

In vitro spermatogenesis of shark minnow fish (*Osteochilus hasselti* Valenciennes 1842) as a potential fish reproductive biotechnology

by Gratiana E Wijayanti

Submission date: 09-Apr-2023 12:20PM (UTC+0700)

Submission ID: 2059377510

File name: Umami_2020_IOP_Conf._Ser._Earth_Environ._Sci._457_012081.pdf (3.46M)

Word count: 5164

Character count: 28021

1

***In vitro* spermatogenesis of shark minnow fish (*Osteochilus hasselti* Valenciennes 1842) as a potential fish reproductive biotechnology**

M Umami^{1,2*}, Y Sistina¹ and G E Wijayanti¹

¹Faculty of Biology, Jenderal Soedirman University, Jl. dr. Suparno 63 Grendeng, Purwokerto, Banyumas, Central Java, 53122 Indonesia

²Departement of Biology Education, FITK IAIN Syekh Nurjati Cirebon, Jl. Perjuangan, Kesambi Cirebon, West Java, 45132, Indonesia

*Corresponding author: muhimatul.umami92@gmail.com

Abstract. *In vitro* spermatogenesis has many clinical applications and hopefully with improvement of research findings this technique will solve the applied fisheries era 4.0. This research aimed to evaluate the effects of testosterone and type of serum on the *in vitro* spermatogenesis of shark-minnow fish (*Osteochilus hasselti*). In the present research, four concentrations of testosterone (0, 5, 10 and 15 ng/mL) were tested. The types of serum used were autologous serum and Fetal Bovine Serum (FBS). The results showed that the use autologous serum in the culture medium capable of maintaining the consistency of the tissue culture in culture period and maintaining *in vitro* spermatogenesis of shark-minnow. This study shown that stage-specific of spermatogenic cells needed certain testosterone concentration. The stage of secondary spermatocyte needed testosterone concentration of 5ng/mL i.e. 50.81±9.29% in medium with autologous serum and 36.47±15.49% in medium with FBS, while stage of spermatid and spermatozoa needed testosterone concentration of 10ng/mL i.e. 36.66±19.81% and spermatozoa 43.45±23.44% in autologous serum and the proportion of spermatid i.e. 42.35±9.09% and spermatozoa 35.25±14.0% in medium with FBS. This study proven that *in-vitro* spermatogenesis, already demonstrated in shark-minnow fish, offered great promises to cope with reproductive issues in the aquaculture and applied fisheries biotechnology.

Keywords: Spermatogenic cells, *in vitro* spermatogenesis, reproductive biotechnology, *Osteochilus hasselti*.

1. Introduction

The development of spermatogenic cells in shark-minnow fish (*Osteochilus hasselti* Valenciennes, 1842) can be approached by *in vivo* or *in vitro* researches [1-3]. However, *in vivo* research was rendered by the needs to provide a large number of homogenous fish, and thus, *in vitro* model was adopted in this research. Information on the *in vitro* development of spermatogenic cells and concentration of testosterone required to support spermatogenesis in the shark minnow fish has not been available. Spermatogenesis regulation involves hormones such as Follicle Stimulating Hormone (FSH), Luteinizing Hormone (LH) and gonadal steroid hormones [4, 5]. Reports on roles of testosterone on spermatogenesis *in vitro* conditions have been available for some fishes, i.e. eel [6-8], gold fish [9], Japanese huchen [10]. Japanese eel (*Anguila japonica*) spermatogenesis, which was



Content from this work may be used under the terms of the Creative Commons Attribution 3.0 licence. Any further distribution of this work must maintain attribution to the author(s) and the title of the work, journal citation and DOI.

Published under licence by IOP Publishing Ltd

initiated by the 11-Ketotestosterone (11-KT; a typical androgen in fish), gonadotropin stimulate Leydig cells to produce 11-KT, then 11-KT activates Sertoli cells to facilitate all stages of spermatogenesis from spermatogonia proliferation to spermiogenesis [6-8]. 11-KT is an androgen hormone that is most effective in initiating spermatogenesis. 11-KT can induce all stages of spermatogenesis [11]. In this research, development of spermatogenic cells were observed for 36 days of culture in a medium containing 11KT 10 ng / mL. Nine days after culture, spermatogonia begin to proliferate. On the 18th day of the culture period, zygoten stage spermatocytes that experience meiosis I prophase can be observed. Furthermore, spermatids and spermatozoa begin to be observed on the 21st day of the culture period. On the 36th day of culture, all stages of spermatogenic cells can be observed [12]. In the Eel spermatogenesis, spermatogonia proliferation begins on the third day of the culture period, meiotic initiation about 2 weeks after the addition of 11-KT in the culture medium. The role of 11-KT in spermatogenesis has also been studied in goldfish (*Carassius auratus*) [9]. 11-KT hormone induces the initiation of spermatogenesis and spermiation in the testis fragment culture Japanese huchen (*Hucho perryi*). Furthermore, the 11-KT as steroid hormone has the ability to stimulate DNA synthesis, renewal of spermatogonia, and/or create spermatogonia proliferation *in vitro* conditions [10]. Another type of androgen hormones that affects spermatogenesis can include testosterone, 11 β -hydroxytestosterone, 5 β -dihydrotestosterone, dehydroepiandrosterone, androsteron [11-12].

Testosterone is the main androgen, which plays the role in spermatogenesis during the reproductive cycle of the male halibut (*Hippoglossus hippoglossus* L) [13]. Information on the *in vitro* development of spermatogenic cells and concentration of testosterone required to support spermatogenesis in the shark minnow fish has not been available, and thus, research is urgently needed. In addition, it is necessary to examine the involvement of micro environmental factors on the success of shark minnow fish spermatogenesis. These factors include the suitability of the medium, growth factors and culture period [14, 15]. The aims of this research were to evaluate effectiveness of *in vitro* induction of spermatogenesis, processes of spermatogenic cells development and to evaluate the effects of testosterone and type of serum on the shark minnow fish (*Osteochilus hasselti* Valenciennes, 1842) spermatogenic cells. In the present research, four concentrations of testosterone were tested, namely 0 ng/mL, 5 ng/mL, 10 ng/mL and 15 ng/mL using a randomized block design. The types of serum used were the autologous shark minnow serum and Fetal Bovine Serum (FBS) serum at a concentration of 10% (v/v).

2. Materials and methods

2.1. Materials

2.1.1. Experiment object: Male of shark minnow fish (*Osteochilus hasselti* Valenciennes 1842) of day 2 post spawning with weight 28 – 29 g and length 13 – 14 cm. Testes weight were 0.79 – 0.93 g and GSI 2.79%.

2.1.2. Materials. Testosterone (17 α -Methyl Testosterone; Polaris), aquabidest, GnRH analog plus Domperidone (Ovaprim, Syndel Laboratory, Vancouver), Fetal Bovine Serum (FBS, Cai 27n Labs), autologous serum, antibiotic (Penicillin Streptomycin, Gibco no. 15140), L-12 glutamin, Dulbecco's Modified Eagle Medium (DMEM, Gibco), Purified Agar (Oxoid, L28), Dulbecco's Phosphate-Buffered Saline solution (DPBS) pH 7.4, steril aquadest and clean paper.

2.1.3. Materials for histology preparat. Bouin solution as fixative, alcohol 70%, alcohol 80%, alcohol 90%, alcohol 100% as dehydran, Xylol (or xylene) solution as clearing agent, paraffin (Sigma, p3558), aquadest, gelatin 1%, stain Carazzi's Hematoxylin, eosin 1% aquosa, and mounting agent as Entellan new. The research equipment for preparation of shark minnow fish testes fragments included digital scales accurate to 0.01 g, Graph paper, dissecting set. The equipment for the made of fish serum was 1 mL injection syringe and needle size 26 G x 1/2" (Terumo Syringes), centrifuge (Eppendorf model

5415). Equipment for fragment culture included sterile tweezers, scalpel blades, spatula, 24-well plates, cell culture dishes 35 x 10 mm, petri dish, sterilizers, Laminar Air Flow (LAF), CO₂ incubator (Binder CB-150), mercury thermometer, falcon tube 15 mL and 50 mL (Conical centrifuge tube. Fisher Scientific), micropipette with tip (Eppendorf), masker, gloves and alcohol 70%. Equipment for made histology preparations included scissors, tweezers, sample bottles, fixative bottles, clean paper, stopwatch, dropping pipette, Glass beaker 50 mL and 100 mL (Pyrex, Asahi glass), incubator oven, paraffin block mold made of cardboard paper with size 1.5 x 1.5 x 1.5 cm, staples, specimen holder, spiritus lamp, label, rotary microtome, and cutter. Equipment for affixing and staining: Paintbrush, hot plate, object glass, cover glass, staining jar and label. Equipment for data collection: light microscope (Olympus Cx40), eye piece micrometer, eyepiece graticule grid, digital camera, digital microscope (Optilab Profesional: Miconos) and software of ImageJ 1.46r.

2.1.4. Preparation of culture environment. The dissecting set, microcentrifugation tubes 1.5 mL, falcon tube, glass petri dish and micropipette tip were sterilized by using autoclave at a temperature of 121°C, 2 atm for 30 minutes, while the dropping pipette was sterilized in a sterilizer. Micropipette and test tube rack were sterilized with tissue paper which had been spread with 70% alcohol. The culture room was sterilized a day before use by irradiating using a UV lamp for 12 hours.

2.2. Research material preparation

2.2.1. Boiun's solution [16]. Made of 75 mL saturated aqueous picric acid, 25 mL formalin (40% formaldehyde) and 5 mL of glacial acetic acid.

2.2.2. Stain Carazzi's Haematoxylin [17]. This solution was prepared by dissolving 0.5 g Haematoxylin in 100 mL glycerol, 0.01 g Potassium Iodate 25 mL of the distilled water and 25 g potassium alum in 375 distilled water. The haematoxylin and potassium alum solutions were mixed then carefully the potassium iodate solution was added to the mixture. The staining solution was filtered through a filter paper. The haematoxylin solution can be used immediately, it lasted for about three months [18].

2.2.3. Eosin Y 1% stain. Eosin Y 1 g was dissolved in 100 mL distilled water, stirred up to homogeneous and then filtered with a paper filter. Solution must be kept at room temperature until use.

2.2.4. Handling medium and culture medium formulation [19]

1) Handling medium (1000 µL):	2) Culture medium (for 1000 µL):
DPBS 950µL	DPBS..... 800µL
Antibiotic..... 50µL	Serum..... 100µL
	Antibiotic..... 50µL
	L-glutamine..... 50µL

2.2.5. Agar solution 1%: Agar solution 1% 10 mL (v/v) make from dissolved 0.1 g purified agar to the distilled water 100 mL (v/v). The mixture was heated to boiling and the agar solution ready to use.

2.3. Procurement of shark minnow serum [20]

Fish serum was obtained from male shark minnow fish with an average body weight of 53.45 ± 16.78g and GSI 8.05 ± 5.26%, blood was taken from the caudal vein at 8 – 12 a.m. The blood was collected in a microcentrifugation tube 1.5 mL and allowed the blood to clot at room temperature for 15-30 minutes then stored in the refrigerator for 8-24 hours to optimize blood clotting. Blood clots and serum were centrifuged at 3000 rpm for 10 minutes. The serum was taken using a micropipette, then put into

a microcentrifuge tube and labelled, then the serum was stored in the freezer until the use. Serum thawed and filtered using sterile syringe 0.2 μm filter before being used for culture.

2.4. Testicular fragment preparation

Male shark minnow fish was weighed, then the fish paralyzed with the damage the nerve tissue. The abdominal wall was cleaned using alcohol 70% swab. The abdominal part was dissected using a sterile surgical technique, the testes and liver were isolated, weighed and recorded to determine aspect the Gonadosomatic Index (GSI) and Hepatosomatic Index (HSI). The testes were washed using DPBS solution to remove blood and were placed in a sterile Petridish containing handling medium to prevent the testes from dehydration. The testes were brought into the culture room and cut in a sagittal orientation followed by transversally orientation to form fragments of $\pm 2 \text{ mm}^3$ in size as many as 48 pieces of fragments were used for culture (figure 1).

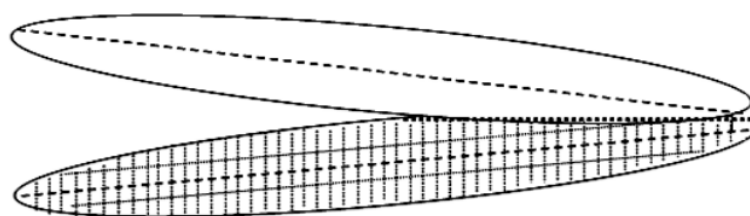


Figure 1. Scheme of direction for cut off testicular fragments of shark minnow fish (*O. hasselti* Valenciennes, 1842) for *in vitro* spermatogenesis.

2.5. Organ culture system preparation:

A 1% agar solution was poured into a petri dish, and allowed to solidify for ± 10 minutes, the sterile blue pipette tip were inserted with the wide opening facedown, on the top of the agar to create circular agar block (figure 2.1). A concave surface was made at the center of each agar block using a concave spatula with diameter $\pm 3 \text{ mm}$ (figure 2.2). The agar block were the put into 24 well-plate. The tested hormone was added to the culture medium. The well-plates containing medium and hormones were equilibrated for 1 hour in the CO_2 incubator.

2.6. Testes fragment culture

The testes fragment was put on concave surface of the agar in the equilibrated culture medium. The testes fragments were incubated at the temperature of 28°C , O_2 95% and CO_2 5%. Sterile aquadest was filled into the most left and right of the well plate to maintain humidity. Aseptic technique was performed throughout the experimental procedure.

2.7. Culture condition monitoring

Culture condition was monitored by observing the temperature of incubator and colour of the culture medium. Culture was good if medium colour was pink and pure colour (similar to the first culture condition). Culture medium was refreshed every third days by replacing 80% culture medium. In addition, testes fragment was also observed on the microscope. The testes fragment was good indicated consistent of tissue's well. Development of spermatogenic cells evaluated on day 5th, 10th, and day 15th of culture.

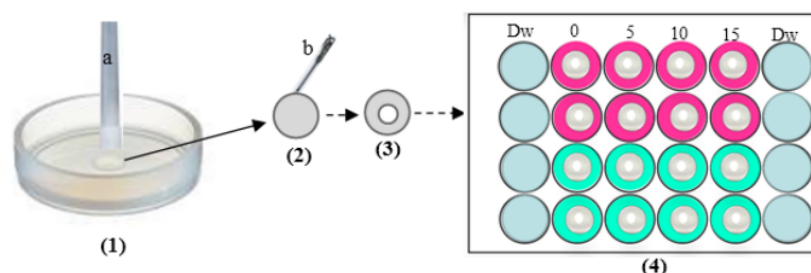


Figure 2. Diagrams showing organ culture system preparation; (1), (2) and (3) = the step of make agar block; (4) = step of equilibration medium and hormone in 24 well-plates; Dw = Distilled water; 0, 5, 10 and 15 ng / mL showed the testosterone concentrations tested; The pink color indicated the treatment group with the addition of FBS; The green color showed the treatment group with the addition of shark minnow fish serum

2.8. Histology preparation with modification [21]

The cultured testes fragments¹⁷ was harvested on day 5th, 10th, and 15th day of culture, washed 3-5 times with DPBS medium, then were fixed in Bouin's solution in 4 hours at room temperature¹⁸. The testes fragments were processed for standard paraffin embedded section, cut at 5µm then sections were stained with Carazzi's Haematoxylin and Eosin.

2.9. Mitotic activity identification

Mitotic activity was identified based on the result of the histological evaluation that referring to the criteria of mitotic cells [22]. The mitotic index (Y) was identified by observing cells in histological samples, and the percentage was calculated by calculating the number of spermatogonia undergoing mitotic division by the total number of spermatogonia found, then multiplied by 100%. In each sample, 5 sections were evaluated, each slice was evaluated in 5 view fields so that each sample was evaluated as many as 25 fields of view.

2.10. The proportion of lobular area and number of cells

Morphology, cell number and proportion²³ of lobules area containing stage-specific spermatogenic cells were evaluated histologically. Stages of spermatogenic cells included spermatogonia, spermatocytes, spermatids and spermatozoa in histological preparations were evaluated using a digital microscope (Optilab Professional: Miconos). The identification of spermatogenic stages was done by comparing the description of spermatogenic cells in *Cyprinidae* fish [23]. The proportion of lobules area occupied by each particular spermatogenic stage (X) was calculated using a calibrated ocular graticule using the Cavalieri's principle with modification [24]. The number of squares in graticule, occupied by the lobules area with stage-specific spermatogenic cells was calculated as followed: condired as 1 if fully (1) or > ½ of the square is occupied by lobules area; as ½ if ½ of the square is occupied by lobules area; as ¼ if ¼ of the square is occupied by lobules area and as 0 if <¼ of the square is occupied by lobules area, then use formula as below it:

$$X = \frac{\sum \text{Lobula area with stages of spermatogenic cells}}{\sum \text{lobula area observed}}$$

In addition, to the proportion of lobules area with stage-specific¹⁷ of spermatogenic cells, the number of spermatogenic cells of each stage in each lobules was calculated. The cell count was calculated by positioning the eyepiece graticule grid in the lobe that the cell counts. If the number of eyepiece graticule grid surrounding the lobe was less than 3 squares, thus the number of cells in each squares

was calculated and then averaged and the results are recorded in the number of cells / mm². If the number of squares that covered the lobules were counted for more than 3 squares, 3 squares were randomly selected, then the number of cells in each square was calculated, then averaged and the results were noted in the number of cells / mm².

2.11. Analysis

Data of mitotic index and proportion of spermatogenic cells in each lobules were analyzed using two-way ANOVA at 95% accuracy level [25]. The analysis was carried out using SPSS software version 18.0, while morphological data of stage of spermatogenic cell developmental and post-culture development based histological feature were analyzed descriptively.

4

3. Results and discussion

3.1. Development of testis fragment post-culture

Testicular fragments cultured with various combinations of testosterone concentration and type of serum in the culture medium can grow well. Evaluation on day 15th of culture showed that there was an out grow of the cells at the periphery of the fragment and the testicular fragment tend to flatten. The colour of the culture medium indicated that the culture condition was suitable to support the culture. This is in accordance with the statement of other researchers that changes in fragment morphology and cell polarity are influenced by the existing of epithelial cells cultured and nutrient supply Basolaterally [14]. Testicular fragments cultured for 15th days on a medium containing autologous serum had more compact fragments than fragments cultured on medium containing FBS (figure 3).

The results showed that the autologous serum was better in supporting the development of shark-minnow spermatogenic cell development compared to FBS. The different components and the concentration of each component in both types of serum possibly cause variations in culture results. Serum from the same organism as the source of fragments can support culture growth better than serum from other organisms. The addition of human serum in a culture medium results in a better human cartilage than that in the medium supplemented by FBS [26]. The addition of 0.25% salmon serum in the medium culture of spermatogonia cell of rainbow trout (*Oncorhynchus mykiss*) can improved the cell viability, the number of cells and spermatogonia mitotic activity during *in vitro* spermatogenesis [27]. Testicular fragment tissue of mice is cultured in α -Minimum Essential Medium (α -MEM) medium plus 10% FBS shows spermatogenesis activity with release of spermatids. However, it is not found in fragments cultured with medium without FBS [28].

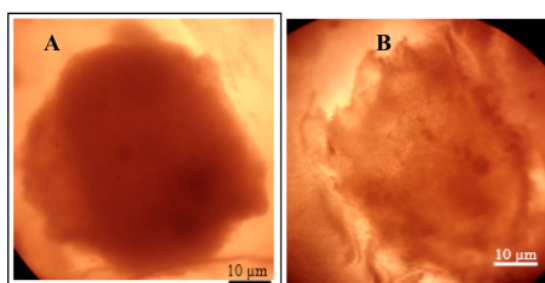


Figure 3. Micrograph of Testicular fragment of shark minnow fish (*O. hasselti* Valenciennes 1842) that cultured in autologous serum (A) and FBS (B).

3.2. Mitotic index of spermatogenic cells

Assessment from result of the histological preparations from pre-culture testicular fragments group shown an average of mitotic spermatogonia cells $20.85 \pm 15.49\%$ and an average of spermatogonia

cells that are not mitotic $79.15 \pm 15.53\%$. Statistical analysis showed that mitotic activity in all fragments were no significant different or equal ($p > 0.05$). After the 5th day of culture, the testicular fragment with the addition of FBS in the medium showed that spermatogonia cell activity was not mitotic, with the number of spermatogonia are $0.86 \pm 1.26 \times 10^3$ cell / mm². The results of fragments cultured on a medium contain autologous serum, however, showed that spermatogonia cells could be observed undergoing cell division (figure 4), the average mitotic index at testosterone concentration of 0 ng/mL was $30.47 \pm 31.03\%$, at concentration of 5 ng/mL was $33.45 \pm 27.92\%$, at concentration of 10 ng/mL was $37.62 \pm 22.06\%$ and at concentration of 15 ng/mL was $40.12 \pm 23.01\%$. These data shown that the higher the concentration of testosterone added to the culture medium, is the higher the mitotic activity of spermatogonia (figure 5). However, all treatments were not significantly different ($p > 0.05$). Similar studies on eel (*A. japonica*) testicular fragments cultured on a medium contained 11-KT with a concentration of 10 ng/mL show proliferative activity through mitotic division type A spermatogonia to become late type B spermatogonia on day 9 of culture indicated mitosis 50-70% of cyst [1]. Thus, the addition of testosterone on the 5th day of culture shows that the result of culture are relatively the same as that of the 9th day culture with the addition of 11-KT. In short, testosterone is relatively more effective than 11-KT which gives a shorter time of 4th days compared to 11- KT.

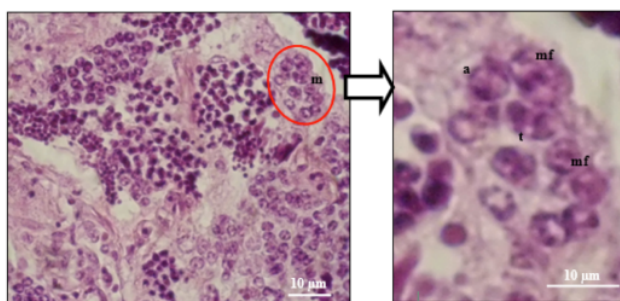


Figure 4. Micrograph of testicular fragments of shark minnow fish (*O. hasselti* Valenciennes, 1842) with Paraffin method and Hematoxylin-Eosin stain. m = mitotic phase of spermatogonia cells; mf = metaphase; a = anaphase; t = telophase.

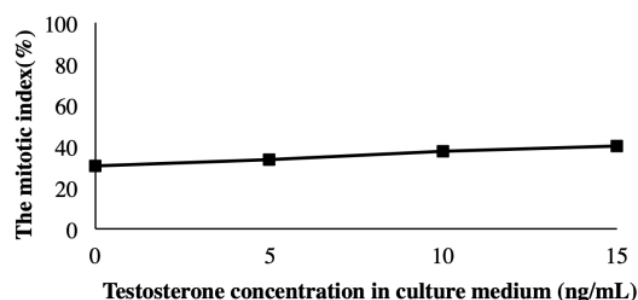


Figure 5. The mitotic index of shark minnow spermatogonia (*O. hasselti* Valenciennes 1842) cultured on day 5th on medium contained autologous serum and various testosterone concentrations.

3.3. Proportion of lobules area with stage-specific spermatogenic cells

Based on histological observation, the spermatogenic stages can be observed, including type A spermatogonia, type B spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids, and spermatozoa (figure 6). Type A spermatogonia has the largest size compared to other spermatogenic cells, large and homogeneous nuclei contain one or two nucleoli (figure 6A). Type B spermatogonia is a spermatogenic cell with more advanced cell stages, cell size is smaller than type A spermatogonia but larger than other spermatogenic cells, these cells formed a cyst of two or four cells surrounded by Sertoli cells (figure 6B). Primary spermatocytes have the character of round cells with a clear cytoplasm, the nucleus is relatively smaller than the spermatogonia nucleus (figure 6C). Secondary spermatocytes have a round shape and are smaller than primary spermatocytes, the nucleus looks more colorful and located at the periphery of the cell. Therefore, it has a chromosome configuration like an umbrella (figure 6D). Spermatids are round in shape, smaller than the spermatogonia and spermatocytes, and have heterogeneous nuclei (figure 6E). Spermatozoa have the smallest size than that of other spermatogenic cells, they have round shape of cells with condensed nucleus. Therefore, it is more violet and has a tail (figure 6F).

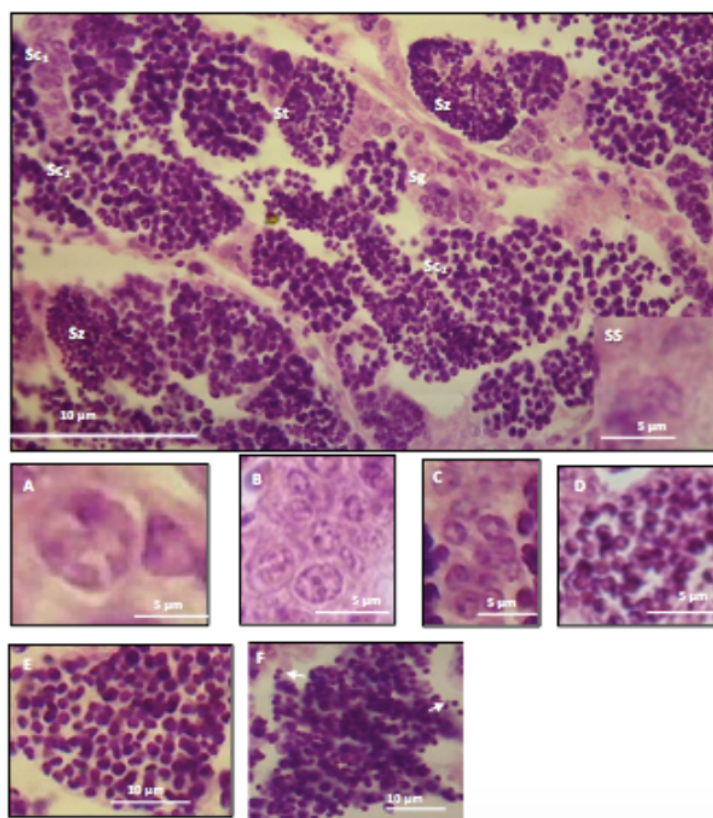


Figure 6. Micrograph of testicular fragments of Shark minnow fish (*O. hasselti* Valenciennes, 1842) cross-cut after paraffin method and stained with Hematoxylin-Eosin. A(Sg_A) = Spermatogonia A; B(Sg_B)= Spermatogonia B; C(Sc₁) = Primary Spermatocytes; D(Sc₂) = Secondary Spermatocytes; E(St) = Spermatids; F(Sz) = Spermatozoa (the arrow shows the spermatozoon's head); SS = Sertoli cells.

The proportion of spermatogenic cells in the pre-culture is the same which is dominated by secondary spermatocytes for $34.47 \pm 3.56\%$ (figure 7). On the day 5th, the addition of testosterone in the culture medium increased the proportion of lobules with the stage-specific of spermatogenic cells ($p < 0.05$) (figure 8). The stage of secondary spermatocytes needed concentration of testosterone of 5 ng/mL i.e. $50.81 \pm 9.29\%$ in medium with autologous serum and $36.47 \pm 15.49\%$ in medium with FBS, while stage of spermatid and spermatozoa needed concentration of testosterone of 10 ng/mL i.e. $36.66 \pm 19.81\%$ and spermatozoa $43.45 \pm 23.44\%$ in autologous serum and the proportion of spermatid i.e. $42.05 \pm 9.09\%$ and spermatozoa $35.25 \pm 14.01\%$ in medium with FBS, and then stage of spermatid needed concentration of testosterone 15 ng/mL i.e. $43.62 \pm 12.12\%$ in medium with FBS and the proportion of spermatozoa $48.60 \pm 14.00\%$ in medium with autologous serum. It was short, and the development of spermatogenic cells need the testosterone concentration for stage-specific spermatogenic cells during process *in vitro* spermatogenesis. These results support previous report that various dissolved components include testosterone added to the culture medium capable entering it through cell fusion [29]. Testosterone is accepted by receptors on the Sertoli cells, then the presence of paracrine interactions between Sertoli cells and spermatogenic cells can facilitate the process of spermatogenesis [30].

The results of histological evaluation show that administration of autologous serum into the medium, lead to a higher percentage of spermatogonia ($0.63 \pm 0.8\%$) compared to FBS ($0.07 \pm 0.10\%$). The t-test analysis shows that the type of serum given a significant effects on shark minnow spermatogenesis ($p < 0.05$). This indicates that autologous serum and FBS have different activities in the testicular fragment culture *in vitro*. Autologous serum contains testosterone which plays a role in maintaining *in vitro* spermatogenesis.

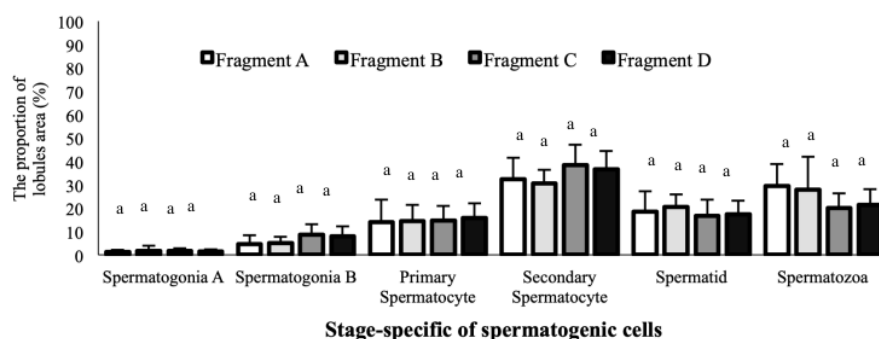


Figure 7. The proportion of lobules area with stage-specific of spermatogenic cells of shark minnow (*O. hasselti* Valenciennes, 1842) testicular fragments at pre-culture conditions ($p > 0.05$).

The shark minnow testes fragments on day 10th and 15th of cultured shown that the observed testes fragments are predominantly spermatozoa cells in each lobe despite poor environmental conditions for the development of spermatogenic cells. It was marked on several fragments after histology evaluation makes some fragments indicating the condition of the testicular lobes that are not intact. It was short, culture period and other factors influenced development of shark minnow fish spermatogenic cells *in vitro* conditions. The serum added in the culture medium can be damaging in the long term cultured tissue [31]. The culture period of up to 15th day causes the microenvironment most likely unable to support spermatogenesis of shark minnow fish further *in vitro* conditions. In the culture period of the 10th and 15th day, spermatozoa have possibly developed, but due to physiological conditions, it was not used to fertilize the oocytes, causes the spermatozoa to die, and it effects the quality of culture. Thus, it is necessary to conduct further investigation on *in vitro* for the duration of spermatogenesis of shark

minnow fish that is from the stage of spermatogonia until spermatozoa. In addition, the analysis of serum content of shark minnow fish also needs to be carried out to determine other factors such as growth factors that play a role during *in vitro* spermatogenesis.

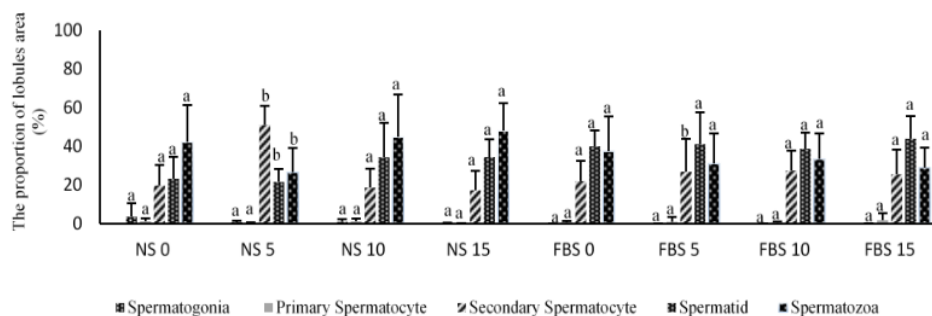


Figure 8. The proportion of lobules area with stage-specific of spermatogenic of shark minnow (*O. hasselti* Valenciennes 1842) testicular fragments on the 5th day of culture with various testosterone concentrations (0; 5; 10 and 15 ng / mL) added and different type of serum (autologous serum (NS) and FBS) ($p < 0.05$).

4 Conclusion

In vitro spermatogenesis in shark minnow fish (*O. hasselti* Valenciennes 1842) can be carried out well for 5th days culture with testosterone at the concentration of 5 ng/mL plus autologous serum better than FBS to get spermatocyte stage. Further culture period of 10th and 15th days with higher testosterone concentration of 10 ng/mL gave spermatozoa stages, possibly required more optimal culture environment. Shark minnow fish (autologous) serum added in the culture medium can maintain better *in vitro* spermatogenesis than FBS. Since the Spermatogenesis *in vitro*, is in place, so this offers a great promise to be applied in solving reproductive issues in aquaculture and/or fisheries biotechnology.

Acknowledgements

This research was funded by Basic Incentive Research Strategic National Scheme of The Directorate General of Higher Education 2013 to G.E. Wijayanti.

References

- [1] Supiyanto 2013 *Effect of Progesterone and GnRH Analog on Spermatogenic Activity in shark minnow (Osteochilus hasselti C.V)* (Purwokerto: Jenderal Soedirman University) (In Indonesian Language)
- [2] Gassei K and Schlatt S 2007 *Annals. New York. Academy. Sci.* **1120** 152–167
- [3] Wijayanti G E and Sugiharto 2006 *Omni. Akuatika.* **1** 37-43 (In Indonesian Language)
- [4] Nagahama Y 1994 *Int. J. Dev. Biol.* **38**, 217-229
- [5] Huhtaniemi I T and Themmen A P 2005 *Endocrine.* **26** 207–217
- [6] Miura T, Yamauchi K, Takahashi H and Nagahama Y 1991 *PNAS.* **88** 5774-5778.
- [7] Miura C, Miura T, Yamashita M, Yamauchi K and Nagahama Y 1996 *Dev. Growth. Differ.* **38** 257-262
- [8] Miura C, Miura T, Kudo N, Yamashita M and Yamauchi K 1999 *Dev. Growth. Differ* **41** 463-471
- [9] Kobayashi M, Aida K and Stacey N E 1991 *Zoo. Sci* **8**(2) 389-393
- [10] Amer M A, Miura T, Miura C and Yamauchi K 2001 *Biol. Reprod* **65** 1057-1066
- [11] Miura C and Miura T 2011 *Aqua-BioSci. Monographs.* **4** 105–129
- [12] Miura T, Yamauchi K, Takahashi H and Nagahama Y 1992 *J. Exper. Biol.* **261** 359–366

- [13] Weltzien F A, Taranger G L, Karlsen Ø and Norberg B 2002 *Comparative Biochemistry and Physiology Part A: Mol. Integrative. Physiol.* **132**, 567-575
- [14] Freshney R I 2005. *Culture of Animal Cells: A manual of Basic Technique* 5th ed (San Fransisco: John Wiley and Sons Inc)
- [15] Brunner D, Frank J, Appl H, Schöffl H, Pfaller W and Gstraunthaler G 2010 *Altex.* **27** 53-62
- [16] Preece A 1972 *A Manual for Histologic Technicians* 3rd ed (Boston: Little Brown & Co)
- [17] Avwioro G 2011 *J. Pharm, Clin, Sci.* **1** 24-34.
- [18] Llewellyn B D 2013 *Hematoxylin Formulae*. <http://stainsfile.info>
- [19] Wijayanti G E 2003 *Pre and Post Natal Development of the Germ cells in a Marsupial Tammar wallaby* (Melbourne: The University of Melbourne)
- [20] Wijayanti G E and Soeminto 2007 *Reproduction performance of shark minnow fish (Osteochilus hasselti C.V.) under photoperiod manipulation* (Purwokerto: Jenderal Soedirman University (In Indonesian Language)
- [21] Suntoro S H 1988 *Histological and Histochemical Staining Methods* (Jakarta: PT. Bhratara Aksara) (In Indonesian Language)
- [22] Sommer C, Fiaschi L, Hamprecht F A and Gerlich D W 2012 *IEEE Proceeding* 2306-09
- [23] Takashima F and Hibiya T 1995 *An Atlas of Fish Histology. Normal and Pathological Features* Second Edition (Tokyo: Gustav Fischer Verlag) pp: 146-149
- [24] Michel R P and Cruz-Orive L M 1988 *J Micros* **150** 117-136
- [25] Steel R G. and Torrie J H 1993 *Principles and procedures of statistics, a biometrical approach 2nd edition* (Jakarta: PT Gramedia Pustaka Utama) (In Indonesian Language)
- [26] Chua K H, Aminuddin B S, Fuzina N H and Ruszymah B H *Sin. Med. J.* **48** 324-332.
- [27] Shikina S and Yoshizaki G 2010 *Biol. Rep.* **83** 268-276.
- [28] Sato T, Katagiri K, Gohbara A, Inoue K, Ogonuki N, Ogura A, Kubota Y and Ogawa T 2011 *Nature* **471**(7339) 504-507
- [29] Brunner D, Frank J, Appl H, Schöffl H, Pfaller W and Gstraunthaler G 2010 *ALTEX.* **27** 53-62
- [30] Skinner M. K and Fritz I B 1985 *PNAS.* **82** 114-118
- [31] Creemers LB, Ouden KD, Van Pelt AM and De Rooij DG 2002 *Reproduction.* **124** 791-799

In vitro spermatogenesis of shark minnow fish (*Osteochilus hasselti* Valenciennes 1842) as a potential fish reproductive biotechnology

ORIGINALITY REPORT

9%

SIMILARITY INDEX

8%

INTERNET SOURCES

5%

PUBLICATIONS

%

STUDENT PAPERS

PRIMARY SOURCES

1

www.researchgate.net

Internet Source

3%

2

research.aalto.fi

Internet Source

1%

3

www.qucosa.de

Internet Source

<1%

4

www.esb.ucp.pt

Internet Source

<1%

5

Sureshkumar, S.V.. "Hepatoprotective effect of extracts from *Pergularia daemia* Forsk.", *Journal of Ethnopharmacology*, 20060919

Publication

<1%

6

etd.auburn.edu

Internet Source

<1%

7

repo.knmu.edu.ua

Internet Source

<1%

8

josr-online.biomedcentral.com

Internet Source

<1 %

9

"Meeting abstract", In Vitro Cellular & Developmental Biology - Animal, 1991

Publication

<1 %

10

joe.endocrinology-journals.org

Internet Source

<1 %

11

N. Sofikitis. "Efforts to create an artificial testis: culture systems of male germ cells under biochemical conditions resembling the seminiferous tubular biochemical environment", Human Reproduction Update, 03/24/2005

Publication

<1 %

12

espace.library.uq.edu.au

Internet Source

<1 %

13

webmineral.com

Internet Source

<1 %

14

Mochida, K.. "Effects of endocrine-disrupting chemicals on expression of ubiquitin C-terminal hydrolase mRNA in testis and brain of the Japanese common goby", Aquatic Toxicology, 20041118

Publication

<1 %

15

journal.isv.org.ir

Internet Source

<1 %

16	Chiemi Miura, Takeshi Miura, Nozomi Kudo, Masakane Yamashita, Kohei Yamauchi. "cDNA cloning of a stage-specific gene expressed during HCG-induced spermatogenesis in the Japanese eel", Development, Growth and Differentiation, 1999 Publication	<1 %
17	Tomomi Nishie, Aika Komaru, Syota Shiroguchi, Takako Yamaizumi et al. "Nonylphenol reduced the number of haploids in in vitro spermatogenesis of the endangered cyprinid <i>Gnathopogon caerulescens</i> ", Toxicology in Vitro, 2023 Publication	<1 %
18	archive.org Internet Source	<1 %
19	bilbo.edu.uy Internet Source	<1 %
20	complete.bioone.org Internet Source	<1 %
21	dspace.jcu.cz Internet Source	<1 %
22	scholars.wlu.ca Internet Source	<1 %
23	www.oncotarget.com Internet Source	<1 %

24

T. Miura, M. Higuchi, Y. Ozaki, T. Ohta, C. Miura. "Progestin is an essential factor for the initiation of the meiosis in spermatogenetic cells of the eel", Proceedings of the National Academy of Sciences, 2006

Publication

<1 %

25

Takeshi Miura, Nobutaka Ando, Chiemi Miura, Kohei Yamauchi. " Comparative Studies between and Spermatogenesis of Japanese Eel (*Anguilla japonica*) ", Zoological Science, 2002

Publication

<1 %

26

"Eel Biology", Springer Nature, 2003

Publication

<1 %

27

Gaye Çetinkaya, Anıl Sera Kahraman, Menemşe Gümüşderelioğlu, Sezen Arat, Mehmet Ali Onur. "Derivation, characterization and expansion of fetal chondrocytes on different microcarriers", Cytotechnology, 2011

Publication

<1 %

Exclude quotes

On

Exclude matches

< 5 words

Exclude bibliography

On