

**Effect of Ascorbic Acid (Vitamin C) on H<sub>2</sub>O<sub>2</sub> Induced Oxidative DNA Damage in Human Lymphocytes Estimated by Comet Assay****Hanaa Naji Abdullah, Sura Ali Al Asadi and Mohammed Abduldaim Saleh****Effect of Ascorbic Acid (Vitamin C) on H<sub>2</sub>O<sub>2</sub> Induced Oxidative DNA Damage in Human Lymphocytes Estimated by Comet Assay****Hanaa Naji Abdullah<sup>1</sup>, Sura Ali Al-Asadi<sup>2</sup> and Mohammed Abduldaim Saleh<sup>3</sup>**<sup>1</sup>Research Unit-College of Health and Medical Technology-Middle Technical University<sup>2</sup>Radiology Technology-College of Health and Medical Technology-Middle Technical University<sup>3</sup>Biotechnology Department - College of Science- Diyala University[Dr.Hanaa\\_gene2010@healthtech.edu.iq](mailto:Dr.Hanaa_gene2010@healthtech.edu.iq)[Assistprof.salih@sciences.uodiyala.edu.iq](mailto:Assistprof.salih@sciences.uodiyala.edu.iq)**Received: 9 June 2017****Accepted: 20 August 2017****Abstract**

"Oxidative DNA damage can be induced by reactive oxygen species and free radicals. Reactive Oxygen Species which is induced by oxidative damage plays a key role in DNA damage". The aim of our study is to identify the protective effect of ascorbic acid (AA, vitamin C) on hydrogen peroxide which induced oxidative damage in DNA by using the Comet assay. Lymphocytes pretreated with or without antioxidants, incubated at 37°C for 30 minutes, then H<sub>2</sub>O<sub>2</sub> (100μM) was added & incubated again at 37°C for 1 hour (60 minutes). Viability of cells was detected by trypan blue stain exclusion method. The decrease in viability brought about by H<sub>2</sub>O<sub>2</sub> when the cells incubated for 60 minutes and the viability was present to be 39±3% from 80±4% and it was highly developed by the found of AA at 100 μM which appeared 72 ± 1%. These findings indicate that the activity of ascorbic acid as an antioxidant is evidenced by its ability to suppress the oxidative effect against H<sub>2</sub>O<sub>2</sub> and protect the lymphocytes. Estimation of comet tail moment and tail length in human lymphocyte treated with 100μM of hydrogen peroxide as positive control showed that 13± 4.5% of the cells showed no DNA damage while the DNA damage from low to very high damage were 19± 3.5%, 12.2± 2.3%, 14± 3.2% and 37± 3.0%, respectively. In contrast, treatment of the cells with 100 μM H<sub>2</sub>O<sub>2</sub> in combination with 10, 25, 75

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and 100  $\mu$ M of AA reduced the percentage of DNA damage according to the concentrations used and they were  $9 \pm 2.3\%$ ,  $4.5 \pm 1.6\%$ ,  $6 \pm 0.5\%$  and  $10 \pm 1.4\%$ , respectively. In addition, H<sub>2</sub>O<sub>2</sub> induced DNA damage at percentage of 78% at concentration of 100  $\mu$ mol/L without the addition of ascorbic acid. while the treatment of human lymphocyte with ascorbic acid was able to reduce oxidative DNA damage by 17% in comparison with control group.

**Keywords:** Oxidative DNA damage, Ascorbic acid, Hydrogen peroxide, Comet assay.

### تأثير حامض الاسكوريك على تدمير الحمض النووي التأكسدي المحث بواسطة بيروكسيد الهيدروجين في الخلايا اللمفاوية البشرية والمقاسة بواسطة فحص المذنب.

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#### الخلاصة

مركبات الاوكسجين الفعالة والجذور الحرة تحت تلف الحمض النووي التأكسدي . تلعب تلك المركبات دور رئيسي في تلف الحمض النووي التأكسدي. هذه الدراسة تهدف الى التعرف على تأثير الوقائي لحمض الاسكوريك ضد بيروكسيد الهيدروجين المحث للتلف التأكسدي للحمض النووي في الخلايا اللمفاوية باستخدام مقايصة المذنب. تم معاملة الخلايا اللمفاوية مع مضادات الاكسدة او بدونها وحضنت بدرجة حرارة 37 درجة مئوية ولمدة 30 دقيقة، ثم اضيف بيروكسيد الهيدروجين وحضنت لمدة 60 دقيقة. تم التحري على حيوية الخلايا بواسطة طريقة صبغة تريبان الازرق. ان انخفاض حيوية الخلايا  $39 \pm 3$  بأضافة بيروكسيد الهيدروجين وحضن الخلايا لمدة 60 دقيقة من  $80 \pm 4$  وهناك زيادة عالية بوجود حمض الاسكوريك عند 100 مايكرومولر تظهر  $72 \pm 1\%$ . هذه النتائج تظهر نشاط حمض الأسكوريك كمضادات الأكسدة اظهرت حماية عالية ضد بيروكسيد الهيدروجين. وتم قياس ذيل وطول المذنب في الخلايا اللمفاوية بوجود بيروكسيد الهيدروجين (100 مايكرومولر) كسيطرة موجبة و اشارت النتائج عدم وجود تحطم للحمض النووي ( $4.5 \pm 13$ ) بينما تباينت نسب تحطم الحمض النووي من الواطء الى العالي جدا على التوالي ( $19 \pm 3.5\%$ ،  $12.2 \pm 2.3\%$ ،  $14 \pm 3.2\%$ ،  $37 \pm 3\%$ ) على التوالي. بينما عند اضافة حامض الاسكوريك بتركيز (100 مايكرومولر) الى بيروكسيد الهيدروجين بتركيز (100، 75، 50، 25، 10 مايكرومولر) اظهرت النتائج انخفاض في نسبة تحطم الدنا على مختلف الدرجات. وان الانخفاض العالي. في حين عند معاملة بيروكسيد الهيدروجين (100 مايكرومولر) لتركيز مختلفة من حمض

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الاسكوربيك (10,25,50,75,100 مايكرومولر) و اظهرت انخفاض في نسب تلف الحمض النووي بمختلف الدرجات. وانخفاض عالي لتلف الحمض النووي بتركيز 100 مايكرومولر لحمض الاسكوربيك من تلف الواطىءالى العالي (2.3±9، 1.6±4.5، 0.5±6، 1.4±10) على التوالي. بالاضافة الى ذلك، بيروكسيد الهيدروجين بتركيز 100 مايكرومولر تحت تلف الحمض النووي بنسبة 78% بدون اضافة حمض الاسكوربيك. بينما عند معاملة الخلايا للمفاوية مع حمض الاسكوربيك ينخفض تلف حمض النووي بتركيز 17% مقارنة بمجموعة السيطرة. **الكلمات المفتاحية:** تلف الحمض التأكسدي، حمض الاسكوربيك، بيروكسيد الهيدروجين، مقياس المذنب.

### Introduction

Protection of the cell DNA by ascorbic acid (AA, vitamin C) from the damage resulted by mutagens and free radicals. It prevents genetic alteration within cells from chromosomal mutations (1) and could have a preventive or therapeutic effect against various diseases. Ascorbic acid is an important antioxidant which prevents other compounds from being oxidized (2). In addition, AA is considered a very sensitive index of oxidative stress (3) which involves production of reactive oxygen species (ROS) and also responsible for aging and neoplastic progressions (4). Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) is widely recognized to produce reactive oxygen species which cause damage to nucleic acids, proteins and disintegrate plasma membrane. It is also responsible for aging and neoplastic progressions (5). H<sub>2</sub>O<sub>2</sub> is utilized to induce oxidative DNA damage (6) and found to be related to the induction of most cancers in animals (7), carcinogenesis and mutagenesis (8). importance originates from its role in the induction and progress of carcinogenesis (9). Hydrogen peroxide induces its harmful effect endogenously by various physiological strategies during oxidative phosphorylation in time of inflammatory respiratory burst. The hydrogen peroxide is a critical source of oxidative damage in cells, resulting in DNA lesions, together with ss and ds breaks (10). Ascorbic acid (Vitamin C) act as antioxidant and has long been known for its anti-cancer properties (11). The comet assay is a defined genotoxicity assay (in vitro and in vivo) of chemicals (12,13) and it is a simple, rapid, and sensitive method for detecting DNA breaks in individual cells, and is commonly used in genotoxicity assays, primary researches in DNA damage and repair and is also being increasingly more used in human biomonitoring (14,15,16). The aim of the present was to detect

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the protective effect of AA on H<sub>2</sub>O<sub>2</sub> that induced oxidative damage in DNA by using Comet assay.

### Materials and Methods

#### **Blood collection**

Blood samples were obtained by vein puncture and poured into heparinized tubes from apparently healthy male individuals (nonsmokers, non-alcohol, non-drug consumers, non-exposure to mutagen such as X-ray). After collection, blood samples were coded and processed within 2 hours.

#### **Isolation of human lymphocytes**

Heparinized whole blood (5 ml) was diluted one to one with phosphate buffer saline (PBS), then cautiously layered on top of a lymphocyte separation medium in a centrifuge tube. After centrifugation for 20 minutes at 2000 rpm, lymphocytes were gradient separated [aqueous solution of Ficoll, 57 g/L; density of 1.077 g/mL], then diluted with PBS & centrifuged again at 1500 RPM for 10 minutes. The cellular pellets were re-suspended in 500 mL of PBS & the cells were counted by Neubauer chamber and then cell concentrate was adjusted to 5000 cells/mL to be ready for the Comet assay (17). The trypan blue dye exclusion method was used to determine cell viability. However, only cell suspensions with more than 96% viabilities were used to determine DNA damage (18). The viability (%) was calculated by using following equation:

$$\% \text{ viability} = \frac{\text{Total no. of viable cells}}{\text{Total no. of viable cells} + \text{dead cells}} \times 100$$

#### **Ascorbic acid pretreatment**

Cells were incubated in a dark incubator with different concentrations of ascorbic acid 10, 25, 50, 75 and 100  $\mu$ M (Sigma, Aldrich®) at 37°C for 30 minutes together with the untreated control samples. Samples were then spun at 200 r.p.m for 3 minutes at 48°C. Cells were centrifuged and washed for two times with PBS (0.01 Mol) at 200 r.p.m for 3 minutes at 48°C after pretreatment.



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### Hydrogen peroxide treatment

Cells had been suspended in PBS with 100  $\mu$ Mol H<sub>2</sub>O<sub>2</sub>/L for 5 minutes on ice at dark. Samples were then centrifuged at 200 rpm for 3 minutes at 48°C. Control samples had been incubate with PBS only without hydrogen peroxide.

### The Comet assay

#### Preparation of Slides

Depending on the method described by Singh and coworkers (19), the comet assay was carried out under alkaline conditions. The slides were with two layers of 1% agarose. While this layer was solidified, a second layer containing 10  $\mu$ l samples of WBCs was mixed with 75  $\mu$ l (1% low melting agarose) and placed on the slides. After putting the slides on ice for 10 minutes, they were covered with coverslips and stored at 4°C.

#### Cell Lysis

The coverslips have been removed and the slides were immersed of slides in a newly prepared lysis solution [10 mM Tris-HCl, 100 mM EDTA, 2.5 M NaCl & NaOH [pH 10] with one percent Triton X-100 (Sigma) and 10% DMS were added to the lysed cells and DNA unfolding. After removing the slides from the lysis solution they were placed in electrophoresis tank.

#### Electrophoresis

The slides were then placed in a horizontal gel electrophoresis tank, dealing with the anode. The unit turned into packed with TBE buffer, and then electrophoresis was performed at 4°C under dim light conditions. At 25 V (300 mA), electrophoresis was carried out for 30 minutes. Staining was done with ethidium bromide stain (20  $\mu$ g/ml) and slides were covered with coverslips to be stored at 4°C in sealed bins until evaluation.

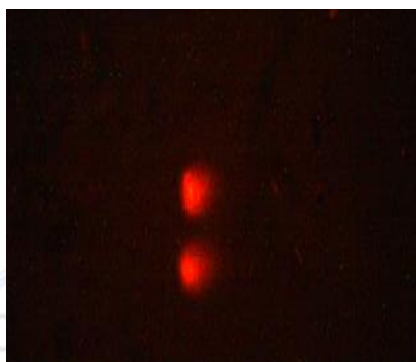
#### Comet analysis

Fifty captured comets from each slide were examined at 400 $\times$  by fluorescence microscope (Comet Assay II; UK). Tail moment (TM, length of DNA migration) was estimated in order to

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quantify the DNA damage tail length (TL, length of DNA migration). Mean and standard errors (SE) are used for analysis results for the fifty cells (twenty five cells/ slide).



**Figure 1:** DNA damage after treatment with hydrogen peroxide (100  $\mu$ mol/L) in the comet assay.

### Statistical analysis

All statistical analyses had been carried out using SPSS software and the results were described as mean  $\pm$  SD. By using ANOVA test. When the P value was less than 0.05, the difference was considered significant.

### Results and Discussion

Comet assay was used for evaluation of the protective effect of AA against the oxidative damage in DNA induced by Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). Hydrogen peroxide produced oxidative damage in DNA of human lymphocytes. Lymphocytes pretreated without or with antioxidants, incubated at 37°C for 30 minutes., then H<sub>2</sub>O<sub>2</sub> (100 $\mu$ M/L) was added, incubated at 37°C for 60 minutes. The viability of the cells was detected using Trypan blue exclusion method, and the proportion of viable cells was calculated as shown in Table 1.

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**Table1:** Estimation of cellular viability stimulated by H<sub>2</sub>O<sub>2</sub> and protection by using ascorbic acid.

Treatment	Viability %
Lymphocytes alone (10μl)	80±4
Lymphocytes (10μl) + H <sub>2</sub> O <sub>2</sub> (100μM/L)	39±3
Lymphocytes + H <sub>2</sub> O <sub>2</sub> (100μM/L) +50μM/L ascorbic acid	45±5
Lymphocytes + H <sub>2</sub> O <sub>2</sub> (100μM/L) +75μM/L ascorbic acid	55±3
Lymphocytes + H <sub>2</sub> O <sub>2</sub> (100μM/L) +100μ/L ascorbic acid	72±1

The results of the current study indicated that the viability of lymphocytes on pretreatment of H<sub>2</sub>O<sub>2</sub>, simultaneous with a time period examine was skilled. As shown in Table 1, H<sub>2</sub>O<sub>2</sub> was to reduce the viability of the lymphocytes and after incubation for 60 minutes and the viability was 39±3%. In contrast, the viability of the cells increased after the addition of AA at a concentration of 100 μ Mand became 72 ± 1%. These results indicate the efficiency of ascorbic acid as antioxidant as evidenced by its ability to protect lymphocytes against the harmful effect of H<sub>2</sub>O<sub>2</sub>. Consequently, the protective mechanism by AA against the oxidative DNA damage might be due to the ability to scavenge the free radicals especially ROS or hydroxyl radicals. The protective effect of ascorbic acid against pro-oxidants stimulated oxidative DNA damage in lymphocytes became more pronounced at the highest concentration (100μM/L). Our findings disagree with the results of Beevi and his colleagues (20) who reported that H<sub>2</sub>O<sub>2</sub> induced the DNA damage in human and the maximum cell death was observed at a concentration of 25 μM. Ascorbic acid produced dose-dependent reductions in oxidative DNA damage as assessed by Comet assay and the results of the alkaline Comet assay are in Table 2, which shows the percentages of tail length and tail moment for the cells treated with H<sub>2</sub>O<sub>2</sub> alone or treated with H<sub>2</sub>O<sub>2</sub> plus different concentrations of ascorbic acid.

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**Table 2:** Percentage of oxidative DNA damage in human lymphocytes by using comet assay.

Treatment conditions	Percentage of DNA damage (tail length and tail moment)				
	No damage <5%	Low damage 5-25%	Medium damage 25%-50%	High damage 50-75	very High damage >75
100 μmol H <sub>2</sub> O <sub>2</sub> /L (without ascorbic acid)	13± 4.5	19± 3.5	12.2± 2.3	14± 3.2	37± 3.0
10 μmol ascorbic acid /L + 100 μmol H <sub>2</sub> O <sub>2</sub> /L	25± 6.3	20± 3.5	10± 1.5	12± 1.3	29.3± 2.5
25 μmol ascorbic acid /L + 100 μmol H <sub>2</sub> O <sub>2</sub> /L	35± 3.5	21 ± 1.5	8.2±1	11.5± 1.0	24± 2.0
50 μmol ascorbic acid /L + 100 μmol H <sub>2</sub> O <sub>2</sub> /L	45.7± 4.2	21± 2.3	7.5± 1.5	10.8± 2	23.1±2.3
75 μmol ascorbic acid /L + 100 μmol H <sub>2</sub> O <sub>2</sub> /L	64.2±2.3	16± 3.1	6.5±1.3	8± 1.5	16± 1.3
100 μmol ascorbic acid /L + 100 μmol H <sub>2</sub> O <sub>2</sub> /L	70± 1.3	9± 2.3	4.5± 1.6	6± 0.5	10± 1.4

P<0.05 (Significant as compared to untreated)

As it can be seen from Table 2, the Comet tail lengths and tail moments measured in human lymphocyte exposed to hydrogen peroxide at 100μM as a positive control showed that 13± 4.5% of the cells showed no DNA damage while the percentage of DNA damage from low to very high damage were 19± 3.5%, 12.2± 2.3%, 14± 3.2% and 37± 3.0%, respectively. In contrast, lymphocytes treated with 100 μM H<sub>2</sub>O<sub>2</sub> in combination with 10, 25, 75 and 100 μM of AA showed lower in the percentages of DNA damage and they were 9± 2.3%, 4.5± 1.6%, 6± 0.5% and 10± 1.4%, respectively (Table 2). In the present study, we found that vitamin C had significant protective effect against the H<sub>2</sub>O<sub>2</sub> induced genotoxicity. We observed that the human lymphocytes exposed to 100μM of H<sub>2</sub>O<sub>2</sub> had shown increase in comet frequency. The results of the present study confirm that the alkaline comet assay is a highly sensitive technique to detect DNA damage induced by H<sub>2</sub>O<sub>2</sub>. The increase in the comet tail length and tail moment of the H<sub>2</sub>O<sub>2</sub> treated lymphocytes may be caused by DNA strand breaks induction.

Our results disagree with that of Bhat and his colleagues (21) and Harrés and his colleagues (22) who found in their tests that AA is capable of generating large DNA degradation at concentrations of 100 – 200 μM.

In the current study we showed that the comet assay can be used to give reproducible results in estimating the extent of oxidative DNA damage to human lymphocytes. It thus provide a good

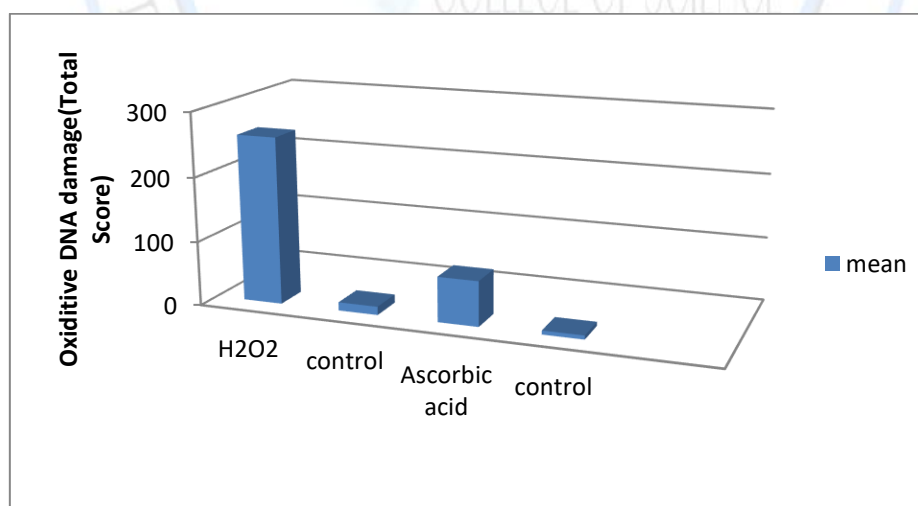


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assay for the determination of the potency of the antioxidant agents tested with high confidence. Several studies have targeted modulating the effect of antioxidant Vitamins, including diet Vit. C on DNA damage (in vivo & in vitro) cells, brought about through the factors which triggered the free radical reactions (23). Nevertheless, in comparison with the positive control, when the lymphocytes were exposed to ascorbic acid in the presence of hydrogen peroxide, we detected a low-level damage of DNA in cells prompted by using H<sub>2</sub>O<sub>2</sub>. In addition, high concentrations of AA resulted in reduced damage of DNA in the tails of comet. However, ascorbic acid has been found to be involved within the Fenton reaction that resulted in OH radical generation, and thus responsible for in vitro oxidative damage of DNA (24).

In another experiment, the antioxidant effect of ascorbic acid was assessed at 100 µmol /L and, the results are shown in Figure 1. Hydrogen peroxide induced 78% DNA damage at concentration (100 µmol/L) without the addition of ascorbic acid, while when human lymphocyte were treated with ascorbic acid, the oxidative DNA damage was reduced by 17% in comparison with control group 0%. Our results showed that the protective effects of ascorbic acid was significant and that the interaction between them was insignificant.



**Figure 1:** Antioxidant activity of Ascorbic acid (100 µmol /L) on human lymphocyte in the comet assay.

Total score: Out of 400, H<sub>2</sub>O<sub>2</sub>: 100 µmol H<sub>2</sub>O<sub>2</sub>/L (without ascorbic acid), Control sample: no H<sub>2</sub>O<sub>2</sub>, Ascorbic acid: 100 µmol ascorbic acid /L + 100 µmol H<sub>2</sub>O<sub>2</sub>/L.

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Our results agree with the results of Lee and colleagues (25) who suggested that ascorbic acid reduced the endogenous level of oxidative damage in DNA of cells and may assist to preclude diseases originated from damage of tissue by free radicals. Even though several reports represent protective action of AA of cells exposed to ROS, reports that mentioned the genotoxic & pro-oxidative action of ascorbic acid cannot be disregarded (26). Commitments of free radicals with deoxyribonucleic acid can stimulate the expansion of single strand breaks. Many studies have been concentration directing the effect of antioxidative operators, for example, an ascorbic corrosive (vitamin C) on the level of DNA damage in cells *in vitro* and *in vivo*, started by parts initiating free-radical reactions (27). Genotoxicity testing (comet assay) gives human a hazard appraisal. An expansion in the genotoxic harm is related with an expanded general danger of malignancy (27, 28). The results of the present investigation demonstrated that the ascorbic corrosive is sufficiently powerful to decrease the genotoxic harm of H<sub>2</sub>O<sub>2</sub> and subsequently lessening the odds of creating growths, as the high level of H<sub>2</sub>O<sub>2</sub> prompts the tumor induction in animals because of the DNA harm (29). Antioxidant vitamins can inert profoundly receptive particles, for example, free radicals, which are produced within different biochemical procedures in the cells (30). Vitamins behave as cell antioxidants and free radical foragers, and consequently act as anticarcinogenic, anticlastogenic and antimutagenic operators. Of these vitamins C and  $\alpha$ -tocopherol are among the best-known cancer prevention agents utilized as *in vivo* animal models (26). Ascorbic acid is a nonenzymatic antioxidant and is in this manner conceivably included in ensuring cells against oxidative stress. Additionally it behaves as a free radical scavenger and its essence helps different systems in diminishing various problematic free radical procedures (31). The co-organization of ascorbic acid with H<sub>2</sub>O<sub>2</sub> diminished lipid peroxidation. The results demonstrate the helpful impacts of ascorbic acid to defeat oxygen dependent cytotoxicity in animals. Despite the fact that the mode of action of ascorbic acid is not completely comprehended, it is trusted that the ascorbic acid act as antioxidant may invigorate the 7- $\alpha$ -hydroxylation of lipids and cholesterol cores in this way improving their corruption to bile acids, which could be discharged from the body (31, 32). Vitamin C has a significant nucleophilic character and it has been proposed that ascorbate may ensure against electrophilic assault on cell DNA and cell layers by catching receptive specialists

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(29) or that ascorbyl anion radical, with the high degree of unpaired electron delocalisation, is in charge of the searching of free radicals or ascorbic acid may focus its role as a chain breaking inhibitor of the peroxidation procedure by rummaging middle person peroxy and alkoxy radical (33, 34).

### Conclusion

The results of the present study confirmed the protective effect of ascorbic acid which is evidenced by its ability to act as an antioxidant and protect the lymphocyte against the was more efficient on oxidants induced lymphocyte oxidative DNA damage, especially at a high concentration (100µM/L).

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**Effect of Ascorbic Acid (Vitamin C) on H<sub>2</sub>O<sub>2</sub> Induced Oxidative DNA Damage in Human Lymphocytes Estimated by Comet Assay**

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