MODELING OF DILATED CARDIOMYOPATHY WITH A FOCUS ON ENZYMATIC PHENO- AND GENOTYPING

Katerina Todorova[1] *, Any Georgieva[1], Rositsa Milcheva[1], Zdravka Petrova[1], Inna Sulikovska[1], Volodymyr Balatskyi[2], Viktor Navrulin[2], Wiktoria Laprus[2], Paweł Dobrzyń[2]

1. Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria
2. Nencki Institute of Experimental Biology, Polish Academy of Sciences, 02-093 Warszawa, Poland

*corresponding author: katerinagencheva@yahoo.com

The cardiomyopathy is a disease of the heart muscle and is a leading cause of heart failure or dangerous arrhythmias both, in animals and humans. This is mainly primary progressive genetically determined condition, that reflexes to damages of the heart tones and reduced pumping of blood to the rest parts of the body. In the development of the condition proper nutrition and restriction to high-intensity activities are typically necessary to reduce the risk of sudden cardiac death, mostly important to humans, breeding and sporting animals. Cardiomyopathy is commonly manifested as dilated cardiomyopathy (DCM) in dogs and horses and hypertrophic cardiomyopathy (HCM) in cats. In humans it was accepted that cardiomyopathies are a group of diseases characterized by genetic heterogeneity. As is well known the contractions of the myocardium are subsequence of multiple cell surface glycoproteins including calcium, potassium and sodium ion channels that propagate the electrical impulses and action potentials. In the process of sialylation – the modification of proteins, where glicoproteins are formed, several enzymes from the family of sialyltransferases take role. There are some data about altered glycoprotein sialylation, particularly in human DCM. The data explained above and the prevalence of DCM cardiomyopathy forms in animals and hunam, provoked our interest to assess the role of several factors in glycoprotein sialylation in the pathogenesis of cardiomyopathy via simulated *in vivo* and *in vitro* models, that are not genetically predisposed and to compare with research data published on the topics of pathophysiological and clinical heterogeneity of DCM.

Key words: Dilated cardiomyopathy, experimental models, sialyltransferases

Aim: Assessment of the role of several factors in glycoprotein sialylation in the pathogenesis of cardiomyopathy via simulated *in vivo* and *in vitro* models, that are not genetically predisposed and to compare with research data published on the topics of pathophysiological and heterogeneity of DCM.

Materials and methods: ***In vivo model of DCM*** **(permissive №459/18.12.2025)**: The role of some sialyltransferases activity was tested in a non-genetically created DCM in vivo mouse model. In mice the DCM was produced by acute exposure to adrenalin, followed by 5-fluorouracil (5-FU) administration. Adrenalin stimulates β1-adrenoceptors, causing tachycardia, enhanced contractility and muscle fatigue. In appropriate doses 5-FU can induce new-onset severe cardiomyopathy with insufficient left ventricular contractility. The experimental design is based on the model of isoproterenol (isoprenaline, ISO) followed by 5-FU administration, proposed by **Salerno et al., 2023**. ICR female mice (3 months of age) will be obtained, individually hosted with supply of water and food ad libitum. 2 groups of female mice (1 gr., n=10; 2 gr., n=5) were formed: first group received one-time 0,5mg adrenalin s.c. ,the second was not treated - control group. Three days after the adrenalin application, the surviving mice from 1 group were randomly assigned to saline (1A group, n =5) or 5-FU administration (1B group, 15 mg/kg/day, n = 5) for 10 days. The animals sacrificed under anesthesia and the hearts were fixed in 10% buffered formalin. **Salerno, N., Scalise, M., Marino, F., Filardo, A., Chiefalo, A., Panuccio, G., ... & Cianflone, E. (2023). A mouse model of dilated cardiomyopathy produced by isoproterenol acute exposure followed by 5-Fluorouracil administration. Journal of Cardiovascular Development and Disease, 10(6), 225.**

Isolation and culture of neonatal murine cardiomyocytes: Neonatal cardiomyocytes were isolated from 0-3-day-old mice using an enzymatic dissociation protocol with minor modifications. Cardiomyocytes were enriched using a two-step purification procedure consisting of differential centrifugation and pre-plating on uncoated culture dishes for 1–3 h at 37°C to allow preferential attachment of fibroblasts. Cells were counted and seeded onto 1% gelatin-coated culture plates at a density of 1.3 × 105 cells/cm2. Cardiomyocytes were initially cultured in DMEM high glucose supplemented with 15% fetal bovine serum (FBS) and 100 U/mL penicillin, and 100 µg/mL streptomycin. After 24 h, the medium was replaced with maintenance medium consisting of DMEM high glucose supplemented with 4% FBS and penicillin/streptomycin. Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

Cell Viability Assay: The cytotoxicity of 5-fluorouracil (5-FU) and adrenaline was assessed using the Neutral Red Uptake (NRU) assay. Neonatal cardiomyocytes were exposed to 5-FU in concentrations of 0.5, 5, 50 and 500 µM and adrenaline in concentrations of 1, 10, 100 µM for 24 h. Absorbance was then measured at 540 nm using a TECAN microplate reader (TECAN, Grödig, Austria). Cell viability was calculated using the following formula: Cell viability (%) = (Absorbance of treated cells/Absorbance of control cells) × 100.

Cytomorphology: May Grunwald-Giemsa and Fluorescent Microscopy/ Acridine orange/propidium iodide staning: Cells were cultured on 13 mm-diameter cover glasses in 24-well plates and treated with 1 µM adrenaline and 5 µM 5-FU for 24 h. To observe morphological changes, native preparations of control and treated cardiomyocytes were stained with May Grunwald-Giemsa (Diapath) or fluorescent dyes acridine orange (AO 1 mg/mL) and propidium iodide (PI 1 mg/mL) in PBS and images were taken using a Leica DM 5000B microscope.

Assessment of Mitochondrial Membrane Potential (ΔΨm) by JC-1 staining: Neonatal murine cardiomyocytes were seeded in 24-well plates and treated with 1 µM adrenaline and 5 µM 5-FU for 24 h. After treatment, cells were incubated with JC-1 dye (5 µg/mL) for 20 min at 37°C in the dark. Fluorescence images were taken using a fluorescence microscope (Leica DM 5000B, Leica Microsystems, Wetzlar, Germany). The changes in the mitochondrial membrane potential was quantified with ImageJ software by measuring the mean fluorescence intensity of red (JC-1 aggregates) and green (JC-1 monomers) channels, and the red/green (R/G) ratio was calculated. A decrease in the R/G ratio indicated mitochondrial depolarization.

Molecular biological analysis: Cardiomyocytes were plated in 25 cm2 tissue culture flasks at a density of 2.5 x 10_4 cells/mL and incubated in growth medium for 24 hours. Next day the cells were treated with 1 µM adrenaline and 5 µM 5-FU. Untreated cells incubated at the same culture conditions were used as control. After 24 h incubation control and treated cell cultures were washed twice with cold phosphate buffered saline (PBS), scratched using a rubber policemen, centrifuged for 5 min at 250 x g and after supernatant removal were frozen at - 80°C and stored until use for molecular biological analysis.

Gene expression analyses: Total RNA was extracted using RNeasy Mini Kit (Qiagen), according to the provided protocol. The yield and purity of the collected RNA were measured using S-300 spectrophotometer (Boeco). Approximately 2 µg of total RNA from each sample were used for first strand cDNA synthesis, as previously described (Milcheva et al., 2019). The generated cDNA was quantified, and the samples were stored at -80°C. Real-time PCR was performed on 1 µl of RT-product, containing approximately 500 ng cDNA as a template, in 20 µl total volume of reaction using RotorGene SYBR Green PCR Kit (Qiagen) following the recommendations of the producer. Three real-time PCR reactions/sample in triplicate were performed for amplification of Gapdh, Casq2, St3gal4, and St6galnac1, using RotorGene™ 6000 Real-time Analyzer (Corbett Life Science-Qiagen). The data were analyzed using Rotor Gene Q Series Software (Qiagen) and the relative quantification of the sialyltransferase expression was calculated by the ΔΔCt method (Zhang et al., 2021) versus Gapdh as a reference gene. After each run, a High-Resolution Melting Curve Analysis (HRM) was performed to verify the specificity of the amplified products, which were visualized on 2.5% agarose gel supplemented with Simply Safe nucleic acid stain (EurX®) versus 100-1000 bp DNA Ladder (EurX®), and the gels were photographed with a gel documentation system Vision (Scie-Plas Ltd).

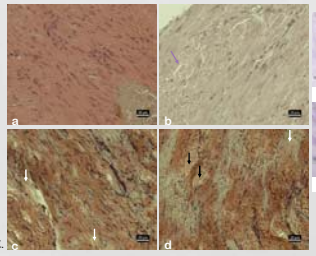
Statistical analysis: GraphPad Prism 5.03 software (San Diego, CA, USA). Non-parametric one-way analysis of variance (Kruskal-Wallis test) with Dunn's Multiple Comparison Test (significance level 0.05) was computed to detect statistically significant differences between the Ct values of the qPCR products between the control and infected samples, and the results were interpreted as follows: P < 0.001 = highly significant (***), P < 0.01 = very significant (**), P < 0.05 = significant (*).

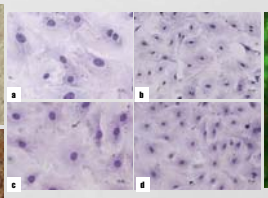
RESULTS:

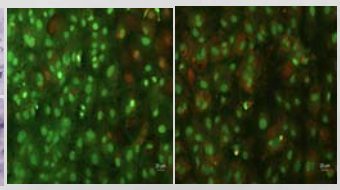
Investigated genes and their primers sequences used in this study:

Gene	Abbreviation	Primer sequences (5'-3')	Product size
Glyceraldehyde-3-phosphate dehydrogenase	Gapdh	TCCTCGTCCCGTAGACAAAATG – F AATCTCCACTTTGCCACTGC – R	103
Calsequestrin 2	Casq2	ACATAGGCTTTGTGATGGTGGA – F CCCGTCAAACTCAATCCTGC – R	119
ST3 beta-galactoside alpha-2,3-sialyltransferase 4	St3gal4	TGGGTAAAAGCCCATCCAC – F TCAAGGCTCTTTATGCTCTCAG – R	119
ST6 beta-galactosamide alpha-2,6-sialyltransferase 2 (putative)	St6galnac1	TCCTACTTCTCAGCTGTGTTGGCA – F TCTCCTGGGCACTTGCGTCA – R	117

Histopathology: a) control; b) 0,5 mg adrenalin, myofibril damage (blue arrow) ; c and d) 0,5 mg adrenalin and 15 mg/kg/day (myocyte disarray): myocyte hypertrophy, mixed with myocyte atrophy and stretching (white arrows), interstitial collagen deposition (black arrows); H&E.



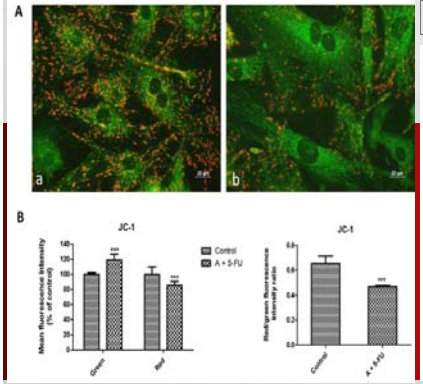


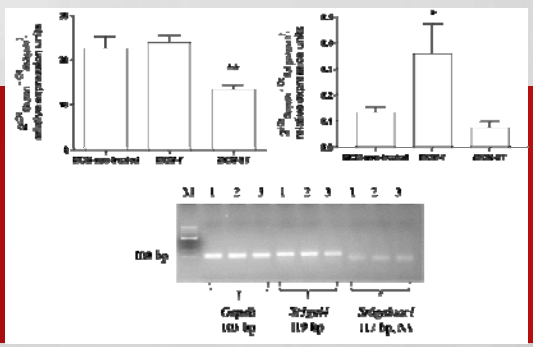


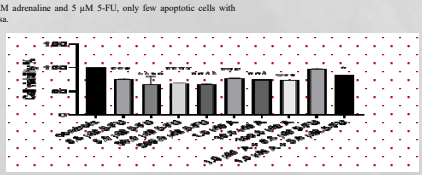
Cytomorphology: Treated (a, b) vs control (c, d) with 1 µM adrenaline and 5 µM 5-FU, only few apoptotic cells with pyknotic nuclei (b) in the treated model; May Grunwald-Giemsa.

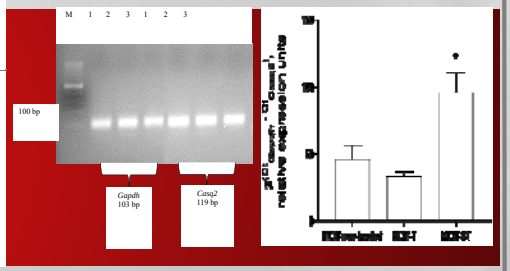
Cytopathology: control vs treated with 1 µM adrenaline and 5 µM 5-FU: slightly reduced number of vital cells, lack of mitotic figures and isolated cases of nuclear blebbing; AO&PI.

Cardiomyocytes (MCM-T and MCM-ST) display profound genetic heterogeneity and fractionate into specialized subtypes based on their exact function, micro-environment and gene expression profiles.



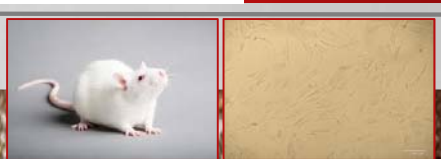






Acknowledgments: This work was supported by the project № ІС-РЦ/16/2026-20 27, between BAS and PAS.

Mouse photo from https://www.taconic.com/products/mouse-rat/standard-immunodeficients/icr-scid



Neonatal myocardiocytes - IEMPAM-BAS & NENCKI INSTITUTE of experimental biology