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ULTRASTRUCTURE CHANGES OF THE NEURONAL COMPONENT IN THE DETRUSOR MUSCLE FOLLOWING SACRAL ROOT STIMULATION OF DECENTRALIZED DOGS

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ABSTRACT

Ultrastructural changes in the neuronal component of the detrusor muscle during the spinal shock phase and following early electric neurostimulation were studied in 12 dogs decentralized at the levels from S1 to S3. Three animals were provided as normal controls. The decentralized animals were divided into 4 groups according to the method of bladder voiding: Group 1A: intermittent catheterization without neurostimulation, Group 1B: intermittent catheterization followed by neurostimulation, Group 2A: early prolonged neurostimulation and Group 2B: early neurostimulation followed by intermittent catheterization. From the different groups, bladder tissue blocks were dissected, fixed in glutaraldehyde and processed for electron microscopic study. Early prolonged sacral root neurostimulation demonstrated a significant reduction of degenerated axons (mean = 28.9%±1.7), an increased frequency of cholinergic axons (mean = 16%±0.75) and an exhibited significant narrowing of the neuroeffector gap junctions (mean = 117.25±22.6nm) in comparison to non-stimulated animals (mean values were 80.5%±0.75, 5.4%±1.7, and 281±62.6nm, respectively) which were voided with intermittent catheterization. However, early sacral root stimulation followed by intermittent catheterization displayed a significant reduction of axonal degeneration (mean=48.3%±1.8) in comparison to late stimulation following the intermittent catheterization (mean= 83.3%±2.8). In conclusion, early sacral root electric stimulation decreased the incidence of neuronal degeneration in decentralized detrusor muscle, together with improving the regenerative potential of cholinergic axons.

INTRODUCTION

Immediately following spinal cord injury, there is a loss of both somatic and autonomic reflexes during the spinal shock phase. Detrusor areflexia with poor compliance is the common initial result and remains for a period of several weeks up to several months before detrusor activity returns[1-3]. During the spinal shock phase, the urinary bladder is converted into an inert flabby bag which fills up and then starts to leak with overflow incontinence[4].

The bladder is merely kept empty during this phase by either indwelling catheterization, intermittent catheterization or suprapubic cystostomy[2]. Urethritis, traumatic urethral stricture and cystitis are common complications of catheterization[5]. Silent deterioration of the upper urinary tract and renal failure remain the main causes of mortality in patients with serious neurogenic vesical dysfunction[1,2]. Moreover, squamous metaplasia[6], transitional cell carcinoma[7] and squamous cell carcinoma[8] have been described in different studies.

Electrical neurostimulation of the atonic bladder has generated a great deal of interest during the last two decades. Tanagho et al. (1989)[9] and Brindley (1990)[10] pursued neurostimulation as a recent modulation in controlling voiding disorders and catheter complications during the spinal shock phase. Experimental detrusor decentralization comprises obvious histological, ultrastructural and isolated muscle strip behaviour changes[3,11]. The clinical relationship between these changes and bladder function is still under investigation.

The reaction of neurons to injury has been studied in experimental animals, with findings confirmed in humans. After an injury the axons undergo both retrograde and anterograde degeneration. The neuron may carry the potentiality for regeneration by growth of axon sprouts, which receive their requirements of certain proteases and neurite growth factors to permit their advance through the tissue[12]

In the present article, we studied the ultrastructural changes of the neuronal component of the detrusor muscle following early prolonged electric sacral root stimulation in decentralized dogs.

MATERIAL AND METHODS

Animal Model:

Fifteen male mongrel dogs weighing between 20 and 35 kg were used for this study. Three dogs were kept as normal controls, while 12 dogs were subjected to spinal section and divided into 2 main groups according to the method of bladder voiding.

Group 1: (6 dogs) subdivided into:

1A) Voiding by intermittent catheterization without electric stimulation (3 dogs).

1B) Voiding by intermittent catheterization for 4-6 weeks, followed by neurostimulation (3 dogs).

Group 2: (6 dogs) subdivided into:

2A) Early electric stimulation only (3 dogs).

2B) Early electric stimulation for 4-6 weeks, followed by intermittent catheterization (3 dogs).

The dogs of both main groups underwent a supra-sacral spinal section under direct vision. In the electric stimulation groups (1B, 2A, and 2B), the sacral roots were exposed extracaudally through a sacral laminectomy, and the nerve roots that gave the highest intravesical pressure were chosen as described by El Refaei et al. (1989)[13] and Li et al. (1992)[14]. Then, a platinum helical electrode (Urosystem Inc.) was wrapped around the roots and connected to a receiver (Avery). All dogs ran the experiment for a period of 6 to 8 weeks under complete medical care.

Specimen Collection:

Under general anaesthesia, the bladder was removed through a midline suprapubic incision. The bladder was opened sagittally and preserved in crushed ice until immediate tissue processing.

Electron Microscopic Tissue Processing:

A tissue block of 1 cm size was obtained from the dome of each bladder and prepared for electron microscopic study. In a few drops of 2.5% glutaraldehyde, the muscularis layer was separated from the mucosa and dissected into fine tissue blocks of 1 mm size. Fixation was accomplished in 2.5% glutaraldehyde in Millonig buffer at 4°C for 2 hours[15]. The blocks were put in phosphate buffer (pH 7.2) with sucrose 7% at 4°C for 12 hours. Then the blocks were washed in veronal buffer with sucrose 4.5% at 4°C for 2 days. Postfixation was done using osmic acid 1.67% in veronal buffer with sucrose at 4°C for 2 hours. Then the blocks were processed for dehydration in ascending grades of alcohols at 4°C, followed by embedding in Epon 812 after intermediary treatment in propylene oxide. Semithin sections (1μm) were prepared from each of the 6 –8 blocks which had been trimmed from each bladder specimen. Each of these sections was stained by toluidine blue method and examined by light microscope to select 4-5 blocks containing the neuromuscular compartment belonging to the longitudinally oriented muscle cells. From each selected Epon block, 10 –20 ultrathin (silver-gray to silver) sections were mounted on uncoated 100-mesh copper grids. The grids were stained by standard uranyl acetate/lead citrate sequence and preserved until photographed with a Phillips EM-300 electron microscope. Each field occupied by

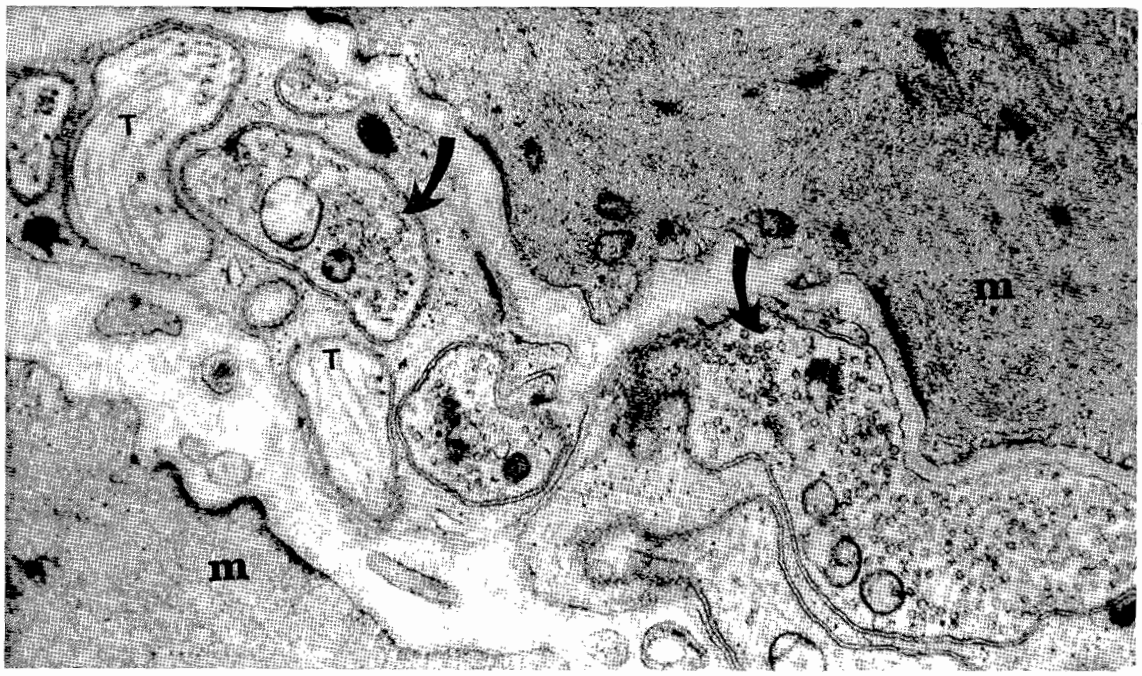

Fig. 1: Electron micrograph showing the neuromuscular component in the normal detrusor muscle. Schwann cell ensheathed axons are in close proximity to two smooth muscle cells (m). Cholinergic axons contain clear-core vesicles (arrows). Non-cholinergic axons demonstrate neurotubules (T) intermixed with fine neurofilaments. (13942X)

the neuronal component was photographed. The magnification ranged from 3,390X up to 19,180X for negative plates.

Morphometric Analysis:

On each electron micrograph (8"X10"print size), the neuroeffector junction gaps between Schwann cell partially ensheathed axons and corresponding muscle cell were measured.The magnification varied between 8,990X and 50,830X, the true gap width was calculated to be determined in nanometer (nm). All data were assessed for statistical analysis, using Analysis of Variance (Anova) and a significance of variance was accepted if $P< 0.05$.

RESULTS

The neuronal component of the detrusor muscle was detected in a total of 360 electromicrograph prints of the normal and different experimental groups. A total of 2019 axons were subjected to morphologic and morphometric analysis.

The normal detrusor muscle demonstrated terminal axons in varicosities at the neuromuscular sites (Fig 1). The Schwann cells accommodated numerous axons that were variable in shape and size. Each axon was bounded by axolemma. The axoplasm contained neurofilaments, neurotubules and mitochondria. The cholinergic axons were packed with clear-core cholinergic vesicles and accounted for a mean of 34.5%$\pm$0.50 of all studied axons. Adrenergic axons were infrequent and defined by the presence of dense-core adrenergic vesicles. Neuroeffector gap junctions measured a mean of 167.4$\pm$27.38 nm. However, close contact (en passage junction) was very infrequent and the gap junction exhibited a mean of 28$\pm$1.6nm. Schwann cell sarcoplasm was rich in mitochondria and free ribosomes.

In the 4 experimental groups of decentralized dogs, ultrastructural morphologic changes including axonal degeneration were defined. These axons demonstrated fragmentation and dissolution of the neurofilaments and neurotubules together with degenerated and/or swollen mitochondria (Fig. 2). Focal or complete depletion of cholinergic and adrenergic vesicles was noted. The neuroeffector junction gaps were significantly increased, and the axolemma was focally ruptured. Partial or com-

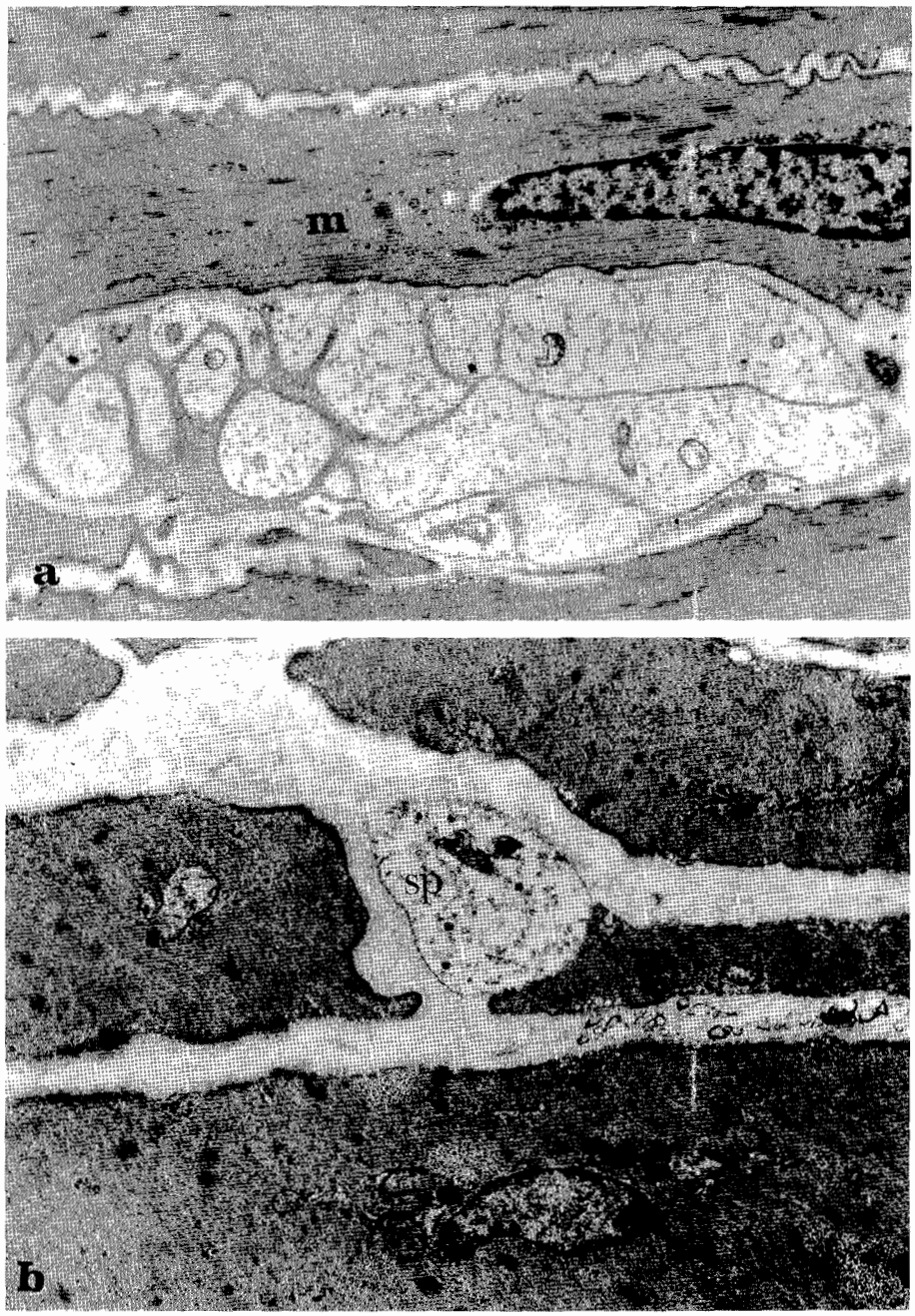

Fig. 2: A: Electron micrograph demonstrating decentralized detrusor muscle. Note the axonal degeneration with depletion of cholinergic vesicles and disruption of neurotubules, neurofilaments and mitochondria. The axons were partially ensheathed with Schwann cells (10630X). **B:** Non-ensheathed sprouts (sp) containing few cholinergic vesicles which perform en passage neuroeffector junction with the muscle (m). Intermittent catheterization followed by neurostimulation (11427X).

plete Schwann cell degeneration was also detected.

Individual axons that were corresponding to sprouts (Fig. 2B & 3) were detected in different groups. These sprouts demonstrated a rounded morphology, non- or partially unsheathed with Schwann cell processes. Their axoplasm contained few cholinergic vesicles, primitive neurotubules and neurofilaments. However, more developed sprouts contained numerous cholinergic vesicles (Fig 3C). They were in close proximity to muscle cells and performed en passage effector junction.

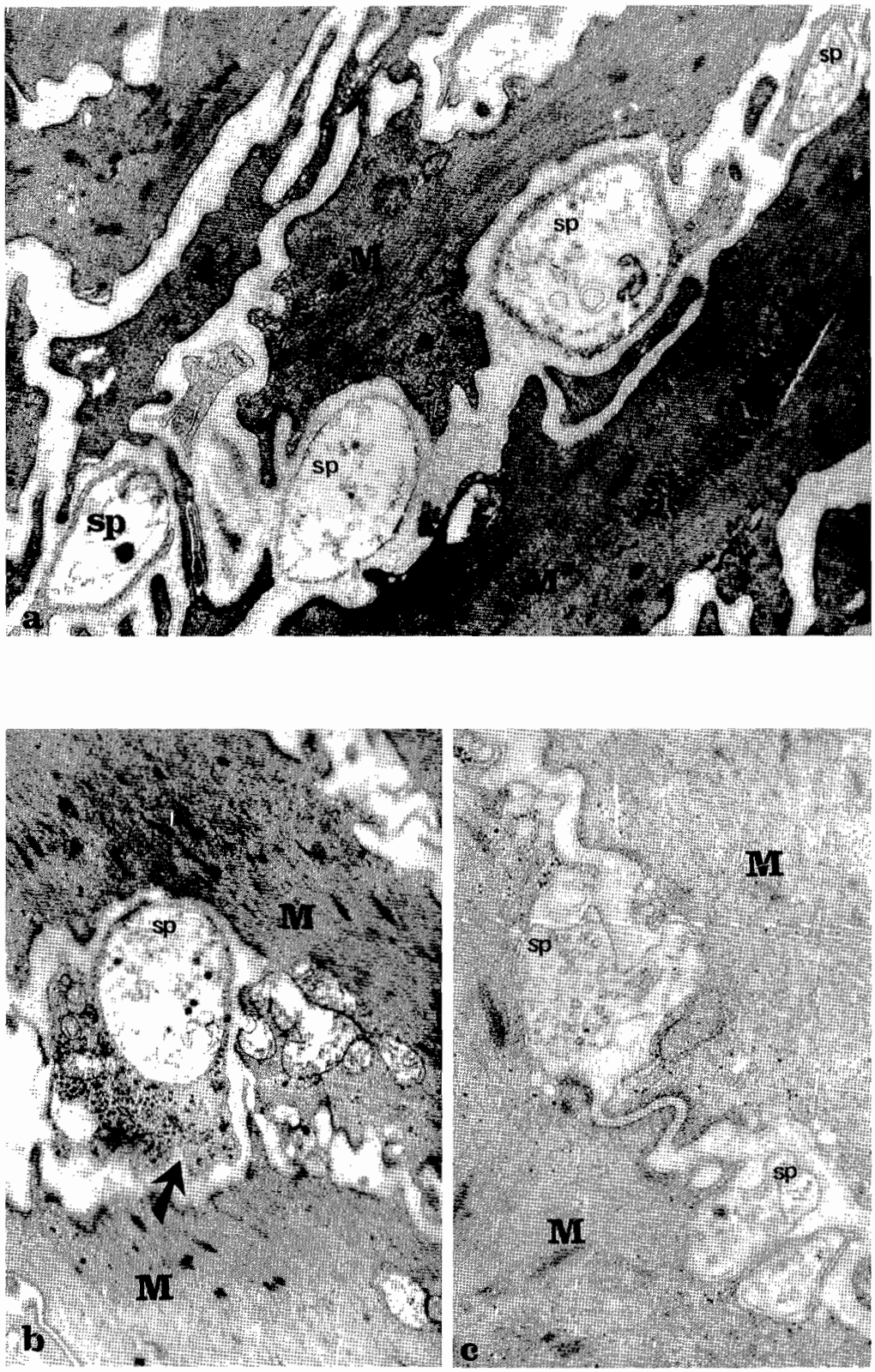

Fig. 3: Electron micrograph showing a *neurostimulated detrusor muscle*. **A:** Numerous partially or non-ensheathed sprouts (sp) with Schwann cell processes. Neurotubules and neurofilaments started to appear in few sprouts. (14299X). **B:** Partially ensheathed sprouts with active Schwann cell (*arrow*) that directs sprout to the nearest target muscle (m) (17589X). **C:** Intact sprouts performing en passage neuroeffector junction with muscle cell (m), occupied with cholinergic vesicles (12396X).

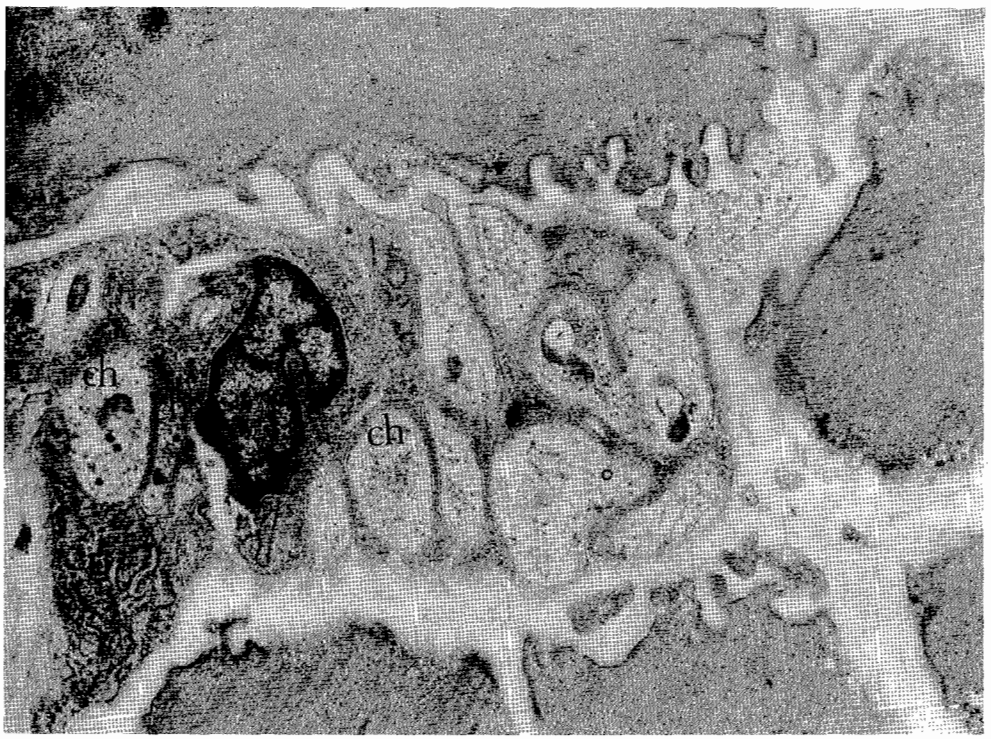

Fig. 4: Electron micrograph showing neurostimulated detrusor. Note active Schwann cell ensheathing numerous intact cholinergic axons (ch) and non-cholinergic axons. The Schwann cell was rich in rough endoplasmic reticulum (E r) together with free ribosomes and intact mitochondria. The neuroeffector gap junction measured 115 nm. (12371X)

Active Schwann cells were determined by the presence of well developed endoplasmic reticulum, free ribosomes and numerous intact mitochondria (Fig 4).

Semiquantitation of ultrastructural changes was correlated with different experimental groups and described in Tables (1 & 2).

Group 1A: Intermittent catheterization (3 dogs):

The detrusor muscles of the animals in this group demonstrated numerous axonal degeneration, representing a mean of 80.5%±0.75 of all examined axons. Cholinergic axons were significantly decreased if compared to the normal detrusor (mean was 5.4%±1.7 and 34.6%±0.5, respectively). The neuroeffector gap junctions were observed to be decreased (mean of 281±63.6 nm) in comparison to the normal controls (mean 167.4±27.3 nm). Sprouts were frequently observed in this group and comprised 84.4% out of intact axons in one animal, while the other 2 animals showed less numerous sprouts (14.7% and 8% of intact axons).

Group 1B: Intermittent catheterization followed by electric stimulation (3 dogs):

No significant difference was detected between Groups 1A and 1B regarding the extent of degenerated axons (the mean was 48.5%±2.8), the mean of cholinergic axons represented 5.6%±2.1. On the other hand, the sprouts were decreased and comprised a mean of 51.4% of intact axons. The neuroeffector junction was significantly wider (mean 225.22±33.1nm) than in the normal control.

Group 2A: Early electric stimulation (3 dogs):

The detrusor muscle of these animals demonstrated a significant reduction of degenerated axons, representing a mean of 28.95±1.7 of all studied axons. Cholinergic axons were significantly increased (mean 16%±0.75), if compared to the previous 2 groups (5.4% and 5.6%, respectively). In contrast, there was a significant reduction compared to the normal control (34.5%±0.5). In addition, adrenergic axons were frequently detected. (Fig 5).

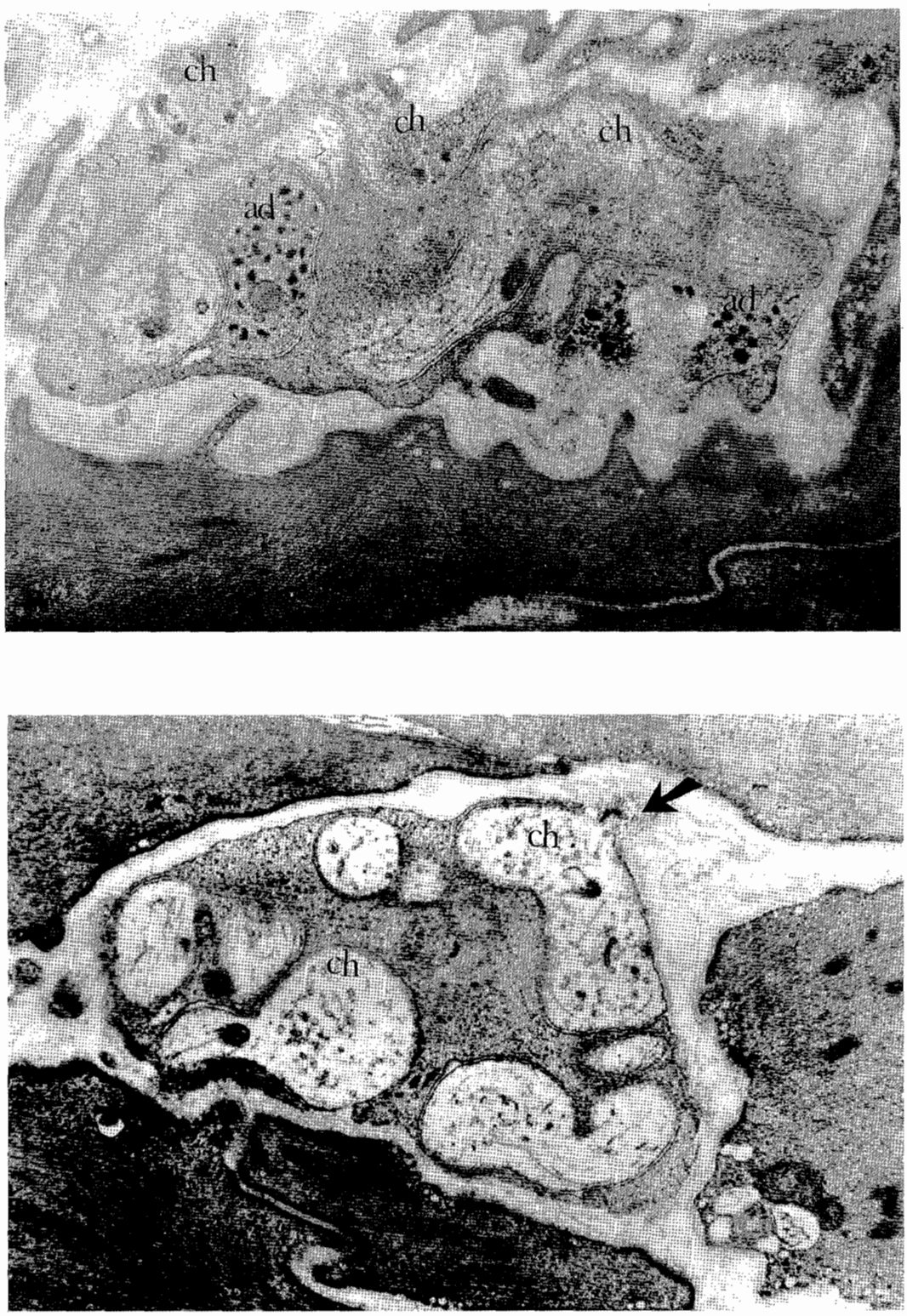

Fig. 5: A: Electron micrograph showing a neurostimulated detrusor with both cholinergic (ch) and adrenergic (ad) axons, ensheathed with active Schwann cell. (15570X) **B:** Neurostimulated detrusor showing Schwann cell ensheathed intact cholinergic axons with rupture of axolemma (arrow) and release of the cholinergic vesicle at the neuromuscular gap (14660X).

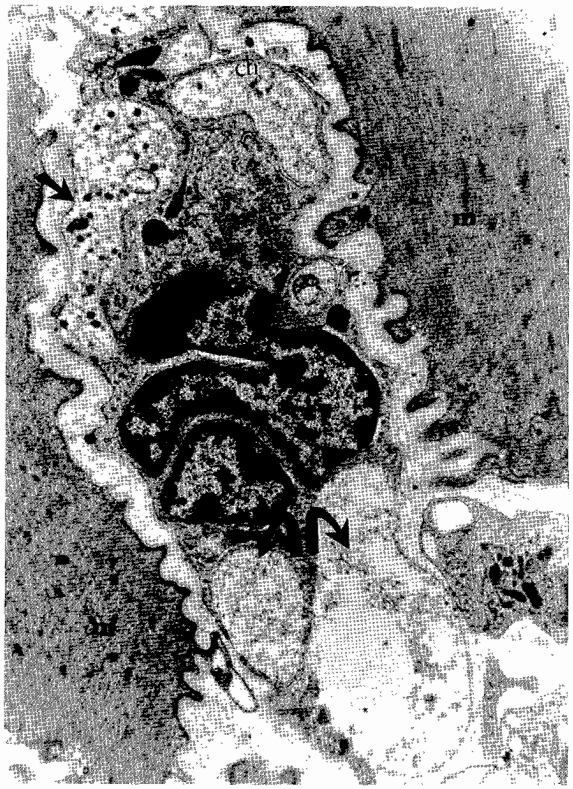

Fig. 6: Electron micrograph showing Schwann cell ensheathed axons between muscle cells (m), containing both intact (ch) and degenerated axons (curved arrows) after neurostimulation of the detrusor followed by intermittent catheterization. An intact axon contains adrenergic vesicles (arrow). (11677X)

Sprouts were infrequently detected, the mean was 10.67% of intact axons. The neuroeffector gap junction was significantly decreased (mean 117.2±22.6 nm) in comparison to all experimental groups with an insignificant difference compared to the normal control (167±27.3 nm).

Group 2B: Early electric stimulation followed by intermittent catheterization (3 dogs):

Axonal degeneration (Fig. 6) of the detrusor in this group was noticed to be significantly decreased (a mean of 48.4%±1.8) compared to the non-stimulated group 1A, or the late stimulated group 1B. However, there was a significant increase of degenerated axons in comparison to the early stimulated group 2A. The cholinergic axons demonstrated a significant reduction (9.8%±2.0) in comparison to both the normal control and early stimulated groups (2A). Numerous sprouts were also observed in this group. The neuroeffector gap junction was significantly increased (a mean of 253.5±60.2nm).

DISCUSSION

Spinal cord transection at S2 and S3 level induces suppression of the sacral reflexes that control bladder evacuation. The innervation of the bladder has been demonstrated to be related to nerve cells located in the intermediate column at the level of S1 to S3 with a maximum condensation at S2[16]. During the early spinal shock phase, the bladder remains flaccid for a period of several weeks up to several months before the detrusor regains activity[17]. One modality of management during this stage is intermittent catheterization to keep the bladder empty[1,4,14]. The decentralized neuron loses an adequate source of stimulation that induces trans-neuronal degeneration and / or atrophy[18]. Tissue blocks obtained from the normal bladder body in this experimental study were rich in cholinergic axons which is similar to that noticed in the detrusor of the human bladder[19].

In the present study, the detrusor muscle that was managed by intermittent catheterization (Group 1A) demonstrated axonal degeneration in the neuronal component. These changes were similar to those described by Elbadawi et al. (1984)[14] and Burt (1993)[18]. The onset of degeneration development is variable. In the study of Feher et al. (1980)[20], ultrastructural changes occurred rapidly as early as on the first day of neuronal injury and were well established by the 7th day, while Elbadawi et al. (1984)[15] reported a full development of these changes by the third week postoperatively. In our experiment, assessment was done 8 weeks after decentralization and about 80% of the axons were noticed to be degenerated.

The variability in the degree, distribution and nature of axonal degeneration could be contributed to the trans-synaptic nature of electric or chemical transmitters which were altered by neuronal dissection. Moreover, the distance between the degenerated axon and its cell origin or mural vesicourethral ganglia could be considered another factor[15,18].

In the non-stimulated group (1A) of the present study, the increased incidence of axonal degeneration together with the significant reduction in the axons occupied by cholinergic vesicles, were in conformity with

Table 1: Axonal Degeneration and Sprouts of the Neuronal Component in the Detrusor Muscle of the Dogs used in our Experiment

Group	No. of Prints	Total Axons	No. of Degenerated Axons	Sprouts No/Intact Axons
Normal	(47)	(335)		
No. 1	15	134	3 (2.2%)	-
No. 2	17	104	2 (1.9%)	-
No. 3	15	97	2 (2.1%)	-
Mean			2.06% ± 0.15	-
Group 1A	(94)	(532)		
No. 4	32	235	190 (80.8%)	38/45 (84.4%)
No. 5	32	167	133 (79.6%)	5/34 (14.7%)
No. 6	30	130	105 (81.0%)	2/25 (8.1%)
Mean			80.5 ± 2.8	
Group 1B	(74)	(411)		
No. 7	22	91	74 (81.3%)	12/17 (70.5%)
No. 8	27	170	147 (86.5%)	11/23 (47.8%)
No. 9	25	150	123 (82.0%)	3/7 (37.0%)
Mean			83.3 ± 2.85	
Group 2A	(85)	(432)		
No. 10	27	193	58 (30.0%)	7/135 (5.0%)
No. 11	23	115	31 (26.9%)	9/84 (18.7%)
No. 12	25	124	37 (29.8%)	8/87 (9.1%)
Mean			28.9% ± 1.7	
Group 2B	(60)	(309)		
No. 13	19	156	77 (49.3%)	13/79 (16.4%)
No. 14	26	101	50 (49.5%)	5/51 (9.8%)
No. 15	15	52	24 (46.2%)	3/28 (10.7%)
Mean			48.3% ± 1.8	

the undetectable detrusor activity that was estimated in the study of Li et al. (1992)[14]. Furthermore, the mean tissue acetylcholine content was significantly decreased in the decentralized dogs[14]. However, the significant widening of the neuro-effector gap junction which was present in the decentralized animals added another factor that could compromise the proper diffusion of chemical transmitters and its capturing to the corresponding muscle receptors.

Electrical neuro-stimulation enabling the bladder to contract has recently been introduced for the management of neurogenic bladder[4]. In patients with urge incontinence, sacral root neurostimulation has been noted to improve the functional bladder capacity and lead to a marked reduction in leakage episodes[21].

Among the early stimulated animals of the present work (Group 2A), ultrastructural

Table 2: Cholinergic, Adrenergic Axons and Neuroeffector Gap Junctions in the Different Groups

Group	Total Axons	Cholinergic Axons P = 0.0001	Adrenergic Axons	Mean Neuroeffector Gap (nm)
Normal Group	(335)			
No. 1	134	47 (35.1%)	2 (1.4%)	177.6
No. 2	104	36 (34.6%)	1 (0.9%)	167.0
No. 3	97	33 (34.0%)	2 (2.0%)	157.0
Mean		34.5% ± 0.5		167.4 ± 27.3
Group 1A	(532)			
No. 4	235	9 (3.8%)	12 (5.1%)	277.0
No. 5	167	12 (7.2%)	1 (0.5%)	221.0
No. 6	130	7 (5.3%)	0	256.0
Mean		5.4% ± 1.7		251.3 ± 28.2
Group 1B	(411)			
No. 7	91	6 (6.6%)	5 (5.5%)	212.6
No. 8	170	12 (7.1%)	5 (3.5%)	205.0
No. 9	150	5 (3.3%)	4 (2.7%)	236.0
Mean		5.6% ± 2.1		215.8 ± 19.4
Group 2A	(432)			
No. 10	193	31 (16.1%)	7 (3.6%)	137.7
No. 11	115	14 (15.2%)	18 (15.6%)	115.2
No. 12	124	17 (16.7%)	12 (9.7%)	98.75
Mean		16.0%±0.75		117.2 ± 22.6
Group 2B	(309)			
No. 13	156	10 (9.9%)	4 (2.6%)	236.0
No. 14	101	12 (86.5%)	13 (12.9%)	203.0
No. 15	52	0	3 (5.8%)	234.0
Mean		10.85 ± 1.7		224.3 ± 18.5

evaluation demonstrated a significant reduction of degenerated axons in comparison to the non-stimulated group (1A). These observations were in accordance with the promising early spontaneous detrusor contraction after one week which was accompanied by complete voiding in the experiment of Li et al. (1992)[14]. Probably, early stimulation protects the axons against degeneration. The excitatory impulses of neuronal stimulation maintain a neuronal membrane depolarization that enhances the opening of ion channels (specifically, calcium ion channels). Upon reaching the synapse, the action potential triggers a sequence of molecular events culminating in the release of neurotransmitters[18]. The transmitter molecules diffuse in all directions exciting whichever smooth muscle cells reached[22].

In our study, the detrusor exhibited degeneration of most cholinergic axons, however, early electric stimulation protected about 47% of axons containing cholinergic vesicles from degeneration. This is in accordance with the

significantly increased acetylecholine tissue content following stimulation reported by Li et al. (1992)[14]. Probably, early stimulation has an input in maintaining the synthesis potential and storage in spared axons, which in turn are transported via intact neurofilaments to the interior of the vesicles to be stored until release[23]. Denervation supersensitivity may develop after sympathetic or parasympathetic nerve destruction[23]. The resultant supersensitivity could be manifested by: (1) the maximum response with no change in the usual concentration of transmitters; (2) a decrease in the transmitters without change in the maximum response and (3) by both an increase in the maximum response with decrease in the transmitters [22].

In the neurostimulated group of our study, there was a significant increase in the frequency of cholinergic axons relative to the non-stimulated group, although it remained to be significantly reduced compared to the normal control. The supersensitivity phenomenon associating denervation and improved frequency of cholinergic axons could explain the effective detrusor contraction and complete voiding in the stimulated animals reported in the study of Li et al. (1992)[14]. In addition, up-regulation by increasing smooth muscle receptors could participate in the development of the supersensitivity phenomenon[23].

In the present work, late neurostimulation following intermittent catheterization (Group 1B) exhibited axonal degeneration with an insignificant difference compared to the non-stimulated group 1A and early stimulation followed by intermittent catheterization was accompanied by axonal degeneration with an insignificant difference compared to the early stimulated group 2A. It seems that proper timing of neurostimulation is considered a critical factor to replace the normal excitatory impulses signaled from the sacral root that was abolished by decentralization. In addition, neurostimulation could provide specific neurite growth factors, together with the intact axonal basal lamina containing laminin and fibronectin which have been shown to promote neurite growth and regeneration[12]. Electric stimulation of the pelvic or pudendal nerves has been noticed to be associated with an increased expression of C-fos protein growth factor[24].

In the present work, active Schwann cells were frequently seen in the different experimental groups to establish their normal function. During neuronal regeneration, Schwann cell synthesis experienced some promoting growth factors and they encircled the degenerated axons to act as a guide to reach the nearest target smooth muscle cell, perform a functional connection and narrow the neuro-effector gap junction[18].

Neuronal regeneration has been evidenced by the appearance of sprouts[18]. In the present work, the detrusor of the animals in the intermittent catheterization group (non-stimulated group) demonstrated frequent immature sprouts, which is in accordance with the presence of sprouts following detrusor decentralization[15]. Among the intermittent catheterization group, the sprouts were underdeveloped to perform an adequate neuromuscular function and detrusor contraction during the spinal shock phase. Meanwhile, the difference in frequency of sprouts in different animals could be attributed to the individual variable regenerative potential. On the other hand, the decreased number of sprouts in the early stimulated detrusor (Groups 2A & 2B) could be explained, primarily, by the decreased number of degenerated axons. Secondarily, neurostimulation could contribute to the maturation and complete regeneration of sprouts to imitate the normal axons, and to be unsheathed with other axons by Schwann cells.

Our results proved that the ultrastructural changes correlated well with the pathophysiologic alterations related to the decentralization and neurostimulation of the detrusor muscle. Early neurostimulation could avoid extensive neuronal degeneration following decentralization and improve the regenerative potential of degenerated nerves through enhancing the development of sprouts and activation of Schwann cells. Using sacral neurostimulation as a treatment modality for serious refractory voiding dysfunction leads to a significant reduction in frequency and an increased functional bladder capacity as well as a decrease in leakage episodes[21,25,26]. In addition, early sacral root neurostimulation shortens the spinal shock phase, with an early return of detrusor activity and avoidance of most of the complications developing during intermittent catheterization.

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