



Lublin, July 14, 2023

Ph.D. Thesis Evaluation Report / Assessment Report on Dissertation

Thesis Title:

“Hyperthermia and Immune Response: exploring the immunoregulatory potential of fever-range temperatures”

Author: Henryk Mikołaj Kozłowski

Supervisor: dr hab. Sylwia Wrotek, prof. NCU

The concept of the PhD thesis proposed by Henryk Mikołaj Kozłowski is very interesting. The dissertation presents original research results related to effects of hyperthermia on the immune system.

Body temperature has been of great interest among physicians since ancient times and fever was regarded as a positive sign for infected individuals, with Greek philosopher Parmenides (540-470 BC) famously stating "Give me the power to produce fever and I will cure all diseases". In modern times, the study of fever has continued to be of great interest to medical professionals, who observed i.e. that infectious fever may contribute to cancer remission. After therapies involving injecting bacteria or other pathogens were discontinued due to their potential risk, the observation of the beneficial effects of elevated body temperature led to the development of new methods for artificially raising body temperature. As a result, fever-range hyperthermia (FRH), which is often utilized as a therapeutic approach, has been increasingly used as a model to study the effects of fever on the immune system without the confounding effects of an active infection. Thus, this model allows for the study of the effects of elevated temperature itself on e.g., cell signaling and gene expression patterns. However, there is still much to be learned about how FRH specifically affects the immune system. Protocols for disease prevention are particularly desirable, so this PhD thesis primarily aimed to investigate the potential utility of FRH in healthy organisms/cells. The objectives of this dissertation were as follows:

- 1) to comprehensively investigate how FRH treatment affects hematological profile, and regulatory molecules such as cytokines, and miRNA at the level of entire organism,
- 2) to accurately identify the FRH-induced macrophage phenotype, including the diversity of macrophage subsets, and
- 3) to explore the potential of FRH to modify the effects of the TLR-4-dependent stimulators.

Therefore, the scientific problem undertaken by Henryk Mikołaj Kozłowski is important and relevant, and the proposed approaches are innovative and valuable. Henryk Mikołaj Kozłowski did his PhD thesis at the Department of Immunology, Faculty of Biological and Veterinary Sciences, Nicolaus Copernicus University in Toruń, under the supervision of Professor Sylwia Wrotek, who is an expert in the field of hyperthermia and inflammation.

General description of the thesis

The PhD thesis contains all the required sections, which are arranged in the following order: Introduction, Aim of the study, List of publications containing the characteristics of three articles, Discussion, Conclusions and future perspectives, Acknowledgments, References, and Abstracts in English and Polish. The dissertation begins with introduction to the subject-matter. Next, the main aims and three detailed objectives are described. The main part of the thesis includes experimental data, which are





presented in the form of three papers: two of them are already published and one is submitted to scientific journal:

- 1) **Kozłowski HM**, Sobocińska J, Jędrzejewski T, Maciejewski B, Działuk A, Wrotek S. *Fever-range whole body hyperthermia leads to changes in immune-related genes and miRNA machinery in Wistar rats*. Int J Hyperthermia. 2023;40(1):2216899. doi: 10.1080/02656736.2023.2216899. 100 points, IF= 3.753.
- 2) **Kozłowski HM**, Sobocińska J, Jędrzejewski T, Maciejewski B, Działuk A, Wrotek S. *Fever-range hyperthermia can effectively switch macrophage polarization towards regulatory phenotype*. Article sent to journal (not peer-reviewed version).
- 3) **Kozłowski HM**, Pawlikowska M, Sobocińska J, Jędrzejewski T, Działuk A, Wrotek S. *Distinct Modulatory Effects of Fever-Range Hyperthermia on the Response of Breast Cancer Cells and Macrophages to Mistletoe (Viscum album L.) Extract*. Pharmaceuticals (Basel). 2021 Jun 8;14(6):551. doi: 10.3390/ph14060551. 100 points, IF= 5.711.

Specific comments

FRH is a medical treatment that involves heating of the body to a temperature between 39°C to 41°C. The use of FRH as an immunomodulatory therapy has been studied extensively in clinics for recent years, and has shown promising results in the treatment of various medical conditions, including viral infections, autoimmune disorders and to support cancer treatment. Although studies have shown that FRH can have a range of beneficial effects on the immune system, further research is still needed to fully understand the underlying mechanisms. Therefore, Henryk Mikołaj Kozłowski prepared 3 original articles:

Article #1 (Published online: 06 Jun 2023) *Fever-range whole body hyperthermia leads to changes in immune-related genes and miRNA machinery in Wistar rats*. Here, the PhD student developed a novel, fast rat model of infrared-induced FRH. The body temperature of animals was monitored using biotelemetry. FRH was induced by the infrared lamp and heating pad. White blood cell counts were monitored using Auto Hematology Analyzer. In peripheral blood mononuclear cells (PBMC), spleen and liver expression of immune-related genes (IL-10, MIF and G-CSF, IFN- γ) and miRNA machinery (DICER1, TARBP2) was analyzed with RT-qPCR. Furthermore, RT-qPCR was used to explore miRNA-155 levels in the plasma of rats. The authors observed a decrease in the total number of leukocytes due to lower number of lymphocytes, and an increase in the number of granulocytes. Furthermore, they observed elevated expressions of DICER1, TARBP2 and granulocyte colony-stimulating factor (G-CSF) in the spleen, liver and PBMCs immediately following FRH. FRH treatment also had anti-inflammatory effects, evidenced by the downregulation of pro-inflammatory macrophage migration inhibitor factor (MIF) and miR-155, and the increased expression of anti-inflammatory IL-10. In conclusion, the authors stated that FRH affects the expression of molecules involved in inflammatory processes leading to alleviated inflammation and supposed these effects may be miRNAs-dependent and FRH can be involved in therapies where anti-inflammatory action is needed. Henryk Mikołaj Kozłowski is the first author of the manuscript, which indicates his dominant role in its preparation, but I did not find in the submitted documentation signed statements of the co-authors of the work about the exact percentage contribution of the PhD student to this publication.

Article #2 (Article sent to journal; not peer-reviewed version) *Fever-range hyperthermia can effectively switch macrophage polarization towards regulatory phenotype*. Among cells that display an





increased susceptibility to heat, macrophages arouse the most interest. It is known that their diverse functions depend on their polarization state, but it is not well recognized whether this process can be modulated by FRH. To address this, Henryk Mikołaj Kozłowski used two different macrophage cell lines that were treated with FRH. To define macrophage phenotype, he and co-authors examined several functional surface cell markers: CD80 and CD163, and intracellular markers such as inducible nitric oxide synthase (iNOS), arginase-1 (Arg-1). Additionally, in FRH-treated cells the authors analyzed an expression of Toll-like receptor 4 (TLR-4) and its role in macrophage polarization. Authors also checked whether FRH can switch the polarization of macrophages in pro-inflammatory condition triggered by lipopolysaccharide (LPS). Their findings demonstrate that FRH induces M2 polarization, as evidenced by significant increase in expression of CD163 and Arg-1. However, the increased expression of cyclooxygenase 2 (COX-2) and TLR-4 shows that these cells may still display pro-inflammatory properties, what suggest polarization towards M2b phenotype. Interestingly, FRH is able to shift lipopolysaccharide (LPS)-induced M1 polarization towards M2 phenotype and reduces the levels of anti-microbial molecules such as ROS and NO. In summary, the presented results suggest that FRH is a strong modulator of macrophage polarization that favors M2 phenotype even in pro-inflammatory condition. Henryk Mikołaj Kozłowski is again the first author of this publication, but I did not find in the submitted documentation signed statements of the co-authors of this manuscript about the exact percentage contribution of the PhD student to this article.

Article #3 (Published online: 08 Jun 2021) *Distinct Modulatory Effects of Fever-Range Hyperthermia on the Response of Breast Cancer Cells and Macrophages to Mistletoe (Viscum album L.) Extract*. Mistletoe extract (ME) is an adjunctive medication prescribed to cancer patients. The increase in body temperature is frequently observed in patients following ME administration. Nevertheless, the impact of this fever on the effectiveness of therapy is unknown. Therefore, Henryk Mikołaj Kozłowski aimed to investigate the effect of fever-range temperatures on ME-treated breast cancer cells and macrophages. The cells were simultaneously stimulated with ME and subjected to FRH (39 °C or 41 °C). After co-treatment, the cell viability, generation of reactive oxygen species (ROS), cell cycle distribution, and production of pro-inflammatory factors (interleukin (IL)-1 β , IL-6, and cyclooxygenase (COX)-2) were evaluated. The results showed that the exposure of ME-treated breast cancer cells to FRH at 39 °C resulted in a slight decrease in their viability, whereas FRH of 41 °C enhanced this effect. Only FRH of 41 °C induced minor changes in ROS level in ME-treated breast cancer cell lines. In ME-treated macrophages, FRH stimulated cell proliferation. The cell cycle distribution analysis showed a difference between cells cultured at 39 °C and 41 °C in all examined cell lines. Moreover, hyperthermia at 41 °C completely inhibited the ME-induced increase in IL-1 β and IL-6 expression in MCF-7 breast cancer cells, whereas this effect was not observed in 4T1 breast cancer cells. In contrast, in ME-treated macrophages, FRH of 41 °C strongly up-regulated expression of the pro-inflammatory factors. The authors of this paper concluded that fever is an important component of ME therapy that differentially affects cancer and immune cells. Henryk Mikołaj Kozłowski is the first author of this article, but again I did not find in the submitted documentation signed statements of the co-authors of this paper about the exact percentage contribution of the PhD student to this manuscript.

In summary, the data presented in the abovementioned original manuscripts are interesting. Henryk Mikołaj Kozłowski utilized several research methods, including in vivo studies in rats and in vitro experiments using mammalian cells lines (in accordance with 3R principle). He employed the following research techniques: flow cytometry (macrophage polarization, cell cycle, reactive oxygen species), colorimetric assay (nitric oxide level, MTT assay), Dot-blot and Western blot assay (protein expression), real-time PCR (gene expression, miRNA level), surgical procedures (anesthetizing and implantation of biotelemetry devices), deep body temperature measurement (biotelemetry), hematological analysis





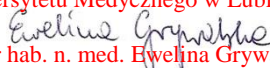
(impedance principle), whole body fever-range hyperthermia (induced by infrared lamp). I appreciate the high effort, time-consuming work and complexity of all performed tests.

Final evaluation statement

The candidate for the PhD degree has performed a large amount of research and obtained new original results. The dissertation work was performed at good scientific level. Henryk Mikołaj Kozłowski succeeds in formulating the motivation and goals of his studies satisfactorily. The research results of this PhD thesis contributes significantly to the knowledge and understanding of the important and highly relevant topic. In his dissertation, Mr. Kozłowski can clearly differentiate between his own and new contributions as well as the results already known in the literature. In addition, he clearly demonstrates the relevance of his results for the area of his expertise. The candidate can correctly assess the scientific significance of his results and place it in the context of existing knowledge in the field of area of immunology. Mr. Kozłowski's work clearly shows signs of independence, expresses critical thinking and clear signs of originality. As part of his doctoral thesis, Henryk Mikołaj Kozłowski demonstrated that he can do research independently. The dissertation shows the candidate's ability to be sufficiently familiar with the relevant research techniques and methods. The candidate for the PhD degree manages to show in his dissertation, that he can critically deal with relevant literature. In my opinion, the material is presented clearly, systematically and logically. The linguistic, stylistic and technical processing of the dissertation was also of high quality.

In summary, my conclusion is that the PhD thesis of Mr. Henryk Mikołaj Kozłowski presents original research results of great importance and high quality. Therefore I recommend without hesitation that the candidate is awarded the doctoral degree.

I, hereby, declare that the reviewed PhD thesis titled "Hyperthermia and Immune Response: exploring the immunoregulatory potential of fever-range temperatures" by MSc Henryk Mikołaj Kozłowski meets the criteria pursuant to art. 187 Act of 20 July 2018 The law on Higher Education and Science (Journal of Laws of 2018, item 1668, as amended) and I propose to allow MSc Henryk Mikołaj Kozłowski to proceed with further stages of the doctoral dissertation.

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