Accuracy of intraoperative tissue staining in delineating deep surgical margins in oral carcinoma surgery

Hiroshi Kurita, Hironori Sakai, Takahiro Kamata, Takeshi Koike,

Hiroichi Kobayashi and Kenji Kurashina

Department of Dentistry and Oral Surgery, Shinshu University School of Medicine,

3-1-1, Matsumoto, 390-8621, Japan

Corresponding author: Hiroshi Kurita

Department of Dentistry and Oral Surgery

Shinshu University School of Medicine

3-1-1, Asahi, Matsumoto, 390-8621

Japan

Tel.: +81 263 37 2677; Fax: +81 263 37 2676

e-mail: hkurita@hsp.md.shinshu-u.ac.jp

Abstract:
The purpose of this study was to assess the accuracy of this tissue staining assessment of surgical specimens in delineating deep surgical margins in oral cancer surgery. Fifteen patients who underwent surgery for oral carcinoma were included in the study. Once the tumor was resected, a vertical section of the surgical specimen was taken from the central part of the tumor. The section was consecutively stained with 0.4% indigo carmine and 0.5% Congo red, and deep surgical margins were assessed using a digital microscope with a magnification power of 25 to 175x. The results of tissue-staining analysis were compared with the corresponding results of conventional histopathological analysis with HE staining, which is considered the gold standard. The extent of carcinoma invasion could be visualized after the application of tissue-staining solutions. Tissue-staining analysis was accurate in 12 of the 15 patients (80%) in evaluating the closest deep surgical margin. There was no significant difference in the tumor-margin distance between tissue staining and histopathological assessment in these 12 patients (Wilcoxon signed-ranks test, $P>0.63$). The results of this study showed that intraoperative tissue staining of surgical specimens permitted visual inspection and assessment of tumor spread to surgical margin, although the method has some limitations. The method had a possible ability in controlling the deep surgical margin.

Key words: intraoperative, margin assessment, cancer surgery, tissue staining

Introduction

One of the most important but difficult aspects of cancer surgery is ensuring complete removal of the tumor at the primary site. It has been shown that failure to achieve a clear surgical margin results in increased risk of local recurrence and a subsequent reduced chance of survival.[1-4] To ensure complete resection of the tumor, frozen-section analysis, including Moh's micrographic technique,[5] is employed in some units. Some researchers have advocated that frozen-section examination for margin assessment is a reliable technique to control the extent of cancer surgery;[6-9] however, this approach has some problems as it is time consuming, costly, and sometimes stressful.

In oral cancer surgery, intraoperative margin assessment is usually undertaken using either the mucosal or deep surgical margin. Vital staining with iodine solution has been employed with success in determining the extent and precise border of the cancerous/dysplastic epithelium, while ensuring radical resection of the tumor at the mucosal surface.[10] On the other hand, no reliable intraoperative procedure, other than frozen-section analysis, has yet been developed to assess the deep surgical margin. Intraoperative macroscopic margin assessment has been a useful technique to ensure the extent of tumor resection. In macroscopic assessment, the distinction between carcinoma and the surrounding tissue is basically founded on a difference in their colors; therefore, the differentiation between carcinoma and the surrounding healthy tissue is sometimes difficult if there is little difference in their colors. An additional method allowing clear distinction of tumor tissues from surrounding healthy tissues is necessary.

Recently, we have found that consecutive application of two staining solutions could produce a better distinction of the invaded tumor from surrounding healthy tissues in the resected surgical specimen. This staining was expected to yield better visualization of the invaded tumor and, thus, better ability to control surgical margins. The purpose of this study was to assess the accuracy of intraoperative tissue staining assessment of surgical specimens in delineating deep surgical margins in oral cancer surgery.

Methods

A prospective study of 15 patients who underwent surgery for oral carcinoma was undertaken. Patients with lesions on the gingiva and hard palate were excluded because intraoperative sectioning of the resected hard tissue (bone) was difficult. Informed consent was obtained from each patient. The patients were six women and nine men, with a mean age of 67.8 years (range 49 – 84). Clinical and histopathological characteristics of the tumors are shown in Table 1. Two patients with recurrent tumor after interstitial radiotherapy were included. Five additional patients underwent platin-based multi-agent neoadjuvant chemotherapy four weeks prior to the planned surgery. Informed consent was obtained from all subjects and/or gradients.

A flow chart of the series of examinations is presented in Figure 1. All patients underwent surgical resection of their oral tumor under general anesthesia. Once the tumor was resected, a 5 mm-thick vertical section of the surgical specimen was taken from the central part of the tumor. The section was then pinned to a board and prepared for tissue staining.

After examination of the section, tissue staining was performed as shown in Table 2. The stained section was examined macroscopically using a digital microscope

(VHX-1000, KEYENCE Co., Osaka, Japan). The VHX-1000 has a 1/2-inch, 2.1-million-pixel CCD image sensor and a high-definition, 15-inch HD TFT color LCD monitor (1,600 x 1,200 pixels). In this study, a standard zoom lens with a magnifying power of 25 to 175 (VH-Z25, KEYENCE, Osaka, Japan) was used. The extent of the invaded tumor was assessed and the foremost site of deep tumor invasion was identified, and the closest distance between the tumor and the surgical resection surface ("A" in Fig. 1) was measured using the measurement function of the VHX-1000. The vertical dimension of the section ("D1" in Fig. 1) was also measured to calculate shrinkage of the section later.

The sliced section was then processed for conventional histopathological evaluation of surgical specimens. The section was subjected to formalin fixation, after which it was embedded in paraffin and a horizontal slice was prepared for conventional microscopic examination with hematoxylin and eosin (HE) staining. The prepared specimen was examined using a light microscope with magnification powers of 40 and 100. The foremost site of the deep tumor invasion was identified and the closest distance between the tumor and the surgical resection surface ("B" in Fig. 1) was measured with an ocular micrometer. The vertical dimension of the microscopic section ("D2" in Fig. 1) was also measured to calculate the shrinkage occurring from digital microscopy to the final histopathological assessment.

It was determined whether the closest point of the deep surgical margin was correctly detected with tissue staining; that is, the closest point of the deep surgical margin was compared between the result of tissue staining and that of histopathological examination. Next, the tumor-margin distance measured on digital microscopy was compared with the corresponding result on histopathological assessment by considering the individual shrinkage of each microscopic section; that is, the tumor-margin distance on microscopic assessment was corrected by computing shrinkage as follows: (the tumor-margin distance on microscopic examination) = ("B") x ("D1") / ("D2") (Fig. 1).

Results

The extent of tumor invasion could be visualized after application of tissue-staining solutions (Fig. 2). Tissue staining produced a brown-black stain on normal muscle, connective, salivary, and most part of adipose tissues but not on tumor and epithelial tissues. Staining had no ability to differentiate tumors from normal epithelium tissue, and the detection of intraepithelial spread of the tumor was impossible. Cutting a vertical slice from the unfixed specimen and the tissue staining had no negative influence on following routine histopathological examination with HE staining.

As a result, the closest point to the deep surgical resection margin by digital microscopic assessment agreed with the corresponding result by histopathological assessment in 12 of the 15 patients (80%). In two cases in which the closest surgical deep margin was incorrectly diagnosed with tissue-staining examination, remnant vital tumor cells in scar tissue after neoadjuvant chemotherapy could not be detected (Fig. 3). In another case, adipose tissue scattered in the muscle tissue was misidentified as carcinoma tissue (Fig. 4).

A comparison of the tumor-margin distance between tissue-staining assessment and permanent histopathological assessment is shown in Table 3. The comparison was made using 12 patients, excluding three cases in which the definition of the closest point of tumor invasion differed between the types of analysis. Shrinkage from digital

microscopy to final microscopic examination showed a median value of 90.1% (ranging from 83 to 100%). There was no significant difference in the tumor-margin distance between histopathological and digital microscopic examination (Wilcoxon signed-ranks test, $P>0.63$). The deviation ranged from 0.4 to 4.1 mm with a median absolute difference of 1.7 mm.

Discussion

The purpose of this study was to assess the accuracy of intraoperative tissue staining of surgical specimens to identify the deep surgical margin in oral carcinoma surgery. The results of tissue-staining examination were compared with those of conventional histopathological analysis with HE staining, which is considered the gold standard.

Recently we have found that tissue staining with 0.4% indigo carmine and 0.5% Congo red was useful in demonstrating the extent or border of tumor invasion. As shown in this study, a consecutive application of 0.4% Indigo carmine and 0.5% Congo red produced brown-black stain on normal muscle, fibrous/scar, salivary, and most part of adipose tissue but not invaded tumor, and, therefore, it enabled to demarcate the extent of the tumor invasion. Indigo carmine is a conventional contrast dye used for vivid accentuation of the intestinal mucosa.[11, 12] If indigo carmine is applied on the surface of the epithelium, the blue color dye is not absorbed and collects in the sulci and grooves of the mucosa, highlighting the topography of the stained mucosa. Congo red is a reactive pH-dependent coloring agent and it identifies acid-secreting gastric cells. Congo red causes color change (from red to dark blue) in these acid-secreting mucosal regions.[11, 12] In addition, it is reported that a consecutive application with methylene blue enables distinction stomach areas altered by the tumor from their surroundings.[13] Therefore, in our study, Congo red is applied after application of indigo carmine. With this staining, carcinoma tissue was bleached out, while surrounding connective and muscle tissues were stained as brown- black. As a result, the consecutive staining enabled to demarcate the extent of the tumor invasion. Thereafter, intraoperative tissue staining of surgical specimens was assessed using consecutive application of these two agents.

The results of this study showed that the tissue staining examination was accurate in 13 of the 15 patients for evaluating the closest deep surgical margin. Further, there was no significant difference in the tumor-margin distance between tissue-staining and histopathological assessment in these 13 patients. These results indicate that the tissue staining examination is useful to control deep surgical margins when the tumor tissue is accurately delineated. In the treatment of head and neck cancer, intraoperative frozen section analysis is thought to be a useful guide to control free surgical margins. Ribeiro et al. studied the usefulness of frozen sections in the resection of oral carcinoma and reported that there was a failure to achieve free surgical margins of the invasive tumor in 15% of cases, despite favorable results on frozen section analysis.[8] It was suggested that deep margins are particularly difficult to assess intraoperatively. The accuracy of tissue-staining assessment appears be equal to that of frozen-section analysis; however, intraoperative tissue staining has some major advantages over conventional frozen-section assessment. Firstly, the procedure is simpler and more expedient; secondly, a wider area of the resection margin can be assessed; thirdly, the procedure allows for better orientation of the surgical specimens; and fourthly, is not time consuming (needs only several minutes).

Previously, we assessed the possible ability of intraoperative digital microscopic analysis in controlling the deep surgical margin in oral carcinoma surgery. In the results, there was no significant difference in the tumor-margin distance between digital microscopic and histopathological assessment.[14] The result suggested that digital microscopic examination was useful to assess deep surgical margins when the tumor was accurately delineated; however, the procedure has some problems, the most major of which is its low ability to distinguish tumor tissues from fibrous tissues. In this study, fibrous and scar tissues were stained with the staining solutions and could be distinguished from tumor tissue; therefore, it can be said that the ability to delineate tumor invasion is more accurate if tissue staining is applied before digital microscopic examination.

On the other hand, in the results of this study, it was sometimes difficult to distinguish between carcinoma and adipose tissue. Adipose tissues scattered in the muscle tissue were misdiagnosed as invaded tumor in one of the 15 cases and the method could not show different staining. Before application of the staining solutions, differentiation between the tumor (white or cream-colored) and adipose (yellow-colored) tissue was easy, because they are different in color; therefore, careful examination of un-stained tissues helps to distinguish between them. However, if adipose tissues are scattered and adjacent to tumor tissues, distinguishing between them is sometimes difficult. Additional methods that can clarify the difference between carcinoma and adipose tissue will be necessary.

The results of this study also showed that remnant vital tumor cells in scar tissue after neoadjuvant chemotherapy were difficult to detect using tissue staining in this study. Microscopic nests of infiltrating tumor cells could not be visualized by tissue staining even with digital microscopic examination. Although histopathological examination with HE staining produces a clearer perception of the shape and/or the structure of the cell, digital microscopic examination could not provide such information. Regarding oral squamous cell carcinoma, it was assumed that almost all of the tumor existed as one entity. In the results of this study, untreated and fresh invaded carcinoma was observed as a continuous mass or nest. On the other hand, if the continuous structure of the invaded tumor had been destroyed with preoperative chemotherapy, microscopic tumor cells remaining in destroyed tissues were difficult to detect by tissue staining of surgical specimens. Previous treatment that produces tumor necrosis might have a major influence on assessment of the deep surgical margin by tissue staining.

In conclusion, the results of this study showed that intraoperative tissue staining of surgical specimens permitted visual inspection and assessment of tumor spread to surgical margin, although the method has some limitations. The method could not detect microscopic nests of infiltrating carcinoma and could not clarify the difference between the invaded carcinoma and scattered adipose tissue. However, this method had possible ability to suggest deep surgical margins and to select a site for additional frozen-section assessment. The numbers involved in this study are small and the stains need some refinement to improve their sensitivity and specificity. Further studies are required.

References

1. Jacobs JR, Ahmad K, Casiano R, Schuller DE, Scott C, Laramore GE, et al. Implications of positive surgical margins. Laryngoscope 1993; 103:64-68.
2. Loree TR, Strong EW. Significance of positive margins in oral cavity squamous carcinoma. Am J Surg 1990; 160:410-414.
3. Ravasz LA, Slootweg PJ, Hordijk GJ, van de Smit F, Twell I. The status of the resection margin as a prognostic factor in the treatment of head and neck carcinoma. J Craniomaxillofac Surg 1991; 19:314-318.
4. Sutton DN, Brown JS, Rogers SN, Vaughan ED, Woolger JA. The prognostic implications of the surgical margin in oral squamous cell carcinoma. Int J Oral Maxillofac Surg 2003; 32:30-34.
5. Davidson TM, Haghighi P, Astarita R, Baird S, Seagren S. Mohs for head and neck mucosal cancer: Report on 111 patients. Laryngoscope; 98:1078-1083.
6. Lee JG. Detection of residual carcinoma of the oral cavity, oropharynx: a study of surgical margins. Trans Am Acad Ophthalmol Otolaryngol 1974; 78:49-53.
7. Byers RM, Bland KI, Borlase B, Luna M. The prognostic and therapeutic value of frozen section determinations in the surgical treatment of squamous carcinoma of the head and neck. Am J Surg 1978; 136:525-528.
8. Ribeiro NFF, Godden DRP, Wilson GE, Butterworth DM, Woodwards RTM. Do frozen sections help achieve adequate surgical margins in the resection of oral carcinoma? Int J Oral Maxillofac Surg 2003; 32:152-158.
9. Gooris PJJ, Vermey B, de Visscher JGAM, Roodenburg JLN. Frozen section examination of the margins for resection of squamous cell carcinoma of the lower lip. J Oral Maxillifac Surg 2003; 61: 890-894.
10. Kurita H, Kurashina K. Vital staining with iodine solution in delineating the border of oral dysplastic lesions. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 1996; 81:275-280.
11. Jung M, Kiesslich R. Chromoendoscopy and intravital staining techniques. Baillere's Clinical Gastroenterology 1999; 13:11-19.
12. Fennerty MB. Tissue staining (chromoscopy) of the gastrointestinal tract. Can J Gastroenterol 1999; 13:423-429.
13. Tatsua M, Okuda S, Tamura H, Taniguchi H. Endoscopic diagnosis of early gastric cancer by the endoscopic Congo red-methylene blue test. Cancer 1982; 50:2956-2960.
14. Kurita H, Uehara S, Funamoto S, Nakatsuka A, Kobayashi H, Kurashina K. Intraoperative digital microscopic assessment of the deep surgical margins in oral carcinoma surgery: a preliminary report. Am J Surg 2006; 191:84-88.

Figure Captions

Figure 1. Flow chart of an examination series. Tissue staining and subsequent histopathological assessment were carried out using a vertical section of the surgical specimen

Figure 2. Section of surgical specimen of tongue carcinoma before (A) and after (B) tissue staining. The extent of tumor invasion could be visualized after the application of tissue-staining solutions. Tissue staining produced a brown-black stain on normal muscle, connective, and salivary tissues but not tumor and epithelial tissues.

Figure 3. Photographs of surgical specimens of tongue carcinoma with tissue staining (A) and histopathological examination (B, HE staining). Remnant vital tumor cells in scar tissue (arrows) after neoadjuvant chemotherapy could not be detected by tissue-staining examination (arrow heads).

Figure 4. Photographs of surgical specimen of tongue carcinoma with tissue staining (A) and histopathological examination (B, HE staining). Adipose tissue (arrows) was misidentified as carcinoma in the tissue-staining examination (arrow heads).

Table 1. Clinical and histopathological characteristics of the tumor

Primary site	
Tongue	11
Oral floor	3
Cheek mucosa	1
Tumor stage (TNM classification of UICC)	
T1	7
T2	2
T4	4
rT2	2
Hisopathological diagnosis	
Well-differentiated SCC	9
Moderately differentiated SCC	3
Poorly differentiated SCC	1
Early invasive SCC	2
Previous and/or preoperative treatment of the tumor	
None	8
Neoadjuvant chemotherapy	5
Interstitial radiotherapy	2

SCC: squamous cell carcinoma

Table 2. Sequence of dye application

1. Direct examination
2. Gently wash with saline solution and dry with a cotton bud
3. Apply 0.4% indigo carmine with a cotton bud (10 to 20 seconds)
4. Apply 0.5% Congo red with a cotton bud (10 to 20 seconds)
5. Wait for 1 to 2 minutes
6. Interpret stain reaction

Table 3. Comparison of the tumor-deep margin distance between digital microscopic and histopahological assessment

Patient No.	**Tumor-margin distance on histopathological examination (B x D1/D2)**	**Tumor-margin distance on vital staining examination (A)**	**Deviation**
No. 1	1.2 mm	5.3 mm	4.1 mm
No. 2	4.0	4.6	0.6
No. 3	16.1	19.0	2.9
No. 4	2.2	5.7	3.5
No. 5	0.6	1.0	0.4
No. 6	5.2	7.7	2.5
No. 7	10.6	9.0	1.6
No. 11	19.4	16.5	2.9
No. 12	12.4	11.7	0.7
No. 13	22.9	22.5	0.4
No. 14	24.8	23.0	1.8
No. 15	10.1	9.0	1.1
Median	10.4	9.0	1.7
IQR	3.5-16.9	5.6-17.1	0.7-2.9

There was no significant difference in the tumor-margin distance between histopathological and vital staining examination (Wilcoxon signed-ranks test, $P>0.63$).

Figure 1

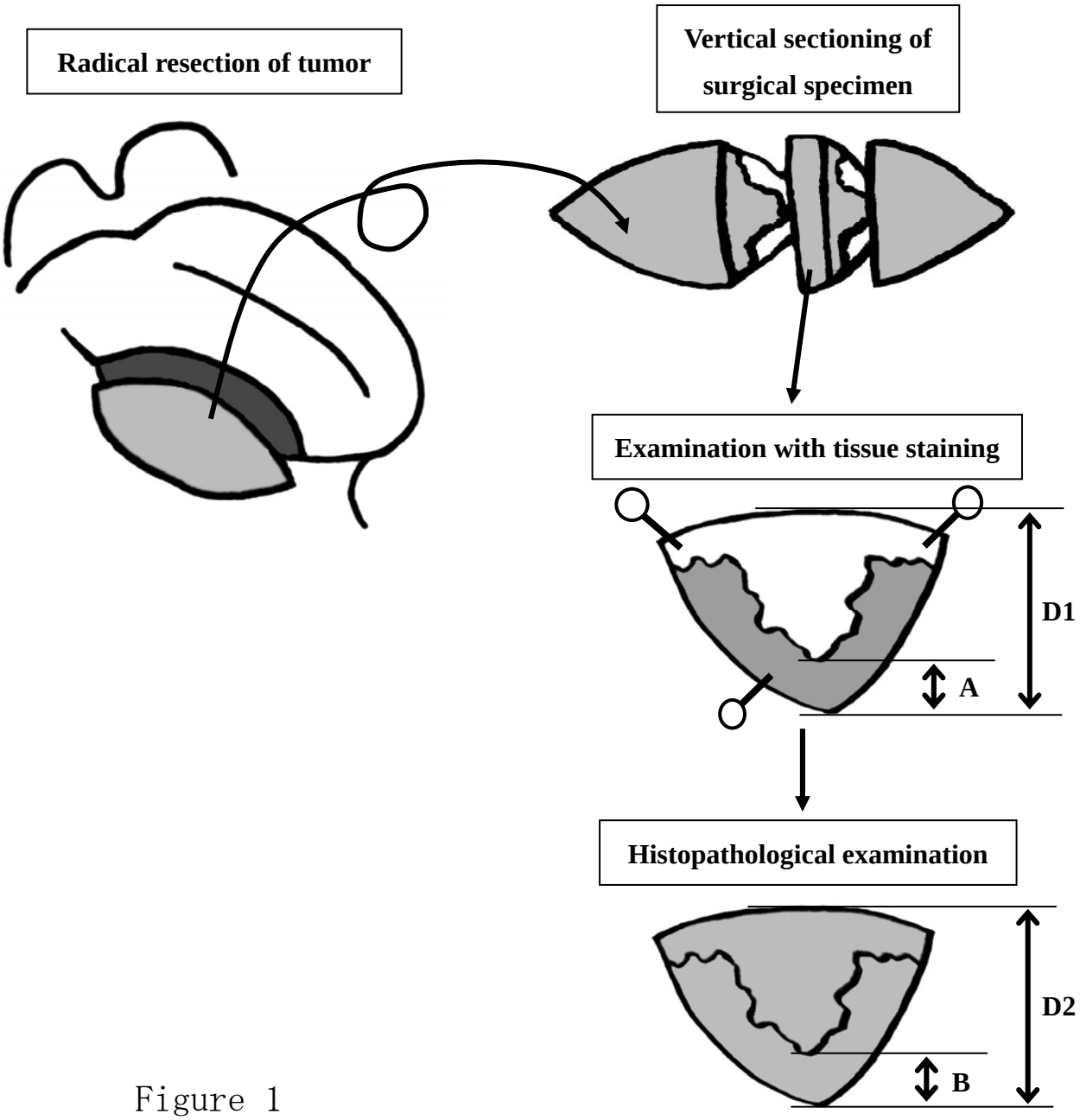

Figure 1

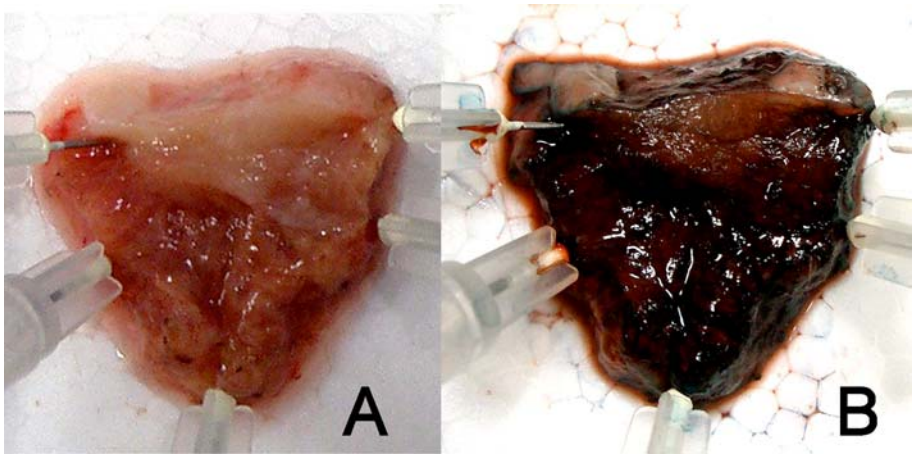

Figure 2

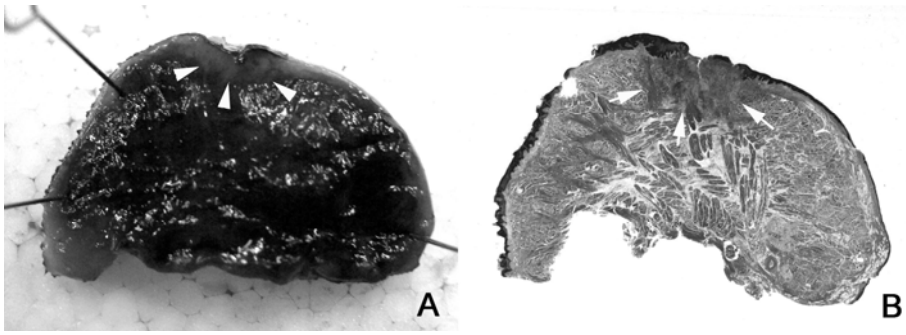

Figure 3

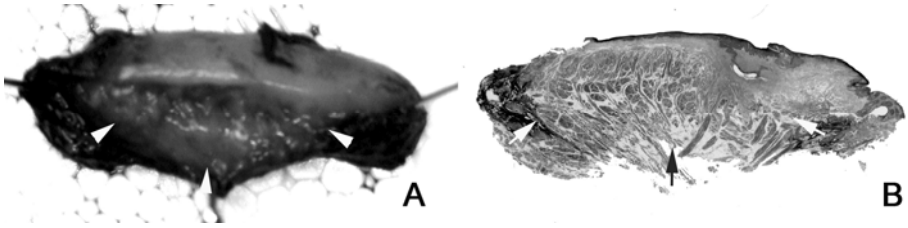

Figure 4