REVIEW

Prepared by: Prepared by Prof. Plamen Todorov, DSc, from the Department of Reproductive Biotechnology and Cryobiology of Gametes, Institute of Biology and Immunology of Reproduction, BAS

Subject: Dissertation on "Study of Cell Death Mechanisms and the Role of the Effector Gasdermin D in the Induction of the NLRP3 Inflammasome: Implications for Male Fertility," presented by Ilka Tsvetanova Tsvetkova-Ivanova for the award of the educational and scientific degree "Doctor" in professional field 4.3. Biological Sciences, specialty "Immunology," cipher 01.06.23.

Information on the procedure: The dissertation was discussed and scheduled for defense following the proposal from the "Reproductive OMIX - Technologies" section at IBIR-BAS. The procedure was accepted, and the jury approved by the Scientific Council of IBIR. All necessary documents related to doctoral studies were presented. I believe that all the requirements of the "Law for the Development of Academic Staff in the Republic of Bulgaria and the Regulations for its application have been met.

Brief biographical data of the candidate: Ilka Tsvetanova Tsvetkova-Ivanova was born in 1994 in Lovech. She received her higher education at the Faculty of Biology at Sofia University "St. Kliment Ohridski", where in 2017 she graduated as a Bachelor in Molecular Biology and in 2019 as a Master in Developmental Biology. Since 2019, she has been enrolled as a full-time PhD student at IBIR-BAS and concurrently works as an assistant at the Institute under an additional employment contract. She is married.

My personal impressions of the candidate are positive. I dare say that Ilka Tsvetkova is a highly qualified young scientist, proficient in modern research methods, cell culture techniques, and genetic analyses. She is respected by her colleagues.

Topicality of the dissertation topic: Infertility is a significant medico-social problem, affecting 10-12% of reproductive-age couples, often due to reduced seminal fluid quality. This is mostly attributed to anomalies in spermatogenesis, where Sertoli cells play a crucial role. They participate in forming the blood-testicular barrier, creating an immune-privileged environment, and ensuring normal testicular functioning through complex hormonal regulation. The dissertation focuses on studying the factors initiating cell death in Sertoli cells, exploring molecular mechanisms of apoptosis and pyroptosis, and related signaling pathways. I believe that the topic is timely and such studies are essential not only in molecular biology and immunology but also for reproductive biology and medicine.

Analysis and evaluation of the dissertation

Structure: The dissertation spans 136 printed pages and is organized as follows: Introduction (3 pages), Literature Review (25 pages), Purpose and Tasks (2 pages), Materials and Methods (15 pages), Results (50 pages), Discussion (12 pages), Conclusions (1 page), Contributions (1 page), Bibliography (17 pages), and a List of Publications and Participations in Scientific Forums related to the dissertation (1 page).

Introduction: In the introduction, the applicant examines the actuality of the problem, justifying the need to conduct the studies underlying the dissertation.

Literature review: The review is extensive, comprehensively covering all aspects of the problem and providing information that serves the chosen scientific topic and is of a contributing nature. In

the beginning, it provides information about the structure and functions of male gonads and the regulation of spermatogenesis. Logically, in view of the thesis, much of the review is devoted to Sertoli cells – their essence and functions, proteins synthesized by them. Information is presented about the five main classes of hazard signal receptors (PRRs) and the mechanisms provided by them for resisting, tolerating damage and restoring normal state. The functions and mechanisms of action of various adaptor proteins, cytokines, caspases and gasdermines are described. Much of the review is devoted to the different types of cell death – autophagy, apoptosis, necrosis, pyroptosis, with greater attention being given to pyroptosis and to the mechanisms of switching the type of cell death.

The review is illustrated with 3 appropriate schemes, which makes it more understandable and easy to perceive. On the basis of the problems identified in the review, the author justifies the purpose and tasks of this dissertation.

Aim and objectives: The aim of the dissertation is to investigate the mechanisms of cell death in Sertoli cells and related signaling pathways involving the main caspases and Gasdermin D. To fulfill the set goal, the author develops 7 tasks that are clearly and precisely formulated.

Materials and methods: The studies were conducted at IBIR-BAS using Sertoli cells from the 15P-1 cell line, isolated from the testicular cells of transgenic 6-month-old mice, sourced from the American Type Culture Collection (ATCC).

A wide range of research methods were used in the experiments: cell culture, microscopy (including fluorescence), flow-through flow-through flow-through flovitometry, Real-time qPCR, nanopore sequencing, ELISA, etc. The methodologies are described clearly and in detail, which allows the reproduction of the experiments. The use of modern equipment and environments and consumables of leading manufacturing companies guarantees the reliability of the results obtained.

There is no doubt about the personal participation and contribution of the author in the experiments. In this respect, it is clear that the PhD student has mastered and successfully applied a number of methods.

Results and discussion: The findings of the dissertation can be categorized into several groups corresponding to the set objectives.

The candidate demonstrated that treating cells with LPS and ATP leads to the activation of both caspase-1 and caspase-3, resulting in cells primarily undergoing apoptosis, with a notable occurrence of pyroptosis. It is likely that the activation of caspase-1 is attributable to the operation of multiple inflammasomes. Cells undergoing cell death do so via the Nlrp3-Asc dependent pathway of caspase-1 activation, while in living cells, the activity follows an axis independent pathway. The activation of caspase-3 is entirely dependent on the Nlrp3-Asc axis.

CD300a acts as a bidirectional regulator of caspase-1 activation. Its presence restricts the onset of pyroptosis via a caspase-1 dependent pathway, and its absence limits robust caspase activity in living cells. Additionally, its influence on caspase-3 activity is two-fold – it stimulates the activation of caspase-3 in living cells and limits its activity in cells directed towards pyroptosis.

The activation of the Nlrp3-Asc inflammasome pathway amplified the expression of total GSDMD (inactive tetramer) but did not significantly increase its active truncated form (C-terminal fragment). The expression of the CD300a receptor is essential for the normal expression of total GSDMD (inactive tetramer) and for its subsequent activation.

GSDMD has divergent effects on caspase-1 and caspase-3 – it inhibits caspase-1 activity while activating caspase-3.

LPS-induced signaling diminishes specific mitophagy, favoring nonspecific macroautophagy under stress conditions with reduced nutrients (glucose).

The results are supported by well-selected and presented evidence. The photographic documentation is of very high quality, and the statistical analysis is impressively presented through graphs. The candidate has adeptly interpreted her results and compared them with findings from other researchers, demonstrating a profound understanding of the subject.

I am confident that the results are original and there is no evidence of plagiarism. An attached reference confirms that the dissertation was checked for plagiarism using specialized software, and none was detected.

Conclusions and contributions: The research led to the formulation of six well-founded conclusions, all of which I endorse, and the identification of three significant contributions. These conclusions accurately encapsulate the results obtained from the study.

Literature: The dissertation is supported by a comprehensive bibliographic list of 213 references, all in Latin. Notably, 47 of these sources are from the past five years, demonstrating the dissertation's relevance and engagement with current research. These sources are cited appropriately throughout the dissertation in alignment with its objectives.

Publications in connection with the dissertation: The candidate has impressively contributed two publications to journals with impact factors and quartiles, satisfying the national minimum requirements for the educational and scientific degree of Doctor as per the Law on Educational and Social Sciences and its implementing rules. In both publications, Ilka Tzvetkova is the primary author, underscoring her active and substantial role in the research. Moreover, the results of this research have been presented at various scientific forums through posters and reports, further disseminating the findings and their implications in the field.

Autoreferat: The autoreferat, encompassing 42 pages, is meticulously prepared in accordance with the stipulated requirements. It comprehensively reflects all the principal results and contributions of the dissertation, succinctly summarizing the key findings and their implications.

Remarks to the dissertation: My primary observation concerns the dissertation's title. The phrase "meaning for male infertility" seems extraneous. The research and its results are fundamentally theoretical, conducted on an immortalized line of mouse cells. It remains uncertain how these findings would translate to a primary line of human Sertoli cells and their relevance for the diagnosis and treatment of male infertility.

Additionally, I have some technical observations. For instance, I believe the terms "diagram" and "scheme" used in the dissertation are redundant, and the term "figure" should suffice in their descriptions.

It is important to note that these critiques are minor and do not detract from the overall merit and quality of the dissertation presented for review.

Conclusion: The dissertation by Ilka Tsvetkova-Ivanova is both scientifically and practically relevant. It exhibits a well-balanced structure, effectively integrating various aspects of the

scientific work and appropriately emphasizing the volume of scientific results and their discussion. Given the dissertation's merits and its adherence to the necessary quantitative and qualitative criteria, I propose to the esteemed members of the scientific jury that Ilka Tsvetkova-Ivanova be awarded the academic and scientific degree of "Doctor" in the specialty of Immunology (01.06.23).

Date: 09.01.2024

Prepared the review:

Prof. Plamen Todorov, DSc