Original Article

Helicobacter Pylori –Infected Patients

Isam Elshaikh Eltayeb and Mohammed Ali M. Marie

Abstract

Background: The role of Helicobacter pylori on gastric carcinogenesis is still unclear but it is considered to predispose carriers to gastric cancer.

Objective: The aim of this study is to investigate the relationship between the extent of DNA damage of normal gastric epithelial cells and H. Pylori positive & negative gastritis according to the histological diagnosis.

Methods: We compared the percentage of cometed cells on the surface of gastric epithelial cells to the cells beneath gastric mucosal cells by the process of serial incubations times. DNA damage was evaluated by the alkaline single cell gel electrophoresis (comet assay) for 52 cases ,compared 19 normal individuals with 19 patients of H.Pylori positive & negative gastritis and further 14 mixed cases with different histology grading were tested by comet procedure for detection of the relationship of histological diagnosis with DNA damage (comet percentage).

Results: There was a relationship between the comet percentage and the histological diagnosis. Comet percentages in specimens from case with normal histology were significantly higher than comet percentages in specimen from case with H. pylori positive gastritis.

Conclusion: There was a significant increase in the percentage of cometed cells on the surface of gastric epithelial cells in both normal and infected H. pylori cells compared to the same specimen in the subsequent gastric cell layers.

Key words: Comet assay, gastric cancer.

Gastric cancer is the second most common malignancy in the world, accounting for 9.9% of all cancer incidence and 12.1% of all cancer death[1]. Carcinogenesis is a multistep process and steps involved on it relate to initiation-promotion sequences. Initiation causes permanent DNA damage (mutation). It therefore potentially leads to cancer development. Promoters can induce tumours in initiated cells, but they are non-tumourgenic by themselves[2]. Gastritis and Helicobacter pylori (H. pylori) are considered to be risk factors for gastric carcinoma[3]. There is growing evidence supporting an association between H. pylori and gastric cancer.

1. Senior Lecturer of Clinical Biochemistry
Riyadh College of Health Sciences.
Clinical Laboratory Department.
King Saud University.
Email: eltayeb@hotmail.co.uk
2. Associate Professor of Microbiology.
College of Applied Medical sciences.
Clinical Laboratory Department.
Email: mmarie@ksu.edu.sa

H. pylori infection induces chronic inflammation that can progress to gastric atrophy, intestinal metaplasia and gastric adenocarcinoma[4].

The objective of this study is to investigate the relationship between the extent of DNA damage and the histological grading of the gastric epithelium from which the biopsy was taken. Secondly for the comparison of percentages of gastric epithelia cells of H. pylori positive and negative gastritis with the normal gastric cells. Finally the study compared the percentages of DNA damaged on the surface of gastric epithelial cells with the cells underneath in both normal individuals and patients with H. pylori positive and negative gastritis patients.

Materials

Reagents used in the comet assay:

1. Lysis Buffer consisting of: 146.1g NaCl, 36.2g EDTA, 1% Triton (added immediately before lysis) and 1% sodium sarconisate. The volume was adjusted to one litre and the final pH (10-13). Sodium chloride, tris and sodium hydroxide pellets A.R. were purchased from

Vickers Laboratories Ltd. (Burley-in-Wharfedale, West Yorkshire, England).

2. Electrophoresis Running Buffer consisting of: 12g NaOH and 0.3722g EDTA

The above contents were dissolved in one litre of distilled water.

3. Neutralising Buffer consisting of: 400 mol/L Tris pH 7.5, Triton X-100 and EDTA from BDH Ltd. (Poole, England), Dulbecco's modified Eagle medium from Gilbico Ltd. (P.O. Box 35, Paisley, Scotland), Phosphate-buffered saline (PBS), sodium sarcosinate, ethidium bromide, low-melting point agargose, collagenase and pronase from Sigma Chemical Co. (P.O. Box 14508, St Louis, MO63178, U.S.A).

The horizontal gel electrophoresis tank was purchased from Gallenkampf Ltd. The power-pack from Biorad Ltd. 76 x 26mm microscope slides and 22 x 32mm coverslips from Mattrand, Germany.

Methods

The study population consisted of 52 unselected patients attending endoscopy clinic suffering from dyspepsia at Al Iman General hospital, Riyadh, Saudi Arabia. At endoscopy unit, biopsies were taken from the gastric antrum for histopathology and for H. pylori urease testing. After the comet and histopathology were performed, we selected and compared the results showing the percentages of DNA damage of gastric cells of 19 normal individuals with 19 patients with positive and negative H. Pylori gastritis and other 14 cases of different histological diagnosis were tested.

Two biopsies were also taken for each comet assay. Biopsies for the comet assay were placed immediately into Dulbecco's modified Eagle Medium DMEM (Sigma) and stored at 48°C until processing. The delay between biopsy and assay varied but was never longer than 24 h. The study was approved by the local Research Ethics at hospital.

Preparation of the single-cell suspension

The comet assay procedure was carried as follow: two biopsies were suspended in 800 ml of DMEM with 100 ml of 0.1% pronase (Sigma) and 100 ml of 0.03% collagenase (Sigma), and incubated at 37 C for 25 min with gentle shaking. Incubation was limited to 25 min to avoid complete digestion of the biopsies, the aim being to release cells predominantly from the epithelial layer. At the end of incubation, the biopsy remnants were removed, the cell suspension centrifuged at 2400 X *g* for 7 s, and the supernatant was discarded.

The yield of cells released from the biopsy following enzyme digestion was determined by staining 50 ul aliquots of the cell suspensions with 150 ul methyl green followed by microscopic examination in a counting chamber.

The comet assay

The comet assay was performed according to Singh's original description with minor modifications[5]. Slides were coated with a thin film of 1% w/v normal melting point agarose dissolved in phosphate buffered saline (PBS). Low melting point agarose (300 ml of 1% w/v in PBS, kept at 37°C) was added to 60ul cell suspension at room temperature, giving a final agarose concentration of 0.83% w/v and an average concentration of cells in the agarose of 0.37x105 /ml. 162ul of cells suspended in agarose was aliquoted onto each of the two slides and covered with a 22x32 mm cover slip (162 ml had been determined previously as the volume that gives the appropriate gel thickness and cell density beneath a cover slip of this size). Slides were placed on a cold block to allow the agarose to set, and then the cover slips were removed.

All subsequent steps were carried out in subdued lighting to prevent DNA damage from UV light. Slides were placed in lysis buffer (2.5 M NaCl, 100 mM Na EDTA, 10 mM Tris pH 10 and 1% Triton 2(Sigma)) at 48C for 1 h. They were then placed in running buffer (300 mM NaOHq1mM EDTA, pH 13, 48C) for 20 min to allow DNA unwinding, followed by 15 min electrophoresis at a constant voltage of 23 V (0.36 V/cm, 310–315 mA). These electrophoresis conditions had been identified in prior experiments as giving a suitable range of DNA damage in

gastric cells. Afterwards, slides were carefully removed from the tank and placed in a dish with ice cold neutralising buffer (400 mM Tris pH 7.5. for 5 min. Slides were dried gently and stained with 25 ml of 20 mlrml Ethidium Bromide (Sigma). By using a fluorescence microscope, count of 100 cells was carried out at least twice for each slide and the percentage of cells that were comets was recorded. Figure 1 and 2 illustrate of what could be observed in a comet assay.

Figure 1 Fluorescent photomicrograph showing a comet. The fluorescent core is located in the centre of the picture, with a zone of fluorescence extending towards the anode.

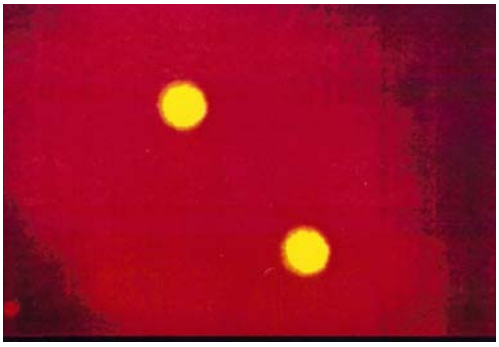

Fig 2 Fluorescent photomicrograph- showing two normal nuclei with no DNA damage

The gastric biopsies were incubated for 25 minutes (1st 25m incubation) and the percentages of DNA damaged cells (cometed cells) were calculated, then the same biopsies were washed out with distilled water and incubated for a further 25 minutes (2nd 25m incubation) and cometed cells percentages were calculated for comparison with the first 25 minutes incubation as in tables (1,2,3).

Table 1: Comparison of comet percentage after the first (1st) 25 minutes and the second (2nd) 25 minutes incubation period of normal gastric mucosa.

Case number	Comet percentages after serials of cell incubations	
	1st 25 minutes incubation	2nd 25 minutes incubation
1	52	28
2	58	36
3	62	47
4	45	39
5	48	29
6	52	38
7	49	36

Table 2: Comparison of comet percentage after the first (1st) 25 minutes and the second (2nd) 25 minutes incubation period of H. Pylori positive active gastritis.

Case number	Comet percentages after serials of cell incubations	
	1st 25 minutes incubation	2nd 25 minutes incubation
8	46	25
9	35	30
10	48	32
11	40	35
12	38	27
13	39	28
14	42	31

The gastric biopsies were incubated for 25 minutes (first 25 minutes incubation) the cells obtained were ignored and discarded, and then the same biopsies were washed out and incubated for further 25 minutes (2nd 25m incubation), the percentages of cometed cells were calculated and the biopsies were washed out and incubated for a further 25 minutes (3rd 25m incubation) and percentages of cometed cells were calculated for comparison with the second 25 minutes incubation (table (4)).

Table 3: Comparison of comet percentage after the first (1st) 25 minutes and the second (2nd) 25 minutes incubation period of four normal individuals &four patients of H.Pylori positive chronic gastritis.

Case number	Comet percentages after serials of cell incubations		Histological diagnosis
	1st 25 minutes incubation	2nd 25 minutes incubation	
15	54	35	Normal gastric mucosa
16	59	48	Normal gastric mucosa
17	46	32	Normal gastric mucosa
18	55	43	Normal gastric mucosa
19	52	45	H. Pylori positive chronic gastritis
20	40	32	H. Pylori positive chronic gastritis
21	45	22	H. Pylori positive chronic gastritis
22	43	28	H. Pylori positive chronic gastritis

Table 4: Comparison of comet percentage after the second (2nd) 25 minutes and third (3rd) 25 minutes incubation period of four normal individuals &four patients of H.Pylori negative active gastritis

Case number	Comet percentages after serials of cell incubations		Histological diagnosis
	2nd 25 minutes incubation	3rd 25 minutes incubation	
23	35	13	Normal gastric mucosa
24	25	12	Normal gastric mucosa
25	42	8	Normal gastric mucosa
26	55	15	Normal gastric mucosa
27	29	13	H. Pylori negative active gastritis
28	28	6	H. Pylori negative active gastritis
29	35	9	H. Pylori negative active gastritis
30	32	12	H. Pylori negative active gastritis

Table 5: Comparison of comet percentage after the third (3rd) 25 minutes and fourth (4th) 25 minutes incubation

Case number	Comet percentages after serials of cell incubations.		Histological diagnosis
	3rd 25 minutes incubation	4th 25 minutes incubation	
31	16	5	Normal gastric mucosa
32	9	0	Normal gastric mucosa
33	21	2	Normal gastric mucosa
34	18	3	Normal gastric mucosa
35	15	0	H.Pylori negative chronic gastritis
36	17	4	H.Pylori negative chronic gastritis
37	16	0	H.Pylori negative chronic gastritis
38	8	2	H.Pylori negative chronic gastritis

The comparison of the percentage of DNA damaged cells (cometed cells) in two different incubation times (3rd and 4th 25m incubations) was illustrated in table (5). The 1st and 2nd 25 minute incubated gastric cells were ignored and discarded. Then the same biopsies were washed out and incubated for a further 25 minutes (3rd 25m incubation), for which the cells' percentages were calculated and then the same biopsies were washed out again and incubated for the last 25 minutes (4th 25m incubation) and cometed cells' percentages were calculated for comparison with the 3rd 25 minute incubation.

Results

Comet percentage (DNA damage) in gastric cells of 19 normal individuals & 19 patients of gastritis under different incubation times

The comparison between the percentages of DNA damaged gastric epithelial cells in seven normal individuals with seven patients with H. Pylori positive active gastritis under first and second 25 minutes incubations was shown in table (1) and (2), while that between the percentage of DNA damaged gastric epithelial cells in four normal individuals with four H. Pylori positive chronic gastritis under first and second 25 minutes incubation was illustrated in table (3).

In table (4) we compared the percentage of DNA damaged gastric epithelial cells of four normal individuals with four patients with H. Pylori negative active gastritis under second and third 25 minutes incubations.

The comparison between the percentage of DNA damaged gastric epithelial cells of four normal individuals with four patients of H. Pylori negative chronic gastritis under third and fourth 25 minutes incubations was presented in table (5).

In Table (6) the DNA damaged gastric cells percentage (Comet percentages) was allocated to different histological groups, five normal individuals had a mean comet percentage of 71%. Five patients with H. Pylori positive active gastritis had a mean comet percentage of 53%, two patients with H. Pylori negative active gastritis had a mean comet percentage of 62.5% and two patients with H. Pylori negative chronic gastritis had a mean comet percentage of 56.5%.

The mean comet percentage was highest in the normal individuals' histology while the lowest comet percentage was in the group of H. Pylori positive active gastritis.

There was a significant difference between the comet percentage in the samples of normal histology compared to H. Pylori positive active gastritis (p=0.0006).

Table (6) : Comparison of comet percentages in gastric cells after a continuous 50 minutes incubation period, together with its comparative histological diagnosis.

Case Number	Comet percentages (% comet)	Histological Diagnosis
39	72	Normal gastric mucosa
40	61	Chronic H. Pylori negative gastritis
41	76	Normal gastric mucosa
42	58	H. Pylori positive active gastritis
43	69	Normal gastric mucosa
44	47	H. Pylori positive active gastritis
45	52	Chronic H. Pylori negative gastritis
46	74	Normal gastric mucosa
47	68	H. Pylori negative active gastritis
48	52	H. Pylori positive active gastritis
49	49	H. Pylori positive active gastritis
50	57	H. Pylori negative active gastritis
51	65	Normal gastric mucosa
52	60	H. Pylori positive active gastritis

Statistics

For comparisons of parametric data, *t*-tests were used and 95% confidence intervals were expressed. Two-tailed *p* values were calculated in all cases; a value of less than 0.05 was taken to imply statistical significance. Calculations were performed using SPSS version 16.

Statistical analysis of these groups of patients that were assessed is given below In the seven normal individuals (table 1) the mean comet percentage was high in the first 25 minute comet (a mean of 52%) compared to the second 25 minute comet (a mean of 36%). There was a significant difference in the comet percentage between the first and second 25 minute comets (p=0.0002).

In a group of patients with the histological diagnosis of Helicobacter pylori positive active gastritis (HPAG) the mean comet percentages were 41% in the first 25 minutes incubation and 29.7% in the second 25 minutes incubation Table (2).

There were a significant difference in the comet percentages between the normal individuals in Table (1) and patients of H. Pylori positive active gastritis in Table (2) (p=0.0002).

In Table (3) four normal individuals were compared to four patients with H. Pylori positive chronic gastritis. The mean comet percentages of the normal individuals were 53.5% and 39.5% in the first 25 minutes and second 25 minutes respectively, also the mean comet percentages of patients with H.Pylori positive chronic gastritis were 45% and 31.8% in the first 25minutes incubation and second 25 minutes incubations respectively. There was a significant difference in the comet percentages between the normal individuals and patients of H. Pylori positive chronic gastritis in the first 25 minutes incubation, (p=0.0004) .

In Table (4) four normal individuals compared to four patients with H.Pylori negative active gastritis. The mean comet percentages in normal individuals were 39% and 12% in the second 25 minutes and third 25 minutes incubations respectively. The mean comet percentages in patients with H.Pylori negative active gastritis were 31% and 1 0% in the second 25 minutes incubation and third 25 minutes respectively. There was a high comet percentages (a mean of 39%) of normal individuals compared to the patients with H. Pylori negative active gastritis (a mean of 31%). There was a significant difference in the comet percentage between the second and third 25 minutes incubation in normal individuals, (p=0.0008).

In Table (5) four normal individuals compared to four patients with H. Pylori negative chronic gastritis, the mean of comet percentages of normal individuals were 16% and 2.5% in the third and fourth 25 minutes incubations respectively. There was a high mean comet percentages of 16 % of normal individuals compared to that one of 14% of the patients with H. Pylori negative chronic gastritis in the third 25 minutes incubation.

The mean comet percentages of patients with negative H. Pylori chronic gastritis were 14% and 1.5% in the third and fourth 25 minutes incubation respectively. There was a significant difference in the comet percentage between the third and fourth 25 minute comet in patients with H. Pylori negative chronic gastritis (p=0.0014).

Discussion

The comet assay was first reported in 1988 by Singh et al[5] and is now established as a rapid, efficient and reproducible measure of DNA damage in single cells. It has been applied extensively to conditions where single cells are freely available in suspension, but less frequently to solid tissues. In order to perform in vitro studies of epithelial cells, it is necessary to create a cell suspension from solid biopsy material.

It is possible that this process could lead to spurious, ex vivo, DNA damage. For instance, both trypsinisation and scraping of cell cultures prior to performing the comet assay have been shown to cause DNA damage[6].

We discovered that the high comet percentages in cells from normal specimens may reflect the true levels of DNA damage that occur during physiological processes. The theory supports our results which state

that the cells die due to senescence in normal processes of cell turnover.

We have interpreted our results to state that the normal surface gastric epithelial cells accumulating for a longer time results in higher rate of DNA damage to be detected experimentally compared to the rapid turnover of H. Pylori infected cells.

Secondly, the decreased repair mechanism of DNA damaged by physiological cell death leads to a higher rate of DNA damage; the DNA repair mechanism does not take place in the normal cells and so DNA damaged cells percentage is highly seen.

It also could be the case that the gastric epithelial cells which became infected with H. Pylori died more quickly which resulted in less cometed cells due to generation of younger cells with less DNA damage. Also, these H. Pylori gastric infected cells might develop a mutation which can result in cell phagocytoses and early gastric cells death.

Our results were in contrast with results published by Marcelo et al[4] and Michal et al[12]. Both authors used comet assay to show that H. pylori infected patients have significant DNA damage than non-infected patients.

The above authors support their results with the theory of increased rates of cellular exfoliation[7] and cell proliferation[8] that have been demonstrated in tissues infected with H. pylori. This implies that there is a higher rate of cell death than in normal tissue.

Also they suggested that a normal cell dies quickly, and so it will be less likely that the DNA damage will be detected experimentally or possibly accumulated, thus showing a lower comet percentage which represents less DNA damage in the normal cell than what is actually occurring.

Furthermore, the use of biopsy specimens for estimation of DNA damage may select different types of cell populations associated with different histological diagnosis. Exfoliation of cell into the gastric lumen occurs at a faster rate in H. pylori infected tissue than in normal tissue[9], so the majority of cells with DNA damage may not be included in the biopsy specimen. Only cells with single strand breaks caused by reactive oxygen species (ROS) which have not resulted in cell death would remain in the tissue[10]. The H. Pylori infected gastric cells are susceptible to mutation, also if they have a high degree of DNA damage they are more likely to die or be phagocytosed by inflammatory cells, resulting in a fewer cells with DNA damage to be detected.

The mutation and a higher DNA damage in specimens from H. Pylori infected tissue can be detected experimentally unless progression to later stages of the preneoplastic sequence is occurring.

When there is H. pylori infection in the gastric tissue there will be an accompanying inflammatory cell infiltrate, consisting of neutrophil polymorphs, monocytes, lymphocytes and plasma cells.[9] When the infection is active neutrophil predominate and will be found locally at the site of inflammation[11], so they may be collected with the biopsy specimen. These additional cells would not be present in normal gastric tissue. Therefore, biopsies taken from tissues with different histological profiles can be expected to have heterogonous cell population with varying proportion of different cells. In tissue infected with H pylori the proportion of gastric epithelial cell present in the assay may have been less than in normal tissues.

The comet assay itself is not selective. Any cell with single strand breaks will form a comet. This will result in a distortion of the comet percentage from that which would have occurred if only gastric epithelial cells were present. This would reflect the average level of DNA damage in all of the cells present, instead of only that of the gastric epithelial cells. To overcome this disadvantage the cells in the specimen could be separated according to cell type before the assay is performed. This would be difficult to achieve and could cause artefactual DNA damage. Alternatively, extra biopsies of tissue from the same biopsy taken for the comet assay could be taken from each patient in order to investigate in detail the proportions of different cell types in each biopsy. Such an investigation was done at standard incubation time of 40 minutes and

no difference in cell types was found.

The gradual reduction in DNA damage throughout the gastric layer cells in normal individuals may be due to the fact that the gastric epithelial cells are old and have accumulated damaged DNA with age. Also, the younger cells should be synthesized and are present at the crypts and muscular layer in a few days. The younger cells move towards the mucosa in a gradual manner. Although these younger cells are present at the crypts and muscular layer initially, after a few days these cells are present at the mucosa.

The results of this study suggest that in normal individuals the highest degree of DNA damage was in the mucosa. The lowest degree of DNA damage was observed in the muscular layer and these finding may be due to the reasons mentioned above.

In H. pylori patients the low degree of DNA damage throughout the gastric layer cells could be due to rapid turnover of the gastric cells as a result of inflammation and hence younger cells with low DNA damage are present in infected tissue. The younger cells in H. pylori patient are present through the gastric layer cells due to the processes of gastric cell turnover. These cells move towards the mucosa more rapidly in H. pylori patients compared to normal individuals.

In summary, the results of this study have shown that in normal individuals the high degree of DNA damage from the surface epithelium of gastric mucosa could be due to the fact that these cells are older and had accumulated damaged DNA with age. If cells are undergoing DNA damage as a result of physiological cell death, then DNA repair mechanisms will not be activated. The process leading to cell death is slower in normal cells, giving more time for DNA damage to be measured experimentally. This may explain the high degree of DNA damage from the surface epithelium of the gastric mucosa in normal individuals. In H. Pylori, the low degree of DNA damage from the surface epithelium of the gastric mucosa could be due to rapid turnover of the epithelial cells as a result of inflammatory response and hence younger cells with low DNA damage are present in the gastric mucosa.

Increased rates of cellular exfoliation and cell proliferation have been demonstrated in tissues infected with H pylori[8]. In H pylori patients some gastric cells with DNA damage may not be included in the biopsy specimen, and if they have a high degree of DNA damage they are more likely to die or be phagocytosed by immune cells. Therefore, part of the gastric cells with DNA damage might not be detected in H pylori infected tissue. This may explain the low degree of DNA damage from the surface epithelium of gastric mucosa in H pylori patients.

In conclusion, there was a relationship between comet percentage and histological diagnosis. Comet percentages in specimens from case with normal histology were significantly higher than comet percentages in specimen from case with H. pylori gastritis. Finally, there was a significant increase in the percentages of surfaced cometed gastric epithelial cells compared to the subsequent gastric epithelial cells and the following gastric layers.

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