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A Novel Experimental Rat Model for the *In Vivo* Assessment of Myocardial Ischemia Based on Single Photon Emission Computed Tomography

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Abstract. *Background/Aim: Myocardial infarction, an acute medical situation with a high mortality rate worldwide, has been extensively studied in modern cardiovascular research, using different experimental models. However, a deep understanding of myocardial activity loss has not been fully investigated. We have developed a novel experimental rat model for noninvasive assessment of myocardial ischemia based on single photon emission computed tomography (SPECT/CT), in order to further understand and evaluate myocardial activity before and after surgical induction of myocardial ischemia. Materials and Methods: Thirty adult female Wistar rats underwent open thoracotomy with (n=20) or without (n=10) surgical ligation of the left anterior descending coronary artery (LAD). The myocardial ischemia was confirmed with ECG and myocardial viability was evaluated via SPECT/CT at 7 days before as well as at 7 and 14 days post-surgery, after which animals were sacrificed and myocardial ischemic injury was further assessed histologically. Results: All animals were evaluated with anatomical and functional criteria based on the SPECT/CT imaging results. A successful surgical technique causing ischemia and loss of myocardial function in all animals undergoing a LAD ligation was established. Furthermore, evaluation of the viable myocardium with SPECT/CT confirmed the reduction of functional myocardial cells of the left ventricle post-infarction, which was also documented histologically. Conclusion: Using our technique, the validity of this animal model to induce and evaluate myocardial ischemia was demonstrated. Our choice to apply SPECT-CT qualitative and quantitative evaluation of myocardial function leads to a new approach in experimentation with an anticipated significant impact in the ongoing cardiovascular laboratory research.*

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Myocardial infarction is one of the leading causes of mortality creating the necessity of deep understanding of the mechanism underlying myocardial cells' damage, as well as evaluating their function in a qualitative and quantitative way. The understanding of pathophysiological mechanisms of the cardiovascular system is one of the greatest fields of modern

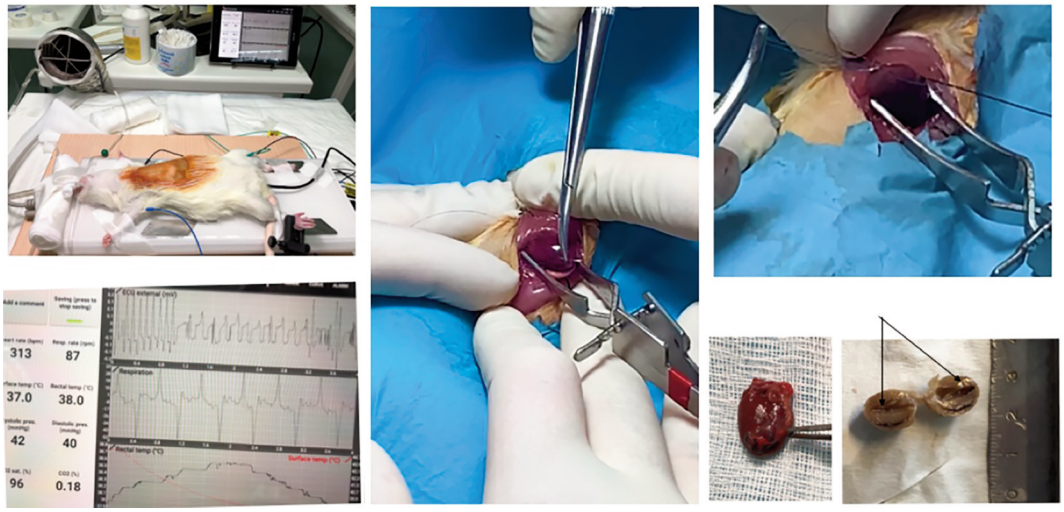

Figure 1. *Illustrations depicting the surgical technique, the electrocardiogram (ECG) changes, and the macroscopic anatomo-morphological changes in ischemic myocardium. Under sterile conditions, ischemia is caused by ligation of the left anterior descending (LAD) coronary artery at the first 1/3 of its course after the bifurcation of LAD leading to a stable, reliable, and reproducible myocardial infarction which is confirmed via ECG monitoring operatively (ST elevation on ECG, i.e., the trace in the ST segment is abnormally high above the baseline), macroscopic features of ischemia on the day of euthanasia and tissue harvesting and loss of cardiac muscle fibers in the macroscopic appearance after formaldehyde fixation.*

medicine. Therefore, basic, and translational research is needed to better understand its underlying mechanisms and consequences for cardiac structure and function (1-3).

Rodents have been intensely used in pre-clinical trials as an accepted animal model. Their use has been steadily increasing, especially in protocols related to cardiac activity. Myocardial ischemia is evaluated experimentally with both surgical and pharmaceutical methods causing acute infarction which is mainly evaluated *post mortem* (3, 4). *In vivo* experiments in such protocols are highly challenging due to the increased mortality rate. Surgical techniques such as ligation of the left anterior descending coronary artery (LAD) has been widely used to establish a model of myocardial infarction (4, 5). There are some disadvantages in this technique including the high mortality rates after ligation and the variations in the infarct size (5, 6). Therefore, improving the techniques causing ischemia is required, while non-invasive imaging methods in living animals post-infarction is extremely valuable for evaluating many aspects of the ischemic myocardium *in vivo* (2, 7-9).

Myocardial ischemia and left ventricular defect causing heart failure can be associated with viable and non-viable myocardial tissue (3, 7, 8). This ischemic myocardium that has lost viability can be evaluated using many approaches, such as *in vitro* histopathological measurements or *in vivo* imaging techniques, including ultrasound or magnetic resonance with different sensitivities and specificities.

Single photon emission computed tomography (SPECT) and computed tomography (CT) are highly sensitive and specific methods for analyzing both physiological and anatomical aspects. Therefore, their use in preclinical trials is highly recommended (10-12).

We report here a novel reliable and reproducible rat model, in which myocardial ischemia and infarction were induced by ligation of the left anterior descending coronary artery, and SPECT/CT imaging, along with histology and biochemistry, were used for *in vivo* evaluation and assessment of the infarction in a qualitative and quantitative manner.

Materials and Methods

Animals and study protocol. The study protocol was approved and authorized by the Greek General Directorate of Veterinary Services (License No: 1870/23-04-2018) and was conducted in accordance with the Greek legislation regarding ethical and experimental procedures. Thirty adult female Wistar rats underwent general anesthesia and mechanical ventilation. Tracheal intubation was achieved by a 17G tube and maintained with sevoflurane. A special temperature controlling mattress and a rat specific ECG monitoring combined with temperature and oxygen measurement were applied throughout the procedure. Perioperative care was taken with subcutaneous adhesion of dolorex (0.06 ml in h 0, 8 and 24; Ktiniatriki, Athens, Greece), intramuscular adhesion of terramycin (0.03 ml; Pfizer Hellas S.A., Athens, Greece), subcutaneous hydration with 3 ml of normal saline 0.9% and eye protection with gel tobrex. Subsequently, under sterile conditions, a left mini thoracotomy between the 3rd and 4th intercostal space was undertaken and the pericardium was opened. A Prolene 6-0 stitch (Ethicon, Raritan, NJ, USA) was used for handling the heart apex and a silk 4-0 for ligation of LAD in 20 animals, while 10 were used as sum n0-ligation group (Figure 1). Myocardial ischemic time was 45 min and ECG continuous recording for confirmation of ischemia was utilized (Figure 1). Afterwards, animals were

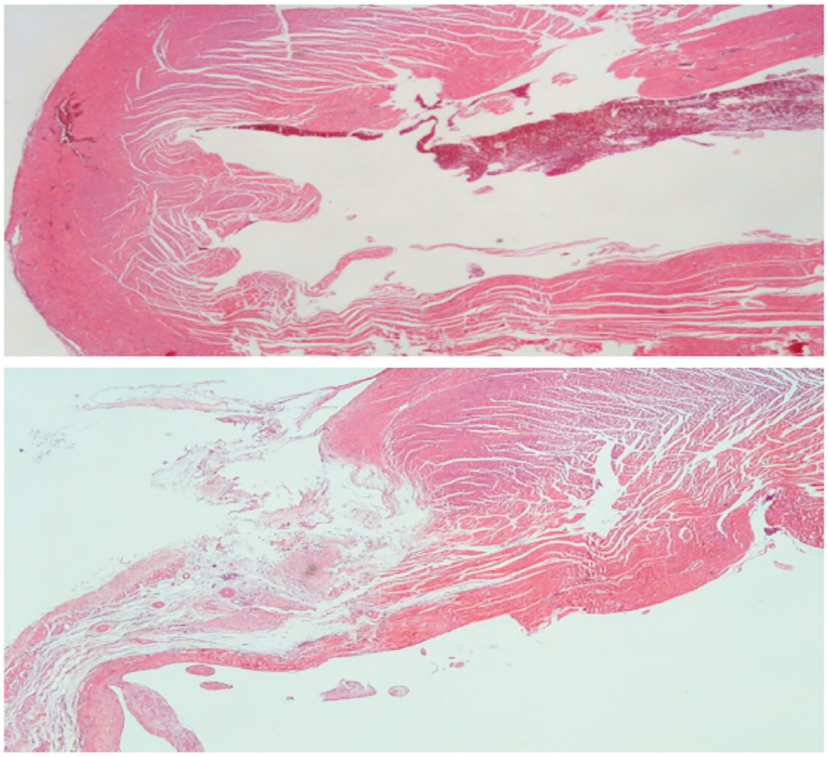

Figure 2. *Myocardium histology in no left anterior descending coronary artery (LAD) ligation and LAD ligation groups. Hematoxylin/eosin 2× magnification without ligation of LAD. Normal cardiac muscle fibers (top image), Hematoxylin/eosin 2× magnification with ligation of LAD. Ischemic changes in myocardium with loss of cardiac muscle fibers (lower image).*

extubated, and all had an uneventful recovery and were able to undergo SPECT-CT imaging at 7 and at 14 days after surgery. On the 15th postoperative day, the rats were sacrificed, and tissue and organ harvesting was performed. Euthanasia introduced by injecting KCl in the inferior vena cava (IVC).

SPECT/CT evaluation. All animals underwent a preoperative SPECT-CT imaging prior to carrying out myocardial ischemia, in order to analyze the functional myocardium of the healthy heart. A novel technique for SPECT evaluation was developed especially for small rodents and the imaging chamber was formulated to focus on the thoracic cavity of the animal (Figure 1). 200 μl of 1.5 mCi-3 mCi of 99 mTc-Sestamibi were injected in the rat *via* the inferior vena cava and subsequently, imaging was taking place at 20 min post injection. The SPECT-CT imaging was made with x-Cube and γ-Cube (Molecubes, Gent, Belgium). SPECT acquisition was done with local scan spiral acquisition for 30 min and maximum likelihood expectation maximization (MLEM) reconstruction with 250 μm voxel size and 500 iterations. SPECT post-processing had to do with normalization of images on myocardium uptake [myocardium having the same maximum number of counts, removal of all other organs and smoothing median filter (1.8 mm full width at half maximum (FWHM), isotropic)]. Furthermore, CT acquisition and post processing for the whole body was performed using a general purpose protocol (50kVp), utilizing an ISRA reconstruction with 200 μm voxel size. In all images, a scale was used for analyzing the absorption of the radiation particles from the myocardial cells, in order to evaluate the cellular activity in voxels. Axial slices (short, long and horizontal) and 3D rendering with color indication scale was used to show the myocardial activity 7 and 14 days post-surgery, in order to analyze the changes in myocardial absorption of the radioactive particles after infarction. The post processing and the quantification of all images was performed with Vivo Quant 3.0 (Invicro, Boston, MA, USA). Results were measured in voxels from each cardiac chamber and the left to right ventricular ratio difference was used to identify the changes of myocardial activity after LAD ligation and the presence of ischemia in the left ventricle (Figure 2).

Histology. Blood sampling was taken from the IVC and, from the same route of administration, excessive dose of KCl was administered for euthanasia. Hearts were rapidly excised and placed in 10% formaldehyde. The samples were afterwards transferred to the histology laboratory, were fixated at room temperature using 10% formaldehyde in a ratio of 2:1 formaldehyde:tissue (v/v) for 48 h and were then dehydrated and embedded in paraffin. In order to keep the long axis of the heart intact, we created slices in the perpendicular plane. Hematoxylin and eosin (H&E) staining was used to identify the fibrotic areas of the myocardium (Figure 2).

Statistical analysis. Data were expressed as mean±standard deviation (SD) for quantitative variables and as frequencies and percentages for qualitative variables. The Kolmogorov-Smirnov test was used for normality analysis of the quantitative variables. Comparison of

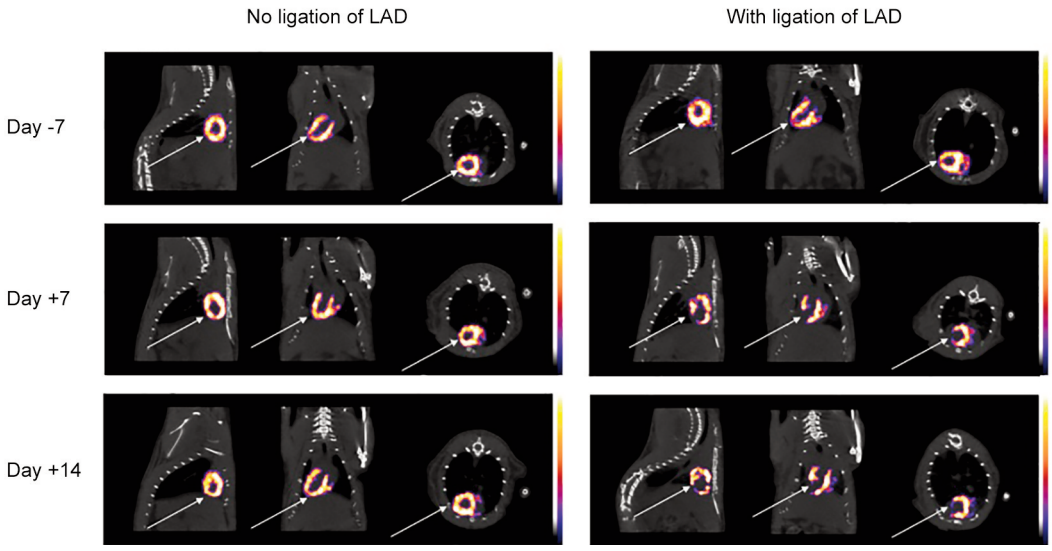

Figure 3. *Single photon emission computed tomography (SPECT/CT) images in groups with and without left anterior descending coronary artery (LAD) ligation. The color bar indicates the difference in accumulated activity (deep blue being the lowest and white the highest). All tomography views show the myocardium 7 days before surgery (day -7) and 7 and 14 days after surgery (day +7 and day +14). The white arrows show the area supplied by LAD (heart apex). The difference between the animals without ligation (left column) and the animals with LAD ligation (right column) is pointed on days +7 and +14 where there is no absorbance of radioactive particles from ischemic myocardium (no cellular activity).*

quantitative and qualitative variables for pairwise comparisons between groups was performed using one way ANOVA with Bonferroni correction and the Chi-square test with Bonferroni correction, respectively. All tests were two-sided. A p-value <0.05 was defined as the level of statistically significant difference. Statistical analysis was performed using the statistical package SPSS version 21.00 (IBM Corporation, Armonk, NY, USA).

Results

In this experiment, all animals had comparable imaging results and no animal was lost neither perioperatively nor during the imaging procedures indicating a very reliable *in-vivo* experiment (5). The initial images were evaluated after removal of all other organs and by performing a whole-body spiral acquisition in a high-resolution protocol (Figure 3). The territories between normally functioning myocardial tissue and ischemic parts were easily recognizable after the axial and 3D reconstruction leading to a physiological and anatomical map of the rat's heart. The standardization of the procedure by scanning all animals preoperatively helped to visualize the distribution of the radioactive tracer after the establishment of the ischemia and the changes occurring after 7 and 14 days respectively.

Accumulated activity was recognized in highly metabolic myocardial cells while decreased or no activity was shown in ischemic areas.

The distribution of the radioactive particle was measured *via* the differentiation of the left ventricular to right ventricular area (LV/RV) which is a mathematical ratio without being influenced by any other parameters of the experimental method, such as animal weight, dosage or radioactive substance, absolute number of voxels. Specifically, in healthy individuals the ratio LV/RV 7 days prior to surgery is about 8.7±0.3 (Day -7) and remains stable in the animals which underwent only thoracotomy without ligation. The group of 20 animals with ischemia established by ligation of LAD, showed same values preoperatively but had significant decrease of the active myocardium with values of LV/RV ratio 7.5±0.2 post surgically (Figure 4, Table I). Confirmation of the ischemic areas were further completed by histological analysis.

Discussion

Myocardial infarction is a leading cause of death worldwide. Therefore, development of new therapies and diagnostic approaches are urgently needed (1, 2). This has led to the creation of numerous experimental protocols, as well as guidelines for experimental models, in order to establish more reliable pre-clinical trials (5).

The aim of experimental models of myocardial ischemia is to offer better mechanistic understanding that cannot be gained from a clinical situation and may have a low direct applicability to the clinical practice. Furthermore, an experimental model could provide mechanistic insight from an experimental study for translation to the clinical situation, and for this purpose models must imitate the clinical situation, as close as possible (7). We consider our model to be in this latest category.

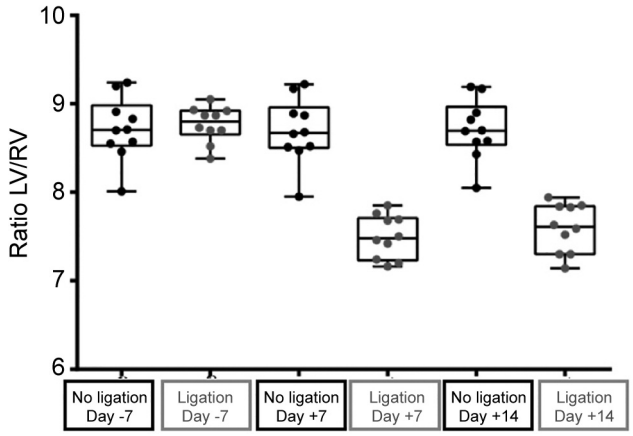

Figure 4. *Histogram presenting the left ventricular to right ventricular area (LV/RV) ratio between the two groups. In healthy individuals the LV/RV ratio 7 days prior to surgery is 8.7±0.3 (day -7) and remains stable in the animals which underwent only thoracotomy without ligation (black color). The group of animals with ischemia (grey color) established by ligation of left anterior descending coronary artery (LAD), showed same values preoperatively (day -7) but had significant decrease of the active myocardium with values of LV/RV ratio equal to 7.5±0.2 post-surgically (day +7 and day +14).*

Table I. *Statistical results and differences in left ventricular to right ventricular area (LV/RV) ratio among the no-ligation (n=10) and the ligation (n=20) animal groups.*

	Group		*p*-Value
Variables	No ligation	Ligation of LAD	
Ratio LV/RV(-7d)	8.72±0.36	8.77±0.20	0.480
Ratio LV/RV(7d)	8.69±0.37	7.50±0.24	<0.005*
Ratio LV/RV(14d)	8.71±0.34	7.59±0.28	<0.005*

*$p<0.005$ *vs*. sham-treated controls.

The possibility of having trustworthy results in living animals in comparison to *post mortem* analysis is of major importance. Until now, most experimental models were based on histological analysis of heart tissue and identification of fibrotic areas, and only few experimental protocols have been developed for *in vivo* analysis of the myocardial activity. (3-5).

Rodents are highly comparable animals to human physiology and anatomy concerning the heart. Therefore, the analysis of rat heart tissue post infarction can lead to a solid knowledge which can easily be repeated in laboratories worldwide for the *in-vivo* evaluation of many novel surgical, pharmaceutical, or other treatments. In this particular model, a well-established experimental animal model was used to produce myocardial ischemia and infarction by ligating the LAD. This procedure is associated with an increase mortality rate, above 50%, due to the great stress for the animals. Consequently, most of the reported experimental rat models are "acute" experiments that permit only *in vitro* myocardial evaluation of the infarction postmortem. In contrary, we managed not only to decrease the overall mortality rates to below 20% by a meticulous and standardizing technique for LAD ligation *via* a left thoracotomy, but also to perform *in vivo* imaging analysis with a special developed for small animals SPECT/CT, as all animals survived until the 15 post-operational day of final evaluation, after which euthanasia followed. Until now, such *in vivo* experimental studies often use an intravascular or pharmaceutical method to achieve myocardial infarction in order to minimize the losses associated with open surgical interventions (5-9).

As shown in Figure 2 and Figure 3, the presence of ischemia was well recognized and documented in the left ventricle and, specifically in the area supplied by LAD, with loss of the ability to absorb radioactive particles. The areas and the territories between healthy and ischemic tissue are greatly seen in every axis of the heart and the 3D reconstruction in a combination of SPECT and CT imaging lead to a full anatomical and physiological map of the rat's heart (10-12). The ability to combine SPECT/CT imaging with x-cube and γ-cube creating a high-resolution outcome, although considered as a great challenge, in our model, it was completed very smoothly and effectively.

This new infarction model was developed keeping in mind the increasing awareness of the need for rigor and reproducibility in designing and performing scientific research to ensure validation of results, as postulated by the reported guidelines (5).

Based on the results illustrated in Figure 4 and Table I, the validity of our model was established in every single animal used with a clear imaging of heart chambers and visualization of myocardial activity by SPECT-CT, which is able to evaluate the functional and the non-functional areas and transform the qualitative results into quantitative so that precise myocardial changes can be evaluated.

We conclude that our model of an *in-vivo* evaluation of myocardial ischemia is highly reliable, and our evaluation protocol can be used with high accuracy in various trials in cardiovascular experimental research.

Conflicts of Interest

The Authors declare that they have no conflicts of interest in relation to this study. George Loudos and Maritina Rouchota work for Biomtech laboratories that produces the x-cube and γ-cube.

Authors' Contributions

A.K. and A.E.P. conceived the study. G.L. and M.R provided the original SPECT evaluation. A.K, M.R. and G.V. performed the

animal experiments. D.K., G.Z., I.M.G., AEP and DD provided scientific advice. A.K. wrote the paper and D.D. with A.E.P. revised the manuscript.

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