An exploration of the surgical modality of sentinel lymph node biopsy in patients with cN0 tongue carcinoma: An animal study

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Objective. Lymphatic mapping with vital dye is often exploited during a sentinel lymph node biopsy (SLNB); however, the time restraints associated with blue dye may limit its efficacy in tongue carcinoma. Using an animal study, this exploration attempted to achieve a rational surgical modality to circumvent this hindrance.

Study design. Forty-eight rabbits were divided into 2 equal groups: A and B; 0.2 mL methylene blue (MB) was injected into 4 parts of the tongues of the rabbits in A just after elevating the cervical flap and into the tongues of the rabbits in B just before the elevation. The times when the lymphatic vessel and lymph node turned blue and then returned pale were recorded.

Results. Cases in group A had significantly more time left for tracing blue-stained lymphatic vessels than those in group B.

Conclusions. Elevating the cervical flap before injecting MB can alleviate the time restraint of MB as a lymphatic tracer in SLNB with tongue carcinoma.


The tongue is the most common site for oral squamous cell carcinoma,1 and tongue carcinoma has a high rate of metastasis to the cervical lymph nodes because of its constant functional movement and abundant lymphatic drainage. Reviews have showed that even in patients with a clinically negative neck (cN0), the incidence rate of micrometastases of the cervical lymph nodes may be as high as 50% (cN0 pN1).1 Although the pathologic status of regional lymph nodes is an independent prognostic factor for patients with cN0 tongue carcinoma and is the basis for deciding treatment, there is not yet a clinical staging modality or biological marker that can reveal the presence of micrometastases.2,3

In theory, the sentinel lymph node biopsy bears the promise of staging regional lymph nodes of early carcinoma before treatment. In practice, this method helps manage regional lymph nodes in cN0 breast carcinoma.4 Recently published studies that applied sentinel lymph node biopsy as a staging tool in head and neck carcinoma also reported a high reliability and accuracy,5,6 leading to some consensus on the methodological requirements of its use in head and neck staging.7 However, the consensus is not complete, and there is still a long way to go before sentinel lymph node biopsy can be widely used to clinically manage head and neck carcinomas.8 The elaborate topography and complex lymphatic drainage system in the head and neck usually make the complete detection of sentinel lymph nodes in carcinomas in this area a difficult and time-consuming task. This contributes largely to the low application of sentinel lymph node biopsy to carcinomas in this area, especially when using a blue dye as a lymphatic tracer.8

Three methods are currently available to identify cancerous sentinel lymph nodes before biopsy. They are preoperative lymphoscintigraphy, with or without the intraoperative use of a gamma probe, lymphatic mapping with the preoperative injection of blue dye, and a combination of these two. It is well known that the latter has the highest rate of identification of sentinel lymph nodes, and a relatively high success rate of applying sentinel lymph node biopsy to head and neck cancer would thus be achieved if performed properly. However, blue dye as a lymphatic tracer is not often used in the head and neck because completely detecting sentinel lymph nodes in this area is a time-consuming task. Therefore, this pilot study has explored the time constraints of methylene blue in sentinel lymph node biopsy with cN0 tongue carcinoma and attempted to achieve a rational surgical modality to circumvent this limitation.
MATERIALS AND METHODS

Animals and group design

Forty-eight healthy adult Japanese Big-Ear rabbits were obtained from the Ninth People’s Hospital, Shanghai Jiao Tong University, and kept in standard laboratory conditions. All experimental procedures were approved by the Animal Research Committee of Shanghai Jiao Tong University. The rabbits were grouped as follows: first, they were randomly divided into 2 equal groups, each with 24 rabbits. Group A received an injection of methylene blue just after the elevation of the cervical flap, and group B received an injection of methylene blue just before the elevation of the cervical flap. The animals in both group A and group B were further divided into 4 equal site groups, the tongue-tip, tongue-lateral, tongue-center, and tongue-root groups, according to the part of tongue where methylene blue would be injected during the experiment.

Animal experiments

Experiments were carried out on the rabbits in group A as follows. After being administered 3% amobarbital (Sheng Q Plant, Shanghai Pharmaceutical Inc., Shanghai, China; 30 mg/kg) through the ear-marginal vein as anesthesia, the rabbits were fixed facing up. The neck was exposed by incising along the midline and turning over the bilateral cervical flaps just over the superficial layer of the deep cervical fascia to reveal the whole area of the submental region, the submandibular region, and the anterior and lateral cervical regions. Next, with a fine needle punctured vertically into the center of the part of the tongue according to site group to which the experimental rabbit belonged to, 0.2 mL methylene blue was injected into the tongue tissue. Preliminary tests had revealed that this was the right dose to stain the draining node blue. Immediate dissection of the neck was then performed to identify the blue-stained lymphatic vessel and lymph nodes. The sites of blue-stained nodes and the time (counting from the moment the methylene blue was injected) taken for the lymphatic vessels and nodes to turn blue and to return pale were recorded.

The procedure of the experiment on rabbits in group B was the same as those in group A, except that the injection of methylene blue occurred just before the elevation of the cervical flap.

A technician who was not involved in the procedure was hired specifically to judge whether the lymphatic vessels and nodes were blue or pale.

Statistical analysis

All data were expressed as (sample mean ± standard deviation). SPSS 10.0 for Windows software (SPSS Inc, Chicago, IL, USA) was used to analyze the data, and differences were considered significant when \( P \) was less than .05.

RESULTS

As in the human neck, all the submental lymph nodes, submandibular lymph nodes, superficial cervical lymph nodes, and deep cervical lymph nodes can be found in the rabbit neck (Fig. 1). Blue-stained lymph nodes were successfully identified in all 48 experimental rabbits. However, each of the 12 cases in the group tongue-tip had blue-stained lymph nodes on both sides of the neck (Fig. 2), whereas this was not observed in any of the other groups (Fig. 3).

For all the 24 experiments in group A, the time taken for the lymphatic vessels and nodes to turn blue was recorded, whereas this could not be done in any experiments in group B (Table I). The times taken for the lymphatic vessels and lymph nodes to turn blue in group A were 1.313 ± 0.283 minutes and 1.655 ± 0.311 minutes, respectively.

For all 48 cases in this exploration, the recorded times for the lymphatic vessels and lymph nodes to become pale were 33.748 ± 7.973 minutes and 328.453 ± 14.712 minutes, respectively. The time taken for raising the cervical flap was controlled at 22.048 ± 2.356 minutes, which is similar to neck dissections on the humans (Table I).

There were no significant differences of the times for blue-stained lymphatic vessels to return pale among the 4 site groups or between the cases with blue-stained lymph nodes on 1 side of the neck versus cases with blue-stained lymph nodes on both sides of the necks (Tables II and III). However, cases in group A had significantly more time left for tracing blue-stained lymphatic vessels than those in group B.
DISCUSSION
The motives to explore a rational surgical modality for sentinel lymph node biopsy in patients with cN0 tongue carcinoma

For the management of the necks of patients with cN0 tongue carcinoma, a uniform and reasonable procedure is still eagerly anticipated. For years, head and neck surgeons have been trying to find a reliable procedure to reveal the presence of nodal micrometastases (cN0 pN1), with the objective of deciding between operation or irradiation for treatment.1,9

As the first node that drains lymph from the tumor, the sentinel lymph node, in theory, can generally predict the regional lymph nodal status of all solid cancers, and sentinel lymph node biopsy has achieved great success in managing early breast cancer.4 As for head and neck cancers, with experience and technical adaptations, the sentinel lymph node biopsy has also been reported to have a high reliability and accuracy in some recently published studies.5,6 However, reviews also suggest that the complete detection of sentinel lymph nodes of head and neck cancers demands further technical adaptations.10

When combined with lymphoscintigraphy, lymphatic mapping with vital dye is often exploited to help improve the identification rate of sentinel lymph nodes, yet this technique is exquisitely time sensitive. The blue dye is nonparticulate and labels the draining lymphatic vessels, even the sentinel lymph nodes, in minutes. Thus, the color rapidly disappears from the lymphatic vessel and nodes by dilution.11,12 This reflects the time restraint to completely detect blue-stained vessels and nodes in areas of complex nodal basins, such as the head and neck.

In a pig study, Nason et al.8 described using carbon dye to prolong the staining of sentinel lymph nodes and their draining lymphatic vessel to circumvent this type of time restraint. For the same reason, this investigation, using rabbits as the experimental subject, explored a rational surgical modality for sentinel lymph node biopsy in patients with cN0 tongue carcinoma. The results showed that this surgical modality could shorten the times taken in tracing the blue lymphatic vessels and identifying the sentinel lymph nodes.

A rational experimental subject—rabbit tongue in vivo

As shown in this investigation, although there were fewer lymph nodes in the rabbits’ necks, all the submental nodes, submandibular nodes, superficial cervical nodes and deep cervical nodes could be found there (Fig. 1), as in humans. Considering the high similarity

Table I. Times taken for raising the cervical flaps and for lymphatic vessels and lymph nodes to be stained blue or return to pale

<table>
<thead>
<tr>
<th></th>
<th>Cases</th>
<th>Sample mean ± SD (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time for the lymphatic vessel to be stained blue</td>
<td>24</td>
<td>1.31 ± 0.28</td>
</tr>
<tr>
<td>Time for the lymph node to be stained blue</td>
<td>24</td>
<td>1.66 ± 0.31</td>
</tr>
<tr>
<td>Time for the lymphatic vessel to return pale</td>
<td>48</td>
<td>33.75 ± 7.97</td>
</tr>
<tr>
<td>Time for the lymph node to return pale</td>
<td>48</td>
<td>328.45 ± 14.71</td>
</tr>
<tr>
<td>Time for raising the cervical flap</td>
<td>48</td>
<td>22.05 ± 2.36</td>
</tr>
</tbody>
</table>

All the times were counted from the moment the methylene blue was injected and are expressed as sample mean ± SD. The times for lymphatic vessels and lymph nodes to be stained blue could only be recorded in 24 cases in group A.
Comparison of the times taken for the cervical lymphatic vessels to return pale among the 4 site groups

<table>
<thead>
<tr>
<th>Site groups</th>
<th>Cases</th>
<th>Mean ± SD</th>
<th>MS</th>
<th>U</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tongue-tip</td>
<td>12</td>
<td>35.58 ± 8.10</td>
<td>7.7372</td>
<td>3</td>
<td>0.1149</td>
<td>&gt;.05</td>
</tr>
<tr>
<td>Tongue-lateral</td>
<td>12</td>
<td>31.60 ± 8.93</td>
<td>67.3243</td>
<td>44</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tongue-center</td>
<td>12</td>
<td>36.46 ± 8.53</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tongue-tip</td>
<td>12</td>
<td>30.94 ± 6.95</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

One-way analysis of variance: the difference was considered significant when $P < .05$; $F = 0.1149 < F_{0.05} (3, 44) = 2.82$, so $P > .05$, there were no significant differences between the times for lymphatic vessels to return pale among the 4 site groups.

Table III. Comparison of the times taken for the cervical lymphatic vessels to return pale between cases with blue-stained lymph nodes on both sides of the neck (group tongue-tip) and those on 1 side of the neck (remaining site-groups)

<table>
<thead>
<tr>
<th>Site group</th>
<th>Cases</th>
<th>Mean ± SD</th>
<th>t</th>
<th>U</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cases in group</td>
<td>12</td>
<td>35.58 ± 8.10</td>
<td>0.9885</td>
<td>46</td>
<td>&gt;.05</td>
</tr>
<tr>
<td>tongue-tip</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cases in remaining</td>
<td>36</td>
<td>33.00 ± 8.03</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>site groups</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

Two-sample/group $t$ test for independent samples (under equal variances situation); the difference was considered significant when $P < .05$. There were no significant differences between the times taken for lymphatic vessels to return pale between the tongue-tip group and the remaining site-groups.

in the function and development of the lymphatic systems of advanced vertebrate animals and those of human beings, a rabbit’s tongue in vivo should have a similar lymphatic drainage pattern to that of humans.

No cN0 tongue carcinoma model had been used in this exploration in view of the following facts. According to current knowledge on the mechanisms of cancer metastasis from the primary lesion to regional lymph nodes in the early stages, the presence of cN0 tongue carcinoma supports the idea of a denser reticulum of lymphocapillary vessels in the primary tumor that facilitates the metastasis of tumor cells to regional lymph nodes. For the collective lymphatic vessel, no change occurs; thus, the regional lymph nodes that drain lymph from the tumor tissue would be the same as before. In other words, whether cN0 tongue carcinoma is present or not, the outcomes of this experiment would not change. Still, it would take a relatively long time to set up an animal model of cN0 tongue carcinoma. A rational experimental design should achieve a good outcome as simply as possible, so it can be safely concluded that using rabbit tongues in vivo as subjects in this investigation is both practical and rational.

The time restraint of methylene blue as a tracer in sentinel lymph node biopsy

At present, 3 vital dyes, isosulfan blue, patent blue V, and methylene blue, are commonly used as lymphatic tracers in sentinel lymph node biopsies. Each of these dyes has displayed different degrees of time restraint: isosulfan blue is capable of keeping sentinel lymph nodes stained for 30 to 60 minutes, and patent blue V allows the operator a little less time (about 30-50 minutes) to identify blue-stained sentinel lymph nodes than isosulfan blue after 1 injection. Repeat injection of these dyes every 20 minutes was once advocated to avoid fading of the color in the sentinel lymph nodes, which was later found to affect the results. In this exploration, the time for regional lymph node staining by methylene blue to destain pale was 328.45 ± 14.71 minutes (Table I), which is 5 to 10 times longer than that of isosulfan blue or patent blue V and is consistent with the results (up to 6 hours) reported by Jasinski et al. However, as for “tracing blue lymphatic vessel successfully,” an important factor for forecasting that sentinel lymph nodes would be identified successfully, no record on this kind of time restraint of blue dye has been found published previously; so it is the first time for this exploration to reveal that time restraint of methylene blue for “tracing blue lymphatic vessel successfully” in sentinel lymph node biopsy was 33.75 ± 7.97 minutes (Table I).

The degree to which the time restraint of the vital dye affects the outcome of identifying sentinel lymph nodes in sentinel lymph node biopsy depends on the blue dye used, the site of the tumor, and the technique used. According to the results shown previously, of the 3 blue dyes, methylene blue provides the longest time for identifying sentinel lymph nodes. Recent comparison studies also showed that methylene blue could be an effective alternative to isosulfan blue, which has been considered the gold standard dye for sentinel lymph node evaluation in identifying the sentinel lymph nodes of breast cancer, with a success rate more than 90%. The site of the tumor also has a role in the effects of the time restraint of the blue dye on identifying the sentinel lymph nodes. In recent research on melanoma, Yuan Liu et al. performed a comprehensive comparison of the effectiveness of isosulfan blue versus methylene blue in sentinel lymph node biopsies, and the results demonstrated that methylene blue was more effective.
efficient than isosulfan blue for the exposure of sentinel lymph nodes in both the cervical and groin regions \((P \leq .01)\), whereas they were equally effective in the axilla \((P = .919)\). Because there is a less complex lymphatic drainage system in the axilla compared with the neck and a less intricate topography in the axilla compared with the groin, dissecting and completely detecting sentinel lymph nodes should need less time in the axilla than in the cervical or groin regions for the same operator. If the time the operator needs in the axilla is within the time restraints of both methylene blue and isosulfan blue, and the time needed in the groin and neck is beyond the time restraint of isosulfan blue but within that of methylene blue, then the time restraint will have little effect in the axilla whether the blue dye used is methylene blue or isosulfan blue; however, in the cervical or groin regions he tends to achieve a higher identification rate of sentinel lymph node using methylene blue than using isosulfan blue. Dr Yuan Liu was probably in such a case.

The performance of sentinel lymph node biopsy using blue dye could be traced down as early as 1991.24 To date, the list of cancers that have been studied exploiting it for managing regional lymph nodes includes almost all parts of the human body.23,25-27 As they all have a site-specific topography, a suitable blue dye and site-specific technique details, such as the surgical modality, the method and dose for injecting the blue dye are needed to diminish the effects of the time restraint of the blue dye so that a high identification rate of sentinel lymph nodes may be achieved.7,16,28,29

**Significance of elevating the cervical flap before the injection of methylene blue**

Lymphatic mapping with blue dye is simple and cheap and presents few complications. However, the time restraint of blue dye is sometimes associated with a poor sentinel lymph node identification rate, especially when a complex nodal basin and intricate topography, as in the human neck, are involved. This is the primary hindrance to the wide application of this technique in clinical management. Thus, as many patients with cN0 carcinoma would benefit from this technique, the time restraint of the blue dye should be addressed. For this reason, this study has explored the possibility of addressing the effects of the time restraint of methylene blue in sentinel lymph node biopsy for managing cN0 tongue carcinoma. Methylene blue was chosen here for its availability, decreased allergic response, and high performance.20-23

As shown previously, the time restraint of blue dye will affect the operator tracing blue-stained lymphatic vessels and lymph nodes less if a less complex topography exists. However, the sentinel lymph nodes of the cN0 tongue carcinoma are deep and dispersed in the neck, which hinders the exposure of blue-stained lymphatic vessels and sentinel lymph nodes. However, what if one exalts the superficial cervical flap before injecting methylene blue?

In this investigation, the time for elevating the cervical flap was controlled to approximately 20 minutes, as during neck dissection on patients (Table I). The results showed that in all 48 cases, the time for the lymphatic vessel to return pale was 33.75 ± 7.97 minutes (Table I). There was no significant difference in terms of the part of tongue where methylene blue was injected or the sites where the blue-stained lymph nodes were located (Figs. 2 and 3, Tables II and III). Because the rabbit’s neck has very little connective tissue surrounding the node (Fig. 1), after elevation of the cervical flap, the operation for exposing the blue-stained nodes and lymphatics consists only of incising the superficial layer of the deep cervical fascia and turning over the submaxillary gland, which takes approximately 1 minute. Thus, for all the cases in group A, we were able to record the time needed for lymphatic vessels and nodes to turn blue (Table I, time for methylene blue to move from the tongue to cervical lymphatic vessels and nodes was more than 1 minute).

Blue-stained lymphatic vessels were visible in group A for approximately 30 minutes, whereas these vessels were visible in group B for approximately 10 minutes. Thus, the cases in group A had significantly more time left for tracing blue-stained lymphatic vessels than those in group B (Table IV). Patients with cN0 tongue carcinoma should undergo elevation of the cervical flaps because of the intricate topography and complex lymphatic drainage pattern, as it would be difficult for one to completely detect blue-stained lymphatic vessels in fewer than 10 minutes. However, 30 minutes would be enough time to do so. Because tracing lymphatic vessels successfully is the best forecasting factor for a successful identification of the sentinel lymph node,19 the necessity for elevating the cervical flap before injecting methylene blue is that it increases the chance of

**Table IV.** Comparison of the times left for dissecting and tracing blue-stained cervical lymphatic vessels after the elevation of the cervical flaps between group A and group B

<table>
<thead>
<tr>
<th>Group</th>
<th>Cases</th>
<th>Mean ± SD</th>
<th>t</th>
<th>U</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>24</td>
<td>32.96 ± 6.75</td>
<td>12.42</td>
<td>46</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>B</td>
<td>24</td>
<td>12.10 ± 4.82</td>
<td>6.75</td>
<td>12.42</td>
<td>46.001</td>
</tr>
</tbody>
</table>

Pairwise/matched t tests for dependent samples: the difference was considered significant when \(P < .05\). There was a significant difference between the times left for tracing blue-stained lymphatic vessels in group A and group B.
successfully detecting sentinel lymph nodes within the time restraint of methylene blue, by the way of greatly improving the success rate of tracing the lymphatic vessels and shortening the time needed to identify blue-stained lymph nodes.

The question is now that is this flap practical, or would it not damage the lymph drainage system of the tongue? In this study, all rabbits were subjected to elevation of the same thick flap as in the neck dissection just over the superficial layer of deep cervical fascia. It would be impractical if this flap contained sentinel lymph nodes or their draining lymphatic vessels because it would damage the latter and lead to a failure in identifying sentinel lymph nodes. However, because this exploration has achieved a 100% (48/48, result 1) detection rate of blue-stained lymph node contradicts this presumption.

In summary, it would be not only necessary but also practical to elevate the cervical flap in neck dissection before injecting methylene blue in sentinel lymph node biopsy of cN0 tongue carcinoma.

CONCLUSIONS

Lymph mapping with vital dye is often used in sentinel lymph node biopsy to stage the regional lymph nodes of cN0 cancer. However, the time restraint of blue dye is the main hindrance for its wide application, especially in intricate areas, such as the head and neck. Among the commonly used blue dyes, methylene blue can keep lymphatic vessels and nodes stained for the longest amount of time, and it should thus be the first choice for sentinel lymph node biopsy of cN0 tongue carcinoma. A rational surgical modality of elevating cervical flaps before injection of methylene blue can help to circumvent the time restraint of methylene blue in sentinel lymph node biopsy of cN0 tongue carcinoma.

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REFERENCES


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