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Review of the doctoral dissertation of M.Sc. Simona Bisogno entitled:
"Risk evaluation of neurodevelopmental disorders in offspring conceived by Assisted Reproduction Technologies"

Assisted reproductive technologies (ART) are widely used to overcome infertility-related problems in humans. Since the birth of the first ART-conceived baby in 1978, almost 10 million babies have been born using different ART procedures. Despite its inarguable usefulness, concerns about the health consequences of ART-conceived babies have been raised. For this reason, undertaking research aimed at estimating the risk of neurodevelopmental disorders in offspring born through ART, using an animal model, is strongly justified.

1. **Scientific merit of the thesis**

The subject of the PhD thesis concerns the effect of assisted reproductive technologies on offspring, with a particular emphasis on the survival of embryos and neonates, the occurrence of neurodevelopmental disorders and their intergenerational transmission. In addition, PhD student made an attempt to explain the mechanisms underlying the observed phenomena. Since such studies using animal models have not been performed on this scale before, I consider the subject of the work to be original and the results obtained to be a substantial extension of the current knowledge on the long-term consequences of ART on offspring conceived through this method.

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2. Formal evaluation of the PhD thesis

The doctoral dissertation has a classical layout. It is preceded by a list of abbreviations, a list of figures and an Abstract in English and Polish languages. The main part of the doctoral dissertation begins with an Introduction outlining the background of the research. Then PhD student moved to Materials and Methods and to the Results, divided into chapters and sub-chapters. The thesis ends with a Discussion, Final conclusions and the list of cited References. Generally, I find the content of the dissertation correct. My only reservation concerns the lack of a chapter "Aim of the study". The lack of a research hypothesis and clearly defined research goals (which appear only in the Abstract) prevents me from assessing the completeness of the experiments performed.

The dissertation is written in clear, concise and communicative language. It was a real pleasure to read it. Typos, linguistic errors and unclear formulations were very rare. The graphic side is beyond question. The charts follow a single convention and are mostly provided with careful legends. In a few places, individual graphs within the figure are arranged in a different order than they appear in the text (*e.g.* on Figs 11 and 12) or are incorrectly assigned (*e.g.* Fig. 7a refers to females, not males, as written in the text). There are also errors in statistical significance in the figure captions (*e.g.* Fig. 5, 12, 13, 19). Additionally, more attention should have been paid to the record of references to keep one template. The results are divided into chapters and sub-chapters. However, it is not clear why their titles are in a different convention than the other chapters in the dissertation (Roman *vs.* Arabic numerals). Moreover, some microscope photographs lack a scale bar, and where it is present, there is no description of its value in the figure caption. There are also some errors in the numbering of chapters (*e.g.* 2.4.1 instead of 2.4.2 on page 31; III.I instead of III.III on page 55).

3. Substantive evaluation of the thesis

The topic undertaken by PhD student is of special importance due to the social aspect. Given the increasing use of ART to treat infertility in humans, it is pivotal to develop animal models addressing the effects of this technology on offspring health and determining its safety. Besides the difficulty in controlling confounding factors, human retrospective studies cannot delineate the long-term effects of ART itself from those attributed to other factors, such as advanced maternal and paternal age *etc.* This highlights the importance of using experimental animal models.

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During this study PhD student used a mouse as an animal model to study the health consequences of ART. She performed experiments using a wide range of techniques - from those typical of experimental embryology, but technically challenging, such as embryo transfer, through behavioural tests, to molecular biology, Elisa, proteomics and spectroscopic techniques. This deserves the highest recognition because it testifies to the extensive experimental skills and qualifications of the PhD candidate.

3A. Evaluation of Introduction

The Introduction of the dissertation was prepared in a synthetic form but contains all the information necessary for understanding the content discussed. It provides a very good compendium of knowledge about brain structure and development, different procedures of ART, and its consequences on the health of offspring, with emphasis on neurodevelopmental disorders. I appreciate that PhD student emphasised the ambiguity of the results regarding the health consequences of ART on offspring, and cited works presenting contradictory reports. It is also worth noting that PhD student described both the epidemiological data on humans and the results of the research obtained using animal models. I have no reservations about this part, apart from the previously mentioned lack of the hypothesis and objectives of the experiment.

3B. Evaluation of Materials and Methods

The methods used to achieve the objectives of the work were generally correct. However, animal model should correspond as closely as possible to what happens in humans. Therefore, I am curious why the PhD candidate did not use *in vitro* fertilization instead of natural mating of mice. Moreover, in the case of human (and also other mammals) ART, embryo transfer is performed noninvasively, by inserting the catheter with the embryos into the uterine cavity through the cervical canal. In this study, embryo transfer was performed by traditional surgical method. The surgical transfer is an invasive procedure that requires anaesthetizing the mouse, cutting skin and muscles, as well as inserting a pipet into the organs of the reproductive tract. By the way, it is worth noting that Simona Bisogno planned a control in her experiment consisting of the impact of the transfer itself on the analysed parameters. Did PhD student consider alternative non-invasive transcervical route of transfer (Bin Ali *et al.*, 2014)?

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The Materials and Methods section, describing the procedures and techniques used in this study, should contain all the information required to reproduce the experiments performed. Unfortunately, I noticed some omissions in this part of the thesis.

1. There is no information about human material (oocytes) used in this study: their origin, permissions required, and method of storage. It is also unknown whether they were vitrified and what the procedure of thawing looked like.

2. This section lacks information about the statistical tests and the names of the computer programs used for the analyses. The p-value, at which differences are considered statistically significant, is also not provided.

3. In case of reagents used (*e.g.* media, mineral oil *etc.*) and equipment, the manufacturer's information and country of origin should have been provided.

4. Generally, the schemes are informative, which makes it easier for the reader to follow the course of the experiment. However, they are not consistent with the information in the text. According to Fig. 2, in the case of the ET variant, 4-cell embryos were transferred into the uterus of the recipient and the text suggests that blastocyst-stage embryos were transplanted. According to Fig. 4, IVC female and IVC male lines were obtained in the II generation, whereas the text shows that both IVC and ET II generation groups were obtained.

5. Regarding embryo transfer: Which strain of mouse was used as recipients? What does "F1" mean (there is no information about this hybrid mouse in the "Animals" sub-section)? What was the dose of ketamine and xylazine (it should be given in mg per kg of body weight)? What was the route of administration of the anaesthetic? Did you administer any analgesic after the embryo transfer? There are some reports showing the importance of postoperative analgesia for implantation success (Koutroli *et al.*, 2014; Schlapp *et al.*, 2015). What threads were used for sewing? How many embryos did you transfer into each horn of the uterus? Did you transfer the embryos regardless of their stage or select only blastocysts? How was the course of pregnancy monitored?

6. Regarding *Three chamber Social recognition test*: What was the sex of the stranger? What was used as the non-social stimuli? Was it just an empty compartment? There is also no broader

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description of how the results of this test were recorded and analysed, which makes their interpretation difficult for non-specialists.

7. What method was used for euthanasia of E19.5 foetuses?

8. What was the age of the females which served as advanced maternal age model?

3C. Evaluation of Results

The presentation of the results within chapters was structured in a way that does not necessarily establish a logical sequence. As an example: behavioural tests performed on adult mice followed by assessing embryo survival and development to term or adult brain data followed by the prenatal brain analysis.

The results are demonstrated in 18 figures, mostly adequately described and cited in the text. Quantitative results were analysed using correct statistical tests. However, the exact number of samples (*e.g.* the number of mice) taken for analysis should have been specified in the text or on the graphs (the term "n≥9" is not sufficient).

The PhD used *Three chamber social recognition* test and the *Light/dark box* test, which are commonly used to assess sociability and anxiety-like behaviour, respectively. Simona Bisogno obtained interesting results indicating reduced sociability in offspring generated by ART. In contrast, she did not notice alterations in terms of anxiety-like behaviours. For me, as a non-specialist, the results raise further questions. Therefore, I would like to ask the PhD student to clarify some aspects:

1. How do you interpret the lack of difference in interaction time between S IVC and NS IVC groups (Fig. 5A)?

2. Bearing in mind that the mother's age ranged from 3 to 5 months in this study and that maternal age might account for some disorders in offspring - did you look at the results in terms of the different age of mothers?

3. Did you consider using another behavioural test to study anxiety-like behaviour, such as *Elevated Plus Maze* just to be sure that there are no differences between experimental and control groups (especially in the light of alterations observed in the second generation)?

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4. Another interesting observation was reduced embryo survival and development to the term of ART-conceived offspring. In the case of this part of the results, I also have a question. The birth rate for the IVC group was low, but comparable with the ET group. How did you distinguish between a failed embryo transfer and a failed pregnancy? Did you perform vaginal smears to confirm implantation and possible resorptions?

5. I consider the examination of the influence of intracellular lipid changes in an *in vitro* cultured embryo as a cause of neurodevelopmental disorders to be a particularly valuable finding of the research. Can these data serve as a starting point for therapeutic solutions regarding indicators of the developmental potential of embryos obtained as a result of ART?

6. I do not fully understand the rationale behind the part of the thesis regarding AMA oocytes and embryos, both human and mouse. The influence of maternal age has not been taken into account in earlier aspects analysed. Moreover, the methodology for this part of the dissertation is very poorly described, *e.g.* it is unknown how old the females – oocyte donors were.

3D. Evaluation of Discussion

All the results obtained are discussed based on the relevant literature. It should be noted that the PhD student's polemic against the previous data was carried out in accordance with the current state of knowledge. It should be appreciated that M.Sc. Simona Bisogno is critical of the results obtained. I find this discussion very mature and multi-threaded. Generally, this section does not raise objections. However, again, as a non-specialist, I have a few questions. It is written that "*in vitro culture procedures may lead to deficits in social behavior and motivation which are a core symptom of NND and a widely recognized characteristic of ASD phenotype*". In light of these interesting observations, I miss comparing the scope of alterations in social behaviours of ART-conceived mice shown using relevant tests with the alterations observed in mice, which are models of autism spectrum disorder or other types of neurodevelopmental disorders. Moreover, I miss the reference to the work of Ecker and colleagues (2004) who demonstrated for the first time the detrimental effect of i*n vitro* culture of embryos on the development to term and behaviour of adult mice: anxiety and spatial memory. Could the PhD candidate discuss the possible reasons for the discrepancies between this study and her own results, highlighting the limitations of the animal model?

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5. **Final grade**:

The critical comments listed above do not significantly affect my positive evaluation of Simona Bisogno's dissertation. It is an interesting and extensive study, properly planned and carried out with appropriate methods. The aspect that I found particularly valuable was the attempt to elucidate the molecular mechanism behind the observed neurodevelopmental disorders. As a result, it provides completely new and original insights into the impact of ART on neurodevelopmental disorders.

I, hereby, declare that the reviewed PhD thesis by Simona Bisogno meets the criteria pursuant to art. 187 of Act of 20 July 2018 (Journal of Laws of 2018, item 1668, as amended) and request that the Research Discipline Council of Biological Sciences of the Jagiellonian University in Kraków accepts Simona Bisogno for further stages of doctoral proceedings.

Na podstawie przedstawionej rozprawy stwierdzam, że Rozprawa Doktorska spełnia warunki określone w artykule 187 Ustawy z dnia 20 lipca 2018 r. Prawo o szkolnictwie wyższym i nauce (Dz. U. z 2018 r. poz. 1668 z późn. zm.). W związku z powyższym przedkładam Radzie Dyscypliny Nauki Biologiczne Uniwersytetu Jagiellońskiego wniosek o dopuszczenie mgr Simonę Bisogno do dalszych etapów przewodu doktorskiego.

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