

## Application Value of in Vivo Nerve Staining in Nerve-Sparing Radical Hysterectomy

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### Abstract

**Objective:** To stain pelvic autonomic nerve in nerve-sparing radical hysterectomy (NSRH) using sodium thiosulfate-methylene blue (DMB), and discuss the clinical significance of NSRH and application of intra-operative nerve staining.

**Methods:** DMB was used to stain uterine ligaments. Some blue tissue was initially identified for nerve tissue by HE staining, and confirmed by immunohistochemistry using S-100, TH, and VIP staining analysis.

**Results:** The nerve volume density of sacrouterine ligament stump tissues in the NSRH group was less than the control group. Sympathetic and parasympathetic nervous volume densities in the cardinal ligament stump tissues in the NSRH group were less than the control group.

**Conclusion:** DMB staining of nerves was facilitated identification of pelvic autonomic nerves. DMB staining can effectively guide and assess the distribution and preservation of pelvic autonomic nerves. The method is worthy of wide application.

**Keywords:** Cervical cancer, NSRH, RH, In-vivo nerve staining, DMB

### Introduction

Cervical cancer is one of the most common gynecologic malignancies. In 2008, the International Agency for Research on Cancer (IARC) estimated that the incidence and mortality of cervical cancer in developing countries was higher than developed countries [1]. Moreover, patients showed a trend towards a younger age at the time of diagnosis [2]. Persistent human papilloma virus (HPV) infections have been confirmed to be associated with the occurrence of cervical cancer, especially in patients with high-risk subtype HPV16 infections [3,4]. The main treatment of early invasive cervical carcinoma is radical hysterectomy or radiotherapy, both of which have a similar cure rate [5,6] and a 5-year survival rate > 90%. Radiotherapy can lead to a series of complications which can negatively impact the patient's quality of life. Therefore, surgery is the preferred treatment for early invasive cervical cancer.

The traditional radical surgery for cervical cancer is RH III, in which the ligaments need to be respected as close as possible to the pelvic wall to provide sufficient exposure of the hypogastric nerves (HNs), pelvic splanchnic nerves (PSNs), and pelvic plexus bladder branch (VB) for excision [7]. The quality of life after RH is poor. Urinary dysfunction occurred in 74% of patients, such as a loss of sensation for micturition, urinary incontinence, and urinary retention. The incidence of urinary retention and urinary frequency was 70% ~ 85% [8,9]. Therefore, RH with wide resection and trauma is associated with many complications, such as a post-operative neurogenic bladder, bowel dysfunction, and sexual problems. To improve the patient's quality of life, Japanese scholars have suggested a nerve-sparing radical hysterectomy (NSRH) [10].

Nerves widely exist in adipose tissue and generally accompany the course of blood vessels. Bulky nerves can be observed directly, but it is difficult to identify small nerves and nerve plexuses without magnification. Vital staining involves the use of a non-toxic dye to show natural structures inside the cell without affecting normal cell activities. Thus, we made the bold assumption that we could preserve nerves by specific neural coloring to differentiate nerves from surrounding tissues. On the basis of a large number of animal experiments, autopsies, surgical practice, and experience

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in gynecologic surgery, we decided to stain the pelvic autonomic nerves during NSRH using DMB, and evaluated the surgical effect compared with the RH group and discuss the clinical significance of NSRH and application of intra-operative nerve staining.

## Materials and Methods

### Patients

Forty-four patients with cervical cancer (Stage Ib<sub>1</sub>-Ia) were enrolled in this study; all of the patients had undergone gynecologic surgery at Liaocheng People's Hospital between October 2012 and October 2014. Twenty patients were randomly selected for the NSRH group in which one or two sides of the pelvic autonomic nerves were preserved, which were stained intra-operatively with DMB. Twenty-four patients underwent the RH operation. There were no significant differences in age ( $p=0.431$ ) and the body mass index ( $p=0.424$ ) between the two groups. The clinical stages were as follows: Ib<sub>1</sub>, 18 cases; Ib<sub>2</sub>, 16 cases; and Ia, 10 cases. The pathologic diagnoses were as follows: squamous cell carcinoma, 40 cases; adenocarcinoma, 3 cases; and adenosquamous carcinoma, 1 case.

### Methods

**DMB preparation method:** Compound 10 ml of 0.1% methylene blue solution. Take 0.4% methylene blue solution (2.5 ml), 7.5 ml diluted with distilled water. Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> · 5H<sub>2</sub>O 800 mg was added to the methylene blue solution, heated until the dark blue receded, and the PH was adjusted to approximately 3.5. The above solution was placed in a glass bottle with a rubber stopper, the bottle was wrapped with aluminum foil, sealed from light, placed in a -20 °C refrigerator after autoclaving before surgery could be performed.

**Surgical-specific surgery and intra-operative nerve staining:** Operative points: Surgery was performed based on classical Piver III in the RH group. We isolated the hypogastric plexus when dissecting the superficial vein, and isolated the pelvic splanchnic nerves and the inferior hypogastric plexus when dissecting the uterine deep vein during NSRH. We stained the pelvic autonomic nerves with DMB (Invitrogen, USA), then retained the nerves in part. Intra-operatively, DMB liquid was sterilized by autoclaving. The bottle was wrapped with silver paper to shield the liquid away from light. Then, a cotton ball was

dipped into the DMB liquid and used to paint the operative field, and repeated at least 3 times. Then operative field was thrice-washed with vitamin C. After the color of the surrounding tissue faded, the preserved nerves were identified.

**Histologic analysis of stained tissue:** The stained tissues were observed without magnification, and noted to have a sheaf-like and mesh arrangement. We recorded the observations with a camera, marked the tissues with thread at the section edge of the ligaments, and placed the specimens in 10% neutral formalin for fixed 24 hours. Then we tested the tissues with HE and S-100 (Santa Cruz Biotechnology, Inc, USA) analytic methods. We obtained specimens from sacrouterine and cardinal ligament stump tissues to detect nerves.

**Quantitative and qualitative analysis of nerves in specimens:** Every section was subjected to immunohistochemistry testing using TH and VIP (Creative Diagnostics, USA) staining analysis. We randomly selected 5 views, then measured the volume density of nerves (S-100 marks), sympathetic nerves (TH marks), and parasympathetic nerves (VIP marks). These images were inputted into Imageproplus 6.0 medical image analysis software to measure the area of positively expressed protein and the area of each image. Using macro processing, the generated data were inputted to Excel for summation processing. The volume density (Vv) of nerves in these ligaments was obtained.

## Results

### The stability of dying liquid

DMB was colorless, transparent, and remained sealed at -20°C. After melting, reagents did not take on obvious changes. DMB was colorless after high temperature and high pressure sterilization, and there was a small amount of white precipitate in the bottle. Thus, DMB was stable in character and adaptable to save (Figure 1).

### Neural dye and quantitative and qualitative analysis of tissue samples

**Dying effect to the naked eye:** Specimens exhibit a wide blue after DMB dying. After fully washing with physiologic saline containing vitamin C, we found that most blue faded, and the beam light blue or reticular tissue was gradually highlighted, which was directly observed to the naked eye and without the

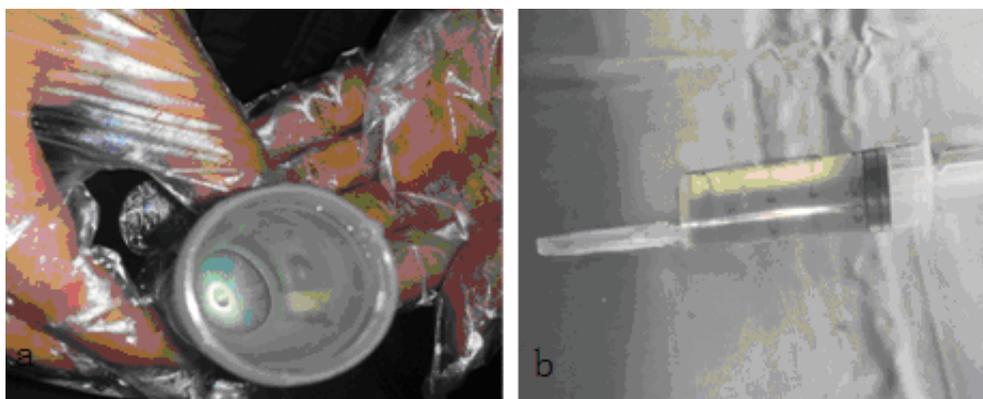


Figure 1 a: DMB before autoclaving; b: DMB after autoclaving.

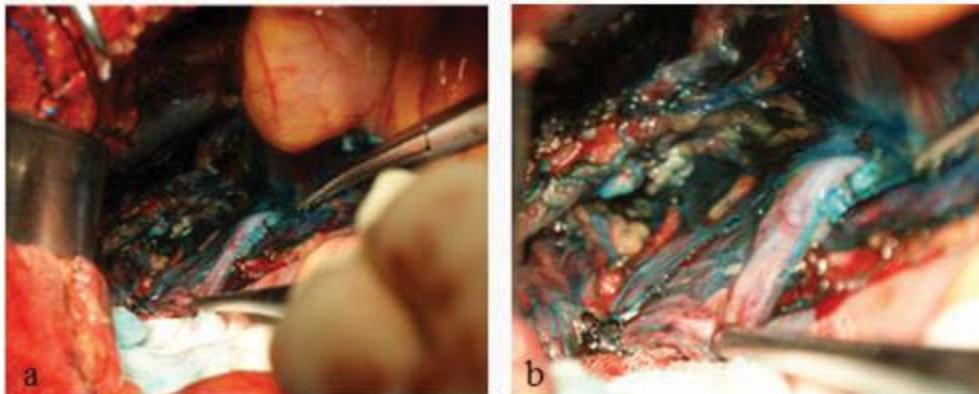


Figure 2: Wide blue after DMB nerve dying

aid of other auxiliary equipment. We initially determined the light blue for nerves, which will be retained intra-operatively (Figure 2).

**Consistency of the nerve dying judgment:** After DMB dying, blue-stained sections near the sacral ligament were visually identified as nerve tissues. All 8 inspections confirmed nerve tissue by HE and specific nerve staining antibody of S-100. We initially determined that the neural specificity of DMB was good, and it could be used as a nerve tracer.

**Sympathetic nerve and parasympathetic nerve staining and quantitative analysis in the sacral uterine ligament and the cardinal ligament:**

**Sympathetic dyeing of the sacral uterine ligament:** As shown in Figure 3, the sympathetic ganglion and sympathetic nerve fibers with positive TH protein was observed in the sacral uterine ligament.

**Quantitative analysis of the sympathetic nerve in the sacral uterine ligament:** As shown in Table 1, after S-100 marking, the volume densities (Vv) of the sacral uterine ligament tissues were measured with Imagepluspro 6.0 image processing software. The Vv value of the NSRH and control groups was  $0.0159 \pm 0.0011$  and  $0.0417 \pm 0.0048$ , respectively ( $P = 0.005$ ). They were shown to be sympathetic nerves.

**Sympathetic and parasympathetic nerve dyeing and quantitative analysis of the cardinal ligament**

**Sympathetic and parasympathetic nerves dyeing of the cardinal ligament:** As shown in Figure 3, the sympathetic ganglion and sympathetic nerve bundled with positive TH protein had been observed in the cardinal ligament of the uterine specimen. The parasympathetic ganglion and parasympathetic nerve bundled with positive VIP protein was observed in the broken end of the cardinal ligament of the uterine specimen.

**Quantitative analysis of nerve, sympathetic nerve, and parasympathetic nerve in the cardinal ligament of the uterine specimen:** As shown in Table 2, the Vv value of the cardinal ligament stump tissues of the NSRH and control groups was  $0.0049 \pm 0.0058$  and  $0.0176 \pm 0.0180$ , respectively ( $P = 0.019$ ). The nerve content of the cardinal ligament stump

	S-100 (Vv)	TH (Vv)
NSRH	$0.0159 \pm 0.0011$	$0.0092 \pm 0.0056$
RH	$0.0417 \pm 0.0048$	$0.0146 \pm 0.0237$
t	3.352	2.465
P	$P=0.005$	$P=0.016$

Table 1: The quantitative and qualitative analysis of sacral uterine ligament.

	S-100 (Vv)	TH (Vv)	VIP (Vv)
NSRH	$0.0049 \pm 0.005$	$0.0069 \pm 0.0148$	$0.0041 \pm 0.0037$
RH	$0.0176 \pm 0.0180$	$0.0189 \pm 0.0139$	$0.0162 \pm 0.0119$
t	2.635	3.236	2.998
P	$P=0.019$	$P=0.005$	$P=0.007$

Table 2: The quantitative analysis of the cardinal ligament.

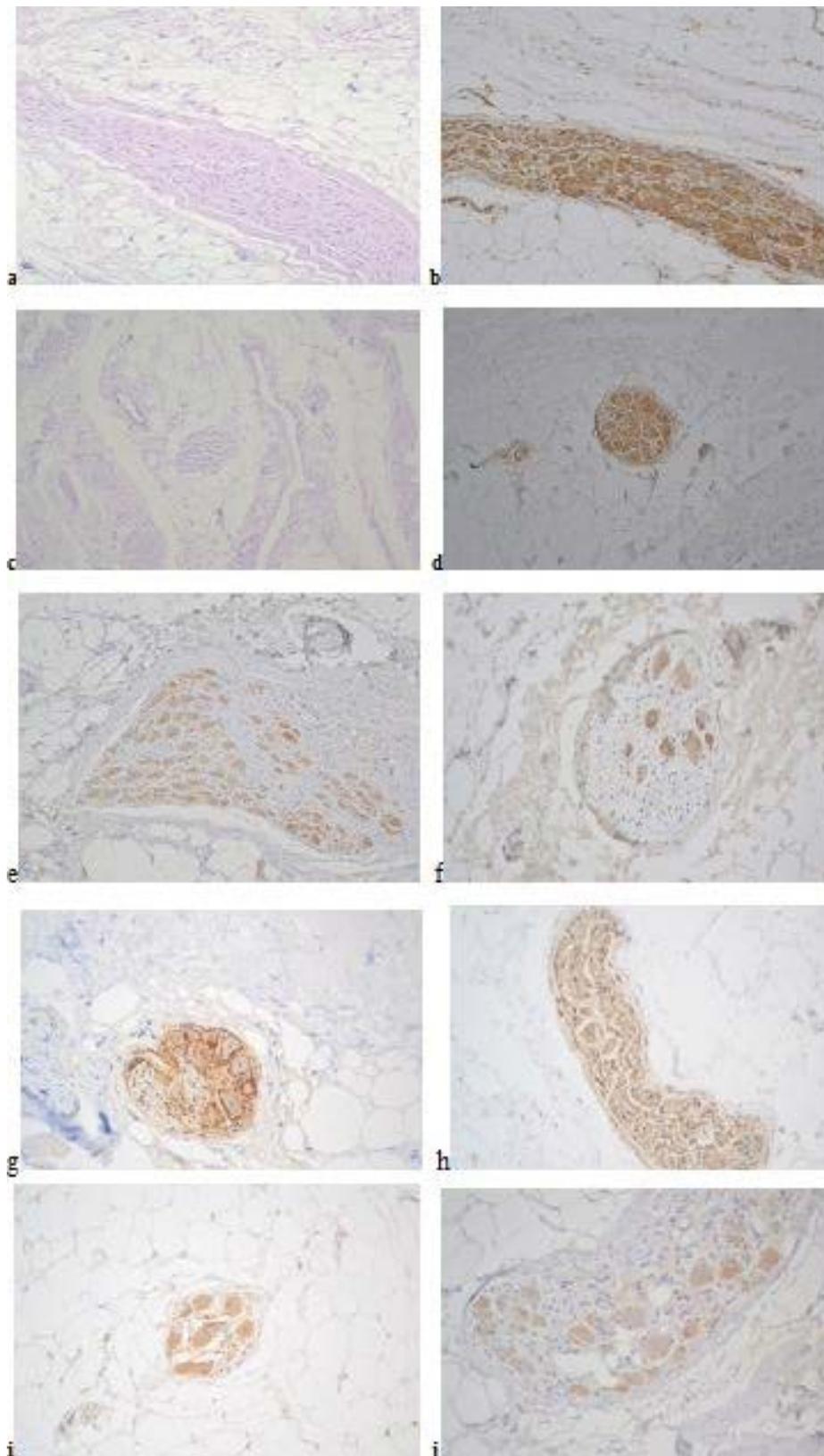
	n	operation time (min)	blood loss (ml)	pelvic lymph node (n)
NSRH	20	$237.50 \pm 30.15^*$	$405.80 \pm 100.12$	$22 \pm 6$
RH	24	$179.76 \pm 33.74$	$466.36 \pm 215.22$	$19 \pm 6$
t		5.276	1.787	1.677
P		$P=0.000$	$P=0.089$	$P=0.550$

Table 3: Comparison of curative effect.

tissues in neural NSRH decreased significantly compared to the control group. The Vv value of the sympathetic nerve and control group was  $0.0069 \pm 0.0148$  and  $0.0189 \pm 0.0139$ , respectively ( $P = 0.005$ ). The parasympathetic and control group Vv was  $0.0041 \pm 0.0037$  and  $0.0162 \pm 0.0119$ , respectively ( $P=0.007$ ). The sympathetic and parasympathetic nerve content in the cardinal ligament stump tissues of the NSRH group was significantly reduced compared to the control group.

**Intra-operative clinical pathologic observations**

As shown in Table 3, the operative time of the NSRH and control groups was  $237.50 \pm 30.15$  min and  $179.76 \pm 33.74$  min, respectively ( $P= 0.000$ ). The operative step of the NSRH group was relatively complex because of intra-operative careful dissection, exposure, and nerve dying, but the intra-operative vital signs were normal. The intra-operative blood loss of the NSRH group was  $405.80 \pm 100.12$  ml, which was less than the control group ( $466.36 \pm 215.22$  ml;  $P = 0.089$ ). The median number of intra-operative pelvic lymph node cleaning of the NSRH and control groups was 18 (range, 13 ~ 32) and 16 (range, 13 ~ 28;  $P = 0.550$ ).



**Figure 3:** a: Nerve fiber by HE(2x100); b: Nerve fiber by S-100(4x100); c: Ganglion by HE(2x100); d: Ganglion by S-100(4x100); e: Positive TH of sympathetic nerve fibers (4x100); f: Positive TH of sympathetic ganglion (4x100); g: Positive TH of sympathetic ganglion (4x100); h: Positive TH of sympathetic nerve fibers (4x100); i: Positive VIP of parasympathetic ganglion (4x100); j: Positive VIP of parasympathetic nerve fibers (4x100).

## Discussion

Sakamoto [11] summed up the Fujiwara keynotes and introduced NSRH to the world in 1988, which was referred to as the "Tokyo operation." Sakamoto [11] pointed out that the cardinal ligament was the key part of this technology, and divided the cardinal ligament into blood vessels and nerves, thus providing a theoretical basis for further improvement of this technique [12].

The surgical indication, scope, safety, and long-term efficacy of NSRH has been the focus of gynecologic experts. The 2-year survival rates for the NSRH and RH groups are 95.5% and 100%, respectively. Sakuragi stated that nerve preservation had no significant effect on the survival rate. Vanden [13] compared the 2-year recurrence and survival rates in the NSRH and RH groups, and confirmed that NSRH is as safe as RH. Three studies [13,14] determined the post-operative tumor recurrence rates in the NSRH and RH groups. A meta-analysis showed that there are no differences between the 2- and 4-year recurrence rates in the two groups {RR=0.54 [95% CI (0.04, 7.80); p=0.65]; RR=0.78 [95% CI (0.04, 16.89); p=0.88]}.

Hockel [15] offered a new interpretation, showing that cervical cancer metastasis is usually limited to the extent of the uterine Mullerian tube unit, including the sub terminal vagina, cervix, uterine body, uterine membrane (uterine blood supply and lymph drainage), and the uterine ligament. Pelvic autonomic nerve and vaginal tissues did not belong to the disease unit.

The most common method of nerve staining is the Lee test, which has the advantage of identification without magnification and special instruments, and convenience. Seif [16] used an animal model to demonstrate the use of methylene blue staining to protect small nerve fibers. Our study used this method to dye pelvic autonomic nerves in the NSRH for the first time, which labeled nerves more intuitively, and preserved the nerves to a greater degree. We used and improved the formula of Lee to blend DMB staining solution, which should be sealed from light. DMB can be stored for a long time at -20°C in the refrigerator and remain colorless after rewarming naturally, sterilization at a high temperature, and high pressure. We also observed that DMB had a good staining effect and good specificity for nerves.

We used DMB to dye the operative field, and found that tissues were widely stained blue. Most of the tissues gradually faded after being flushed with saline containing vitamin C. Pelvic autonomic nerves stained light blue and were distributed as bundles and reticulation with different thicknesses and individual differences. These nerves had a difference between the surrounding tissues, which was visible without magnification. We chose part of the blue-stained tissue near the stumps of the cardinal and sacrouterine ligaments, which made HE chips after fixing and were examined using S-100 immune markers; these blue-stained tissues were nerves.

Some scholars have studied the retention of nerves in RH, and deemed that patients with stage IIb and higher are not recommended to undergo NSRH. We gradually realized that the locally advanced cervical cancer was not suitable for NSRH. We should pay attention to specific conditions during the operation. We found that 4 cases had met the criteria, but could only undergo unilateral nerve sparing due to intra-operative bleeding, obesity, local adhesions, and indeterminate anatomic factors.

We also qualitatively and quantitatively analyzed nerves in the stumps of ligaments. The content of nerves in the sacrouterine ligament of the NSRH group was less than the control group, which was shown to be sympathetic nerves, and the cardinal ligament stump tissue of the NSRH group was less than the control group. The sympathetic and parasympathetic nerve content in the NSRH group was significantly less, thus the nervi hypogastricus was mostly comprised of sympathetic nerves. The pelvic plexus was comprised of nervi hypogastricus and pelvic splanchnic nerves, and consisted of sympathetic and parasympathetic nerves. The results showed that the content of nerves in the stumps of ligaments were reduced after being dyed with DMB and retained. DMB staining can effectively guide and assess the distribution and preservation of pelvic autonomic nerves. DMB as an ideal nerve tracer is worthy of wide application. Our study found no significant side effects at present, but the sample size is little and the observation time is shorter, further research is still needed in the later work.

## Appreciation

In the process of learning NSRH surgery, Professor Chen Chunlin, Liu Ping, Wu Ming, Liu Kaijiang, Yao Shuzhong had given much careful guidances and edifications. I cherish deep gratitude to them.

## Conflict of interest

None to declare.

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