2. MATERIAL AND METHODS

2.1. Materials

2.1.1. Animals

This study has been undertaken in female Wistar rats (130-140 g) in strict accordance with institutional animal care and use guidelines. The rats were obtained from the Animal Facility of the Faculty of Pharmacy, Alexandria University, Egypt, and were kept at an ambient temperature and given free access to rat chow (16% proteins, Tanta Oil and Soap Co., Egypt) and water. International guidelines for the care and use of laboratory animals were strongly adhered to and followed.

2.1.2. Drugs

Phosphatidyl choline (PC)

Phosphatidyl choline (50 mg/ml) derived from soy bean Lecithin (Lipostabil ® N by A. Nattermann and Cie. GmbH Germany, member of the Sanofi-Aventis Group). The ampule contains sodium deoxycholate (solubilizer), benzyl alcohol (preservative), sodium metabisulfite (antioxidant and preservative) and double distilled water for dissolution. A daily dose of 0.1 ml/rat of Lipostabil ® [118] was injected subcutaneously in the groin of the right side of the rat near the femoral nerve for 4 consecutive days.

Sterile Saline

Normal saline (Sodium Chloride 0.9% w/v) in sterile filtered double-distilled water, without any additives (El-Nasr Pharmaceutical Chemicals Co ADWIC®). A daily dose of 0.1 ml/rat of sterile normal saline was injected subcutaneously in the groin of the right side of the rat near the femoral nerve (control group) for 4 consecutive days.

Thiopentone sodium

Thiopental® (Sandoz, Basel, Switzerland) was stored at room temperature. A fresh solution (50 mg/ml) was prepared daily by dissolving 500 mg thiopentone sodium lyophilized powder in 10 ml sterile saline. A dose of 50 mg/kg body weight was administered i.p. to induce anesthesia.[119]

2.1.3. Reagents

Neutral buffered formaldehyde, pH = 7.4 [120]

Sodium dihydrogen phosphate, monohydrate ($NaH_2PO_4.H_2O$) …….	4 g
Disodium hydrogen phosphate, anhydrous (Na_2HPO_4) ……..	6.5 g
40%Formaldehyde …….....	100 ml
Distilled water ……...…..…….....	900 ml

4% Formaldehyde–1% glutaraldehyde ($_4F_1G$), pH = 7.4 [121]

Sodium dihydrogen phosphate, monohydrate ($NaH_2PO_4.H_2O$) …….	1.16 g
Sodium hydroxide (NaOH)	0.27 g
40%Formaldehyde …….....	10 ml
50% Glutaraldehyde …….....	2 ml
Distilled water ……...…..…….....	88 ml

Sorensen phosphate buffers [122]

- Solution (A): 14.2 g of disodium hydrogen phosphate were dissolved in 1 liter of deionized water to produce one liter of 0.1 M Na_2HPO_4.
- Solution (B): 13.6 g of potassium dihydrogen phosphate were dissolved in 1 liter of deionized water to produce one liter of 0.1 M KH_2HPO_4.
- To prepare 100 ml Sorensen phosphate buffer (0.1 M, pH=7.2): 72 ml of solution A were mixed with 28 ml of solution B.
- The pH of the buffer was measured using Jenway 3305 pH-meter (Jenway Ltd., Essex, England). The pH was adjusted when necessary using few drops of either solution A (slightly alkaline) or solution B (slightly acidic).

Buffered 1% osmium tetroxide solution, pH = 7.2 [121]

Osmium tetroxide (OsO_4) ……	0.25g
Phosphate buffer 0.1 M, pH = 7.2 ……	25 ml

Preparation of Toluidine blue for semi-thin sections [123]

Borax ……..……..…….....	1 g
Toluidine blue ….....……..…….....	1 g
Distilled water ……..….....….....…...	to 100 ml

Preparation of Uranyl acetate [124]

Saturated solution in 70% alcohol was used after centrifuged for 10 minutes at 5000 rpm. Urany acetate should be stored in dark bottles and used within one month. The advantage of an alcoholic stain is that it will penetrate more easily into the plastic embedded tissue and thus give a high contrast requiring only a short staining time

Preparation of Reynold's lead citrate [125]

- 1.33 g lead nitrate ($Pb(NO_3)_2$), 1.76 g sodium citrate ($Na_3(C_6H_5O_7).2H_2O$) and 30 ml carbon dioxide-free double-distilled water (carbon dioxide is removed by boiling the water and leaving it to cool down) were added together in the 50 ml volumetric flask.
- The solution was shacked vigorously for several minutes, and then 5–6 times over a 30 min period to complete the conversion of lead nitrate to lead citrate. The suspension was stirred on hot plate and allowed to cool down.
- 8 ml of 1 N NaOH (40 g sodium hydroxide NaOH in one liter of distilled water) were added to the suspension while stirring till the milky suspension turn clear. If not, few more drops of NaOH were added. The volume was completed to 50 ml with distilled water and the pH was checked using a pH meter. The pH was 12.0 +/- 0.1.
- The flask was tightly stoppered. The solution has a shelf life up to 6 months if sealed tightly. Before use, the solution was centrifuged 10 minutes at 5000 rpm.

2.2. Methods

2.2.1. Treatment protocol

Under aseptic technique, rats were injected subcutaneously with 0.1 ml/rat saline (Control group, n = 10) and by 0.1 ml/rat of Lipostabil ® (PC/DC group, n=10) in the groin of the right side of the rat near the femoral nerve for 4 consecutive days. Injections were made using sterile 30-gauge needles.

2.2.2. Acquiring the specimens for histopathological studies

On the 5th day, the rats were anesthetized using thiopental (50 mg/kg) administered via intra-peritoneal injection. A longitudinal incision was performed along the groin crease of the right side of the rat. Gentle dissection of the femoral bundle was performed and about 1 cm of the bundle was excised and flushed with ice cold saline. Five specimens were immediately immersed in $_4F_1G$ for histopathological studies using electron microscopy (Figure 9). The other five specimens were immediately immersed in neutral buffered formalin for light microscopy study.[126] All rats were euthanized with overdose of the anesthetic.[119]

2.2.3. Histpathological Examination of rat femoral nerve under light microscope:

The left femoral nerve was cut into small pieces (about 0.5 cm in diameter) to allow good penetration of the fixatives and fixed in neutral buffered formaldehyde solution and embedded in paraffin blocks within 24 hours through the following process[120]:

- Dehydration of specimen in ascending concentrations of alcohol, ending with absolute alcohol.
    - Clearing by the use of multiple changes of xylene.
    - Wax impregnation by the use of paraffin wax.
    - Embedding in paraffin wax using mold of suitable size
- Wax blocks containing specimens were cut into small sections each of thickness 5μm by the use of rotary Bright 5030 microtome (Bright instrument Co Ltd, England) and selected sections were mounted on glass slides
- The preparation of paraffin sections for staining included the following steps:
    - Removal of the paraffin wax by the use of xylene
    - Removal of xylene by the use of absolute alcohol
    - Treatment with descending grades of alcohol
    - Washing the section on the glass slide with distilled water.
- Sections were stained by Haematoxylin and Eosin stain (H and E) and examined under the light microscope

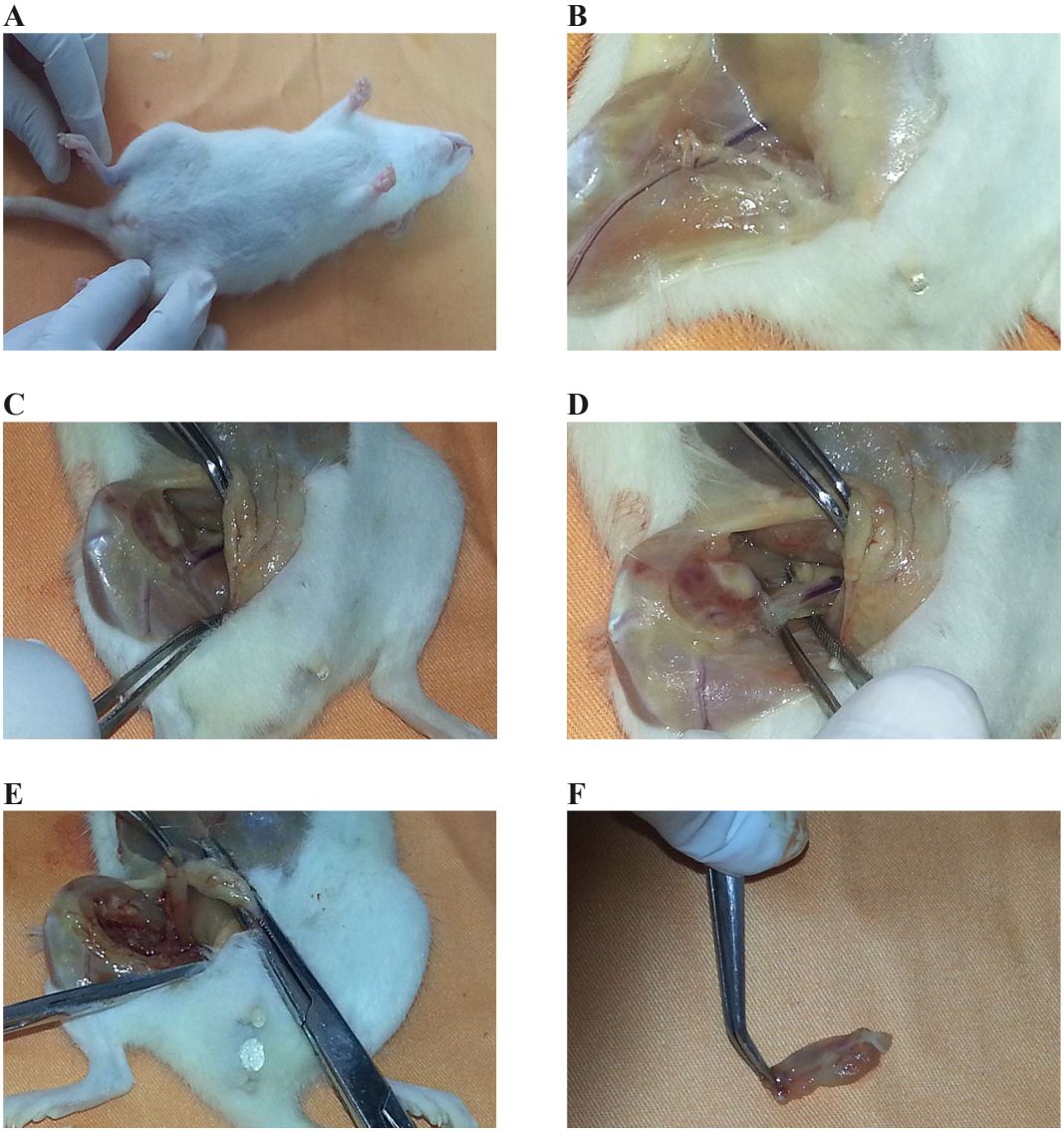

Figure 9: Surgical procedure of acquiring the femoral nerve. (A) Anesthetized rat lying supine. (B, C) An incision performed along the groin crease of the right side of the rat to expose the femoral artery, vein and nerve. (D) gentle dissection of the femoral bundle was performed. (E, F) about 1 cm of the bundle was excised and flushed with ice cold saline.

2.2.4. Preparation of specimen for transmission electron microscope

Principle:[127]

Biological material contains large quantities of water. Since the transmission electron microscope (TEM) works in vacuum, the water must be removed. To avoid disruption as a result of the loss of water, the tissue is immediately fixed in $_4F_1G$ solution and kept at 4 ^oC. This treatment cross-links molecules with each other and traps them together as stable structures. It's important to fix a sample as quickly as possible because, as soon as tissue is removed from its natural environment, it starts to change. The tissue is then dehydrated in alcohol or acetone. This treatment helps the sample to tolerate the high energy of the electron beam and the vacuum. It is worth noting that the vacuum inside an electron microscope is important for its function. Without a vacuum, electrons being aimed at the sample would be deflected off their course when they hit air particles.

The principle of the preparation of the samples for TEM is that the specimen must be of low density that it allows electrons to travel through the tissue. For this purpose specimens are embedded in hard resin that polymerize into a solid hard plastic block. The block is cut into thin sections by a diamond knife in an ultra-microtome. Each section is only 50-100 nm thick.

The sample can also be stained to identify different cellular organelles. Staining is usually done by heavy metals such uranium and lead, which scatters electrons well and improves the contrast in the microscope. The parts of the sample that interact strongly with the metals show up as darker areas.

Procedure:[121]

- Small blocks from tissues (1 mm^3) of both groups (control and PC) were removed from the rat and dropped as soon as possible into $_4F_1G$, buffered with 0.1M phosphate buffer, pH=7.4 and the samples were kept at 4^oC. Fixation is usually started at room temperature and after 15-30 minutes, and then continues for 16-24 hour at 4 °C.
- After rinsing with 0.1 M phosphate buffer of pH= 7.2 (3 times for 10 minutes each), post-fixation was carried out with 1% osmium tetroxide (oxidizing agent) for 2 hr at 4°C.
- The specimens were washed again in buffer (3 times for 10 minutes each), and then they were dehydrated in ascending grades of acetone solution in water 30%, 50%, 70%, 80%, 90% (15 minutes each) and 100% (3 times for 30 minutes each).
- Propylene oxide is more volatile than acetone or ethanol. Therefore, tissues were cleaned from residual acetone in propylene oxide solution (2 times for 25 minutes each).
- Specimens were embedded in a mixture of 1:1 of Epon-Araldite epoxy resins in pre-dried (at 37 ^oC) gelatin capsule for 1 hr before use. Polymerization was done in oven at 65 ^oC for 24 hours.[128]

- The epoxy resin block is trimmed and the target tissue is selected using a light microscope by viewing semi-thin sections (1μm) stained with toluidine blue. The sections were mounted on glass slide, dried, warmed, and the toluidine blue dye is added on the warm slide for 2 minute. The slide was rinsed with distilled water and dipped twice in 95% ethanol. The slide was washed with absolute ethanol (2 times each for 1 minute) and passed in xylene and then slide cover was fixed with resinous material and examined.[123]
- The block is then re-trimmed and the specific area for observation is ultra-thin sectioned (50 nm) using a glass knife on a LKB ultratome (LKB-Produkter AB, Sweden), and then mounted on copper grids (mesh), double stained with uranyl acetate and lead citrate, and investigated on a JEOL 100CX TEM (JEOL Ltd., Tokyo, Japan). Uranyl acetate is negative stain in electron microscopy. This procedure must be carried in the dark because uranyl acetate is photo sensitive and will precipitate if exposed to light. Also, lead citrate is sensitive to carbon dioxide and will precipitate as lead carbonate if excessively exposed to air. Although the use of one of these stains alone could be quite practical for routine purposes, the highest contrast is obtained when both of these stains are used in sequence, "double contrasting".[125]

2.2.5. Histopathological scoring

Evaluation of all four categories inflammation, necrosis, fibrosis and nerve damage was done in a semi-quantitative scoring for H and E stained slides. Each category was scored into one of four grades per microscopic field (×100): grade 1 (<25%), grade 2 (26% to 50%), grade 3 (51% to 75%), and grade 4 (76% to 100%).[129, 130]

2.3. Statistical Analysis

Statistical analysis used the Mann-Whitney test to characterize the differences and similarities between control group treated with saline and group treated with PC/DC. The analysis was performed using Graph Pad Prism, software release 3.02. Probability levels less than 0.05 were considered significant.

3. RESULTS

The aim of this study is to identify the effect of PC/DC combination (Lipostabil®) on various tissues at the injection site including the nervous tissue. The effect of Lipostabil® was evaluated histopathologically at the 4th day after injection of the drug in the groin of the right side of the female Wistar rats near the femoral nerve for 4 consecutive days. The effect of the drug was compared to control rats treated only with saline. Biopsies from 6 rats in each group were evaluated using H and E staining. Samples were scored for inflammation, necrosis, and fibrosis as well as nerve damage. The rest of the samples (4 samples in each groups) were used for electron microscopic (TEM) studies for assessment of neural structures.

Histopathological examination using H and E stained sections of the tissues at the site of injection in the control group revealed minor inflammation and focal hemorrhage as a result of the trauma due to repeated injection. However, there were no signs of skeletal muscle degeneration or neural damage (Figures 11-13). On the other hand, injection of Lipostabil® in the groin of the right side of the female Wistar rats near the femoral nerve for 4 consecutive days caused fat necrosis as manifested by ruptured fat globules and fat cysts (Figure 14). This was associated with intense infiltration of leukocytes in the injection area causing inflammation invading the skeletal muscles (Figure 15). The deposition of collagen fibers amongst the inflammatory background is considered as an early sign of fibrogenesis (Figures 16-17). The skeletal muscles in the injected area also demonstrated prominent myofiber necrosis, myophagocytosis and regeneration (Figure 18). The presence of basophilic sarcoplasm with prominent nuclei and visible nucleoli depicted regeneration of skeletal muscles fibers (Figures 19-20). Concerning the neural tissue, the repeated injection of Lipostabil® caused intense inflammation adjacent to the nerve leading to entrapment of the nerve bundles within the infiltrating leukocytes (Figures 21-23).

Table 1 and 2 and figure 24 summarize the histopathological scoring of tissues inflammation, necrosis, fibrosis and nerve damage. Compared to the control group injected with saline, injection of 0.1 ml Lipostabil® for 4 consecutive in the groin of the right side of the female Wistar rats near the femoral nerve caused significant intense leukocytes infiltration in the skeletal muscles causing severe inflammation and muscle damage leading to the deposition of collagen fibers as an early sign of fibrosis. These leukocytes cuffed the nerve bundle causing significant neural damage. The intense inflammation caused significant necrosis in the adipose tissue and skeletal muscles.

Examination of the ultra-structures of the neural tissues in the injected area using TEM revealed the presence of intra-neural fibroblasts abutting the nerve fibers along with marked myelin degeneration (Figure 26). The intra-neural fibroblasts and myelin degeneration were associated with the deposition of intra-neural collagen fibers as an early sign of neural fibrosis (Figure 27-30). The local injection of Lipostabil® also affected the Schwann cells causing degeneration of myelin (Figure 31). Moreover, the injection of Lipostabil® affected the intra-neural blood vessels causing thickening of its walls (Figure 32). The sections also demonstrated endoneural inflammation (Figure 33).

Normal Rat Neurovascular Bundle

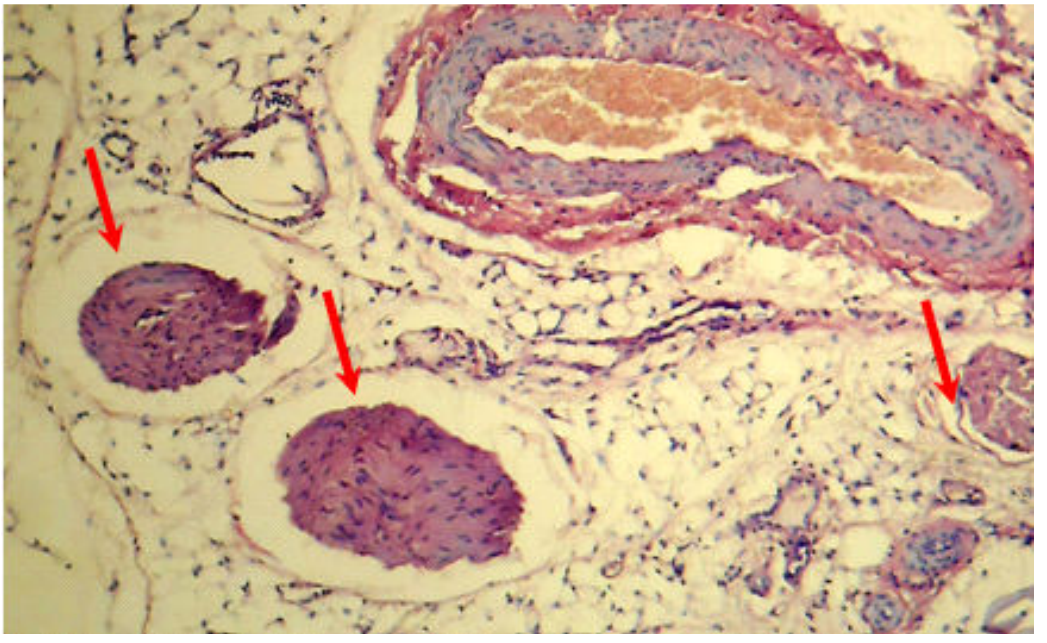

Figure 10: Photomicrograph (200 ×) of normal neurovascular bundle stained with H and E stain. The red arrows point to nerves.

Effect of Repeated Saline Injection on Neural Tissues at the Injection Site

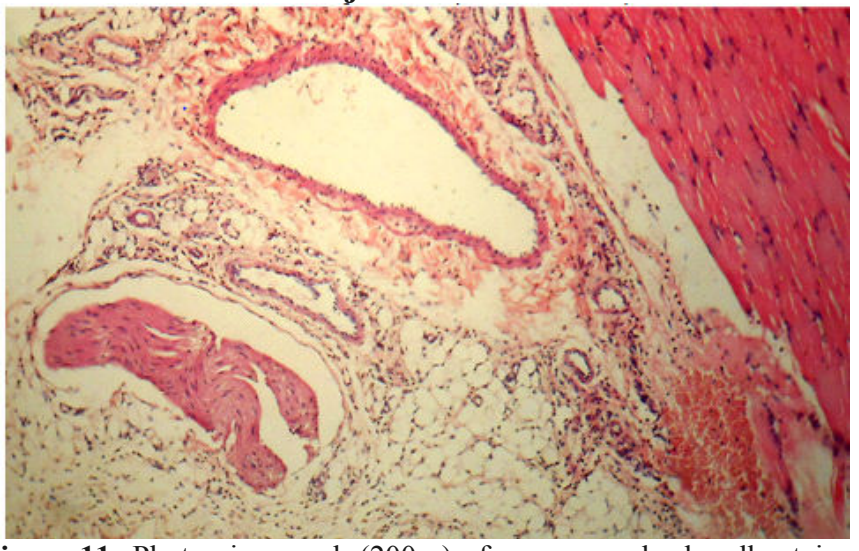

Figure 11: Photomicrograph (200 ×) of neurovascular bundle stained with H and E stain from control female Wistar rats injected with 0.1 ml/day/rat saline for 4 consecutive days showing minor inflammation and focal hemorrhage at the site of repeated injection.

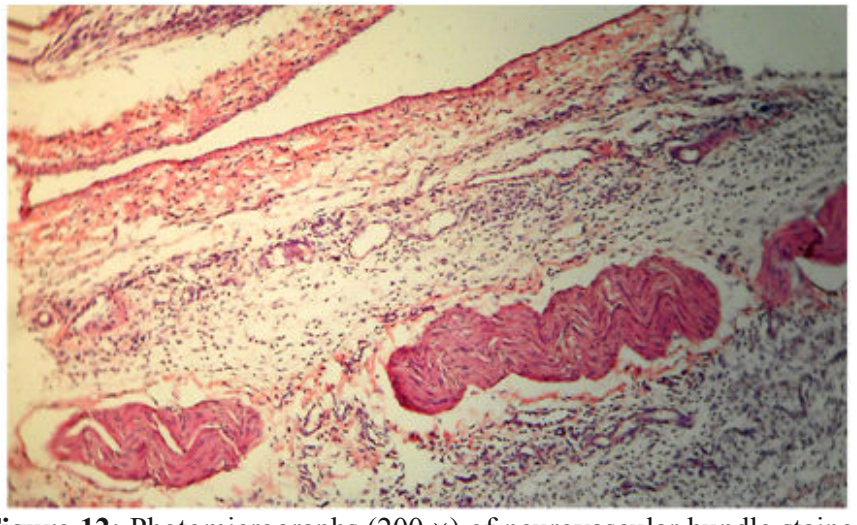

Figure 12: Photomicrographs (200 ×) of neurovascular bundle stained with H and E stain from control female Wistar rats injected with 0.1 ml/day/rat saline for 4 consecutive days showing minor inflammation and focal hemorrhage at the site of repeated injection.

Effect of Repeated Saline Injection on skeletal Muscles at the Injection Site

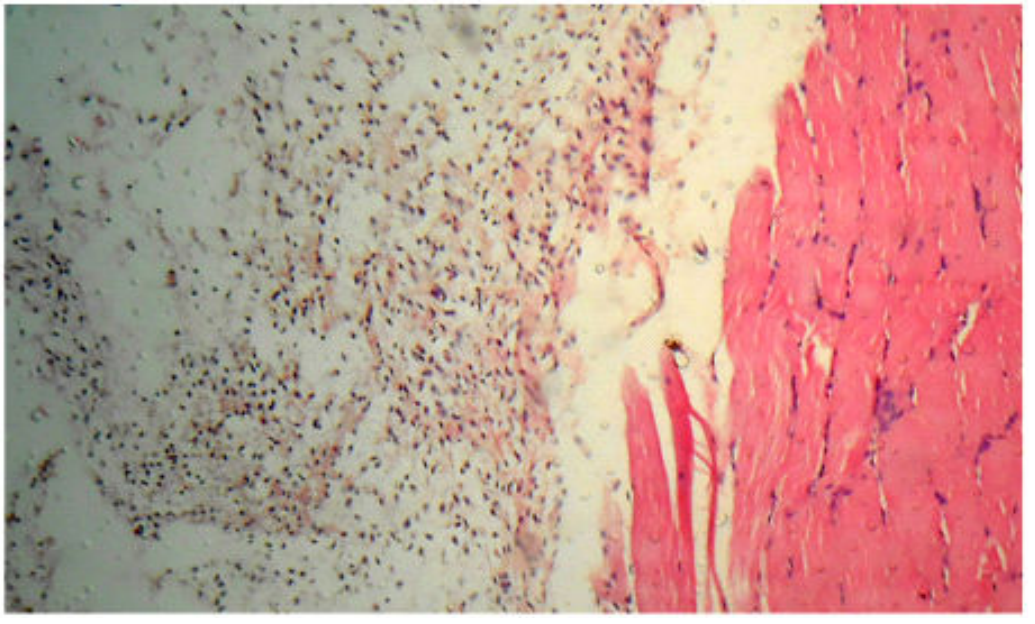

Figure 13: Photomicrographs (200 ×) of skeletal muscles stained with H and E stain from control female Wistar rats injected with 0.1 ml/day/rat saline for 4 consecutive days showing minor interstitial inflammation and intact skeletal muscles fibers at the site of injection.

Effect of Repeated Lipostabil® Injection on adipose tissue at the Injection Site

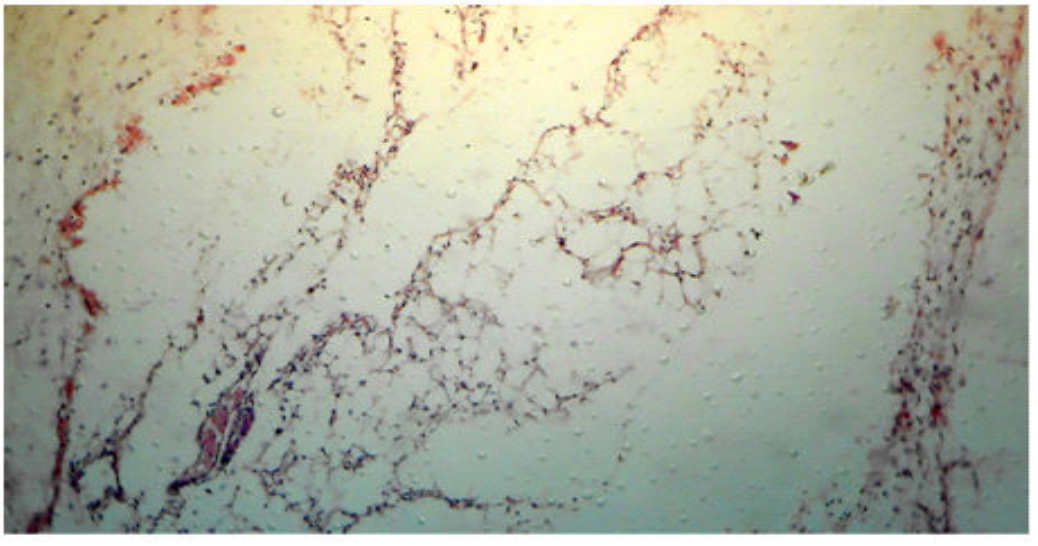

Figure 14: Photomicrographs (100 ×) of adipose tissue from rats injected with Lipostabil® (0.1 ml/day/rat) for 4 consecutive days. The section is stained with H and E stain. The photomicrograph shows ruptured fat globules and fat cysts, an indication of fat necrosis.

Effect of Repeated Lipostabil® Injection on skeletal muscles at the Injection Site

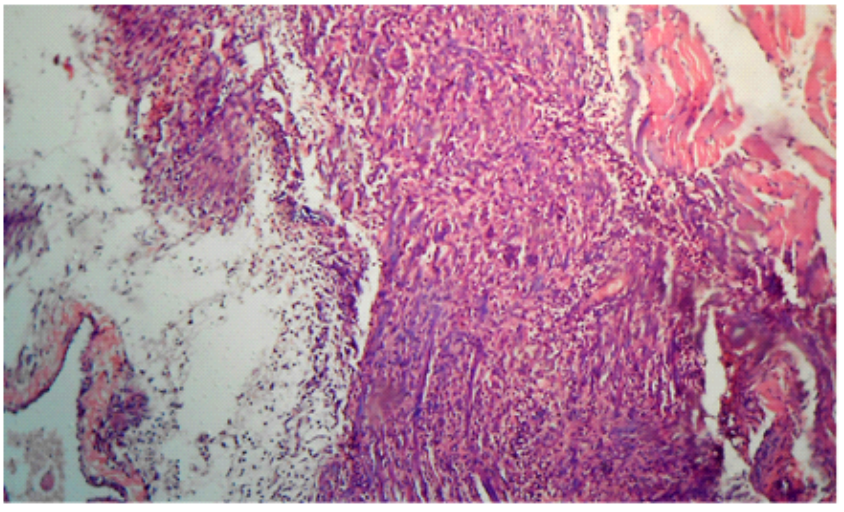

Figure 15: Photomicrographs (100 ×) of skeletal muscles stained with H and E stain from female Wistar rats injected with 0.1 ml/day/rat Lipostabil® for 4 consecutive days showing marked interstitial inflammation invading the skeletal muscles at the site of injection.

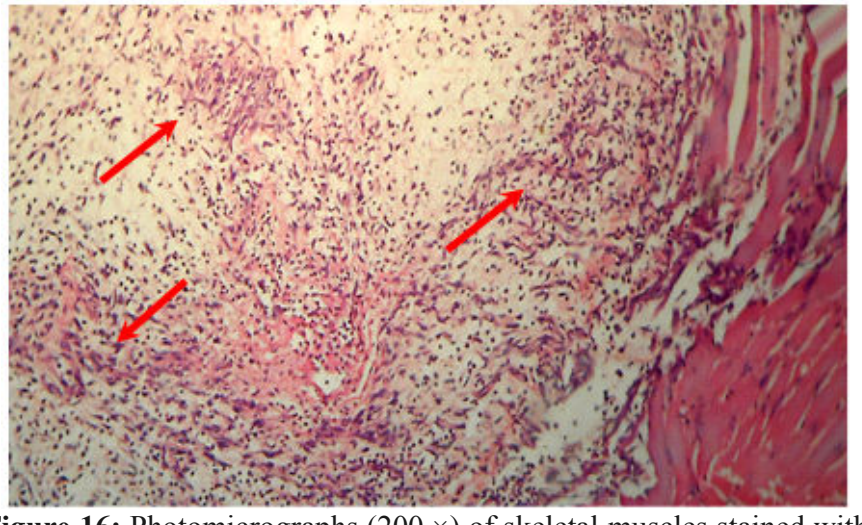

Figure 16: Photomicrographs (200 ×) of skeletal muscles stained with H and E stain from female Wistar rats injected with 0.1 ml/day/rat Lipostabil® for 4 consecutive days showing deposition of collagen fibers as an early sign of start of fibrosis (red arrows) along with inflammation at the site of injection.

Effect of Repeated Lipostabil® Injection on skeletal muscles at the Injection Site

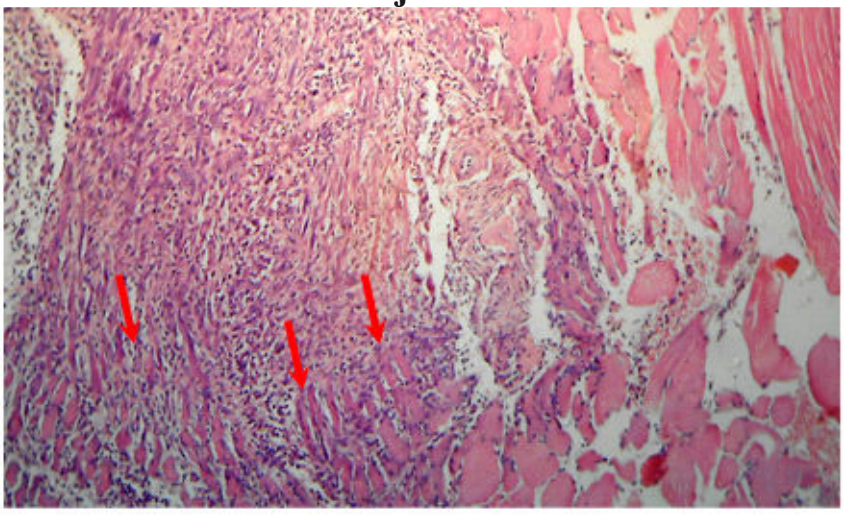

Figure 17: Photomicrographs (200 ×) of skeletal muscles stained with H and E stain from female Wistar rats injected with 0.1 ml/day/rat Lipostabil® for 4 consecutive days showing inflammatory cellular infiltration through pre-existing skeletal muscle fibers (red arrows) at the site of injection.

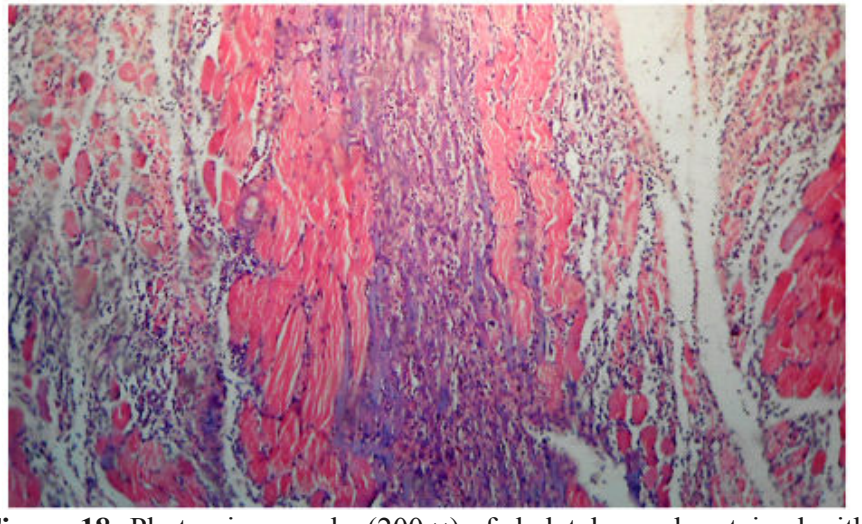

Figure 18: Photomicrographs (200 ×) of skeletal muscles stained with H and E stain from female Wistar rats injected with 0.1 ml/day/rat Lipostabil® for 4 consecutive days showing prominent myofiber necrosis, myophagocytosis, and regeneration at the site of injection.

Effect of Repeated Lipostabil® Injection on skeletal muscles at the Injection Site

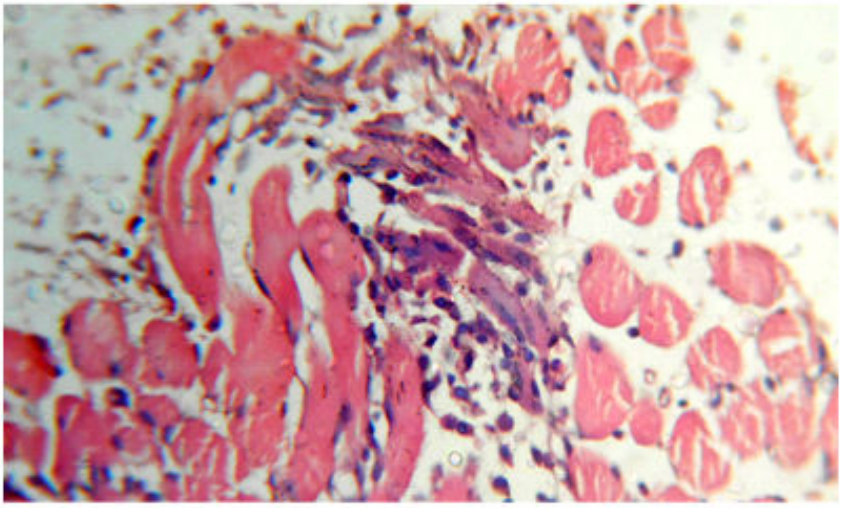

Figure 19: Photomicrographs (400 ×) of skeletal muscles stained with H and E stain from female Wistar rats injected with 0.1 ml/day/rat Lipostabil® for 4 consecutive days showing basophilic sarcoplasm that depicts regenerating skeletal muscle fibers with prominent nuclei and visible nucleoli.

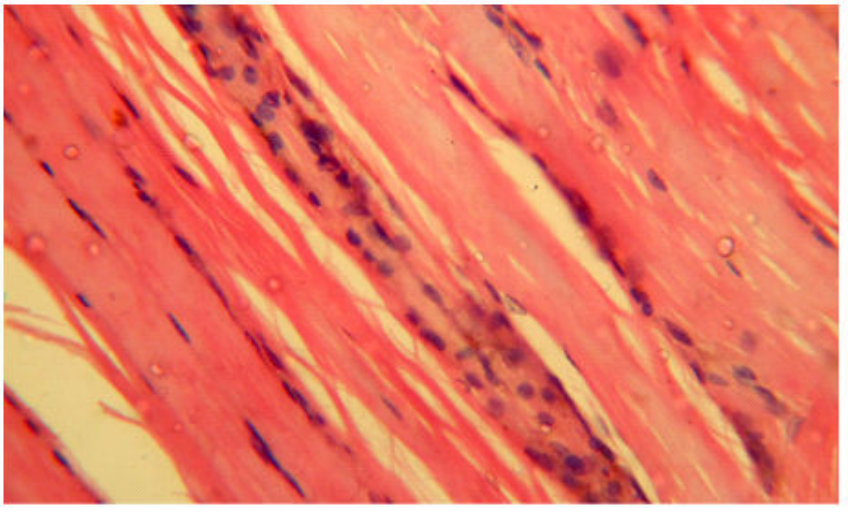

Figure 20: Photomicrographs (400 ×) of skeletal muscles stained with H and E stain from female Wistar rats injected with 0.1 ml/day/rat Lipostabil® for 4 consecutive days showing regenerating muscle fiber.

Effect of Repeated Lipostabil® Injection on Neural Tissues at the Injection Site

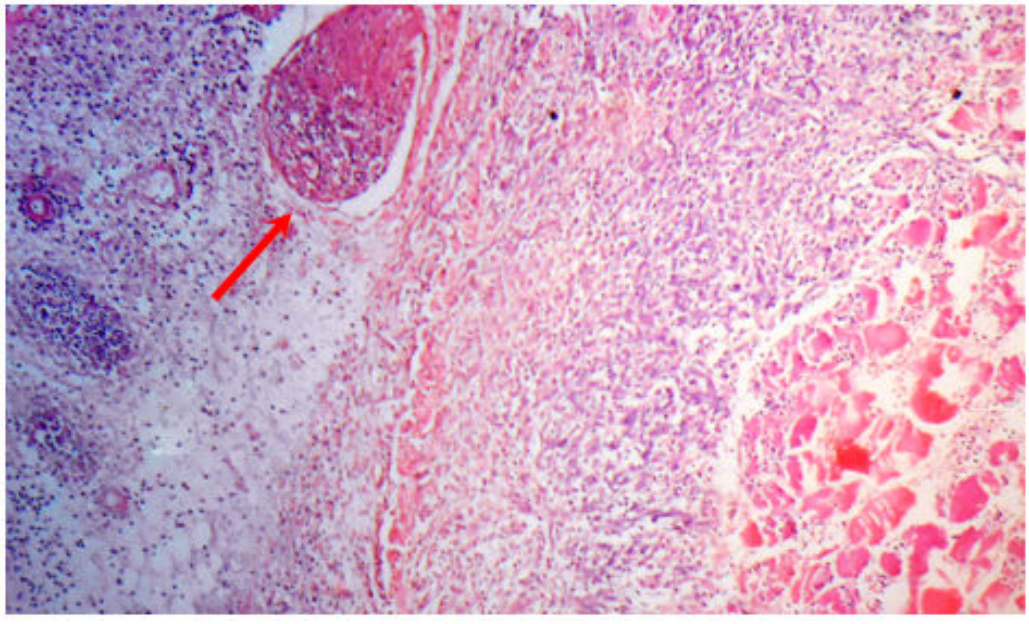

Figure 21: Photomicrographs (200 ×) of neural tissues stained with H and E stain from female Wistar rats injected with 0.1 ml/day/rat Lipostabil® for 4 consecutive days showing nerve bundle (red arrow) entrapped within intense inflammation at the site of injection

Effect of Repeated Lipostabil® Injection on Neural Tissues at the Injection Site

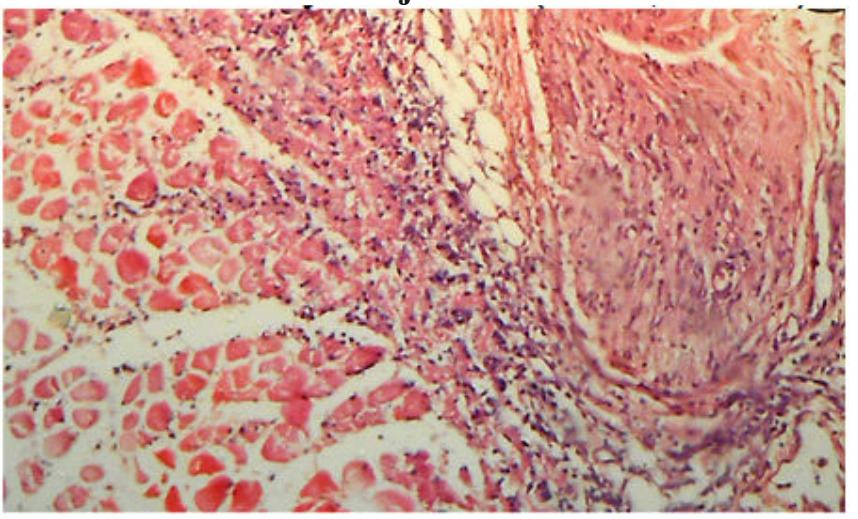

Figure 22: Photomicrographs (300 ×) of nerve bundle stained with H and E stain from female Wistar rats injected with 0.1 ml/day/rat Lipostabil® for 4 consecutive days showing nerve bundle entrapped within intense inflammation at the site of injection.

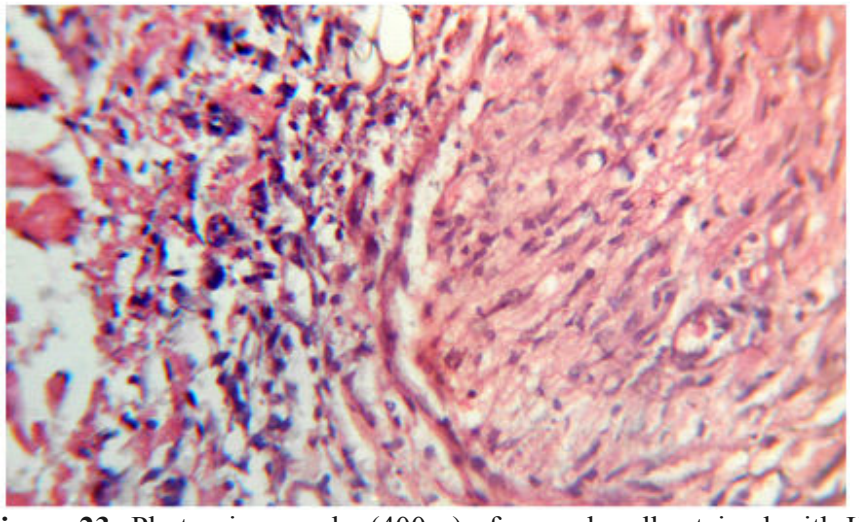

Figure 23: Photomicrographs (400 ×) of nerve bundle stained with H and E stain from female Wistar rats injected with 0.1 ml/day/rat Lipostabil® for 4 consecutive days showing nerve bundle entrapped within intense inflammation at the site of injection.

Table (I) : Effect of local normal sterile saline injection (0.1 ml/rat for consecutive 4 days) on different tissues at the injection site

Control

Saline	Inflammation	Necrosis	Fibrosis	Nerve damage
Rat 1	1	1	1	1
Rat 2	1	1	1	1
Rat 3	1	1	1	1
Rat 4	1	1	1	1
Rat 5	1	1	1	1
Rat 6	2	2	2	2
Min	**1**	**1**	**1**	**1**
Max	**2**	**2**	**2**	**2**
Median	**1**	**1**	**1**	**1**

Table (II) : Effect of local Lipostabil® injection (0.1 ml/rat for consecutive 4 days) on different tissues at the injection site

Treated

Lipostabil	Inflammation	Necrosis	Fibrosis	Nerve damage
Rat 1	1	1	1	2
Rat 2	2	2	2	2
Rat 3	2	2	2	2
Rat 4	3	2	3	3
Rat 5	3	2	3	3
Rat 6	4	3	3	3
Min	**1**	**1**	**1**	**2**
Max	**4**	**3**	**3**	**3**
Median	**2.5**	**2**	**2.5**	**2.5**

Effect of local Lipostabil® injection versus saline on different tissues at the site of injection

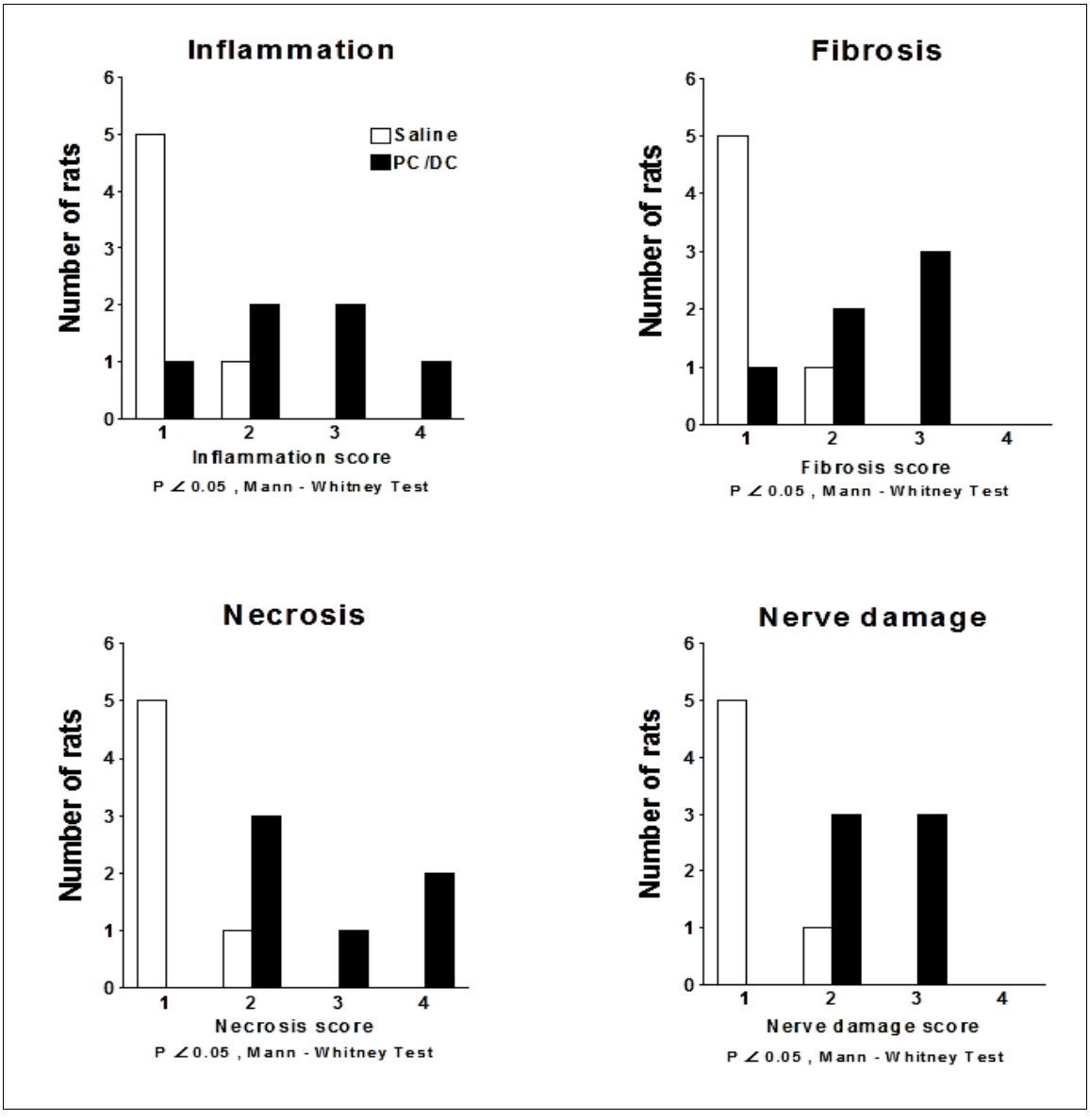

Figure 24: Bar graphs showing histopathological tissue changes due to local injection of 0.1 ml/day/rat PC/DC (black columns) compared to rats injected with 0.1 ml/day/rat saline (white columns) for 4 consecutive days. The *y*-axis represents the number of rats and the *x*-axis represents the histopathological score. P value less than 0.05 is considered significant.

Normal Rat Nerve Ultra-structures Using Transmission Electron Microscope (TEM)

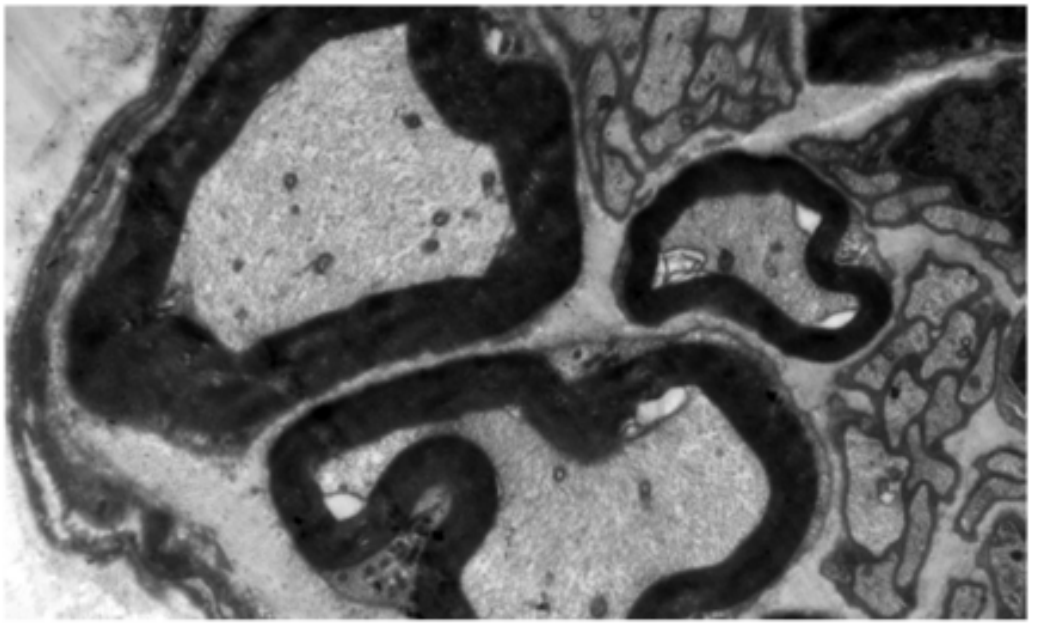

Figure 25: Photomicrographs (34,400 ×) of normal nerve ultrastructure (Osmium tetroxide stain) using TEM.

Effect of Repeated Lipostabil® Injection on Ultra-structures of the Neural Tissues at the Injection Site

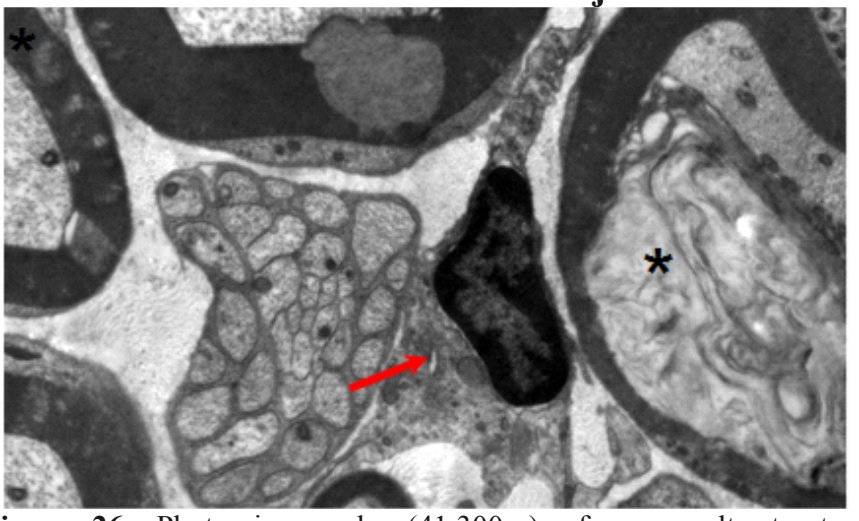

Figure 26: Photomicrographs (41.300 ×) of nerve ultrastructure (Osmium tetroxide stain) using TEM from female Wistar rats injected with 0.1 ml/day/rat Lipostabil® for 4 consecutive days. Arrow pointing to intra-neural fibroblast.

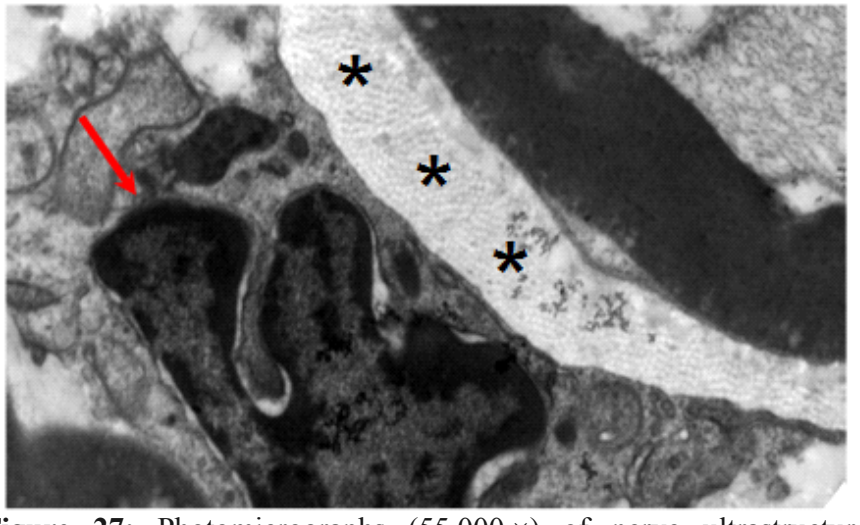

Figure 27: Photomicrographs (55,000 ×) of nerve ultrastructure (Osmium tetroxide stain) using TEM from female Wistar rats injected with 0.1 ml/day/rat Lipostabil® for 4 consecutive days showing intra-neural fibroblast (red arrow) and intra-neural collagen fibers (asterisks)

Effect of Repeated Lipostabil® Injection on Ultra-structures of the Neural Tissues at the Injection Site

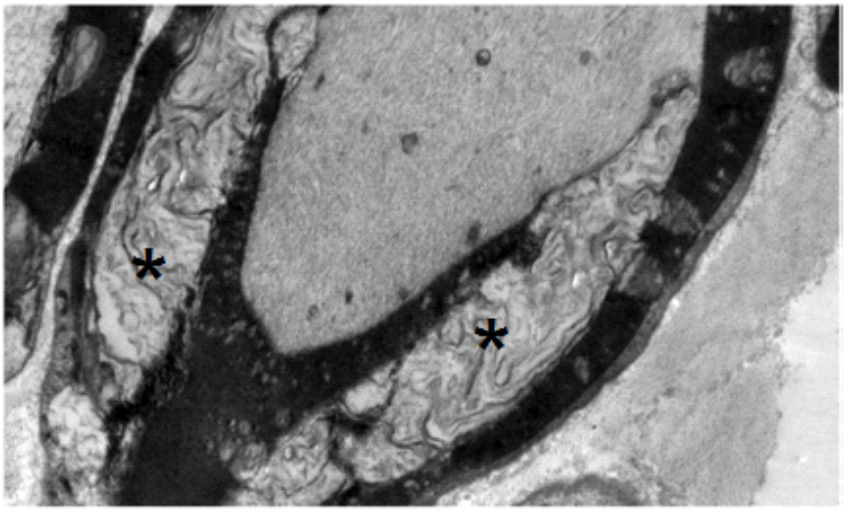

Figure 28: Photomicrographs (34,400 ×) of nerve ultrastructure (Osmium tetroxide stain) using TEM from female Wistar rats injected with 0.1 ml/day/rat Lipostabil® for 4 consecutive days. The section demonstrates large nerve fiber showing myelin disintegration (asterisks).

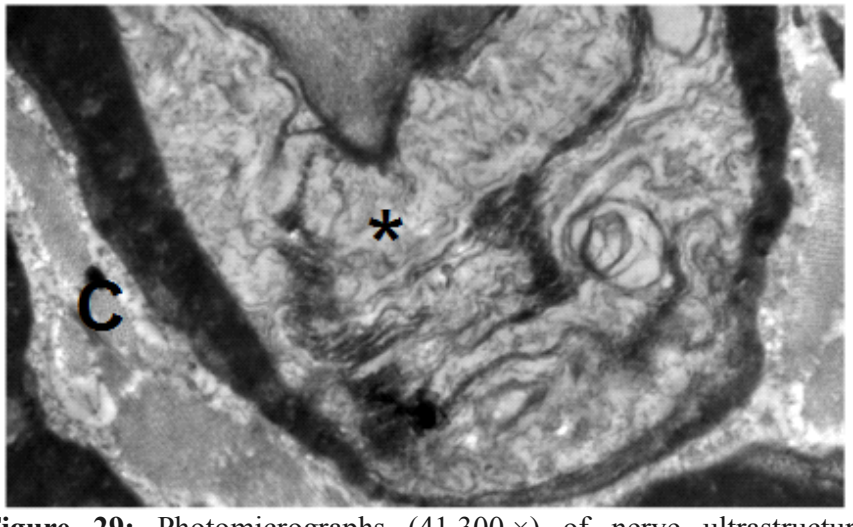

Figure 29: Photomicrographs (41,300 ×) of nerve ultrastructure (Osmium tetroxide stain) using TEM from female Wistar rats injected with 0.1 ml/day/rat Lipostabil® for 4 consecutive days. The section demonstrates another case with advanced myelin degeneration (asterisk) and intra-neural collagen fiber deposition C.

Effect of Repeated Lipostabil® Injection on Ultra-structures of the Neural Tissues at the Injection Site

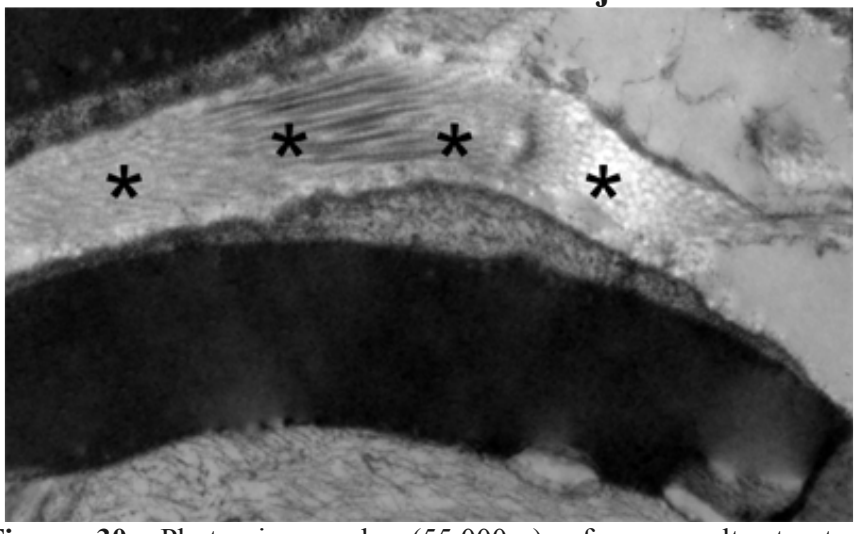

Figure 30: Photomicrographs (55,000 ×) of nerve ultrastructure (Osmium tetroxide stain) using TEM from female Wistar rats injected with 0.1 ml/day/rat Lipostabil® for 4 consecutive days. The section demonstrates intra-neural collagen fibers (asterisks).

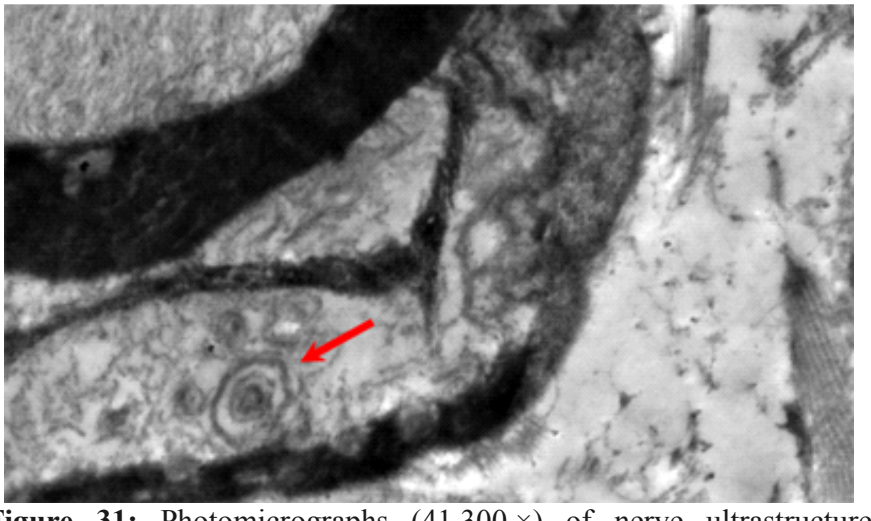

Figure 31: Photomicrographs (41,300 ×) of nerve ultrastructure (Osmium tetroxide stain) using TEM from female Wistar rats injected with 0.1 ml/day/rat Lipostabil® for 4 consecutive days showing degenerated myelin whorls within the cytoplasm of Schwann cells.

Effect of Repeated Lipostabil® Injection on Ultra-structures of the Neural Tissues at the Injection Site

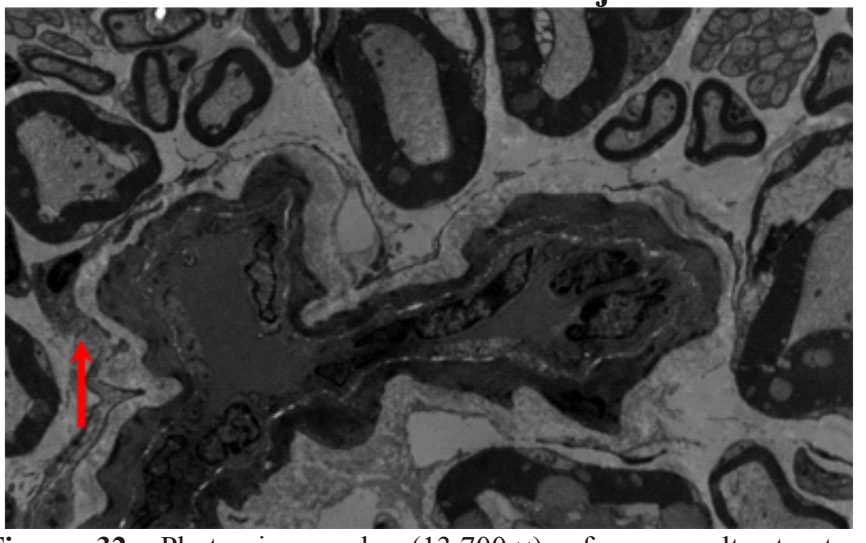

Figure 32: Photomicrographs (13,700 ×) of nerve ultrastructure (Osmium tetroxide stain) using TEM from female Wistar rats injected with 0.1 ml/day/rat Lipostabil® for 4 consecutive days. The section demonstrates intra-neural thick walled blood vessel (central field) and surrounding fibroblast (arrow).

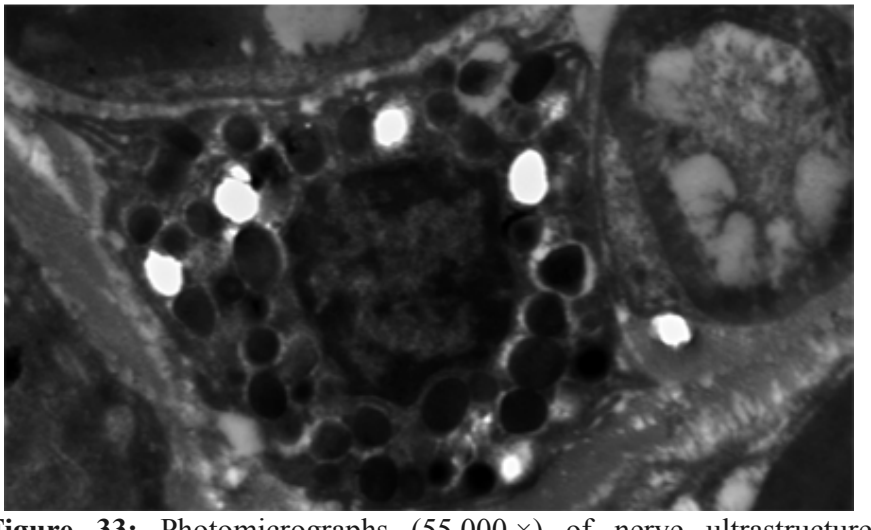

Figure 33: Photomicrographs (55,000 ×) of nerve ultrastructure (Osmium tetroxide stain) using TEM from female Wistar rats injected with 0.1 ml/day/rat Lipostabil® for 4 consecutive days. The section demonstrates endo-neural inflammation, mast cell with characteristic granules.