Evaluation of ex-vivo methylene blue injection into the inferior mesenteric artery to improve lymph node harvest in rectal cancer

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Purpose: *Exact lymph node staging is essential for prognosis estimation and treatment stratification in rectal cancer. Recent studies showed that ex-vivo injection of methylene blue dye into the inferior mesenteric artery of rectal cancer specimens might improve lymph node harvest. This study evaluates pathological lymph node assessment using this recent technique compared to conventional pathological techniques.*

Methods: *Methylene blue solution was injected ex-vivo into the inferior mesenteric artery of 25 rectal cancer specimens and lymph node assessment was performed after formaline fixing overnight. The results were compared to data obtained from a control group of 25 rectal cancer specimens which underwent conventional pathological lymph node assessment.*

Results: *Methylene blue injection was successfully performed in all patients in the stained group. A total number of 383 (15.32 ±4.28) and 157 (6.28 ±2.79) lymph nodes were detected in the stained and unstained groups respectively ($p< 0.001$). The difference was most pronounced in lymph nodes measuring ≤4 mm in diameter ($p< 0.001$). Metastases were found in 154 (6.16 ±6.76) and 37(1.48 ± 2.86) lymph nodes occurring in 18 and 10 patients in the stained and unstained groups, respectively ($p= 0.003$). Lymph node ratio (LNR) was calculated for the patients who were finally staged as stage III showing no significant difference between the two groups.*

Conclusions: *Ex-vivo methylene blue injection into the inferior mesenteric artery is a simple, easy and safe method that significantly improves lymph node harvesting in rectal cancer, especially small-sized lymph nodes.*

Key words: *Methylene blue, lymph node, rectal cancer.*

Introduction:

In Egypt, colorectal cancer is the fourth most commonly diagnosed cancer in both men and women.[1] Rectal cancer comprises approximately 30% of the malignancies arising in the large bowel.[2] Total mesorectal excision (TME) is the standard treatment of cancer rectum and the total number of lymph nodes harvested in the surgical resection specimen greatly impacts staging accuracy and prognosis.[3-4] TNM stage of rectal cancer with exact nodal staging correlates with survival rates and prognosis estimation and it is the foundation on which all treatment regimens are based.[5]

Complete surgical resection for stage I rectal cancer is considered curative therapy, and the five-year relative survival rate is around 90%. However, stage II and III rectal cancers have five-year survival rates of only 67 and 54%, respectively.[6] A hypothesis for the relative decrease in survival in stage II rectal cancer is that some patients may be under staged as node-negative when they are actually node-positive.[7] Moreover, in many patients who are not treated with preoperative

radiation or radio-chemotherapy the decision for adjuvant chemotherapy is made by the lymph node status.[8,9] Evidence suggests that patients, particularly in stage II rectal cancer, with a reduced lymph node harvest have a worse prognosis. In addition, 5-year overall survival and disease-free survival are significantly reduced in patients with low lymph node harvest.[10-11] Also, several studies have demonstrated a similar association between survival and lymph node harvest in stage III disease.[12-13]

Therefore, Lymph node assessment is an essential part of staging in rectal cancer. In fact, nodal involvement is the single most important prognostic factor in colorectal carcinoma.[10,14] The accuracy of (N) staging increases with the number of lymph nodes examined.[15] Numerous studies have demonstrated an improvement in the overall survival and/or disease-free survival of colorectal cancer patients with increasing number of lymph nodes retrieved for examination.[16,17] Furthermore, the number of lymph nodes examined in rectal cancer is considered an independent factor for locoregional disease whether metastases are present or not.[18]

There is debate regarding the optimal number of lymph nodes required for adequate staging. The evaluation of at least 12 lymph nodes following colorectal cancer resection is widely cited in clinical guidelines. This number was first proposed in 1990 by the Working Party Report to the World Congress of Gastroenterology in Sydney.[19] However, this is not a scientific biological figure and is a grade C recommendation based on level III-IV evidence.[20-22]

Ex-vivo intra-arterial injection of methylene blue solution in order to improve the lymph node harvested in the pathological examination of colorectal cancer specimens was initially introduced by Bruno Märkl et al.[23] The injected methylene blue stains arterial blood vessels and capillaries. This is one of the main reasons for choosing methylene blue solution to improve the visualization of lymph nodes because the density of vessels is much higher in lymph nodes in comparison to the surrounding fat. It is not entirely clear how the solution passes into the lymph nodes when injected into the specimens ex-vivo after the blood stream is disjuncted. The most likely mechanism could be the increase of interstitial pressure caused by the injection and therefore induced lymphatic flow[24,25] several studies concluded that this method is effective, simple and time and cost effective.[23-25]

This study evaluates pathological lymph node assessment using ex-vivo injection of methylene blue dye into the inferior mesenteric artery of rectal cancer specimens compared to conventional pathological techniques.

Patients and methods:

This study was carried out on 50 patients with resectable rectal cancer who were allocated into two groups, stained group with ex vivo methylene blue injection then pathological assessment and unstained group with conventional pathological assessment. Patients who underwent emergency surgery, palliative surgery and patients with recurrent rectal carcinoma were excluded. All the patients included in this study were subjected to history taking, thorough clinical examination and collection of available data from the investigations. Total mesorectal excision (TME) was done to patients with middle and lower rectal tumors while patients with upper rectal tumors were subjected to tumor specific mesorectal excision. All specimens were sent fresh to pathology after randomly assigned to undergo injection of the inferior mesenteric artery with methylene blue dye (stained group) or a gross pathologic dissection of the lymph nodes (unstained group).

The inferior mesenteric artery of each specimen of the stained group was cannulated ex-vivo by the surgeon with a standard 16-20G intravenous catheter using only the plastic tube portion, followed by the injection of 15 to 20 ml of methylene blue solution (50 mg diluted with 0.9% saline in the ratio 1:3). Subsequently, all specimens were fixed in 10% buffered formalin for 24 hours. (23-25)

Figures (1-3).

Beginning from the proximal end, the specimens were cut in 5 to 7 mm thick slices until the end of the tumor region was reached and representative areas were embedded using the whole mount technique. The fat of the remaining parts was then dissected and examined by palpation. The fatty tissues were then sliced and stretched to create thin, transparent layers. The cut surfaces were screened for lymph nodes.

After paraffin embedding, 3 μm sections were cut and stained with hematoxylin and eosin. Slides were then examined for the number and size of lymph nodes and the presence of metastases.

Statistical analysis: Data were fed to the computer and analyzed using IBM SPSS software package version 20.0. Qualitative data were described using number and percent. Quantitative data were described using mean and standard deviation, median, minimum and maximum.

Comparison between different groups regarding categorical variables was tested using Chi-square test. When more than 20% of the cells have expected count less than 5, correction for Chi-square was conducted using Fisher's Exact test or Monte Carlo correction.

For normally distributed data, comparison between two independent population were done using independent t-test while more than two population were analyzed F-test (ANOVA) to be used. For abnormally distributed data, comparison between two independent population were done using Mann Whitney test Correlations between two quantitative variables and ordinal data were assessed using Spearman coefficient. Multivariate Linear regression was assessed. Significance test results are quoted as two-tailed probabilities. Significance of the obtained results was judged at the 5% level.

Results:

This study included 50 patients with resectable rectal cancer who were divided into two groups, stained group whit ex vivo methylene blue injection then pathological assessment and unstained group with conventional pathological assessment. 56% (14) in each group were males with mean age 51.68 ±11.60 years (range, 25-74) and 51.04 ±13.45 years (range, 26-74) for the stained and unstained groups, respectively. The mean body mass index (BMI) was 27.56 ±1.94 for the stained group, compared to 27.16 ±1.65 for the unstained group. There was no statistically significant differences between both groups in demographic data, tumor site, histological criteria and preoperative staging. In the stained group, 17 patients (68%) received neoadjuvant therapy, while in the unstained group, 18 patients (72%) received neoadjuvant therapy. Low anterior resection (LAR) was performed in 18 patients (72%) in the stained group and 17 patients (68%) in the unstained group and rest of the patients underwent abdominoperineal resection. Operations were performed by 5 colorectal consultant surgeons and pathological assessment was performed by two pathologists. Differences between the two groups regarding the surgeons performing the operations and pathologists examining the resection specimens were statistically insignificant.

Although there was no significant difference between both groups regarding preoperative clinical nodal staging (cN) (P 0.844), this difference become statistically significant after pathological nodal staging (pN) (p= 0.003). In the stained group, 14 patients (56%) were finally staged as pN2, while in the unstained group, only 3 (12%) patients were staged as pN2 and 15 patients (60%) were finally staged as pN0 **Table (1)**. On studying the comparison between cN and pN in each group, a statistical significant difference was identified only in the stained group (p= 0.009) **Table (2)**.

In the stained group, a total of 383 lymph nodes were identified with a mean of 15.32 ±4.28, compared to a total number of 157 lymph nodes identified in the unstained group with a mean of 6.28 ±2.79. The difference between the two groups was highly statistically significant (p< 0.001). At least 12 lymph nodes were identified in 21

out of 25 patients (84%) in the stained group. However, in the unstained group, adequate nodal harvest was found in only 3 out of 25 patients (12%) **Table (3)**.

Metastases were found in 154 lymph nodes in 18 out of 25 patients (72%) in the stained group with a mean of 6.16 ±6.76 and in 37 lymph nodes in 10 out of 25 patients (40%) in the unstained group with a mean of 1.48 ±2.86. The difference between the two groups was statistically significant (p= 0.003) **Table (3)**.

LNR was calculated for these patients with positive lymph nodes and a mean of 0.53 ±0.32 and 0.41 ±0.32 were found for the stained and unstained groups respectively, the difference was not statistically significant. Lymphovascular invasion was detected in 16 out of 25 patients (64%) and 15 out of 25 patients (60%) in the stained and unstained groups respectively.

The lymph nodes were measured and categorized by size. The difference in nodal harvest between the two groups was most pronounced in lymph nodes measuring ≤4 mm in diameter. The difference for the <2 mm and 3-4 mm categories was statistically highly significant (p< 0.001). For lymph nodes in the 5-6 mm category there was also a statistical significant difference between the two groups (p= 0.008). However, for the lymph nodes in the >6 mm category the difference between the stained and unstained groups was not statistically significant **Figure (4)**.

On studying the relation between neoadjuvant therapy and lymph node harvest in the two groups, the effect of neoadjuvant therapy on reducing the nodal harvest was statistically significant only in the stained group (p< 0.001) with a mean nodal harvest of 19.88 ±2.95 in the patients who didn't receive neoadjuvant therapy compared to 13.18 ±2.90 in the patients treated with neoadjuvant therapy. In the unstained group there was no significant difference in lymph nodes harvest (p=0.752) in patients who received neoadjuvant therapy and those who directly underwent surgery (6.17 ±3.17 vs6.57 ±1.62).

In the present study, out of 17 patients in the stained group who received neoadjuvant therapy 13 patients (76.5%) had adequate nodal harvest. Whereas, none of the 18 patients in the unstained group who received neoadjuvant therapy had adequate nodal harvest.

The relationships of other factors with lymph node harvest were also studied as these factors can affect lymph node retrieval. These factors include surgeon variation, pathologist variation, type of the operation, tumor site, histological type and grade of the tumor and lymphovascular invasion. However, none of these factors had a statistically significant effect on nodal harvest neither in the stained nor in the unstained groups.

Linear regression for the effect of neoadjuvant therapy and methylene blue injection on lymph node harvest was studied revealing a more powerful impact of methylene blue injection on nodal harvest than neoadjuvant therapy **Table (4)**.

Discussion:

It was proved by Wong et al that accuracy of lymph node status increases with the number of examined lymph nodes.[26] Additionally, Swanson et al. showed a very impressive linear correlation between increasing numbers of lymph nodes examined and 5-year survival rates in T3N0 colorectal cancer.[27]

Many studies tried to improve lymph node harvest by increasing sensitivity of metastasis detection using sentinel lymph node technique, immunohistochemical analysis and polymerase chain reaction (PCR).[28-30] Immunohistochemical is expensive and failed to prove a significant difference concerning 3-year disease-free survival.[31] In contrast, confirmation of micrometastasis using RT-PCR showed a significant smaller 3-year disease-free survival and reduced overall survival in positive patients. Fat clearance protocols are an effective way to improve lymph node recovery. Despite that, many of these protocols are difficult and time consuming. Even more problematic is the additional necessity of poisonous chemicals that need to be handled. Both are circumstances that limit their widespread

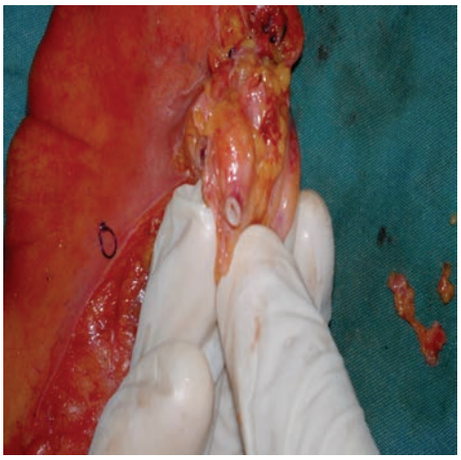
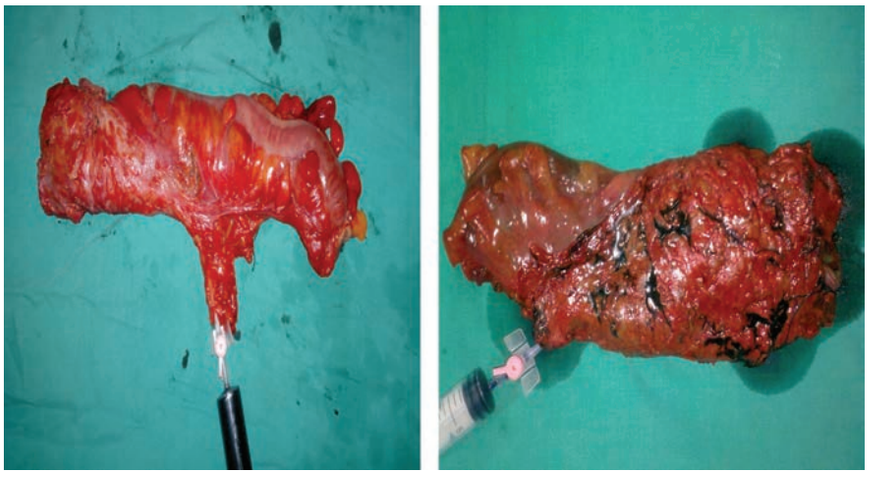
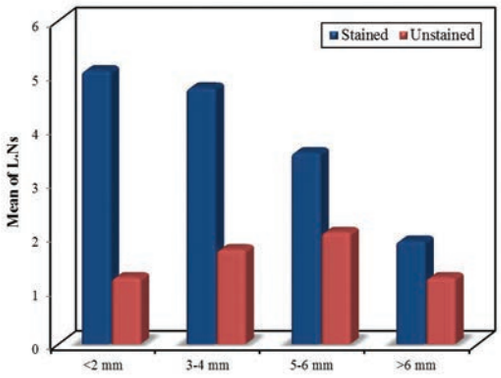


Figure (1): Identification of the IMA.



Figures (2-3): Injection of methylene blue solution into the IMA.



Figures (4): Comparison between both groups according to size of L.Ns harvest.

use.[32, 33]

A new and simple technique was described to stain lymph nodes in rectal cancer specimens by ex vivo methylene blue injection into the inferior mesenteric artery making them easier to detect by conventional pathological dissection. This technique was first described by Märkl et al.[23]

Neo-adjuvant therapy, surgeon and pathologist variation and type of operations are factors that affect number of lymph nodes harvested. In this study there was no significant difference between the two groups regarding those factors.[34-38]

Table (1): Comparison between the studied groups regarding clinical and pathological nodal staging.

	Stained (n=25)		Unstained (n=25)		p
	No.	%	No.	%	
cN					
cN0	6	24.0	6	24.0	
cN1	9	36.0	10	40.0	0.844
cN2	10	40.0	9	36.0	
pN					
pN0	7	28.0	15	60.0	
pN1	4	16.0	7	28.0	0.003*
pN2	14	56.0	3	12.0	

Table (2): Comparison between clinical and pathological nodal staging in each group.

		cN					
		cN0		cN1		cN2	
		No.	%	No.	%	No.	%
Stained	pN						
	pN0	4	66.7	2	22.2	1	10.0
	pN1	1	16.7	2	22.2	1	10.0
	pN2	1	16.7	5	55.6	8	80.0
	rs (p)	0.509* (0.009)					
Unstained	pN						
	pN0	5	83.3	6	60.0	4	44.4
	pN1	1	16.7	4	40.0	2	22.2
	pN2	0	0.0	0	0.0	3	33.3
	rs (p)	0.367 (0.071)					

rs: Spearman coefficient

*: Statistically significant at $p \leq 0.05$

Table (3): Comparison of lymph node harvest in both groups.

	Stained	Unstained	Test of sig.
Total L.Ns	(n=25)	(n=25)	
Min.- Max.	8.0 – 24.0	2.0 – 12.0	tp< 0.001*
Mean ± SD.	15.32 ± 4.28	6.28 ± 2.79	
Metastatic L.Ns	(n=25)	(n=25)	
Min. - Max.	0.0 – 21.0	0.0 – 12.0	MWp= 0.003*
Mean ± SD.	6.16 ± 6.76	1.48 ± 2.86	

t: Student t-test

MW: Mann Whitney test

Table (4): Linear regression for total L.Ns.

	B	SE	Beta	t	p	95% CI	
						LL	UL
Constant	19.366	1.97		9.823*	< 0.001	15.400	23.332
Neoadjuvant therapy	3.672	0.99	0.293	3.700*	0.001	1.675	5.668
Methylene blue injection	-8.893	0.91	0.775	9.777*	< 0.001	7.063	10.723

R= 0.840, R2= 0.706, F= 56.320*, p< 0.001

In the present study, the process of methylene blue injection into the inferior mesenteric artery was simple, easy and safe method as it was successful in all patients in the stained group with an average time of less than 4 minutes. In addition to the availability and low cost of methylene blue dye possesses no hazardous or poisonous effects. This coincides with the results of similar studies assessing this technique. Kerwel et al. described this technique to be simple, time and cost-effective and should be reproducible in other institutions, particularly where inadequate nodal harvests are problematic.[39]

This study shows improvement in the mean lymph node harvest in the stained group as compared with the unstained group (15.32 ±4.28 vs. 6.28 ±2.79, p< 0.001). In 84% of patients in the stained group, lymph node recovery was more than 12 whereas this was achieved in only 12% of patients in the unstained group. This major improvement in nodal harvest following methylene blue staining of lymph nodes was similarly found by other published studies. for example, in the original retrospective study by Märkl et al. including 24 rectal cancer specimens, the average number of lymph nodes examined was 27 ±7 and 14 ±4 for the stained and unstained groups, respectively.[23] An average lymph node harvest of 30 ±13.5 for the methylene blue stained group compared to 17 ±11 for the unstained group were found in a prospective study by Kerwel et al. involving 50 patients with primary resectable rectal cancer.[39] A study by Klepšytė et al. including 40 rectal cancer specimens also demonstrated an improvement in lymph node harvest following methylene blue staining with an average lymph node numbers per specimen of 18 ±5 and 14 ±6 in the stained and unstained groups, respectively.[24] However, on comparing the results of the present study with other published results, it seems clear that nodal harvest in this study is the lowest among all, whether before or after methylene blue lymph node staining. This indicates a more obvious need to improve lymph node harvests aiming at a more accurate rectal cancer staging.

Looking at the additional lymph nodes identified in the stained group, the increase was attributed to identifying more of the small lymph nodes. Kerwel et al. found similar results as the largest improvement was found in size groups between 1 and 4 mm causing a shift in size distribution toward smaller nodes.[39] The significance of the smaller lymph nodes is that studies have reported that 66 to 78% of metastatic lymph nodes are smaller than 5 mm.[28,40-42]

Other finding in this study was improvement of the number of metastatic lymph nodes identified in the stained group. Kerwel et al., found no significant improvement in the detection of nodal metastasis. This was attributed to the finding that nodal harvest in the unstained group was already very good and well above the reported averages obtained with conventional techniques.[39]

To date, thorough examination of postoperative specimens in trial to obtain the maximum number of lymph nodes is the optimal method for accurate nodal staging. Several authors claim that an insufficient number of identified and examined lymph nodes is one of the main causes of understaging in colorectal cancer. The stage migration theory is based on this concept.[43] In the present study, nodal stage

migration was significant in the stained group (p=0.009). Two patients in the stained group were preoperatively staged as node-negative and ended up to be node-positive and 5 other patients were upstaged from N1 to N2 after pathological assessment. While in the unstained group, only one case was upstaged from N0 to N1.

A study by Märkl et al. involving 53 rectal and 1 colon cancer patients who had received neoadjuvant therapy demonstrated that adequate lymph node harvest can be achieved after methylene blue staining even in patients treated with neoadjuvant therapy. The mean number of lymph nodes was 29 ±11 and 10 ±4 for the methylene blue stained and unstained groups, respectively.[44] Similarly, in the present study, there was a significant improvement in nodal harvest in patients received neoadjuvant therapy in stained group 13.18 ±2.90 compared to unstaining group 6.17 ±3.17, ($p< 0.001$).

Baxter et al. have reported that only 20% of patients treated with neoadjuvant therapy have adequate nodal sampling.[34] In the present study, 76.5% of patients in the stained group who received neoadjuvant therapy had adequate nodal harvest whereas, none of the 18 patients in the unstained group who received neoadjuvant therapy had adequate nodal harvest.

Lymph node harvest in the patients in the stained group who received neoadjuvant therapy was significantly lower than patients who didn't receive neoadjuvant therapy (13.18 ±2.90 vs. 19.88 ±2.95, $p< 0.001$), which is consistent with published studies documenting a reduced lymph node harvest in patients treated with neoadjuvant therapy.[34-36] Interestingly, a similar trend was not observed in the unstained group. This finding maybe attributed to the assumption that neoadjuvant therapy doesn't cause the lymph nodes to disappear but rather become smaller and therefore more difficult to detect.[44] However, in the present study, methylene blue injection proved to have a more powerful impact on nodal harvest than neoadjuvant therapy.

Conclusion:

Ex-vivo methylene blue injection into the inferior mesenteric artery is a simple, easy and safe method that significantly improves lymph node harvesting in rectal cancer, especially small-sized lymph nodes. This improvement in nodal harvest can be even achieved in patients treated with neoadjuvant therapy. This eventually leads to a more accurate rectal cancer staging via improved detection of metastatic lymph nodes.

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