ULTRSTRUCTURAL CHANGES IN THE SPINAL GANGLIA AFTER IMMOBILIZATION OF THE HINDLIMB OF THE ALBINO RAT

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ABSTRACT
Prolonged bed rest and immobilization of joints for long periods of time are frequently prescribed for intensive care and orthopedic patients. Abundant scientific evidences in the past 50 years have demonstrated that long-term immobilization lead to multiple pathological changes in most organs and systems of the body, with particular in the nervous and musculoskeletal systems. Moreover, critical illness neuropathy with defect in sensory perception is recognized more frequently in intensive care patients due to immobilization and bed rest for long time. The present investigation was, therefore, designed to study the ultrastructural changes in the primary sensory neurons in the dorsal root ganglia of the albino rat after a long period of immobilization of both the knee and ankle joints by using above knee plaster cast. These changes may shed some light on the possible mechanism underlying the immobilization neuropathy and the defect in sensory perception.

Ten adult male albino rats were used in this investigation. The left knee and ankle joints were immobilized, in the resting position, by the application of an above knee plaster cast for 12 weeks. The contralateral right side was used as control. After 12 weeks, the plaster cast was removed and the rats were sacrificed by intracardiac perfusion with a mixture of 2% paraformaldehyde and 2%
glutaraldehyde. The left (experimental immobilized side) and right (control side) lumbar dorsal root ganglia (L4, L5 and L6) were exposed, carefully dissected out, kept in the same fixative overnight and then processed for electron microscopic examination.

Electron microscopic examination of the DRG of the immobilized side revealed that the affected sensory neurons showed aggregation of distorted cytoplasmic organelles with dissolution of Nissl substance and formation of vacuoles of variable sizes. The most noticeable observation was marked hyperplasia of the neurofilaments and dilatation of the rough endoplasmic reticulum.

Changes similar to apoptosis were seen in the nucleus of the affected neurons including abnormal enfolding of the nuclear membrane and fragmentation and condensation of the nuclear chromatin. Nucleolar margination, nucleolar hypertrophy and triple nucleoli were also observed. On the other hand, the cytoplasmic and nuclear changes observed on the immobilized side were not observed in the ganglia of the control mobilized side.

Examination of the myelinated axons in the dorsal root ganglia of the immobilized side revealed wide distention of myelin sheaths with splitting of the myelin lamellae and subsequent formation of large vacuoles and vesicular degeneration of myelin. These changes were not observed in the control ganglia.

INTRODUCTION

Prolonged bed rest and immobilization of joints for long periods of time are amongst the most frequently prescribed therapies for intensive care and orthopedic patients (Allen et al., 1999). Unfortunately, abundant scientific evidence in the past 50 years has demonstrated that this long-term inactivity, elimination of gravity and immobilization lead to multiple pathological changes in most organs and systems of the body generally known as "immobilization syndrome" (Denes, 1996; Okun et al., 2002).

Critical illness neuropathy is an axonal polyneuropathy recognized more
frequently in intensive care patients due to immobilization (Zifko, 2000). Despite careful nursing and physiotherapy, wasting, myopathy and neuropathy are commonly seen in sedated or comatose intensive care patients undergoing long-term bed rest. Wiis and Qvist (1999) described four cases of peroneal nerve lesion with drop foot in patients with up to eight weeks of immobilization. Petersen et al. (1999) reported two pediatric intensive care patients with flaccid tetraparesis.

More recently, Fletcher et al. (2003) reported neurophysiologic evidence consistent with critical illness polyneuropathy in more than 90% of long-stay patients after intensive care unit discharge. The neurological changes due to immobilization are more pronounced in younger than in older ones, indicating that longer periods of immobilization in younger animals lead to a greater degree of harmful effects (Urso et al., 2006). Moreover, in contrast to nerve lesions in adult animals, retrograde neuronal death is accelerated when nerve injury occurs in immature animals such as newborn rats (Bondok and Sansone, 1984-a; Bondok and Sansone, 1984-b; Yip et al., 1984; Devor et al., 1985). Gawish (2004) revealed that long-term immobilization of the rat hind limb resulted in a significant reduction in the cross-sectional area of the sciatic nerve and in the total number of the myelinated nerve fibers.

Searching the literature for any structural changes in the sensory neurons in the dorsal root ganglia that may explain the effects of this immobilization-associated neuropathy revealed only electrophysiologic studies and clinical reports. In the light of the previous investigations on the effects of immobilization and prolonged bed rest, the present investigation was designed to study the ultrastructural changes in the dorsal root ganglia of the albino rat after a long period of immobilization of both the knee and ankle joints by the use of an above knee plaster cast, which is one of the most widely used methods for the immobilization (Coutinho et al., 2002). The cast is popular and inexpensive method for the immobilization. These changes in the dorsal root ganglia may explain the possible mechanism
underlying the immobilization neuropathy.

MATERIAL AND METHODS

Animals:
Ten adult male albino rats weighing 285 ± 28 gm were used in this investigation. The animals were housed in plastic cages, one rat in a separate cage, under controlled environmental conditions, with free access to standard food and water.

Fixation:
Rats were anesthetized by intraperitoneal injection of chloral hydrate (300 mg/kg) before the immobilization procedure. The knee and ankle joints of the left hind limb were immobilized in a semiflexed position, by the application of an above knee plaster cast for 12 weeks. The contralateral right side was used as a control. After a period of 12 weeks in the plaster cast, the rats were anaesthetized with chloral hydrate. Then the thorax was opened, a cannula was inserted into the left ventricle of the heart, the right atrium was incised and blood was washed out by intracardiac perfusion with 500 ml of normal saline followed by 500 ml of a mixture of 2% parafor-

maldehyde and 2% glutaraldehyde in 0.1M cacodylate buffer at pH 7.4.

Obtaining the Specimens:
An incision was made in the back of the thigh on both the left (immobilized) and right (control) sides and the left and right sciatic nerves were exposed near the greater sciatic foramen. The sciatic nerve was followed to its roots (L4, 5, 6) in the intervertebral foramina where the dorsal root ganglia are located. The L4, L5 and L6 dorsal root ganglia were carefully dissected out and kept in the same fixative in the refrigerator overnight.

Processing the Specimens:
The specimens were rinsed in cacodylate buffer, postfixed with 1% osmium tetroxide for 2 hours, dehydrated in ascending series of ethanol, cleared in propylene oxide, infiltrated in a mixture of propylene oxide and Epon resin and then embedded in Epon resin. Semithin sections (1 mm thick) were cut with the ultramicrotome, stained with toluidine blue and examined with a light microscope for orientation and selection of the areas containing nerve cells. Ultrathin sections (70 - 80 nm) were cut, dou-
ble stained with uranyl acetate and lead citrate and examined with a JEOL electron microscope.

RESULTS

A. CONTROL GANGLIA (MOBILIZED SIDE)

The present observations on the ultrastructure of the control dorsal root ganglia (Figs. 1, 2 and 3) showed that the spinal ganglion neurons had well-organized organelles and a centrally placed nucleus. The cell bodies of the primary sensory neurons appeared surrounded by a layer of satellite cells. The nerve cells appeared separated by bundles of myelinated axons with regularly arranged myelin lamellae.

Nissl substance consisted of well-organized parallel cisternae of rough endoplasmic reticulum. Free ribosomes were present in small clusters between the cisternae of the endoplasmic reticulum. The mitochondria were randomly distributed. Well-developed Golgi apparatus surrounded the nucleus and was associated with numerous vesicles. The neurofilaments and the neurotubules were dispersed throughout the perikaryon around Nissl bodies and the mitochondria.

The nucleus was large, rounded and centrally located. The nuclear membrane had a smooth undulating profile and the nucleus contained uniformly dispersed chromatin granules.

B. EXPERIMENTAL GANGLIA (IMMOBILIZED SIDE):

Electron microscopic examination of the dorsal root ganglia 12 weeks after immobilization revealed a spectrum of structural damage ranging from loss of Nissl substance and cytoplasmic degradation of organelles to frank necrosis on the immobilized side (Figs. 4, 5, 6). The most noticeable observation was marked hyperplasia of the neurofilaments, dilatation of the rough endoplasmic reticulum and marked enfolding of the nuclear membrane (Figs. 6, 7, 9, 10, 11).

Frequently, affected primary sensory neurons exhibited perinuclear aggregation of distorted cytoplasmic organelles with dissolution of Nissl substance, increased numbers of

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neurofilaments, dilatation of the rough endoplasmic reticulum, vacuoles of variable sizes, formation of large peripheral vacuoles, increased number of lysosomes and decreased size of the mitochondria (Figs. 7, 8, 9). Some degenerated neurons were transformed into large electron-dense multivesicular body (Fig. 6). Polysomes appeared dissociated into single free ribosomes and appeared scattered around the neurofilaments and the dilated rough endoplasmic reticulum (Figs. 8, 9). Lamellar bodies, small myelin figures and dense bodies appeared interspersed among the neurofilaments (Fig. 9).

The degenerated neurons appeared engulfed by satellite cells (Figs. 5, 6). The degenerative changes observed in the sensory neurons of the immobilized side were not observed in the ganglia of the control mobilized side.

Changes similar to apoptosis were seen in the nucleus of some neurons including abnormal enfolding of the nuclear membrane and fragmentation and condensation of the nuclear chromatin (Figs. 6, 7, 11). In some nerve cells, nucleolar hypertrophy, nucleolar margination and triple nucleoli were observed (Figs. 12 & 13).

Examination of the myelinated axons in the dorsal root ganglia of immobilized side revealed striking ultrastructural alterations. They appeared in the form of wide distention of myelin sheaths of the myelinated nerve fibers with splitting of the myelin lamellae and subsequent formation of large vacuoles and vesicular degeneration of myelin (Fig. 14).
Fig. 1: Electron micrograph of a control L5 dorsal root ganglion showing a part of sensory neuron containing a rounded nucleus (N), well-organized rough endoplasmic reticulum (RER), Golgi apparatus (G) in the perinuclear zone and randomly distributed mitochondria (M) and lysosomes (L). X 6,000.

Fig. 2: Electron micrograph of a control L4 dorsal root ganglion showing a part of sensory neuron containing a nucleus (N) with regular nuclear membrane, well-organized rough endoplasmic reticulum (RER), Golgi apparatus (G) and numerous mitochondria (M). The spaces between the RER and other organelles contain neurofilaments. X 5,000.
Fig. 3: Electron micrograph of a control dorsal root ganglion showing a part of sensory neuron containing well-organized rough endoplasmic reticulum (RER), numerous randomly distributed mitochondria (M), other organelles and a nucleus (N) with mild irregularity in the nuclear membrane. Minimal amount of neurofilaments is present between the different organelles. X 5,000.

Fig. 4: Electron micrograph of a dorsal root ganglion 12 weeks after inimmobilization showing a completely degenerated neuron containing apoptotic nucleus (N) and numerous degenerated organelles. X 10,000.

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Fig. 5: Electron micrograph of a dorsal root ganglion on the immobilized side showing a completely degenerated neuron (N) engulfed by a satellite cell (S). X 5000.

Fig. 6: Electron micrograph of a dorsal root ganglion 12 weeks after immobilization showing 2 degenerated neurons (1 & 2) engulfed by satellite cells (S). Nerve cell 1 has a nucleus (N) with markedly enfolded nuclear membrane. Nerve cell 2 is transformed into a large multivesicular body. X 10,000.
Fig. 7: Electron micrograph of a dorsal root ganglion 12 weeks after immobilization showing a degenerating neuron containing apoptotic nucleus (N) with large electron-dense mosaic-shaped nucleolus (Nu) and markedly enfolded nuclear membrane (Nm). The cytoplasm contains distorted organelles and dense bodies (arrows). X 11,000.

Fig. 8: Electron micrograph of a dorsal root ganglion 12 weeks after immobilization showing a degenerating neuron containing dissolved ribosomes (R) from the rough endoplasmic reticulum, degenerated nucleus (N), increased number of lysosomes (L) and distorted mitochondria (M). X 12,000.

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Fig. 9: Electron micrograph of a dorsal root ganglion 12 weeks after immobilization showing a primary sensory neuron containing dissociated ribosomes (R) from the rough endoplasmic reticulum and their dissolution into free ribosomes, marked increase in the neurofilaments (NF) and myelin figures (My). X 12,000.

Fig. 10: Electron micrograph of a dorsal root ganglion 12 weeks after immobilization showing a primary sensory neuron containing marked increase in the neurofilaments (NF) and enfolding of the nuclear membrane (arrow). X 15,000.
Fig. 11: Electron micrograph of an affected primary sensory neuron 12 weeks after immobilization showing a nucleus that appears divided into 3 pieces (N) by marked enfoldling of the nuclear membrane (arrows). X 9,000.

Fig. 12: Electron micrograph of a sensory neuron 12 weeks after immobilization showing a nucleus (N) containing 3 nucleoli (Nu) which are located at the margin of the nucleus (nucleolar margination) and are attached to the nuclear membrane. X 10,500.

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Fig. 13: Electron micrograph of a sensory neuron 12 weeks after immobilization showing a nucleus (N) containing 3 nucleoli (Nu). X 4,500.

Fig. 14: Electron micrograph of L5 dorsal root ganglion of the immobilized side 12 weeks after immobilization showing several myelinated axons. The myelin shows multiple splitting and disruption of the myelin lamellae with formation of vacuoles (*) between the lamellae. X 6,000.
DISCUSSION

The effect of immobilization on the structure of the peripheral nervous system is not so clear and has not been fully investigated in the recent literature. At the present time, there is a vague knowledge concerning the structural bases for the immobilization neuropathy syndrome. Searching the literature for any histological evidence that might explain the immobilization-associated polyneuropathy revealed only clinical reports and electrophysiologic studies. Almost all the earlier reports on immobilization on the nervous system have dealt only with the clinical manifestations of long-term bed rest. Unfortunately, the ultrastructural changes in the primary sensory neurons which might explain the sensory deficit in the immobilization syndrome have not been investigated. The only available ultrastructural report on the effect of immobilization on the nervous system is that of Gawish (2004) who presented the morphometric and the ultrastructural changes in the sciatic nerve of the albino rat as a model of long-term immobilization.

According to the results of the present investigation and of previous investigations (Gawish, 2004), it is obvious that the structural changes in the dorsal root ganglia and in the peripheral nerves could be, in part, responsible for the sensory manifestations of the immobilization neuropathy syndrome.

Gawish (2004) reported that long-term immobilization of the hind limb resulted in dramatic changes in the sciatic nerve in the form of a highly significant reduction in the cross-sectional area of the sciatic nerve (43%) and in the total number of the myelinated fibers (33%). Similar to the present observations, immobilization resulted in splitting of the myelin lamellae, loss of the compact lamellar structure of the myelin sheath and in degenerative changes in the myelin. Therefore, it is reasonable to suggest that the changes in the sensory neurons are most probably due to the degenerative changes and loss of their peripheral branches in the sciatic nerve; a degeneration called retrograde degeneration.

In the present study, long-term immobilization of both the knee and ankle joints for 12 weeks resulted in a
spectrum of ultrastructural damage to the primary sensory neurons on the immobilized side. The most remarkable observations were marked hyperplasia of the neurofilaments, marked enfolding of the nuclear membrane and dilatation of the rough endoplasmic reticulum. The affected primary sensory neurons showed distorted organelles, numerous lysosomes, dissolution of Nissl substance and formation of vacuoles of variable sizes. Nuclear changes similar to apoptosis were seen in the affected neurons with condensation and fragmentation of the nuclear chromatin. Nucleolar hypertrophy, multinucleoli and nucleolar margination were also observed. Moreover, unusual cytoplasmic inclusions filled with neurofilaments were observed in the affected neurons. Degenerative changes in the myelinated axons in the spinal ganglia of immobilized side with splitting of the myelin lamellae and formation of large vacuoles were observed. These neuronal changes were not observed in the control ganglia of the mobilized control side. The ultrastructural changes after immobilization may indicate a definite structural neural involvement in the immobilization-associated polyneuropathy.

The dissociation of ribosomes from polysomes and rough endoplasmic reticulum to single ribosomes has been also described in degenerating neurons in the facial nucleus of adult mice following facial nerve transection (Torvik and Skjorten, 1971) and in the trigeminal ganglion of adult rats following infraorbital nerve transection (Aldskogius and Arvidsson, 1978). Since the free ribosomes are involved in the production of proteins for local use by the cell and not for transport to the axon terminals, this dissociation into single ribosomes may be a response to increase protein production needed for the survival of cell body.

Abnormal increase in the amount of neurofilaments was a prominent feature of the affected neurons. Similar observations were seen in the spinal ganglia of adult rat after transection of the brachial plexus (Zelena, 1971), in the facial neurons of newborn rabbit after transection of the facial nerve (Torvik, 1972) and in young rats after sciatic nerve crush at birth (Bondok and Gawish, 1994). More recently, the present changes after im-
mobilization is almost similar to those reported in the dorsal root ganglia after exposure to neuropathy-inducing organophosphorous compounds (Massicotte et al., 2003).

The presence of nuclear changes similar to apoptosis, nucleolar hypertrophy, multinucleoli and nucleolar margination raise the question of DNA damage. The nucleolar margination is defined as the presence of nucleoli touching the nuclear membrane. Yin et al. (2003) have indicated that the incidence of nucleolar hypertrophy and nucleolar margination is an indication that chronic atrophic gastritis can develop into gastric cancer. Hussein et al. (2003) revealed multinucleation in the irradiated skin cells and that ultraviolet-B irradiation caused an increase in the apoptotic activity and reflecting DNA damage.

The results of the present study are of interest from two points of view. Firstly, they extend our knowledge on the ultrastructure of the retrograde neuronal degeneration. Secondly, they add new information relevant to the reaction of primary sensory neurons to immobilization. Disintegration and vacuolation of cell organelles and apoptosis may indicate a stage of "no return" and inevitable cell death.

It is also questionable whether these changes in the nerve cells are primary to immobilization or secondary to the changes in the muscles or the peripheral nerves and whether these changes are reversible or not. Grana et al. (1996) presented evidence that muscle disuse due to cast immobilization of one leg can result in a reversible dysfunction of neuromuscular transmission. On the other hand and more recently, Fletcher et al. (2003) reported persistent neuromuscular and neurophysiologic abnormalities in long-term survivors of prolonged critical illness.

It is concluded in this study that long-term immobilization has a damaging effect on the structure of the primary sensory neurons and subsequently on the sensory perception. These ultrastructural changes after immobilization may indicate a definite structural neural involvement in the immobilization-associated polyneu-
ropathy. This conclusion supports the lay wisdom "use it or lose it". Therefore, early mobilization and shortening of the period of immobilization and bed rest are highly recommended to avoid structural damage of the peripheral nerves and their associated ganglia. Encourage movement and gentle exercises to improve blood flow to the affected area. Physiotherapy and electrical stimulation of immobilized muscles can prevent many of the complications of inactivity and bed rest.

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إن عدم الحركة لفترات زمنية طويلة وكذلك الراحة السريرية الطويلة يستخدمان بطريقة
شاملة لمرضى العناية المركزية ومرضى العظام. وهذا يؤدي إلى العديد من التغيرات المرضية في
معظم أعضاء واجهزة الجسم خاصة الجهاز العصبي والجهاز الحركى.

واوذلك فقد صممت هذه الدراسة لبحث التغيرات التركيبية فائقة الدقة بالخلايا العصبية
الحساسة الأولية في العقد العصبية للجدار الخلفي للعصب الشوكي للجردان البيضاء بعد تثبيت
الطرف الخلفى الأيسر لدورة طويلة، وشملت تثبيت مفصلي الكاحل (رست القدم) والركبة وذلك
باستخدام قابل جبس، تم تثبيت حتى أعلى مفصل الركبة، وهذه التغيرات رما تسلط الضوء على
طريقة حدوث الاعتلال العصبي الطرفى المتعدد المصاحب لعهد الحركة وكذلك سبب الخلل في
الإدراك الحسي.

استخدم في هذا البحث عشرة ذكور من الجردان البيضاء البالغة. تم تثبيت مفصلي الكاحل
والركبة بالطرف الخلفى الأيسر لمدة اثنتي عشر أسبوعًا باستخدام قابل جبس امتد حتى أعلى
مفصل الركبة واستخدمت الجهة اليمنى المتحركة كجهة ضابطة. وبعد مضي اثنتي عشر أسبوعًا تم
إزالة قابل الجبس وتم التضحية بالجردان ببعضهما بالقلب بنصف لتر من محلول ملح طبيعي
تغسل الدورة الدموية تنبعه نصف لتر من محلول مثبت مكون من خليط من 2% بارافورمالهيد
و 2% جلوكارالدزيد. تم استكشاف واستئصال العقد العصبىة القطنية الرابعة والخامسة

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والسادسة لكل من الجهة اليسرى المثبتة والجهة اليمنى المتحركة (الضابطة) وتم وضعهم في نفس المثبت بال الثنائية لدة ليلة واحدة وتم تجهيزهم للدراسة بالمجهر الإلكتروني. تم تقسيم العينات قطاعات فائقة الزق وصبغت صبغة مزدوية بأسائط البيريليوم وسترات الرصاص وتم فحص التغييرات فائقة الدقة بالمجهر الإلكتروني.

أظهر فحص العقد العصبية الشوكية بالمجهر الإلكتروني بعد اثنين عشر أسبوع من التثبيت أن الخلايا العصبية الحسية الأولية المتضررة ظهر بها تشوه بالعصم ذاتية وظهور تغيرات متعددة الطبقات وجميعها ضخمة وأجسام داكنة بين الشعرات العصبية. وكانت الملاحظة الأكثر وضوحًا هي زيادة غير عادية في الشعرات العصبية وانتشار خزائنز الشبكة الأندوبلازمية المحببة. وظهرت أجسام سيتوتوبلازمية ميتة على شكل كرات من الشعرات العصبية المتلاصقة.

وسوف تظهر تغييرات مشابهة لوح الخلايا البشري بنواة الخلايا العصبية المتضررة نماذج انتفاخات
بغراف النواة مع تجذير وتوكيد كروماتين النواة. وتم تلاحظ مثل تلك التغييرات بنواة وسيتوتوبلازم العقد العصبية الضابطة بالجانب الأيمن المتحرك.

أظهر فحص الحاور العصبية المغمدة بالعقد العصبية بالجانب المثبت وجود إنتفاخات واسعة بالنمد النخاعي مع انفصال وظهار وتكون فجوات كبيرة واضحة حتوي على الغمد النخاعي.

ولقد استنتج من هذه الدراسة أن عدم الحركة لفترة طويلة له تأثير ضار على تركيب الخلايا العصبية الحسية الأولية وبالتالي على الإدراك الحسوي. ومن المؤكد إن هذه التغييرات التركيبية فائقة الدقة التي تنتج عن عدم الحركة لها دور مؤثر في حدوث إعتلال الأعصاب الطرفية المتعدد والمصاحب لمعد الحركة ولذلك توصي هذه الدراسة بتقليل فترة عدم الحركة أو الحركة المبكرة وكذلك تقليل الراحة السريرية بقدر المستطاع.

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