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Establishment of a primary cell culture system from heart of *Schizothorax esocinus*

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Abstract
The present study reports the establishment of a primary cell culture system from heart of *Schizothorax esocinus*, an indigenous cold-water fish of Indian Himalayas. The study opens a window for development of cell culture system from schizothoracids of Indian Himalayas. The standardization of the primary cell culture was done using L-15 media at different FBS concentration and temperature range. Explant method used for tissue preparation was found to be more reliable than the enzymatic dissociation technique. The primary cell culture system of heart showed consistent growth at an optimum FBS concentration of 15% and an optimum temperature of 24 ºC. Characterization of newly developed primary cell culture was done by karyotyping. Chromosome number of the primary cell culture system was found to be 98 which is consistent with the chromosome number of *S. esocinus*.

Keywords: Primary cell culture, *Schizothorax esocinus*, Heart, FBS, L-15

1. Introduction
Schizothorax esocinus is an important fish belonging to family cyprinidae which commonly thrives in Himalayas of India, China, Pakistan, Afghanistan and Nepal [1]. The fish is found in cold-water rivers and snow laden streams. It continues to make a significant contribution to the India's fish basket, economic growth, and development of cold-water fisheries sector. Furthermore, *S. esocinus* serves as a bioindicator of aquatic pollution as it is extremely sensitive to changes in the environment [2]. Development of an *in-vitro* culture system is needed to conduct various toxicological, physiological and pathological studies prior to bioassay experiments. A cell culture platform is also useful for *in-vitro* localization of fish viruses [3]. Cell culture has also proven to be the gold standard for the detection and isolation of fish viruses [4].
The establishment of the first stable fish cell line (RTG-2) was from rainbow trout gonad tissue by Wolf and Quimby, 1962 [5]. Since then, continued efforts have been made towards the development of fish cell lines from a wide range of species [6]. However, a limited number of cell lines have been developed from cold-water fishes. In this line, the present study was conducted to develop a cell culture system from heart of *S. esocinus*, an indigenous cold-water fish of Indian Himalayas.

2. Materials and Methods
2.1 Sample collection
Forty fish specimens of *S. esocinus* with an average weight of 70-80g (±0.5g) were purchased and transported in sterile fish tanks where they were acclimatized and quarantined in 30L aquarium tank containing fresh water with continuous aeration at room temperature.

2.2 Decontamination of fish
In order to minimize the possible routes of contamination, the donor fish was starved for about 24h to reduce the gross contamination from faecal matter and disgorged feed. Further, the fish were monitored continuously for 7 days and 50% water exchange was done on daily basis. Decontamination was done following the protocol of Lai *et al*., 2003 [7] by subjecting the fish to antibiotic treatment with 100 IU/ml penicillin and 1000µg/ml streptomycin; and, 10 µg/ml amphotericin B as an antifungal drug for 24h prior to culture.

2.3 Primary cell culture
Sampling was done by aseptically removing heart from decontaminated fish. Afterwards, primary cell culture was carried out either by enzymatic dissociation or explant method. Enzymatic digestion of the tissues was done by following the protocol of Goswami *et al*., 2013 [8]. Explant method of primary cell culture was done by following earlier protocol of Majeed *et al*., 2013 [9].

2.4 Passaging of cells
Cells were observed for their attachment and proliferation, and morphological details were also recorded on daily basis using inverted microscope (EVOS FLC, Life Technology). The optimum pH of the cell culture was maintained at 7.4. Once the confluency was achieved, the subculture of cells was done using trypsinization method [9]. The old cell culture media was removed and the flask was washed 2-3 times with PBS. Then 0.25% trypsin-EDTA solution (Cell Clone Genetix Brand, India) was added to detach the confluent cells and afterwards, the flasks were tapped gently to dislodge the cells. The detached cells were then suspended in fresh medium along with other supplements to a final volume of 3ml, sub-cultured and incubated at 24 ºC.

2.5 Growth studies
In order to estimate the ideal conditions required for growth and maintenance of cells, FBS and temperature optimization was done as described here under:

2.5.1 FBS optimization: To standardize the optimum concentration of FBS for the growth of *S. esocinus* heart cells, the tissue explant was seeded in a standard 25 cm^2 cell culture flask. The media supplemented with different concentrations of FBS ranging from 5-20% was added to culture the cells as described previously by Goswami *et al*., 2012 [10]. The flasks were observed daily under an inverted microscope (EVOS FLC by Life Technologies) until a confluency of 60-70% was achieved.

2.5.2 Temperature optimization: Temperature optimization was carried out following the protocol of Nambi *et al*., 2015[11]. To ascertain the optimum temperature for the maximizing the growth of cells, *S. esocinus* tissue flasks were incubated at different temperatures (4-28 ºC) for 20 days at seeding concentration of 1×10^5 cells in 25cm² tissue culture flasks. L-15 media supplemented with the optimum FBS concentration of 15% was used to grow the tissue explants. Cells were observed at different intervals to record the growth and level of proliferation, and cell counting was carried out using a hemocytometer.

2.6 Characterization of primary cell culture
In the present study, karyotyping was used to characterize the newly developed primary cell culture from the heart of *S. esocinus*. Chromosomal analysis was done by following the protocol of Majeed *et al*., 2013 [9] from the cell culture flasks showing a confluency of 60-70%.

In order to prepare the slides, small quantity of mixture was pipetted and dropped onto grease free, pre cleaned slides from a height of 1.5 ft. Slides were then air dried, and kept in dark place for 1-3 days to ensure complete ageing. Staining was carried out using 2% Giemsa stain in PBS at various time points (5 min, 10min, 15min and 20 min). Subsequently, the slides were washed with double distilled water, air dried and observed under oil immersion objective (100X) in bright field microscope. Photographs of metaphase spreads were recorded for further analysis.

3. Results and Discussion
3.1 FBS optimization
The effect of different FBS concentration on the growth of *S. esocinus* heart cells is shown in Table 1 and Fig.1. The attachment and the growth of the cells increased with increase in the concentration of serum from 5% up to a maximum of 20%. The optimum growth of the cells was recorded at 15% FBS concentration wherein confluency was attained after 27days. A further increase in serum concentration up to 22% did not yield consistent results.

These results are in accordance with the findings of Suryakodi *et al.*, 2021 [12] where the authors established a primary cell culture from different organs of rainbow trout (*Oncorhynchus mykiss*) using L-15 medium supplemented with 15% FBS. The concordance of their findings with the current results is presumably due to the fact that both the species studied were cold water fishes thriving at temperatures below 20-25 °C [13].

Table 1: Number of cells (1x105) at different FBS concentration observed at different time intervals.

FBS Concentration (%) / Time (days)	5	7	10	15	18	20	22	*p*-value
8	0	0	1.33±0.94	5.66±2.62	4.33±0.94	11±2.94	2.33±2.05	<0.0001
12	0	0.66±0.94	3±1.41	10.66±4.02	8±0.81	15±3.55	2.66±1.88	<0.0001
18	0	3.33±0.47	7±1.63	14.66±3.39	9.33±1.88	18.66±6.18	3±2.16	<0.0001
21	0.33±0.47	3.66±1.69	8.33±1.88	15.66±2.86	14.33±2.86	25±4.96	3.66±1.24	<0.0001
25	0.66±0.94	1.66±1.24	10.33±1.24	22.33±4.18	16±2.94	30.33±1.69	3.33±1.24	<0.0001
27	0.66±0.94	0.33±0.47	13.33±2.62	28.66±5.90	22±5.71	35.66±2.49	2.33±3.29	<0.0001
32	0	0.33±0.47	15.33±6.59	47.33±3.09	26.33±4.64	38.33±2.49	0	<0.0001

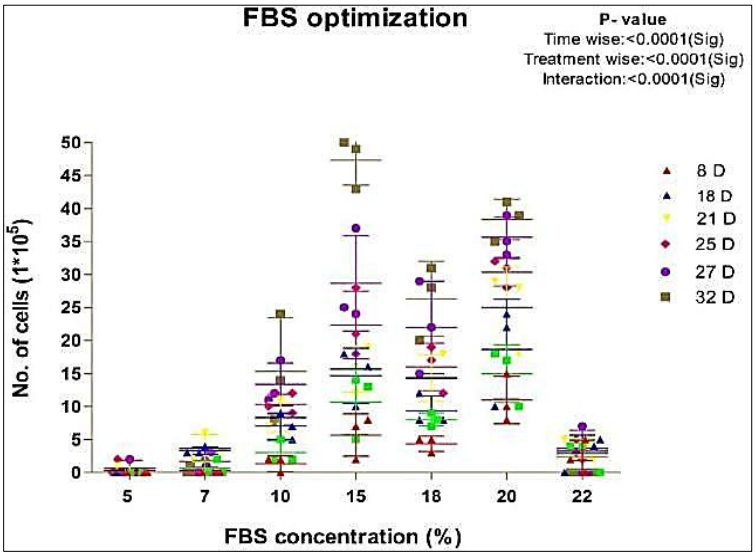

Fig 1: A Scatter-plot diagram showing the effect of varying FBS concentration on the growth of cells.

3.2 Temperature optimization

Optimization of temperature was done on the basis of number of cells growing at different temperatures for 7 days of incubation in cell culture medium. Different temperatures tested for the development of primary cell culture from the heart of *S. esocinus* is reflected in the Table 2 and Fig. 2. The optimum temperature for the growth of cells was found to be 24 ºC.

Goswami *et al*., 2013 [8] documented similar findings on the development of caudal fin cell line from *S. richardsonii*, a cold water schizothoracid fish, where in, optimum temperature for cell growth was recorded at 24 °C. The current results regarding temperature optimization are in contrast to the findings of Kumar *et al*., 2020 [14] where the optimum temperature for the development of a cell line from the eye of *S. richardsonii* was found to be 28 °C. Species-specific disparity in optimized temperatures of schizothoracids might be due to difference in tissues used for development of a primary cell line.

Table 2: Number of cells (1x105) at different tested temperatures at different time intervals

Temperature (ºC) / Time (days)	4	10	18	24	26	30	p- value
3	0	0.33±0.47	1±0.81	0.33±0.47	0	0	<0.0001
8	0	2.66±0.47	5±1.63	13±1.41	7±3.55	1.66±1.24	<0.0001
12	1±0.81	2±1.63	8.66±3.29	22.33±4.18	9.33±2.05	3±1.63	<0.0001
15	1.33±1.24	5±2.44	13±0.81	30.33±1.69	10.66±2.86	3.33±1.24	<0.0001
20	0	6.33±1.24	19±0.81	41±7.11	13.33±1.24	5±0.00	<0.0001
24	0	5.33±1.69	22±0.81	51.66±6.94	21±1.63	7±1.63	<0.0001

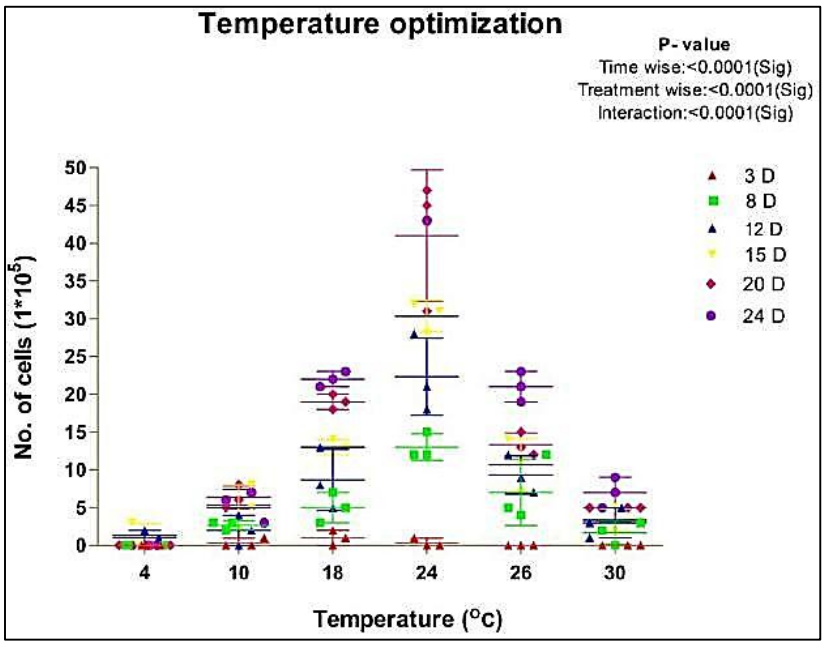

Fig 2: A Scatter-plot diagram showing the effect of varying temperatures on the growth of cells.

3.3 Tissue preparation and culture method

In this study, explant method and enzymatic dissociation of tissues was done for the development of primary cell culture. After 20 days of culture, maximum adherence of cells was observed in the explant method of tissue preparation as shown in Table 3 and Fig. 3. The cells become confluent within 18 days with a confluency of 45-50%. However, the enzymatic dissociation method of tissue preparation resulted in suspension of cells in culture flask and cells showed negligible proliferation.

Explant and enzymatic dissociation method of tissue preparation were compared to observe their *per se* efficiency for cell attachment and growth. There is less damage to tissue in explant method compared to enzymatic dissociation due to the use of trypsin [15]. This is supported by earlier findings wherein authors found a pronounced cell attachment using tissue explant compared to enzymatic dissociation [7] [16] [10] [9].

Table 3: Attachment of cells using different culture methods

Mode of culture	1st seeding	2nd seeding	3rd seeding	4th seeding	5th seeding	Number of cells (1x105) (After 1month)
Explant method	8	11	4	8	5	36
Enzymatic Dissociation	2	3	0	1	3	9

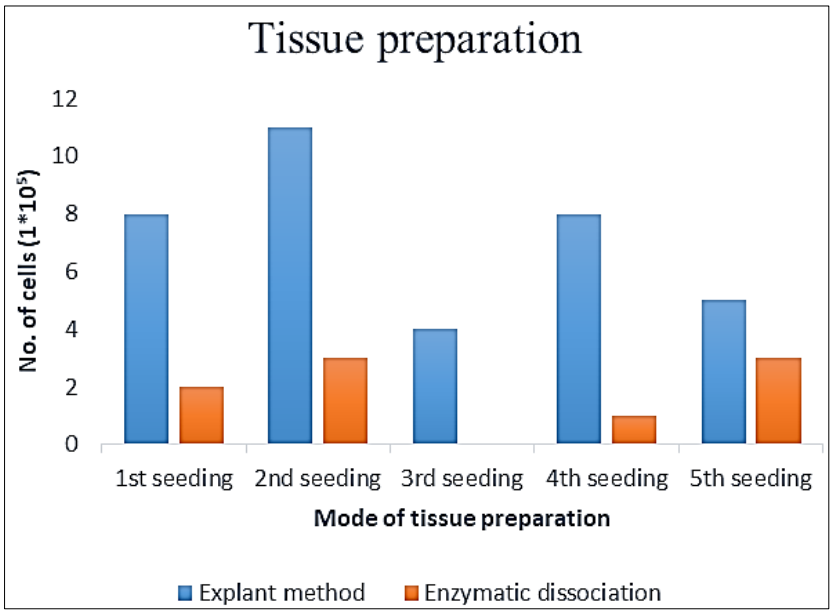

Fig 3: Attachment of cells using explant and enzymatic dissociation method of tissue preparation

3.4 Proliferation of cells

The heart explant of *S. esocinus* was seeded onto the cell culture flask on day- 1. Afterwards, the seeded culture was observed daily for growth. The attachment of the tissue explant was evident at day- 3. The outgrowth of cells from the attached tissue explant was noted at day-5. The proliferation of fibroblast- like cells was observed at day- 12. About 70% of cell confluency was evident at day- 19. Interestingly, the confluent monolayer was observed at day 25-28. The various developmental stages observed in primary cell culture of heart tissue of *S. esocinus* is presented in Fig. 4.

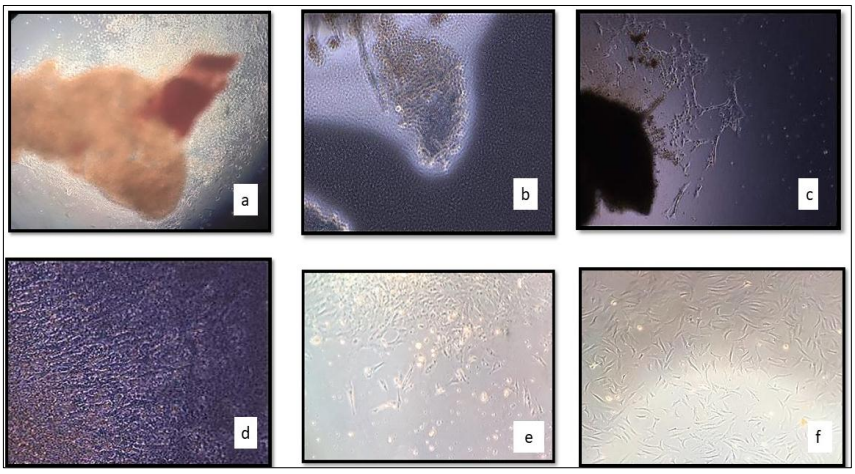

Fig 4: Developmental stages in primary cell culture of heart tissue of *S. esocinus*: (a) Attachment of explant at day-1; (b) Attached explant at day-3; (c) Outgrowth of cells from tissue explant at day-5; (d) Proliferation of fibroblast-like cells at day-12; (e) Pre-confluent cells at day-19; (f) Confluent cells at 25-28 day.

3.5 Characterization (chromosomal analysis)

Chromosomal analysis of the newly developed primary culture from the heart tissue of *S. esocinus* was done as depicted in Fig. 5. Visualization of the prepared slides after 2% Giemsa staining was done under 100X light microscope (Olympus) where in chromosome number of *S. esocinus* heart was found to be 98.

While studying the 50 metaphasic plates of *S. esocinus*, the chromosome number was ascertained to be 98 which is consistent with the findings of Ganai *et al*., 2014 [17]. Previous studies have also used karyotyping as a method of cell characterization [18, 19, 20].

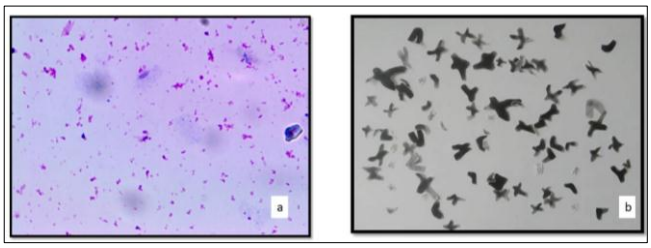

Fig 5: Karyotyping of established primary cell culture from heart of *S. esocinus*: (a) Chromosomal threads after staining with 2% Giemsa stain; (b) karyotype of *S. esocinus*

4. Conclusion

In the present study two important parameters *viz*., FBS and temperature were optimized for the development of a primary cell culture of heart of *S. esocinus*. FBS is an important component of cell culture medium. It is known to contain various growth factors which aid in survival, maintenance and proliferation of cell. Whereas, temperature is an important physical parameter which sustains the growth of cells in a cell culture medium. With the change in FBS concentration of 15%, the cells showed a great extension in growth. At the same time, on standardizing the incubation temperature, the maximum cell growth was observed at 24 ºC. It was also observed that the explants method of cell culture results in the optimum growth and proliferation of cells. The chromosome analysis by karyotyping revealed a chromosome number of 98 confirming the species identity of the developed primary cell culture with *S. esocinus*. The present study offers a great scope for future experiments to further refine the development of cell lines from cold water fishes.

5. Acknowledgement

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