Uniwersytet Przyrodniczy w Poznaniu
Wydział Medycyny Weterynaryjnej i Nauk o Zwierzętach
Katedra Genetyki i Podstaw Hodowli Zwierząt
ul. Wołyńska 33, 60-637 Poznań
tel. +48 61 848 71 87, e-mail: genetyka@up.poznan.pl

Prof. dr hab. Dorota Cieślak tel. +4861 846117 email dorota.cieslak@up.poznan.pl

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A review of the doctoral dissertation of **Simona Bisogno, MSc**
entitled " *Risk evaluation of neurodevelopmental disorders in offspring conceived by Assisted Reproduction Technologies"* supervised by Prof. Dr. Hab. Grazyna Ptak and co-supervised by Dr. Maria Florencia Heber

Summary of the Dissertation

The doctoral thesis presents a study conducted on the mouse model involving oocytes, blastocysts, fetuses, and animals that aims to verify the extent of neurodevelopmental alterations that can be induced by assisted reproductive techniques (ART). Some of the procedures also involved human oocytes and blastocysts. As an embryologist, I will concentrate my opinion on issues related to assisted reproductive techniques and their possible impact on oocyte and embryo quality. The aspects related to neurobiology are out of the scope of my expertise.

The hypothesis assumes that embryo modifications caused by ART procedures are associated with an increased risk of neurodevelopmental afflictions. To document this hypothesis 2 generations of mice were obtained. The first generation (F1) contained 3 categories of animals: 1) NM animals produced by natural mating, 2) ET animals derived from embryos produced from oocytes matured, fertilized, developing in vivo for 4 days and transferred after flushing and 3) IVC animals derived from embryos produced from oocytes matured, fertilized and developing in vivo for 2 days, flushed and cultured in vitro for another 2 days and finally transferred on Day 4. Animals of the first generation (=F1) were subjected to two behavioral tests and their brains were analyzed. The F1 animals were used to produce five lines of the F2 mice: 1) F2 IVC female line, 2) F2 IVC male line, 3) F2 ET female line, 4) F2 ET male line, and 5) F2 IVC line. The F2 fetuses were utilized as donors of brain, hypothalamus, and hypothalamus tissue. The study also includes analysis of the lipid droplets in murine and human oocytes and blastocysts. Several parameters attributed to fetal and animal development were monitored bodyweight, offspring survival, development to term, fertility, selected brain parameters, and lipid profile in oocytes and blastocysts.

The most interesting achievements of this work include the following findings:

- A 2-day culture of murine embryos in vitro (IVC group) exerts a negative impact on growth during the preweaning period and behavioral traits of F1 generation
- the behavioral alterations depend on the parental site e.g. decreased social motivation is a consequence of ART applied to fathers;
- prenatal brain development is impaired in the IVC group as shown by increased oxidative stress, elevated lipid peroxidation, and, reduced expression of genes associated with neurogenesis
- lipid profile is altered in IVC blastocysts and shows similarities to oocytes derived from aged donors
- embryo transfer (ET) procedure can negatively impact the survival and development to the term of embryos regardless of their origin in vivo or in vitro

General comments on the dissertation

In my opinion, any deliberation on a possible negative impact of ART on the progeny status should consider an excellent work presented by Romundstadt et al in the Lancet in 2008. The authors investigated selected parameters of gestation, delivery, and infants of women who had conceived spontaneously and after assisted fertilization. They concluded that adverse outcomes of assisted fertilization compared with those in the general population could be attributed to the factors leading to infertility, rather than to factors related to reproductive technology. The author of this dissertation has also mentioned this aspect at the beginning of the discussion on page 67.

I would like to address two human studies referred to by the author of this dissertation: the first one of Barberet et al. 2022 who performed a metanalysis on DNA methylation in IVF offspring based on 51 records from the 928 initially identified. The authors stated that differences in DNA methylation after ART conceptions are modest, and the functional relevance in adult tissues is unknown. This study also suffers from a small sample size. The second study by Cannarella et al. 2022 describes a metanalysis of DNA methylation patterns in offspring derived from IVF embryos and conceived spontaneously based on 50 records from the 949 initially identified. The authors stated that the methylation pattern of the two offspring categories may be different. However, due to the heterogeneity of the data and the small sample size, further population studies are needed. Although the authors have demonstrated a possible relation between the ART and DNA methylation profile they were aware of the limitations of their studies.

With respect to the ART techniques applied to this experiment, only a 2-day embryo culture in vitro was performed and no other manipulations were utilized. I want to stress that the experimental model does not reflect what is routinely used to obtain human IVF embryos. The standard protocol for obtaining human embryos in vitro is based on oocytes matured in vivo which are aspirated from the ovary, fertilized in vitro and the resulting embryos are cultured in vitro for 3-5 days. The standard procedure of obtaining bovine embryos in vitro is even more complex because it includes in vitro maturation and fertilization of oocytes and in vitro culture of the resulting embryos for 7 days. It is crucial to the developing embryo and the conceptus of whether the particular stages of in vitro embryo production occur in vivo or in vitro. Each step is characterized by unique processes that may affect the quality of the blastocyst and the fetus's health. In the described experiment IVC embryos were flushed on day 2. In the mouse activation of the embryonic genome crucial for further development occurs already at the zygote/2-cell stage. On day 2 the embryo reaches the morula stage containing 4-16 blastomeres and till the compact stage, it stays in the oviduct. Besides, at this stage, the first signals of differentiation occur whether a blastomere will give rise to ICM or TE. On day 4 the blastocyst is already hatched and zona pellucida free so I suspect that embryos were flushed earlier on day 3.5 when the zp still persists.

Therefore, it is necessary to discuss all the processes occurring during preimplantation development (e.g. activation of the embryonic genome, morula compaction, blastocyst cavitation, cell line differentiation) and their hypothetical relations to the neurodevelopmental alterations in the fetus or adult animal. To discuss the importance of obtained results it is also necessary to provide information on the scope of experiments: how many animals were utilized, how many samples e.g. brains, oocytes, and blastocysts were analyzed.

Detailed comments on the dissertation

- The doctoral dissertation is written in English and presented in the form of a monograph which includes: abstracts in English and Polish, a list of abbreviations, a list of 22 figures, an introduction (17 pages), material and methods (8 pages), results (28 pages), discussion (7 pages), final conclusions (2 pages), list of 175 references.

- The introduction presents a summary of the opinions on whether application of the ART can be associated with an increased risk of neurodevelopmental alterations. Some of the referred studies support this hypothesis by demonstrating a possible link whereas some studies do not support this. The main attention is focused on human studies whereas information about animal studies is scarce. The present experiment was based on an animal model and thus more attention should be paid to animal studies. The author also provides a summary of brain development and the role of lipids in this process. Characterization of brain structures like the hippocampus and hypothalamus is also provided. The last subject of the introduction concerns the mouse as a model for neurodevelopmental disorders. There is no information on the preimplantation development of murine embryos and the critical processes that take place during that time and are crucial to the developing embryo. Selected examples of neurodevelopmental disorders described in animals should be discussed e.g. large offspring syndrome in cattle.

- Material and methods subsection contains a description of selected but not all procedures utilized in this study. There is no description of the methodology related to the following data presented in the "results" section:
  - Source and characterization of murine and human oocytes and embryos (p.36)
  - Bodyweight (p.41 and 51)
  - Offspring survival (p.43)
  - Offspring survival and development to term (p.44)
  - Fertility (p. 46)
  - Survival rate (p.47)

  Generally, the author does not provide data on the number of animals, oocytes, and embryos utilized in the experiment.

- The subsections presenting results called „Chapters" are well written and documented. The scope of the experiment presented in this subsection is larger than the material and methods that is not coherent.
- Discussion addresses all aspects presented in the Result subsection. There is however one issue that needs more attention: in my opinion, the ART applied to this experiment is limited to a 2-day culture of in vivo produced embryos and this aspect should be underlined and compared with more advanced technologies like a complex procedure of in vitro embryo production or even animal cloning. The authors state on page 69 *"that even the least invasive ART procedures (e.g. embryo transfer) can negatively impact the survival and development to the term of embryos produced either in vivo (ET) or in vitro (IVC)*". It means that embryos of in vivo origin flushed from the uterus and transferred to the foster mother may suffer from some disorders. This finding needs more attention since the commercial ET technology has been widely applied to cattle breeding and several generations of animals have been produced to date. Interestingly the large offspring syndrome has been identified among calves derived from in vivo and in vitro-produced embryos. Although it was significantly frequent among in vitro progeny, the size of the monitored populations differed a lot in favor of the in vivo group. Thus, special care should be taken when referring results obtained on animal models to humans. More attention should be also addressed to the issue of the age effect.

Detailed comments on the experimental design

The description of the experimental design concerning generating the first (F1) and the second (F2) generation of experimental animals is not clear and sometimes not coherent. According to the author the founder animals (P = parents) were mated for producing Day 2 embryos for IVM and TE, and for achieving pregnancies following natural mating (NM group). It is not explained whether the embryo donors of the P generation were sacrificed by embryo flushing but I assume they were. According to the author: "*Day 4 embryos (IVC and ET) were transferred to pseudo-pregnant asynchronous F1 females*". The mentioned Day 4 embryos produced by the P generation are in fact the F1 generation. They cannot be transferred to F1 females but to recipient mice of the P generation so the F1 animals can be produced.

The offspring of the P animals was the first generation (F1 IVC, F1 ET, F1 NM) subjected to two behavioral tests and adult brain analyses, and also used for producing the second generation (F2). According to the author: "*After completion of behavioral tests and subsequent mating for II generations, adult mice offspring (I Generation) were ethically sacrificed*". The F2 generation was achieved by natural mating of the appropriate F1 animals whereas embryo flushing and transfer were not included. Assuming the pregnant F1 females were sacrificed after the tests, how the F2 offspring was produced?

The pregnant F1 females were donors of fetuses on day 19 pc. The fetal brain and hypothalamus were subjected to selected protocols. I do not know whether the brain and hypothalamus of the adult F2 animals were also collected and analyzed. Besides, at this point, the term "hippocampus" is not introduced.

The experiment also included analysis of **lipid droplets in oocytes and blastocysts** (2.12 subsection, p.36). There is no information on the origin of this material (species, stage of development) and also the reader is not informed about the cellular structures analyzed. At the end of this subsection, the abbreviation LD appeared but the appropriate explanation was provided on page 7. More detailed information can be found in the Results subsection on page 60. Lipid droplets were also analyzed in oocytes and embryos of donors at an advanced age (AMA) of mice and humans. The category of AMA material has not been described in the M&M section and I was also surprised with the information that human material was included which was not previously described. Some information is provided on page 62. Also, Figure 22 (pages 64-65) presents images of mouse and human oocytes stained for lipid visualization with important information on the analyzed material provided in the legend of this picture. Such information as the age of the oocyte donors would be expected in the M&M section.

p.32 The mice of the natural mating (NM) group were used as controls in the experiment. The NM embryos were neither flushed from the uterus nor cultured in vitro. In my opinion, a more appropriate control for the IVC group would be the ET mice. The author mentioned on page 38 that "*a part of the NM embryos were transferred to pseudo-pregnant females*" and were considered "*an additional control*". I would like to stress that the procedure of embryo flushing and embryo transfer in mice requires surgery that involves a local inflammation (stress). IVC embryos were derived from ovulated oocytes fertilized in vivo and cultured in vivo till Day 2 when they were flushed and cultured in vitro for 2 days. ET embryos were derived from ovulated oocytes fertilized in vivo and cultured in vivo till Day 4 when they were flushed from the uterus and transferred to the foster mothers without culture in vitro.

Some discrepancies were also discovered in the description of the nervous system analysis:

- p. 33 The 2.9 subsection - ELISA-based assays – concerns the prenatal brain and adult hypothalamus. On the same page, another test of the Superoxide dismutase (SOD) activity was mentioned which concerned the prenatal brain and hippocampus. The term "hippocampus" was used here for the first time in the M&M section and besides I do not know which generation of mice (F1 or F2) was the donor of this material.
- p. 34 The 2.10 subsection - qPCR- prenatal brain - Gene expression analysis performed on mouse prenatal brain tissues obtained from CTR, IVC, and ET groups. The abbreviation CTR is not explained and not mentioned in the M&M section. The explanation is provided on page 62 (results) "*CTR group (oocytes from mouse young donors)*" but no detailed information about the animal generation and their age is provided.
- P. 34 The 2.11 subsection - Liquid chromatography and tandem mass spectrometry- concerns the proteomic analysis of adult hippocampus collected from 4- 4-month-old mice. No information is provided on the origin of this material concerning animal generation.

I recommend considering the comments listed above when preparing the manuscripts.

In conclusion, I would like to emphasize that the presented doctoral thesis of Simona Bisogno, MSc presents the results of a panel of experiments concentrated on the development of murine fetuses and individuals derived from embryos of three categories to demonstrate whether a 2-day embryo culture in vitro may induce neurodevelopmental alterations. The outcome of this study extends the current state of knowledge by adding new information on reproductive traits, and the development of fetuses and animals. The work is very well documented. From the embryology point of view, the crucial aspect of the thesis that needs more attention is the short period of in vitro culture of embryos. I suggest the author consider the following before publication of the results: the scope of the experiment and its limitations related to the duration of in vitro culture (2 days) and the stage of in vitro cultured embryos. All my comments and doubts have been presented to stress the importance of the topic to human medicine and general science and convince the author to extend the spectrum of issues that have to be considered when preparing manuscripts and discussing the data.
In my opinion, the work submitted for review contains original data that can be used to verify the hypothesis and meets the requirements for doctoral theses in the discipline of biology.

Considering the above, I declare that the doctoral dissertation submitted for evaluation meets the requirements set out in Article 187 of the Act of 20 July 2018 Law on Higher Education and Science (Journal of Laws of 2018, item 1668, as amended .) and I apply to the Discipline Council of Biology of the Jagiellonian University in Krakow to admit Simona Bisogno MSc for further stages of the doctoral procedure.

D. Cieślak

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Prof. dr hab. Dorota Cieślak