the Young's moduli of elasticity in the small deformation region, ultimate stress and collagen amount for the AAHV arterial wall samples. The pulmonary leaflets differed only in the ultimate stress between groups. The aortic leaflets differed in the ultimate strain and the collagen amount between groups. Aortic ventriculo-arterial junctions differed only in the ultimate stress between groups. The differences are summarized in Table 6.

Difference between each two storage time groups was tested by the Mann–Whitney *U*-test. The statistics is summarized in Table 7a, b.

Table 4 The mechanical and structural properties of the aortic allograft heart valves in different time groups

	Group 0	Group I	Group II	Group III
E_0 (MPa)				
Wall	0.24 (0.08–0.34)	0.34 (0.16–1.54)	0.18 (0.13–0.25)	0.10 (0.06–0.12)
Leaflet	0.87 (0.46–2.56)	2.33 (0.69–4.99)	1.59 (0.80–4.04)	1.16 (0.20–2.15)
V-A junction	0.29 (0.07–0.38)	0.23 (0.22–0.85)	0.16 (0.09–0.24)	0.10 (0.07–0.18)
E_1 (MPa)				
Wall	2.31 (1.01–3.97)	3.94 (1.28–9.95)	1.82 (1.19–4.34)	1.26 (0.44–1.59)
Leaflet	20.53 (15.77–47.35)	19.55 (12.61–29.01)	24.17 (16.63–34.72)	13.02 (10.16–22.38)
V-A junction	0.84 (0.58–1.80)	2.21 (0.86–2.85)	1.17 (1.00–1.27)	0.93 (0.80–1.32)
Ultimate strain				
Wall	0.36 (0.21–0.45)	0.45 (0.35–0.67)	0.50 (0.47–0.59)	0.38 (0.31–0.72)
Leaflet	0.19 (0.17–0.29)	0.33 (0.20–0.37)	0.22 (0.16–0.31)	0.34 (0.27–0.40)
V-A junction	0.25 (0.13–0.56)	0.43 (0.23–0.51)	0.32 (0.26–0.43)	0.45 (0.38–0.56)
Ultimate stress (MPa)				
Wall	0.49 (0.30–0.60)	0.83 (0.37–5.69)	0.54 (0.36–1.03)	0.16 (0.05–0.50)
Leaflet	3.38 (2.22–4.14)	4.76 (2.34–8.13)	3.98 (3.04–4.24)	3.32 (2.33–4.75)
V-A junction	0.18 (0.16–0.22)	0.34 (0.31–0.48)	0.25 (0.18–0.31)	0.28 (0.25–0.41)
A_A(elastin)				
Wall	0.47 (0.46–0.60)	0.43 (0.38–0.44)	0.45 (0.40–0.49)	0.44 (0.41–0.50)
Leaflet	0.15 (0.11–0.16)	0.14 (0.08–0.18)	0.11 (0.08–0.16)	0.18 (0.13–0.22)
V-A junction	0.23 (0.20–0.30)	0.29 (0.22–0.35)	0.29 (0.25–0.34)	0.33 (0.25–0.40)
A_A(collagen)				
Wall	0.07 (0.02–0.18)	0.18 (0.10–0.25)	0.03 (0.01–0.11)	0.12 (0.08–0.33)
Leaflet	0.21 (0.13–0.33)	0.38 (0.31–0.48)	0.27 (0.12–0.41)	0.48 (0.41–0.58)
V-A junction	0.12 (0.11–0.29)	0.27 (0.17–0.32)	0.13 (0.06–0.40)	0.32 (0.25–0.37)
MT (μm)				
Wall	1941.14 (1681.14–2137.03)	1763.41 (1682.01–2116.60)	1914.17 (1638.27–2207.02)	2230.03 (1717.23–2803.79)
Leaflet	479.33 (417.00–540.00)	540.52 (431.42–637.44)	501.00 (365.00–668.00)	506.69 (470.34–602.42)
V-A junction	911.18 (702.89–1458.44)	1209.07 (997.10–1395.08)	1052.06 (897.63–1370.51)	1081.31 (896.78–1191.41)

Median values (interquartile range). E_0 and E_1—Young's modulus of elasticity in the small and large deformation region, respectively

A_A, area fraction; MT, medium thickness; V-A junction, ventriculo-arterial junction; group 0, fresh, group I, cryopreserved for 0.1–4.9 years, group II, cryopreserved for 5–9.9 years, group III, cryopreserved for more than 10 years

Aortic AHV

The Young's moduli of elasticity in the small as well as in the large deformation regions for all aortic AHV samples (arterial wall, leaflet, ventriculo-arterial junction) did not differ between fresh (group 0) and cryopreserved (group I, II, III) AAHV significantly, indicating that the stiffness was not influenced by

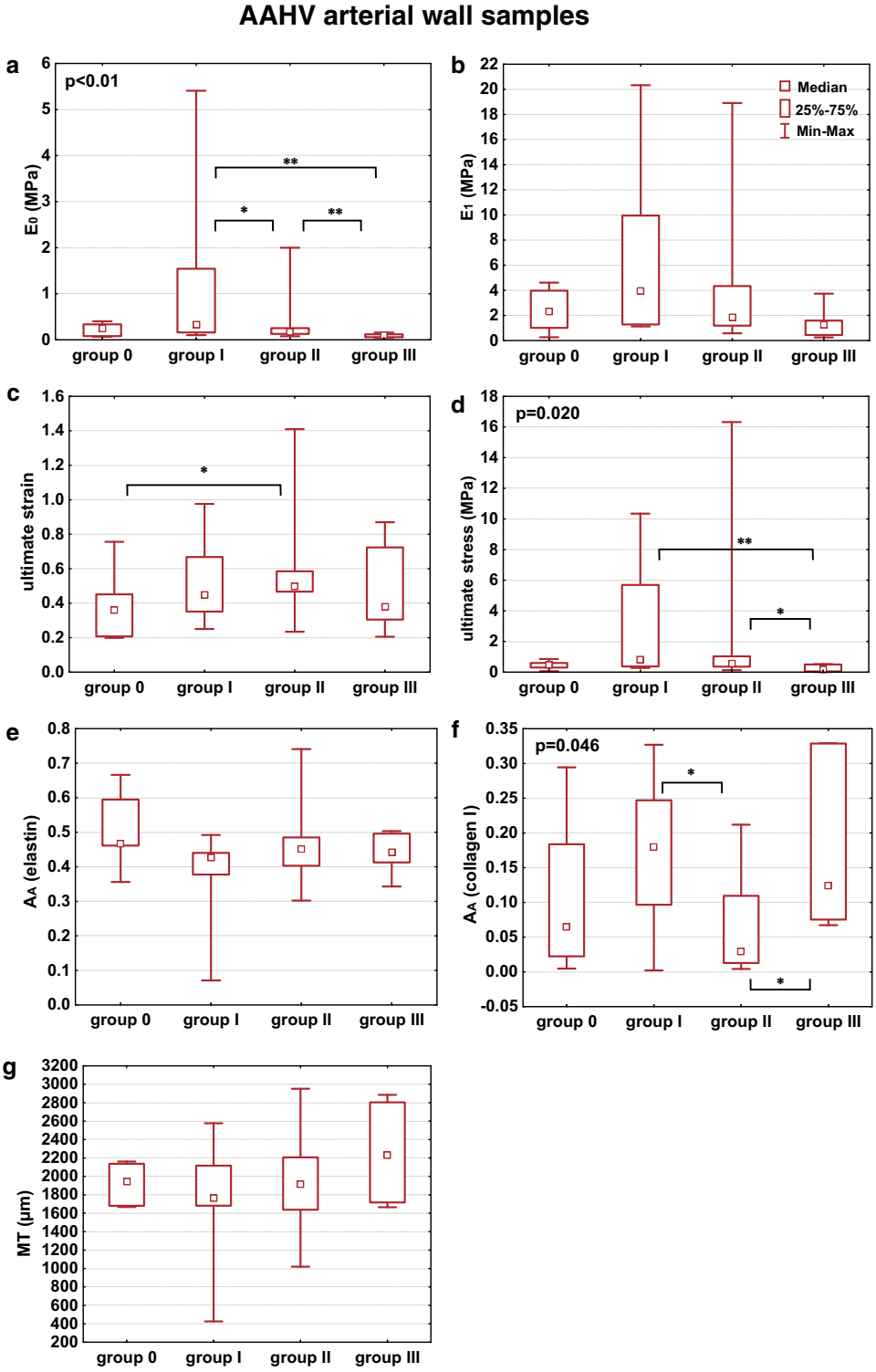

Fig. 4 The mechanical and structural parameters of the arterial wall samples of aortic allograft heart valves (AAHV): the Young's modulus of elasticity in the small deformation region (E_0, **a**), the Young´s modulus of elasticity in the large deformation region (E_1, **b**), ultimate strain (**c**), ultimate stress (**d**), area fraction of elastin [A_A(elastin), **e**], area fraction of collagen I [A_A(collagen I), **f**], and medium thickness (MT, **g**). The square dot denotes the median, the rectangle spans the interquartile range, the whiskers denote the limit values, *p* value denote the significant differences between groups (Kruskal–Wallis ANOVA), and $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ (Mann–Whitney *U* test). The results showing significant differences between groups I–II, I–III and II–III in E_0 and no differences in E_1. Ultimate strain differed between groups 0–II, ultimate stress differed between groups I–III and II–III. A_A(collagen I) differed between groups I–II and II–III. No differences were found in A_A(elastin) and MT

cryopreservation. Changes in the Young's modulus of elasticity in the small deformation region showed that AAHV in the first 5 years of cryostorage were more rigid than the ones stored for the longer period.

The strain at the point of rupture differed only between groups 0–II for the arterial wall and 0–III for aortic leaflet samples. Median value of ultimate strain in the leaflet samples was smaller after 5 years of storage (group II) than that less than 5 years of storage (group I) but did not dropped under fresh aortic AHV initial values. Samples of the aortic leaflets cryostored longer than 10 years showed higher ultimate strain values than the group II samples.

The solidity of AAHV samples interpreted as the ability to sustain the highest load at the point of rupture (ultimate stress) was higher in all cryopreserved samples.

No differences in the elastin amount in all tissues were found in AAHV samples stored less than 10 years (group 0, I, II).

The type I collagen fibres amount for all AAHV samples did not differ between the fresh and cryopreserved tissue until 10 years of cryopreservation. The exception was the higher collagen amount in aortic wall in the group II when compared with group I.

There were no significant changes in the medium thickness of all AAHV samples.

Pulmonary AHV

The statistics showed differences in the Young's moduli of elasticity in the small deformation region between group 0–II, I–II and II–III for arterial wall samples. The stiffness of the pulmonary AHV was higher after 5 years of cryopreservation (group II) than those ones of other groups. No difference was observed in the Young's moduli of elasticity in the large deformation region. The pulmonary leaflet and V-A junction samples did not show any significant changes as well.

The ultimate strain values for wall samples was smaller in group II compared to group 0 and group I. Meaning that the samples of arterial wall in group II looked to be stiffer than the group 0 and group I. The leaflet samples differed in the ultimate strain between group 0–I (near level of significance) and group 0–III. The V-A junction samples ultimate strain varied only between group I–III, where the value of group III was higher than that of group II.

No difference was observed in the ultimate stress for the pulmonary artery wall and V-A junction samples. The leaflet samples solidity was higher in group III compared with all other groups.

The area fraction of elastin in wall samples was higher in group II and sustained higher in group III compared to group I. The leaflet and V-A junction samples elastin amount did not differ between groups.

The area fraction of type I collagen fibres was smaller in group II and higher in the group III of the wall samples when compared with group 0 and group I. The amount of collagen was also higher in the pulmonary leaflet in group I when compared to group 0. The V-A junction samples did not differ between time groups.

There were no differences in the medium thickness in all tissue samples. The exceptions were: the smaller MT in the leaflet in group II when compared with the group I; and the smaller MT in the wall in group III when compared with group I.

Correlation between the duration of cryopreservation and mechanical and structural properties of AHV

The Spearman's rank correlation coefficients are summarized in the Supplement 2.

The aortic AHV wall and leaflet samples showed significant inverse relationship between the length of cryopreservation and the Young's moduli of elasticity (r_s E_0 − 0.48, r_s E_1 − 0.30 and r_s E_0 − 0.30, r_s E_1 − 0.27 respectively). The negative correlation was found also in E_0 for the aortic V-A junction samples (r_s = − 0.53). Another inverse relationship was observed between the length of cryopreservation and the ultimate stress (r_s = − 0.31) in the aortic wall.

The ultimate strain correlated medium positively with the collagen amount in the pulmonary leaflet, wall and V-A junction samples (r_s = 0.44, 0.60, 0.44, respectively) and with the collagen amount in the aortic leaflet (r_s = 0.48).

Discussion

The authors are aware of the complexity of the issue. Nevertheless, they do believe that the basic mechanical properties and morphology of semilunar AHV represent the most important characteristics of the

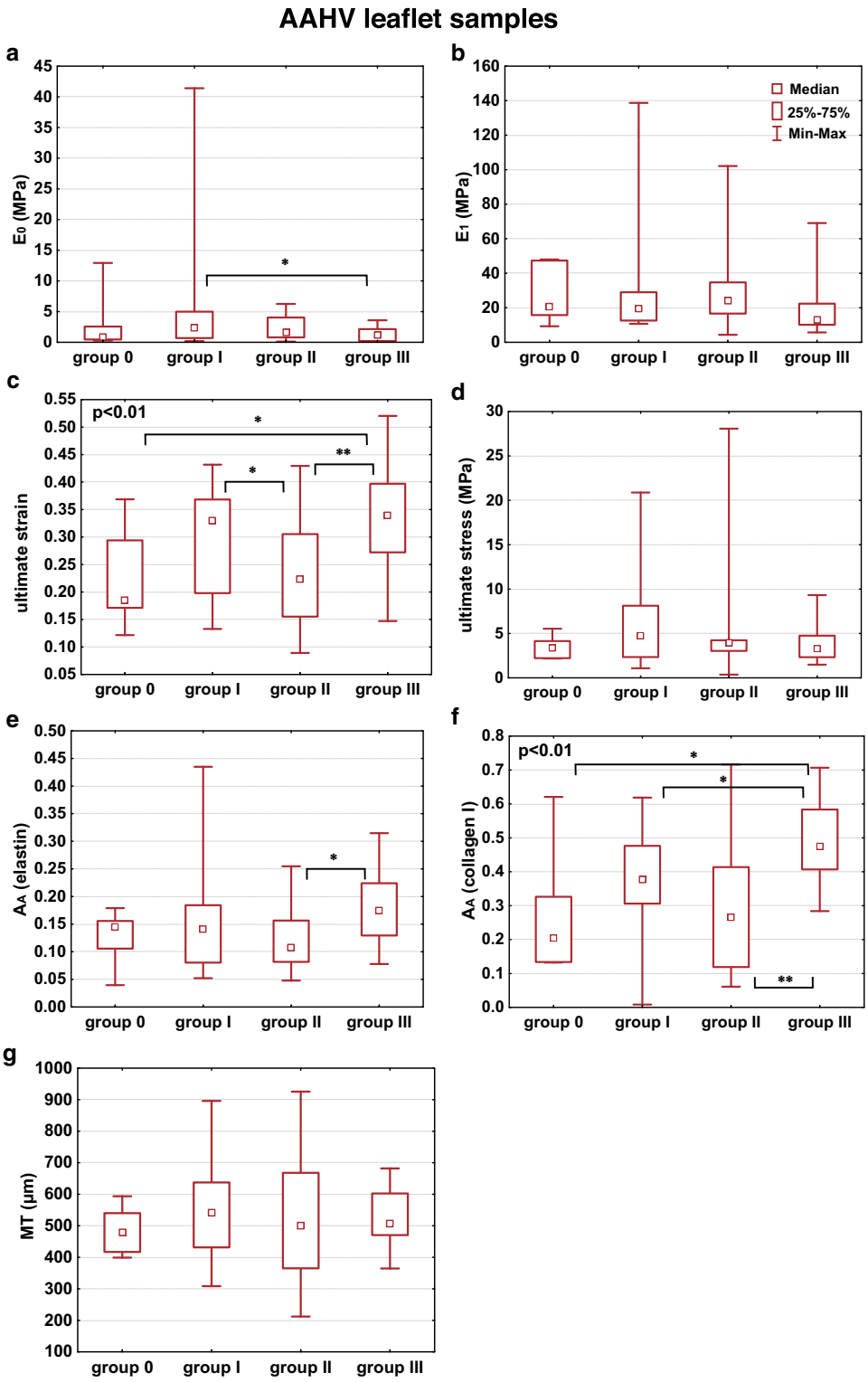

Fig. 5 The mechanical and structural parameters of the leaflet samples of aortic allograft heart valves (AAHV): the Young's modulus of elasticity in the small deformation region (E_0, **a**), the Young´s modulus of elasticity in the large deformation region (E_1, **b**), ultimate strain (**c**), ultimate stress (**d**), area fraction of elastin [A_A(elastin), **e**], area fraction of collagen I [A_A(collagen I), **f**], and medium thickness (MT, **g**). The square dot denotes the median, the rectangle spans the interquartile range, the whiskers denote the limit values, *p* value denote the significant differences between groups (Kruskal–Wallis ANOVA), and $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ (Mann–Whitney *U* test). The results showing significant differences between groups I–III in E_0 and no differences in E_1. Ultimate strain differed between groups 0–III, I–II and II–III. No differences were observed in ultimate stress. A_A(elastin) increased between group II and III. A_A(collagen I) differed between groups 0–III, I–III and II–III. No differences were found in MT

AHV tissue from the surgical point of view. Time limit of AHV cryopreservation means one of the important factors limiting the AHV availability in tissue banks. The possibility to prolong the expiration AHV limits is of importance beyond doubt from the point of view of daily practice.

All the tissue specimens taken from the aortic as well as from pulmonary AHV exhibited stiffening due to increasing loading, i.e., the stress–strain curve was nonlinear. This behaviour is typical for soft biological tissue (Meyers et al. 2008). The initial loading in the region of small deformation was characterized by

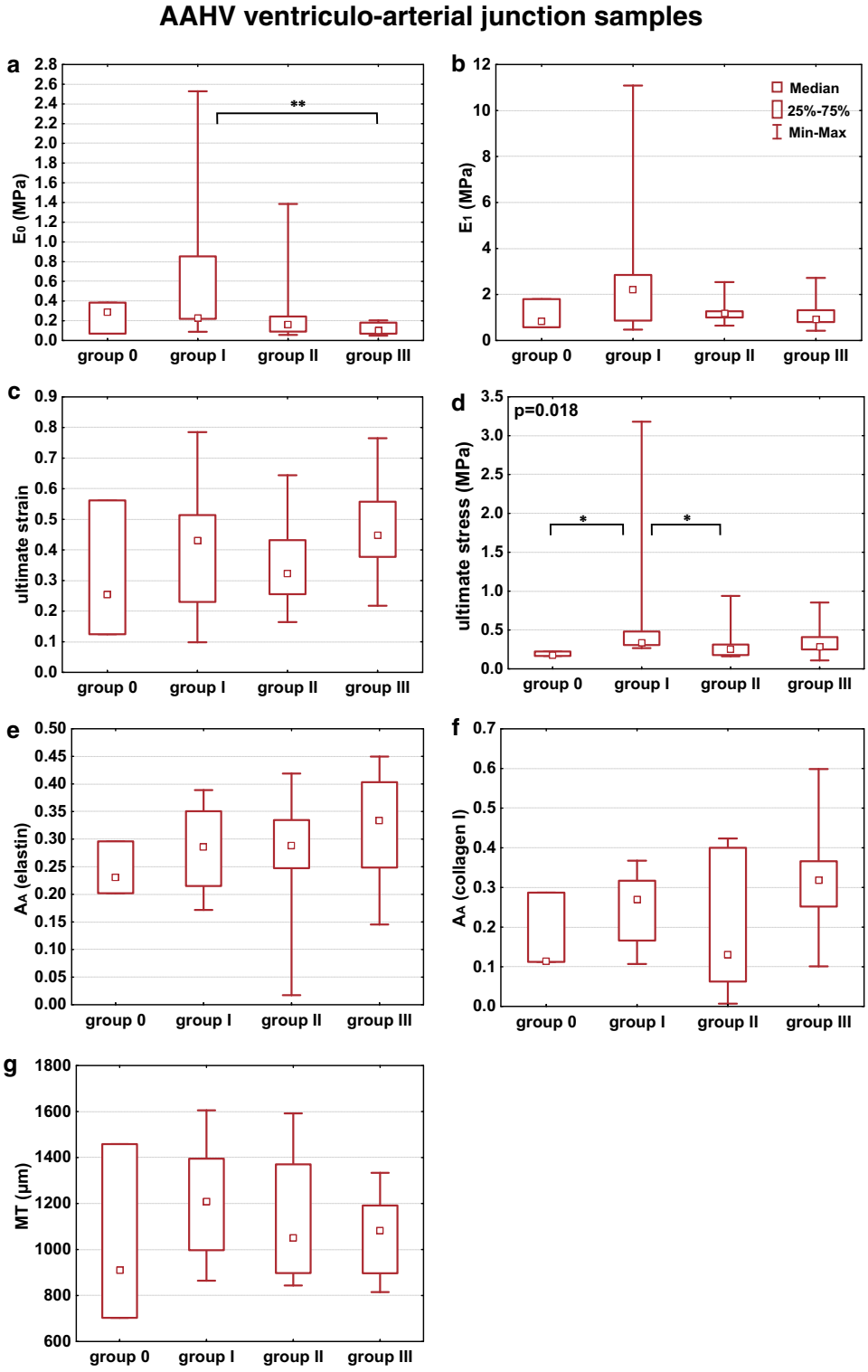

Fig. 6 The mechanical and structural parameters of the ventriculo-arterial junction samples of aortic allograft heart valves (AAHV): the Young's modulus of elasticity in the small deformation region (E_0, **a**), the Young´s modulus of elasticity in the large deformation region (E_1, **b**), ultimate strain (**c**), ultimate stress (**d**), area fraction of elastin [A_A(elastin), **e**], area fraction of collagen I [A_A(collagen I), **f**], and medium thickness (MT, **g**). The square dot denotes the median, the rectangle spans the interquartile range, the whiskers denote the limit values, p value denote the significant differences between groups (Kruskal–Wallis ANOVA), and $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ (Mann–Whitney U test). The results showing significant differences between groups I–III in E_0 and no differences in E_1. Ultimate strain did not differ. Ultimate stress differed between groups 0–I and I–II. No differences were observed in A_A(elastin), A_A(collagen I) and MT

small Young's modulus of elasticity, while the further loading led to tissue stiffening and the linear region of large deformations was characterized by high Young's modulus of elasticity. In general, the stiffening can be explained by collagen crimping. The collagen is at normal non-stretch state in waving relaxed configuration and thus in the initial phase of loading contributes only marginally to tissue mechanical response. In this state contributes to the tissue mechanical response mostly the elastin with the Young's modulus of elasticity about 0.6 MPa (in ligamentum nuchae, (Vincent 1990). During the

Table 5 The mechanical and structural properties of the pulmonary allograft heart valves in different time groups

	Group 0	Group I	Group II	Group III
E_0 (MPa)				
Wall	0.09 (0.06–0.12)	0.13 (0.09–0.21)	0.32 (0.20–0.49)	0.14 (0.05–0.22)
Leaflet	1.17 (0.40–6.32)	2.33 (0.90–4.98)	1.61 (0.71–6.77)	2.12 (0.59–7.32)
V-A junction	0.36 (0.19–0.54)	0.21 (0.14–0.33)	0.43 (0.18–0.83)	0.22 (0.15–0.31)
E_1 (MPa)				
Wall	0.97 (0.91–1.17)	0.99 (0.58–1.95)	2.03 (1.18–3.04)	1.23 (1.10–2.07)
Leaflet	15.51 (11.13–30.02)	15.48 (10.77–24.49)	18.49 (8.59–34.02)	22.66 (12.32–43.74)
V-A junction	2.10 (1.21–2.83)	1.54 (0.67–2.09)	2.47 (1.47–4.08)	1.30 (0.64–1.53)
Ultimate strain				
Wall	0.45 (0.34–0.81)	0.46 (0.31–0.67)	0.20 (0.15–0.25)	0.41 (0.33–0.48)
Leaflet	0.14 (0.11–0.23)	0.25 (0.17–0.39)	0.15 (0.12–0.31)	0.29 (0.17–0.37)
V-A junction	0.21 (0.15–0.34)	0.18 (0.16–0.21)	0.13 (0.12–0.18)	0.25 (0.22–0.33)
Ultimate stress (MPa)				
Wall	0.33 (0.27–0.36)	0.31 (0.20–0.45)	0.31 (0.19–0.46)	0.39 (0.34–0.42)
Leaflet	2.23 (1.01–2.95)	3.07 (2.04–3.84)	2.34 (1.08–4.38)	4.69 (3.00–5.50)
V-A junction	0.26 (0.22–0.33)	0.16 (0.09–0.25)	0.23 (0.17–0.38)	0.20 (0.13–0.29)
A_A(elastin)				
Wall	0.37 (0.32–0.39)	0.36 (0.27–0.40)	0.40 (0.37–0.43)	0.41 (0.34–0.42)
Leaflet	0.08 (0.05–0.13)	0.12 (0.10–0.18)	0.13 (0.09–0.16)	0.10 (0.08–0.27)
V-A junction	0.22 (0.19–0.25)	0.21 (0.14–0.29)	0.24 (0.22–0.25)	0.25 (0.25–0.29)
A_A(collagen)				
Wall	0.08 (0.05–0.19)	0.18 (0.03–0.28)	0.03 (0.02–0.07)	0.14 (0.07–0.17)
Leaflet	0.21 (0.18–0.25)	0.32 (0.26–0.43)	0.36 (0.17–0.43)	0.31 (0.18–0.39)
V-A junction	0.13 (0.08–0.21)	0.36 (0.06–0.43)	0.08 (0.07–0.27)	0.24 (0.10–0.33)
MT (μm)				
Wall	1316.86 (1017.65–1368.02)	1239.31 (1150.27–1701.19)	1370.92 (1160.06–1606.94)	1005.53 (852.56–1395.19)
Leaflet	317.00 (283.00–375.00)	427.91 (329.78–505.36)	325.72 (248.00–416.00)	382.50 (287.00–454.51)
V-A junction	709.33 (662.18–808.45)	823.56 (687.78–888.51)	819.95 (657.74–892.81)	868.60 (822.42–1098.59)

Median values (interquartile range). E_0 and E_1—Young's modulus of elasticity in the small and large deformation region, respectively

A_A, area fraction; MT, medium thickness; V-A junction, ventriculo-arterial junction; group 0, fresh, group I, cryopreserved for 0.1–4.9 years, group II, cryopreserved for 5–9.9 years, group III, cryopreserved for more than 10 years

increasing mechanical loading, the collagen straightens the waving. After stretching, the collagen fibres with their high modulus of elasticity (1–2.5 GPa for collagen in rat-tail tendon, (Meyers et al. 2008) start to contribute significantly to the tissue mechanical response and thus the tissue becomes stiffer.

The aortic and pulmonary leaflets had higher Young's moduli of elasticity and ultimate stress and

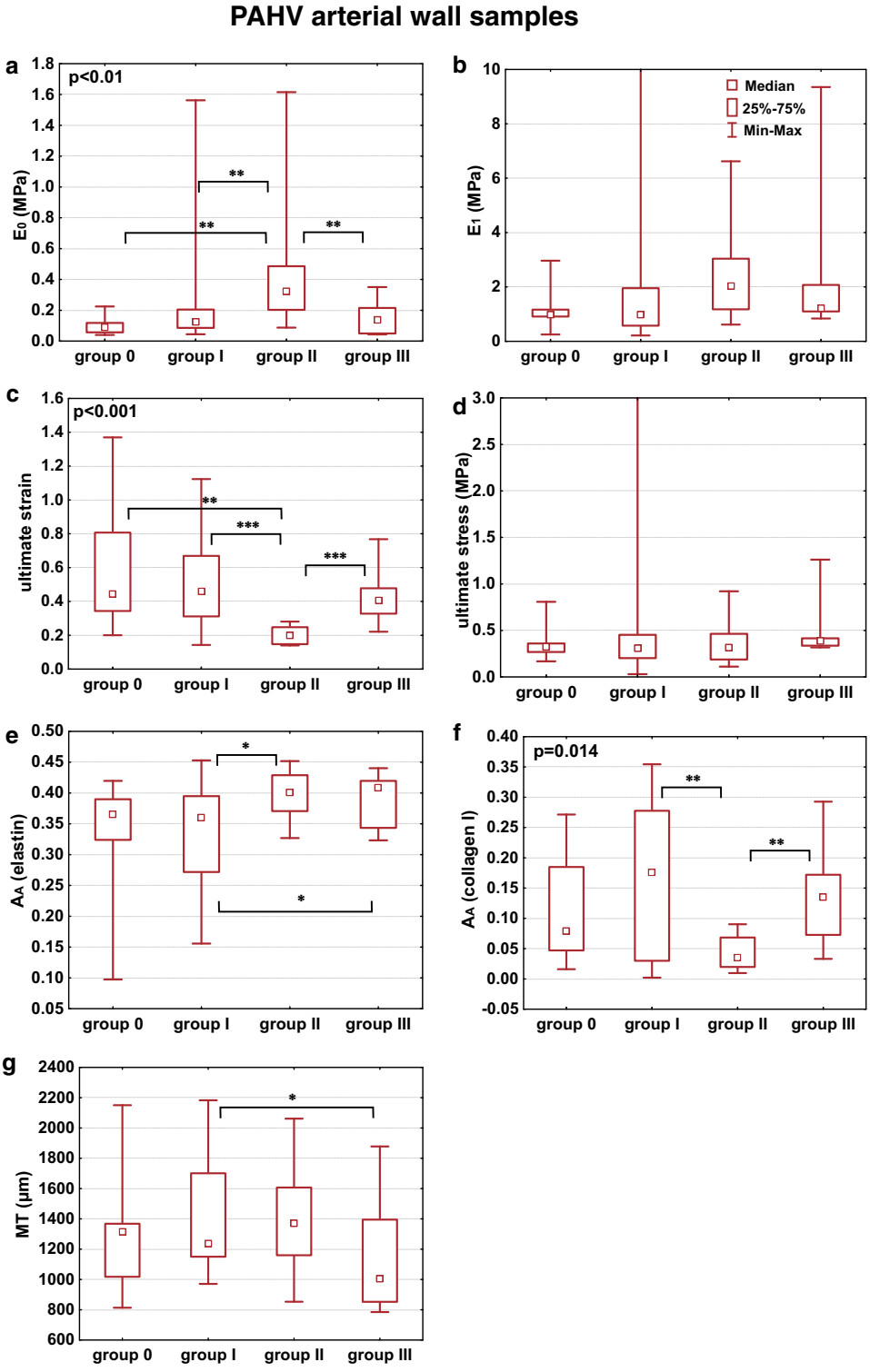

Fig. 7 The mechanical and structural parameters of the arterial wall samples of pulmonary allograft heart valves (PAHV): the Young's modulus of elasticity in the small deformation region (E_0, **a**), the Young's modulus of elasticity in the large deformation region (E_1, **b**), ultimate strain (**c**), ultimate stress (**d**), area fraction of elastin [A_A(elastin), **e**], area fraction of collagen I [A_A(collagen I), **f**], and medium thickness (MT, **g**). The square dot denotes the median, the rectangle spans the interquartile range, the whiskers denote the limit values, p value denote the significant differences between groups (Kruskal–Wallis ANOVA), and $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ (Mann–Whitney U test). The results showing significant differences between groups 0–II, I–II and II–III in E_0 and no differences in E_1. Ultimate strain differed between groups 0–II, I–II and II–III. No differences were found in ultimate stress. A_A(elastin) differed between groups I–II and I–III, A_A(collagen I) differed between groups I–II and II–III, MT between groups I–III

oppositely smaller ultimate strain than the artery wall and ventriculo-arterial junction in both AHV. It could be explained by the fact, that the leaflets had much higher amount of collagen then the arterial wall samples.

Like many other mechanical studies, the design of the mechanical part of our study mimics the physiological loads to which the valves are subjected by transvalvular pressure in diastole but does not involve the shear stress of flexural forces exerted on the valve in its natural function. We suppose that the ultimate strain, ultimate

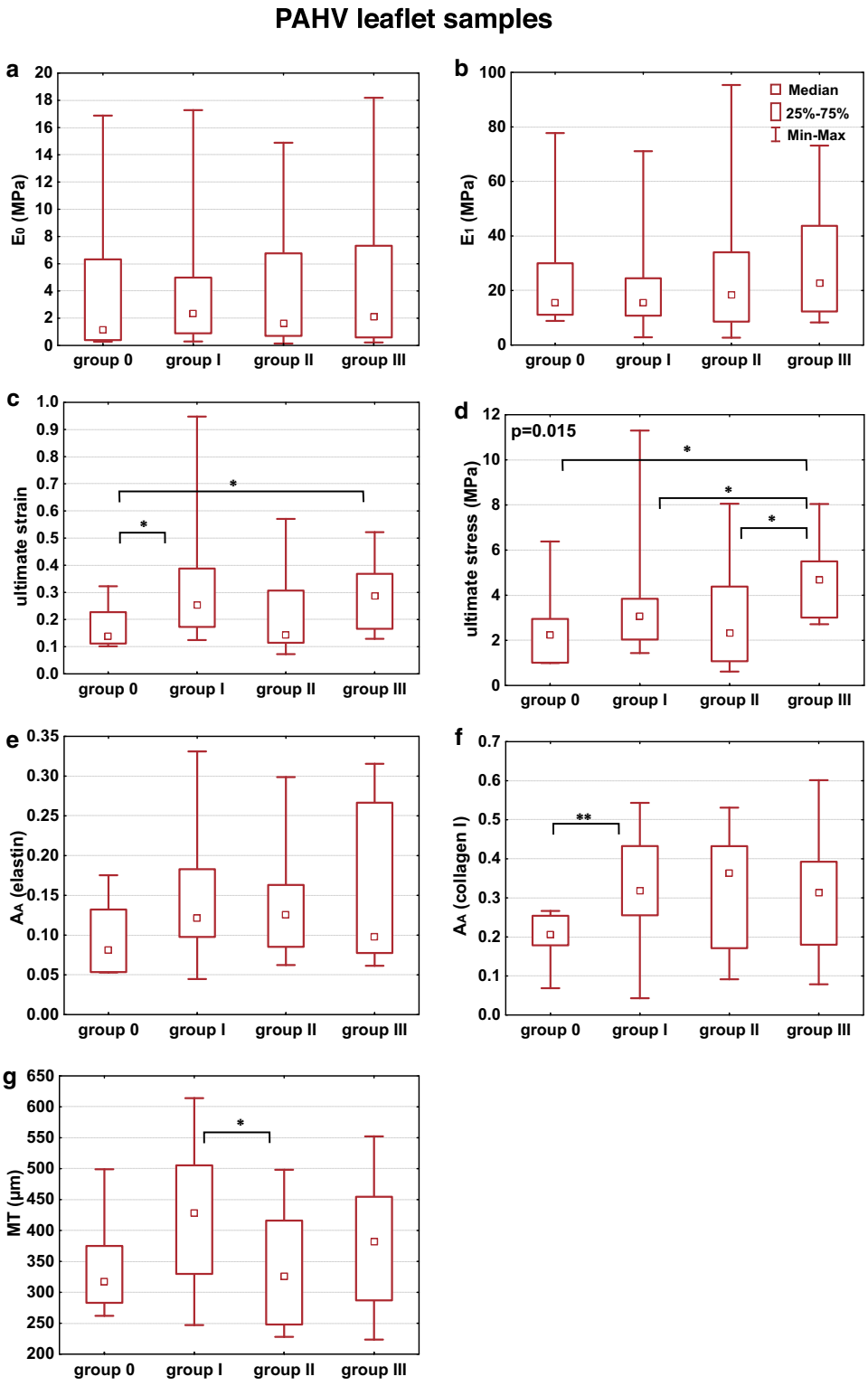

Fig. 8 The mechanical and structural parameters of the leaflet samples of pulmonary allograft heart valves (PAHV): the Young's modulus of elasticity in the small deformation region (E_0, **a**), the Young's modulus of elasticity in the large deformation region (E_1, **b**), ultimate strain (**c**), ultimate stress (**d**), area fraction of elastin [A_A(elastin), **e**], area fraction of collagen I [A_A(collagen I), **f**], and medium thickness (MT, **g**). The square dot denotes the median, the rectangle spans the interquartile range, the whiskers denote the limit values, *p* value denote the significant differences between groups (Kruskal–Wallis ANOVA), and $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ (Mann–Whitney *U* test). The results showing no differences in E_0 and E_1. Ultimate strain differed between groups 0–I and 0–III. Ultimate stress differed between groups 0–III, I–III and II–III. No changes were observed in A_A(elastin). A_A(collagen I) differed between groups 0–I. MT differed between groups I–II

stress and stiffness characterized by Young's moduli at the large deformation region (E_1) are the main mechanical variables that should be considered.

In aortic AHV, the physical deformation response of wall samples to stress did not changed significantly neither during the process of cryopreservation nor during the first 10 years of storage. The decline in ultimate strain values of aortic leaflet in group II did not drop under initial value of fresh AHV samples. The pressure needed to rupture the tissue samples was

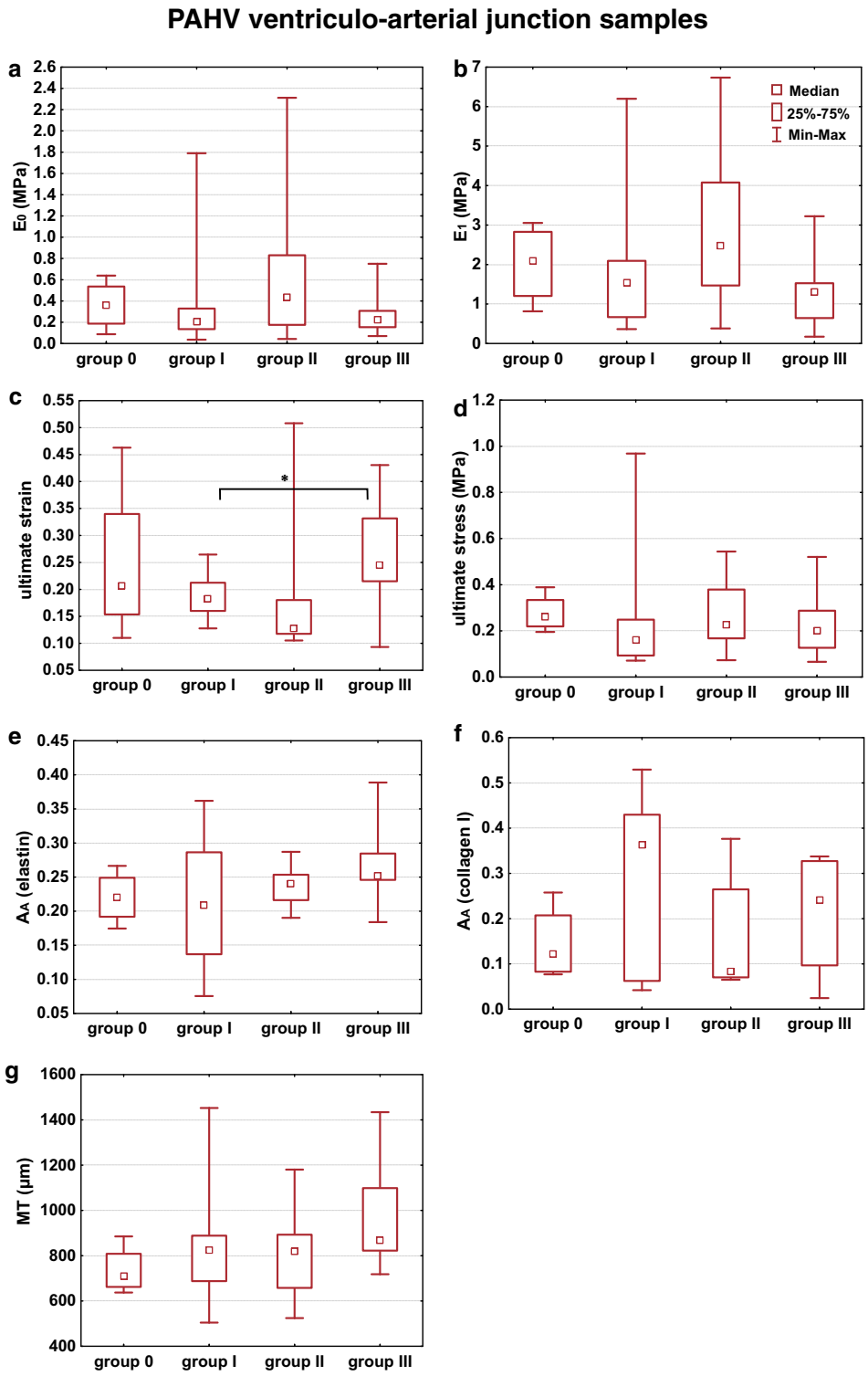

Fig. 9 The mechanical and structural parameters of the PAHV ventriculo-arterial junction samples of pulmonary allograft heart valves (PAHV): the Young's modulus of elasticity in the small deformation region (E_0, **a**), the Young's modulus of elasticity in the large deformation region (E_1, **b**), ultimate strain (**c**), ultimate stress (**d**), area fraction of elastin [A_A(elastin), **e**], area fraction of collagen I [A_A(collagen I), **f**], and medium thickness (MT, **g**). The square dot denotes the median, the rectangle spans the interquartile range, the whiskers denote the limit values, p value denote the significant differences between groups (Kruskal–Wallis ANOVA), and $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ (Mann–Whitney U test). The results showing differences in ultimate strain between groups I–III. No differences were observed in the other variables

approximately equal for all aortic AHV samples in groups 0, I and II. No significant changes in Young's modulus of elasticity at the large deformation region (E_1) were found in all tissue samples across the study groups indicating that the stress–strain curve gradient or in other words the stiffness of AHV was not markedly impaired during the process of cryopreservation and storage. The significant decrease in type I collagen fibres area fraction in the aortic AHV wall samples in group II is unclear as it is then followed by increase again in group III. Note, that the group II had the higher ratio female/male samples than the other

Table 6 The Kruscal–Wallis ANOVA = significant differences of parameters between time groups (group 0, I, II, III)

	E0	E1	Ultimate strain	Ultimate stress	A_A(elastin)	A_A(collagen I)	MT
Aortic AHV							
Arterial wall	$p < 0.01$	n.s.	n.s.	$p = 0.020$	n.s.	$p = 0.046$	n.s.
Leaflet	n.s.	n.s.	$p < 0.01$	n.s.	n.s.	$p < 0.01$	n.s.
V-A junction	n.s.	n.s.	n.s.	$p = 0.018$	n.s.	n.s.	n.s.
Pulmonary AHV							
Arterial wall	$p < 0.01$	n.s.	$p < 0.001$	n.s.	n.s.	$p = 0.014$	n.s.
Leaflet	n.s.	n.s.	n.s.	$p = 0.015$	n.s.	n.s.	n.s.
V-A junction	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

E_0 and E_1—Young's modulus of elasticity in the small and large deformation region, respectively

A_A, area fraction; MT, medium thickness; AHV, allograft heart valve; V-A junction, ventriculo-arterial junction; group 0, fresh, group I, cryopreserved for 0.1–4.9 years, group II, cryopreserved for 5–9.9 years, group III, cryopreserved for more than 10 years; n.s., means non-significant

groups. But for a deeper gender statistic we need more specimens. No significant changes were found in the area fraction of elastin and median thickness.

In the pulmonary AHV, the ultimate strain dropped in group II wall samples compared to group I, indicating that the pulmonary artery after 5 years of cryostorage was significantly less deformable at the point of the rupture. On the other hand, the ultimate stress in wall samples was equal during the first 10 years of cryostorage. The E_1 values did not change significantly as in the aortic AHV groups. The PAHV wall samples showed significant decrease in collagen area fraction after 5 years of cryostorage too, whereas the elastin fraction increased in the same group. The median thickness of leaflet samples was smaller in group II than group I but did not decline under the fresh PAHV median thick values.

The AHV cryostored longer than 10 years (group III) showed heterogenous results in many variables indicating that prolonged storage may influence the mechanical and structural characteristics to a greater extent.

The vast majority of mechanical and structural studies has been performed with porcine or ovine heart valves (Hlubocky et al. 2011; Stemper et al. 2007; Vesely 1998). The influence of cryopreservation on structural, chemical and immunoenzymatic properties of human aortic valve allografts were studied by Pfitzner et al. (2018). He came to the conclusion that the morphologies of fresh and cryopreserved aortic valve grafts were comparable, and no difference was demonstrated between short- and long-term storage of cryopreserved valves. Human heart valves are very precious study material. Sampling of fresh and cryopreserved AHV within their expiration period (less than 5 years) collides with both economic and ethical issues as they can be used for transplantation. Therefore, the number of samples in our control group (group 0) is limited. This project was granted by the courtesy of the National AHV Bank of the Department of Transplantations and Tissue Bank, Motol University Hospital, Prague, Czech Republic.

According to Vesely (1998) elastin serves as the tensioner of the collagen fibres during unloading. We have found no diminution in the elastin or collagen area fraction during cryopreservation. Stemper et al. (2007) states that the refrigeration of porcine aorta significantly reduced the mechanical attributes (primarily the ultimate stress and Young's modulus of elasticity). No such difference was observed in freezing at − 20 °C or − 80 °C. The ultimate strain was not affected by storage technique. The cryopreservation process with cryoprotectant agent (10% DMSO) used in AHV however, differs significantly from common refrigerating and freezing described in his study design, therefore the results are not comparable. The mechanical characteristics of fresh and frozen human aorta (descending) were studied by Adham et al. (1996). According to his results, cryopreservation did not alter the stress–strain characteristics of the samples. The cryopreserved aorta had an elastic modulus almost identical to that of control (fresh) tissue. Similar results were observed also in decellularized rabbit carotid arteries (Fonck et al.

Table 7 Mann–Whitney *U*-test = significant differences of parameters between two storage time groups (group 0–I, 0–II, 0–III, I–II, I–III, II–III) of (a) aortic allograft heart valve (AHV) samples and (b) pulmonary allograft heart valve (AHV) samples

	0–I	0–II	0–III	I–II	I–III	II–III
(a) Aortic AHV						
Aortic wall						
E_0	n.s.	n.s.	n.s.	$p = 0.030$	$p < 0.01$	$p < 0.01$
E_1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Ultimate strain	n.s.	$p = 0.031$	n.s.	n.s.	n.s.	n.s.
Ultimate stress	n.s.	n.s.	n.s.	n.s.	$p < 0.01$	$p = 0.015$
A_A(elastin)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
A_A(collagen)	n.s.	n.s.	n.s.	$p = 0.030$	n.s.	$p = 0.023$
MT	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Aortic leaflet						
E_0	n.s.	n.s.	n.s.	n.s.	$p = 0.044$	n.s.
E_1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Ultimate strain	n.s.	n.s.	$p = 0.025$	$p = 0.041$	n.s.	$p < 0.01$
Ultimate stress	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
A_A(elastin)	n.s.	n.s.	n.s.	n.s.	n.s.	$p = 0.014$
A_A(collagen)	n.s.	n.s.	$p = 0.020$	n.s.	$p = 0.031$	$p < 0.01$
MT	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Aortic ventriculo-arterial junction						
E_0	n.s.	n.s.	n.s.	n.s.	$p < 0.01$	n.s.
E_1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Ultimate strain	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Ultimate stress	$p = 0.016$	n.s.	n.s.	$p = 0.019$	n.s.	n.s.
A_A(elastin)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
A_A(collagen)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
MT	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
(b) Pulmonary AHV						
Pulmonary artery wall						
E_0	n.s.	$p < 0.01$	n.s.	$p < 0.01$	n.s.	$p < 0.01$
E_1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Ultimate strain	n.s.	$p < 0.01$	n.s.	$p < 0.001$	n.s.	$p < 0.001$
Ultimate stress	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
A_A(elastin)	n.s.	n.s.	n.s.	$p = 0.039$	$p = 0.033$	n.s.
A_A(collagen)	n.s.	n.s.	n.s.	$p < 0.01$	n.s.	$p < 0.01$
MT	n.s.	n.s.	n.s.	n.s.	$p = 0.039$	n.s.
Pulmonary leaflet						
E_0	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
E_1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Ultimate strain	$p = 0.49$	n.s.	$p = 0.040$	n.s.	n.s.	n.s.
Ultimate stress	n.s.	n.s.	$p = 0.012$	n.s.	$p = 0.023$	$p = 0.015$
A_A(elastin)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
A_A(collagen)	$p < 0.01$	n.s.	n.s.	n.s.	n.s.	n.s.
MT	n.s.	n.s.	n.s.	$p = 0.022$	n.s.	n.s.
Pulmonary ventriculo-arterial junction						
E_0	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

Table 7 continued

	0–I	0–II	0–III	I–II	I–III	II–III
E_1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Ultimate strain	n.s.	n.s.	n.s.	n.s.	$p = 0.042$	n.s.
Ultimate stress	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
A_A(elastin)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
A_A(collagen)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
MT	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

E_0 and E_1—Young's modulus of elasticity in the small and large deformation region, respectively

A_A, area fraction; MT, medium thickness; group 0, fresh, group I, cryopreserved for 0.1–4.9 years, group II, cryopreserved for 5–9.9 years, group III, cryopreserved for more than 10 years; n.s., means non-significant

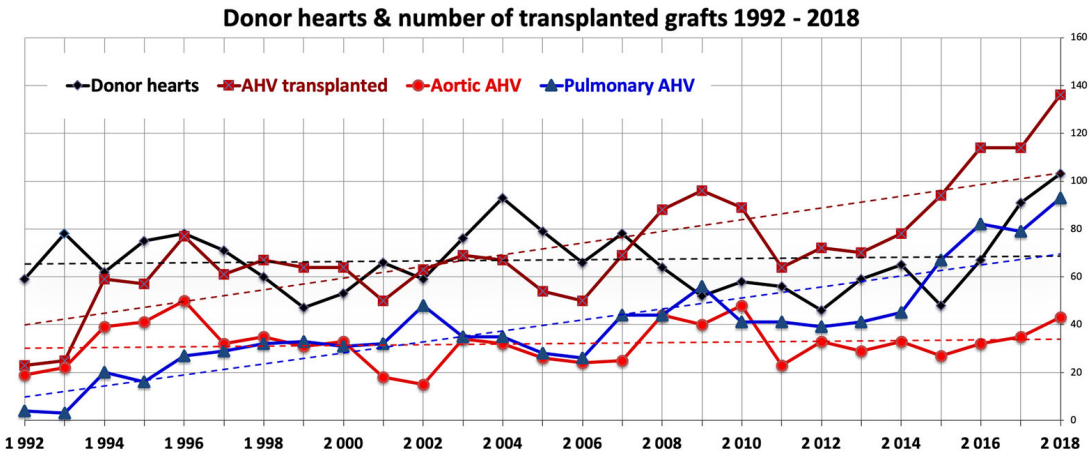

Fig. 10 The number of donor hearts and transplanted allograft heart valves (AHV) over a period of last 26 years in National AHV Bank of the Department of Transplantations and Tissue Bank, Motol University Hospital, Prague, Czech Republic shows the steady trend of donor hearts in contrast with increasing demand for AHV transplantation. The X-axis represents the years, the Y-axis the numbers, dashed lines represent the trends

2008), indicating that cryopreservation does not affect the structure and mechanical properties of the arterial samples. Experimental work on the mitral sheep allografts deploying different model to characterize the tissue's mechanical behaviour (Hlubocky et al. 2011) also came to the conclusion that the tissue processing and cryopreservation do not alter the structural characteristics and elasticity.

One of the major limitations in the use of AHV is the disproportion between availability and request in many countries. The upward trend in the number of transplantations is in our settings caused by raising demand for pulmonary AHV whilst the number of transplanted aortic AHV remains at the same level (Fig. 10).

Other disadvantages include more demanding implantation technique than prosthetic valve replacement and degeneration and calcification of the graft. Our project does not address structural deterioration of implanted allografts that occur over time and is probably connected with the immune response. One of the research topics discussed in recent literature are decellularized AHV containing only connective tissue which may be repopulated with host cells and therefore reducing immunogenicity (Bibevski et al. 2017; Da Costa et al. 2006).

The endothelium plays a critical role in regulating valve mechanics and valve function and perhaps long-term durability (Simmons 2009). The role of the endothelium was not studied as it was proved that human valve allografts show severe endothelial destruction arising already in the initial steps of tissue processing at least in our Tissue Establishment—donor heart harvesting and antibiotic decontamination (Burkert et al. 2008).

This study has several limitations. The most important limitation was small number of fresh AHV due to their scarcity and high economical cost. A possible limitation of our protocol was not controlling for the gender as a factor that may influence heart valve mechanics. From our experience, as well as according to other heart valve bankers it is evident that female cardio-vascular tissue tends to be better preserved than the one from male donors of the same age. Balanced gender composition of groups or comparison of AHV from the same donor age and gender would reduce the risk of misinterpretation of the final results of evaluation. However, the number of samples for research purposes is limited. Future projects should involve more tissue establishments to get large numbers of the AHV, especially the aortic which remain in stock much longer than the pulmonary one and expire each year.

There are many other characteristics of histological and structural changes, which could be searched, but for our purposes we used just collagen and elastin fraction. It's obvious that the elastin and collagen content alone do not determine mechanics, their mutual organization plays a major role (Vesely 1998). There is also extensive discussion about the role of proteoglycans glycosaminoglycans (Eckert et al. 2013; Hopkins 2005) in connective tissue mechanics. Although histological quantification has some limitations (such as tissue shrinkage), it provides information about the spatial distribution of collagen I and elastin relatively to the other tissue components. The results of mechanical measurements carried out in the open air could be influenced by dehydration of the tissue. The dehydration could lead to a slight stiffening of the tissue during experimentation (Viidik 1979). This potential source of bias was, however, minimized by the short duration of the experiments (about 15 min) and by spraying the tissue with saline solution (1–2 mL of 0.9% solution of sodium chloride/min).

The results of our study offer a unique insight into human semilunar heart valves, aorta and pulmonary artery mechanics and histology. The mechanical data show that AHV cryostored longer than present expiration period have no reasonable defects. Their structure concerning the area fraction of type I collagen and elastin fibres does not change significantly. We conclude that the mechanical and structural properties are not deteriorated significantly neither during the process of cryopreservation (programmed cooling) nor during the first 10 years period of storage. These results will contribute to advocating the changes in cryopreserved AHV expiration period policy. Taking into account the group II cryostorage characteristics (median 5.26 for aortic and 7.48 years for pulmonary AHV), we do consider our data as validation for extending the expiration period of AAHV to 6 years, and of PAHV to 8 years.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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Structural changes arising from different thawing protocols on cryopreserved human allograft's aortic valve leaflets

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D – writing the article; E – critical revision of the article; F – final approval of the article

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Abstract

Background. The aim of our experimental work was to assess the impact and morphological changes that arise during different thawing protocols on human aortic valve (AV) leaflets resected from cryopreserved aortic root allografts (CARAs).

Objectives. Two thawing protocols were tested: 1. CARAs were thawed at a room temperature (23°C); 2. CARAs were placed directly into a water bath at a temperature of 37°C. After all the samples were thawed, non-coronary AV leaflets were sampled from each specimen and fixed in a 4% formaldehyde solution before they were sent for morphological analysis.

Material and methods. All the samples were washed in distilled water for 5 min and dehydrated in a graded ethanol series (70%, 85%, 95%, and 100%) for 5 min at each level. The tissue samples were then immersed in 100% hexamethyldisilazane (HMDS) for 10 min, and then air-dried in an exhaust hood at room temperature. Processed samples were mounted on stainless steel stubs and coated with gold. Histological analysis was performed with the use of an electron microscope on a scanning mode operating at 25 kV – BS 301.

Results. Thawing protocol 1 (room temperature at 23°C): 6 (100%) samples showed loss of the endothelial covering of the basal membrane with no damage to the basal lamina. Thawing protocol 2 (water bath at 37°C): 5 (83%) samples showed loss of the endothelial covering of the basal membrane with no damage to the basal lamina. One (17%) sample showed loss of the endothelial covering the basal membrane with significant damage to the basal membrane.

Conclusions. Based on our experimental work, we can clearly conclude that cryopreserved AV leaflet allografts show identical structural changes at different rates of thawing.

Key words: aortic valve, allograft, thawing, cryopreserved, structural changes

Introduction

The first allograft transplants in cardiac surgery were freshly harvested aortic valves (AVs). The first fresh AV allograft transplant was performed by Murray in 1956.[1] Despite the imperfect hemodynamic outcome of the operation, the allograft performance was outstanding, with perfect leaflet function. Other early experimental and clinical trials, such as Heimbecker, Lam et al. and Kerwin et al., supported the superior properties of fresh AV allografts.[2–4] Nevertheless, the first successful operation with a patient surviving the fresh AV allograft transplant was performed by Ross in 1962, based on Brewin's experimental work.[5,6]

Many cardiac centers started to implement cryopreservation of fresh AV allografts due to the shortage of donors. Cryopreservation of AVs led to a significant decrease of allograft durability, and between the 1960s and the early 1970s this led almost to the abandonment of these types of procedures.[7] This was primarily due to irreversible damage to cell viability and loss of the structural integrity caused by thawing, resulting in the loss of allograft toughness and elastic properties.[8–10] Technical advances in tissue handling led to the reintroduction of allograft transplants back into use in cardiac surgery.[11] To date, there have been no recommended guidelines for cryopreservation and subsequent thawing of cryopreserved allografts that would eliminate damage to the cellular structures.

Material and methods

Allograft harvest and characteristics

All the allografts were harvested in the operation theater from patients that were organ donors and were pronounced "clinically dead" in compliance with the transplant laws of the Czech Republic.

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

Allograft processing cryopreservation protocol

All human aortic roots (ARA) underwent an initial decontamination according to the standard protocol of the tissue bank. Afterward, all allografts were stored in an antibiotic cocktail comprised of Cefuroxime 0.2 mg/mL + Piperacillin 0.2 mg/mL + Netilmicin 0.1 mg/mL + Fluconazole 0.1 mg/mL in the tissue culture nutrient medium E 199 for 24 h at 37°C (Altimed Pharmaceutical, Mississauga, Canada). Subsequently, all ARA were moved into a cryoprotectant solution in a sterile laminar flow cabinet; they were packed using a double layer technique (sealed in Gambro Hemofreeze bags; NPBI BV; Gambro, Utrecht, the Netherlands). The cryoprotectant used was 10% dimethyl sulfoxide in the nutritional source for cell culture E 199. All ARA were then cooled at a controlled rate of –1°C/min from 10°C to –60°C, and next rapidly cooled and stored in cryo-containers with a liquid phase of liquid nitrogen at –196°C.

Table 1. Thawing protocol 1 – basic allografts characteristics

Gender	Donor age [years]	Aorta diameter [mm]	ABO, Rh compatibility
Female	55	21	A+
Female	41	21	A+
Male	55	25	AB+
Female	56	24	A+
Male	57	27	B+
Male	59	28	O-

Table 2. Thawing protocol 2 – basic allografts characteristics

Gender	Donor age [years]	Aorta diameter [mm]	ABO, Rh compatibility
Male	34	21	A-
Female	51	24	B+
Male	44	24	B+
Male	44	25	O-
Male	42	27	AB+
Female	37	27	A+

Thawing protocols

Experimental work was based on investigating 12 cryopreserved aortic root allografts (CARAs). They were randomly divided into 2 groups, each group consisting of 6 samples. All allografts were thawed in their original packaging (packed using double layer technique and immersed in 10% dimethyl sulfoxide). Two thawing protocols were tested:

- protocol 1: 6 human CARAs thawed at room temperature of 23°C; thawing times were as follows: min 2 h 49 min, max 4 h 5 min (median: 3 h 19 min);
- protocol 2: 6 human CARAs were placed directly into a water bath at 37°C; thawing times were as follows: min 26 min, max 41 min (median: 32 min).

After all the CARAs were thawed, non-coronary AV leaflets were sampled from each specimen and fixated in a 4% formaldehyde solution before they were sent for morphological analysis. The time variability in both thawing protocols was given by different allografts sizes (Tables 1,2), as well as different amounts of cryoprotectant used for each allograft during the cryopreservation process.

Table 3. Scoring system for electron microscope sample analysis

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

Microscopic slide preparation

After the thawing protocols were completed, non-coronary AV leaflets were resected and fixed in Baker's solution. Each sample was divided into 5–10 mm subsamples. In order to prevent artificial mechanical damage to the cellular structures, no mechanical stretching of the samples was performed. All samples were washed in distilled water for 5 min, and dehydrated in a graded ethanol series (70%, 85%, 95%, and 100%) for 5 min at each level. The tissue samples were then immersed in 100% hexamethyldisilazane (HMDS) (CAS No. 999-97-3; Fluka Chemie AG, Buchs, Switzerland) for 10 min and air-dried in an exhaust hood at room temperature.

Processed samples were mounted on stainless steel stubs, coated with gold and stored in a desiccator until they were studied and photographed by an electron microscope on scanning mode operating at 25 kV – BS 301. A special scoring system (from 1 to 6) was introduced to analyze the morphological changes of the arterial wall of ARA under the electron microscope (Table 3).[12]

Results

Histological analysis of the ARA arterial wall was as follows:

– thawing protocol 1 (thawing at room temperature of 23°C): 6 (100%) non-coronary AV leaflets showed loss of the endothelial cells covering the basal membrane with no damage to the basal lamina (score 5) (Fig. 1);
– thawing protocol 2 (water bath at 37°C): 5 (83%) non-coronary AV leaflets showed loss of the endothelial cells covering the basal membrane with no damage to the basal lamina (score 5); 1 (17%) non-coronary AV leaflet showed significant damage to the basal membrane (score 6) (Fig. 2).

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

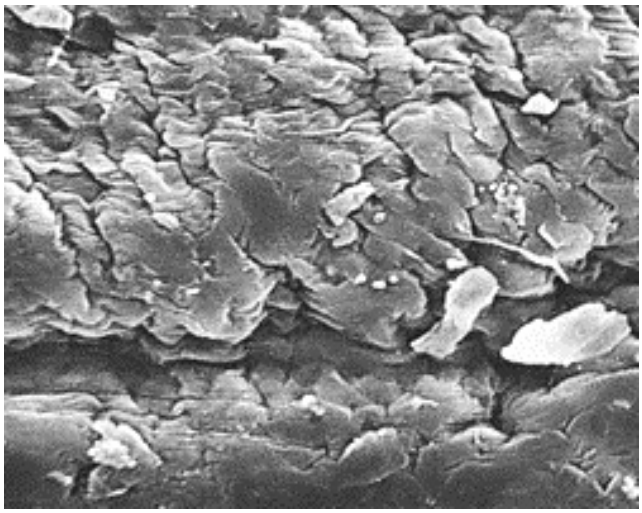

Fig. 1. Non-coronary AV leaflet (magnification: ×520); thawing at a room temperature (23°C)

AV – aortic valve.

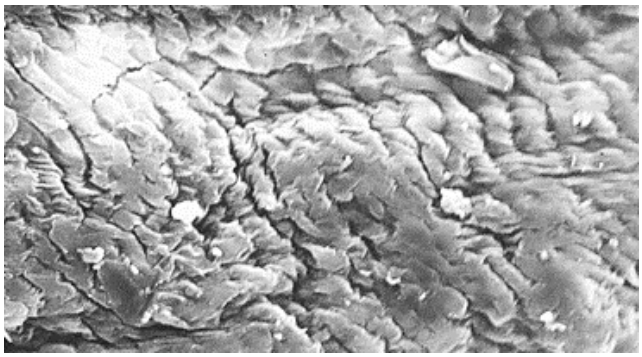

Fig. 2. Non-coronary AV leaflet (magnification: ×520); water bath at 37°C

AV – aortic valve.

Discussion

Since the first successful AV allograft transplant performed by Ross in 1962, over 25000 AV allografts have been implanted to date.[13] Over the time, the procedures of sterilization and storage have evolved immensely; from fresh aseptic harvest with immediate transplantation, through antibiotic sterilization and wet storage at 4°C, up to current antibiotic sterilization and cryopreservation.[14] Even though the durability

of fresh AV allografts is superior to cryopreserved AV allografts, the lack of donors has forced most of the cardiac centers to focus on allograft cryopreservation. Cryopreservation plays a major role in the degeneration of AV allografts, which subsequently leads to progressive calcification and fibrosis, affecting up to 1/4 of all implanted AV allografts.[13] Despite the negative impact of cryopreservation on AV allografts, Fukushima et al. showed that cryopreserved AV allografts were durable for over 15 years.[15] They also showed that allograft durability was closely associated with and affected by obesity and age of the recipient and donor. The most important factor was the surgical technique used during the allograft transplantation.[15] Our experimental results show identical structural changes in both examined thawing protocols; therefore, a faster rate of thawing theoretically does not necessarily mean that AV leaflets will be more structurally damaged or compromised; they would not require more frequent observation after implantation.

Another aspect that is thought to contribute to the cryopreserved AV allografts failure is gender mismatch. However, evidence behind this theory is imprecise, as gender matching is not done routinely before such transplants. Böll et al. demonstrated that gender-mismatched vs gender-matched allografts showed no significant difference in regard to death, need for reoperation and allograft function.[16]

Experimental work by Brockbank et al. showed significantly reduced extracellular matrix damage and well-preserved cellular structures in ice-free leaflets. They also demonstrated that cryopreservation of the transplants of the heart valves at –80°C prevents ice formation, and tissue cracking, and preserves extracellular matrix.[8,17] Improvements in modern antibiotic treatment of AV allografts before cryopreservation have had a significant impact on the infection resistance of AV allografts, as shown in their enhanced bacterial resistance.[18]

The use of cryopreserved allografts has become a gold standard in surgical procedures, such as Ross procedure, or in cases of bacterial endocarditis. However, there is growing evidence that decellularized engineered allografts may be superior to cryopreserved allografts.[19] Decellularized AV allografts have shown outstanding mid-term results after their implantation in terms of their stable structural integrity, low rate of calcification and hemodynamic properties.[20] Despite the promising short and mid-term results, long-term results are still not known.

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

Conclusions

Our experimental work, based in structural changes occurring during different thawing protocols in cryopreserved AV leaflets, showed that different rates of thawing indicated identical structural changes. Therefore, the rate of thawing does not play a significant role in minimizing structural changes that occur during the thawing of cryopreserved AV leaflets.

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Histological Composition and Mechanical Properties of Cryopreserved Samples of Aortic and Pulmonary Valves

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Abstract. Human cryopreserved allografts of pulmonary and aortic valves are routinely used as total valve replacement. For successful surgery it is needed to sufficiently preserve biomechanical properties and histological structures of allografts. However, it is not known how the mechanical properties of these allografts relate to their histological composition. The aim of our study was to compare the histological composition and mechanical properties of the valves. From allografts we prepared 2 valve cusps and samples of aorta or pulmonary trunk. In a previous study we had measured following parameters: ultimate stress, ultimate strain, Young's moduli of elasticity, intima-media thickness, wall thickness, area fraction of elastin and area fraction of collagen in the whole wall. We found weak positive correlation between ultimate stress and Young's modulus in small and large deformation with wall thickness in the valve cusps. In the arteries we found positive correlation between Young's modulus in large deformation with intima-media thickness and ultimate strain with intima-media thickness and area fraction of collagen, and negative correlation between ultimate strain with area fraction of elastin. In our study we quantified also the other components of wall with mechanical significance, such as the fraction of smooth muscle cells and chondroitin sulfate, which belong to glycosaminoglycans. We did not find correlation between these components and mechanical properties of these valves. Therefore, it is recommended to perform both mechanical and histological analysis to further characterize cryopreserved allografts.

Introduction

Human cryopreserved allografts of pulmonary and aortic heart valves and the roots of these major elastic arteries are routinely used for valve replacement. Together with surgical technique, biomechanical sufficiency and preservation of the histological structures of the grafts are considered to be necessary for a proper outcome for reconstructive surgery [1].

The aortic and pulmonary valves prevent the reversal of blood flow to the heart ventricles in diastole. Valve function normally relies on 3 cusps, as well as the annular dense collagenous connective tissue and the arterial root geometry. The cusps have similar semilunar shapes. The valves are primarily composed of endothelium and connective tissue cells and extracellular matrix components of connective tissue. The main constituents of the connective tissue are collagen fibrils, elastic fibres and proteoglycans, such as chondroitin sulfate (CS) [2].

Elastic arteries such aortic root and pulmonary trunk receive blood flow directly from the heart ventricles in systole. Elastic arteries consist of three layers as follows: (i) The tunica intima, which

is composed of the vascular endothelium and a subendothelial layer; (ii) the tunica media, which is the thickest layer, which contains concentrically elastic fenestrated membranes and smooth muscle cells; (iii) the tunica adventitia, which is the most abluminal layer of the elastic arteries. It is composed of elastic and collagenous fibres that are interspersed with connective tissue cells, blood vessels (the vasa vasorum) and nerve fibres (the nervi vasorum).

The microstructure and the mechanics of the mitral valve have been thoroughly studied [3,4], but similar results for cryopreserved aortic and pulmonary valves are lacking in the literature.

Our hypotheses were as follows:

H_A: There is no difference between the area fraction of smooth muscle actin in wall *(A_A(SMA, wall)* of aortas and *A_A(SMA, wall)* of pulmonary trunks; there is no difference between the area fraction of chondroitin sulfate in wall *(A_A(CS, wall)* of aortas and *A_A(CS, wall)* of pulmonary trunks; there is no difference between *A_A(CS, wall)* of cusps of aortic valves and *A_A(CS, wall)* of cusps of pulmonary valves.

H_B: There is no correlation between *A_A(SMA, wall)* or *A_A(CS, wall)* and the mechanical properties of the aortas and the pulmonary trunks; there is no correlation between *A_A(CS, wall)* and mechanical properties of the aortic and the pulmonary cusps.

Materials and methods

Samples for our study were obtained from Heart Valve Bank of the Department of Transplantation and Tissue Banking, University Hospital Motol, Czech Republic after their expiration (five years). The allograft heart valves are cryopreserved at -196 °C according to the established protocol [5]. We prepared eight rings of aortas, eight rings of pulmonary trunks, twelve cusps from aortic valves and twelve cusps from pulmonary valves.

Mechanical loading

All specimens were 5 mm wide. The 5 mm wide circumferential strips of the cups were loaded at room temperature by a uniaxial tensile test using a Zwick/Roell Z50 traction machine equipped with a 1-kN load cell to obtain the mechanical parameters, namely Young's moduli of elasticity, ultimate stress and ultimate strain. Uniaxial ring test was used for measuring mechanical properties of elastic arteries. The Young's moduli of elasticity were determined in the linear regions of the stress-strain curve, namely in the small deformation region and in the large deformation region, using linear regression. The strains were approximately 0-20% and 40-60% for elastic arteries (0-10% and 8-60% for cusps) for the two regions, respectively, depending on the shape of the curve. The ultimate stresses and the ultimate strains were determined at the start of the rupture. The stress was defined as force divided by initial area. The strain was defined as the actual circumference change divided by its initial circumference for elastic arteries, respectively, as the elongation of the specimen divided by its initial length for cusps. Our own software – Elfpy [6], was used for the evaluation.

Histological analysis

Tissue samples were fixed with 4% buffered formalin and embedded in paraffin blocks. In each sample, two 5-μm-thick histological sections of elastic arteries were processed immunohistochemically in order to reveal the presence of smooth muscle actin (SMA) and two sections of elastic arteries or cusps of valves were processed immunohistochemically in order to reveal the presence of CS. Antibodies and protocols used for immunohistochemistry are in Table 1.

Table 1. Antibodies, their dilution, technique of pretreatment and detection of immunoreactions used for immunohistochemistry.

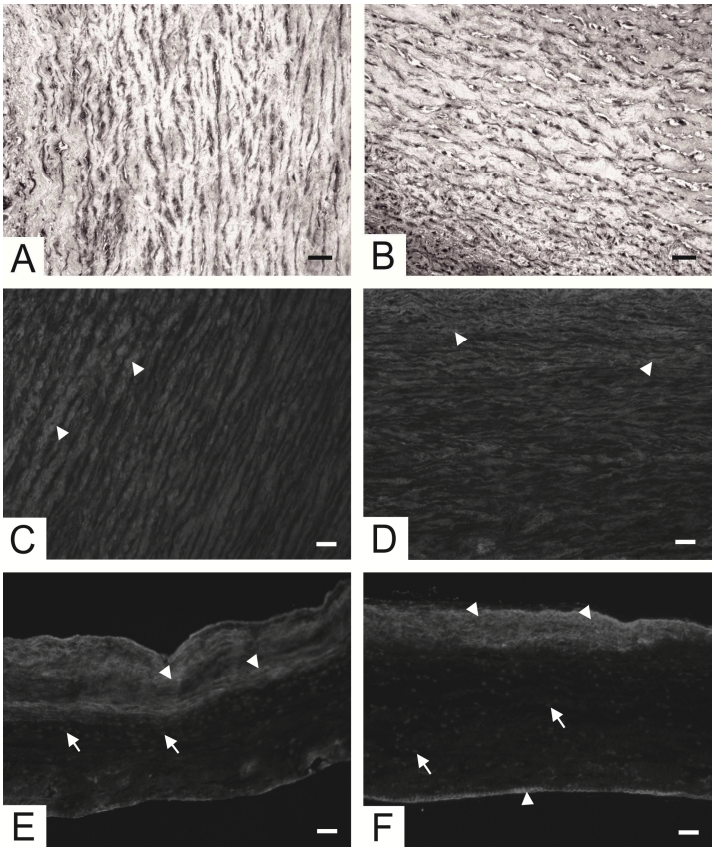
Antibody	Dilution	Pretreatment	Detection of immunoreaction
Monoclonal Mouse Anti-Actin (Smooth Muscle) Antibody Clone 1A4 (M0851, Dako, Glostrup, Denmark)	1:200	20 minutes with Target pretreatment (Dako, Glostrup, Denmark) at 96°C	Immununoperoxidase technique by N-Histofine (Nichirei Biosciences, Tokyo, Japan) vizualitation reaction by diaminobenzidine (Dako, Glostrup, Denmark)
Monoclonal Mouse Anti-Chondroitin Sulfate Antibody Clone CS-56 (C8035, Sigma Aldrich, Missouri, USA)	1:500	5 minutes with Proteinase K (Dako, Glostrup, Denmark)	Immunofluorescence technique by secondary antibody Goat Anti-Mouse IgG H&L (Alexa Fluor® 488) (ab150117, Abcam, Cambridge, UK)

Eight microphotographs were taken from each sample and each method. The area fraction of SMA and CS were evaluated using a stereological grid point [7]. All quantitative estimates were calculated using well established stereological methods [7] and Ellipse software (ViDiTo, Košice, Slovakia). The data were processed using STATISTICA 10 (StatSoft, Inc., Tulsa, OK, USA). The results had normal distribution. The t-test was used when comparing samples.

Results

We did not find significant difference between $A_A(SMA, wall)$ of aortas and $A_A(SMA, wall)$ of pulmonary trunks. No significant difference was found between $A_A(CS, wall)$ of aortas and $A_A(CS, wall)$ of pulmonary trunks. Histological comparison of elastic arteries is in Figure 1A-D. We did not find correlation between mechanical properties and histological composition in aortas or pulmonary trunks.

We did not find significant difference between $A_A(CS, wall)$ of cusps of aortic valves and $A_A(CS, wall)$ of cusps of pulmonary valves. Histological comparison of cusps is in Figure 1E-F. We did not find correlation between mechanical properties and histological composition in cusps of aortic or pulmonary valves.



Summary

Histological composition of aortic and pulmonary valves did not correlate with their mechanical properties. Therefore, characterization of these allografts during cryopreservation requires both histological assessment as well as mechanical tests.

Fig. 1 Comparison of aortic and pulmonary valves components using immunochemical reaction. **A** Smooth muscle cells contain smooth muscle actin (dark objects in wall of aorta; **B** smooth muscle cells (dark objects) in wall of pulmonary trunk; **C** chondroitin sulfate (white arrowheads) in wall of aortas; **D** chondroitin sulfate (white arrowheads) in wall of pulmonary trunk; **E** chondroitin sulfate (white arrowheads) in wall of cusps of aortic valves; **F** chondroitin sulfate (white arrowheads) in wall of cusps of pulmonary valves; nuclei of cells (white arrows), scale 50 μm.

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