THE DISTRIBUTION OF SP-LIKE AND SOT-LIKE IMMUNOREACTIVE NERVE FIBERS IN THE RAT MOLAR PULP

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DEDICATION

This thesis is dedicated to my parents Eleni and Anastasios and my brothers Sofronios and Giorgos for their love, support and guidance throughout my life. I especially dedicate my thesis to my mother, who not only encouraged and financially supported my efforts but has also been the person I most deeply admired in my life for her bravery, courage and dedication.

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INTRODUCTION

Pulpal inflammation is a subject of special interest to the endodontist and pain is the most characteristic clinical expression of this complex process. What underlies a pulpitis state histologically, is a complicated dynamic mechanism still unknown in its details. Much progress has been made in this area during the past two decades, especially after the isolation of various neuropeptides in the dental pulp.

Substance P (SP) is one of the first neuropeptides identified in the dental pulp. It has a sensory nature, originating from the trigeminal ganglion and it serves an active vasodilatory function, critical for the phenomenon of neurogenic inflammation. Somatostatin (SOT) or growth hormone release-inhibiting factor (GHRIF) is a peptide with diverse biological activities in various tissues. Its presence in the dental pulp has been only recently reported in the dental literature. An indirect role in pulpal hemodynamics by inhibition of the release of SP, has been suggested.

The purpose of the present study was to immunohistochemically identify the presence of SP and SOT in the dental pulp of rat molars. We further investigated the effect of a direct pulp capping type of injury on SP- and SOT-containing, pulpal nerve fibers.

REVIEW OF THE LITERATURE

Neuroanatomy of Teeth

The classification of nerve fibers is based on their function, diameter, direction of transmission, myelinization and conduction velocity.[1]

The dental pulp organ is supplied by both the somatosensory and autonomic nervous systems.[2,3,4] The somatosensory nerve fibers originate from the trigeminal ganglion[4,5,6] and possibly the mesencephalic nucleus[7] and the cervical dorsal root ganglia[6,8] and are distributed in the upper and lower teeth by the maxillary and mandibular nerves, respectively[4]. In the teeth, they are histologically identified as myelinated (Aβ and Aδ fibers) and unmyelinated (C-fibers).[9] Their conduction velocity (cv) ranges as follows: Aδ fibers > 2 m/s and < 30 m/s, C fibers$\leq$ 2 m/s.[10,11] The myelinated fibers are generally associated with pain while the unmyelinated axons represent both pain sensory and autonomic fibers.[9]

The majority of myelinated nerve fibers found in the pulp, are Aδ fibers. They are myelinated, fast-conducting (mean cv 13.4 m/s), low-threshold (mean threshold 8.4-13.4 mA with 20 ms pulse), large-diameter fibers.[9,12] Larger diameter, fast-conducting Aβ fibers have also been found, but their role is still unresolved.[12] It is hypothesized that they respond to noxious mechanical stimuli on intact teeth and participate in masticatory regulation and the jaw opening reflex after electrical stimulation and load-induced deformation of the tooth crown.[11,13,24] C-fibers are unmyelinated, slow-conducting (mean cv 1 m/s), high-threshold (mean threshold 37.4-40.4 mA with 10 ms pulse), small-diameter fibers.[9,12]

Functionally, the Aδ afferents respond to mechanical (drilling, probing and air drying), mild thermal and osmotic stimulation of dentin[11,12] as well as application of serotonin close to or into the pulp.[11] The C-fibers are polymodal and react to intense heat (~ 440 ^{0}C) and application of bradykinin and histamine.[1,11,12] Likewise, the character of pain mediated by the Aδ fibers is sharp, immediate and lingering as clinically seen in the initial stages of pulpal inflammation while C-fibers activate the slow, dull, burning pain of the last phase of pulpitis.[1,12] The Aδ fibers are most probably activated by the hydrodynamic mechanism.[11] Pulpal blood flow changes that result to ischemia and hypoxia affect primarily the Aδ fibers.[1,10-12]

Intradentally, the morphology of innervation has been studied in the man, monkey, dog, cat and rat.[11] Regardless of species, the nerves accompanied by vessels enter the pulp through the apical and accessory foramina in neurovascular bundles.[9,11,14-16] Less than 10% of them terminate in the root.[17] During their ascent coronally, the myelinated axons lose their myelin sheath and diverge until they reach the cell-rich zone where they form a network called the subodontoblastic nerve plexus (plexus of Rashkow).[14-16] The plexus presents differences according to age[15,18] and tooth region.[15] According to the pattern of distribution, Gunji[15] and later Maeda and others in 1986[16] classified the nerve fibers into four types: 1) marginal pulpal fibers that end in as far as the odontoblastic layer, 2) simple predentinal fibers that enter the predentin following a straight, spiral, transverse or loop course, 3) complex predentinal fibers that arborize intensely and may cover a surface area of up to 100,000 μ^2 in the predentin and 4) dentinal fibers that penetrate into dentin within 100 μ from the odontoblast-predentin border.

The depth of nerve penetration into the dentine has been investigated extensively by means of light microscopy (LM), electron microscopy (EM) and immunohistochemistry (IHC). Innervation of the peripheral root dentin has been reported[19,20] but most studies seem to agree that only the inner 100-200 μm of dentine is innervated.[10,11,16,17,21-23,26] Similar evidence exist regarding the extent of odontoblastic process penetration into the dentinal tubules,[21,27,28] although recent studies postulate its extent to the dentinoenamel (DE)[29,30] and dentinocemental (DC) junctions.[20]

The most densely innervated areas in the tooth correspond to the pulp horns or tips[17-19,22,25,27] paralleling the concentration of the dentinal tubules.[9] The range of odontoblastic processes or tubules accompanied by nerve fibers, in the predentin and dentin of this area has been reported to be 10%, 27%, 40% and 50%.[17,18,22,25,27] Reparative dentin, interradicular dentin and pulp stones on the other hand, show lack of innervation.[22,25,31]

The relationship of the odontoblastic process and unmyelinated intradentinal nerve fibers has been investigated by many EM studies. Thus, nerve axons are encased in a surface concavity by the process, following a straight or helical course around it[32-34] and Gunji[15] described a "swelling" pattern of association (large cap-shaped and small elliptical swellings) between them. Complex infoldings of "corkscrew" fibers into the process suggest a functional association of the two.[32,33,35] Cellular appositions of nerve terminals have been identified, with each other or with Schwann cells in the subodontoblastic layer and the cell-free zone, with fibroblasts in the cell-free and odontoblast layers and with odontoblasts in the odontoblastic layer, predentin and dentin.[36] Axo-axonic appositions possibly related to the axon reflex mechanism,[37] were seen in the apical area,[38] the plexus of Rashkow,[36,39] the cell-free zone and the predentin and dentin.[36]

The intercellular space varies between same or different cells, ranging from 5-35 nm.[36,40] Cellular attachments of the type of gap junctions between nerve endings and odontoblast or odontoblast processes,[34] as well as tight and intermediate type junctions between axons and odontoblast processes[32] have been postulated. Byers[41] reported 3 types of junctions between intrapulpal cellular elements and/or nerve terminals: a) Gap junctions between fibroblasts and between odontoblasts or odontoblast processes b) Wide appositions similar to desmosomes with an intracellular space of 20-35 nm, seen between neural axons and odontoblasts, and nerves and fibroblasts and c) Close appositions with a smaller intercellular space of 100-150 nm, between terminal axons. Current studies however deny the existence of axon-odontoblast gap junctions between the mesodermally derived odontoblasts and the ectodermally derived sensory terminals.[15,36,41] Instead they describe a parallel apposition of plasma membranes separated by a 20-40 nm intercellular cleft.[36]

Sympathetic and Parasympathetic Innervation of Teeth

The autonomic system is divided into sympathetic and parasympathetic. Furthermore, the preganglionic and postganglionic autonomic nerve fibers are characterized as adrenergic or cholinergic according to the type of chemical mediator, Norepinephrine (NE) or Acetylcholine (ACh) respectively, that facilitates their ganglionic and neuroeffector transmission activity.[42] The preganglionic sympathetic and parasympathetic, as well the postganglionic parasympathetic and some of the postganglionic sympathetic neurons are generally cholinergic in nature.[43] Recent findings of the neuropeptides NPY and VIP in the pulp,

suggest their dual coexistence with NA and ACh in sympathetic and parasympathetic nerve terminals, respectively.[3,44]

The sympathetic fibers of the teeth originate from the superior cervical ganglion (SCG) and enter the pulp through two neuronal pathways.[4,45] The major pathway consists of solely sympathetic nerves that after leaving the SCG, travel around the common and external carotid arteries, the maxillary artery and finally the superior and inferior alveolar arteries.[4,45] The other pathway is characterized by coexistence of sympathetic and sensory fibers. It initiates from the SCG and enters the radicular pulp after joining the trigeminal ganlion.[4,45] Sympathetic nerves are thought to comprise only about 10% of the total innervation in mature teeth.[9,46,47] It is noteworthy that trigeminectomy and cervical sympathectomy have been shown to have an excitory and an inhibiting effect respectively, on the odontoblastic differentiation of growing teeth.[48]

The parasympathetic innervation of teeth is considered controversial.[3,14] Indirect conclusions have been reached after positive localization of VIP-like immunoreactivity (VIP-LI) in the dental pulp and reduction of the pulpal VIP content after electrical stimulation of the lingual nerve and extirpation of the sphenopalatine ganglion. The assumption is based on the colocalization of ACh and VIP in parasympathetic nerves, in the salivary glands and nasal mucosa.[3]

The presence and differentiation of nerve terminals intrapulpally, has been investigated by numerous researchers with controversial results. Current sophisticated ultrastructural, histochemical, autoradiographical as well as methods of chemical and mechanical sympathectomy report the presence of four types of nerve endings in the pulp: sensory, adrenergic,

cholinergic and non-adrenergic/non-cholinergic (purinergic or peptidergic).[43]

Presence of adrenergic nerve endings has been reported at the apical area,[49] the subodontoblastic capillary plexus,[50] the odontoblast/predentin border and adjacent to the odontoblast processes.[3,45] Suggestions of their functional role include maintenance of pulpal homeostasis[42] by blood flow regulation,[43,45,50] pain transmission,[45] odontoblast response regulation and recruitement of progenitor cells after injury,[43] dentinogenesis regulation,[45,49] collagen formation[45] and participation in the axon reflex mechanism.[14,50,51]

Cholinergic nerve endings have been identified by some, adjacent to the adrenergic terminals in the predentin close to the odontoblast processes.[43,45] Additionally, choline acetyltransferase and cholinesterase have been identified in the odontogenic layer,[43] the odontoblast nucleus,[45] the subodontoblastic nerve plexus,[43,45] the central pulp and the dentinal tubules within calcified dentin.[45] Ten Cate and Shelton however, reported lack of cholinesterase activity or cholinergic nerves in the pulp immediately below the odontoblasts.[45] It has been hypothesized that the cholinergic axons assume sympathetic, parasympathetic or sensory function in the dental pulp.[22]

Quantification of Nerve Fibers

The majority of studies that attempted quantification and dimensional measurements of the intradental neuronal axons, refer to the apical crossectional area, a few mm past the apical foramen.[10] Most of those studies were comparable regarding the sensory innervation of

different mammalian species, however the interpretation of the results should be carried carefully considering parameters such as the inability of the LM to visualize the unmyelinated fibers,[52] the method of fixation used,[53] the difference between different species, permanent and deciduous teeth[54,55] of various ages,[10,56] the level of sections[10] as well as interanimal[56] and intaanimal deviations.[10]

In summarizing those results, Graf and Bjorlin[57] found 151-1296 myelinated and 40-650 unmyelinated nerve fibers in human permanent teeth. Johnsen and Johns[55] estimated mean values of 359 myelinated and 1591 unmyelinated fibers in human permanent incisors, 361 myelinated and 2240 unmyelinated axons in human permanent canines, 247 myelinated and 2083 unmyelinated fibers in human deciduous incisors, and 439 myelinated and 2521 unmyelinated axons in human deciduous canines. Reader and Foreman[46] found 72% unmyelinated and 28% myelinated nerves at the apices of human premolars. According to Hirvonen,[38] Johnsen et al. 1983 found a mean of 500 myelinated and 1830 unmyelinated fibers in 15 years old and older, human premolars.

Johnsen and Karlsson[54] that looked at feline incisors, found means of 22 and 126 myelinated and 149 and 432 unmyelinated axons in deciduous and permanent pulps, accordingly. When Fried and Hildebrand[22] counted axons in feline permanent incisors 3 months to 9 years old, they found myelinated and unmyelinated fibers ranging from 2-60 and 8-385 in number, respectively. The same investigators[53] found a corresponding range of 10-20 myelinated and 80-90 unmyelinated axons in 2 months old feline deciduous incisors. In feline permanent canines, Jones and Anderson (1982)[22] counted 153 myelinated and 192 unmyelinated fibers.

Finally, in feline permanent canines Holland and Robinson[56] reported a 56-79.6 percentage of nonmyelinated axons.

Hirvonen[38] that looked at the apical pulps of dog permanent canines, found a range of 62.2-77.9 % unmyelinated fibers. Bueltman, Karlsson and Edie[52] quantified the axons in marmoset permanent incisors and canines and found a total of 1099 unmyelinated and 889 myelinated fibers in the core, and 984 unmyelinated and 164 myelinated fibers in the periphery of apical cross-sections.

Very few investigations have been conducted regarding the quantification of nerve fibers in the peripheral pulp. Beasley and Holland[58] attempted that in a single specimen composed of electron microscopical montages of several pulpal cross-sections from the midcrown area, 4-5 mm from the tooth tip and they found 81% unmyelinated and 19% myelinated axons (ratio 4:1) on a total sample of 3,407 fibers. Holland and others[59] that studied the innervation of the feline pulp-predentinal border and predentin tubules after denervation and reinnervation, found in the operated side, 2 days after, lack of axons in the border area while 0-0.6% of the predentinal tubules were innervated. For the same interval, the control sides showed 0.04-0.64 axons (n/μm of border) and 12.7-91.1% innervated tubules. The corresponding numbers after 12/15 weeks were, in the operated sides, a mean of 0.20-0.30 axons (n/μm of border) and 4.7-16.5 % innervated tubules and in the control side 0.17-0.29 axons (n/μm of border) and 12.9-54.5% innervated tubules.

Finally, Bishop[60] examined various positions of rat incisors and reported a mean number of less than 1 myelinated axons and 275 unmyelinated axons, as derived from 7 sections.

Nerve Axons at the Root Apex

Reference	Method	Tooth	Number of axons M	Number of axons NM	Diameter M	Diameter MN
Graf &	LM	Human permanent				
Bjorlin		Incisors	257-991	200-300	1-10[a]	-
(1951)[57]		Cuspids	390-1296	40-250	1-9	
		Bicuspids	1135	400	1-13	-
		Bicuspids	151-457	95-650	1-7	-
Johnsen &	EM/CS	Human permanent				
Johns		Incisors	359 ± 46	1591 ± 728	.5-6.7[a]	-
(1978)[55]		Canines	361 ± 82	2240 ± 966	.5-6.7	-
		Human deciduous				
		Incisors	247 ± 19	2083 ± 1031	.5-6.7	-
		Canines	439 ± 71	2521 ± 722	.5-6.7	-
Reader &	TEM/CS	Human permanent				
Foreman		Premolars	195-358	348-913	3.36[b]	.79
(1981)[46]			m: 256	m: 670		
Johnsen et al.	EM/CS	Human permanent				
(1983)[38]		Premolars				
		15 years or older	m: 500	m: 1830		

Reference	Method	Tooth	Number of axons M	Number of axons NM	Diameter M	Diameter MN
Johnsen & Karlsson (1974)[54]	EM/CS	Cat permanent Incisors	126 ± 31	432 ± 63	-	-
		Cat deciduous Incisors	22 ± 16	149 ± 110	-	-
Fried & Hildebrand (1981)[22]	EM/CS	Cat permanent Incisors	2-60	8-385	1-9[a]	.1-1.6
		Cat deciduous Incisors 2 mo after birth	10-20	80-90	1-5	.1-1.2
Jones & Anderson (1982)[22]		Cat permanent Canines	153	192	.75-15[a]	.2-3
Holland & Robinson (1983)[56]	EM/CS	Cat permanent Canines	193-529	375-1376	-	-
Hirvonen (1987)[38]	EM/CS	Dog permanent Canines	437-671	772-2363	-	-

Reference	Method	Tooth	Number of axons M	Number of axons NM	Diameter M	Diameter MN
Bueltman et al. (1972)[52]	EM/CS	Marmoset permanent				
		Incisor core	53-230	50-228	.5-6.5[a]	.1-1.5
		Incisor periphery	9-77	45-121	.5-6.5	.1-1.5
		Canine core	55-137	105-155	.5-6.5	.1-1.5
		Canine periphery	38-41	382-391	.5-6.5	.1-1.5
Bishop (1981)[60]	LM & EM CS & SS	Rat Mand/lar Incisor apical, central & incisal sections	0-7	0-328	-	.1-2.5

[a] Diameter derives from data on axonal circumference.[22]

[b] Average equivalent area diameter.[46]

m: mean value

CS: Cross-sectional specimens

SS: Sagittal sections

LM: Light microscopy

EM: Electron microscopy

TEM: Transmission electron microscopy

Nerve Terminals Intracoronally

Reference	Method	Tooth	Number of axons M	Number of axons NM	Diameter M	Diameter MN
Koling (1985)[61]	EM	Human permanent Molars & Premolars SOP		only NM		.1-1.2
Beasley & Holland (1978)[58]	EM/CS	Cat permanent Canine Midcrown (4-5 mm from tooth tip)				
		Core	63	209	3.5	.35
		Middle	92-126	206-687	2.5	.35
		Periphery	22-57	93-296	2.5	.35
Holland et al. (1991)[59]	EM/CS	Cat permanent[c]				
		Pulp-Predentin Border				
		2 ds after/Oper side		0 n/nm		
		2 ds after/Control side		.04-.64 n/nm		
		12/15 wks after/Oper side		.20-.30 n/nm		
		12/15 wks after//Control side		.17-.29 n/nm		
		Predentin tubules innervated				
		2 ds after/Oper side		0-3		
		2 ds after/Control side		19-236 (continues)		

Reference	Method	Tooth	Number of axons M	NM	Diameter M	MN
(continues)		12/15 wks after/Oper side		10-35		
		12/15 wks after//Control side		35-250		
Bishop (1981)[60]	LM & EM CS & SS	Rat Mand/lar Inc/or apical, central & incisal sections	0-7	0-328	-	.1-2.5

[c] Control and operated sides combined. 2 days after and 12/15 weeks after denervation and reinnervation. Axonal densities as n/nm of border.

SOP: Subodontoblastic plexus

CS: Cross-sectional specimens

SS: Sagittal sections

LM: Light microscopy

EM: Electron microscopy

Pulpal Vasculature

The architecture of the pulpal vasculature has been studied by various methods on teeth of humans and other mammalian animals. Lepkowski was the first investigator to report on pulpal blood supply, in 1901[62]. Earlier studies by Hopewell-Smith (1918), Boling (1942), Kramer (1960), Saunders (1957), Kindlova and Matena (1959), Han and Avery (1961) used methods such as the "Prussian blue" injection and the Indian ink

suction techniques, microradiography with projection X-ray microscopy, and the latex cast technique to provide information for the structural arrangement of the pulpal vessels.[63] More recent investigations, mainly during the last decade by Kishi and Takahashi used the low-viscosity resin cast technique to give a more detailed three-dimensional description of the vascular architecture, under normal and inflammatory conditions with the aid of Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM).[62-64]

In terms of volume, recent investigations of the cat pulp have shown that overall, 14.4% of the pulpal area consists of blood vessels with an average of 42.9% in the central pulp and 5-10% close to the odontoblastic layer.[65] The average capillary density was found to be 1402/mm^2 which is significantly high as compared to other tissues.[65] Morphological changes of the vascular network have been investigated in relation to aging and inflammatory conditions. The former were divided into four stages: the completion stage of crown formation, the root formation stage, the root completion stage and finally the maturation stage. The latter changes were examined during two stages of the inflammatory process: acute inflammation and chronic inflammation.[62,66,67]

Morphological Changes during Aging

During the end of the crown formation stage, the dental papilla is supplied by branches of the inferior alveolar and superior alveolar arteries. The division continues at the base of the tooth bud and a group of branches is distributed in the coronal area while another, over the area close to the reduced enamel epithelium. Numerous arterioles and venules course the

central pulp longitudinally with characteristic curves and bends along their path. The terminal branches of the arterioles form the "terminal capillary network" (TCN) at the periphery.[62]

During the root formation stage, numerous arterioles and venules closely related to periodontal vessels, run parallel at the area of the root opening. The superficial capillary network has a thickness of 400-500 μm in the roof of the pulp chamber, and it diminishes gradually in thickness as it approaches the chamber floor.[62]

At the root completion stage, three distinct layers of vessels are seen coronally, underneath the dentin: the TCN composed of flat true capillaries, located in the odontoblastic layer, the capillary network (CN), approximately 500 μm in length that is composed of parallel running precapillaries and postcapillaries, and the venular network (VN) with a lattice-like appearance.[62,64,66] During the continuously progressing maturation stage, the pulpal cavity diminishes in size due to the deposition of secondary dentin, and the pulpal vasculature conforms to the changes by remodelling itself.[64,67] In each root of the mandibular first molar, 7-8 main arterioles enter and 5-6 venules exit the tooth, via the main and smaller apical foramina of 20-150 μm in diameter.[62,63,66] Generally venules exit the large foramina and arterioles enter through the smaller ones.[63] Only the TCN is observed at this stage and its capillaries drain directly into the main venule. The appearance of the TCN in the areas of pulp horns and between them, resembles hairpin loops and flat network, respectively.[62,66] The apical portion area presents a flat cross-fence network formation.[63] The physiological significance of the continuous reconstruction of the pulpal vessels with age is related to a lower resistance of this protective system and compromise of the pulpal vitality in elderly persons.[64,67]

Earlier investigations on human and mouse teeth by Harris and Griffin,[68] Dahl and Mjor[69] and Corpton and others[70] showed a dental vascular system comprising fenestrated capillaries with and without pericytes and non-fenestrated capillaries,[68-71] small arteries,[68] terminal arterioles[68,69,72] and venules.[69] More recently, Iijima and others described five types of vessels in the human pulp as observed with SEM: muscular arterioles (20-45 μm in diameter), terminal arterioles (10-30 μm in diameter), precapillary arterioles (4-12 μm in diameter), capillaries (3-6 μm in diameter) and postcapillary venules (8-50 μm in diameter).[73] Zhang and his associates added a sixth segment, the muscular venule (10-7 μm in diameter).[74]

Some distinct structural features of the pulpal vasculature associated with regulatory role in the blood flow, are also observed. They are the veno-venous (VVA) anastomoses seen between two pulp horns,[62,63] the arteriovenous (AVA) anastomoses[62,63,66,68,69,75] and the U-turn loops which are small arterioles that give off 2-3 fine arteriolar branches.[62,63,66] The latter are seen mainly in the root canals and they are connected with their branches with sphincters.[62,63]

Morphological Changes during Inflammation

According to Takahashi,[67] during the first stages (within 4 hrs after application of noxious stimulus) of acute inflammation, vascular permeability increases mainly in the venous system and leakage is seen in the postcapillaries of the CN and the venules of the VN. Under more severe conditions the resin casts show a Chinese brush-like pattern of the pulp horn and presence of resin in empty dentinal tubules deriving from the

leaking CN and VN. During the second stage of the acute reaction, migrating leukocytes with extending pseudopods are seen adhered on the vascular walls. They later complete their migration through the intercelullar epithelial junctions.[67]

Chronic inflammatory changes related to chronic ulcerative pulpitis include the presence of abscesses that lack vascular supply, surrounded by an almost intact area of normal vasculature. Chronic pulp polyps present themselves as mushroom-shaped areas covered laterally by dilated tortuous vessels. In areas of perforations of the chamber floor, the periodontal vessels partially invade and substitute the pulpal vessels in the area of perforation.[67]

On a more microscopic level, the morphological changes of blood vessels under conditions of caries pulpitis show a range from minor endothelial changes to total cell necrosis, lysis, alterations of the basement membrane, opening of the tight junctions and erythrocyte extravasation in the extravascular tissue leading to increased vascular permeability and eventually edema.[76,77] Most of these degenerative changes have been attributed to the action of collagenase and histamine.[77]

Pulpal Hemodynamics

Regarding the pulpal blood flow (PBF) responses to applied external stimuli, they have been investigated experimentally by techniques using radiosotope-labeled microspheres,[78] radioisotope tracers,[12] laser Doppler flowmetry[79] and plethysmography, and three types of responses have been described.[12]

The type I response, characterized by significant decrease of PBF is due to activation of α-receptors in pulpal vessels and activation of sympathetic adrenergic fibers.[12,78] Direct electrical stimulation of the cervical sympathetic nerve, indirect stimulation of the sympathetic nervous system and intraarterial infusion of 5-HT, NE and $PGF_{2\alpha}$ produce type I response.[12]

The type II response is biphasic in nature, initiated by an increase that is followed by an immediate decrease in PBF. The vasodilators SP, PGE_2, bradykinin, papavarine, VIP and isoproterenol are known to induce this effect. The type II response of gradual decrease of PBF is due to the increase in capillary permeability and subsequent increase in the tissue pressure and is seen after intraarterial infusion of histamine.[12,81]

The mechanism underlying the type III response has been ascribed to an initial vasodilation that in the low compliance intrapulpal environment leads to a passive compression of the venules due to active arteriolar dilation and increase of intrapulpal pressure.[12,78,80,81]

Neuropeptides

The firmly held, for many years belief that synaptic transmission is mediated only by the so-called today "classical neurotransmitters" NE and ACh was gradually deserted during the past three decades, after the isolation of various amino acids and peptides that have been shown to possess neurotransmitter or neuromodulator activity. Furthermore, the initial hypothesis of Dale that one neurotransmitter corresponds to one neuron, has given its place to the belief that multiple compounds are released by a single nerve ending. The significance of this phenomenon is

related to the variable number and type of information transfered by a single fiber.[1]

According to the definition by Takagaki and Nagatsu[82] the neurotransmitter is a substance that is synthesized biologically in the pre-synaptic area of nerve terminals, and transmits information between neurons. It is released upon the arrival of a nerve impulse, binds to specific receptors and carries excitatory or inhibitory potential in the post-synaptic area.[82] Neuromodulators are neuropeptide substances that alter or assist the synaptic transmission.[82]

The proposed criteria for the characterization of a substance as a neurotransmitter are generally the following[83]: 1) the neurotransmitter and its production mechanism are present presynaptically 2) the neurotransmitter should be released after physiological stimulation of the presynaptic neuron 3) both the exogenous neurotransmitter candidates and the endogenously released neurotransmitter should have the same effect postsynaptically and should be antagonized by the same pharmacological agents and 4) an inactivation or metabolic mechanism should exist for the neurotransmitter. Therefore, the substantiation of a neuropeptide as a neurotransmitter requires physiological, histochemical and pharmacological evidence.

The neuropeptides and neuromodulators identified until now, by various methods in the dental pulp include: SP,[84,85] CGRP,[85,86] VIP,[85,87] NKA,[44] NKB,[12] NPK,[88] NPY,[85,89] Enkephalin (Enk),[82] Somatostatin (SOT),[90] Peptide histidine isoleucine amide (PHI),[85] Gastrin-Releasing Peptide (GRP)[1] and Gamma-Amino Butyric Acid (GABA).[1] SP, NKA, NKB and NPK belong to the same family of tachykinins that share a similar amino-acid sequence and biological activities.[88]

According to a review work by Wakisaka[4] SP, NKA and CGRP are sensory in nature, originating from the trigeminal ganglion and serve a vasodilatory function in the dental pulp.[4,86,91] NPY is a sympathetic neuropeptide, that originates from the superior cervical ganglion.[4] It coexists and coacts with NA to produce vasoconstriction and thus to maintain the pulpal vascular tone.[4,91] VIP fibers in the dental pulp are hypothesized to be parasympathetic in nature, and although the lingual nerve and the sphenopalatine ganglion are suggested as possible sources of those fibers, their exact nature and origin are still unknown.[91] The peptide induces vasodilation with a different mechanism than that of SP, NKA and CGRP.[4]

Generally, the various neuropeptides found in the dental pulp seem to play primarily a role in the regulation of the pulpal blood flow, the neurogenic vasodilation phenomenon and possibly in pain transmission mechanisms.[4,12,82,88]

Other more broad physiological activities attributed to the sensory neuropeptides, include effects on wound healing,[92-97] enhancement of phagocytosis of macrophages and neutrophils,[95,98] stimulation of angiogenesis,[94,95] promotion of connective tissue growth,[94,95] stimulation and inhibition of lymphocytes,[94,95] induction of release of the inflammmatory cytokines IL-1, TNF-a and IL-6 from blood monocytes,[99] growth effects on the development of atherosclerosis and desmoplasia in neoplasias,[94] pathogenesis of allergy,[100,101] trophic effects on dentin formation[95] and facilitation of local immune responses.[94,95,97,100]

SUBSTANCE - P (SP)

Introduction

Substance-P (SP) was discovered over 60 years ago by Euler and Gaddum.[102] They isolated it from intestinal muscle and brain tissue extracts and it was found capable of reducing the arterial blood pressure by peripheral vasodilation and stimulation of the intestinal tone in rabbits, after preliminary treatment with atropine. The letter P refers to the use of powder extracts of equine brain and gut.[103]

Neurochemistry and Metabolism

SP is an undecapeptide, chemically identified by Chang and Leeman only recently.[104,105] It is composed of Lys1, Arg1, Glx2, Pro2, Gly1, Leu1, Met1 and Phe2 with Arginine as the NH_2-terminal residue and its molecule has the following structure[104-106]:

Arg-Pro-Lys-Pro-Cln-Gln-Phe-Phe-Gly-Leu-Met-NH_2

Most of the known biological activities of the SP molecule are associated with the C-terminal heptapeptide portion. The N-terminal tetrapeptide (Arg-Pro-Lys-Pro) fragment stabilizes the molecule against degradation by aminopeptidases and stimulates the phagocytic activity of macrophages and polymorphoneuclear leucocytes (PMNs).[107] Regarding the allergic skin reactions induced by intradermal injection of SP, it has been shown that the N-terminal aminoacid sequence of the molecule is related to the vasodilation and flare reactions while the C-terminal residue sequence posesses a wheal-producing property.[101]

It has a molecular weight greater than 1,000 and less than 2,000 as obtained by gel filtration, and 1,340 as calculated by its amino acid composition. Physalaemin and eledoisin, 2 other undecapeptides isolated from the skin of a South American amphibian and the salivary glands of cephalopods respectively, have been found to have similar chemical composition and biologic activities with SP such as smooth muscle stimulation, vasodilation and sialagogic effects. Due to the above similarities it is suggested that all three peptides had a common origin during evolution.[104] The exact mechanism of inactivation of the released peptide is not clear yet, but it has been suggested that a metabolic pathway is more likely than a reuptake mechanism.[103] Lembeck and associates[108] have suggested three possible ways of elimination of SP from the blood stream: 1) enzymatic degradation in blood or tissues 2) binding to, or reuptake into tissues and 3) excretion by urine. A postproline cleaving enzyme found in bovine and rat brain, has been shown to cleave SP to an N-terminal tetrapeptide and a C-terminal heptapeptide. The C-terminal heptapeptide was later shown to be actively taken up into rat brain and rabbit spinal cord slices.[83]

Another suggested pathway is the enzymatic degradation by an enzyme bound to brain membranes, capable of cleaving SP in the area of the its 7 and 8 positions.[83] Benuck and his colleagues[109] have shown that the purified from calf brain lysosomal enzyme, Cathepsin D could cleave the Phe-Phe linkages of both SP and SOT. Earlier, they had suggested the implication of brain neutral proteinases that were capable of cleaving bonds at the 7,8 and 10 positions.[110] Neutral endopeptidase, dipeptidyl peptidase IV and aminopeptidase M have been shown to possess a SP-hydrolysing action in the kidneys.[111]

Central and Peripheral Localization

It is well established today, that SP has a widespread distribution throughout the body. Although a comprehensive review of the peripheral and central localization of SP is beyond the purpose of this work, a summary of its occurrence in various organs and areas will be presented.

Thus SP has been localized in the following areas of various animals and man:

1) All parts and layers of the digestive tract[112,113]

2) All areas of the central and peripheral nervous system such as:

- Somato-sensory system, especially in :

  Dorsal horns (laminae I, II and less in III)[113-122]

  and less in the ventral horns (laminae V-VII and X) of the spinal cord[113,115,121] and Trigeminal nerve nucleus[117]

- Dorsal roots and ventral roots[112]
- Spinal ganglia[116]
- Visual system[117]
- Limbic system[117]
- Hypothalamo-hypophyseal system[112,117]
- Pyramidal system[117]
- Basal ganglia,[112] especially in substantia nigra[117]
- Cerebellar system[117]
- Preganglionic sympathetic fibers[112]

3) The kidneys,[111] ureters[106,112] and urinary bladder[112]

4) The uterus[112]

5) The skin[113,123]

6) The temporomandibular joint (TMJ) (joint capsule, peripheral articular

disc, synovial membrane and periosteum)[98]

7) The periodontal membrane (PDL)[124] and the

8) The tooth pulp[84,85]

Neurophysiology

Currently available experimental data shows that SP is a predominantly excitory neurotransmitter in central nervous system neurons.[83,113,114,116,125-127] It has been found associated with primary afferent neurons that terminate profusely in the laminas I and II of the dorsal horn and correspond to the small diameter, C fibers that are functionally related to nociceptive transmission.[83,115,128] Its synthesis occurs in the cell bodies of the primary afferents and from there it is transported to the peripheral and central terminals of the sensory axon.[83] Iontophoretic application of SP causes depolarization of spinal neurones resulting in selective excitation of mechanically-nociceptive, chemically-nociceptive and thermally-nociceptive neurones in the dorsal horn, as well as non-nociceptive neurones in the dorsal horn and cuneate nucleus.[83]

Capsaicin, a pungent constituent of red peppers, is a neurotoxin that when given systemically causes destruction of C fibers and in higher doses, of Aδ sensory fibers.[83] Capsaicin treatment causes a calcium-dependent release of SP from primary afferents, depletes SP from dorsal horn, dorsal root ganglia and peripheral nerves, produces thermal and chemical analgesia and a SP-mediated depolarization on spinal motoneurons.[83,121,129-134] This suggests that SP plays a role in mediating nociceptive information that is carried by small diameter primary afferents.[83,121,132] In addition, it has been shown that opiate analgesics

such as morphine, produce a naloxone-reversible inhibition of SP release from primary sensory neurons.[130,135,136]

Electrophysiological and neuropharmocological experiments using SP antagonists in various peripheral tissues have provided additional information regarding the functional role of SP in the peripheral nervous system. These include the peripheral vasodilation and plasma extravasation caused by antidromic stimulation of sensory nerves or by SP itself,[83,129,137] the neurogenic edema caused by electrical stimulation of the saphenous nerve[138] and the elevation of nociceptive thresholds after systemic administration of SP antagonists.[83]

SP can be released presynaptically from spinal cord specimens in a calcium-dependent mechanism[83,118] by increased extracellular potassium levels, as well as in vitro and in vivo after electrical stimulation of peripheral nerves with intensities that are capable to sufficiently activate Aδ and C fibers.[83] The decrease in the potassium and sodium conductances, has been suggested as the ionic mechanism underlying the postsynaptic excitory action of SP on spinal cord neurons.[132]

SP AND THE DENTAL PULP

Distribution and Origin

The dental pulp has been shown to be one of the richest tissues in SP concentration, outside the CNS.[127,139] SP fibers enter the dental pulp through the apical foramen[140] and are distributed unevenly in its mass[85,141] mainly accompanying the middle and apical large blood vessels.[4,140,142,143] In the radicular pulp, they are localized around blood

vessels in a network arrangement, of a diameter up to 100 μm around the vessel wall or running along the vessels or without any association with them.[140,143] In the incisal portion, many unrelated to blood vessels, SP fibers are seen heading towards the pulp horn, and close to the odontoblast layer they run parallel to the pulp.[140,143] At the subodontoblastic layer, they branch and penetrate the predentin and dentin in various distribution patterns.[4,140] In the human molar, some fibers accompany the odontoblast processes up to the dentin border, and can be traced at about 60 μm from the odontoblast-predentin border or course transversely at various directions within the predentin.[144] In the rat molar pulp, they are traced in the predentin at about 20 μm from the pulpodentinal border.[142]

Unlike superior cervical ganglion (SSG) sympathectomy, inferior alveolar nerve (IAN) sectioning leads to total disappearance of SP fibers in the dental pulp and this is indicative of their sensory nature and origin from the trigeminal ganglion.[84,142] Double immunostaining reveals a similar distribution of SP, CGRP and NKA fibers in the pulp and it is suggested that all three of them are contained within the same nerve fibers.[4,140]

SP immunoreactivity has been reported for the trigeminal ganglion of the cat,[115] rat[145] and rabbit as well as the SSG.[82] Investigations of the distribution of the above three tachykinins in the trigeminal ganglion have shown that the SP-containing ganglion cells are small and medium-sized with a diameter of 15-50 μm[82,140] while their number is the next larger after that of CGRP-containing ganglion cells,[140] comprising approximately 10-30% of all ganglionic cells.[82] Finally, the three peptides may coexist in the same ganglion cells and it is speculated that those cells are the ones actually supplying the dental pulp with SP, CGRP and NKA.[4,140]

SP positive nerve fibers have been also identified in the SCG. According to some investigators they are supplied from other ganglia while others that detected a SP precursor in the SCG, claim that SP is synthesized there. The latter observation provides an argument against the Dale's "one neuron - one neurotransmitter" hypothesis.[82]

Functional Role of SP in the Dental Pulp

SP is released in the dental pulp from the nerve terminals of unmyelinated C-fiber afferents, after electrical stimulation with a stimulus that exceeds the activation threshold for Aδ fibers as it has been first shown by Olgart and others in 1977, in the cat dental pulp.[91] The substitution of the released peptide via axonal transport is very slow, a minimal amount of it is re-uptaken, while the largest amount is rapidly destroyed in the extraneuronal environment of the pulp, thus leading to an eventual drop of its content in the pulp. According to the above observations, SP has a local role in the pulp.[91]

SP is the first neuropeptide found in the feline dental pulp by Olgart and associates in 1977.[84] They observed SP-positive fibers in the central pulp and in relation to the odontoblasts in the form of single, unmyelinated or fine caliber fibers or small bundles of them running close to myelinated nerves, blood vessels or without any association to any of those structures.[84] Later, SP immunoreactivity was reported in the pulp of dog, mouse embryo, human molar and rat molar.[82]Generally, the two patterns of distribution of SP fibers in relation to blood vessels (positive relation and no relation to them), suggest two types of local action in the dental pulp: the SP fibers in association to blood vessels have a vasodilative action, and the SP fibers that

penetrate in the odontoblastic layer and predentin may have a sensory role.[82]

Vasodilative Action

IAN and mandibular nerve stimulation were first used by Kroeger in 1968 and Tonder and Naess in 1978 to show an increase in the intrapulpal pressure and blood flow in dogs.[91,146,147] Further studies using the feline pulp, slowed that electrical stimulation of the IAN, of sufficient intensity to stimulate the high threshold C-fibers caused a SP release[148] and a vasodilatory response resistant to propranolol, atropine and the combination of mepyramine and cimetidine.[147] Experiments with somatostatin showed that this peptide was able to inhibit the release of SP and to reduce the intrapulpal vasodilation induced by IAN stimulation.[149,150] The administration of a SP antagonist resulted in inhibition of the antidromic vasodilation induced by electrical stimulation of the mandibular nerve.[151] Based on the above findings, it is suggested that the sensory neuropeptide SP is involved in the antidromic vasodilation phenomenon and it is an important mediator of the nerve-induced vascular response in the dental pulp.[91]

Furthermore, local capsaicin pretreatment of the feline IAN resulted in decreased SP content in ipsilateral pulps and a reduced vascular response after nerve stimulation, thus suggesting that the release of neuropeptides from unmyelinated C-fibers plays a regulatory role in the pulpal haemodynamics.[91]

Sensory Role

SP fibers have been identified in the subodontoblastic region,[85] and the predentin and dentinal tubules that are areas lacking blood vessels.[142,144] In addition, the fact that external stimuli of mechanical, chemical and thermal nature applied to the teeth are perceived as pain,[4,82,142,144] led to the hypothesis that SP plays a role in the pain sensation.[4,82,85,142,144] However, their low number suggests only an assistant function in the pain transmission.[82]

It is well established today that the action of SP in central neurones is excitatory.[127,152] Olgart and associates have suggested that the SP fibers of the dental pulp are peripheral afferent branches associated with central neurons in the trigeminal nucleus caudalis and therefore, they serve a nociceptive function by transferring chemical noxious inputs from the pulp to the trigeminal nociceptive system.[84,153,154] The involvement of 5-HT pathways originating from the raphe nucleus and their interaction with SP at the level of superficial layers of subnucleus caudalis, have been recently shown after inhibition of the release of SP as induced by electrical stimulation of the pulp, by 5-HT at the superficial areas of the subnucleus caudalis, in rabbits.[155]

However a direct excitatory effect on the peripheral nerve endings in the skin and the cat dental pulp has not been shown experimentally, and therefore such an action seems unlikely.[127,152] Local application of synthetic SP in pharmacological doses was not able to cause excitation of the Aδ sensory neurones in the feline pulp.[127]

Physiological Role and Neurogenic Inflammation

It is currently believed that the vasodilatory response induced by sensory nerve activation, and the release of SP as well as other neural and chemical mediators represents a defensive reflex reaction in the dental pulp[91] similar to other tissues and organs such as the skin, the mucosa of the respiratory tract and the eye.[156] Noxious stimuli induced by strong orthodontic stimulation of the crown, cavity preparation in the outer half of dentin,[157] mechanical deformation of enamel and dentin, low intensity ultrasonic stimulation, electrical stimulation of the crown and chemical irritation with capsaicin, bradykinin and hypertonic salt solutions applied in a deep cavity, can activate the pulpal C-fibers and initiate a fast vasodilative response.[91,157,158]

This haemodynamic response is different in mature and immature pulps. The observed variability concerns mainly the reactions of the terminal capillary network and those of the larger blood vessels as they have been investigated by experiments using laser Doppler flowmetry in combination with the local ^{125}I clearance technique.[159] The difference between young and adult pulps has been attributed to the almost complete disappearance of the terminal capillary network[67] and the increase in density of the SP-fiber network due to the decrease of the total supporting tissue with aging, in the latter.[127] Therefore, the local vasodilative reactions are considered to be a beneficial reflex mechanism against noxious stimulation and this is of important value in the case of mature pulps that reside in a narrower space and a low-compliance environment.[91]

The effect of excitation of the intrapulpal sensory fibers on pulpal microcirculation, is basically related to stimulation of adequate intensity for

activation of C-fibers. Various studies have shown that antidromic stimulation of peripheral chemosensitive C-fibers causes vasodilation, plasma extravasation and edema formation, initiated by SP release.[129,160] High intensity antidromic stimulation of the IAN after application of a hypertonic solution in dentinal cavities caused a biphasic neural response consisting of an increased nerve response that was followed by a decreased responsiveness.[160] Similarly, electrical stimulation of the IAN after α-adrenergic blockade with phentolamine resulted in increase of the PBF followed by a continuous progressive drop, caused by increased vascular permeability and protein extravasation.[147] The neurogenic vasodilation after antidromic vasodilation is thought to be mediated by release of SP from C-fibers although later, other neuropeptides such as NKA, NKB and CGRP were shown to exert similar effects.[44,161-163] Potent coaction of neuropeptides has been shown experimentally when SP and CGRP were used together in the dental pulp. It was found that the vasodilatory effect of CGRP was 10 times greater when given after SP, than before it.[162] In addition, CGRP and to a lesser degree SOT were able to potentiate the actions of SP, through inhibition of its degradation by of a SP endopeptidase.[164]

Generally the biphasic neural response after repeating IAN stimulation has been related to the initial vascular and neural effects of SP, and to the subsequent release of histamine from mast cells. The indirect action of SP on vascular permeability, via histamine release has also been shown after intraarterial infusion of SP.[165] Histamine acts directly on pulpal blood vessels to increase the vascular permeability and fluid extravasation and therefore the intrapulpal pressure[12,166] and furthermore to block the impulses transmitted by Aδ fibers.[166] The involvement of

histamine in the inflammatory process, has been suggested by various studies.[129,156,160,165] Other chemical mediators implicated in the biological process of pulpal injury include serotonin, bradykinin, kalikrein, PGs[82,160,166] and ATP.[160]

The mechanism of neurogenic inflammation as described by Olgart[166] and Kim[12] is as follows: An initial insult to the pulp causes the release of inflammatory tissue mediators such as PGs and bradykinin, that act on sensory nerve fibers and lead to the release of various kinins and SP.[167] These neurokinins act directly on vessels and induce vasodilation and vascular leakage. At the same time, serotonin and vascular kinins act on mast cells and initiate the release of histamine that further increases the vascular leakage. The combined effect of the various mediators results in an increase of the tissue pressure that unlike other tissues, in the special pulpal environment causes a decrease of PBF. The removal of inflammatory products is therefore impaired, vessel damage occurs and a viscious cycle begins that leads to areas of pulpal inflammation and finally pulpal necrosis.[12]A mechanism suggested for the suppression of the above process is that mediated by a met-enkephalin-like peptide and opioid peptides that suppress the formation of bradykinin and the release of SP respectively, in the pulp.[91,152,166,168] It has been recently suggested that immunocompetent cells are a major source of opioid peptides in inflamed tissues.[169] Inhibition of SP release by somatostatin is another possible mechanism.[150,166,170]

SOMATOSTATIN (SOT) or GROWTH HORMONE RELEASE - INHIBITING FACTOR (GHRIF)

Introduction

Krulich and his associates in 1967 were the first to discover a growth hormone release-inhibiting factor (GHRIF) in fractions of sheep hypothalamus on Sephadex columns, during routine screening for GH-releasing factor (GHRF).[171] They later reported the presence of GHRIF in purified hypothalamic extracts from rat pituitary, in vitro.[172] When the two factors were used together, GHRIF inhibited the stimulatory effects of GHRF on release and synthesis of the growth hormone (GH).[173]

Neurochemistry, Metabolism and Somatostatin Analogues

The active factor was isolated, characterized and reproduced in 1973 by Brazeau and his colleagues.[174] In the same article they proposed the name somatostatin (SOT) for the characterized tetradecapeptide. The determined structure is that of a 14-amino acid cyclic polypeptide with an intrachain disulfite bridge:

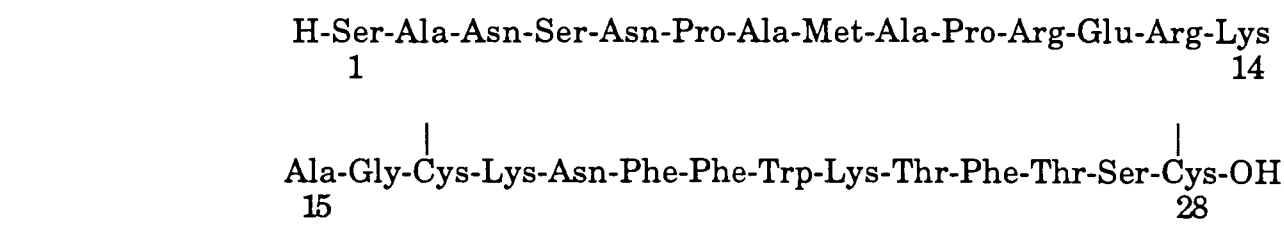
H-Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys-OH

In vivo studies have shown that SOT is a peptide with a very short half-life (< 4min) in mice, rats and man.[175,176] Studies on distribution and excretion, using a SOT analogue showed that the molecule is mainly

concentrated in the pineal and posterior pituitary and after 24 hrs, 70% of it is excreted in the urine. Available studies on the inactivation mechanism of SOT report enzymatic cleavage by brain Cathepsin D at the Phe-Phe and Trp-Lys linkages and pepsin at the Ala-Gly, Phe-Phe, Trp-Lys and Thr-Phe sites.[109]

After the original identification of the tetradecapeptide SOT-14, by Brazeau and his coinvestigators, studies showed that tissue SOT comprises other than SOT-14 (molecular weight 1,600) forms of SOT with molecular weights 25,000, 15,000, 12,000, 10,000, 6,000, 4,000, 3,000 and 2,000 daltons.[177-181] The form with the molecular weight of 25,000 daltons corresponds to the larger molecule reported so far, and it is considered to be a prohormone for SOT-14.[178]

Pradayol and his associates in 1980, were able to isolate an N-terminal extension octacosapeptide form of SOT-14, (prosomatostatin or SOT-28) with the following structure[180]:



It is suggested that SOT-28 is an initial or intermediate precursor of SOT-14[182] and although they both seem to exert a similar spectrum of action on various tissues experimentally,[180,182,183] it has been suggested that SOT-28 possesses distinct biological activities from SOT-14.[183] The potency of the two forms was found to be different in various tissues.[180,183,184]

In addition to SOT-28, Bohlen and others in 1980 have isolated and characterized another somatostatin-like peptide (SOT-25), from ovine

hypothalamus.[185] SOT-25 was considered to be a metabolic product of SOT-28 and precursor of the final form of the tetradecapeptide. The synthetic forms of both were shown to be 10 times more potent in vitro and in vivo than SOT-14.[185]

Pituitary Effects and Inhibitory Activities

Laboratory studies substantiated the inhibitory effect of SOT on GH secretion induced by insulin-hypoglycemia, sleep, exercise, meals, hyperthermia, liver and renal failure, sodium pentobarbital, chlorpromazine, dibutyryl cAMP, arginine, L-Dopa, morphine, catecholamine infusion in the third ventricle and electrical stimulation of the hypothalamus and amygdala.[176,186] SOT partially inhibits the release of thyrotropin hormone (TSH) induced by thyrotropin-release factor (TRF). It has no effect on the secretion of prolactin, luteinizing hormone (LH), follicle-stimulating hormone (FSH), and adrenocorticotropin (ACTH) in normal patients.[175,176,186]

Regarding the broad spectrum of its inhibitory activities throughout the body, SOT can inhibit the secretion of both pancreatic hormones insulin and glucagon, gastrin, secretin, pancreozymin, gastric HCl, pepsin, gastric inhibitory polypeptide (GIP), intestinal glucagon-like immunoreactivity (GLI), motilin, vasoactive intestinal polypeptide, ACTH (only in Nelson and Cushing syndromes and Addison disease) and renin.[175,176,186,187]

Modulatory action on immunologic responses is expressed with stimulation of macrophage activity in vitro and inhibition of histamine release from basophils without any effect on mast cells.[100]

Therapeutic Applications and Side Effects

A summary of the therapeutic applications of SOT will only be presented here. For a detailed analysis the reader is referred to review works by Guillemin et al. (1976) and Lundbaek (1978). Thus the disorders and disease states associated with potential therapeutic use of SOT include: acromegaly, diabetes mellitus, juvenile diabetes, brittle diabetes, ketoacidosis in insulin-dependent diabetics, acute pancreatitis, pancreatic islet cell tumors (insulinomas and glucagonomas), gastrinomas, VIPomas, gastric and duodenal ulcers, Zollinger-Ellison syndrome, Nelson's disease, mental disorders, epilepsy and Parkinson disease.[175,176,187-189]

Serious toxic effects associated with the long-term use of SOT in man have been reported. Side effects encountered are: transient nausea, abdominal cramps, diarrhea, interference with GI motility and food absorption, transient cardiovasular effects such as rise in the blood pressure and fall in the pulse rate, hypoglycemia in diabetic patients treated with insulin and finally abnormal platelet function, blood disorders and lung hepatization reported only in baboons.[176,189,190]

Peripheral and Central Localization

The localization of SOT has been extensively investigated in various animal species since its discovery. An outline of the reported peripheral and central areas of localization, includes the following organs and tissues:

- Hypothalamus (highest concentrations in the nerve endings of median eminence and arcuate nuclei)[186,191-195]

- Extrahypothalamic areas such as cerebral cortex, brain stem, olfactory lobe, cerebellum, pineal gland, septum, preoptic area, amygdala, thalamus,spinal cord, primary afferent neurons and autonomic ganglia[186,191,193,195]
- GI system (stomach, pylorus, duodenum, jejunum and ileum)[175,176,188,193,195,196]
- Pancreas (exclusively in a_1 or D cells. Normal rat islets containinsulin, glucagon and SOT in a ratio 660/50/1, on a molar basis)[175,176,193,195,197]
- Retina[198-200]
- Thyroid gland[193,201]

Localization and Functional Role of Somatostatin in the Peripheral Nervous System

Regarding the role of SOT in the CNS, it is currently unknown but available evidence such as the variable distribution in SOT-containing neurons,[193] its localization in brain synaptosomes and nerve terminals,[83,186] its direct depressant effects on electrical neuronal activity[202] and its behavioral effects[203] suggest a neurotransmitter or neuromodulator role.

SOT has been localized peripherally in both the primary afferent neurons and autonomic nervous system.[193] SOT-containing small diameter afferent neurons have their cell bodies in the dorsal root ganglia and the trigeminal ganglion while their peripheral processes extend to the skin and viscera.[193] Their central processes end in the substantia gelatinosa of the dorsal horn of the spinal cord or in the spinal nucleus of the trigeminal nerve.[193] Positive SOT immunoreactivity has been observed in the dorsal

horn with the highest concentration in lamina II.[113] In vitro and in vivo experiments of SOT release from the spinal cord, showed that the peptide is released in a Ca^{++} - dependent way, by increased extracellular potassium levels[83,131] and it acts both pre- and post-synaptically as a neuromodulator.[132,204]

The localization of SOT in small diameter afferent neurons that terminate in the substantia gelatinosa suggests a sensory nociceptive role, similar to that of SP.[132,193] Studies using capsaicin, point as well to an involvement in the nociceptive transmission.[83,131,205] The finding of enkephalin-containing neurons that interact with neurons containing SP and SOT is also suggestive of the role of both neuropeptides in the mechanisms of pain and analgesia.[193] Experiments with SOT and SP showed that the two neuropeptides transmit different information, related to noxious thermal and mechanical stimuli respectively, in the spinal dorsal horn.[205] An inhibitory transmitter role extended to the "gate control theory" mechanism, similar to the general inhibitory or depressant action of SOT in other tissues has been postulated.[113,204,206] Lembeck and his associates have shown that SOT acted as a strong inhibitor of the neurogenic vasodilation and plasma extravasation induced by saphenous nerve stimulation and it did not inhibit the effects of infused SP. According to these observations, SOT was found to be a very potent inhibitor of the SP release from peripheral sensory nerve endings.[170] Other investigators however reported a predominantly excitatory role.[83,132]

In the autonomic nervous system, positive SOT immunoreactivity has been identified in the GI system particularly in the ganglionic cells of the plexus of Auerbach, the submucous plexus and the base of glands and crypts,[113] as well as in autonomic ganglia such as the coeliac-superior

mesenteric ganglion and the inferior mesenteric ganglion in guinea pigs.[207] Single sympathetic neurons were shown to contain both SOT and NA.[207]

SOMATOSTATIN AND THE DENTAL PULP

Unpublished experimental results by Gazelius and others, first reported the presence of SOT immunoreactivity in the cat dental pulp.[82,90] Studies by Akai and Wakisaka in the past, using immunohistochemical means, were not able to report a positive presence of the neuropeptide.[82] Currently, there is only one study in the dental literature that provides evidence for the presence of SOT-immunoreactive nerve fibers in the dental pulp, using the indirect immunofluorescence technique.[90] The distribution pattern described, is as follows: SOT-positive fibers, localized within large nerves enter the pulp through the apical foramen, and they are distributed both apically and coronally up to the plexus of Rashkow and the peripheral pulpal area. The immunoreactive fibers were seen encircling blood vessels of various sizes, or without any association with them. Due to lack of demineralization, no observations were made for the area of predentin and dentin.[90] According to the similar to other neuropeptides, perivascular distribution pattern observed, a role in the regulation of pulpal blood flow is suggested. Experimental evidence showing lack of a direct vasoactive role of SOT in the feline dental pulp[150] and inhibition of the release of SP and the antidromic vasodilation mediated by SP[150] may be suggestive of an indirect action of SOT on the regulation of pulpal blood flow by pre-synaptic inhibition or modification of the release of SP from sensory nerve terminals.[90,132,170]

MATERIALS AND METHODS

Operative Procedures

Sixteen, 239-250 g. male Sprague-Dawley rats were used. The operative procedure was divided into three parts, and five animals (with the exception of the last series that included 6 animals) were operated each time. In each of the three series, all animals were anesthetized with Ketamine hydrochloride (Ketaset, Aveco Co., Inc., Fort Dodge, IA) and Xylazine (Rompun, Mobay Corp., Shawnee, KS) (0.1 ml/100g of body weight), injected intraperitoneally (IP), and received class V cavity preparations on the buccal surface of both first maxillary molars, using a sterile 1/4 round bur at low speed, with sterile saline solution as an irrigant for the removal of debris. All preparations resulted in pulpal exposures of 0.2-0.3 mm in diameter and the exposed pulps were capped with a fast set calcium hydroxide-containing material (Life, Kerr Manufacturing Co., Romulus, MI) and a silver glass-ionomer restoration (Chelon-Silver, Esp-Premier Sales Corp., Norristown, PA) on the surface.

After intervals of 5, 7 and 21 days, the rats of each series were anesthetized with sodium pentobarbital (2X0.1 ml/100 g body weight) injected IP and perfused through the left cardiac ventricle with phosphate buffer, followed by Zamboni fixative consisting of 4% p-formaldehyde, 0.05% glutaraldehyde and 0.2% picric acid.[208,209] The maxilla was removed and separated into two halves, to be used as experimental specimens for detection of SP-like and SOT-like immunoreactivity (IR), respectively. In addition, the mandibles were removed and cut into two halves to be used as control specimens for the same series. Spinal cord specimens with the

surrounding tissue, were also taken from each animal and the dorsal root ganglia were later isolated from 6 specimens, in order to serve as positive controls. The maxillary and mandibular halves were stored in Zamboni fixative at 4^0C for 2-3 days and were subsequently demineralized in a mixture of EDTA and Zamboni fixative containing 7.5% EDTA, 4% p-formaldehyde, 0.05% glutaraldehyde and 0.2% picric acid, for 5-6 weeks at 4^0C. The demineralizing agent was changed every week. The spinal cord specimens were treated in the same way and after a demineralization period of 2-3 weeks, the dorsal root ganglia of 6 specimens were separated and stored in Zamboni fixative, until they were histologically processed.

Paraffin Embedding and Preparation of Frozen Sections

After completion of demineralization as evidenced radiographically, the tooth specimens were separated into right (R) and left (L) series, each consisting of one maxillary and one mandibular half corresponding to each animal (#1 through #16). The specimens of the R side were embedded in paraffin wax and examined for SP-immunoreactivity (SP-IR) while the specimens of the L side were frozen and examined for SOT-immunoreactivity (SOT-IR).

The maxillary and mandibular specimens of the R side were cut buccolingually, at the cervical level of the crown into two parts, one consisting of the crown portions (CRN) and the other of the radicular and alveolar bone portions (RT). All the subspecimens were then dehydrated through ascending series of ethanols at 4^0C, cleared with xylene at room temperature and embedded in paraffin wax. Sections at 5 μm thick were cut longitudinally in the mesio-distal plane for the CRN specimens and

crossectionally in the bucco-lingual plane for the RT specimens, using a rotary microtome, and were collected on aminoalykylsilane-treated glass slides.

The maxillary and mandibular specimens of the L side were cut into CRN and RT portions as above, rinsed in PBS-X buffer containing 30% sucrose for 24 hours, embedded in Tissue Tek OCT compound (Miles Inc., Elkhart, IN) and rapidly frozen by liquid nitrogen. Sections at 5, 7, 8 and 10 μm thick were cut in the cryostat, longitudinally in the mesio-distal plane for the CRN specimens, and crossectionally in the bucco-lingual plane for the RT specimens and were collected on aminoalykylsilane-treated glass slides.

Dorsal root ganglia specimens #1, #5 and #3 were routinely processed for paraffin embedding and sectioned at 5 μm by rotary microtome. Ganglia specimens #2, #4 and #6 were processed for frozen sectioning, at 5 μm in the cryostat.

Avidin - Biotin Complex Immunohistochemistry

The method of avidin-biotin-peroxidase complex (ABC) immunohistochemistry (IHC), as adapted by Bourne (1983),[210] was employed for both SP and SOT demonstration. The method is essentially qualitative and was chosen for its sensitivity and specificity and for its morphological localization of the ligand.[1] The avidin-biotin method represents one of the most recent developments in immunoperoxidase staining[210] and it is considered to be 8-40 times more sensitive than the conventional peroxidase/anti-peroxidase (PAP) techniques.[1]

In the technique employed, the commercial Vectastain ABC Kit (Rabbit IgG,Vector Laboratories, Burlingame, CA) was utilized. The staining procedure was performed as described in the Appendix I where the second and third steps were omitted for the frozen sections.

SP Immunostaining

For the determination of the optimal dilution, the primary antibody for SP was subjected to serial dilutions of 1:100, 1: 250, 1:500, 1:750, 1:1000 and 1:1500 that were incubated with their respective controls for 1 hour at room temperature and overnight (22-26 hours) at 4^0C. Rabbit anti-SP polyclonal antibody (Calbiochem, San Diego, CA) was diluted in PBS-X buffer. The 1:750 dilution, incubated overnight (22-26 hours) at 4^0C was considered to produce the best immunostaining.

During staining according to the described ABC-IHC method, three to five slides were selected based on the position of the exposure site for the maxillary CRN series and randomly for the mandibular CRN, maxillary RT, mandibular RT and dorsal root ganglia series. Slides were incubated with primary antibody for 22-26 hours, at 4^0C in a humidifying chamber. Each staining trial included one tooth section, randomly selected, that was treated in parallel with the tooth specimens. This section served as negative control for observation of non-specific staining in the teeth as well as in the ganglia through the substitution of normal blocking serum for the primary antibody. Separate staining trials were run for the ganglia sections and were treated exactly as the tooth series. All sections were counterstained, dehydrated in alcohol, cleared in xylene and coverslipped with Permount.

SOT Immunostaining

Similarly to the SP series, the primary antibody for SOT was subjected to serial dilutions of 1:50, 1:100, 1:250, 1:500, 1:750, 1:1000 and 1:1500 that were incubated with their respective controls for 1 hour at room temperature and overnight (10-20 hours) at 4^0C, for determination of the optimal dilution. Rabbit anti-SOT polyclonal antibody (Dako Corporation, Carpinteria, CA) was diluted in PBS-X buffer. The 1:750 dilution, incubated overnight (22-26 hours) at 4^0C gave the best immunostaining.

The ABC-IHC method was applied as described before, but the second and third stages were omitted. Two to six slides were selected with criterion the position of the exposure site for the maxillary CRN series, and randomly for the mandibular CRN, maxillary RT, mandibular RT and dorsal root ganglia series. All primary antibody incubations were carried out for 22-26 hours, at 4^0C in a humidifying chamber. Each staining trial included one randomly selected tooth section that served as negative control via omission of the primary antibody. Separate staining trials were run for the ganglia sections and all sections were counterstained, dehydrated and coverslipped with Permount.

Examination and Analysis of Data

Two to five slides from both series were examined with light microscopy (25-100X) for the presence or absence of the specific brown-black reaction product of the DAB substrate. For the RT specimens, the number of positively stained nerve fibers was counted and recorded for the first two molars, in every slide examined. More specifically, the teeth examined

were the first maxillary molar (T1), the second maxillary molar (T2), the first mandibular molar (T3) and the second mandibular molar (T4). Due to the inconsistency in the number of slides examined in each series, and the available number of roots in each molar, we decided to compare average values only. Thus, mean values of the number of positively stained nerve fibers were obtained for each molar examined in each slide (Avg), and the mean number (Ma) of Avg values was finally used for the statistical analysis of the data.

The two factors investigated in this study were: 1) Presence of statistically significant differences in the mean number of SP-IR and SOT-IR nerve fibers among the three time groups, in the tooth subjected to the experimental procedure (T1). The three groups compared were assigned as T1a (3-day interval), T1b (7-day interval) and T1c (21-day interval) 2) Intraanimal variation of the number of IR nerve fibers among the four groups of teeth (T1, T2, T3 and T4) examined for each animal.

Two statistical tests were used. The one-way analysis of variance (ANOVA) was used to examine the first hypothesis and the paired t-test was chosen for the second investigation. For the second hypothesis, four comparisons were made between the following groups: T1 was compared to T2 in order to detect differences in the number of immunoreactive nerve fibers between the operated tooth (T1) and a maxillary intact control tooth (T2). T1 was compared with T3 for differences between the experimental tooth (T1) and its mandibular respective unoperated control (T3). T3 was compared with T4 for differences between the first (T3) and second (T4) mandibular molars and finally, T2 was compared to T4 in order to find out differences between the second molars, used as representative controls from each jaw.

The CRN slides were examined for positive immunoreactivity in the same teeth as in the RT series (T1, T2, T3 and T4) and when present, specific observations were made regarding the relationship of SP-immunoreactive (SP-IR) and SOT-immunoreactive (SOT-IR) nerve fibers to blood vessels and the odontoblast layer, and the localization of unrelated to any other structure, nerve fibers.

The data for the RT series was reported in Appendices II through IX. The results of the statistical analysis are reported in Tables I through VI. Graphic presentation of the data is displayed in Figures 1 through 4. Representative photomicrographs were taken with Kodak Gold Plus ASA 100 and 400 color slide film and are displayed in Figures 5 through 32.

RESULTS

This study demonstrated specific SP-like immunoreactivity (SP-LI) and SOT-like immunoreactivity (SOT-LI) within the dental pulp of Group A [SP-ROOT] (Figures 9 through 14) and Group B [SOT-ROOT] (Figures 15 through 20) series of teeth. Positive immunoreactivity was also demonstrated in the Group C [SP-CROWN] (Figures 21 through 26) and Group D [SOT-CROWN] (Figures 27 through 32) series. Figures 5 through 8 present positive immunoreactivity for SP and SOT within specimens of dorsal root ganglia, as opposed to their controls.

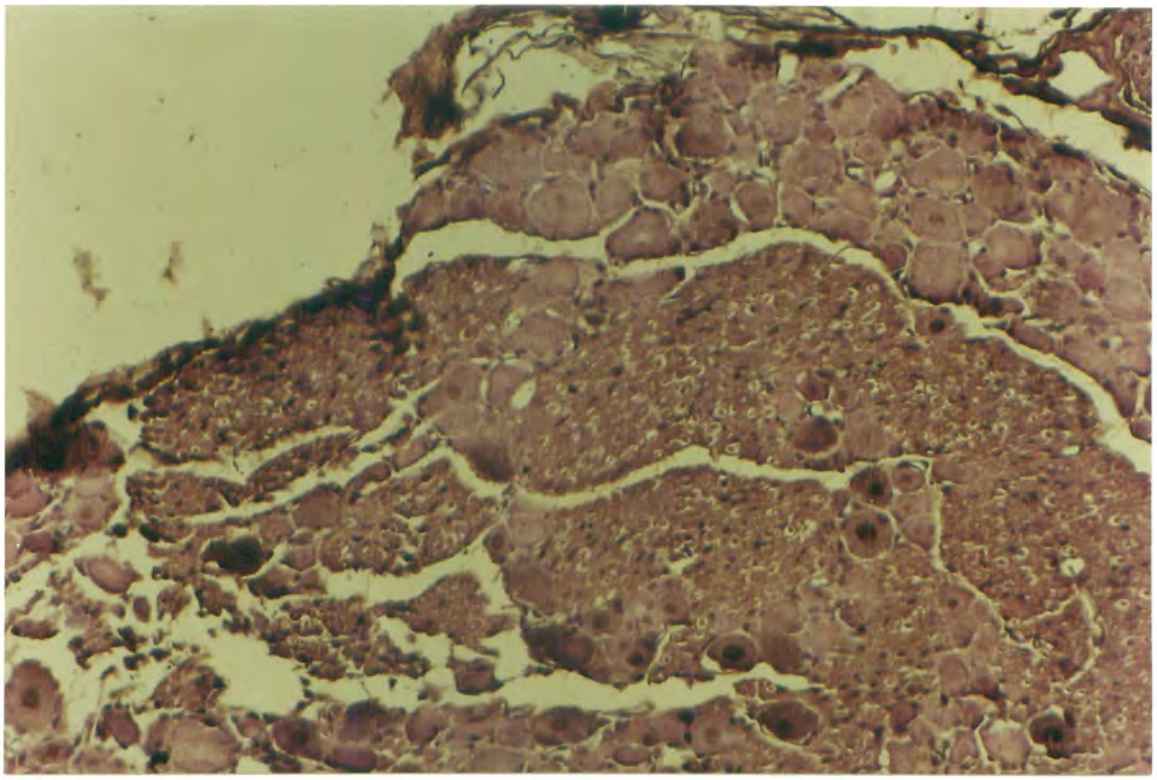


Fig. 5. Cell bodies of sensory neurons in specimen of dorsal root ganglion. Areas of clear cells with dense black-brown staining are indicative of SP-like immunoreactivity. Original magnification X50.

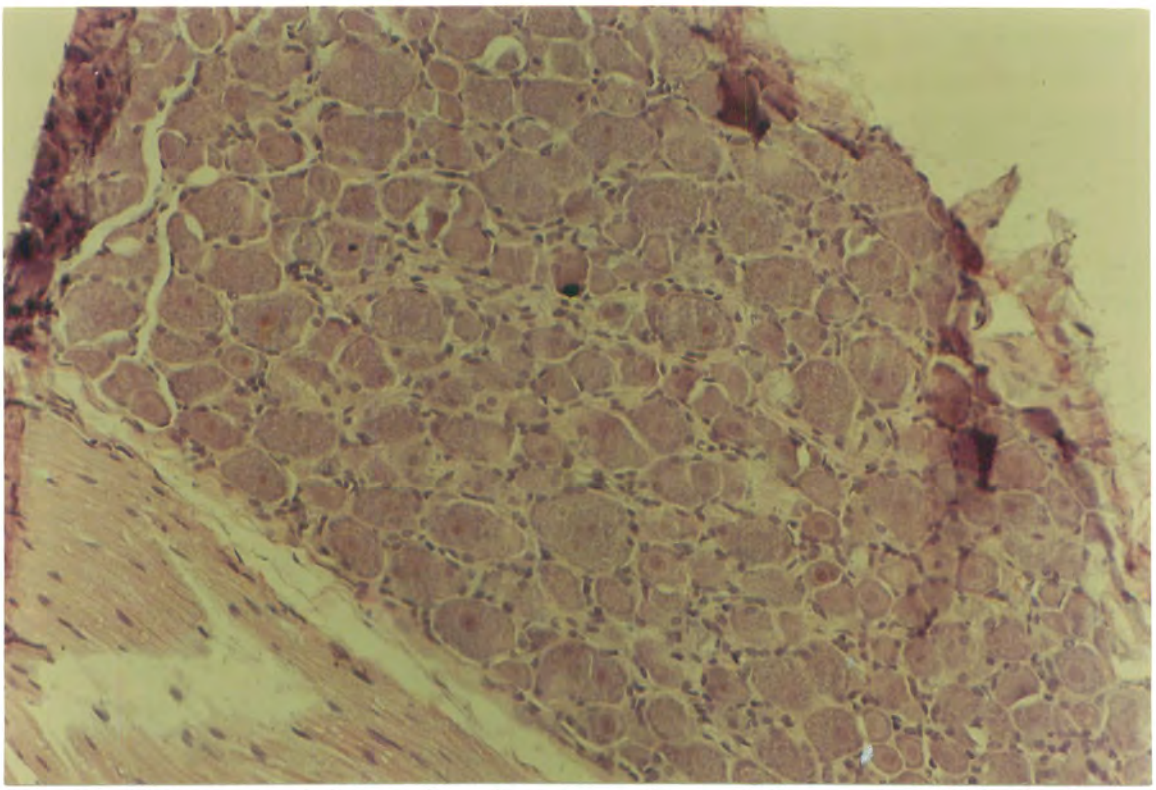


Fig. 6. SP immunostaining, dorsal root gangion negative control. Note the lack of immunostaining compared to Figure 5. Original magnification X50.

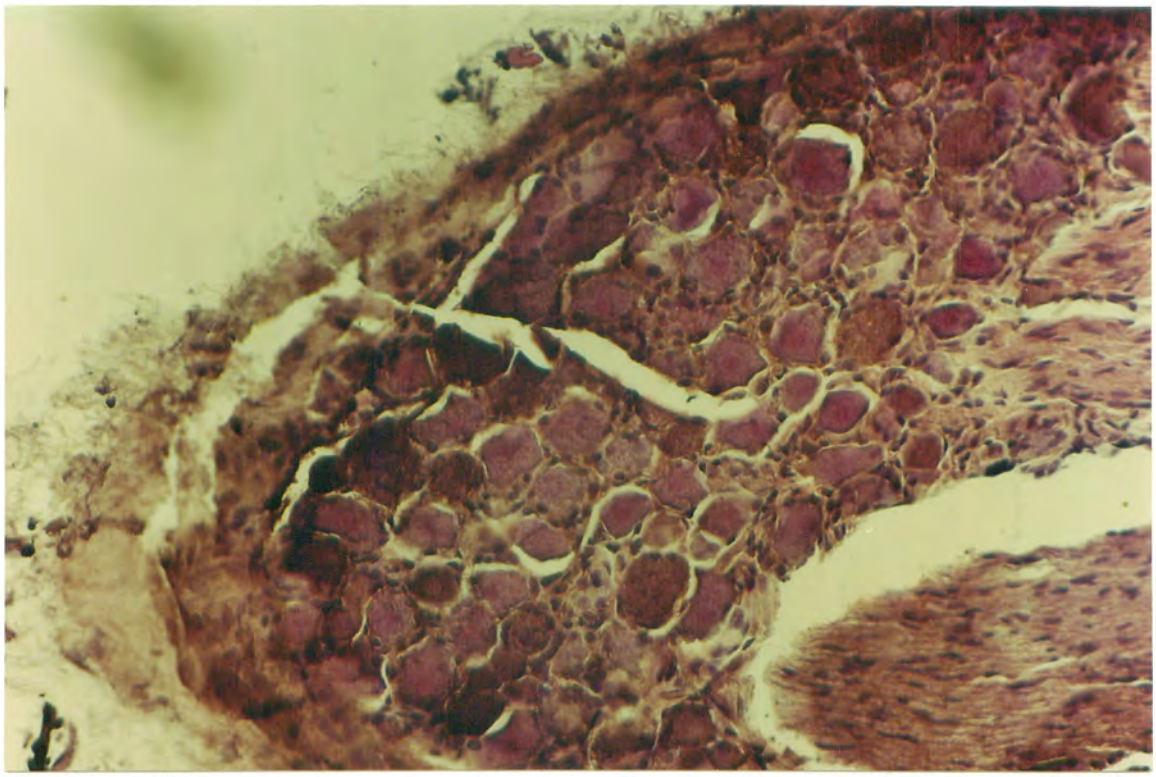


Fig. 7. SOT immunostaing, dorsal root ganglion positive control. Multiple cell bodies with dense, specific SOT-like immunoreactivity, are seen. Original magnification X50.

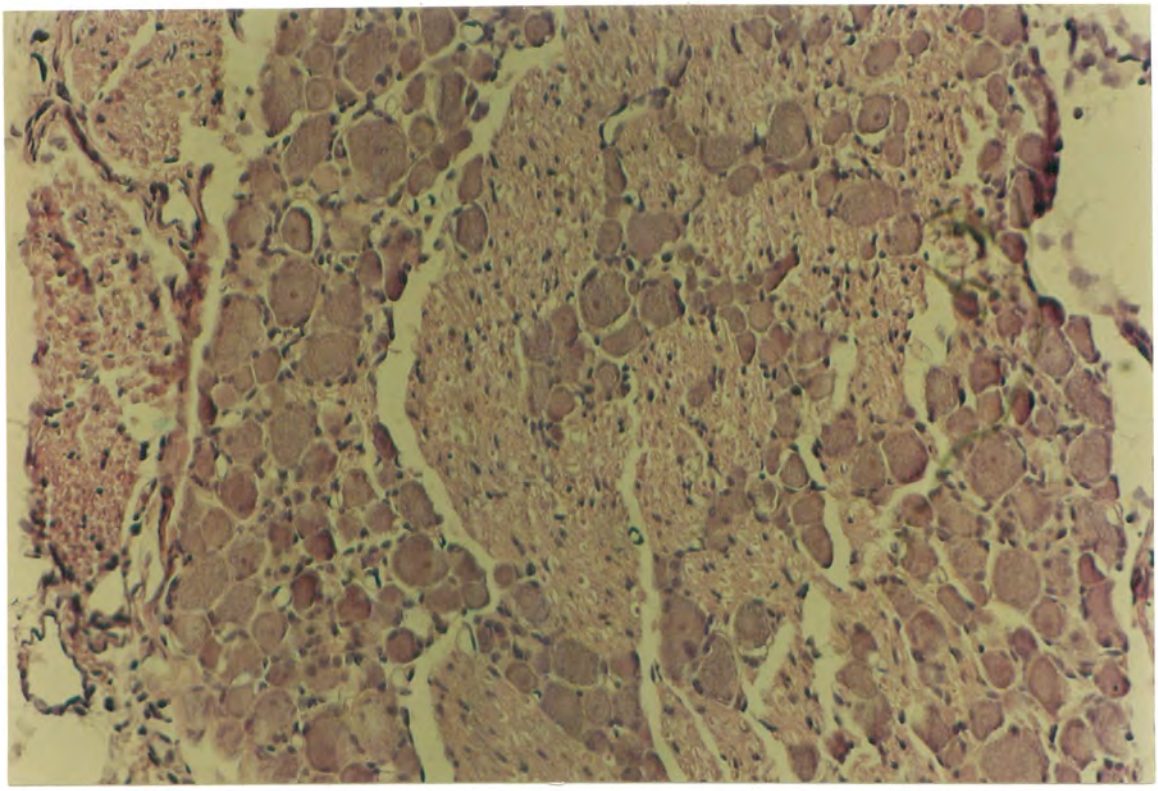


Fig. 8. SOT immunostaing, dorsal root ganglion negative control. Note the absence of staining as compared to Fig. 7. Original magnification X50.

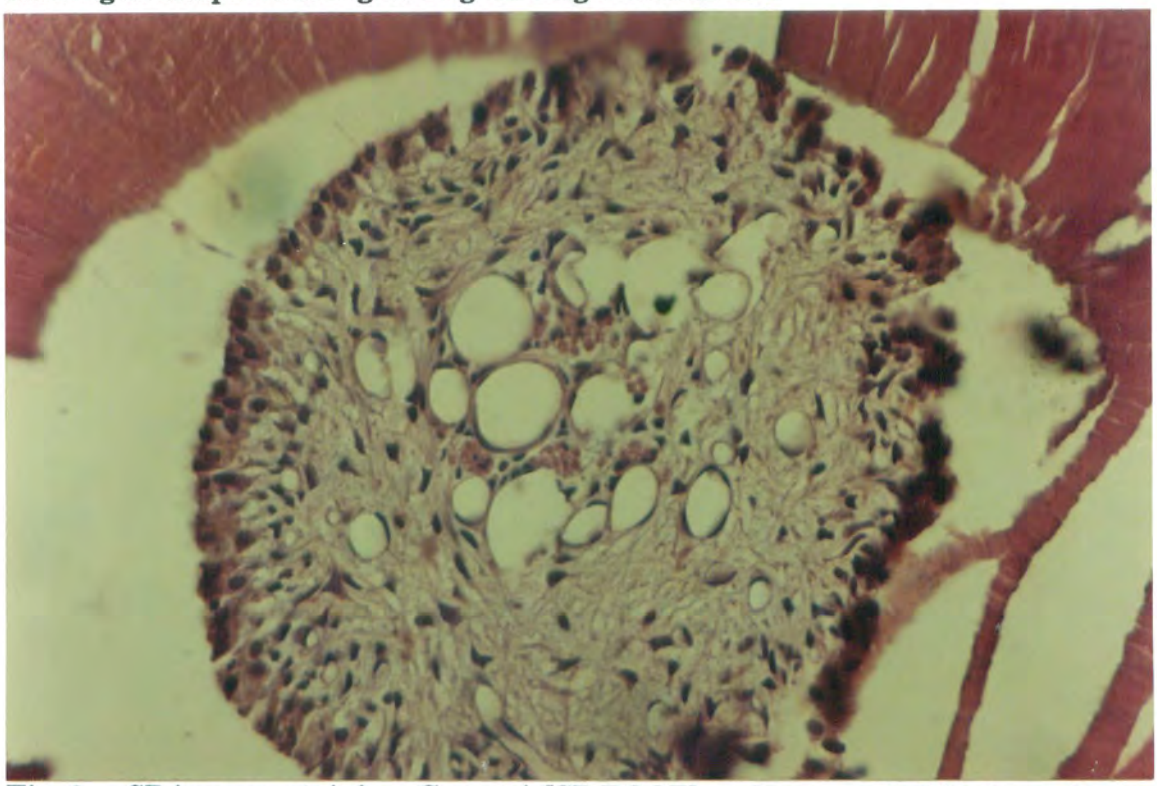


Fig. 9. SP immunostaining. Group A [SP-ROOT] series, operated first maxillary molar (T1) at 3rd postoperative day. Crossections of SP-IR nerve fibers ascending coronally with blood vessel trunks.Original magnification X100.

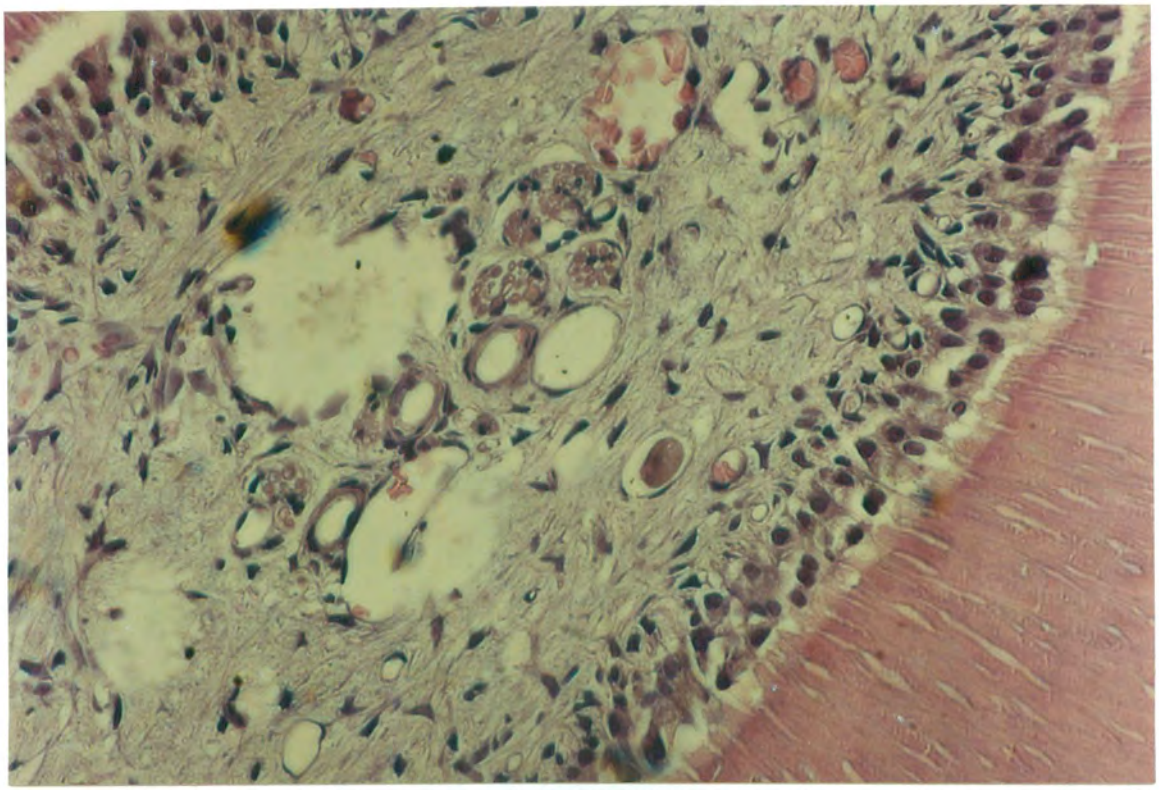


Fig. 10. SP immunostaining. Group A [SP-ROOT] series, first mandibular molar (T3) control at 3rd postoperative day. Original magnification X100.

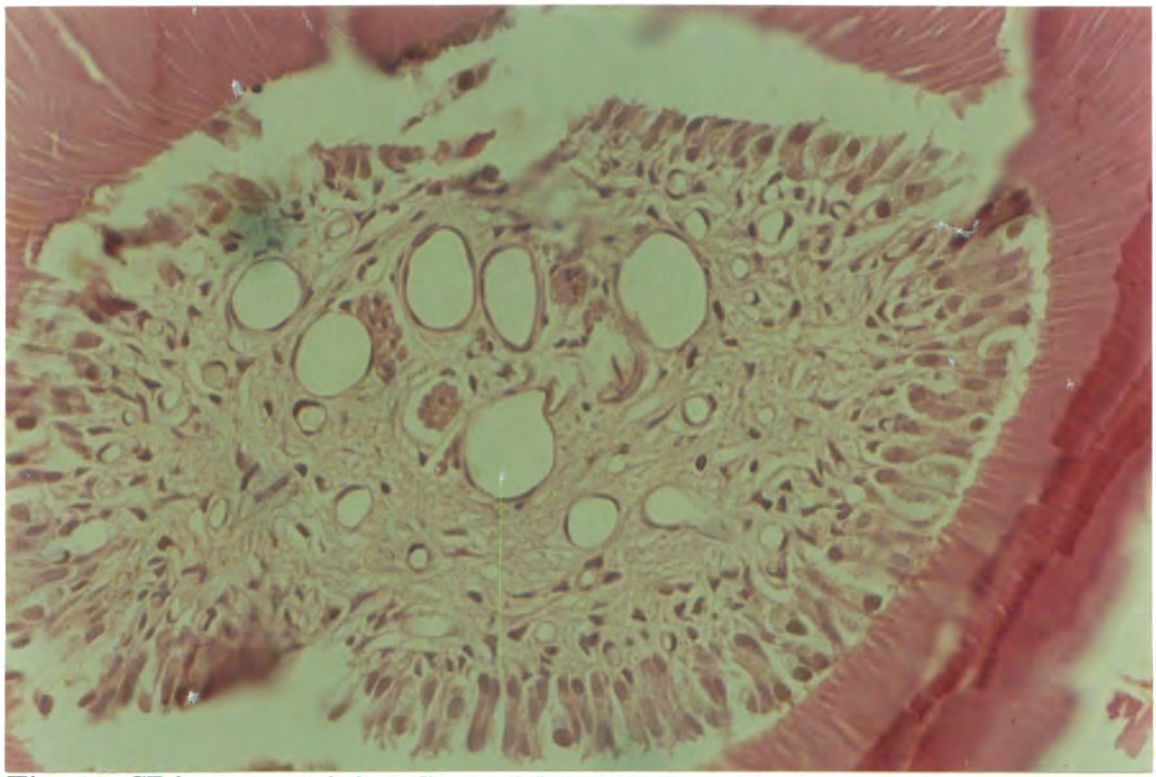


Fig. 11. SP immunostaining. Group A [SP-ROOT] series, first maxillary molar (T1) at 7th postoperative day. Although staining is not good, some aggregations of SP-IR nerve fibers at the periphery of blood vessels are discerned. Original magnification X100.

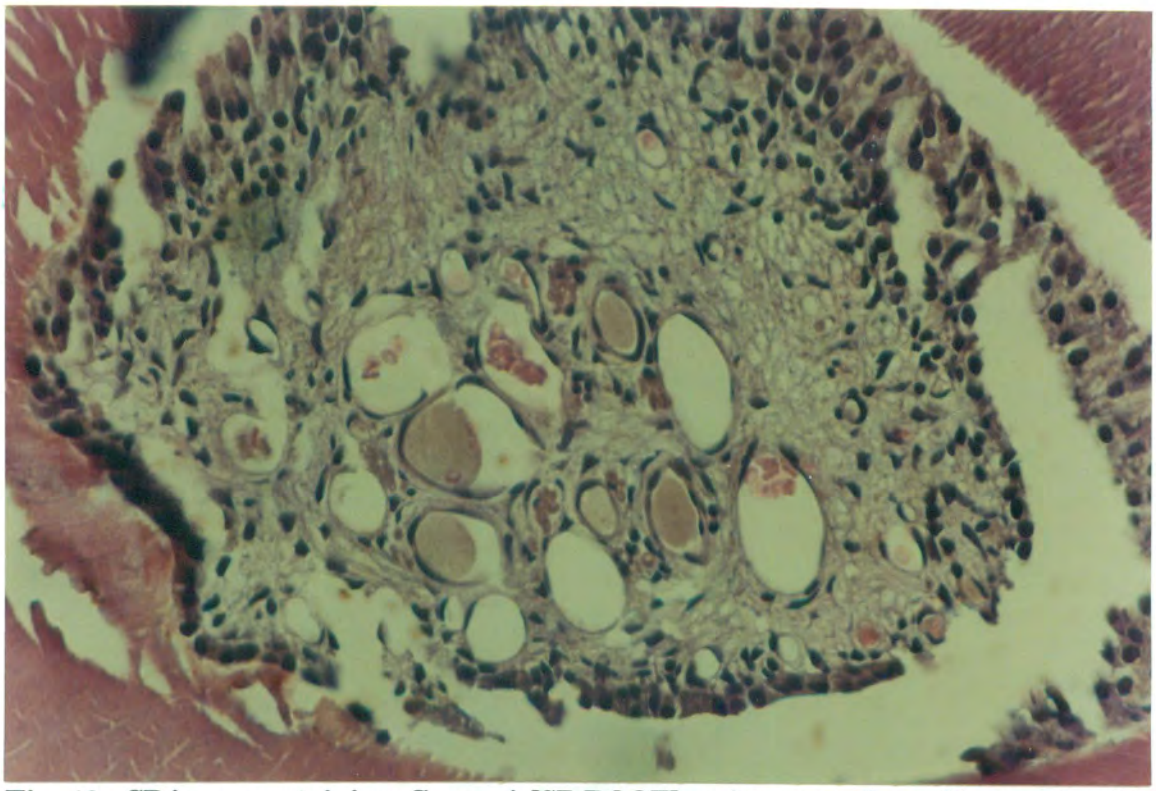


Fig. 12. SP immunostaining. Group A [SP-ROOT] series, second maxillary molar (T2) control at 7th postoperative day. Red blood cells and perfused fixative are seen within some blood vessels. Original magnification X100.

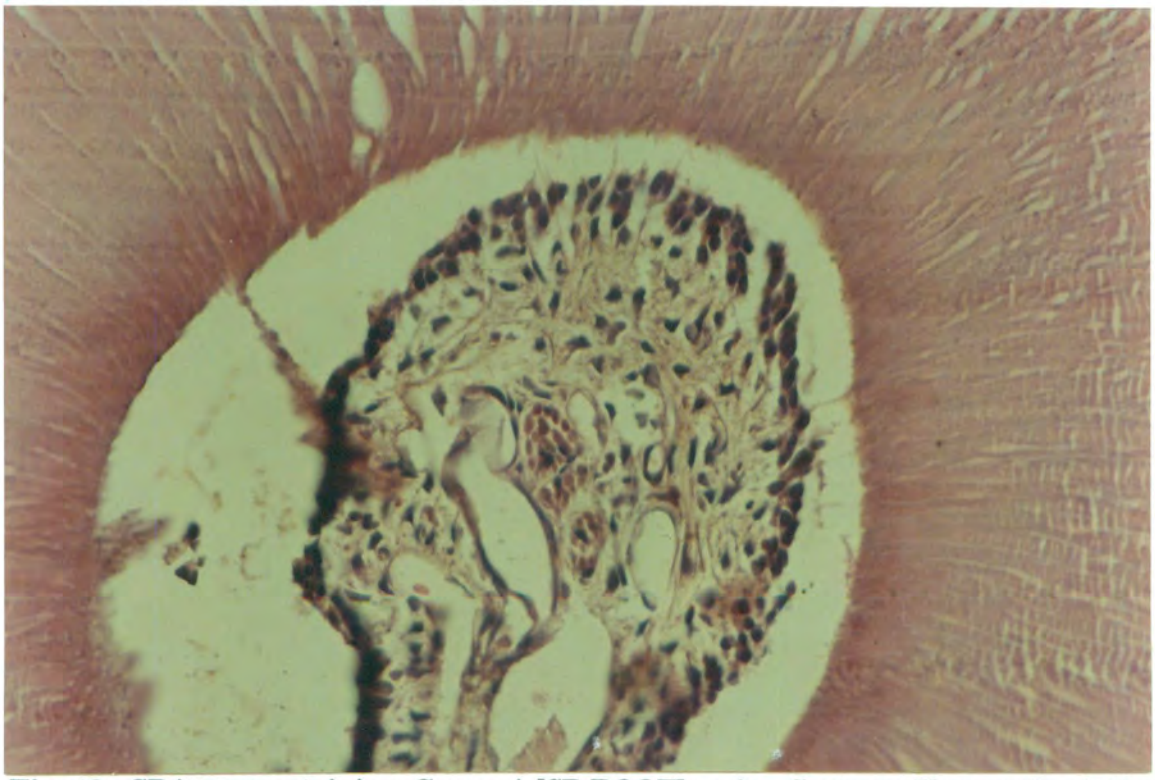


Fig. 13. SP immunostaining. Group A [SP-ROOT] series, first maxillary molar (T1) at 21st postoperative day. Original magnification X100.

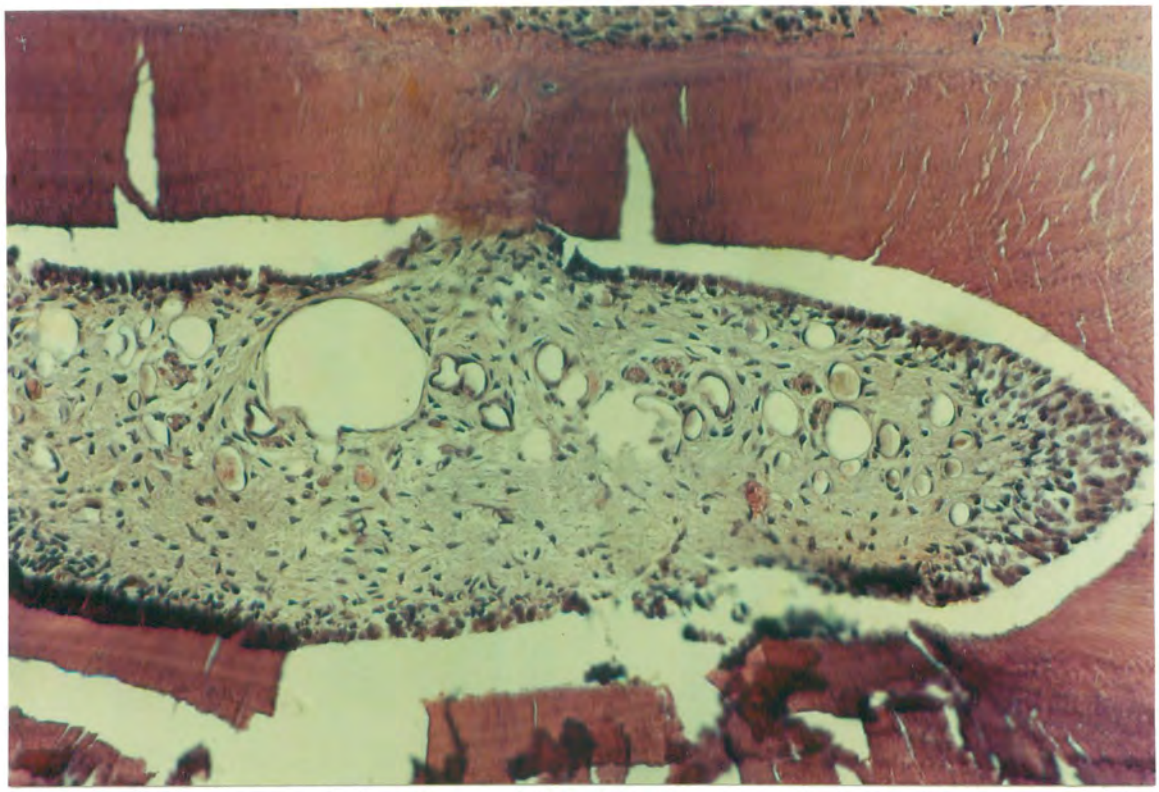


Fig. 14. SP immunostaining. Group A [SP-ROOT] series, first mandibular molar (T3) control at 21st postoperative day. Original magnification X50.

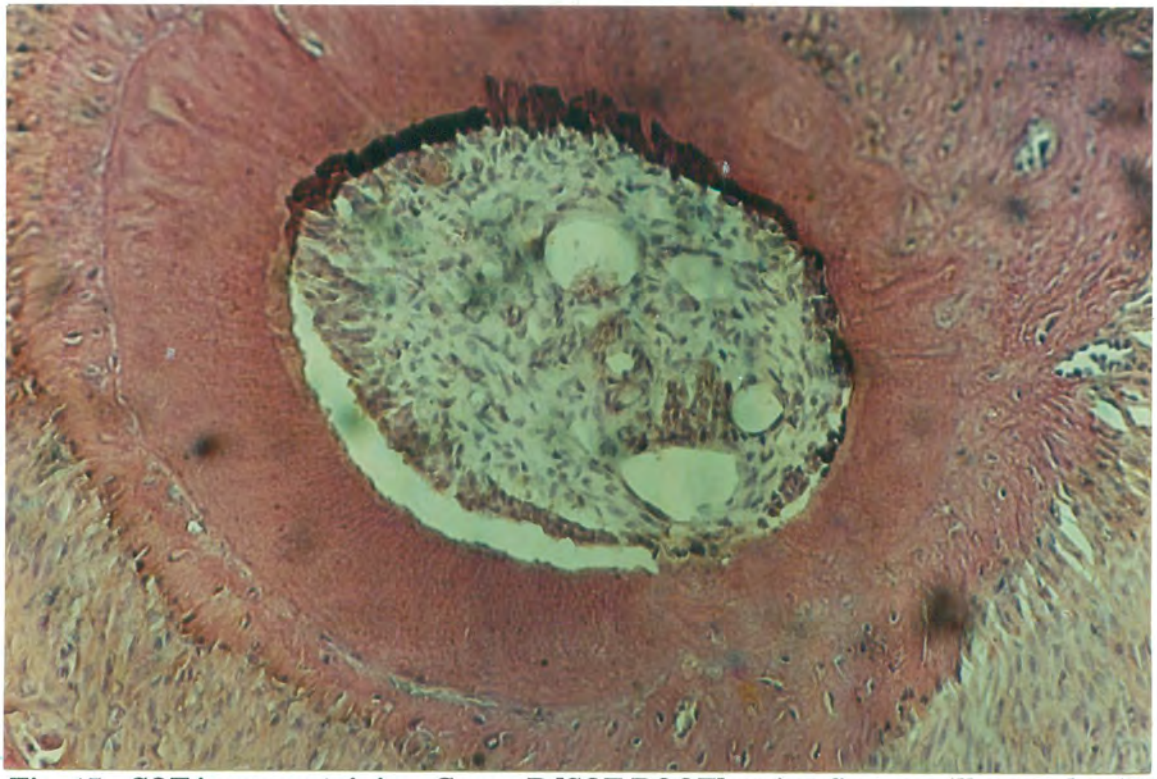


Fig. 15. SOT immunostaining. Group B [SOT-ROOT] series, first maxillary molar (T1) at 3rd postoperative day. Perivaslular distribution of SOT-IR nerve fibers. Original magnification X50.

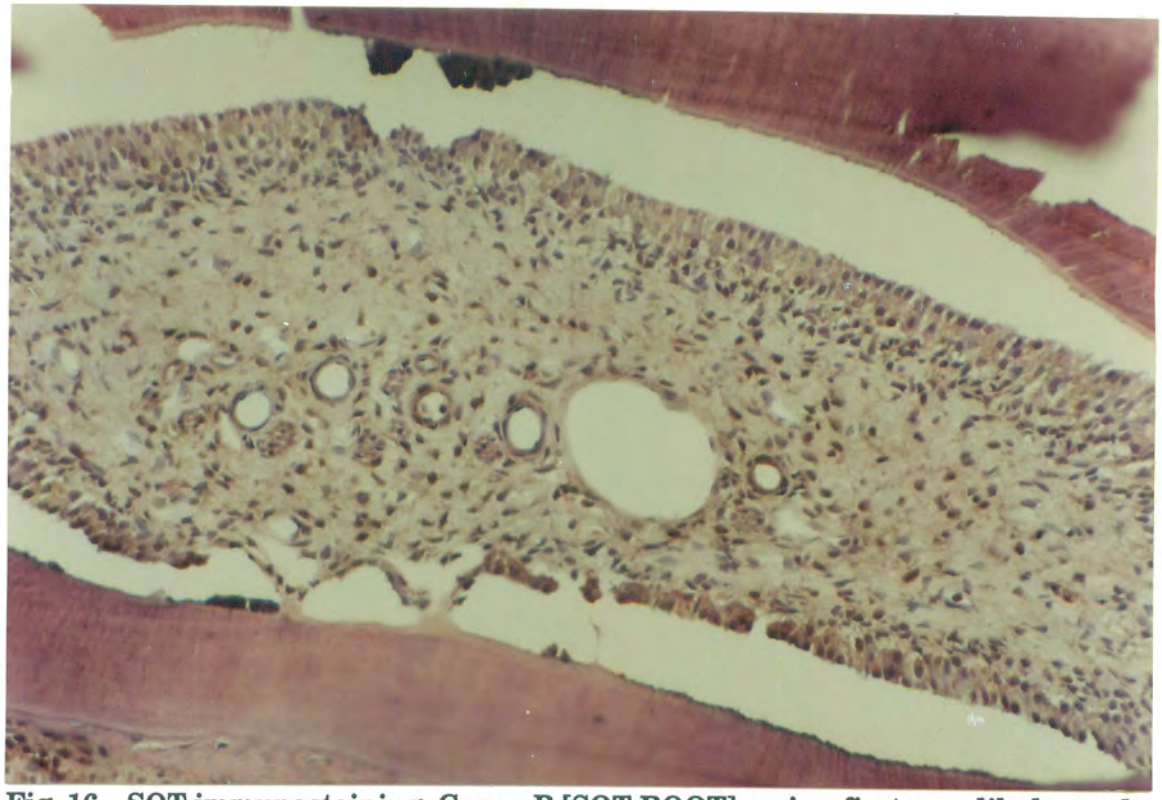


Fig. 16. SOT immunostaining. Group B [SOT-ROOT] series, first mandibular molar (T3) control at 3rd postoperative day. Original magnification X50.

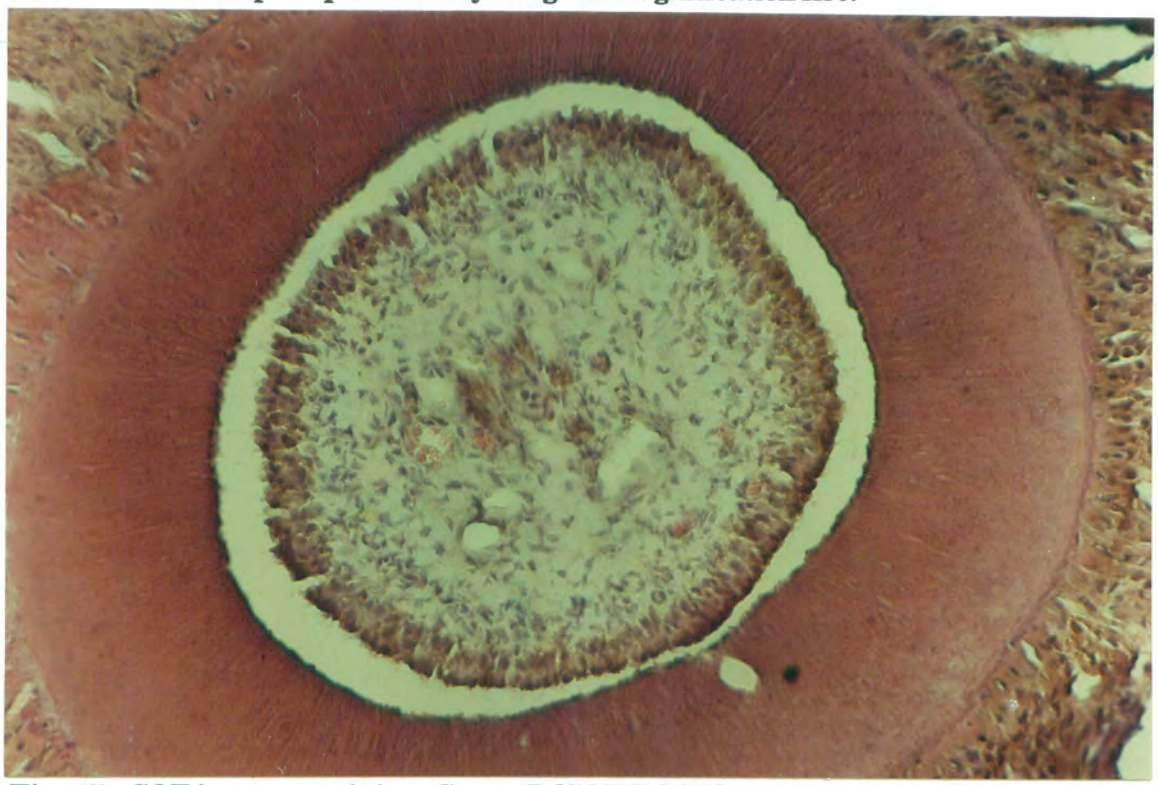


Fig. 17. SOT immunostaining. Group B [SOT-ROOT] series, first maxillary molar (T1) at 7th postoperative day. Specimen is overstained, but central areas of brown staining are indicative of SOT-like immunoreactivity. Original magnification X50.

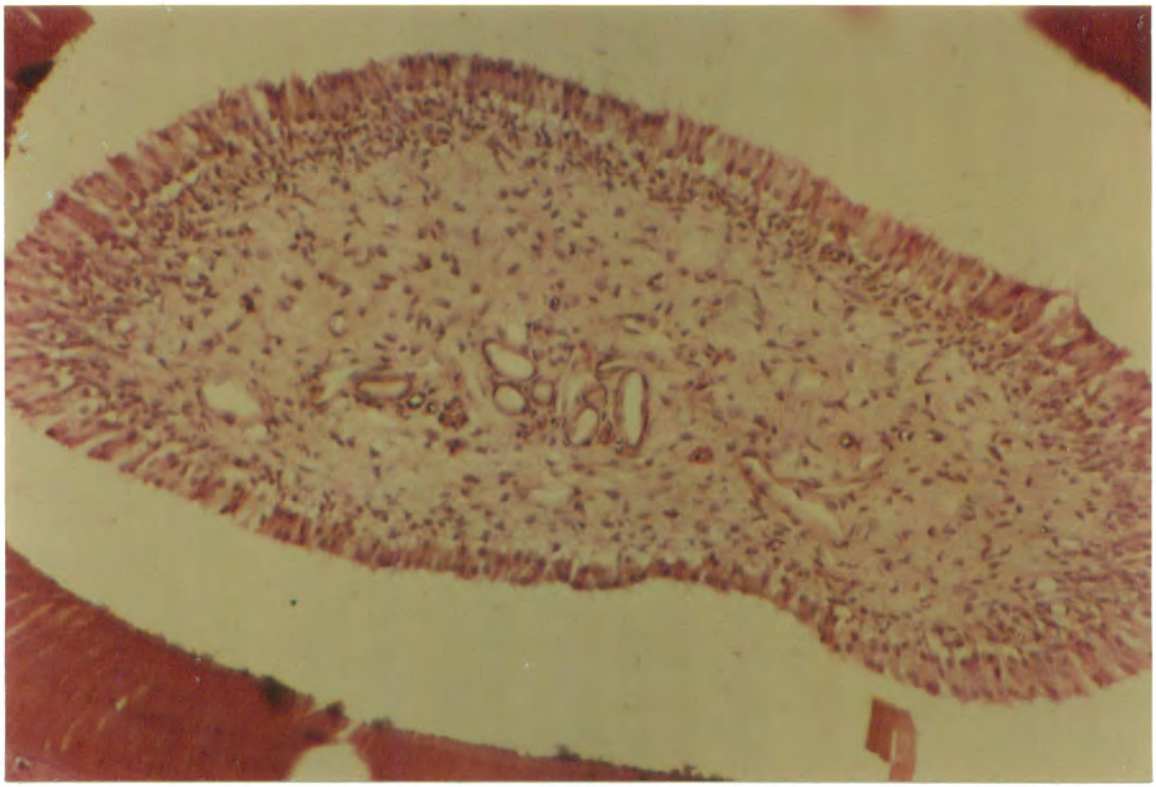


Fig. 18. SOT immunostaining. Group B [SOT-ROOT] series, first mandibular molar (T3) control at 7th postoperative day. Light SOT-like immunoreactivity is seen in central areas, closely associated with blood vessels. Original magnification X50.

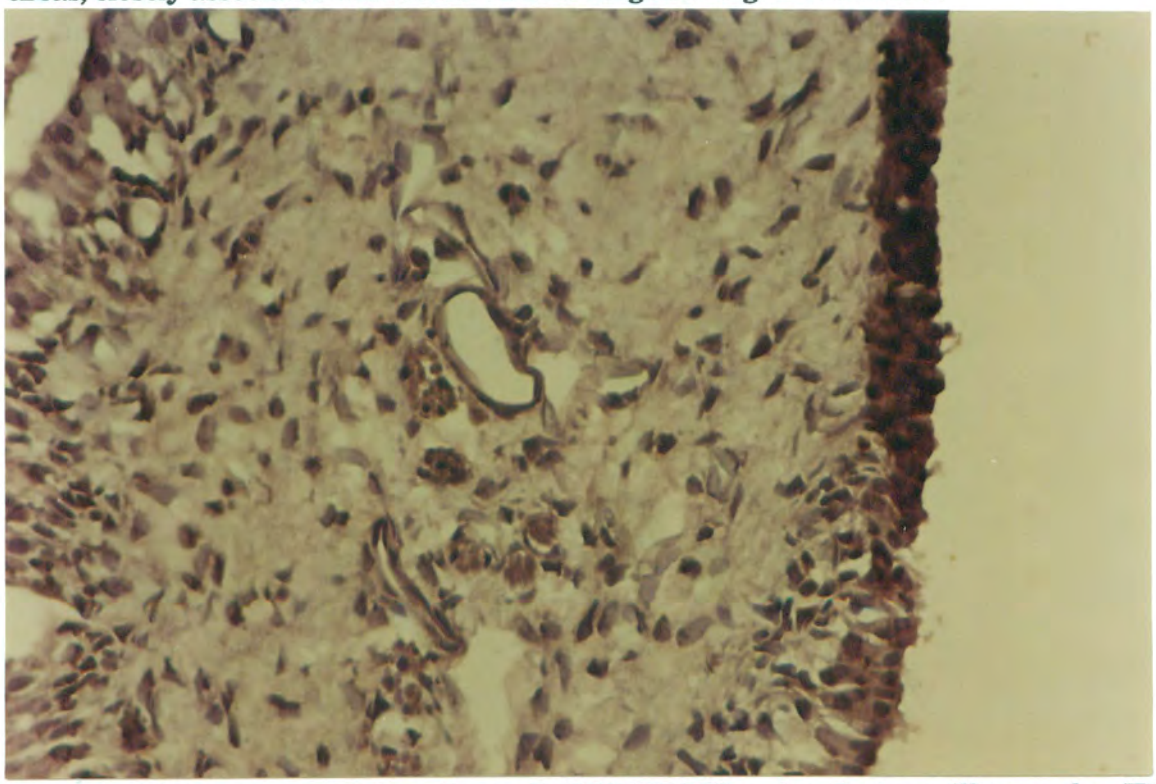


Fig. 19. SOT immunostaining. Group B [SOT-ROOT] series, first maxillary molar (T1) at 21st postoperative day. Original magnification X100.

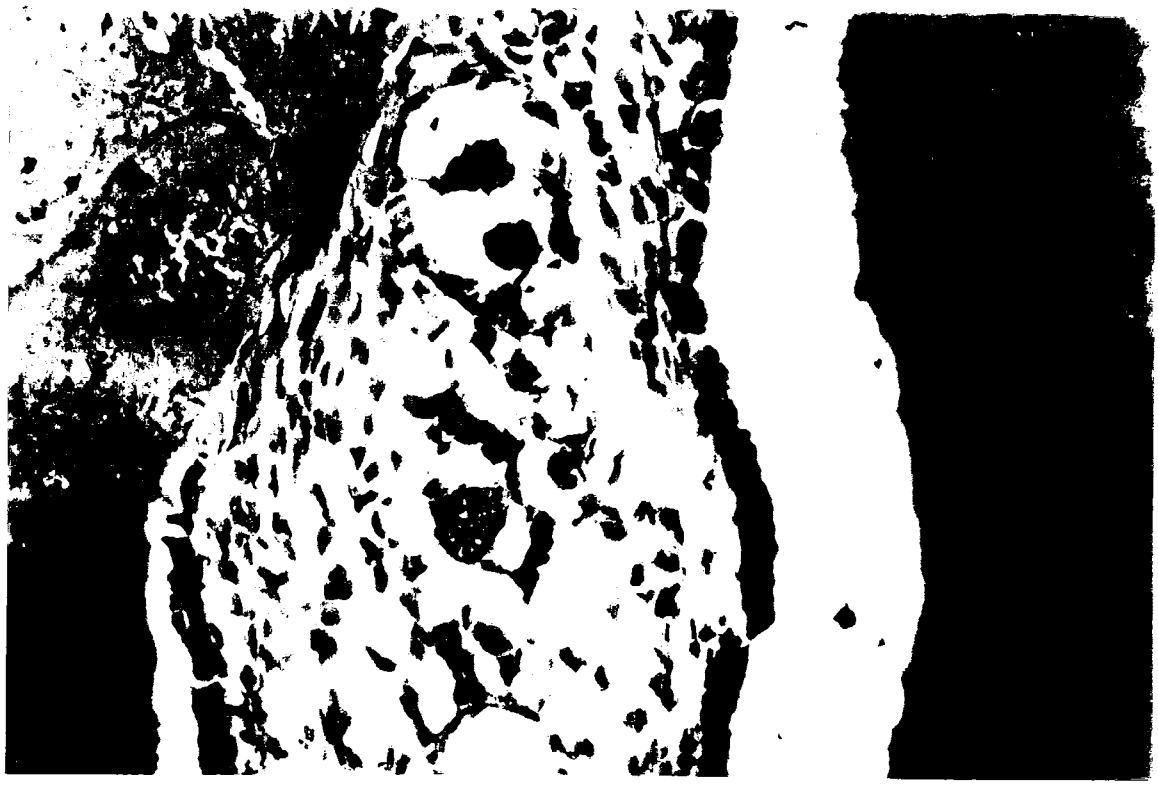


Fig. 20. SOT immunostaining. Group B [SOT-ROOT] series, first mandibular molar (T3) control at 21st postoperative day. Lightly stained SOT-IR nerve fibers are projected within the lumens of blood vessels. Original magnification X100.

GROUP A [SP-ROOT SERIES] and GROUP B [SOT-ROOT SERIES]

Differences in the mean number of SP-IR and SOT-IR nerve fibers among the three time groups, in the experimental tooth (T1) were examined by the one-factor analysis of variance (ANOVA) test.

For the group A of SP root series, the mean number of nerve fibers obtained for each group were 34.975 for the T1a group (3 days), 44.18 for the T1b group (7 days) and 40.983 for the T1c group (21 days) (Figure 3). An F value of 0.637 and a p value of 0.546 revealed no significant differences among the three groups at the significance level α=0.05 (Table I).

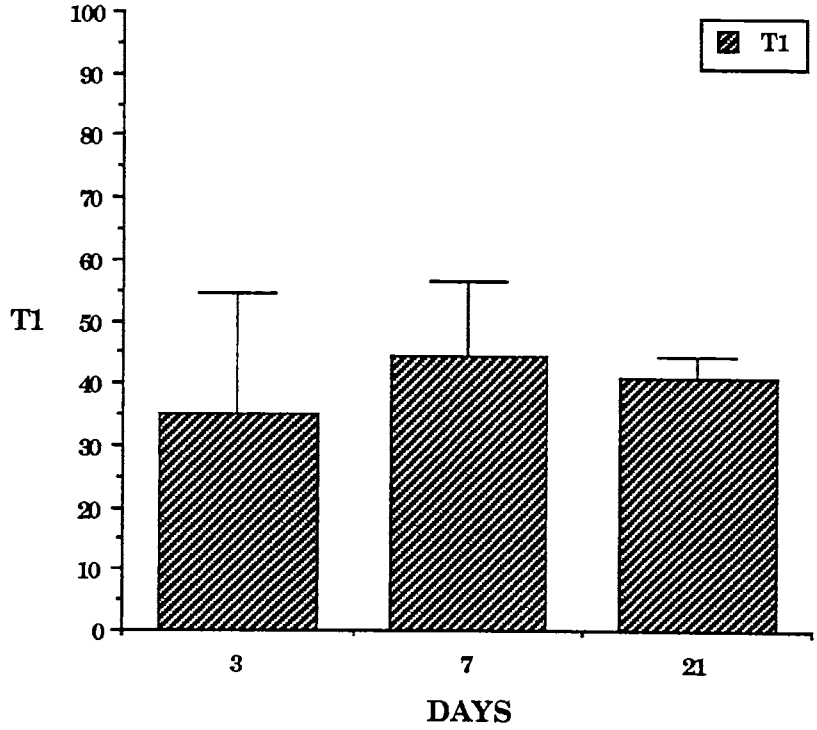


Fig. 3. Mean and standard deviation values of SP-IR nerve fibers in the experimental tooth T1 for the 3, 7 and 21-day time intervals (groups T1a, T1b, T1c).

TABLE I
ANALYSIS OF VARIANCE FOR GROUP A [SP - ROOT SERIES]
[T1a, T1b, T1c GROUPS]
ONE - WAY ANOVA

VARIATION SOURCE	DF	SUM SQUARES	MEAN SQUARES	F-TEST	PROB/LITY
BETWEEN GROUPS	2	191.174	95.587	0.637	p = 0.546 NS
WITHIN GROUPS	12	1801.404	150.117		
TOTAL	14	1992.577			

NS: not statistically significant

* Significance Level $\alpha = 0.05$

Pairwise comparisons of the three groups were examined with the Scheffe F-test. The comparisons of T1a to T1b, T1a to T1c and T1b to T1c gave a Scheffe F value of 0.627, 0.289 and 0.093 respectively. No significant differences were obtained for any of the three contrasts at the significance level α=0.05 (Table II).

TABLE II
ANALYSIS OF VARIANCE FOR GROUP A [SP - ROOT SERIES]
[T1a, T1b, T1c GROUPS]
ONE - WAY ANOVA

COMPARED GROUPS	MEAN DIF/RENCE	SCHEFFE F-TEST
T1a vs T2a	-9.205	0.627 NS
T1a vs T3a	-6.008	0.289 NS
T2a vs T3a	3.197	0.093 NS

NS: not statistically significant
*** Significance Level α = 0.05**

In the group B of SOT root series, the mean number of nerve fibers were 56.06 for the T1a group (3 days), 64.4 for the T1b group (7 days) and 51.6 for the T1c group (21 days) (Figure 4). The F value obtained was 0.6151 and no significant differences were found among the three groups because the p value was 0.5598 (Table III).

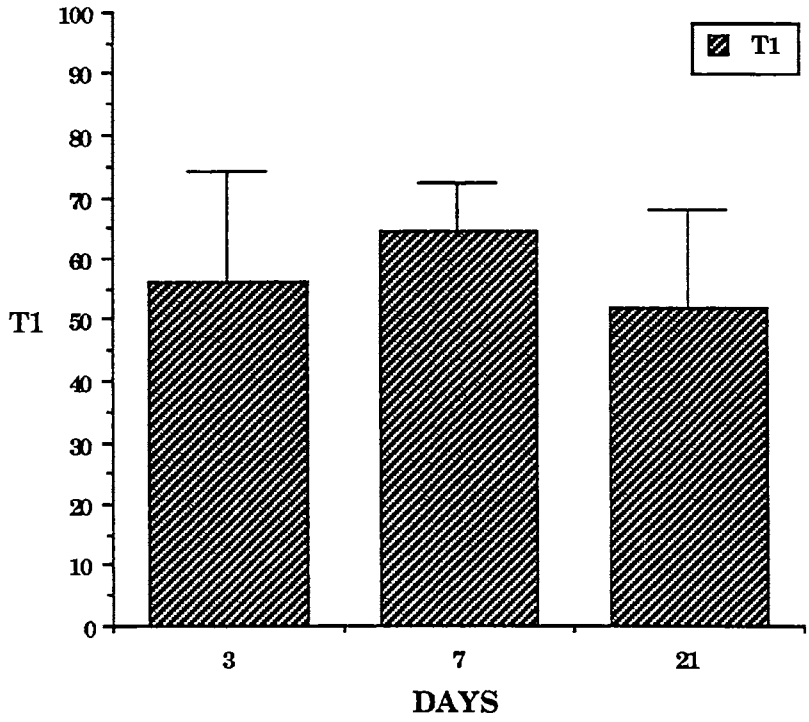


Fig. 4. Mean and standard deviation values of SOT-IR nerve fibers in the experimental tooth T1 for the 3, 7 and 21-day time intervals (groups T1a, T1b, T1c).

TABLE III
ANALYSIS OF VARIANCE FOR GROUP B [SOT - ROOT SERIES]
[T1a, T1b, T1c GROUPS]
ONE - WAY ANOVA

VARIATION SOURCE	DF	SUM SQUARES	MEAN SQUARES	F-TEST	PROB/LITY
BETWEEN GROUPS	2	307.5557	153.7778	0.6151	p = 0.5598 NS
WITHIN GROUPS	10	2500.072	250.0072		
TOTAL	12	2807.6277			

NS: not statistically significant

* Significance Level $\alpha = 0.05$

The pairwise comparisons of T1a to T1b, T1a to T1c and T1b to T1c groups gave Scheffe F values of 0.2608, 0.0995 and 0.6144 respectively. No statistically significant differences were found in any of the three comparisons at the significance level α=0.05 (Table IV).

TABLE IV
ANALYSIS OF VARIANCE FOR GROUP B [SOT - ROOT SERIES]
[T1a, T1b, T1c GROUPS]
ONE - WAY ANOVA

COMPARED GROUPS	MEAN DIF/RENCE	SCHEFFE F-TEST
T1a vs T2a	-8.34	0.2608 NS
T1a vs T3a	4.46	0.0995 NS
T2a vs T3a	12.8	0.6144 NS

NS: not statistically significant

*** Significance Level α = 0.05**

Intraanimal variation of the mean number of IR nerve fibers among the four groups T1, T2, T3 and T4 which represented one experimental and three control teeth (Figures 1 and 2), were examined with the paired t-test (Tables V and VI).

In the group A of SP root series, the difference of means of the T1 and T2 groups was 0.053 and the paired t value was 0.018. No statistically significant difference was found because the p value was 0.9861. Likewise, the comparison of T1 and T3 groups gave values of 12.443 and 1.473 as difference of means and paired t value respectively. A p value of 0.1912

revealed no significant difference between the two groups. When the T3 and T4 groups were compared, the difference of means and the paired t values were 16.6 and 2.851 respectively. The difference was statistically significant at the probability level of $p<0.05$. Finally, the comparison of T2 and T4 groups gave a 3.35 value of difference of means, a 0.544 paired t value and a 0.5994 p value that showed no significant difference between T2 and T4 groups (Table V).

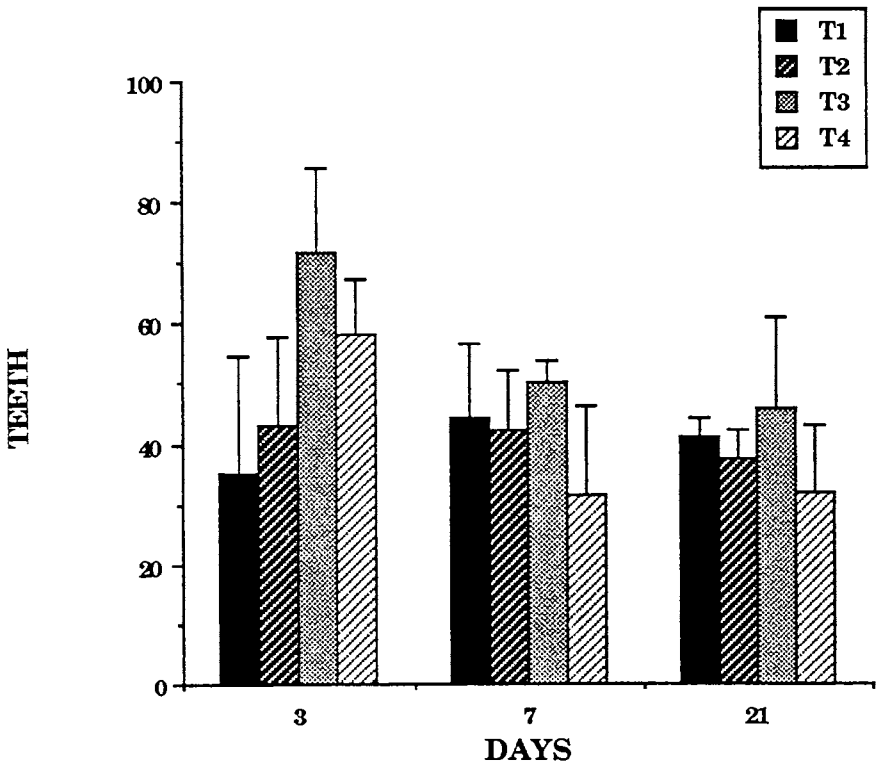


Fig. 1. Mean and standard deviation values of SP-IR nerve fibers in the SP Root Group.

TABLE V

INTRAANIMAL COMPARISONS IN THE GROUP A [SP - ROOT SERIES]

PAIRED t - TEST

COMPARED GROUPS	MEAN (X - Y)	PAIRED t VALUE	PROB/LITY
T1 - T2	-0.053	-0.018	0.9861 NS
T1 - T3	-12.443	-1.473	0.1912 NS
T3 - T4	16.6	2.851	0.0247 $p < 0.05$
T2 - T4	-3.35	-0.544	0.5994 NS

NS: not statistically significant

*** Significance Level $\alpha = 0.05$**

In the group B of SOT root series, the comparison of T1 and T2 groups gave values of 3.9182, 1.2713 and 0.2324 as difference of means, paired t value and p value respectively. No significant difference was found between the two groups. The T1 and T3 comparison showed a 9.5583 difference of means value, a paired t value of 1.9266 and a p value of 0.0802. Therefore, no statistically significant difference was found between these two groups. When the T3 and T4 groups were compared, the difference of means and the paired t values were 13.9 and 2.7581 respectively. The difference between these groups was statistically significant at $p<0.05$. Finally, in the comparison of T2 and T4 the difference of means value was 0.14, the paired t value was 0.0268 and the p value was 0.9792. No significant difference was determined (Table VI).

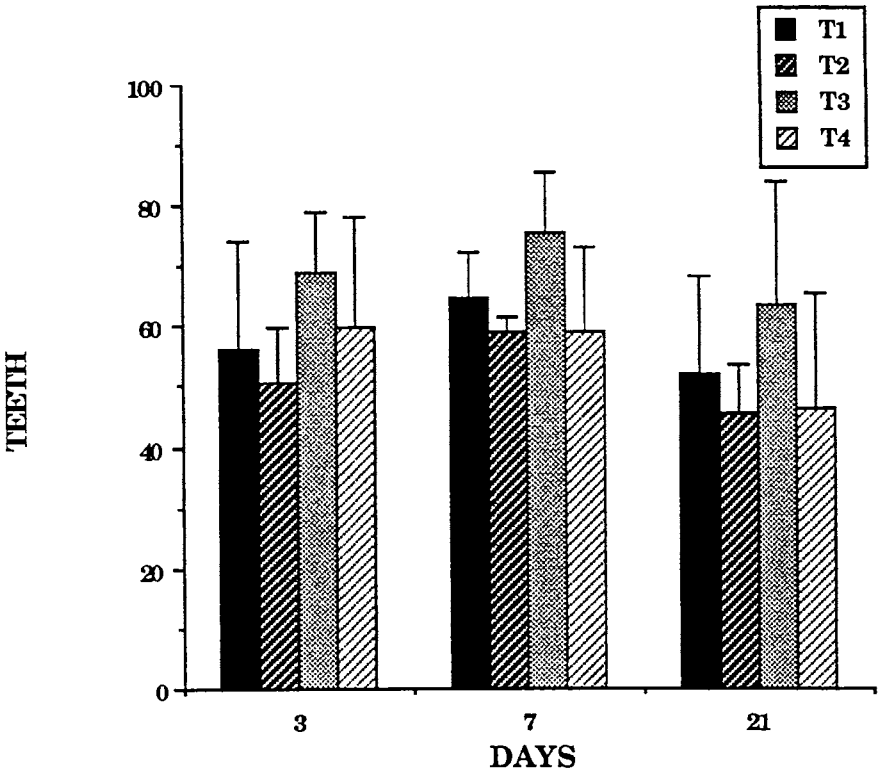


Fig. 2. Mean and standard deviation values of SOT-IR nerve fibers in the SOT Root Group.

TABLE VI
INTRAANIMAL COMPARISONS IN THE GROUP B [SOT - ROOT SERIES]
PAIRED t - TEST

COMPARED GROUPS	MEAN (X - Y)	PAIRED t VALUE	PROB/LITY
T1 - T2	3.9182	1.2713	0.2324 NS
T1 - T3	- 9.5583	- 1.9266	0.0802 NS
T3 - T4	13.9	2.7581	0.0163 p < 0.05 *
T2 - T4	0.14	0.0268	0.9792 NS

NS: not statistically significant

*** Significance Level $\alpha = 0.05$**

GROUP C [SP-CRN SERIES] and GROUP D [SOT-CRN SERIES]

The examination of the groups C of SP-CRN series and D of SOT-CRN series revealed positive immunoreactivity in both cases, with characteristic patterns.

GROUP C [SP-CRN SERIES]

In the group C [SP-CRN series], the cavity preparation area of the 3-day experimental tooth (T1) showed a layer formed of dentin pieces, debris, silver particles and inflammatory cells underneath the Ketac filling material, that was underlied by an area of secondary dentin formation, inflammatory cellular changes of acute nature and dilated blood vessels (BVs). No SP-IR nerve fibers were observed in close proximity to or into this area, in any case. The rest of the coronal pulp was normal and similar in appearance to that of the control teeth (T2, T3, T4) as described below (Figure 21).

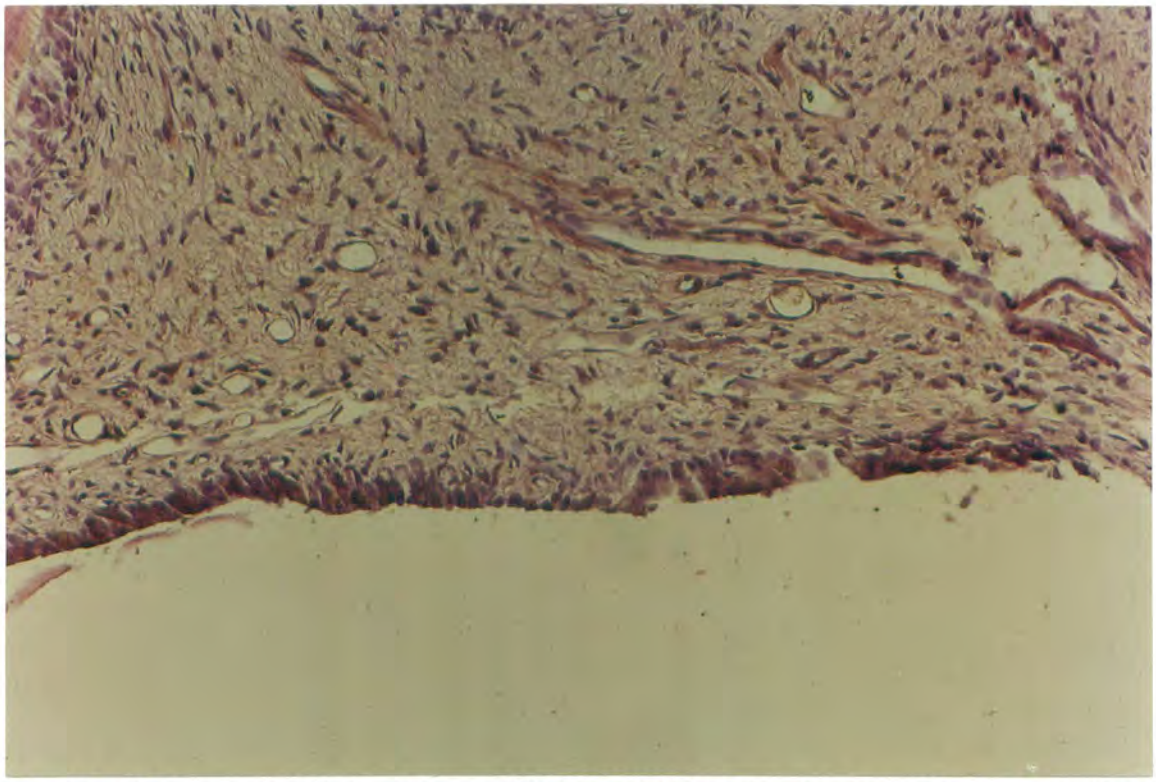


Fig. 21. SP immunostaining. Group C [SP-CROWN] series, first maxillary molar (T1) at 3rd postoperative day. Longitudinal sections of a blood vessel, accompanied by SP-IR nerve bundle as it ascends coronally.Original magnification X100.

The 7-day experimental tooth (T1) presented a similar layer orientation underneath the preparation area, with some differences. Under the capped cavity, the area that consisted of silver particles, dentin chips, inflammatory cells and debris was in many cases encapsulated and separated from the underlying pulpal tissue that in turn exhibited a characteristically higher amount of secondary dentin, more moderate inflammatory changes and somewhat fibrotic appearance. Osteodentin formation was suggested by the presence of isolated hard tissue nuclei in the more central area of the inflamed pulp. Rarely, isolated SP-IR nerve fibers were seen in the area of dilated vessels with discernible Schwann cell nuclei (Figure 22). The remaining coronal pulp was normal and the SP-fiber distribution was similar to that of the control teeth (T2, T3, T4).

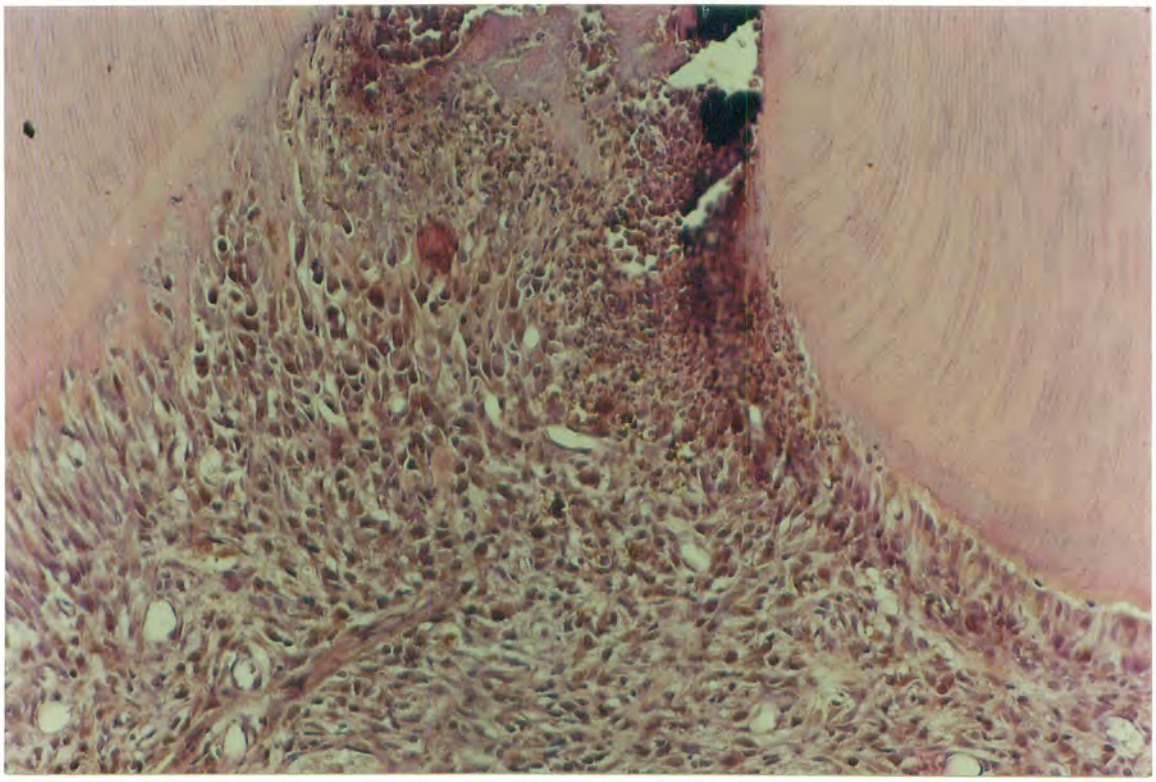


Fig. 22. SP immunostaining. Group C [SP-CROWN] series, first maxillary molar (T1) at 7th postoperative day. Area of preparation with silver particles, dentin chips, inflammatory cells, and secondary dentin at the periphery. Notice SP-IR nerve bundle with discernible Schwann cell nuclei. Original magnification X50.

Finally, the 21-day experimental molar (T1) showed a distinguished architecture at the preparation area. The previously described layer of debris, dentin plugs and silver particles was clearly walled off by an overstained rim of osteodentin that was appositioned in the form of a bridge, extending into the mesial pulpal tissue mass, to a considerable distance. Secondary dentin was also widely appositioned, with inflammatory cells entrapped in its mass more peripherally. The chronic inflammatory area was considerably reduced in extent and the rest of the pulp assumed its normal architecture right underneath it (Figure 23).

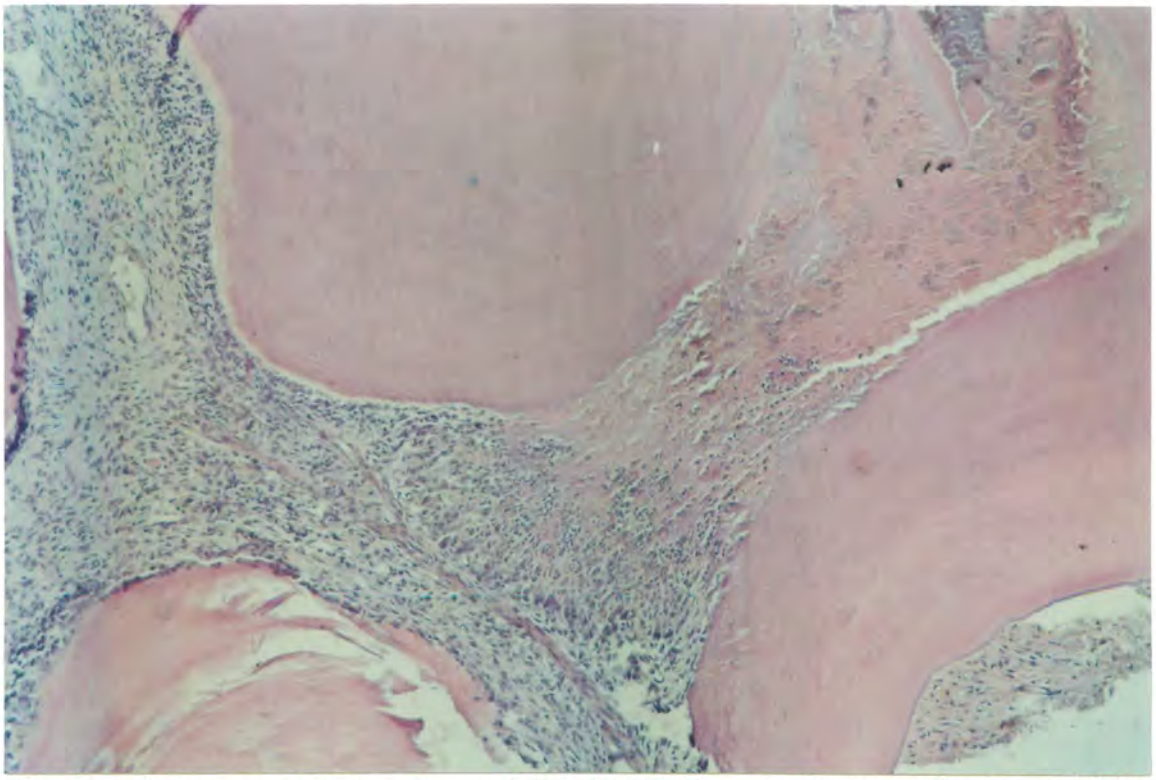


Fig. 23. SP immunostaining. Group C [SP-CROWN] series, first maxillary molar (T1) at 21st postoperative day. Dentin bridge with entrapped inflammatory cells peripherally. A SP-IR nerve bundle, without any association to blood vessel structures gives off two smaller branches as it ascends coronally. Notice the termination of one branch into the cell-rich zone area.Original magnification X25.

In the control teeth of the SP-CRN group, regardless of the time period (3-day, 7-day or 21-day) or the type of tooth (T2, T3 or T4) the SP-IR fibers entered each root in large trunks, accompanying the large BVs as it was seen in the crossections of the root series. They continued in the same way as they ascended coronally, and entered the main pulp above the cervical level (Figures 25 and 26). At this point they followed the branching of the smaller BVs, surrounding them individually or running alongside with them. Some of them followed that course further, to the pulpal horn areas while some others seemed to run as single smaller branches, without any association to BVs (Figure 24). The latter were in many cases clearly seen, ending into the cell-rich zone. We were unable to identify their further

course because of their smaller size that made their staining indistinguishable from that of the other structures of these areas and the high number of cellular elements into this area. The course of those smaller nerve branches was in most cases straight. End coursing in the form of U-turns was occasionally seen centrally and peripherally, before their entrance into the para-odontoblastic area.

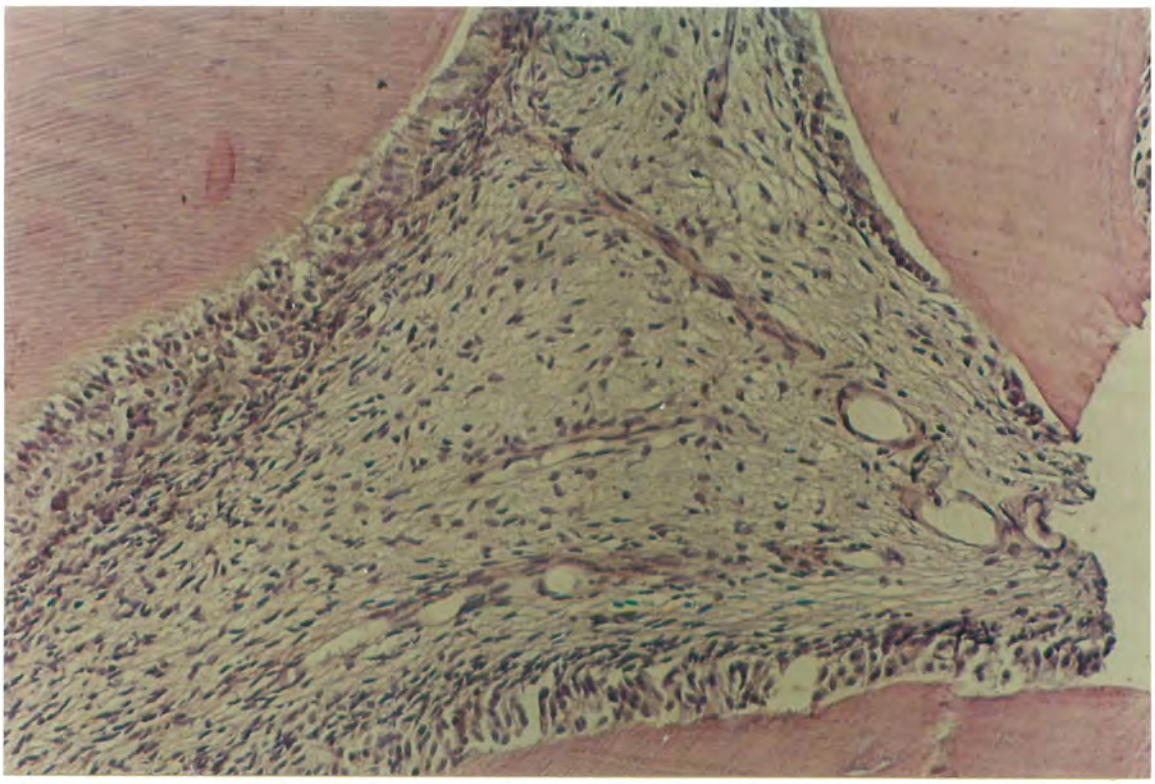


Fig. 24. SP immunostaining. Group C [SP-CROWN] series, second maxillary molar (T2) at 3rd postoperative day. SP-IR nerve bundles accompany blood vessels coronally. A small branch follows an independent course and enters the cell rich zone at the periphery. Original magnification X50.

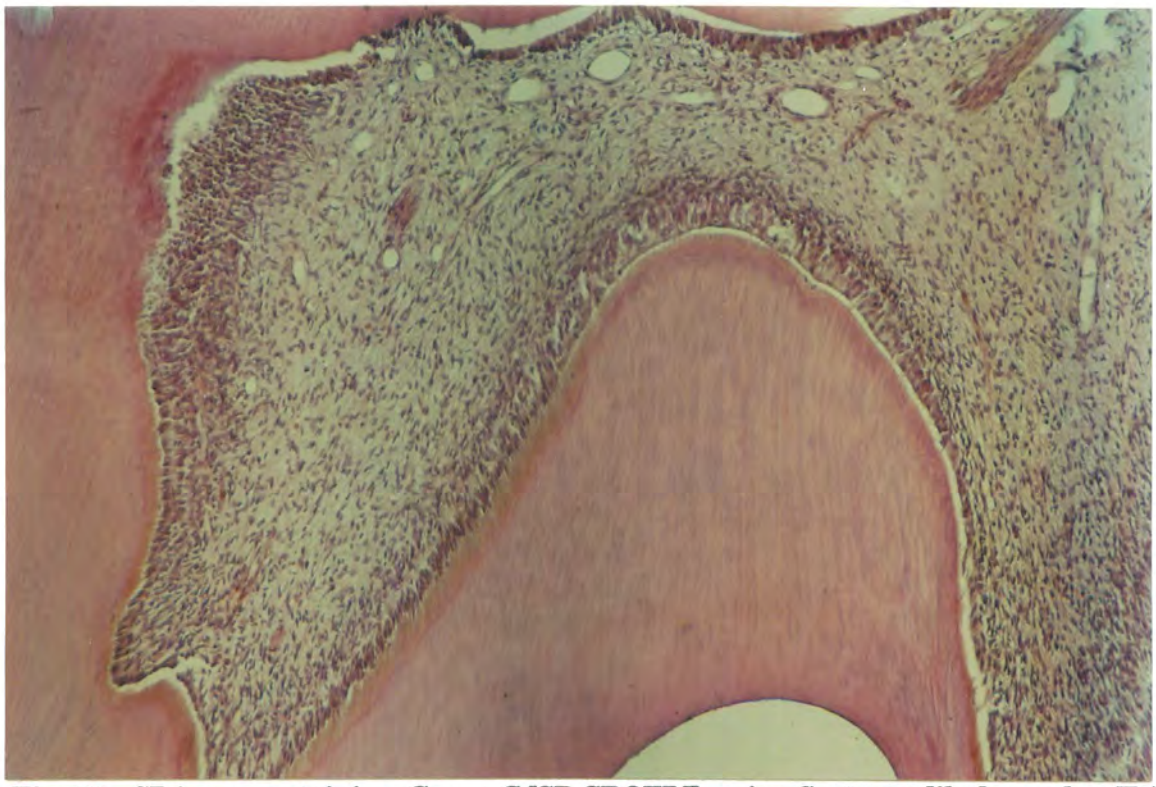


Fig. 25. SP immunostaining. Group C [SP-CROWN] series, first mandibular molar (T3) control at 7th postoperative day. Two single branches are seen, heading towards the peripheral areas. Original magnification X25.

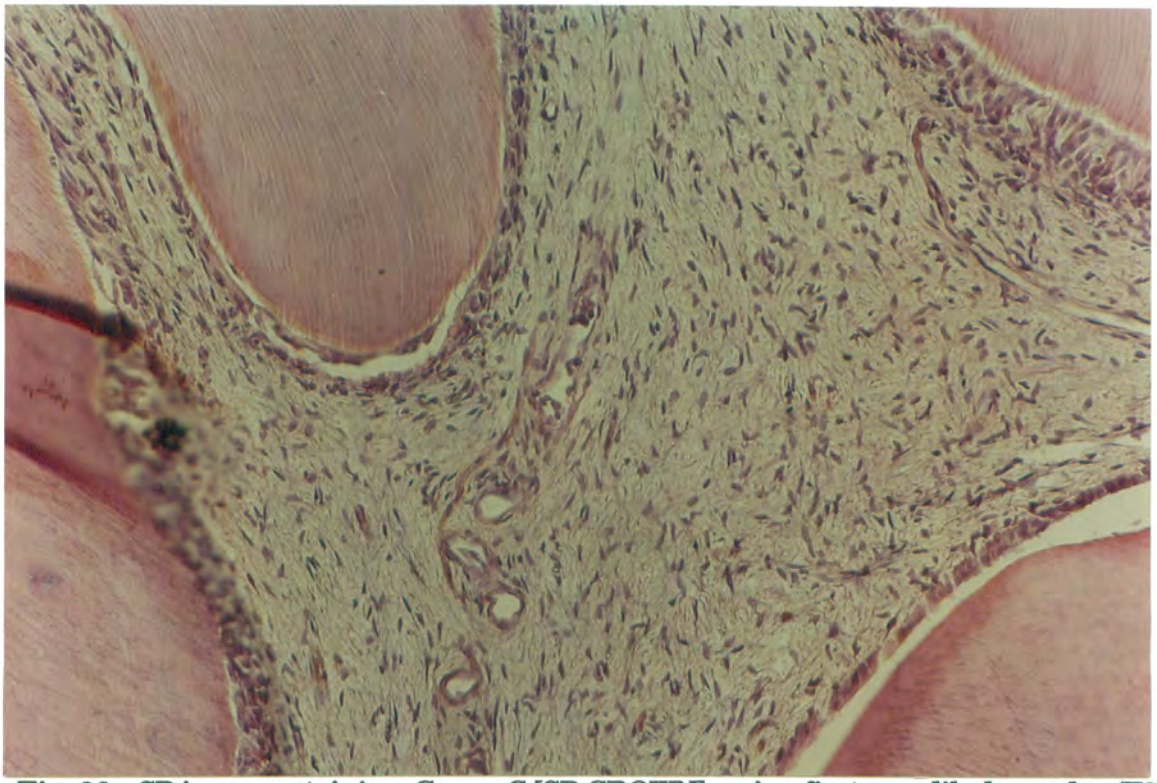


Fig. 26. SP immunostaining. Group C [SP-CROWN] series, first mandibular molar (T3) control at 21st postoperative day. Original magnification X50.

GROUP D [SOT-CRN SERIES]

The 3-day experimental tooth (T1) of the group D [SOT-CRN series] showed a parallel appearance to the same tooth of the SP-CRN series. In many cases however, the pulpal tissue showed evidence of irreversible changes with minimal amount of secondary dentin formation, more extensive areas of acute inflammation and sometimes, a wide area of cellular death that appeared highly hyalinized. Dilated blood vessels were still present at the periphery and in one case SOT-IR nerve fibers accompanied them at the immediate to the inflammatory area, pulp. Beyond the limits of the disorganized area, the pulpal appearance was similar to that of the control teeth (T1, T2, T4) as described below (Figure 27).

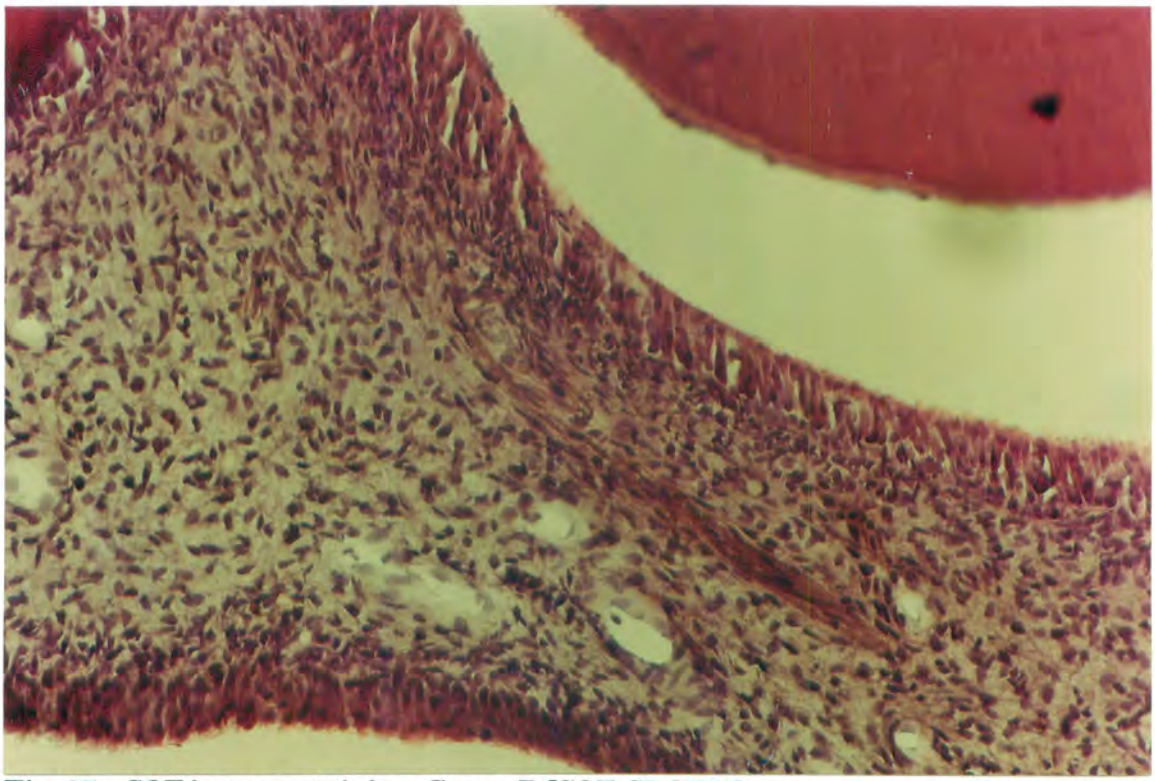


Fig. 27. SOT immunostaining. Group D [SOT-CROWN] series, first maxillary molar (T3) at 3rd postoperative day. Specimen is overstained, but a large SOT-IR nerve bundle is seen ascending and encircling a blood vessel, close to the cell-rich zone. Original magnification X50.

The 7-day experimental molar (T1) resembled in architecture the same tooth of the SP-CRN series, although many specimens were lost during the immunohistochemical processing or during the cryostat cutting. Generally, the amount of the secondary dentin was higher than the 3-day series, osteodentin nuclei were not clearly seen and in the cases of irreversible changes, microabscess formation was evidenced. The remaining pulp was similar to that of the control teeth.

Finally, the 21-day first maxillary molar (T1) did not, in any case permit specific observations because only one of the three available slide series offered an adequate amount of pulpal tissue for observation. However, even in this case the general appearance was that of extensive pulpal necrosis with hyalinized tissue in the place of osteodentin bridge, abscess formation and peripheral secondary dentin formation unable to wall off the necrotized area that extended far into the more distal pulpal areas. In the other two slide series, the inadequacy of the pulpal tissue amount was due to understaining of its major mass.

The control teeth of all SOT-CRN series (3-day, 7-day and 21-day) again presented similar findings, regardless of the tooth type (T2, T3 and T4). The SOT-IR fibers, entered the central mass of the apical pulp in large bundles of myelinated nerves as seen with the optical microscope, in close association with the large BVs (Figure 28 and 31). They ascended coronally with them, with minimal branching up to the cervical level where both types of structures gave off smaller branches closely associated with each other (Figure 30). Their course, further continued towards the pulpal horn tips in the same manner (Figure 32) and sometimes single, unassociated to BVs SOT-IR nerve fibers were running towards the odontoblastic areas of the pulpal horn and the in-between-cusps areas, where they were seen

entering the cell-rich zone (Figure 29). Their further course was unidentifiable for the same reasons explained for the SP-CRN series.

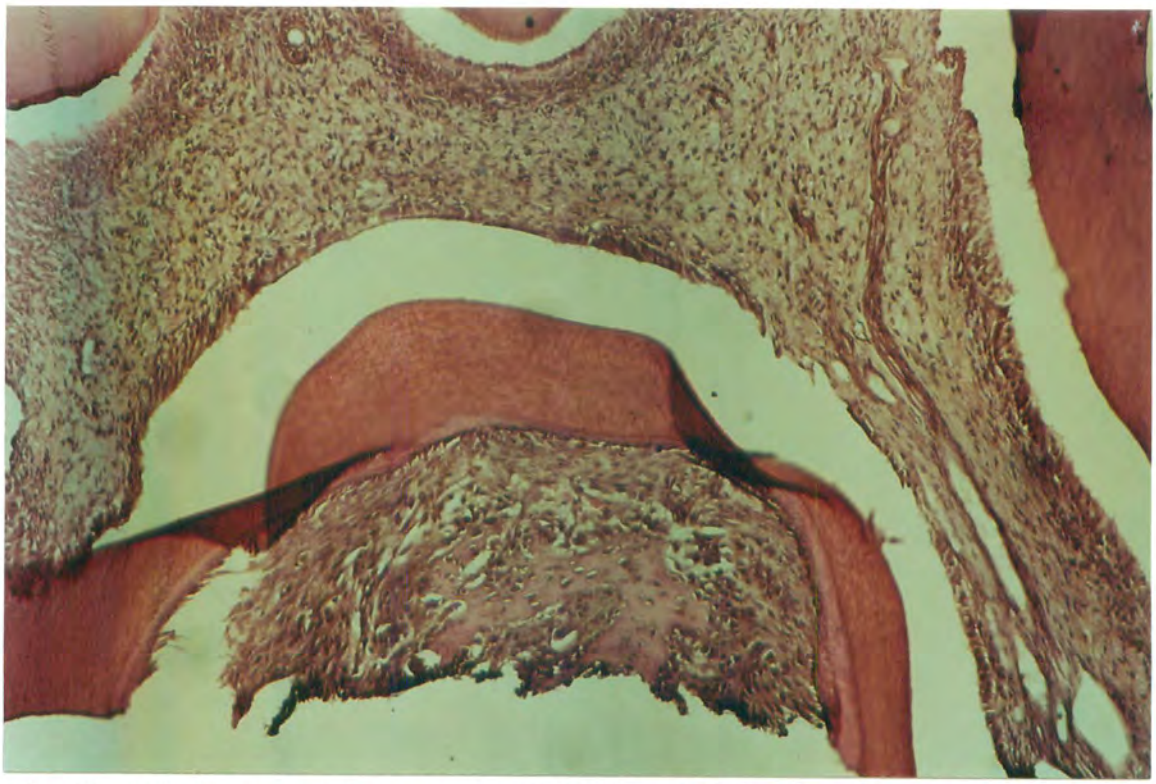


Fig. 28. SOT immunostaining. Group D [SOT-CROWN] series, second maxillary molar (T2) control at 3rd postoperative day. SOT-IR nerve bundles run along with large blood vessel trunks towards the coronal pulp. Original magnification X25.

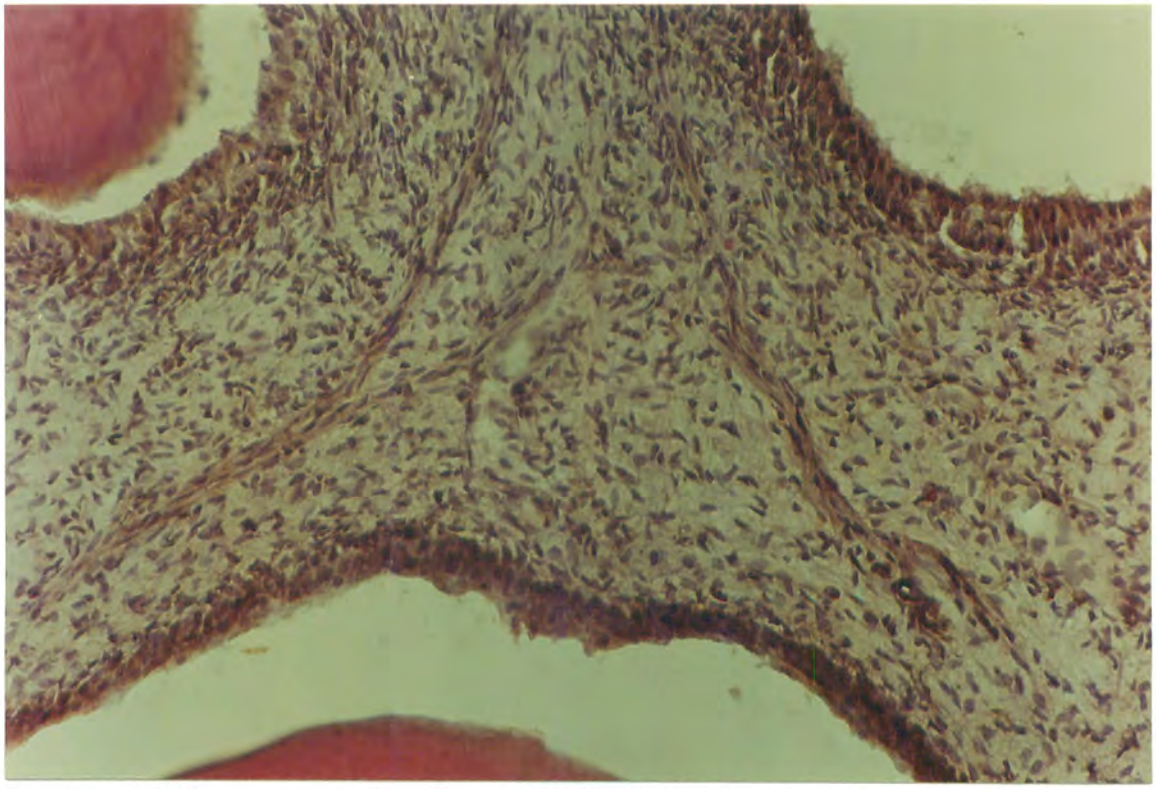


Fig. 29. SOT immunostaining. Group D [SOT-CROWN] series, second maxillary molar (T2) control at 7th postoperative day. Two main SOT-IR nerve bundles branch off and head independently, towards the pulpal horn tip. Original magnification X50.

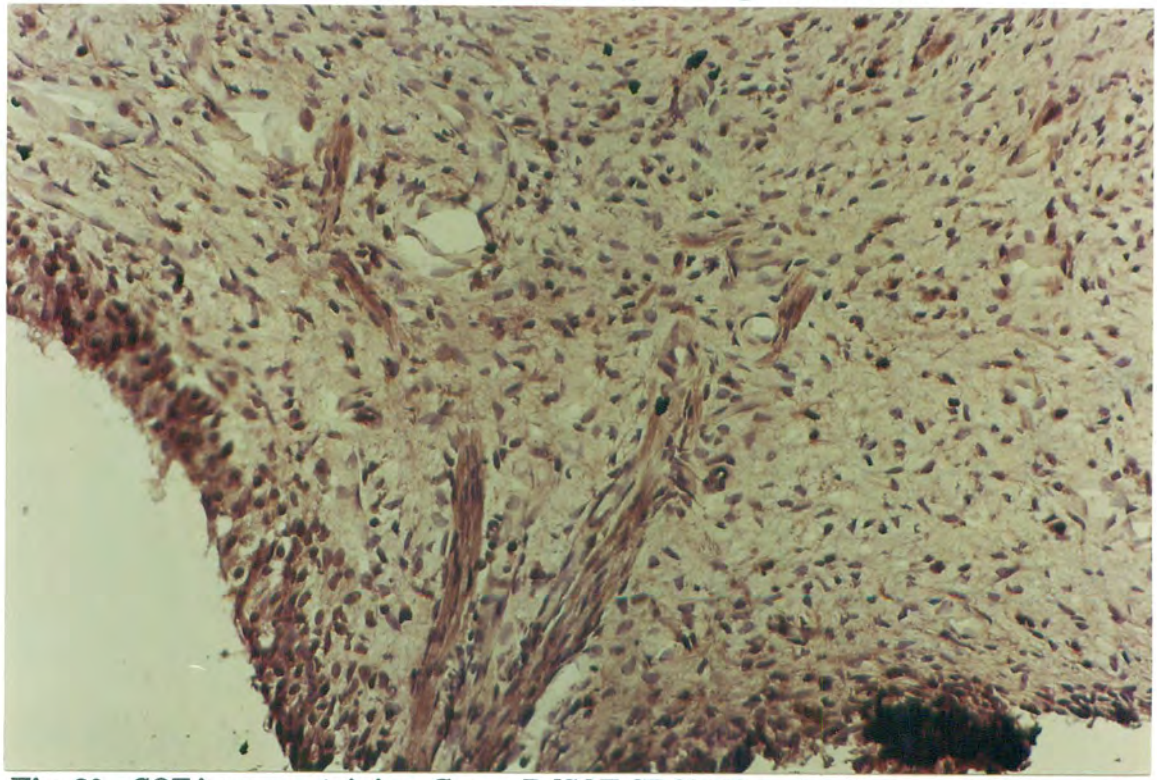


Fig. 30. SOT immunostaining. Group D [SOT-CROWN] series, first mandibular molar (T3) control at 7th postoperative day. Original magnification X50.

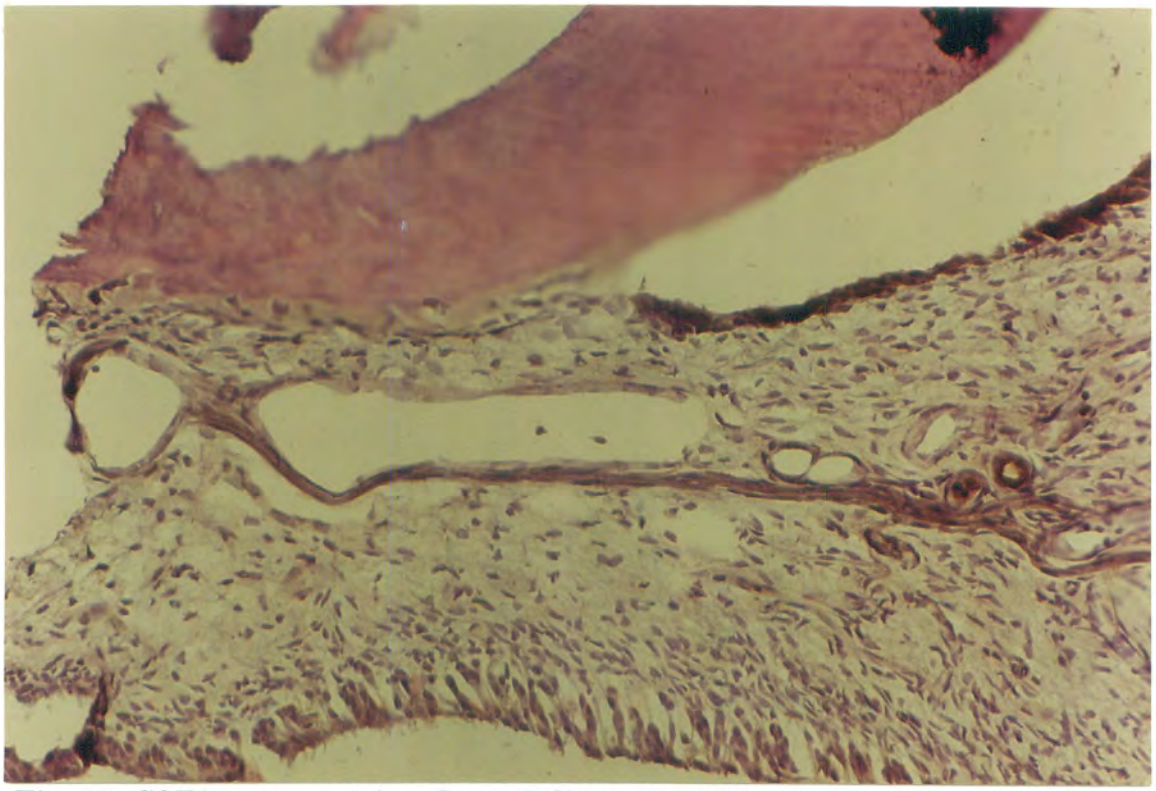


Fig. 31. SOT immunostaining. Group D [SOT-CROWN] series, second mandibular molar (T4) control at 21st postoperative day. A large vessel is accompanied by a SOT-IR bundle, up to the cervical level where branching begins. Original magnification X50.

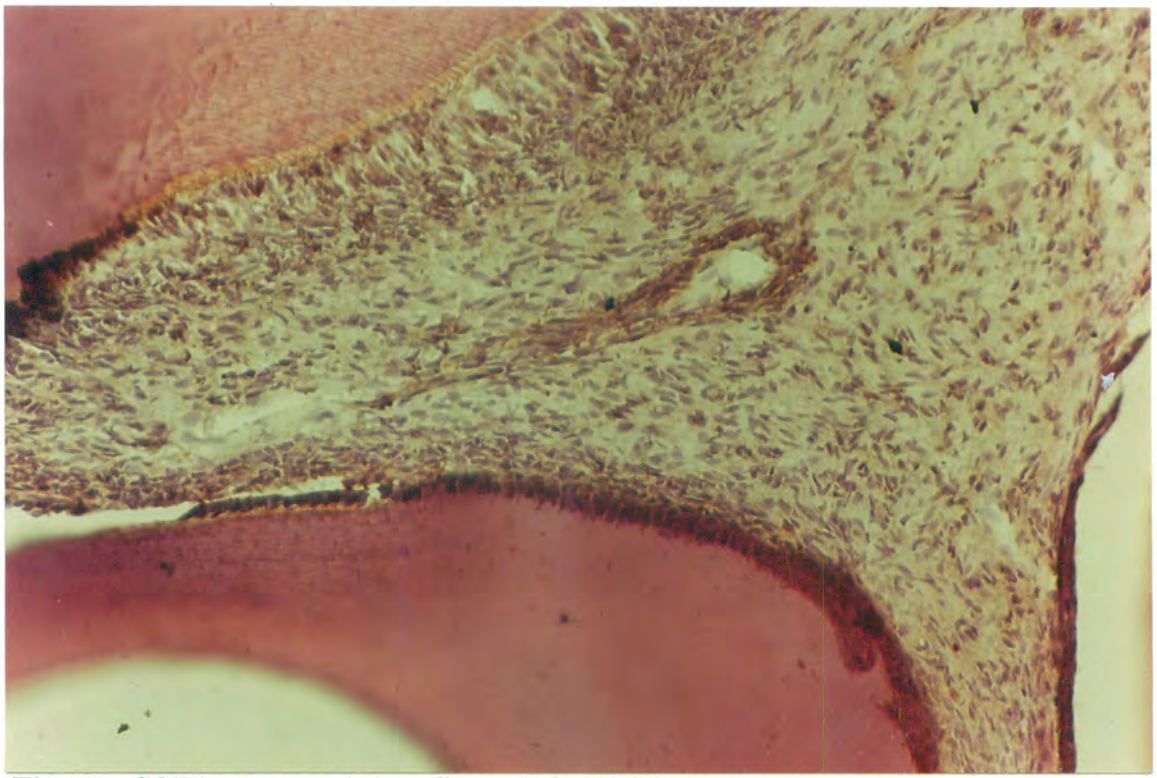


Fig. 32. SOT immunostaining. Group D [SOT-CROWN] series, second mandibular molar (T4) control at 21st postoperative day. Original magnification X50.

DISCUSSION

Although the results of the present study appear intriguing, we should emphasize the preliminary nature of this research. A statement of positive isolation of SP and SOT within the dental pulp has to be further confirmed by other methods, such as biochemical assay. The definitions of SP-like and SOT-like immunoreactivity (SP-LI and SOPT-LI) are more preferable because we cannot exclude the possibility that molecular configurations similar to SP and SOT may be present into the pulp, and this could result in false-positive staining.

Overall, SP-LI and SOT-LI were demonstrated in both the RT and CRN series of the two experimental groups. Specific immunostaining for both ligands was also clearly demonstrated in the dorsal root ganglia specimens that served as positive controls.

The experimental procedure employed in this study was chosen, based on the observations of current research regarding the effects of injury and inflammation on the reactions of the dental sensory innervation. Various types of injury such as Class V cavity preparation,[96] pulpal exposure,[211] combination of cavity preparation, acid etching and desiccation,[212] orthodontic tooth movement,[124] cut and crush injury to the IAN,[213] IAN denervation in conjunction with pulpal exposure,[97] tooth replantation[214] and traumatic occlusion[214] have been induced in order to study the relationship of type of stimuli and pulpal nerve reactions. The observed changes were characterized by nerve sprouting in the vital tissue portions and periapex. Its extent was dependent on the time interval, the area of remaining vital tissue and the intensity of the inflammation.[211,215,216]

In our design, we decided to use the combination of pulpal exposure, direct pulp capping with a calcium hydroxide agent and glass-ionomer restoration, to study the effect of this type of injury and the resultant inflammation during the healing phase, on the response of SP- and SOT-containing nerves in the rat molar pulp. According to this model, we expected to see an acute inflammatory reaction in the 3rd post-injury day, that would progressively subside, with concurrent initiation of a dentin bridge formation at the 7th post-injury day and considerable healing with a more complete bridge formation, at the 21st day. The relationship between the above pattern of healing and the changes of the SP- and SOT-containing fibers was assessed, in the root crossections, by pairwise comparison of the positively stained nerve counts among the three experimental time groups, and in the crown sections, by morphological changes observed in the neural distribution.

Based on the results of studies on the sensory neuropeptide CGRP,[96] the expected reactions of the SP-containing fibers in the 3rd day, would be proliferation of terminal branches close to the inflamed area and throughout the radicular pulp, and innervation of dentinal tubules, not seen in intact teeth. In the 7th day, the inflammatory reaction would progress to milder levels, reparative dentin would form peripherally to the induced lesion and the nerve fibers would sprout beyond the area of new dentin. Finally, in the 21st day, the healing would progress with isolation of the injured area by a more complete reparative dentin bridge and nerve distribution similar to that of uninjured teeth. When the outcome would be progressive destruction, the sprouting would persist and the amount of reparative dentin would be minimal.

Our results failed to show any statistically significant difference between the three time groups that would be interpreted as sprouting, in both cases of SP- and SOT-containing nerve fibers. No positively stained fibers were observed in the predentin and dentin areas, although all specimens were subjected to demineralization. In the crown portions, terminal nerve proliferation, although not included in our initially intended series of observations, was not one of our positive observations in any case, whether the inflammatory reaction persisted throughout the 21-day period or reached a clear stage of healing. When the overall results of the experimental root group were compared to those of the three control teeth, T3 and T4 groups were the only ones that showed a statistically significant difference in both SP and SOT nerve counts. No such difference was observed morphologically, in the crown groups of either neuropeptide.

Possible explanations for the difference between our results and those of other similar studies could be our small sample size, the fact that we used crossections for our root specimens and the immunohistochemical method used for nerve identification. A larger sample size could possibly identify differences more reliably, if they were present, use of longitudinal radicular sections could enable us to trace the pattern of distribution more clearly and finally, the higher sensitivity of RIA techniques might provide more information.

Nevertheless, SP-LI and SOT-LI were found immunohistochemically in the rat dental pulp, in our study. Although, SP-LI in the dental pulp has been previously confirmed by numerous studies using various methods, it is the first time that SOT-LI is shown by means of IHC. Gazelius and others[82,90] have found (in unpublished data), SOT immunoreactivity in the feline pulp using a radio-immunoassay technique, Akai and Wakisaka[82]

were unable to confirm its presence immunohistochemically and recently, Casasco and his associates[90] were the first to report in the dental literature, positive SOT-LI in the human pulp by means of the indirect immunofluorescence technique.

Common finding among all the above studies on SP and SOT and our study, was the pattern of perivascular distribution of both SP-IR and SOT-IR nerve fibers and the branching of single, unrelated to any other structure fibers that run towards the peripheral para-odontoblastic areas. The close association of those nerves to the vascular structures could support a role in the neurogenic inflammation phenomenon as explained previously. The mechanism of action of SP is thought to be both direct on the blood vessel wall and indirect through the release of histamine from mast cells. Regarding the mode of action of SOT, it has been shown that the peptide does not possess a similar direct vasoactive action but it can inhibit the release of SP and the antidromic vasodilation mediated by SP.[150] According to the above findings, an indirect action on the regulation of pulpal blood flow has been suggested, through pre-synaptic inhibition or modification of the release of SP from the sensory nerve terminals.[90,132,170]

Regarding the effects of the two neuropeptides on the inflammatory cells, SP stimulates the proliferation of T lymphocytes,[217] enhances the chemotaxis of neutrophils,[218] the margination and endothelial adhesion on PMNs and monocytes in venules,[100] their phagocytotic activity [107] and the the mast cell degranulation.[100] SOT on the other hand, inhibits the release of histamine from basophils but not mast cells, stimulates the macrophage function, suppresses the T lymphocyte activation[100] and inhibits the neutrophil chemotaxis caused by SP.[218] Although the effects of the two neuropeptides seem opposing, it is suggested that their antagonistic action

during the process of immunological phenomena, continues until a level of tachyphylaxis is achieved.[100] It is believed that at this level, both neuropeptides reach physiologically meaningful concentrations, able to modify the development and functions of immunocompetent cells at desirable pathways of interaction.[100]

The effect of sensory neuropeptides on immune responses[100] and wound healing,[92-94] has opened new fields of investigation regarding the role of sensory neuropeptides on pulpal would healing. Recently, Byers and Taylor (1993)[97] found that innervated teeth have a better chance of survival and heal more rapidly than denervated teeth, when both are subjected to occlusal exposures. Nerve sprouting of CGRP-IR fibers was a characteristic of the healing process and the authors suggested a mechanism where a nerve growth factor (NGF) is produced by injured fibroblasts, that facilitates the nerve sprouting in order to increase the amount of released sensory neuropeptides in the area, and thus promote the process of neurogenic inflammation and therefore healing. According to a study by Kessler and Black,[219] treatment with NGF and capsaicin resulted in significant increase and decrease respectively, of the content of both SP and SOT in dorsal root ganglia and spinal cord tissue specimens. The authors suggested that SP and SOT neuronal populations develop simultaneously and their neurons possess NGF receptors.

Finally, it is apparent that both peptides possess a diversity of actions at various levels of investigation and that further research is required in order to elucidate their individual actions, interactions and role in pulpal inflammation and pain mechanisms.

Results and Study Design

The perfusion fixation of all animals (#1-#16) was considered satisfactory and ranged from good to excellent. The cold fixation that followed, was also adequate as confirmed histologically on H & E stained sections, and overfixation with consequent loss of antigenicity was not a concern. Improvements such as trypsin incubation could be utilized to uncouple protein cross-linkages and therefore, uncover antigenic sites. Decreased staining as it appeared in some cases, could be due to fixation or decalcification. Possibly, use of frozen sections of freshly extracted teeth could provide additional information. Heavy staining of the peripheral odontoblastic and para-odontoblastic areas as well as areas of inflammatory infiltrate was characteristic in both experimental groups (SP and SOT). Possible nonspecific staining due to endogenous peroxidase activity was a concern and hydrogen peroxide was used to minimize this threat.

In the SP root series, the specimens #2 of maxillary molars and # 6, 8, 10, 14 and 16 of mandibular molars were lost during the sectioning part of the histological procedure. Similarly, the specimens #6, 10 and 12 of maxillary molars and #10 and #16 of mandibular molars of the SOT root series as well as the specimens #6, 10, 12 and 15 of maxillary molars and #10, and 12 of the SOT crown series were considered lost due to inadequate sectioning or poor adhesion on the aminoalykylsilane-treated slides, that resulted in loss of the major part of the soft tissue during the immunohistochemical processing.

Certain points that appear controversial in the methodology chosen, together with a collective presentation of the currently available routes of investigation, were considered appropriate for discussion.

First, the use of polyclonal antibodies that can be viewed as a flaw of the design, was chosen in order to increase the likelihood of detecting positive immunoreactivity, if present. Monoclonal antibodies have higher specificity, however they may paradoxically result in loss of specificity, at a higher expense as well. Use of a combination of two monoclonal antibodies, specific for different epitopes on the ligand molecule is also a suggestion. Second, the preabsorption of normal blocking serum for the primary antibody, that served as a negative control could have been supplemented with additional negative controls in order to eliminate the potential of cross-reactivity. Such a control would use preabsorption of the primary antibody by the known and other closely related ligands. Third suggestion could be the use of a negative control for each sample that would offer a better comparison of positive, negative and background stainings.

Further research regarding the pathophysiological interactions of the two neuropeptides, especially in the case of SOT that is the least investigated in the dental pulp, can be achieved through the following routes:

1) Immunohistochemical study using monoclonal antibodies,
2) radioimmunoassay or receptor autoradiography for quantitation of the peptides,
3) biochemical study by enzyme assay or high performance liquid chromatography,
4) detection of mRNA gene expression by DNA in situ hybridization,
5) nerve conduction study following application of dilute ligands to exposed dentin and pulp,

6) study of axonal transport of the ligands after treatment with colchicine or capsaicin,

7) electron microscopic IHC for cellular and ultrastructural localization and

8) study of the peptides in developing dental papilla.

SUMMARY

The purpose of this study was to investigate the occurrence and distribution of Substance P (SP) and somatostatin (SOT) in the dental pulp of rat molars, by means of immunohistochemistry (IHC). The reactions of SP- and SOT-containing nerve fibers to a combination of pulpal exposure and direct pulp capping type of injury, were further examined.

The two maxillary first molars of sixteen male rats were subjected to pulpal exposure injuries, capped with a fast set calcium-hydroxide agent and a silver glass-ionomer restoration. After intervals of 3, 7 and 21 days, five animals (with the exception of the last series that included six animals) were killed after anesthetization and intracardial perfusion with Zamboni fixative. The maxillas and mandibles were removed, fixed, decalcified and cut into root (RT) and crown (CRN) parts. The maxillary and mandibular halves of the right side of each animal, were embedded in paraffin, sectioned at five microns and subjected to Avidin-Biotin-Peroxidase Complex Immunohistochemistry (ABC-IHC) for SP. The left side halves were frozen in liquid nitrogen, cut on a cryostat at five to ten microns and subjected to ABC-IHC for SOT. Specimens of dorsal root ganglia served as positive controls for the SP and SOT analysis. Positive SP- and SOT-IR nerve fibers were counted in crossections of the RT series and their pattern of distribution was examined morphologically, in the CRN series. The results of the RT series were tabulated and examined statistically.

The results of this study showed the presence of specific SP-like and SOT-like immunoreactivity within the pulp of operated and intact rat molars. No statistically significant difference in the number of either SP- or SOT-positive fibers was found between the three time groups (3,7 and 21

days). The number of SP-IR and SOT-IR fibers did not differ significantly between experimental and control groups. Both types of fibers showed a perivascular distribution pattern and occasionally single, unrelated to any other structure fibers were seen running towards the para-odontoblastic area, up to the level of the cell-rich zone.

A role in pulpal hemodynamics is suggested by our series of experiments for both peptides. A direct action on the pulpal vessel wall and an indirect one through the release of histamine from mast cells, are currently believed to consist the mechanism of action of SP in the dental pulp. The implications of SOT localization in the pulp are still unknown, but based upon its physiology in other tissues, SOT may play an indirect role in pulpal haemodynamics by inhibiting the release of SP and the antidromic vasodilation mediated by SP.

CONCLUSIONS

1. SP-like and SOT-like nerve fibers were immunohistochemically identified in the rat molar pulp of both injured and intact teeth.

2. There was no statistically significant difference in the number of SP-IR and SOT-IR nerve fibers between the 3-day, 7-day and 21-day time groups.

3. There was no statistically significant difference in the number of SP-IR and SOT-IR nerve fibers, between the experimental group of first maxillary molar (T1) and the control groups of second maxillary molar (T2), first mandibular molar (T3) and second mandibular molar (T3).

4. There was a statistically significant difference ($p< 0.05$) in the number of SP-IR and SOT-IR nerve fibers, between the control groups of mandibular first molar (T3) and mandibular second molar (T4).

5. SP-IR and SOT-IR nerve fibers showed a characteristic perivascular pattern of distribution in both root and crown series.

6. In some specimens of the crown series, single, unrelated to any other anatomical structure SP-IR and SOT-IR nerve fibers, were running towards the para-odontoblastic area up to the level of the cell-rich zone.

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APPENDIX I

Avidin - Biotin Complex Immunohistochemistry

AVIDIN - BIOTIN COMPLEX IMMUNOHISTOCHEMISTRY

1. Prepared paraffin embedded sections and frozen sections as described above.
2. Deparaffinized in xylene and hydrated through graded alcohol series.
3. Rinsed in distilled water for 5 minutes.
4. Quenched endogenous peroxidase activity with incubation for 30 minutes in 0.3% H_2O_2 (Mallinckrodt Specialty Chemicals Co., Paris, KY) in absolute methanol.
5. Washed in PBS-X buffer for 20 minutes.
6. Incubated sections for 20 minutes with diluted normal serum (Normal Goat Serum, Vector Laboratories, Burlingame, CA).
7. Blotted excess serum from sections.
8. Incubated paraffin sections overnight (22-26 hours) at 4^0C with primary anti-SP Rabbit polyclonal antibody (Calbiochem, San Diego, CA), at 1: 750 dilution in PBS-X buffer. Incubated frozen sections overnight (10-20 hours) at 4^0C with primary anti-SOT Rabbit polyclonal antibody (Dako Corporation, Carpinteria, CA), at 1: 750 dilution in PBS-X buffer (see below).
9. Washed slides in PBS-X buffer for 30 minutes.
10. Incubated sections for 30 minutes with diluted biotinylated secondary antibody (anti-Rabbit Biotinylated IgG)
11. Washed slides for 10 minutes in PBS-X buffer.
12. Incubated sections for 60 minutes with VECTASTAIN ABC Reagent (Vector Laboratories, Burlingame, CA).
13. Incubated sections in peroxidase substrate solution (3,3' diaminobenzidine or DAB) (Vector Laboratories, Burlingame, CA) for 8-10 minutes, for both SP and SOT.
14. Washed sections for 5 minutes in tap water and
15. Counterstained in hematoxylin and eosin and mounted, dehydrated and coverslipped with Permount.

APPENDICES II THROUGH IX

Data

APPENDIX II

GROUP A [ROOTS]
SP ANALYSIS

Animal	Sl.#	T1	Avg	T2	Avg	Sl.#	T3	Avg	T4	Avg
#1	1	33,22,19	24.7	50,26,23,22	30.3	1	90,86,38,11	56.3	95,81,20	65.3
	2	40,16,15,9	20	31,25,24	26.7	2	119,107,33,8	66.8	83,80,32	65
	3	58,15,11,4	22	41,29,28,22	30	3	114,76,36,12	59.5	112,95,37	81.3
	4	48,34,26,14	30.5	46,37,26,13	30.5	4	87,84,36,7	53.5	92,50,25	55.7
	5					5				
Ma±SD			24.3±4.6		29.4±1.8			59±5.7		66.8±10.6
#2	1					1	139,102,22	87.7	67,20	43.5
	2					2	138,134,25	99	136,23	79.5
	3					3	101,70,28	63.3	32	32
	4					4	126,79,17	74	Eliminated	
	5	Lost during histological processing				5				
Ma±SD								81±15.6		51.7±24.8
#3	1	Eliminated		Eliminated		1	Eliminated		96,19	57.5
	2	39,23,11,9,7	17.8	39,34,30,6	27.3	2	Eliminated		86	86
	3	20,12	16	50,46	48	3	Eliminated		108	108
	4	22,17	19.5	34,27	30.5	4	Eliminated		19	19
	5					5				
Ma±SD			17.8±1.8		35.3±11.1					67.6±38.4
#4	1	65,56,31	50.7	97,71,21	63	1	69,65,52	62	110,57,15	60.7
	2	77,71,66	71.3	99,72,30	67	2	66,60,46	57.3	87,61,15	54.3
	3	66,63,54	61	119,59,21	63.3	3	69,64,63	65.3	82,75,14	57
	4	68,64,57	63	88,75,17	60	4	66,66,65	65.7	112,70,19	67
	5	70,61,56	62.3	81,80,25	62	5	62,61,49	57.3	72,65,19	52
Ma±SD			61.7±7.3		63.1±2.6			61.5±4.1		58.2±5.9

APPENDIX III

GROUP A [ROOTS]
SP ANALYSIS

Animal	Sl.#	T1	Avg	T2	Avg	Sl.#	T3	Avg	T4	Avg
#5	1	60,40,39,34,18	38.2	59,58,45,18	45	1	105,56	80.5	30,26,25	27
	2	55,40,37,27,26	37	58,49,35,21	40.8	2	95,42	68.5	45,21,20	28.7
	3	43,36,35,25,15	30.8	59,55,36,32	45.5	3	127	127	82,66,32	60
	4	53,51,47,23,18	38.4	54,51,42,30	44.3	4	69	69	119,73,20	70.7
	5					5				
Ma±SD			36.1±3.6		43.9±2.1			86.2±27.7		46.6±22.1
#6	1	36,27	31.5	77,56	66.5	1				
	2	47,34	40.5	66,38	52	2				
	3					3				
	4					4				
	5					5	Lost during histological processing			
Ma±SD			36±6.4		59.2±10.3					
#7	1	112,84,53,34	70.8	58,38	48	1	83,38,24,17	40.5	104,51,19	58
	2	88,65,44,40	59.3	73,55,44,34	51.5	2	122,44,11	59	74,34,22	43.3
	3	72,61,57,52	60.5	44,37,36	39	3	84,83,10	59	38,19,17	24.7
	4	95,73,51	73	21	21	4				
	5					5				
Ma±SD			65.9±7		39.9±13.6			52.8±10.7		42±16.7
#8	1	62,37,36,32,10	35.4	55,44,36,29	41	1				
	2	57,50,32,29,11	35.8	50,40,33,29	38	2				
	3	62,53,46,31,20	42.4	55,44,43,28	42.5	3				
	4	56,55,42,25,17	39	47,36,34,32	37.3	4				
	5					5	Lost during histological processing			
Ma±SD			38.1±3.3		39.7±2.5					

APPENDIX IV

GROUP A [ROOTS]
SP ANALYSIS

Animal	Sl.#	T1	Avg	T2	Avg	Sl.#	T3	Avg	T4	Avg
#9	1	70,60,17	49	39,36,32,22	32.3	1	79,76,34,27	54	51,35,10	32
	2	67,30,25	40.7	43,34,29,25	32.8	2	82,78,33,25	54.5	18,18	18
	3	61,39,39	46.3	45,28,21,18	28	3	38,36,26	33.3	14,11	12.5
	4	69,34,28	43.7	40,26,25,23	28.5	4	56,40	48	Eliminated	
	5	64,41,32,8	36.3	50,43,38,34	41.3	5				
Ma±SD			43.2±4.9		32.6±5.3			47.5±9.9		20.8±10.1
#10	1	53,47,38,14	38	45,32	38.5	1				
	2	32	32	42,33	37.5	2				
	3	Eliminated		49,46	47.5	3				
	4	43,43	43	34	34	4				
	5					5	Lost during histological processing			
Ma±SD			37.7±5.5		39.4±5.8					
#11	1	36,35	35.5	56	56	1	106,40,22	56	81	81
	2	39,37	38	67,14	40.5	2	87,52,11	50	15	15
	3	40,23,20	27.7	32,16	24	3	90,44,20	51.3	15	15
	4	64,39,34	45.7	51,10	30.5	4	94,57,14	55	16	16
	5					5				
Ma±SD			36.7±7.4		37.8±13.9			35.1±2.9		31.8±32.8
#12	1	53,42,35,32	40.5	59,37,36,31	40.8	1	Eliminated		Eliminated	
	2	65,54,36,31	46.5	53,49,46,45	48.3	2	Eliminated		9	9
	3	65,49,47,32	48.3	50,43,36,29	39.5	3	Eliminated		30	30
	4	49,43,28,20	35	50,39,37,29	38.8	4	Eliminated		22	22
	5					5				
Ma±SD			42.6±6.1		41.9±4.4					20.3±10.6

APPENDIX V

GROUP A [ROOTS]
SP ANALYSIS

Animal	Sl.#	T1	Avg	T2	Ms	Sl.#	T3	Avg	T4	Avg
#13	1	33	33	37,26	31.5	1	Eliminated		Eliminated	
	2	48	48	30,20	25	2	Eliminated		Eliminated	
	3	60,32	46	43,32	37.5	3	Eliminated		Eliminated	
	4					4	Eliminated		47	47
	5					5				
Ma±SD			42.3±8.1		31.3±6.3					47
#14	1	47,47,46,27,11	35.6	66,48,31,23	42	1				
	2	51,45,41,36	43.3	41,31,26,14	28	2				
	3	35,34,30,27	31.5	33,28,26	29	3				
	4	61,30,25,25,20	32.2	40,33,32,21	31.5	4				
	5	58,37,37,29	40.3	31	31	5	Lost during histological processing			
Ma±SD			36.6±5.1		32.3±5.6					
#15	1	59	59	47,31	39	1	93,88,28,23	58	54,51,24	43
	2	66,55,50,20	47.8	58,51,50	53	2	86,29	57.5	30,21	25.5
	3	49,48,35,19	37.8	49,37,34,24	36	3	93,71,35	66.3	42,32,14	29.3
	4	34,32,30,24	30	47,39,39,26	37.8	4	63,50,20	44.3	21,19,14	18
	5					5				
Ma±SD			43.7±12.6		41.5±7.8			56.5±9.1		29±10.5
#16	1	65,44,41,22	43	55,41,40,36	43	1				
	2	63,32	47.5	41,38,37	38.7	2				
	3	75,43,18	45.3	58,43,31,28	40	3				
	4	55,50,15	40	47,42,29	39.3	4				
	5					5	Lost during histological processing			
Ma±SD			44±3.2		40.2±1.9					

APPENDIX VI

GROUP B [ROOTS]
SOT ANALYSIS

Animal	Sl.#	T1	Avg	T2	Avg	Sl.#	T3	Avg	T4	Avg
#1	1	31,27,15	24.3	71,32	51.5	1	128,78,47,28	70.3	109	109
	2	58,39,32,16	36.3	32	32	2	91	91	22	22
	3	40,39,16	31.7	53,38,33	41.3	3	102,89	95.5	Eliminated	
	4					4				
	5					5				
Ma±SD			30.8±6.1		41.6±9.8			85.6±13.4		65.5±61.5
#2	1	107,69,42,40,19	55.4	57	57	1	66	66	67,21	44
	2	64,47,32	47.7	80,41,30	50.3	2	Eliminated		Eliminated	
	3	110,80,45	78.3	89	89	3				
	4	107,74	90.5	53,32	42.5	4				
	5					5				
Ma±SD			68±19.9		59.7±20.4			66		44
#3	1	97,37	67	Eliminated		1	Eliminated		Eliminated	
	2	115,51	83	Eliminated		2	110,63,42,39	63.5	80,78,65	74.3
	3	108,41	74.5	Eliminated		3	98,80,41,21	60	107,97,65	89.7
	4	85	85	Eliminated		4	93,82,26,13	53.5	104,102,83	96.3
	5					5				
Ma±SD			77.4±8.3					59±5.1		86.8±11.3
#4	1	78,46	62	Eliminated		1	93,45	69	47	47
	2	Eliminated		Eliminated		2	71,15	43	77,13	45
	3	58,50,43	50.3	Eliminated		3	69	69	64	64
	4	Eliminated		Eliminated		4	98	98	87,86	86.5
	5					5				
Ma±SD			56.2±8.3					69.8±22.5		60.6±19.2

APPENDIX VII

GROUP B [ROOTS]
SOT ANALYSIS

Animal	Sl.#	T1	Avg	T2	Avg	Sl.#	T3	Avg	T4	Avg
#5	1	54	54	44	44	1	109,84,21	71.3	58,56,24	46
	2	Eliminated		Eliminated		2	84,67,13	54.7	52,50,15	39
	3	51,42,38	43.7	65	65	3	103,70,30	67.7	43	43
	4	54,50,34	46	53,38,35	42	4	84,68,37	63	58,18	38
	5					5				
Ma±SD			47.9±5.4		50.3±12.7			64.2±7.2		41.5±3.7
#6	1	Lost during histological processing				1	114,74	94	101,71	86
	2					2	107,70,18	65	79,60,10	49.7
	3					3	94,88,43	75	98,61	79.5
	4					4	115	115	88	88
	5					5				
Ma±SD								87.2±22.1		75.8±17.8
#7	1	Eliminated		53,51	52	1	105,59	82	99,27	63
	2	Eliminated		46,9	27.5	2	119,71	95	84,53	68.5
	3	Eliminated		76,66	71	3	115,69,34	72.7	103,26	64.5
	4	86,63	74.5	77	77	4	81,53	67	56,28	42
	5	72	72							
Ma±SD			73.2±1.8		56.9±22.3			79.2±12.2		59.5±11.9
#8	1	Eliminated		Eliminated		1	75,66	70.5	84,16	50
	2	87,70,26	61	74,43	58.5	2	60,59	59.5	64	64
	3	Eliminated		Eliminated		3	76,59	67.5	53,50	51.5
	4					4	78,64	71	80,62	71
	5					5				
Ma±SD			61		58.5			67.1±5.3		59.1±10.1

APPENDIX VIII

GROUP B [ROOTS]
SOT ANALYSIS

Animal	Sl.#	T1	Avg	T2	Avg	Sl.#	T3	Avg	T4	Avg
#9	1	Eliminated		Eliminated		1	72,25	48.5	Eliminated	
	2	Eliminated		Eliminated		2	Eliminated		Eliminated	
	3	49	49	59	59	3	102	102	39	39
	4	69	69	64	64	4	68,52,32	50.7	69	69
	5					5	Eliminated		18	18
Ma±SD			59±14.1		61.5±3.5			67.1±30.3		42±25.6
#10	1					1				
	2					2				
	3					3				
	4					4				
	5	Lost during histological processing				5	Lost during histological processing			
Ma±SD										
#11	1	86,68,35	63	82,32	57	1	Eliminated		54	54
	2	97,85	91	51,42	46.5	2	96	96	Eliminated	
	3	Eliminated		60	60	3	90	90	43,43,24	36.7
	4					4				
	5					5				
Ma±SD			77±19.8		54.5±7.1			93±4.2		45.3±12.2
#12	1					1	79,70	74.5	Eliminated	
	2					2	84,34	59	25,20	22.5
	3					3	80,17	48.5	33	33
	4					4	88,73	80.5	Eliminated	
	5	Lost during histological processing				5				
Ma±SD								65.6±14.6		27.8±7.4

APPENDIX IX

GROUP B [ROOTS]
SOT ANALYSIS

Animal	Sl.#	T1	Avg	T2	Avg	Sl.#	T3	Avg	T4	Avg
#13	1	102,45	73.5	50,35	42.5	1	64,59	61.5	66	66
	2	48	48	67	67	2	62,54,30	48.7	75	75
	3	70	70	61	61	3	77,44	60.5	97	97
	4	48	48	53	53	4	80,68	74	66	66
	5	47	47	34	34	5	68,67	67.5	88,34	61
Ma±SD			57.3±13.3		51.5±13.4			62.4±9.4		73±14.3
#14	1	Eliminated		Eliminated		1	96,63,34	64.3	76,39	57.5
	2	50	50	21	21	2	70,67,34	57	50	50
	3	47	47	53	53	3	68,67,32	55.7	74	74
	4					4	88,59,38	61.7	43	43
	5					5				
Ma±SD			48.5±2.1		37±22.6			59.7±4		56.1±13.3
#15	1	52	52	44,30	39.5	1	Eliminated		23,20	21.5
	2	56	56	Eliminated		2	Eliminated		40,18	29
	3	20	20	58	58	3	Eliminated		Eliminated	
	4	22	22	40	40	4	38,33	35.5	50,28	39
	5					5				
Ma±SD			37.5±19.1		45.8±10.5			35.5		29.8±8.8
#16	1	Eliminated		64	64	1				
	2	42,26	34	18	18	2				
	3	54,37	45.5	50,32,25,23	32.5	3				
	4	Eliminated		41,31,27	33	4				
	5	35,32	33.5	45,36	40.5	5	Lost during histological processing			
Ma±SD			37.7±6.8		37.6±16.9					