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Behavior of chicken primordial germ cells injected into the bloodstream of recipient embryo

Zachowanie się pierwotnych komórek płciowych kury, wprowadzonych do naczyń krwionośnych zarodka biorcy

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I would like to thank my Supervisor, Professor Marek Bednarczyk, for scientific care, kindness, his time and also for support and understanding. I also thank dr Paweł Łakota for his valuable substantive support and comprehensive help.

Thanks for the whole team from the Department of Biochemistry and Animal Biotechnology for their smile, support and help through all four years.

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LIST OF COMMONLY USED ABBREVIATIONS

PGCs - primordial germ cells
E-G&K - Eyal-Giladi and Kohav (1976)
H&H - Hamburger and Hamilton (1951)
rpm - revolutions per minute
PGCs $^{\mathbf{PKH26[-]}}$ - primordial germ cells unlabeled with PKH26 fluorochrome
PGCs $^{\mathbf{PKH26[+]}}$ - primordial germ cells labeled with PKH26 fluorochrome
GP - Green-legged Partridgelike
R - Ross 308
I - injection of treosulfan into subgerminal cavity
II - injection of treosulfan into egg yolk
PBS - Phosphate Buffered Saline
PBS[-] - Phosphate Buffered Saline without calcium (Ca^{2+}) and magnesium (Mg^{2+}) ions
PAS - Peridic Acid Solution
FBS - Fetal Bovine Serum
Pen-Strep - Penicillin Streptomycin - antibiotic
OPTI-MEM® I - OPTI-MEM I Reduced Serum Medium
PKH26 - Paul Karl Horan 26 fluorescent dye
TREO - treosulfan - cytostatic

1. INTRODUCTION

1.1. PREFACE

Primordial germ cells (PGCs) are the only cells capable of transferring information stored in genetic material from generation to generation. PGCs can be isolated, cultured *in vitro* and genetically modified with maintaining potential of creating a germline (van de Lavoir et al., 2006). It has been shown that PGCs can be transferred from one embryo to another and create functional germ cells in recipient embryos (Sang, 2009). This phenomenon has been used in the production of transgenic birds through germline chimeras, while additional genetic modification of chicken PGCs can influence the efficiency of poultry production and even provide an excellent tool for production of human therapeutic proteins (Park and Han, 2012).

The main aim of the studies on chimeras and transgenic birds is to produce the embryo consisting of cells having and expressing transgene, and cells ensuring the proper development of embryo (Ishii et al., 2004). Therefore, worldwide research on development of transgenic animals are developed in two main directions: i) efficient manipulation of as many different types of cells as possible, preserving their high survival rates and ii) modifying certain exogenes without interfering with the other genes of animals (Han, 2009). Accordingly, many studies focus on development of techniques for obtaining PGCs, maintaining their high viability, and optimization of obtaining germline chimeras (by PGCs injection) with a high potential of germline transmission. One of the most important factors determining the efficiency of obtaining chimeras, or transgenic offspring is percentage of introduced PGCs in relation to the total population of endogenous PGCs in gonads of recipients (Li and Lu, 2010). This ratio can be modified by elimination of endogenous PGCs belonging to recipient embryos, and at the same time increasing the number and capacity of integration of introduced exogenous PGCs.

This paper presents the behavior of PGCs isolated from gonads of donor embryos and introduced into embryos acting as recipients. The research hypothesis assumes that it is possible to increase the number of PGCs introduced and integrated with recipient organism by developing an appropriate method of their isolation, selection of donor-recipient cell genotypes, and reducing the population of endogenous PGCs of recipient.

1.2. THE USE OF TRANSGENIC CHICKENS

Domestic chicken (*Gallus gallus domesticus*) has become an important subject of biotechnological research, particularly dealing with transgenesis. The reason why birds are increasingly being subjected to genetic manipulation is a free access to embryos and an ease of carrying out microsurgical procedures (Ishii et al., 2004). One of the most important groups of transgenic research in

poultry is implementation of a chicken model to produce biotherapeutics (Rashidi and Sottile, 2009; Song and Han, 2011). These expensive and complicated to manufacture drugs are an important part of many therapies, including treatment of tumors, multiple sclerosis, hepatitis C, autoimmune diseases, and many others. Apart from birds, production of biotherapeutics also uses mammals, e.g.: cows, sheeps, goats, or even rabbits (Sang, 2003). Compared to transgenic mammals, avian bioreactors have advantages, such as a short reproductive cycle, a small gap between generations, high laying performance, easy breeding (Li and Lu, 2010), presence of protease inhibitors in eggs (Rapp et al., 2003), glycosylation protein pattern similar to the human, possibility of production in oviduct and deposition in egg proteins toxic to mammals (Lillico et al., 2005). Also, the phylogenetic position and compact structure of chicken genome make birds an easier study object than mammals (Han, 2009). These advantages make the costs of biotherapeutics production in eggs much smaller than that produced in mammary glands. The high content of albumin in egg makes the generated amount of therapeutic protein up to several hundred milligrams (Ivari, 2006). Chicken bioreactors provided, among others, human erythropoietin (Koo et al., 2010), interferon alpha 2b (Rapp et al., 2003), interferon beta-1a (Lillico et al., 2007), monoclonal antibodies (Kamihira et al., 2005), granulocyte colony stimulating factor (G-CSF) (Kwon et al., 2008), parathyroid hormone (Lee et al., 2007), and so on.

It is speculated that, in addition to creating chicken bioreactors, genetic manipulations in birds can be used to increase the quantity and improve the quality of poultry products, such as meat and eggs. Genetic interference into the biosynthetic pathways can lead to changes in the feed conversion, growth rates, composition and weight of body (Cyriac et al., 2012). For example, induction of the increased expression of growth factors will result in a faster growth rate in birds. On the other hand, reducing the expression of myostatin can increase the muscle growth with a decrease in a fat content and effect on improved quality of meat (Sang, 2003).

Transgenic technologies in poultry production have also found applications in increasing or inducing the resistance of birds against some viral diseases. Hence, one of the main objectives of genetic programs in poultry production is to identify natural resistance genes or genes that could make it stronger. Tools to achieve this effect include interspecific and interracial exogen transfers, as well as modifications and enhancing the resistance gene expression (Sławińska et al., 2014). Replication of avian influenza virus has been already blocked through a direct inactivation of viral genes by short RNA fragments encoded by an artificially introduced gene (Ge et al., 2003). Moreover, *in vitro* studies suggest that the poultry produced using transgenic methods, also in an indirect way, can effectively inhibit infections caused by virus of infectious bursal disease, through the production of previously not produced proteins (Sang, 2009).

Genetic modifications may also find use in reducing the negative impact of poultry production on environment condition. Transgenic adding of phytase to the pool of digestive enzymes in chicken can solve the problem of environmental pollution with phosphorus, by forcing its distribution in the body of animal (Sang, 2003).

An important element is the use of chicken model in study of developmental biology (Rashidi and Sottile, 2009). An easy access to the developing chicken embryo makes that it can be successfully used to carry out various types of manipulations, which in mammals is much more difficult (Mozdziak and Petitte, 2004). Moreover, maintenance of potential of chicken PGCs to create germline, even after a long time of *in vitro* culture and, carried thereon genetic manipulation, combined with the ability to acquire somatic features make these cells a successful new model in the study of developmental biology (van de Lavoir et al., 2006). The aforementioned ease of an access to embryo and possibility of re-entry of PGCs into the bloodstream of embryo at early stages of its development favour this model. These advantages create new opportunities of chicken genome manipulation giving the perspective of implementation of the results into agriculture and pharmaceutical industry (Mahdi and Fariba, 2012).

1.3. PRODUCTION METHODS OF TRANSGENIC CHICKENS

Transgenic chickens may be produced in several ways by introduction of a transgene into cells that give rise to germ cells (oocytes and sperms) or cells which are contained in the fertilized egg or in embryo at early stages of its development. Different stages of development of germ cells and their precursors provide further opportunities for a direct intervention in host genome. Currently, it is assumed that there are two main production methods of transgenic chickens based on destination of depositing transgene. These are: i) direct modification of embryo by using a DNA or viral vector, and ii) indirect action on blastodermal cells, embryonic stem cells, primordial germ cells and spermatogonial stem cells (Li and Lu, 2010). More detailed methods for generation of genetically modified birds are presented in Table 1.

Tab. 1. Methods used in production of transgenic chickens (modification of Cyriac et al., 2012).

method	description
Virus Mediated Gene Transfer	- retro- and lentiviral vectors - the most efficient method - exploiting natural abilities of retrovituses to combinate their own genetic material with host DNA - transfection of BCs, PGCs, testicular cells
Microinjection of DNA	- injection of exogenous DNA into pronucleus of freshly fertilized egg - minimal usage because of difficult procedure - low efficiency
Embrionic Stem Cell/ Primordial Germ Cell/ Blastodermal Cell Mediated Gene Transfer	- require to obtain the cells (ESCs, PGCs, BCs) - cells are maintained in *in vitro* conditions that prevent them from differentiation - exogen introduction by lipofection, electroporation, nucleofection - modified cells require re-introduction into the embryo
Nuclear Transfer	- nucleus transfer into an enucleated cell - low efficiency - limited use for poultry due to yolk abundance and difficult access to single-celled embryo
Artificial Chromosomes	- can carry very large DNA fragments (1Mb or even more) - minimal use in chickens
Testis Mediated Gene Transfer	- exogenous DNA with lipofectant are injected directly into the nucleus - exogenes bind to spermatogonia or spermatozoa DNA, which are then used for artificial insemination - low efficiency in chickens
Sperm Mediated Gene Transfer	- utilizes ability of sperm to bind and internalize foreign DNA and to transfer it to oocyte during artificial insemination - sperm cells are subjected to transfection by: electroporation, lipofection, REMI, etc.

Most of methods for production of transgenic chickens involve an introduction of the previously designed, "new" genes into cells which will give rise to germ cells. Consequently, most of these techniques produce a hen (or rooster) exhibiting a mosaic presence of transgene in gonads. Offspring with presence of transgene in all cells of the body can be obtain only after the crossbreeding of chimeras. Therefore, the appropriate modification and targeting of cells giving rise to the germ line is essential to success of experiment (Mozdziak and Petitte, 2004).

A transfer of exogens using viruses is the most efficient production technique of transgenic chickens (Petitte and Mozdziak, 2007; Nischijima and Iijima, 2013). It was observed that one hundred percent of hatched chickens, transfected by this method, showed the presence and expression of introduced gene (Koo et al., 2004; Kwon et al., 2004; Koo et al., 2006; Lee et al., 2007; Kwon et al ., 2008; McGrew et al., 2010). However, for technical and safety reasons, the use of viruses for agriculture is limited; other non-viral methods were developed for effective cell transfection and production of transgenic chickens using germline chimeras. Many of these methods use primordial germ cells as a vector of the transgene. It is believed that the most safe, effective and promising method is obtaining transgenic chickens by transfer of genetically modified PGCs to recipient embryos using the electroporation method and/or lipofection. Despite their safety, non-viral methods, however, have some shortcomings, which elimination is the main objective of research on genetically modified chickens. An analysis, which was conducted on the basis of literature (146 items) accumulated in the Web of Science database, aimed at the compilation of shortcomings and imperfections of each of the two basic methods: viral and non-viral (cell-based), the results are presented synthetically in Table 2.

Tab. 2. Problems in transgenic chickens production.

production methods of transgenic chickens	problems	authors
nonviral (cell-mediated)	difficult to control differentiation process of PGCs, BCs and testicular cells in long-term *in vitro* cultures	Heo et al., (2011)
	very small number of PGCs can be isolated from gonads, and in particular from germ bloodstream	Chang et al., (1997)
	very low efficiency of exogenous DNAs integration into host genome by lipofection	Nishijima and Iijima, (2013)
	difficult to isolate germ or embryonic stem cells	Li and Lu (2010)

	injection of transgenes or nuclear transfer at early stages of embryo development is particularly complicated due to large size and fragility of egg produced by a hen	Lillico et al., (2005)
	achieving a sufficient hatchability of chicks; direct introduction of exogen into fertilized chicken egg is difficult because of multiplicity of cells included in blastoderm; difficult to generate F1 offspring due to the mosaic expression of transgene in F0 generation; transgenic lines are generated approximately after 1 year	Mozdziak and Petitte, (2004)
	transgene expression may occur in other cells	Mahdi and Fariba, (2012)
	chicken egg in contract to mammalian oocyte is not transparent, making it a challenge to view for injection and manipulation	Petitte and Mozdziak, (2007)
viral	introduced transgene size is limited to about 10 kb when viral vector systems are used; tissue specific promoters with strong activity are required	Byun et al., (2011)
	high susceptibility of transgene silencing; danger of recombination with retroviruses that are widespread in commercial poultry flocks	Esmaeilzadeh and Farhadi, (2011)
	viral transduction technique has relatively low and unpredictable rates of germline transmission and production of transgenic chickens	Park and Han, (2012b)
	expression of exogenes transferred by retroviral vectors can be changed in different tissues and at various stages of development	Song and Han, (2011)
	exogenes can only integrate into dividing cells and specific target cells must be properly recognized; possible unwanted retroviral integration in immediate vicinity to potential oncogenes - activation of carcinogenesis process	Li and Lu, (2010)
	transgene silencing in next generations	McGrew et al., (2004)

Our observations confirm the above informations. Moreover, with respect to the PGCs, the biggest problems in production of chicken chimeras for transgenic tests using the method of isolation and PGCs injection include: a small number of PGCs isolated from gonads and free of aggregates, low survival of isolated cells in short-term *in vitro* culture, a small number of exogenous PGCs integrated with recipient embryos, low survival rate of recipient embryos after microinjection of cells, a small percentage of obtained germline chimeras, and the presence of endogenous PGCs, which hinders the implantation of introduced cells in gonads of recipient embryos. In order to reduce the above issues we should improve methods for isolating PGCs from gonads towards their larger number and survival rate, optimize the selection of PGCs donors and recipients for the low mortality of recipient embryos, increase a share of chimeras with exogenous PGCs and minimize the participation of endogenous PGCs in gonads of recipient embryos through their sterilization.

1.4. PRIMORDIAL GERM CELLS AS A TOOL IN CREATING TRANSGENIC CHICKENS

Primordial germ cells are increasingly being used in research on the development of transgenic birds. Their genetic modification takes place using both viruses (Shin et al., 2008; Kim et al., 2010; Motono et al., 2010) and non-viral techniques (Naito et al., 1998; van de Lavoir et al. , 2006; Macdonald et al., 2012; Park and Han, 2012; Glover et al., 2013; Tyack et al., 2013; Jordan et al., 2014; Chojnacka-Puchta et al., 2015), however, the latter are preferred, particularly when it comes to the practical use in poultry breeding programs.

Previous studies have shown that isolated chicken PGCs can successfully be introduced into recipient embryos at stage 12-18 (by H&H), where they circulate freely in the blood of developing embryo without losing ability to transform into germ cells at later stages of development (Naito et al., 1998; Naito et al., 1999; Kim et al., 2004; Mozdziak et al., 2006; Macdonald et al., 2012; Park Han, 2012a and 2012b, Sawicka et al., 2015). This unique property provides the possibility of applying PGCs in the production of germline chimeras and transgenic chickens.

The big advantage of PGCs is that they can be cultured and modified *in vitro* (Kuwana et al., 1996), and therefore, the production method of transgenic chickens using PGCs appears to be particularly attractive, because integration and expression of transgene can be checked before an introduction into genome of recipient embryo (Mozdziak and Petitte, 2004). With this solution, number of birds, of which only transgenic are chosen, is reduced and time required to assess the phenotype is shortened. In addition, it increases the likelihood that resulting transgenic animals will have targeted changes within their genome. Using the PGCs is primarily aimed to improve the performance of exogen transmission to offspring, as these cells are the precursors of an egg and sperm (Li and Lu, 2010). However, further studies are required to optimize the

production of transgenic birds, taking into account, among others, more detailed expertise of the behavior of exogenous PGCs in recipient embryos, an increase of amount of isolated PGCs and optimizing the selection of PGCs donor and recipient genotypes.

1.5. MORPHOLOGICAL AND BIOLOGICAL CHARACTERISTICS OF PRIMORDIAL GERM CELLS

Chicken primordial germ cells viewed under the microscope are large, round granular cells with the size of 14-19μm (Nakajima et al., 2011). Initially, PGCs were identified based on morphological features, such as: presence of a large spherical nucleus with a diameter of approx. 8μm, very well developed Golgi apparatus and endoplasmic reticulum (Fujimoto et al., 1976), presence of refractive lipids in cytoplasm and numerous grain reserve substances (Zhao and Kuwana, 2003). At present, identification of PGCs most often uses: i) Peridic Acid Solution (PAS) (Meyer, 1960) - after the stage 4, grains containing reserve material in the form of glycogen are stained, and ii) immunohistochemical markers such as SSEA-1 antibody (stage-specific embryonic antigen-1), EMA-1 (embryonic mouse antigen-1) (Karagenc et al., 1996), which recognize glycoprotein antigens on cell surface (Nakamura et al., 2007) and CVH (chicken vasa homologue) exhibiting expression characteristics for germ cells (Tsunekawa et al., 2000; Bernardo et al., 2012; Nakamura et al., 2013). In addition to these markers, Jung et al., (2005) proposed a method of PGCs double staining with antibodies SSEA-3, SSEA-4, integrin α6 and β1, lectins STA (*Solanum tuberosum agglutinin*) and DBA (*Dolichos biflorus agglutinin*).

Primordial germ cells exhibit features of pluripotency, i.e. they can differentiate into many cell types and have the ability to self-renew (Wu et al., 2008). In contrast to mammalian PGCs, chicken PGCs with successive stages of migration during embryonic development continuously decrease the expression of pluripotency genes and exhibit a gradual loss of characteristics associated with this property. This phenomenon is accompanied by a reduction in PGCs ability to form colonies in the *in vitro* culture (Naeemipour et al., 2013).

Primordial germ cells can be identified at an early stage X, and their further differentiation is dependent on the formation of the embryo (Ginsburg and Eyal-Giladi, 1987). They arise from epiblast (Eyal-Giladi et al., 1981) and at stage X of embryonic development they are located in the centre of area pellucida (Eyal-Giladi and Kohav, 1976). Initially, they are attached to the posterior side of hypoblast, but with the growth (stage XI to XIV) they move to the anterior part to finally reach the region of the germinal crescent (stage 4-6) (Fig.1). The number of PGCs at stage X is approx. 130 and increases nearly four times at stage 10 (Nakamura et al., 2007).

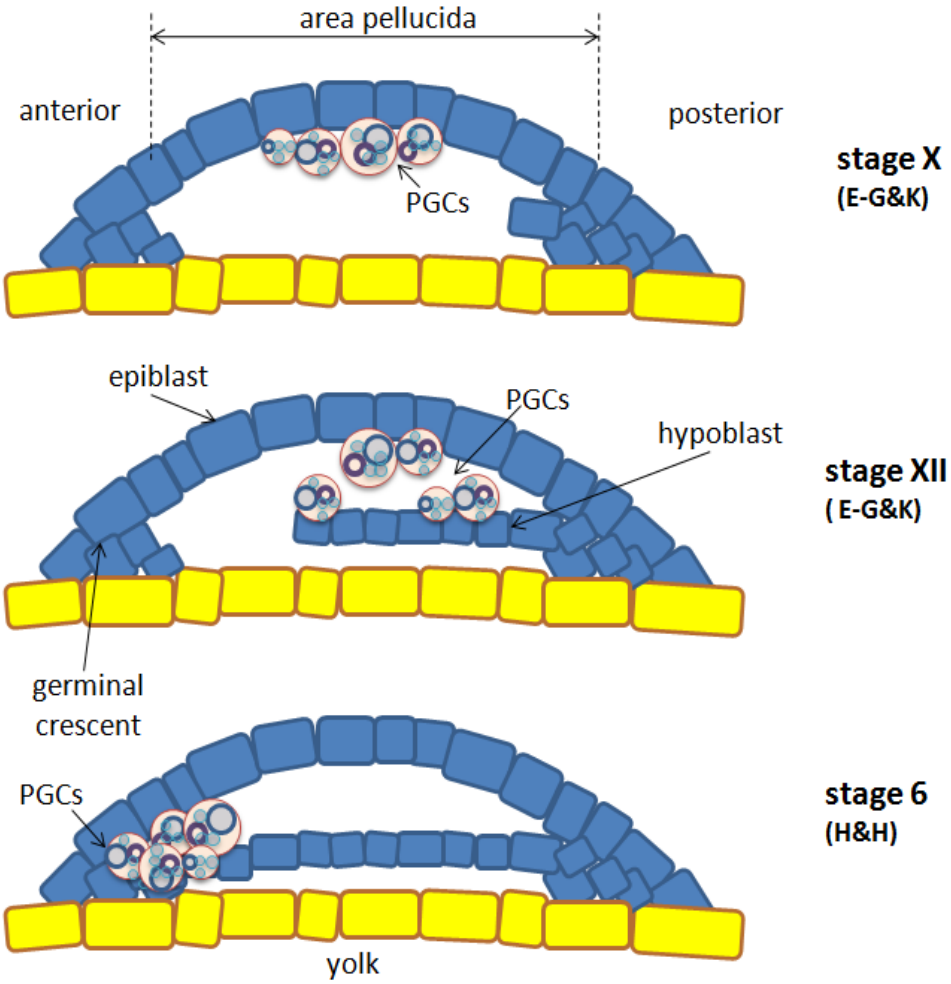


Fig.1. Formation and migration of primordial germ cells in early stages of chick embryo development (own compilation based on Simkiss, 1994; Tagami and Kagami, 1998; Han, 2009 and Bernardo et al., 2012). Roman numerals are designated by Eyal-Giladi and Kohav (1976), while Arabic are designated by Hamburger and Hamilton (1951).

At the stage 10-12, when blood vessels are formed (Fig.2), PGCs pass into the bloodstream of embryo, from which through bloodstream they enter the genital ridges (stage 20-24), there they settle, proliferate and begin to differentiate into male and female reproductive cells (Chojnacka-Puchta et al., 2012). Before the start of the 14th day of embryonic life, PGCs mitotic divisions cease (at this time we can observe in the gonads approx. 26000 PGCs) and the process of gametogenesis begins (Mochizuki and Matsui, 2010).

There are several possible mechanisms by which PGCs can move (Kuwana, 1993). These include: their own movement, the movement caused by

the physical contact with the surrounding somatic cells, the affinity to certain cells or extracellular matrix, such as fibronectin, passive migration by morphogenetic movement and migration induced by chemotactic agents. There was also a supposition that the circulating PGCs were chemically attracted by the compounds released from the gonads (Kuwana and Rogulska, 1999). Migration itself is assisted by extracellular matrix, whose compounds facilitate adhesion of PGCs during their way through the bloodstream to the gonads (D'Costa et al., 2001) and the chemokine SDF-1, whose presence is essential to the migration process (Stebler et al., 2004). These unique migration properties enable the production of germline chimeras by injection of donor PGCs into the bloodstream of recipient embryos on the 3-3.5th day of their development.

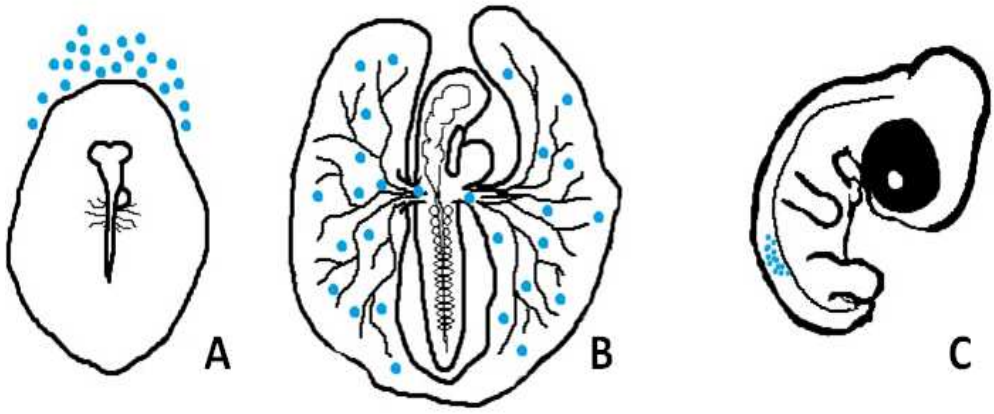


Fig.2. Migration of PGCs in chicken embryo; A-stage 12; B- stege 17; C- stege 28; developmental stages according to Hamburger and Hamilton (1951) (own compilation based on Simkiss, 1994; Hong et al., 1995; Tagami amd Kagami, 1998 and Han, 2009).

1.6. METHODS FOR ISOLATION OF PRIMORDIAL GERM CELLS

The unique migratory properties of PGCs to the gonadal primordia preceded by their free circulation in the bloodstream of embryos provide opportunities for the isolation of these cells from: i) blastoderm at stage X; ii) the blood of 2.5-3-day-old embryos (stages 13-17), and iii) the gonads of 5-7 day-old embryos (stages 26-31) (Chojnacka-Puchta et al., 2012). The following describes all three methods for the isolation of chicken PGCs to create germline chimeras.

Isolating PGCs from blastoderm at stage X Petitte et al. (1990) produced for the first time chicken germline chimera by separating blastodermal cells containing PGCs and putting them under the blastoderm of recipient at the same stage of embryonic development. The resulting rooster had functional sperms.

However, due to the very low hatching of chickens and a small number of PGCs from stage X above described chimera production is not very popular.

In contrast to mammals, avian PGCs use the circulatory system of forming embryo, as a means of transport from the region of germinal crescent to the future gonads. Accordingly, embryo blood at stages 13-17 becomes a potential source of PGCs for the production of chicken chimeras. In particular, blood coming from stage 14 appears to be the most suitable for this purpose, as concentration of circulating PGCs is then the highest (Tajima et al., 1999). Given the above, Tajima et al. (1993) successfully isolated primordial germ cells from blood of donor embryos, by using a glass micropipette, and introduced them into the bloodstream of recipient embryos at the same stage of development, thus producing germline chimeras. Further, Naito et al., (2004) showed that PGCs isolated from the blood and introduced into the blastoderm at stage X successfully enter the bloodstream of recipient embryos, from where they migrate towards gonads. A disadvantage of the method of isolation of PGCs from the embryonic blood is a difficulty of application and very small number of obtained PGCs (approximately 0.048% of total blood) (Yasuda et al., 1992), which limits the use of this method of isolation for more technically, advanced transgenic manipulation. It should be emphasized that germline chimeras obtained by isolation of PGCs from embryonic blood have a higher degree of germline transmission (11.3-96%) compared to chimeras obtained by isolation of PGCs from blastoderm (Han, 2009).

The most common way of obtaining PGCs is their isolation from the gonads of 5-7-day-old embryos. In order to release cells, the gonads are fragmented and/or digested using 0.25% trypsin-EDTA solution. Compared to other two methods, the number of PGCs per one embryo obtained by this method is the highest, thus increasing the success of chimera generation. It was demonstrated that germline chimeras can be produced by an introduction of PGCs derived from gonads of 5-day-old donor embryos to blood vessels of recipient embryos at stage 15-16 (Tajima et al., 1998). It has also been proven that cells isolated from gonads (stage 27) and injected into embryos in stage X and 17 also undergo the normal development and form gametes (Mozdziak et al., 2006). These studies easily show that PGCs obtained from gonads are capable of remigration and differentiation into the functional germ cells in recipient embryos, even if they underwent the migration phase in donor embryos.

1.7. SELECTION OF DONOR AND RECIPIENT GENOTYPE OF PRIMORDIAL GERM CELLS

Primordial germ cells can be transferred between embryos in an allogenic or xenogenic way, i.e. between donors and recipients of the same species (e.g., from a "chicken" to a "chicken"), and between donors and recipients of different species (e.g. from a "quail" to a "chicken"). In the first case, there are two

additional options including transfer between the same or different races and/or types of hens. Literature contained in the database Web of Science was used to analyze the selection of donors and recipients of PGCs cells, which is summarized in Table 3. It is believed that selection of genotypes for the role of donors and recipients of PGCs has a significant impact on migration and population growth of introduced PGCs (Ishiguro et al., 2009; Kang et al., 2009), and hence a degree of germline transmission (Nakamura et al., 2013).

Interspecies and interracial germline chimeras were successfully produced by an injection of PGCs derived from different species (Kang et al., 2008; Ishiguro et al., 2009; Kang et al., 2009; Wernery et al., 2010; Kang et al., 2011; van de Lavoir et al., 2012; Park and Han, 2013) and breeds of birds (Tajima et al., 1998; Park et al., 2003; van de Lavoir et al., 2006; Kim et al., 2010 , Nakamura et al., 2010; Nakajima et al., 2011; Nakamura et al., 2011; Nakamura et al., 2012; Park and Han, 2012, Miyahara et al., 2014) to recipient embryos. It has been shown thus that PGCs of different species can migrate towards gonads of recipients and normally proliferate in gonads (Park et al., 2014). Still, however, there are no studies verifying the suitability of various breeds and types of chickens for the production of germline chimeras.

The production of germline chimeras involves incorporation of exogenous PGCs into endogenous gonadal tissue of recipient embryos. Therefore, there is a specific competition between the two populations of primordial germ cells - endogenous and exogenous, which leads to the production of two types of germ cells - derived from a donor and recipient (host). Thus, the proportion of exogenous gametes is determined by the ratio of the number of germ cells of the host to the cells artificially introduced into the gonads of recipient embryos (Nakamura et al., 2013). This number may be affected by two factors. The first one is the ability of germ cells to mitosis, which varies depending on chicken breed. For example, chicken breeds, such as the White Leghorn, Barred Plymouth Rock and Fayoumi differ in their ability to accept foreign PGCs, with the best results for the first of these breeds and the worst for the Barred Plymouth Rock. The second factor regulating the ratio of number of host germ cells to artificially introduced cells is the number of exogenous PGCs, which reach the gonads and colonize them as well as the number of endogenous PGCs, already present there. The proportions between them can be increased by the partial or complete removal of endogenous PGCs by sterilization. Various sterilization techniques of recipient embryos are presented in the next section.

Tab. 3. Combinations of donors and recipients in generation of avian chimeras.

authors	**species / breeds** **donor → recipient**
Xenogenic transfer	
Gao et al., (2011)	Japanese Quail (*Coturnix japonica*)→ Peking Duck (*Anas platyrhynchos*)
Ishiguro et al., (2009)	Chicken (*Gallus domesticus*)→ Japanese Quail (*Coturnix japonica*)
Kang et al., (2008)	Pheasant (*Phasianus colchicus*) → Chicken (*Gallus domesticus*)
Kang et al., (2009)	Pheasant (*Phasianus colchicus*) → Chicken (*Gallus domesticus*)
Ono et al., (1996)	Japanese Quail (*Coturnix japonica*)→ Chicken (*Gallus domesticus*)
van de Lavoir, (2012)	Chicken (*Gallus domesticus*) →Guinea fowl (*Numida meleagris*)
Wernery et al., (2010)	Houbarda bustard (*Chlamydotis undulata*)→ Chicken (*Gallus domesticus*)
Yasuda et al., (1992)	Chicken (*Gallus domesticus*) → Japanese Quail (*Coturnix japonica*)
Allogenic transfer	
Chang et al., (1997) Jung et al., (2010) Kim et al., (2010) Lee et al., (2006) Park et al., (2003b) Park et al., (2010) Jeong i Han,(2002)	Korean Ogol Chicken → White Leghorn
Han et al., (2002) Park and Han,(2000) Park et al., (2003a)	White Leghorn →Korean Ogol Chicken
Nakajima et al., (2011)	White Leghorn → Rhode Island Red
Shiue et al., (2009)	White Leghorn → Taiwan Country Chicken
Zhang et al., (2013)	Black Boned Chicken →Suqin Yellow Chicken
Kagami et al., (1997) Miyahara et al., (2014) Naito et al., (1994) Naito et al., (2001) Nakamura et al., (2010) Nakamura et al., (2012) Song et al., (2005) Speksnijder et al., (1999) Tagami et al., (1997) van de Lavoir et al., (2006)	Barred Plymouth Rock → White Leghorn
Naito et al., (1994) Naito et al., (1998) Tagami et al., (1997) Tajima et al., (1998)	White Leghorn → Barred Plymouth Rock
Kim et al., (2005)	Black Japanese Quail → Wild-type Japanese Quail

1.8. STERILIZATION METHODS OF RECIPIENT EMBRYOS

In order to increase the efficiency of germline transmission and genetic modification a number of methods of inactivation and removal of endogenous PGCs from recipient embryos have been developed (Wentworth et al., 1989). The most important of these include: i) surgical removal (Naito et al., 1994; Kagami et al., 1997), ii) inactivation using UV radiation (Reynaud, 1976), X-rays (Lim et al., 2006; Nakamura et al., 2012) and γ radiation (Carsience et al., 1993), and iii) chemical treatment with busulfan (Aige-Gil and Simkiss, 1991; Furata and Fujihara, 1999; Song et al., 2005; Nakamura et al., 2009; Lee et al., 2013) and tamoxifen (Mohsen et al., 2002).

Mechanical removal of blastodermal cells from central part of area pellucida at stage X is technically difficult, however, as compared to methods based on radiation, it gives much better results (Motono et al., 2009). Another commonly used method is the removal of part of blood from bloodstream of developing 2-3.5-day embryos immediately prior to an injection of exogenous PGCs. This method, however, requires great precision and number of PGCs removed in this way is very small (Naito et al., 1994). On the other hand, gamma radiation, X-ray or UV damage DNA by acting on a target cell, which disrupts the continuity of all cell cycles, and consequently leads to cell death. Germ cells in contrast to somatic cells are more susceptible to radiation, which is used in sterilization of recipient embryos (Nakamura et al., 2013). Another method of depriving the embryo of gonadal PGCs uses chemicals. Tamoxifen is an agent which was studied in chicken embryos for sterilizing properties, but its effectiveness in elimination of endogenous PGCs is small (Mohsen et al., 2002). A cytostatic called busulfan is most commonly used for this purpose (Furata and Fujihara, 1999; Song et al., 2005; Nakamura et al., 2009; Nakamura et al., 2010; Lee et al., 2013). This is an alkylating agent causing germ cells apoptosis. In addition to sterilizing properties, the treatment with busulfan can cause many side effects, such as teratogenicity, infertility, or death (Lee et al., 2013). Despite the high effectiveness of busulfan to reduce endogenous number of PGCs, its side effects are still a problem, as it can cause abnormalities in development of embryos and increase their mortality rate. Structural analogue of busulfan-treosulfan appears to be an excellent alternative in chemosterilization of chicken embryos, because of its low toxicity and good solubility in water (Brink et al., 2014). However, to date, this cytostatic agent was not used in such studies.

1.9. STUDY OBJECTIVE

The aim of this study was to develop a comprehensive method to create chicken germline chimeras by optimizing of isolation methods for primordial germ cells (PGCs), selection of donors and recipients of cells and sterilization of recipient embryos as factors influencing the behavior of donor primordial germ cells in the body of a recipient.

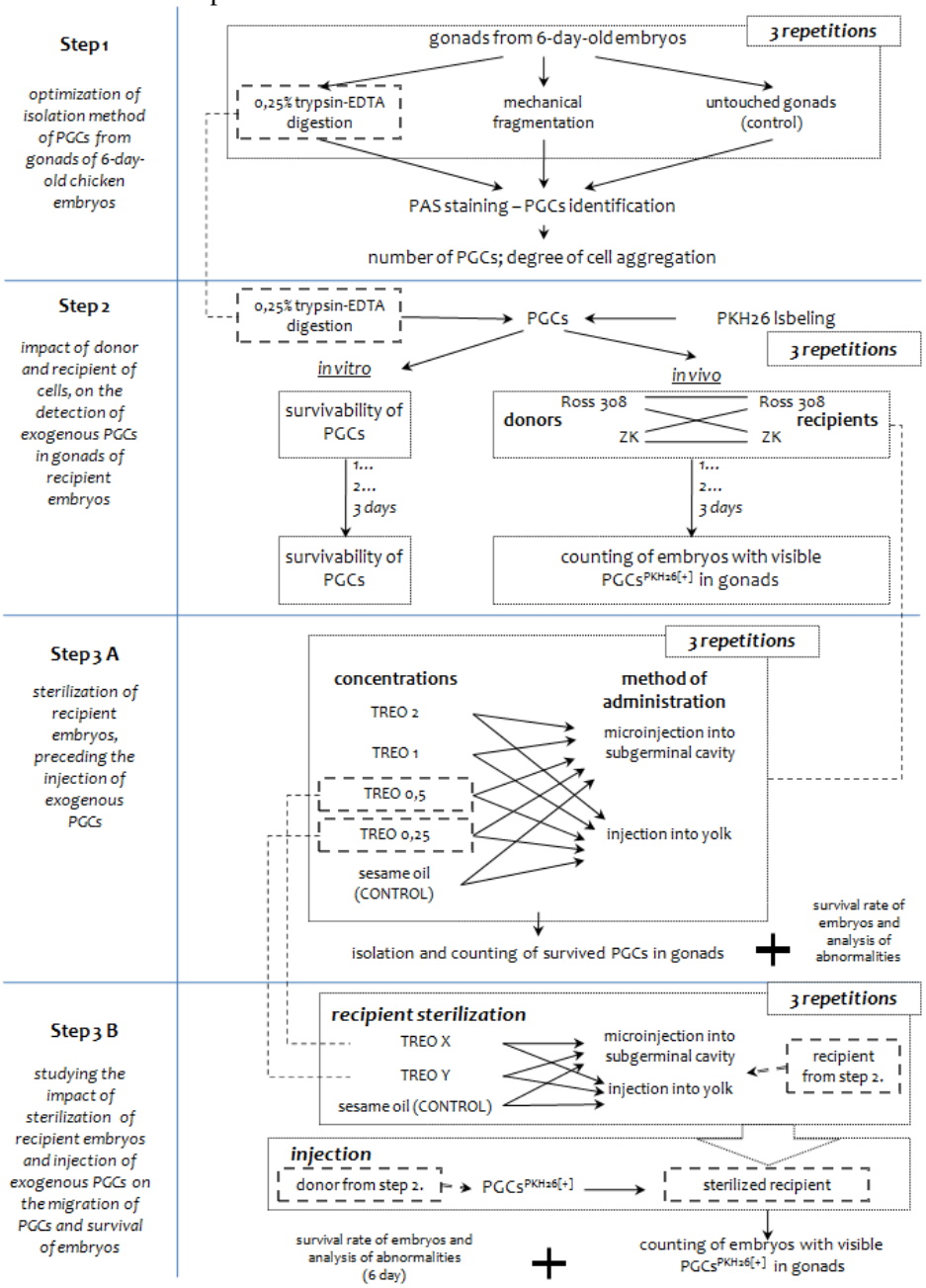
Carrying out this work includes the following tasks:

1. Optimization of a method of isolating PGCs from the gonads of 6-day-old chicken embryos,
2. Examination of the impact of donor and recipient cells on detection of exogenous PGCs in gonads of recipient embryos,
3. Sterilization of recipient embryos, preceding an injection of exogenous PGCs,
4. Sterilization of recipient embryos and an injection of fluorochrome-labeled PGCs in order to track the migration of PGCs in gonads of recipients and assess their impact on survival and development of embryos.

2. MATERIALS AND METHODS

2.1. RESEARCH SCHEME

Scheme shows the plan of carried studies:



2.2. RESEARCH MATERIALS

2.2.1. Eggs

The research material used in the work were fertilized chicken eggs (Gallus gallus domesticus) obtained from broilers Ross 308 (Drobex Agro; Makowiska) and from polish native breed green-legged Partridgelike chickens - ZS-11 (G. Skórnicki; Duszniki Wielkopolskie).

2.2.2. Laboratory equipment

- incubator for eggs - Fest hatching apparatus; ALMD1-N3
- stereoscopic microscope - MST 132
- incubator CO_2 for cell cultures - Smart Cell HF-90; Heal Force
- heating block - AccuBlock Digital Dry Bath D1200; Labnet
- Bürker counting chamber - Mareinfeld
- inverted microscope with fluorescent lamp - Axiovert 40 CFL; Zeiss
- fluorescence microscope - MN800 FL; OPTA-TECH
- set for micromanipulation - InjectMan® NI2; CellTram® Oil; Eppendorf
- glass microcapillaries for injection - MAS lab
- photo camera - Olympus u820

2.2.3. Reagents

- PBS - Phosphate Buffered Saline; tablets; Fisher Scientific; POCH
- PBS[-] - Phosphate Buffered Saline 1x; [-] Calcium Chloride; [-] Magnesium Chloride; Gibco® Invitrogen
- PKH26 - Red Fluorescent Cell Linker Kits for General Cell Membrane Labeling; Sigma-Aldrich
- FBS - Fetal Bovine Serum; Gibco® Invitrogen
- Pen-Strep - Penicillin-Streptomycin; Sigma-Aldrich
- Trypan Blue - 0,4%; Sigma-Aldrich
- PAS & Schiff reagent - PAS staining system; Sigma-Aldrich
- Trypsin -EDTA - 1x; Sigma-Aldrich
- OPTI-MEM® I Reduced Serum Medium - 1x; Gibco® Invitrogen
- Treosulfan - 1000mg; Ovastat®; Medac
- Aqua pro injectione - Polpharma
- sesame oil - Haitoglou Bros

2.2.4. Other materials

- parafilm - Parafilm® M; Sigma-Aldrich

– needles - Microfine + U40; 0,3 x 8mm; BD
– needles - 0,6 x 25mm i 0,9 x 40; Terumo
– 4-well cell culture plates - SPL Life Sciences
– 96-well cell culture plate - BD Falcon

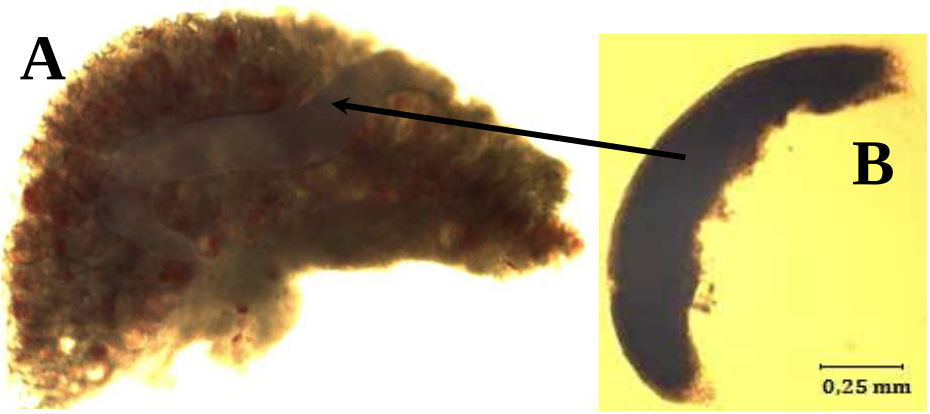
2.3. OPTIMIZATION OF ISOLATION METHOD OF PRIMORDIAL GERM CELLS FROM THE GONADS OF 6-DAY-OLD CHICKEN EMBRYOS

2.3.1. Eggs incubation

Eggs were incubated for 6 days at a temperature of 37.8°C and relative humidity 62-65%. Gonads and PGCs were isolated when embryos obtained 28-29 developmental stage according to the system of Hamburger & Hamilton (1951).

2.3.2. PGCs isolation from gonads of 6-day old embryos

Embryos were randomly divided into three groups, each consisting of 24 animals. Then, under a stereoscopic microscope by using a microinstruments (0.3 x 8 mm needles, scissors) mesonephromas were isolated (Fot. 1).



Fot.1. Mesonephromas (A) with visible gonad and isolated gonad (B); MN800 FL OPTA-TECH.

Subsequently, gonads were separated from mesonephros and placed in 0,1 ml of PBS solution (Phosphate Buffered Saline) (Fot. 2) without Ca^{2+} and Mg^{2+} ions - hereinafter PBS[-].

Fot.2. Isolated gonads in PBS[-] solution.

Obtained gonads were divided into three groups (Fig.3):

- **A -** gonads were mechanically fragmented, then placed in 0.5 ml PBS[-] and incubated in 4-well flat-bottom plates at 37.8°C and 5% CO_2 saturation for 1 hour - 3 replicates of 8 embryos (16 gonads).
- **B -** gonads were partially digested after isolation (digestion for 1 minute at 37°C) with 0.25% trypsin-EDTA solution (enzyme was inactivated by addition of 10% FBS - Fetal Bovine Serum), purified from trypsin-EDTA residues (centrifugation for 5 minutes at 2500 rpm), then placed in 0.5 ml PBS [-] and incubated in 4-well flat-bottom plates at 37.8°C and 5% CO_2 saturation for 1 hour - 3 replicates of 8 embryos (16 gonads).
- **C -** untouched gonads - control group - placed in 0.5 ml PBS[-] and incubated in 4-well flat-bottom plates at 37.8°C and 5% CO_2 saturation for 1 hour - 3 replicates of 8 embryos (16 gonads).

After incubation, the content of wells was pipetted several times, and checked for presence of PGCs aggregates under inverted microscope.

2.3.3. Determination of number and survival rate of PGCs

After incubation obtained PGCs were stained with 0.4% trypan blue. Then dead (stained blue) and viable cells (unstained) were counted in Bürker counting chamber under inverted microscope to determine the survival rate of PGCs.

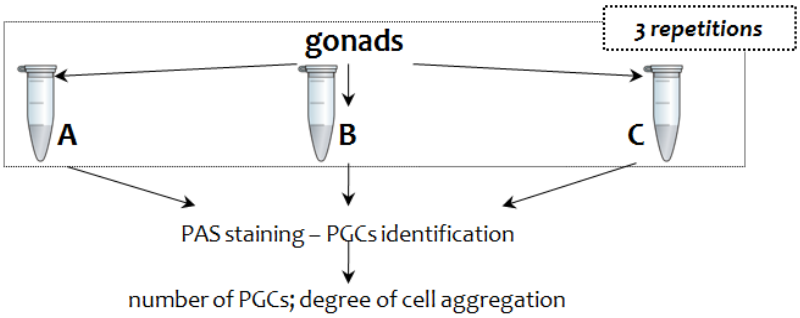


Fig.3. Scheme of PGCs isolation from gonads.

2.3.4. Identification of PGCs - PAS staining

Obtained cells were standard stained with PAS reaction (kit - PAS staining system) for the presence of supplementary material in the form of glycogen grains. It is a characteristic reaction for primordial germ cells (Meyer, 1960).

For PAS staining cells were fixed in 4% formaldehyde, washed twice in PBS solution and incubated at room temperature for 5 minutes in the presence of PAS reagent. After this time cells were again washed twice with PBS solution and treated with a Schiff's solution for 15 minutes at room temperature. This reagent was removed by subsequent washing in PBS solution. Slide was analyzed under OPTA-TECH microscope.

2.4. IMPACT OF DONOR AND RECIPIENT OF CELLS, ON DETECTION OF EXOGENOUS PRIMORDIAL GERM CELLS IN GONADS OF RECIPIENT EMBRYOS

2.4.1. General plan of trial

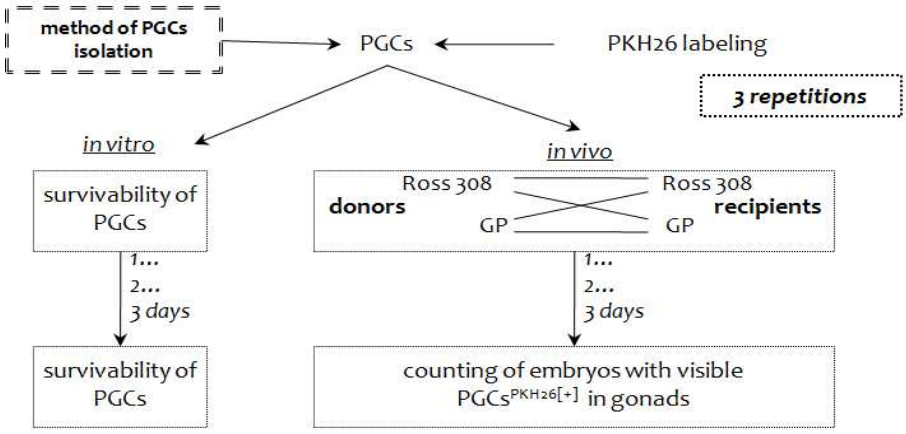


Fig.4. Scheme of second experiment.

Based on previous results we selected optimal method to isolate PGCs from the gonads of embryo donors. Isolated cells were cultured in a CO_2 incubator at full humidity or were injected into the bloodstream of recipients embryos in developing stage 15-19 according to H&H (Fig. 4). Study was performed in 3 replications.

Number of injected embryos in different systems was various due to the health status of embryo. Some of them were not suitable for treatment. Initially, in each group 60 embryos were assigned for injection.

2.4.2. Eggs incubation

Eggs of donors were incubated for 6 days while eggs of recipients for 3.5 days at a temperature of 37.8°C and 62-65% humidity until the stage 28-29 for donors and 15-19 (H&H) for recipients. Then embryos were used for the isolation and injection of PGCs respectively.

2.4.3. Isolation of PGCs from gonads of 6-day-old donor embryos

The gonads were collected under sterile conditions from 6-day-old chicken embryos using microinstruments under stereoscopic microscope and placed in 100µl of PBS solution with Pen-Strep antibiotic (100:1; Penicillin Streptomycin). Subsequent procedures were held in a chamber with laminar air flow, wherein the biological material was maintained on heating block at 37.8°C. In order to release PGCs, gonads were predigested for 1 minute with 0.25% trypsin-EDTA solution (37°C), as described previously in earlier experiment. Digestion was stopped by adding 10% FBS, and trypsin-EDTA solution was removed by centrifugation (2500 rpm; 5 min). Then, PGCs were incubated for another hour in a volume of 0.5 ml PBS[-] solution, at 37.8°C and 5% CO_2. After this, the resulting precipitate was centrifuged (2500 rpm, 5 min) and resuspended in 0.5 ml of OPTI-MEM® I medium. PGCs were counted in a Bürker chamber under inverted microscope.

2.4.4. PGCs labeling with fluorochrome PKH26

PGCs suspension was divided into 2 parts (Fig.5.):

1. group $PGCs^{PKH26[-]}$- for 3-day *in vitro* cultivation in order to evaluate survival rate of cells unlabeled with PKH26; 100µl of cell suspension (approx. 1/5 of the total volume) were transferred into a 4-well plate. Then, cell culture was supplemented with 400µl OPTI-MEM® I medium, 5% FBS and 1% Pen-Strep antibiotic. Cells were incubated (until the day of fluorescence detection in gonads of recipient embryos) at 37.8°C and 5% CO_2 for 3 days. Simultaneously with fluorescence detection an assessment of PGCs *in vitro* culture was conducted. Cells were collected from wells and counted as in subsection 2.3.3.

2. group $PGCs^{PKH26[+]}$- for injection into embryos, and to evaluate cell survival rate after PKH26 labeling; remained PGCs (approx. 4/5 of the total

volume) were centrifuged (2500 rpm; 5 min), the supernatant was removed and cells were subjected to PKH26 labeling according to manufacture instruction. Labeled PGCs were then suspended in 500µl OPTI-MEM® I medium and counted in Bürker chamber under inverted microscope. As in the case of a $PGCs^{PKH26[-]}$ group, cell suspension was supplemented with 5% FBS and 1% Pen-Strep. Such labeled PGCs were used for injection into recipient embryos.

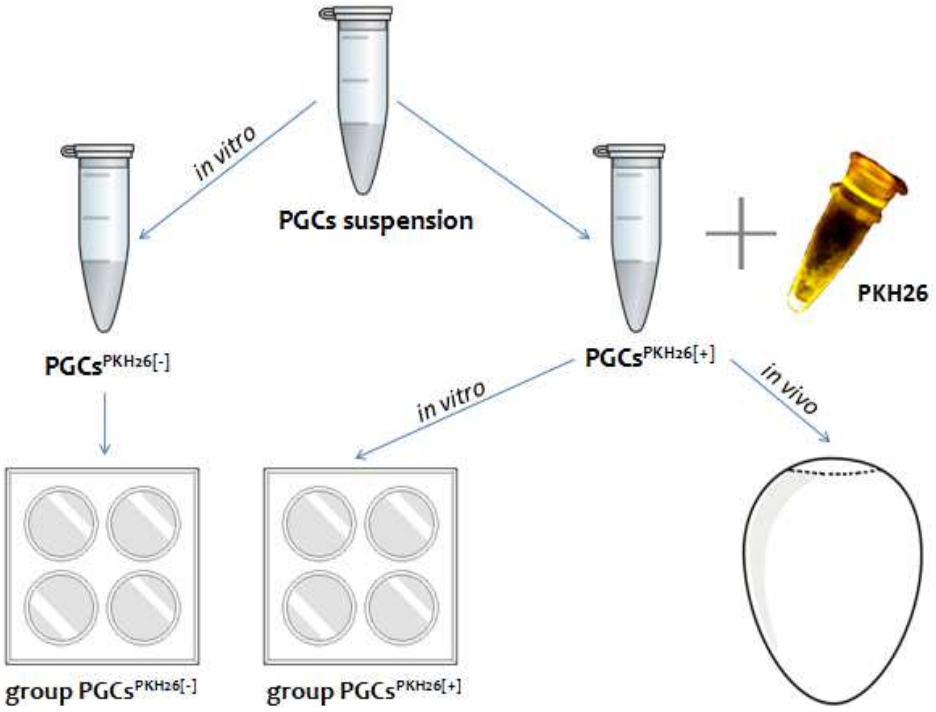


Fig. 5. Schematic distribution and use of PGCs suspension.

2.4.5. Injection of PGCs into 3.5-day-old recipient embryos

Disinfected eggs from 3.5-day-old recipients were drilled in the blunt end (⌀ of window - 1cm), in a place where air chamber is located, so as not to damage the inner membrane of egg. $PGCs^{PKH26[+]}$ were centrifuged (2500 rpm; 5 min) and resuspended in 100µl of fresh OPTI-MEM® I medium. To cell suspension 10µl of FBS and 2µl of Pen-Strep antibiotic were added. With a set for micromanipulation 1-2 ml of PGCs suspension was introduced into the bloodstream of embryos (dorsal aorta). After injection, eggs were sealed with parafilm and incubated for 3 subsequent days at 37.8°C and 62-65% humidity.

PGCs that remained after microinjection were re-counted, transferred to a 4-well culture plate and cultured after refilling in OPTI-MEM® I medium, 5% FBS and 1% Pen-Strep (group $PGCs^{PKH26[+]}$ established in order to evaluate cell survival rate after PKH26 labeling).

2.4.6. Evaluation of survival rate of cultured *in vitro* PGCs before and after PKH26 labeling

After 3 days of *in vitro* cultivation, unlabeled cells (group $PGCs^{PKH26[-]}$) and labeled with PKH26 cells (group $PGCs^{PKH26[+]}$) were counted in Bürker chamber under inverted microscope in order to evaluate cell viability after incubation in CO_2 chamber. To distinguish dead and living PGCs 0.4% trypan blue was used. Cells during the assessment were 9 days (isolated from 6-day-old embryos + 3 days of *in vitro* cultivation).

2.4.7. Detection of PGCs fluorescence in the gonads of recipients embryos

Gonads isolated from the 6-day-old recipient embryos were placed in a drop of PBS solution on a microscope slide (each pair of gonads was placed in a separate drop) (Fig.6.)

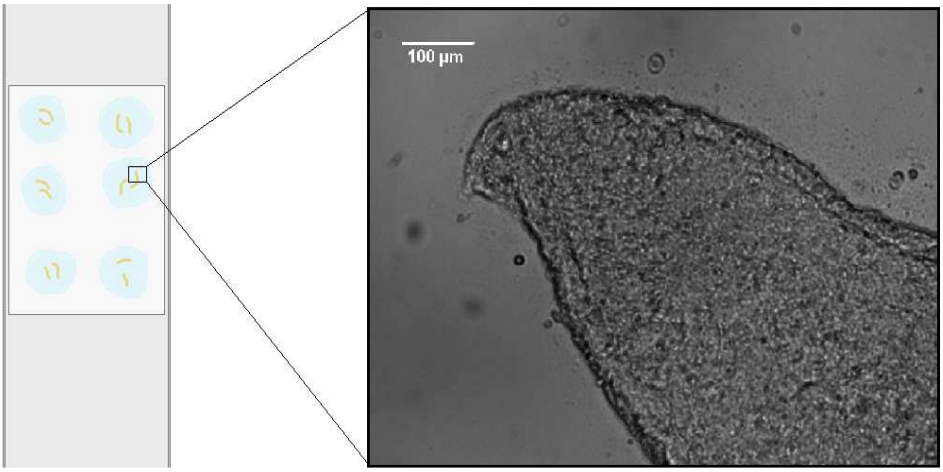


Fig.6. Observation of gonads under an inverted microscope; Axiovert 40 CFL Zeiss

Fluorescence was detected under inverted microscope with a fluorescent lamp (wavelength range: 551-567 nm). Only embryos with visible, introduced, labeled with PKH26 primordial germ cells in the form of round, single points with a diameter of 14 to 19µm or points in the form of aggregates were counted.

2.5. STERILIZATION METHOD OF RECIPIENT EMBRYOS AND ITS EFFECT ON THEIR SURVIVABILITY AND DEVELOPMENT

2.5.1. Optimization of treosulfan dose and site of its injection

2.5.1.1. General plan of trial

Based on the findings of previous trial, recipients were green-legged Partridgelike embryos which were sterilized in two different ways (injection into subgerminal cavity; injection into yolk) with four different treosulfan dilutions - 8 combination, plus 2 control groups (Fig.7). When embryos reached 6 day of development, viability rate of embryos and number of survived PGCs were counted. Experiment was repeated three times.

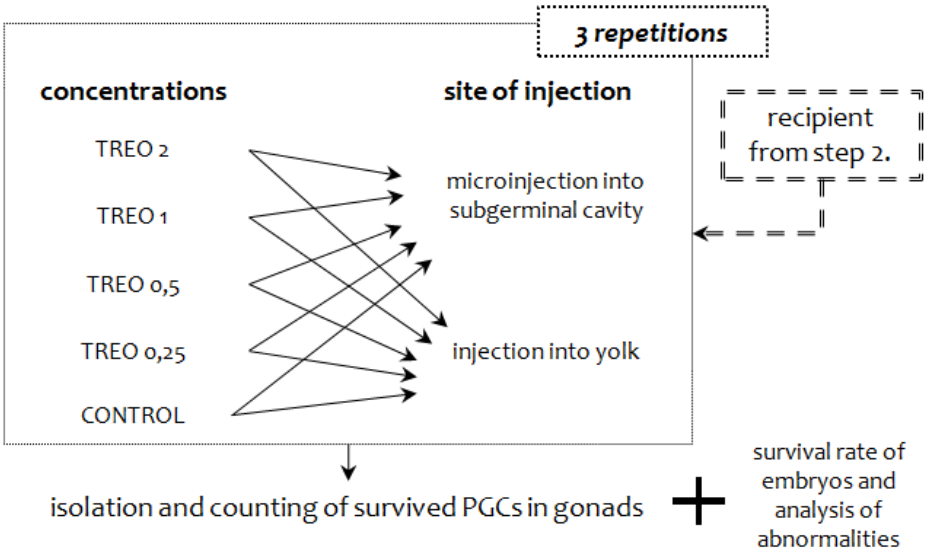


Fig.7. Scheme of third A experiment.

2.5.1.2. Preparation of eggs for sterilization procedure

Eggs were divided into two groups and maintained at room temperature for 2-3 days before the experiment was start in order to stabilize the position of blastoderm. The eggs were placed in the appropriate position:

- blunt end up - for group I, where cytostatic agent was injected into subgerminal cavity,
- horizontal - for group II, where cytostatic agent was administered into egg yolk.

This procedure was intended to displace and to stabilize blastoderm location (Fig.8). After this eggs were cleaned and disinfected. The first group of

eggs was drilled in the blunt end to expose blastoderm. The second group of eggs punctured with a needle (0,9 x 40mm) in the sharp end.

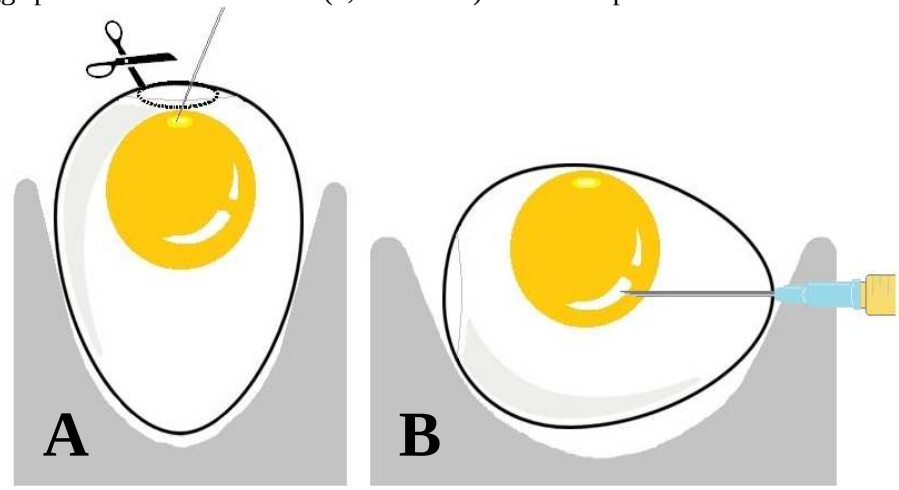


Fig.8. Methods of treosulfan insertion into a fertilized eggs (**A** - group I - injection into subgerminal cavity; **B** - group II - injection into yolk).

2.5.1.3. Preparation of treosulfan dilutions

Treosulfan was dissolved in 10 ml of Aqua pro injectione solution to form a stock with a concentration of 100mg/ml. Then this stock was diluted 50, 100, 200 and 400 times in sesame oil. There was thus obtained 4 groups of dilutions in which the concentration of treosulfan was respectively: 2, 1, 0.5 and 0.25mg/ml. In the control group was used Aqua pro injectione100 times diluted with sesame oil.

2.5.1.4. Methods of treosulfan injection

Emulsion of cytostatic solutions and control emulsion (Aqua pro injectione) were administered into eggs in two different ways (Fig. 8A and 8B).

A total of 10 groups of embryos was obtained:

- I TREO 0.25,
- I TREO 0.5,
- I TREO 1,
- I TREO 2,
- I control,
- II TREO 0.25,
- II TREO 0.5,
- II TREO 1,
- II TREO 2,
- II control.

Injection holes in egg shells was sealed with parafilm.

2.5.1.5. Embryos incubation

Sterilized eggs were incubated for 6 consecutive days at 37.8°C and 62-65% humidity. On the third day control embryo proper development was performed. During this control, dead embryos were removed in order to protect remaining embryos against contamination. Then incubation was continued until 6 day of embryonic development.

2.5.1.6. Evaluation of embryos survival rate

After incubation, dead and alive embryos were counted, along with individuals removed on the third day of incubation. Based on these data, we estimated survival rate for each of the ten groups of embryos.

2.5.1.7. Isolation and counting of donor PGCs

Gonads were isolated using a stereoscopic microscope and surgery microinstruments and placed on a 96-well culture plate. Each pair of gonads was placed in a separate well filled with 300µl of PBS[-] solution. Plate was incubated for 2 hours at 37.8°C and 5% CO_2 according to the method of Nakajima et al. 2011. This method is designed to discharge PGCs from somatic cells of gonads and migration of PGCs towards PBS[-] solution on the basis of a difference in osmotic pressures. After two hours of incubation PGCs were counted for each group using a Bürker chamber and inverted microscope.

2.5.2. Effect of sterilization of recipient embryos and injection of donor derived primordial germ cells on survival rate and development of recipients

2.5.2.1. General plan of trial

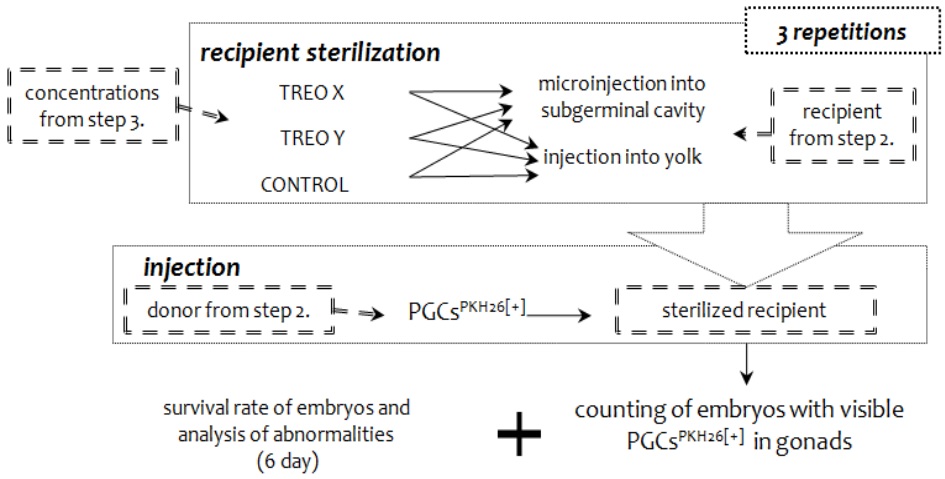


Fig.9. Scheme of third B experiment.

The last step is a combination of sterilization and injection of embryos (Fig.9) and their impact on death of embryos and presence of developmental abnormalities. 3 repetitions of experiments were performed.

2.5.2.2. Preparation of recipient eggs for sterilization procedure

Recipient eggs (GP) for 2-3 days before sterilization were placed in an appropriate position in order to stabilize blastoderm - blunt end up or horizontally (see subsection 2.5.1.2). After this time eggs were disinfected and drilled in blunt end (⌀ of window - 1cm) or punctured in sharp end of egg and subjected to sterilization procedure with treosulfan. Cytostatic was administered into subgerminal cavity or yolk respectively.

2.5.2.3. Selection of treosulfan dilution and sterilization of recipient embryos

On the basis of previous experience two dilutions of treosulfan were selected: 200x and 400x. As before, dilutions of treosulfan (stock: 100mgTreosulfanu/1ml Aqua pro injectione) were administered into the eggs in form of sesame oil emulsion. Control group was also injected with Aqua pro injectione in sesame oil emulsion (100x dilution). Fertilized eggs were injected with 1-2µl of treosulfan dilutions into subgerminal cavity or injected (using insulin syringes) with 50µl of dilutions into the yolk. Eggs were sealed with parafilm and incubated.

2.5.2.4. Incubation of donor eggs and preparation of recipient embryos to injection procedure

Donor eggs were incubated for 6 days at 37.8°C and 62-65% humidity. Then eggs were broken and embryos (stage 28-29 according to H&H) were obtained to isolate PGCs.

In turn, the treated with treosulfan recipient eggs were subjected to 3.5-day incubation until the stage 15-19 according to H&H. Incubation of donor and recipient embryos was synchronized in such a way that when PGCs were isolated from 6-day-old embryos, the second group - sterilized recipients of PGCs were in 15-19 developmental stage according to H&H. In this stage, after disinfection, eggs containing sterilized recipients were re-opened and assessment of condition state of embryos was performed. For $PGCs^{PKH26[+]}$ injection only viable embryos were used - showing no visible body and cardiovascular system defects. Dead embryos were counted and causes of death were also determined (see subsection 2.5.2.8.a.).

2.5.2.5. Isolation of PGCs and PKH26 fluorochrome labeling

PGCs were isolated from 6-day-old donor embryos by method described previously in subsection 2.3.4.. Briefly, obtained suspension was labeled with PKH26 fluorochrome according to methodology described by manufacturer.

Next, PGCs$^{PKH26[+]}$ were suspended in 500µl OPTI-MEM® I medium and counted under inverted microscope by using Bürker chamber. The medium was supplemented with the addition of 5% FBS and 1% Pen-Strep antibiotic. Cells were maintained at 37.8°C and 5% CO_2 until injection procedure.

2.5.2.6. Injection of PGCs$^{PKH26[+]}$ into sterilized recipient embryos and incubation of embryos

Eggs with 3.5-day-old recipient embryos sterilized by cytostatic were re-opened and disinfected. Only eggs with centrally localized embryos were selected for injection. Remaining embryos (displaced or incorectly located), that did not meet this criterion were removed due to inconvenient or even impossible access to the blood vessels.

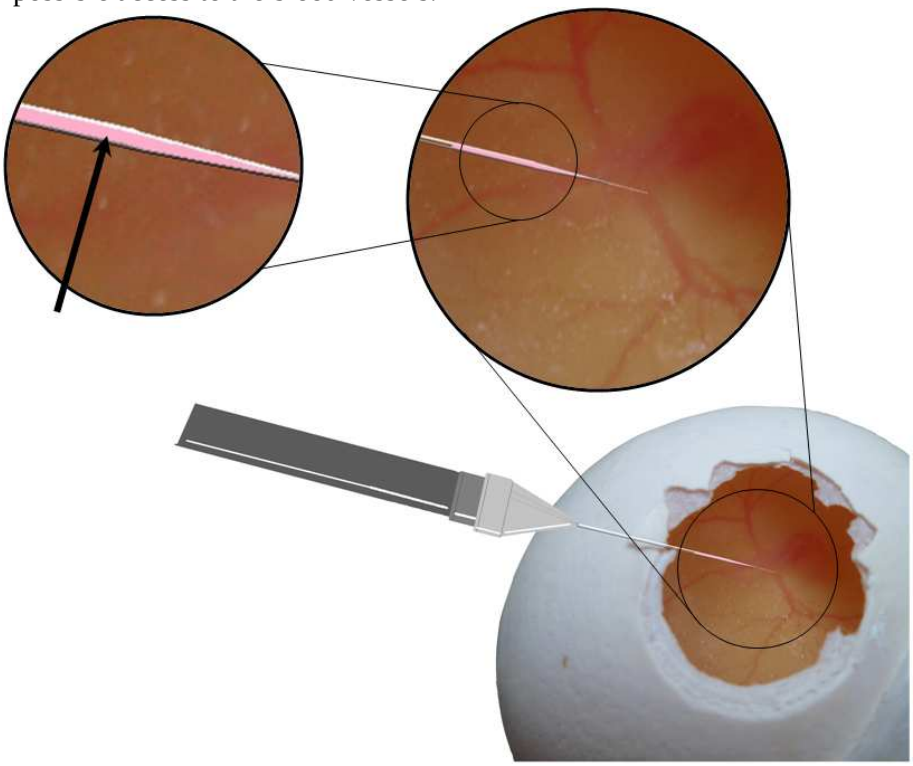


Fig.10. Injection of PGCs$^{PKH26[+]}$ into the bloodstream of recipient embryos. Arrow indicates a microneedle containing suspension of PGCs$^{PKH26[+]}$.

PGCs$^{PKH26[+]}$ were centrifuged (2500 rpm; 5 min) and transferred to fresh OPTI-MEM® I medium. To 100µl of medium 10µl of FBS and 2µl of Pen-Strep antibiotic were added. Amount of 1-2 µl of PGCs$^{PKH26[+]}$ suspension (mean concentration - 293 333/1ml) was introduced by micromanipulator into the bloodstream sterilized recipient embryos (Fig.10). After completion of

injection eggs were again secured with parafilm and incubated for a further 3 days at 37.8°C and 62-65% humidity.

2.5.2.7. Detection of fluorescence in gonads of sterilized and injected with $PGCs^{PKH26[+]}$ recipients embryos

The fluorescence was detected in the same manner as in subsection 2.4.7. Gonads were obtained in a 6th day from the all living recipient embryos. Each pair of gonads were placed in a separate drop of PBS solution on a microscope slide. Then, gonads gently flattened using glass coverslip and placed under an inverted microscope with fluorescence lamp (wavelength range from 551 to 567 nm). Only those embryos in which gonads were visible, labeled with PKH26 primordial germ cells in the form of round, single points with a diameter of 14 to 19μm or points in aggregates form were counted.

2.5.2.8. Assessment of sterilization and injection procedures on embryos development

2.5.2.8.a. Survival rate of embryos and causes of early death

Viability of recipient embryos were counted separately for each group of dilutions and for each group of administration methods of treosulfan, ie. for sterilized into subgerminal cavity or into yolk. Embryos mortality was determined for 3.5 day after sterilization treatment and for 6 day-old embryos (after $PGCs^{PKH26[+]}$ injection). Day and probable direct cause of death were determined (eg. no signs of development, blood rings etc.).

2.5.2.8.b. Developmental abnormalities in 6-day-old embryos

In the day of fluorescence detection (6th day of recipient development) embryos were checked for the presence of body abnormalities. All abnormal individuals were counted and photographed. Abnormalities were determined separately for each treosulfan dilution and for each method of sterilization.

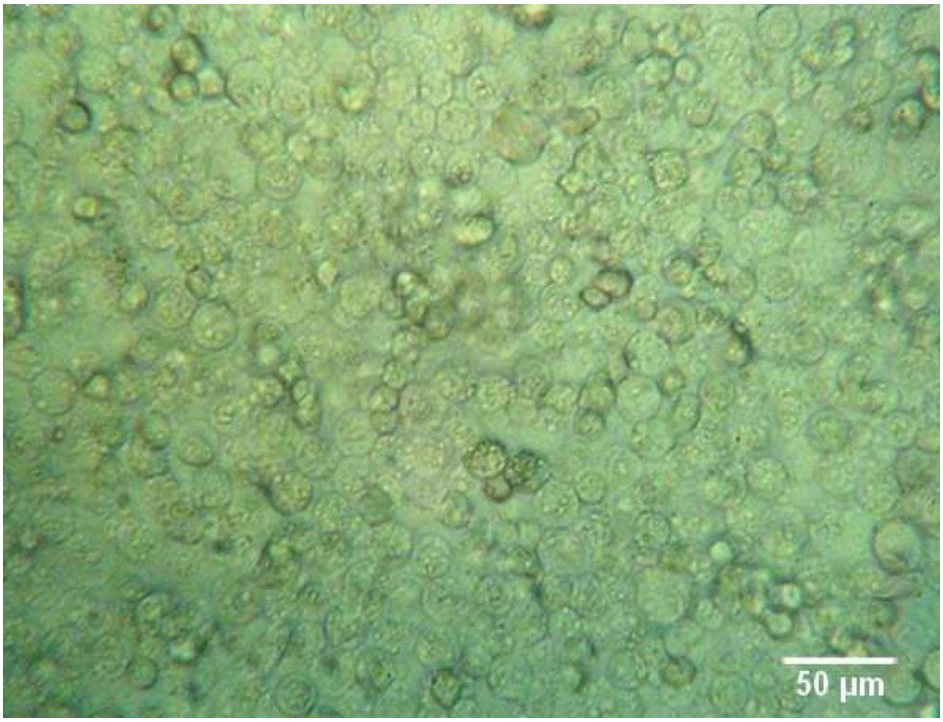
2.6. STATISTICAL ANALYSIS

SAS Enterprise Guide 4.3. was used for statistical calculations. With the help of this program, the analysis of variance (single and multi-factor), contingency analysis, and the Paerson's correlation were performed. Individual application of these analyzes and the use of post-hoc tests are described in following sections.

3. RESULTS

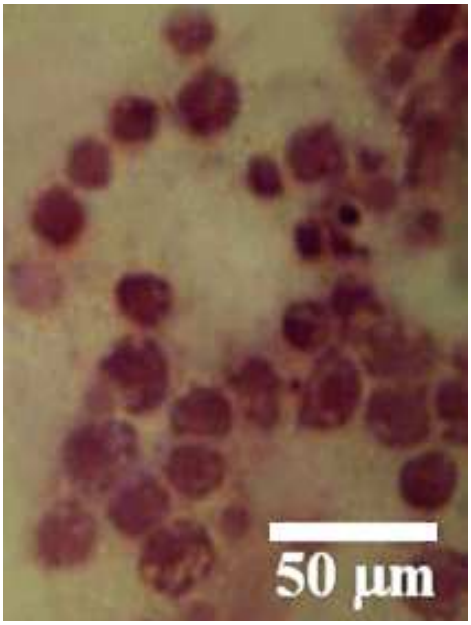
3.1. OPTIMIZATION OF ISOLATION METHOD OF GONADAL PRIMORDIAL GERM CELLS FROM 6-DAY-OLD CHICK EMBRYOS

Isolated during the study cells (Fot.3) have morphological features typical of primordial germ cells, that is round shape, numerous granules in cytoplasm, diameter from 14 to 19μm and large cell nucleus. Total number of discharged primordial germ cells was 7.118 x 10^6 (mean 5.932x 10^5) out of which 3.33 x 10^6 of cells derived from Ross 308 chickens (mean 5.550 x 10^5), and 3.788 x 10^6 of cells derived from Green-legged Partridgelike chickens (mean 6.313 x 10^5). Obtained differences were not statistically significant ($P > 0.05$).



Fot.3. Primordial germ cells isolated from gonads; Axiovert 40 CFL Zeiss

Behavior of cells treated with PAS staining was also examined. The aim of this staining was to visualize supplementary material of PGCs in a form of glycogen. Stained PGCs are shown in photography 4.



Fot.4. Identification of PGCs (PAS reaction); MN800 FL OPTA-TECH

Primordial germ cells were recovered from gonads in three different ways - by mechanical fragmentation (A), by partial digestion with 0.25% trypsin-EDTA (B) and by free migration of cells from untouched gonads in PBS[-] solution (C). Last of the aforementioned groups was used as a control. A total of 144 gonads were isolated from 72 chicken embryos. A total of 1.46 million cells were collected. Table 4 shows the results of each PGCs isolation method. The highest survival rate (76.17%) and the highest number of viable PGCs was observed in control group. The lowest cells viability was in group B, where gonads were partially digested with a trypsin-EDTA solution (71.55%). In turn, the lowest number of PGCs were obtained from mechanically fragmented gonads. Above differences between groups were found to be statistically significant ($P < 0.05$).

Behavior of donor primordial germ cells was also evaluated in terms of tendency to form cell aggregates. Only PGCs isolated with B method did not created agglomerates in the form of PGCs aggregates, and obtained material was high purity and homogeneity. In other two methods A and C aggregates were obtained. Chi-square test confirmed that method of PGCs isolation significantly affect the presence of conglomerates in these two groups ($P < 0.05$). Primordial germ cells conglomerates isolated with method A are shown in picture 5.

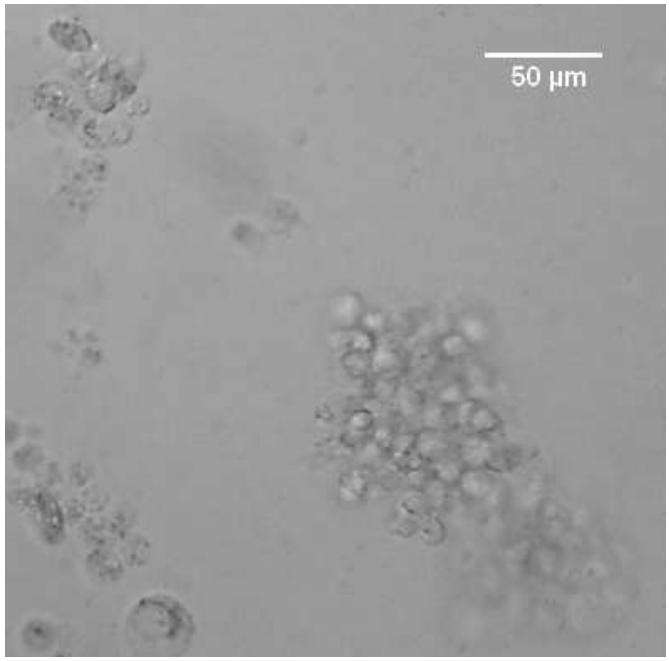
Tab. 4. Effect of different isolation methods of PGCs on number and viability of cells.

method	n	number of recovered PGCs	$\bar{x}$	SD	agregates
A	24	viable in1ml dead in 1 ml total survival rate [%]	93333.33 [c] 35333.33 128666.67 [c] **72.54**	9.22	yes*
B	24	viable in1ml dead in 1 ml total survival rate [%]	114000.00 [b] 45333.33 159333.33 [b] **71.55**	9.29	no*
C (control)	24	viable in1ml dead in 1 ml total survival rate [%]	151333.33 [a] 47333.33 198666.67 [a] **76.17**	8.03	yes*

$\bar{x}$ - mean values; SD - standard deviation;

a,b,c - values in same column with different letters differ statistically ($P < 0.05$)

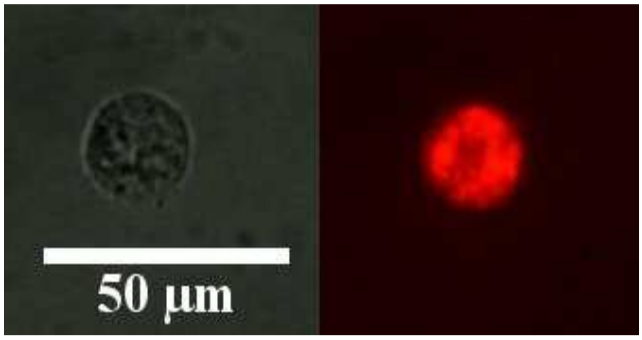
* - statistically significant differences ($P < 0.05$)



Fot.5. Cell aggregates in group A - PGCs were recovered by mechanical fragmentation of gonads; Axiovert 40 CFL Zeiss

3.2. EFFECT OF DONOR AND RECIPIENT COMBINATION ON DETECTION OF EXOGENOUS PRIMORDIAL GERM CELLS IN GONADS OF RECIPIENT EMBRYOS

For PGCs isolation 180 Ross 308 embryos and 180 Green-legged Partridgelike embryos were used. Based on the findings from previous trial cells were isolated by partial digestion of gonads with 0.25% trypsin-EDTA solution. To be able to observe the behavior of donor derived PGCs, cells were labeled with fluorochrome PKH26 (Fot. 6) and were microinjected into bloodstream through the dorsal aorta of 143 Ross 308 and 162 Green-legged Partridgelike recipient embryos.



Fot. 6. Primordial germ cell labeled with PKH26 in white light (left) and ultraviolet - 551-567 nm excitation filter (right); Axiovert 40 CFL Zeiss

3.2.1. Survivability of unlabeled and labeled with PKH26 primordial germ cells in *in vitro* culture

Behavior of primordial germ cells during short-term *in vitro* culture was also examined in this studies. Assessment of PGCs viability on the day of isolation and after three days of *in vitro* cultivation was carried out using trypan blue. Results of PGCs survivability on the first day of *in vitro* cultivation are presented in Table 5., while after 3 days of *in vitro* cultivation in Table 6.

A total of 3 330 000 PGCs were obtained from Ross 308 embryos and 3 788 000 PGCs from Green-legged Partridgelike embryos, however, these differences were not statistically significant ($P > 0.05$). Respectively 2 696 000 and 2 230 000 cells were labeled with fluorochrome. The remaining part of cells were *in vitro* cultured in short-term. On the day of PGCs isolation, in the case of unlabeled with PKH26 dye cells, higher survival rate have PGCs derived from Green-legged Partridgelike and it was 66.31 %. While in the case of labeled with PKH26 dye cells higher survival rate have PGCs derived from Ross 308 embryos (53.68 %). However, those findings were not statistically confirmed ($P > 0.05$).

Tab. 5. Survivability of unlabeled [-] and labeled [+] PGCs originating from Ross 308 and Green-legged Partridgelike embryos on the day of isolation.

donor		survival rate of $PGCs^{PKH26[-]}$ %		survival rate of $PGCs^{PKH26[+]}$ %
R	$\bar{x}$	64.14	$\bar{x}$	53.68
	SD	±21.25	SD	±15.82
GP	$\bar{x}$	66.31	$\bar{x}$	48.92
	SD	±27.74	SD	±16.06

$\bar{x}$ - mean values; SD - standard deviation

R - Ross 308; GP - Green-legged Partridgelike

After 3 days of *in vitro* cultivation total number of unlabeled primordial germ cells was 420 000 for Ross 308 and 443 000 for Green-legged Partridgelike, whereas the number of PKH26 labeled cells amounted to 1 670 000 and 962 000 PGCs respectively. Survival rate of unlabeled with PKH26 dye PGCs after 3 days of cultivation in *in vitro* conditions was similar in both groups - 86.63 % (22.49 % increase) for Ross 308 and 86.34 % (20.03 % increase) for Green-legged Partridgelike. In the case of unlabeled cells 62.87 % (9.19 % increase) and 73.54 % (24.62 % increase) survival rates were obtained respectively, but these differences were not statistically significant ($P > 0.05$).

Tab. 6. Survival rate of unlabeled [-] and labeled [+] PGCs originating from Ross 308 and Green-legged Partridgelike embryos after three days of *in vitro* cultivation.

donor		survival rate of $PGCs^{PKH26[-]}$ %		survival rate of $PGCs^{PKH26[+]}$ %
R	$\bar{x}$	86.63	$\bar{x}$	62.87
	SD	±6.98	SD	±24.07
GP	$\bar{x}$	86.34	$\bar{x}$	73.54
	SD	±9.49	SD	±15.50

$\bar{x}$ - mean values; SD - standard deviation

R - Ross 308; GP - Green-legged Partridgelike

The impact of PKH26 factor on behavior of PGCs was also evaluated. Survival rate of PGCs after labeling is listed in Table 7. On first day of *in vitro* cultivation higher survival rate have unlabeled PGCs than labeled cells, however, this result was not statistically significant ($P > 0.05$). A similar tendency was found after three days of *in vitro* cultivation. However this time, higher survival rate (86.48%) of unlabeled cells compared to labeled (68.20%) differed significantly ($P < 0.05$).

Tab. 7. Survival rate of unlabeled [-] and labeled [+] PGCs in the day of isolation and after three days of *in vitro* cultivation.

PKH26	day	survival rate [%]	
+	1.	$\bar{x}$ SD	51.04 [a] ±23.59
-	1.	$\bar{x}$ SD	65.22 [a] ±15.40
+	3.	$\bar{x}$ SD	68.20 [a] ±20,09
-	3.	$\bar{x}$ SD	86.48 [b] ±7.94

a,b - values in same column with different letters differ statistically: $P < 0.05$

$\bar{x}$ - mean values; SD - standard deviation

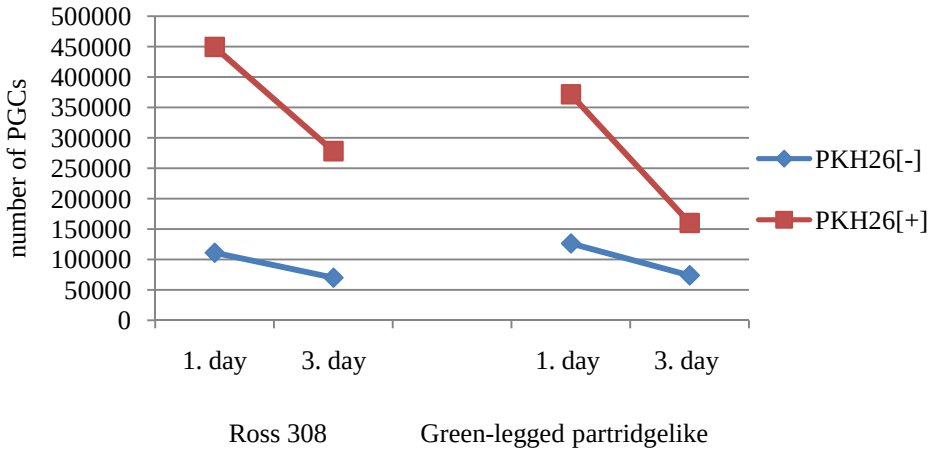


Fig.11. Effect of PKH26 labeling on the number of PGCs during short-term *in vitro* cultivation.

Number of labeled and unlabeled cells during 3-day cultivation was compared in Figure 11. Regardless of procedure there was a decrease in cell number. Assuming, that the number of PGCs (unlabeled and labeled with fluorochrome) on the first day was equal to 100%, changes in the number of cells on third day of cultivation were as follows:

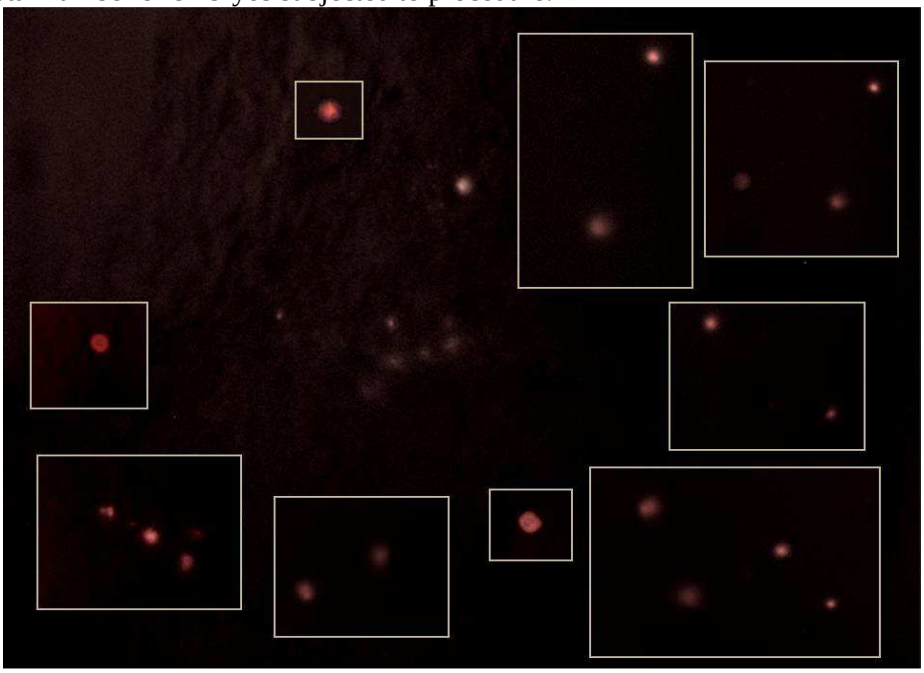
a) for PGCs isolated from Ross 308 embryos:
36.94% decrease in the total amount of $PGCs^{PKH26[-]}$ (100% → 63.06%),
38.06% decrease in the total amount of $PGCs^{PKH26[+]}$ (100% → 61.94%),
b) for PGCs isolated from Green-legged Partridgelike embryos:
41.53% decrease in the total amount of $PGCs^{PKH26[-]}$ (100% → 58.47%),

56.86% decrease in the total amount of $PGCs^{PKH26[+]}$ (100% → 43.14%).

If we analyze these data regardless of cell donors genotype, then the number of fluorochrome-labeled PGCs during three days of *in vitro* cultivation was reduced by 46.57% (100% → 53.43%), while unlabeled by 39.40% (100% → 60.60%).

3.2.2. Fluorescence detection in gonads of recipient embryos

Gonads of recipients were checked for the presence of introduced, labeled with PKH26 dye primordial germ cells. Occurrence of points within gonads of about 14 to 19μm, that emits fluorescence in wavelength range of 551-567 nm, proved the presence of exogenous PGCs (Fot. 7). Survival rate of embryos after injection was determined based on percentage of viable embryos in relation to total number of embryos subjected to procedure.



Fot. 7. Gonad of Green-legged Partridgelike embryo (background 200x magnification; small images 400x magnification) with visible, labeled with PHK26 day primordial germ cells that derived from Ross 308 donors; Axiovert 40 CFL Zeiss

Table 8. shows the results of donor / recipient impact on the percentage of recipients embryos with exogenous PGCs. The largest percentage of embryos having exogenous PGCs (54.50%) was noted in group where donor of cells was Ross 308, and Green-legged Partridgelike was the recipient of cells ($P < 0.01$). The lowest percentage of embryos with visible exogenous PGCs (3.30%) was observed in a group where donor of cells was Green-legged Partridgelike and

the recipient was Ross 308, similarly (8.30%) in group where Ross 308 was donor and recipient ($P < 0.01$). Homogenous combination of GP / GP was middle value (19.10%). Genotype combination had no significant effect on survival rate of embryos ($P > 0.05$).

Tab. 8.Effect of donor/recipient combination on survival rate of embryos after PGCs $^{PKH26[+]}$ injection and on fluorescence detection in recipient gonads

donor→recipient	**survival rate of embryos in control group - noninjected* [%]**		**survival rate of injected embryos [%]**		**embryos with visible PGCs $^{PKH26[+]}$ [%]**
	n	$\bar{x}$ **SD**	**n**	$\bar{x}$ **SD**	$\bar{x}$ **SD**
R→R	28	100.00 ±0.00	78	92.30 ±3.40	8.30 ^{B} ±0.13
GP→GP	30	96.70 ±5.77	73	93.20 ±8.82	19.10 ^{AB} ±1.70
GP→R	30	96.70 ±5.77	65	93.80 ±2.59	3.30 ^{B} ±0.27
R→GP	30	100.00 ±0.00	89	86.50 ±3.34	54.50 ^{A} ±1.40

A,B - values in same column with different letters differ statistically: $P < 0.01$

$\bar{x}$ - mean values; SD - standard deviation

R- Ross 308; GP - Green-legged Partridgelike

* - only recipients with drilled out window in egg shell

Calculated linear Pearson correlation coefficients between the percentage of embryos with visible, labeled PGCs and the number of dead embryos as well as survival rate were highly statistically significant and statistically significant respectively. Dependencies relating to the number of dead embryos were high (0.72240) according to Guillford scale, and correlation was positive. In turn, dependencies about survivability were moderate, and correlation was negative (Tab. 9).

Tab. 9. Pearson correlation between percentage of embryos with visible exogenous PGCs and survival rate of those embryos.

	live embryos	dead embryos	survival rate [%]
% of embryos with visible PGCs $^{PKH26[-]}$	0.38970	0.72240**	-0.65148*

* - statistically significant correlation coefficient; $P < 0.05$

** - highly statistically significant correlation coefficient; $P < 0.01$

Effect of injection on survival rate of recipients is shown in Table 10. In shells from both groups of eggs the injection windows were drilled, however injection were made only among experimental groups. Embryos from control group - noninjected have higher viability (98.31%), compared to those of which labeled primordial germ cells were introduced into bloodstream (91.15%). Difference (7.16%) was confirmed to be statistically significant ($P < 0.01$).

Tab. 10. Effect of injection on survival rate of recipient embryos

procedure	n	live embryos	dead embryos	survival rate [%]	
injection	305	278	27	$\bar{x}$ SD	91.15^{B} ±5.38
no injection (control)	118	116	2	$\bar{x}$ SD	98.31^{A} ±3.98

A,B - values in same column with different letters differ statistically: $P < 0.01$

$\bar{x}$ - mean values; SD - standard deviation

3.3. INJECTION OF EXOGENOUS PRIMORDIAL GERM CELLS PRECEDED BY STERILIZATION OF RECIPIENTS AND ITS EFFECT ON SURVIVALBILITY AND DEVELOPMENT OF RECIPIENT EMBRYOS

3.3.1. Selection of treosulfan concentration injected into egg

In this part of experiment the behavior of donor derived primordial germ cells was examined after treating with sterilizing substance - treosulfan. For this purpose a 127 of Green-legged Partridgelike embryos were used among which 105 of embryos were subjected to sterilization procedure and 22 of embryos were randomly selected for control group. In general survival rate of embryos was 47.24%. Among 127 embryos, 61 were treated into subgerminal cavity and 66 into yolk. Taking into account the different concentrations of cytostatic, 23, 22, 30 and 30 embryos were injected with 0.25, 0.5, 1 and 2 mg/ml solutions respectively.

Results of Chi-square test (Table. 11) indicates that there is a very high dependence between the method of sterilization and survivability of embryos (P < 0.001). Within experimental groups a greater viability was found in embryos sterilized into subgerminal cavity (72.73%) as well as into yolk (83.33%) both with TREO 0.25. It was also noted that, with increasing concentration of administered cytostatic survival rate of embryos decreased, giving the lowest values in group were concentration of treosulfan was 2 mg/ml (20.00% in group I and 13.33% in group II) (Fig.12).

Tab. 11. Effect of treosulfan administration on embryos survival rate (Chi-square test).

statistics	**value**	**probability**
Chi-square	36.3975	<.0001
likelihood ratio Chi-square	39.8738	<.0001

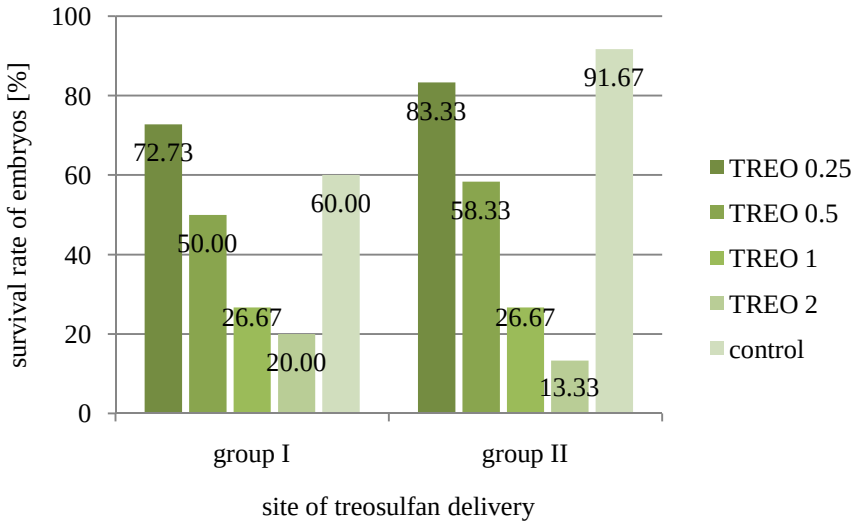


Fig.12. Effect of treosulfan delivery on survival rate of embryos.

I - embryos treated with cytostatic into subgerminal cavity

II - embryos treated with cytostatic into egg yolk

control - embryos that received Aqua pro injectione emulsion in sesame oil

Contingency analysis showed no significant correlation (P> 0.05) between mortality of embryos and injection site of treosulfan, without division into concentrations (Tab. 12). Embryos mortality depending on the site of treosulfan delivery is shown in Figure 13.

Tab. 12. Effect of treosulfan delivery site on survival rate of embryos (Chi-square test).

statistics	value	probability.
Chi-square	1.0057	0.3159
likelihood ratio Chi-square	1.0073	0.3155

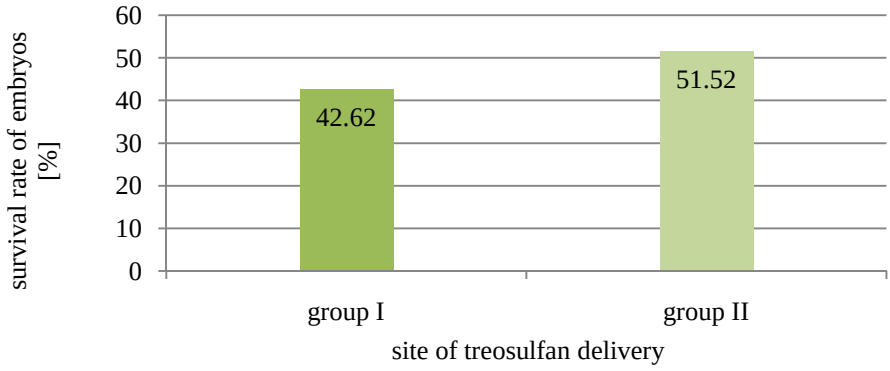


Fig.13. Effect of treosulfan delivery site on survival rate of embryos.

I - embryos treated with cytostatic into subgerminal cavity

II - embryos treated with cytostatic into egg yolk

Based on results of Chi-square test it was shown that there is a very high statistical correlation between the mortality of embryos and concentration of injected treosulfan solution, regardless of administration site (Tab. 13). The highest survival rate of embryos (78.26%) has group, where 0.25mg/ml concentrations of cytostatic were used. Control group has slightly lower survivability than TREO 0.25 group (77.27%). The highest mortality was found in group where the most concentrated treosulfan (2mg/ml) was used (16.67%). It was noted that survival rate of embryos decreased along with increasing concentrations of treosulfan ($P < 0.001$) - Figure 14. shows this dose-dependent relations.

Tab. 13. Effect of treosulfan concentration on survival rate of embryos (Chi-square test).

statistic	value	probability.
Chi-square	33.6582	<.0001
likelihood ratio Chi-square	35.8612	<.0001

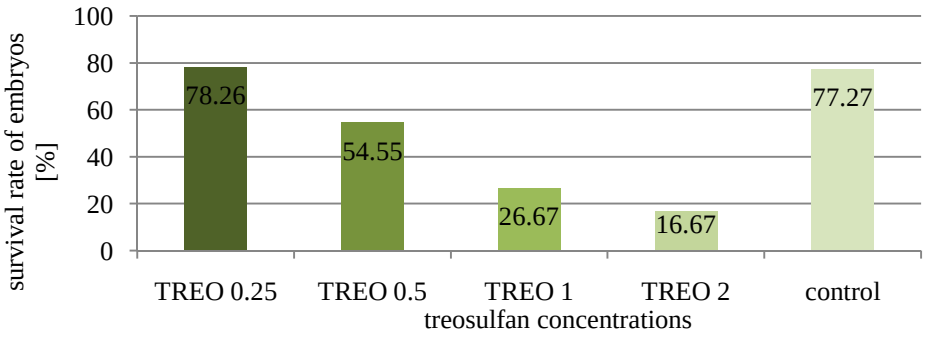


Fig.14. Effect of treosulfan concentration on survival rate of embryos.

control - embryos that received Aqua pro injectione emulsion in sesame oil

Number of survived primordial germ cells after sterilization procedure is shown in Figure 15. The highest number of cells within experimental groups was obtained at 0.25mg/ml concentration, but it was about 50% lower (2535.71 – 2775.0) as compared to control group (5041.67 – 5386.36). The lowest number of PGCs was obtained when 2mg/ml treosulfan concentration was applied, both in group of eggs treated into subgerminal cavity (183.33) as well as into egg yolk (375.0).

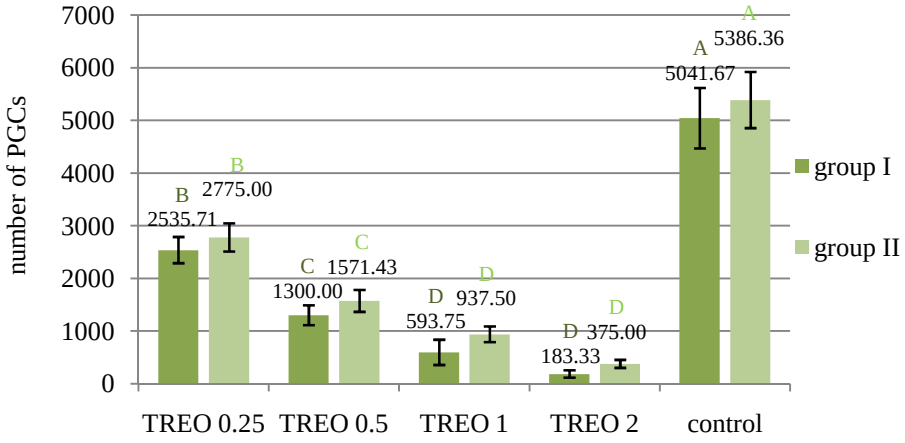


Fig.15. Effect of injection site and applied treosulfan concentration on the number of survived - endogenous PGCs in embryonic gonads at day 6.

I - embryos treated with cytostatic into subgerminal cavity

II - embryos treated with cytostatic into egg yolk

control - embryos that received Aqua pro injectione emulsion in sesame oil

A, A, B, B, C, C, D, D - values in same columns with different letters differ statistically: $P < 0.001$

Analysis of variance, presented in Table 14., showed that the behavior of endogenous PGCs in embryonic gonads is affected by both method of treosulfan injection (P = 0.0119) as well as concentration of delivered cytostatic (P < 0.001). Test results for interactions between examined experimental factors i.e. site of injection and applied concentration were insignificant (P = 0.9888).

Tab. 14. Variance analysis of effect of injection site and applied treosulfan concentration on the number of survived - endogenous PGCs in gonads of 6-day-old embryos.

	sum of squares	**mean square**	**F**	**Pr. > F**
site	888717.1	888717.1	6.83	0.0119
concentration	174673867.0	43668466.8	335.55	<.0001
site*concentration	40328.0	10082.0	0.08	0.9888

Scheffe test results are shown in Table 15. This test confirmed the significant differences in number of survived PGCs in embryonic gonads, depending on the site of treosulfan administration (into subgerminal cavity or into egg yolk). Treosulfan injected into subgerinal cavity caused a depletion of endogenous PGCs in a greater extent than cytostatic injected into egg yolk - 2297.0 or 3014.71 cells respectively.

Tab. 15. Effect of injection site of treosulfan on the number of survived - endogenous PGCs in gonads from 6-day-old chick embryos (Scheffe test).

site of treosulfan delivery	$\bar{x}$
group I	2297.00[b]
group II	3014.71[a]

I - embryos treated with cytostatic into subgerminal cavity

II - embryos treated with cytostatic into egg yolk

a,b - values in same column with different letters differ statistically: P < 0.05

$\bar{x}$ - mean values

Results of Scheffe test for different treosulfan concentrations, regardless of the site of administration, are shown in Figure 16. Only differences between concentrations 2 and 1 mg/ml were not statistically significant, other cases differed significantly (P < 0.001).

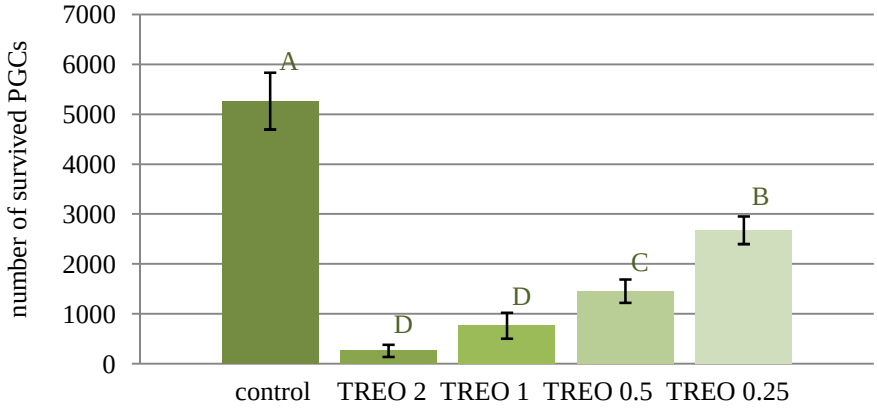


Fig.16. Relation between the number of endogenous PGCs and treosulfan concentrations.

control - embryos that received Aqua pro injectione emulsion in sesame oil

A,B,C,D - values in same column with different letters differ statistically: $P < 0.001$

Photos of isolated, survived, endogenous primordial germ cells after sterilization procedure are presented in Table 24. in chapter 6.

3.3.2. Sterilization of recipient embryos followed up injection of primordial germ cells and their effect on survivability of embryos

In present study the behavior of donor PGCs introduced into sterilized recipient embryos was also examined. Survival rate of recipients and ability of exogenous PGCs labeled with PKH26 dye, to incorporate into embryos gonads were evaluated. Based on the results of previous step, for sterilization 0.5 and 0.25mg/ml concentrations of treosulfan were selected. Cytostatic agent was introduced into subgerminal cavity or into yolk of fertilized eggs (Fig. 17). In total, 208 embryos derived from Green-legged Partridgelike representing the recipient of PGCs, were sterilized. In turn, for isolation of PGCs, 90 donor embryos - derived from Ross 308 chickens, were used. PGCs recovered from these embryos, were detected in 67 recipient embryos.

Control group (Fig.17) had the highest survivability of embryos (84.44% in group I and 91.67% in group II), the highest number of normally positioned recipient embryos (81.11% in group I and 83.33% in group II), the highest number of embryos that survived injection (71.11% in group I and 77.78% in group II) and finally the highest number of embryos with exogenous PGCs labeled with flurochrome dye (45.56% in group I and 33.33% in group II). Among groups TREO 0.25 and TREO 0.5, the highest number of recipients, in which exogenous PGCs labeled with flurochrome PKH26 were detected, was observed when more diluted treosulfan was administered into subgerminal

cavity (39.90%), and the lowest (22.22%) in case of less diluted treosulfan introduced into egg yolk. However, all differences turned out to be statistically insignificant (P > 0.05).

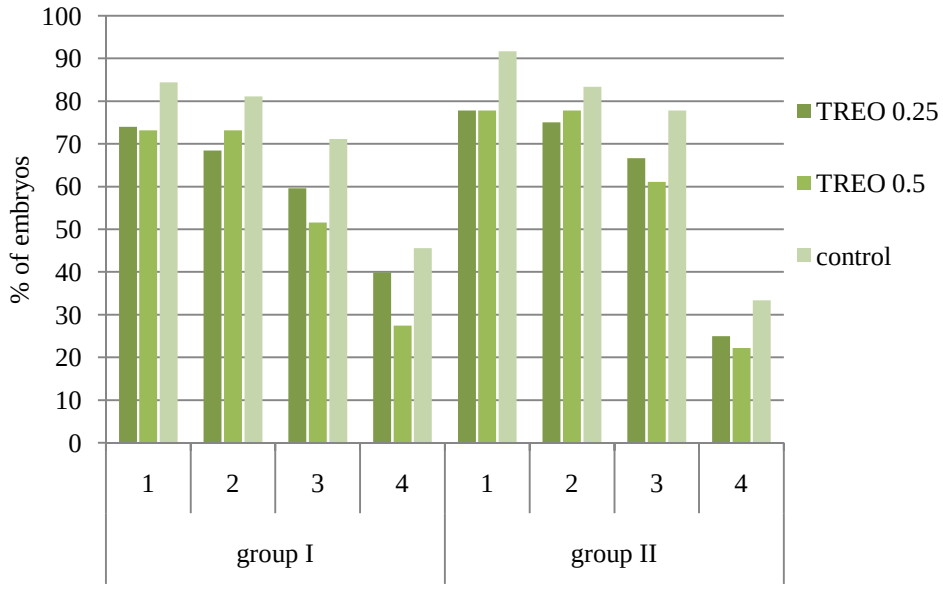


Fig.17. Effect of treosulfan injection into subgerminal cavity (group I) or egg yolk (group II) on survival rate of recipient embryos and presence of exogenous PGCs labeled with PKH26 fluorescent dye.

1 - embryos that survived after treosulfan sterilization

2 - embryos normally positioned (subjected to further injection)

3 - embryos that survived injection of exogenous PGCs

4 - embryos with visible, introduced PGCs $^{PKH26[+]}$

I - embryos treated with cytostatic into subgerminal cavity

II - embryos treated with cytostatic into egg yolk

control - embryos that received Aqua pro injectione emulsion in sesame oil

For a better visualization of embryos survivability and fluorescence detection, these results were grouped in terms of injection site and used concentration - Figures 18 and 19 respectively. However, there were no statistically significant differences (P > 0.05).

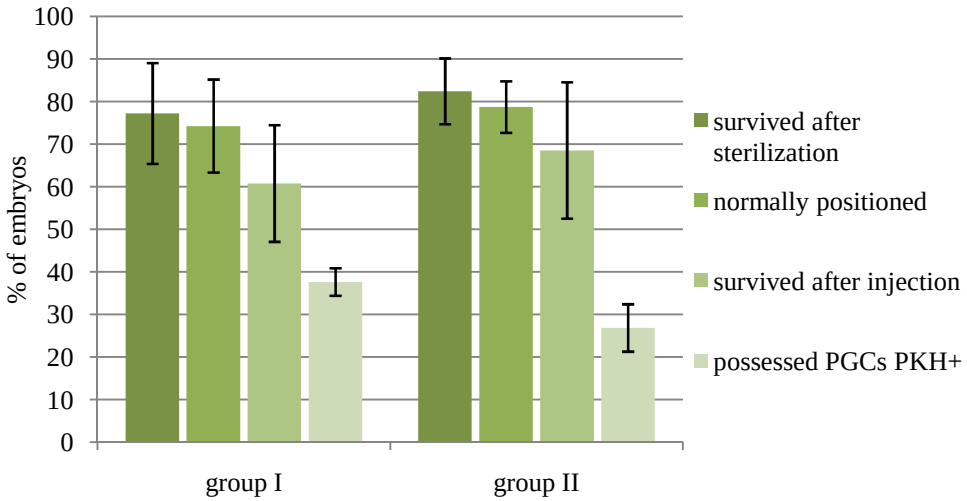


Fig.18. Effect of different injection site of treosulfan (into subgerminal cavity or into egg yolk) on survival rate of recipient embryos and presence of exogenous PGCs labeled with PKH26 fluorescent dye.

I - embryos treated with cytostatic into subgerminal cavity

II - embryos treated with cytostatic into egg yolk

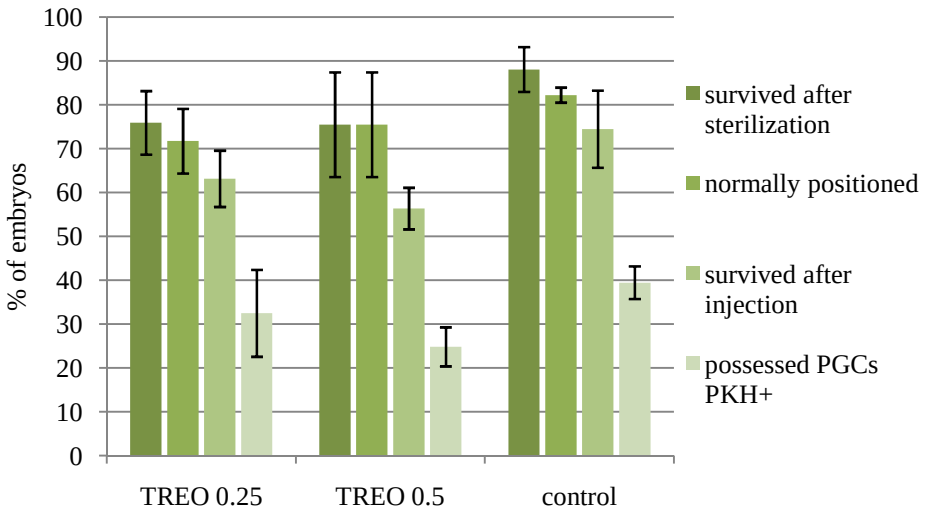


Fig.19. Effect of different concentrations of treosulfan on survival rate of recipient embryos and presence of exogenous PGCs labeled with PKH26 fluorescent dye.

control - embryos that received Aqua pro injectione emulsion in sesame oil

Behavior examination of exogenous PGCs, in terms of fluorescence emission in gonads of Green-legged Partridgelike embryos, was carried out on 6-day-old recipients. Among 208 individuals subjected to sterilization, followed by injection of PGCs labeled with PKH26, 67 (32.21%) have visible primordial germ cells in gonads. Photos of those gonads under a microscope with fluorescent lamp (Tab. 25) are shown in chapter 6.

The data in Table 16.summarize the effect of injection site and treosulfan concentration on percentage of embryos with visible in gonads exogenous $PGCs^{PKH26[+]}$. Among groups treated with treosulfan the highest percentage of embryos was in group I TREO 0.25 (40.00%), while the lowest in group II, where higher concentration of cytostatic was given (22.22%). I and II control groups were respectively 46.88% and 33.33%. However, there was no interactions and statistically significant differences between groups ($P > 0.05$).

Tab. 16. Fluorescence detection in gonads of sterilized recipient embryos injected with $PGCs^{PKH26[+]}$ - percentage was calculated relative to initial number of embryos.

group	n	embryos with $PGCs^{PKH26[+]}$
I		%
TREO 0.5	33	27.27
TREO 0.25	35	40.00
control	32	46.88
II		%
TREO 0.5	36	22.22
TREO 0.25	36	25.00
control	36	33.33

I - embryos treated with cytostatic into subgerminal cavity

II - embryos treated with cytostatic into egg yolk

control - embryos that received Aqua pro injectione emulsion in sesame oil

Regarding to effect of treosulfan concentrations, it was demonstrated that there are differences between control group, where the highest number of embryos with exogenous $PGCs^{PKH26[+]}$ was observed - 27 individuals, and sterilized with 0.5mg/ml treosulfan group, where number of embryos with visible in their gonads labeled PGCs was the lowest - 17 individuals. However, there was no statistically significant correlations ($P > 0.05$). Results are shown in Table 17.

Tab. 17. Fluorescence detection in gonads of recipient embryos sterilized with different treosulfan concentrations and injected with PGCs$^{PKH26[+]}$.

group	n	embryos with PGCs $^{PKH26[+]}$ [%]
TREO 0.5	69	24.82
TREO 0.25	71	32.45
control	68	39.44

control - embryos that received Aqua pro injectione emulsion in sesame oil

Taking into account injection site of cytostatic emulsion (Tab. 18), it was noted that higher number of embryos with visible PGCs$^{PKH26[+]}$ were obtained when treosulfan was administered into subgerminal cavity - 38 individuals. Group injected into egg yolk had 29 such cases, however above differences were found to be statistically insignificant ($P > 0.05$). Photos of gonads with visible PGCs$^{PKH26[+]}$ are shown in chapter 6. in Table 25.

Tab. 18. Fluorescence detection in gonads of recipient embryos sterilized into different sites and injected with PGCs$^{PKH26[+]}$.

group	n	embryos with PGCs $^{PKH26[+]}$	
		number of individuals	[%]
I	100	38	38.00
II	108	29	26.85

I - embryos treated with cytostatic into subgerminal cavity

II - embryos treated with cytostatic into egg yolk

3.3.2.1.Effect of sterilization and injection procedures on embryos development

Mortality rate of embryos subjected to influence of cytostatic was determined in 3.5 day of embryo development. Embryos that showed normal signs of life (heart beating, properly filled blood vessels, lack of blood rings etc.) were subjected to PGCs$^{PKH26[+]}$ injection. Photos of dead embryos are shown in chapter 6 (Tab. 26).

Among hundred embryos treated into subgerminal cavity (Tab. 19), 23 were considered as dead. The lowest percentage mortality (15.65%) was noted in control group, and the highest (27.27%) in group where 0.5mg/ml concentration of treosulfan was administered. It was observed that with increasing concentrations of cytostatic, the mortality rate of embryos increase, due to hemorrhage (blood rings) on second day. These differences proved to be statistically significant ($P < 0.05$).

Tab. 19. Mortality of embryos up to 3.5 days of development - embryos were treated with treosulfan emulsion into subgerminal cavity (Chi-square test).

group	dead/sterilized mortality	day of embryo death	
TREO 0.5	9/33 27.27%	6 blood rings on 2nd day 3 no signs of development	*
TREO 0.25	9/35 25.72%	5 blood rings on 2nd day 4 no signs of development	*
control	5/32 15.65%	4 blood rings on 2nd day 1 no signs of development	*

* - statistically significant differences (P <0.05)

control - embryos that received Aqua pro injectione emulsion in sesame oil

In group II, where eggs were injected with treosulfan into yolk (Tab. 20) 108 embryos were subjected to sterilization, of which 16 embryos did not survive up to 3.5 day of embryonic development - more in experimental groups than in control. Additionally, three eggs proved to be unfertilized. There was a statistically significant relationship between used concentration and the day of embryos death (P < 0.05). Most of blood rings on the second day were observed in group that received a higher concentration of treosulfan. In turn, more deaths on the third day were observed when lower concentration of treosulfan was used.

Tab. 20. Mortality of embryos up to 3.5 days of development - embryos were treated with treosulfan emulsion into egg yolk (Chi-square test).

group	dead/sterilized mortality	day of embryo death	
TREO 0.5	8/36 22.22%	(1 unfertilized) 5 blood rings on 2nd day 2 deaths on 3rd day	*
TREO 0.25	8/36 22.22%	(2 unfertilized) 2 blood rings on 2nd day 4 deaths on 3rd day	*
control	3/36 8.33%	2 blood rings on 2nd day 1 death on 3rd day	*

* - statistically significant differences (P <0.05)

control - embryos that received Aqua pro injectione emulsion in sesame oil

Sterilized 3.5-day-old recipient embryos with normal position (Fot.15. in chapter 6.) were qualified for PGCs$^{PKH26[+]}$ injection, remaining embryos (Fot. 14. in chapter 6.) were rejected due to impossible access. Injected embryos were incubated for further 3 days after which mortality and day of death were determined (on the 6th day of embryonic development - day of fluorescence detection in gonads). In order to check whether there is a relationship between the death of embryo, and used concentration and site of treosulfan administration, Chi-square test was performed. Photos of dead embryos are shown in Table 26. in chapter 6.

In the case of group I - sterilized into subgerminal cavity (Tab. 21), 74 embryos out of 77 live embryos were centrally positioned and were suitable for injection PGCs$^{PKH26[+]}$. Only in group, where 0.5mg/ml concentrated treosulfan was administered, all embryos were properly positioned. Nevertheless, obtained differences were not statistically significant ($P > 0.05$). The highest mortality rate after injection of cells was observed in group TREO 0.5 - 7 embryos out of which 4 died on 3-4 day. In each of experimental and control groups there were two cases of deaths on 5-6 day. However, the Chi-square test did not confirm a statistically significant relationships ($P > 0.05$).

Tab. 21. Effect of treosulfan sterilization into subgerminal cavity and injection of PGCs$^{PKH26[+]}$ on survivability of recipient embryos (Chi-square test).

group	normally positioned/live embryos subjected to injection*	dead/ injected embryos mortality	day of embryo death
TREO 0.5	24/24 100.00%	7/24 29.17%	4 deaths on 3-4 day 1 no signs of development 2 deaths on 5-6 day
TREO 0.25	24/26 92.31%	3/24 12.50%	1 death on 3-4 day 2 deaths on 5-6 day
control	26/27 96.30%	3/26 11.54%	1 death on 3-4 day 2 deaths on 5-6 day

* embryos with incorrect position were removed - impossible to inject

control - embryos that received Aqua pro injectione emulsion in sesame oil

In the case of group II - sterilized into egg yolk (Tab. 22), among 89 embryos, that survived sterilization 4 were improperly positioned and therefore were not qualified to the next step of studies. Similarly to group I, only in group

were higher treosulfan concentration was used, all of embryos were centrally positioned. In this respect there were no statistically significant differences ($P > 0.05$). The highest mortality of embryos after injection of cells was in group TREO 0.5 and it was equal to 6 embryos, out of which 3 died on 5-6 day and 3 did not undertake further growth after injection of $PGCs^{PKH26[+]}$. In control group number of dead embryos was the lowest among other experimental groups and it was equal to 2 individuals, that did not undertake development after injection of $PGCs^{PKH26[+]}$ into bloodstream. However, results proved to be statistically insignificant ($P > 0.05$).

Tab. 22. Effect of treosulfan sterilization into egg yolk and injection of $PGCs^{PKH26[+]}$ on survivability of recipient embryos (Chi-square test).

group	normally positioned/live embryos subjected to injection*	dead/ injected embryos mortality	day of embryo death
TREO 0.5	28/28 100.00%	6/28 21.43%	3 no signs of development 3 deaths on 5-6 day
TREO 0.25	27/28 96.43%	3/27 11.11%	2 deaths on 3-4 day 1 no signs of development
control	30/33 90.91%	2/30 6.67%	2 no signs of development

* embryos with incorrect position were removed - impossible to inject

control - embryos that received Aqua pro injectione emulsion in sesame oil

On the day of fluorescence detection (6th day of incubation) in gonads of Green-legged Partridgelike embryos developmental abnormalities were also determined. The highest number of embryos with pathological lesions was observed in group, in which 0.5mg/ml concentration of treosulfan was administered into egg yolk (4 embryos - 18.18%). Control groups have the lowest percentage of embryos with pathological lesions (2 embryos in group I - 8.70% and 1 in group II - 3.57%). Among groups treated with cytostatic, the lowest number of embryos with developmental abnormalities was noted in group treated into subgerminal cavity and it was equal to 9.52% (2 embryos) (Tab. 23). However, Chi-square test did not confirmed statistically significant differences between groups ($P > 0.05$).

Tab. 23. Developmental abnormalities in 6-day-old recipient embryos after treosulfan sterilization and injection of $PGCs^{PKH26[+]}$ (Chi-square test).

group	normal embryos	embryos with abnormalities
I TREO 0.5	88.24%	11.76%
I TREO 0.25	90.48%	9.52%
I control	91.30%	8.70%
II TREO 0.5	81.82%	18.18%
II TREO 0.25	87.50%	12.50%
II control	96.43%	3.57%

I - embryos treated with cytostatic into subgerminal cavity

II - embryos treated with cytostatic into egg yolk

control - embryos that received Aqua pro injectione emulsion in sesame oil

Percentage of embryos with pathological lesions were grouped in terms of applied treosulfan concentrations. Those results are shown in Figure 20. The highest number of embryos with developmental abnormalities was found in TREO 0.5 group (15.38%), while the lowest in control (5.88%). Group TREO 0.25 had intermediate value (11.11%). In this case, the Chi-square test also did not confirm statistically significant dependences ($P > 0.05$).

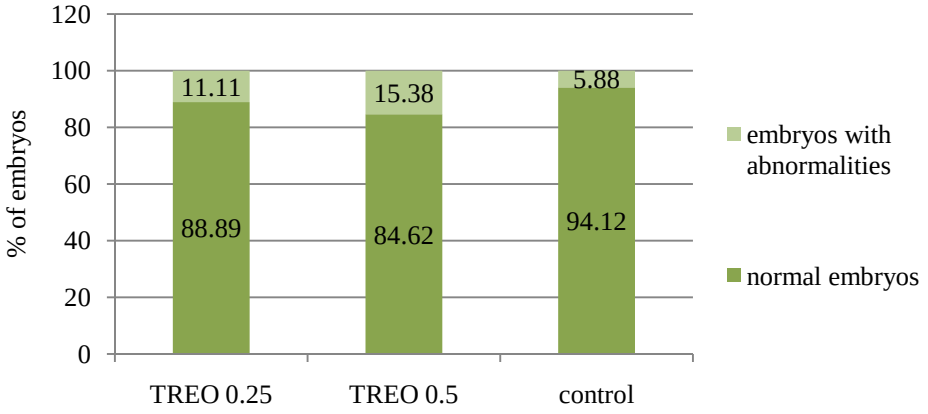


Fig.20. Developmental abnormalities in 6-day-old recipient embryos after sterilization with different treouslfan concentrations and injection of $PGCs^{PKH26[+]}$ (Chi-square test).

control - embryos that received Aqua pro injectione emulsion in sesame oil

Percentage of pathological defects in embryos were grouped also in terms of administration sites of cytostatic. Results were similar, but slightly more embryos with developmental abnormalities were noted in group II, where treosulfan was administrated into egg yolk and were equal to 10.81% (Fig. 21). Obtained differences proved to be statistically insignificant after conducted statistical analysis using Chi-square test ($P > 0.05$).

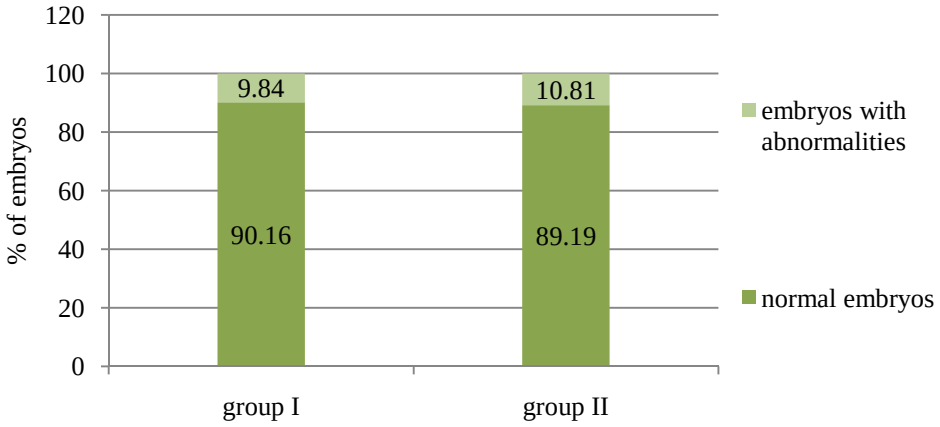


Fig.21. Developmental abnormalities in 6-day-old recipient embryos after treosulfan sterilization into different sites and injection of $PGCs^{PKH26[+]}$ (Chi-square test).

I - embryos treated with cytostatic into subgerminal cavity

II - embryos treated with cytostatic into egg yolk

All embryos with pathomorphological defects were grouped, and then photographed. Their photos are presented in Table 27. in chapter 6.

4. DISCUSSION

New technologies of transgenic birds production have been developed all around the world and they include use of chimeras which, if properly crossbreeded, lead to production of birds - bioreactors (Bednarczyk et al., 2003; Sang, 2006; van de Lavoir et al., 2006;). In this approach genetically modified primordial germ cells, which are carriers of a foreign DNA, are introduced into chicken embryos in order to integrate them with host genome (Speksnijder et al., 1999). Besides, PGCs and avian chimeras may be used for biopreservation of endangered bird species (Naito, 2003) through their artificial generation (introduction of PGCs of endangered bird species into sterilised chicken embryos and as a consequence "production" of these birds by recipients of cells), in animal breeding and meat industry (Song et al.,2005) by changing proportion of sex (preliminary sexing of exogenous PGCs) and also as a tool to understand complex mechanisms of birds embryonic development (Tagami and Kagami, 1998). Unique properties of PGCs speak in favour of the use of PGCs in chimera's production. It is related to behavior of PGCs and their migration pathway from germinal crescent, through bloodstream to gonads which allows for repeatable and effective production of germline chimeras.

Production of avian germline chimeras is significantly hindered by the necessity to become familiar with difficult, time-consuming and labour-intensive methods of identification, isolation and culturing of primordial germ cells, their potential genetic modification/labeling, effective introduction into recipient embryos, securing of optimum conditions for their migration, proliferation and transformation into functional gametes. Only when all these conditions have been meeting and after hutching, rearing and crossbreeding of chimeras, can heritance of PGCs donor genotype (enriched with potential transgene) be expected in the following generations.

No descriptions of similar and extended studies are found in the available literature except for the articles focusing on individual aspects-methods, which have been considered in preparation of this thesis. It can be exemplified by studies on ways of isolation and purification of PGCs (Tajima et al., 1998; Li et al., 2005; Możdziak et al., 2005, Nakajima et al., 2011, Naeemipour and Bassami, 2013b), application of different systems of genotypes in a role of donors and recipients of PGCs (Yasuda et al., 1992; Kagami et al., 1997; Park et al., 2003a and 2003b; Ishiguro et al., 2009; Wernery et al., 2010; Kang et al., 2011; van de Lavoir et al., 2012; Park et al., Han, 2013) or sterilization of recipient embryos (Mohsen and Ahmed, 2002; Song et al., 2005; Motono et al., 2009; Nakamura et al., 2010; Park et al., 2010; Lee et al., 2013).

The studies on behavior of primordial germ cells of donors and recipients, which are presented in this publication, focus on four main tasks. Their findings are discussed in relation to previous studies in this area.

4.1. OPTIMIZATION OF ISOLATION METHOD OF GONADAL PRIMORDIAL GERM CELLS FROM 6-DAY-OLD CHICK EMBRYOS

Primordial germ cells were isolated from gonads of 6-day-old chicken embryos. In order to confirm the character of PGCs, the isolated suspension of cells was stained with peridic acid solution (PAS). Staining with PAS is a standard, simple and cheap method for chicken PGCs identification (Tagami and Kagami, 1998). This method has been widely used for years in studies on chicken PGCs (Meyer, 1964; Yasuda et al., 1992; Kagami et al., 1997; Jung et al., 2005; Nakajima et al., 2011; Naeemipour et al., 2013). It should be emphasized, however, that it is a reaction typical of chicken PGCs. By comparison, quail PGCs cannot be identified with this method since they do not have particles of glycogen (Chang et al., 2010). Morphological analysis of freshly isolated PGCs, which was carried out in own studies, showed presence of big, round cells, 14-19 μm in diameter. It was shown (Fot. 3 and 6), that cytoplasm of these cells contained numerous granules of a storage material in a form of glycogen which are specifically stained with magenta in PAS reaction. Microscopic observation, under a greater magnification, showed presence of a great cellular nucleus, approximately 9 μm in diameter, which was located eccentrically and did not show affinity to PAS stains. Some of PGCs had pseudopodia and irregular shape which was also characteristic of these cells at this stage of development (Kuwana and Rogulska, 1999).

Literature often describes also additional procedures of cleaning the suspension of PGCs (isolated from blood and gonads) from contaminations such as little background particles and other undesirable somatic cells such as morphotic elements of blood. They include Percoll density gradient centrifugation (Oishi 2010), Ficoll density gradient centrifugation (Yasuda et al., 1992; Park et al., 2003b), Nycodenz density gradient centrifugation (Zhao and Kuwana, 2003), lysis of erythrocytes with ACK buffer (ammonium chloride-potassium) (Yamamoto et al., 2007), immunomagnetic separation of cells with MACS (magnetic-activated cell sorting) (Ono and Machida, 1999; Wei et al., 2001; Kim et al., 2004; Kang et al., 2009; Naeemipour and Bassami, 2013) and use of FACS (fluorescence-activated cell sorting) system based on reaction of monoclonal anty-SSEA-1 antibodies (Mozdziak et al., 2005; Motono et al., 2010). FACS and MACS methods are also proved to be effective in purifying of gonadal PGCs. Although, the above mentioned methods are effective because obtained suspension of PGCs is characterized with higher purity and homogeneity, the quoted studies show that a great number of PGCs is irreversibly lost, and those which survive are weakened and show reduced vitality. Therefore, in these studies we resigned from applying the procedures of PGCs purification. However, in order to secure a good quality, gonads were collected to a microtube with PBS solution which was kept on a heating block (37.8 °C) throughout the experiment time.

PGCs, for production of bird chimeras, may be isolated from blastoderm in stage X or from blood of 2.5-3-day-old embryos. However, only very few PGCs can be obtained from these tissues. The highest number of PGCs for biotechnological manipulations can be collected by their isolation from gonads of 5-7-day-old embryos. Therefore, in this study, PGCs were isolated from gonads of 6-day-old chicken embryos. Behavior of primordial germ cells, in terms of their ability to migrate from gonads, was analysed with three different methods. The described methods of PGCs isolation from gonads of 6-day-old chicken embryos were developed on the basis of Nakajima et al., (2011) studies. Authors suggest a new and simple method of collecting highly purified (about 50%) gonadal PGCs with high vitality by incubation of gonads (in a temperature of 37.8°C and 5% CO_2) from 7-day-old embryos in PBS solution deprived of calcium and magnesium ions (PBS[-]). At present, it is the most effective method of PGCs isolation from gonads of chicken embryos because it allows, in a short time, to collect a high number of well-purified and viable PGCs (Nakamura et al., 2013).

In presented here study, this method was expanded by mechanical fragmentation of gonads (A) or their digestion with 0.25% trypsin-EDTA solution (B) and was, in an unchanged form, used as a control group (C). Thus, numerous PGCs were obtained in presented studies. The highest number, more than 198 thousand cells, was obtained in a control group and the lowest number – in a group A (more than 128 thousand cells), in which gonads were mechanically fragmented. Almost 160 thousand cells were released in a group of gonads digested with trypsin-EDTA. A very low number of PGCs were obtained in a group of mechanically fragmented gonads and this can be attributed to sticking the cells together and their adhesion to the cut gonad tissues in a form of big PGCs aggregates. Intense detachment of PGCs from gonads in two other groups and their migration to PBS [-] may indicate a very poor connection of PGCs with somatic cells of gonads in 6-day-old embryos. It is worth emphasizing that a mean number of PGCs in an embryo varied from about 5.3 to about 8.2 x 10^3 PGCs, and was significantly higher than data provided in literature [e.g. Motono et al., (2010) isolated 1.0 x 10^3 PGCs per one embryo and Mozdziak et al., (2006) about 0.7 x 10^3]. This discrepancy, apart from the method of isolation, may also be related to the quality of embryos, their origin, breed and type of use. However, in the first place, it depends on the degree of gonad development. Available studies are very diverse and ununified in this aspect. Primordial germ cells are usually collected from gonads in 27-28 H&H stadium of development which can be translated to 5-5.5 day of incubation (Chang et al., 1997; Han et al., 2002). In the studies, which are presented in this thesis, eggs were incubated for 6 days which contributed to a higher number of PGCs in gonads, since they had more time for proliferation. The mentioned heating block which was used during time-consuming isolation of gonads from embryos might also have supported the continued proliferation of PGCs.

Li et al., (2005) showed that the highest number of PGCs can be obtained after their isolation from stage 28 (according to H&H). This isolation must be combined with digestion of the tissues with 0.25% trypsin-EDTA solution in order to obtain a suspension of single PGCs, which are free from aggregates and other somatic cells which form gonads. Trypsin-EDTA solution enables releasing of PGCs which are entrapped in a dense tissue of gonads. Nevertheless, digestion time cannot be too long because PGCs may become partially digested or too short because not all PGCs will be released from tissues. After considering the results of Chojnacka-Puchta et al., (2015) and Sawicka et al., (2015), time of digestion with trypsin-EDTA was set for 60 seconds. Survivability of PGCs digested with trypsin-EDTA (about 72%) in own study significantly differs from the result provided by Li et al., (2005) (about 90%). This difference may be related to a one-hour incubation of cells in CO_2 chamber and PBS solution deprived of calcium and magnesium ions which was performed after isolation of cells. Storing of cells for one hour without any medium or nutritional compounds might have contributed to a higher mortality of PGCs. It seems to be confirmed by similar results of PGCs survivability in a case of cells in mechanically fragmented (about 73%) and untouched (a control) group (about 76%).

Behavior of gonadal PGCs was evaluated also in reference to the capacity of PGCs to adhere to each other i.e. to form aggregates. Controlling of PGCs aggregation is one of the key factors which are essential to maintain high number of cells which can be submitted to further manipulations (Yu et al., 2011). After leaving gonads and under *in vitro* conditions, PGCs start rapidly to form cellular aggregates (Di Carlo and De Felici, 2000). It is caused by presence of cell adhesion molecules (CAMs) on the surface of PGCs, which act like a specific factor which sticks adjacent cells together (Karagenc and Petitte, 2000). The available literature, however, does not provide any information on the relationship between a method of PGCs isolation from gonads and formation of cellular aggregates. A method of partial digestion with trypsin-EDTA, was the only one which allowed preventing formation of such conglomerates in all three repetitions of the experiment which may suggest inactivating properties of trypsin-EDTA on CAMs. It is also suspected that in other groups (A and C), a high number of gonadal somatic cells, which were not submitted to digestion, made PGCs stick together.

4.2. EFFECT OF DONOR AND RECIPIENT COMBINATION ON DETECTION OF EXOGENOUS PRIMORDIAL GERM CELLS IN GONADS OF RECIPIENT EMBRYOS

Migration of PGCs transferred within chickens of the same breeds/types cannot be so far measured. The reason behind it is that a specific and easy-to-detect genetic marker allowing for differentiation between cells of donors and recipients has not been discovered yet (Motono et al., 2010). Preliminary

modification of cells with a marker gene – green fluorescent protein (GFP) is the only way to identify this gene in recipient embryos (Chojnacka-Puchta et al., 2015). It is, however, a difficult, time-consuming and expensive procedure. Therefore, it has been decided to label PGCs with PKH26 fluorochrome dye in the described study.

The PKH26 dye is a widely used marker of gonadal and circulating PGCs in studies on formation of bird chimeras (Jeong and Han, 2002; Park et al., 2003a; Minematsu et al., 2004; Yamamoto et al., 2007; Kang et al., 2008; Kang et al., 2009; Jung et al., 2010; Nakamura et al., 2010; Kang et al., 2011; Nakajima et al., 2011; Miyahara et al., 2014). It binds, irreversibly, to a cellular membrane of labeled PGCs which allows to follow the migration of exogenous cells and sites of their settlement in recipient gonads. Phototoxicity of PKH26 was evaluated on a human line of hematopoietic cells (Oh et al., 1999). It was shown then that the very fluorochrome does not adversely influence the viability and development of cells. It is the exposure to fluorescent light of the microscope which dramatically reduces these two parameters.

The cells which were isolated in presented here study were either labeled or unlabeled with PKH26 fluorochrome and placed on OPTI-MEM® I growth medium in order to assess behavior of PGCs in a short-term *in vitro* culture. Earlier it was shown that PGCs which were isolated from 5-day-old chicken embryos were able to proliferate *in vitro* even during the first two days of a primary culture (Allioli et al., 1994) and grow 3.8 times by the fourth day of culture (Chang et al., 1995). Besides, it was shown that even short-term *in vitro* maintenance of PGCs may increase the efficiency of germinal transmission (Park et al., 2003b). In own study, the somatic cells, a few hours after placing a culture plate, started to adhere to the surface of well bottom and constitute a fibroblast-like layer on which non-adherent PGCs gathered. After the first day of culture, PGCs started to form agglomerates consisting of a few or several cells and adhere to a fibroblast layer. On the third day of culture in OPTI-MEM® I medium, cells suddenly started to shrink and their cytoplasm was becoming darker. The $PGCs^{PKH26[+]}$ and $PGCs^{PKH26[-]}$ cells died probably due to lack of nutrients, growth factors or other supplements which were essential for growth.

It can be also explained by unavoidable physical and physicochemical injuries which were sustained during isolation of PGCs and during further procedures, which in consequence lead to death of these cells. Reduction of the overall population in a short-term culture may be contributed, as it was already mentioned, to lack or growth factors in a medium which resulted in death of cells. Li et al., (2005), came to the same conclusions. The researcher used TCM-199 medium with 10% calf serum in his short-term culture of gonadal PGCs and reported a reduction of vitality from 80.5% to 7.9% of freshly isolated PGCs after a 72-hour *in vitro* culture.

In presented study, a 17.6% increase of survivability of PGCs labeled with PKH26 was reported as well as 21.6% increase of survivability of PGCs which

were not labeled with fluorochrome, between the first and the third day of *in vitro* culture of PGCs. However, an overall number of PGCs significantly decreased within those 3 days. This reduction is even more significant in case of cells labeled with fluorochrome. Their number decreased by 46.75% on the third day when compared to the first day. A slightly lower mortality (39.4%) was observed in a group of unlabeled PGCs. Lower survivability of $PGCs^{PKH26[+]}$ vs. $PGCs^{PKH26[-]}$ may be, thus, consistent with the above mentioned phototoxic effect of fluorochrome. The labeled cells were submitted to UV radiation under the microscope during their counting and observation on culture plate. Then, the phototoxic effect of PKH26 and increased mortality of PGCs might have taken place.

Kim et al., (2005) came to interesting conclusions. The researcher analyzed behavior of quail PGCs which were recently isolated from a 5-day-old embryos and submitted to a 3-day *in vitro* culture in medium which had been optimized earlier for chicken PGCs. The uncultured PGCs were proved to be more effective in terms of hatchability, proportion of chimeric chickens and a degree of germinal transmission when compared to PGCs which were cultured for 3 days. Given that and taking into consideration a reduced survivability of PGCs on the third day of culture, in presented here study we resigned from studying of the impact of a short-term *in vitro* culture on migration abilities of already weakened PGCs by their re-injection into the recipient bloodstream.

Available literature provides also information on behavior of PGCs in long-term *in vitro* cultures (Kuwana et al., 1996; Macdonald et al., 2010; Naito et al., 2010; Miyahara et al., 2014) which however, were based on much more complex media than the medium which was used in own study. The goal was to sustain steady growth and proliferation of PGCs without adversely affecting migration and biological activity. Observations of the migration capacity of PGCs from long-term culture *in vitro* are contradictory. Han et al., (2002) believes that PGCs retain their migration capacity, biological activity and germinal transmission potential even after a 2-month *in vitro* culture. Besides, they do not lose their potential for expression of pluripotency markers and integrations with the recipient gonads (Shiue et al., 2009). Park et al., (2003b) also believes that a proper *in vitro* preparation of PGCs before their introduction into the recipient embryos should enhance germinal transmission in chimeras. On the other hand, Miyahara et al., (2014), believes that a long-term culture of PGCs reduces their migration capacity which translates into a reduced number of exogenous PGCs in gonads when compared to freshly isolated PGCs which are introduced into recipient embryos. It is known, however, that PGCs retain their capacity to migrate in a bloodstream of recipient embryos, to colonize their gonads and to form functional gametes even after freezing them in a liquid nitrogen (Tajima et al., 1998) or purifying in Ficoll (Jeong et al., 2002) or Percoll (Chojnacka-Puchta et al., 2015) gradient.

The following step of this study involved assessment of behavior of chicken primordial germ cells in terms of the migration capacity of donor PGCs

in a recipient organism. On the basis of proportion of embryos in which visible and fluorochrome labeled PGCs were detected, the most effective combination of genotypes - Ross 308 → Green-legged Partridgelike was selected. In theory, this result might have been affected by many factors. In presented study, factors which might have been important included proliferative potential (which was genetically conditioned) and migration capacity of donor PGCs and ability of recipients to incorporate exogenous PGCs which may differ between breeds that were used in own study. It can be also explained by variable and breed-related activity of enzymes and other substances involved in PGCs migration, as well as adjustment of donor and recipient developmental stages.

Obtained results of own study related to the donors and recipients combination might have been also affected by breed-dependent proliferative potential of exogenous PGCs which settled in the recipient gonads and competitiveness with endogenous PGCs for space in gonads. No information can be found in the literature on how PGCs of Green-legged Partridgelike and Ross 308 could behave in relation to the above. The team of Nakamura (2011) used PGCs originating from three distinct chicken breeds: White Leghorn, Barred Plymouth Rock and Fayoumi. By introducing the same number of cells into the bloodstream of recipient embryos they proved that the number of exogenous PGCs that settle in the gonads of 6-day-old recipient embryos is statistically different between the three chicken breeds that were examined. Those differences affected the later ratio between donor- and recipient-derived germ cells. It is therefore known that the proliferation potential of PGCs in gonads *in vivo* can differ between chicken breeds. The ability of PGCs to proliferate can also differ in *in vitro* conditions (Miyahara et al., 2014) – PGCs isolated from Barred Plymouth Rock embryos achieved a higher degree of proliferation than PGCs isolated from White Leghorn. However, in own study, there was no statistically significant correlation between *in vitro* proliferation of PGCs isolated from Ross 308 and Green-legged Partridgelike embryos. This does not exclude such correlation in *in vivo* conditions or in precisely adjusted growth medium.

The size of PGCs population can differ depending on species and type of donor, and even between different poultry flocks. However, in our experiments no statistically significant differences in the number of cells obtained from Green-legged Partridgelike or Ross 308 embryos were found (3.788×10^6 vs 3.33×10^6, respectively). No publications that would compare the number of PGCs between chicken breeds were found in the available literature, but there are single reports about breeds matching of donors and recipients of these cells. Naito and coworkers (1994) proved that the efficiency of germinal transmission can be increased even 3.5-fold if the breeds of donors and recipients are properly matched. In their experiments, better results were obtained taking White Leghorn as donor and Barred Plymouth Rock as recipient of PGCs than in reversed combination. Also other authors (Ono et al., 1998a and b; Park et al., 2003b) indicate that a proper combination of breeds used as donors and

recipients can influence improvement or deterioration of degree of chimerism and of germinal transmission. Furthermore, the efficiency can also be changed by combining bird lines within one breed (Nakamura et al., 2010). The above studies, as well as our findings prove that the problem of low number of exogenous PGCs integrated with recipient embryos can be solved by proper matching of donor and recipient genotypes.

Similar conclusions were drawn in this study. The combination of Ross 308 as PGCs donor and Green-legged Partridgelike as recipient turned out to be more than 16.5-fold more effective (54.5% vs 3.3%) than the reversed combination. Homogenous combinations also yielded significant differences. Green-legged Partridgelike chickens were characterized in this respect by a higher number of embryos in which exogenous PGCs were detected in recipient gonads (19%), while in the group of Ross 308 only 8.3% chimeric embryos were obtained.

In case of chickens asymmetric migration of PGCs is observed – more cells migrate in the direction of the left gonad than of the right one (Ono et al., 1996; Nakamura et al., 2007), which in consequence can result in a higher number of proliferating PGCs in the left gonad (Nakajima et al., 2011). In our experiments, the number of exogenous PGCs in individual gonads was not measured; a further optimization in this direction is therefore needed.

The decrease of migratory potential of germ cells in various genotype combinations can also result from differences in their chemical composition between chicken breeds and types. In this work, no studies comparing the chemical composition of PGCs isolated from Ross 308 or Green-legged Partridgelike were conducted. It is nevertheless known that in the migration of PGCs towards genital ridges are involved such substances as integrins, CXCR4 (Stealer et al., 2004; Kunwar et al., 2006; Motono et al., 2008), extracellular matrix molecules, such as laminins, fibronectins, chondroitin sulphate, collagen type IV (Uren et al., 1989) and tenascin C (Angstrom and Tucker, 1996). It is supposed that the expression levels of those substances differ between Ross 308 and Green-legged Partridgelike breeds.

The results obtained in this study can also be explained by asynchrony of development level of donors and recipients of PGCs that are belonging to different breeds. Although our studies used PGCs derived from 6-day-old embryos that were introduced into 3.5-day-old embryos, the development degree of Ross 308 embryos was substantially different from Green-legged Partridgelike. This could influence the behavior of PGCs. The later, as an old, native breed with little genetic modifications, is characterized by a slower embryonic development than Ross 308 chicken that were under a strong selection pressure during many years (Buzała et al., 2015). In other words, we observed in our experiments that 3.5-day-old and 6-day-old Ross 308 embryos are bigger and more developed than 3.5-day-old and 6-day-old Green-legged Partridgelike embryos. This anatomical and physiological difference could have an effect on the results. More precisely, in stage 13-15 (H&H), PGCs leave the

heart of developing embryo and continue their migration in direction of genital ridges. During these stages, the ventral aorta develops first cranially and later caudally (Bernardo et al., 2012). In relation with the above, when exogenous PGCs microinjection will be conducted before the abovementioned blood vessel will develop or when it will be impossible to insert the needle in the abdominal branch of aorta, the cells can get trapped in the network of head capillaries– this could have happened in own study. It seems that such possibility can be confirmed by the studies conducted by Nakamura and coworkers (1991), where PGCs accumulate close to the head in embryos deprived of abdominal part. It was shown that 8.7% to 20% of total PGCs population accumulate in head region of embryo as so called extragonadal PGCs and it is a natural phenomenon (Nakamura et al., 1988; Yasuda et al., 1992; Kuwana, 1993; Ishiguro et al., 2009). It therefore seems that the efficiency of germline chimera production obtained in the study is influenced by the level of synchronization of donors and recipients belonging to different breeds and the ratio and localization of donor PGCs migrating towards recipient embryo gonads. It is interesting that gonadal PGCs have a lower potential to settle in the gonads after introduction to recipient embryo that PGCs isolated from blood (Naito et al., 1994; Han et al., 2002; Park et al., 2003b).

In our study, we have resigned from the isolation of PGCs from blood, because it is a much more difficult and labor-intensive method that yields a low number of PGCs. However, when PGCs are isolated from gonads of 5-7-day-old embryos and introduced into the bloodstream of 2.5-3.5-day-old embryos, the germinal transmission rate of gametes originating from donor of cells reaches less than 28%. The same indicator reaches much higher values (nearly 98%) when donor PGCs are at the same developmental stage as recipients (Naito et al., 2007). Minematsu and coworkers (2004) evaluated the migration abilities of PGCs isolated from chicken embryos at different developmental stages using two indicators – percent of recipient embryos with fluorescently labeled with PKH26 PGCs present in gonads and the total number of labeled PGCs in these gonads. The first of mentioned indicators was 100% when PGCs derived from 6.5- and 10.5-day-old embryos were used as donors, while the other showed lower values when the cells were isolated from 14.5-day-old and older embryos. However, in spite of decreasing (together with subsequent developmental stages) ability of exogenous PGCs to settle in recipient gonads, it is not entirely hampered even in case of PGCs obtained from 20-day-old donors (Naito et al., 2007). The potential to differentiate into gametes is not deteriorated even if PGCs are isolated from chicken embryos at different developmental stages (Jung et al., 2010). The presented here study confirms the above. In the combination R → ZK (most advantageous), cells were isolated from 6-day-old Ross 308 embryos and introduced into 3.5-day-old Green-legged Partridgelike embryos. It is supposed that recipients were in the most advantageous (out of tested combinations) developmental stage to incorporate exogenous PGCs. In the reversed combination (ZK → R), the recipients were

already "too old" to ensure optimal settlement of cells in the gonads, as they are characterized by a faster embryonic development.

It is unlikely that factors such as sex combination of donors and recipients, number of injected PGCs, interference in PGCs structure and their preparation for injection could influence the % of obtained chimeric embryos, as those elements were constant during the study and did not introduce differentiation between the groups.

According to literature data, sex combination of donors and recipients is one of the factors that may influence the efficiency of exogenous PGCs settlement into recipient gonads. The efficiency of bird germline chimera production and degree of germline transmission are higher when the donor and the recipient are the same sex (Tagami et al., 1997; Naito et al., 1999; Macdonald et al., 2010; Yamaguchi et al., 2000; Kang et al., 2009; Park and Han, 2013). In presented here study, the cells used to create chimeric embryos were derived from both sexes, i.e. they were a mix of males and females. Therefore, if sex-specific differences of chicken PGSs had an influence on the results, they would affect all combinations with a similar degree.

Another factor that was constant in experiment and had no influence on results was the number of injected PGCs. This factor is considered as one of the most important elements influencing the percent of chimerism. Injection of a large number of exogenous PGCs into recipient embryos increases the number of PGCs migrating in the direction of genital ridges (Kim et al., 2010). On the other hand, it is supposed that a large number of injected PGCs can have a negative effect on their migration abilities (Motono et al., 2010), and in consequence lead to lower percent of chimerism. The number of PGCs introduced to recipient embryos is very variable among literature data – from 50 (Nakajima et al., 2011), via 900 (Kim et al., 2010) and up to 3600-4000 PGCs/embryo (Kang et al., 2008). In our study, the number of injected PGCs per one embryo was the same for all four donor/recipient combinations and was equal to around 1000 cells, which also resulted in chimeric embryos. These findings support the assumption that a high number of injected PGCs have no influence on decreasing migration abilities of PGCs.

PGCs treatment in all tested genotype combinations was the same, so interference into PGCs structure and *in vitro* management did not influence the results obtained in this study. Manipulations, such as fluorochrome labeling used in this work (that was proved to be able to decline PGCs) or genetic modifications (Kim et al., 2010) can impair migration abilities of PGCs, which will cause their exclusion from their migration through bloodstream toward gonads. Of manipulated PGCs, only those with high degree of proliferation can migrate into the genital ridges. However, a lot of fluorochrom labeled PGCs cells were settled in gonads, which suggests that cells derived from donor maintained high biological activity.

In our study, we also investigated how the interference into bloodstream of recipient embryos by PGCs injection and the presence of a drilled window in

the eggshell influence the survival of recipient embryos. In order to prevent embryo death caused by infection, we closed the windows drilled in the eggshell with sterile parafilm (size 2x2 cm). A significant difference (7.16%) was subsequently obtained in survival rate of 6-day-old embryos subjected or not to injection with fluorochrom labeled PGCs. The survivability obtained in this study differ significantly from those obtained by Zing and coworkers (2013). These authors compared among others the hatchability of chickens from eggs with drilled window and of chicken additionally injected with transfected exogenous cells into blastoderm. A significant difference in chicken hatchability was shown (35% and 5.21%, respectively). The decreasing embryo survival rate can therefore suggest that both injection of exogenous PGCs and the drilled window itself influence embryonic development. In presented here study, the stage of hatching was not reached, nevertheless the significantly higher embryo survival on the 6th day of incubation as compared with the abovementioned studies (91.15% for injected embryos and 98.31% for non-injected embryos, but with drilled windows) seem to point out to a negative influence of the interference into bloodstream rather than of the drilled window. Other authors report that place of injection has an influence on survival of manipulated embryos (Han et al., 1996; Bednarczyk et al., 2000). In our studies, we chose to drill the egg on the blunt end. Han and coworkers (1996) tested three injection localizations – blunt end, sharp end and side of egg – the first method proved to be the most efficient, and the preserved and not disturbed air chamber as well as double parafilm layer were to ensure the correct development of embryo and survival of 81% on the 5th day (Chang et al., 1997). Opposite conclusions were reached by Bednarczyk and coworkers (2000), they proved a higher hatching level in case of drilling in blunt rather than sharp end (41 and 9.8%, respectively). High sterility of the procedure is a more important factor than the place of drilling. In our study, high sterility was achieved due to the use of a laminar flow chamber, disinfection of the eggshell with 75% ethanol, washing of the drilled surface with antibiotic solution and use of UV-sterilized parafilm.

4.3. INJECTION OF EXOGENOUS PRIMORDIAL GERM CELLS PRECEDED BY STERILIZATION OF RECIPIENTS AND ITS EFFECT ON SURVIVALBILITY AND DEVELOPMENT OF RECIPIENT EMBRYOS

4.3.1. Optimization of treosulfan concentration injected into egg

The aim of this step was to propose a novel efficient sterilization method of recipient embryos, permitting better conditions for competition of exogenous PGCs. Embryo sterilization procedure is conducted in order to deprive them of endogenous PGC. It was shown that PGCs removal from blastoderm stage X led to lower host PGCs number and increased germinal transmission after the

transfer of donor blastodermal cells (Kagami et al., 1997). Removal of PGCs circulating in the blood immediately before introducing "new" cells led to a minor increase of germinal transmission level. This is due to the low amount of blood drawn from the embryo (around one third) and leaving of a large number of unaffected endogenous PGCs (Naito et al., 1994). UV-irradiation of embryos leads to a decrease of the number of endogenous PGCs to less than 30% of cells in the non-sterilized control group (Aige-Gil and Simkiss, 1991a) and increases mortality and incidence of developmental abnormalities of embryos.

Similar effects are obtained with γ radiation. It has to be noted that the results vary according to radiation dose (Park et al., 2010). Irradiation of chicken and quail eggs with different strengths of X-rays gives similar results (Li et al., 2001; Atsumi et al., 2008; Nakamura et al., 2012). It was proven that micromagnetic waves inhibit mitotic activity of endogenous PGCs resulting in increased proliferation of exogenous PGCs in the gonads of chimeric embryos (Atsumi et al., 2009). This results in increased ratio of introduced PGCs to recipient PGCs and in increased germinal transmission level (Nakamura et al., 2012).

The degree of germline chimerism and transmission rate can be increased by sterilizing recipient embryos using cytostatic chemical substances (Nakamura et al., 2010). Regarding this aspect, only busulfan (Song et al., 2005) and tamoxifen (Mohsen and Ahmed, 2002) has been tested. Busulfan sterilization efficiency is high, but very variable due to difficulties in administration of a constant and reproducible dose to the embryo. Moreover, residual busulfan remaining in the embryo can lead to apoptosis of exogenous PGCs (Nakamura et al., 2013). Other studies have shown that busulfan administration to the embryo in early developmental stages will block the elimination of exogenous PGCs, allowing normal development of the embryo (Nakamura et al., 2009; Tagami and Nakamura, 2011). Tamoxifen is another cytostatic substance used to sterilize chicken embryos (Mohsen and Ahmed, 2002). It is an anti-estrogen drug with reduced capacity to cause serious side effects. When administered to 1-day-old chicken embryos, it slightly inhibited the migration of PGCs from germinal crescent region, leading to a 16% decrease in the PGCs population in gonads of 10-day-old embryos as compared with control group.

In our study, we resigned from the above methods due to high costs of equipment for radiation-based sterilization, technical and manual difficulties related with surgical removal of PGCs, high toxicity of the abovementioned chemical substances, low busulfan solubility, low embryo survival and numerous developmental abnormalities occurring after applying all listed sterilization methods. We therefore tried to set up a new chemosterilization method of chicken embryos using a chemical substance called treosulfan.

Treosulfan used in this study is a structural analog of busulfan. Even though both of those compounds show alkalizing properties, they differ in the mechanism of action and cytotoxic activity. We did not found in the literature

any examples of the use of this cytostatic to eliminate endogenous PGCs. Its choice came from previous information about its use in human medicine for elimination of slowly and fast proliferating cells (Krzemieniecki and Zygulska, 2011). We decided to verify its cytotoxic activity relative to the behavior of chicken primordial germ cells and, at the same time, examine the survival rate of embryos after treosulfan application.

When analyzing the results of embryo survival six days after treosulfan administration, a clear correlation can be observed between increasing treosulfan concentration and increased embryo mortality. Administration of the cytostatic at a concentration of 2 mg/ml increased the number of dead embryos from 3 to 6.88 times, as compared with the control group (Aqua pro injectione). Administration of treosulfan at a concentration of 1 mg/ml caused a 2.25- to 3.44-fold decrease of embryo survivability. The groups TREO 0.5 and TREO 0.25 gave results that were most comparable with literature data (Nakamura et al., 2010; Lee et al., 2013). Embryo mortality in the TREO 0.5 and TREO 0.25 group was respectively 1.2- and 1.57-fold higher than in the control group. Only in the case of TREO 0.25 injected into subgerminal cavity, a slight 1.2-fold increase of survival was observed, as compared with the control. In the group injected into yolk with the same treosulfan dilution, we obtained a slight 1.1-fold increase of mortality compared with the control group. In other studies, in case of chicken embryos treated with 100μg busulfan previously emulsified or not emulsified with 1% polyglycerol polyricinoleate, the survival rate on the seventh day of incubation was ranged from 68.2% (1.4-fold decrease as compared with control embryos) to 77.65% (1.2-fold more than in control group), resulting in hatching from 62% (Lee et al., 2013) to 36.4% (Nakamura et al., 2010). To compare, Mohsen and Ahmed, (2002) showed that the mortality of embryos treated with tamoxifen was from 1.05- to 1.31-fold higher than in the control group. It is therefore supposed than residual treosulfan at a concentration higher than 0.25 mg/ml present in the egg is not undergoing fast inactivation and can negatively influence embryo survival. The influence of sesame oil is also unknown. Further analyses that would confirm this supposition are therefore required.

In own study, a strong correlation is observed between the concentration of treosulfan and the decreasing number of surviving PGCs. This result indicates that a constant and reproducible amount of the cytostatic was introduced into subgerminal cavity. It is possible thanks to complete solubilization of the substance, which is not easy in the case of busulfan (Aige-Gil and Simkiss, 1991b). A correlation between the site of injection and the number of endogenous PGCs was also observed. In the presented study, treosulfan was dissolved in a nontoxic injection medium - Aqua pro injectione, and emulsified using sesame oil. This oil worked as a transporter for the cytostatic - when introduced into the yolk, which is characterized by a higher density, it moved upward in the direction of the blastoderm. Direct injection into subgerminal cavity and faster (than in case of injection into yolk) contact of treosulfan

emulsion with the small PGCs population in the blastoderm eliminated more PGCs than in the other group where the cytostatic had to move through the yolk in order to finally reach (via density difference) the PGCs located inside the blastoderm.

It was calculated that sterilization with treosulfan led to 48.5 % - 96.4% lower number of PGCs as compared with control group. Song and coworkers (2005) report that embryos treated with busulfan had 63.4 - 83.7% less PGCs than the non-sterilized group. The embryos of Nakamura and coworkers (2010) had 99.4% less PGCs compared with control. In case of tamoxifen, the embryos have around 16% less PGCs (Mohsen and Ahmed, 2002). Further studies on the influence of treosulfan on somatic cells of the blastoderm are nevertheless required.

4.3.2. Sterilization of recipient embryos followed up injection of primordial germ cells and their effect on survivability of embryos

In the last part of this study, we examined the behavior of fluorescently labeled primordial germ cells of the donor in sterilized gonads of recipient embryos. Interestingly, the number of embryos, with visible exogenous $PGCs^{PKH26[+]}$, that underwent sterilization was lower (by 14.5% - 32.28%) than in the case of embryos from second step, where even 54.5% chimeric embryos were obtained (Table 8). Even in the control group, not treated with treosulfan, a lower (by 7.62% - 21.17%) number of embryos with incorporated PKH26 positive PGCs as compared with second step was observed. No statistically significant differences between the site of treosulfan introduction as well as its concentration and the number of embryos with exogenous $PGCs^{PKH26[+]}$ present in gonads were shown. It indicates that at the moment of exogenous PGCs injection, the cytostatic did not have any lethal influence on cells introduced in the third day. Such result permitted to rebuild PGCs population by exogenous cells and can yield in future a high germinal transmission rate. In the study conducted by Nakamura et al., (2010) test crossing of chicken treated with busulfan showed that exogenous PGCs got successfully incorporated into sterilized gonads of recipient embryos and that busulfan had no cytotoxic effect on exogenous PGCs. The current study did not take into consideration the influence of treosulfan on the degree of germline transmission, as it would require conducting hatching of germline chimeras, maintaining them until sexual maturity and test crossings, which would significantly prolong the duration of experiment. Nevertheless, such studies are planned in future.

Increasing doses of treosulfan injected into subgerminal cavity or into yolk of fertilized chicken eggs had an inhibitory influence on embryos development, which was manifested by a higher number of mortality during the first 24 hours, higher number of blood rings on the second day and third day as compared with the control group that was not treated with cytostatic. More cases of embryo death in the first three days of development were observed in group injected

into subgerminal cavity (23 cases) than in group II (16 cases). It can also be explained by the fact that interference into integrity of blastoderm through mechanical damage by microcapillary can be fatal for the delicate blastoderm and as a consequence lead to embryo death on current or later stages of development. Yolk puncture in case of group II also turns out to have a negative influence on embryo survival rate in first three days of development. Fluorochrom labeled PGCs injection was an additional treatment on the third day of embryonic life so it can also contribute to a slight increase of embryo mortality, but it was not confirmed statistically. It is therefore concluded that administration of increasing cytostatic concentrations and not the injection of exogenous PGCs leads to increased embryo mortality in above case.

In the sixth day of embryonic life, the morphology of embryos was evaluated with respect to incidence of developmental pathologies. Most abnormalities were related with incorrect morphology of the eyeball, lack of beak and total body deformation, which is similar to studies reporting on tamoxifen and busulfan (Mohsen and Ahmed, 2002; Song et al., 2005; Yu et al., 2012). In Mohsen and Ahmed (2002) studies, it was reported that doses of 200 and 400μg of tamoxifen could be used to chemosterilization of chicken embryos without influencing to a significant degree their mortality and the incidence of developmental anomalies (0.07% cases), which are very common in case of busulfan injection. In own study the percent of embryos with developmental anomalies in the control group was much higher than in the case of tamoxifen (5.88%) and nearly 10% lower than in the case of 0.5 mg/ml concentration of treosulfan. Although the group that was treated with treosulfan at concentration of 0.25 mg/ml was characterized by intermediate values with respect to percent of embryos with morphological abnormalities, no statistically significant correlation between the cytostatic concentration and percent of developmental abnormalities in embryos was observed. It is also supposed that the sesame oil used as carrier for treosulfan solution could have contributed to observed anomalies. However, further studies in this direction are required.

5. CONCLUSIONS

1. Behavior of primordial germ cells depends on: the isolation method, combination of cell donor/recipient, cell labeling and embryo sterilization method.

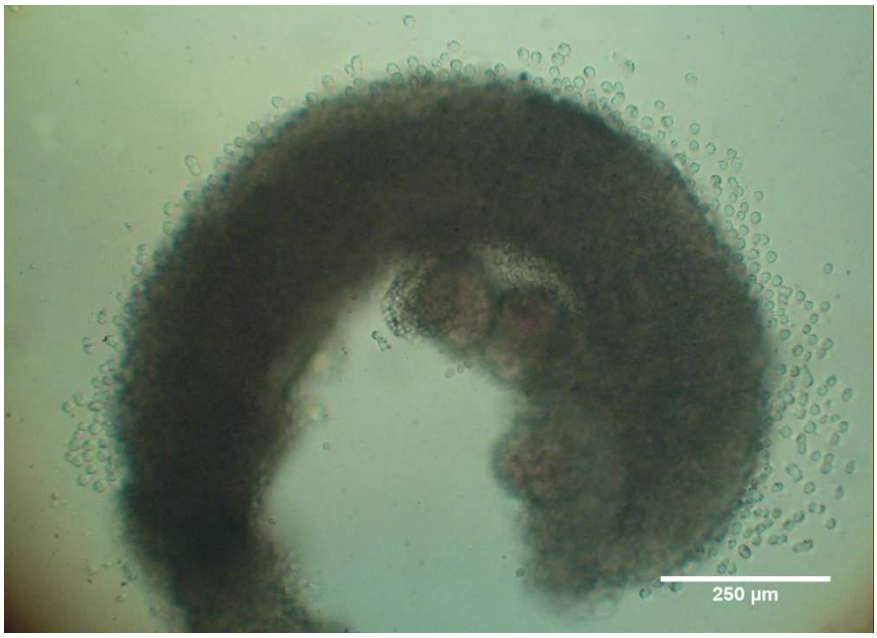
2. The highest number of morphologically normal primordial germ cells (114 000; $P < 0.05$) that did not form aggregates was obtained by incubating gonads digested with 0.25% trypsin-EDTA in a PBS solution without calcium and magnesium ions.

3. The highest percent (54.5; $P < 0.01$) of embryos with exogenous PKH26 labeled primordial germ cells were present in the group Ross 308 → Green-legged Partridgelike, as cell donor → recipient, respectively.

4. Cytostatic treosulfan can be used for chemical sterilization of chicken embryos, especially when administrated into subgerminal cavity. With its increasing concentration (from 2 to 0.25 mg/ml), there is an increased elimination of endogenous primordial germ cells (from 48.5% to 96.4%), as compared with control group and a decrease of recipient embryo survival rate. The percent of embryos with exogenous donor cells does not change.

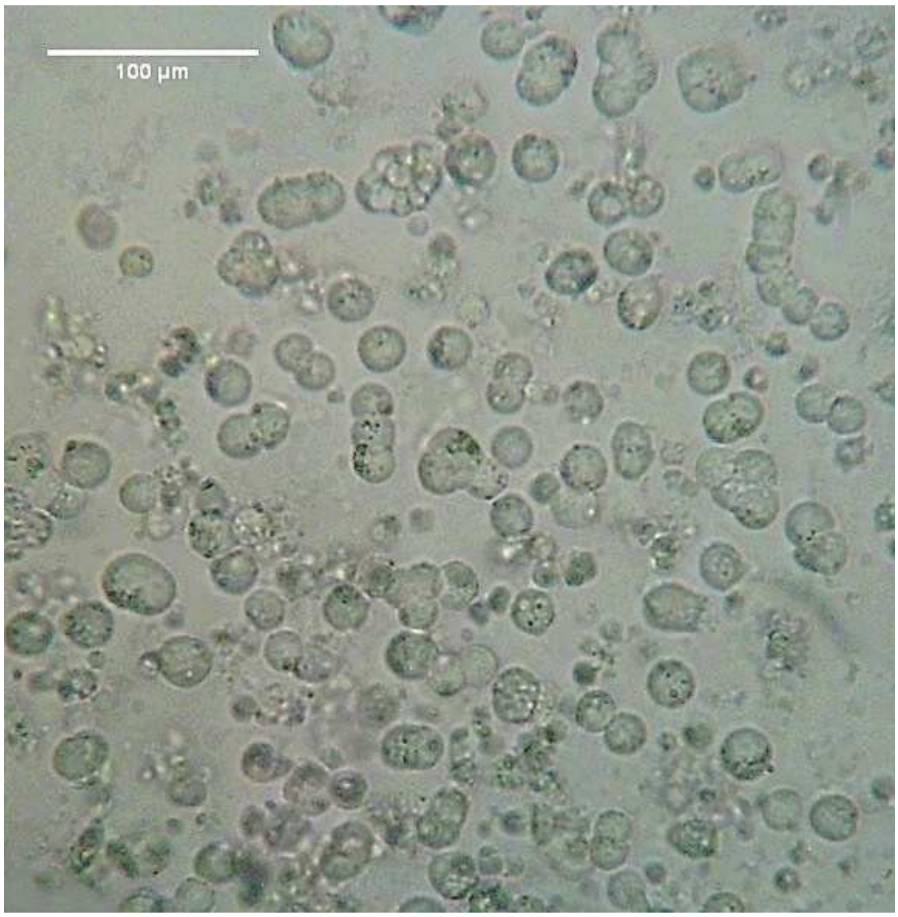
5. Cytostatic treosulfan does not significantly influence the incidence of developmental abnormalities in treated embryos.

6. PHOTOS

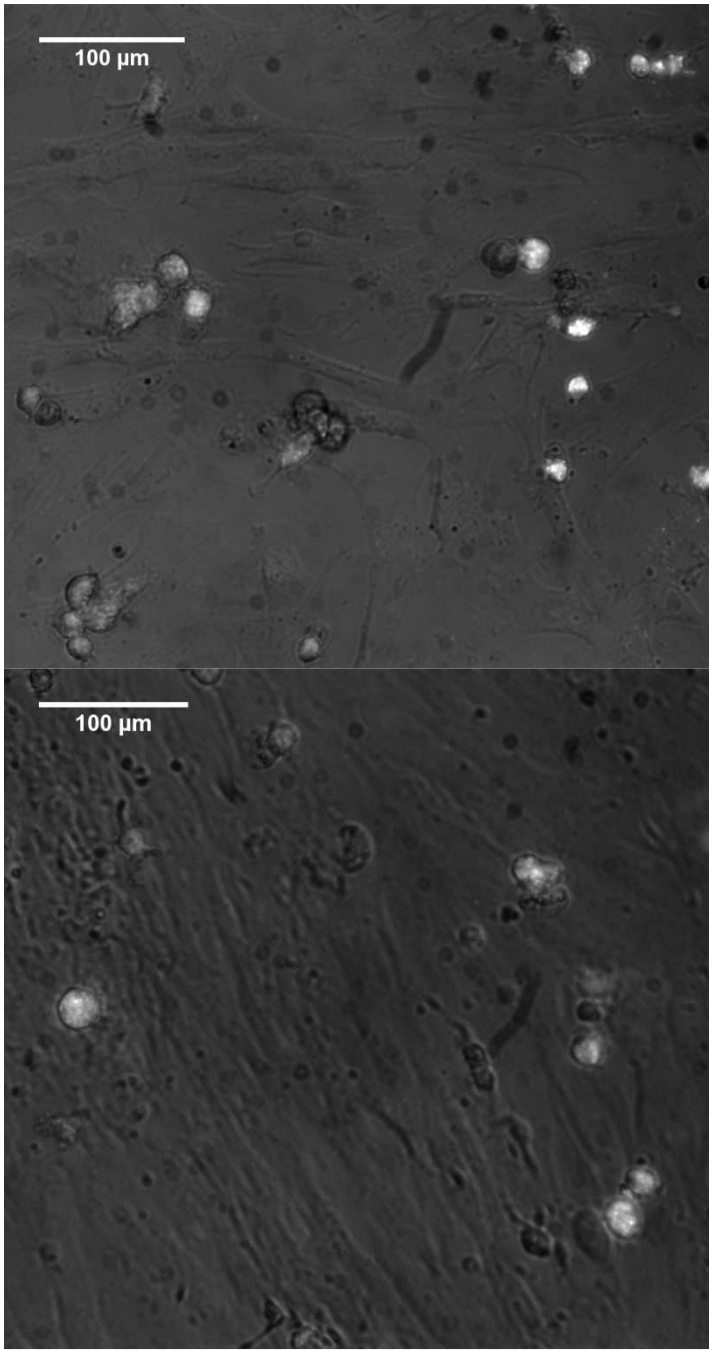


Fot.8. Discharge of primordial germ cells from embryonic gonads (control group); Axiovert 40 CFL Zeiss.

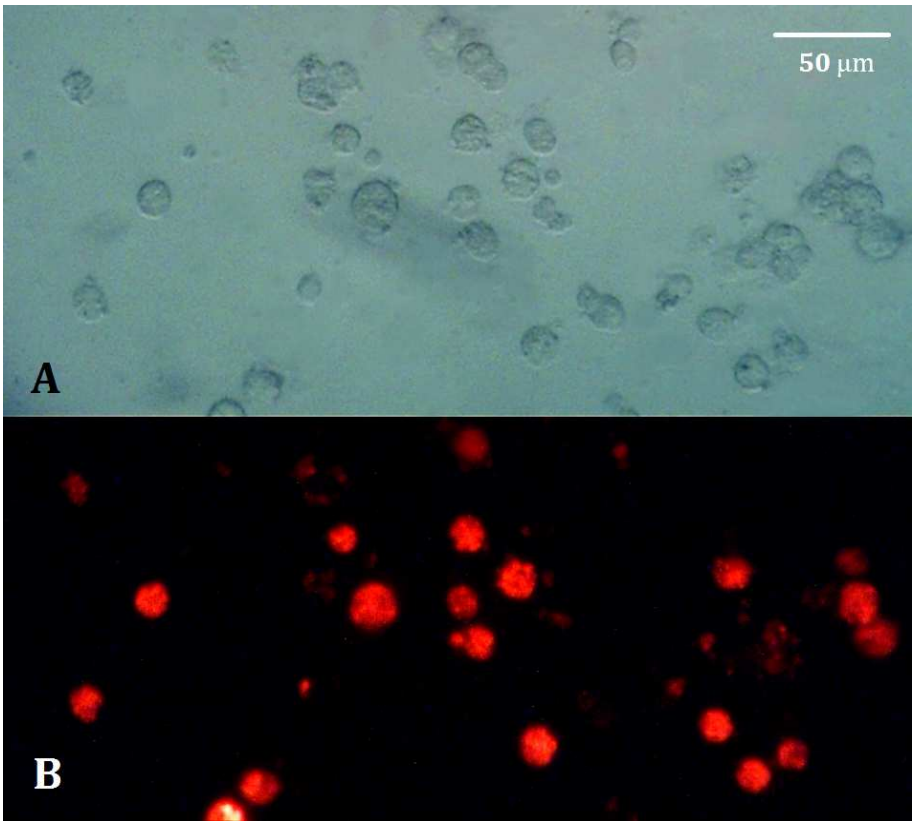
Fot. 9. Migrating PGCs from untouched gonads (control group); Axiovert 40 CFL Zeiss.



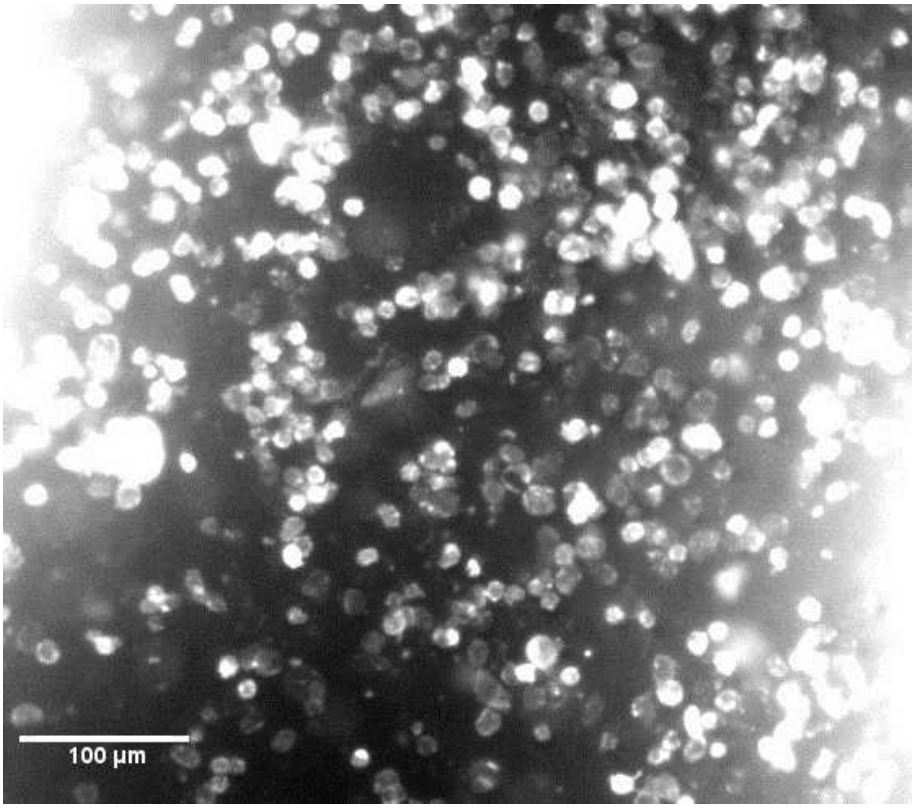
Fot. 10. Unlabeled cells (group $PGCs^{PKH26[-]}$) - first day of *in vitro* cell culture; Axiovert 40 CFL Zeiss.



Fot. 11. Labeled cells (group $PGCs^{PKH26[+]}$) - second (top) and third (bottom) day of *in vitro* cell culture. Fibroblast-like cells visible in the background; Axiovert 40 CFL Zeiss.

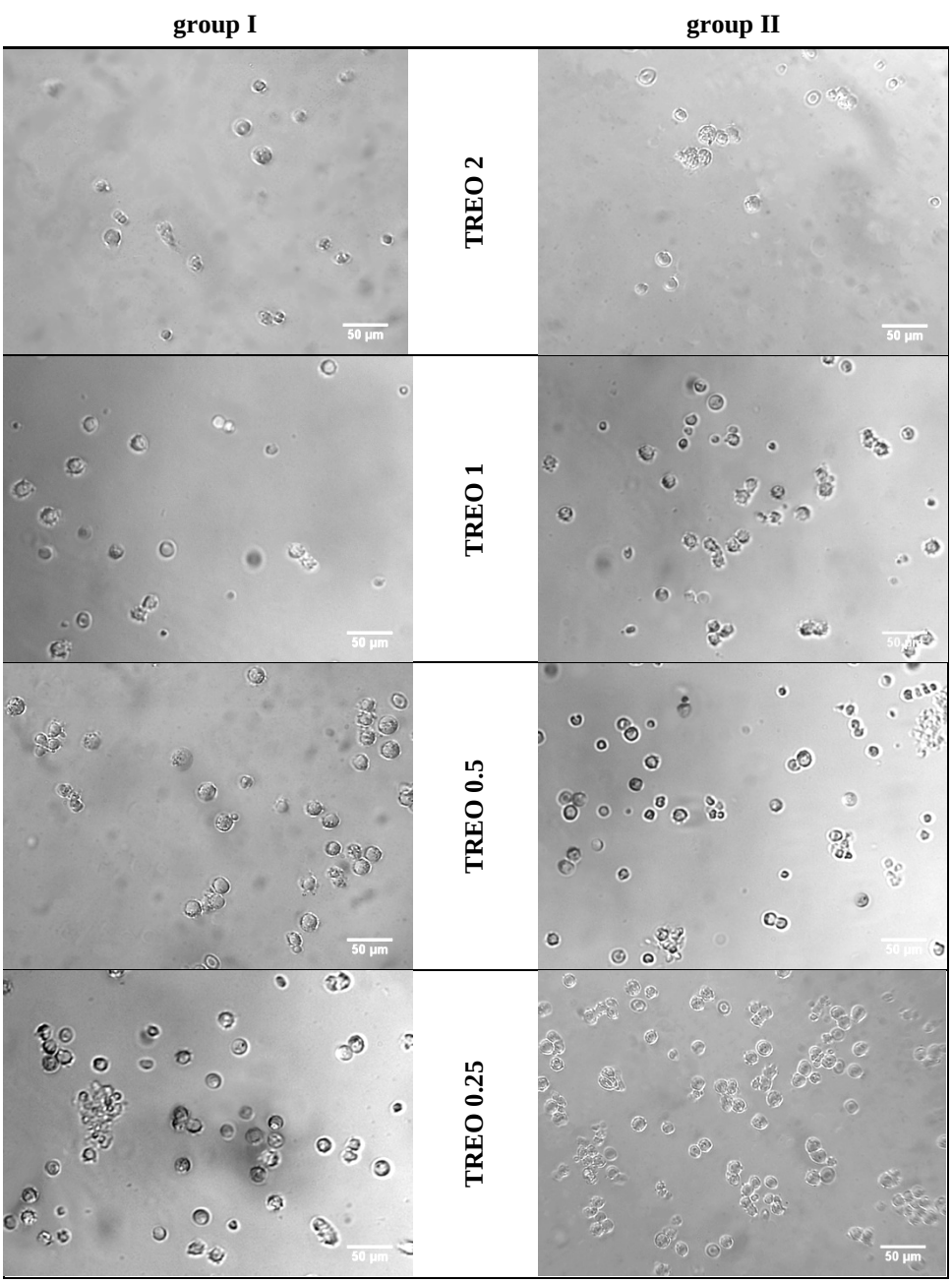


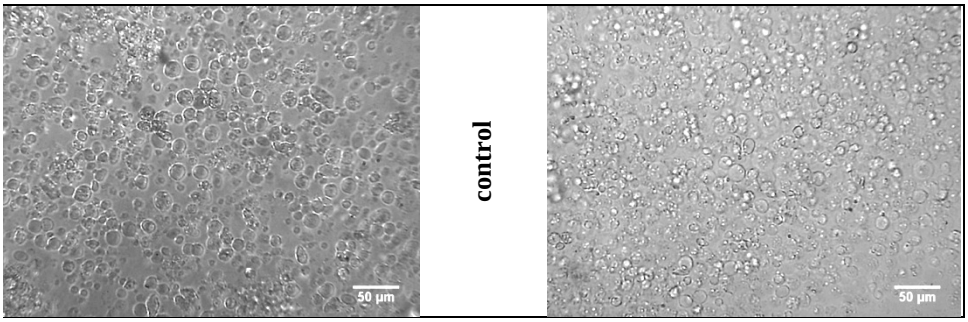
Fot. 12. PGCs isolated from gonads after PKH26 labeling; A - white light; B - fluorescence (551-567 nm excitation filter); Axiovert 40 CFL Zeiss.



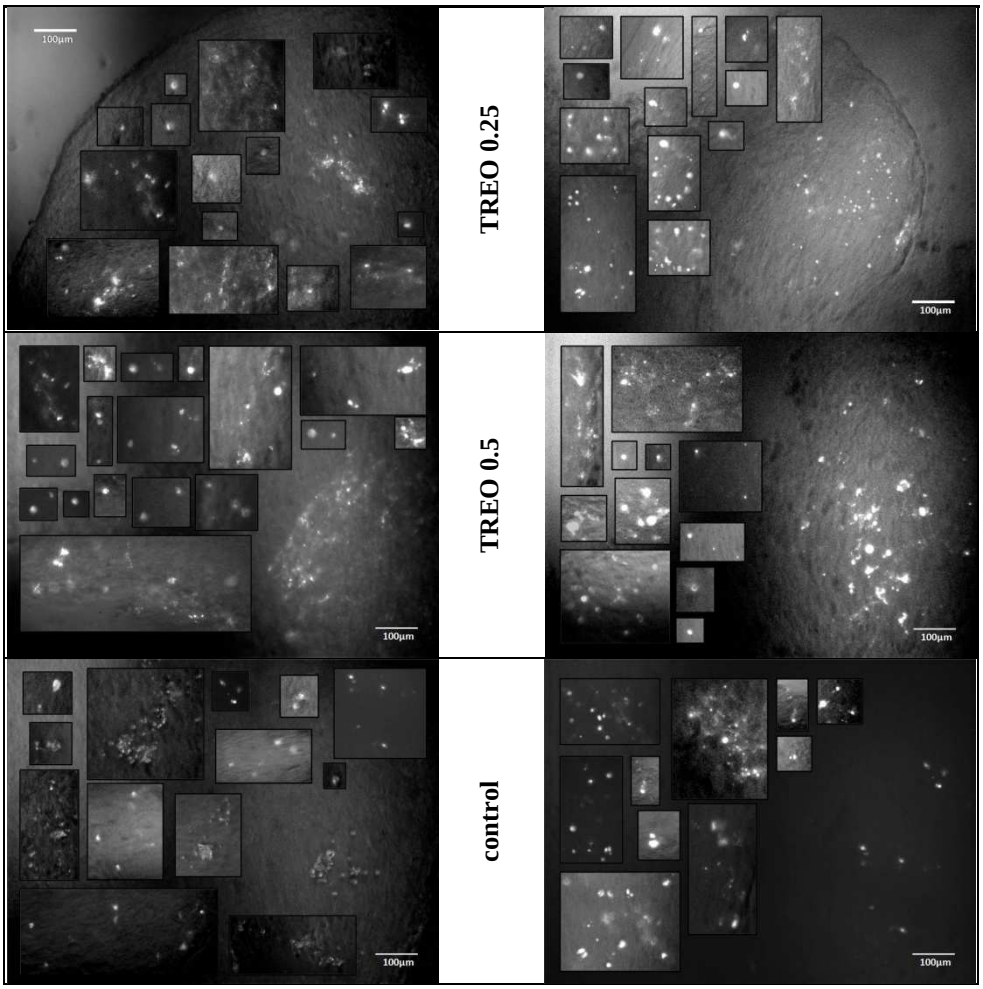
Fot. 13. Primordial germ cells in a drop of injection suspension; Axiovert 40 CFL Zeiss.

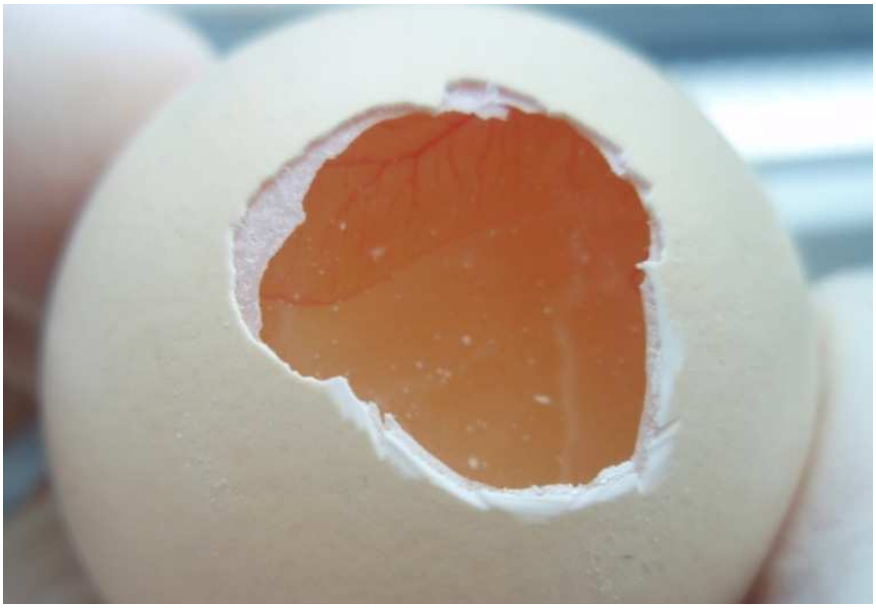
Tab. 24. Recovered PGCs from gonads of sterilized embryos; Axiovert 40 CFL Zeiss.



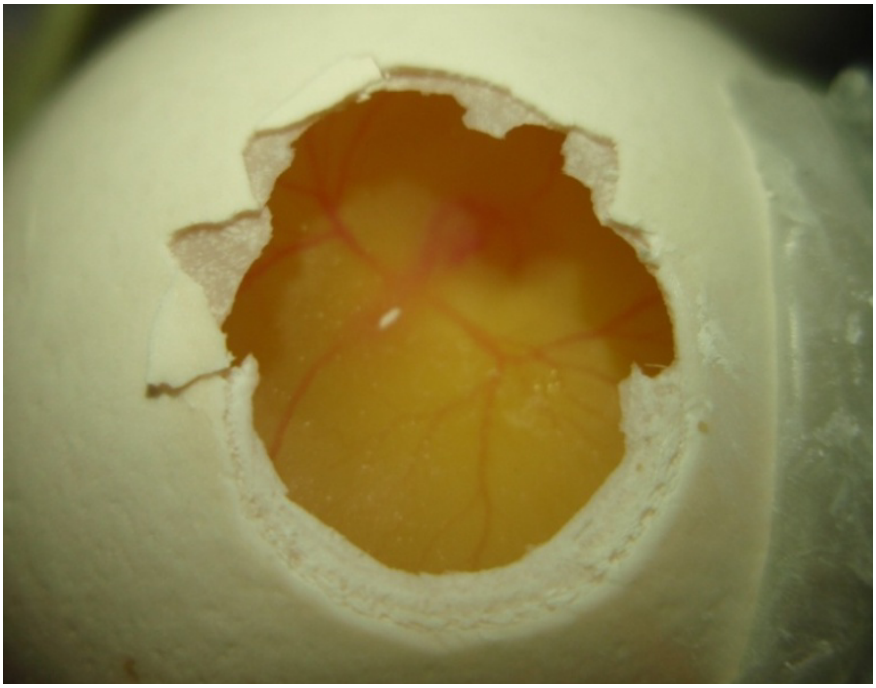


Tab. 25. Gonads of sterilized chicken recipient embryos with visible PGCs$^{PKH26[+]}$; Axiovert 40 CFL Zeiss.

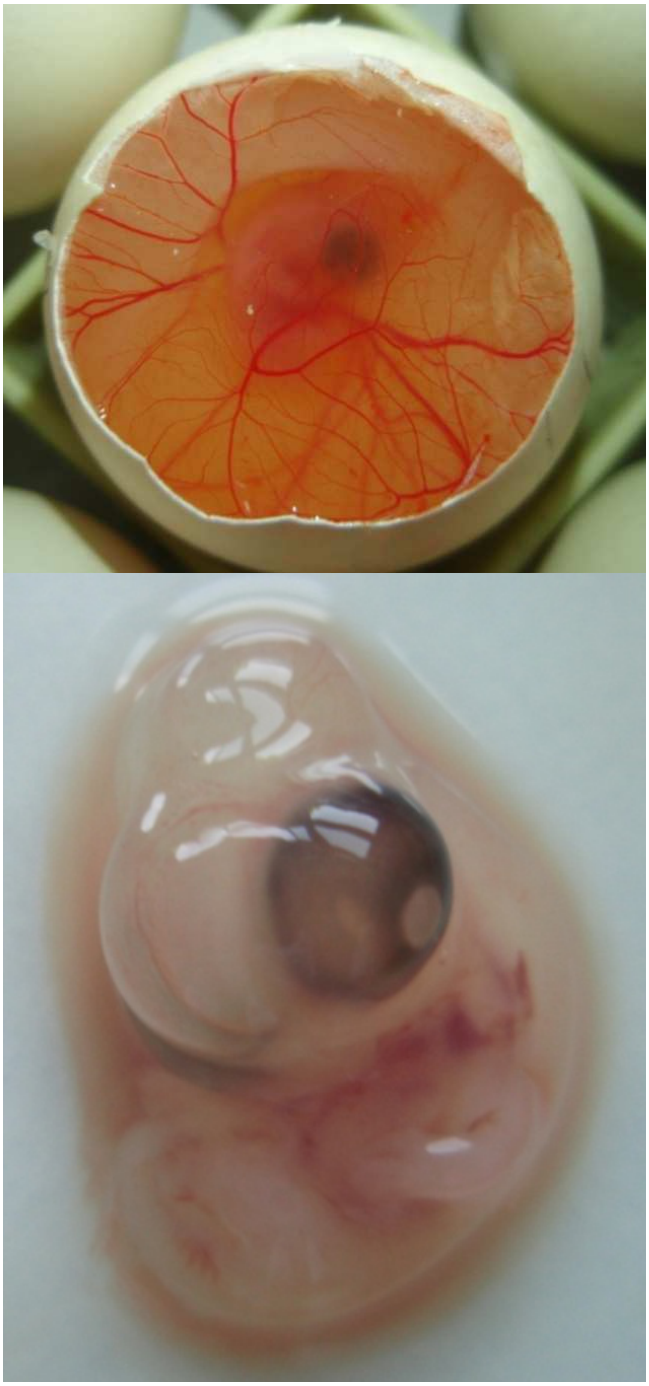




Fot. 14. Incorrect position of embryo - impossible injection.

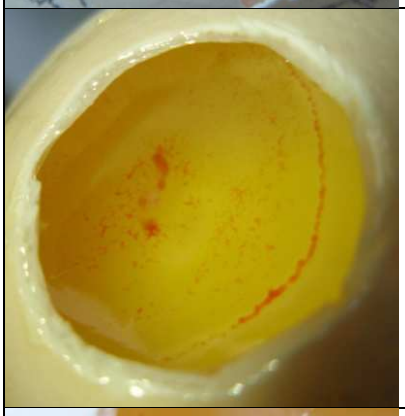
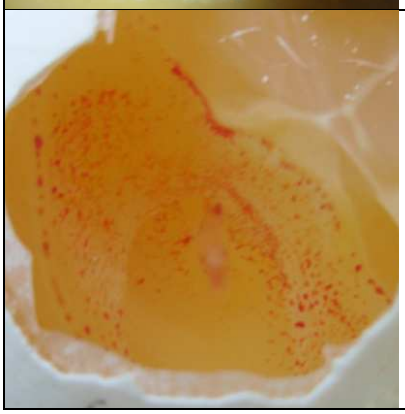
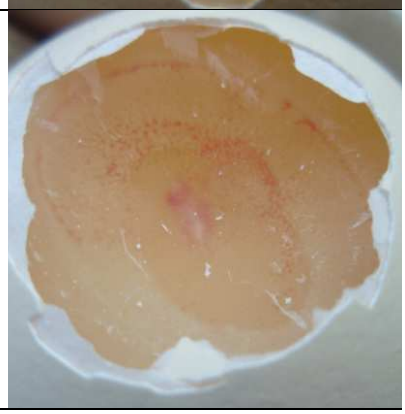


Fot. 15. Normal position and development of embryo - 3rd day.

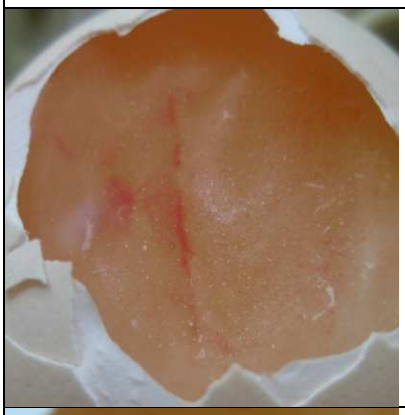
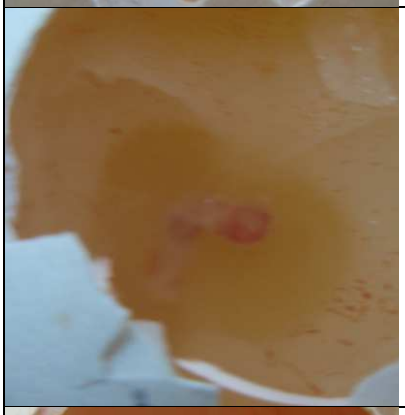
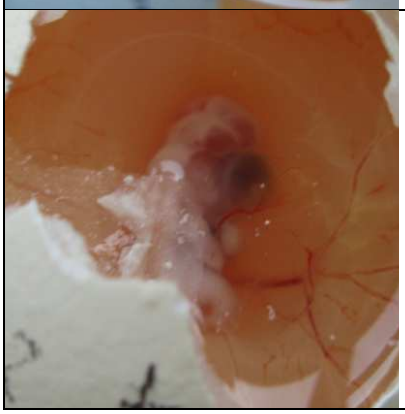
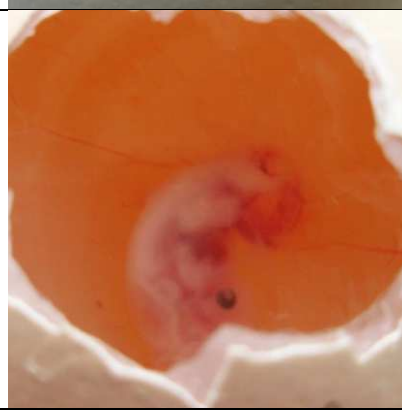


Fot. 16. Normally developed embryo - 6th day.

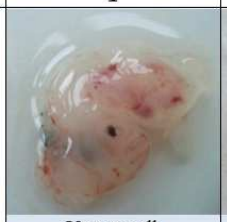
Tab. 26. Death of sterilized and injected with PGCs$^{PKH26[+]}$ embryos.

group I		**group II**
STERILIZATION		
	no signs of develop-ment after treosulfan treatment	
	blood rings on 2nd day	
	blood rings on 3rd day	

Tab. 26. Death of sterilized and injected with PGCs$^{PKH26[+]}$ embryos.

group I		**group II**
INJECTION OF PGCs$^{PKH26[+]}$		
	no signs of develop-ment after injection of cells	
	death on 3-4 day	
	death on 5-6 day	

Tab. 27. Developmental abnormalities of 6-day-old recipient embryos after sterilization and injection of PGCs$^{PKH26[+]}$.

DEVELOPMENTAL ABNORMALITIES				
	1	**2**	**3**	**4**
I TREO 0.25	 Very small, underdeveloped eyes	 Total deformation		
I TREO 0.5	 Reduced body and limbs	 Very small, underdeveloped eyes		
control	 Three eyes	 Total deformation		 Lack of beak
	4	**3**	**2**	**1**
II TREO 0.25		 Small, underdeveloped eyes	 Lack of beak	 Reduced mesencephalon
II TREO 0,5	 Very small size of embryo	 Lack of beak	 Small, underdeveloped eyes	 Small, underdeveloped eyes

7. REFERENCES

[1] Aige-Gil V., Simkiss K., 1991a. Sterilising embryos for transgenic chimaeras. Br. Poult. Sci. 32, 427-438.

[2] Aige-Gil V., Simkiss K., 1991b. Sterilisation of avian embryos with busulphan. Res. Vet. Sci. 50, 139-144.

[3] Allioli N., Thomas J.L., Chebloune Y., Nigon V.M., Verdier G., Legras C., 1994. Use of retroviral vectors to introduce and express the beta-galactosidase marker gene in cultured chicken primordial germ cells. Dev Biol. 165(1), 30-7.

[4] Anstrom K.K., Tucker R.P., 1996. Tenascin-C lines the migratory pathways of avian primordial germ cells and hematopoietic progenitor cells. Dev. Dynamics. 206, 437-446.

[5] Atsumi Y., Yazawa S., Usui F., Nakamura Y., Yamamoto Y., Tagami T., Hiramatsu K., Kagami H., Ono T., 2009. Depletion of primordial germ cells (PGCs) by X-irradiation to extraembryonic region of chicken embryos and expression of xenotransplanted quail PGCs. J. Poult. Sci. 46, 136-143.

[6] Bednarczyk M., Łakota P., Siwek M., 2000. Improvement of hatchability of chicken eggs injected by blastodermal cells. Poult. Sci. 79, 1823-1828.

[7] Bednarczyk M., Płucienniczak G., Płucienniczak A., Łakota P., Sochanik A., Dłużniewska P., Grajewski B., 2003. Expression of exogenous genes in blastodermal cells of chicken *in vitro*. Folia Biol. 51, 189-194.

[8] Bernardo M. A., Sprenkels K., Rodrigues G., Noce T., Chuva de Sousa Lopes S. M., 2012. Chicken primordial germ cells use the anterior vitelline veins to enter the embryonic circulation. Biol. Open 000, 1-7.

[9] Brink M.H., Zwaveling J., Swen J.J., Bredius R.G.M., Lankester A.C., Guchelaar H.J., 2014. Personalized busulfan and treosulfan conditioning for pediatric stem cell transplantation: the role of pharmacogenetics and pharmacokinetics. Drug Discov. Today 19(10), 1572-1586.

[10] Buzała M., Janicki B., Czarnecki R., 2015. Consequences of different growth rates in broiler breeder and layer hens on embryogenesis, metabolism and metabolic rate: A review. Poult. Sci. 94(4), 728-733.

[11] Byun S.J., Kim S., Kim K.W., Kim J.S., Hwang I.S., Chung H.K., Kan I.S., Jeon I.S., Chang W.K., Park S.B., Yoo J.G., 2011. Oviduct-specific enhanced green fluorescent protein expression in transgenic chickens. Biosci. Biotechnol. Biochem. 75(4), 646-649.

[12] Carsience R.S., Clark M.E., Gibbins A.M.V., Etches R.J., 1993. Germline chimeric chickens from dispersed donor blastodermal cells and compromised recipient embryos. Development 117, 669-675.

[13] Chang G., Cheng X., Li B., Ji D., Liu X., Qin Y., Chen R., Chen G., 2010. Migration and accumulation of primordial germ cells of early embryo in quail. Ital. J. Anim. Sci. 9, 45.

[14] Chang I.K., Tajima A., Chikamune T., Ohno T., 1995. Proliferation of chick primordial germ cells cultured on stroma cells from the germinal ridge. Cell Biol Int. 19(2), 143-9.
[15] Chang I., 1992. Simple method for isolation of primordial germ cells from chick embryos. Cell Biol. Int. Rep. 16, 853-857.
[16] Chang I., Jeong D.K., Hong Y.H., Park T.S., Moon Y.K., Ohno T., Han J.Y., 1997. Production of germline chimeric chickens by transfer of cultured primordial germ cells. Cell Biol. Int. 21(8), 495-499.
[17] Chojnacka-Puchta L., Kasperczyk K., Płucienniczak G., Sawicka D., Bednarczyk M., 2012. Primordial germ cells (PGCs) as a tool for creating transgenic chickens. Pol. J. Vet. Sci. 15(1), 173-180.
[18] Chojnacka-Puchta L., Sawicka D., Łakota P., Płucienniczak G., Bednarczyk M., Płucienniczak A., 2015. Obtaining chicken primordial germ cells used for gene transfer: *in vitro* and in vivo results. J. Appl. Genet., Article in Press.
[19] Cyriac S., Churchil R.R., Gibin G., 2012. Transgenic chicken: methods and applications. J. Indian Vet. Associ. 10(2), 67-71.
[20] D'Costa S., Pardue S.L., Petitte J.N., 2001. Comparative development of avian primordial germ cells and production of germ line chimeras. Avian Poult. Biol. Rev. 12, 151-168.
[21] Di Carlo A., De Felici M., 2000. A role for E-cadherin in mouse primordial germ cell development. Dev. Biol. 226, 209-219.
[22] Esmaeilzadeh M., Farhadi A., 2011. An overview of the basics of generate transgenic hen 'Bioreactors' . Webmed Cent. Biol. 2(10), 1-9.
[23] Esmaeilzadeh M., Kazemzadeh F., 2012. Overview on the efforts to generate transgenic chicken. Afr. J. Biotech. 11(36), 8784-8789.
[24] Eyal-Giladi H., Kochav S., 1976. From cleavage to primitive streak formation: a complementary normal table and a new look at the first stages of the development of the chick. I. General morphology. Dev. Biol. 49, 321-337.
[25] Eyal-Giladi H., Ginsbury M., Fabarov A., 1981. Avian primordial germ cells are of epiblastic origin. Embryol. Exp. Morph., 65, 139-147.
[26] Fujimoto T., Ukeshima A., Kiyofuji R., 1976. The origin, migration and morphology of the primordial germ cells in the chick embryo. Anat. Rec. 185, 139-153.
[27] Furuta H., Fujihara N., 1999. Proliferation of exogenously injected primordial germ cells (PGCs) into busulfan-treated chicken embryos. Asian J. Androl. 1, 187-190.
[28] Gao J., Yuan F., Tang X., Han H., Sha J., Yuan J., Shao Y., Jin X., Liu H., Rui L., Li Z., 2011. Contribution of blastoderm cells to Japanese quail (Coturnix coturnix japonica)-Peking duck (Anas platyrhynchos) chimerasas. Anim. Sci. J. 82, 729-734.
[29] Ge Q., McManus M.T., Nguyen T., Shen C.H., Sharp P.A., Eisen H.N., Chen J., 2003. RNA interference of influenza virus production by directly

targeting mRNA for degradation and indirectly inhibiting all viral RNA transcription. Proc. Natl. Acad. Sci. U.S.A. 100, 2718-2723.
[30] Ginsburg M., Eyal-Giladi H., 1987. Primordial germ cells of the young chick blastoderm originate from the central zone of the area pellucida irrespective of the embryo-forming process. Development 101, 209-219.
[31] Glover J.D., Taylor L., Sherman A., Zeiger-Poli C., Sang H. M., McGrew M. J., 2013. A novel piggyBac transposon inducible expression system identifies a role for akt signalling in primordial Germ cell migration. PLoS ONE 8(11), 77222.
[32] Hamburger V., Hamilton H.L., 1951. A series of normal stages in the development of the chick embryo. J. Morphol. 88, 49-92.
[33] Han J.Y., Seo D.S., Shoffner R.N., Guise K.S., 1994. Manipulation of chicken primordial germ cells for transgenic chicken. Animal Cell Technology: Basic & Applied Aspects The Sixth International Meeting of Japanese Association for Animal Cell Technology JAACT'93 6, 317-324.
[34] Han J.Y., 2009. Germ cells and transgenesis in chickens. Comp. Immunol. Micro. 32, 61-80.
[35] Han J.Y., Park T.S., Hong Y.H., Jeong D.K., Kim J.N., Kim K.D., Lim J.M., 2002. Production of germline chimeras by transfer of chicken gonadal primordial germ cells maintained *in vitro* for an extended period. Theriogenology 58(8), 1531-9.
[36] Han Y.B., Zhou Z.K.Y.Q., 1996. Studies on the cultural and isolation of primordial germ cells from blood of chick embryos. Biol. Tech. 6, 11-13.
[37] Heo Y.T., Lee S.H., Yang J.H., Kim T., Lee H.T., 2011. Bone marrow cell-mediated production of transgenic chickens. Lab Invest. 91(8), 1229-40.
[38] Hong Y.H., Seo D.S., Jeong D.K., Choi K.D., Han J.Y., 1995. Migration of the primordial germ cells and gonad formation in the early chicken embryo. Am. J. Appl. Sci, 8(6), 557-562.
[39] Ishiguro S., Minematsu T., Naito M., Kanai Y., Tajima A., 2009. Migratory ability of chicken primordial germ cells transferred into quail embryos. J. Reprod. Develop. 55(2), 183-186.
[40] Ishii Y., Reese D.E., Mikawa T., 2004. Somatic transgenesis using retroviral vectors in the chicken embryo. Dev. Dynam. 229, 630-642.
[41] Ivarie R., 2006. Competitive bioreactor hens on the horizon. Trends Biotech. 24, 99-101.
[42] Jeong D.K., Han J.Y., 2002. Migration activity of chicken gonadal primordial germ cells (gPGCs) and post-transfer localization of LacZ-transfected gPGCs in the embryonic gonads. Asian Australas. J. Anim. Sci. 15(9), 1227-1231.
[43] Jeong D.K., Hong Y.H., Han J.Y., 2002. Simple separation of chicken gonadal primordial germ cells with and without foreign genes. Cell Biol. Int. 26(7), 647-651.

[44] Jiang F.X., Clark J., Renfree M.B., 1997. Ultrastructural characteristics of primordial germ cells and their movement to the gonadal ridges in the tammer wallaby. Anat. Embryol. Berl. 195, 473.
[45] Jordan B.J., Vogel S., Stark M.R., Beckstead R.B., 2014. Expression of green fluorescent protein in the chicken using in vivo transfection of the piggyBac transposon. J. Biotech. 173, 86-89.
[46] Jung J.G., Kim D.K., Park T.S., Lee S.D., Lim J.M., Han J.Y., 2005. Development of Novel Markers for the Characterization of Chicken Primordial Germ Cells. Stem Cells 23, 689-698.
[47] Jung J.G., Lee Y.M., Kim J.N., Kim T.M., Shin J.H., Kim T.H., Lim J.M., Han J.Y., 2010. The reversible developmental unipotency of germ cells in chicken. Reproduction 139, 113-119.
[48] Kagami H., Tagami T., Matsubara Y., Harumi T., Hanada H., Maruyama K., Sakurai M., Kuwana T., Naito M., 1997. The developmental origin of primordial germ cells and the transmission of the donor-derived gametes in mixed-sex germline chimeras to the offspring in the chicken. Mol. Reprod. Dev. 48, 501-510.
[49] Kamihira M., Ono K., Esaka K., Nishijima K., Kigaku R., Komatsu H., Yamashita T., Kyogoku K., Iijima S., 2005. High-level expression of single-chain Fv-Fc fusion protein in serum and egg white of genetically manipulated chickens by using a retroviral vector. J. Virol. 79(17), 10864-10874.
[50] Kang S.J., Choi J.W., Kim S.Y., Park K.J., Kim T.M., Lee Y.M., Kim H., Lim J.M., Han J.Y., 2008. Reproduction of wild birds via interspecies germ cell transplantation. Biol. Reprod. 79, 931-937.
[51] Kang S.J., Choi J.W., Park K.J., Lee Y.M., Kim T.M., Sohn S.H., Lim J.M., Han J.Y., 2009. Development of a pheasant interspecies primordial germ cell transfer to chicken embryo: Effect of donor cell sex on chimeric semen production. Theriogenology 72, 519-527.
[52] Kang S.J., Sohn S.H., Kang K.S., Lee H.C, Lee S.K., Choi J. W., Han J. Y., 2011. Molecular and biological aspects of early germ cell development in interspecies hybrids between chickens and pheasants. Theriogenology 75, 696-706.
[53] Karagenc L., Cinnamon Y., Ginsburg M., Petitte J.N., 1996. Origin of primordial germ cells in the prestreak chick embryo. Dev. Genet .19(4), 290-301.
[54] Karagenc L., Petitte J. N., 2000. Soluble factors and the emergence of chick primordial germ cells *in vitro*. Poult. Sci. 79, 80-85.
[55] Kim J.N., Kim M.A., Park T.S., Kim D.K., Park H.J., Ono T., Lim J.M., Han J.Y., 2004. Enriched gonadal migration of donor-derived gonadal primordial germ cells by immunomagnetic cell sorting in birds. Mol. Reprod. Dev. 68, 81-87.
[56] Kim J.N., Park T.S., Park S.H., Park K.J., Kim T.M., Lee S.K., Lim J.M., Han J.Y., 2010. Migration and proliferation of intact and genetically

modified primordial germ cells and the generation of a transgenic chicken. Biol. Reprod. 82, 257-262.
[57] Kim M.A., Park T.S., Kim J.N., Park H.J., Lee Y.M., Ono T., Lim J.M., Han J.Y., 2005. Production of quail (Coturnix japonica) germline chimeras by transfer of gonadal primordial germ cells into recipient embryos. Theriogenology 63, 774-782.
[58] Koo B.C., Kwon M.S., Choi B.R., Kim J.H., Cho S.K., Sohn S.H., Cho E.J., Lee H.T., Chang W., Jeon I., Park J.K., Park J.B., Kim T., 2006. Production of germline transgenic chickens expressing enhanced green fluorescent protein using a MoMLV-based retrovirus vector. FASEB J. 20, 2251-2260.
[59] Koo B.C., Kwon M.S., Choi, B.R., Lee H.T., Choi H.J., Kim J., Kim N., Jeon I., Chang W., Kim T., 2004. Retrovirus-mediated gene transfer and expression of EGFP in chicken. Mol. Reprod. Dev. 68, 429-434.
[60] Koo B.C., Kwon M.S., Lee H., Kim M., Kim D., Roh J.Y., Park Y., Cui X., Kim N., Byun S.J., Kim T., 2010. Tetracycline-dependent expression of the human erythropoietin gene in transgenic chickens. Transgenic Res. 19, 437-447.
[61] Krzemieniecki K., Zygulska A.L., 2011. Treosulfan in treatment of advanced ovarian cancer and metastatic cutaneous and eyeball malignant melanoma. Onkol. Prak. Klin. 7(3), 127-131.
[62] Kunwar P.S, Siekhaus D.E, Lehmann R., 2006. In vivo migration: a germ cell perspective. Annu. Rev. Cell. Dev. Biol. 22, 237-265.
[63] Kuwana T., 1993. Migration of avian primordial germ cells toward the gonadal anlage. Develop. Growth & Differ. 35 (3), 237-243.
[64] Kuwana T., Hashimoto K., Nakanishi A., Yasuda Y., Tajima A., Naito M., 1996. Long-term culture of avian embryonic cells *in vitro*. Int. J. Dev. Biol. 40(5), 1061-1064.
[65] Kuwana T., Rogulska T., 1999. Migratory mechanisms of chick primordial germ cells toward gonadal anlage. Cell Mol. Biol. 45, 725-736.
[66] Kwon M.S., Koo B.C., Choi B.R., Lee H.T., Kim Y.H., Ryu W., Shim H., Kim J., Kim N., Kim T., 2004. Development of transgenic chickens expressing enhanced green fluorescent protein. Biochem. Bioph. Res. Co. 320, 442-448.
[67] Kwon M.S., Koo B.C., Choi B.R., Park Y., Lee Y.M., Suh H.S., Park Y.S., Lee H.T., Kim J., Roh J.Y., Kim N., Kim T., 2008. Generation of transgenic chickens that produce bioactive human granulocyte-colony stimulating factor. Mol. Reprod. Dev. 75, 1120-1126.
[68] Lee H.C., Kim S.K., Park T.S., Rengaraj D., Park K.J., Lee H.J., Park S.B., Kim S.W., Choi S.B., Han J.Y., 2013. Compensatory proliferation of endogenous chicken primordial germ cells after elimination by busulfan treatment. Stem Cell Res. Ther. 4, 136.
[69] Lee S.H., Gupta M.K., Han D.W., Han S.Y., Uhm S.J., Kim T., Lee H.T., 2007. Development of transgenic chickens expressing human

parathormone under the control of a ubiquitous promoter by using a retrovirus vector system1. Poult. Sci. 86, 2221-2227.
[70] Lee Y.M., Jung J.G., Kim J.N., Park T.S., Kim T.M., Shin S.S., Kang D.K., Lim J.M., Han J.Y., 2006. A testis-mediated germline chimera production based on transfer of chicken testicular cells directly into heterologous testes. Biol. Reprod. 75, 380-386.
[71] Li B.C., Chen G.H., Qin J., Wu X.S., Wu S.L., Cai Z.T., 2005. Suitable stages for isolation and culture PGC-s from chicken embryos. Int. J. Poult. Sci. 4 (11), 885-890.
[72] Li H.C., Kagami H., Matsui K., Ono, T., 2001. Restriction of proliferation of primordial germ cells by the irradiation of Japanese quail embryos with soft X-rays. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 130, 133-140.
[73] Li J., Lu L., 2010. Recent progress on technologies and applications of transgenic poultry. Afr. J. Biotech. 9(24), 3481-3488.
[74] Lillico S.G., McGrew M.J., Sherman A., Sang H.M., 2005. Transgenic chickens as bioreactors for protein-based drugs. Drug Discov. Today 10, 191-196.
[75] Lillico S.G., Sherman A., McGrew M.J., Robertson C.D., Smith J., Haslam C., 2007. Oviduct-specific expression of two therapeutic proteins in transgenic hens. Proc. Natl. Acad. Sci. USA 104, 1771-1776.
[76] Lim J.M., Kwon H.M., Kim D.K., Kim J.N., Park T.S., Ono T., Han J.Y., 2006. Selective decrease of chick embryonic primordial germ cells in vivo and *in vitro* by soft X-ray irradiation. Anim. Reprod. Sci. 95, 67-74.
[77] Macdonald J., Glover J.D., Taylor L., Sang H.M., McGrew M.J., 2010. Characterisation and germline transmission of cultured avian primordial germ cells. PLoS ONE 5, 15518.
[78] Macdonald J., Taylor L., Sherman A., Kawakami K., Takahashi Y., Sang H. M., McGrew M. J., 2012. Efficient genetic modification and germ-line transmission of primordial germ cells using piggyBac and Tol2 transposons. Proc. Natl. Acad. Sci. USA 109, 1466-1472.
[79] McGrew M.J., Sherman A., Ellard F.M., Lillico S.G., Gilhooley H.J., Kingsman A.J., Mitrophanous K.A., Sang H., 2004. Efficient production of germline transgenic chickens using lentiviral vectors. EMBO Rep. 5, 728-733.
[80] McGrew M.J., Sherman A., Lillico S.G., Taylor L., Sang H., 2010. Functional conservation between rodents and chicken of regulatory sequences driving skeletal muscle gene expression in transgenic chickens. BMC Dev. Biol. 10, 26.
[81] Meyer D.B., 1960. Application of periodic acid-Schiff technique to whole chick embryos. Stain Technol. 35, 83-89.
[82] Meyer D.B., 1964. The migration of primordial germ cells in the chick embryo. Dev. Biol. 10, 154-190.

[83] Minematsu T., Tajima A., Kanai Y., 2004. The migratory ability of gonadal germ cells in the domestic chicken. J. Poult. Sci. 41, 178-185.
[84] Miyahara D., Mori T., Makino R., Nakamura Y., Oishi I., Ono T., Nirasawa K., Tagami T., Kagami H., 2014. Culture conditions for maintain propagation, long-term survival and germline transmission of chicken primordial germ cell-like cells. J. Poult. Sci., 51: 87-95.
[85] Mochizuki K., Matsui Y., 2010. Epigenetic profiles in primordial germ cells: Global modulation and fine tuning of the epigenome for acquisition of totipotency. Develop. Growth Differ. 52, 517-525.
[86] Mohsen G.A.M., Ahmed R.A.L., 2002. Tamoxifen inhibits the migration of the primordial germ cells of the chick embryos with less mortality rate. Pakistan J. Biol. Sci. 5(10), 1060-1062.
[87] Motono M., Komatsu H., Kawabe Y., Nishijima K., Iijima S., 2009. Sterilization of chicken primordial germ cells. Anim. Cell Tech. Basic & Applied Asp. 15, 163-168.
[88] Motono M., Ohashi T., Nishijima K., Iijima S., 2008. Analysis of chicken primordial germ cells. Cytotechnol. 57(2), 199-205.
[89] Motono M., Yamada Y., Hattori Y., Nakagawa R., Nishijima K., Iijima S., 2010. Production of transgenic chickens from purified primordial germ cells infected with a lentiviral vector. J. Biosci. Bioeng. 109(4), 315-321.
[90] Mozdziak P.E., Angerman-Stewart J., Rushton B., Pardue S.L., Petitte J.N., 2005. Isolation of chicken primordial germ cells using fluorescence-activated cell sorting. Poult. Sci. 84, 594-600.
[91] Mozdziak P.E., Petitte J.N., 2004. Status of transgenic chicken models for developmental biology. Dev. Dynam. 229, 414-421.
[92] Mozdziak P.E., Wysocki R., Angerman-Stewart J., Pardue S.L., Petitte J.N., 2006. Production of chick germline chimeras from fluorescence-activated cell-sorted gonocytes. Poult. Sci. 85, 1764-1768.
[93] Naeemipour M., Bassami M., 2013. Isolation, culture and characterization of chicken primordial germ cells. J. Cell Mol. Res. 5(2), 48-53.
[94] Naeemipour M., Hesam Dehghani H., Bassami M., Bahrami A., 2013. Expression dynamics of pluripotency genes in chicken primordial germ cells before and after colonization of the genital ridges. Mol. Reprod. Dev. 80, 849-861.
[95] Naito M., 2003. Development of avian embryo manipulation techniques and their application to germ cell manipulation. Anim. Sci. J. 74, 157-168.
[96] Naito M., Harumi T., Kuwana T., 2010. Long term *in vitro* culture of chicken primordial germ cells isolated from embryonic blood and incorporation into germline of recipient embryo. J. Poult. Sci. 47, 57-64.
[97] Naito M., Matsubara Y., Harumi T., Tagami T., Kagami, H., Sakurai, M., Kuwana T. 1999. Differentiation of donor primordial germ cells into functional gametes in the gonads of mixed-sex germline chimaeric chickens produced by transfer of primordial germ cells isolated from embryonic blood. J. Reprod. Fertil. 117, 291-298.

[98] Naito M., Minematsu T., Harumi T., Kuwana T., 2007. Testicular and ovarian gonocytes from 20-day incubated chicken embryos contribute to germline lineage after transfer into bloodstream of recipient embryos. Reproduction 134, 577-584.
[99] Naito M., Sakurai M., Kuwana T., 1998. Expression of exogenous DNA in the gonads of chimaeric chicken embryos produced by transfer of primordial germ cell transfected *in vitro* and subsequent fate of the introduced DNA. J. Reprod. Fertil. 113(1), 137-43.
[100] Naito M., Sano A., Harumi T., Matsubara Y., Kuwana T., 2004. Migration of primordial germ cells isolated from embryonic blood into the gonads after transfer to stage X blastoderms and detection of germline chimaerism by PCR. Br. Poult. Sci. 45, 762-768.
[101] Naito M., Sano A., Matsubara Y., Harumi T., Tagami T., Sakurai M., Kuwana T., 2001. Localization of primordial germ cells or their precursors in stage X blastoderm of chickens and their ability to differentiate into functional gametes in opposite-sex recipient gonads. Reproduction 121, 547-552.
[102] Naito M., Tajima A., Yasuda Y., Kuwana T., 1994. Production of germline chimeric chickens, with high transmission rate of donor-derived gametes, produced by transfer of primordial germ cells. Mol. Reprod. Dev. 39, 153-161.
[103] Nakajima Y., Minematsu T., Naito M., Tajima A., 2011. A new method for isolating viable gonadal germ cells from 7-dayold chick embryos. J. Poult. Sci. 48, 106-111.
[104] Nakamura M., Kuwana T., Miyayama Y., Fujimoto T., 1988. Extragonadal distribution of primordial germ cells in the early chick embryo. Anat. Rec. 222, 90-94.
[105] Nakamura M., Kuwana T., Miyayama Y., Yoshinaga K., Fujimoto T., 1991. Ectopic colonization of primordial germ cells in the chick embryo lacking the gonads. Anat. Rec. 229, 109-115.
[106] Nakamura Y., Kagami H., Tagami T., 2013. Development, differentiation and manipulation of chicken germ cells. Develop. Growth Differ. 55, 20-40.
[107] Nakamura Y., Usui F., Atsumi Y., Otomo A., Teshima A., Ono T., Takeda K., Nirasawa K., Kagami H., Tagami T., 2009. Effects of busulfan sustained-release emulsion on depletion and repopulation of primordial germ cells in early chicken embryos. J. Poult. Sci. 46, 127-135.
[108] Nakamura Y., Usui F., Miyahara D., Mori T., Ono T., Kagami H., Takeda K., Nirasawa K., Tagami T., 2012. X-irradiation removes endogenous primordial germ cells (PGCs) and increases germline transmission of donor PGCs in chimeric chickens. J. Reprod. Dev. 58, 432-437.
[109] Nakamura Y., Usui F., Miyahara D., Mori T., Watanabe H., Ono T., Takeda K., Nirasawa K., Kagami H., Tagami T., 2011. Viability and

functionality of primordial germ cells after freeze-thaw in chickens. J. Poult. Sci. 48, 57-63.
[110] Nakamura Y., Usui F., Ono T., Takeda K., Nirasawa K., Kagami H., Tagami T., 2010. Germline replacement by transfer of primordial germ cells into partially sterilized embryos in the chicken. Biol. Reprod. 83, 130-137.
[111] Nakamura Y., Yamamoto Y., Usui F., Atsumi Y., Ito Y., Ono T., Takeda K., Nirasawa K., Kagami H., Tagami T., 2008. Increased proportion of donor primordial germ cells in chimeric gonads by sterilisation of recipient embryos using busulfan sustained release emulsion in chickens. Reprod. Fert. Develop. 20, 900-907.
[112] Nakamura Y., Yamamoto Y., Usui F., Mushika T., Ono T., Setioko A. R., Takeda K., Nirasawa K., Kagami H., Tagami T., 2007. Migration and proliferation of primordial germ cells in the early chicken embryo. Poult. Sci. 86, 2182-2193.
[113] Nishijima K., Iijima S., 2013. Transgenic chickens. Develop. Growth Differ. 55, 207-216.
[114] Oh D.J., Lee G.M., Francis K., Palsson B.O., 1999. Phototoxicity of the fluorescent membrane dyes PKH2 and PKH26 on the human hematopoietic KG1a progenitor cell line. Cytometry. 36(4), 312-318.
[115] Oishi I., 2010. Improvement of transfection efficiency in cultured chicken primordial germ cells by percoll density gradient centrifugation. Biosci. Biotechnol. Biochem. 74(12), 2426-2430.
[116] Ono T., Machida Y., 1999. Immunomagnetic purification of viable primordial germ cells of japanese quail (Coturnix japonica). Comp. Biochem. Physiol. Part A Mol. Integr. Physiol. 122, 255-259.
[117] Ono T., Yokoi R., Aoyama H., 1996. Transfer of male or female primordial germ cells of quail into chick embryonic gonads. Exp. Anim. 45(4), 347-352.
[118] Ono T., Yokoi R., Maeda S., Nishida T., Aoyama H., 1998a. Settlement of quail primordial germ cells in chicken gonads. Anim. Sci. Technol. 69, 546-555.
[119] Ono T., Yokoi R., Maeda S., Nishida T., Aoyama H., 1998b. Transfusion of chick primordial germ cells into quail embryos and their settlement in gonads. Anim. Sci. Technol. 69, 911-915.
[120] Park K.J., Kang S.J., Kim T.M., Lee Y.M., Lee H.C., Song G., Han J.Y., 2010. Gamma-irradiation depletes endogenous germ cells and increases donor cell distribution in chimeric chickens. *In vitro* Cell. Dev. Biol. Anim. 46, 828-833.
[121] Park T.S., Han J.Y., 2012b. PiggyBac transposition into primordial germ cells is an efficient tool for transgenesis in chickens. PNAS 109 (24), 9337-9341.
[122] Park T.S., Han J.Y., 2000. Derivation and characterization of pluripotent embryonic germ cells in chicken. Mol. Reprod. Dev. 56(4), 475-482.

[123] Park T.S., Han J.Y., 2012a. Genetic modification of chicken germ cells. Ann. NY Acad. Sci. 1271, 104-109.
[124] Park T.S., Han J.Y., 2013. Conservation of migration and differentiation circuits in primordial germ cells between avian species. J. Reprod. Dev. 59(3), 252-257.
[125] Park T.S., Hong Y.H., Kwon S.C., Lim J.M., Han J.Y., 2003a. Birth of germline chimeras by transfer of chicken embryonic germ (EG) cells into recipient embryos. Mol. Reprod. Dev. 65(4), 389-395.
[126] Park T.S., Jeong D.K., Kim J.N., Song G.H., Hong Y.H., Lim J.M., Han J.Y., 2003b. Improved germline transmission in chicken chimeras produced by transplantation of gonadal primordial germ cells into recipient embryos. Biol. Reprod. 68, 1657-1662.
[127] Park T. S., Lee H. C., Rengaraj D., Han J. Y., 2014. Germ cell, stem cell, and genomic modification in birds. J. Stem. Cell. Res. Ther. 4, 201.
[128] Petitte J.N., Mozdziak P.E., 2007. The incredible, edible, and therapeutic egg. PNAS 104(6), 1739-1740.
[129] Petitte J.N., Clark M.E., Liu G., Verrinder Gibbins A.M., Etches R.J., 1990. Production of somatic and germline chimeras in the chicken by transfer of early blastodermal cells. Development 108, 185-189.
[130] Rapp J.C., Harvey A.J., Speksnijder G.L., Hu W., Ivarie R., 2003. Biologically active human interferon α-2b produced in the egg white of transgenic hens. Transgenic Res. 12, 569-575.
[131] Rashidi H., Sottile V., 2009. The chick embryo: hatching a model for contemporary biomedical research. BioEssays 31,459-465.
[132] Rehimi R., Khalida N., Yusuf F., Dai F., Morosan-Puopolo G., Brand-Saberi B., 2008. Stromal-derived factor-1 (SDF-1) expression during early chick development. Int. J. Dev. Biol. 52, 87-92.
[133] Reynaud G., 1976. Localization study of primordial germ-cells in embryos of Japanese quail by means of ultraviolet-irradiation. C. R. Hebd. Seanc. Acad. Sci. D 282, 1195-1198.
[134] Sang H.M. 2009. Genetic modification of the chicken: new technologies with potential applications in poultry production. [W:] Genetic modification and poultry production. Biology of breeding poultry, Hocking P. M. (red.). CAB International
[135] Sang H., 2003. Genetically modified livestock and poultry and their potential effects on human health and nutrition. Trends Food Sci. Tech. 14, 253-263.
[136] Sang H., 2006. Transgenesis sunny - side up. Nat. Biotechnol. 24, 955-956.
[137] Sang H., 2012. Disease resistance and other applications of transgenesis in the chicken. Reprod. Fert. Develop. 25(1), 320-320.
[138] Sawicka D., Chojnacka-Puchta L., Zieliński M., Płucienniczak G., Plucienniczak A., Bednarczyk M., 2015. Flow cytometric analysis of apoptosis in cryoconserved chicken primordial germ cells. Cell. Mol. Biol. Lett. 20(1), 143-159.

[139] Shin S.S., Kim T.M., Kim S.Y., Kim T.W., Seo H.W., Lee S.K., Kwon S.C., Lee G.S., Kim H., Lim J.M., Han J.Y., 2008. Generation of transgenic quail through germ cell-mediated germline transmission. FASEB J. 22, 2435-2444.
[140] Shiue Y-L., Tailiu J-J., Liou J-F., Lu H-T., Tai C., Shiau J-W., Chen L-R., 2009. Establishment of the long-term *in vitro* culture system for chicken primordial germ cells. Reprod. Dom. Anim. 44, 55-61.
[141] Simkiss K. 1994. Transgenic birds. [W:] Animals with novel genes, Maclean N. (red.). Cambridge University Press.
[142] Sławinska A., Siwek M.Z., Bednarczyk M.F., 2014. Effects of synbiotics injected in ovo on regulation of immune-related gene expression in adult chickens. Am. J. Vet. Res. 75(11), 997-1003.
[143] Song G., Han J.Y., 2011. Avian biomodels for use as pharmaceutical bioreactors and for studying human diseases. Ann. N.Y. Acad. Sci. 1229, 69-75.
[144] Song Y., D'costa S., Pardue S.L., Petitte J.N., 2005. Production of germline chimeric chickens following the administration of a busulfan emulsion. Mol. Reprod. Dev. 70, 438-444.
[145] Speksnijder G.J., Etches R.J., Verrinder Gibbins A. M., 1999. Germline chimeric chickens from facs-sorted donor cells. Mol. Reprod. Dev. 52, 33-42.
[146] Stebler J., Spieler D., Slanchev K., Molyneaux K.A., Richter U., Cojocaru V., Tarabykin V., Wylie C., Kessel M., Raz E., 2004. Primordial germ cell migration in the chicken and mouse embryo: the role of the chemokine SDF-1/CXCL12. Dev. Biol. 272, 351-361.
[147] Swartz W., 1980. Response of early chick embryos to busulfan. Teratology 21, 1-8.
[148] Tagami T., Kagami H., 1998. Developmental origin of avian primordial germ cells and its unique differentiation in the gonads of mixed-sex chimeras. Mol. Reprod. And Dev. 50, 370-376.
[149] Tagami T., Matsubara Y., Hananda H., Naito M., 1997. Differentiation of female chicken primordial germ cells into spermatozoa in male gonads. Dev. Growth Differ. 39, 267-271.
[150] Tagami T., Nakamura Y., 2011. Improving rates of germline transmission in chimeric chickens using busulfan solubilized sustained-release emulsion. Avian Biol. Res. 4, 74-77.
[151] Tajima A., Hayashi H., Kamizumi A., Ogura J., Kuwana T., Chikamune T., 1999. Study on the concentration of circulating primordial germ cells. J. Exp. Zool. 284(7), 759-64.
[152] Tajima A., Naito M., Yasuda Y., Kuwana T., 1993. Production of germ line chimera by transfer of primordial germ cells in the domestic chicken (Gallus domesticus). Theriogenology 40, 509-519.

[153] Tajima A., Naito M., Yasuda Y., Kuwana T., 1998. Production of germ-line chimeras by transfer of cryopreserved gonadal primordial germ cells (gPGCs) in chicken. J. Exp. Zool. 280, 265-267.
[154] Tsunekawa N., Naito M., Sakai Y., Nishida T., Noce T., 2000. Isolation of chicken vasa homolog gene and tracing the origin of primordial germ cells. Development 127(12), 2741-2750.
[155] Tyack S.G., Jenkins K.A., O'Neil T.E., Wise T.G., Morris K.R., Bruce M.P., McLeod S., Wade A.J., McKay J., Moore R.J., Schat K.A., Lowenthal J.W., Doran T.J., 2013. A new method for producing transgenic birds via direct in vivo transfection of primordial germ cells. Transgenic Res. 22, 1257-1264.
[156] Urven L.E., Abbott U.K., Erickson C.A., 1989. Distribution of extracellular matrix in the migratory pathway of avian primordial germ cells. Anat. Rec. 224, 14-21.
[157] van de Lavoir M.C., Collarini E.J., Leighton P.A., Fesler J., Lu1 D. R., Harriman W.D., Thiyagasundaram T.S., Etches R.J., 2012. Interspecific germline transmission of cultured primordial germ cells. PLoS ONE 7(5), 35664.
[158] van de Lavoir M.C., Diamond J.H., Leighton P.A., Mather-Love C., Heyer B.S., Bradshaw R., Kerchner A., Hooi L.T., Gessaro T.M., Swanberg S.E., Delany M.E., Etches R.J., 2006a. Germline transmission of genetically modified primordial germ cells. Nature 441, 766-769.
[159] van de Lavoir M.C., Mather-Love C., Leighton P., Diamond J.H., Heyer B.S., Roberts R., Zhu L., Winters-Digiacinto P., Kerchner A., Gessaro T., Swanberg S., Delany M.E., Etches R.J., 2006b. High-grade transgenic somatic chimeras from chicken embryonic stem cells. Mech. Dev. 123, 31-41.
[160] Wang Y., Hou L., Li C., Guan W., Chen L., Li X., 2010. Isolation, culture and biological characteristics of primordial germ cells from Beijing fatty chicken. J. Reprod. Develop. 56, 303-8.
[161] Wei Q., Croy B. A., Etches R. J., 2001. Selection of genetically modified chicken blastodermal cells by magnetic-activated cell sorting. Poult. Sci. 80, 1671-1678.
[162] Wentworth B. C., Tsai H., Hallett J. H., Gonzales D. S., Rajcic-Spasojevic G., 1989. Manipulation of avian primordial germ cells and gonadal differentiation. Poultry Sci. 68 (7), 999-1010.
[163] Wernery U., Liu C., Baskar V., Guerineche Z., Khazanehdari K. A., Saleem S., Kinne J., Wernery R., Griffin D. K., Chang I. K., 2010. Primordial germ cell-mediated chimera technology produces viable pure-line Houbara bustard offspring: potential for repopulating an endangered species. PLoS ONE 5, 15824.
[164] Wu Y., Ge C., Zhang C., 2008. Epidermal growth factor promotes proliferation of chicken germ cells (PGCs) and differentiation potency of embryoid bodies derived from PGCs. Cell Res. 18, 45.

[165] Yamaguchi H., Xi Y., Fujihara N., 2000. Inter embryonic (homo- or hetero-sexual) transfer of primordial germcells between chicken embryos. Cytotechnology 33, 101-108.
[166] Yamamoto Y., Usui F., Nakamura Y., Ito Y., Tagami T., Nirasawa K., Matsubara Y., Ono T., Kagami H., 2007. A novel method to isolate primordial germ cells and its use for the generation of germline chimeras in chicken. Biol. Reprod. 77, 115-119.
[167] Yasuda Y., Tajima A., Fujimoto T., Kuwana T., 1992. A method to obtain avian germ-line chimaeras using isolated primordial germ cells. J. Reprod. Fertil. 96, 521-528.
[168] Yu M., Guan K., Zhang C., 2011. The promoting effect of retinoic acid on proliferation of chicken primordial germ cells by increased expression of cadherin and catenins. Amino Acids 40, 933-941.
[169] Yu Y., Ping G., Yanping F., Yongping Y., Shijun L., Xiuli P., Yanzhang G., 2012. Busulfan influence the survival rate of chicken embryos and the morphology of gonads. J. Anim. Vet. Adv. 11, 561-565.
[170] Zhang Y., Yang H., Zhang Z., Shi Q., Wang D., Zheng M., Li B., Song J., 2013. Isolation of chicken embryonic stem cell and preparation of chicken chimeric model. Mol. Biol. Rep. 40, 2149-2156.
[171] Zhao D.F., Kuwana T., 2003. Purification of avian circulating primordial germ cells by Nycodenz density gradient centrifugation. Br. Poult. Sci. 44, 30-35.

STRESZCZENIE

Biologia i zachowanie się pierwotnych komórek płciowych (PGCs- ang. primordial germ cells) ptaków znacznie różni się od tych u ssaków, co w efekcie daje unikalną właściwość- możliwość tworzenia ptasich chimer płciowych poprzez wprowadzenie PGCs dawców do układu krwionośnego 3.5- dniowych zarodków biorców. Celem pracy było zbadanie zachowań PGCs dawcy i biorcy.

Praca skupia się na trzech aspektach: 1) sposób izolacji PGCs z gonad zarodków biorców w stadium 28-29 (H&H) w kierunku uzyskania jak najwyższej liczby nie tworzących agregatów komórek; 2) badanie możliwości migracyjnych PGCs pochodzących od dwóch różnych ras/typów kury domowej, dostępnych na terenie kraju (Ross 308 - R i zielononóżka kuropatwiana - ZK) w roli dawcy i biorcy komórek - na tym etapie efektywność kombinacji dawca/biorca była sprawdzana poprzez detekcję fluorescencji w gonadach zarodków biorców; 3) opracowanie nowej metody częściowej sterylizacji zarodków biorców z wykorzystaniem cytostatyka - treosulfanu (TREO), polegającej na eliminacji endogennych PGCs i połączeniu najwydajniejszej metody sterylizacji oraz kombinacji dawców i biorców w celu stworzenia chimerycznego zarodka, a także zbadanie wpływu czynnika sterylizującego na prawidłowy rozwój zarodka biorcy.

Rezultatem wykonanych badań jest opracowanie kompleksowego systemu, który może stanowić alternatywę dla obecnie istniejących metod produkcji kurzych chimer płciowych w szczególności znajdujących zastosowanie w badaniach transgenicznych, jak i również w konserwacji zagrożonych gatunków ptaków oraz w hodowli i biologii zwierząt. W trakcie badań wyizolowano łącznie 9,688 x 10^6 PGCs pochodzących od 581 6-dniowych dawców i iniekowano je do 513 zarodków biorców. Uzyskano 130 zarodków wykazujących cechy chimery płciowej. Największą liczbę prawidłowych morfologicznie pierwotnych komórek płciowych (114 000; $P < 0,05$), nie tworzących agregatów uzyskano inkubując gonady nadtrawione 0,25% trypsyną-EDTA, w roztworze PBS[-]. Najwięcej (54.5; $P < 0,01$) zarodków posiadających egzogenne, znakowane PKH26 PGCs odnotowano w grupie R(dawca komórek)/ZK (biorca komórek). Liczba wyeliminowanych endogennych PGCs zwiększała się (od 48,5% do 96,4%) wraz ze wzrostem stężenia TREO (koncentracja od 2 do 0,25 mg/ml), zwłaszcza podanego do jamy podzarodkowej. W wyniku podania TREO maleje przeżywalność zarodków biorców, natomiast procent zarodków z egzogennymi komórkami dawcy nie ulega zmianie.

Zachowanie się PGCs zależy od sposobu ich izolacji, doboru dawcy/biorcy komórek, ich wyznakowania oraz sposobu sterylizacji zarodka. Wdrożenie wyników powyższej pracy, w szczególności dotyczących działania treosulfanu, pozwoli w przyszłości rozszerzyć wiedzę na temat mechanizmu jego działania, a także zwiększyć wydajność produkcji transgenicznych ptaków z wykorzystaniem PGCs.

I know, it's just another PhD dissertation, but without you it wouldn't be possible... So, special thanks to Franuszek ♬ and Paweł - you are people borne of sound, the songs are in your eyes, and you wear them like a crown - thanks to you I 'm growing stronger every day and I found "the courage to walk out into the street with arms out ❤". Danusia, Johnny, Krystyna, Maniek, Kołek, Borys and Tolki ☺ I will always remember how you helped me - love you so much!

Anita, Agata M., Agata D., Mateusz, Asia, Konrad, Agata Sz. and Michalina, thank you very much for the help you gave me. I know how busy you are, so I really appreciated the time you spent for me. You are the kind of people that makes this university a worth place to work.