Abstract of the doctoral thesis of mgr inż. Natalia Dzięgiel entitled:

"Development of a rabbit zygotes transfection method with the use of nanoparticles"

Supervisor of the thesis: dr hab. Jacek Jura, prof. IZ

Date of the abstract preparation: 19.04.21

Doctoral thesis was carried out in the National Research Institute of Animal Production.

The currently used animal transgenesis methods are complicated and labour-intensive. Furthermore, their efficiency is often unsatisfactory. The transfection methods used for *in vitro* cell cultures, the procedures of which are standardised and simple, do not work the same way when used for the transfection of zygotes. The nanoparticles are potential carriers for nucleic acids and proteins in the biotechnological applications and controlled drug delivery systems. This way of using nanoparticles is based on their properties, including small size combined with the ability to bind particles on their surface, and the ability to avoid the negative side effects to the carried substance during its intracellular transport. Nonetheless, the possibility to use them as carriers in zygote transfection has not been sufficiently studied yet.

Likewise, the literature review shows that there is not much literature on using high hydrostatic pressure as a part of the zygote transfection procedure. However, it was proven that the high hydrostatic pressure has a positive effect on gamete and embryo *in vitro* maturation, and improves gamete and embryo survival after cryopreservation. It was also proven that the high hydrostatic pressure increases the cloning efficiency.

The aim of the presented thesis was to establish an efficient method of the rabbit zygote transfection using nanoparticles and DNA complex. The second goal was to investigate the effect of the high hydrostatic pressure used as a preconditioning factor during the procedure of the rabbit zygote transfection using nanoparticles/DNA complex.

First, based on the literature review, the spherical gold nanoparticles with the surface modification of polyethyleneimine (PEI), measuring 10 nm (purity: BioPure, manufacturer: Nanocomposix, USA) were chosen as a potential DNA carrier. The gold nanoparticles were found to show higher biocompatibility than the nanoparticles made of other materials. Further, they are inert, and it is possible to produce them in various sizes and shapes,

maintaining this process as a low-cost one. Using nanoparticles with surface modified with polyethyleneimine enables binding negatively charged DNA particles, as well as the condensation of nucleic acids and formulation of the stable nanoparticle-DNA complexes.

The aim of the fist experiment was to find the concentration of the previously selected gold-PEI nanoparticles which would not be toxic to the rabbit zygotes, nor would they affect the rabbit embryo development. In order to do it, the aqueous solution of the spherical, 10 nm gold-PEI nanoparticles were injected into the perivitelline space of the rabbit zygotes at the concentration of 5, 25, 50 and 100 ng/µL. Next, the embryos were cultured until reaching the blastocyst stage. The injection of the nanoparticles solution at the concentration of 100 ng/µL resulted in lowering the blastocyst percentage share, which was three times lower than in the control group. Moreover, the percentage share of the degenerated embryos in this group was higher in comparison to the control group (unmanipulated zygotes). In the group injected with the nanoparticle solution at the concentration of 100 ng/µL, the blastocyst percentage share was 22,23%, while it was 66,67% in the control group. In the groups injected with the nanoparticle solution at concentration of 50, 25, 5 ng/µL, the blastocyst percentage share was 86,57%; 41,94% and 76,19%, respectively. Based on the obtained results, the concentration of 50 ng/µL was chosen as the optimal concentration which will not be harmful to the rabbit zygotes, enabling effective transfection at the same time.

Next, the proportions of the nanoparticles and the plasmid DNA necessary for efficient nanoparticle/DNA complex formation was investigated using gel retardation assay. Pmax FP Red-N DNA plasmid (Lonza, USA) coding the red fluorescent protein was chosen as a reference for the transfection efficiency. The results of the gel retardation assay showed that the proportion of the nanoparticles to Pmax FP Red-N plasmid allowing the proper complex formation was 2:1. According to that, 50 ng/µL and 25 ng/µL nanoparticle concentration of the plasmid solution were selected for further experiments. The incubation conditions necessary for the nanoparticle-DNA complex formation were also investigated. Subsequently, equal solution volumes of the nanoparticles and the DNA were mixed at the previously established proportions, and DNase- and RNase-free water was used for the dilutions. Next, mixed solutions were vortexed (5 seconds, level: 2) and incubated at room temperature for 45 minutes. Selected, morphologically normal rabbit zygotes (two visible polar bodies, visible pronuclei) were transfected by microinjection of the solution containing nanoparticles/DNA complexes into the perivitelline space, until zona pellucida expansion was observed. After the injection procedure, zygotes were morphologically evaluated again. Zygotes with morphologically normal cytoplasm were transferred to the culture medium: TCM199 with a

10% supplementation of foetal bovine serum. The embryos were cultured for 5 days, at 37 °C and the CO2 level was 5%. The embryo culture was monitored every 48 h and culture medium was changed. After the embryo culture was completed, examination of their morphology and selection was performed to the following groups of developmental stage: blastocyst, morulae and degenerating embryos. The expression of the red fluorescent protein was examined using fluorescent microscopy equipped with 510-560 nm wave length filter. The evidence of the red fluorescence in blastocyst-stage embryos was qualified as positive transfection effect. The obtained transfection efficiency was 18,7%. The blastocyst percentage rate in the group of the transfected embryos was similar to the one which was reported in the control group consisting of the unmanipulated embryos.

The aim of the next study was to evaluate the effect of the high hydrostatic pressure on zygotes transfected with nanoparticles/DNA complexes and non-manipulated zygotes. In both groups which underwent the high hydrostatic pressure influence, the reported percentage blastocyst rate was higher than in the control group. In the group with 20 MPa HHP applied, the percentage rate of the embryos at the blastocyst stage was 65.4 %, whereas in the group were HHP at 40MPa was used, this rate was 72.9%. At the same time, the control group demonstrated the percentage share of embryos at the blastocyst stage at only 40.75%.

In the groups which underwent the high hydrostatic pressure procedure before being transfected with nanoparticles, the obtained transfection efficiency was lower than in the group transfected only with nanoparticle/DNA complexes. The transfection efficiency was 13.47% in the group treated with the HHP at 20 MPa, and 13.67% in the group treated with HHP at 40 MPa. Moreover, the high hydrostatic pressure procedure led to decreasing the blastocyst percentage rate in both groups of the embryos developed from the transfected zygotes which was 31.65% (HHP 20 MPa), and 23.02% (HHP 40 MPa). In both groups this rate was lower than in the control group.

The last part of the study was comparing OCT4 (the gene responsible for embryo development and cell pluripotency), CASP7 and BCL2 (the genes involved in regulating the apoptosis) expression level between the blastocyst groups obtained in the presented experiments. The OCT4, BLC2 and CASP7 expression analysis showed that the high hydrostatic pressure increases the expression of all studied genes. The increased expression of genes responsible for the embryonic development, the cell proliferation and the regulation of the apoptosis had positive effect on rabbit embryo development. On the other hand, in the groups of blastocysts obtained from the zygotes which had underwent the high hydrostatic pressure procedure before the transfection using the nanoparticles, the higher level of the

CASP7 and BCL2 gene expression might suggest that these combined procedures induce the apoptosis process. The effectiveness of the transfection of rabbit zygotes using the complex: 10 nm gold spherical nanoparticles with polyethyleneimine modification (AuPEI)/plasmid DNA (Pmax FP Red-N, Lonza) combined at the ratio of 2:1 (50 ng/µL: 25 ng/µL) was 18.7%. Efficiency of the transfection method based on nanoparticle/DNA complex is much higher than the standard DNA microinjection. Its efficiency shows a level similar to possibly achieved by using the CRISPR/Cas9 system. Moreover, the method of transfection using nanoparticle/DNA complex is less complicated and easier to perform than both aforementioned methods. The high hydrostatic pressure (HHP) applicated for non-manipulated zygotes before *in vitro* culture had a positive effect on increasing the percentage of obtained blastocysts. The obtained results matched those reported by the other authors for gametes and embryos of other mammalian species. The application of HHP at 20 and 40 MPa before the transfection with nanoparticle/DNA complexes decreases its efficiency.