

TO THE CHAIRMAN OF THE SCIENTIFIC JURY

Standpoint

of *Prof. Krassimira Olegova Todorova-Hayrabyan, Ph.D., DSc.*

Head of the Laboratory of Reproductive OMICs Technologies at the Institute of Biology and Immunology of Reproduction "Acad. Kiril Bratanov" - BAS

Regarding the defense of the dissertation of *Lyuboslava Dimitrova Valkova*, Senior Clinical Embryologist at Hospital "Dr. Shterev" LLC

For conferring the **educational and scientific degree "Philosophy Doctor" (PhD)** in subject of higher education **4. Natural Sciences, Mathematics and Informatics, professional field 4.3 Biological Sciences, scientific specialty Cell Biology 01:06:18**

On the thesis entitled "**Evaluation and optimization of vitrification for human preimplantation embryos and oocytes**"

With Supervisors:

Prof. Dr. Atanas Shterev, MD

Corresponding member Prof. Rumen Pankov, DSc

1. General presentation of the procedure and the doctoral student

The presented set of materials on paper / electronic media is in accordance with the Procedure for acquiring **educational and scientific degree "Philosophy Doctor" (ESD "PhD/Doctor")** at the Faculty of Biology (BF) of Sofia University "St. Kliment Ohridski" (SU) and the respective Rules of the BF of SU.

The doctoral student has submitted 9 scientific publications in indexed and peer-reviewed journals, 3 of which in impact factor / impact rank (SJR) journals, and also 15 scientific forums presentations/lectures are presented, of which 3 are presented orally by the author. The dissertation is diligently designed.

The doctoral student Lyuboslava Dimitrova Valkova graduated from the Master's Program "Cell Biology and Developmental Biology" at the Faculty of Biology of the Sofia University "St. Kliment Ohridski", Sofia, Bulgaria, with excellent success.

Currently, Lyuboslava Valkova is a Senior Clinical Embryologist at SAGBAL "Dr. Shterev" EOOD (Hospital "Dr. Shterev" LLC), after working for 1 year as a biologist-specialist in the Department "Structure and function of chromatin", at the Institute of Molecular Biology, Bulgarian Academy of Sciences, where she had training in the RT-PCR method. She has also 1 and 16 years respectively in AIP SMP "Dr. Atanas Shterev" EOOD, and in SAGBAL "Dr. Shterev" EOOD, as an embryologist at different expert levels. There she became acquainted with the processing of sperm, eggs, and embryos for the purpose of

intrauterine insemination, in vitro fertilization, ICSI, embryo transfer, freezing, storage, thawing, and use of sperm, eggs, and embryos. During this period Luboslava Valkova became a member of the following professional organizations in the field of assisted reproduction: the European Society of Human Reproduction and Embryology (ESHRE), Bulgarian Association of Infertility and Reproductive Health (BASRZ), Bulgarian Society of Human Reproduction and Embryology (BSHRE). She is fluent in written and spoken English at an excellent level, as well as Spanish at an intermediate level.

2. Relevance of the research topic

In recent years, there has been a change in the demographic distribution of the Bulgarian population, with an increase in the average age for first reproduction due to career development, while at the same time "rejuvenating" cases of cancer, which would lead to loss of ovarian function, as well as to sterility due to chemo- or radiation- therapy. This is a particularly immense problem for unborn women without a partner and has not only biomedical, but also a social dimension. In these and other cases, when a further application of assisted reproductive technologies are to be expected, such as the donor egg programs, gametes and embryos cryopreservation efficiency improvement is an utmost necessity, as well as is the subsequent optimization of the process of embryo transfer (ET).

There are a number of indications for freezing of untransferred embryos, such as providing an additional option for subsequent transfer in case of failure, or if one would wish for a subsequent pregnancy at a later stage, in order to avoid hormonal stimulation and follicular puncture. The procedure is also required due to arisen embryo transfer contraindications, such as the development of ART complications - ovarian hyperstimulation syndrome (OHSS), a polyp in the uterine cavity during hormonal stimulation, uterine bleeding, inability to penetrate the uterus, etc. Cryopreservation of oocytes is an important stage of the so-called " Freeze all " program, as well as prenatal genetic diagnosis and screening for mono-genetic diseases and aneuploidies.

The present dissertation is a very valuable research project, lasting for many years and covering a significant number of clinical cases, which aims to optimize a number of factors influencing the cryopreservation method " *vitrification* " applied to human pre-implantation embryos and oocytes.

3. PhD student knowledge of the problem

The PhD student has been working and researching the problem for 17 years in her role as an embryologist at an obstetrics, gynecology and assisted reproduction clinic. Data were collected from 2453 preimplantation human embryos, collected out of 941 cycles, from 844 patients. In the PhD thesis a 274 literary source are presented, as though literature review tightly encompasses 20 pp., it uses a didactic approach and has a professional structure, without unnecessary comments, reflecting the approach of the clinical researcher. About 25 different topics are described in detail. The research data described have been presented at 15 scientific forums during a period of 9 years (2011-2020), as well as in 9 scientific publications. In 11 of the 15 forums the doctoral student is the first author, as well as in 7 of the 9 scientific papers on the dissertation.

These data show a very good knowledge of the problem, which is evident from the elegant and accurate introduction to the problem in the *introduction* and *literature review* chapters, as well as in the professional comments in the *discussion* chapter. In the *discussion* chapter, the doctoral student made 72 citations of literature sources when commenting on her data.

4. Research methodology

The methodological approach used is extremely professional and shows a very well conducted clinical study. In this PhD thesis 844 patients were studied, having 941 cycles of frozen-thawed embryo transfer (FTET), where a total of 2453 preimplantation human embryos were used. About 10 standard assisted reproduction methods were used, as well as some new approaches took place. Methods used: classic *in vitro fertilization* (IVF), *denudation* of the oocytes and *intracytoplasmic injection of sperm* (ICSI), a combination of the two methods for fertilization of oocytes (IVF / ICSI), *biopsy* for the isolation of autologous endometrial cells, *co-culture of embryos* with autologous endometrial cells before vitrification, *fresh embryo transfer* (ET), *artificial collapse* (AC), *vitrification* and thawing of preimplantation embryos, vitrification and thawing of oocytes, frozen-thawed embryo transfer (FTET).

5. Characteristics and evaluation of the dissertation and contributions

The dissertation is written on 129 pages, illustrated with 31 original figures, 34 tables and 7 appendices; 274 literature sources are cited. The chapters *Introduction* and *Literature review* are written on 22 pages, chapter *Materials and methods* is written on 10 pages, chapter *Results* is written on 43 pages, with 4 major topics - Reporting the survival of human preimplantation embryos after vitrification, achieved clinical pregnancies (CP) after application of the cryopreservation method vitrification and subsequent frozen-thawed embryo transfer (FTET), analysis of the survival of the embryos and developing pregnancies in "freeze all" program. The analysis of the results of vitrification of oocytes or preimplantation embryos by 24 parameters are presented in detail in the chapter *Results*. In the chapter *Discussion* on 15 pages the connection of the obtained data with those in the published literature is presented. The work is written and structured very well, very easy to read and very understandable. The logic of the study is easily traceable, doctoral student is critical of her own data and on the literature data. The chapters literature review, materials and methods, results and discussion are impressively detailed and informative. The obtained own data are significant and sufficient for the conclusions made. The author's contributions are presented in an elegant way.

The study examines the extent to which survival is affected after thawing of human preimplantation embryos frozen by vitrification depending on 24 different factors, incl. co-culturing the embryos with autologous endometrial cells prior to vitrification, implementation of an open and closed system for vitrification, application of artificial collapse (AC) before vitrification. The data were validated by analyzing the clinical pregnancies rate (CPR), especially when used together with co-culture of embryos with autologous endometrial cells prior to vitrification, open and closed system for vitrification, assisted hatching after thawing of the embryos before FTET or artificial collapse was applied before blastocyst vitrification. Investigated was the effect of the "freeze all" strategy.

The obtained by the doctoral student and her supervisors results confirm the efficiency and safety of application of embryo and oocyte vitrification, especially in combination with additional techniques – FTET, etc. The implementation of the "freeze all" program provides an opportunity, in certain groups of patients, to delay the embryo transfer, which will increase the chances of pregnancy and childbirth.

Of great importance is the successful implementation of this program in women of advanced reproductive age. The number of patients in the mentioned group is constantly increasing due to the

delay of pregnancy and motherhood. This group of women is a challenge to achieve clinical pregnancy and live birth, which is why all methods that could help to achieve these results are extremely important.

There are also plans for further research proposed in the thesis - a follow-up study of the newborns obtained after using vitrification and their individual development, in order to assess the safety of the method.

In this dissertation the following **conclusions** were made regarding the factors that influence the survival of embryos after FTET: the quality of the in vitro developed embryos on the day of vitrification, human serum albumin content increase in the freezing and thawing solutions during cryopreservation of blastocysts, as well as the conducting of an artificial collapse before vitrification; the application of assisted laser hatching (AHL) to the embryos after thawing leads to a statistically significant increase in the CPR in vitrified blastocysts; the patient's age (under 35 years) is related to the percentage of achieved CP; during FTET, the of embryos should have more than 50% of the cells survival, otherwise no clinical pregnancy is reached; the vitrification of blastocysts results in a statistically significantly increase in CPR, when compared to embryos on day three, in patients ≤ 35 years. For the "freeze all" program, the age of the patients does not have a negative effect on the CPR.

According to data obtained from this thesis, the finding of factors that have a positive effect on cryopreservation of embryos and their routine application in practice have developed a statistically significant increase of 36% in CPR during the period 2003-2015 years.

Contributions of the thesis are unmistakable and focused in the following **three major achievements**: first in the country study on the application and optimization of the method *vitrification*, aimed on determination of the factors influencing the survival of embryos after thawing, which was clinically validated by tracking achieved clinical pregnancy rate of frozen-thawed embryo transfer; validation of the optimization of the method vitrification – its application resulted in statistically significant and substantial increase (over 31%) of the clinical pregnancies after putting into practice the modified method for cryopreservation of blastocysts, quality control with the introduction of criteria for selection of frozen embryos (with or maximum quality for the day) and criteria for conducting thawed embryo transfer (only thawed embryos with over 50% of the cells viable); routine application of artificial collapse before cryopreservation of blastocysts and assisted laser hatching after thawing of embryos before thawing of embryo transfer. The application of the "freeze all" strategy in the cryopreservation of blastocysts has led to a significant increase in the number of achieved clinical pregnancies.

6. Evaluation of the publications and personal contribution of the doctoral student

The results are presented in a dissertation, abstract and 9 scientific papers in English and Bulgarian, as 3 are published in indexed and peer-reviewed editions with an impact factor. The research data were presented at 15 scientific forums for a period of 9 years (2011-2020), as well as in 9 scientific publications. In 11 of the 15 forums the doctoral student is the first author, as well as in 7 of the 9 articles on the dissertation, which shows the high level of personal participation of the doctoral student.

7. Abstract

The abstract is detailed, illustrative and reflects the main results achieved in the dissertation.

CONCLUSION

The dissertation work includes applied research results, which represent an original contribution to science and meet all the requirements of the Law for the development of academic staff in the Republic of Bulgaria (ZRASRB), its Implementation Regulations and the Rules of ZRASRB of Sofia University " St. Kliment Ohridski " . The presented materials and dissertation results fully comply with the specific requirements adopted in connection with the Regulations for application of ZRASRB.

The dissertation shows that ***Lyuboslava Dimitrova Valkova*** has in-depth theoretical knowledge and professional skills in the scientific specialty " Cell Biology", as a doctoral student on full-time education, demonstrating qualities and skills for independent research.

Due to the above, I confidently give my positive assessment of the research presented by the above reviewed dissertation, abstract, results and contributions, and I propose to the esteemed scientific jury to award the educational and scientific degree " Doctor " (PhD) to ***Lyuboslava Dimitrova Valkova*** in PhD program in the scientific specialty " Cell Biology", in professional field 4.3. Biological sciences, from the area of higher education 4. Natural sciences, mathematics and informatics.

24 .11.2020

Prepared the opinion:

Prof. Krassimira Todorova, Ph.D., DSc.