Background: The objective of this study was to describe a small animal aortic conduit model that could analyze long-term conduit valve (CV) function by echocardiography.

Methods and Results: Recipient Wistar rats (200–250 g, n=20) underwent aortic leaflet injury of their native aortic valve under echocardiographic control. After 2 weeks, U-shaped decellularized CVs obtained from other rats were implanted onto the abdominal aorta. Implanted CVs were analyzed via pulsed-wave echocardiography at day 0, 4 and 12 weeks. CV stenosis was assessed as systolic flow velocity (post-pre CV)/flow velocity in the ascending aorta. CV regurgitation was assessed as the ratio of the amount of reversed diastolic flow to forward systolic flow in post-pre CV. The endpoint was set at 12 weeks. Three rats died immediately after aortic valve injury and all surviving rats received CV implantation (n=17, 85%). The survival rate after conduit implantation was 100% at 4 weeks and 88% (15/17) at 12 weeks. Regarding the CV function at 0, 4 and 12 weeks, the average observed value of CV stenosis was 3.8±7.9%, 3.1±4.1% and 14±10% (P<0.01), respectively. The average value of CV regurgitation was 0%, 12±27% and 52±43%, respectively (P<0.001).

Conclusions: By using this model, the degeneration of implanted CV could be assessed not only qualitatively, but also quantitatively. (Circ J 2013; 77: 2295–2302)

Key Words: Echocardiography; Small animal model; Valves

Cardiovascular calcification remains a global health burden with unmet challenges to health-care systems globally. Currently available aortic valvular prostheses and aortic valve conduits have several limitations. Mechanical valves require lifelong anticoagulation, which might cause bleeding or thromboembolic complications and bear a risk for prosthetic infection, and tissue valves are prone to progressive degeneration. Tissue engineering might bear promising solutions for overcoming the limitations of biological and mechanical heart valve substitutes. One popular concept of heart valve tissue engineering bases itself on decellularized biological matrices. The removal of cellular components is practiced to reduce immunological reactions, which are thought to be responsible for accelerated valvular graft deterioration, and for subsequent repopulation of the transplanted valves with autologous cells. We and others have performed heart valve implantation studies in vivo using large animal models, involving decellularized valves in the pulmonary and in the aortic position. Similarly, several groups have published results about small animal models of heterotopic aortic valve interposition, as well as end-to-side transplantation. However, in small animal aortic position models, the physiological function of the implanted valves remains questionable.

In order to preserve the functional hemodynamic load on a heterotopic aortic valve implant, there must be some turbulence and reversal of blood flow in the aorta that permits leaflet closure. Légaré et al. have previously reported on the experimental creation of native aortic regurgitation (AR) for allowing flow reversal in the abdominal aorta in rats. However, a major problem observed in this model is the rather high mortality rate due to cardiac failure associated with the acute AR. In addition,
Creation of AR was performed with a modification to a previously reported method. In anesthetized animals, a right lateral neck incision was used to expose the right carotid artery. The distal common carotid artery was ligated with 4-0 nylon suture. An arteriotomy was then performed to allow the insertion of a 0.9-mm guide wire. The echocardiographic probe was positioned on the thorax to obtain a good view of the left ventricle, the aortic valve and the ascending aorta equivalent to a parasternal long-axis view in standard echocardiography in humans. Under continuous echocardiographic guidance, an arterial leader catheter was advanced towards the aortic valve in a retrograde manner (Figure 1A). The sonographer guided the position and advancement of the catheter while it was pushed through a leaflet of the aortic valve into the left ventricle (Figure 1B). The resulting tear in the leaflet induced an acute AR. AR was considered moderate in the instance of following echocardiographic criteria at the time of surgery; color Doppler ratio of the regurgitation jet width to LVOT diameter was 50–70%, and pulsed-wave Doppler confirming reversed diastolic flow in the abdominal aorta. Leaflet perfusion was repeated when the severity of the regurgitation jet in the abdominal aorta was considered insufficient by echocardiographic criteria (Figures 2A, B). When the generation of AR was completed, the proximal carotid artery was ligated with 4-0 nylon sutures.

**Donor Graft Harvesting and Engineering**

The harvesting of the donor graft was conducted as we have previously described. In brief, donor rats (n=17) were euthanized by CO2 insufflation. After thoracotomy, the heart and the conduit valve (CV). Moreover, because the implanted CV is too small in these small animal models, one of the major limitations is the difficulty to perform measurements by directly targeting the CV for a quantitative analysis of the valve function, that is, as it is regularly done in the clinical field.

Considering these studies, we hypothesized that a further improvement of the described aortic conduit model with controlled AR, generated by using improved echocardiographic techniques during the operation and the follow-up time, might more soundly: (1) reduce short- and long-term mortality; (2) demonstrate a complete closure of the valve; and (3) allow for a quantitative analysis of the CV function. We tested this hypothesis using our standardized small animal aortic conduit implantation model.

**Methods**

**Animals**

Male rats (donors: Lewis, recipients: Wistar, n=20) weighing 200–250 g were purchased from the in-house breed of animals from the local animal care facility and used for the investigation. All experiments were approved by the State animal care committee and conducted according to the National Animal Welfare Act. During the whole study, all animals were fed with a procalcific diet supplemented with 300,000 U/kg vitamin D, 1.5% calcium phosphate and 2% cholesterol.

**Echocardiography**

The recipient rats were intubated and anesthetized with 2.0–2.5% isoflurane. Standard 2-dimensional, M-mode, color- and pulsed-wave Doppler transthoracic and transabdominal measurements were conducted with a Philips HDX11 ultrasonography system equipped with a 15 MHz transducer. Pulsed-wave Doppler recordings were taken. Left ventricular (LV) dimensions, ejection fraction (EF), ratio of AR jet width to LV outflow tract diameter (AR jet/LVOT), ratio of time-velocity integral of reversed diastolic flow to forward systolic flow (RVTI), and maximum forward systolic flow velocity (MSV) in the ascending thoracic (Asc. Ao) and the abdominal aorta (Abd. Ao) were evaluated as previously reported. All images were recorded digitally for later analysis.

**Creation of AR**

Creation of AR was performed with a modification to a previously reported method. In anesthetized animals, a right lateral neck incision was used to expose the right carotid artery. The distal common carotid artery was ligated with 4-0 nylon suture. An arteriotomy was then performed to allow the insertion of a 0.9-mm guide wire. The echocardiographic probe was positioned on the thorax to obtain a good view of the left ventricle, the aortic valve and the ascending aorta equivalent to a parasternal long-axis view in standard echocardiography in humans. Under continuous echocardiographic guidance, an arterial leader catheter was advanced towards the aortic valve in a retrograde manner (Figure 1A). The sonographer guided the position and advancement of the catheter while it was pushed through a leaflet of the aortic valve into the left ventricle (Figure 1B). The resulting tear in the leaflet induced an acute AR. AR was considered moderate in the instance of following echocardiographic criteria at the time of surgery; color Doppler ratio of the regurgitation jet width to LVOT diameter was 50–70%, and pulsed-wave Doppler confirming reversed diastolic flow in the abdominal aorta. Leaflet perfusion was repeated when the severity of the regurgitation jet in the abdominal aorta was considered insufficient by echocardiographic criteria (Figures 2A, B). When the generation of AR was completed, the proximal carotid artery was ligated with 4-0 nylon sutures.
Thoracic aorta were dissected from surrounding tissue. Using a stereomicroscope (Nikon, Duesseldorf, Germany), a U-shaped aortic conduit with a small myocardial cuff was prepared. The coronary arteries were ligated by using a 8-0 monofilament (Ethicon, Norderstedt, Germany). Soon after the harvesting, the aortic graft conduits were decellularized according to a detergent-based protocol consisting of 4 cycles of 12 h incubation with 0.5% sodium dodecyl sulphate, 0.5% sodium deoxycholate and 0.05% (g/v) sodium azide (Sigma-Aldrich, Taufkirchen, Germany), followed by 24 h rinsing with distilled water containing 0.05% sodium azide, and 3 subsequent cycles of 24 h with PBS containing 1% penicillin/streptomycin.

**Graft Implantation Procedure**

Two weeks after the induction of AR, the recipient rats (n=17) were intubated, anesthetized with 2.0–2.5% isoflurane, and a central venous jugular vein catheter was inserted. The graft implantation procedure was performed as previously described. All animals underwent a median laparotomy, lateralization of intestines, and dissection of the infrarenal aorta from the inferior vena cava. After systemic administration of 300IU/kg heparin and aortic clamping, a U-shaped, decellularized aortic conduit was sutured to the infrarenal aorta in an end-to-side manner, using a 10-0 monofilament, non-absorbable polypropylene suture (Ethicon, Norderstedt, Germany). Intermittent reperfusion was used to keep limb ischemia times below 30min. Following the release of blood flow through the conduit, the native aorta between the 2 anastomoses was ligated to improve perfusion of the implant.

**Functional Evaluation of the Implant**

Before recovery from the anesthesia and after completion of the implantation, the function of the implanted CV was examined by using a novel improved method by transabdominal aortic color- and pulsed-wave Doppler.

\[ \text{CV regurgitation grade} = \frac{(\text{RVTI in post-conduit})}{(\text{RVTI in pre-conduit})} \]

Peptide Productions and Biological Functions

**Figure 2.** Control of the aortic regurgitation (AR) grade. Both images were taken during the operative generation of AR. (Upper panel) Color Doppler of the ascending aorta. (Middle panel) Pulsed-wave Doppler of the ascending aorta. (Lower panel) Pulsed-wave Doppler of the abdominal aorta. (A) After the insertion of the catheter through the left ventricular outflow tract (LVOT), the resulting tear in the leaflet induced an acute AR in the ascending aorta. But many cases did not reach moderate AR and sufficient reversed diastolic flow in the abdominal aorta by a single perforation (R1 arrow). (B) After repeated attempts for the creation of AR, we could confirm ample reversed diastolic flow in the abdominal aorta (R2 arrow). P-wave Doppler, pulsed-wave Doppler; Asc. Ao, ascending aorta; Abd. Ao, abdominal aorta.
All statistical comparisons were performed using Stat View J-5.0 software (SAS Institute, Cary, NC). All values are expressed as the mean ± standard deviation. Comparisons between the 2 groups were made using an unpaired t-test for parametric values. By simple linear regression analysis, the correlation of the color Doppler ratio of regurgitation jet width to LV outflow tract diameter was evaluated using these comparisons. Differences were considered statistically significant at P<0.05. Estimation of survival was performed with Kaplan-Meier’s curves with a 95% confidence interval.

Results

Creation of AR in the Recipients

Catheter-induced aortic valve injury resulted in significant acute AR with a large volume of reversed diastolic flow in the abdominal aorta in all animals. AR was graded as moderate (in...
n=16, 80%) to severe (in n=4, 20%) on the basis of the width of the AR jet. Three rats were subsequently euthanized (n=2 after 1 day, n=1 after 3 days) because of clinical deterioration caused by acute congestive heart failure (15% short-term mortality). Based on the acute survival result, animals were divided into 2 groups; the acute death group (n=3) and the surviving group (n=17). Serial measurements recorded are shown in Table 1. Follow-up echocardiography data. All surviving rats showed movement of the conduit valve at all follow-up times, but 5 rats (29%) at 4 weeks and 14 rats (93%) at 12 weeks appeared with new conduit valve regurgitation. MSV, maximum forward systolic flow velocity; pre-conduit, pre-conduit abdominal aorta; post-conduit, post-conduit abdominal aorta. All other abbreviations as per Table 1. Values are mean ± SD. †P<0.05 vs. AR, *P<0.05 vs. implantation, **P<0.05 vs. 4 weeks.

No significant differences were observed regarding the operation time and LV echocardiography data between the 2 groups. With respect to AR, the animals in the death group showed a significantly greater severity than those in the surviving group (color Doppler ratio of regurgitation jet width to LV outflow tract diameter and number of rats with severe AR, P<0.001). There was a significant difference in the ratio of time-velocity integral of reversed diastolic flow to forward systolic flow in the descending aorta; RVTI in Abd. Ao, ratio of time-velocity integral of reversed diastolic flow to forward systolic flow in the abdominal aorta).
at the time of AR creation. The reason for this observation might be that RVTI in the abdominal aorta does not correlate well (r=0.50) with the AR severity in the moderate AR group.

**Detection of Effective AR in Recipients’ Abdominal Aorta**

In order to analyze the relationship between AR severity and reversed flow in the abdominal aorta in the recipient rats, single line analysis was performed. We have established the correlation of color Doppler ratio of regurgitation jet width to LV outflow tract diameter and ratio of time-velocity integral of reversed diastolic flow to forward systolic flow in the ascending aorta (Figure 4A) or in the abdominal aorta (Figure 4B) at the time of AR creation. The reason for this observation might be that RVTI in the abdominal aorta does not correlate well (r=0.50) with the AR severity in the moderate AR group.

**Implantation Operative Results**

Seventeen surviving rats received an infrarenal decellularized CV implant. The implantation was performed with a mean operative duration of 128±15 min. Aortic cross-clamp times for the proximal anastomosis varied between 20 and 35 min (23±5 min), and aortic cross-clamp times for distal anastomosis ranged from 10 to 25 min (18±4 min). All animals recovered without signs of neurological problems and/or ischemic symptoms of the lower limbs.

**Figure 5.** Evolution of conduit valve (CV) destruction. The function of the implanted CV was quantitatively examined using our new method of aortic color and pulsed-wave Doppler analyses. The left column depicts the results obtained at implantation (Imp); the middle column depicts the results obtained at 4 weeks after implantation (4wks); and the right column demonstrates results after 12 weeks (12wks). NS, no significant difference between these 2 groups. (A) At implantation, all CV showed complete functional competence and no regurgitation. However, 5 rats at 4 weeks and 14 rats at 12 weeks after implantation were revealed to have developed new CV regurgitation. At analysis of these regurgitation phenomena, there was a significant difference between the 2 follow-up points (**P<0.01, ***P<0.001). (B) In the first 4 weeks after implantation, the average CV stenosis grade remained stable, whereas at the time point of 12 weeks after implantation, there was a significant increase as compared to previous time points (*P<0.05, **P<0.01).

**Figure 6.** Hematoxylin/eosin (A), Movat’s pentachrome (B) and von Kossa staining (C) of cross sections of decellularized aortic conduit valves 12 weeks after implantation. Calcification of the leaflets as well as the aortic sinus wall was observed.
Long-Term Survival

All rats survived the implantation of conduits for 4 weeks (100%). At 12 weeks, however, the survival rate decreased to 88%. A detailed investigation of the effect of controlled moderate AR on survival for short- and long-term periods was performed, comparing the subgroups with severe AR (Group S; n=4, 20%) and moderate AR (Group M; n=16, 80%) with Kaplan-Meier’s curves (P<0.001). At both 4 and 12 week time points, the actual survival was significantly higher in the moderate AR group as compared to the severe AR group (Group S: 25% vs. Group M: 100% at 4 weeks and Group S: 0% vs. Group M: 90% at 12 weeks, P<0.001; Figure S1).

Evaluation of Long-Term LV and CV Function

Table 2 summarizes the long-term echocardiography data. EF was significantly different between the time point of the creation of AR and the 4 and 12 weeks follow-up time points, but there were no significant differences between implantation and the 2 follow-up time points.

After 4 and 12 weeks following implantation, all surviving rats showed movement of the CV leaflets. With regard to the CV function, 5 rats at 4 weeks and 14 rats at 12 weeks appeared to develop a new CV regurgitation with an average valve regurgitation of 0.12±0.22 at 4 weeks and 0.66±0.26 at 12 weeks according to the applied definition (P<0.05). Average CV stenosis was demonstrated to be 0.04±0.07, 0.03±0.04 and 0.19±0.15 at each follow-up point (P<0.05; Figure 5). Representative cross sections through the CV 12 weeks after implantation showed partially preserved and partially calcified leaflets (Figure 6). Moreover, due to the severely procalcific diet, the aortic wall of the conduits exhibited progressive calcification during follow up (Figure S2).

Discussion

In the present study, a standardized small animal model of heterotopic aortic conduit implantation allowing for preserved implant valve function was developed. This goal was achieved by echocardiography-controlled generation of moderate AR of the native aortic valve, additionally resulting in reduced short- and long-term mortality. The functional preservation of the implant valve makes our model suitable for small animal studies of native as well as tissue-engineered heart valve prostheses.

Aortic valve allografts (AVA) have been used for more than 50 years with satisfactory clinical outcome. Although cryopreservation is widely accepted as the gold standard method for processing and storage of AVA, novel technologies, such as decellularization and tissue engineering have been introduced to overcome the limitations of AVA. Clinical feasibility and safety of decellularization as a method of improvement of aortic valve implantation have been demonstrated, but comparable results are scarce. For this reason, in vivo scenarios for the preclinical analysis of decellularized AVA in well characterized small animal models present a valuable tool. Small animal models are favored in the biomedical research due to lower costs and a wide range of histological and molecular biological analytic tools.

Current rat models of infrarenal aortic valve implantation are well described and have provided important advances in our understanding of decellularized allograft failure. However, these previous studies have focused on the valve conduit rather than the leaflets, despite having leaflets present in the blood stream and despite the evidence of the leaflet movement. We consider, in many cases, these leaflets remaining not completely functional in the physiological manner. Because of this, the leaflets are prone to thrombosis against the adjacent aorta and fail usually rather early when compared to the preclinical and clinical experience with decellularized valves implanted in the orthotopic position. Thus, the availability of a functional CV model is of critical importance.

Under these premises, Légaré et al reported on an aortic conduit model involving native AR for allowing flow reversal in the abdominal aorta. We believe that this concept is the most suitable to analyze CV failure as it provides a functional valve to assess the interaction between immunological and mechanical injury in AVA failure. But this aortic conduit model involving native AR has a few drawbacks such as high mortality due to generated regurgitation and a lack of long-term evaluation. Moreover, previous reports did not evaluate complete CV function. In the present study, after analyzing the aforementioned drawbacks, we propose solutions to improve the involved problems.

One of the major limitations to the aortic conduit model with AR is the rather high mortality in the short term. The study by Légaré et al reported 3 deaths (33%) in the short term due to acute heart failure and 1 death (11%) due to sinus of valsalva aneurysm and right arterial fistula. We suspect that the outcome was due to the fact that AR in their study was created not under continuous echocardiographic guidance. Arsenault et al reported aortic valve leaflet injury models using echocardiography for studying congestive heart failure in the presence of severe AR. Echocardiography was straightforward in the rat AR model during their procedure, as is the case in studies on the native (orthotopic) heart. The perforating catheter was easily identified and controlled, thus the AR was clearly visible. This might explain why in their study a low acute mortality rate was demonstrated (17%). In our current series, we found a comparable outcome, confirming the observations from the latter study with an acute mortality rate of 15% in the present study. Moreover, a high rate of controlled AR generation (76% by this study for generating severe AR and 80% by our study to generate moderate AR) became feasible, when high quality live echocardiography imaging was used. Based on these results, we therefore suggest that echocardiography should be used during aortic valve leaflet injury via a catheter.

A second major limitation generally adherent to the presented model is its unsuitability for long-term follow-up studies because of the creation of AR, which often results in progressive congestive heart failure from volume overload. All severe AR rats in our study died during the follow-up period suggesting that possibly creating a lower degree of AR would have been a solution to avoid this problem, which then could enable long-term follow-up studies to be performed.

However, on the other hand, a lower AR grade could not preserve CV function in our study. Tani et al reported in their study that the presence of reversed flow in the abdominal aorta suggested an AR greater than a moderate grade. In our study, single-line analysis was performed (Figure 4), which confirmed that if the rats demonstrated an AR greater than a moderate grade, we sometimes could not find an increased reversed flow in the abdominal aorta. Following this report and our analysis, we hypothesized that moderate AR is not only enough to obtain an acceptable result for implanted valves, but it might also be necessary to reduce the long-term mortality. Here we summarize our positive results for the approach of controlled moderate AR: (1) achievement of a fine 12 weeks’ long-term survival (88%); and (2) successful accomplishment of CV function.

Regarding CV function, previous reports have demonstrated only the movement of CV leaflet by transabdominal M-Mode.
echocardiography with direct imaging of the CV.\textsuperscript{9,11} Otherwise, in these small animal models, one of the major limitations is the difficulty to perform measurements by directly targeting the CV for a quantitative analysis of the valve function, either by measuring the regurgitant fraction or the venous outflow. Our study is the first report involving a small animal model to evaluate CV regurgitation and stenosis via pulsed-wave Doppler measurements in the recipient abdominal aorta in positions post and pre CV. The goal of our study was to analyze CV function in a greater depth and in a longitudinal follow-up manner. We believe that these echocardiographic measurements should be used in aortic conduit studies because of their relatively easy and low invasive application, providing a highly valid and reproducible assessment of the valve function.

In most of the explants, calcification of the valve was observed after 12 weeks. The main underlying cause for the fast implant calcification is the application of a procalcific diet resulting in accelerated cardiovascular calcification, which is supposed to have contributed to the partial destruction of the implant valves after 12 weeks. However, the procalcific diet allows for examination of prosthesis degeneration in short time periods. Combined with the present small animal implantation model of echocardiography-controlled moderate AR of the native valve, we present a tool worthwhile to elucidate and influence pathophysiological mechanisms involved in the degeneration of aortic valve prostheses, which will be subject to future studies.

**Study Limitations**

There are several limitations to the present study. The findings of this study are based on a limited number of small animals and certainly need further validation in upcoming trials, ideally involving other animal species. From the clinical standpoint, the herein employed model of heterotopic valve implantation should be considered with caution as it does not entirely reflect the hemodynamic scenario of orthotopically implanted valve prostheses. Although this small animal model provides a number of advantages for the preclinical screening and in vivo evaluation of novel engineered heart valves, the results should be further validated in animal models of orthotopic valve implantation. Considering very recent technological developments, a further enhancement of the employed echocardiography instruments, for example, by using systems equipped with 30 or 35 MHz transducers, might provide superior imaging quality and thereby probably even further improve the study results, as it has been suggested for the clinical scenario.\textsuperscript{22}

**Conclusion**

Generation of controlled moderate AR using echocardiography can effectively reduce short- and long-term mortality in our standardized combined AR and conduit implantation model. In addition, by assessing the reversed diastolic flow behind the implanted CV, a quantitative analysis of the CV function was demonstrated. Therefore, we conclude that this model might represent an ideal model for studying aortic CVs in small animal models.

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**Supplementary Files**

**Supplementary File 1**

**Figure S1.** Effects of controlled moderate aortic regurgitation (AR) on short- and long-term survival in a completely functional conduit valve implantation model.

**Figure S2.** von Kossa staining of cross sections of the aortic conduit wall 4 weeks (A) and 12 weeks (B) after implantation. Please find supplementary file(s) at http://dx.doi.org/10.1253/circj.CJ-12-1439