The Journal of Applied Science **Vol. 19 No. 2: 1-10 [2020]**
วารสารวิทยาศาสตร์ประยุกต์ doi: 10.14416/j.appsci.2020.02.001
ISSN 1513-7805 Printed in Thailand ISSN 2586-9663 (Online)

Research Article

Ultrastructure of the granulopoietic cells in head kidney of the Barbour's seahorse, *Hippocampus barbouri* Jordan & Richardson, 1908 in captivity

Sinlapachai Senarat[1,*], Jes Kettratad[2,3], Chanyut Sudtongkong[1], Theerakamol Pengsakul[4], Pisit Poolprsert[5], Koraon Wongkamhaeng[6], Woranop Sukparangsi[7]

[1]Department of Marine Science and Environment, Faculty of Science and Fisheries Technology, Rajamangala University of Technology Srivijaya, Trang Campus, Sikao, Trang, 92150
[2]Department of Marine Science, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand
[3]Marine Ecology and Marine Resources Utilization Research Unit, Aquatic Resources Research Institute, Chulalongkorn University, Bangkok 10330, Thailand
[4]Faculty of Medical Technology, Prince of Songkla University, Songkhla, 90110, Thailand
[5]Program of Biology, Faculty of Science and Technology, Pibulsongkram Rajabhat University, Phitsanulok 65000, Thailand
[6]Department of Zoology, Department of Zoology, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand
[7]Department of Biology, Faculty of Science, Burapha University, Chon Buri 20131, Thailand
***E-mail**: Senarat.s@hotmail.com; Sinlapachai.s@rmutsv.ac.th
Received: 28/04/2020; Revised: 13/10/2020; Accepted: 25/10/2020

Abstract

The Barbour's seahorse, *Hippocampus barbouri* is one of the vulnerable-category species based on IUCN's Red List (CITES Appendix II); therefore, *H. barbouri* has been in focus for conservation aquaculture in Thailand. Since morphologic or structural alterations of hematopoietic cells of fish under captive system have not been extensively documented, here the ultrastructure of the granulopoietic series in the head kidney of aquacultured *H. barbouri* was initially revealed through transmission electron microscopy (TEM). Results from our finding presented that the granulopoietic cells of this seahorse could be classified into six sub-series including myeloblast, promyelocyte (two sub-stages: early and late promyelocytes), myelocyte, metamyelocyte and mature granulocyte. The highest proportion of granulopoietic cells among these six sub-series was the late promyelocyte. Noticeably, the first appearance of granule like-structure was identified in the early promyelocyte and this granular structure greatly accumulated in the mature granulocyte. This study also offers an insight into developmental series of granulopoiesis related to the prominent granules, which will provide better understanding of cellular structure of the hematopoietic cells and immune system in the seahorse under captivity condition.

Keywords: Granulopoietic cells, Head kidney, Hematopoietic cells, seahorse, TEM

Introduction

Definitive head kidney (or anterior kidney), a unique teleost organ, plays important roles in regulation of fish homeostasis including a part of endocrine system (analogous to mammalian adrenal gland and thyroid gland) and formation of blood cells related to immune system, in particular as "kidney marrow" for lifetime hematopoiesis where hematopoietic stem cell (HSC) finally reside and differentiate (Willett et al., 1999; Rombout et al., 2005; Avagyan and Zon, 2016; Geven and Klaren 2017). According to Boomker (1979), structural and ultrastructural features of the kidney tissues (pronephros and mesonephros) in some teleost fishes, i.e. *Clarias garipinus* and *Sarothrodon mossambicus* were described as a major organ forming blood elements, equivalent to mammalian red bone marrow. It is well-defined based on most ultrastructural studies that formation of hematopoietic cells and blood cell lineages in the kidney marrow can be morphologically classified into different series. Sites for developing multiple blood cell lineages including erythropoiesis, thrombopoiesis and granulopoiesis in the head kidney were found in *Dicentrarchus labrax* (Esteban et al., 1989). Developmental steps of granulopoiesis in telost fishes were characterized in some literatures (Zapata, 1979; Savage, 1983; Zuasti et al., 1987). In *D. labrax*, the granulopoietic progenitors can give rise to promyelocytes, myelocytes, metamyelocytes and the mature cells including heterophils, eosinophils and basophils. The main criteria of identifying these cell types in granulopoietic series are based on cell size, cell shape and granular features, as shown in maturational steps of *Oreochromis niloticus* (Abdel-Aziz et al., 2010). However, ultrastructural alteration during developmental steps in granulopoietic series has not been in focus. Understanding cellular changes during this process will uncover differentiation stages of teleost leucocytes.

Barbour's seahorse *Hippocampus barbouri*, an economically valuable marine fish, is listed for the marine aquarium trade and used in Chinese traditional medicine (Lourie et al., 2004; IUCN, 2006). Recently, the population of this seahorse species is decreasing and thus *H. barbouri* is classified as "Vulnerable" on the IUCN's Red List of threatened Species and CITES Appendix II (Lourie et al., 2004; IUCN, 2006). To preserve *H. barbouri* in Thailand, Phuket Marine Biological Center (PMBC) has been working on developing the optimal condition for aquaculture of the seahorse. Immune cells responding to the artificial condition in this seahorse is still under investigated. Hence, in the present study, ultrastructural details related to immune cells, in particular the granulopoiesis from the head kidney of *H. barbouri* were described using transmission electron microscopy (TEM), aiming to provide in-depth information on the process of the granulopoiesis of *H. barbouri* and hematopoietic niche in kidney marrow of the seahorse. Certainly, our observation provided for better understanding of its granulopoietic cell, which would display possible interspecific comparisons on hematopoietic cell among seahorses and use as a creating potential indicator of hemato-ecological impacts.

Materials and methods

Ten hatchery-captive *Hippocampus barbouri* (1 month of age with 65.56±0.98 mm in total lenght) were collected, as described in Kamnurdnin (2017) during October to December 2017. This seahorse was reared by adequate captive conditions using natural seawater at the Phuket Marine Biological Center (PMBC), Phuket Province, Thailand. The experimental protocol was approved by the Animal Care and Use Committee of Faculty of Science in accordance with the guide for the care and use of laboratory animal prepared by Chulalongkorn University (Protocol Review No. 1623004).

A small pieces of the head kidney (1x1 mm^3) were immersed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4 at 4 °C and subsequently post-fixed in 1% osmium tetraoxide (OsO_4). The fixed tissue samples were then processed using TEM sample preparation techniques. The fixed kidney tissue in semithin sections was stained with toluidine blue and examined with light microscopy. Ultrathin section of 90 nm was cut, lightly stained with uranyl acetate and lead citrate and analyzed with transmission electron microscope (JEM-2100 at 200kV). The features and series of granulopoiesis were identified using criteria and guidelines from from Esteban et al. (1989) and Abdel-Aziz et al. (2010). Also, the determination of granulopoietic proportion was performed using 50 randomly selected cells per each TEM image. The measurement of cell size, nuclear size and granule size was examined using 10 randomly selected cells per TEM image, where the number of their granule was also counted.

Results and discussion

Few works concerning the granulopoiesis in some fish have been addressed (Yamamoto & Iuchi 1976; Mattisson & Fange 1977). In this study, we therefore provided a new insight into the granulopoietic series of *H. barbouri*, which is identified based on size, morphological traits, cellular organelle complexity and granular features.

The differentiation of the granulopoiesis involved a series of maturational steps by transition from myeloblasts to mature granulocyte (Figures 1-3). A series of successive granulopoietic population in *H. barbouri* was further divided into five steps: myeloblast, promyelocytes, myelocytes, metamyelocytes and mature granulocytes (Figures 1-3).

Myeloblast was considered as an undifferentiated progenitor or presumptive blast cell for granulopoietic cell series. This was the first recognizable granulocytic lineage. In the kidney marrow, the size of myeloblast was approximately 5.17±0.87 μm in diameter and served as 10.66 percent of the total cell proportion. In general, the myeloblasts were grouped as clusters of 2-3 cells (Figure 1B). Each cell had a large nucleus (about 3.68±0.97 μm in diameter) without distinct clumps of condensed heterochromatin (Figure 1C). The nuclear membrane outline was clearly appeared and surrounded by several organelles including free ribosomes, endoplasmic reticulum (ER) and mitochondria (Figure 1C).

Promyelocyte was a larger cell type, compared to the myeloblasts, about 6.76±0.98 μm in diameter. The proportion of the promyelocyte population was very high (61.33 percent) and thus this cell type was observed occupying most of the area in the thin sections (Figure 1A). The ultrastructure of promyelocyte can be characterized in two sub-stages (early and late promyelocytes). Early promyelocyte exhibited an irregular nuclear shape with prominent heterochromatin (Figure 2A). Remarkedly, formation of granule like-structure can be initially observed at this stage although it was not easy to identify this cellular structure even under TEM observation (Figure 2B). Similarly, granulopoietic cell with this structure can also be found in human bone marrow (Bainton and Farquhar, 1966), emphasizing the equivalent of fish anterior kidney to mammalian bone marrow. This stage further differentiated into the late promyelocyte exhibiting the nucleus with abundance of heterochromatic clumps along the nuclear membrane (Figure 2A). The concentration of dense-cored vacuoles was clearly identified to be a vacuolated granule like-structure in the cytosol (Figure 2B). A development of cytoplasmic organelles including ER and mitochodria was also easily observed (Figure 2B).

Myelocyte had slightly bigger cell size (6.92±1.2 µm in diameter) than the promyelocyte. This cell exhibited irregular cell shape. Myelocyte population was about 18 percent of the total granulopoietic cells, which was higher than those of myeloblast and metamyelocyte. The shape of centrally located nucleus (3.64±0.91 µm in diameter) was similar to that of the late promyelocyte (Figure 2C), but the arrangement of compacted heterchromatin was observed along the nuclear membrane. At this late stage of promyelocyte, numerous granules (average number: 7.8±0.66 granules/cell and size of granule: 0.25±0.81 µm in diameter) can be found and these granules exhibited electron-dense and dispersed throughout the cytosol (Figures 2E-2F). The characters of developing granules at this cell stage agreed well with the presence in high amount of other cellular organelles including packed mitochondria, ER and ribosome (Figure 2D), suggesting that the formation of electron-dense granules perhaps required high amount of energy and protein production.

Metamyelocyte displayed about 5.33 percent of total cell population. It had an eccentric nucleus (Figure 2E). A prominent specific granules were still recognized (Figures 2E-2F), but number (35.2±0.76 granules/cell) and size (0.34±1.10 µm in diameter) of specific granules was higher than what found in the myelocyte (Figure 2G). Small mitochondria were dispersed among the granules (Figure 2E). Several pseudopodial formations were noted during this stage (Figures 2E-2F). The metamyelocyte was then differentiated into the mature granulocytes (Figure 3). The proportion of the mature granulocyte was rarely (3.33 percent). The granulocytes presented a large amount of specific granules (Figure 3).

TEM imaging of *H. barbouri* seahourse head kidney clearly visualized the differentiation stages of the granulopoietic series, enabling to classify them from the youngiest myeloblasts to the mature granulocytes. The presence of these cells in the kidney marrow of the seahorse was similar to those investigated in other teleosts (Esteban et al., 1989; Meseguer et al., 1990; Abdel-Aziz et al., 2010). In addition, the granulopoietic series in the seahorse can be explained as the percent proportion or percentage of cell populations. By this approach, it is interesting that our rared seahorses exhibited the highest proportion of the late promyelocytes compared to other granulocytic cells. This is constrast to other literatures describing teleost granulopoiesis. It was postulated that the increased number of promyelocytes was observed during an infectious disease and significantly correlated with the fever index (Marsh et al., 1967). Our previous data also showed the appearance of kidney histopathology of *H. barbouri* (Kamnurdnin et al. in press). Thus, peak in promyelocyte population in this study might be the early sign for renal inflammation. This also implies that the development of immune cells in our rared seahorse was effective in response to the presence of infectious agents.

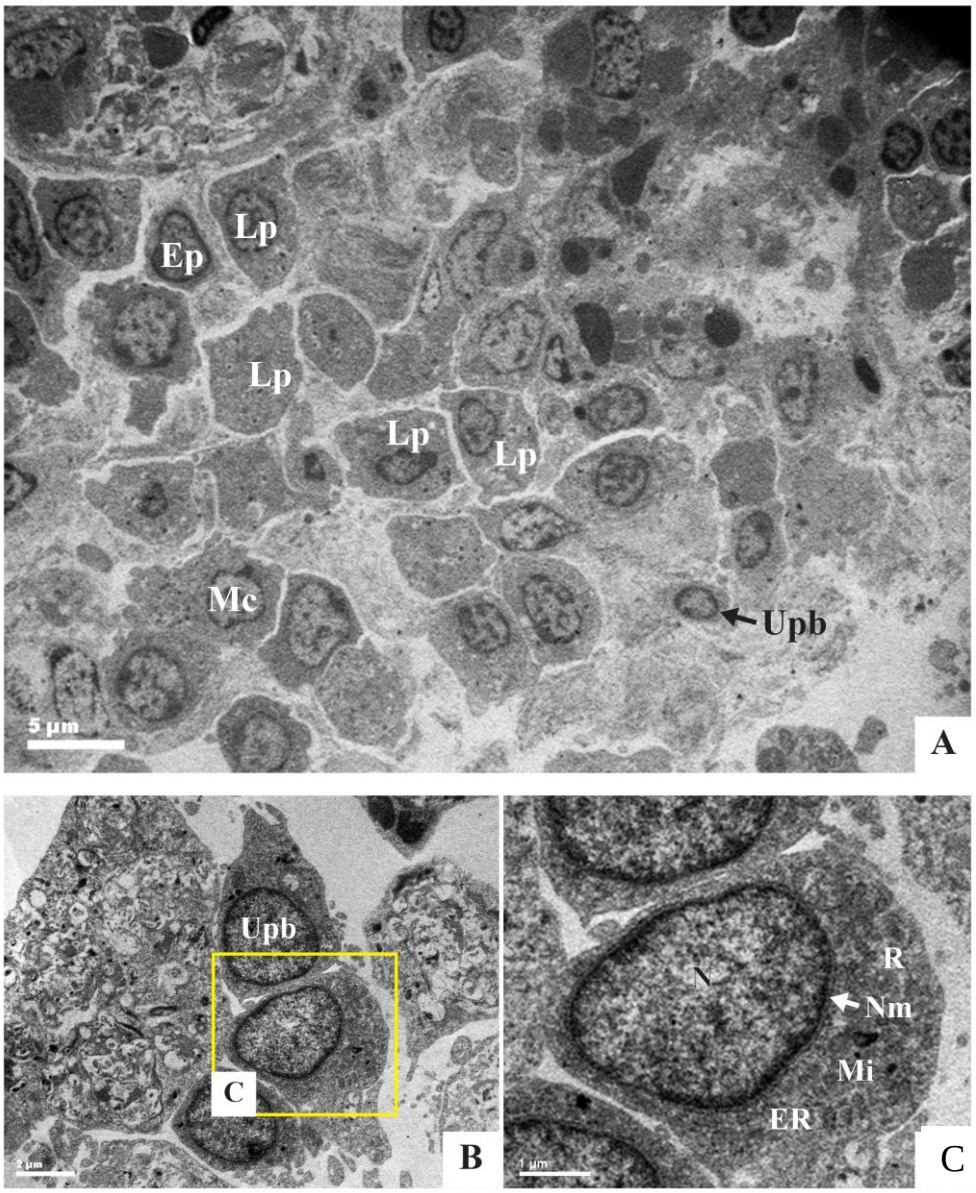

Figure 1 Electron micrographs showing the granulopoiesis in the head kidney of *Hippocampus barbouri.* A. Ultrastructural overview of granulopoietic cells B. Undifferentiated progenitor of granulopoietic series or myeloblast C. Higher magnification of myeloblast. Abbrevations: Ep = early promyelocyte, ER = endoplasmic reticulum, Lp = late promyelocyte, Mc = myelocytes, Mi = mitochondria, Nm = nuclear membrane, R = ribosome, Upb = undifferentiated presumptive blast cell.

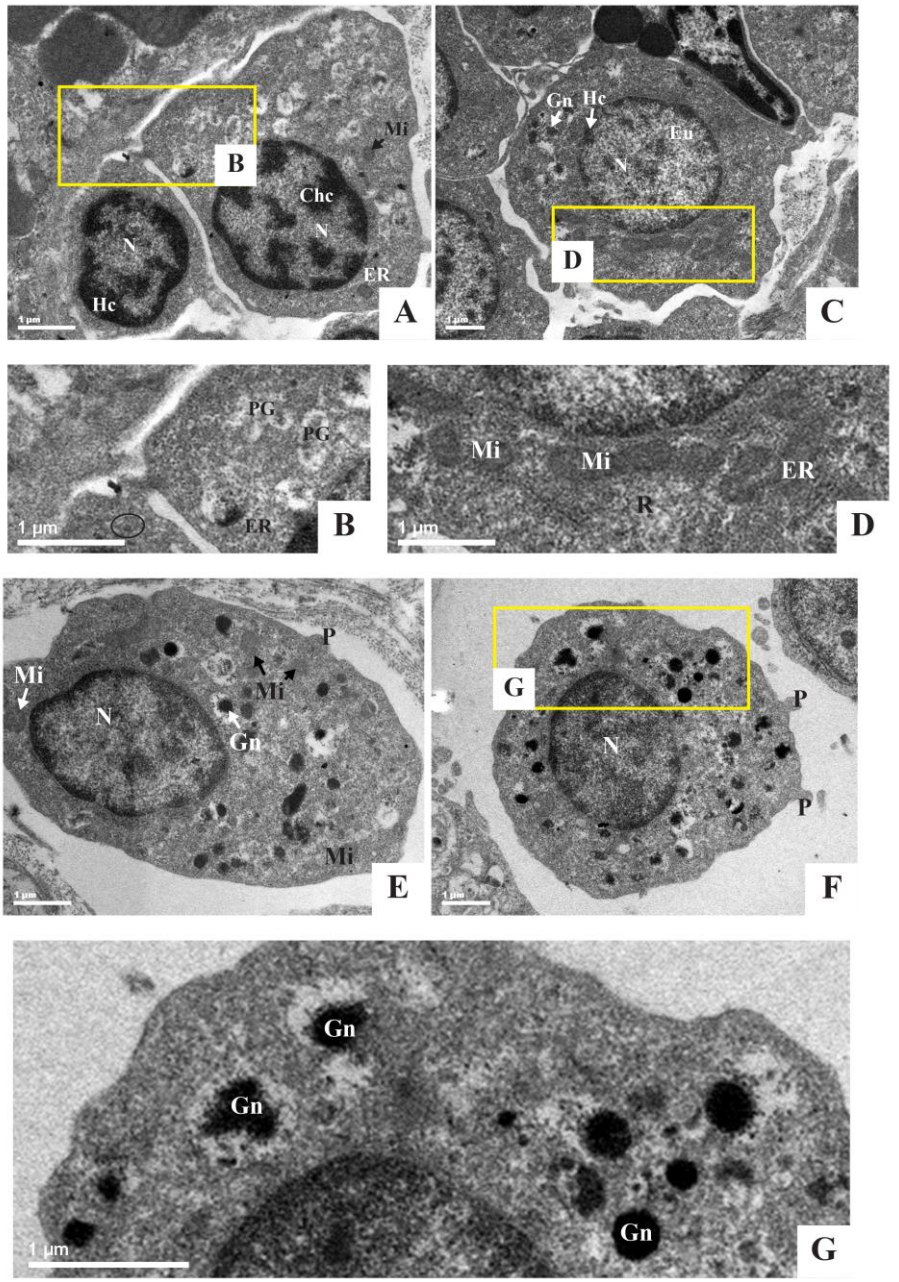

Figure 2 Electron micrographs showing the granulopoietic series in the head kidney of *Hippocampus barbouri*. Different stages of granulopoietic cells are shown: early promyelocyte and late promyelocyte (A-B), myelocyte (C-D) and metamyelocytes (E-G). Abbreviations: Chc = clumped heterochromatin, ER = endoplasmic reticulum, Eu = euchromatin, Gn = granules, Hc = heterochromatin, Mi = mitochondria, N = nucleus, P = pseudopodia, PG = pre-granules or vacuolated granule like-structure, R = ribosome, cycle = granule like-structure.

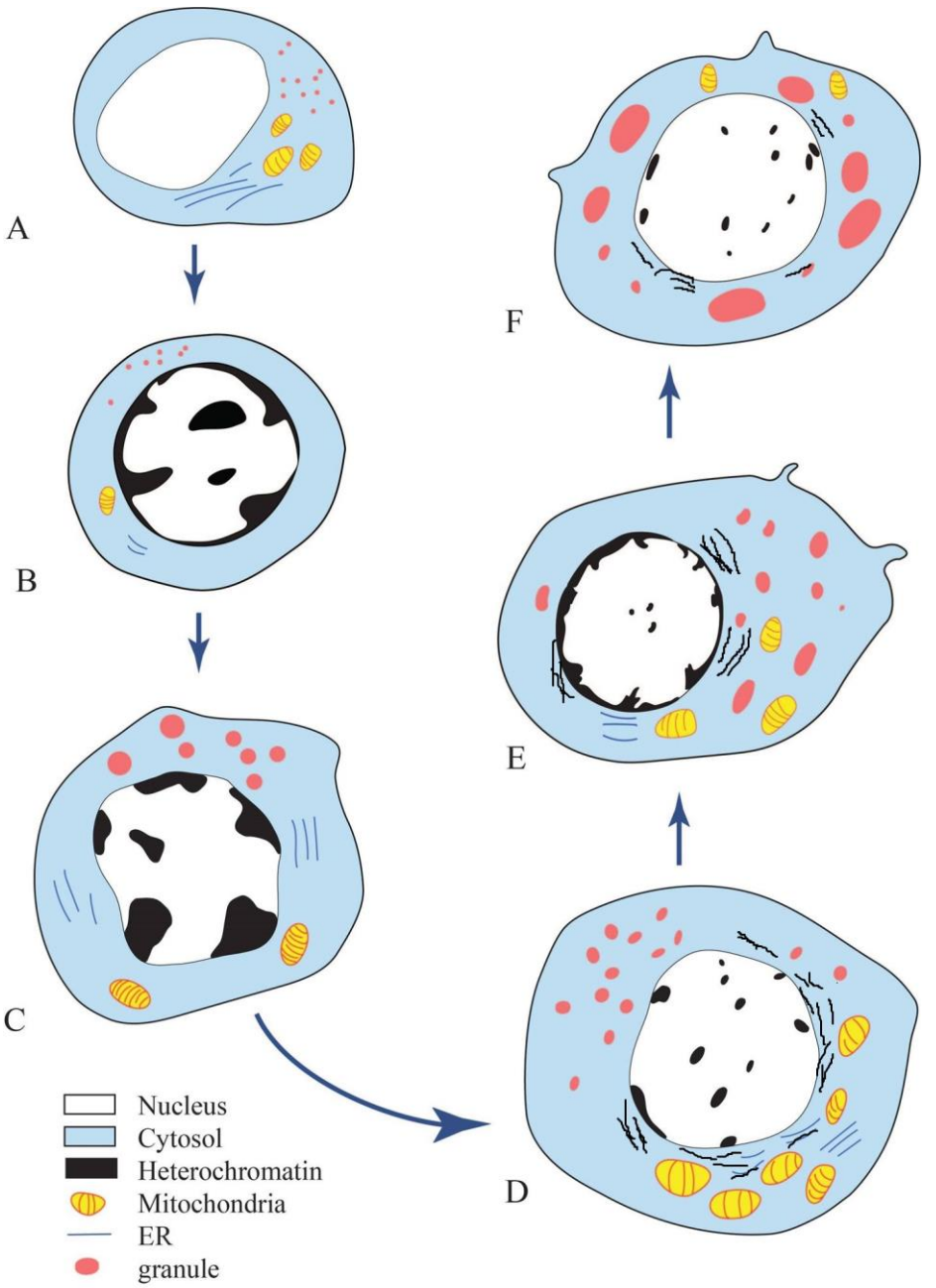

Figure 3 Schematic diagram showing the characterizations of the granulopoietic series of *Hippocampus barbouri* including myeloblast (A), early promyelocyte (B), late promyelocyte, (C), myelocyte (D), metamyelocyte (E) and mature granulocyte (F).

As important function of specific granules is to play roles in immunity, the granules contain hydrolytic enzymes and cytotoxic contents to support of leucocyte function (De duve, 1963; Hirsch and Cohn, 1964; Cohn & Hirsch, 1960). The formation of specific granules in granulopoietic cells is a complex mechanism involving many pathways in cell signaling and cellular mechanism of protein synthesis and transportation via trans-Golgi network for granular formation (Borregaard, 1966; Borregaard et al., 1987; Lawrence et al., 2018). Mitochondria also plays some part in the intracellular membrane system connecting to endoplasmic reticulum–Golgi complex network and this kind of intracellular network ultrastructure can also be seen in granulopoiesis of a teleost fish (sea bass), as shown in Meseguer et al., 1990. Our finding also showed similar observation to these literatures that the condensation of the granule content in the developing promyelocyte came at the same period of large accumulations of mitochondria and ER in the cells.

The presence of different granular cell series in our observation showed that the head kidney of seahorse definitely can be considered as kidney marrow, an equivalent structure to human bone marrow (Bainton, and Farquhar, 1966). The intial stage of granular cell population in sea bass were reported by Meseguer et al. (1990) and they found that myelocyte was the first cell type in the series producing the granules while our study first showed that it was the earlier stage - promyelocyte in the seahorse firstly generating specific granules. Other aspects of development from myelocyte to mature cells in term of density/number of the specific granules were consistent to other reports of telost fishes (Esteban et al., 1989; Abdel-Aziz et al., 2010) and other vertebrates (Curtis et al., 1979; Brederoo et al., 1986), suggesting the well-conserved program of immune cell formation among vertebrates.

Conclusion

This study highlights the first ultrastructural detail of granulopoietic series in the head kidney of *H. barbouri* seahorse under captivity. Under differentiation from the myeloblast to the mature myelocyte, here we reported key findings including the late myelocyte as the highest proportion and the myelocyte as an initial cell type producing specific granules. The presence of similar cell morphology, nucleus character and organelle complexity in the granulopoietic cells in the seahorse agreed well to other literatures reporting teleost and vertebrate granulopoiesis and emphasized the analogy of teleost head kidney to mammalian bone marrow. Hopefully, this informative data above will present making possible interspecific comparisons between seahorses/other fishes and use as an indicator of hemato-ecological impacts.

Acknowledgements

We would like to thanks to the Department of Marine Science and Environmnet, Faculty of Science and Fisheries Technology, Rajamangala University of Technology Srivijaya, Trang Campus, for their technical support in a laboratory and informative discussion.

References

Abdel-Aziz, E. H., Abdu, B. B. S., Ali, T. E. & Fouad, H.F. (2010). Haemopoiesis in the head kidney of tilapia, Oreochromis niloticus (Teleostei: Cichlidae): a morphological (optical and ultrastructural) study. *Fish Physiology and Biochemistry*, *36*(3), 323-36. https://dx.doi.org/10.1007/s10695-008-9297-z

Avagyan, S. & Zon, L. I. (2016). Fish to Learn: Insights into blood development and blood disorders from zebrafish hematopoiesis. *Human Gene Therapy*, *27*(4), 287–294. https://dx.doi.org/10.1089/hum.2016.024

Bainton, D. F. & Farquhar, M. G. (1966). Origin of Granules in Polymorphonuclear Leukocytes. *The Journal of Cell Biology*, *98*(2), 277-301. https://dx.doi.org/10.1083/jcb.28.2.277

Boomker, J. (1979). The haemocytology and histology of the haemopoietic organs of *Clarias gariepinus* and *Sarotherodon mossambicus*. Onderstepoort Journal of Veterinary Research, 46, 217-222.

Borregaard, N. (1996). Current concepts about neutrophil granule physiology. *Current Opinion in Hematology*, *3*(1), 11-18. https://dx.doi.org/10.1097/00062752-199603010-00003

Borregaard, N. & Miller, L. M. (1987). Chemoattractantregulated mobilization of a novel intracellular compartment in human neutrophils. *Science,* 237(4819), 1204-1206.

Brederoo, P., Van Der Meulen, J. & Deams, W.T. (1986) Ultrastructural localization of peroxidase activity in developing neutrophil granulocytes from human bone marrow. *Histochemistry*, *84*(4-6), 445–453

Curtis, S. K., Cowden, R. R. & Nagel, J. W. (1979). Ultrastructure of the bone marrow of the salamander Plethodon glutinosus (Caudata: Plethodontidae). *Journal of Morphology*, *159*(2), 151-184.

Cohn, Z. A. & Hirsch, J. G. (1960) The isolation and properties of specific cytoplasmic granules of rabbit polymorphonuclear leucocytes. *Journal of Experimental Medicine*, *112*(6), 983-1004. https://dx.doi.org/10.1084/jem.112.6.983

De duve, C. (1963). The lysosome concept. In A. V. S. de Reuck & M. P. Cameron (Eds), *Foundation Symposium on Lysosomcs*, pp. 1-35. Boston, Little, Brown & Co Publisher.

Esteban, M. A., Meseguer, J., García A. A. & Agulleiro, B. (1989). Erythropoiesis and thrombopoiesis in the head kidney of the sea bass (*Dicentrarchus labrax* L.). An ultrastructural study. *Archives of Histology and Cytology*, *52*(4), 407-419. https://dx.doi.org/10.1679/aohc.52.407

Geven, E. J. W. & Klaren, P. H. M. (2017). The teleost head kidney: Integrating thyroid and immune signaling. *Developmental & Comparative Immunology*. *66*, 73-83. https://doi.org/10.1016/j.dci.2016.06.025

Hirsch J. G. & Cohn, Z. A. (1964). Digestive and autolytic functions of lysosomcs in phagocytic cells. *Federation Proceedings*, *23*, 1023-1025.

The International Union for Conservation of Nature (IUCN). (2006), IUCN Red list of Threatened Species.

Kamnurdnin, T. (2017). *Effects of food on growth and gonadal development of seahorse Hippocampus* sp. (M.Sc Thesis) Department of Marine Science, Chulalongkorn University, Bangkok. (in English)

Kamnurdnin, T., Senarat, S., Kettratad, J., pengsakul, T., Jiraungkoorskul, W., Sooksuwan, R., Sukparangsi, W. & Sudtongkong, C. (in press). Histopathological observation and health status of captive *Hippocampus barbouri* Jordan & Richardson, 1908. *Songklanakarin Journal of Science and Technology*. https://rdo.psu.ac.th/sjstweb/Ar-Press/2020Jan/12.pdf

Lawrence, S. M., Corriden, R. & Nizet, V. 2018. The ontogeny of a neutrophil: mechanisms of granulopoiesis and homeostasis. *Microbiology and Molecular Biology Reviews*. *82*(1), 7-17. https://doi.org/10.1128/MMBR.00057-17

Lourie, S., Foster, S., Cooper, E. & Vincent, A. (2004). A Guide to the Identification of Seahorses. Washington DC: Project Seahorse and TRAFFIC North America. Accessed March 10, 2011 at http://www.traffic.org/species-reports/traffic_species_fish29.pdf.

Mattisson, A. G. M. & Fange, R. (1977). Light and electron microscopic observations on the blood cells of the Atlantic hagfish, *Myxine glutinosa* (L). *Acta Zoologica (Stockholm)*, *58*(4), 205-221. https://doi.org/10.1111/j.1463-6395.1977.tb00257.x

Marsh, J. C., Boggs, D. R. & Cartwright, G. E. (1967). Neutrophil kinetics in acute infection. *The Journal of Clinical Investigation*, *46*(12), 1943–1953.

Meseguer, J., Esteban, M. A., Garcia, A. A., Lopez, R. A. & Agulleiro B. (1990). Granulopoiesis in the head kidney of the sea bass *Dicentrarchus labrax* L. An ultrastructural study. *Archives of Histology and Cytology*, *53*(3), 287-296. https://doi.org/10.1679/aohc.53.287

Rombout, J.H.W.M., Huttenhuis, H.B.T., Picchietti, S. & Scapigliati, G. (2005). Phylogeny and ontogeny of fish leucocytes. *Fish and Shellfish Immunology*, *19*(5), 441-455. https://doi.org/10.1016/j.fsi.2005.03.007

Savage, A. G. (1983). The ultrastructure of the blood cells of the pike *Esox lucius* L. *Journal of Morphology*, *178*(2), 187-206. https://doi.org/10.1002/jmor.1051780209.

Willett, C., Cortes, A., Zuasti, A. & Zapata, A. (1999). Early hematopoiesis and developing lymphoid organs in zebrafish. *Developmental Dynamics*, *214*(4), 323-336.

Yamamoto, M. & Iuchi, I. (1976). Electron microscopic study of erythrocytes in developing rainbow trout Salmo gairdneri with particular reference to changes in the cell line. *Journal of Experimental Zoology*, *191*(3), 407-426. https://doi.org/10.1002/jez.1401910311

Zapata, A. (1979) Estudio ultraestructural de la mielopoiesis en peces teleosteos. Morfol Normative Patology Sec A, *3*, 737-747

Zuasti, A., Ferrev, C., Ballesta, J. and Pastor, L.M. 1987. Ultrastructure of the tubules nephron of Testudoaracea (Chelonia). A comparison between hibernation and non hibernation animals. Histol. Histopath. 2, 391–400.