CHAPTER II
MATERIAL AND METHODS
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MATERIAL

The material consisted of:

1. Skeletal muscle biopsies from 81 patients submitted for routine diagnosis by the Department of Neurology NIMHANS, and from other institutions in Bangalore, during 1985-1991.

2. Skeletal muscle from 18 foetuses of different age groups and

3. Spinal cord from an autopsied case of Werdnig-Hoffmann Disease (WHD) (SMA-type.1).

SELECTION OF PATIENTS

1. Infants (<1 year) who presented with hypotonia, delayed acquisition of motor milestones and motor weakness.

2. Children (up to 12 years) with history of hypotonia and motor weakness dating back from infancy.

3. Adults with history suggestive of congenital myopathies, were included in the study.

Infants who presented with hypotonia of skeletal muscle but with clinical features suggestive of chromosomal abnormalities, cerebral palsy, connective tissue disorders and injury to the cervical spinal cord or plexuses were excluded from the study.
The clinical assessment and the biopsy were performed by a competent neurologist.

CLINICAL ASSESSMENT

In the clinical assessment of the patient information regarding prenatal intrauterine movements and the chronology of acquisition of motor milestones was obtained from the mother. The presence of congenital markers such as low set ears, high-arched palate, skeletal deformity such as scoliosis and contractures were noted. Evidence of cardiomegaly, hepatomegaly, retinitis pigmentosa and patterns of respiration were recorded. Ptosis, extraocular, facial and bulbar muscle weakness were carefully looked for and special effort was made to note fasciculation of tongue muscles. The attitude of the child was studied with reference to the "frog position". The power of the muscles were assessed by Medical Research Council grade and the deep tendon reflexes were graded. Electromyography and nerve conduction studies were carried out. Biochemical assay of serum for creatine phosphokinase (CPK) and lactate dehydrogenase (LDH) was carried out in addition to other routine investigations.

Open muscle biopsies (usually quadriceps) were done under dissociative anaesthesia using Ketamine for children and under local anaesthesia for adults. The muscle specimens were processed for histological, histochemical and electron microscopic studies.
Follow-up studies were available in 8 cases of Infantile spinal muscular atrophy (ISMA) and 4 cases of congenital muscular dystrophy (CMD).

HUMAN FOETUSES

Human foetuses were collected to study the morphological features of skeletal muscle during development and compare with features in infantile neuromuscular disorders.

The foetuses were collected from Vanivilas Hospital, Bangalore and Gunasheela Nursing Home, Bangalore. They were obtained fresh following medical termination of pregnancy with informed consent. None of the mothers had any neurological illness. The crown-rump and crown-heel lengths and weights of the foetuses were recorded in each case. The gestational age of each foetus was noted down as provided by the gynaecologist taking the last menstrual period into consideration. This was corroborated with the estimated gestational ages based on the data of Arey (1965). Fifteen foetuses were collected 1/2 - 2 hours after pregnancy termination. Three foetuses of gestational ages 28 weeks, 31 weeks and 36 weeks could be collected 12 hours after the termination of pregnancy.

A morphological study of deltoid, biceps, quadriceps and gastrocnemius muscles from the foetuses was carried out, as these muscles are the ones usually studied in patients with neuromuscular disorders.
SPINAL CORD

Spinal cord specimen available from a case of Werdnig-Hoffmann Disease (WHD) was fixed in 10% buffered formalin. Different segments of the spinal cord viz, cervical, thoracic, lumbar and sacral were processed for histological and immunohistochemical studies.

METHODS

The muscle biopsies from the patients were sent immediately following the biopsy procedure to the department of Neuropathology, NIMHANS, in a polythene cover with a drop of saline to keep the tissue moist so as to avoid drying artefacts. Human foetuses were collected fresh and the different muscles as stated earlier were taken.

Soon after collecting the specimen, a small portion of the biopsy material was flash frozen in isopentane precooled in liquid nitrogen for histochemical studies. Tiny pieces of the tissue were fixed in 3% gluteraldehyde in 0.1M-cacodylate buffer (pH 7.4) for electron microscopy. The rest of the tissue was fixed in 10% buffered formalin for histological studies.

HISTOLOGY

The histological study on muscle biopsies was undertaken by employing the following stains:
Haematoxylin and Eosin (H&E): to demonstrate sarcoplasmic components, the position of muscle cells nuclei, the general architecture of stroma, the blood vessels and the inflammatory cells.

Masson's trichrome (MAT): to assess the distribution and content of the interstitial connective tissue.

Mallory's phosphotungstic: to demonstrate cross striations in acid haematoxylin (PTAH) muscle.

Gomoris Silver Reticulin: to demonstrate reticulin framework around each muscle fibre.

The different spinal segments of the spinal cord from a case of Werdnig-Hoffmann disease were stained with -

Kluver-Barrera method: to demonstrate myelin and nerve cells.

Bodian's silver method: to study the cell bodies, cytoskeletal framework and the axis cylinder.

Vogt Cresyl Echt method: to demonstrate Nissl bodies in the neurons.

Loyez Haematoxylin method: for myelin

PTAH: to demonstrate glial fibres.

The above staining procedures were followed as described in the manual of histologic staining methods of the Armed Forces Institute of Pathology. The tissues fixed in 10% buffered formalin were processed in an automatic tissue processor Histokinette (Hendrey Relays limited, England) and embedded in paraffin wax. 5-8 micron thick sections from
muscle tissue were collected on albuminised slides. Spinal cord sections were collected on polylsine coated slides for histological and immunohistochemical studies.

**ENZYME HISTOCHEMISTRY**

The application of enzyme histochemical methods on muscle tissue helped in recognition of fibre types, structural abnormalities in the muscle (which appears normal in the conventional histology) and detection of specific enzyme deficiencies.

The following histochemical stains were employed in our study:

1. **Oxidative enzymes**
   a. Diphorases - Nicotinamide adenine dinucleotide tetrazolium reductase (NADH-TR).
   b. Dehydrogenases - Succinic dehydrogenase (SDH).
   c. Oxidases - Cytochrome C oxidase (COX).

2. **Phosphatases**
   a. Adenosine triphosphatase (ATPase)
   b. Acid phosphatase.

3. **Phosphorylase**

Oxidative enzymes: By the demonstration of oxidative enzymes, the two major muscle fibre types can be distinguished. In addition, structural abnormalities such as central and multi cores can be detected in NADH-TR reaction.
SDB reaction is specific to identify mitochondrial abnormalities.

Adenosine Triphosphatase: The pH lability of this reaction allows the differentiation of muscle fibre types at pH 9.5, 4.6 and 4.3 (see Table 2).

Acid Phosphatase: This is a lysosomal enzyme marker and is used to demonstrate the hydrolytic enzyme in the autophagic vacuoles.

Phosphorylase: This cytoplasmic enzyme indicates the capability of glycogen utilization and metabolism in the muscle fibre. By the application of this method to muscle sections, absence or variation in the activity of the enzyme is noted.

ENZYME HISTOCHEMICAL METHODS: A small portion of the skeletal muscle biopsy (2-3 mm cube) was cut and flash frozen in isopentane precooled in liquid nitrogen (-170°C). 8 micron thick transverse sections were cut on a cryostat (MINITOME - International equipment company, USA) at -20°C. The cryostat cut sections were stained for various enzymes stated above by standard histochemical methods (Pearse-1972).

1. Oxidative enzymes
   Incubating stock solution for NADH-TR and SDH:
   
   To 2.5 ml of 0.2M-Tris (hydroxy methyl amino methane) HCl buffer (pH 7.4), 1 ml. 5mM-Magnesium chloride, 2.5 ml.
Nitro-BT (4mg/ml) 3 ml distilled water was added, filtered and stored at 4°C, as the indicator - stock solution.

a) NADH-TR : To 1 ml. of the above stock solution, the co-enzyme Beta-nicotinamide adenine dinucleotide reductase (beta-NADH₂) (2 mg) was added, thoroughly mixed and used fresh.

b) SDH : To 1 ml. of the stock solution 2 mg. of disodium succinate was added, thoroughly mixed and used fresh each time.

The cryostat sections were incubated with the respective substrate solution at 37°C for 30 minutes in a moist environment. The slides were dipped in 10% formal saline to stop reaction, washed in distilled water, air dried and mounted in glycerin jelly.

c) Cytochrome Oxidase : The sections were incubated in the medium containing 5 mg. 3,3'-diaminobenzidine tetrahydrochloride (DAB) in 9 ml. of 50 mM-phosphate buffer (pH 7.4), 1 ml. catalase solution (20 mg/ml), 10 mg. Cytochrome C and 750 mg. sucrose for 60 mts at 37°C. The slides were washed briefly in distilled water, air dried and mounted in glycerin jelly.

2. Phosphatases :

a) Adenosine triphosphatase

Pre-incubating medium

i) 0.02M acetate buffer (pH 4.6)

ii) 0.1M Calcium barbital buffer (pH 9.5)
The slides were first incubated in the above solutions at 37°C for 20mts, in a moist environment. The solutions were shaken and further incubated in 0.005M ATP substrate (pH-9.5) solution containing 5mg. adenosine 5'-triphosphate di sodium salt, 2 ml 0.1M-Sodium barbitone, 0.18M-calcium chloride and 3ml distilled water, for 45 mts. at 37°C. The frozen sections, following incubation in the enzyme reaction mixture were washed in 1% calcium chloride, followed by 2% cobalt chloride and distilled water. The histochemical reaction was visualised by developing the colour in 1% yellow ammonium sulphide solution. After washing in distilled water, the sections were air dried and mounted in glycerin jelly.

b) Acid Phosphatase

The sections were incubated at 37°C for 60mts in a freshly prepared solution of 0.01M-sodium beta-glycerophosphate in 0.05M-acetate buffer (pH 5.0), containing 0.004M-lead nitrate. The slides were briefly washed. The histochemical reaction was developed in 1% yellow ammonium sulphide. After washing in distilled water, the sections were air dried and mounted in glycerin jelly.

3. Phosphorylase

100 mg glucose-1-phosphate, 10 mg adenosine-5-phosphate, 2 mg glycogen, 180 mg sodium fluoride, 900 mg polyvinyl pyrrolidone and 1 drop of insulin (40 i.u/ml) were dissolved
in 10 ml of 0.1M acetate buffer (pH 5.9) and thoroughly mixed. The slides were incubated in the above solution at 37°C for 60 mts. They were washed in 40% ethanol, fixed in absolute ethanol for 30 mts. and stained with Gram's iodine (1:10) for 5 mts. The slides were mounted in glycerin jelly.

The other stains used on the cryostat sections were:

1. Modified Gomori's trichrome (MGT) - to demonstrate ragged red fibres.

2. Periodic acid schiff (PAS) - to demonstrate glycogen.

3. Oil red O - to demonstrate neutral fat deposition in muscle fibres.

ELECTRON MICROSCOPY

The biopsies of muscle specimens were processed for fine structural studies.

METHOD: The tissues fixed in 3% gluteraldehyde in 0.1M cacodylate buffer (pH-7.4) were rinsed in 0.1M sodium cacodylate buffer (pH-7.4), post fixed in 1% buffered osmium tetroxide for 1 hour and dehydrated in ascending grades of alcohol. En bloc staining was carried out using 2% uranyl acetate in 95% alcohol. The tissues were cleared in propylene oxide and after infiltration in 1:1 propylene oxide araldite-Cy212 mixture (Synthetic resin TAAB), the tissues were embedded in araldite in flat embedding moulds. The specimens were oriented in longitudinal and transverse
planes. The moulds were left undisturbed in an oven at 60°C for 2 days to allow polymerisation. Seven blocks were made from each specimen.

SECTIONING AND STAINING: The blocks were cut on an ultramicrotome (ULTRACUT-E-Reichert-Jung-Austria). Semithin sections stained with 1% toluidine blue were used to select the area of interest. Ultrathin sections corresponding to grey-silver interference colour were collected on copper grids. The ultrathin sections were stained by double staining method (Frasca and Parks, 1960). Saturated uranyl acetate in 70% methanol and 1% lead citrate (pH 11.9) were used. The grids were viewed under Jeol Transmission electron microscope (JEM-100 C X II).

Detailed morphological features were noted and suitable areas were photographed using kodak film sheets (4489) and 35mm kodak fine grain release positive film (5302).

IMMUNOHISTOCHEMISTRY

Muscle: The cryostat sections from three cases of congenital muscular dystrophy (CMD) were fixed in cold acetone. Immunohistochemical staining was carried out using mouse monoclonal antibodies to dystrophin [Ab "2-5 E2" gifted by Dr.K.Arahata], as the primary and HRP-conjugated rabbit anti-mouse (Dakopatts) serum as the secondary antibody carried out by the indirect immuno peroxidase technique.
Spinal cord: Sections from different segments of the spinal cord from a case of Werdnig-Hoffmann disease (WHD) were stained using monoclonal antibodies to phosphorylated neurofilaments (SMI-31), HRP tagged rabbit anti-mouse (Dakopatts) by the indirect immuno peroxidase method. The sections were stained for aberrate expression of phosphorylated neurofilament 150, 200 Kd antigens in the spinal neurons.

The histological features noted in various disorders of the skeletal muscle were evaluated in conjunction with the clinical and biochemical features in the patients, to arrive at a definitive diagnosis.

The types of muscle disorders encountered in our study and the number of patients in each category is indicated in Table-1.
<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Number of patients n = 81</th>
<th>Percentage</th>
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</thead>
<tbody>
<tr>
<td><strong>I. Infantile spinal muscular atrophy</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Spinal muscular atrophy-1 (Werdnig-Hoffmann Disease)</td>
<td>22</td>
<td>27.16</td>
</tr>
<tr>
<td>2. Spinal muscular atrophy-2 (intermediate type)</td>
<td>13</td>
<td>16.05</td>
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<tr>
<td><strong>II. Congenital muscular dystrophy</strong></td>
<td>13</td>
<td>16.05</td>
</tr>
<tr>
<td><strong>III. Cogenital myopathies</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Central core disease/multicore disease</td>
<td>3</td>
<td>3.70</td>
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<tr>
<td>2. Centronuclear myopathy</td>
<td>6</td>
<td>7.41</td>
</tr>
<tr>
<td>3. Myopathy with type II fibre hypoplasia</td>
<td>2</td>
<td>2.47</td>
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<tr>
<td>4. Myopathy with type I fibre predominance</td>
<td>4</td>
<td>4.94</td>
</tr>
<tr>
<td>5. Benign congenital hypotonia</td>
<td>7</td>
<td>8.64</td>
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<td><strong>IV. Metabolic myopathies</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Mitochondrial myopathies</td>
<td>6</td>
<td>7.41</td>
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<tr>
<td>2. Carnitine deficiency</td>
<td>2</td>
<td>2.47</td>
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<tr>
<td>3. Mitochondria-lipid-glycogen-myopathy</td>
<td>1</td>
<td>1.23</td>
</tr>
<tr>
<td>4. Acid maltase deficiency</td>
<td>2</td>
<td>2.47</td>
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