REVIEW

By: Assoc. Prof. Ivanka Georgieva Tsacheva, PhD

Department of Biochemistry, Sofia University "St. Kliment Ohridski",
member of the scientific jury appointed by order No RD-38-260/27.05.2025
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regarding the dissertation of RUMIANA RUMENOVA GANEVA entitled: "**Importance of the Endometrial Immune Profile for the Success of Assisted Reproduction**" for awarding the educational and scientific degree "PhD" in a professional field 4.3. Biological Sciences, Scientific specialty "Cell Biology"
Scientific supervisor: Assoc.Prof. Veselina Moskova-Doumanova, PhD

I declare that I have no conflict of interest within the meaning of Article 4, paragraph 5 of the Academic Staff Development Act in the Republic of Bulgaria (ASDA). This review has been prepared in accordance with the regulatory documents - the Law on Academic Academic Degrees and the Recommendations of the BF Faculty Council on the criteria for the award of academic degrees.

Rumiana Ganeva received her education at the Faculty of Biology of Sofia University "St. Kliment Ohridski". In 2016, she graduated as a Bachelor in Molecular Biology, and in 2018 she obtained a Master's degree in MP Developmental Biology. She gained additional skills through Nature Masterclasses online courses in Scientific Writing and Publishing; Experiments: from Idea to Design; Scientific Communication and Scientific Presentations, and Statistics in Public Health through a Johns Hopkins University online course.

The dissertation is structured according to the requirements for this type of scientific work. It is written on 193 A4 pages and contains the following main sections: 1. Introduction (2 pages); 2. Literature review (59 pp.); 3. Aim and objectives (1 p.); 4. Materials and Methods (19 p.); 5. Results and discussion (43 p.); 6. Conclusions (1 p.); 7. Conclusion (2 p.); 8. Contributions (1 p.). Information related to publications and participations of the PhD student at scientific forums is attached, as well as six appendices certifying the opinions of the Ethics Committees of Nadezhda Medical Center and of Sofia University St. Kliment Ohridski, the informed consent of the patients included in the study and the raw data from the ROC analysis. The excellent technical design of the work makes a very good impression.

Relevance of the problem. The periods of pregnancy create the seemingly paradoxical situation of the immune status of the maternal organism, in which a tolerant environment must be maintained to the fetus and at the same time immune protection against pathogens must be maintained, an essential part of which is realized through cellular mechanisms. Control over the functions of cellular players in the immune response is achieved by a complex network of cytokine stimuli synthesized with the participation of uterine immune cells. The regulatory effect of local and systemic cytokine stimulus during the period of embryo implantation is still poorly understood, with the role of T and B cells, NK cells and macrophages being the focus of this topic. Appropriate cellular and molecular markers are being sought to establish the parameters of immune status that ensure successful implantation on one hand, and a successful pregnancy outcome on the other. In this sense, Rumiana Ganeva's dissertation work is particularly valuable given the concrete clinical application of such knowledge in assisted reproduction.

The literature review sequentially presents the role of the endometrium in reproductive processes, the role of the immune system in implantation processes, markers for the recognition of immune cells in the endometrium, the role of immune cells in reproductive pathologies, methods for the diagnosis of immune-mediated RIF (recurrent implantation failure) and immunotherapy in *in vitro* patients. The review of the literature is comprehensive, informative and logically connected. Each immune cell type has been characterized functionally in the systemic immune response and in the endometrium in particular.

Rumiana has tackled the complex task of representing immune cells as a network of sources of molecular stimuli characterizing the dynamic environment in the uterus before and after implantation. Some factual errors and ambiguous notations (Tk instead of Tc; terminal maturation instead of terminal differentiation; communication instead of interaction; CD3 co-receptor instead of signaling subunit; monocytes represented as lymphoid instead of myeloid cells, etc.) are found in the exposition. Despite these gaps, the literature review gives a good impression of the level of preparation of Rumyana for real experimental work in this complex topic. My recommendation to this section is to avoid the use of foreign words.

The aim was to establish immune markers of endometrial receptivity to assess the effectiveness of local immunotherapy in RIF patients *in vitro*.

A comprehensive analysis of the scientific literature on the subject was performed to compile the experimental design and study preparation, and 344 sources were cited.

Experimental design (tasks)

The research work is divided into two parts, which are interrelated. Initially, in a group of 116 women, the immune populations present in the endometrial stroma during the mid-luteal phase were analyzed quantitatively and qualitatively. This was followed by a comparative analysis of immune populations in the endometrial stroma of patients with successful and failed embryo implantation to derive immune indicators of endometrial receptivity as ratios between different pairs of immune cells.

In the second part, the aim is to determine, on the basis of the derived immune indicators, the effect of administered PBMC treatment on embryo transfer success, endometrial receptivity, including a comparative determination of the effect of treatment on receptivity before and after PBMC placement according to embryo transfer outcome.

Results

In the first part of the experimental work, the percentage distribution of immune cells and the correlations between them were determined in biopsy samples from the entire study group of 116 patients by immunohistochemical analysis, and it was found that all the immune cells examined in the endometrial stroma correlated significantly. The highest correlation coefficient was found between macrophages and T lymphocytes ($R=0.563$, $p<0.001$), NK cells and monocytes ($R=0.588$, $p<0.001$) and NK and B cells. The amounts of immune cells in relation to the outcome after embryo transfer were determined in the two groups with successful implantation and with failed implantation, as well as the correlations between them. The ratios of different pairs of immune cells were calculated and additionally subjected to ROC analysis, which was used to select 6 immune indicators for the assessment of endometrial receptivity: the ratios of NK/T (CD56/CD3), Tc/T (CD8/CD3), Th/T (CD4/CD3), Maph/T (CD68/CD3), Th/Tc (CD4/CD8) and Th/Maph (CD4/CD68), which showed a high discriminative value.

The second part of the work included 18 patients with recurrent implant failures. No differences in the concentrations of IL-6, TNF-α, IL-4 and IL-10 were found in their plasma samples when compared with the concentrations of the same cytokines in a control group of healthy women. Peripheral blood mononuclear cells (autologous PBMCs) were isolated from each patient and the type and amount of peripheral blood lymphocytes isolated was determined flow cytometrically. A preliminary experiment on the choice of culture method for isolated PBMCs with hCG was performed, in which, on the basis of an assessment of the survival of cultured cells and the concentrations of major proinflammatory (TNF-α and IL-6) and anti-inflammatory (IL-4 and IL-10) cytokines secreted into the medium over 24 hours of incubation, it was determined that the hormone was not critically required.

By measuring the amount of proinflammatory acute phase opsonin CRP in blood plasma, it was found that intrauterine placement of autologous PBMCs did not result in the manifestation of systemic inflammation. It was also found that after PBMC placement, the amount of CD4+ T cells increased significantly, which was evaluated as a positive effect for successful implantation.

The success rate of embryo transfer after intrauterine insertion of autologous PBMCs was determined; of the 18 patients included, 11 underwent embryo transfer 1 month after treatment and 36.4% (4/11) of patients had successful embryo implantation.

A comparative analysis of immune indices after autologous PBMC placement in patients with successful and failed implantation was performed. In women with successful embryo transfer after intrauterine placement of autologous PBMCs, the Maph/T ratio showed a near significance ($p=0.068$) increase, which may be due to a decrease in total T lymphocytes and an increase in macrophages.

Immune indices prior to placement of PBMC in patients with successful and failed implantation were comparatively analyzed to distinguish those in whom treatment would alter the endometrial environment in the desired direction, leading to successful implantation. There were no significant differences between the successful and unsuccessful implantation groups according to these endometrial immune indices. Given that the number of patients is not large, it is likely that these results should be validated in a group including a larger number of patients.

The Maph/T, NK/T, Th/T and Th/Tc ratios showed high positive predictive potential as most of the successful cases had values of these ratios above the cut-off values defined in stage 1. Similarly, Maph/T, Th/Tc, and Tc/T showed high negative predictive potential, as most of the unsuccessful cases had values of these ratios below the cutoff values defined in stage 1.

The analysis of the dissertation results in a two-part discussion, where they are also discussed in the context of already published evidence by other authors, is excellent. This discussion confirms my belief about the relevance of the topic and the extreme difficulty of analysing such a large amount of data. I particularly appreciate the schemes created to compare the endometrial immune environment in successful and failed implantation. This type of illustration adequately represents the understanding Rumiana has reached in the course of her work.

The work on this dissertation has scientific and theoretical contributions. Particular high evaluation of the dissertation work give the contribution results achieved for the first time in Bulgaria.

Conclusions

On the basis of the experiments, 6 conclusions are formulated - 3 from the results of the first part and 3 more from the results of the second part. The conclusions are correctly formulated and fully correspond to the results obtained.

Sciencemetrics

The results related to this dissertation have been published in 3 articles, two of which are in established, reputable journals with impact factor and Q1. They have already been cited at least 7 times. Rumiana has presented her work in 4 international forums and posters in 14 forums.

The abstract contains 58 pages and reflects reliably the most significant results obtained in the development of the dissertation. It is very well structured, contains summarized data and scientific interpretation of the obtained results. The conclusions and contributions are unchanged. I believe that the abstract meets all the necessary requirements.

Questions

I have the following questions for the doctoral student:

1. How do you explain the lack of correlation between CD56+ NK and CD3+ T cells (Fig. 4.5) in the group with successful implantation, while the NK/T ratio has a statistically significant high value (Tables 4.3 and 4.4) and is one of the selected indicators?

2. Why is it possible that in some RIF patients the number of CD3+ T cells matters for successful implantation, while in others it does not matter, as written in discussion part 1?

Conclusion

The submitted dissertation, in terms of its volume, form and content, fully meets all requirements and Regulations for its implementation and the Regulations of SofiaUniversity “Kliment Ohridski”. It is a complete study that provides valuable scientific information. The research has been conducted methodologically correctly, a wide range of modern methods has been mastered, a serious research activity has been carried out in terms of volume and scale, and the ability to interpret the obtained results and draw conclusions and contributions has been demonstrated. The dissertation is original, with scientific and applied contributions and is the work of the author.

Because of the above, I confidently give my positive assessment of the conducted research, the achieved results and contributions, and I propose to the honorable scientific jury to award the educational and scientific degree "PhD" to RUMYANA RUMENOVA GANEVA.

04.07.2025 Assoc.prof. I.Tsacheva