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Original Article

EVALUATION OF DNA DAMAGE IN THE ESOPHAGEAL MUCOSA AND PERIPHERAL BLOOD OF PATIENTS WITH GASTROESOPHAGEAL REFLUX DISEASE

Avaliação dos danos do DNA na mucosa esofágica e sangue periférico de portadores da doença do refluxo gastroesofágico

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ABSTRACT – ***Background*** - The gastroesophageal reflux disease is the most prevalent digestive disorder. Patients with it may present some complications during its development, and Barrett's esophagus is the most important in view of its potential malignancy. However, the inflammatory processes of the gastrointestinal tract may show malignant degeneration. ***Aim*** - To assess possible DNA damage in patients with gastroesophageal reflux esophagitis of various degrees and to evaluate the application of the Comet assay in its detection. ***Methods*** - Twenty-five patients were studied. They were divided into four groups: control (n=5), mild esophagitis (n=8), severe esophagitis (n=5) and cancer (n=7). The Comet assay was performed on peripheral blood cells (lymphocytes) and biopsy of the distal esophagus. ***Results*** - The Comet assay detected DNA damage in patients with mild and severe esophagitis (peripheral blood and biopsy), and damage intensity was greater in severe esophagitis ($p<0,05$). DNA damage in patients with severe esophagitis and cancer did not show significant difference, and its intensity corresponds to class-4 Comet assay (greater than 95% of damage). ***Conclusions*** - 1) The frequencies of DNA breakage in the esophageal mucosa and lymphocytes are directly related to inflammation level; 2) severe esophagitis shows virtually the same DNA damage frequency as that of esophageal cancer; 3) the Comet assay showed to be very sensitive for DNA damage detection.

HEADINGS – Esophagitis, peptic. DNA Damage. Comet assay.

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RESUMO - ***Racional*** - A doença do refluxo gastroesofágico é a afecção digestiva de maior prevalência. Os portadores podem apresentar na evolução algumas complicações, sendo o esôfago de Barrett a de maior importância, tendo em vista seu potencial de malignidade. Todavia os processos inflamatórios do trato gastrointestinal podem apresentar degeneração maligna. ***Objetivos*** - Avaliar os possíveis danos do DNA em portadores de esofagite de refluxo gastroesofágico de vários graus e verificar a aplicação do ensaio Cometa na detecção dos mesmos. ***Métodos*** - Foram estudados 25 pacientes distribuídos em quatro grupos: controle (n=5), esofagite leve (n=8), esofagite severa (n=5) e câncer (n=7). O ensaio Cometa foi realizado no sangue periférico (linfócitos) e biópsia do terço distal do esôfago. ***Resultados*** - O ensaio Cometa detectou danos no DNA nos pacientes com esofagite leve e severa (sangue periférico e biópsia), sendo que na esofagite severa a intensidade dos danos foi maior ($p<0,05$). Os danos do DNA dos pacientes com esofagite severa e câncer não mostraram diferença significativa e a intensidade dos mesmos corresponde ao ensaio Cometa classe 4 (maior que 95% de danos). ***Conclusões*** - 1) As frequências de quebras do DNA da mucosa esofágica e linfócitos estão diretamente relacionadas ao grau de inflamação; 2) a esofagite severa apresenta praticamente a mesma frequência de danos no DNA do câncer esofágico; 3) o ensaio Cometa mostrou-se muito sensível para a detecção dos danos do DNA.

DESCRITORES - Esofagite péptica. Dano ao DNA. Ensaio em Cometa.

INTRODUCTION

The gastroesophageal reflux disease (GERD) is an important disease of the gastrointestinal tract, in view of the high and increasing incidence, the intensity of symptoms and severity of complications, among which Barrett's esophagus (BE) stands out as the most important[3]. BE represents the last stage of esophagitis triggered by GERD, in which the distal squamous epithelium chronically exposed to gastroduodenal contents is replaced by metaplastic columnar epithelium[15]. The risk of developing cancer in BE patients is not well established and the rates range from 0.4 to 1.5%[17] by year of follow-up.

However, any inflammation of the gastrointestinal tract predisposes to malignant degeneration[5], a fact which may occur not only with the BE but in less advanced stages of esophagitis[1].

In addition, bacterial products released at the site of inflammation can cause DNA damage and these may represent the early stage of carcinogenesis[12].

Our objectives were to study the possible DNA damage in esophageal mucosa and peripheral blood of patients with reflux esophagitis in different degrees and verify the application of the Comet assay in the diagnosis of DNA damage and the risk of esophageal cancer.

METHOD

The design of this study was submitted to the Ethics Committee of the Faculdade de Medicina de Botucatu and approved (letter 187/2001). All patients signed a consent form.

Was studied 25 subjects (21 men and four women), aged between 15 and 85 years (mean: 57.32 ± 18.00 years) (Table 1).

TABLE 1 - Demographic aspects observed in patients of the four groups studied

Group	N	Average age	Male	Female
Control	5	53 ± 20.63	3	2
Mild esophagitis	8	50.12 ± 18.58	6	2
Severe esophagitis	5	51.8 ± 18.07	5	0
Cancer	7	65.42 ± 14.08	7	0
Total	25	57.6 ± 18.0	21	4

After clinical assessment, patients underwent endoscopy and divided into four groups, depending on the endoscopic finding[18] control (normal mucosa), mild esophagitis (reflux esophagitis grade I and II), severe esophagitis (esophagitis reflux grades III and IV) and cancer (squamous cell carcinoma).

During endoscopy four biopsies from the distal esophagus and 5 ml of peripheral blood were taken. Two samples from the biopsies were placed in Hanks balanced salt solution and the other in freezing midst.

All samples were sent for analysis of DNA damage using the Comet assay[20].

Esophageal mucosal cells and lymphocytes were isolated by digestion of proteins and collagenase, using the technique described by Pool-Zobel, et al.[16]. Then, the cells were subjected to the test of viability with ethidium bromide solution.

Determination of DNA damage

The technique used for determination of DNA damage was described by Singh, et al.[20], modified by Klaude, et al.[11], according to protocol Speit and Hartmann[22].

The study of the slides allowed the blind analysis of 5000 cells, 50 per sheet totaling 200 cells per individual.

The cell analysis was made by the imaging system in epiflurescency microscope (Axiophot II Zeiss) and 400X magnification. Images were viewed with circular shapes (undamaged DNA - Class 0) and structures in the form of "Comets" (with DNA damage - Classes 1 to 4). The Comet assay grade 4 represents DNA damage in a percentage higher than 95%.

The length of each image means the distance of migration of damaged DNA strand. In each slide, the number of comets was multiplied by the value of its class, giving rise to the scores of each blade, which can range from 0 (no damage) to 200 (maximum damage). The scores were added to each slide, resulting in final scores.

The quantification of DNA damage of cells was performed by the Image Analysis System Internative Comet Assay II (Percentive Instruments). The parameters measured were the tail moment (tail length x tail intensity or frequency of DNA migrated) and total scores in groups.

For statistical analysis was used the Tukey test, which allowed the comparison of tail moment (CM) and total scores in the four groups.

RESULTS

Table 2 presents the averages evaluated parameters in the blood, according to groups and statistical tests.

TABELA 2 - Médias dos parâmetros avaliados no sangue segundo grupos e testes estatísticos

	Parâmetros	
Grupos	Momentos da cauda	Escores
Controle	0,004 ± 0,008 a	1,400 ± 1,14 a
Esofagite leve	0,018 ± 0,024 a	42,250 ± 28,64 b
Esofagite severa	0,605 ± 0,402 b	143,600 ± 14,82 c
Câncer	1,183 ± 1,129 b	167,286 ± 22,60 c

Nota: média de grupos seguidos de letras iguais não diferem significativamente (0,05)

Table 3 presents the means of the parameters evaluated in the second biopsy groups and statistical tests.

TABLE 3 - Biopsy parameters in all groups

Parameters		
Groups	**Moments of the tail**	**Scores**
Control	0,014 ± 0,029 a	2,00 ± 2,00 a
Mild esophagitis	0,305 ± 0,374 b	77,88 ± 28,00 b
Severe esophagitis	4,962 ± 1,116 c	220,00 ± 12,64 c
Cancer	4,838 ± 3,296 c	256,42 ± 12,38 d

Note: Group average followed by same letter do not differ significantly (p> 0.05)

Considering the parameter of tail moment (Figure 1) was observed in biopsies significant difference between the control group, mild esophagitis, severe esophagitis and cancer, but the severe esophagitis and cancer groups showed no significant difference (p>0.05). Table 2 shows that in blood, control and mild esophagitis groups showed no significant difference when considering the tail moment parameter. However the values observed in these groups are smaller than those of severe esophagitis and cancer groups (p <0.05).

For scores (Figure 2) showed that when considering the biopsies, the four groups differ (Table 3). With respect to the blood, control groups, mild esophagitis and severe esophagitis are different (p <0.05), but the severe esophagitis and cancer groups showed no significant difference (Table 2).

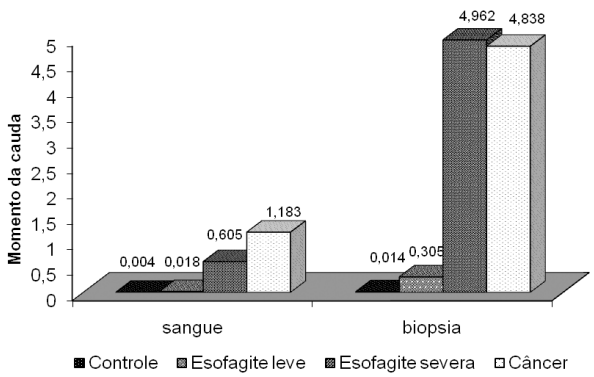

FIGURE 1 - Analysis of the parameter tail moment (lymphocytes, biopsies) in Comet assay in the four groups

DISCUSSION

In this research there was studied eight individuals with mild esophagitis and five with severe esophagitis. The average age of patients with severe esophagitis was 61.8 ± 18.07 years, higher than that observed in those with mild esophagitis (50.12 ± 18.58 years), suggesting that the esophageal mucosa suffered the harmful action of

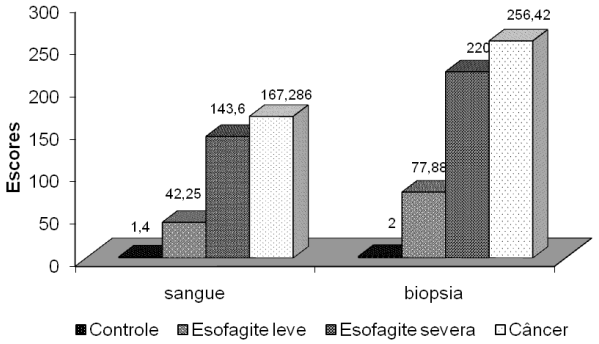

FIGURA 2 - Analysis of the parameter scores (lymphocytes, biopsies) in Comet assay in the four groups

the stomach juice for a longer time, resulting in severe inflammation. A similar result was observed by Tseng, et al.[26], in epidemiological study conducted in China.

In addition to patients with esophagitis, were also studied seven patients with squamous cell carcinoma of the esophagus. In this group all patients were male, confirming literature data, according to which the disease has a predilection for male subjects[6,8,27]. The age of patients with esophageal cancer (65.42 ± 14.08 years) does not differ from that by most authors[6,27]. The patients in this group received no neodjuvant therapy (chemotherapy or radiotherapy) before collection of blood or biopsy of the distal esophagus, conduct that would cause DNA damage[23].

This research was conducted to evaluate DNA damage of the esophageal mucosa and peripheral blood of patients with gastroesophageal reflux esophagitis. DNA is not a stable molecule and is frequently exposed to various agents, natural or artificial, which may cause damage3. Under normal conditions, about 99% of the damaged DNA can be repaired, but about 1% can remain in the genome of the cell[7]. The unrepaired DNA damage can result in loss of genetic information, or interference with transcription and replication, and is therefore deleterious to the host[4].

Another important aspect is that DNA damage can induce mutations[10,24]. Such genetic instability may be linked to several diseases, including cancer[2,9].

Thus, detection of DNA damage is important to conduct research related to genetic toxicology and molecular epidemiology[19,25].

In this research was used the Comet assay or Single Cell Gel Eletrophoresis test indicator of genotoxicity, because quantifies DNA damage. The Comet assay was used by several researchers[12,14].

The assessment of DNA damage in peripheral blood (Table 2 - tail moment), showed that in control group and mild esophagitis values did not differ. However in severe esophagitis and cancer groups values are higher than those of the previous two groups (significant difference). In severe esophagitis and cancer groups observed values show no significant difference. When

considering the parameter scores (Table 2) was observed than in control, mild esophagitis and severe esophagitis values differ, with most significant damage in the severe esophagitis group (143.6 ± 14.82) than in group mild esophagitis (42.5 ± 28.64). Severe esophagitis and cancer groups observed values show no significant difference (143.6 ± 14.82 X167.28 ± 22.60). The comparison of these results with the literature was hampered because no similar paper was found.

Table 3 shows that when analyzing DNA damage in esophageal mucosa, the values observed in control groups, mild esophagitis and severe esophagitis differed, being higher in the severe esophagitis group (4.96 ± 1.11) than in mild esophagitis (0.305 ± 0.37). The severe esophagitis and cancer groups were not significantly different (4.96 ± 1.11 X 5.24 ± 3.29). These results were observed when considering the tail moment parameter, in agreement to those published by Ladeira, et al.[12]. These authors studied gastric mucosal biopsies of patients with mild and severe gastritis and found that the greater the severity of inflammation greater is the intensity of DNA damage.

In groups severe esophagitis and cancer DNA damage corresponds to the Comet assay class 4, with percentages above 95%.

When considering the parameter scores, the values observed in biopsies of patients control, mild esophagitis, severe esophagitis and cancer differ, being higher in severe esophagitis (220 ± 12.64) than in mild esophagitis (77.88 ± 28).

Inflammation, a variety of phagocytes (neutrophils, monocytes and macrophages) can generate free radicals in response to proinflammatory mediators[21]. Free radicals induce harmful effects to the cell's DNA and can induce DNA breaks and other genetic changes that are potentially carcinogenic[3].

In this study, by analyzing the data obtained and taking into account the degree of mucosal inflammation, it can be seen that increasing the frequency of DNA breaks is related to increased intensity of mucosal inflammation, with significant differences between DNA damage in normal mucosa, mild esophagitis and severe cancer.

It should be noted that the Comet assay is a very sensitive technique that allows assessment of DNA damage in different cell populations, in addition to detecting differences in performance of the engine repair.

Based on these data, the authors suggest the possibility that inflammation itself exerts a strong genotoxic effect and consequent genomic instability in cells, increasing the likelihood of malignant degeneration.

CONCLUSIONS

The frequencies of DNA strand breaks in the esophageal mucosa and peripheral blood lymphocytes are directly related to the degree of inflammation; severe esophagitis presents almost the same frequency of DNA damage as in esophageal câncer; the Comet assay was highly sensitive for detecting DNA damage and appears to be promising as a prognostic test of susceptibility to malignancy.

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