Differences in innervated neurons of the internal anal sphincter based on age and sex: a histological study

Running title: Sex, age difference of anal nerve

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Abstract

Aim

Previous studies indicate sex and age differences in anal sphincter function, but few morphological studies have focused on the quality and quantity of the nerves that control the sphincter muscles. This study aimed to determine whether there are morphological and quantitative sex and age differences in the nerves in the conjoined longitudinal muscle.

Methods

This was a single-center, retrospective study using surgical specimens from 44 patients who underwent abdominoperineal resection between 2003 and 2012. Hematoxylin-eosin- and S-100-stained peripheral nerves (nerve fibers and ganglion cells) in the conjoined longitudinal muscle beneath the dentate line were observed microscopically. A qualitative examination assessed the degeneration score, which was based on the presence or absence of karyopyknosis, vacuolar degeneration, acidophilic degeneration of the cytoplasm, denucleation, and adventitial neuronal changes. For quantitative examinations, each neuronal and muscular area was traced to calculate the neuronal area ratio in S-100- immunostained photomicrographs at the observation site.

Results

Women had significantly fewer in quantity of nerves than men. Elderly individuals (aged ≥80 years) had significantly fewer in quantity of nerves than young ones. Furthermore, elderly individuals tended to show greater morphological changes that appeared to be due to degeneration.

Conclusions

Our findings suggest that anal hypofunction in female and older individuals may result from differences in the quantity and quality of the neurons controlling the anal sphincter muscle.

Keywords: aging, anal nerve, anal sphincter, fecal incontinence, sex difference

Introduction

Fecal incontinence is a relatively common anorectal disorder, with an incidence ranging from 11% to 15% according to research of a community-based sample of adults[1]. Many reports have indicated that age is a risk factor for fecal incontinence[2-5]. The incidence of fecal incontinence based on sex is controversial. Some reports have stated that there is no difference in fecal incontinence between males and females[2, 3]. In contrast, other reports have suggested that fecal incontinence is more common in women than in men[4, 5]. However, examination of anal function using manometry has shown a difference in fecal incontinence associated with age, as well as sex[6, 7]. Some morphological changes, such as tearing of muscle fibers due to childbirth[8] and an increase in thickness of the anal sphincter muscle due to aging[9], might explain differences in anal function.

In recent years, introduction of sacral nerve stimulation, a novel treatment method for fecal incontinence, has drawn attention to the underlying neuronal pathology of fecal incontinence[10]. As indicated by the efficacy of neural mediation[11], anal sphincter hypofunction appears to be caused by changes not only in the muscle tissue, but also in the neurons controlling the anal sphincter. Several reports have indicated that the amount of neurons in the intestine declines with age[12, 13]. However, only one study has examined neurons of the anal sphincter in the mouse. There have been no studies using human materials focusing on neurons of the anal sphincter[14]. Therefore, in the present study, we histologically and immunohistologically examined the nerves toward the internal anal sphincter using rectally amputated specimens. We investigated differences in the amount and morphology of nerves of the anal sphincter based on sex and age.

Methods

Patients

Between 2003 and 2012, 44 patients underwent abdominoperineal resection (males: n = 34; females: n = 10; age: 41–90 years; median: 70 years) at Toho University Medical Center Omori Hospital, Japan. A total of 43 patients had rectal cancer and one patient was operated on for peritoneum dissemination of gastric cancer. To avoid the effect of therapy, we excluded patients with a history of irradiation and chemotherapy, including therapies for other diseases. Clinicopathological features summarized in Table 1.

Tissue preparation and staining

Surgical specimens that were obtained during surgery were fixed in 10% buffered formalin for 36 hours. Specimens were cut longitudinally and the section that was farthest from the tumor, including the dentate line, was obtained. Samples were embedded in paraffin and then cut at a thickness of 3 µm. Sections were stained with hematoxylin and eosin (HE) and S-100 immunoperoxidase stain. We used rabbit polyclonal anti-human S-100 protein (1:2400; DAKO Z0311; Dako, Glostrup, Denmark) for the primary antibody. Immunostaining was performed using the BenchMark XT auto-stainer (Ventana Medical Systems, Tucson, AZ) without antigen retrieval.

Area of evaluation

We evaluated peripheral nerves in conjoined longitudinal muscle. We selected a 5-mm length of area in the muscle located beneath the histological anal transitional zone[15], corresponding to the dentate line of macroscopic observation.

Observation sites

The internal anal sphincter is considered the most important element of anal function because it is responsible for 75% of the maximum resting pressure, and thus performs a major role in maintaining continence[16]. However, in the current study, we did not observe nerves in the internal sphincter directly because we can observe only a limited number of neurons in the visual field. There is a lower neuronal density in the internal anal sphincter than in the lower rectal wall[17]. This fact made it difficult to observe and compare the amount of neurons. Therefore, in the present study, we decided to use the conjoined longitudinal muscle for the observation site. Anatomically, there is a large amount of neurons running towards the internal anal sphincter in this observation site[17, 18].

Morphological examinations

We modified Nishizawa's method[19] for evaluation. Ten nerves in HE-stained sections were selected at the evaluation area and observed under high-power magnification (40 × 10). A degeneration score was then created based on the following features: karyopyknosis, vacuolar degeneration, acidophilic degeneration of the cytoplasm, denucleation, and adventitial neuronal changes (Fig. 1). We assigned 1 point for each feature and added the total points for the degeneration score. Therefore, the degeneration score ranged from 0 to 5. We then examined the association between the score and age or score and sex.

Quantitative examinations

S-100 immunoperoxidase-stained sections of surgical specimens were used for quantitative evaluation. In this study we used the neuronal area ratio (neuronal

area/longitudinal muscle area×1000), not neuronal density (number of neuron/muscle area), as an indicator of quantity of nerves. The reason for using this ratio is because precisely counting the number of neurons is difficult when neurons are cut parallel or diagonally.

Conjoined longitudinal muscle was observed in S-100-immunostained sections under mid-power magnification fields (10 × 10) and images were digitally captured (4080 × 3070 pixels). S-100-immunolabeled nerves were traced and each area was measured. The total sum of the areas was then calculated in the images to determine the area of nerves (Fig. 2). We performed this process for three points and calculated the mean, which was used for analysis of the quantity of nerves. Image analysis was performed by NIH ImageJ (National Institutes of Health, Bethesda, Maryland, USA）[20]. The sections were evaluated by two authors (T.K and T.N) who were blinded to the clinical information of the patients. We also examined the relationship between results of histological examinations and the status of diabetes mellitus of the patients. In addition, we examined the relationship between location (anterior, posterior, and lateral walls) and histological findings.

Statistical analysis

The Mann–Whitney U test was used to examine the relationships between amount of nerves and sex, age, and status of diabetes. The Kruskal–Wallis test was used to examine the relationships between the amount of nerves and the circumferential direction of the anal canal (anterior, posterior, and lateral walls). All statistical analyses were performed using SPSS for Windows, v13.0J (SPSS, Japan Inc., Tokyo, Japan). A $p < 0.05$ was considered to be significant.

Results

Morphological examinations

In morphological examinations, vacuolar degeneration was observed in 10 specimens (23%), adventitial neuronal change was observed in three (7%), acidophilic degeneration was observed in two (5%), and karyopyknosis was observed in one (2%). However, denucleation was not observed in any of the specimens. Degree of each morphological change is mild.

The mean value of the degeneration score of each individual was 0.432 ± 0.618 (median value: 0 [0–2]). The mean degeneration score was 0.441 ± 0.660 in men (n = 34) and 0.400 ± 0.267 in women (n = 10). There was no significant difference in the mean degeneration score between the two groups ($p = 0.95$). We also compared the degeneration score by age. The median value of the degeneration score in patients aged ≥80 years (n = 8) tended to be higher than that in those aged <80 years (n = 36, 0.875 ± 0.835 versus 0.333 ± 0.535), but there was no significant difference between the two groups ($p = 0.0501$).

Quantitative examinations

When we determined the quantity of nerves as a neuronal area ratio, the mean total value of all individuals was 7.353 ± 5.126 (median: 5.74; range: 1.05–21.7). The mean quantity of nerves was significantly lower in women than in men (3.714 ± 3.114 versus 8.424 ± 5.215, $p = 0.003$; Fig. 3a).

Moreover, to investigate whether the quantity of nerves changes with age, we compared the neuronal area ratio between the age groups of ≥80 years and <80 years. There were significantly fewer nerves in the ≥80 years group compared with the <80 years group ($p = 0.026$; Fig. 3b).

In this study, we regarded the ratio (of the neuronal area/conjoined longitudinal muscle area) as the quantity of neurons innervating the internal sphincter. It is possible that conjoined longitudinal muscle area differs according to age or sex. To exclude this possibility, we examined the relationship between area and age and sex. The results of the Mann–Whitney U test showed no significant age difference in area ($p = 0.287$) and no significant sex difference in area ($p = 0.80$).

To confirm homogenous distribution of the nerves according to direction (anterior/ posterior/ lateral), we examined 28 patients in whom the location of nerves could be identified. The quantity of nerves was not significantly different among the anterior, posterior, and lateral walls ($p = 0.42$). In addition, to investigate the effect of diabetes on the quality and quantity of nerves, glycated hemoglobin (HbA1c) (National Glycohemoglobin Standardization Program) levels were examined in 26 patients and compared with histological results. There were only two patients in the poor control group of diabetes (HbA1c levels >6.5%). The neuronal area ratios of these patients were 5.53 and 19, and the degeneration scores were 0 and 1. The Mann–Whitney U test showed no significant difference in the degeneration score ($p = 0.34$) and amount of nerves ($p = 0.77$) between the poor control diabetic group and the other groups, including good control diabetic and non-diabetic individuals.

Discussion

Our study showed that the quantity of anal nerves in women was significantly less than that in men. One of the explanations for anal dysfunction in women is tearing of the sphincter muscle fibers during delivery[8]. However, our findings suggest that another reason for this decline in women's anal function is having a lower amount of nerves in the anus. Some reasons, such as anatomical differences, traumatic nervous disorder by delivery[21], and secondary atrophic changes after muscle disorders by delivery have been suggested for the difference in amount of anal nerves in women compared with men. However, the actual reason is still not clear. Further studies to clarify this reason are required, such as comparing the quantity of nerves of multiparas with nulliparas.

Age-related changes have been described in several reports that examined the anal sphincter. In an investigation using ultrasonography, Abe et al. reported that the thickness of the internal sphincter increases with age[9]. However, histological observation of the internal sphincter muscle showed that the number of smooth muscle cells is decreased and collagen fibers are increased with age[22, 23]. Our study showed that the quantity of nerves toward the internal sphincter muscle in the older group was less than that in the younger group. This result suggests that not only changes in muscle, but also changes in nerves, are associated with anal dysfunction in aging.

Several investigations using gastrointestinal tract samples other than the anal sphincter have shown a decrease in quantity of nerves with age[12, 13]. Bernard et al. performed immunostaining of neurons in the myenteric plexus of the descending and sigmoid colon

in 16 human cases and showed that the number of HuC/D-positive and choline acetyltransferase-positive neurons decline with age[12]. Similarly, Gomes et al. analyzed the human colon and showed less neuron density and increased fibrous components in myenteric ganglia in older people (>65 years old) compared with younger people (20–35 years old)[13]. To the best of our knowledge, this is the first report to show a decrease in volume of nerves related to the anal region of humans.

With regard to morphological changes in neurons, Hanani et al.[24] reported that vacuolation increased with age in human enteric ganglia. Eliahu et al.[25] conducted a histomorphological examination of pelvic ganglia in rats and reported that more vacuolated neurons were observed in older rats than in young rats. In the present study, morphological changes based on vacuolation tended to be more common in the older group of ≥80 years.

In the present study, there was a significant difference in the quantity of nerves between the older and younger groups. However, we could not find a linear correlation between age and quantity of nerves. The quantity of nerves may be maintained up to the age of approximately 80 years, but further examination with a greater number of younger subjects is required to clarify the issue.

Several types of neurons, which express different mediators, can be recognized in the intestinal wall, and they are thought to play an important role in maintaining anal function. In a study of the human colon, the number of HuC/D-positive and ChAT-positive neurons declined with age with sparing of neuronal nitric oxide synthase-positive neurons[12]. A

study on older mice that used the immunohistochemical technique showed that immunoactivity to neuronal nitric oxide synthase and substance P in neurons of the internal anal sphincter was decreased[14]. Both of these studies used immunofluorescence with frozen sections. However, analyzing formalin-fixed paraffin-embedded material is difficult. Evaluating which type of nerves or mediators decrease by aging in human anorectal material may be necessary.

This study has some limitations. We checked the medial records of patients and confirmed that there were no specific complaints indicating anal dysfunction, but we did not examine anal function of each case physiologically. We also could not evaluate the clinical history of hemorrhoids, anal fistula or vaginal delivery and so on, which may induce anal dysfunction.

Although stereology is an ideal technique for quantification of neurons, it is not often used in analysis of surgically resected materials. It was difficult to use stereology in this study, because we could not use whole mount tissue samples. Instead, we used paraffin-embedded collected samples. The use of paraffin-embedded tissue enabled us to consistently identify precise targeted areas. Stereological confirmation of the results is required.

Recently, preoperative chemoradiation therapy (pCRT) has been established as the standard treatment in the Health Center Controlled Networks guidelines for rectal cancer[26]. However, radiation therapy appears to be the major cause of anal dysfunction[27, 28]. Nishizawa et al. histologically examined peritumoral neurons in specimens obtained

by intersphincteric resection[19]. They showed that degeneration of nerves was more severe and Wexner scores were higher in the pCRT group than in the surgery-only group. In the internal sphincter, degeneration of nerves after pCRT for rectal cancer has also been reported[29]. In a study of prostatic cancer series, a relationship between the degree of degeneration of nerves in the rectum by irradiation and anal dysfunction was shown[30]. Therefore, radiation therapy likely causes histological degeneration to nerves, which can induce anal dysfunction. Our results suggest that, when planning irradiation therapy, careful consideration is required for older and female patients.

This study shows that in the anal region, women have fewer nerves than men, and older individuals aged over 80 years also have fewer nerves than younger individuals. These results suggest that the cause of anal dysfunction in women and older individuals is due to neuronal factors, as well as changes in the sphincter muscles.

Disclosure Statement

No potential conflicts of interest were disclosed.

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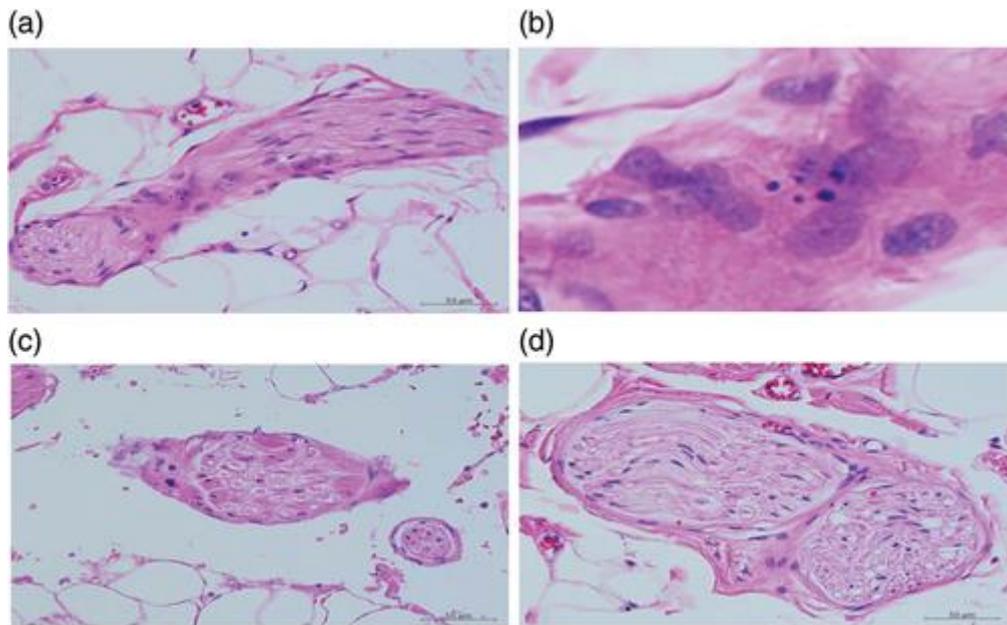
Figure legends

Fig. 1 Features of neuronal evaluation. Hematoxylin- and eosin-stained sections were assessed. Magnification of the objective lens was ×40. Peripheral nerve tissue with karyopyknosis is shown in (a). Higher magnification image of pyknotic/ apoptotic nucleus is shown in (b). Acidophilic degeneration shown in (c). Vacuolar degeneration and adventitial neuronal change were shown in (d).
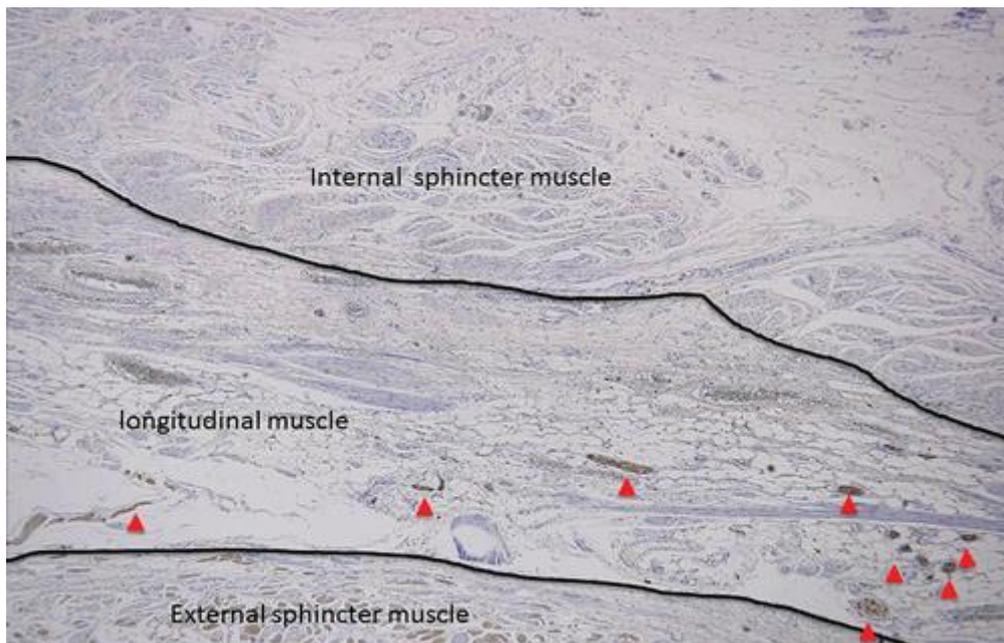
Fig. 2 Representative observation site used for evaluating the quantity of nerves. The conjoined longitudinal muscle area is enclosed by a black line. S-100-immunolabeled nerves are indicated by arrowheads
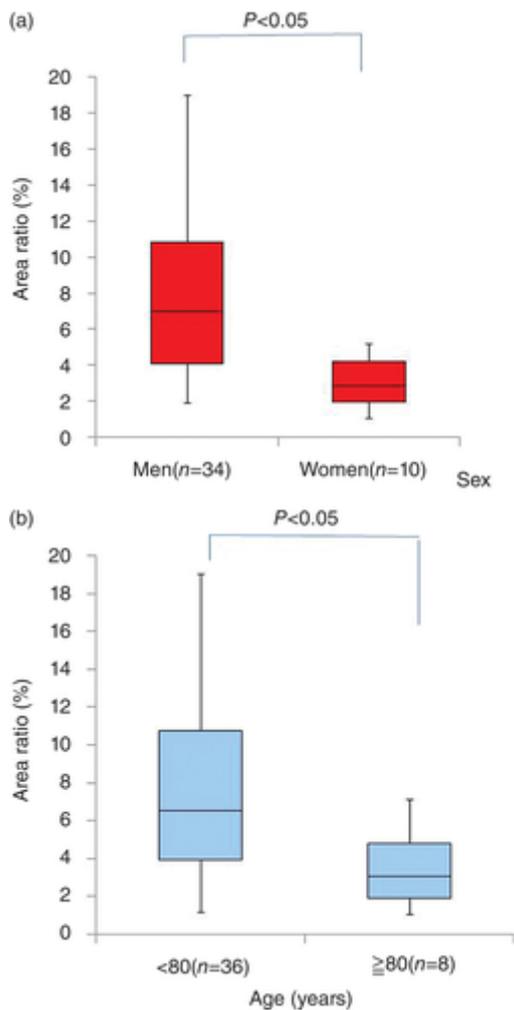
Fig. 3 Sex and age differences in the quantity of nerves. (a) The mean amount of nerves shown as the neuronal area ratio was 8.424 ± 5.215 in men and 3.714 ± 3.114 in women. The neuronal area ratio of nerves in women was significantly less than that in men ($p = 0.003$). (b) The mean neuronal area ratio of nerves was 4.159 ± 3.426 in older patients (>80 years) and 8.063 ± 5.276 in younger patients (≤80 years). The neuronal area ratio of nerves in older patients was significantly less than that in younger patients ($p = 0.026$)

Table 1.　Clinicopathological characteristics of the patients

	Age 80 or older (n=8)	Age less than 80 (n=36)	*p* value
Female/Male (female ratio)	4/4 (50.0%)	6/30 (16.6%)	0.064
Histology of cancer			
well	3	5	–
mod	5	27	–
por	0	2	–
muc	0	2	–
Pathological stage of cancer			
I	2	5	–
II	3	11	–
III	1	14	–
IV	0	5	–
Unclassified	Recurrent tumor 1 Unknown 1	Dessemination of gastric cancer 1	
Degeneration score	0.875 ± 0.835	0.333 ± 0.535	0.0501
Neuronal area ratio	4.159 ± 3.426	8.063 ± 5.276	0.026

well= well differentiated tubular adenocarcinoma

mod=moderately differentiated adenocarcinoma

por=poorly differentiated

adenocarcinoma

muc=mucinous adenocarcinoma