ESTIMATION OF PROTEIN BY LOWRY’S METHOD

**Reagent A:** 2% sodium carbonate in 0.1 N sodium hydroxide.

**Reagent B:** 0.5% copper sulphate in 1% potassium sodium tartarate.

**Reagent C:**
Alkaline copper solution - Mixed 50ml of solution A and 1ml of solution B prior to use.

**Reagent D:**

**Folin ciocalteau reagent:** Reflux gently for 10 hrs a mixture consisting of 100g sodium tungstate, 25g of sodium molybdate 700ml water, 50ml of 85% phosphoric acid and 100ml of concentrated hydrochloric acid in 1.5l flask. Added 150g lithium sulfate, 50ml water and few drops of Bromine water. The mixture was boiled for 15 minutes without condenser to remove excess bromine, cooled, diluted to 1litre and filtered. The reagent should not have greenish tint.

SODIUM DODECYL SULPHATE – POLY ACRYLAMIDE GEL ELECTROPHORESIS (SDS- PAGE)

1. **4X stacking buffer:**
   - Tris HCL - 12.10 g
   - Distilled water - 80.00 ml
   - Adjust the pH to 6.8
   - Add 0.8 g of SDS (Sodium Dodecyl Sulphate)
   - Make up to 200ml.

2. **4X separation buffer:**
   - Tris HCL - 91 g
   - Distilled water - 300 ml
   - Adjust the pH to 8.8 by HCl
Add 2 g of SDS (Sodium Dodecyl Sulphate)  
Make up to 500ml.

3. **2X sample buffer:**

   62.5mM Tris HCL (pH 6.8) - 25 ml  
   (Stacking Buffer)  
   4% SDS - 3g  
   20% glycerol - 10 ml  
   Make up to 100ml with distilled water.  
   **Note:** Glycerol must be added at the last only.

4. **10% Ammonium Per Sulphate (APS)**

   0.1 g APS is taken and dissolved in 1 mL distilled water

5. **Acrylamide and Bisacrylamide**

   Acryl amide - 30g  
   Bisacrylamide - 0.3g  
   Distilled water - 100mL

6. **10X running buffer:**

   Tris HCL - 250 mM  
   Glycine - 2 M  
   SDS - 1%  
   Make up to 1000 ml with distilled water.

7. **Staining solution:**

   Coomassie Brilliant Blue - 200mg.  
   Methanol - 50ml.  
   Glacial acetic acid - 7ml.  
   Distilled water - 43ml.  
   The above mixture was filtered using Whatman No 1 filter paper.
8. Destaining solution:

- Methanol: 30ml.
- Glacial acetic acid: 7ml.
- Distilled water: 63ml

9. 10X Running Buffer

- 250 mM Tris HCl: 39.4g
- 2M Glycine: 150.14g
- 1% SDS: 10g

Make up to 1 litre with distilled water.

Preparation of 1X running buffer:

Take 100 ml of 10X running buffer and make up to 1 litre with distilled water.

10. Gel loading Dye

10 mg of Bromophenol blue was dissolved in 1mL of distilled water.

11. Separating gel (for 15ml)

<table>
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<tr>
<th>S.NO</th>
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<th>5%</th>
<th>6%</th>
<th>7%</th>
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12. Stacking Gel

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IMMUNO HISTOCHEMISTRY- ANALYSIS OF BACTERIAL REPLICATION

1. 10X PBS (Phosphate Buffered Saline)

NaCl - 80.00 g
KCl - 2.00 g
Na₂HPO₄ - 14.4 g

Add H₂O - 800 ml.

Adjust the pH to 7.4 with HCl. Add distilled water upto 1000ml.

Autoclave for 20 mins and store at room temperature.

2. Preparation of 1X PBS (Phosphate buffered Saline):

Take 100 ml of 10X PBS and make upto 1 litre with distilled water.

3. Bromodeoxyuridine (BrdU)

Stock solution - 100mg of BrdU in powder form is taken and dissolved in 1mL 1X PBS.

Working solution - 10μL of stock solution of BrdU + 90 μL of 1X PBS
4. **Crude cork**

Stock solution – 5mg crude cork was taken and dissolved in 50 µL methanol.

Working solution - 5 µL (50 µg) of stock solution + 45 µL methanol

5. **α-D-glucopyranosyl- chromone**

Stock solution – 5mg of α-D-glucopyranosyl- chromone was dissolved in 50 µL methanol.

Working solution - 5 µL (50 µg) of stock solution + 45 µL methanol

6. **Ciprofloxacin**

Stock solution – 5mg of antibiotic ciprofloxacin was dissolved in 50 µL methanol.

Working solution - 5 µL (50 µg) of stock solution + 45 µL methanol

7. **Preparation of 1X TBST**

1X PBS - 10 mL

Tween 20 - 10 µL

8. **Dilution of primary antibody**

Primary Antibody - 1 µL

(Monoclonal anti BrdU antibody produced in mouse)

Bovine Serum Albumin (BSA) in 1X TBST - 1000 µL

[0.2 g BSA in 10mL TBST]

9. **Dilution of secondary antibody**

Secondary Antibody - 1 µL

(Goat Anti- mouse IgG HRP)

Bovine Serum Albumin (BSA) in 1X TBST - 1000 µL

[0.2 g BSA in 10mL TBST]
10. Preparation of DAPI developing solution

DAP - 250 mg
H₂O₂ - 25 µL
H₂O - 10 mL

AGAROSE GEL ELECTROPHORESIS

1. TE Buffer

Tris HCl -
EDTA -

2. Preparation of 6x loading dye

Bromophenol blue (W/V) - 0.25%
Xylene cyanol FF (W/V) - 0.25%
Glycerol in H₂O (V/V) - 30%
The dye was stored at 4 °C for further analysis.

3. Preparation of Agarose

Agarose - 0.5 g
1X TE buffer - 100 mL.

TOXICOLOGY

1. Preparation of Stock solution using cork extract

5 mg of crude cork extract was dissolved in 50 µL ethanol.
2. Preparation of working solution

From the stock solution, 5 µL was taken and diluted with 45 µL of ethanol.

1 µL of working solution will contain 10 µg of crude.

HISTOLOGY

Preparation of various grades of 2-propanol

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<th>% of alcohol</th>
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<td>90%</td>
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</table>
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Unique Phenotypes in the Sperm of the Earthworm *Eudrilus eugeniae* for Assessing Radiation Hazards


*Department of Biotechnology, Manonmaniam Sundaranar University, Alwarkurichy, Tamilnadu, India.*

*Corresponding author – Dr.S.Sudhakar, Associate professor and Head, Dept. of Biotechnology, Manonmaniam Sundaranar University, Alwarkurichi – 627 412.

E-mail: sudhakarmsu@yahoo.com
**Abstract** The earthworm, *Eudrilus eugeniae* is a segmented worm. It has two pairs of testes whose cells are highly proliferative. It was found that the earthworm, which is irradiated with X-ray, shows the following phenotypic changes in its sperm: Fragmented acrosome in the head, break in the tail, and the appearance of zigzag sperm tail. Sperm morphology can be used as a tool to study radiation hazards in local areas. These three phenotypes were not observed in the sperm of worms exposed to different concentration of toxic chemicals such as sodium arsenate, lead acetate, and mercuric chloride. In contrast, exposure of worms to ethidium bromide caused fragmented acrosome in the head of their sperm cells.

**Key words** Earthworm. Testis. Radiation hazards. Sperm.
Introduction

Earthworms are ecologically important soil-dwelling organisms. They benefit the soil by decomposing the organic matter in it. They are used as model organisms for testing toxicity in the soil (Otitolouju, 2005; Steenbergen et al. 2005) and in other environmental soil pollution studies (Van Gesteland and Ma, 1993; Heimbach, 1992) because of their ecological importance, ubiquitous nature and sensitivity.

The testis of the earthworm is highly proliferative, containing progenitor cells, from which mature sperm cells are produced. After four mitotic divisions, the spermatogonia cells enter the seminal vesicle (Troyer, 1980) and undergo the following stages: morula (16-cell stage), spermatocytes (32- and 64-cell stage), spermatids (128-cell stage) and ultimately, the mature sperm (Troyer and Cameron, 1980; Kruger et al. 2008). The mature sperms are stored in the seminal vesicle (Troyer and Cameron, 1980). During copulation, mature sperm cells move back into the testes sacs and are ejaculated into the spermiducal funnels (Troyer, 1980). Then, through the vasa deferentia (tube-like structures connecting the seminal vesicle with the genital pore), the sperm passes out through the male genital pores (Kruger et al. 2008).

The various reports describe the effects of different chemicals on the reproduction and sperm of the earthworm. In case of pentachlorophenol cocoon production and hatching are affected. The chemical mercury is more toxic to Octochaetus pattoni (Abbasi and Soni, 1983). These metals besides having toxic effect on earthworms can also have sublethal effects on growth and reproduction. Exposure of cadmium nitrate to Lumbricus terrestris exhibited significant reduction in spermatozoa from testes and seminal vesicles (Cikutovic, 1993). Corpas et al. (1995) reported that exposure of lead to Eisena fetida causes damage in the spermatogonia and spermatocytes. The reproductive parameters of earthworms exposed to toxic chemicals seems to be useful bioindicators of soil pollution and promising tools to predict genotoxic damage of the germ cell elements. Hence, earthworms have a wide range of characteristics features that make them the most suitable animals to be used as a bioindicator for testing soils pollution (Beyer and Cromartie, 1987; Goats and Edward, 1988; Calahan, 1988; Corp and Morgan, 1991).

A disintegrating atom emits alpha, beta and gamma rays, or a combination of these types. X-rays and gamma rays are called ionizing radiations while alpha and beta rays are non-ionizing in nature. Exposure to radiation causes severe damage to the living system (Mullenders et al. 2009). Hence, it is important to monitor levels of radiation in the soil. The earthworm is apt living system to study the hazards of soil radiation. Hertel-Aas et al. 2007 studied the effect of radiation on the reproduction of the earthworm, Eisenia fetida, and found that chronic irradiation reduces the reproductive capacity of E. fetida (Nakamoril et al. 2009).

The above expressed reports illustrates the reproduction of the earthworm can be affected by both chemical and the radiation hazards in the soil. Since the world
depends on atomic power stations for the energy, it is essential to expand the knowledge of monitoring radiation hazard. In the circumstances, finding of radiation specific phenotypes in the irradiated earthworm will be constructive for screening the impact of radiation on living systems of soil.

In the present study, earthworm Eudrilus eugeniae is used to monitor the radiation hazard. The mild irradiation on worm caused the following unique phenotypes in the structure of the sperm: fragmented acrosome in the head, break in the tail of the sperm and formation of the zigzag sperm tail. The studies of the sperm morphology can be used as a tool to access radiation hazard in the local area.

Material and methods

Rearing of earthworms

The earthworm, Eudrilus eugeniae was used as a test animal for this study. E. eugeniae was cultured and maintained in the laboratory in a plastic tub containing soil, cow dung and leaf litters at room temperature (Viljoen and Reinecke, 1992). The earthworm E. eugeniae, is a segmented worm containing 80-100 segments. The segments 1-13 and a thick cylindrical collar region from 13th to 19th constitute the preclitellar region and clitellum, respectively. The segments downstream of clitellum comprise the postclitellar region. Reproductive organs such as the seminal vesicle and testis are located in segments 10-11 and 11-12, respectively.

Injection of Chemicals

Healthy earthworms of similar sizes, ranging from 1.0g to 1.2g in body weight, were carefully selected for the experiments. Chemicals such as Glucose, Sodium arsenate, Mercuric chloride, Lead acetate and Ethidium bromide (purchased from HiMedia Laboratories Pvt.Ltd. India) at three different concentrations (5, 10 and 25µg in 5µl volume) were injected into the worms using a 30-gauge needle. In these experiments, glucose was used as negative control. After 24hrs, the sperms were collected and their morphology was observed. Earthworms injected with Phosphate Buffer Saline (PBS) were considered the control group.

Irradiation by X-ray

Worms of 1.0 to 1.2 g body weight was irradiated with X-rays for 25, 50 and 75 millisieverts (mSv). Morphological changes in the sperm at 24th hr were documented.

Collection of Sperm

The anterior part, including the clitellum of the worm, was cut off and dissected. The testes was taken out and gently macerated with a fine-tipped forceps. Samples were suspended in saline at room temperature, and the cells were fixed with freshly
prepared solution of methanol-glacial acetic acid in 3:1 ratio. After mounting, they were stained with 2.5% giemsa in the phosphate buffer for 10 minutes. The slides were then viewed under the microscope.

Immunofluorescence staining
Sperms were fixed with methanol-glacial acetic acid mixture (3:1) and the fixed samples were treated with 0.05% Triton X-100 in 1X PBS. They were incubated at room temperature with 0.5 µg/ml of DAPI (4’, 6-diamidino-2’-phenylindole, dihydrochloride) in 1X PBS for 20 minutes, followed by three washings with 1X PBS to remove excess DAPI. DAPI selectively binds to the minor groove of the A-T regions of DNA and fluoresces under the UV light (Morikawa and Yanagida 1981; Lawrence and Possingham 1986; Kubista et al. 1987). The samples were finally mounted and were viewed under the fluorescence microscope (Nikon Ti S) using phase-contrast mode. The mature sperm cells of *Eudrilus eugeniae* have a 20±4 µm head and an 80±4µm long tail. One hundred sperms of the worms subjected to the different doses of X-rays and chemicals were examined carefully under the microscope.

Results and discussion
The earthworm, *Eudrilus eugeniae* is shown in fig. 1a. It is commonly referred to as West African night crawler, which occurs all over the world (Shagoti, 1985, Segun, 1998). *E. eugeniae* is usually referred as epigeic species (Domínguez et al. 2001) because they reside in 10 cm depth of the soil surface and recycle the organic waste materials (Lee, 1985). The chemicals and radiation hazards made by the humans is on the surface of the soil in the depth of about 15cm- 1m (Chowdhury et al. 2005; Zaini et al. 2008). Hence, the earthworm *E. eugeniae* is suitable for monitoring both radiation and chemical hazards.

Sperm and its production are sensitive for chemical and radiation exposure (Friedler, 1996), and radiation affects rapidly dividing cells (Neel et al. 1990). The sperm is produced in testis, and stored at the seminal vesicle. The earthworm, *E. eugeniae* was dissected as described in materials and materials, and the reproductive parts of the earthworm were shown in Fig. 1b. Then, the sperm was collected, and it was observed under phase contrast microscope (Fig. 2a). To confirm this, the sperms were stained with DAPI and observed in the fluorescence microscope. DAPI selectively bound with the nuclei of the sperm head and fluoresced as shown in Fig. 2b. The tip and the bottom of the sperm head were not stained with DAPI. The tip resembles the acrosome of the human sperm, and the bottom (unstained by DAPI) resembles the collar of the human sperm (Jockenhovel et al. 1990). Thus, it is confirmed that the sperm of *E. eugeniae* is morphologically similar to the human sperm. Figure 2c is the merged image of phase-contrast (Fig. 2a) and fluorescence (Fig. 2b) microscopies in order to study the nucleated region of the sperm.

It was found that the morphology of the sperms collected from the worms injected with different concentration of glucose were normal (Fig. 3a) as the sperm shown in
fig. 2a. Since the glucose is an important molecule for the living system from bacteria to human, it was injected for the purpose of negative control. Then, different toxic chemicals Mercuric chloride, Sodium arsenate and Lead acetate were injected, and the sperms were observed as described in the materials and methods. The same data were obtained in the sperms of worms injected with 5, 10, 25µg concentration of Mercuric chloride, Sodium arsenate and Lead acetate. The fig. 3b shows the sperm obtained from 25µg concentration of Sodium arsenate injected worm. There was no phenotypes was noted on the sperm morphology in the studies of worms injected with different concentration of Mercuric chloride, and Lead acetate individually (data not shown).

The data confirm that the toxic chemicals, Mercuric chloride, Sodium arsenate and Lead acetate have no effect on morphology of sperm. Similarly, Rongquan and Canyang, 2009 also reported that the earthworm *Pheretima guillelmii* does not show any morphological phenotype in the sperm except sharp decrease in the body weight of the worm upon the exposure of lead. But chronic exposure to heavy metals caused a reduced sperm production (Reinecke and Reinecke 1997). The exposure of worm *Eisenia fetida* to sublethal concentration of lead with manganese, it was observed that the cellular damage of spermatozoa including breakage and loss of nuclear and flagella, thickening of cell membranes, malformed acrosomes and loss of nuclear material (Reinecke and Reinecke, 1997). Reinecke et al. (1995) reported that exposure of *Eudrilus eugeniae* to the pesticide dieldrin resulted in structural damage, particularly to the nucleus, of the sperm. Mercuric chloride exposure has caused significant reduction in the sperm motility in the mummichog, *Fundulus heteroclitus* (Abu and Judith, 1987). Similarly, reports have been illustrated that the chemicals exposure affects the sperm production in mammals. Al-Omar et al. (2000) reported that lead causes decrease in seminiferous tubules diameter in adult rats. Corpas et al. (2002) showed that lead acetate causes decrease in the diameter and epithelial thickness of rat seminiferous tubules. The potential toxicity of metals, such as lead, cadmium, chromium, selenium and arsenic, caused alteration in sperm morphology, count, motility as well as biochemical disruptions of enzymes and hormones. Acharya et al. (2003) described an increase in the number of sperm with abnormal morphology and a decrease in sperm counts in mice after a single intraperitoneal injection of 100 mg lead acetate/kg of body weight. They reported that significant decline in sperm count is due to the genotoxic activity of lead.

In contrast, the sperm of worms exposed to ethidium bromide at a concentration of 25 µg caused fragmented acrosome in the head (Fig. 3c). Ethidium bromide is an intercalating agent that binds in between the two strands of DNA (Lunn, 1990). The intercalation of ethidium bromide in DNA could cause DNA damage by interfering DNA replication and transcription effectively. The severe DNA damage activates the following varieties of cellular responses: 1. Cell cycle arrest, 2. DNA repairing pathways, 3. Express stress related genes, and 4. Apoptosis. Hence, the ethidium bromide-induced fragmented acrosome in the head of the sperm could be either direct or indirect effect. The Indirect effect may be due to the any one of the cellular responses induced by the ethidium bromide to keep the gene pool from the damaged or
mutated sperm which could ground the birth of defective young one. However, the sperm of worms injected with ethidium bromide at concentrations of 5 µg and 10 µg showed no morphological changes. The data suggest that the fragmented acrosome in the head depends on the dose of ethidium bromide. The graph in Fig 3d indicates that, of all the substances tested, only ethidium bromide damages the sperm head. Hence, the studies of sperm morphology could be used to assess the carcinogen pollution of the soil. Similarly, Rodriguez and Bustos-Obregon (2000) observed that Malathion inhibit DNA synthesis in the seminiferous epithelium and are cytotoxic for spermatogenic cells in mice. Atef et al. (1995) have demonstrated that Malathion elicit morphophysiological damage of sperm, with cytogenetic damage of male germ cells (Bustos-Obregón and Díaz, 1999).

The earthworms were subjected to different doses of X-rays and chemicals, were examined carefully under the microscope. The sperms of these worms had different morphological phenotypes, as shown in fig. 4. The altered sperm morphologies were as follows: 1. Fragmented acrosome in the head (Fig. 4b); 2. A break in the tail (Fig. 4c) and 3. Zigzag tails, as shown in Fig. 4d, and those with the above three morphological phenotypes were counted (Fig. 4e, f & g). The data clearly shows that frequency of the observed phenotypes in the sperm increases with enhanced doses of X-ray radiation. Similarly, Rikmenspoel and Van Herpen (1969) found that, at low doses of radiation, a fraction of the sperm of bull was killed, and their survival rate of the sperm was reduced significantly. The phenotype, fragmented acrosome in the head has been observed in the ethidium bromide injected worms and also in the X-ray irradiated worms. The break and zigzag in the tail are the phenotypes observed only in the irradiated worms. Hence, these unique phenotypes are easy to observe with minimum laboratory facilities, can be used as a tool to assess radiation hazards in soil.

Acknowledgement

This project was financially supported by UGC (University Grants Commission), New Delhi and the Department of Biotechnology (DBT), New Delhi.

References


Legend

Fig. 1 a The earthworm *Eudrilus eugeniae*. M- mouth, C- clitellum and A- anus. b Internal anatomy of *Eudrilus eugeniae* showing the major reproductive organs. T- testes, S- seminal vesicles and O- ovaries. The numbers indicate the segments.

Fig. 2 The mature sperm of *Eudrilus eugeniae*. a Mature sperm under the phase-contrast microscope (image at 40X). b DAPI-stained head region of the sperm under the fluorescence microscope (image at 40X). c. Merged image of both phase-contrast and fluorescence microscope images.

Fig. 3 Phenotypes of sperm of worm after 24 hours of injection with different chemicals. a Control sperm (image at 40X). b Sperm of worm injected with 25µg of sodium arsenate (image at 40X). c Sperm of worm injected with 25µg of ethidium bromide: d Phenotypes of sperm of worm injected with 25µg of various chemicals. H – head, T- tail; the white arrow indicates the fragmented acrosome in the head of sperm after ethidium bromide injection.

Fig. 4 Phenotypes of sperm of X-ray-irradiated worms. a Control (image at 40X). b Fragmented acrosome in head region of sperm (image at 40X). c Break in the tail region (image at 40X). d Zigzag tail region. e Frequency of the various abnormalities observed upon exposure to X-ray irradiation for 25, 50 and 75 mSv. H – head, T- tail; the white and black arrows indicate the fragmented acrosome in the head and break in the tail, respectively, following exposure to X-rays.