Materials and methods

1. **Materials:**
2. **Animals:**

Seventy adult female albino rats (obtained from Faculty of Veterinary Medicine, Benha University) were used in this study. Their weight ranged from 200-250g each. Animals were placed in quarantine for one week prior to breeding and housed in separate plastic cages in a controlled environment at 23-25 0C and light – dark cycle (14-10 H) with free access to water and balanced diet which consists of milk, vegetables and bread. All rats were kept under the same circumstances throughout the experiment.

After acclimatization to laboratory conditions, two females were kept overnight with one male to allow mating; the presence of sperms in vaginal smear in the next morning indicates the day zero of pregnancy.

1. **Drugs:**

*Two drugs were used in this study:-*

1. **The cisplatin** is **present in** the form of vial containing 50 mg liquid cisplatin. The drug is produced by **BRISTOL-MAYERS-SQUIB**. The vial was to be diluted by 500 ml of distilled water, so every 1 ml of the solution will contain 0.1 mg of cisplatin. Every rat was injected intraperitoneally by 1 mg cisplatin /kg once every other day and this was equivalent to the human low therapeutic dose **(Apfel et al., 1992).**

**2-**The folic acid is in the form of 800 microgram tablets prepared for oral use is produced by **AVENTIS PHARMA**. This was powdered and dissolved in 10 ml of distilled water, so every 5 ml of the solution will contain 400 microgram of folic acid. Each rat was given 5ml of the solution once daily through a nasogastric tube. **(Deborah et al, 2012).**

1. **Methods:**

Sixty pregnant rats were used in this study and were divided into two equal groups:

**I- Prenatal group:** This group contained thirty pregnant rats and was subdivided into three equal subgroups. Each subgroup consisted of ten pregnant rats.

1. **Prenatal control subgroup:** The rats received saline from 1st day of pregnancy till the time of sacrifice at 18th day& 20th day of pregnancy.
2. **Prenatal cisplatin subgroup:** The rats were injected intraperitoneally with the therapeutic dose (l mg/Kg) of Cisplatin on alternate days from 1st. day of pregnancy till the time of sacrifice at 18th day& 20th day of pregnancy.
3. **Prenatal cisplatin & folic acid subgroup:** The rats were injected intraperitoneally with (I mg/Kg) of Cisplatin (every other day) Plus folic acid in a dose of (400microgram) daily by the nasogastric tube from 1st day of pregnancy till the time of sacrifice at 18th day& 20th day of pregnancy.

The fetuses were obtained by caesarian section as follows,

1. The pregnant female rat was anesthetized by Ether
2. The anaesthetized animal was fixed through its 4 limbs
3. The abdominal wall was incised to explore the uterus
4. The uterus was opened to get the fetuses

Then the specimens were obtained as follows;

The fetuses were decapitated. The back of thigh was then dissected under a dissecting microscope to explore the sciatic nerve then the nerve was removed gently. The back of the fetus was also dissected under a dissecting microscope, to explore the vertebral column at the thoracic and lumbar levels. The later was then opened to get the spinal cord &the dorsal root ganglia. The specimens of the spinal cord, dorsal root ganglion and sciatic nerve were then prepared for light and electron microscopic examination

**II- Postnatal group:** This group contained thirty pregnant rats and was subdivided into three equal subgroups; each subgroup consisted of ten pregnant rats.

1. **Postnatal control subgroup:** The pregnant rats were allowed to complete pregnancy. Their pups received distilled water from 1st day of delivery till being sacrificed on the 7th Day,14th Day & 21st day postnatally
2. **Postnatal cisplatin subgroup:** The pregnant rats were allowed to complete pregnancy. Their pups were injected intraperitoneally with the therapeutic dose of Cisplatin (l mg/Kg) on alternate days from 1st day of delivery till being sacrificed on the 7th Day, 14th Day & 21st day postnatally.
3. **Postnatal cisplatin & folic acid subgroup:** The pregnant rats were allowed to complete pregnancy. Their pups were injected intraperitoneally with the therapeutic dose of Cisplatin(I mg/Kg) every other day plus folic acid in a dose of (400microgram) daily given to the pups through a nasogastric tube from 1st day of delivery till being sacrificed on the7th Day,14th Day & 21st day postnatally .

The pups were anaesthetized by ether inhalation and the specimens were obtained by the same way as in the prenatal groups. The specimens of the spinal cord, dorsal root ganglion and sciatic nerve were prepared for light and electron microscopic examination

 I- Light microscopic preparation: **(Drury and Wallington, 1980)**

1. The specimens were fixed in 10% buffered neutral formalin for at least 7 days.
2. They were, then, transferred to ascending grades of ethyl alcohol (50%, 70%, 96% and absolute alcohol) for dehydration.
3. The specimens were cleared in xylol.
4. The tissues were, then, impregnated with soft paraffin by putting them in several changes of melted wax (melting point 50°C) in an oven.
5. Finally, embedding in hard paraffin was done by putting the tissue 'in melted wax (melting point 55°C), poured into a mold and then cooled down to form paraffin blocks containing the tissues.
6. Serial sections 5-7 µm thick were sliced, mounted on slides with an adhesive, stained with Heamatoxylin & Eosin and silver for the prenatal specimens and with Heamatoxylin & Eosin and Masson's Trichrome for the postnatal specimens of the sciatic nerve but with Heamatoxylin & Eosin only for postnatal specimens of the spinal cord and the dorsal root ganglion **(Bancroft and Gamble, 2002).**
7. The stained slices were then examined with an Olympus light microscope equipped with an automatic photo micrographic camera system.

II- Electron microscopic preparation

**I - Fixation:**

The fresh specimens were cut into small pieces in 3% phosphate buffered gluteraldehyde (pH 7.4) at 4 °C for two hours. Washing for 2 hours at 4°C in 3 changes of phosphate buffer (pH 7.4) was done (**Meek, 1970)**. The specimens were then post- fixed in a freshly prepared 1% osmium tetroxide in phosphate buffer at 4°C for 1-2 hours **(Peter, 1970).**

**2- Dehydration:**

This was done by ascending grades of ethanol concentration (30%, 50%, 70%, 80%, 90%, and 95%) for 10 minutes each. Finally, dehydration was done in absolute ethanol (2 changes, each for 15 minutes). The dehydration was completed by propylene for two changes; ten minutes each **(Glauert and Beesley, 1986).**

**3- Embedding:**

The specimens were then embedded in pre-labeled plastic capsule.

Embedding mixture, (**Glauert and Beesley , 1986).**

- 10 ml of araldite (the embedding material).

- 8 ml of the hardener dodenyl succinic anhydride DDSA both were mixed properly and kept in a 60°C oven for 10 minutes to eliminate air bubbles.

- 0.4 ml (about 18 drops of accelerator tridimethyl aminomethyl phenol DMP was then added and mixed well.

**4- Polymerization:**

Capsules were polymerized in a temperature controlled oven at 60°C for 48 hours **(Meek, 1970).**

**5- Trimming:**

The polymerized block was trimmed into a pyramid with a small trapezoid surface **(Meek, 1970).**

**Sectioning:**

Semithin sections were cut at 1 um thickness with a glass knife, and then the sections were stained with 1% toluidine blue stain dissolved in 1% borax for proximately 30-60 seconds at 60-70°C and examined by the Olympus microscope for general orientation **(Bancroft and Gamble, 2002).**

**Mounting**

Ultra-thin sections; 50 nm thick were obtained from the selected blocks and mounted on copper grids (mesh size 200), **(Meek, 1970).**

**Staining**

Grids were kept in Petri-dishes and stained with uranyl acetate for minutes **(Stempak, 1964)** followed by lead citrate for 10 minutes **(Hall et al., 2012).**

**Examination:**

The stained grids were examined by Philips 201-transmission electron microscope at 60-80 kV in transmission Electron microscope Unit at Medical Military Academy. Finally, the electron photographs were taken from the selected areas.