Improved detection of lymph nodes in cases of rectal cancer using combined methylene blue injection and fat clearance compared with fat clearance alone

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Introduction
Evaluation of lymph nodes (LNs) in rectal cancer is a fundamental component of all staging systems. Fat clearance and ex-vivo injection of methylene blue into the inferior mesenteric artery are known methods that can improve LN yield in patients with rectal cancer especially after neoadjuvant chemoradiation. Both techniques were widely compared with routine manual palpation of LNs. The question is that 'Do adding ex-vivo injection of methylene to fat clearance as a single combined technique give further superiority on fat clearance alone regarding detection of nodal harvest and status?'.

Patients and methods
This study was carried out through comparing clinicopathological data of 40 patients whose specimens were subjected to combined ex-vivo injection of inferior mesenteric artery and xylene fat clearance (group I) with that of 30 patients whose specimens underwent only xylene fat clearance. All patients presented with resectable rectal cancer and have received neoadjuvant chemoradiotherapy.

Results
There was a statistically significant difference regarding total nodal harvest in group I compared with group II (17.52±6.32 vs. 14.56±5.64; $P<0.05$). Similarly, detection of at least 12 LNs was statistically different (87.5 vs. 63.3%; $P<0.05$). However, it was not the case regarding detection of nodal metastases (55 vs. 56.7%), which was not significantly different ($P=0.085$).

Conclusion
Using ex-vivo methylene blue injection into the inferior mesenteric artery and xylene fat clearance as a single combined technique shows a significant difference when compared with xylene fat clearance alone regarding total LN harvest and detection of the optimal number of LNs in cases of rectal cancer. However, it did not show such significance regarding detection of nodal metastases.

Keywords:
fat clearance, lymph node, methylene blue, rectal cancer

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Introduction

Lymph nodes (LNs) number and status in colorectal cancer present one of the most important pathologic predictors of patient outcome that can also determines the degree of benefit from adjuvant chemotherapy [1–3]. Approximately 68% of patients with negative nodal involvement will survive 5 years, compared with only 40% of those with metastases [4]. For accurate staging, the evaluation of at least 12 LNs in colorectal resection specimens is widely reported in clinical guidelines [1,5–10]. Neoadjuvant chemoradiotherapy can affect getting the maximal LN harvest with subsequent inaccurate staging and prediction of the patient's prognosis. Efforts to maximize nodal harvest seem to be a good clinical practice to overcome the inefficient routine manual palpation [8,11,12]. Fat clearance techniques are widely used to improve LN harvest particularly in patients receiving neoadjuvant therapy [13]. However, Cohen *et al.* [14] and Jass *et al.* [15] reported that fat clearance alone does not improve significantly LN harvest, so there is still a need for further methods to achieve this job. Sanchez *et al.* [16] modified the fat clearing method by injecting methylene blue into the inferior mesenteric artery of rectal cancer resection specimens, *ex vivo*, to stain LNs blue followed by fat clearance. These advanced methods showed significant differences regarding LN harvest when compared with routine manual palpation but with little data regarding comparing these methods with each other.

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Patients and methods

This study was carried out through a review of medical records of two groups of patients who underwent elective surgery for resectable rectal cancer with intent to cure after receiving neoadjuvant chemoradiotherapy. All patients were operated at either Department of surgery, Faculty of medicine, or Department of Surgery, Medical Research Institute, University of Alexandria. Group I, as a retrospective study group, included data collected from 40 patients operated between June 2013 and June 2015 as a part of previous research [17]. Specimens of these patients were subjected to ex-vivo injection of inferior mesenteric artery followed by xylene.

Group II, as a prospective study group, included 30 patients who were operated between January 2014 and January 2017 as a part of ongoing study. Specimens of this group were subjected only to xylene fat clearance. Surgical procedures for both groups were done by two expert surgical teams, and pathological studies were done by two expert pathologist one for each group.

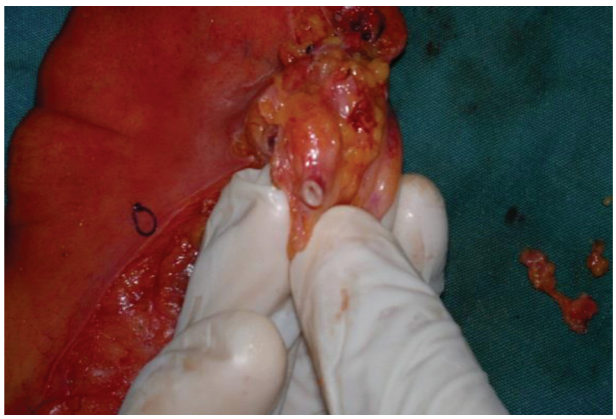
Review of medical records involved data regarding history taking, clinical examination, and laboratory and radiological findings. Surgery was performed at least 6 weeks after completing neoadjuvant therapy. Using total mesorectal excision technique, surgical procedures were either abdominoperineal resection or low anterior resection.

Ex-vivo injection of methylene blue was done by the surgeon. After identification of the inferior mesenteric artery stoma in the fresh specimen, cannulation of the artery was done using the plastic tube portion of a standard 16–20 G intravenous catheter, followed by injection of 15–20 ml of methylene blue solution (50 mg diluted with 0.9% saline in the ratio 1 : 3). Subsequently, the specimens were fixed in formalin for 24 h. Serial sectioning of the mass was done together with mouting of radial, proximal, and distal margins. The regional fat was dissected and cut into less than 0.5-cm sections and processed in ascending concentration of alcohol, then placed overnight in xylene. After clearing, meticulous picking of visible LNs was preformed followed by cutting, staining, and examination by microscopy (Figs 1–6). In group II, specimens were subjected to the previous steps without injection of methylene blue.

Statistical analysis

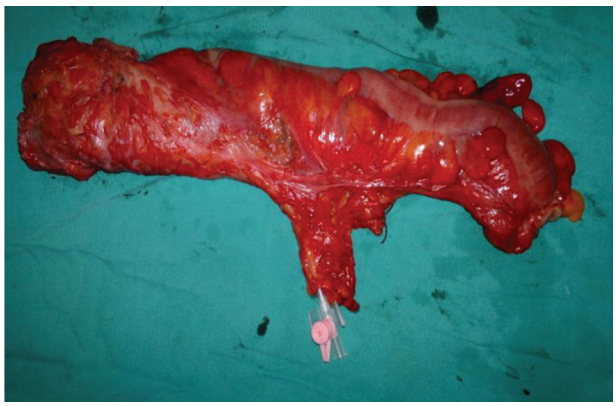
It was performed using IBM SPSS statistics for Windows (version 20.0; IBM Corp., Armonk, New York, USA). Qualitative data were described using number and percent. It was compared using χ^2-test. Numeric data were expressed in mean±SD and compared using student *t*-test or the Mann–Whitney rank-sum test, depending on the

Figure 1



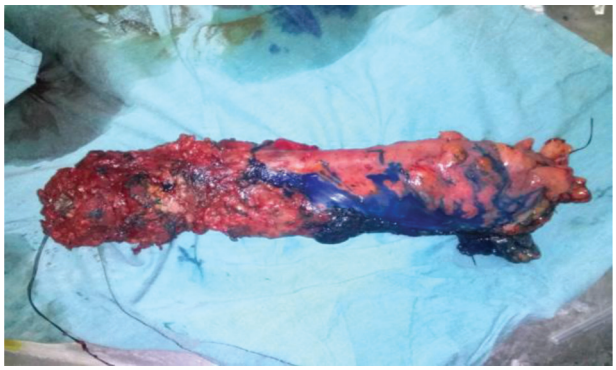
Identification of inferior mesenteric artery.

Figure 2



Cannulation of inferior mesenteric artery.

Figure 3



Blue staining of mesorectal surface.

results of the distribution test. Statistical significance was considered at P 0.05 or less.

Results

Both groups were matched according to demographic, anatomical, surgical, and tumor histopathological data without statistical significance (Table 1). A total number of 701 (17.52±6.72) LNs were identified in group I compared with 437 (14.3.7±5.63) LNs in group II, which was a statistically significant difference (P=0.019).

At least 12 LNs were identified in 35 (87.5%) patients of group I compared with 19 (63.3%) patients in group II, which was also statistically significant (P<0.001). Metastases were detected in 22 (55%) patients in group I and 17 (56.7%) patients in group II with (P=0.988) (Table 2). Retrieved LNs were measured and categorized according to their size. The difference in nodal harvest between the two groups was most pronounced and statistically significant in LNs measuring up to 2 mm and more than 2 to up to 4 mm categories. However, for the

Figure 4

After fixation in formalin for 24 h.

Figure 5

Regional fat dissection.

Figure 6

After xylene fat clearance.

Table 1 Comparison between the two studied groups according to demographic data, tumor location, surgical procedures, and histological criteria.

	Group I (n=40)	Group II (n=30)	Test of significance	P
Sex [n (%)]				
Male	26 (65.0)	18 (60.0)	χ^2=0.213	0.644
Female	14 (35.0)	12 (40.0)		
Age (years) [n (%)]				
Minimum–Maximum	23.0–77.0	26.0–72.0	t=0.568	0.572
Mean±SD	52.35±14.84	50.60±12.62		
BMI (mean±SD)	26.80±2.19	26.90±1.74	t=0.013	0.981
Tumor location [n (%)]				
Lower rectum	16 (40.0)	11 (36.7)	χ^2=0.053	0.818
Middle rectum	24 (60.0)	11 (63.3)		
Surgical procedure [n (%)]				
Abdominoperineal resection	11 (27.5)	9 (30)	χ^2=1.458	0.483
Low anterior resection	29 (72.5)	21 (70)		
Histological type [n (%)]				
Adenocarcinoma	32 (80.0)	25 (83.3)	χ^2=0.366	0.416
Mucinous carcinoma	8 (20)	5 (16.7)		

χ^2: χ^2-test. t: Student t-test.

LNs in the more than 4 mm categories, the difference was statistically insignificant (Table 3).

Discussion

For decades, fat clearance techniques were adopted by many centers to improve nodal harvest in cases of colorectal cancer [18–21]. Methylene blue injection into the superior rectal or the inferior mesenteric artery has been introduced as a simple effective alternative technique [22–24]. Each of these methods was widely compared with the routine manual palpation showing significant differences regarding nodal harvest especially after neoadjuvant chemoradiation. Fat clearing techniques have been claimed to be a time consuming method, so addition of methylene blue injection technique to fat clearance may help in identification of LNs by intense blue staining of them, decreasing the time consumed in LNs identification [16]. Sanchez *et al.* [16] reported evident significant difference regarding LN harvest comparing combined ex-vivo methylene blue

Table 2 Comparison between the two studied groups according to lymph node harvest

	Group I (n=40)	Group II (n=30)	Test of significance	P
Optimal harvest [n (%)]				
≤11	5 (12.5)	11 (36.7)	χ^2=33.826*	<0.001*
≥12	35 (87.5)	29 (63.3)		
Total harvest				
n	701	437		
Minimum–Maximum	8.0–23.0	7.0–24.0		
Mean±SD	17.52±6.2	14.57±2.34	Z=2.348	0.019*
Median	17.0	14.0		
Metastatic LN				
Number (% of total)	216 (30.81)	147 (30.20)		
Minimum–Maximum	2.0–11.0	1.0–12.0		
Mean±SD	9.82±6.74	8.65±5.40	Z=0.037	0.08
Median	5.0	5.0		
Metastatic cases [n (%)]	22 (55)	17 (56.7)	χ^2=0.015	0.988

χ^2 for χ^2-test. Z for Mann–Whitney test. LN, lymph node. *Statistically significant at P≤0.05.

Table 3 Comparison between the two studied groups according to size of lymph nodes

	Group I	Group II	Z	P
L.Ns ≤2 mm				
Number (% of total)	74 (10.56)	29 (6.73)		
Min.–Max.	0.0–8.0	0.0–4.0		
Mean±SD.	1.85±1.17	0.97±1.12	3.399*	<0.001*
Median	3.0	1.0		
Positive LNs (% of +ve)	5 (2.32)	4 (3.03)		
L.Ns >2 to ≤4 mm				
Number (% of total)	89 (12.7)	40 (9.28)		
Min.–Max.	0.0–9.0	0.0–4.0		
Mean±SD.	2.25±1.51	1.33±0.91	3.828*	<0.001*
Median	5.0	2.0		
Positive LNs (% of +ve)	12 (5.56)	9 (6.82)		
L.Ns >4 to ≤6				
Number (% of total)	196 (27.96)	137 (30.86)		
Minimum–Maximum	2.0–11.0	2.0–12.0		
Mean±SD	4.9±1.86	4.57±1.44	0.029	0.88
Median	7.0	7.0		
Positive LNs (% of +ve)	79 (36.56)	54 (40.90)		
LNs >6				
Number (% of total)	342 (48.79)	225 (52.2)		
Minimum–Maximum	4.0–15.0	4.0–13.0		
Mean±SD	8.55±2.34	7.5±2.15	0.015	0.988
Median	8.0	9.0		
Positive LNs (% of +ve)	120 (55.56)	65 (49.25)		

+ve: for total number of positive lymph nodes. Z: Mann–Whitney test. LN, lymph node. *Statistically significant at P≤0.05.

injection and fat clearance with manual palpation of surgical specimens of rectal adenocarcinoma in patients receiving neoadjuvant chemoradiotherapy. In the present study, we tried to demonstrate the possible superiority of using combined methylene blue injection and fat clearance in comparison with the popular technique of using only fat clearance taking into consideration the superiority of both techniques on routine manual palpation regarding total nodal harvest especially after neoadjuvant therapy. Both groups were matched regarding demographic, anatomical, surgical, and tumor histopathological criteria.

In the present study, the total nodal harvest was statistically superior in group I (17.52±6.72 vs. 14.3.7±5.63; P=0.031). This can be explained by the visualization and detection of a significant higher number of smaller LNs in group I. This is an evident finding regarding LNs measuring up to 2 mm and more than 2 to up to 4 mm categories, which was not the case regarding larger LNs categories. This increase in individual nodal harvest reflects a significant detection of an optimal number of at least 12 LNs in group I compared with group II (87.5 vs. 63.3%; P<0.05).

Although there was a presence of a significant difference in total nodal harvest and detection of the optimal number of LNs, the difference between the two groups regarding the rate of patients with nodal metastasis was not statistically significant (55.00 vs. 56.70%; P=0.98), with also no significant difference regarding the mean of affected nodes (9.82±6.74 vs. 8.65±5.40; P=0.08). Taking into consideration that fat clearance technique by itself has improved detection of total and metastatic nodes, so it may be accepted to say that no further significant detection of metastatic nodes could be achieved using other methods, and subsequently, nonsignificant difference in this study can be easily explained. However, there are several studies that revealed absence of a significant difference regarding detection of nodal metastases even when these studies compared advanced techniques with routine manual palpation of LNs [22,25,26]. Parson *et al.* [25] in their SEER database reported evident increase in LN harvest over time which was associated with the outcome but with no increase in LN positivity.

Markle and colleagues studied a group of 669 cases of colorectal cancer using special methods to improve nodal harvest (methylene blue injection alone in 559 cases, fat clearance alone in 55 cases, and a combined methylene blue injection followed by fat clearance in another 55 cases). Although they compared this group with a cohort of 663 historical cases using only conventional manual dissection, they reported no significant difference regarding nodal positivity rates, which were even absolutely identical at 37% (P=0.98), with only trends toward higher rates of nodal metastases in subgroups of high-grade cancers and rectal cancers without neoadjuvant therapy [22]. They compared their results with Ricciardi *et al.* [26], who conducted a detailed analysis of more than 120 000 cases, and concluded that the poor nodal harvest did not significantly cause detection of lower rate of metastases. Märkl *et al.* [22], depending on their sequential LN preparation and examination, detected the first metastatic node among the first nine detected node in close proximity with the tumor in 86% of cases.

In another publication, Märkl and colleagues found that in only two of 81 cases the largest metastatic node was less than 4 mm whereas the largest LN in all other cases was large enough to be missed during routine examination. They concluded that the pathologists may show a poor performance regarding detecting high number of LNs, but they are much more effective regarding crucial nodes [27]. Herrera *et al.* [28] noticed that though metastases in LNs from rectal adenocarcinoma occur frequently in small LNs (≤5 mm), but mostly of perirectal distribution rendering them easily expected by the pathologists. Kim and colleagues even reported that fat clearance did not increase metastatic LNs yield in both neoadjuvant and nonneoadjuvant groups. This might reflect the fact that one certified pathologist who is specialized in colorectal disease can perform precise pathologic assessment by manual dissection, and this may explain their relatively small additional benefit of fat clearing in detecting more metastatic LNs [29]. Back to our study, the significance of small LNs was the key to explain absence of significant difference regarding detection of nodal metastases though significant difference regarding total nodal harvest. Analysis of detected metastatic LNs in group I revealed that categories of LNs size less than 2 and 2–4 mm contained only 2.32 and 5.56 % of total metastatic nodes, respectively, though these two size categories were responsible for the significant difference regarding total nodal harvest and detection of optimal number of LNs.

Conclusion

Though adding methylene blue injection technique to widely used fat clearance method gave superiority

over using fat clearance alone regarding total nodal harvest and detection of optimal number, it did not show similar statistical significance regarding detection of nodal metastases. Nevertheless, we recommend this combined technique as it can achieve a sufficient nodal harvest in patients with rectal cancer treated with neoadjuvant chemoradiation, as this total harvest is strongly associated with outcome of the patients.

Limitations of this study include the small number of cases and absence of randomization between the two groups. Moreover, although being experts, presence of two pathologists is another limitation.

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Conflicts of interest

There are no conflicts of interest.

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