Original Article

Effect of Propolis on Dentin Regeneration and the Potential Role of Dental Pulp Stem Cell in Guinea Pigs

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Abstract

Objective: Evaluation of the effect of Propolis as a bioactive material on quality of dentin and presence of dental pulp stem cells.

Materials and Methods: For conducting this experimental split-mouth study,a total of 48 maxillary and mandibular incisors of male guinea pigs were randomly divided into an experimental Propolis group and a control calcium hydroxide group. Cutting the crowns and using Propolis or calcium hydroxide to cap the pulp, all of the cavities were sealed. Sections of the teeth were obtained after sacrificing 4 guinea pigs from each group on the 10th, 15th and 30th day. After they had been stained by hematoxylin and eosin (H&E), specimens underwent a histological evaluation under a light microscope for identification of the presence of odontoblast-like cells, pulp vitality, congestion, inflammation of the pulp and the presence of remnants of the material used. The immunohistochemistry (IHC) method using CD_{29} and CD_{146} was performed to evaluate the presence of stem cells and the results were statistically evaluated by Kruskal-Wallis, Chi Square and Fisher tests.

Results: In H&E stained specimens, there was no difference between the two groups in the presence of odontoblast-like cells, pulp vitality, congestion, inflammation of the pulp and the presence of remnants of used material($p>0.05$). There was a significant difference between the quality of regenerative dentin on the 15th and 30th days ($p<0.05$): all of the Propolis cases presented tubular dentin while 14% of the calcium hydroxide cases produced porous dentin. There was no significant difference between Propolis and calcium hydroxide in stimulation of dental pulp stem cells (DPSCs).

Conclusion: This study which is the first one that documented the stimulation of stem cells by Propolis, provides evidence that this material has advantages over calcium hydroxide as a capping agent in vital pulp therapy. In addition to producing no pulpal inflammation, infection or necrosis this material induces the production of high quality tubular dentin.

Keywords: Dental Pulp Stem Cell, Dentin, Guinea Pigs, Propolis, Regeneration

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Introduction

Dental pulp is sometimes exposed during clinical procedures, such as cavity preparation or caries removal. In this situation, the pulp is involved in a process called reparative dentinogenesis, where some of the cells deposit a new matrix as a barrier in the injured site. It has been shown that adult dental pulp contains precursor cells capable of forming odontoblast-like cells in response to appropriate signals and materials (1). So, in certain cases, using direct pulp capping to save pulpal health and function is recommended. Since the selection of the capping material is a critical factor to produce the best treatment outcome, many studies of capping materials are carried out by researchers. The ideal properties of pulp capping agents are infection

control, ease of handling, prevention of micro leakage and promotion of hard tissue formation (2). During the reparative process in exposed pulp primary odontoblasts that were lost are replaced with newly differentiated odontoblast-like cells. This process is known to follow the sequential steps of proliferation, migration and differentiation of progenitor or stem cells (3). It has been suggested that these newly formed cells were the pulp cells and undifferentiated mesenchymal cells. However Gronthos has reported the presence of a unique population of postnatal dental pulp stem cells (DPSC) with self-renewing, highly proliferative capacity and multipotential differentiation into odontoblast-like cells which formed the dentin matrix with some tubular features in vivo (4). Some other researchers have also identified a potential mesenchymal stem cell population derived from exfoliated deciduous human teeth, named as stem cells of human exfoliated deciduous (SHED) teeth, capable of extensive proliferation and multipotential differentiation to these cells (4, 5).

Various materials have been used in vital pulp procedures, especially direct pulp capping. Calcium hydroxide has been extensively and regularly used for direct pulp capping in modern clinical dentistry. As it is known to have a potential role in inducing hard tissue repair, this material has been applied to the exposed pulp and the hard tissue is expected to be regenerated over the pulp. The antimicrobial effect of calcium hydroxide relates directly to its high pH (12.5), which has destructive effect on cell membranes and protein structures. The action of calcium hydroxide is dependent on its dissociation and the release of hydroxyl ions (OH-), which diffuse into the surrounding tissues and result in the formation of a necrotic layer (6). The reparative dentin which is formed by calcium hydroxide is porous and is not a complete barrier. As the result, development of biocompatible materials that induce normal dentin-pulp complex is preferred (7).

Recently Propolis has been recognized as a useful material for human health and veterinary medicine. Made by the honeybee, it is a potent antimicrobial and anti-inflammatory agent. Honeybees collect the resin from cracks in the bark of trees and leaf buds (8). In general, Propolis is composed of 50% resin and vegetable balsam, 30% wax, 10% essential and aromatic oils, 5% pollen and 5% other various substances, including organic debris depending on the place and time of its collection (6, 9). The constituents of Propolis vary widely due to climate, season and location; so its chemical formula is not stable (10, 11). The most important pharmacologically active constituents in Propolis are flavonoids, which are well-known plant compounds that have antioxidant, antibacterial, antifungal, antiviral, and anti-inflammatory properties (6, 9-12). Studies of Propolis applications have increased because of its therapeutic and biological properties (9, 10). Current research involving Propolis in dentistry spans many fields, particularly in cariology, oral surgery, periodontics and endodontics due to its properties, especially its biocompatibility (9, 11-13).

The main aim of this study was to evaluate the effect of Propolis on dentin regeneration and on the potential role of DPSCs.

Materials and Methods

For conducting this experimental split-mouth study, dried Propolis collected in spring (from Azerbaijan) was subjected to exhaustive maceration, filtered using aqueous ethanol (96%) and concentrated using a rotary evaporator. The alcoholic extraction of Propolis was accomplished by repeating the above process three times. A total of 48 mandibular and maxillary incisors from 12 male guinea pigs (age 8-10 weeks, weight 200-250 g) were randomly divided into two groups; Propolis as the experimental and calcium hydroxide as control groups, each containing 24 teeth. The animals underwent general anesthesia with ketamine 60 mg/kg (ROTEXMEDICA, Germany) and xylazine 2%, 10 mg/kg (ALFASAN, Woerden, Holland) intra peritoneally. After the incisors had been cleaned and disinfected with 3% hydrogen peroxide followed by swabbing of the mouth with 0.2% chlorohexidinegluconate, the teeth were cut perpendicularly just above the level of the gingiva with a disk rotated by a low speed engine. Pulp exposure was performed using a number of 0.5 round dental burs and cavities of 1mm in diameter and 2mm in depth were prepared. During cavity preparation the tooth and cutting instruments were irrigated with sterile saline to prevent any heat damage generated by the process. The exposed pulp tissues of each group were directly capped with Propolis and calcium hydroxide, (Aria dent, Iran) respectively. The cavities were subsequently sealed with glass ionomer cement (Fuji II, GC, Japan). Four animals were sacrificed at 10, 15 and

30 days. Resected teeth and surrounding bone were fixed in 10% neutral formalin, demineralized in formic acid for 15 days, embedded in paraffin and sectioned serially in 4µm thickness parallel to the long axis of the tooth. The sections were stained using hematoxylin and eosin (H&E) and viewed by light microscopy. Histological evaluation was carried to determine vascular congestion, pulp vitality, presence of inflammatory cells, level of inflammation, presence of odontoblast-like cells, presence of dentinal bridge, quantity and quality of newly formed dentin and particles remaining from the capping material.

For the detection of DPSCs, the en-vision immunehistochemistry (IHC) method was conducted using the CD_{29} antibody, which is a specific marker for DPSC, and CD_{146}, which is another DPSC and perivascular marker (14).

Statistical analysis

Statistical analyses were conducted using the Kruskal-Wallis, Chi Square and Fisher tests. The analyses were performed using SPSS17.

Ethical considerations

All procedures were conducted in accordance to the animal guidelines of the Pasteur Institute, Tehran, IRAN.

Results

A total of 48 incisors from 12 guinea pigs were treated randomly with Propolis or calcium hydroxide as a pulp capping agent; with 24 teeth receiving each treatment. Vascular congestion was evident in both the experimental cases treated with Propolis and in the controls within different time periods. Throughout the experimental period all the cases treated with Propolis were observed to have vital pulp without any sign of necrosis, in contrast to the controls in which 75% had vital pulp during the same period. Chronic inflammation with dominance of lymphocyte and plasma cells was detectable in both groups at a level below 10%.

In all cases, odontoblast-like cells were present at every interval and there was no significant difference between the two groups ($p>0.05$). These cells were in an organized layer which could be seen either on the pulp chamber walls or under the formed reparative dentin.

True dentinal bridge did not form during the experimental period in either group. The dentin was formed in an irregular manner and in abundance.

Although there was no significant difference between the quantities of dentin produced in both groups over the experimental period, this was not true of its quality. By the 10th day there was no significant difference in the formation of tubular or porous dentin between the two groups. On day 15 those treated with Propolis had 70% tubular dentin while 90% of the control group had ?only? porous dentin (Fig 1). However, by day 30 all the cases (100%) with Propolis had tubular dentin (Fig 2) while only 86% of the control group had tubular dentin ($p=0.005$).

Within 15 days only 20% of the cases had remnants of Propolis while 40% of the controls had remnants of calcium hydroxide. However, by the 30th day 17% of the experimental group still had remnants of Propolis, while there were no remnants of calcium hydroxide in the control group by this time.

The following results were obtained in the IHC evaluation, using CD_{29} and CD_{146} as markers for the detection of DPSCs. In both groups the presence of DPSCs either within the dentin or around the vessels was evident at the 10, 15 and 30-day examinations in both groups. Based on CD_{29} and CD_{146} markers there were more perivascular stem cells than stem cells in the dentin in both the experimental and the control group during the study.

Using information from both markers, fewer stem cells were detected in the calcium hydroxide group over time i.e. decreasing from 62.5% on the 10th day to 20% in 30th day and 54.5% to 20% for CD_{146} and CD_{29}, respectively (Table 1). There was no specific pattern in the detection of stem cells in the Propolis group over time.

Table 1: The presence of DPSCs in Propolis and calcium hydroxide groups identified by CD_{146} and CD_{29} markers at different intervals over the experimental period

	CD_{146}		CD_{29}	
	DPSC presence		DPSC presence	
Time	**Propolis**	**$Ca(OH)_2$**	**Propolis**	**$Ca(OH)_2$**
10th day	25.00%	62.50%	37.50%	54.50%
15th day	36.25%	54.50%	33.00%	36.00%
30th day	16.75%	20.00%	30.00%	20.00%

Although fewer stem cells were found in the

Propolis groups compared to the calcium hydroxide controls at each time point, there was no significant difference between the two groups except at day 10 at which time there was a dominance of stem cells in calcium hydroxide control group.

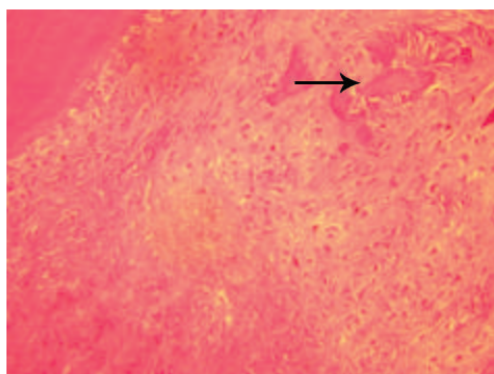

Fig 1: Porous dentin formation (arrow) in calcium hydroxide group (Magnification ×200).

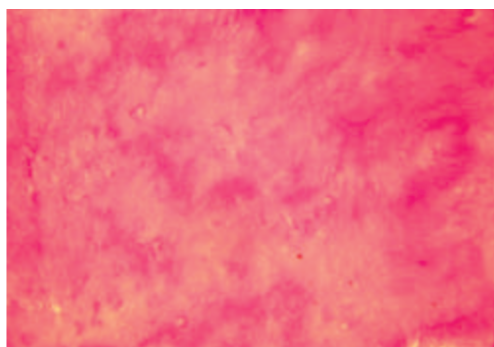

Fig 2: Tubular dentin formation in Propolis group (Magnification×200).

Discussion

Knowledge of pulp physiology has increased considerably in recent years and led to a better understanding of pulp healing. Nonetheless the criteria agreed to characterize successful direct pulp capping vary among authors. The clinical criteria for successful pulp capping are that the tooth is free of symptoms, there is adequate reaction to sensitivity tests and the tooth has a normal radiographic appearance. As inflammation complicates the healing of pulp, a critical evaluation of the results of pulp capping can only be made histologically. Following injury to mature tooth pulp, progenitor cells recruit the repair processes and differentiate into second generation odontoblasts. Although in general practice direct pulp capping (DPC) is usually considered to be a temporary treatment, it has been suggested as a permanent treatment (3, 7).

Calcium hydroxide compounds are the gold standard for vital pulp therapy in human teeth. The procedure is carried out on pulps which are contaminated with bacteria and where there is a potential risk of bacterial leakage along the restoration margins (15). Evidence shows limited effectiveness of calcium hydroxide to eliminate bacteria from human root canals completely (16, 17). Propolis is a good antimicrobial and antifungal agent (18). It breaks down bacterial cell walls, cytoplasm and prevents bacterial cell division (19).

According to Silva et.al Propolis compared to other experimental materials was the least irritant one which can make it a valuable alternative in endodontics (20). Shaher et.al treated the fibroblasts of the pulp and periodontal ligament with Propolis and concluded that this material is not toxic (21). Scheler et.al showed regeneration of the pulp by applying Propolis on injured dental pulp, (22) while calcium hydroxide caused necrosis of the pulp chamber (7). During the present study, there was no sign of pulp necrosis with Propolis, while necrosis was observed in about 25% of the control calcium hydroxide group.

In present study specimens capped with Propolis showed no inflammation which could be related to the anti-inflammatory property of this material. Flavonoids and caffeic acid present in Propolis are known to play an important role in reducing the inflammatory response by inhibiting the lipoxygenase pathway of arachidonic acid. Flavonoids and caffeic acid also aid the immune system by promoting phagocytic activities and stimulating cellular immunity (23).

Ansorge has shown the ability of Propolis to stimulate the production of transforming growth factor (TGF) Beta 1 which is important for the differentiation of odontoblasts (24). It also induces the synthesis of collagen by dental pulp cells (25). However in this study there was no significant difference in the presence of odontoblasts between the two materials.

Bretz et.al reported formation of reparative dentin in DPC with Propolis (25). In another study Sabir et.al found partial dental bridge formation after 4 weeks with Propolis (26). Parolia et.al conducted a study on permanent teeth. They concluded that dentinal bridge formation and tubular dentin were more evident in Propolis and MTA (Mineral Trioxide Aggregates), whereas most of the calcium

hydroxide specimens showed incomplete bridge formation with amorphous and non-tubular dense dentin (27). In other studies incomplete formation of dentinal bridge and tunnel defects have been reported in cases treated with calcium hydroxide (3, 7). Treatment with Propolis in the present study was associated with the formation of tubular dentin with no pores or connective tissue and which was similar to primary dentin (Fig 1). In contrast, the dentin formed by calcium hydroxide was porous, was filled with loose connective tissue and blood vessels and was similar to in structure to bone (Fig 2). Probably, this is due to the rapid cell turnover in guinea pig that results in the changing of SCs to mature cells after using bioactive Propolis on pulp during the experimental period.

Since dentin formed under two capping materials has different qualities, it seems that there are different odontoblast-like cells in each group, which may be due to the variable origins of their stem cells. This result is in agreement with Ji et al who observed that calcium hydroxide can stimulate DPSCs and periodontal ligaments (PDL) stem cells, producing hard tissue in exposed pulp tissue(3). Horsted-Bindslev et al proposed that perivascular cells and other cell populations, including bone marrow stem cells, which migrate via the blood stream may act as progenitor cells (7).

Stem cells can not be identified with certainty in any tissue: scientists rely on indirect properties such as the expression of repertoire surface proteins, clonogenicity or an undifferentiated state (1, 28). In this study presence of stem cells was evaluated using CD_{29} and CD_{146} markers which are specific for DPSCs (29).

Alliot showed that signals from calcium hydroxide can precipitate the differentiation of stem cells to odontoblasts (30). In the present study, stem cells were similarly detected using specific markers after capping by calcium hydroxide during the experimental period. To date there appears to have been no study published that has documented the stimulation of stem cells by Propolis. Thus, to our knowledge this is the first time that this finding has been reported.

This study showed that although more stem cells were found in the calcium hydroxide control group, compared with the Propolis group at each time point, the dentin which is formed by Propolis is of better quality. This may be due to the longevity of Propolis in situ compared with calcium hydroxide, which enables it to act as a stimulant for stem cell differentiation over a longer period.

Tecles et al. demonstrated that pulpal injury stimulated the proliferation of stem cells localized in the perivascular area (31). This result has been confirmed by other researchers showing that pericytes could also differentiate into odontoblasts (5, 29, 30 , 32). The present study showed that the number of perivascular stem cells at any given time was more than the number of dentin stem cells in both the Propolis and calcium hydroxide groups. Also there were some cases in which stem cells were completely absent even on the 10th day. However, in these cases a dentinal barrier had already been formed. This may be explained by the known, rapid formation of dentin in guinea pigs.

Conclusion

To our knowledge this is the first study that has documented the stimulation of stem cells by Propolis. It also provides evidence that Propolis has advantages over calcium hydroxide as a capping agent in vital pulp therapy. In addition to producing no pulpal inflammation, infection or necrosis, this material induces the production of a tubular and high quality dentin.

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Original Article

Comparison of Morphometric Aspects of Light and Electron Microscopy of the Hypoglossal Nerve between Young and Aged Male Wistar Rats

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Abstract

Objective: Age-related changes occur in many different systems of the body. Many elderly people show dysphagia and dysphonia. This research was conducted to evaluate quantitatively the morphometrical changes of the hypoglossal nerve resulting from the aging process in young and aged rats.

Materials and Methods: Through an experimental study ten male wistar rats (4 months: 5 rats, 24 months: 5 rats) were selected randomly from a colony of wistars in the UWC. After a fixation process and preparation of samples of the cervical portion of the hypoglossal nerve of these rats, light and electron microscopic imaging were performed. These images were evaluated according to the numbers and size of myelinated nerve fibers, nucleoli of Schwann cells, myelin sheath thickness, axon diameter, and g ratio. All data were analyzed by Mann-Whitney, a non-parametric statistical test.

Results: In light microscope, numbers of myelinated nerve fibers, the mean entire nerve perimeters, the mean entire nerve areas and the mean entire nerve diameters in young and aged rats' were not significantly different between the two groups.
In electron microscope, numbers of myelinated axons, numbers of Schwann cell nucleoli and the mean g ratios of myelinated axon to Schwann cell in young and aged rats were not significantly different. The myelinated fiber diameters, the myelin sheath thicknesses, myelinated axon diameters and the mean g ratio of axon diameter to myelinated fiber diameter in young and aged fibers were significantly different

Conclusion: The mean g ratio of myelinated nerve fibers of peripheral nerves stabilizes at the level of 0.6 after maturation and persists without major change during adulthood. This ratio of axon diameter to fiber diameter (0.6) is optimum for normal conduction velocity of neural impulses. Our study indicated that the g ratio of myelinated nerve fiber of the hypoglossal nerve decreased prominently in aged rats and can be a cause of impairment in nerve function in old age. Thus, prospective studies concerning electrophysiological and conductive properties of the peripheral nerve could be useful to clarify further the effects of aging on peripheral nerves.

Keywords: Hypoglossal Nerve, Myelinated Nerve Fiber, Aging, Rat

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Introduction

The hypoglossal nerve contains motor neurons of the tongue. There is a limited number of studies on aging-related changes in the morphological composition of the cranial nerves, mainly the hypoglossal nerve, in different species, in comparison to somatic peripheral nerves, perhaps on account of the relative ease and practicality of obtaining biopsies of the latter (1), such as the sural nerve (2, 3). Peripheral nerve function is significantly affected by maturation and aging (4-6). The aging process triggers modifications in the human body that are responsible for many different types of clinical manifestations, represented in the upper aero digestive tract as vocal disorders and swallowing disorders (7).

Oropharyngeal dysphagia is a frequent symptom in the elderly, especially in men aged over 60, and

it is normally associated with an increase in the duration of the oropharyngeal phase of swallowing (8). Many different authors have demonstrated that the aging process is also related to reduction of pharyngeal and supraglotic sensitivity and is considered a factor responsible for the onset of dysphagia, aspiration and repetitive pneumonia in the elderly, owing to reduction of reflexes that protect the lower airway (9). A study on tongue muscle contractile power demonstrated that the aging process affects protrusive contraction of the tongue muscles in rats and so decreases tetanic forces of the tongue. These changes are similar to human models and may be associated with age-related changes in the swallowing function (10). So, it is important that information on the hypoglossal nerve's morphology and axonal morphometric parameters in older age is available in order to determine whether there may be a peripheral contribution to age-associated changes in motor function of the tongue, pharynx and upper aerodigestive tract. The present morphometric study of the hypoglossal nerve has been undertaken in order to investigate whether morphological changes occur in this nerve in rats.

Materials and Methods

Tissue preparation

This research is approved by the Ethical Committee of Cardiff University. Male white wistar rats, from a colony of wistars at the University of Wales, Cardiff (UK), kept under constant conditions of temperature and humidity, fed water and chow ad libitum, and maintained under barrier conditions, were used in this study (11). Animals of two age groups: 4- and 24- months (n=5 per group) were perfused via the left ventricle under ether anaesthesia as follows: perfusion with 200 ml of phosphate buffered saline containing heparin (25 units/ml) at 37°C for eight minutes was followed by perfusion with 3% glutaraldehyde in 100 mM sodium cacodylate buffer, pH= 7.3 at 4°C for 30 minutes. The left and right hypoglossal nerves were sectioned at the upper cervical level, then diced into small pieces and transferred to fresh fixative for two hours at 4°C. After rinsing in 100 mM sodium cacodylate buffer, osmication in 1% osmium tetroxide in cacodylate buffer and dehydration, the tissue was embedded in Spurr's resin. For electron microscopy, ultrathin sections were cut on a Reichert Ultrcut, stained with lead citrate and uranyl acetate and examined with a Phillips 400 electron microscope. For light microscopy, the blocks were sectioned at 2 μm with every tenth section being saved and mounted in order on glass slides. Each set of sections was stained with 1% toluidine blue.

Morphometry

Photomicrographs of the 2μm sections were printed to make montages of the entire nerve at a final magnification of ×220. Ten montages, five from each side, were made of each two age groups. The cross-sectional areas and perimeters of the entire sections of the hypoglossal nerves of both age groups were measured by a computerized tissue-image analyzer software called Motic Image Plus; 2005. Numbers of the myelinated nerve fibers were counted manually (11).

For electron microscopy, electron micrographs of six areas from each five hypoglossal nerve - a total of 30 areas from each two age groups (sampled by a standardized random protocol designed to give every part of the nerve an equal chance of being sampled) - were taken at ×1500 and printed at a final magnification of ×5250. This represented an area of 2465μm².

The total numbers of myelinated fibers were counted in each micrograph. In this protocol the myelinated fibers overlying the upper and left margins were included in the counts whereas those overlying the lower and right margins were excluded. Myelinated fiber/axon diameters were determined by measurement of their smallest diameters. Myelin sheath thickness was determined using a radiating set of straight lines separated by 60° on a myelin sheath, overly superimposed on the centre of each myelinated axon with the result that the lines transected the myelin sheath at six points. At the points of intersection of each line with the innermost lamella of the sheath, the thickness was measured perpendicular to that point manually, and averaged. Axonal diameters were calculated by subtracting ×2 the mean myelin sheath thickness from the measured 'fiber' diameter. Ideally the myelin sheath thickness measurement should be made at the point showing the smallest sheath thickness but in older age groups the inner and outer lamellae of the myelin sheaths were not always as distinct as in the young group. The ratio of the axon diameter to the total fiber diameter (g ratio) was also calculated. The numbers of axons measured were a mean of 1000 for each group. All data were analyzed by Mann-Whitney,

Table 1: Counts obtained from light microscopic montages of transverse sections of the cervical hypoglossal nerve

Age(months)	4	24	P-value
Total numbers of myelinated fiber	3357.5 ± 406.18	3272.4 ± 396.69	0.572
Entire nerve perimeter; transverse section(mm)	1.559 ± 0.288	1.507 ± 0.194	0.787
Entire nerve area; transverse section(mm²)	0.188 ± 0.071	0.181 ± 0.036	0.880
Entire nerve diameter; transverse section(mm)	0.450 ± 0.129	0.435 ± 0.087	0.726

Note: $p < 0.05$ is significant.

a non-parametric statistical test and a $p < 0.05$ was considered significant.

Results

A Light microscopy

The cervical hypoglossal nerve trunk was enclosed within a collagenous epineurium but not subdivided into fascicles (Fig 1). The mean values for the entire nerve cross-sectional diameter, perimeter, and area and the total numbers of myelinated fibers are given in table 1. No differences were observed in comparison between groups. Although total numbers of myelinated fibers decreased with age, difference was not statistically considerable (p=0.572). There were also slight but not a significant decreases in the entire nerve cross-sectional diameter (p=0.726), perimeter (p= 0.787) and area (p= 0.880) with aging (Table 1).

Electron microscopy-1

The mean values for the numbers of myelinated axons, numbers of Schwann cell nucleoli and ratio of myelinated axon to Schwann cell nucleous per mm² (Fig 2) are given in table 2. Although these parameters decreased in 24-month-old rats compared with 4-month-old rats, there were no significant changes with aging (p>0.05).

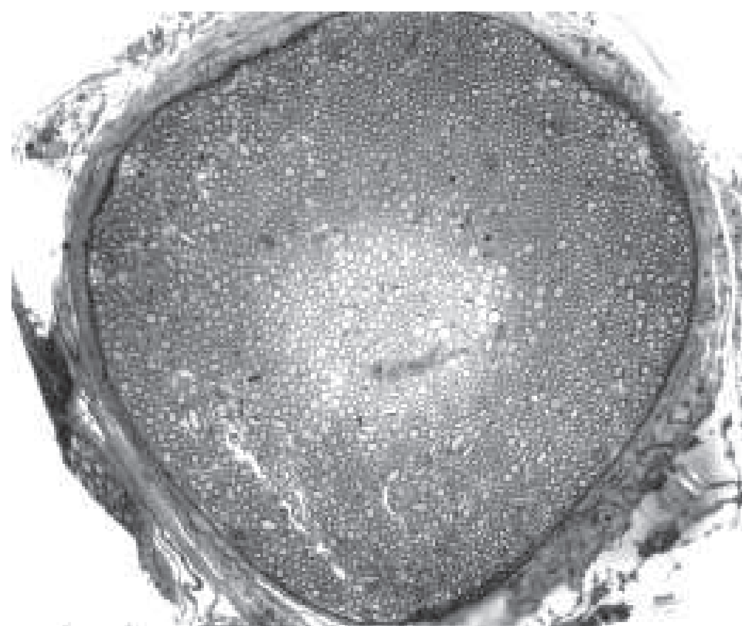

Fig 1: Representative light microscopic transverse section of the 4-month old hypoglossal nerve (×132).Stained with Toluidine blue.

Table 2: Counts obtained from electron microscopic micrographs; per 1 mm²

Age(months)	4	24	P-value
Numbers of myelinated axons	22888.49 ± 7776.88	21906.72 ± 108.94	0.367
Numbers of Schwann cell nucleoli	957.40 ± 685.59	957.40 ± 827.58	0.147
Ratio of myelinated axon to Schwann cell	11736.32 ± 6227.18	11314.41 ± 668.60	0.74

Note: $p < 0.05$ is significant.

Table 3: Myelinated fiber measurements from electron micrographs of the cervical hypoglossal nerve

Age(months)	4	24	P-value
Myelinated fiber diameter (µm)	3.7 ± 1.34	3.24 ± 1.55	0.005
Myelin sheath thickness (µm)	0.69 ± 0.27	0.73 ± 0.33	0.002
Myelinated axon diameter (µm)	2.37 ± 0.98	1.77 ± 1.05	0.005
g ratio	0.62 ± 0.096	0.52 ± 0.12	0.000

Note: $p < 0.05$ is significant.

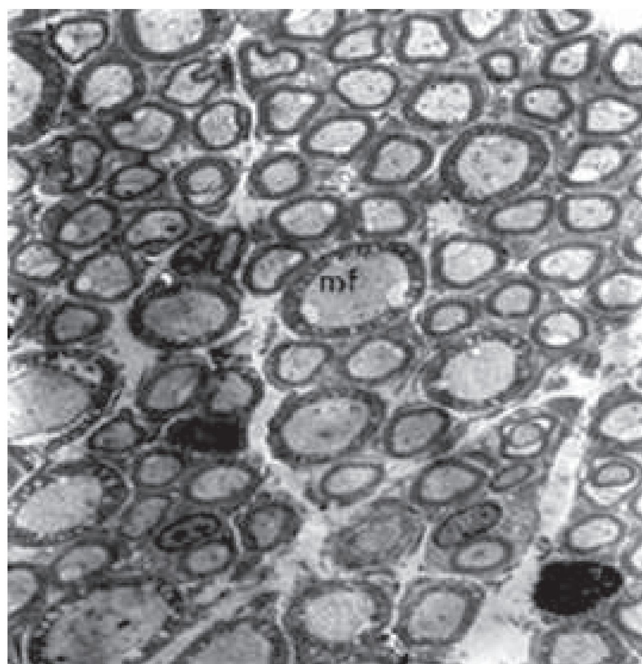

Fig 2: Representative electron micrograph from a transverse section of the 4-month-old hypoglossal nerve (×2625). mf; Myelinated fiber.

Electron microscopy-2

The mean values for myelinated fiber diameter, myelin sheath thickness, myelinated axon diameter and g ratio of axon diameter to myelinated fiber diameter as shown in table 3, decreased significantly ($p< 0.05$) with aging.

The distributions of measurements made on the nerve fiber populations are presented in figures 3-6.

In the two age groups, the measurement of the myelinated fiber diameter did not present a similar unimodel distribution (the majority of the fibers had a 2 to 5 µm diameter in the 4-month group but

1 to 4 µm diameter in the 24-month group), and all showed significant decrease between 4 and 24 months ($p<0.05$).

The distribution of myelin sheath thickness, myelinated axon diameter and g ratio distribution were all unimodal but not similar in myelinated axon diameter and g ratio in both age groups (majority of the axons had a 1 to 3 µm diameter in the 4-month group, but 0.5 to 2.5 µm diameter in the 24-month group and the majority of the g ratios were 0.6 to 0.7 at 4 months, but 0.5 to 0.6 at 24 months).

Discussion

There are many studies in literature about age-related changes in the structure and function of the nervous system. But this study provided more extensive data about age-related morphometric changes in the cervical hypoglossal nerve of rats.

As previously mentioned, peripheral nerve function is significantly affected by maturation and aging (4). Rats are less susceptible to spontaneously peripheral neuropathy (12), so we selected them for the present study.

The present study showed that in rats cross-sectional perimeter, area and diameter and total numbers of myelinated fibers change slightly but not to a statistically significant extent ($p>0.05$). This finding is compatible with other results (13, 14). In a previous study we showed that morphology of the cervical vagus nerve of the rat is maintained without overt deterioration throughout the adult lifespan. Although decreases in myelinated and unmyelinated nerve numbers calculated from electron micrographs were statistically significant, they were in fact only small changes (15).

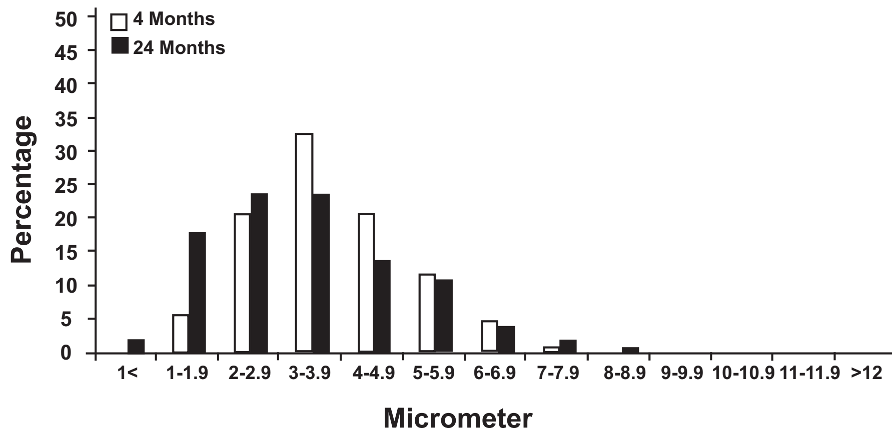

Fig 3: Distribution of myelinated fiber diameter in electron micrographs of the hypoglossal nerve.

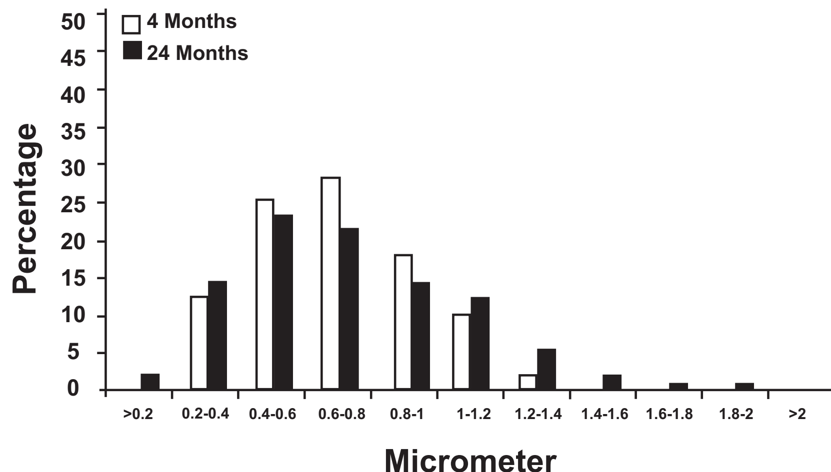

Fig 4: Distribution of myelin sheath thickness in electron micrographs of the hypoglossal nerve.

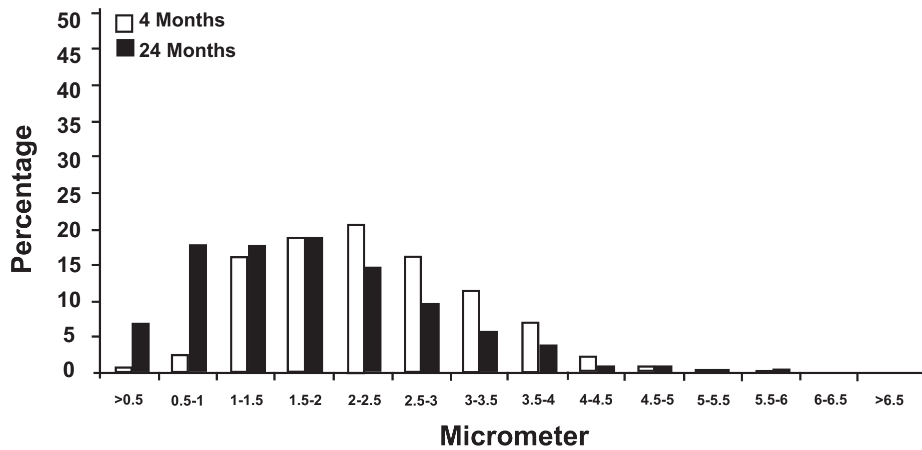

Fig 5: Distribution of axon diameter in electron micrographs of the hypoglossal nerve.

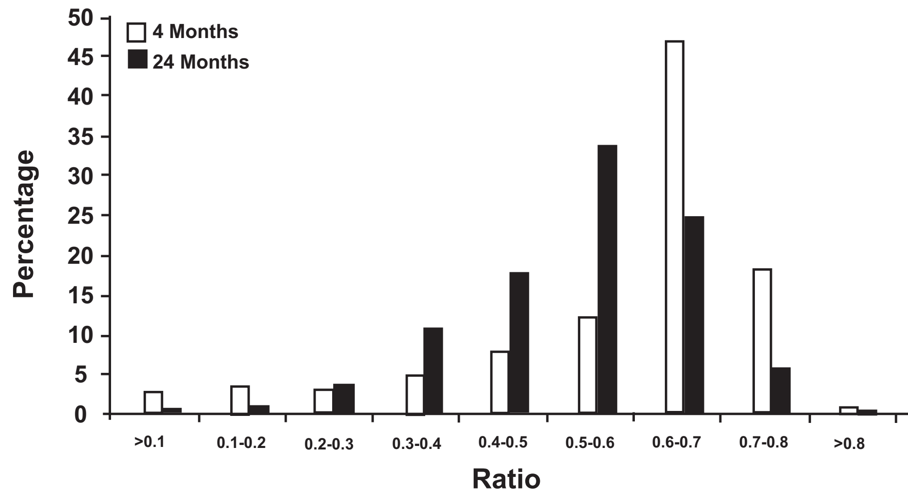

Fig 6: Distribution of g ratio in electron micrographs of the hypoglossal nerve.

A study on tongue contractile power showed a decrease in tongue muscle protrusive forces (10) and another study demonstrated an increase in the duration of the oropharyngeal phase of swallowing (8) through aging, which causes dysphagia, aspiration and other clinical manifestations of the upper aerodigestive tract in the elderly (9).Rats have a short lifespan compared with humans. This could explain why minimal alterations have been seen in our study.

Jeronimo et al. in a study on the rat's sural nerve, showed that in rats there is a postnatal growth spurt between the first and third months of life (30 and 90 days), as judged by increases in body weight, which is associated with changes in myelinated fiber diameter in peripheral nerves. His study also indicated that fiber population distribution changes with increasing body weight (4). This finding is compatible with the result obtained by Jacobs and Love in human sural nerves (16). Body weight continued to increase at a less rapid rate up to the age of six months (180 days) but nerve parameters tended to stabilize between the ages of three and six months (90 and 180 days) (4). This is also in agreement with studies showing that morphometric parameters of nerve fibers are stable from six months to older ages (11).

Fascicle cross-sectional area of sural nerve, in Jeronimo et al. study, showed a significant increase in three and six month old rats compared with one month old rats. In contrast, myelinated fiber density decreased significantly in three and six month

old rats compared with one month old rats, but there was no significant difference between three and six month old rats (4). Although according to the present study cross-sectional area and perimeter of the hypoglossal nerve did not change significantly after maturation and during aging up to 24 months, slight changes in these parameters may be causes of some considerable changes after 24 months.

In the study of Jeronimo et al. the increase in the fascicle area observed from one to three months was largely related to the increase in myelinated fiber size; by contrast from three to six months, this increase was related to an increase in the connective tissue components (4). Although in our study the myelinated fiber diameter of the hypoglossal nerve decreased significantly through aging up to 24 months, there was no significant change in the cross-sectional area of the nerve. The amount of endoneurial connective tissue increases with aging and this causes the cross-sectional area of the hypoglossal nerve to be constant during aging (4, 16, 17).

Jeronimo et al. demonstrated that total number of myelinated fibers and Schwann cell nucleoli are similar on both sides and age groups of premature and matured sural nerves. We found that there were slight but not significant decreases in total numbers of myelinated fiber and Schwann cell nucleous after maturation, during aging up to 24 months. It can be hypothesized that these changes may cause more considerable changes in older ages, after 24 months.

According to the present study, a significant decrease in g ratio can cause dysfunction in nerve impulse conduction in aged rats, so it is necessary to design another study to investigate age-related changing of electrophysiological properties and nerve conduction velocity of the hypoglossal nerve. In the present study the g ratio in myelinated fibers of the hypoglossal nerve reached 0.6 at the age of four months. This is quite similar to our previous study in which we demonstrated that the vagus nerve of the male wistar rat reached0.6 in g ratio of myelinated fibers at four months of age (11). But in Jeronimo et al. study, it was shown that the g ratio of myelinated fibers of the sural nerve reach to 0.6 at the age of 6 months (4).

So it can be hypothesized that the peripheral nerves originating from the brain, such as the hypoglossal nerve, have earlier maturation compared with the peripheral nerves originating from the spinal cord, such as sural nerves.

Correlation between the myelin sheath and diameter of the respective axon has been known since 1905 (18) and may differ significantly between nerves and also between large and small fiber classes within individual nerves (19). Our results are in accordance with those describing a thicker myelin sheath in large axons (19- 21).

Conclusion

The results of the present study showed that the gross morphometric aspects (light microscopy) of the cervical hypoglossal nerve of the rat are maintained without overt change throughout aging. Although the decreases in myelinated nerve numbers and g ratios measured from electron micrographs are statistically significant, they may in fact be small changes, so more electrophysiological and nerve function studies are needed to prove it.

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There is no conflict of interest in this article.

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