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AQUATIC ANIMAL REPORTS 2(1) (2024) : 31-42
DOI: 10.5281/zenodo.10683936
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Journal homepage: https://scopesscience.com/index.php/aqar

Received: 28 July 2023; Received in revised form: 14 February2024
Accepted: 16 February 2024; Available online:21 February 2024

RESEARCH PAPER

Citation: Atmaca, S. & Ekici, A. (2024). Isolation of early stage testicular germ cells of rainbow trout by percoll gradient centrifugal method. *Aquatic Animal Reports*, 2(1), 31-42. https://doi.org/10.5281/zenodo.10683936

ISOLATION OF EARLY STAGE TESTICULAR GERM CELLS OF RAINBOW TROUT BY PERCOLL GRADIENT CENTRIFUGAL METHOD

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Abstract

The purpose of this study was to isolate germ cells from rainbow trout (*Oncorhynchus mykiss*) testicular tissue by employing a percoll gradient centrifugation method. Therefore; testicular tissue samples from rainbow trout were incubated with 0.5% trypsin (2 h, 20°C) in phosphate-buffered saline (PBS) solution for enzymatic separation. Gradient centrifugation method was performed using percoll at different rates (45% - 10%, 50% - 10% and 55% - 10%). Following centrifugation, three layers were formed. In the uppermost layer, debris and spermatids; early stage germ cells in the middle layer and a small amount of spermatids; and in the pellet located in the bottom layer, erythrocytes and spermatids were detected intensively. In addition; both histological and immunofluorescence methods were utilized to identify testicular and somatic cells. Hematoxylin-eosin staining of histological sections from testicular tissue revealed spermatogonia types A and B, and spermatid cells. Fluorescent staining of tissue sections revealed early stage germ cells labeled with primary antibody (DDX4) and the secondary antibody (FITC).

Keywords: Early stage germ cell, Rainbow trout, spermatogonia, percoll gradient, isolation

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Introduction

Currently, various living aquatic populations face the imminent threat of extinction, largely due to the detrimental impacts of human activities. Among these populations are Salmonid and trout species, which have witnessed a steady increase in production over the years. These fish populations are at risk due to pollution of water resources and climate change, and even a few species are extinct. To ensure the continuity of fish species, applications to protect endangered species and use in aquaculture are becoming more important (Okutsu et al., 2008; Lee et al., 2015). The conservation of these living organisms remains the foremost approach employed for this purpose. Nevertheless, it's important to note that this strategy is not without its share of risks and challenges.

Assisted reproductive techniques (induction of ovulation, in vitro gametogenesis, sperm cryopreservation, gene transfer, chromosome manipulation techniques) are being used to protect genetic resources and increase the quantity and diversity of species produced in parallel with technological advancements. Germ cell studies have also become part of these techniques (Yoshizaki et al., 2003; Okutsu et al., 2008; Mylonas et al., 2010; Lacerda et al., 2013; Kawasaki et al., 2015). Isolation and cryopreservation studies of germ cells that differentiate into both gamete types are currently in use (Brinster, 2002; Yoshizaki et al., 2003; Kobayashi et al., 2007; Okutsu et al., 2007).

Studies on the isolation of fish germ cells, initiated by Gamo in 1961 and Hamaguchi in 1982 on medaka fish, later continued on fish species such as *Oncorhynchus mykiss*, *Oreochromis niloticus*, *Osphronemus gouramy*, *Labeo rohita*, *Marosatherina ladigesi*, *Acipenser baerii*, *Tinca tinca*, *Stizostedion lucioperca*, *Salmo trutta*, *Thymallus thymallus* in the following years (Gamo, 1961; Hamaguchi, 1982; Takeuchi et al., 2002; Kobayashi et al., 2004; Okutsu et al., 2006; Lacerda et al., 2006; Lacerda et al., 2010; Andriani et al., 2010; Panda et al., 2011; Andriani et al., 2012; Andriani, 2012; Andriani, 2013; Linhartova et al., 2014; Güngör, 2015; Psenicka et al., 2015; Lujic et al., 2017).

The aim of this study is to initiate investigations into surrogate production within the realm of fish in our country. To achieve this, the focus has been directed towards the rainbow trout, which stands as the most extensively cultivated species in our region.

Material and Methods

Fish Used in the Experiment

This study was carried out at Istanbul University, Faculty of Aquatic Sciences, Sapanca Inland Aquaculture Production Research and Application Unit. In the study, 15 male rainbow trout aged 13-14 months were used. The average length of the fish was 24.8±1.34 cm, and the average weight was 171.9±29.10 g.

Enzymatic Separation of Testicular Cells

The fish were kept in high doses (200 mg/l) in tricaine methanesulfonate (MS 222) for a long time and caused to die (Carter et al., 2011). Subsequently, an abdominal incision was performed on the fish, allowing for the removal of two fragments of testicular tissue. Each extracted tissue was then placed into separate Petri dishes. Three different buffers were used to wash the testicular tissues: phosphate buffered salt (PBS), physiological saline (0.9% NaCl), and Hank's balanced salt solution (HBSS) after this stage. In the study, it was determined which buffer was

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suitable for enzymatic separation. For each buffer solution, 0.5% trypsin was added to the pieces of tissue (Takeuchi et al., 2002). Samples incubated in a shaking incubator at 20°C for 2 h (Takeuchi et al., 2002) were filtered through a 50 µm filter. 40 µg/ml DNase I was added to the 4 ml filtered sample. At the next step, 1% bovine serum albumin (BSA) was added (Psenicka et al., 2015).

Enzymatic Isolation with Percoll Gradient Centrifuge Method

After enzymatic separation, percoll gradient step was performed to separate early stage germ cells from other testicular (spermatids and sperm) and somatic cells. Percoll was diluted separately with HBSS, PBS and physiological salt (0.9% NaCl) buffers to establish gradient ratios.

With HBSS, which is the first of these buffers, the lower layer-upper layer ratios are prepared as, respectively, 33%-5%, 30%-10% and 45%-10%. The PBS buffer, which is used as a buffer in the second group, and the lower layer-top layer ratios are prepared as, respectively, 30%-10%, 30%-5%, 33%-5%, 45%-10%, 50%-10% and 55%-10%. In the third group, physiological salt buffer (0.9% NaCl) used as a buffer and the lower layer-top layer ratios were prepared as, respectively, 30%-10%, 45%-10% (Linhartova et al., 2014, Psenicka et al., 2015; Güngör, 2015). After the filtration process, 1 ml of the samples were taken and transferred to the percoll gradient layers that were made using different buffers. After centrifuging at 4°C for 30 minutes to 500g, the samples were examined under a light microscope (Figure 2.1).

Histological Analysis

The testicular tissue of fish, where germ cell isolation was also done, was fixed in 10% neutral buffered formalin. A routine tissue processing procedure was then performed. Manual microtome (Leica RM 2125 RT) was used to take 5 µm thick sections from paraffin blocks of testicular tissue and transfer them onto the slide. Hematoxylin-Eosin staining procedure was then performed (Culling, 1963; Hinton, 1990). In order to determine the process of spermatogenesis and the cell types in this process, the samples were examined under light microscopy.

Immunofluorescent Applications

Immunofluorescence Marking of Cells from the Percoll Gradient Layer

The standard procedure for immunofluorescence labeling was used to distinguish germ cells from somatic cells (Linhartova et al., 2014; Psenicka et al., 2015). The middle layer testicular cells (50 µl) were taken on the slide and used for immunofluorescence marking processes. Samples fixed to the slide were washed with PBS and then treated with permeabilization buffer. After these procedures; samples were incubated with the primary antibody (DDX4) for 1 night at 4°C. It was then incubated with secondary antibody (FITC) at room temperature for 2 hours. After incubation, the cell nuclei were stained with DAPI at a concentration of 300 nM for 10 minutes at room temperature in order to mark them. The samples were then sealed with Dabco 33 and examined under an inverted microscope (Nikon Eclipse TI).

Procedures of Immunofluorescence Marking in Tissue Sections

Histological sections of testicular tissue were transferred to poly-L-lysine coated slides. The immunofluorescence method of tissue sections was performed by modifying the methods of Kayalar and Öztay (2014) and Psenicka et al., (2015). The samples were then sealed with a concealer (Mountat, Permafluor) and examined under an inverted microscope (Nikon Eclipse TI).

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Results

Findings of Isolation by Percoll Gradient Method

Germ cell isolation proved to be successful exclusively within the groups utilizing the PBS buffer. No observable gradient formation was observed in the groups involving HBSS and 0.9% NaCl. Therefore, in experiments, PBS-diluted percoll was used to separate testicular cells. Germ cell isolation was achieved at rates of 45%-10%, 50%-10%, and 55%-10% in percoll groups diluted with PBS buffer. After centrifugation, samples of percoll gradient layers (top, middle and bottom) were examined and photographed under a microscope.

The microscopic analysis unveiled that early-stage germ cells exhibited a spherical configuration and were characterized by one or more nuclei. In contrast, spermatids displayed a distinct spherical shape coupled with a substantial cytoplasmic presence. 50%-10% and 55%-10% percoll gradient upper layer debris, spermatids and a small amount of early stage germ cells (Figure 1); In the middle layer of 45%-10%, 50%-10% and 55%-10%, early stage germ cells and a small amount of spermatid were detected (Figure 2). In the pellet located in the bottom layer; erythrocytes, spermatids and debris were detected (Figure 3).

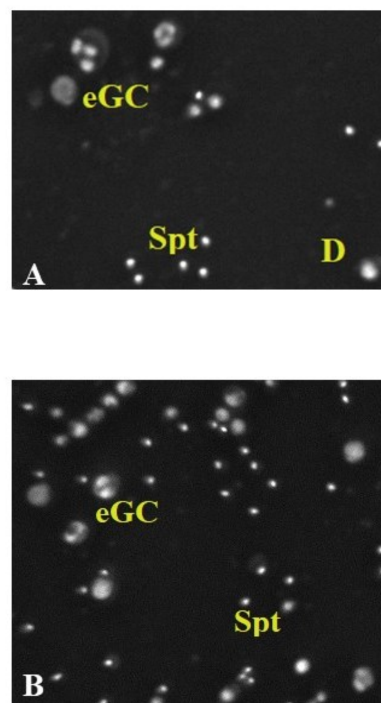

Figure 1. Upper layer images of percoll gradient ratios, A: 50%-10%, B: 55%-10% (Spt; spermatid, D; debris, eGC; early stage germ cells).

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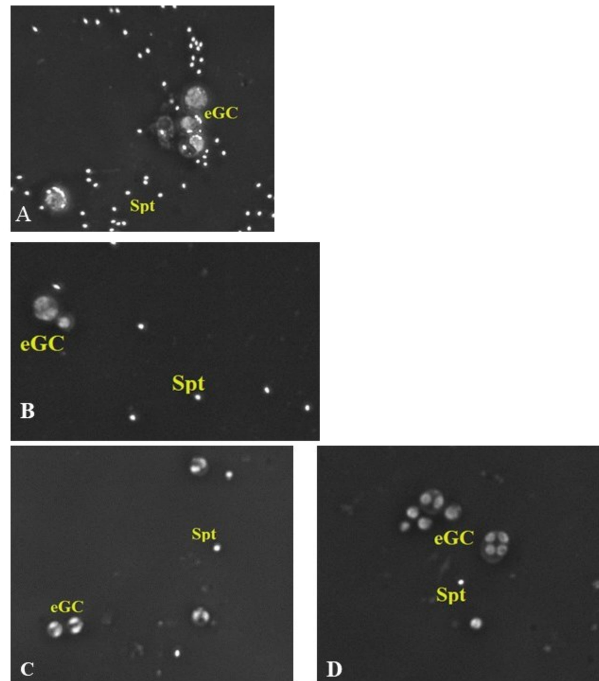

Figure 2. Mid-layer images of percoll gradient ratios, A: 45%-10%, B: 50%-10%, C, D: 55%-10% (Spt; spermatid, eGC; early stage germ cells).

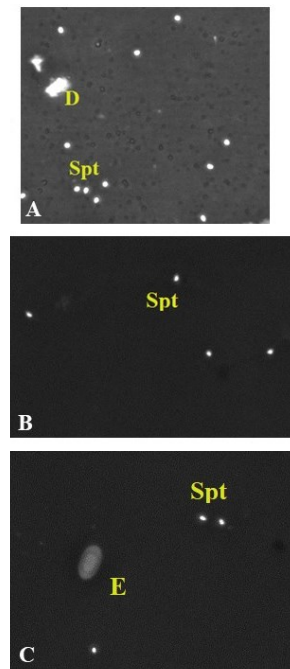

Figure 3. Bottom layer images of percoll gradient ratios, A: 45%-10%, B: 50%-10%, C: 55%-10% (Spt; spermatid, D; debris, E; erythrocyte).

Findings of Testicular Tissue Histology

In the histological sections of the testicular tissue of rainbow trout, three different cell forms were observed: Type A and Type B spermatogonia and spermatid. Type A spermatogonia have been found to be 5-6 µm in diameter. Type B spermatogonia have been found to have a diameter

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of 3 µm, spherical structure and one or more nuclei. It was determined that spermatids, which have a spherical structure and have a dark color, have a smaller morphology than Type A and Type B spermatogonia (Figure 4).

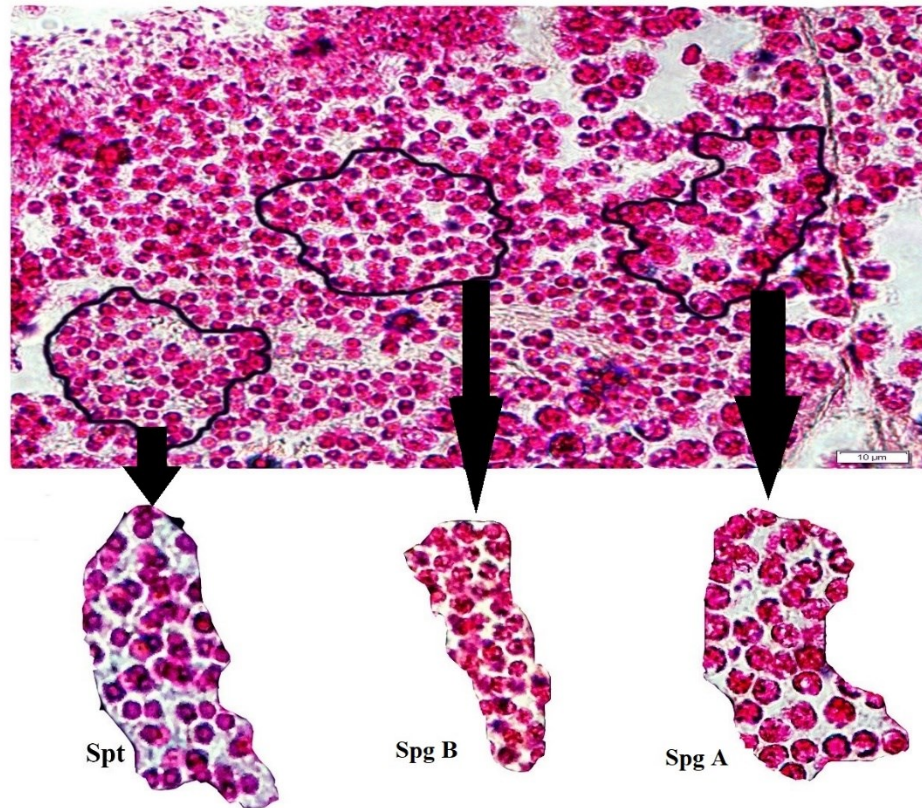

Figure 4. Histological section of rainbow trout testicular tissue, Spt; spermatid, Spg A; Type A spermatogonia, Spg B; Type B spermatogonia (Hematoxylin-Eosin staining,100x).

Findings of Immunofluorescence Applications

Findings of Immunofluorescence Marking of Percoll Gradient Layer Samples

Following the isolation process, the early germ cells that were identified unfortunately couldn't be successfully labeled using DDX4-FITC. In these instances, the images captured were solely those of cells that had been stained with DAPI. DAPI's ability to stain the nuclei of all testicular cells makes it impossible to obtain specific distinctions (Figure 5).

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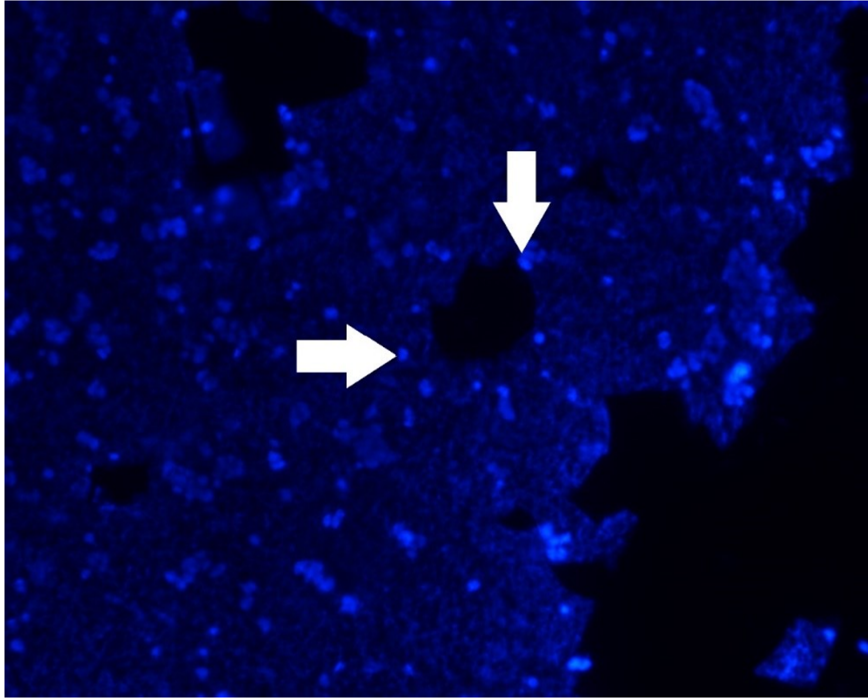

Figure 5. Image of testicular cell nuclei after staining with DAPI on immunofluorescent staining (indicated by arrows) (40x) (Nikon Eclipse TI).

Findings of Immunofluorescence Marking in Tissue Sections

As a result of staining the sections of rainbow trout testicular tissue with primary and secondary antibodies (DDX4-FITC), green early stage germ cells were detected. The cells were photographed under an inverted microscope (Figure 6).

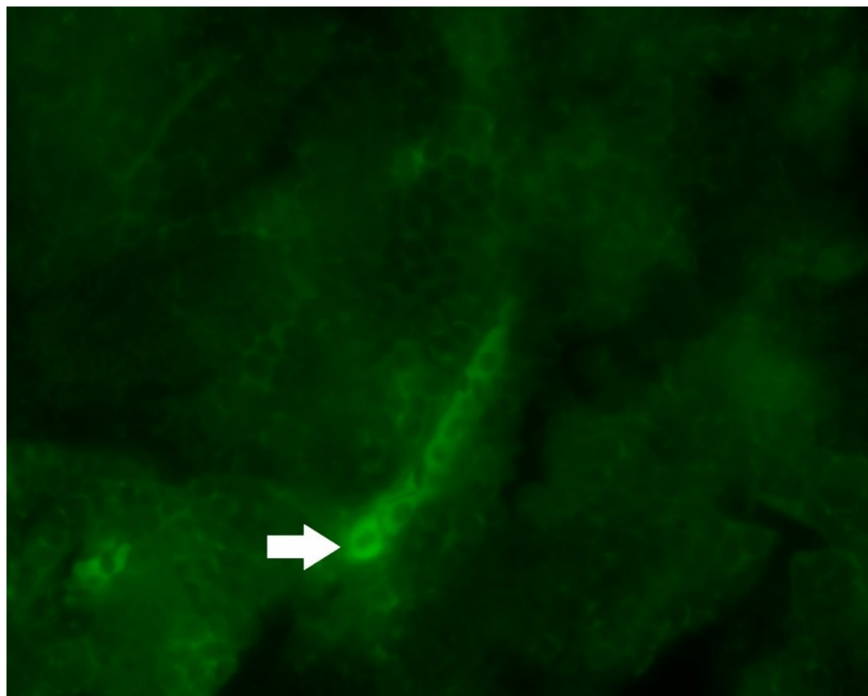

Figure 6. Image of testicular cells after staining with DDX4-FITC as a result of immunofluorescence application in tissue sections (shown with white arrow) (60x) (DDX4: 1-100 FITC: 1-400) (Nikon Eclipse TI).

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Discussion

One of the most prominent issues today are germ cell transplantation applications, which can be used for aquaculture species, in addition to protecting genetic resources. Germ cell isolation, which is the first step of this application, was performed using testicular tissue of rainbow trout in this study.

Lacerda et al. (2006) in their studies on the Nile tilapia (*Oreochromis niloticus*), they modified and used the method used by Bellve et al. (1997) for the separation of spermatogonial cells. In the study by Lacerda et al. (2006), testicular cells were obtained after enzymatic (collagenase, trypsin and DNase) separation. As a result of the separation of these cells with percoll gradient, four layers were formed. The first two layers were spermatogonia, the third layer was spermatocytes and spermatids, and the pellet in the lower layer was erythrocytes and spermatozoa. The percoll gradient ratios used in our study were taken as a reference to the rates used by Ercan and Ekici (2016) in their studies, and the lower layer-upper layer was formed as 45%-10%, 50%-10% and 55%-10% respectively. Three layers were observed after centrifugation. At the top of these layers, debris and spermatids; early stage germ cells were detected in the middle layer and erythrocytes and spermatids were detected in the pellet in the lower layer. These results were obtained by Linhartova et al. (2014) and Güngör (2015) are similar to the results obtained in their studies.

Psenicka et al. (2015) isolated early stage germ cells from gametes belonging to individuals of Siberian sturgeon (*Acipenser baerii*). They performed isolation of ovarian and testicular cells using Leibovitz medium (L-15), PBS and HBSS buffers. HBSS, physiological saline (0.9% NaCl) and PBS were utilized in this study, but isolation with a PBS buffer was the only way to achieve success. In this context, in the trials conducted for this study, only PBS was used as a buffer.

Linhartova et al. (2014) incubated testicular tissue samples using 0.1% trypsin and 0.1% collagenase enzymes for 1.5 h at 25°C in the isolation of early stage germ cells of tench (*Tinca tinca*) by the percoll gradient method. In our study, we used temperature, enzyme ratios and incubation time used by Linhartova et al. (2014). However; cellular separation could not be detected since the isolation conditions performed by Linhartova et al. (2014) in tench did not produce gradient formation in early stage germ cell isolation of rainbow trout in this study. Güngör (2015) conducted the enzymatic separation step by incubating testicular tissue samples of pikeperch (*Sander lucioperca*) with 0.3% trypsin enzyme for 1.5 hours at 25°C. In this study, cellular separation could not be performed because the temperature, enzyme ratios, incubation period and percoll gradient ratios applied by Güngör (2015) in the study did not provide gradient formation when used. In our study; It is thought that the failure to achieve success in the isolation conditions used by Güngör (2015) and Linhartova et al. (2014) may be due to the need for different isolation conditions depending on the species of fish. In isolation conditions, optimizations were made by decreasing the incubation temperature and increasing the enzyme ratio. Our study achieved successful results by using the enzyme ratios (Andriani et al., 2010 and 2012; Andriani, 2012) and incubation time (Adriani et al., 2010; Andriani, 2013) used in *Osphronemus gouramy* and *Marosatherina ladigesi* species. Based on these findings, it can be asserted that the enzymatic ratio and the incubation time used at the isolation stage are not dependent on the type of fish. Takeuchi et al. (2002) incubated rainbow trout PGH for 2 h at

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20°C using the enzyme 0.5% trypsin in enzymatic separation. In our study, successful results were obtained when these isolation conditions were used. Takeuchi et al. (2002) identified cells by forming a transgenic line. It is thought that the enzyme ratios (0.1% trypsin 0.3% collagenase, 0.05%, 0.1%, 0.4%, 0.6% trypsin) and incubation degree (25°C) used in our study are not suitable for rainbow trout and therefore successful results cannot be obtained.
Immunofluorescence staining of tissue sections revealed early stage germ cells marked with primary (DDX4) and secondary (FITC) antibodies under inverted microscopy (Figure 3.6). The formation of transgenic lines by marking the germ cells with vasa-GFP and the isolation of the cells by flow cytometry method are expensive and require a long process to implement. The percoll gradient method is preferable because it is relatively easier to apply and takes place in a shorter time than other methods.

Conclusion

In conclusion; with the percoll gradient centrifugation method, success has been achieved in the isolation of rainbow trout germ cells and the enzyme rates and temperature values used in the enzymatic decomposition stage. However, it was understood that optimization of percoll gradient ratios was needed for more intensive acquisition of early stage germ cells. The isolation of rainbow trout germ cells, which have the feature of differentiating into both gamete types was carried out. In the future, it is thought that cryopreservation and transplantation applications of these cells can be carried out as well as studies on the conservation of fish genetic resources and also the cultivation of alternative species.

Acknowledgements

We would like to express our endless thanks to Didem Demircan, Çiğdem Ürkü, Füsun Öztay, Özgecan Kayalar, Ece Sönmez, İlker Keskin, Ege Güngör and the staff of Sapanca Inland Aquaculture Production Research and Application Unit who did not spare their help in laboratory studies as well as scientific sharing during the study.

The data of this study include a part of the master's thesis project. This study was carried out with the experience gained from the courses on "germ cell isolation, cryopreservation and transplantation" organized within the scope of COST Action FA 1205-Aquagamete and supported by this action.

Ethical approval

The animal study protocol was approved by the Istanbul University Animal Experiments Local Ethics Committee dated 5/11/15 and numbered 2015/88.

Informed consent
Not available

Data availability statement
The authors declare that data can be provided by corresponding author upon reasonable request.

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Conflicts of interest
There is no conflict of interests for publishing this study.

Funding organizations

This study was supported by the Scientific Research Project Commission of İstanbul University (project ID number: 59902, dated: 2015; and project ID number: 20249, dated: 2017)

Contribution of authors
Author 1: Conceptualization, Investigation, Methodology, Visualization, Writing original draft.
Author 2: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Validation, Writing original draft, Review and editing.

"All authors have read and agreed to the published version of the manuscript."

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