View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Repositório Institucional UNIFESP

Sentinel lymph node biopsy in rats. Comparison between paraffin and frozen section analysis[1]

Ivan Dunshee de Abranches Oliveira Santos Filho[I], Milvia Maria Simões e Silva Enokihara[II], Janaina Namba Shie[III], Andrea Fernandes de Oliveira[IV], Ivan Dunshee de Abranches Oliveira Santos[V], Lydia Masako Ferreira[VI]

DOI: http://dx.doi.org/10.1590/S0102-86502014001300011

[1]MD, Post graduate student, Post-graduation Program in Translacional Surgery, UNIFESP, Sao Paulo, Brazil. Acquisition and interpretation of data, statistical analysis, manuscript writing.
[II]MD, PhD, Pathology Department, UNIFESP, Sao Paulo, Brazil. Acquisition and interpretation of data.
[III]MD, Resident Physician in General Surgery, Department of Surgery, UNIFESP, Sao Paulo, Brazil. Acquisition and interpretation of data.
[IV]MD, Post graduate student, Post-graduation Program in Translacional Surgery, UNIFESP, Sao Paulo, Brazil. Manuscript writing.
[V]PhD, Head of Division of Plastic Surgery, Department of Surgery, UNIFESP, Sao Paulo, Brazil. Conception and design of the study, critical revision.
[VI]PhD, Full Professor, Chairwoman Plastic Surgery Division, Department of Surgery, UNIFESP, Sao Paulo-SP, Brazil. Critical revision.

ABSTRACT

PURPOSE: To simulate a lymph node metastasis in an animal model using activated carbon, assess their identification in frozen section analysis and compare with histopathological examination in paraffin.

METHODS: Thirty two adult female rats were used. They received the carbon injection on its hind legs. Half of the rats was sacrificed on day one, and the other half after 21 days. Thus, 64 lymph nodes were dissected and split longitudinally. One half of the lymph node was sent immediately to frozen section analysis. The other half was fixed in 10% formaldehyde to be cut in paraffin. Slides were divided into quadrants and classified by the presence of carbon in these four quadrants_ They were also classified by the carbon staining intensity.

RESULTS: Comparing the slides obtained in the first day and 21 days, there was a tendency of carbon to spread over time, but without statistical significance. The intensity did not alter over time.

CONCLUSION: There was no concordance between the two methods of pathological analysis, however the actived carbon was seen in all lymph nodes.

Key words: Lymph node biopsy. Frozen sections. Paraffin embedding. Charcoal. Rats.

Introduction

Currently, sentinel lymph node biopsy (SLNB) is the gold standard for staging cutaneous melanoma, and was the greatest advancement that emerged over the past two decades to a more reliable and early staging. The technique was described in 1992, and indicates the first lymph node that can receive metastatic cells from the primary tumor[1]. Many studies have demonstrated that histological analysis of sentinel node reflects the state of the corresponding lymphatic basin[2,3]. With the use of patent blue associated with nuclear medicine, the percentage of detection of lymph nodes increased up to over 97% and the percentage of detection of micrometastases increased considerably[4,5].

After a lymphadenectomy, the analysis of lymph node metastases usually examines a slice of 5 mm thick of each lymph node dissected. This slice is stained by hematoxylin-eosin. This sampling is considered insufficient for the detection of micrometastases, particularly in melanoma, because their metastatic cells can penetrate the lymph node in small clusters or even as isolated cells[6]. The analysis of the sentinel lymph node should be meticulous, especially in the subcapsular sinus, most frequent site of micrometastases[7].

The frozen section analysis is not routinely performed by concerns that freezing could compromise the analysis in paraffin. In other words, the small fragment of the lymph node used in the frozen section analysis could contain the only metastases, and if not visualized could lead to a false negative diagnosis[8]. On the other hand, if it were possible to perform a frozen section examination during surgery, and it indicated that a lymph node dissection is necessary, there would be no need for a second hospitalization for lymphadenectomy, thus reducing morbidity, the cost of a second surgery and the stress of two hospitalizations.

Oliveira Filho described an experimental model in the adult rats demonstrating that sentinel lymph node biopsy is feasible[8]. Using this experimental model, Junqueira demonstrated that activated carbon can also be used as a marker, helping the pathologist in the search for metastases, without compromising the histological analysis of lymph nodes[9].

The aim of this study was to simulate a lymph node metastasis in an animal model using activated carbon at different concentrations, assess their identification in frozen section histopathology and compare with standard histopathology in paraffin.

Methods

This study was approved by the Ethical Committee under protocol number 0582/10.

In the experiment 32 adult Wistar EPM-1 rats, weighing between 250 and 300 grams, were used. Each animal was anesthetized with an intra peritoneal injection of tiletamine hydrochloride (25 mg / kg) and zolazepam hydrochloride (25 mg / kg). After sedation, 0.08 ml of 0.25% activated carbon was injected in the ventral portion of a hind paw. In the contralateral paw, the same volume of 0.125% activated carbon was injected. The paw that received the 0.25% activated carbon solution was alternated for each animal. 16 rats received in each hind paw 0.08ml of patent blue (to assist in the immediate location of the lymph node). Thirty minutes after the injection, the SLN was dissected and removed for examination.After excision of the lymph nodes, the rats were sacrificed with extremely high doses of the same anesthetic.

Each lymph node was identified, numbered and split along its bigger diameter. One half of the lymph node was sent immediately to frozen section pathological examination. Two slides were prepared. The other half of the lymph node was placed in 10% formaldehyde for tissue fixation and preparation of final histopathology in paraffin using HE staining[7].

The remaining sixteen animals were kept in cages under the same conditions of temperature, humidity, lighting and noise, receiving commercial ration and water ad libitum for 21 days. After this period, the animals underwent the same anesthetic and surgical procedure. The sentinel nodes removed underwent the same technique of frozen section histopathology and paraffin with HE staining, similar to the initial group.

The slides obtained from the two histological techniques, frozen section and paraffin were examined by two independent pathologists. Slides were divided into 4 quadrants, and the presence of carbon in each of the quadrants was evaluated on a score from 1 to 4. For example, if there were carbon present in only 1 of the 4 quadrants, the slide would receive a grade 1. If 2 quadrants were stained, the slide would receive a grade 2.

The slides were also evaluated for carbon staining intensity, receiving a score of 1 to 4. A slide with low staining intensity of carbon received a grade 1. A strongly stained slide, received the highest score. For this classification, pathologists used a table as a reference with photos containing images of slides with 4 intensities.

Quadrants and intensities scores were described according to days in each concentration and method of evaluation using absolute and relative frequencies and compared between days using the Mann-Whitney test.

The results of quadrants and intensities at each concentration and evaluation method are described by joining the two days of sacrifice and verified the agreement/reproducibility between evaluation methods using Kappa coefficient of Fleiss.

The tests were performed with a significance level of 5%.

Results

The lymph nodes that received the 0.25% activated carbon injection had more quadrants impregnated in the frozen section technique on day 21 than on day 0 ($p < 0.001$). In the remaining situations there was no statistically significant difference from day 0 to day 21 ($p > 0.05$). There was also no statistical difference when analyzing the intensity. (Table 1)

TABLE 1 - Quadrants and intensity description according to days and comparisons results.

Variable	Category	Day 0 (N = 16)		Day 21 (N = 16)		p
		n	%	n	%	
Quadrant 0.25 Parafinn	1	0	0.0	0	0.0	0.239
	2	1	6.3	0	0.0	
	3	3	18.8	0	0.0	
	4	12	75.0	16	100.0	
Quadrant 0.25 Frozen Section	1	0	0.0	0	0.0	<0.001
	2	6	37.5	0	0.0	
	3	7	43.8	0	0.0	
	4	3	18.8	16	100.0	
Quadrant 0.125 Parafinn	1	0	0.0	1	6.3	0.780
	2	0	0.0	0	0.0	
	3	0	0.0	0	0.0	
	4	16	100.0	15	93.8	
Quadrant 0.125 Frozen Section	1	0	0.0	0	0.0	0.138
	2	1	6.3	0	0.0	
	3	4	25.0	0	0.0	
	4	11	68.8	16	100.0	
Intensity 0.25 Parafinn	1	2	12.5	0	0.0	0.128
	2	5	31.3	2	12.5	
	3	3	18.8	5	31.3	
	4	6	37.5	9	56.3	
Intensity 0.25 Frozen Section	1	3	18.8	2	12.5	0.539
	2	7	43.8	4	25.0	
	3	2	12.5	8	50.0	
	4	4	25.0	2	12.5	
Intensity 0.125 Paraffin	1	0	0.0	1	6.3	0.780
	2	6	37.5	3	18.8	
	3	4	25.0	9	56.3	
	4	6	37.5	3	18.8	
Intensity 0.125 Frozen Section	1	1	6.3	2	12.5	0.381
	2	8	50.0	4	25.0	
	3	6	37.5	7	43.8	
	4	1	6.3	3	18.8	

Mann-Whitney test results

The comparison between the frozen section technique and paraffin technique did not show similar results in the analysis of the quadrants, independent of the concentration of activated carbon. The same was valid for the intensity analysis (Table 2).

TABLE 2 - Quadrants and description of intensities at each position and results of concordance coefficients.

Variable	Category	Parafinn (N = 32)		Frozen section (N = 32)		Fleiss' Kappa	p
		n	%	n	%		
Quadrant 0.25	1	0	0.0	0	0.0	0.190	0.159
	2	1	3.1	6	18.8		
	3	3	9.4	7	21.9		
	4	28	87.5	19	59.4		
Quadrant 0.125	1	1	3.1	0	0.0	-0.076	0.582
	2	0	0.0	1	3.1		
	3	0	0.0	4	12.5		
	4	31	96.9	27	84.4		
Intensity 0.25	1	2	6.3	5	15.6	0.048	0.655
	2	7	21.9	11	34.4		
	3	8	25.0	10	31.3		
	4	15	46.9	6	18.8		
Intensity 0.125	1	1	3.1	3	9.4	0.038	0.741
	2	9	28.1	12	37.5		
	3	13	40.6	13	40.6		
	4	9	28.1	4	12.5		

Table 2 shows that the frozen section analysis and paraffin did not show similar results on the quadrants or intensity, because the Kappa coefficients of Fleiss were very low and statistically equal to zero ($p > 0.05$).

Discussion

Before sentinel lymph node technique was described, metastases that were not clinically detectable, were only detected when the surgeon made an elective lymphadenectomy. Metastases were diagnosed in approximately 20% of cases. In the remaining cases, the surgeon was with a impression of having made a greater than necessary procedure, associated with high morbidity[10]. The development of sentinel node biopsy technique was one of the great advances of recent years in the treatment of melanoma, because it provided a better staging of patients[11].

Several studies have demonstrated that histological analysis of sentinel lymph node reflects the state of the corresponding lymph node basin[2,3,10]. In a lymphadenectomy, the

anatomo-pathological study of lymph node metastases usually examines a slice 5 mm thick on each dissected lymph node. Such sampling is considered insufficient for the detection of micrometastases, especially in cutaneous melanoma because their metastatic cells in the lymph node can penetrate in small groups[6].

The sentinel lymph node is fixed in 10% formaldehyde, embedded in paraffin and sectioned in its longest axis, ie, longitudinal sections from the hilum, sliced 1-2 mm thick, according to the size of the lymph node[7].

At first, the technique of SLNB described by Morton was carried out only using patent blue dye during surgery. The technique was simpler and less expensive, but there was still a percentage of 10 to 20% where the surgeon could not find the sentinel lymph node. Thus, the method has been improved with the use of nuclear medicine and the percentage of detection of lymph nodes increased significantly[4,5].

The frozen section examination of the lymph node is controversial due to its low sensitivity, ie, micrometastases can not be diagnosed. Furthermore, there is the possibility of material deterioration and loss of tissue that would be used in standard histopathological examination[4,5].

Some studies were conducted to analyze the sensitivity and specificity of the frozen section analysis. The difficulty in finding small deposits of tumor cells in lymph nodes is an important disadvantage of this technique and its indication is controversial[12-14]. On the other hand, some authors found high accuracy in performing the intraoperative frozen section examination of SLNB and the method did not prolong the duration of surgery[15,16].

Due to the controversy found in the literature, a study design was conducted based on an experimental animal model. Initially it has been described the feasibility of sentinel lymph node biopsy in the inguinal region[7]. Following the same method, activated carbon was used at various concentrations to mimic the presence of metastases in the lymph node. The authors concluded that the lowest concentration used (0.5%) was the most suitable as it was possible to identify the carbon in all evaluations, without changing the visualization of the structures of the lymph node[8].

In the present study the authors utilized the activated carbon solution, with half and one-quarter the concentration used in the previous study, that is, 0.25% and 0.125%, respectively. It was possible to visualize the carbon in all slides analyzed.

The first analysis is taken regarding the time. Would there be any difference between the analysis made in first and 21 days? After 21 days there was a tendency to more easily identify the carbon in the 4 quadrants when compared with the analysis from the first day. However this trend was not statistically significant (except in frozen section 0.25%). A possible explanation would be that the greater time available would allow the carbon to reach all parts of the lymph node.

The purpose of analyzing the quadrants was to discover how metastasis occurs in this model that simulates metastatic melanoma. Metastases occur in only a small portion or do they occur in a widespread manner throughout the lymph node? Based on this response we could initiate the following thought: if we remove a small fragment of the lymph node for intraoperative frozen section analysis, there is a chance we are removing the only fragment in which metastasis was present (leaving nothing for the analysis in paraffin)? If the frozen section fails to detect the metastasis, we could classify this patient as negative, when in fact he is a false negative? Observing the results of the first day, we see that the distribution is quite varied, thereby it would be risky conduct a frozen section analysis. However, very rarely in clinical practice, we obtain the sentinel lymph node on the same day he received metastasis. Perhaps a later biopsy is more consistent with reality. Observing the results obtained with 21 days, we can see that in 63 of 64 lymph nodes was possible to visualize carbon in all 4 quadrants, which makes us confident to perform a frozen section analysis. If the frozen section is an inferior method, we would not cause an iatrogenicity. Clearly we can not transpose these results to clinical practice. Besides being a simulation model, it is an animal model.

Regarding the intensity of activated carbon, no difference between day one and day 21 was observed. The intuition may suggest that the presence of activated carbon would increase cumulatively over time, and intensity after 21 days would inevitably greater than the first day. Unlike that the intensity remained. We must remember that we were careful to use extremely low concentrations of carbon, in order to simulate the conditions of an initial metastasis (not a massive metastasis).

Finally comparing the frozen section analysis and the paraffin, the low Kappa Fleiss levels indicate us that there was no concordance between the methods, both in the quadrant and in the intensity evaluation. In a subjective analysis, pathologists found greater difficulty in visualizing the carbon in the frozen section analysis, either by a limitation of the method, as by the difficulty of obtaining a quality slide when freezing a lymph node smaller than one centimeter. It was considered that the visualization and recognition of structures in general, is made more easily and accurately in the analysis of material embedded in paraffin.

Conclusions

In an animal model, it was possible to identify the activated carbon in all analyzes, either paraffin or frozen section

and at all moments analyzed. It was evidenced a tendency of carbon to spread on the lymph node over time. In this study, the different forms of analysis (paraffin x frozen section) did not obtained concordance.

References

1. Morton DL, Wen DR, Wong JH, Economou JS, Cagle LA, Storm FK, Foshaq LJ, Cochran AJ. Technical details of intraoperative lymphatic mapping for early stage melanoma. Arch Surg. 1992 Abr;127(4):392-99.
2. Reingten D, Cruse CW, Wells K, Berman C, Fenske N, Glass F, Schroer K, Heller R, Ross M, Lyman G. The orderly progression of melanoma nodal metástases. Ann Surg. 1994 Dec;220(6):759-67.
3. Thompson JF, McCarthy WH, Bosch CM, O'Brien CJ, Quinn MJ, Paramaesvaran S, Crotty K, McCarthy SW, Uren RF, Howman-Giles R. Sentinel Lymph Node status as an indicator of the presence of metastatic melanoma in regional lymph nodes. Melanoma Res. 1995 Aug;5(4):255-60.
4. Morton DL, Cochran AJ, Thompson JF, Elashoff R, Essner R, Glass EC, Mozzillo N, Nieweg OE, Roses DF, Hoekstra HJ, Karakousis CP, Reintgen DS, Coventry BJ, Wang HJ, Multicenter Selective Lymphadenectomy Trial Group. Sentinel node biopsy for early-stage melanoma: accuracy and morbidity in MSLT-I, an international multicenter trial. Ann Surg. 2005 Sep;242(3):302-13.
5. Morton DL, Thompson JF, Cochran AJ, Mozzillo N, Elashoff R, Essner R, Nieweg OE, Roses DF, Hoekstra HJ, Karakousis CP, Reintgen DS, Coventry BJ, Glass EC, Wang HJ, MSTL Group. Sentinel-Node Biopsy or Nodal Observation in Melanoma. N Engl J Med. 2006 Sep;355(13):1307-17.
6. Silveberg SG. Sentinel node processing: recommendations for pathologists. Am J Surg Pathol. 2002 Mar;26(3):383-5.
7. Cochran AJ. Surgical pathology remains pivotal in the evaluation of 'sentinel' lymph nodes. Am J Surg Pathol. 1999 Oct;23(10):1169-72.
8. Oliveira AF, Santos ID, Tucunduva TC, Sanches LG, Oliveira Filho RS, Simões e Silva Enokihara MM, Ferreira LM. Sentinel lymph node biopsy in cutaneous melanoma. Acta Cir Bras. 2007 Sep-Oct;22(5):332-6.
9. Junqueira RP, Oliveira Filho RS, Ranauro CZ, Videira RVS, Enokiahara MMSS, Pinheiro LGP. Solução de carvão ativado e corante vital para a biópsia do linfonodo sentinela em ratos. Einstein. 2008;6(4):463-6.
10. Veronesi U, Cascinelli N, Adamus J, Balch C, Bandiera D, Barchuk A, Bufalino R, Craig P, De Marsillac J, Durand JC, van Geel NA, Holmstrom H, Hunter JA, Jorgensen OG, Kiss B, Kroon B, Lacour J, Lejeune F, MacKie R, Mechl Z, Mitrov G, Morabito A, Nosek H, Panizzon F, Prade M, Santi P, Van Slooten E, Tomin R, Trapeznikov N, Tsanov T, Urist M, Wozniak KD. Thin stage I primary cutaneous malignant melanoma. Comparison of excision with margins of 1 or 3 cm. N Engl J Med. 1988 May;318(18):1159-62.
11. Morton DL, Wen DR, Foshag LJ, Essner R, Cochran A. Intraoperative lymphatic mapping and selective cervical lymphadenectomy for early-stage melanomas of the head and neck. J Clin Oncol. 1993 Sep;11(9):1751-6.
12. Koopal SA, Tiebosch AT, Albertus Piers D, Plukker JT, Schraffordt Koops H, Hoekstra HJ. Frozen section analysis of sentinel lymph nodes in melanoma patients. Cancer. 2000 Oct;89(8):1720-5.
13. Tanis PJ, Boom RP, Koops HS, Faneyte IF, Peterse JL, Nieweg OE, Rutgers EJ, Tiebosch AT, Kroon BB. Frozen section investigation of the sentinel node in malignant melanoma and breast cancer. Ann Surg Oncol. 2001 Apr;8(3):222-6.
14. Stojadinovic A, Allen PJ, Clary BM, Busam KJ, Coit DG. Value of frozen-section analysis of sentinel lymph nodes for primary cutaneous malignant melanoma. Ann Surg. 2002 Jan;235(1):92-8.
15. Ariyan S, Ariyan C, Farber LR, Fischer DS, Flynn SD, Truini C. Reliability of identification of 655 sentinel lymph nodes in 263 consecutive patients with malignant melanoma. J Am Coll Surg. 2004 Jun;198(6):924-32.
16. Alkhatib W, Hertzenberg C, Jewell W, Al-Kasspooles MF, Damjanov I, Cohen MS. Utility of frozen-section analysis of sentinel lymph node biopsy specimens for melanoma in surgical decision making. Am J Surg. 2008 Dec;196(6):827-32.

Correspondence:
Ivan Dunshee de Abranches Oliveira Santos Filho
Rua Napoleão de Barros, 715/4º andar
04024-002 São Paulo – SP Brasil
Tel: (55 11)98346-3650 / 5571-6579
ivandunshee@gmail.com

[1]Research performed at Unit of Skin Tumors, Plastic Surgery Division, Surgery Department, Federal University of Sao Paulo (UNIFESP), Brazil.