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Development and characterization of a cell line WAF from freshwater shark *Wallago attu*

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Abstract A new epithelial cell line, WAF was developed from caudal fin of freshwater shark, *Wallago attu.* The cell line was optimally maintained at 28 °C in Leibovitz-15 (L-15) medium supplemented with 20 % fetal bovine serum. The cell line was characterized by various cytogenetic and molecular markers. The cytogenetic analysis revealed a diploid count of 86 chromosomes at different passages. The origin of the cell lines was confirmed by the amplification of 547 and 654 bp sequences of 16S rRNA and cytochrome oxidase subunit I genes of mitochondrial DNA, respectively. WAF cells were characterized for their growth characteristics at different temperature and serum concentration. Epithelial morphology of the cell line was confirmed using immunocytochemistry. Further cell plating efficiency, transfection efficiency and viability of cryopreserved WAF cells was also determined. Cytotoxicity and genotoxicity assessment of cadmium salts on WAF cells by MTT, NR and comet assay illustrated the utility of this cell line as an in vitro model for aquatic toxicological studies. The cell line will be further useful for studying oxidative stress markers against aquatic pollutants.

Keywords *Wallago attu* · Cell line · Freshwater shark · Characterization

Introduction

Development of fish cell lines from different fish species is essential for studying various aspects of cellular physiology, molecular biology, genetics, immunology, endocrinology, toxicology, nutrition and biotechnology [1–12]. Since the first report of a fish cell line [13], two hundred eighty three fish cell lines from different fish species have been reported [12]. In Indian scenario, approximately 30 cell lines from *Etroplus suratensis* [14], *Labeo rohita* [15], *Puntius densonii* [16], *Puntius sophore* [17], *Tor tor* [18] and *Puntius chelynoides* [19] have been established.

Wallago attu, commonly known as freshwater shark, is one of the large freshwater catfish inhabiting the rivers, reservoirs and connected watersheds of the Indian subcontinents [20]. It is a bony fish, belonging to the family Siluridae, grows about 2 m, weighing more than 45 kg [20]. The rapid growth, majestically elongated, silvery body, and high nutritional quality of flesh makes this species a potential candidate species for aquaculture. Presently the species has been declared as an endangered fish species due to over-exploitation of natural stock and destruction of their natural habitat due to various anthropogenic activities, use of agricultural insecticides and ichthyotoxic materials and fish diseases (epizootic ulcerative syndrome) [21, 22].

The establishment of a cell line from *W. attu* would be useful for testing its susceptibility to infectious agents and for toxicological and exogenous gene expression studies. In the present study, we report the establishment and characterization of WAF cell line derived from caudal fin of *W. attu*.

Materials and methods

Specimens

Healthy juveniles of *W. attu* (5–10 g) were collected from the Gomti river, Lucknow and were kept in clean

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$6 \times 3 \times 2$ ft^3 fibre reinforced plastic tanks at the wet lab of National Bureau of Fish Genetic Resources, Lucknow. The juveniles were acclimatized in hygienically maintained freshwater with proper aeration and frequent water exchange for a minimum period of 1 month. They were fed twice a day @2 % of their body weight.

Explant preparation

One month old fingerlings of about 4–6 cm were selected for the study. They were starved in sterile, aerated water containing 1,000 IU/ml penicillin and 1,000 µg/ml streptomycin for 24 h at room temperature. Fish was sterilized by dipping in 0.5 % w/v iodine solution for 5 min and then euthanized in ice before explant preparation. Caudal fin was excised aseptically and washed with PBS containing 500 IU/ml penicillin and 500 µg/ml streptomycin and 2.5 mg/ml fungizone. The tissue samples were then minced with sterile dissecting blades and scissors at room temperature. The minced tissues were washed four times with PBS containing antibiotic and antimycotic solution and then seeded into 25 cm^2 cell culture flasks (Nunc, Denmark) with 50 µl fetal bovine serum (FBS) (Invitrogen). The flask was incubated overnight at 28 °C for successful attachment of the explant to the flask surface. L-15 medium (Invitrogen) supplemented with different concentration of serum (10, 15, and 20 %) was added and half the medium was changed after every 5 days. The flasks were observed daily for explants attachment, proliferation and migration of cells. Morphology of cells radiating from explants was observed regularly using an inverted microscope (Olympus Optical Co., Ltd.).

Subculture

After reaching 90–95 % confluency, the cells were carefully dislodged by treating with 0.25 % trypsin solution and 0.2 % ethylenediaminetetraacetic acid (EDTA) in PBS for 30 s. The detached cells were suspended in 5 ml of fresh L-15 with 20 % FBS and seeded in 25 cm^2 tissue culture flask. A split ratio of 1:2 was maintained thereafter for subculturing. In the initial 10 subcultures, 50 % of the culture medium was replaced with the fresh medium.

Growth studies

The effect of different temperatures and FBS concentrations on cell growth was examined at 20th passage. Cells were seeded at a concentration of 1×10^5 cells in 25 cm^2 tissue culture flasks and incubated at 18, 20, 24, 28 and 32 °C for 7 days. Cells from triplicate flasks at each temperature were trypsinized and counted using a hemocytometer for a period of 1 week. Cell growth in different concentrations of FBS (5, 10, 15 and 20 %) was studied at 28 °C using the same procedure described above.

Immunofluorescence staining

Immunotyping of the WAF cells was performed with monoclonal antibodies directed against vimentin and cytokeratin (C-18) at 50th passage. In brief, cells were grown on round coverslips in 12 well tissue culture plates (Nunc). Upon reaching 90–95 % confluency, cells were washed with PBS and fixed in 4 % *p*-formaldhyde. The fixed cells were washed twice in PBS, permeabilized with 0.1 % Triton X-100, blocked with PBS containing 5 % sheep serum and then incubated for 40 min at 37 °C. Block was removed and 100 µl of 1:40 dilution anti vimentin clone V9 (V6630-CLONE 9 Sigma), and a 1:400 dilution of anti pan cytokeratin clone-11 (C2931-Clone C-11Sigma) was added in duplicate wells and incubated for overnight at 4 °C. Then, the cells were washed with PBS and were incubated for 30 m with 100 µl of 1:300 dilution of FITC-labelled anti-mouse IgG. The cells were then washed in PBS, mounted with 50 % glycerol in PBS and were observed under fluorescence microscope. Appropriate controls for autofluorescence and secondary antibodies were included.

Cell plating efficiency

The plating efficiency of cell line was determined at 35th passage. Plating efficiency for the cell line was determined by seeding 200, 500, and 1,000 cells (z) per flask (25 cm^2 tissue culture flask) at 28 °C in L-15 medium with 20 % FBS. After 12 days, the medium was discarded and the cells were fixed with 5 ml of 1 % crystal violet in 25 % formalin stain-fixative solution for 15 min, rinsed with tap water and air-dried. The experiment was done in triplicate. The colonies were then counted (x) under the microscope, and plating efficiency(y) was calculated using the formula $y = 100 \times z^{-1}$ [23].

Chromosomal analysis

Chromosomal counts were established at passage 15, 25, 35 and 45. Cells were seeded in 75 cm^2 tissue culture flasks in L-15 medium with 20 % FBS. After 24 h incubation, medium was replaced with 10 ml of fresh medium containing 0.1 ml colcemid solution (1 µg ml^{-1}), (Sigma, St Louis, MO, USA) and incubated at 28 °C for 2 h. After harvesting by centrifugation (700 g, 5 min), the cells were suspended in a hypotonic solution consisting of 0.5 % KCl for 10 min and fixed in methanol: acetic acid (3:1). Slides were prepared following the conventional drop-splash technique [23]. The chromosomes were counted under a microscope, after staining with 5 % Giemsa for 10 min.

Molecular characterization

Template DNA for PCR assays was prepared by extraction from cultured fin cells. Briefly, the sample was homogenized in lysis buffer (100 mM NaCl, 10 mM Tris–HCl, pH 8.0, 25 mM EDTA, pH 8.0, 0.5 % sodium dodecyl sulphate, 0.1 mg/ml proteinase K) and then incubated at 65 °C for 1 h. 5 M NaCl was added to the solution to a final concentration of 0.7 M. 1/10 volume of N-cetyl *N*,*N*,*N*-trimethyl ammonium bromide (CTAB)/NaCl solution (10 % CTAB in 0.7 M NaCl) was slowly added to the mixture and the solution was incubated at 65 °C for 2 h. After incubation the digest was deproteinized by successive phenol/chloroform/iso-amyl alcohol extraction and DNA was recovered by ethanol precipitation, drying and resuspension in TE buffer. The concentration of isolated DNA was estimated at wavelength of 260 nm using a UV spectrophotometer. The DNA was diluted to get a final concentration of 100 ng/µl.

The 578 bp fragment of mitochondrial 16S rRNA gene was amplified in a 50 µl reaction volume with 5 µl of 1X Taq polymerase buffer, 0.2 mM of each dNTP, 0.4 µM of each primer, 2.5 U of Taq polymerase and 100 ng genomic DNA using the thermal cycler C-1000(BIORAD). The primers used for the amplification of the partial 16S rRNA gene were 16SAR (5′-CGCCTGTTTATCAAAAACAT-3′) and 16SBR (5′-CCGGTCTGAACTCAGATCACGT-3′) [47]. The thermal profile used was 35 repetitions of a three-step cycle consisting of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1.5 min including 4 min for initial denaturation at 94 °C and 7 min for the final extension at 72 °C.

The 655 bp fragments of cytochrome oxidase subunit I (COI) gene was also amplified in a final concentration of 50 µl volume with a final concentration of 1X Taq polymerase buffer, 2 µl of $MgCl_2$ (50 mM), 0.25 µl of each dNTP (0.05 mM), 0.5 µl of each primer (0.01 mM), 0.6 U of Taq polymerase and 100 ng of genomic DNA. The primers used for the amplification of the COI gene were FISHF1-5′TCAACCAACCACAAAGACATTGGCAC3′ and FISH R1-5′TAGACTTCTGGGTGGCCAAAGAATCA3′ [48]. The thermal regime consisted of an initial step of 2 min at 95 °C followed by 35 cycles of 40 s at 94 °C, 40 s at 54 °C and 1 min 10 s at 72 °C followed by final extension of 10 min at 72 °C.

The PCR products were visualized on 1.2 % agarose gels and the most intense products were selected for sequencing. Products were labeled using the BigDye Terminator V.3.1 Cycle sequencing Kit (Applied Biosystems, Inc) and sequenced bidirectionally using an ABI 3730 capillary sequencer following manufacturer's instructions. The obtained sequences of PCR fragments were compared to the known sequences of the species.

Transfection with GFP reporter gene

Sub confluent monolayers (70–80 % confluency) of WAF cells at 44th passage were transected with pEGFP-C1 plasmid using LTX and Plus Reagents (Invitrogen). In brief, WAF cells were seeded at a density of 1×10^5 cells per well in 12-well plates individually and incubated for 18 h at 28 °C. Before transfection, cells were rinsed with PBS and supplemented with 500 µl of fresh L-15 medium devoid of serum. The plasmid (200 ng of pEGFP-C1) was dissolved in 100 µl of OptiMEM and then 0.5 µl of plus reagent was added. The mixture was incubated for 5 min at 30 °C. 2 µl of lipofectamine LTX was then added to the mixture and incubated for 30 min. The mixture was then added dropwsie on 70–80 % confluent WAF cells in 12 well plates. The medium was replaced after 6 h. The green fluorescence signals were observed after 18 h under a fluorescence microscope (Olympus).

Cadmium cytotoxicity and genotoxicity on WAF cell line

In order to assess the applicability of WAF cell line for cytotoxicity and genotoxicity assays two cadmium salts, $CdSO_4$ and $CdCl_2$ were used. Cytotoxicity was assessed by MTT reduction assay and neutral red (NR) uptake assay in 96-well plate. Following 24 h exposure of cadmium salts at a concentration range of 100–0.00001 mg/l, the media was aspirated off and fresh L-15/ex solution was added to each well for 4 h.

10 µL of MTT solution (5 mg/ml) was added in each well and the plate was incubated for 4 h at 28 °C. Following incubation the MTT containing medium was aspirated off from the microtiter plate and the intracellular formazan crystals were extracted and solubilized in solubilization buffer. The plates were gently shaken for 10 min and the absorbance was recorded with the help of ELISA reader (Sunrise™, Tecan) at 570 nm.

NR uptake assay was performed by incubating the cadmium exposed cells with 100 µl of 33 µg/ml of neutral red solution prepared in L-15/ex solution at 28 °C for 2 h. The cells were then washed with NR fixative solution (0.5 % (v/v) formaldehyde and 1 % (w/v) $CaCl_2$ in deionized distilled water). 100 µl/well of NR extraction solution (1 % (v/v), acetic acid and 50 % (v/v) ethanol in deionized distilled water) was then added to solubilize the lysosomal neutral red. The plates were gently shaken for 10 min and the absorbance was recorded with the help of ELISA reader (Sunrise™, Tecan) at 530 nm.

Cytotoxicity experiments were performed in triplicates with six replicates for each exposure concentration. Data was analyzed with Graph Pad Prism 5. The individual data

points of the concentration–response cytotoxicity charts are presented as the arithmetic mean percent inhibition relative to the control ± standard deviation (SD).

Comet assay was used for the assessment of genotoxicity in cadmium exposed cells at a concentration equal to its IC50 value obtained from the cytotoxicity assays. Cells were trypsinized using TPVG solution (0.1 % Trypsin, 0.2 % Ethylene diamine tetra-acetic acid; EDTA and 2 % glucose in 1× PBS) before being used in the comet assay. Comet assay was carried out following the protocol of Singh et al. (1988) with minor modifications. Treated cells were embedded in 0.5 % low melting agarose layer on slide, precoated with 1.0 % normal melting agarose, coated with an additional layer of 0.5 % normal melting agarose. The cells were lysed with high salt and detergent concentrations (100 mM EDTA, 2.5 M NaCl, 10 mM tris base, 1 % Triton X-100, adjusted to pH 10) for 1 h. DNA was allowed to unwind (1 mM EDTA, 10 % dimethyl sulfoxide [DMSO]; 300 mM NaOH, pH 13) for 20 min and then subjected to electrophoresis in the same solution as for unwinding (25 V, 300 mA) for 15 min. After electrophoresis, the alkalis in the gels were neutralized by rinsing the slides in a neutralization buffer (0.1 M Tris pH 7.5) for 5 min. The slide was treated with methanol for 10 min, stained with 45 µl of 20 µg/ml ethidium bromide solution and viewed under a fluorescent microscope (Olympus Optical Co. Ltd.).

Cryopreservation

The viability of cells following storage in liquid nitrogen (LN_2) was evaluated in freezing medium at 20, 30, 40 and 50 passages. In brief, cells growing logarithmically were harvested by trypsinization and concentrated by centrifugation. The pellet was washed with PBS and cell count was adjusted to 3×10^6 cells per ml of L-15 medium with 20 % FBS and 10 % DMSO. Aliquots (1.0 ml) were dispensed into 2.0 ml sterile cryovials (10 numbers) (Nunc) held at 4 °C for 2 h, −20 °C for 1 h, −80 °C overnight and then transferred into liquid nitrogen containers (−196 °C). After 6 months of storage, the vial was thawed quickly in a water bath at 28 °C. Freezing medium was removed by centrifugation. The cells were suspended in L-15 medium with 20 % FBS and seeded into 25 cm^2 tissue culture flasks. The viability of the cells was measured by trypan blue staining and the number of cells was counted using haemocytometer.

Results

Morphological observation

Morphological observation under the inverted microscope revealed that cell radiation started from the explant after 7–8 days of explant preparation. The cells outgrew from the explants and proliferated well to form a confluent monolayer during the first month of culture (Fig. 1a). Most of the cells proliferating from caudal fin explants were fairly heterogeneous in nature and were composed of both epithelial and fibroblast-like cells (Fig. 1b). At 25th subculture, the cell line showed fibroblast-like morphology (Fig. 1c).

Growth studies

The cells were able to grow at temperature range between 20 and 32 °C. However, maximum growth was obtained at 28 °C (Fig. 2a). Similar growth pattern of cells was observed while increasing FBS concentration from 5 to 20 % at 28 °C. Cells exhibited significant growth at 5 % concentrations of FBS, but relatively good growth was observed at 10–15 % FBS concentration. Maximum growth of the cells was observed with 20 % FBS (Fig. 2b).

Imunocytochemistry

The WAF cells showed positive reaction for cytokeratin and negative reaction for vimentin (Fig. 3).

Plating efficiency

Plating efficiency for each cell line was determined at seeding concentrations of 200, 500 and 1,000 cells. WAF cells showed plating efficiency of 60 % with no significant differences between replicates.

Chromosomal analysis

Chromosomes counts of 107 metaphase slides from WAF cells revealed 66 to 88 diploid chromosomes with a model value of 76 (Fig. 4). Both heteroploidy and aneuploidy were observed in the cell line in small proportion.

Molecular characterization

Amplification of 547 bp 16S rRNA and 654 bp COI genes of WAF cell line confirmed the origin of WAF cell line (Fig. 5). GenBank accession no. for 16S rRNA and COI of WAF cell line was JX983590 and JX983591 respectively.

Transfection efficiency

The expression of pEGFP in the WAF cell line was detected after 18 h of transfection of WAF cell line with pEGFP plasmid. (Figure 6a, b). The transfection efficiency was found to be 20 %.

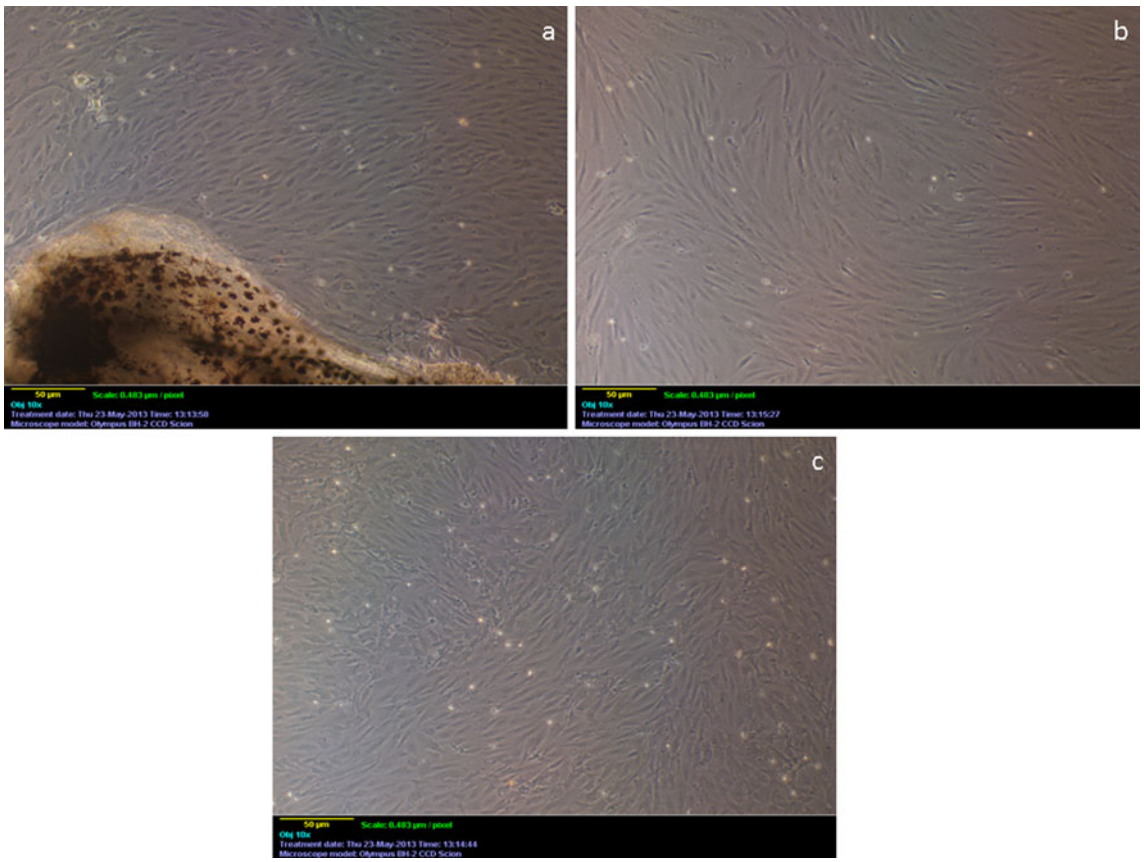

Fig. 1 Derivation of WAF cell line from fin (100×). **a** WAF cells primary culture at day 7. **b** Cells at passage 5. **c** Epithelial cells at passage 25

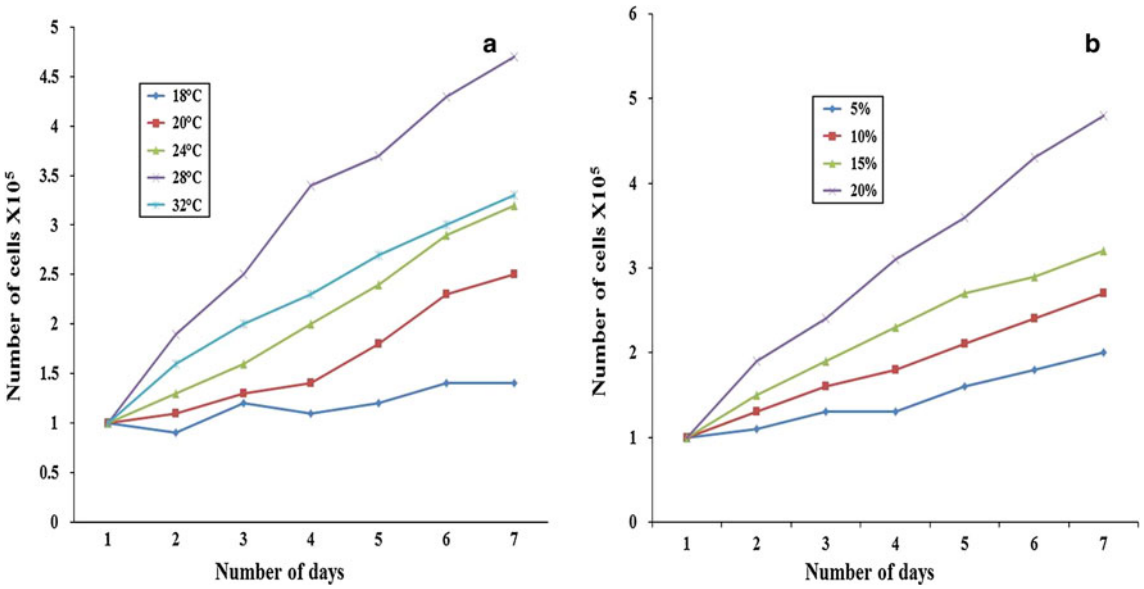

Fig. 2 Growth characteristics of WAF cell line. **a** Growth of WAF cells at different temperature (°C). **b** Growth of WAF cells at different concentration of FBS (*percentage*)

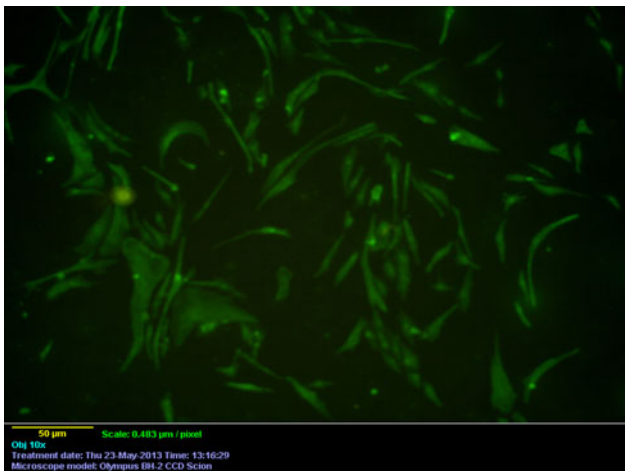

Fig. 3 Morphological characterization of WAF epithilial cell line by cytokeratin-FITC (100X)

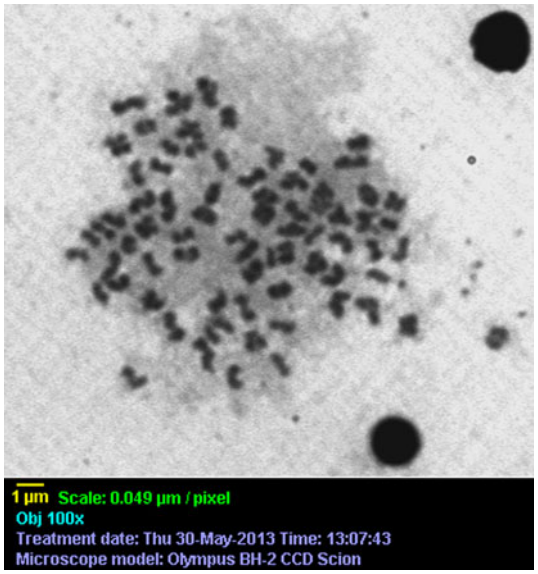

Fig. 4 A standard chromosome spread of WAF cells at passage 40

Cadmium cytotoxicity and genotoxicity assay

The dose response curve obtained from the cytotoxicity assays is given in Fig. 7a, b. IC 50 value obtained from the dose response curve of MTT assay was 0.5,685 mg/l for $CdSO_4$ and 0.4963 mg/l for $CdCl_2$. Similarly, IC50 value from NR assay was 5.724 and 5.060 mg/l for $CdSO_4$ and $CdCl_2$ respectively.

Exposure of WAF cells to cadmium salts demonstrated comet formation in most of the cells with high damage in DNA whereas there was no comet or DNA damage observed in negative control (Fig. 8a, 8b).

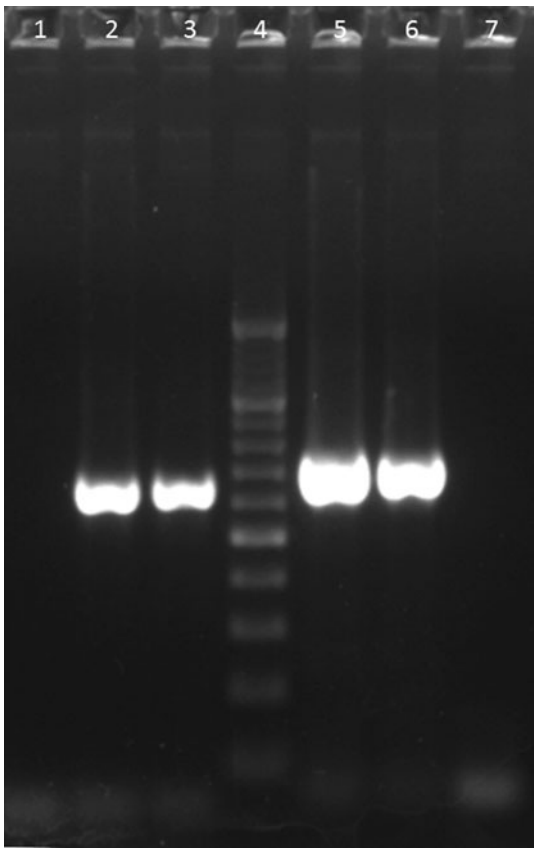

Fig. 5 PCR amplification of 547 bp 16S rRNA and 654 bp COI mt-DNA genes of WAF cells. *Lane 1* 16S rRNA negative control; *lane 2* WAF 16S rRNA; *lane 3* postive control 16S rRNA; *lane 4* 100 bp Generuler express DNA ladder (Fermentas); *lane 5* WAF COI; *lane 6* postive control COI; *lane 7* negative control COI. (negative control: without template; positive control: muscle tissue of *W. attu*)

Cryopreservation

Evaluation of the viability of WAF cells stored in liquid nitrogen (−196 °C) expressed the capability of the cells to survive following 6 months of cryostorage. The cells recovered from liquid nitrogen storage showed 68–70 % viability and grew to confluency within 7 days. Following storage, no alterations in morphology or growth pattern was observed.

Discussion

Fish cell lines have been found to be a powerful tool to study epidemiology, molecular carcinogenesis, toxicology and functional genomics in fish [1, 2]. However, presently very few fish cell lines are available for research in tropical species. The explant technique has many advantages over the trypsinization method in terms of speed, ease and maintenance of cell interactions and the avoidance of

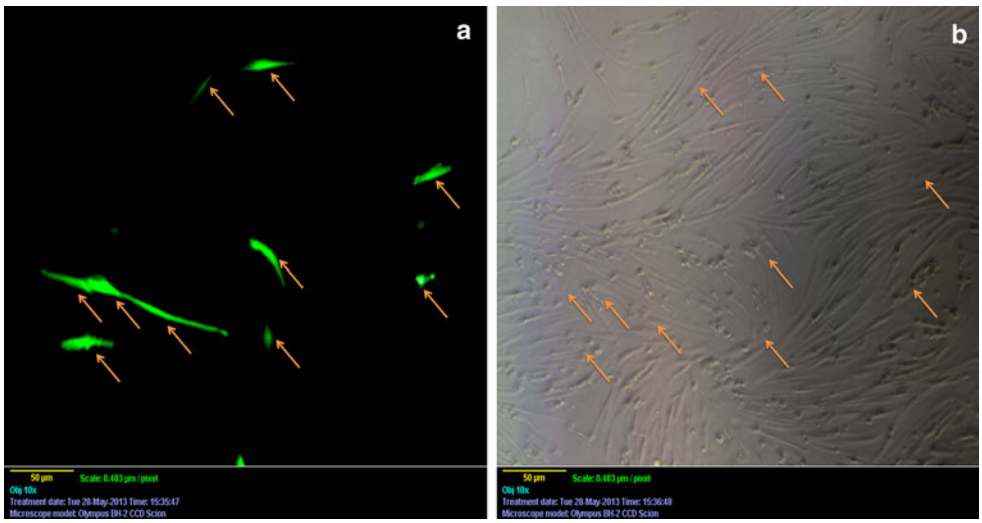

Fig. 6 Expression of green fluorescent protein in WAF cells transfected with pEGFP-C1 vector. **a** Fluorescent view (100×). **b** Phase contrast view (100×)

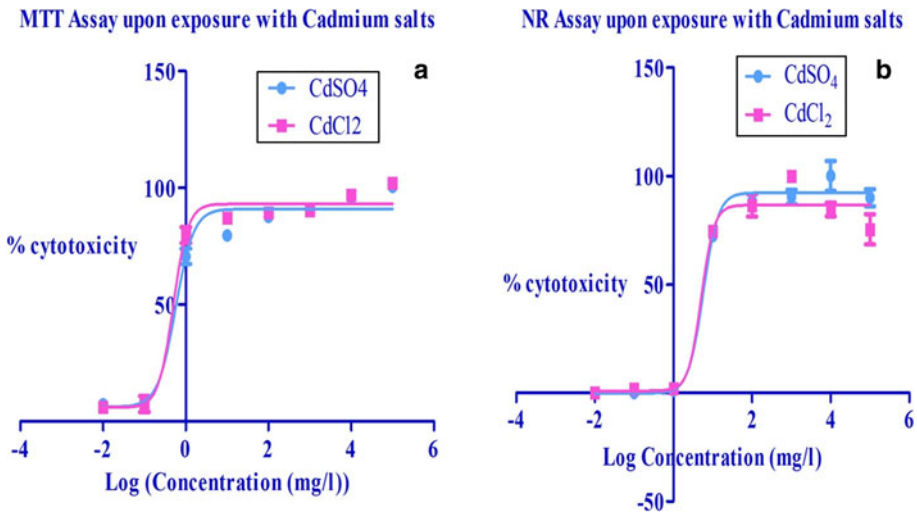

Fig. 7 Dose response curve obtained by cytotoxicity assay of cadmium salts on WAF cells **a** MTT assay. **b** NR assay

enzymatic digestion which can damage the cell surface [24]. In contrast to enzymatic method, the explant technique involves spontaneous migration of cells while retaining the histotypic environment of the tissue. The tissue of choice and optimum physico-chemical environment-like culture medium, FBS concentration, growth supplements, incubation temperature, etc. varies considerably across fish species [25]. The suitability of Leibovitz's L-15 media for supporting the growth of fish cell lines compared with other media has been documented by Fernandez et al. [26] by comparing the growth of many fish cell lines in different culture media at different temperature. FBS is essential for survival and optimal growth of cells. In primary cell cultures, FBS at high concentrations (20 %) was favorable for cell growth and attachment. Relatively good growth of the fish cell line was observed at 10–15 % FBS but maximum growth was observed at 20 % FBS concentration. The cell line could be maintained at 10–15 % FBS to minimize the cost. The growth temperature for WAF ranged between 20 and 32 °C while the optimum growth temperature was 28 °C, which was in conformity with other fish cell lines reported [18, 27]. Unlike human/mammalian cell lines, fish cell line can tolerate a wide temperature range due to high content of unsaturated fatty acids in their cell wall. The highest growth rate of various tropical fish cell lines was observed at 32 °C [28], 28 °C [29] and 20–25 °C [30]. A temperature of 35–37 °C has been reported to be lethal to many fish cells [30]. One of the advantages of cell lines that grow over a wide temperature range is their potential suitability for isolating both warm water and coldwater fish viruses [31]. Mixed population of cells were present in primary culture of *W. attu* fin i.e. both fibroblast-like and epithelial-like cells. While in subsequent sub cultures, elongated epithelial-like cells dominated over fibroblast-like cells. The epithilial morphology of the WAF cells was confirmed by cell-specific marker. Usually, a predomination of fibroblastic cells over epithelioid cells in cell cultures from

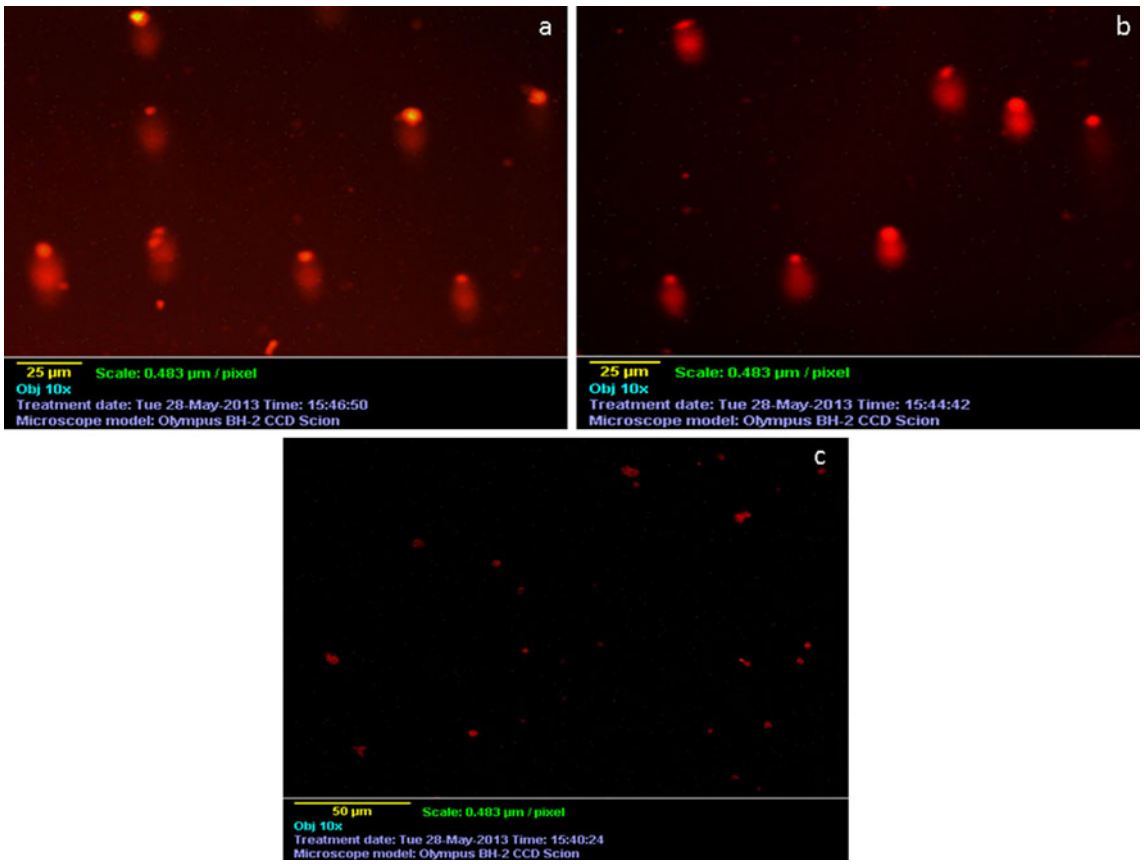

Fig. 8 Comets after single-cell gel electrophoresis of WAF cells. **a** $CdSO_4$ treated WAF cells. **b** $CdCl_2$ treated WAF cells (100×). **c** Untreated WAF cells (negative control) (100X)

fish has been reported [27, 28, 32]. In contrast, sea perch heart (SPH) cells migrating from heart tissue have been reported to be epitheloid in morphology with no change during successive propagation [33].

The WAF cell line showed significant plating efficiency i.e. 60.0 % in L-15 medium with 20 % FBS. Yadav et al. [18] recorded 63.0 % for the cell line TTCF at 20 % FBS. Chi et al. recorded the plating efficiency of 21 % of the GF-1 cells seeded at a density of 100 cells per flask at subculture 50 and this increased to 80 % at subculture 80 [34].

Generally, adaptation of cell line is often associated with changes in chromosome number and/or morphology, which gives rise to cell line specific marker chromosomes [35]. The chromosomal analysis revealed that the number of chromosomes in WAF cell ranged from 76–88 with a model value of 86. Both heteroploidy and aneuploidy were observed in the cell line though they were very small in proportion. Loss of chromosomes or additions from nearby cells during chromosome preparation could be the possible reason for the abnormal chromosome number in a low percentage WAF cells. Chromosomal analysis revealed that WAF cell lines possessed a diploid chromosome number of $2n = 86$, which was identical to the modal chromosome number of *W. attu* suggesting that this cell line is stable [36].

Hebert et al. [37] demonstrated the utility of COI gene as a universal barcode, referred as "DNA barcoding" for the genetic identification of animal life. Cooper et al. [38] used COI region for identification of 67 cell lines used for barcode analysis. Authenticity of WAF cell line was confirmed by using 547 and 654 bp fragments of 16S rRNA and COI respectively which showed 100 and 99 % homology to the voucher sequences of *W. attu* at NCBI.

The successful transfection of WAF cell line with pEGFP-C1 plasmid indicates its potential for gene transfer and expression studies. The estimated transfection efficiency was 20 % which is comparable to TTCF cell line with 7 % efficiency [18] and to other fish cell lines [39, 40]. Zhou et al. [41] reported 2 % transfection efficiency in a CSTF cell line developed from Chinese sturgeon *Acipenser sinensis* gray. This implies that WAF cell line could serve as an in vitro system for evaluation of promoter efficiency in various recombinants constructs along with exogenous gene manipulation and expression studies.

In order to assess the applicability of WAF cell line for in vitro toxicological studies, Cytotoxicity and genotoxicity

assessment was carried out by using Cadmium salts. Cd is regarded as a potential hazard for the fish and other aquatic lives because it is readily absorbed by organisms directly from the water in its free ionic form. It is used in ecotoxicological studies because its concentration rises in the environment due to some industrial and domestic wastes [42, 43]. The adverse effects of Cd on the reproductive, respiratory and hematological systems in many fish species have been reported [44, 45]. MTT and NR assays have commonly being employed in toxicological studies focusing on comparison and identification of established cell lines as sensitive biomarkers for or indicators of initial metal contamination. In the present study the cytotoxicity effect of cadmium salts on WAF cells was found to be statistically significant ($p \leq 0.05$). The study validates the use of WAF cell line for assessment of aquatic toxicity. Comet formation observed in cells treated with cadmium salts establishes the cell lines potential for genotoxicity studies.

The successful revival of cryopreserved WAF cells an interval of 6 months with 68 to 70 % viability without any significant morphological alteration or changes in growth rate would be instrumental in conservation of this important fish species. The viability of cryopreserved cell line has been reported from 50–85 % in fish cell lines (50 % for SAF-1from *S. aurata* [32]; 73 % for GF-1 from *E. coioides* [34]; 80–85 % for SF from *L. calcarifer* [46]; 75 % PSCF from *Puntius sophore* [17]).

The success in establishing new cell line from *W. attu* will be useful for in vitro research in genetics and conservation of this endangered giant fish.

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