A mouse model of myocardial infarction in Filamin B deficient mice

Master thesis in Medicine

Elin Svedlund

UNIVERSITY OF GOTHENBURG

Supervisor Levent Akyürek

Dept of Medical Biochemistry and Cell Biology

Vascular Biology Group

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Abstract

Background Myocardial infarction (MI) is the most common endemic disease and the condition is the leading cause of death in all industrialized countries. Animal models of MI plays a significant role in the development of diagnosis and therapeutic strategies in human MI, as it enables studies of pathobiological and pathophysiological mechanisms. A mechanism that could be interesting as a therapeutic strategy to treat cardiovascular diseases is the induction of angiogenesis. The protein FLNB has been shown to stimulate angiogenesis and it is therefore interesting to investigate its effects on the myocardium post MI since neovascularization is crucial for the healing process.

Objectives My first aim was to develop a method of inducing MI in mice. My second aim was to use the method to induce MI in FLNB deficient mice and in wild types in order to examine differences between the groups regarding the ischemic injury.

Methods When the method was established a baseline echocardiography was performed one day prior to surgery. The echocardiography procedure was repeated at three time points in order to quantify the area of infarction. Ten weeks post-surgery the hearts were fixed for histological sections and staining. Survival, cardiac function and healing process were analyzed.

Results and discussion The protocol of permanent ligation of LAD at the atrioventricular junction ensured a statistically significant and reproducible MI. No difference in size of the infarct area between the groups was indicated by the analysis but the experiment needs to be repeated with a larger number of animals in order to obtain significant statistical basis. A Fisher Exact test suggested that wild-type mice survive myocardial injury to a greater extent

than the mice that are partly deficient of FLNB. Although, death occurred during the critical days post-surgery and no conclusion about survival can therefore be drawn.

Introduction

Cardiovascular disease (CVD) is the most common endemic disease of our time and the conditions (myocardial infarction, congestive heart failure, arrhythmias, stroke etc.) are the leading causes of death in all industrialized countries[1]. Coronary heart disease alone causes 7.2 million deaths each year globally whereof nearly 2 million in Europe[2]. In Sweden 1.4 million people lives with CVD. In 2011 almost 40% of the overall mortality was due to diseases of the heart and blood vessels[3]. According to the National Board Directory of causes of deaths, 34 666 persons were deceased from cardiovascular diseases in 2011 – 18 204 women and 16 462 men. In Europe 25 percent of men and 38 percent of women die within eight years after the onset of a myocardial infarction (MI). Almost half of the men and women diagnosed with MI before the age of 65 will die within the first year[4]. In Sweden in the year of 2010, 31 600 people - 19 000 men and 12600 women - suffered from 33 700 myocardial infarctions[3]. Over 9 000 of the MI:s were fatal, approximately 5 200 men and 3 900 women died[3]. More than 3 600 of the individuals were younger than 80 years of age[5]. One year later almost 13 200 persons had died from the consequences of the MI[6]. The medical and economic burden of mortality and morbidity as a consequence of cardiovascular disease are immense. The total cost of cardiovascular disease rate in Sweden is calculated to approximately 60 billion per year[7]. But the true figure is even higher than that since the statistics do not include the cost of health and social care nor does it include persons who do not have cardiovascular disease as their primary diagnosis[7]. Onset of cardiovascular disease as well as the mortality due to them have decreased in Sweden, but the number of people diagnosed early in life and whose quality of life are limited by disabling sequelae are ever increasing[8]. The development of mortality in acute MI within a year, a month and a day respectively are decreasing at almost the same rate[9]. This positive trend is mostly a result of research that has resulted in refined methods of the acute treatment. Decades of

research have also shown the importance of well-treated hypertension and hyperlipidemia as well as the pronounced negative consequences of smoking on the cardiovascular system. Nevertheless, much research remains to be done. We need to understand more of these complex diseases in order to develop optimal ways to diagnose and treat them. Use of animal models of MI plays a significant role in the development of diagnosis, prognosis and therapeutic strategies of human MI, as it enables studies of pathobiological and pathophysiological mechanisms of both acute injury and in chronic remodeling [10]. Models of MI in mice have proved to be very useful since they are highly reproducible and the animals can be kept at a low cost[11]. The left ascending coronary artery (LAD) in mice passes obliquely across the left ventricular wall similar to the ramus intermedius coronary artery in man. Once ligated, 2-3 mm distal of the border of the left atrial appendage, a large infarction involving anterolateral, posterior and apical regions of the heart will be induced[12]. The permanent ligation causes irreversible damage to the myocardium. Extensive collateralization in some species, such as dogs, limits the size of infarction and the animals are therefore no good subjects for this type of model. MI in animal models can be induced by principally two different methods[13]. The first one is based on the total or partial blocking of coronary arteries by surgical procedures or by drug intervention. This often results in acute ischemia. The surgical procedure is complicated and it requires advanced equipment. There are several different ways of preforming MI-induction in animals but the existing protocols are not detailed enough. They leave room for unnecessary mistakes during the learning process resulting in animal suffering and high mortality rates. Drug-induced MI is a more convenient method and it can easily be achieved reducing the blood flow in the coronary arteries due to artery spasm and increased oxygen consumption of the myocardium. The drugs that are most commonly used for this purpose are Isoproternol, Adriamycin and Ergonoyine[13]. Although, this method is seldom used in research since the accurate

positioning of the infarct is hard to achieve with drugs[13].

Induction of atherosclerosis in coronary arteries is the other way of creating MI:s in a controlled manner. A pro with this method is that it closely mimics the progression of the disease in humans. Yet, the method is very time consuming and it is therefore not used that often in clinical research[14].

The development of biotechnology allows studies of the functional role of overexpression or deletion of specific gene products in cardiac phenotype. Our research group focuses on the role of filamin (FLN) proteins in the healing process after myocardial infarction since these proteins have been shown to stimulate angiogenesis[15]. Angiogenesis is the sprouting process of capillary blood vessels from already existing microvessels. The formation plays a significant role in various biological processes such as embryological development, wound healing, inflammation etc. It also causes the pathogenesis of various diseases – diabetic retinopathy for example[16]. There are three filamin genes in mouse, FLNA, FLNB and FLNC. FLNA and FLNB are ubiquitously expressed while FLNC is expressed in heart and skeletal muscles[17]. FLN are large actin-binding proteins that links actin to cellular membranes during cell movements and the proteins also mediate interactions between actin and transmembrane receptors[18]. FLNA is the most intensively studied protein while much less is known about FLNB. To study FLNB in vivo, our research group generated mice with a disruption in the FLNB-gene. Less than 3% of homozygous embryon were born, which indicates that the protein is an important factor for embryonic development. The homozygous mice that were born were very small and had severe skeletal- and microvascular malformations. No one of them survived more than 4 weeks. Mutations in human FLNB have been found in severe skeletal malformations[19], disorders in the central nervous system and also in the cardiovascular system[20]. Thus, the phenotypes of FLNB-deficient mice resembles those of human FLNB-mutations. However, heterozygous mice could not be

distinguished from wild types. Strong expression of FLNB was found in the chondrocytes and in the endothelial cells. In the acute phase of MI severe endothelial damage occurs within the first 24 hours[21]. Angiogenesis is central to cardiac repair and delayed healing can cause cardiac rupture and severe scar formation[22]. There are many studies that have shown angiogenisis to be of great importance to the healing process of myocardium post MI (Ahn et al., 2008; Boodhwani et al., 2006; Nelson et al., 2000; Pandya et al., 2006). Because of the angiogenic properties of FLNB and the fact that the protein is strongly expressed in endothelial cells we wanted to study the protein in the healing process in myocardium post MI. Our research group hopes that filamins will function as prognostic markers post MI in the future. Otherwise, the studies of the proteins may lead to further important understanding of the heart's complex healing process.

Aims

The purpose of this project was to develop a model of MI in mice and to produce a detailed description of how the surgical method is performed. The protocol of the surgical technique was then going to be used to induce MI in wild type mice (WT) and in mice partially deficient of FLNB (HT) in order to compare survival, cardiac function, and healing process between the groups. Following questions were to be answered:

- Will wild-type mice survive myocardial injury to a greater extent than mice partly deficient of FlnB?
- Does the cardiac function differ between the groups during the healing process?
- Will histological staining show difference in scar size?

Material and methods

Experimental animals

We used 10 week old C57BL/6 mice, weighing approximately 25 grams. 15 male mice were included in the experiment, 6 wild-types (WT) and 9 heterozygous (HT). The animals had free access to standard chow and tap water and they were housed in a 12 hours light-dark cycle. This study and the involved procedures were approved by the local Animal Care and Use Committee. The ethical number of the myocardial infarction model in this project is: 177/11.

Genotyping

I performed the genetic identification of the animals by purification of DNA from excised tissue from ear punching and tail biopsies. The mice were 21 days when the tissues were harvested. The analysis was then performed with polymerase chain reaction (PCR).

DNA extraction

The isolated DNA from the animals was placed in collection tubes and 80 µl lysis buffer were added. After being heated in 95 °C for 20 minutes, 80 µl of neutralizing buffer were added and the tubes were then placed on ice.

PCR

Reaction mixture 4 µl of RNase-free water**,** 1 µl of Reverse primer (RP), 1 µl of Forward primer (FP), 4 µl of DNA and 10 µl of Mastermix (MM).

The solution was mixed well by pulse spin in a microfuge. The volumes listed are for one reaction. The total volume without DNA is 16 µl. 10% extra was made for losses during pipetting.

The tubes were placed in a PCR machine and the following program was run: 94 °C for 15 minutes (initialization), 94 °C for 30 seconds, 60 °C for 30 seconds, 72 °C for 2 minutes, 72 °C for 10 minutes. Step 2 – 5 are run for 34 cycles.

In this project a 2,004-bp wild-type Flnb fragment was amplified with forward primer 5-GCC TCA AAG AGC TAC TGT CCA CGA-3 located on exon 20 and reverse primer 5-GGG TCA GAA TCA CGC AGG TTA CTT-3 located on exon 21. A 2,504-bp mutant Flnb fragment was amplified with reverse primer 5-GAC AGT ATC GGC CTC AGG AAG ATC G-3 located in the -geo insert and the wild-type FlnB forward primer.

Electrophoresis

1.15 g agarose was added into 100 ml TAE (Tris base, aceticacid and EDTA) buffer. The solution was heated for 2 minutes in a microwave machine on high effect and then cooled off in running water to approximately 60 degrees C. 2.5 drops of EtBr were added and the solution was well mixed before it was poured into the form. Combs were put in and the gel was left to solidify for 35 minutes. 2 microliters of dye were added to the samples. The combs were then carefully removed and 20 microliters of the samples were loaded in the wells. The electrophoresis was set for 120 V, 30 minutes. After the electrophoresis the gel was placed in an apparatus that allows the gel to be exposed to ultraviolet light. An image was then taken with a polaroid camera.

Protocol of coronary artery ligation in mice

Pre-surgery The weight of the animal is registered. Generally the procedure is performed in mice 6-10 weeks old, weighing 20-25 grams. Buprenorphine is given at 0.1 mg/kg s.c. Anesthesia is induced by placing the mouse in an induction box filled with 4-5% isoflurane. To place the tube (19-22 –gauge) in right position the tongue is slightly retracted and a fiber optic light is used to illuminate the neck region to help visualize the trachea during the insertion of the tube. Insertion is made carefully through the larynx into the trachea to avoid puncture structures of the pharyngeal region. The tube is then connected to a rodent ventilator (type: Minivent, tidal volume 250 microliters, 2 Hz) to administer positive pressure ventilation. The mouse is ventilated with room air supplemented with oxygen (2 L/min) at a

rate of 120/min and with a tidal volume of 2.1-2.5 ml. Ventilation is confirmed by syncronized chest movements. The tube is taped in place to prevent dislodgment. 1.5-2% MAC of isoflurane is applied during the procedure. If gas anesthesia is not possible Ketamine (100 mg/kg im), Xylazine (5 mg/kg s.c) is used. Sodium pentobarbital is associated with higher mortality. Sterile lubricant is applied to the eyes. The mouse is placed in supine position with the paws taped to the operating table. The mouse is kept as close to 37 °C as possible throughout the procedure using a heating pad or heat lamps. Body temperature can be monitored with a rectal probe connected to a digital thermometer. The fur is removed from the left side of the chest with a depilatory agent. Carefully wipe of the agent to avoid chemical burn and dermatithis. The area is then disinfected with iodine. If available, two electrodes are placed subcutaneously to monitor the ECG.

Surgery Toothted forceps are used to pull the skin up and away from the chest. A 1.5 cm incision is made at the level of the left third and fourth rib. The pectoralis major and pectoralis minor are dissected with a thin forceps and scissor. If they are not retracted spontaneously the area is freed by gently retracting them with a 6-0 silk. The thoracotomy is made by a small incision into the intercostal muscle and pleura, 2 mm lateral of the sternum. The distance from sternum makes the risk of bleeding from the internal thoracic artery minimized. The third rib is lifted with a forceps and the opening is gently enlarged. Care must be taken to avoid damage of the lungs. The ribs are separated approximately 6 mm with a mouse retractor. It is possible to push the lungs gently aside by using an absorption triangle wetted in sterile 0.9% NaCl. The pericardium is opened with two forceps. The left anterior descending coronary artery is identified. Visualizing LAD is challenging and it takes a lot of practice to become proficient. To gently put intermittent pressure on the area of the heart where the location of LAD is expected the reflux of the blood can help reveal the artery. A perpendicular light source can also be of use. Fade the light to avoid disturbing reflexions from the myocardium

(Fig. 1). A Prolene 6-0 ligature is passed around the proximal end of the artery, 2-3 mm distal to the border of the left atrial appendage. The ligature is tied with two single knots. This site of ligation induces roughly 40% ischemia of the left ventricle (LV). The needle should be curved for easier passage. It is important to not enter the cavity with the needle nor go too superficial as the ligature will cut through the wall. If bleeding occurs gently press a cotton tipped applicator onto the site until the bleeding stops. Successful performance of artery occlusion is verified by visual inspection by noting the development of a pale color in the anterior and distal wall of the LV. If access to ECG a ST-segment elevation and widening of the QRS is observed. If the circumflex artery also is occluded the acute mortality will increase. The long ends of the suture are cut off. The chest is closed by placing two ligatures (6-0) around the third and fourth rib. The pectoralis muscles are attached with a single suture, covering the wound. The chest is pressed to restore negative pressure. The wound is closed with 6-0 silk mattress sutures. Buprenorphine (0.4 mg/kg intra peritoneal (i.p)) is given just before the animal wakes up.

Recovery The isoflurane is stopped and rythmic shallow breathing should soon be observed. The tube is then removed from trachea. 0.5 sterile sailine is injected to the dorsal subcutanous space and the mouse is placed in a cage containing a heating pad until it regains mobility. Extra oxygen is recommended. Give fluids (1.0-1.5 ml of 5% dextrose in water i.p.) to restore hydration due to blood loss and evaporation. Additional anesthesia (buprenorphine 0.1 mg/kg) is repeated 6-24 hours later. The whole procedure takes approximately 15 minutes. In general 30% of the mice die within 24 hours. Sham-operated mice undergo the same procedure without the tying of the suture. These animals recover quickly.

Metabolic changes due to acute injury can be confirmed by infusing 1% (6 microliters) of Evan´s blue dye into the aorta prior to excision. The dye will perfuse the parts of the heart that is not supplied by LAD and therefor will unoccluded regions become stained. Necrotic areas

will remain pale (Fig. 2). TTC staining can be used to stain metabolically viable tissue red.

Echocardiography

One day post MI the hearts were examined by echocardiography (UCG) in order to exclude atypical infarctions and to determine baselines. The equipment used is a Vevo 770 – a high resolution micro-ultrasound imaging system designed for small animals.

The mouse was sedated with isoflurane and placed on a heating plate with electrocardiogram contact pads. The nose was placed into a nose cone supplied with a 1-2% flow of isoflurane. Electrode gel was placed on the paws and they were then taped into position on the contact pads. Body temperature was monitored using a rectal probe and temperature was not allowed to drop under 36.5 °C. The chest was cleansed with distilled water before ultrasound gel was applied.

Protocol

One long axis view (LAX) in EKV (ECG-gated kilohertz visualization generates 2D cine loops), three short axis views (equal distance between – papillary plane, pulmonalis valve, apex) in EKV, one M (motion)-mode in papillary plane and one Doppler in pulmonalis valve plane. Once imaging was completed the mouse was cleaned with water and allowed to recover on a heat pad before returning to cage. The mice were scanned again using the same protocol ten weeks post infarction.

Histology

At a defined time point (10 weeks post MI) the mice were sacrificed and hearts were harvested under deep anesthesia with inhalation of 5% Isoflurane. 0.1 ml saturated KCl was injected into the left ventricle to arrest the heart in diastole.

Hearts were snap-frozened in liquid nitrogen immediately after excision and stored in -80°C for later analysis. After excision the hearts were washed in phosphate buffered saline (PBS), excess tissue was trimmed off and the weight of the hearts was listed. The hearts were then

fixated in paraformaldehyd (PFA) in room temperature. 24 hours later PFA was replaced with 70% Ethanol. The fixed tissues were cut into three pieces – one cut in level with the suture and one between the suture level and the distal apex.

The tissues were then dehydrated, embedded in paraffin and sectioned with a microtome in 6-µm thick sections transversely from the base of the LV to the apex. The levels of analysis had a 300 µm interval between them. Five sections from each level were stored mounted on glass slides. One of them was stained with Masson´s Trichrome stainings (Fig. 3). and the others were stored for eventual future analysis.

Staining

The slides were deparaffinized in 58 °C over night. Then they were put into a rack and dipped in four consecutive stain jars containing xylene - 10 minutes in each jar. The rack was then dipped in a series of 100%, 95%, 80% and last 70% ethanol for 5 minutes each to get Xylene removed. The slides were then rinsed with tap water for 5 minutes to remove ethanol. The tissues were then surrounded with a liquid blocker to prevent the staining solutions from draining off. The sections were kept in 4% PFA at room temperature for the first fixation. For the second fixation the sections were kept in Bouin´s solution over night at room temperature under a hood to prevent the tissues from drying due to evaporation. This step intensifies the colors and increase the contrast between the tissue components. The slides were then washed under running tap water (18-26°C) for 5-10 minutes to remove the all yellow color (picric acid). Then they were rinsed briefly in deionized (destilled) water (ddH2O). The sections were then incubated for 5 minutes with Wiegert´s Hematoxilin Staining, which staines the nucleis black. Then the slides were put in a glass chamber and washed under warm tap water for ~10 minutes to get excess of Hematoxylin removed and to get the black color intensified. The tissues were then rinsed for 1 minute in ddH2O.. Sections were incubated for 5 minutes with Biebrich scarlet-acid fuchsin, which stains cytoplasm and muscle fibers red. Then they

were washed 3x 1minute with ddH2O. The sections were then incubated for 10 minutes in a glass chamber with Phosphotungstic/Phosphomolybdic Acid Solution, which prepared the uptake of the aniline blue stain. After incubation, the slides were drained but not allowed to dry. Then they were incubated directly for 5 minutes with the Aniline Blue Solution and then they were washed 3x 1 minute with ddH2O. Aniline blue stains the collagen blue. Acetic acid renders the shades of color more delicate and transparent. The sections were incubated for two minutes and then washed for 2x 1minute with ddH2O. Afterwards they were drained successively in 70%EtOH for 3 minutes, 90% EtOH for 3 minutes, 100%EtOH for 3 minutes and lastly Xylol for 5 minutes. Lastly the slides were mounted with 1-2 drops of a xylene-based mounting media (Permount or Polymount). The coverslip were dropped gently on to the slides and the drops spread out and cover the tissue. Bubbles were carefully avoided. The slides were then pressed under heavy weight for 10 minutes and then they were stored in room temperature.

Ethics on animal use

Animal research in Sweden is strictly regulated by both EU and Swedish legislation on animal welfare. However, legal and ethical norms do not always coincide and legal norms can sometimes be questioned from an ethical point of view. Consequently, use of animal in research requires reflection of several matters related to the researchers responsibility to both animals as well as research results.

A few examples of reflective questions that need answers in form of well-founded arguments in order to proceed with the intended research model are listed below.

- For whom is the research performed?
- Based on what arguments are animals used in the model?
- What am I ethically and legally entitled to do with an animal?
- What are my ethically and legally duties towards humans and how do they differ towards animals?

By using the two most influential normative theories, utilitarianism and deodontology, argument and decision making can be facilitated. According to utilitarianism an act can be justified if the positive overall outcome is greater than the negative consequences. The fathers of utilitarianism, Jeremy Bentham and J. S. Mills, meant that calculation of consequence should be based on the ability to experience pain and suffering. In contrast Immanuel Kant, the founder of deontology (duty ethics), said that those who are able to formulate a moral law are to be morally considered. According to duty ethics, the consequences of an act are too difficult to estimate. Nor is it the consequences of an action that determines whether it is right or wrong, but the intention behind it. Kant meant that an act is morally right when it is both intrinsically good and also good without qualification (the act does not make the situation

worse).

Legal norms are dependent on the values within a culture. For instance mice, rats and birds are not considered as laboratory animals in USA. According to EU all vertebrate animals that are subject to invasive procedures (at minimum a pinprick) are defined as a laboratory animal whereas the Swedish definition which is even broader – all animals used for scientific purpose, regardless field or methods, are included. Consequently, the annual statistics of the number of animals being used for research each year varies alot depending on which country the figures are provided from.

Even though research based on humans would give more reliable results this is regarded unethical out of respect of human dignity (in animal ethics the view of human superiority is greatly debated) and therefore not an option. The dilemma is called the animal research paradox – we use animals in experiments because they are sufficiently like us and because they are sufficiently different from us (which allows us to motivate the suffering we cause). The uncertainty of interpreting results from animals to humans can be exemplified by the introduction of penicillin to the market. Penicillin was first tested on mice and the found beneficial effects were applied to humans. The substance would probably never have reached the market if it would have been tested on guinea pigs instead. Penicillin causes death in guinea pigs due to suppression of normal enteric flora resulting in enterocolitis and enterotoxemia.

In addition, several studies have shown that despite promising result in animal trials only 5-10% of the drugs reach the market, due to unpredicted safety issues. Pharmaceutical regulators have also reported that 92% of drugs that pass preclinical trials later fails clinical trials because animal studies so often ”fails to predict the specific safety problem that ultimately halts development”(Food & Drug Administration (2004). Challenge and

Opportunity on the Critical Path to New Medical Products. Bethesda, MD: FDA).

Hence - the predictive value of preclinical trials need to be improved and the relevance of results from preclinical trials need to be questioned from both ethical and scientific perspective in order to ensure that animal based research models only are used when absolutely necessary.

This is an example of where research ethics and animal ethics are being crossed. It is crucial to perform as relevant research results as possible both for the sake of research and for the sake of the animals. It is the researchers responsibility to carefully consider available options for the planned research model in order to avoid useless result and to minimize the suffering of animals.

But how is this done? In the book The Principles of Humane Experimental Technique, published by William Russell and Rex Burch in 1959, guiding and ethical principles for the use of animals in research are described and summarized in three Rs. The Rs stands for Replacement, Reduction and Refinement and the concept of them has become very influential widely accepted over the past 50 years.

Replacement – refers to methods available to avoid the use of animals in experiments, both absolute replacements and relative (i.e. replacing more sentinent animals with animals that have a lower pain perception, often invertebrates

Reduction – refers to any strategy that will result in fewer animals being used. This in combination with maximizing the obtained information from each of them can potentially limit future suffering.

Refinement – refers to the modification of experimental procedures to reduce pain, stress and

distress to enhance welfare of the animals.

Approval by an ethics committee

According to EU-directive 2012/63 all research using animals must be ethically evaluated. In Sweden ethical review of animal based research has been mandatory since 1979. The Swedish Board of Agricultures has appointed seven committees who evaluates the research in respect of animal suffering, potential human benefit and also societal interest.

Each committee consists of twelve members – 6 scientists or staff who routinely work with laboratory animals and six laymen, some of them representing animal welfare organizations. .

The layman of the committee represents the general public´s evaluation whereas the scientist´s role is to make an evaluation of the quality of the applied research as well as its potential contribution to knowledge gain. Together they have a legally defined task of ensuring that no animals are used if not necessary and also unnecessary pain or suffering are used.

The researcher need to provide the committee with a detailed project description including information about why animals are being used and why that specific species, the animal situation (housing, handling, end points, lab practice, expected negative effects on them and level of suffering), the choice of method and expected scientific gain, quality of research results and time until expected results.

Does the research apply to a great number of people who all suffers relatively little or is it a concern about a small number of people who have a great deal of suffering that effect their everyday lives? That is examples of questions that the committee must take into account.

Data collection and statistics

Survival data

When an animal died the date was recorded for later analysis. The risk of mortality post MI is at its highest the first seven days due to complications of surgery and rupture of the infarcted area. The survival analysis provides answers to questions such as how many of the animals survived the duration of the experiment and at what rate mortality occurred. In case of, for instance, dropouts or subjects being studied for different length of time the Kaplan-Meier Estimate is the most well-known and simplest way to calculate survival over time. However, if every subject is followed until death or to the closure of the experiment, as in this study, the survival curve is estimated by calculating the fraction surviving at each time point according to:

$$S_t = \frac{\text{Number of subjects living at the start} - \text{Number of subjects died}}{\text{Number of subjects living at the start}}$$

A graph presenting time in the x-axis and the percentage of surviving animals in the y-axis is made by entering the results of the calculations from the raw data into a statistical program, Excel in this case. The data is presented in Fig. 5 and Fig. 6.

Quantification of infarcted area

BioPix is an imaging software for histological quantifications. The program is easy to use following a protocol and it allows rapid quantifications of large number of samples. By selecting the stained areas in the image of a tissue-section, or performing length or area measurements, results of calculations based on raw data will automatically be available for

exportation to a statistical program for graph presentation. The area of infarction is calculated by dividing the sum of infarcted area from all sections by the sum of LV areas from all sections. By multiplying by 100 the area is expressed in percentage. An average value, the standard deviation and a T-test of the ratio between infarction and total area for the two genotypes were also calculated. The null hypothesis was rejected at $p < 0.05$. SD-ratio is a measure of the disperse of data from the mean. A two-sample Student´s T-test examines if the means of two normally distributed populations are equal. In case of a p-value below the threshold of statistical significance the null hypothesis is rejected in favor of the alternative hypothesis. The quantification of infarcted area is presented in Fig. 4.

Results

Establishing the method was very challenging and took more time than I had planned for and that is the reason why the number of experiments became limited.

Survival

Due to a small and atypical infarction confirmed on the ultrasound one day post-surgery one HT was excluded. 5 HT and 4 WT died on the third day post-surgery and one HT died on the fourth day. Four animals survived the entire duration of the experiment, 2 WT and 2 HT. The research group decided it was necessary to repeat the experiment with a larger number of animals since two subjects from each group are too few to draw any conclusions about the data (Fig. 5). The second attempt included 13 females (6 WT and 7 HT) and 10 males (5 WT and 5 HT), 14- 16 weeks of age. Two HT females died during surgery and one HT male was excluded from the study since the ultrasound one day post-surgery indicated that the ligation of LAD had failed and no infarction was induced. One HT female died two days post-surgery and three HT males died respectively three, four and five days post-surgery (Fig. 6).

Cardiac function

The cardiac functions, based on echocardiography, were analyzed by my colleague. The ejection fraction, heart rate and blood pressure did not differ between the groups during the healing process.

Histology

Five HT and eight WT survived the duration of the experiment. The data of average ratio of infarct to total volume of the left ventricle is presented in Fig. 4. No difference between the two groups was notable.

Discussion

In this project I developed a method of inducing myocardial infarction in mice. When the protocol was established the mouse model was used to examine the effects of filamin B proteins on the healing process of the myocardium. The effects were evaluated with echocardiography and histological staining. Our intention was to shed light on the complex healing process after ischemic injury in the myocardium and hopefully contribute to the acquisition of more knowledge to the research area. The healing of the myocardium is a dynamic process including inflammation, tissue formation and tissue remodeling[23]. Neovascularization is an integral component of the remodeling process[24] and it is essential to wound healing. Filamin B has previously been shown to have angiogenic properties[15]. The proteins are generally considered to be cytoplasmatic architectural proteins but also involved in cell signaling and nuclear transcription. Filamins provide mechanical stability by maintaining cell to cell and cell to matrix connections and they also transmit signals to the actin skeleton during cellular locomotion[18]. Mice as well as humans have three filamin genes[17] – FLNA, FLNB and FLNC. FLNC is expressed in skeletal and cardiac muscle while FLNA and FLNB are ubiquitously expressed. Point mutation in the filamin gene results in severe congenital malformations in the skeletal, central nervous, urogenital and the cardiovascular systems in human. Similar malformations have been reported in FLNA-mutated mice[25] and our group for the first time reported skeletal and malformations in FLNB deficient mice[17]. The casual roles of filamin proteins in these congenital disorders are unknown. The knowledge of this could be of great importance in controlling angiogenesis in the treatment of cardiovascular diseases. Generation of in vivo and in vitro models of FLNB deficiency will advance our knowledge and understanding of the importance of FLNB in organogenesis and angiogenesis. Using a gene-trapping technique our group generated a mouse model of FLNB deficiency that was used in this project in order to test the hypothesis that reduced filamin expression will reduce cardiac function and survival after myocardial

infarction in mice due to impaired response to cardiovascular remodeling. Therapies aimed to induce angiogenesis could have a central role in the treatment either as single therapy or in addition to conventional therapies. When it comes to the results of the FLNB-knockout mice study more work must be done before the questions of the aims can be answered. Since four HT died in comparison with zero WT the Fisher Exact test suggests that wild type mice survives myocardial injury to a greater extent than the gene modified. However, since the time of death occurred during the critical first seven days post-surgery no conclusions can be drawn when it comes to survival. The sizes of the infarctions in the deceased animals were not larger than in the wild types and it is thus not a valid explanation to the distributions of deaths. A larger number of animals need to be included in the project to obtain significant statistical basis. The histological analysis indicates that there is no difference in the size of infarcted area between the genotypes 10 weeks post MI. The ultrasound analysis performed by my colleague did not show any difference in infarct size or cardiac function. After the end of the study the idea of HT-mice in some way might compensate for the lack of FLNB were awakened. We therefore performed RT-PCR to study the gene expression and we found a 30% difference in the FLNB protein levels between the genotypes. It is possible that the protein expression may vary between individual mice in the HT-group and if the differences are less than 20% no disparity, when it comes to induced infarct area, from WT will be obtained. Since only one mouse from the HT-group survived 10 weeks the lack of difference in the analysis might be explained by an almost normal protein level in that particular individual. The protein levels can be tested before initiation of the project in order to exclude HT-mice with a protein level close to wild types.

Occlusion of LAD in mice has proved to be a good and efficient model for research in the area of myocardial ischemia[26]. The model can be performed at a low cost and it is highly reproducible, which allows studies of molecular and physiological variables in a controlled

manner. If LAD is permanently occluded no blood flow is permitted to perfuse the area and an infarct will be induced in the left ventricle and apex while the septum will be spared. With a distinct septal artery and a left coronary artery which courses over the LV giving off variable branches the mouse coronary artery differs from human and common large animal models. The impact of this anatomical difference to post-infarct remodeling is yet unknown[27]. The ischemic changes in the hearts of mice is though similar to what is described in human and extensive remodeling occurs at 48 hours and beyond[28]. There are several different methods of performing LAD occlusion but the number of detailed descriptions of the procedure are limited. The surgical operation of inducing MI is quite complicated and it takes a lot of practice to become proficient. That is the reason to the limited number of animals surviving the experiment for analysis. A detailed description of the procedure is of great value for optimizing survival and avoiding pitfalls. A skillful surgeon can obtain a post-surgical survival rate of approximately 70% and the high survival rate is crucial for obtaining statistically significant results at the cost of as small sample sizes as possible[29]. Variations in the infarcted area due to surgery may relate to variations in the distribution of the origin of the LAD. A pool of anomalies in the origin of the main coronary trunks have been observed – for example accessory ostium, high origin and aortic intramural courses. Variations in origin but also variations in distal branching can most probably explain the heterogeneities of infarct sizes[27]. Distal branching often varies among different mouse strains. A small MI is likely due to a ligature placed much more distally than intended or possibly because of an incomplete ligation. An incomplete ligation often gives rise to scar tissue which partially interrupt the blood flow and a non transmural MI will be obtained. Non transmural infarctions does not show significant remodeling and are often removed from published data results. Survival, phenotype, cardiac function and degree of infarct have been shown to correlate on the precise placement of the occluding suture. Occlusion approximately

0.3 mm from the atrioventricular junction results in significant alterations in haemodynamic and histologic end-points without any significant reduction in survival. 50-76% survival 7 days post MI has been reported in this group[30]. A ”too high” placement of the suture will generate an infarct in up to 70% of the LV and the animals will not survive. The mortality increases significantly if the infarct area becomes larger than 50%[30]. It is hard to ethically justify the use of a model with such severe outcome. Low placement (0.6 mm from the atrioventricular junction) of the suture does not differ from sham-operated animals when it comes to haemodynamic end-points[30].

Populärvetenskaplig sammanfattning

Hjärtinfarkt (HI) är vår tids vanligaste folksjukdom och tillståndet utgör den ledande orsaken till dödsfall i alla välfärdsländer. Insjuknande och dödlighet vid HI har minskat i Sverige men antalet människor som insjuknar tidigt i livet och vars livskvalité därmed begränsas under lång tid är ständigt ökande. Den medicinska och ekonomiska bördan av dödlighet och sjuklighet efter hjärt-kärlsjukdom är enorm för samhället. En djurmodell av HI spelar en viktig roll för utvecklingen av diagnos och behandling av mänsklig HI. En sådan modell möjliggör studier av orsakerna till tillståndet och ger också möjlighet att undersöka vilka behandlingsmetoder som kan vara mest fördelaktiga.

Det första syftet med mitt projekt var att utveckla en modell av hjärtinfarkt i levande möss. Genom att sätta ett litet stygn runt ett av hjärtats större kärl hindras blodflödet från att avge syre till hjärtmuskeln. Syrebristen leder till att de delar av hjärtat som försörjs av det avstrypta kärlet skadas. I beskrivningen av ingreppet berättar jag i detalj hur operationen skall utföras eftersom det är ett tekniskt mycket svårt ingrepp. En noggrann beskrivning underlättar inlärningen för blivande kirurger och det gör att onödigt lidande för mössen kan förhindras. Mitt andra syfte med projektet var att använda modellen till att operera möss som delvis saknar ett visst protein som kallas för filamin B (FLNB). FLNB har I tidigare studier visats stimulera nybildandet av blodkärl. Denna process är av stor betydelse för läkningsprocessen i hjärtat efter en infarktskada. Genom att jämföra dessa genmodifierade möss med möss som har normala FLNB-nivåer kan mer information om hjärtats läkningsprocess inhämtas. Min forskningsgrupps förhoppning är att filaminproteiner skall kunna användas till att förutspå prognosen för infarktdrabbade människor och kanske även användas vid behandling. I projektet utfördes ett hjärtultraljud dagen innan operation för vi skulle ha ett utgångsläge att senare jämföra hjärtat med. 24 timmar samt en respektive tio veckor efter operationen upprepades ultraljudsundersökningen för att analysera hjärtats pumpförmåga och eventuella

förändringar i skadeområdet. Tio veckor efter operationen togs hjärtat ut och skivades i tunna snitt innan det färgades i olika färger för att hjärtskadan skulle kunna analyseras ytterligare. En statistisk analys visade att möss med normala FLNB-nivåer överlever infarktskada i större utsträckning än de genmodifierade. Döden inträffade dock under de första kritiska dagarna efter operationen och slutsatser vad gäller överlevnad kan därför inte dras. Ultraljuden och de histologiska analyserna visade inte på någon skillnad i pumpförmåga och infarktstorlek mellan grupperna. Experimentet behöver dock upprepas med ett större antal djur för att definitiva slutsatser skall kunna dras.

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References

1. Gaziano, T., et al., *Cardiovascular Disease*, in *Disease Control Priorities in Developing Countries*, D.T. Jamison, et al., Editors. 2006: Washington (DC).
2. 2002, W.H.O.W.H.R.
3. *Socialstyrelsen: Dödsorsaker 2011, sid 23.*
4. Heart & Stroke Statistical Update, A.H.A.
5.

3 Socialstyrelsen: Hjärtinfarkter 1987- 2010, tabell 6 och 11.
6. *Socialstyrelsen: Hjärtinfarkter 4 Ibid, tabell 22.*
7. Hjärt-Lungfonden, H.-.
8. The National board of health and welfare, A.S.R., Sweden 2013).
9. *Socialstyrelsen: Hjärtinfarkter Ibid, tabell 16, 18 och 22.*
10. Kolk, M.V., et al., *LAD-ligation: a murine model of myocardial infarction.* J Vis Exp, 2009(32).
11. Salto-Tellez, M., et al., *Myocardial infarction in the C57BL/6J mouse: a quantifiable and highly reproducible experimental model.* Cardiovasc Pathol, 2004. **13**(2): p. 91-7.
12. Kumar, D., et al., *Distinct mouse coronary anatomy and myocardial infarction consequent to ligation.* Coron Artery Dis, 2005. **16**(1): p. 41-4.
13. Ou, L., et al., *Animal models of cardiac disease and stem cell therapy.* Open Cardiovasc Med J, 2010. **4**: p. 231-9.
14. Russell, J.C. and S.D. Proctor, *Small animal models of cardiovascular disease: tools for the study of the roles of metabolic syndrome, dyslipidemia, and atherosclerosis.* Cardiovasc Pathol, 2006. **15**(6): p. 318-30.
15. Del Valle-Perez, B., et al., *Filamin B plays a key role in vascular endothelial growth factor-induced endothelial cell motility through its interaction with Rac-1 and Vav-2.* J Biol Chem, 2010. **285**(14): p. 10748-60.
16. Zhao, T., et al., *Vascular endothelial growth factor (VEGF)-A: role on cardiac angiogenesis following myocardial infarction.* Microvasc Res, 2010. **80**(2): p. 188-94.
17. Zhou, X., et al., *Filamin B deficiency in mice results in skeletal malformations and impaired microvascular development.* Proc Natl Acad Sci U S A, 2007. **104**(10): p. 3919-24.
18. Stossel, T.P., et al., *Filamins as integrators of cell mechanics and signalling.* Nat Rev Mol Cell Biol, 2001. **2**(2): p. 138-45.
19. Krakow, D., et al., *Mutations in the gene encoding filamin B disrupt vertebral segmentation, joint formation and skeletogenesis.* Nat Genet, 2004. **36**(4): p. 405-10.
20. Zhou, X., J. Boren, and L.M. Akyurek, *Filamins in cardiovascular development.* Trends Cardiovasc Med, 2007. **17**(7): p. 222-9.
21. Chung, N.A., et al., *Angiogenesis in myocardial infarction. An acute or chronic process?* Eur Heart J, 2002. **23**(20): p. 1604-8.
22. Barandon, L., et al., *Repair of myocardial infarction by epicardial deposition of bone-marrow-cell-coated muscle patch in a murine model.* Ann Thorac Surg, 2004. **78**(4): p. 1409-17.
23. Singer, A.J. and R.A. Clark, *Cutaneous wound healing.* N Engl J Med, 1999. **341**(10): p. 738-46.

24. Kocher, A.A., et al., *Neovascularization of ischemic myocardium by human bone-marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function.* Nat Med, 2001. **7**(4): p. 430-6.
25. Feng, Y., et al., *Filamin A (FLNA) is required for cell-cell contact in vascular development and cardiac morphogenesis.* Proc Natl Acad Sci U S A, 2006. **103**(52): p. 19836-41.
26. Wang, J., et al., *A simple and fast experimental model of myocardial infarction in the mouse.* Tex Heart Inst J, 2006. **33**(3): p. 290-3.
27. Fernandez, B., et al., *The coronary arteries of the C57BL/6 mouse strains: implications for comparison with mutant models.* J Anat, 2008. **212**(1): p. 12-8.
28. Sutton, M.G. and N. Sharpe, *Left ventricular remodeling after myocardial infarction: pathophysiology and therapy.* Circulation, 2000. **101**(25): p. 2981-8.
29. Gao, E., et al., *A novel and efficient model of coronary artery ligation and myocardial infarction in the mouse.* Circ Res, 2010. **107**(12): p. 1445-53.
30. Degabriele, N.M., et al., *Critical appraisal of the mouse model of myocardial infarction.* Exp Physiol, 2004. **89**(4): p. 497-505.

Figures and tables

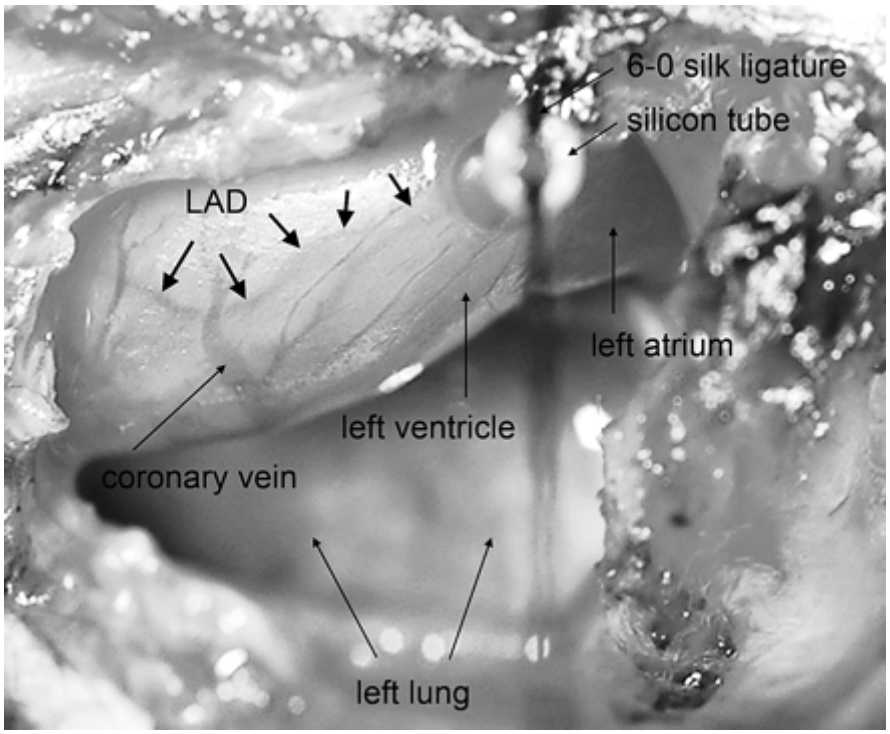

Figure 1. Anatomy of mouse heart and coronary vasculature.

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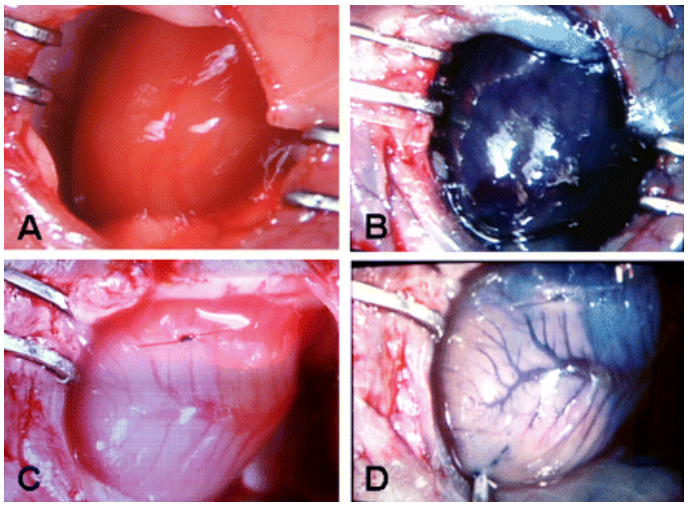

Figure 2. Macroscopic view of hearts after ligation of LAD. A and C shows hearts prior to injection while B and D shows hearts after injection of Evan´s blue.

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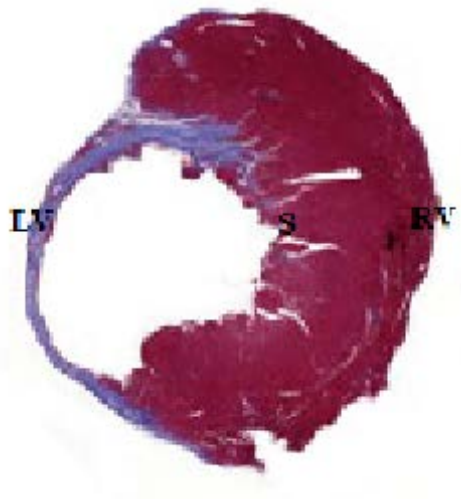

Figure 3. Masson´s trichrome staining of an infarcted heart from FlnB knock-out.

LV (left ventricle), S (septum), RV (right ventricle)

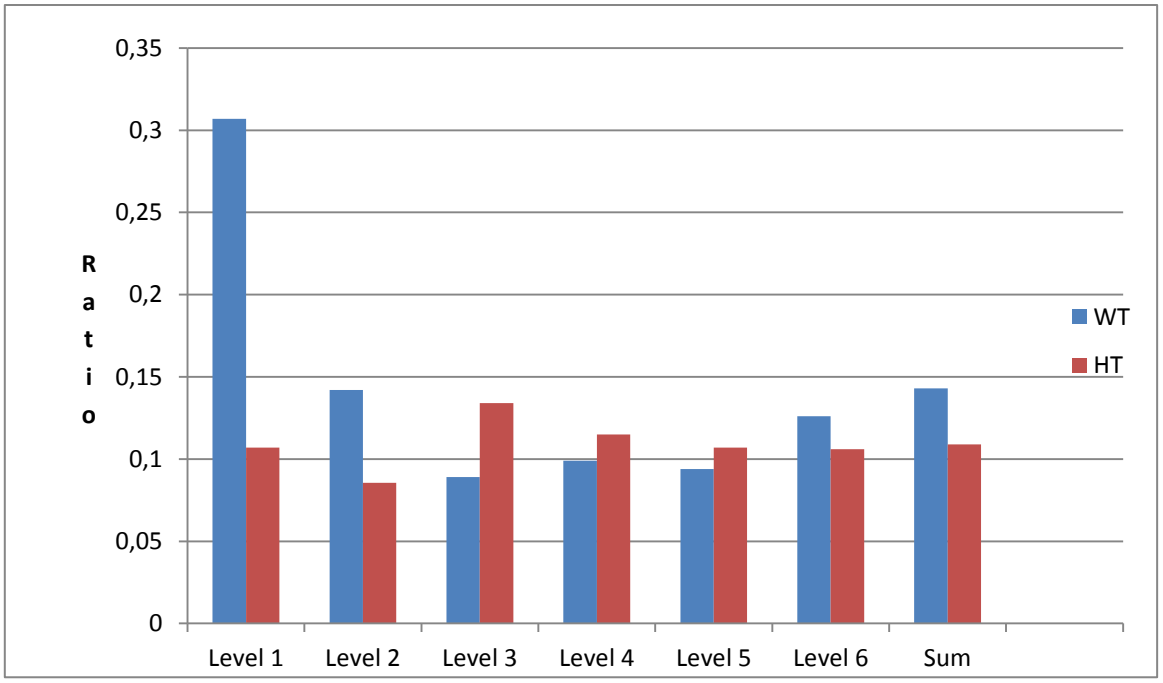

Figure 4. Avarage ratio of infarct to total area in the different levels of sections.

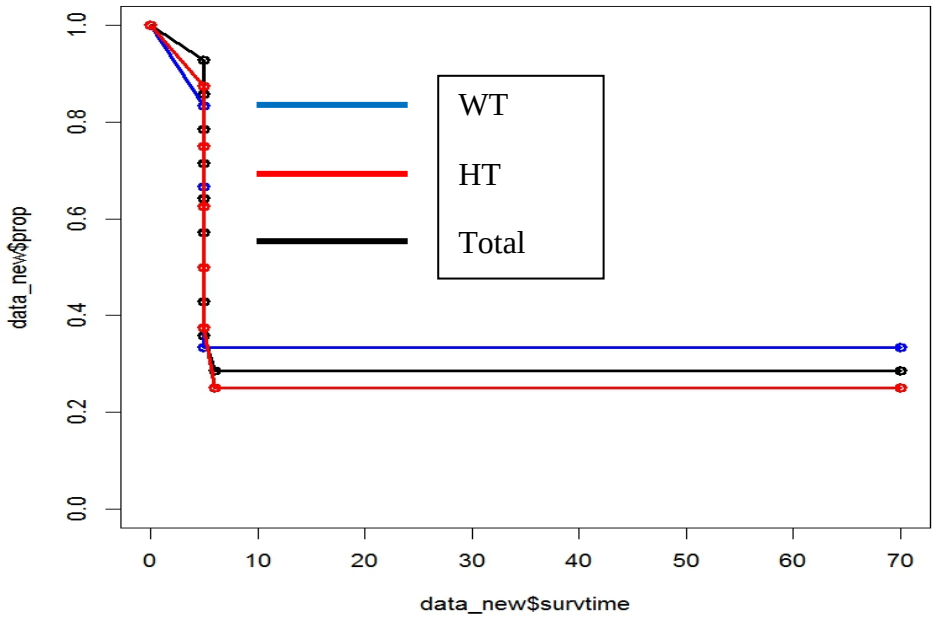

Figure 5. Survival curves for heterozygous, wild-type and all combined. First infarction experiment. The X-axis shows the number of days survived. The Y-axis shows surviving proportion.

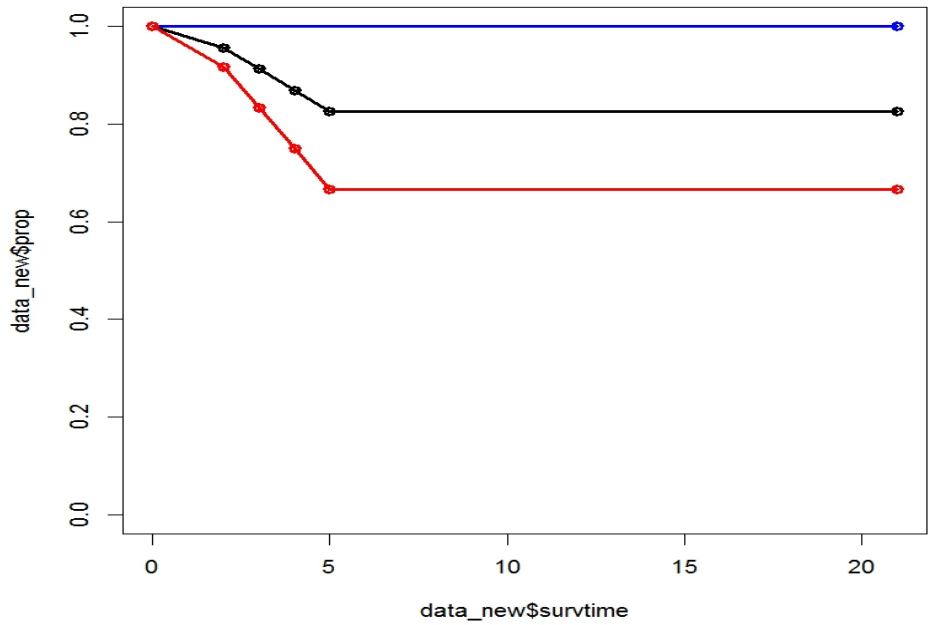

Figure 6. Survival curves for HT, WT and all combined. Second infarction experiment.