J Interv Gastroenterol1:2, 59-63; April/May/June 2011; © 2011 Landes Bioscience

Effects on confocal laser endomicroscopy image quality by different acriflavine concentrations

Chang-Qing Li[1], Tao Yu[1], Xiu-Li Zuo[1], Xiang-Jun Xie[1,2], Wen-Bo Li[1], Chuan-Lian Chu[1], Fang Zuo[1], Yan-Qing Li[1]

[1]Department of Gastroenterology, Shandong University Qilu Hospital, Jinan, China; [2]Department of Gastroenterology, Qingdao Municipal Hospital, Qingdao, China

Key words: acriflavine, confocal laser endomicrosopy, diagnosis, concentration

Abbreviations: CLE, confocal laser endomicroscopy

Background: Acriflavine is one of the commonly used staining agents in confocal laser endomicroscopy (CLE), a newly developed technique allows for real time histological observation of gastrointestinal mucosa, but the concentration is not unified. This study aimed to evaluate the effects of acriflavine with different concentrations on the CLE image quality and to find a sound concentration in clinical practice.
Methods: Twenty four consecutive patients who underwent upper gastrointestinal CLE were enrolled into this study. The patients randomly accepted acriflavine in four different concentrations which were the conventional 0.05% and 3 lower ones respectively: 0.02%, 0.01% and 0.005% spraying onto the same focal antrum mucosa during CLE procedures. Differences of Image quality were demonstrated by an objective score system.
Results: There was no significant difference about image quality among acriflavine concentrations: 0.05%, 0.02% and 0.01%, but 0.005% decreased image quality significantly (P=0.012). And 0.005% was also the only one which decreased general assessment significantly (P=0.01). For the 3 diagnostic value assessment indices, there was no significant difference about nonspecific and even staining, while 0.02% showed significant better polar staining (P=0.03).
Conclusions: Acriflavine concentration 0.02% is the best one applied in CLE with the best nuclei staining ability and preserved image quality.

Introduction

Confocal laser endomicroscopy (CLE) is a newly developed endoscopic device for in vivo diagnosis of gastrointestinal disease.[1] For its optical principles some agents with reflective properties are necessary, of which fluorescein sodium and acriflavine are the most common staining agents. Fluorescein sodium is administered intravenously to show tissue structure and microvessels, and acriflavine is administered topically, which is one of the specific nuclei staining agents which allows diagnosis depending on assessment of nuclei, such as dysplasia.[2] But unlike fluorescein sodium with uniformed administration, application of acriflavine was not unified including the doses or concentrations. Some suggested 0.05% as the standard concentration[3] and others suggested 0.02%.[4] In our practices we found in many cases image quality of these two concentrations were both satisfactory, and 0.02% of acriflavine was better than 0.05% in assessment of epithelial cell nuclei. This study was to test whether 0.02% or lower concentrations of acriflavine was better than 0.05% by a randomized blinded controlled trial.

*Correspondence to: Yan-Qing Li; Email: liyanqing@sdu.edu.cn
Submitted: Jul/12/2010; Revised: Jul/14/2010; Accepted: Jul/16/2010
Previously published online: www.landesbioscience.com/journals/jig
DOI: 10.4161/jig.1.2.16828

Patients and methods

Patients inclusion and exclusion

Patients who underwent upper gastrointestinal CLE examination from September 20, to October 17, 2008 were enrolled into this study. Those with endoscopic normal mucosa or minor gastritis were included. Those with severe gastritis, ulcer, remnant stomach, tumor located in stomach antrum were excluded. Patients were randomly divided into four groups accepting four different concentrations of acriflavin. Randomization of patients' group was performed according to a randomized number series automatically generated by Microsoft Excel (Microsoft Inc., USA). All the patients were informed about the purpose of this study and the written informed consents were obtained from the patients. This study was approved by the local ethics committee (the Clinical Ethics Committee, Qilu Hospital, Shandong University).

Acriflavine

Acriflavine stock solution with the concentration 0.2% was stored in low temperature 4 celsius and kept in dark place. The candidate concentrations of acriflavine were routine 0.05%, lower 0.02%, 0.01% and 0.005% respectively according to our pilot study which determined concentration lower than 0.005% was generally unable to show tissue structure. Once endoscopic investigation decided a patient to be included, acriflavine solution used

to spray was made up immediately according to the patient's randomized group assignment. A 1 ml syringe and a 10 ml volume syringe were used to extract acriflavine stock solution and saline respectively. Acriflavine stock solution was diluted and stored in another 20 ml syringe and kept away from light.

CLE procedures

Preparation before CLE (EC-3870K; Corp., Tokyo, Japan) was the same as conventional upper gastrointestinal endoscopy. Patients fasted for at least 8 hours and orally took chymotrypsin to avoid effects by mucus and bubble in stomach prior to examination. Anisodamine 10 mg was routinely administered to reduce gastric peristalsis before examination except for those with urinary retention, glaucoma or some other contraindications. CLE was performed by one single endoscopist who was familiar with CLE operation and diagnosis. Once gastric antrum was clearly visible, another researcher promptly prepared acriflavine according to the patient's group which was kept blind to the endoscopist. The greater curvature side of antrum was selected as the observation spot for its good stability and convenience for touching mucosa with CLE laser probe. The observation spot was washed with water to minimize the effect of gastric juice, and 10 ml of acriflavine solution in the syringe mentioned above was sprayed onto mucosa via the biopsy channel immediately. The observation spot was washed with water again one minute after acriflavine spraying and observed with CLE. The endoscopist was asked to assess the image quality simultaneously as images were shown on the CLE screen. Every CLE procedure was under fixed brightness and laser volume located mid in the control panel on CLE display and operation screen to avoid the effects by laser and brightness. CLE images of each patient were stored in a specific folder for later analysis.

Image quality assessment

Image quality was assessed from 2 aspects respectively, the general assessment and the diagnostic value assessment. In general assessment, CLE images were classified into 3 grades: poor, almost invisible tissue structure; mild, visible tissue structure with too dark vision; good, clearly visible tissue structure. General assessment was scored 1, 2 or 3 when at least 2/3 of the patient's images were classified as poor, mild or good respectively.

In diagnostic value assessment, there were 3 false items to be determined: A. Whether the epithelial cells were equably stained? B. Whether the nuclei of epithelial cells were nonspecifically stained? C. Whether the nuclei of epithelial cells were stained as basal polarity? True to item A was scored 2 and false was 1. True to item B was scored 1 and false was 2. True to item C was scored 2 and false was 1. Diagnostic value was assessed by the best images of each patient. Score of image quality was the sum of general assessment and diagnostic value assessment. Indices of image quality assessment were shown in **Table 1**. When the mucosa is stained nonspecifically, all cells would be stained completely which makes it difficult to distinguish nuclei from cytoplasm. Since the epithelial cells of gastrointestinal tract are simple columnar epithelium, of which the nuclei locate basally, in CLE images nuclei are shown as a bright line in basal epithelium when CLE focal plane is in deeper layers of mucosa. The principle of diagnostic value assessment is illustrated in **Figure 1**.

In addition to the real time assessment, all images of each patient were reassessed by another researcher who was also blind to the patients' groups and corresponding acriflavine concentrations.

Statistical analysis

Differences between average ages of the four groups were determined by one way-ANOVA test, and differences in image quality scores between four groups were determined by nonparametric test (Kruskal-Wallis test). Differences of diagnostic value categories including the 3 false items were determined by crosstab chi-square test. Correlation statistics (Spearman's test) were performed between real time and afterward assessments to test the reliability of image quality assessments. A P value < 0.05 was determined as significant.Interobserver agreement of the 3 false items in the diagnostic value assessment was determined by kappa value, which the values of 0.1-0.2 were considered as slight agreement, 0.21-0.4 as fair agreement, 0.41-0.6 as moderate agreement, 0.61-0.8 as substantial agreement and 0.81-0.99 as almost perfect agreement. All the statistics were run by SPSS 13.0 software package.

Results

Patients

Twenty four patients meeting the inclusion criteria were recruited in this study. All patients completed investigations without any severe complications except for minor to mild nausea in some cases. Each group included 6 patients as arranged by the number series mentioned previously. The mean ages of four

Table 1. Indices of image quality assessment

General	2/3 of images rated as poor		1
	2/3 of images rated as mild		2
	2/3 of images rated as good		3
Diagnostic value	A. Whether the epithelial cells were equably stained?	True	2
		False	1
	B. Whether the nuclei of epithelial cells were nonspecifically stained?	True	1
		False	2
	C. Whether the nuclei of epithelial cells were stained as polar?	True	2
		False	1

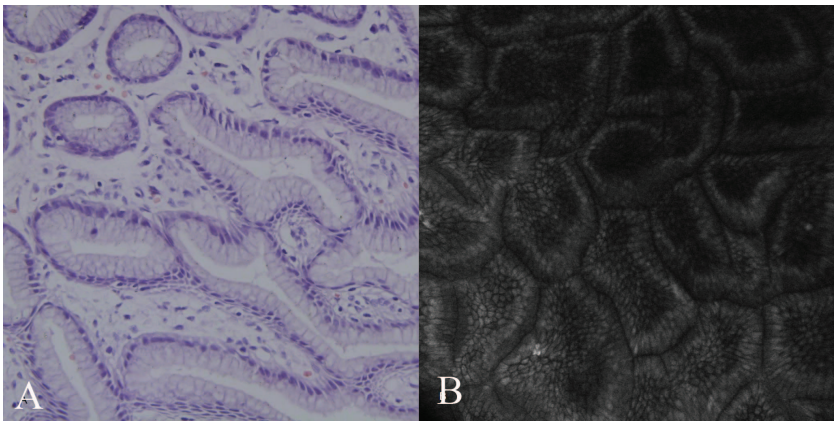

Figure 1. Polarization of gastric epithelial cells. (A) Histopathology shows the nuclei of gastric epithelial cells were basally located. (B) CLE images show the nuclei of gastric epithelial cells as a single bright line in the base of cells near gastric pits.

groups are: group 0.05%, 47.67 years (95% CI, 27.70-67.64); group 0.02%, 52.83 (95% CI, 42.44-63.23); group 0.01%, 59.50 years (95% CI, 46.98-72.02); group 0.005%, 55.50 years (95% CI, 46.19-64.81). There was no significant difference in ages between four groups (P=0.474). For small sample size of every group and few females in all the patients, the gender ratio differences between groups could not reveal any significance.

Real time assessment

There were a total of 3,031 images acquired from the 24 patients. Average numbers and confidential intervals of images in the four groups are: 0.05%, 176.83 (64.94-288.72); 0.02%, 122.50 (91.98-153.02); 0.01%, 104.83 (28.43-181.24); 0.005%, 66.67 (15.24-114.09). Though numbers of images dropped with decreased acriflavine concentrations, one-way ANOVA revealed no significant difference among average image numbers of four groups (P=0.078).

The k independent samples test (Kruskall-Wallis test) showed that image quality significantly decreased following dilution of acriflavine (P=0.01). The 2 independent samples tests (Mann-Whitney test) between the adjacent concentrations revealed that 0.005% was the only concentration decreased image quality significantly compared to the adjacent higher concentration (0.05% vs. 0.02%, P=0.937; 0.02% vs. 0.01%, P=0.699; 0.01% vs. 0.005%, P=0.026). Effects of image quality by different concentrations were shown in **Figure 2**.

The general assessment appeared to be in the same distribution as total sums. Different concentrations of acriflavine surely affected general image quality [P=0.01 (Kruskall-Wallis test)]. And 0.005% was the only one which decreased image quality significantly compared to the adjacent higher concentration (0.05% vs. 0.02%, P=0.241; 0.02% vs. 0.01%, P=0.211; 0.01% vs. 0.005%, P=0.041). In diagnostic value assessment, the only affected assessment item was that about polar staining (P=0.009), which suggested 0.05% and 0.02% the best applied acriflavine concentrations. Details of general assessment and diagnostic value assessment were shown in **Table 2**. Feaures of each concentration were illustrated in **Figure 3**.

Afterward assessment

Afterward assessment revealed the same trend of image quality among the four acriflavine concentrations as the real time

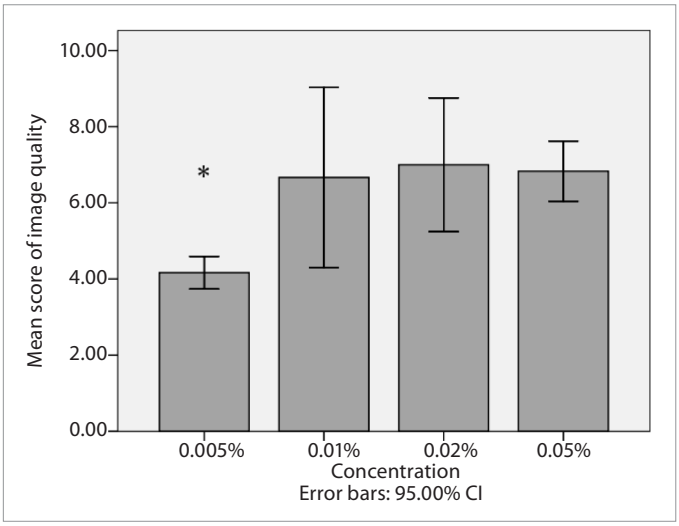

Figure 2. Effect of acriflavine concentration on image quality. Acriflavine applied in 0.005% decreased image quality significantly, while there was no significant difference among the other 3 concentrations [*concentration decreases image quality significantly (P<0.05)].

assessment, suggesting the only significantly decreased image quality existed in concentration of 0.005%. The correlation coefficents test (Spearman's test) analysis between real time and afterward assessment was significant (P<0.001). Although unlike real time assessment, both the afterward general assessment and diagnostic value assessment revealed no significances among four groups, the interobserver agreements of nonspecific staining and polar staining were moderate (kappa=0.471) and substantial (kappa=0.667), respectively. Interobserver agreement of even staining was fair (kappa=0.323). The details of interobserver agreement on diagnostic value assessment were shown in **Table 3**.

Discussion

Acriflavine has been applied in CLE for a few years by endoscopists from Europe[5] and Japan.[6] Clinical trials have shown the reliability of acriflavine in diagnosis of upper and lower gastrointestinal diseases by CLE.[1] The conventional concentration applied in CLE is 0.05%, which was seldom questioned before

Table 2. Diagnostic value assessment of each acriflavine concentration

		Acriflavine concentration(%)				Total	P value (chi-square test)
		0.005	0.01	0.02	0.05		
Nonspecific staining	False	1	1	3	2	7	0.528
	True	5	5	3	4	17	
*Polar staining	False	6	1	2	1	10	0.009
	True	0	5	4	5	14	
Even staining	False	6	3	3	4	16	0.212
	true	0	3	3	2	8	

*Significantly different among the four groups (P<0.05)

Table 3. Interobserver agreement analysis of the diagnostic value assessment

Real time assessment		Afterward assessment		Total	kappa value
		True	False		
Nonspecific stain	True	12	4	16	0.471
	False	2	6	8	
Polar stain	True	10	2	12	0.667
	False	2	10	12	
Even stain	True	13	3	16	0.323
	False	4	4	8	

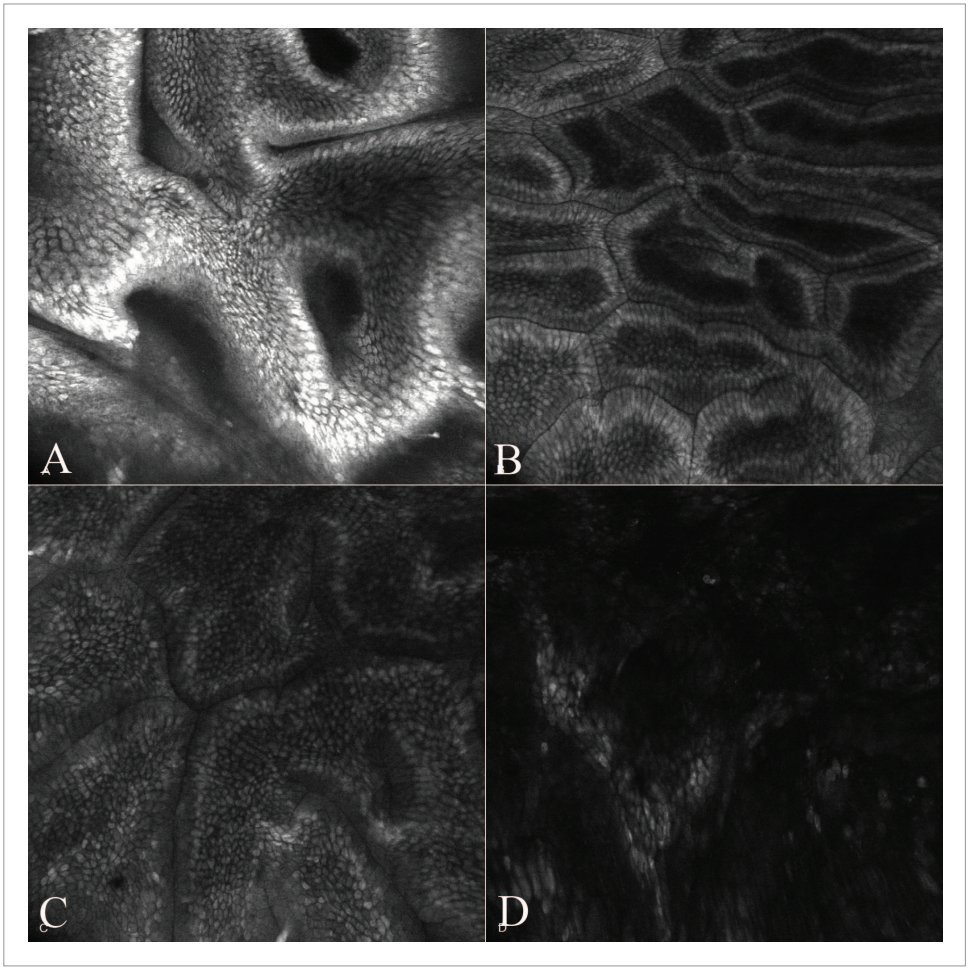

Figure 3. Representative images for each group. (A) Acriflavine in 0.05%, tissue structure was clearly shown and nuclei of some epithelial cells were stained brightly with well polarization and some without. (B, C) Acriflavine in 0.02% and group 0.01% respectively, tissue structures were clearly shown and nuclei of most epithelial cells were stained and showed well polarization. (D) Acriflavine in 0.005%, tissue structure could not be clearly visible.

whether it was the best concentration available. Our pilot study showed that concentrations lower than 0.05% were also satisfactory in many cases. The aim of this study was to find a more preferable dose of acriflavine in the CLE procedures.

In this study, we recommended lower concentrations including 0.02% and 0.01% the alternative concentrations in CLE. Though tissue structure displayed by 0.05% acriflavine in every patient was obviously the clearest, the insignificance between 0.05%, 0.02% and 0.01% might not only be caused by equally satisfactory tissue structure displaying with 0.02% and 0.01%, but also the significance of diagnostic value assessment, in which we introduced polar staining and unspecific staining for evaluation. The introduction of these items was derived from our confusion when we did nuclei assessment in CLE diagnosis with acriflavine staining. The columnar epithelial cells in many images with 0.05% were stained wholly, which made it difficult to identify the nuclei. In cases of diagnosing lesions with apparent tissue structure alterations, it is preferable for conventional acriflavine concentration because of clearer vision. But when it comes to some abnormalities in only a few cells such as focal dysplasia in background of intestinal metaplasia, identification of nuclei position and assessment of polarization of epithelial cells would be very valuable, which is one of the advantages of CLE over other endoscopic investigations.

Though our conclusion suggests difference between concentrations of 0.02% and 0.01% insignificant, 0.02% is more preferable for better subjective judgement by the endoscopists. For the choice between 0.05% and 0.02%, the lower concentration would be more preferable for both endoscopists and patients.

To exclude the influences by laser and brightness volume, we have accomplished all the investigations under the fixed laser and brightness volume. But in our pilot study, we have found either laser or brightness did little on image quality in every acriflavine concentration.

Analysis of interobserver agreement between real time and afterward assessment suggested good reliability of our assessment methods, such as total image quality, nonspecific staining and polar staining. Though general assessment or diagnostic value assessment alone was not significant different in the afterward assessment, interobserver agreement of the important polar staining was substantial, which was the only significant diagnostic value index in the real time assessment. The insignificance might be due to small sample size of each group. Future larger sample studies might be needed.

In this study we have excluded the influences of mucosal conditions such as severe lesions, and all the patients were for upper gastrointestinal endoscopy. Whether our conclusions about lower concentrations are applicable for other conditions such as severe gastritis, ulcer or tumor and other segments of gastrointestinal tract such as colon mucosa are still questionable. We have also applied 0.02% acriflavine on some patients with ulcer or tumor and got satisfactory image quality. Some randomized prospective studies under more conditions should be conducted in the future.

In conclusion, we propose 0.02% as the best acriflavine concentration in CLE, for its best nuclei assessment and preserved image quality.

References

1. Polglase AL, McLaren WJ, Skinner SA, Kiesslich R, Neurath MF, Delaney PM. A fluorescence confocal endomicroscope for in vivo microscopy of the upper- and the lower-GI tract. Gastrointest Endosc 2005; 62:686-95.
2. Tekola P, Baak JP, Belien JA, Brugghe J. Highly sensitive, specific, and stable new fluorescent DNA stains for confocal laser microscopy and image processing of normal paraffin sections. Cytometry 1994; 17:191-5.
3. Kiesslich R, Goetz M, Angus EM, Hu Q, Guan Y, Potten C, et al. Identification of epithelial gaps in human small and large intestine by confocal endomicroscopy. Gastroenterology 2007; 133:1769-78.
4. Leong RW, Nguyen NQ, Meredith CG, Al-Sohaily S, Kukic D, Delaney PM, et al. In vivo confocal endomicroscopy in the diagnosis and evaluation of celiac disease. Gastroenterology 2008; 135:1870-6.
5. Kiesslich R, Burg J, Vieth M, Gnaendiger J, Enders M, Delaney P, et al. Confocal laser endoscopy for diagnosing intraepithelial neoplasias and colorectal cancer in vivo. Gastroenterology 2004; 127:706-13.
6. Kakeji Y, Yamaguchi S, Yoshida D, Tanoue K, Ueda M, Masunari A, et al. Development and assessment of morphologic criteria for diagnosing gastric cancer using confocal endomicroscopy: an ex vivo and in vivo study. Endoscopy 2006; 38:886-90.