Original Article

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An Evaluation of Tissue Destruction Time in Phrenic Nerve after Death and Counting the Number of Nerve Fibers in C3, C4 and C5 Branches

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ABSTRACT

BACKGROUND AND OBJECTIVE: Most studies related to phrenic nerve are conducted based on an anatomical perspective and there is no histological study associated with this nerve. Since detecting the approximate time of death based on tissue destruction and determining the main branch of phrenic nerve based on the number of nerve fibers is extremely important, the histological examination of phrenic nerve was evaluated in the present study.

METHODS: In this experimental study, the left and right phrenic nerves, obtained from 10 male corpses, were described using Grant's method of dissection after cutting the chest. In order to count the number of nerve fibers in the constituent branches (C3, C4 and C5), Hematoxylin and Eosin staining and Bielschowsky staining were used. In addition, to determine tissue destruction time, phrenic nerve of 8 rabbits were examined one to eight days after their death.

FINDINGS: The results regarding tissue destruction time demonstrated that phrenic nerve starts to degenerate on the sixth day after death and is completely degenerated on the seventh and eighth days. Moreover, mean nerve fibers in C3, C4 and C5 were 41.2% (2224), 44.9% (2428) and 13.9% (749), respectively. The differences between the number of nerve fibers in these branches was statistically significant ($p<0.05$).

CONCLUSION: The results of this study demonstrated that a significant part of phrenic nerve fibers are originated from C4 branch. In addition, tissue destruction of this nerve starts on the sixth day after death.

KEY WORDS: *Phrenic nerve, Histology, Tissue destruction.*

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Introduction

The left and right phrenic nerves are the primary driver diaphragm muscle (1). This nerve is mainly formed in anterior branch of forth cervical spinal nerve (C4). However, some branches from anterior branches of C3 and C5 also join it (2). Based on anatomic studies and all available books and articles, C4 branch is introduced as the main branch in the formation of phrenic nerve (3, 4).

Although each nerve, including phrenic nerve, consists of several nerve fibers (5), no study has been conducted to count the number of nerve fibers in this nerve. The number of nerve fibers is significant at least from two perspectives; first, in nerves consisting of several main branches, the main branch can be detected based on the number of branches (6) and second, in damaged nerves, we can evaluate the repair process during interventional treatment by counting the number of branches (7, 8).

Kaya et al. assessed the recovery process by counting the number of branches in the damaged sciatic nerve (9). The pressures and damages to phrenic nerve may be the result of trauma, surgery and tumor masses, which may lead to paralysis of the diaphragm muscle (10-12).

Although C3, C4 and C5 branches are all highly vital, identification of the branch that plays a key role in phrenic nerve formation is very important for preventing damage to this nerve from surgical perspective. Different variations have been reported regarding phrenic nerve in the neck and most studied related to this nerve are based on anatomical perspectives (13-15) and there is no histological study associated with this nerve. Nowadays, different methods such as examination of various body tissues are used in forensics to detect the approximate time of death (16, 17).

Nerve tissue is one of the most sensitive tissues in exposure to damage and has a faster destructive process compared with other tissues (18, 19). Moreover, considering shortage of teaching cadavers in anatomy lessons, being aware of the time of tissue destruction for better and faster fixing of cadavers is essential. Since approximate detection of time of death based on tissue destruction is highly important in forensics and in order to detect the main branch that constitutes the phrenic nerve, the present study was conducted to assess the time of tissue destruction of phrenic nerve after death and count the number of nerve branches in the constituent branches according to histological studies.

Methods

In this experimental study, 20 left and right phrenic nerves, obtained from 10 male corpses, were described using standard and classic method (Grant's method) of dissection after cutting the chest (20). Simple and practical tools were used for anatomy and stereoscope was used for observing tiny branches of nerves.

Cadavers were 22 to 61 years old. Before starting the process of anatomy, a written consent was obtained from the families of the cadavers. After accurate dissection of the neck and chest and reaching phrenic nerve, the constituent branches (C3, C4 and C5) in the neck was isolated before joining and was used for histological evaluation. Then, a part of main trunk of the phrenic nerve right below the hilar was also isolated and was histologically evaluated for counting the number of nerve fibers. All cadavers died in the last 24 hours and their autopsy was performed in the forensics hall in Kerman.

Counting the nerve fibers by hematoxylin – eosin staining: After isolating phrenic nerve from cadavers, it was placed in 10% formaldehyde in order to fix the tissue (21). Then, tissue processing was done on samples and after preparing sections from paraffin blocks containing the phrenic nerve, the samples were stained with hematoxylin and eosin using routine laboratory methods to count the number of nerve fibers in the phrenic nerve (19, 22). Then, four 7µ consecutive sections were prepared and the nerve fibers were counted using optical microscope at magnifications of 200x. For more accurate counting, the image of nerve profile was shown on a whiteboard using a projector attached to the microscope and after framing and specifying each fiber with a dot on the board, counting nerve fibers was started. Then, the average numbers of counted sections were recorded in pre-prepared tables.

Bielschowsky staining: Bielschowsky staining technique was used for specific study of nerve tissue. In this technique, after preparing 7µ sections, the slides were placed in 20% silver nitrate for 30 minutes and after being washed twice with distilled water, samples were immersed in a solution of silver ammonia for 30 seconds. Then, samples were placed in 20% gold chloride for 10 minutes and after being washed twice, they were placed in 5% sodium thiosulfate for 5 minutes. Then, after being washed with absolute alcohol and after tissue transparency, all samples were covered with substantial glass (23, 24).

Determining the approximate time of tissue destruction after death: For this purpose, 8 lab

rabbits were killed after being completely anaesthetized with ether and the phrenic nerve of one of them was used as control sample after sectioning and staining. hen, in the same evening, the phrenic nerve on the opposite side of the same rabbit was isolated and a section was prepared. During the next days, the other dead rabbits were used in the same way and each evening, the phrenic nerve on the opposite side was histologically evaluated. This process continued up to eighth day.

Statistical analysis: The tissue images related to the time of tissue destruction were assessed qualitatively. The data related to the number of nerve fibers in the phrenic nerve branches (C3, C4 and C5) were analyzed using SPSS (SPSS 16, Chicago Inc., IL) and One way ANOVA. In addition, the number of nerve fibers in the main trunk of the phrenic nerve in the lower area of the hilar were compared on the left and right and $p<0.05$ was considered significant.

Results

Counting the number of nerve fibers in the phrenic nerve: Results of counting the number of nerve fibers in the constituent branches of left and right phrenic nerve demonstrated that the fibers in C4 branch are more than C3 and C5 branch (table 1, 2). According to the results of this study, the average nerve fibers in C3, C4 and C5 branches were reported to be 41.2% (2224), 44.9% (2428) and 13.9% (749), respectively and the difference in the number of fibers between these three branches was statistically significant (Fig 1). Results revealed no significant difference in the number of fibers in each branch separately on the left and right (C3 p=0.0911, C4 p=0.852, C5 p=0.96).

The fibers in the left and right phrenic nerve below the hilar (Fig 2) were also counted (table 3). In Bielschowsky staining technique, neurons as well as axons and dendrites were dark or very dark brown (Fig 3). The obtained data showed that the average nerve fibers in the left and right phrenic nerves in these samples were 2690 and 2685, respectively.

In statistical evaluations, no significant difference was observed between the number of fibers in the left and right phrenic nerves below the hilar. The results of this study demonstrated that there is a main nerve trunk in about 48% of all fibers constituting phrenic nerve below the hilar. According to our results, the difference between the maximum and minimum number of fibers in phrenic nerves below the hilar was 930.

Determining the time of nerve destruction: Examining the stained rabbit samples of phrenic nerve and their comparison with control samples demonstrated that the nerve started to degenerate on the sixth day, this process advanced in the evening of the sixth day and the nerve was completely degenerated on the seventh and eighth day (Fig 4).

Table 1. Nerve fibers in C3, C4 and C5 branches of the right phrenic nerve.

Cadaver	1	2	3	4	5	6	7	8	9	10
Branch	N(%)	N(%)	N(%)	N(%)	N(%)	N(%)	N(%)	N(%)	N(%)	N(%)
C3	2300(38.7)	2290(39.4)	2280(41.7)	2100(42)	2240(42.2)	2230(42.1)	2120(41.2)	2285(42.4)	2252(41.8)	2140(40.1)
C4	2800(47)	2730(47)	2500(45.8)	2240(44.8)	2320(43.8)	2300(43.6)	2253(43.8)	2368(44)	2345(43.6)	2438(45.7)
C5	850(14.3)	790(13.6)	680(12.5)	655(13.2)	740(14)	753(14.3)	765(14.8)	717(13.6)	783(14.6)	760(14.2)

Table 2. Nerve fibers in C3, C4 and C5 branches of the left phrenic nerve.

Cadaver	1	2	3	4	5	6	7	8	9	10
Branch	N(%)	N(%)	N(%)	N(%)	N(%)	N(%)	N(%)	N(%)	N(%)	N(%)
C3	2280(39.1)	2275(39.6)	2295(41.6)	2125(42)	2263(42.2)	2215(41.8)	2145(41.5)	2255(42.2)	2225(42.2)	2205(40.3)
C4	2740(46.9)	2680(46.6)	2532(45.9)	2265(44.7)	2345(43.7)	2325(43.9)	2280(44.2)	2377(44.5)	2305(43.7)	2470(45.1)
C5	820(14)	795(13.8)	695(12.5)	672(13.3)	752(14.1)	760(14.3)	740(14.3)	705(13.3)	743(14.1)	802(14.6)

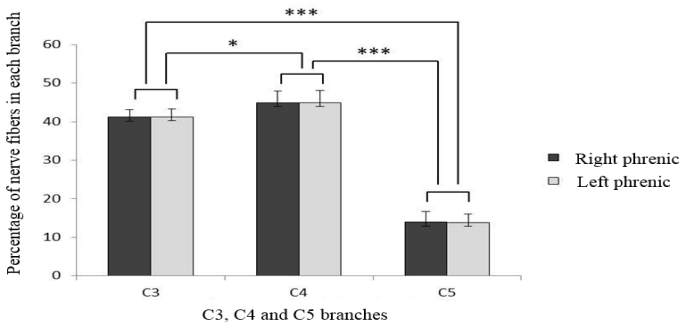

Figure 1. The average number of nerve fibers in C3, C4 and C5 branches (p<0.001*, p<0.05*).**

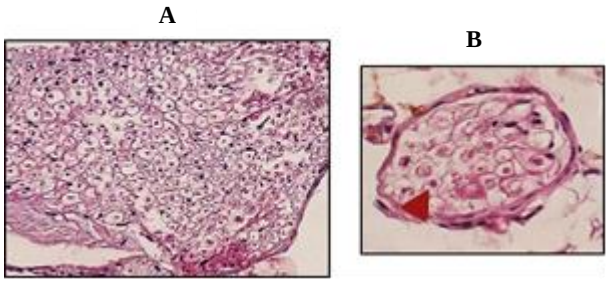

Figure 2. Phrenic nerve, 200x zoom (A), a side branch of phrenic nerve with 31 fibers, 400x zoom (B).

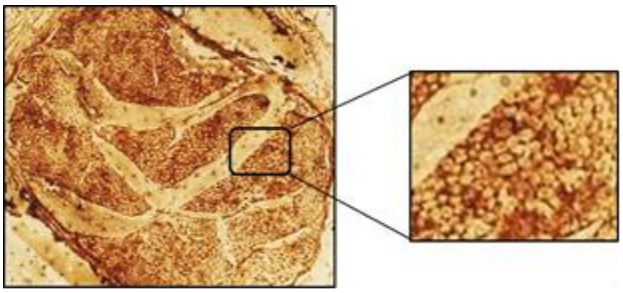

Figure 3. Phrenic nerve profile below the hilar, (Bielschowsky staining, 100x zoom).

Table 3. The number of nerve fibers in the main trunk of the phrenic nerve in the lower area of the hilar.

Cadaver	1	2	3	4	5	6	7	8	9	10
right phrenic nerve	3205	3142	2847	2408	2655	2595	2592	2773	2350	2280
left phrenic nerve	3170	3187	2795	2510	2737	2640	2615	2715	2275	2253

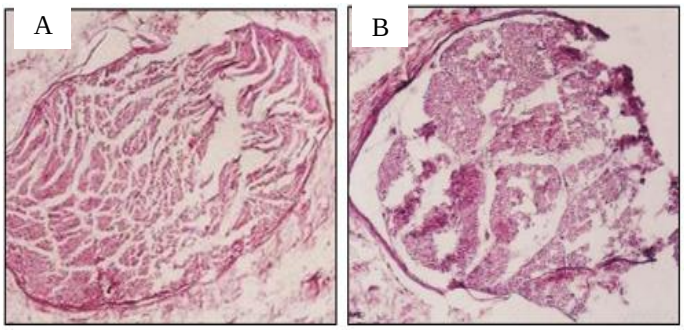

Figure 4. Phrenic nerve profile of rabbit on the sixth (A) and eighth day (B) after death (100x zoom).

Discussion

The present study assessed phrenic nerve from histological perspective, indicating the prominent role of C4 branch in the formation of this nerve, which was in line with previous anatomic studies. Canella et al. demonstrated that C4 branch in the main constituent of phrenic nerve (2). In a report by Nair et al., C4 was introduced as the main branch of phrenic nerve (3). In case a tumor exists in neck and for a surgery in the central area of neck, it is essential to be clinically aware of the main branch in phrenic nerve (11). Moreover, in animal studies using interventional treatments, one can evaluate the process of restoration of damaged nerves by counting the number of nerve fibers (9). Results of this study demonstrated that tissue destruction of phrenic nerve starts from the sixth day after death. Determining the time of tissue destruction after death is essential for obtaining healthy samples in histological studies, fixing the cadavers and determining the approximate time of death for forensics (17). In this regard, all human cadavers died during the last 24 hours. Moreover, the difference between the maximum and minimum number of fibers in phrenic nerves below the hilar is because of the fact that before reaching diaphragm miscle, phrenic nerve grants its sensory branches for pleurodiaphragmatic and peritoneum that covers the lower level of the diaphragm and the isolation spot of these branches are diverse in difference individuals.

This is a vital issue in health and chest surgeries (25). In samples with more fibers, it is possible that these branches are not isolated yet. In other words, they are isolated in lower levels (26, 27). It is also suggested that tissue destruction for other tissues of body be considered in future studies. Results of this study demonstrated that C4 branch plays the most significant role in the formation of phrenic nerve (44.9%). Moreover, the issue destruction in this nerve starts from sixth day after death.

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