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Evaluation of the Genotoxicity in Patients with HBV, HCV, and HCC Using Micronucleus and Comet Assay in Mosul City\Iraq

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***Abstract*---**Hepatitis B virus (HBV) and hepatitis C virus (HCV) have mutagenic effects on somatic cells. HBV and HCV may be showing these mutagenic effects through its viral proteins or through integrating into host DNA. The aim of this study was to determine whether HBV and HCV have a genotoxic effect on the DNA of oral epithelial cells. A cohort of 145 samples have been collected from participants from the date of 5 \ 1 \ 2020 to 15\9\ 2021. Among those samples are (40 healthy controls, HBV 38, 44 HCV, and HCC 23) to make cytogenetic evaluation by observing the micronucleus (MNi) test and comet assay. For each individual, 100 cells were analyzed for comet assay. Around 100 cells were observed and MNi were scored for each individual. Our results showed significantly higher frequencies of MNi in HBV, HCV, and HCC patients groups than in the control group. There was no difference in MNi scores among HBV, HCV, HCC patients groups, and showed a significantly difference of study groups compared to healthy carriers. In conclusion: chronic HBV, HCV, HCC patients have genomic instability as affected as patients because of their levels of DNA damage and MNi.

***Keywords*---**comet assay, HBV, HCC, HCV, micronucleus test.

Introduction

Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer and the second greatest cause of death from cancer globally. HCC is linked with cirrhosis in around 90% of cases. Despite the fact that the incidence of HCC

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caused by alcohol and NASH is increasing, chronic viral hepatitis remains a leading cause of liver cancer globally (Russo et al., 2022).

The infections of Hepatitis B Virus (HBV) and Hepatitis C Virus (HCV) affects millions of human in worldwide. Approximately (10-15%) of HBV infected patients and about (70-80%) of HCV infected patients and developed a chronic form of their respective diseases. The persistent of HBV and HCV infections can be development and progression of liver disease, ranges in severity from minimal lesions throughout the parenchyma liver cells to severe fibrosis, cirrhosis and hepatocellular carcinoma (HCC) which represented a major problem associated with HBV and HCV infection(Szabó et al. 2004). The HBV and HCV may promote potently the processes of mutagenic cellular, such as integration HBV genome into the host DNA and the generation of oxidative stress as outcome the immune response (Sung et al. 2012). HB infects an estimated more than 35 million people and is the ninth reason leading of death worldwide. Chronic infection of HBV can lead to serious dangerous and clinical complications, including cirrhosis, liver failure requiring transplantation and hepatocellular carcinoma (HCC) (Lopez et al., 2002). The role of HBV and HCV in liver carcinogenesis through direct and indirect mechanisms is complex and still being debated. Integration the sequences of HBV DNA into the genome of host cell can activate cellular genes by a cis-acting mechanism. Chromosomal instability may also outcome from integration of HBV DNA. Indirectly, liver cells injury mediated by cellular immune responses may be sufficient to leading to hepatic cancer through promoting proliferation and cell death, and genetic mutations may accumulate in the context of necroinflammatory disease (Sung et al. 2012).

Cytogenetic analysis of micronuclei and binucleated cells in the micronucleus test (MNi) can be used to detection the genotoxicity effects of an agents (Surralles and Natarajan, 1997). MNi is a small separated part of the nucleus that arises during cellular division. MNi generates in inter-phasic cells from chromosomal fragments. The MNi is cytoplasmic structures measuring between 20 to 30% size of a nucleus with the same nucleus staining. In general, the frequency of cells with MN is ranging from 0 to 0.9%. Any chromosomal alterations concomitant increase in MNi count. The number of MNi has been correlated to the degree of carcinogenic effect (Nezhad et al., 2020). Another cytogenetic analysis is Comet assay also known as Single-Cell Gel Electrophoresis (SCGE) is a simple and sensitive technique for assessment of genotoxicity by quantifying the amount of damaged DNA caused in individual cells. Also, SCGE used as an important method for monitoring the health of aquatic animals by detecting damaged DNA in fish, clams shellfish, and mussels (Tasneem and Yasmeen, 2018). In this study, we evaluated the induction of host DNA in the oral and renal epithelial cells of patients infected with HBV or HCV and compared with the healthy control individuals through used both MNi scoring and comet assay.

Materials and Methods

Sampling

A cohort of 145 samples have been collected from participants (73 females and 72 males) from the date of 5 \ 1 \ 2020 to 15\9\ 2021. Among those samples

are (40 healthy controls, HBV 38, 44 HCV, and HCC 23) they have been clinically diagnosed that have signs by specialized physician's. Their ages are ranging from 16- 79 years.

Micronucleus assay in oral epithelial cells

The patients were tested in the clinic to ensure the healthy conditions of the orally tissues under potent light and standard condition. The exfoliated cells of oral mucosa scraping collects according to Bortoluzzi *et al.*, (2014) as the followed steps: A light mouthwash with distilled water was used to reduce debris. Then, with a wooden water soaked spatula were gently scraped the interior surfaces of the right and left cheeks. The samples were applied over two glass slides and air dried at room temperature. After that fixative the slides by using 100 % methanol and allowed to air dried and staining was performed, using May Granwald-Giemsa stain.

Scoring

The selection and counting MNi form followed the rules already described by Thomas *et al.*, (2009) under light microscope with 40x objective magnification.100 cells were count and checked for MN and nuclear abnormalities.

Evaluation of DNA damage in oral epithelial cells by comet assay

This test was conducted according to Trevigen protocol instructions as the followed steps. Lysis Solution was prepared and prior cooled at 4°C for at least 20 minutes. Then, Combined cells at 1 x 105\ml with molten LMA agarose at 37°C and immediately pipetted 50 µl onto CometSlide™. used side of pipette tip to spread agarose/cells over sample area to ensure complete coverage of the sample area. After that, Placed slides flat in refrigerator at 4°C with the dark field for 10 minutes. Increasing gelling time to 30 minutes to improves adherence of samples in high humidity environments. Then, Immersed slides in 4°C Lysis Solution for 30-60 minutes and Drained excess buffer from CometSlide™ . immersed CometSlide™ in freshly prepared Alkaline Unwinding Solution, pH>13 for 1 hour at 4°C, in the dark. After added Alkaline Electrophoresis Solution, placed slides in electrophoresis slide tray as slide label adjacent to black cathode and cover with Slide Tray Overlay. Then, Setting power supply to 21 volts and applied voltage for 30 minutes. Gently drained excess of electrophoresis solution, and gently immersed in D.W. twice for 5 minutes each, then in 70% alcohol (Ethanol) for 5 minutes. The samples was dried at 37°C for 10-15 minutes. Samples may be stored at room temperature or scoring at this stage. The CometSlide™ stained with 80µL 1X Ethidium Bromide, leave for 30 min and then dipped in chilled distilled water to remove excess stain. Then, the slides are scored immediately. The DNA damage was evaluated by reading the slides using 40X lens of fluorescent microscope equipped with digital camera. The analysis system for 4 comet images was applied for 50 cells were randomly chosen as 25 cells per each slides. Compared amount of migration for each cell, number of migrated cells, and migration extension between damaged cells.

Viability assay

A viability assay should be performed to determine the dose of the test substance that gives at least 90% viability. This done through mixed 10µl of 10^6 cells/ml in micro-centrifuge tube with 5µl trypan blue. placed on a slide after standing at least two minutes and then put a cover slip. Score 100 cells and record the number of viable cells (shiny) and dead cells (blue).

Statistical analysis

A one-way ANOVA was used to analyze the data. To overcome the variance between means as means±SD, Duncan's novel multiple range test was applied. The statistical program SPSS 28.0 was used for all statistical investigations (SPSS Ltd., Surrey, UK).

Results

Results of Micronucleus test in oral epithelial cells

The results of mn in oral epithelial cells among groups under study were shown in Table 1). The results revealed that the highest mn were in patients with HCV (4.95 ±1.628) followed by HBV patients and HCC patients as (4.82±1.449, 3.48±1.082) respectively, compared to the healthy control group (0.20±0.464). BN was used to compare the healthy control group to the HBV patients, HCV patients, and HCC patients groups. The results showed that the BN in healthy controls (0.53±0.784) was higher than in HCV patients and HCC patients as (0.41±0.622, 0.30±0.470) respectively. See table (1). In contrast, the results showed that the PN in the healthy controls was lower (0.05± 0.221) than in the HBV patients' group, HCV patients' group, and HCC patients' group as (3.50±1.084, 1.48±1.406, 0.17±0.388) respectively. The same with KR. The results revealed that the KR was lower in the healthy controls (2.85±0.949) than in the HBV patients group, HCV patients group, and HCC patients group (4.61±1.079, 3.82±1.063, 3.43±1.037) respectively. The results showed that the KL was higher in the healthy controls (1.37±1.030) than in the HBV patients' group and the HCV patients' group (1.03±1.174, 0.61±0.895) respectively. while it was lower than HCC patients' group (2.87±0.815). See table (1). Furthermore, the statistical significance of MNi test estimated in oral epithelial cells among all groups under study and healthy controls were compatible with clinical significances and showed clinical significances at ($p < 0.05$) (See Table 1).

Table 1
Micronucleus assay in oral epithelial cells among study groups

Parameters / Study groups		**Total mn**	**BN**	**PN**	**KR**	**KL**	**DIF**
Healthy controls	Mean	0.20	0.53	0.05	2.85	1.37	95.00
	Std. Deviation	0.464	0.784	0.221	0.949	1.030	1.895
	No.	40	40	40	40	40	40

HBV patients	Mean	4.82*	0.53	3.50*	4.61*	1.03	85.53
	Std. Error	0.198	0.054	0.140	0.101	0.103	0.363
	No.	38	38	38	38	38	38
HCV patients	Mean	4.95*	0.41	1.48*	3.82*	0.61	88.73
	Std. Error	0.198	0.054	0.140	0.101	0.103	0.363
	No.	44	44	44	44	44	44
HCC patients	Mean	3.48*	0.30	0.17	3.43*	2.87*	89.74
	Std. Error	0.198	0.054	0.140	0.101	0.103	0.363
	No.	23	23	23	23	23	23

* mean difference is significant at 0.05 level (t-Test), S.E: standard error, Total mn: Total Micronucleus, BN: Binucleated, PN: Pyknotic nucleus, KR:Karyorrhexis, KL:Karyolytic cell, DIF: Normal differentiated cell

Figure 1 showed the results of total mn in oral epithelial cells among HBV, HCV, and HCC patient groups compared to healthy controls. The results revealed that the total mn in HBV, HCV, and HCC patient groups were higher in males more than females whilst the results of total mn in healthy controls was lower in males more than females (Figure 1).

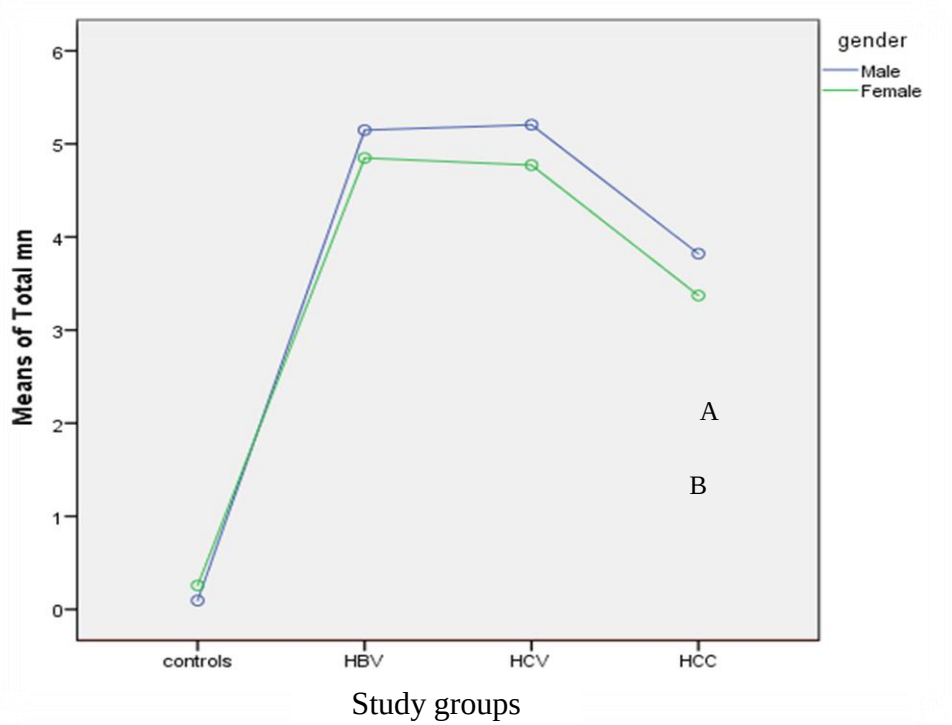

Figure 1. The mean of Total mn in oral epithelial cells in study groups according to gender

A, B: Different letters indicate that there are significant differences at the P≤ 0.05 (t-Test)

Table 2 summarizes the comparison of mn in oral epithelial cells among HBV, HCV, HCC and healthy controls according to age groups. The results revealed a significantly higher mean of mn in group of HBV patients with age group 50-60

years while the lower mean of mn was showed with age group 40-50 years as (6.00±0.644, 4.50±0.465)respectively (Table 2). With HCV patient group, the results showed the highest mean of mn with age 60-70 years was (7.00±.0.644) followed by age group 40-50 years (5.20±0.465) whilst the lower mean of mn was showed with age group 10-20 years (4.00±0.619) compared to healthy controls. See Table 2. In HCC patient group, the results revealed that the mean of mn was highest with age groups (70-80 years, 60-70 years, and 50-60 years) as (4.00±1.577, 4.00±0.644, and 4.00 ±0.644) respectively (Table 2).

Table 2
Total mn in oral epithelial cells in study groups according to age groups

Study groups	**Healthy controls**			**HBV patients**		
Statistical parameters / Age groups (Years)	Mean	Std. Deviation	No.	Mean	Std. Error	No.
10-20	0.45	0.688	11	-	-	-
20-30	0.06	0.236	18	4.73*	0.332	11
30-40	0.20	0.447	5	4.92*	0.362	13
40-50	0.00	0.000	2	4.50*	0.465	8
50-60	0.33	0.577	3	6.00*	0.644	1
60-70	0.00	0.00	1	5.00*	0.644	5
70-80	-	-	-	-	-	-
Total	0.20	0.464	40	4.82*	0.198	38
Study groups	**HCV Patients**			**HCC patients**		
Statistical parameters / Age groups (Years)	Mean	Std. Error	No.	Mean	Std. Error	No.
10-20	4.00*	0.619	2	-	-	-
20-30	4.75*	0.332	16	-	-	-
30-40	5.06*	0.362	16	3.50*	0.362	4
40-50	5.20*	0.465	5	2.75*	0.465	8
50-60	4.33*	0.644	3	4.00*	0.644	5
60-70	7.00*	0.644	2	4.00*	0.644	4
70-80	-	-	-	4.00*	1.577	2
Total	4.95	0.198	44	3.48*	0.198	23

* mean difference is significant at 0.05 level (t-Test), S.E: standard error, Total mn: Total Micronucleus,

Results of Comet assay in oral epithelial cells

Figure (2) showed the results of total cells with DNA damage (TCWD) in oral epithelial cells among the study groups. The results revealed that the TCWD was higher in the HCC patient group followed by HBV patient group. While the lower level of TCWD was shown in the HCV patient group compared to the healthy control group (Figure 2). The word 'comet' is thus applied to the individual cell DNA-migration patterns generated by this experiment, which like starry comets when viewed via a fluorescence microscope (see figure 3). In most cases, 50-100 comets are assessed per cellular sample.

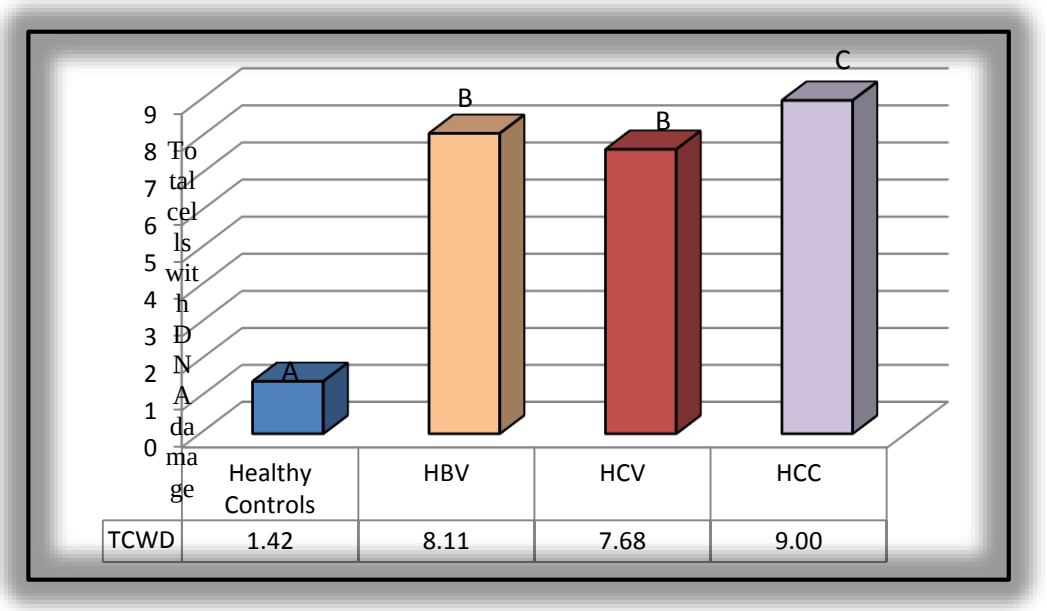

Figure 2: Total cells with DNA damage of oral epithelial cells among study groups
Different letters indicate that there are significant differences at the P≤ 0.05 (t-Test)

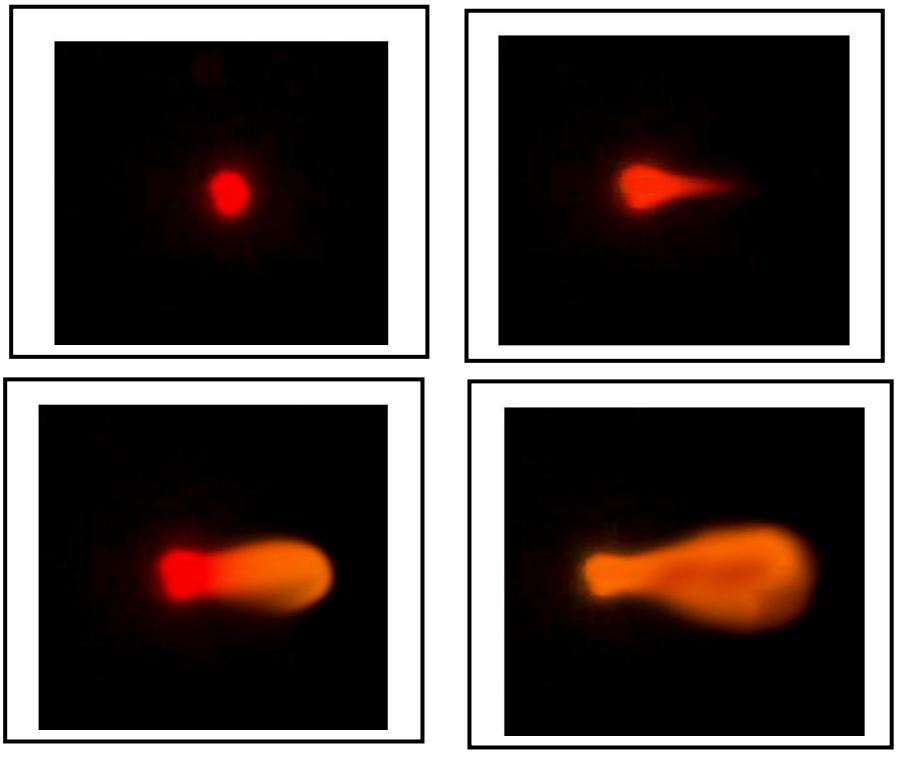

Figure 3. Tails of DNA damage through comet assay

The results of total cell with DNA damage in oral epithelial cells among HBV, HCV, and HCC patient groups compared to healthy controls according to gender were shown in figure (4). The results revealed that the TCWD in HBV, HCV, and HCC patient groups were higher in males more than females. In contrast , the results of TCWD in healthy controls were higher in females than in males (Figure 4).

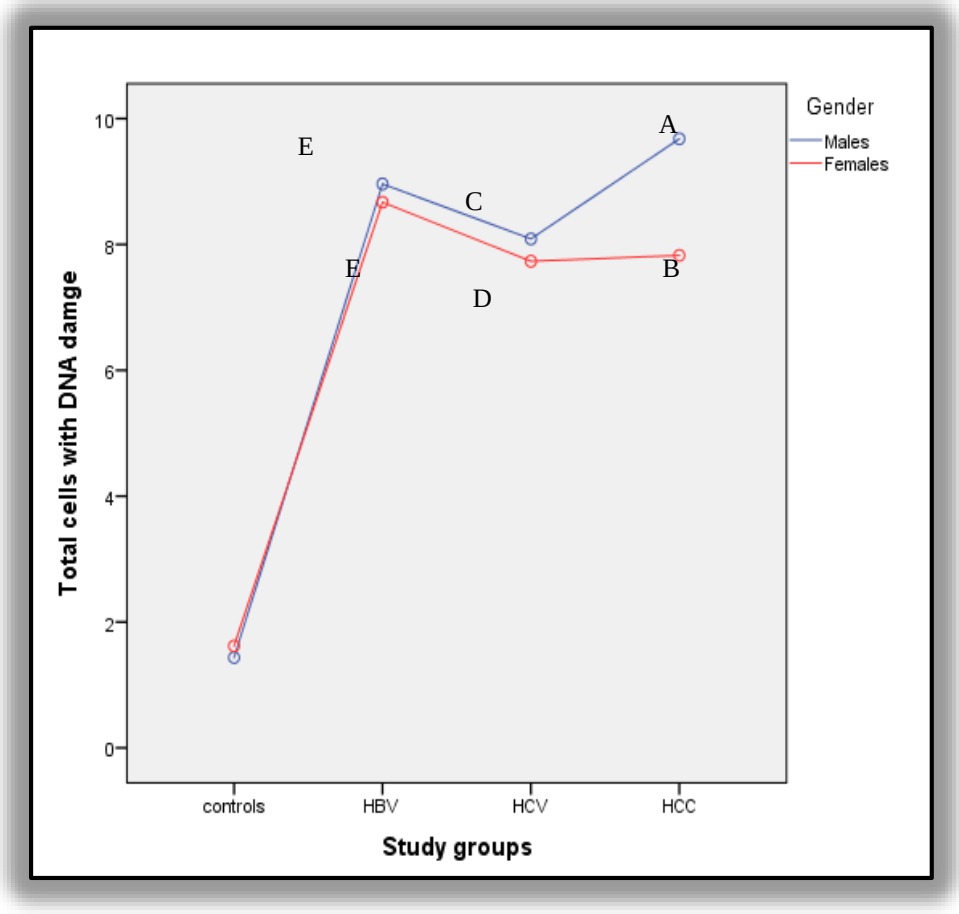

Figure 4. Total cells with DNA damage of oral epithelial cells among study groups according gender

Different letters indicate that there are significant differences at the P≤ 0.05 (t-Test). Similar letters indicate that there are none- significant differences at the P≤ 0.05 (t-Test).

The results of the comet assay as for total cells with DNA damage in oral epithelial cells among HBV, HCV, HCC, and healthy controls according to age groups were summarized in Table 3. The results revealed a significantly higher mean of TCWD in the group of HBV patients with an age group of 50-60 years (11.00± 0.800) followed by the age groups of 60-70 years, and 40-50 years (9.80±0.800, 8.50±0.578) respectively. while the lower mean of TCWD was shown with the age group of 20-30 years as (7.09±0.413). See Table 4-5. With the HCV patient group,

the results showed the highest mean of TCWD at age 60-70 years was (9.50±0.800) followed by the age groups 50-60 years, 40-50 years (8.67 ±0.800; 8.20±0.578) respectively. In comparison to healthy controls, the age group of 10-20 years had a lower mean of TCWD (6.50±0.768) (Table 3).

In the HCC patient group, the results showed the mean of TCWD was highest in the age groups of 60-70 years (10.50±0.800) followed by the age groups 70-80 years and 40-50 years (9.50±1.959, 9.38±0.578) respectively. While the age group of 30-40 years had a lower mean of TCWD (6.50±0.449) when compared to healthy controls (Table 3). Furthermore, the statistical significance of TCWD estimated in oral epithelial cells among all groups under study and healthy controls was compatible with clinical significance and showed clinical significance at ($p < 0.05$)

Table 3
Total cells with DNA damage of oral epithelial cells in study groups according to age groups

Study groups	**Healthy controls**			**HBV patients**		
Statistical parameters / Age groups (Years)	Mean	Std. Deviation	No.	Mean	Std. Error	No.
10-20	1.18	0.751	11	-	-	-
20-30	1.33	0.485	18	7.09*	0.413	11
30-40	1.80	0.447	5	7.85*	0.449	13
40-50	1.50	0.707	2	8.50*	0.578	8
50-60	2.33	0.577	3	11.00*	0.800	1
60-70	1.00	.	1	9.80*	0.800	5
70-80	-	-	-	-	-	-
Total	1.42	0.636	40	8.11*	0.362	38
Study groups	**HCV Patients**			**HCC patients**		
Statistical parameterS / Age groups (Years)	Mean	Std. Error	No.	Mean	Std. Error	No.
10-20	6.50*	0.768	2	-	-	-
20-30	7.38*	0.413	16	-	-	-
30-40	7.56*	0.449	16	6.50*	0.449	4
40-50	8.20*	0.578	5	9.38*	0.578	8
50-60	8.67*	0.800	3	9.00*	0.800	5
60-70	9.50*	0.800	2	10.50*	0.800	4
70-80	-	-	-	9.50*	1.959	2
Total	7.68*	0.362	44	9.00*	0.362	23

* mean difference is significant at 0.05 level (t-Test), S.E: standard error

Discussion

Chronic hepatotropic virus infections of HBV and HCV are characterized by highly mutagenic cellular processes including increased oxidative stress and viral integration into the host cell's DNA (Sung *et al.*, 2012). In this study, we found that oral epithelial cells from HBV- and HCV-infected patients had a higher

frequency of MNi than oral epithelial cells from healthy, non-infected people. This finding is in contrast to what Ozkal *et al.* previously reported (2005).

Micronucleus (mn) originally referred to chromosomes that have been fragmented or completely expelled from the primary nucleus during mitosis. These acentric fragment or broken chromosomes lead up to MNi, which are tiny nuclei that look like the major nuclei when stained. (Bolognesi *et al.*, 2013). As a result, the mn assay is an effective parameter for use as a biomarker (Motgi *et al.*, 2014). Compared to healthy controls, those occupationally exposed to toxic environmental agents such as solvents, polycyclic aromatic hydrocarbons, pollutants from sugarcane straw burning, gasoline, arsenic, and anti-neoplastic pharmaceuticals had higher frequencies of MNi in exfoliated buccal cells. (Ramos *et al.*, 2014). According to Thomas *et al.* (2009), even though buccal cells turn over every 7 to 21 days, it is theoretically possible to observe the genotoxic effects of an acute exposure after this period so that DNA damage in exfoliated cells collected from the oral cavity holds great promise as a minimally invasive method for monitoring exposition to genotoxic agents. In general, the MNi test from exfoliate buccal cells is a helpful biomarker for evaluating the genotoxic impact and, as a result, measuring the damage to human DNA from a variety of sources, but other research have questioned its potential to detect or signal prospective oral cancer development (Lee *et al.*, 2000). Certain lifestyle habits, including as smoking, alcohol intake, vitamin deficits, and supplementing, might cause cytogenetic damage (Agarwal *et al.*, 2014). Furthermore, smoking has been associated with increases in the frequency of MNi in exfoliated buccal epithelial cells (Piyathilake *et al.*, 1995). These authors, on the other hand, observed a greater incidence of chromosomal breakage, which resulted in the creation of acentric chromosome/chromatid fragments, which eventually led to the formation of mn. Furthermore, the mn might be the consequence of entire chromosomes being unable to get to the spindle poles during mitosis (Fenech *et al.* 2011).

The inability to acquire free nucleoids limited the use of buccal epithelial cells to evaluate genotoxicity using the comet assay. Initially, proteinase K enrichment of lysis solution was recommended to remove cellular and nuclear-associated proteins in order to generate nucleoids that would migrate in an electric field during alkaline electrophoresis (Eren *et al.*, 2002). RMPI-1640 was utilized as a carrier to sustain buccal epithelial cells in the prior experiments, and proteinase K was used at identical doses during lysis. On the other hand, comet assay revealed an increase in mean of exfoliated buccal cells in healthcare workers in oncology hospitals regularly handling antineoplastic drug mixtures, even though the difference was not statistically significant, in day hospital nurses (the group handling the highest amount of drugs during the administration process), while ward nurses and pharmacy technicians did not (Ursini *et al.*, 2013).

The comet test is a low-cost, simple, quick, reliable, and sensitive technique for detecting primary DNA damage in single-cell suspensions of any type, and it only takes a little amount of sample material. For these reasons, the comet test (alkaline, neutral, and with lesion-specific enzymes to detect particular forms of DNA damage such as 8OHdG, formamidopyrimidine DNA glycosylase, endonuclease III, T4 endonuclease. V.) has few significant competitors. After lysis, denaturation, electrophoresis, and staining, the amount of DNA damage is

quantified either visually by separating the injured cells into five groups, or using a camera and software imaging program to analyze the picture . Tail length (measured in micrometers), tail intensity or tail DNA percentage (DNA has the form of a comet when damaged), and tail moment are the most often measured characteristics (combination of the first two parameters) (Dusinska and Collins, 2008).

At pH 13, the Comet assay may detect DNA single-strand breaks and alkali labile sites, or double-strand breaks under neutral circumstances (neutral version) (Fairbaim *et al.*, 1995). SCGE is useful since it requires extremely little cell samples and can assess DNA damage in both proliferating and non-proliferating cells (Hartmann and Speit, 1994). Over 90% of malignancies are epithelial in origin, and since DNA damage is a critical process in cancer formation (Weinstein, 1988), DNA damage assessment in buccal epithelial cells might be a suitable indicator of early damage. Rojas *et al.* established the parameters for applying the comet assay in buccal epithelial cells for the first time in their study (Rojas *et al.*, 1996).

There is just one study that uses the SCGE test to evaluate and analyze DNA damage and repair in buccal cells and lymphocytes after a radiation exposure. According to the findings, baseline DNA damage in oral epithelial cells is higher than in lymphocytes (Dhillon *et al.*, 2004). Importantly, the comet assay's use as a predictor of cancer risk. Since there are no prospective studies suggesting an elevated cancer risk, comet assay can be considered a biomarker of exposure rather than a biomarker of impact for the time being. However, the comet is a relatively new method for assessing initial DNA damage in vitro and in vivo test (Suspiro and Prista, 2011). In conclusion, the current study found that chronically infected individuals with HBV or HCV had more chromosomal instability, as evidenced by the existence of MNi and DNA damage in their oral epithelial cells. Although we did not find a statistically significant impact, cirrhosis' likely influence on these parameters should be investigated further.

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