

Relationship between the Extent of DNA Damage and Gastritis in Normal and *Helicobacter pylori*-Infected Patients

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Background/Aims: The role of *Helicobacter pylori* in gastric carcinogenesis is unclear, but *H. pylori* infection is thought to predispose carriers to gastric cancer. The aim of this study was to investigate the relationship between the extent of DNA damage in normal gastric epithelial cells and *H. pylori*-positive and -negative gastritis according to histological diagnosis. We also compared the percentage of cometed cells on the surface of the gastric epithelial cells to the percentage beneath the gastric mucosal cells using serial incubations times. **Methods:** The comet assay is a rapid, efficient and reproducible measure of DNA damage in single cell and it was used in this study. DNA damage was evaluated in 52 cases using alkaline single cell gel electrophoresis (comet assay). Comparisons were made between 19 normal individuals, 19 patients with *H. pylori*-positive and -negative gastritis and 14 mixed cases with different histology gradings to determine if there was a relationship between histological diagnosis and DNA damage (comet percentage). **Results:** The comet percentages in specimens from cases with normal histology were significantly higher than the comet percentages in specimens from cases with *H. pylori*-positive gastritis. In addition, there was a significant increase in the percentage of cometed cells on the surface of gastric epithelial cells in both normal and *H. pylori*-infected cells compared to the subsequent gastric cell layers of the same specimen. **Conclusions:** A relationship between the comet percentage and the histological diagnosis was observed. (*Gut Liver* 2011;5:315-320)

Key Words: Comet assay; *Helicobacter pylori*; Gastric cancer

INTRODUCTION

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 cause permanent DNA damage (mutation). It is 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* are considered to be risk factors for gastric carcinoma.³ There is growing evidence supporting an association between *H. pylori* and gastric cancer. *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 AND 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 14 cases of different histological diagnosis were tested.

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

1. Preparation of the single-cell suspension

The comet assay procedure is as follows: Two biopsies were suspended in 800 mL of DMEM with 100 mL of 0.1% pronase (Sigma Chemical Co., St. Louis, MO, USA) and 100 mL of 0.03% collagenase (Sigma Chemical Co.), and incubated at 37°C for 25 minutes with gentle shaking. Incubation was limited to 100 minutes 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 2,400×g for 7 seconds, 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.

2. 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 60 uL 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 0.37×10⁵/mL. 162 uL of cells suspended in agarose was aliquoted onto each of the two slides and covered with a 22×32 mm cover slip (162 mL had been determined

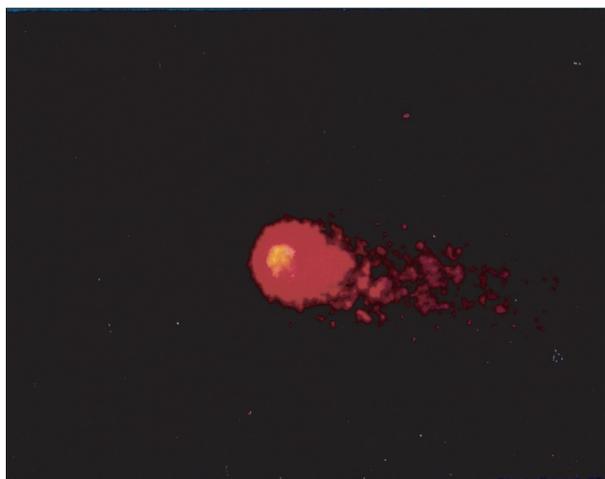


Fig. 1. Fluorescent photomicrograph showing a comet. The fluorescent core is located in the centre of the picture, with a zone of fluorescence extending towards the anode.

previously as the volume that gave 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 Chemical Co.] at 48°C for 1 hour. They were then placed in running buffer (300 mM NaOH, 1 mM EDTA, pH 13, 48°C) for 20 minutes to allow DNA unwinding, followed by 15 minutes 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 minutes). Slides were dried gently and stained with 25 mL of 20 ml/ml Ethidium Bromide (Sigma Chemical Co.). 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 (Figs 1 and 2).

3. Statistics

For comparisons of parametric data, t-tests are used and 95% confidence intervals are expressed. Two-tailed p-values were calculated in all cases; a value of less than 0.05 is taken to imply statistical significance. Calculations were performed using SPSS version 16.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

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

In Tables 1 and 2, we compared the percentage of DNA damaged gastric epithelial cells in seven normal individuals with

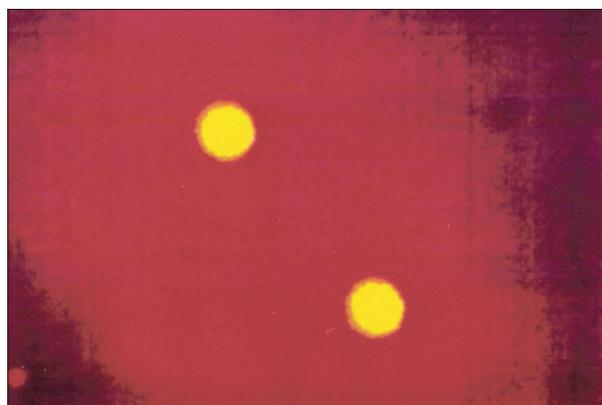


Fig. 2. Fluorescent photomicrograph showing two normal nuclei with no DNA damage.

seven patients with *H. pylori*-positive active gastritis under first and second 25 minutes incubations. While in Table 3, we compared 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 incubations.

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. In Table 5, we compared 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.

The gastric biopsies were incubated for 25 minutes (first 25 minutes 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 (second 25 minutes incubation) and cometed cells percentages were calculated for comparison with the first 25 minute incubation as in Tables 1, 2, and 3.

In Table 4, 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 a further 25 minutes (second 25 minutes incubation), the percentages of cometed cells were calculated and the

biopsies were washed out and incubated for a further 25 minutes (third 25 minutes incubation) and percentages of cometed cells were calculated for comparison with the second 25 minute incubation.

In Table 5, we compared the percentage of DNA damaged cells (cometed cells) in two different incubation times (third and fourth 25 minutes incubations). The first and second 25 minutes incubated gastric cells were ignored and discarded. Then the same biopsies were washed out and incubated for a further 25 minutes (third 25 minutes incubation), for which the cells' percentages were calculated and then the same biopsies were washed out again and incubated for the last 25 minutes (fourth 25 minutes incubation) and cometed cells' percentages were calculated for comparison with the third 25 minutes incubation.

In Table 6, The DNA damaged gastric cells percentage (comet percentages) were allocated to different histological groups, five normal individuals had a mean comet percentage of 71%.

Table 1. Comparison of the Comet Percentages of Normal Gastric Mucosa after the First and Second 25 Minutes Incubation Period

Case no.	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 the Comet Percentages of *H. pylori*-Positive Active Gastritis after the First and Second 25 Minutes Incubation Period

Case no.	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

Table 3. Comparison of the Comet Percentages of Four Normal Individuals and Four *H. pylori*-Positive Chronic Gastritis Patients after the First and Second 25 Minutes Incubation Period

Case no.	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	<i>H. pylori</i> -positive chronic gastritis
20	40	32	<i>H. pylori</i> -positive chronic gastritis
21	45	22	<i>H. pylori</i> -positive chronic gastritis
22	43	28	<i>H. pylori</i> -positive chronic gastritis

Table 4. Comparison of the Comet Percentages of Four Normal Individuals and Four *H. pylori*-Negative Active Gastritis Patients after the Second and Third 25 Minutes Incubation Period

Case no.	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	<i>H. pylori</i> -negative active gastritis
28	28	6	<i>H. pylori</i> -negative active gastritis
29	35	9	<i>H. pylori</i> -negative active gastritis
30	32	12	<i>H. pylori</i> -negative active gastritis

Table 5. Comparison of the Comet Percentages of Four Normal Individuals and Four *H. pylori*-Negative Chronic Gastritis Patients after the Third and Fourth 25 Minutes Incubation Period

Case no.	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	<i>H. pylori</i> -negative chronic gastritis
36	17	4	<i>H. pylori</i> -negative chronic gastritis
37	16	0	<i>H. pylori</i> -negative chronic gastritis
38	8	2	<i>H. pylori</i> -negative chronic gastritis

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 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 ($p=0.0006$).

In the seven normal individuals (Table 1), the mean comet percentage was high in the first 25 minutes comet (a mean of 52%) compared to the second 25 minutes comet (a mean of 36%). There was a significant difference in the comet percentage between the first and second 25 minutes comets ($p=0.0002$). In Table 2, a group of patients with the histological diagnosis of *H. 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. 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 25 minutes 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 of incubation ($p=0.0004$).

In Table 4, four normal individuals compared to four patients

Table 6. Comparison of the Comet Percentages of Gastric Cells after a Continuous 50 Minutes Incubation Period and the Comparative Histological Diagnosis

Case no.	Comet percentages (% comet)	Histological diagnosis
39	72	Normal gastric mucosa
40	61	Chronic <i>H. pylori</i> -negative gastritis
41	76	Normal gastric mucosa
42	58	<i>H. pylori</i> -positive active gastritis
43	69	Normal gastric mucosa
44	47	<i>H. pylori</i> -positive active gastritis
45	52	Chronic <i>H. pylori</i> -negative gastritis
46	74	Normal gastric mucosa
47	68	<i>H. pylori</i> -negative active gastritis
48	52	<i>H. pylori</i> -positive active gastritis
49	49	<i>H. pylori</i> -positive active gastritis
50	57	<i>H. pylori</i> -negative active gastritis
51	65	Normal gastric mucosa
52	60	<i>H. pylori</i> -positive active gastritis

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. While, the mean comet percentages in patients with *H. pylori*-negative active gastritis were 31% and 10% in the second 25 minutes incubation and third 25 minutes incubations, 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 minutes 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 ex-

tensively to conditions where single cells are freely available in suspension, but less frequently to solid tissues. In order to perform *in vivo* 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.

Our results may imply that the normal surface gastric epithelial cells accumulate DNA damage for a longer time resulting in higher rate of DNA damage to be detected experimentally unlike *H. pylori* infected cells with rapid turnover.

Another explanation is that the decreased repair mechanism of DNA damage 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 therefore 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 be develop a mutation which can result in cell phagocytosed and early gastric cells death.

Our results are in contrary with results published by Ladeira *et al.*⁴ and Arabski *et al.*⁷ 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 exfoliation⁸ and cell proliferation⁹ 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 suggest 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,¹⁰ 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.¹¹ 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 few 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.¹⁰ When the infection is active neutrophil predominate and will be found locally at the site of inflammation,¹² 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 heterogenous 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, where they should be accounted for the majority of cells.

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* pa-

tients 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 accumulate 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*.⁹ 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.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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