THESES OF DOCTORAL (PhD) DISSERTATION

ADAPTATION OF A LARGE ANIMAL INFARCTION MODEL TO PANNONIAN MINIATURE PIGS

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1 Research Background and objectives

According to the latest report of the European Union in 2020, nearly 10 million experimental animals are used in research projects every year. 92% of this is laboratory rats and mice, fish, and about 70,000-80,000 are experimental pigs. 90% of the experimental animals used are bred for experimental purposes by registered breeders, which is in line with the objectives of the EU Directive and the principle of 3R (replacement, reduction, refinement) (EUR-Lex, 2020). The most important requirement for experimental animals used in medical research is that their anatomical and physiological parameters are similar to those of humans. The pig meets this expectation in many ways. In recent decades, pigs have become extremely important laboratory animals in several fields of science and the use of pigs is practically unavoidable in cardiovascular research (Smith et al., 2006).

Traditional meat type pigs – typically breeds and hybrids belonging to the large white and landrace breed groups have the body sizes at a young age, 3-4 months, which make them suitable for modeling the human circulatory system. The main limiting factor of their use is rapid increase in body size, which makes experiments that can only be carried out on chronic and adult individuals impossible, or difficult to carry out. Such are e.g. stent implants, when fixed-sized implants fixed in the coronary arteries have to remain in the blood vessels of the same size for a long time, or myocardial infarction research. In these cases, the physical dimensions of pigs weighing up to 100 kg make diagnostic procedures such as magnetic resonance imaging (MRI) impossible to perform. In animal model experiments requiring a long

follow-up, the use of ancient pig breeds with slow growth vigor, such as the Mangalica, can be a solution. However, in the case of Mangalica, the increase in size is also significant, which allows, for example, the follow-up of an implant placed in a blood vessel for 6-12 months. This is one of the reasons why it is justified to carry out animal experiments involving miniature pigs, so-called minipigs.

Several animal studies related to myocardial infarction have been carried out worldwide in recent decades, in which the KMOK Dr. József Baka Diagnostic Oncoradiology Research and Education Center. Over the past 3 decades, the Institute has carried out hundreds of cardiovascular animal model projects on pigs of different genotypes (meat hybrids, Mangalica, Göttingen miniature pigs, Pannonian minipigs). These cardiac catheterization and imaging diagnostic tests have already confirmed several physiological and anatomical differences between genotypes and have also raised new questions (Petrási, 2002; Petrási, 2008; Petrási et al., 2008). In my research, we carried out studies that mapped the usability of Pannonian miniature pigs in cardiovascular research and their influencing properties. During my research, I set the following goals:

1. Investigation of the suitability of a Hungarian minipig breed bred for experimental purposes in the infarction model used in preclinical research on meat hybrid pigs.
2. Examination of breed-specific characteristics of miniature pigs during angiographic examinations and MRI examinations.
3. Examination of cardiac function parameters of minipigs by MRI.
4. Examination of blood parameters and heart-specific necroenzymes of miniature pigs.
5. Investigation of the response of miniature pigs to AMI (acute myocardial infarction) induction.

2 MATERIAL AND METHOD

2.1 Experimental animals

In the experiment, a total of 24 healthy, 55-90 kg, 15-24 month old adult female Pannonian pigs were used, which came from a herd with registered (ENAR) and officially certified animal health status (Pannon Minipig Kft., Kiskorpád). The pedigree stock of the Pannonian miniature pig was kept in small groups (6-8 individuals), in a traditional, closed barn with a solid substrate and littered with straw. They were fed with a diet rich in fibre and low in energy, reducing the breed's tendency to gain weight similar to that of fat pigs. After day 3 of the experiment, they returned to their farm of origin during the follow-up period, where the animals were isolated, but the way they were kept and fed was similar to the previous ones (see below).

2.2 Experimental layout

The design of our experiment was of a "longitudinal research" nature, in which we planned to re-observe the same variables on each individual without randomization, using the animals as their own controls. At the same time, the aim of the study was to test the feasibility of the closed-chested ischemia-reperfusion model of large animals developed for decades in Pannonian miniature pigs. To ensure statistical independence, the animals involved were not in family relationships. All subjects underwent the same procedure, according to which the individuals underwent closed thoracic infarction induction after a baseline MRI scan, followed by a follow-up MRI scan on day 3 and day 30. Figure 1 shows the design of the experiment.

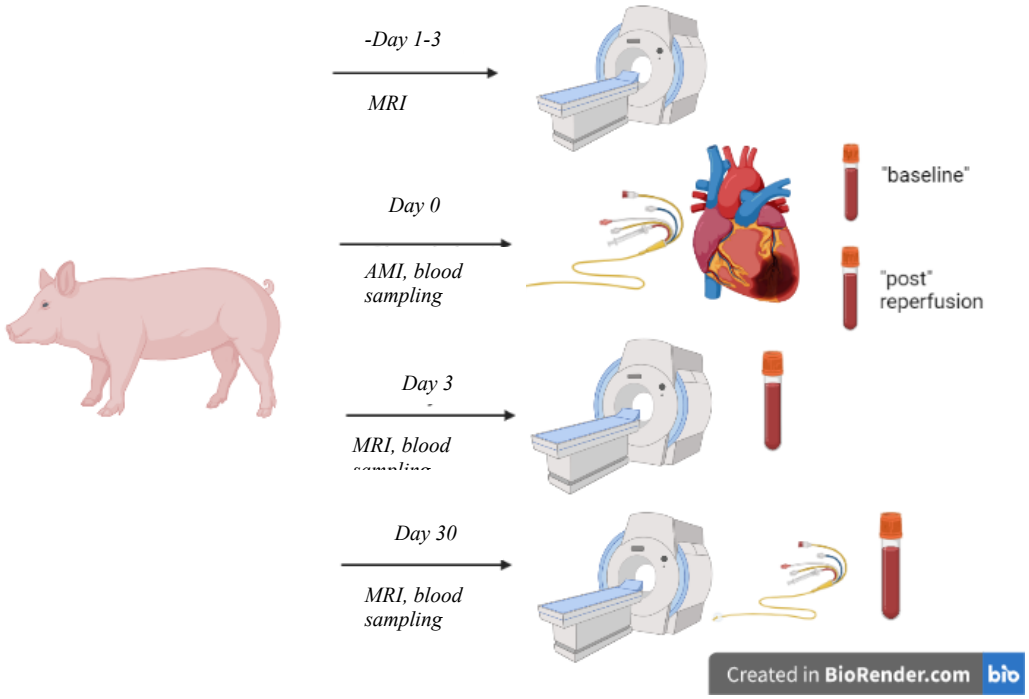

Figure 1: Experimental protocol

The same procedure was performed on all animals without treatment, the experimental unit was one individual.

2.3 Housing of animals

The animals were transported to the Institute in small groups 24 to 72 hours before the start of MRI scans. In the research area of the Institute, in an air-conditioned room, they were randomly placed in individual cages, and each individual was marked with a simply increasing series of numbers. The pigs were also marked with permanent ear tags and microchips. The animals were kept in individual cages until day 3 of the experiment to carry out medication and avoid unnecessary aggression. The animals could see and sense each other, they were in the same airspace. The individual holding and

isolation period, including the catheter intervention, was a total of 4 days, after which they were again placed in small groups.

2.4 cMRI (magnetic resonance examination of the heart)

To assess baseline left ventricular (LV) function, magnetic resonance imaging (cMRI) was performed 24 to 48 hours prior to the onset of myocardial infarction. MRI scans were necessarily performed under total anesthesia, so the animals transported to the institute one to three days before the MRI scan were fasted for 12 hours. As a first step of the anaesthesia methodology, the pigs were treated with 12 mg/kg ketamine hydrochloride (Narkamon100mg/ml injection, Bioveta; Ivanovice na Hané, Czech Republic), 1 mg/kg xylazine (CP-Xylazine 2% injection, CP-Pharma GmbH, Burgdorf, Germany) and 0.04 mg/kg atropine sulphate (Atropine sulfuricum-EGIS 1mg/ml inj., Egis; Budapest, Hungary) combination, administered intramuscularly. Inhaled anesthesia was provided for the entire duration of the imaging study with 1.5-2.5 m/m% isoflurane (Isoflutek 1000 mg/g, Karizoo; Barcelona, Spain) and 2 l/min oxygen. Veins were provided on the animals for administering medication and chest ECG electrodes were placed.

The imaging is performed with a Siemens Magnetom Vision 1.5 Tesla field strength device (Siemens AG; Erlangen) was performed using ECG-guided sequences developed in human medicine, during which we worked in short and long axis views of the heart, with an echo time of 1.2 ms, a flip angle of 50 degrees, a repeat time of 40 ms, a field of view of 300 mm and a slice thickness of 8 mm. During the recordings, we also used 32-channel bed rolls and chest rolls. During cardiac motion picture cine imaging, spontaneous respiration was stopped by intravenous administration of 0.4-0.6 mg/kg

atracrium besylate (Tracrium10mg/ml inj., GlaxoSmithKline, UK) (non-depolarizing, skeletal muscle relaxant) to improve image quality and avoid artifacts. Between two measurements, positive pressure ventilation was performed with a volume of 300 mL, a frequency of 13/min and a pressure of 25-30 mmHg until spontaneous respiration was restored.

By analyzing the images generated during the examination, numerical and quantitative measurements of the function, perfusion and viability of the left ventricle were performed. The primary goal was to collect data on the specific cardiac function parameters of the Pannonian miniature pig. The following parameters have been defined:

- EDV: Final diastolic volume
- ESV: End systolic volume
- SV: the amount of blood expelled from the heart during a systole, stroke volume.
- CO: The amount of blood transmitted by the heart in one minute.
- LVEF: The left ventricular ejection fraction.

All images were analyzed and evaluated using the freeware Segment 3.0 RXXXX (http://segment.heiberg.se) software (Heiberg et al. 2010). In our experiment, in order to eliminate subjectivity, all the recordings were evaluated by the same 3 persons (GR, VA, KD), and the final result was obtained as a consensual summary of the results obtained by these three different persons.

2.5 Pre-intervention interventions

Prior to catheter intervention, in accordance with the ESC STEMI guidelines (Steg et al. 2012, Roffi et al. 2016) and previous protocols, pigs were given 500 mg of acetylsalicylic acid (Aspirin 100 mg tablets, Bayer) and 300 mg of clopidogrel (Trombex 75 mg tablets, Zentiva) as

anticoagulant therapy, followed by 100 mg of aspirin and 75 mg of clopidogrel orally daily. mixed with feed. The catheter intervention was performed under total anesthesia. As with MRI interventions alone, the animals were given 12 mg/kg ketamine hydrochloride (Narkamon100 mg/ml injection, Bioveta) and 1 mg/kg xylazine (CP-Xylazin2% inj., CP-Pharma GmbH) and 0.04 mg atropine (Atropine sulfuricum 1mg) during premedication. /ml inj., Egis) intramuscularly into the muscles of the neck. Subsequently, isofluluran (Isoflutek 1000 mg/g, Karizoo; Barcelona, Spain) (5% vol.) and oxygen (3 l/min) were put to sleep through a face mask, then intubated and kept under isofluranic-oxygen (2% vol and 3 l/min) inhaled anesthesia for the entire duration of the intervention. After the animals were transferred to the operating table, an intravenous cannula was inserted into the ear vein, through which a ringerlactate infusion (Ringer's lactate infusion, 500 ml, Fresenius; Bad Homburg, Germany) was dosed at a rate of ~5 ml/kg/h. The miniature pigs were placed on their backs. The surgical area was isolated, and access to the femoral artery and jugular vein was provided through surgical exploration. A 6F thickness introducer (F = French = 1⁄3 mm) (St. Jude Medical, Little Canada, Minesotta) was inserted into the vessels for the introduction of the catheters used for the procedure. Electrodes were placed on the limbs and continuous monitoring of the animals' heart function with the help of ECG with Einthoven bipolar conduction. In addition, the blood pressure of the animals could be measured invasively through the arterial catheter, and the blood oxygen saturation and respiratory rate were continuously monitored with the help of a pulse oximeter. The above data were recorded in the anaesthesia documentation of the animals. For biochemical tests, blood was taken from a catheter inserted into the jugular vein, and then 5000 IU of heparin was administered through the introducer to prevent thrombus formation.

2.6 Acute myocardial infarction induction

The animals prepared as described above were transferred to the angiography room, where a selective angiographic examination was first performed on the left coronary artery, which was analyzed to plan the site of the expected coronary occlusion. During the angiographic procedure, a Siemens Cios Alpha C-arm, a Siemens Axiom Sensis hemodynamic unit and a Comen C-50V patient monitor were used. The contrast agent used in the examinations is Xenetix 350 mg l/ml (iobitridol); Guerbet), the volume of contrast was 50-100 ml/pig. AMI induction was performed under invasive hemodynamic monitoring by inserting a balloon catheter (2.5-2.75 mm in diameter, 8-15 mm in length) into the left anterior descending coronary artery (LAD) after the origin of the 2nd diagonal branch, and then the balloon was inflated at a pressure of 5-6 atm for a period of 90 min, followed by reperfusion after the balloon was deflated. Immediately prior to the induction of reperfusion, another 5000 IU of unfractionated heparin was administered intracoronarially. The reperfusion was confirmed by coronarography in all cases. Subsequently, the functions of the left ventricle were examined by contrast ventriculography using a 5F pigtail catheter. After the reperfusion, another blood sample was collected from the jugular vein. During the intervention procedure, ECG, oxygen saturation, heart rate, blood pressure and respiratory rate were continuously monitored. The data were recorded every 10 minutes on the animals' anesthesia sheets. Angiography images, blood pressure data and ECG were recorded digitally.

At the end of the procedure, the catheters and introducers were removed and the femoral artery was tied with an appropriately sized absorbable suture. The wound was sealed layer by layer, with appropriate suture material, and the surgical area was covered with a polymer spray containing aluminum. Animals should be given a broad-spectrum, long-acting antibiotic

(procaine, benzylpenicillin 5-5 mg/kg and dihydrostoreptomycin 10 mg/kg) (Shotapen inj., Virbac; Carros, France) and non-steroidal anti-inflammatory and analgesic medication (0.4 mg/kg meloxicam) (Meloxidyl inj., Ceva; Marseille, France) at the start of the surgical procedure.

The animals woke up under constant supervision, and their extubation took place when the pharyngeal reflex returned. After that, they were placed in individual cages in a room with a controlled room temperature (23 °C) until full convalescence. We observed their general condition, checked the area around the surgical wound for signs of bleeding, infection and inflammation. The animals were kept in this room, in individual cages, for the 3 days, until the control MRI scans.

Control cMRI scans were performed on day 3 (72 ± 12 hours) after MI and at the end of the 1-month follow-up period (day 30 ± 2 days). During the MRI scans, the preparation of the pigs and the execution of the examination were the same as the basic measurements. However, during the control examinations, a contrast agent, gadobutrol, was also used intravenously (Gadovist 1mmol/ml inj., Bayer, Germany) at a dose of 0.16 mmol/kg body weight to examine the function of the myocardium damaged by AMI, and the previous sequences were supplemented with late enhancement images during the cMRI scans on days 3 and 30. The basic cMRI was performed without late enhancement examination, as of course scar tissue formation in the muscles was not expected. After the control cMRI scans, functional measurements (EDV, ESV, SV, EF%, CO) were performed at the basic imaging and supplemented with the measurement of myocardial defects (scar %,). To evaluate the image, we used the Segment 3.0 RXXXX software again (http://segment.heiberg.se) (Heiberg, 2010).

2.7 The monitoring period

After the MRI scan on day 3, the animals were transported to the pig farm at their place of origin and kept there in a closed barn. During the keeping, the animals were placed in groups of 6-7 individuals. In the traditional way of keeping, we tried to ensure that they could practice their natural behavioural patterns in order to eliminate the social stress resulting from isolation.

During the experiment, the pigs were fed a special feed containing 88.2% dry matter, 14.01% crude protein, 2.57% crude fat, 10.13% crude fibre and 11.35 MJ/kg (Agroszász Experimental Pig Feed; Szászvár, Hungary). contained digestible energy. The composition of the feed was similar to the previous feed used during the keeping and rearing of the animals. The pigs were fed 1.5% of their body weight twice a day, with the possibility of ad libitum water intake, and litter straw was provided as a manipulable material and hiding place. The animals were under regular veterinary supervision during the 30-day keeping, and no veterinary intervention was necessary during this time.

On the 30th day, we performed a control MRI scan of the animals, and the day before the inspection, the miniature pigs were transported back to the Institute. The preparation and examination method of MRI imaging on Day 30 was the same as on Day 3, in which the animals received gadobutrol intravenously (Gadovist 1 mmol/ml inj., Bayer, Leverkusen, Germany) at a dose of 0.16 mmol/ and tests were performed to measure left ventricular function, perfusion and viability.

At the end of the experiment, on day 30, the animals were gently euthanized by inhaling 5% vol. isoflurasan, and euthanasia was performed by injecting 30 mg/kg of potassium chloride into the jugular vein.

2.8 Blood tests

Blood samples were collected from animals at four points in different stages of myocardial infarction: before infarction induction, immediately after reperfusion, and then on days 3 and 30 of the follow-up period. In the anesthesia used during the control MRI examinations, after the same surgical preparation as the AMI induction, blood was taken from the animals with the help of a venous introducer. until delivery. The laboratory tests were examined in the veterinary laboratory of Praxislab Ltd. (Budapest), during which biochemical parameters were determined: ALT (GPT) (alanine aminotransferase/glutamate pyruvate transaminase), AST (GOT) (aspartate aminotransferase, serum glutamine oxaleacetic acid transaminase), CK (creatine kinase), LDH (lactate dehydrogenase), Hs (high sensitivity) troponin.

ALT, AST, GGT, lipase, CK, LDH, were measured on a Beckman Coulter AU480 autoanalyzer using dedicated reagent kits (Beckman Coulter, Brae, CA, USA). C-reactive protein measurements were performed on the same analyzer using pig-specific immunoturbidimetry assay (Turbovet Pig CRP, Acuvetbiotech, Zaragoza, Spain). Determination of troponin I by high-sensitivity human electro-chemiluminescence immunoassay (Acces hsTnI; B52699) occurred on a Beckman Coulter Access 2 analyzer.

2.9 Statistical analysis

2.9.1 Statistical evaluation of MRI results

In our study, we performed a statistical analysis of the quantitative data of the experimental endpoints. Each animal was its own control. The criteria for inclusion in the experiment were a successful LAD occlusion-reperfusion process and a detectable infarction on cMRI performed 72 hours

after the infarction. Animals that were not suitable for the procedure, did not have a heart attack, or died before or during catheterization were excluded. To test the differences, we used a linear mixed model, where the identification of the animals was used as a random factor. The results are given in mean (M) and standard deviation (SD). A Holm post hoc test was used to compare the results obtained from MRI scans performed at different time points. The results were considered significant if the $p<0.05$. All calculations were made using the R statistics software (R Core Team (2021).

2.9.2 Statistical evaluation of blood results

A linear mixed model was used to test the differences between the results measured at different times. The identifier of the animals was used as a random factor, no other factor was used in the setting. For comparison, marginal averages were estimated using the Restricted Maximum Likelihood (REML) fitting method. To calculate the p-value, Satterthwaite's method was used. Due to the abnormal nature of the blood parameters examined, the data were logarithmicized to perform the calculations. For the results, the data were transformed back and displayed as mean (M) and standard deviation (SD). We used the Bonferonni-Holm post hoc method to compare the results measured at different points in time to control the Family-wise error rate (FWER). The results were considered significant if the $p<0.05$. All calculations were made using the R statistics software (R Core Team (2021). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/), packages lme4 (Bates et al. 2015.) and lmerTest (Kuznetsova et al. 2017.)

3 RESULTS AND EVALUATION

3.1 Preparation and angiography

3.1.1 Anatomy and behavior

In our own experiment, it could be generally stated that miniature pigs were easy to handle compared to meat hybrids and Mangalicas in terms of their habits: unjustified, excessive aggression or agitation was observed only very rarely, which greatly facilitated the work with them (transports, transfers, feeding, watering, etc.). In the experiment, the vascular surgery procedures performed on the thighs during the procedures preparing for catheter interventions were easy to perform despite the thick layer of fat, which was helped by the relatively weak musculature of the limbs. The complete ligation of the blood vessels during the operations, even bilaterally, did not result in severe circulatory disorders in the limbs or even in clinical symptoms lasting more than a few hours. In the case of cervical vessels, due to the extreme amount of cervical fat layer typical of the breed, surgical exploration was a serious challenge for the surgeon, but it was feasible with sufficient practice. According to our experience, the course and anatomy of the coronary arteries of the heart showed great variation in the individual individuals.

Based on our available data, it can be considered that LAD dominance was present in the ventricular blood supply of the majority (70%) of miniature pigs. A smaller proportion, about 30%, of the more powerful, dominant diagonal branch originating from LAD occurred.In the majority of the animals, coronary artery was blocked under the 1st diagonal branch (1 animal also under the 2nd branch), but in 3 animals above the 1st diagonal branch. This was determined on the basis of an individual assessment for

each animal, striving to achieve a similar size of myocardial damage. Based on the angiographic examinations performed, it could be said that the LAD has a shorter course than previously observed in meat-type pigs, so the correct position of the balloon closure of the LAD is more difficult to determine than in meat hybrids. To create a larger area of infarction, a greater blood supply disorder is necessary, however, in minipigs, the occlusion of the relatively short LAD at the first diagonal branch usually results in severe myocardial ischemia, which can lead to fatal arrhythmias, and only the closure of the 2nd or 3rd diagonal branch causes a similar infarction as the obstruction of the 1st diagonal branch used in meat pigs (Schuleri et al., 2008).

3.1.2 Complications, deaths

During the experiment, a total of 6 animals died. All losses occurred during the AMI formation during the catheter procedure or in the term immediately following it. During the follow-up period, there were no deaths. An animal was lost due to a complication of catheter intervention (arterial dissection). The other 5 animals died immediately after AMI due to poorly treatable arrhythmias (ventricular fibrillation, ventricular tachycardia) despite professional antiarrhythmic interventions (24% mortality). In addition to the deceased, 6 more animals required instrumental defibrillation and antiarrhythmic drug treatment after AMI. Antiarrhythmia treatments were performed under the guidance of a cardiologist according to the rules of the profession, based on ECG and pressure values and other monitored parameters. The dosage of the drugs used and the time of their administration (Lidocaine, Cormagnesin, Cordarone Atropine, Tonogen, etc.), as well as the defibrillation shock treatments, were recorded in the documentation of the animals. Out of a total of 24 miniature pigs, 12 (50%)

developed some kind of malignant cardiac arrhythmia, which was fatal in 6 out of 12 despite professional, intensive therapy.

3.2 MRI

3.2.1 Anatomy and behavior

The anesthesia used in the cMRI scans was easy to perform on miniature pigs, although clinical signs such as poor oxygen saturation or mild pulmonary edema were often observed in the animals, partly indicative of cardiac decompensation. These problems did not significantly affect the performance of the scans and disappeared in all cases when the animals were woken up and laid on their stomachs after the end of the cMRI.
MRI scans were performed using ECG-guided, adapted, human sequences in accordance with the decades-old protocol. During the execution of cMRI scans, the proper placement of the ECG electrodes placed on the chest was often more difficult than in the case of a meat pig with a proportionally larger chest compared to its body size, which made the ECG curves of the leads not recognized by the software or with difficulty, thus making it difficult to conduct the scan. The proper positioning and centering in the MRI machine was also difficult due to the body shape of the animals, and as a result, it required more routine from the radiographer to determine the appropriate examination planes and the longitudinal and transverse axes of the heart, although these are often of key importance in the subsequent software analysis of the images.

3.2.2 Software evaluation

The software used for human cardiology examinations for the evaluation of images – in our experiment the evaluation was performed with Segment 3.0

software (Medviso) – provides usable results if the image is of sufficient quality, with special regard to the longitudinal axis (Kramer et al., 2013). The automation provided by the software can greatly help and standardize the evaluation, but human intervention is still essential, i.e. the numerical result carries a certain degree of subjectivity even with the use of software. According to our experience, the numbers characterizing the functionality obtained as a result of the software evaluation (CO, LVEF%, etc.) often showed differences between the evaluations of different persons, so we considered the consensus results of 3 independent persons to be final. The explanation for the individual differences is that the assessment of the papillary muscles in the ventricular cavity, as well as the number of horizontal slices that can be taken into account, and the individual classification of the aortic incision, may be different for each reviewer.

3.2.3 Measurable parameters of the heart

cMRI and late gadolinium stacking imaging are considered the "gold standard" procedure in myocardial infarction research, imaging of myocardial scar tissue and ventricular functionality. In our own experiment, we compared the baseline measurements with the results calculated on days 3 and 30 in order to detect scar tissue formed as a result of AMI and the consequent decreased left ventricular systolic function. By reviewing the MRI image, quantified results were obtained on the specific cardiac function parameters of the Pannonian minipigs at the 3 measurement points of the experiment: end-diastolic volume (EDV), end-systolic volume (ESV), cardiac output (CO), stroke volume (SV), left ventricular ejection fraction (LVEF).

In our experiment, the averages of cardiac functional data obtained during MRI examinations are presented in Table 1.

MRI appointment	element - number	base			72 hours			30 days		
		average	standard deviation	size	average	standard deviation	size	average	standard deviation	size
EDV (ml)	18	57,7	15,5	62	66,0	13,0	40	74,8	14,1	51
ESV (ml)	18	25,9	9,4	35	35,7	9,4	30	35,9	10,6	39
SV (ml)	18	31,9	9,3	29	30,3	7,4	29	38,7	9,3	37
EF (%)	18	55,5	8,3	30	46,0	7,7	27	52,2	8,8	30
CO (l/min)	18	2,6	0,5	2	2,4	0,6	1,9	3,1	0,6	1,8
HR (bpm)	18	84,8	23,4	75	81,1	20,5	66	81,0	17,8	60
Scar (ml)	18	57,7	15,5	62	13,4	5,8	17,8	8,7	4,65	16,8
Scar (%%)	18	25,9	9,4	35	20,0	8,8	27	13,3	6,50	24

1. Table: Results of the MRI evaluation

EDV: end-diastolic volume; ESV: end-systolic volume; SV: stroke volume; CO: cardiac output, cardiac output; LVEF: left ventricular ejection fraction; HR: cardiac valve; Scar: left ventricular scar tissue

3.2.3.1 Size of scar tissue

The successful occlusion of LAD was confirmed by coronarography, but the development of AMI was proven by late gadolinium enchancement cMRI scan performed 72 hours after occlusion. With the software evaluation of the images, we were able to accurately determine the extent and size of the infarction, as edematous, damaged tissues were also well detectable. The scars were mostly located in the anterior, anteroapical, apical anterosseptal and septal segments of the heart. The percentage of myocardial scarring (total scar%) was determined as a percentage of the total myocardium. The damage to the left ventricular myocardium of our experimental animals was 20-9±% on day 3, and the average of the results measured on day 30 was significantly lower, 13-7±%.

3.2.3.2 End-diastolic volume (EDV)

Compared to baseline cMRI scans, end-diastolic volume (EDV) increased steadily on days 3 and 30. Statistically, the Holm post hoc test showed a significant increase in EDV compared to the 72-hour and 30-day baselines ($p=0.012$, <0.001). The additional increase in EDV on Day 30 was also significant compared to Day 3 ($p=0.022$).

3.2.3.3 End systolic volume (ESV)

The ESV showed a significant increase 72 hours after the infarction ($p=0.001$), but it did not differ significantly ($p=0.781$) at day 30 compared to day 3. There was a statistical difference between the baseline value and the 30th day value.

3.2.3.4 Keringési perctérfogat (Cardiac Output; CO)

The circulatory cardiac output changed significantly (increased) by day 30 compared to the baseline value.

3.2.3.5 Displacement SV (Stroke Volume)

Displacement (SV) decreased slightly at 72 hours, but increased significantly at day 30 ($p=0.001$) compared to baseline.

3.2.3.6 Left ventricular ejection fraction (LVEF)

On day 3, left ventricular ejection fraction (LVEF) decreased ($p<0.001$) compared to healthy animals, but there was no statistical difference between baseline and day 30 measurements.

3.2.4 Evaluation of MRI results

3.2.4.1 Evaluation of SV, CO, ESV, EDV

According to our measurements, the mean of the initial, healthy left ventricular stroke (SV) in healthy animals in Pannonian miniature pigs was 32.5±to 9.3 ml, and the cardiac output (CO) was 2.6±to 0.5 l/min in animals with an average body weight of 60.4 kg. In our own experiment, the SV and CO baseline values measured in miniature pigs were generally lower than those previously observed in other similar experiments at the Kaposvár Institute. In other breeds, such as intensively growing meat-type hybrids and slow-growing fat pigs (Mangalica), cardiac outputs (CO) of 41.2 ml and 54 ml were detected, respectively, in an animal model with a similar method (Petrási et al., 2008). When evaluating the results, it should be taken into account that although the above values were also measured at a body weight of around 60 kg, these breeds were younger, growing individuals, compared to the animals currently examined. Based on the above data, we can conclude that miniature pigs at this age may have a significant disadvantage in terms of cardiological functionality compared to young intensive meat hybrids and mangalica.

In our own measurements, the statistically detectable increase in ESV, EDV and SV after the infarction is presumably the result of ventricular remodeling, which can be defined as a progressive increase in size and a change in shape causing deterioration of function in the damaged heart. In animal model experiments, molecular, cellular, and interstitial changes occur after infarction, with or without subsequent reperfusion, which may clinically manifest as changes in LV size, shape, and function. This phenomenon is called myocardial remodeling. The degree of ventricular enlargement after a heart attack is related to the degree of initial myocardial damage, and although an increase in ventricular cavity size helps restore SV despite a persistently low ejection fraction, ventricular dilatation is associated with a decrease in survival. The process of ventricular dilatation can be influenced by three interdependent factors: the size of the infarction,

the healing of the infarction, and the ventricular remodeling after the infarction (Pfeffer et al., 1990). In animal model experiments, induced ischemia also leads to LV remodeling, which is characterized by altered end-systolic and end-diastolic volume (ESV, EDV) and pressure (ESP, EDP). As a prognostic factor, ESV is the primary predictor of survival after myocardial infarction and is more accurate than the ejection fraction, and is therefore an important additional parameter for changes in LVEF (White et al., 1987).

In our experiment, the ESV showed a significant increase in the 72 hours after the infarction, but did not change significantly by day 30. In our experimental animals, we showed a significant correlation between the size of the scar tissue formed and the ESV values. These results are consistent with the current literature data: Based on our results, ventricular remodeling after infarction on the 3rd day after infarction is not a significant factor. Due to the remodelling, a continuous increase in SV was detected in parallel with the increase in the left ventricular end-diastolic volume. By day 30, we also showed a significant improvement in LVEF.

In similar experiments carried out at the Institute, LV ESV was similarly significantly increased in Mangalica pigs as a sign of unfavorable remodeling after AMI (Foinquinos et al., 2020). Brenner et al. (2021) measured the same increase in SV and EDV values in Göttingen miniature pigs and meat-type pigs at 2, 3 and 6 months. The question is also important because in human clinical settings, in addition to LVEF, left ventricular volume also provides important insight into the long-term prognosis and mortality rate of post-infarction patients (Cohn et al., 2000).

In meat-type pigs, these results should of course be interpreted with caution, as the increased ESV, EDV and SV are more likely to be associated with intense increase in body size and heart. In our own study, there was no

significant change in body weight during the follow-up period through animals with adult body size (the mean change in body weight over 30 days was -0.2 kg).

3.2.4.2 Evaluation of LVEF results

LVEF is one of the most important parameters in the diagnosis and treatment of patients with heart failure in human diagnostics (Cikes and Solomon, 2016). In infarction models, LVEF is also the most widely used measure of left ventricular pump function. In the literature, LVEF values measured in large animal experiments under similar conditions (90-minute LAD isolation, followed by reperfusion, long-term follow-up) show varying results.

In our own experiment, there was no significant difference between the baseline and day 30 LVEF values of the miniature pigs. In our previously unpublished study on 8 Pannonian miniature pigs, we were also unable to measure a significant reduction 28 days after the infarction by causing a similar infarction (19 scar%). It is suggested that the adaptation capacity of the Pannonian minipig breed is outstandingly good, which is based on the remodelling caused by the increased volumetric parameters, since in our case there was no significant increase in bodysize due to adult animals during the experiment.

3.3 Bloodtest results

3.3.1 ALT

ALT levels in baseline samples taken prior to AMI induction (M=18.6 U/l SD=9.54) and in samples immediately after reperfusion (M=19.3 U/l, SD=10.99) were within the physiological reference range (5-40 U/l). The

mean value did not increase significantly, with a large individual standard deviation (M=36.6 U/l, SD=54.52, p=0.873) for samples taken within 72 hours. The mean results obtained from blood samples taken at day 30 were statistically lower (p=0.001) than baseline values (M=11.7 U/l, SD=9.08). The modeling/analysis was carried out with logarithmic data.

3.3.2 AST

The mean of AST levels was within baseline in the reference range (10-45 U/L) (M=16.89 U/l, SD=15.61) The results of post-reperfusion (M=27.38 U/l, SD=19.56) and 72-hour (M=37.4 U/l, SD=71.87) blood sampling were not statistically different (p=0.532 and p=0.884, respectively). Day 30 values were lower than baseline values (M=8 U/l, SD=7.66) and the difference was statistically significant (p=0.011). The modeling/analysis was carried out with logarithmic data.

There was a statistically significant (p=0.075) difference between baseline and Day 30 values in the AST/ALT ratio.

3.3.3 LDH

The mean serum level of LDH in the 72-hour samples was significantly increased (M=2334 U/l, SD=2970) p<0.001) compared to baseline (M=601 U/l, SD=208) and post-reperfusion (M=768 U/l, SD=264) values and exceeded the values measured in the pig reference range (50-985 U/l).

LDH values fell significantly below baseline at day 30 (M=420 U/l, SD=210) p=0.013.

3.3.4 CK

CK levels were already above the reference range values at the first blood draw time (M=1606 U/l, SD=832). An increasing trend was observed in the post-reperfusion (M=2400 U/l, SD=844, p=0.389) and 72-hour (M=2349 U/l, SD=2300, p=0.021) samples.

The mean of the 30th day values (M=374 U/l, SD=201) was lower ($p<0.001$) than that of the baseline. CK reference range for pigs (20-200 U/l).

3.3.5 Troponin

Mean c-troponin I values increased significantly immediately after AMI (M=237.2 pg/mL, SD=354.7, $p<0.001$) and increased many times over 72 hours (M=2933 pg/mL, SD=4573, $p<0.001$). By day 30, c-troponin levels had decreased to normal levels of almost zero (M=19.8 pg/mL, SD=70.4, p=0.426) and did not differ from baseline.

Of the necroenzymes measurable in the blood, highly sensitive troponin and lactate dehydrogenase enzyme levels were significantly correlated with LVEF calculated on MRI measurements ($p = 0.008$ and $p = 0.005$).

3.3.6 Evaluation of blood results

It can be assumed that during our experiment, the peri-procedural environment and the stress factors exposed to the animals affected the kinetics of necroenzymes in addition to myocardial infarction. The experimental design used (mostly according to the decades-old proven and established protocol) may influence some biochemical blood parameters during the changing acclimatization of animals, through the stress factors exposed to the animals. ALT and AST are less specific cardiac biomarkers, with elevated levels with high variance in 72-hour samples. In our study,

various factors may have influenced the results with regard to these parameters: intramuscular administration of medicinal products, minor surgical intervention required for the insertion of introducers, and drug-induced liver damage – all of which may have contributed to the increase in values measured at the 72-hour time point in addition to myocardial damage. C-troponin, CK and LDH show more specific results than ALT and AST. Changes in values within 72 hours more clearly reflect myocardial damage and regeneration. It is striking that the results of Day 30, with the exception of c-troponin, were significantly lower than baseline. During the experiment, the animals were kept at the experimental site for 72 hours before the basic blood samples were taken. We placed them in individual cages, they were given medication and had an MRI scan. In contrast, on day 30, the animals spent only 24 hours at the experimental site, and blood samples were taken immediately after the MRI scan, under the same anesthesia procedure. The animals underwent similar medical procedures in terms of both medication and invasive procedures, the only difference during their time in the institute was the length of their stay in individual cages. The increased values may have been caused by physical exposure (injections, minor injuries to skeletal muscles, physical exertion), as well as significant social and other stressors. It can be concluded that stress sensitivity in pigs should be taken into account when designing and interpreting animal model studies. It is essential to determine the optimal time for blood sampling and to prioritize animal welfare in order to achieve correct results.

The level of c-troponin I remained unchanged in the basic blood samples despite the above effects, which is also due to its specificity and rapid kinetics. In contrast, the higher levels of biomarkers with slower kinetics are due to experimental methods. Cardiac-specific c-troponin I (cTnI) is considered a gold standard diagnostic biomarker of acute myocardial injury

because it is found exclusively in the myocardium and is released from necrotic myocardial tissue. In pigs, as in humans, serum c-troponin levels can be used to indicate ischemic damage to the heart muscle (Bertsch et al., 2000; Létienne et al., 2006). In our study, a significant increase in c-troponin levels beyond 24 hours was attributable to acute heart failure following MRI results.

4 CONCLUSIONS, PROPOSALS

Properly adapted, pigs created from Hungarian minibreeds can be used for chronic cardiovascular experiments. Pannonian miniature pigs are easy to handle, and anesthesia and surgical procedures can be easily carried out on them. However, the anatomy of the chest and the position of the heart are different from those of meat-type pigs, which must be taken into account when performing a cardiac MRI scan.

In the case of the large animal model of Pannonian miniature pigs, a higher mortality rate (30-35%) must be expected as a consequence of the infarction compared to other pig breeds. There is a great anatomical variation in the course of the coronary arteries, which can make balloon occlusion difficult. A high percentage of malignant, partially therapy-resistant arrhythmias occurred during the AMI procedure, causing a relatively high mortality rate. Based on the initial SV and CO values, the measurable functionality of the heart of the miniature pigs was weaker compared to pigs of similar size with intensive growth. Changes in the ejection fraction of the left ventricle indicate a high compensatory capacity of the heart of dwarf pigs. In our study, the excellent compensatory ability of the heart of Pannonian miniature pigs was indicated by an increase in SV and CO values after the infarction and a decrease in the area of the infarction during the follow-up period. Left ventricular dysfunction is mild to moderate in most animal model experiments due to the need to strike a balance between the degree of ischemic damage and the size of the resulting infarction and the animals' susceptibility to fatal arrhythmias. Although the model resulted in acute hemodynamic changes consistent with acute AI in the first 72 hours, the longer-term effect affects key hemodynamic indicators of heart failure. Therefore, the large animal model of our experiment should be considered as a model of acute myocardial infarction with mild or moderate LV

dysfunction rather than a model of heart failure. In our experiment, LVEF is practically restored by day 30 despite the ventricular wall movement anomalies (hypokinesis of the infarct area and hyperkinesis of the distant area) observed on the cine images taken during cMRI, from which we can conclude that in addition to the left ventricular ejection fraction, other clinical, e.g. contractility parameters should also be used to model ischemic heart disease.

Functional changes and damage to the heart muscle could be well monitored with the help of necroenzymes (LDH, c-troponin I). The values followed the changes in cardiac function parameters measured during MRI scans. It is hypothesized that the peri-procedural environment and stress factors influenced the kinetics of necroenzymes in addition to the induction of myocardial infarction. The experimental protocol includes inadequate adaptation time of the animals, which can strongly affect some biochemical blood parameters that should be taken into account when designing experiments.

Although long-term follow-up is possible, the high mortality, balloon insertion and cMRI imaging difficulties indicate that the Pannonian Miniature Pig is not necessarily an ideal pig breed for cardiovascular disease examination. The excessive anatomical variability of the Pannonian minipig's heart and the often unpredictable response of the heart (ventricular arrhythmias) could be eliminated in the future by using minipigs bred exclusively for cardiovascular experiments, which could result in a more usable model and compliance with animal welfare regulations. From the point of view of animal husbandry, due to the great variety of the anatomy of the heart, the creation of a more suitable and uniform line for cardiology experiments may be the new breeding target by selection.

5 NEW SCIENTIFIC FINDINGS

1. During the researches, I adapted the heart attack model previously developed in meat hybrid pigs at the Dr. Baka József Center to Pannonian miniature pigs. I determined the breed-specific elements of cMRI and angiographic scans, such as positioning, ECG recording, respiratory stimulation, peripheral vascular availability, anatomical availability of coronary arteries, and evaluation of MRI images. During the cMRI scans I determined the cardiac function parameters of Pannonian miniature pigs. I concluded that the pump functions of the left ventricle of healthy animals are weaker than in intensive breeds of similar size.

2. During the researches, I found that a high proportion of malignant, difficult-to-treat arrhythmias and consequential mortality occur during the infarction of Pannonian miniature pigs.

3. Based on the values of the functional parameters measured as a result of the cMRI scans, I concluded that the long-term compensatory capacity of the heart is extremely good during the response of Pannonian miniature pigs to infarction, so the ejection fraction alone as a measure is not sufficient to evaluate the infarction.

4. I found that the heart-specific necroenzymes LDH and c-troponone I of Pannonian miniature pigs are suitable for monitoring the effects of myocardial infarction, and that the activity of less specific enzymes may be influenced by the conditions of handling and housing of the animals.

6 PUBLICATIONS ON THE TOPIC OF THE DISSERTATION

6.1 Publications in a journal refereed in a foreign language

1. Brenner, G. B., Giricz, Z., Garamvölgyi, R., Makkos, A., Onódi, Z., Sayour, N. V., Gergely, T. G., Baranyai, T., Petneházy, Ö., Kőrösi, D., Szabó, G. P., Vago, H., Dohy, Z., Czimbalmos, C., Merkely, B., Boldin-Adamsky, S., Feinstein, E., Horváth, I. G., Ferdinandy, P.: Post-Myocardial Infarction Heart Failure in Closed-chest Coronary Occlusion/Reperfusion Model in Göttingen Minipigs and Landrace Pigs. J. Vis. Exp. (170), e61901, doi:10.3791/61901 (2021).
2. Kőrösi, D., Vorobcsuk, A., Fajtai, D., Tátrai, O., Bodor, E., Farkas, K., & Garamvölgyi, R. (2023). Adaptation of closed-chest infarction porcine model to adult Pannon minipigs. Journal of pharmacological and toxicological methods, 123, 107469. https://doi.org/10.1016/j.vascn.2023.107469
3. Dénes Kőrösi, András Vorobcsuk, Dániel Fajtai, Tihamér Papp, Emőke Bodor, Rita Garamvölgyi: Closed-chest occlusion of the left anterior descending artery in swine infarction model. ACTA AGRARIA KAPOSVÁRIENSIS (2023) 27 (1), xx–xx; DOI: 10.31914/aak.xxxx
4. Garamvölgyi, R., Kőrösi, D., Tátrai, O., Bodor, E., Fajtai, D., Farkas, K., & Vorobcsuk, A. (2024). dP/dt_{max}: An underestimated prognostic factor in large animal infarction model. Animal models and experimental medicine, 10.1002/ame2.12502. Advance online publication. https://doi.org/10.1002/ame2.12502
5. Dénes Kőrösi, Ágoston Göcző, Noémi Varga, Rita Garamvölgyi*, Nándor Balogh, Kornélia Farkas and András Vorobcsuk: Blood biochemistry changes in a minipig infarction model. Front. Vet. Sci.Sec. Veterinary Experimental and Diagnostic PathologyVolume 12 - 2025 | doi: 10.3389/fvets.2025.1493660.

6.2 Publications in Hungarian refereed journal

1. Kőrösi Dénes, Garamvölgyi Rita, Vorobcsuk András, Szabó András, Petrási Zsolt: Különböző sertéstípusok az orvostudományi kardiovaszkuláris kutatásokban (Irodalmi áttekintés), ACTA AGRARIA KAPOSVÁRIENSIS(2020) 24(2), 47–60; DOI: 10.31914/aak.2442.

6.3 Lectures in Hungarian

1. Nagyállat infarktus modell adaptálása pannon törpesertésekre. dr. Kőrösi Dénes, dr. Vorobcsuk András, dr. Bodor Emőke, dr. Tátrai Ottó, dr.Garamvölgyi Rita: I.MAGYAR AGRÁRTUDOMÁNYI DOKTORANDUSZOK SZIMPÓZIUM, DEBRECEN 2023. FEBRUÁR 24-25.