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Circadian rhythmicity in the head kidney and spleen cells in snakehead teleost, *Channa punctatus*

Sheena Farheen Khan, Sushant Kumar Verma & Manish Kumar Tripathi*

Department of Zoology, School of Studies of Life Science, Guru Ghasidas Vishwavidyalaya, Bilaspur, Chhattisgarh, India

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Abstract- The endogenously occurring circadian rhythm functions as an important signaling mechanism in animals to maintain physiological homeostasis in respect with cyclic environmental variations. Cellularity is one of the most important components in animal's physiology that provides information about overall health of the organism. This study was designed to evaluate the circadian rhythmicity in the cellularity of fish lymphoid organs, head kidney and spleen in the freshwater snakehead teleost, *Channa punctatus*. The fish were sacrificed multiple times with equal time interval during 24-h and head kidney and splenic cellularity was calculated. To analyze the circadian rhythm in the cellularity, cosinor rhythmometry analysis was performed. The significant 24-h variation was observed with elevation in the cellularity during the dark phase of the day in both head kidney and spleen. Additionally, results from cosinor rhythmometry observed significant circadian rhythm in cellularity of the lymphoid organs. These findings contribute to broader understandings of chronobiology in aquatic organisms emphasizing the importance of circadian regulation in immune homeostasis.

Keywords: Circadian rhythm, Cellularity, Fish, Cosinor analysis, *Channa punctatus*

INTRODUCTION

The rotatory phenomenon of earth is responsible for the providing the clock information to the internal rhythmic machinery in animals. Circadian rhythms are endogenously regulated cycles that follows approximately 24-h cycle frequency.[1] The endogenous rhythm is involved in maintaining the synchronization in physiological processes. In fish, the circadian rhythm is controlled through the pineal gland, functioning as the master clock. Additional to this, the cellular components also work as the peripheral clock.[2] The circadian rhythm is present in various physiological processes in fish, including immune system.[3] The circadian rhythm in fish is mainly entrained by the environmental conditions that chiefly includes light-dark cycle, temperature manipulations, acidification of the aquatic ecosystem and hypoxia.[4] The occurrence of these environmental phenomenon could influence the seasonality-induced rhythmicity in animals. The season specific rhythmic responses have also been observed in mackerel tuna (*Euthynnus affinis*).[5]

The chief lymphoid organs in fish are head kidney and spleen that possess plentiful immune cells. The cellularity is a significant immune characteristic phenomenon that is linked with the characterization and functioning of the immune cells within the organism. The distinct functions of diverse immune cells provide better immune-competence in fish. The cellular level processes in fish also gets affected by the circadian rhythm phenomenon with influence in the mitotic activity of intestinal cells in zebrafish (*Denio rario*).[6]

*Corresponding author :
Phone : 9026068226
E-mail : manish10.t@ggu.ac.in

Channa punctatus is known for wide distribution and stress tolerance capacity. This fish is equipped with an accessory respiratory organ that facilitates the fish to survive in the oxygen deficient areas. Fish are a diverse vertebrate group with wide variety of life-style, understanding the cellular rhythmicity is important to anticipate the most functionally active phase in the fish and this could link to the evolutionary relationship with various fish species and higher vertebrates. The present study has been done to evaluate the circadian rhythmicity in the cellularity of head kidney and spleen of the experimental model, *Channa punctatus*.

MATERIALS & METHODS

Fish collection and acclimatization

The experimental fish, *Channa punctatus* was collected during February-March from the outskirts of Bilaspur district. Fish were brought to laboratory, treated with $KMnO_4$ and acclimated for two weeks in 40 L water tanks before the commencement of the experiment. Throughout the acclimation period, fish were fed with commercial food once daily with regular water change.

Sample collection

The fish were weighed, and anesthetized by tricaine methanesulfonate (MS-222). The isolation of head kidney and spleen was done aseptically at different times of the day having equal time interval and organs were weighed separately. For each time, number of fish sacrificed was kept six. Further, cell suspension was prepared by macerating the tissue through nylon strainer under culture medium, RPMI-1640 (Himedia Lab. Pvt. Ltd.). The cell viability was examined using the trypan blue exclusion test and cellularity was determined by calculating the ratio of number of cells in per mL culture media with the weight of the tissue.

Statistical Analysis

The data are presented as mean ± SEM. The comparison was done using one-way analysis of variance test and post hoc analysis was performed using Newman-Keul's multiple range test with p value less than 0.05 (SPSS. 26.0). Further, for circadian rhythm analysis, cosinor rhythmometry was employed.

RESULTS & DISCUSSIONS

The cosinor rhythmometry confirmed the presence of circadian rhythm in the overall cell numbers in head kidney and spleen. The rhythm peak in cellularity was obtained at 23.07 clock hour and 21.98 clock hour in head kidney and spleen, respectively (Table. 1). The 24-h variation in the cellularity of head kidney showed variable results. At night (20:00 h) and midnight (00:00 h), the cellularity was significantly elevated while reduction in cellularity was reported in other times (Fig. 1). Similar pattern was observed in the splenic cellularity with higher cellularity recorded during dark hours (20:00 and 00:00 h) (Fig. 2).

In fish circadian rhythm has been studied in various physiological processes including skin pigmentation, food consumption and oxygen utilization.[7] The cellularity refers to total number of cells present in the tissue which serves as an important indicator of overall physiological status and immune function. Variation in cellularity reflects changes in immune activity, tissue homeostasis and potential pathological conditions. In the present study, distinct 24-h variation in cellularity was observed in head kidney and spleen. Further validation through cosinor rhythm analysis confirmed the presence of circadian rhythm in cellularity within both the lymphoid organs. The presence of circadian rhythm regulation in immune cells dynamics has been well documented in mammalian models. In agreement with our results, the circadian rhythm was confirmed in the human PBMC by Hermann *et al*. (2006)[8]. Similarly, splenic natural killer cells also showed distinct circadian rhythm in the cytotoxic activity in rat.[9,10] The circadian rhythm in the cellularity was also seen in rats with special reference to macrophages.[11,12] Additionally, rhythmic expression in T-cell population in trout was also observed.[13] The rhythm-induced variation has also been studied in cell division, cell composition and clock gene expression in various fish species.[6,14,15] Beyond circadian regulation, seasonality also plays an evident role in manipulating the rhythmicity in the physiology of animals. Seasonal rhythm in the immune functions were also evaluated in striped hamsters (*Cricetulus barabensis*).[16] Similarly, previous studies on *Channa punctatus* have observed annual rhythm is the head kidney and spleen cellularity.[17] The current study contributes to this growing body of evidence by demonstrating circadian modulation in immune cellularity in key lymphoid organs in fish.

The involvement of the melatonin in night-time elevation in the cell numbers could not be ruled out as melatonin is one of the important rhythmically expressed hormone.[3,17] The necessity to evaluate rhythmic expression

in other physiological processes such as immune response will provide better insights into the overall health status of the fish with emphasizing on the chronobiological aspects in aquatic organisms.

Table 1: Cosinor summary of cellularity in head kidney and spleen cells

Tissue	Data Point	p-value	Mesor	Amplitude	Acrophase
Head kidney	36	< 0.001	0.75 ± 0.05	0.41 (0.20, 0.62)	21.79 (19.79, 23.79)
Spleen	36	< 0.001	1.54 ± 0.12	0.79 (0.33, 1.24)	21.98 (19.65, 24.31)

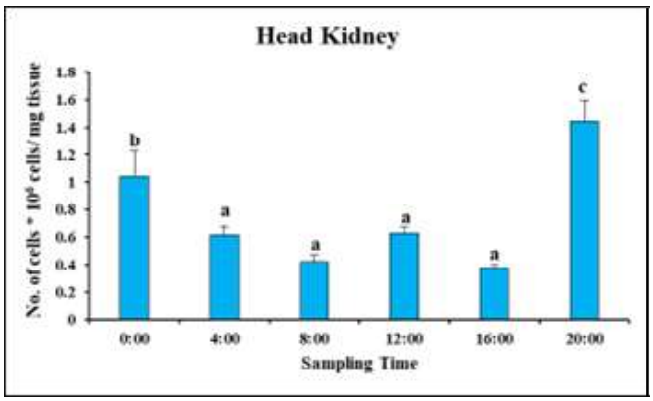

Fig. 1: 24-h variation in head kidney cellularity. Data presented as mean ± SEM. Error bars having different superscripts differ significantly (Newman-Keul's multiple range test, p < 0.05)

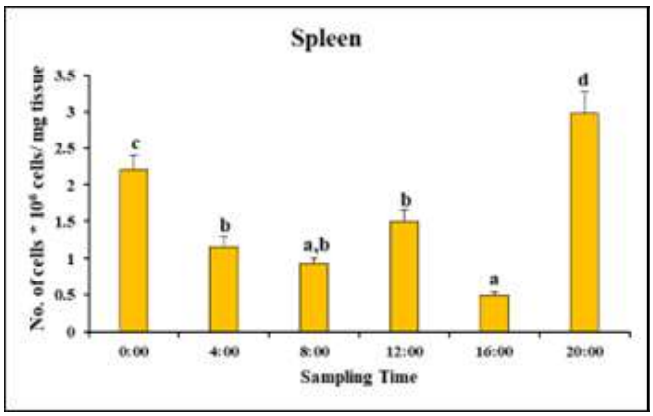

Fig. 2: 24-h variation in spleen cellularity. Data presented as mean ± SEM. Error bars having different superscripts differ significantly (Newman-Keul's multiple range test, p < 0.05)

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