Myelinated Nerve Fibre Analysis of the Human Small Splanchnic Nerve

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Summary: The aim of the present study is to analyse nerve fibres of the human small splanchnic nerve in relation to the ageing process. The analysis was conducted with the use of a new staining method that makes it possible to discriminate various structures of the nervous tissue. An image-analysing digitiser, a microscope with a drawing tube and a personal computer for storing data and performing statistical analyses were employed in this study. We examined 30 human small splanchnic nerves which were taken from cadaver specimens (20 males and 10 females) aged from 44 to 96 years. Our report may provide a first information concerning the ageing process of the human small splanchnic nerve. The results indicate that a decrease in transverse area and perimeter of myelinated axons in one of the important changes occurring in the human small splanchnic nerve with the ageing process.

The autonomic nervous system affects the functions of all other systems and influences human behaviour. It is not surprising, therefore, that clinical manifestations of any illness are influenced by the autonomic nervous system which also affects interactions of individuals with their surroundings^{1,6}. As the nervous system is the most important one in the human body, changes with age in this particular system should be studied in detail. Up to now, several studies on the splanchnic nerve have been performed^{7,10}. However, to our knowledge, the age-related changes in the human small splanchnic nerve have not been studied yet. It is our purpose in the present study to bring age-related changes to light through analysis of numbers, transverse areas and perimeters of myelinated axons of the human small splanchnic nerve.

Material and Methods

Small sections of the splanchnic nerve situated near the diaphragm were removed from 30 human cadavers (20 males and 10 females) aged 44–96 years (average age: 70 years). The causes of death had no direct or indirect connection with the central or peripheral nervous system, so the small

splanchnic nerves were considered to be normal.

Fixation

The fixation procedures consisted of two steps as follows: (1) Primary fixation: the material was kept in 3.7% formaldehyde for at least one week. (2) Secondary fixation: the material was immersed in a mixture of 5% potassium dichromate and 5% potassium chromate (1:4 in volume) for three weeks (two weeks at room temperature, and another week at 37°C). The volume of fixative was at least ten times the volume of the specimen.

Washing

After the fixation, tissue blocks fitted with a nylon-mesh bag or a plastic tissue-basket were washed in running water with a siphon-operated automatic pipette washer for several hours.

Dehydration and celloidin embedding

The tissue blocks were dehydrated with ethanol at 50%, 70%, 90%, 95% and pure, and then were embedded in celloidin. The shrinkage of nerve tissue sections during fixation and embedding amounted to approximately 10% in length.

Sectioning

The blocks were sectioned transversely into 20 μm thick slices.

Staining

The sections were stained with LPH triple stain (Luxol fast blue-periodic acid-Schiff-hematoxylin staining). For more details, see the reference section (Goto, 1987).

Morphometry

The systemic sampling method was employed for the measurement of axons. A sampling site at the centre of the small splanchnic nerve was selected, as shown in Fig. 1. Highly enlarged images (1,600 times with oil immersion) of a square eyepiece grid were selected to count the myelinated axons and to measure the transverse area and perimeter of axons: the area covered was $0.016 \times 0.016 \text{ mm}^2$. We employed (a) a microscope (BH₂, Olympus, Tokyo, Japan), equipped with a drawing tube (BH₂-DA, Olympus, Tokyo, Japan), (b) a digitiser (Crystizer α KC 3300, Graphtec, Yokohama, Japan) and an analysing system software (original programming), and (c) a computer (PC-9801VX₂, NEC, Tokyo, Japan) for storing data on-line, calculation and statistical analysis.

We present the data from every decade age group as the mean value $\pm$ SEM (Standard error of mean). The data were statistically examined by analysis of variance (ANOVA). We use the linear regression analysis, in order to determine the relationships between age and number, average area, total area, average perimeter and total perimeter of axons respectively (Scheffé's multiple comparison test). The significance in the present study (except

for numbers) was assumed at the level of $p < 0.001$ for statistical analysis.

Results

Numbers of myelinated axons

We estimated the total number of myelinated axons in the human small splanchnic nerve as being between 4 and 34 (average 13.63) within a unit area of $0.016 \times 0.016 \text{ mm}^2$. The data for all cases are listed in Table 1. The myelinated nerve fibres appeared as a blue-green myelin sheath surrounding a dark purple or black axon (Fig. 2). According to our data, the number of myelinated axons did not show a marked change with age ($r = -0.17$, $p > 0.10$).

Transverse areas of myelinated axons

The transverse area of myelinated axons in the human small splanchnic nerve ranged from 0.39 to 3.36 (average 1.60) μm^2 . The average transverse area of myelinated axons was smaller in the 90–99 year age group, 80–89 group, 70–79 group and 60–69 group than that in the 40–59 group, ($P < 0.001$): 27.0%, 49.8%, 30.9% and 26.6% of the area in the 40–59 group, respectively (Table 2). A scatter diagram with the regression line between the average transverse area and age is shown in Fig. 3. It reveals that the average area of myelinated axons in the transverse section is reduced with age ($r = -0.56$, $P < 0.001$). The total transverse area also decreases with age ($r = -0.67$, $P < 0.001$, Fig. 4). In the 90–99 and 80–89 groups, the myelinated axons were smaller and thinner compared with those in the 40–49 group (Table 3).

Perimeter of myelinated axons

The average perimeter of myelinated axons in the human small splanchnic nerve was 3.50 μm (ranging from 1.43 to 5.81). The average perimeter of myelinated axons was reduced with age ($r = -0.54$, $P < 0.001$, Fig. 5). It is obvious that there is also a negative correlation between age and the total perimeter of myelinated axons ($r = -0.51$, $P < 0.05$, Fig. 6).

Discussion

It is known that the autonomic nervous system is the orchestrator for adaptive responses to stress from the emotional, environmental or sociologic point of view. Although the human splanchnic nerves supply the viscera, the small splanchnic nerve is considered to affect the function of the su-

Fig. 1. Low-power view of the small splanchnic nerve of 72 year-old man, LPH stain.
Scale bar = 100 μm

Table 1. Fibre analysis of the small splanchnic nerve

Subject	Age	Sex	NAx	MAAx	TAAx	MPAx	TPAx
1395	44	F	7	3.63	23.50	5.81	40.70
1393	45	M	17	1.53	26.10	3.63	61.80
1385	54	M	13	1.59	20.60	3.32	43.20
1371	57	M	16	2.70	45.60	4.90	78.10
1369	58	M	9	2.10	18.80	4.30	38.30
1409	59	M	8	2.40	19.50	4.70	37.50
1427	60	F	12	2.60	30.80	4.60	54.80
1416	64	F	12	2.30	27.30	4.30	51.30
1404	65	M	10	2.10	21.50	4.20	41.70
1378	66	F	21	1.40	29.20	3.50	72.70
1412	67	M	34	0.73	24.90	2.20	75.60
1392	69	M	29	1.10	30.80	2.70	79.00
1382	70	M	15	2.20	32.90	4.20	63.30
1372	73	F	14	1.10	15.90	3.00	42.10
1389	75	M	11	1.60	17.70	3.20	34.90
1377	77	M	11	1.80	19.90	4.20	46.20
1390	77	M	9	1.06	9.53	2.74	24.64
1364	77	F	17	0.95	16.20	2.60	44.18
1414	78	M	16	1.40	21.80	3.20	51.60
1394	81	M	22	1.40	30.20	3.30	73.60
1405	82	M	9	0.99	8.96	2.80	25.01
1375	83	M	19	0.39	7.34	1.43	27.10
1407	84	M	9	1.10	9.98	2.90	25.80
1397	84	F	17	0.60	10.50	2.20	37.40
1368	85	M	8	2.30	18.30	4.30	34.40
1418	85	M	15	0.77	11.60	2.20	33.60
1410	86	F	5	0.60	3.20	2.20	10.90
1383	87	F	10	1.50	15.20	3.40	34.40
1415	91	M	8	1.80	14.60	3.70	29.50
1403	96	F	4	1.60	6.40	3.70	15.50
Mean	72		13.63	1.59	20.73	3.50	45.01
SD	13.11		6.52	0.70	10.00	0.96	17.88

NAx: number of axons in the left small splanchnic nerve, MAAx: mean area of axons, TAAx: total area of axons, MPAx: mean perimeter of axons, TPAx: total perimeter of axons.

Fig. 2. High-power view of the small splanchnic nerve of 60 year-old woman, LPH stain. Axons are stained in dark purple or black, and surrounded by myelin sheath. Scale bar = 0.1 μ m

prarenal body and the kidney through the ramus renalis. Up to now, there have been few reports available on the morphometry of nerve fibres in the human small splanchnic nerve¹¹⁾ – especially with regard to axons. The reason may be due to a lack of discriminative staining method for nerve fibres, and of modern methods for morphometric analyses. For example, various silver impregnation methods have been used for the staining of the nervous system, but they cannot be used for morphometric analyses, except for numbers, and this for two reasons: shrinkage and discrimination. We employed a new staining method, the LPH staining method⁴⁾. The result indicates that this method greatly facilitates morphometric studies on the peripheral nervous system thanks to the clear discrimination of structures, for example axons (blue or dark purple), myelin sheaths (blue-green), neuronal nucleoli (dark blue), other cell nuclei (colour similar to conventional hematoxylin stains), connective tissue

Table 2. Mean area of the small splanchnic nerve

Age group	No.	Average age	Mean area	Atrophy rate
40-49	2	44.5	2.45	—
50-59	4	57.0	2.20	—
60-69	6	65.2	1.71	26.61%
70-79	7	75.3	1.61	30.90%
80-89	9	84.1	1.17	49.79%
90-99	2	93.5	1.70	27.04%

The atrophy rate is a percentile value of the mean area to that of the 40-59 years group.

Fig. 3. Scatter diagram and regression line showing the correlation between the age and the reduction of mean area of axons in the small splanchnic nerve.

Fig. 4. Scatter diagram and regression line showing the correlation between the age and the reduction of total area of axons in the small splanchnic nerve.

Table 3. Total area of the small splanchnic nerve

Age group	No.	Average age	Total area	Atrophy rate
40-49	2	44.5	24.80	—
50-59	4	57.0	37.98	—
60-69	6	65.2	27.40	12.65%
70-79	7	75.3	20.62	34.31%
80-89	9	84.1	11.12	64.58%
90-99	2	93.5	10.50	66.55%

The atrophy rate is a percentile value of the total area to that of the 40-59 years group.

Fig. 5. Scatter diagram and regression line showing the correlation between the age and the reduction of mean perimeter of axons in the small splanchnic nerve.

Fig. 6. Scatter diagram and regression line showing the correlation between the age and the reduction of total perimeter of axons in the small splanchnic nerve.

(faintly pink), amyloid bodies and lipofuscin granules (light purple), etc. The method is also very suitable for morphometric research on the nervous system because of its clear differentiation of structures and minimal shrinkage (only about 10% in length⁴). The fixation method for the morphometric analysis is one of the most important steps in the methodology. The usual formaldehyde fixation may cause a wide range of tissue shrinkages during histologic processing. In our study, we used the secondary fixation with chromic acid after formaldehyde fixation in order to obtain better staining results and to minimize tissue shrinkage^{4,5}.

The next point is the embedding method. For light microscopy, there exist at present two main embedding methods: paraffin and celloidin. The marked shrinkage of tissue that occurs during processing is one of the disadvantages of using paraffin. The paraffin technique is therefore not suitable for morphometric analysis. It was replaced in the present study by the celloidin method after secondary fixation with chromic acid⁴.

As for the microscopic morphometry, a combination of a microscope, drawing tube, digitiser and computer has made this kind of research both easy and accurate, as is described in detail in the "Material and Methods" section above. Otherwise, it would be very difficult or even impossible to measure the areas of irregular shapes with any degree of accuracy.

Several studies about age-related changes in various nerves have been reported since the LPH staining method was introduced. For example, Fujii *et al.* reported that the transverse areas and circularity ratios of the axons in the facial nerve do not change with age, but the numbers of axons become reduced with age ($r = -0.59$)². In another article, the same author reported that the transverse axonal areas of the cochlear nerve is reduced with age ($r = -0.87, p < 0.01$), while the number and transverse areas of amyloid bodies in the vestibulocochlear nerve increase with age (area: $r = 0.76, p < 0.01$; number: $r = 0.77, p < 0.01$). The number of vestibular nerve fibres do not show any change with age³. In Zhang *et al.*¹⁴, 38 human spinal cords aged from 41 to 97 years were used to count the myelinated axons and to measure the axonal transverse area of the posterior funiculus at C6 level. This research revealed that a decrease in size and number of axons was one of the important changes occurring with the ageing process in the human spinal posterior funiculus (number: $r = -0.85, p < 0.001$; area: $r = -0.77, p < 0.001$). As to the human deep peroneal nerve, Yanagisawa *et al.*¹³ reported the number and transverse area of axons measured in 21 males whose ages ranged from 41 to 89 years.

The results indicate that a slenderisation and diminution of axons, and especially a disappearance of large axons are the main picture of the ageing process of the deep peroneal nerve (number: $r = -0.82$, $p < 0.01$; area: $r = -0.75$, $p < 0.01$).

In the clinical field, Fujii *et al.*³⁾ suggested that a presbycusis might be due to a reduction of the axonal area of the cochlear nerve with age. Zhang *et al.*¹⁴⁾ found that an atrophy and a decrease in number of myelinated axons were the main changes connected with ageing occurring in the human spinal cord. However, the clinical appearance of the small splanchnic nerve in relation to the ageing process still remains unknown and should be elucidated in future.

As for the regression lines between age and numbers of axons, ageing processes of several nerves were analyzed and evaluated: the facial nerve²⁾, the vestibulocochlear nerve³⁾, the posterior funiculus of the spinal cord¹⁴⁾, the deep peroneal nerve¹³⁾ and the oculomotor nerve¹²⁾. Furthermore, it can be concluded that changes with the ageing process are more evident in motor nerve fibres than in sympathetic nerve fibres. In the present study, we found that the number of myelinated fibres in the small splanchnic nerve remained almost constant with age, while the axonal areas and perimeters decreased with age (mean area: $r = -0.55$, $p < 0.001$). The results indicate that the vulnerability of the small splanchnic nerve may be related to ageing. It is considered that there may be a correlation between ageing and the visceral hypofunction from a morphometric point of view.

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