Appendix-II

Trypan Blue Solution:
One gram of Trypan Blue (Cl 52040, Spectrochem, India) dissolved in 100 ml of double distilled water.

Semen diluent:
Semen diluent contained 50 g of NaHCO₃, 10 ml of 35% v/v neutral formalin and double distilled water (Total volume 1000 ml)

Haematoxyline stain solution:
Aluminium ammonium sulphate (160 g) (BDH, Glaxo, Mumbai) was dissolved in double distilled water by heating. Eight grams of haematoxylin crystals (C.I. 75290 Loba Chemie, Mumbai, India) were dissolved in 95% ethanol. The haematoxylin solution was added to aluminium ammonium sulphate solution and the mixture was heated to 95 °C. The mixture was removed from heat and 6 g of mercuric oxide was slowly added stirring continuously. The container was immediately plunged into a cold-water bath and filtered when the solution was cold. The solution was allowed to stand for 48 hours and was diluted with equal parts of double distilled water.

Orange G 6 solution:
Stock solution No.1
10% aqueous solution was prepared by dissolving 10 g of Orange G crystals (C.I. 16230 Loba Chemie, Mumbai, India) in 100 ml of double distilled water. The mixture was shaken well and allowed to stand in a dark brown bottle for 1 week.

Stock solution No. 2
50 ml of solution no 1 was made upto 1000 ml with 95% ethanol. 0.15 g of phosphotungstic acid was made to 1000 ml of Stock No. 2. The solution was mixed well and filtered before use.
**EA 50 solution:**
10% stock solutions were prepared of Eosin (Cl 45380, Loba Chemie, Mumbai, India), Light Green SF Yellowish (Cl 42095) and Bismarck Brown (Cl 21000). 50 ml of 10% Eosin (Cl Cl 16230, Loba Chemie, Mumbai), 10 ml of 10% Bismarck Brown (Cl 21010, BDH Chemicals, Glaxo Mumbai) and 12.5 ml of Light Green SF (Cl 42095, BDH Chemicals Glaxo, Mumbai) were mixed and the volume was made to 2000 ml with 95% ethanol 4 g of phosphotungstic acid (Loba Chemie, Mumbai) was added followed by 0.5 ml of saturated lithium carbonate solution (Loba Chemie, Mumbai).

**Acid Ethanol:**
10 ml of concentrated HCl was added to 150 ml of 99.5% ethanol. 50 ml of double distilled water was added to the solution.

**Hypo Osmotic Swelling (HOS) solution:**
The HOS solution was prepared by dissolving 0.735 g sodium citrate (Na₃C₆H₅O₇· 2H₂O) and 1.351 g fructose in 100 mL double distilled water.

**Tyrode’s solution:**
Tyrode’s solution was prepared as described in WHO Manual (1992). The following chemicals were added to 1000 cc of double distilled water 8.0 g of sodium chloride, 200 mg each of potassium chloride (KCl) and calcium chloride (CaCl₂), 200 mg of magnesium chloride (MgCl₂·6H₂O), 50mg of sodium acid phosphate (NaH₂PO₄), 1g each of sodium bicarbonate (NaHCO₃) and glucose

**Carnoy’s fixative solution:**
The fixative was prepared by mixing 3 parts of methanol and 1 part glacial acetic acid.

**Acridine orange Stain:**
10 ml of 1% Acridine Orange (Sigma Chemicals, USA) in double distilled water added to a mixture of 40 ml of 0 1 M citric acid and 2.5 ml of Na₂HPO₄, 7H₂O. The solution was always prepared fresh and filtered before use.
**Zinc Sulphate Solution:** 1.8 grams of ZnSO$_4$ $\cdot 7$H$_2$O was dissolved in 100 ml of double distilled water.

**Preparation of Indol-reagent:** 200 mg of benzoic acid was added to 100 ml of double distilled water and dissolved by repeated shaking in a hot water bath (about 60 °C). When all benzoic acid was dissolved, 25 mg of indol (Sigma Chemicals, USA) was added. The solution was filtered and stored at 4 °C.

**Stock Fructose Standard (2.8 mM):** 50.4 mg of fructose (Qualigens, Fine Chemicals, Mumbai) was dissolved in 100 ml of distilled water.

**Working Fructose Standards:** On the day of analysis one portion of the stock fructose standard solution was diluted to 0.56 mM, 0.28 mM and 0.14 mM concentration standards.

**Preparation of Zn standard:**
Stock standard Solution: (1000 ppm Zn solution E-merck) Stock solution was diluted serially with deionized water to produce 0.5, 1.0, 10.0 mg/L of zinc. The absorbance was recorded and a calibration curve was produced from these concentrations.

**Preparation of Cu Standard:**
Stock standard Solution: (1000 ppm Cu solution E-merck) Stock solution was diluted serially with deionized water to produce 1.0, 10.0 mg/L of copper. The absorbance was recorded and a calibration curve was produced from these concentrations.

**Preparation of Pb standard:**
Stock standard Solution: (1000 ppm Pb solution E-Merck) Stock solution was diluted into standard solutions of 0.5, 1.0, 2.0, 4.0 and 8 mg/L concentrations of lead. All the standards were prepared in 0.2 % HNO$_3$. The
absorbance was recorded and a calibration curve was produced from these concentrations

**Preparation of Cd standard:**

Stock standard Solution (1000 ppm Cd solution E-Merck): The 1000-ppm standard cadmium solution (E-Merck, USA) was diluted into standard solutions of 0.25, 0.5, 2.0 and 4.0 μg/L. All the standards were prepared in 0.2 % HNO₃. The absorbance was recorded and a calibration curve was produced from these concentrations.