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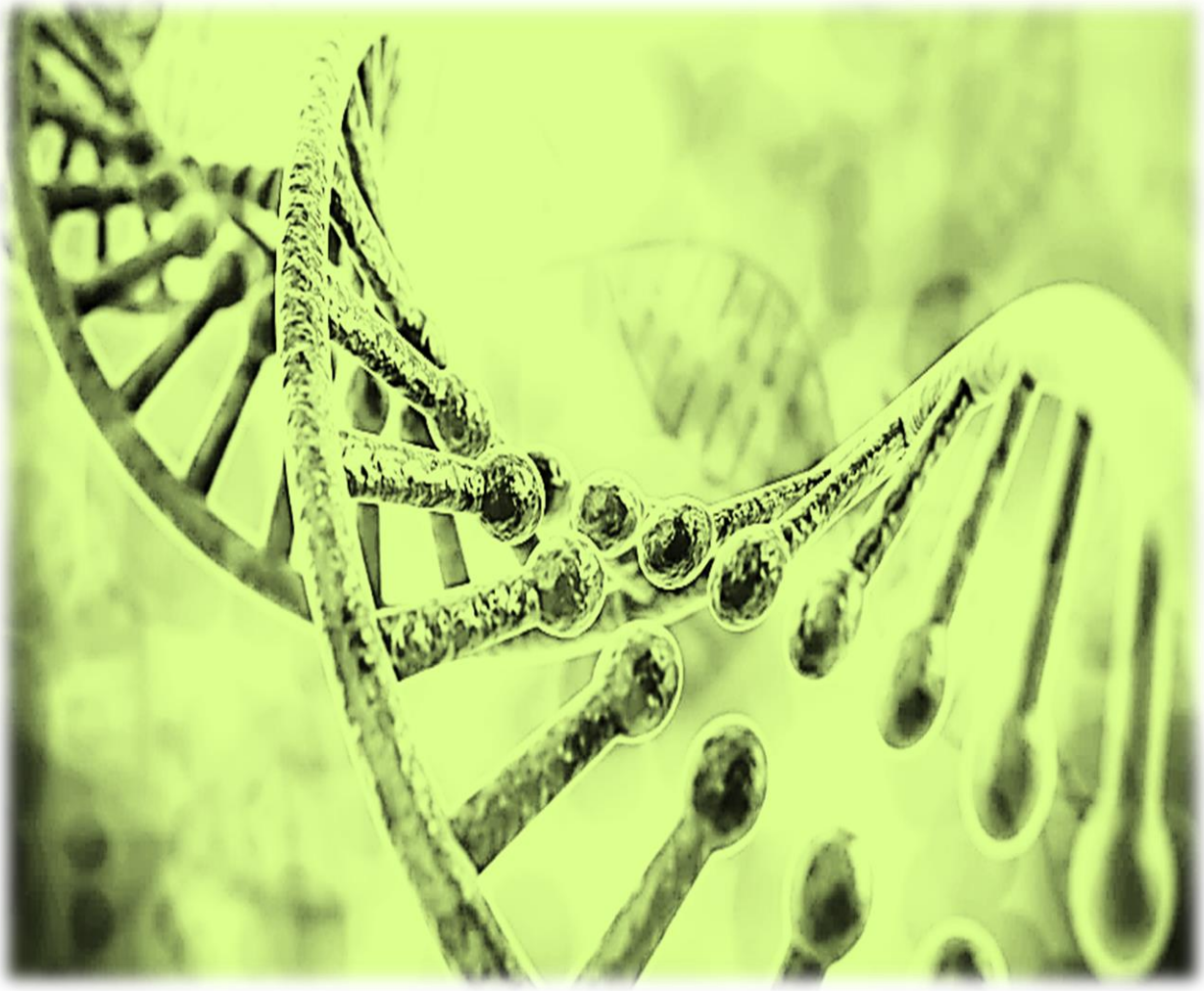
Technical Workshop on Cytogenetic Assays (MN & Comet) Including Animals Handling and Ethical Guidelines

September – 6th & 7th, 2018



Convenor

Dr. Kantha D. Arunachalam

Dean - CENR

SRMIST

Centre for Environmental Nuclear Research · SRMIST ·

PREFACE

Prof. Kantha D. Arunachalam
Dean-CENR, SRM IST

The successful advancement of Center for Environmental Nuclear Research continues! Howevver, this time we have organized the Technical workshop on **Cytogenetic Assays (MN & Comet) Including Animals Handling and Ethical Guidelines** on September 6th and 7th, 2018 at CENR, SRM IST, Chennai. The workshop sessions were expanded to include the new challenges, future trends, directions and most recent techniques in Toxicological studies. The workshop will provide a platform to an in-depth discussion on the techniques cytogenetics and genotoxicity study the effects of chemical and physical agents on genetic material in cells. The most popular and conventional methods are Micronuclei test, Comet assay and Chromosomal aberration. These methods are used in toxicological screening for potential genotoxic compound and measuring deoxyribonucleic acid (DNA) strand breaks in cells. In this workshop, we are providing hands on training of Animal Handling, Cytogenetic assays and will be given plenary session by resource persons from different institutes around India.

We are really happy to see you all in CENR- SRM Institute of Science and Technology, Kattankulathur and we hope that you had a protective and enjoyable experience on the green Institute of SRM-IST!!!!!

Prof. Kantha D. Arunachalam
Convenor

PLENARY SPEAKERS

Dr. Mary N Mohankumar,

Senior Scientist (Formerly), ESD-IGCAR

Dr. Mary N Mohankumar is a former Senior Scientist (from 1986 to 2012) in Environment and Safety Division at Indira Gandhi Centre for Atomic Research (IGCAR). She received her PhD degree from University of Madras in 1997 under the subject of Radiation Biology. She has received prestigious fellowship of the HICARE Fellow in the year of 1999 from Research Institute for Radiation Biology and Medicine (RIRBM), Hiroshima University. Her principal research interests are in the areas of Radiation Biology/Chemistry, Cancer Biology and Environmental Chemistry. She has contributed in scientific societies of Indian Association of Radiation Protection, Environmental Mutagen Society of India. She has authored and co-authored more than 50 Publications in peer-reviewed Journals. She has served as Journal referee in International Journal of Radiation Biology and Aquatic Toxicology.

Dr. S. Anbumani

Scientist (Regulatory Toxicology, CSIR-IITR

Dr. S. Anbumani is a Scientist (since 2014) in regulatory Toxicologist in CSIR-Indian Institute of Toxicology Research, Lucknow, India. Before he joined as Scientist, he worked as Research Fellow (from 2008 to 2012) at Indira Gandhi Centre for Atomic Research (IGCAR), Department of Atomic Energy, Bio-Dosimetry, Radiological Safety Division, India and Senior Research Officer at Jai Research Foundation, India. He is an expertise in Ecotoxicology and his research is focused towards the interaction of emerging contaminants in various ecosystem models at different levels of biological organization. His current research and development is the focuses on combined effects of xenobiotics at environmentally relevant concentrations in aquatic and terrestrial sentinels using biochemical, cytogenetic and molecular end points. He is authored and co-authored more than 25 Publications in peer-reviewed Journals and he is handling the project of Aquatic Radiation Toxicology.

Dr. P. Balakrishna Moorty

Director, IIBAT

Dr. P. Balakrishna Moorty completed his Master of Science in Human Genetics, Physical Anthroplogy, Andhra University, Waltair Associated Press, India, 1975 and Doctor of Philosophy, Managlore (India) University, 1982. He has worked as Project associate chromosones and criminality in All India Institute Medical Sciences, New Delhi, India during 1976-1977 and Jawaharlal Institute of Postgraduate Medical Education & Research, Pondicherry, India from 1977 to 1978. Further, he was worked as Assistant research officer in genetic toxicology, malnutrition National Institute Nutrition, Hyderabad, India from1978 to1982. He went to Hiroshima (Japan) University for Post-doctoral fellow in the cancer and molecular genetics studies during 1982 to1984. Since 1984, he is working as Director, International Institute of Biotechnology and Toxicology (IIBAT), Padappai, Tamil Nadu, India. He is a Member advisory board Faculty biomedical Science Tamil Nadu, Doctor M.G.Ramachandran Medical University, Central Insecticides Board Faridabad, India. He has completed the honourable degree of Doctor of Science, Madras (India) University in 2000. He is the life Member of Toxicology Society of India, Environmental Mutagen Society of India, Association Environmental Biologists of India, Indian Association Aquatic Biologists, Indian Science Congress, Indian Pharmacology Society and National Environmental, Science Academy.

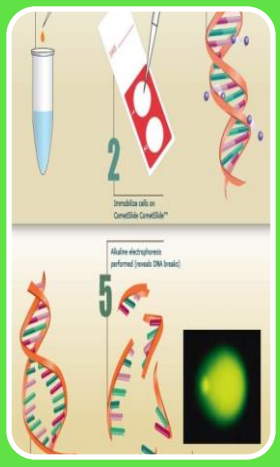
Dr. Ganesh Munusamy-Ramanujam
Asst. Professor (S.G), IIISM-SRM IST

Dr. Ganesh Munusamy-Ramanujam is a full Asst. Professor (S.G) since 2012 at the **Interdisciplinary Insititue of Indian System of Medicine,** SRM Institute of Science and Technology, Chennai. He completed Master degree in Chemistry in Ramakrishna Mission Vivekananda College under University of Madras and completed Ph.D., (2004) in Chemistry at SPIC Science Foundation, University of Madras, Chennai. After, completion of Ph.D., he was completed three Post-Doctoral Fellowship programme from RRI, ON, Canada and University of Florida, FL, U.S (From 2005 to 2011). He was also qualified the CSIR-NET (Lectureship) eligibility in 2000 and GATE in 1998 & 1999. He is an expertise in Microarray and RT-PCR gene analysis of human and animal tissue samples, Designed novel peptides with immune modulating potential and analyzed there anti-inflammatory, anti-atherogenic activity, study the impact of anti-atherogenic Serpin, SERP-1 (under clinical trial) on various immune cell types (Monocyte, Macrophages, T-lymphocytes and their helper subsets TH1, TH2 and TH17). He is actively involved in mice models and recently initiated the projects to identify anti-atherosclerotic potential of proteins (Serine protease inhibitors – Serpins) from mammalian and viral sources. Moreover, he is investigating the molecular mechanism of ayurvedic drugs and Developing novel HTS and chromatography methods. So far, he is completed the Two Major projects and contributed in 24 Research Publications and 6 Book Chapters in reputed Journals. More than, 25 Conferences were attended and presented the Research papers in nationally and international level and he is participating as a reviewer in many reputed journals.

SAMPLE PREPARATION

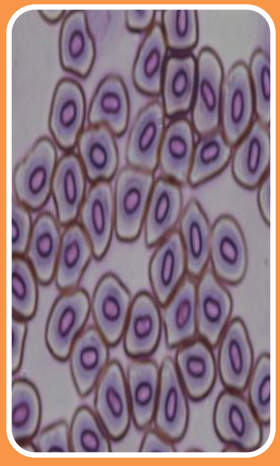
- Animal Dissection
- Blood sample collection
- *Tail*
- *Head*

COMET ASSAY



- Solution Preparation
- Sample preparation
- Methodology
- Observation
- Calculation

MICRONUCLEUS ASSAY



- Chemical Preparation
- Slide Preparation
- Image Observation
- Calculation

FISH ANATOMY AND DISSECTION GUIDELINES

Experimental fish specimens and sample collection

The *Pangasius sutchi* is one of the fast-growing catfishes, cultured in many places due to its market demand and commonly known as freshwater shark belongs to the family pangasidae. The single breed of *Pangasius sutchi* with an average length of 15±1.00 cm and an average weight of 15±1 g could be purchase from the commercial fish seed hatchery centre. After collecting the live fishes, should carefully transported to the laboratory and further will be transferred to the large tank containing oxygenated water and disinfected with potassium permanganate solution.

Study Animal Classification:

Kingdom: Animalia

Phylum: Chordata

Class: Actinopterygii

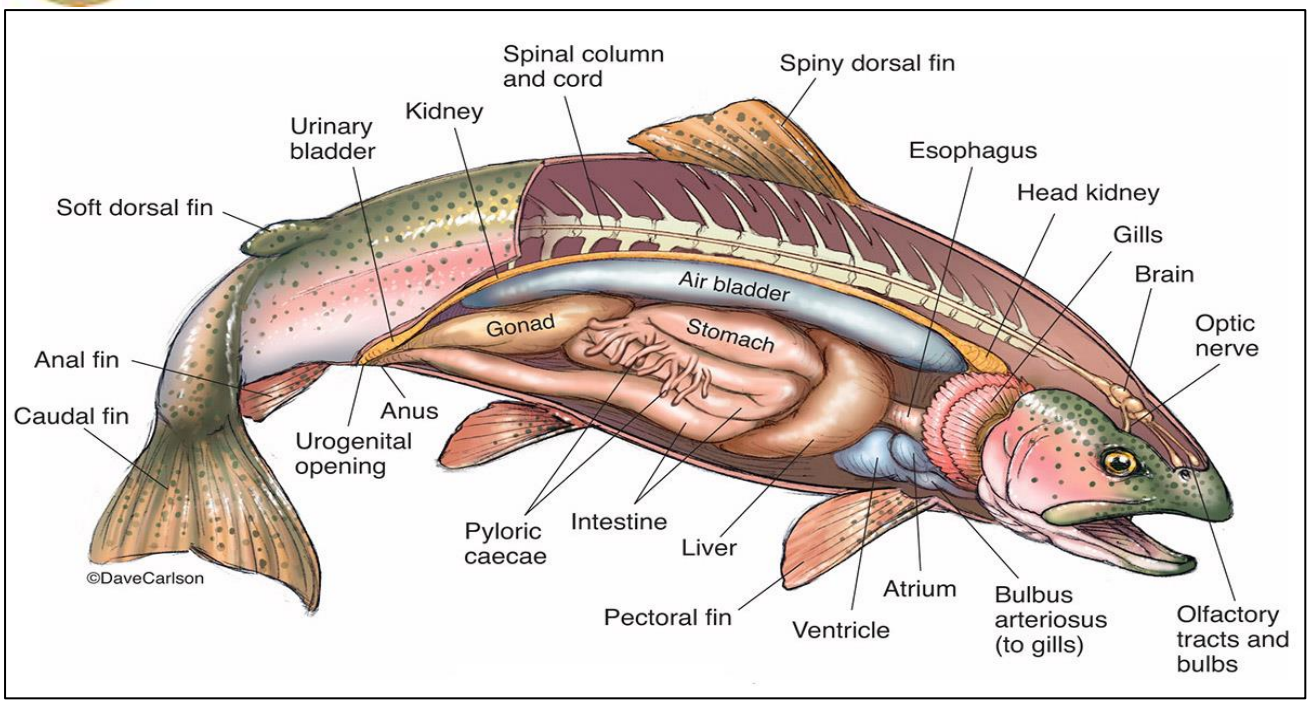
Order: Siluriformes

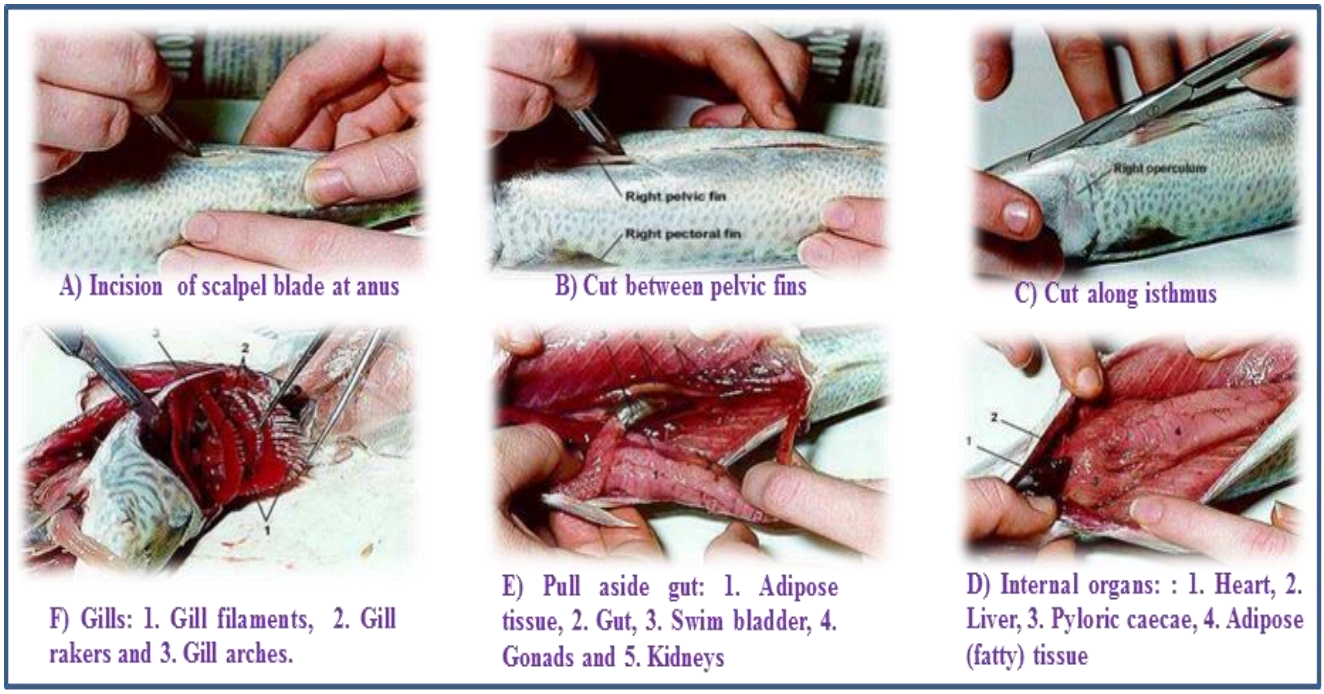
Family: Pangasiidae

Genus: *Pangasius*

Species: *sutchi*

Generally, the fishes will be fed with commercial feed, and the wastes can be removed every day to decrease the ammonia percentage in the water. Before starting any experiments, the fishes will be acclimatized under laboratory conditions of temperature 27 °C, pH 7.5, the hardness of water 220–240 ppm and 12±12 hr Light-dark cycle. As per Standard Operating Procedure (SOP), the blood sample should be collected. Blood may be sampled following four separate procedures: dorsal aorta, cardiac puncture, caudal vein, and advanced method of Heart puncture. Food should be withheld 24 hours prior to sampling to prevent regurgitation or defecation during the procedure. Blood collected for experimental purposes must be taken from living fish. Care must be taken to avoid contamination of the sample with tissue fluids. No more than 0.5-1.0 % of the fish's body weight should be removed from a fish that will be recovered from blood collection. Fish weighing less than 300 grams are blood sampled using a 1 ml syringe is advisable. Fish weighing more than 300 grams are typically blood sampled using a vacutainer needle will be suitable. Fish must be allowed adequate time to recover and regenerate blood volume if serial blood samples are to be collected.





A) Incision of scalpel blade at anus

B) Cut between pelvic fins

C) Cut along isthmus

F) Gills: 1. Gill filaments, 2. Gill rakers and 3. Gill arches.

E) Pull aside gut: 1. Adipose tissue, 2. Gut, 3. Swim bladder, 4. Gonads and 5. Kidneys

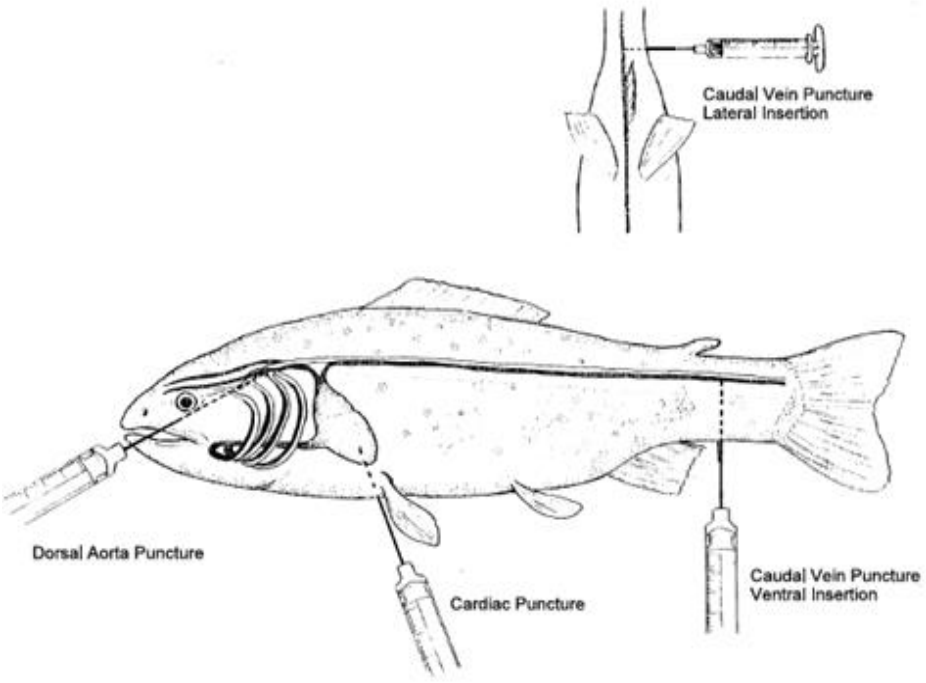
D) Internal organs: : 1. Heart, 2. Liver, 3. Pyloric caecae, 4. Adipose (fatty) tissue

Ways to collect blood in fish:

1. **Caudal vein:** Most commonly used for single puncture collections. The sample is taken midline just posterior of the anal fin. Insert the needle into the musculature perpendicular to the ventral surface of the fish until the spine is reached or blood enters the syringe. If contact with the spine is made withdraw the needle slightly. The vein is ventral to the overlying spine. This blood vessel can also be sampled laterally. In either case, inserting the needle at a 45˚ angle is most effective for

blood collection.

2. **Cardiac Puncture:** Blood is collected from the heart ventricle. Insert needle perpendicular to the ventral surface of the fish in the center of an imaginary line between the anterior most parts of the base of the pectoral fins.
3. **Caudal Severance:** For fish that are too small to bleed with a syringe and needle. Euthanize the fish. Dry the caudal peduncle. Completely sever the tail posterior to the anal fin. For best results, the fish should be held with the head above the tail region and the microhaematocrit tubule touched to the drop of blood pooling at the cut area. Do not squeeze the fish or the sample will be contaminated. The first few drops should be discarded; the rest is collected in one or more microhaematocrit tubules. After the sample is collected return the fish to a separate container of anaesthetic for euthanasia.
4. **Dorsal Aorta (Advanced):** Insert needle on a 30-40° angle into the dorsal midline in the roof of the mouth at about the 3rd to 4th gill arch. Depending upon size and species of fish insertion between the 1st and 2nd arch may be more suitable. Recovering fish tend to bleed at the mouth. This site may be used for indwelling catheterization.



Blood Sampling Methods

COMET ASSAY

The comet assay is called single cell gel electrophoresis. It is a rapid and very sensitive fluorescent microscopic method to assess DNA damage and repair in individual cells. This assay has critically important applications in fields of toxicology ranging from aging and clinical investigations to genetic toxicology and molecular epidemiology. Since the introduction of the comet assay in 1988, a number of advancements have greatly increased the flexibility and utility of this technique for detecting various forms of DNA damage and repair in virtually any eukaryotic cells.

PRINCIPLE:

The principle of the comet assay is based upon the ability of denatured, cleaved DNA fragments to migrate out of the cell under the influence of an electric field, whereas undamaged DNA migrates slower and remains within the confines of the nucleoid when a current is applied. Evaluation of the DNA "comet" tail shape and migration pattern allows for assessment of DNA damage. In this assay, cells are immobilized in a bed of low melting point agarose, followed by gentle cell lysis, treated with alkali to unwind and denature the DNA and hydrolyze sites of damage. The samples are further submitted to electrophoresis and staining with a fluorescent DNA intercalating dye. Finally, the sample will be visualized by fluorescence microscopy.

I. Preparation of Reagents for Comet Assay:

Required materials:

1. Dimethylsulfoxide (DMSO)
2. Disodium EDTA
3. Ethidium Bromide
4. Phosphate Buffered Saline (PBS) (Ca^{++}, Mg^{++} free)
5. Sodium Chloride (NaCl)
6. Sodium Hydroxide (NaOH)
7. Triton X-100
8. Tris-Hel (9.7 g of Tris + 180 ml off dis. H2O, Adjust pH to 7.5 with conc. HCl make upto 200 ml)

Reagent Preparation:

A. Phosphate – buffered saline (1 X PBS): Dissolve 8 g of NaCl, 0.2 g of KCl, 0.61 g of Na_2HPO4 and 0.24 g of KH_2PO_4 in 800 ml of distilled water. Adjust the pH to 7.4 with HCl solution and further add distilled H_2O to makeup 1 litre. Dispense the solution into aliquots

and sterilize them by autoclaving for 20 minutes at 15 lbs. Further, prepared buffer solution should be store at room temperature.

B. Lysis solution:

For 1000 mL of Lysis solution,

Add 2.5 M NaCl

100 mM EDTA

10 mM Tris-HCl; prepare 1 litre, Adjust pH to 0

Add ingredients to about 700 mL distilled water and begin stirring the mixture. Add ~8 gm NaOH and allow the mixture to dissolve (about 20 minutes) completely. Further, adjust the pH to 10.0 by using concentrated HCl or NaOH solution. Quantity sufficient to 890 mL with distilled water (the Triton X-100 and DMSO will increase the volume to the correct amount), store at room temperature.

Final lysing solution: Add fresh 1 % Triton X - 100 and 10 % of DMSO solution, and then should be refrigerate the prepared solution for at least 30 minutes prior to slide addition.

NOTE: The purpose of the DMSO in the lysing solution is to scavenge radicals generated by the iron released from haemoglobin when blood or animal tissues are used. It is not needed for other situations or where the slides will be kept in lysing for a brief time only.

C. Electrophoresis Buffer (300 mM NaOH / 1 mM EDTA)

Prepare from stock solutions:

- 10 N NaOH
- 200 mM EDTA (pH 10)

Add 60 ml of 10M NaOH and 10 ml of 20 mM EDTA then make upto 200 ml. The prepared both stock solutions should be store at room temperature. We recommended the preparation of the NaOH and EDTA stock solutions should be in every 2 weeks.

For l X running Buffer (made fresh before each electrophoresis run): Add 30 mL of NaOH stock solution and 5.0 mL of EDTA (for 1 litre), further, quantity sufficient to 1000 mL and mix well before using (The total volume depends on the gel box capacity). Prior to use, the prepared buffers solution pH should be measure and ensure that pH in >13.

D. Neutralization Buffer: The 0.4 M of Tris Hcl will be added to -800 mL distilled H_2O, adjust the pH to 7.5 with concentrated (>10M) HCI: quantity sufficient to 1000 mL with distilled H_2O, further prepared buffer solution should be stored at room temperature.

E. Staining Solution: Ethidium Bromide (*EtBr: 10X Stock - 20 µg/mL*): Add 10 mg in 50 mL distilled water, store at room temperature. For 1 X stock preparation: Mix 1 mL with 9 mL distilled water.

II. Preparation of Slides for the SCGE/Comet assay

Required materials:

1. Normal Melting Agarose (NMA) 1 % - (1 g of High EEO + 100 ml of PBS)
2. Low Melting Point Agarose (LMPA) 1 % - (1 g of Low EEO + 100 ml of PBS)
3. Methanol
4. Coverslips (No. 1, 24 x 60 mm)
5. Micro centrifuge Tubes
6. Micropipette and Tips
7. Microscope Slides, Conventional/ Micro gel electrophoresis (MGE) slides
8. Coplin jars (Opaque)
9. Horizontal Gel Electrophoresis apparatus
10. Electrophoresis power supply
11. Microscope Slide Tray (Aluminium)

Preparation of Base slides:

A. Prepare 1% of LMPA (250 mg per 50 ml PBS) and 1.0 % of NMA (50 mg per 5 ml in Milli Q water). Microwave or heat until near boiling point and the agarose dissolved. For LMPA, aliquot 5 mL samples into scintillation vials (or other suitable containers) and refrigerate until needed. When needed, briefly melt agarose in microwave or by another appropriate method. Place LMPA vial in a 37 ^{o}C dry/water bath to cool and stabilize the temperature.

B. Dip the slides in methanol and burn them over a blue flame to remove the machine oil and dust.

C. While NMA agarose is hot, dip conventional slides up to one-third the frosted area and gently remove. Wipe underside of slide to remove agarose and lay the slide in a tray on a flat surface to dry. The slides may be air dried or warmed at 500 ^{o}C for quicker drying. Further, store the slides at room temperature until needed and avoid high humidity conditions. We are recommended to prepare the slides one day before to use.

Note: Slides should be labelled before storage.

ALKALINE COMET ASSAY (Flow Chart)

The alkaline comet assay can performed by adding 10µL of cell suspension mixed with 90µL of 2% low melting temperature Agarose at 37°C, then placed on a slide pre-coated with thin layer of 0.5% high melting agarose.

The cell suspension should immediately covered with a cover glass to obtain a uniform layer and the slides will be keep at 4°C for five min, to allow solidification of the agarose (Rojas et al., 1999) and keep slides in an ice tray for a minimum period of 30 minutes.

After 30 minutes, remove the cover glass and lysed the cells in a lysing solution (2.5M NaCl, 100mM EDTA, 10mM Tris, 1% Triton X-100, and pH 10) for 1hr.

After 1hr, wash the slides in redistilled water and placed it in a horizontal gel electrophoresis chamber which filled with cold electrophoretic buffer (1mM EDTA, 300mM NaOH, pH 13) and will be keep at 4°C for 40 min to allow the DNA to unwind. Further, electrophoresis will be performed it for 25 – 30 minutes (1 V/cm, 300 mA).

After Electrophoresis, the slides should be washed three times with neutralization buffer (0.4M Tris, pH 7.5) and further stained with Ethidium Bromide (80 µL EtBr (20 µg mL^{-1})) (Cavallo et al., 2009; Kim et al., 2002).

Analyse the slides with a fluorescence microscope (NIKON Eclipse 400) equipped with a CCD-4230A video camera. The nuclei can be focus by use of a fluorescence microscope. For EtBr, a BP 546/10 nm excitation filter and a 590 nm emission filter will be used. For each slide, 25 randomly chosen nuclei should be analyzed.

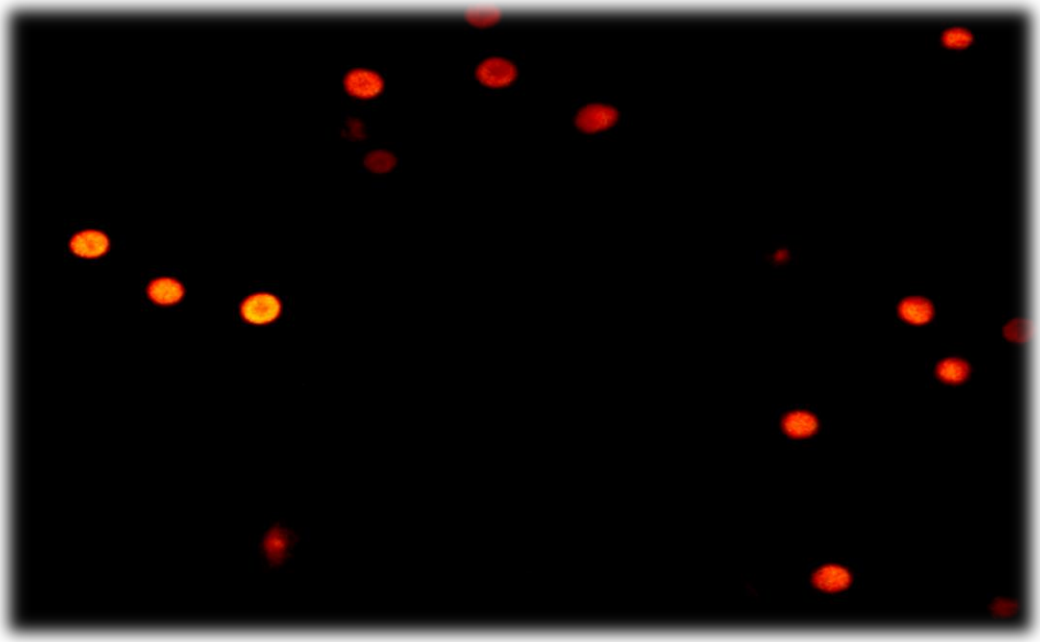
From the repeated experiments, the averaged median percentage of tail DNA as the primary measure of DNA migration should be calculated for each treatment group. Digital images could be acquired and analyzed by the CASP software (Anitha *et al.*, 2000; Emmanouil *et al.*, 2006).

$$\textbf{Genetic Damage Index (GDI)} = \frac{(\mathbf{1} * \mathbf{Class1} + \mathbf{2} * \mathbf{Class2} + \mathbf{3} * \mathbf{Class3} + \mathbf{4} * \mathbf{Class4})}{\boldsymbol{N}}$$

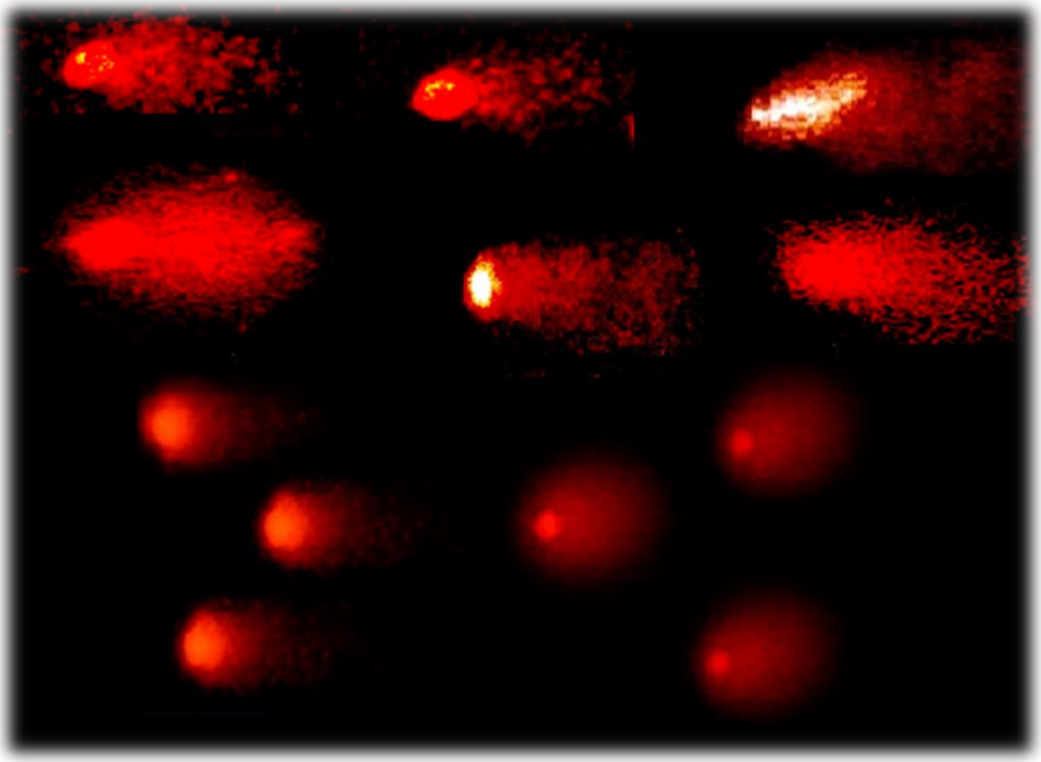
Where N is entire classes (Collins *et al.*, 2004; Odilon *et al.*, 2012).

OBSERVATION:

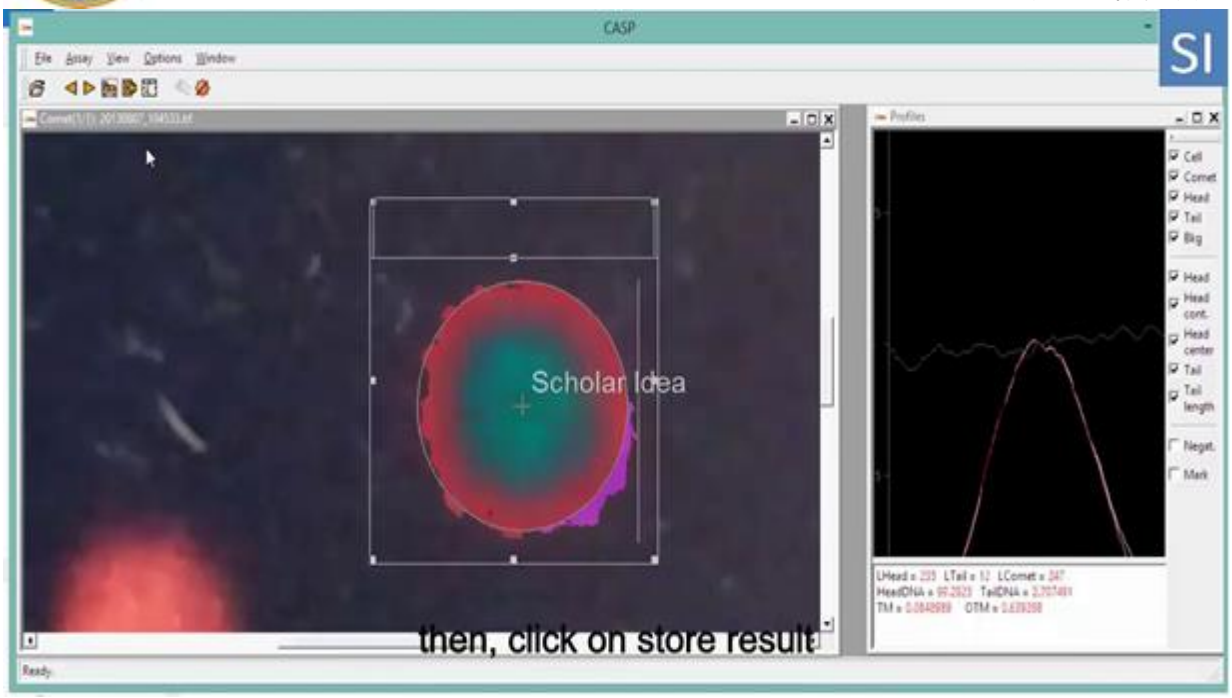
- Slides will be observed under the Fluorescence microscope with 200X magnification. Each comet will be counted with the help of CASP software and the data will be represented.



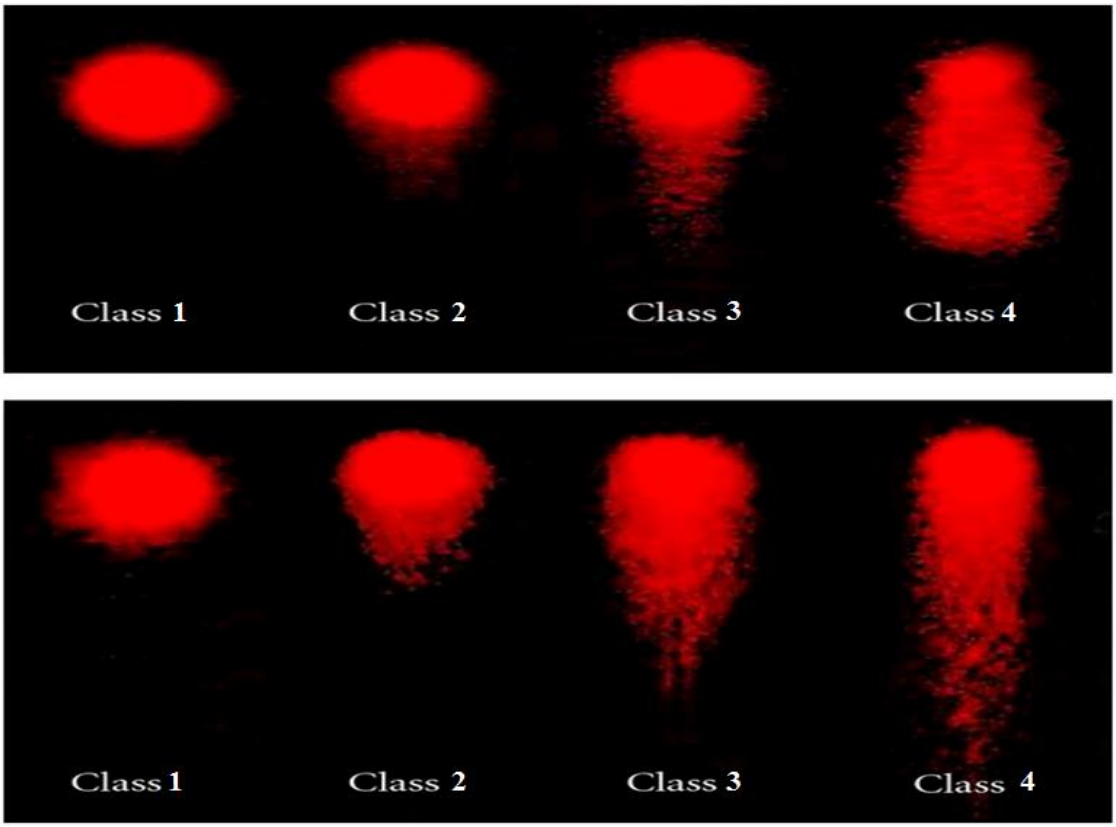
Control sample showed the comet with head and no tail



Treatment sample showed the comet with head and tail damage



CASP Software used for the measurement of Comet via percentage of head and tail damage of cell



Calculation of Genetic Damage Index could be done by categorise the comet cells in class (1-4)

MICRONUCLEUS ASSAY

Mis-joined or unrepaired DNA double stranded bands can produce deletions, translocations and acentric or dicentric chromosomes, thereby leading to significant cellular damage and cell death. Damage to chromosomes is also manifested as breaks and fragments, which appears as micronuclei in the rapidly proliferating cells.

Materials required

1. Glass slides and cover slips.
2. Methanol
3. May–Grunwald stain
4. Giemsa stain
5. Light Microscope.

MICRONUCLEUS CYTOME ASSAY (Flow Chart)

The blood samples should collected by cardiac puncture using the cold hypodermic micro syringes pre-resin with heparin/EDTA (anticoagulant), or citrate buffer (sucrose 250mM, Trisodium citrate 40mM and DMSO 5% with pH 7.6).

The blood samples were immediately smeared onto pre cleaned glass slides, air dried overnight, and then mixed with absolute methanol for 15 min.

Each slide should be stained with May Grunnwald solution for 10-15 minutes and wash it with distilled water and make it dry then stained with 5% Giemsa solution (Medox Biotech Pvt Ltd, India) for 20min. Finally wash it again with distilled water and make it dry.

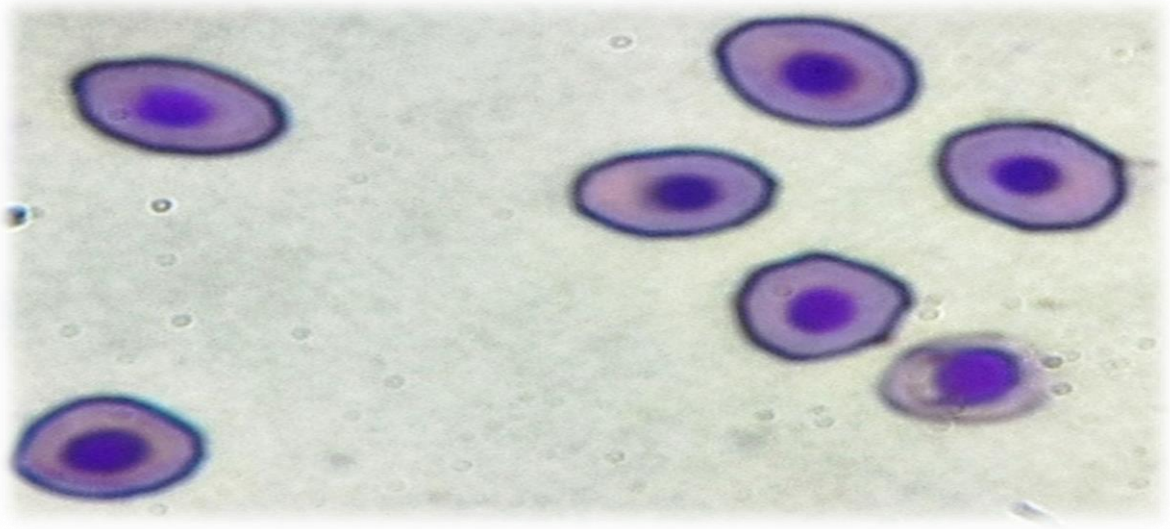
Identify at least 1000 erythrocytes for each specimen, countered and scored microscopically under 1000, X in Carl Zeiss microscope (Bolognesi and Hayashi, 2011).

$$\textbf{Nuclear Division Index} = \frac{(1*M1+2*M2+3*M3+4*M4+5*M5)}{N}$$

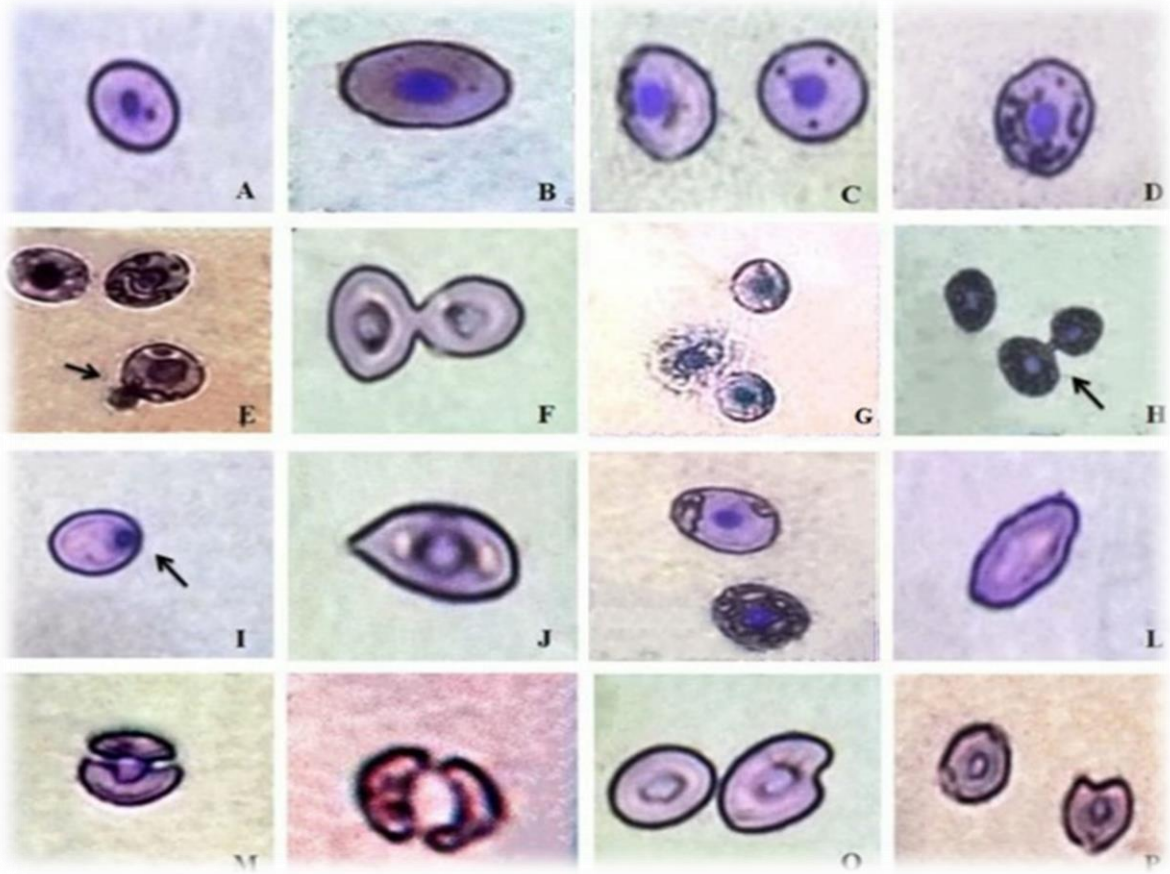
Where M1through M5 represents the number of cells with one to five or multi-nuclei, and N represents the total number of cells scored (Eastmond*et al.*,1989).

OBSERVATION:

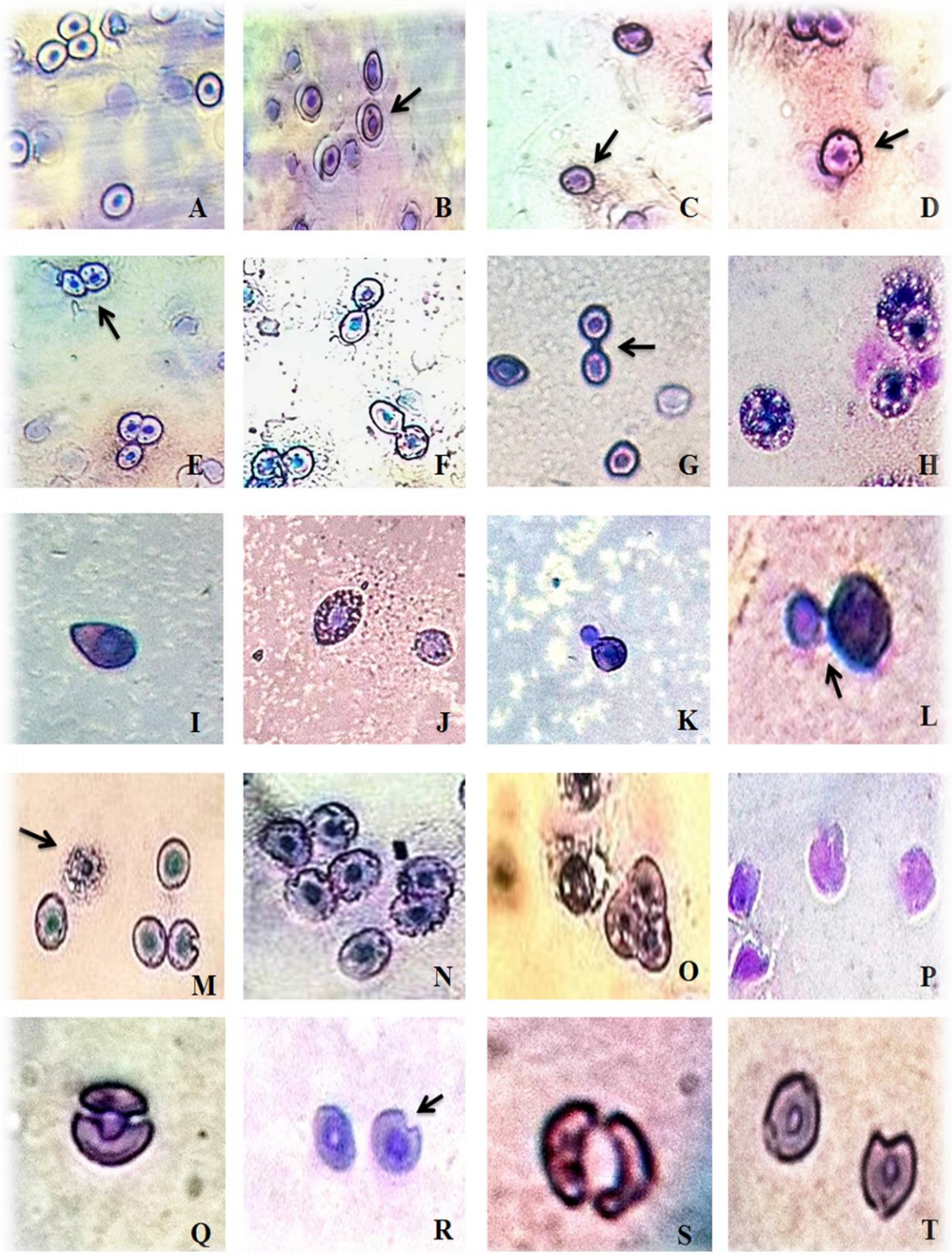
- Slides can be observed under light microscope with 400X magnification.



Control sample shows cells without any Erythrocytic abnormalities



Treatment sample shows cells with many Erythrocytic abnormalities



Erythrocytes abnormalities in fish observed at 400X magnification: (A) Normal cells; (B) Micronuclei; (C) Binucleated cell; (D) Trinucleated cell, (E) Multinucleated cell; (F) Abnormal cell division; (G) Cell contain cytoplasmic bridge; (H) Vacuolated cells; (I) Echinocytes; (J) Cell-wall and cytoplamic damaged cell (K) Budding cell; (L) Budding cell and loop formation; (M) Apoptotic cell; (N) Cell wall damage; (O) Lobed cell; (P) Enucleus cell (Q-S) Other abnormality.

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