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Research Article

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Hydrogen peroxide (H_2O_2) produced by *E. faecalis* in causing genomic instability determined by using comet assay

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Abstract The aim of this study the effect of H_2O_2 produced by *E. faecalis* in causing genomic instability by using comet assay and visualizing the histological alterations in rats colon tissues. The results showed a significant decrease correlated with the decrease of the concentration of *E. faecalis* in each of following characters: the mean % of the length of tail, the mean % of the DNA damage and the mean % of the tail lengthening. These differences were observed in all the cells, exposed to both concentrations of *E. faecalis* and were then compared with that of the control (without *E. faecalis* –treated cells).To determine whether dietary iron causes any genomic instability associated with adenomas or colorectal cancer, the comet assay has been used to detect DNA damage. All intestinal epithelial cells showed an increase in DNA damage after co-incubation with *E. faecalis* (EFCS).This increase was higher in rats which were fed dietary iron for 4 months. The role of catalase in preventing DNA –damage mediated by H_2O_2 was also studied .The results showed a significant decrease ($P \leq 0.01$) in the mean percentage of DNA damage in the treated cells.

Keywords *Enterococcus faecalis;* comet assay; colorectal cancer; DNA damage

Introduction

Reactive oxygen species (ROS), including superoxide anion ($O^{\cdot -}_2$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\cdot}OH$) were generated by *E. faecalis* as a by-product of normal cellular metabolism [1].

Although oxygen is a powerful oxidant that reacts with many molecules such as lipids, proteins, and DNA, over production of ROS from cells were referred to as oxidative stress, which may cause DNA damage and mutations that lead to cancer [2].

The present study investigates *Enterococcus faecalis*, bacteria that are naturally found in human intestinal tract, isolated from various samples of both healthy individuals and patients, suffering from colorectal cancer. Moreover, the study includes the etiological ability of *E. faecalis* in causing genomic instability of colonic epithelial cells of rats due to ability of bacteria in producing extracellular superoxide ($O^{\cdot}_2$), and various reactive oxygen species, such as Hydrogen peroxide (H_2O_2) and Hydroxyl radical ($^{\cdot}OH$).

Several years ago, it has been demonstrated that *E. faecalis*, forms an ionic free radicals depending on the presence of membrane-associated dimethyl menaquinone [3].Such production occurs near the oxygenated luminal surface bofcolonocytes and can be a potential source of chromosomal instability (CIN), which is considered a characteristic feature of colorectal cancer contributing to multi steps of mutations found in this cancer [4]. H_2O_2 generated near the intestinal epithelial surface couldpassively diffuse intocolonocytes and form $^{\cdot}OH$ at DNA sites through iron-

mediated Fenton reaction. This would lead to DNA–protein cross links, DNA breaks and base modifications could cause nucleotide transition and transversion [5].

The comet assay is referred to as the single cell gel electrophoresis (SCGE) assay that provides rapid ,simple ,sensitive and effective method for evaluating genetic damage and repair the *in vivo* and *in vitro* samples of eukaryotic cells [6].

Comet assay developed by Singh *et al*. [7]combines the simplicity of biochemical techniques to detect DNA single strand breaks (SSBs) / Double strand breaks(DSBs), incomplete excision repair sites [8,9] , oxidative DNA bases damage [3] , DNA – DNA / DNA - Protein [10,11], and DNA - Drug - Cross linking [12, 13].It is the only technique that directly measures DNA damage in individual cells. Thus, it has gained rapidly importance in the field of genetic toxicology and human biomonitoring [14]. Furthermore, it is useful in studies of the environmental toxicology [15, 16],Carcinogenesis [17], and aging [18].The aim of this study the effect of H_2O_2 produced by *E. faecalis* in causing genomic instability by using comet assay and visualizing the histological alterations in rats colon tissues.

Materials and Methods

Collection of Samples

A number of samples (98 sample - stool and tissue samples) were collected from different sources; patients [77 member (34 female and 43 male)] and healthy people [21 member (12 female and 9 male)]. Their ages ranged between (38-83) years. Those samples were collected from different hospitals in Baghdad including Teaching Baghdad Hospital, Gastroenterologyand Hepetology Teaching Hospital and Al-Kendi Hospital. These samples included:Stool samples [63 stool sample (14 samples from healthy people and 49 samples from patients)] were put in sterile container that contained 3 ml of normal saline .

Comet Assay: Comet assay was preformed according to Cinghu-Xia., [19] by using Fluorescence microscope (Olympus, Japan).

H_2O_2 Damages Eukaryotic Cells DNA

Colonic epithelial cells of rat's colon, viability (≥95%) incubated well in McCoy medium for 30 min with 0**,** 50, 200 µM H_2O_2. This effect for all H_2O_2 treated cells were assessed regarded as sensitive measures of H_2O_2-mediated damage to genomic DNA [3].

Colonic Cells Co-Incubation Assays

For co-incubation experiments, 0.2 ml of *E. faecalis* at 9×10^8 and 12×10^8C.F.U./ ml phosphate buffer saline, were resuspended in McCoy medium that contained suspension of colonic epithelial cell of rat's colon). Flasks were incubated at 37 °C for 30 mins, tissue culture medium were tested by using comet assay [3].

Catalase Protection Against *E. faecalis* - Induced Damage of Eukaryotic Cells DNA

Colonic epithelial cells and 0.2 ml of *E. faecalis* at 9×10^8 and12×10^8 C.F.U./ ml and 100 unit (U) catalase were added to McCoy's media . After a 30 min co- incubation, comet assays were performed [3].

E. faecalis- Induced Damage of Eukaryotic Cells DNA (*in vivo* assay)

The rats under study were inculcated with 0.2 ml of 9×10^8 and12×10^8 C.F.U./ ml of *E. faecalis* for 7 days , then they were sacrificed to perform comet test.

Iron -Induced Damage of Eukaryotic Cell DNA

Half of the rats that were fed dietary iron, were inoculated with 0.2 ml (9×10^8C.F.U./ ml of *E. faecalis*) and the other was inoculated with 0.2 ml (12×10^8C.F.U./ ml of *E. faecalis*). This process continued for one week, and at the eighth day, the rats of both groups were sacrificed to perform comet test.

Data Analysis of DNA Damage

The percentage of DNA damage was calculated by [(length of tail / (length of tail + length of Nucleus) x 100] for 50cells (25 cells from each end of the slide). The percent of the tail lengthening, was calculated by {[(length of tail + length of Nucleus)- (length of Nucleus)/ (length of Nucleus)]×100}.Data were presented in terms of mean ± standard error (S.E.), and differences among means were assessed by the least significant difference (LSD) and Duncan's test. The assessments were carried out using the computer programme SPSS version 7.5.

Results and Discussion

H_2O_2 –DNA damage was investigated. Table (1) summarizes the data for comet assay analyse for rats colonic epithelial cells had been exposed to 50 µM H_2O_2 and 200 µM H_2O_2 for 30 min.

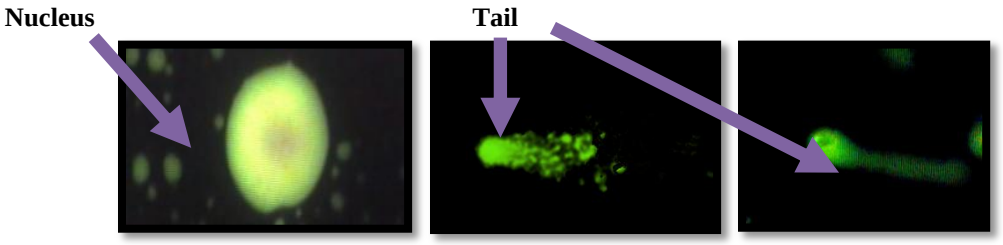
The statistical analysis of the results showed that there was a significant decrease ($P \leq 0.01$) in the length of nucleus between the treatments (50,and 200µM H_2O_2).These decreases were correlated with significant increase in the length of tail ($P \leq 0.01$) which increases with H_2O_2 concentration increase.

Mean percentage of tail lengthening showed a significant increase with the increase of the concentration of H_2O_2. The alkaline lysis single cell gel electrophoresis (comet assay) for measuring single-strand breaks shown in Figure 1. These results in agreement with the results obtained by Hookand Lee, [14] who used 8.8 µM H_2O_2 with different time exposure.

Table 1: Effect of H_2O_2 0^n Rats Colonic Epithelial Cells DNA Using Comet Assay.

Character	Treatment		
	0 H_2O_2 (Control)	**50 µM H_2O_2**	**200 µM H_2O_2**
L.Nucleus (µ)	8.83 ± 0.34^b	7.19 ± 0.30^a	6.87 ± 0.36^a
L. Tail (µ)	0.40 ± 0.17^c	40.98 ± 0.44^b	69.1 ± 0.96^a
Mean % of Comet Score	3.87 ± 1.5^c	84.86 ± 0.66^b	92.7 ± 0.03^a
Mean % of Tail Lengthening.	3.37 ± 2.31^c	653.25 ± 43.10^b	1468.6 ± 95.02^a

Different letters :Significant ($P \leq 0.01$) difference between means of the same row.



A) Control **B) 50 µM H_2O_2C) 200 µM H_2O_2**

Figure 1: Changes of DNA Strand Breaks in Tissue Culture cell Co- Incubation with H_2O_2. A: no Visible DNA Migration from the Nucleus; B: Moderate DNA Migration ;C: Extensive DNA Migration from the Nucleus.

The effects of catalase on reducing the DNA damages by H_2O_2, was investigated by using rats colonic epithelial cells. The addition both of H_2O_2 and catalase to the tissue culture cells in McCoy's media for 30 min led to a significant increase in length of the nucleus ($P \leq 0.01$).

This increase was related to the decrease in the following characters (mean % of length of tail, mean % of comet score, and mean % of the tail lengthening) as shown in table 2 .Because catalase was an antioxidant enzyme that catalyzed the decomposition of H_2O_2 to H_2O and O_2 and leads to decrease of the mean percentage of DNA damage (Fig.2).

These results proved to be different from that attained from Huycke*et al.*,[3] who had shown that the mean % of DNA damage were unchanged in Chinese hamster ovary cells exposed to both200 µM H_2O_2 and catalase.

Table 2: Effects of Catalase and 200 µM H_2O_2 on Rat Cells DNA Using Comet Assay.

Character	Treatment		Probability ≤
	200 µM H_2O_2	**200 µM H_2O_2 with 100 U CAT.**	
L. Nucleus (µ)	5.67 ± 0.36^b	7.31 ± 0.45^a	0.01
L. Tail (µ)	71.60 ± 0.97^a	24.01 ± 1.33^b	0.01
Mean % of Comet Score	92.63 ± 0.42^a	61.50 ± 4.42^b	0.01
Mean % of Tail Lengthening	1474.65 ± 82.9^a	360.93 ± 45.77^b	0.01

Different letters: Significant ($P \leq 0.01$) difference between means of the same row.

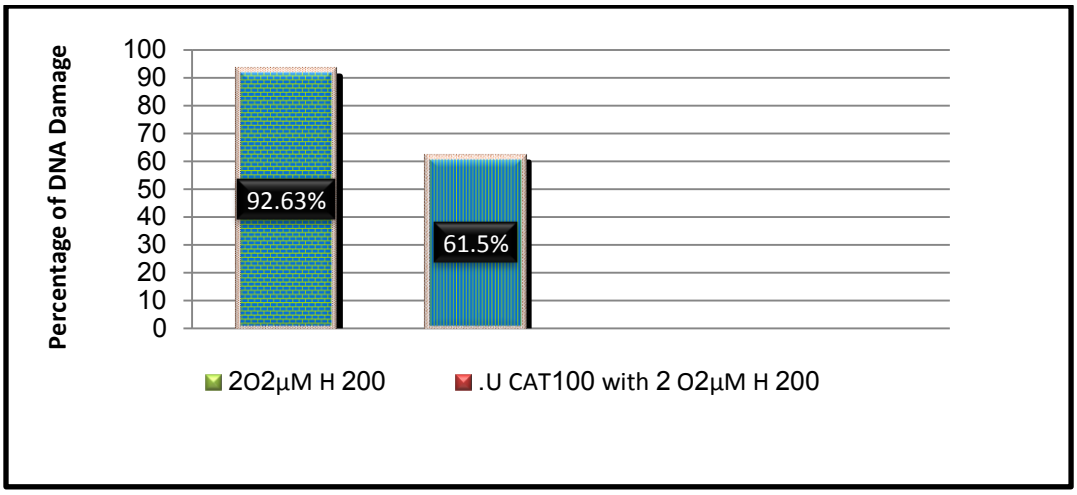


Figure 2: Effects of Catalase and 200 µM H_2O_2 on Rat Cells DNA Using Comet Assay.

Effects of *E. faecalis* on Eukaryotic Cells DNA (*In vitro*)

To determine whether extracellular $O^{\cdot}{}_2^{-}$ released from *E. faecalis* damages eukaryotic cells DNA .*E. faecalis* at 9×10^8 and 12×10^8 C.F.U./ml were co- incubated with rats colonic epithelial cells for 30 min.The results shown in Table 3.

Table 3: Effects of *E. faecalis* on Rats Colonic Epithelial Cells *in vitro* Using the Comet Assay.

Character	**Treatment**		
	0 (Control)	**9×10^8 C.F.U. /ml *E. faecalis***	**12×10^8 C.F.U. /ml *E. faecalis***
L. Nucleus (µ)	5.45±0.22[a]	5.47± 0.27[a]	5.23 ±0.26[a]
L. Tail (µ)	0.71±0.24[c]	13.77 ± 1.11[b]	20.11 ± 1.39[a]
Comet Score %	13.13±2.35[c]	415.88 ±0.02[b]	300.53 ± 1.78[a]
Mean % of Tail Lengthening.	13.14±4.44[c]	299.63 ± 33.77[b]	414.90 ± 42.63[a]

Different letters: Significant ($P\leq 0.01$) difference between means of the same row.

E. faecalis in each of following characters : the mean % of the length of tail ,the mean % of the comet score and the mean % of the tail lengthening (Fig.3). These differences were observed for all the cells exposed to both concentrations of *E. faecalis* compare with that of the control (without *E. faecalis*).The changes of DNA strand breaks *in vivo* using Comet assayare shown in Fig 4. These results shows that *E. faecalis* produced $O^{\cdot}{}_2^{-}$ which disproportionate to H_2O_2 in mildly acidic intocolonic epithelial cells to cause genomic DNA damage [20].

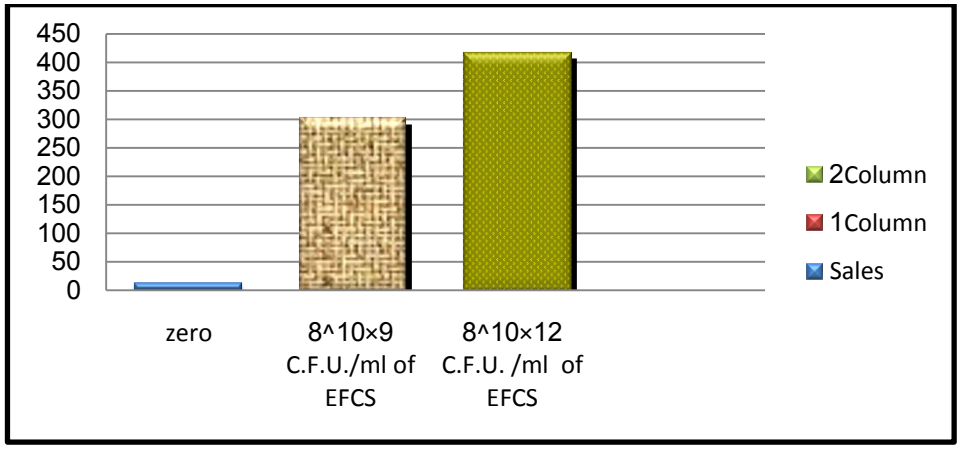
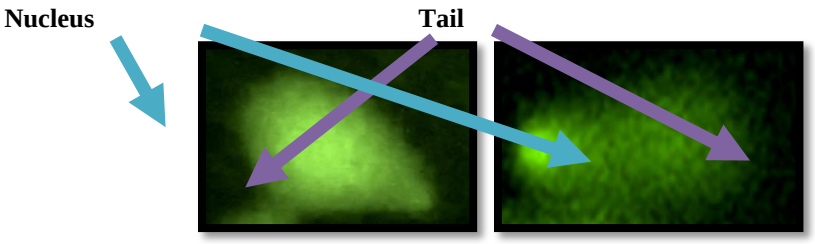


Figure3: Differences in Mean Percentage of Damage DNA In vitro Using Comet assay.



A)9×10^8 C.F.U. /ml*E. faecalis* **B) 12×10^8 C.F.U. /ml*E. faecalis***

Figure 4: Changes of DNA Strand Breaks in vitro Using Comet assay.

Conclusion

Oxidative stress on the colon epithelium due to normal luminal bacteria such as *E. faecalis* is of a low level; this is subjected to modulation by the diet and changing flora. It affords a possible mechanism for CIN and it may explain, at least in part, the genomic instability observed at the earliest stages of colorectal tumor genesis.

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