SIMULATION ASSESSMENT OF THE PROTECTIVE ROLE OF VITAMIN C AND E AGAINST CYTOTOXIC EFFECTS OF CYCLOPHOSPHAMIDE IN RATS TONGUE MUCOSA: HISTOLOGICAL AND IMMUNOHISTOCHEMICAL STUDY

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Abstract. Objectives: Oral mucositis was found to be one of the most common side effects of cyclophosphamide use. The present study was designed to evaluate the effectiveness of vitamin C or E as a treatment for the induced cyclophosphamide tongue mucositis. Materials and Methods: Eighty rats were randomly divided into two equal groups: The control group was intraperitoneally injected by physiological saline and were grouped randomly into four equal subgroups: Distilled water, corn oil, vitamin C (12 mg/kg /day), and vitamin E (40mg/kg/day) treatment groups. For induction of mucositis to the study group, a single dose of cyclophosphamide (300 mg/kg) was administered intraperitoneally to each animal, and the animals were also grouped randomly in to four subgroups in the same manner as in the control group. Five of the animals in each group were sacrificed at day four and the other five at day eight and the tongue was dissected for histological and immunohistochemical analysis. Results: In comparison with the cyclophosphamide /water treated group, vitamin C caused a non-significant increase in epithelial thickness, non-significant decrease in damage score and caspase-3 immune expression at day four (p>0.05), but a significant increase in Proliferating Cell Nuclear Antigen (PCNA) immune expression at day four and eight was seen (p<0.05). While vitamin E cause a significant increase in epithelial thickness, a significant decrease in damage score and caspase-3 immune expression at day four, and a significant increase in PCNA immune expression at day four and eight (p<0.05). Conclusion: Vitamin E is better than vitamin C in decreasing the severity of tongue mucositis induced by cyclophosphamide in rats.

1 Introduction

Cyclophosphamide (CPA) was used as a tumor therapy; it is directly targeting on deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) and interferes with DNA replication and RNA transcription [1, 2]. CPA has been as proved to be effective in the treatment of different types of tumor [3]. Oral mucositis is currently considered to be the most severe complication of anticancer therapy [4,5], characterized by erosive, and ulcerative painful lesions in the oral cavity causing difficulty in eating and/or death [6]. In addition, the increased hospitalization causes significant economic problems [7]. The control of oral mucositis and effective intervention is considered a high priority in cancer patient care. The most common previously used types of treatment for oral mucositis were topical antimicrobial agents, supplementary amino acids, cryotherapy, and low-level laser treatment [8].

Vitamin C is a well-known antioxidant, which can protect the body from the damage caused by free radicals and ameliorate the oxidative damage by decreasing lipid peroxidation and altering the antioxidant defense system [9]. While Vitamin E exhibits anti-oxidant and anti-inflammatory effects, preventing the peroxidation of the polyunsaturated lipids in membranes and prevents lipid peroxidation chain reactions in the cell membrane, by interaction with unsaturated fatty acids, and by protecting the polypeptide chains of proteins [10, 11].
was conflicting information on whether vitamin C or E was beneficial for the prevention of oral mucositis, so the aim of the present study was to evaluate the effectiveness of daily intraperitoneal injection of vitamin C (12mg/kg/day) or vitamin E (40 mg/kg/day) as a treatment for the induced cyclophosphamide tongue mucositis caused by intra peritoneal injection of a high single dose of cyclophosphamide (300mg /kg). As variables to evaluate the grade of protection, we used histological and immunohistochemically methods to clarify its effect on cell apoptosis and proliferation.

2 MATERIALS and METHODS

Eighty Wister-albino rats, age about 7-8 weeks and weighing 120-180 g were used in the study, and cared in the animal house of College of Medicine, Hawler Medical University, Erbil, Iraq, under a standard laboratory conditions. Rats were maintained on a 12-hour light/dark cycle at 30± 5°C and 19%-23% humidity. The animals were kept in standard room conditions and fed with a standard rat chow and allowed to drink water ad libitum. The research project was approved by the Research Ethics Committee under protocol. The rats were randomly divided into two main groups:

Control groups (40 animals): All the animals were intraperitoneally injected by physiological saline (0.9% NaCl) in the same manner and dose like CPA, and grouped randomly in to four groups (10 rats each). The treatment by distilled water, corn oil, 12 mg/kg /day of vitamin C [12], or 40mg/kg/day vitamin E [13] were started one day before intra peritoneal injection of physiological saline and for eight days.

Study groups (40 animals): All the animals were intraperitoneally injected by (300 mg/kg) of CPA [14] and grouped randomly in to four groups (10 rats each). The treatment started one day before intra peritoneal injection of CPA and for eight days as in the control groups.

2.1 Tissue staining with hematoxylin and eosin:

Five animals from each group were sacrificed by over dose of anesthesia at day four from the initiation of treatment and the other five at day eight. The tongue of each animal was dissected from the jaw and a cross section of the middle third was taken, fixed, and processed. From each paraffin block, 4µm thickness sections were obtained and mounted on a clean glass slides for routine hematoxylin and eosin staining. The light-microscopic morphometric analysis was assessed by two independent physicians, the thicknesses of all three layers in the epithelium which include stratum basale, stratum spinosum, and stratum granulosum was measured (the major epithelial thickness) in five photograph fields from each tongue mucosal epithelium section by objective micrometer at high power magnification (x400). The analysis for tongue damage scores were carried out among the groups. The damaged areas were evaluated for the following:

- Cell changes in stratified squamous epithelium such as hyperchromasia, pleomorphism, binucleation, and necrosis.
- Degeneration and vacuolar alteration of basal layer.
- Congestion of blood vessels.
- Inflammatory infiltrate in submucosa.

Five regions in the epithelial area and five regions in the connective tissue were selected (linearly adjacent to each other) and preceded with scoring the damage (mucositis) of these separate location. The changes were assessed by one blind evaluator to the type of the sample with scores of 0 to 5. These scores reflect the animals examined as follows; Grade 0 = Normal, Grade 1(minimal) = < 5%, Grade 2(mild) = 6–20%, Grade 3(moderate) = 21–50%, Grade 4(marked) = 51–75%, and Grade 5(severe) = 76–100%. This method was modified from the method proposed by Üçuncu et al [13] in order to assess the degree of tongue mucositis.

2.2 Immunohistochemical analysis using caspase- 3 and PCNA immunolabeling:

The negative control and positive control tissue specimens were run with each batch of stain. Paraffin embedded tonsil for caspase-3 and oral squamous cell carcinoma biopsies cases for PCNA served as positive controls. Caspase-3-labelled cells were identified by brown nuclear and cytoplasmic staining [15]. Positive PCNA expressing cells were identified by brown nuclei. To ensure the objectivity of the analysis, the evaluation was carried out by two independent observers. Eyepiece mounted with counting grids was used for counting.

Five sections in epithelium were randomly chosen for each animal. Approximately 1000 cells from cell population were counted at a magnification of 400x and the percentages of caspase-3 and PCNA positive cells were calculated. The level of caspase-3 and PCNA expression was evaluated according to the scoring system of Seleit et al [16]. The application of this system gives a score ranging from 0 to 3: The percentage of positively stained cells [absent: < 1%], (mild: 1 - 10%), (moderate: >10 - 50%), and (strong: > 50%).

The potential difference among groups for histopathological data was evaluated using ANOVA test. All statistical calculations were done using computer programs SPSS (Statistical Package for the Social Science;
SPSS Inc., version 15). Statistical significance of differences between the groups was tested with Mann Whitney-U test. P value less than or equal to 0.05 was considered statistically significant.

3 Results

3.1 Hematoxylin and eosin results:

At day four and eight, the Saline/Distilled water (figure 1), Saline/ Corn oil, Saline/Vitamin C, and Saline/Vitamin E treated groups showed normal surface of the tongue. Filiform papillae were evenly distributed, regular in size and conical in shape. The fungiform papillae were also revealed normal mushroom shaped and normal covering epithelium.

![Figure 1](image1.png)

At day four, some areas on the dorsal surface of the tongue of CPA/Water and CPA/Oil treated groups showed complete loss of filiform papillae, and when they are present they are short and cone or abnormal in shape. The epithelium and keratin showed a marked decrease in the thickness. Vacuolation of epithelial cells, separation of keratin, and degeneration of fungiform papillae with its taste bud, inflammatory infiltrate and congestion of blood vessels were also seen. At day eight, increase in thickness of epithelium and keratin were seen in comparison with day four, the filiform papillae appear short with flat toped end and covered by irregular keratinized surface. The fungiform papillae appear lined by columnar cells and the propria contain dilated blood vessels and inflammatory infiltrate (figure 2 and figure 3).

At day four, the dorsal surface of tongue of CPA/Vitamin C treated group showed slight increase in the thickness of epithelium in comparison with CPA/Water treated group at this day, the filiform papillae appeared short with flat top end, the fungiform papillae appeared with degenerated taste bud, the connective tissue shows congestion and inflammatory cells infiltration. At day eight, the surface of the tongue was cover by irregular layer of keratin; the filiform papillae appear nearly with tapered end. Histopathological changes in the connective tissue like congestion and inflammatory infiltrate were also seen (figure 4).

At day four, the dorsal surface of tongue of CPA/Vitamin E treated group showed increase in the thickness of epithelium (acanthosis) and keratin layer, with partial restoration of filiform and fungiform papillae shape, the connective tissue shows congestion and inflammatory infiltrate. At day eight, the epithelium also shows acanthosis, and the fungiform papilla with its taste bud appears nearly normal. Vacuolation of epithelial cells is still present, but it is less than that in CPA/Water treated group (figure 5).

![Figure 2](image2.png)
A

B

C

D

Figure 3: Photomicrographs of the dorsal surface of tongue section of CPA/Oil treated group at day four shows loss of papillae, with thin epithelium and keratin (A: H&Ex100), separation of keratin and cellular changes (B: H&Ex400). At day eight, slight increase in thickness of epithelium and keratin layer with elongation of rete ridges are seen (C: H&Ex100). Separation of keratin and epithelial vacuolation are still present (D: H&Ex400).

A

B

C

D

Figure 4: Photomicrographs of the dorsal surface of tongue section of CPA/Vitamin C treated group, at day four shows increase in the thickness of epithelium and keratin layer in comparison with the CPA/Water treated group, the filiform papillae appear short with flat top end, the fungiform papillae appear with degenerated taste bud, the connective tissue shows congestion and inflammatory cells infiltration (A: H&Ex100; B: H&Ex400). At day eight, the surface of the tongue is cover by thick irregular layer of keratin, the filiform papillae appear nearly with tapered end, and vacuolation of epithelial cells, congestion, and inflammatory infiltrate are seen also (C: H&Ex100; D: H&Ex400).

A

B

C

D

Figure 5: Photomicrographs of the dorsal surface of tongue section of CPA/Vitamin E treated group at day four shows increase in the thickness of epithelium and keratin layer, with partial restoration of filiform and fungiform papillae shape, the connective tissue shows congestion and inflammatory infiltrate (A: H&Ex100; B: H&Ex400). At day eight, the epithelium shows acanthosis, and the fungiform papilla with its taste bud appears nearly normal (C: H&Ex100; D: H&Ex400).

3.2 Epithelial thickness:
At day four, the mean epithelial thicknesses in the Saline/Water, Saline/Oil, Saline/Vitamin C, and Saline/Vitamin E treated groups were 48.2±2.63, 49±3.08, 50.6±1.14, and 52.6±2.88 µm respectively. At day eight, the mean epithelial thicknesses in the Saline/Water, Saline/Oil, Saline/Vitamin C, and Saline/Vitamin E treated groups were 48.8±1.30, 51.2 ±2.77, 51.4±2.80, and 52.2±3.56µm respectively. Statistical analysis showed that there were no significant differences (p>0.05) present between Saline/Water treated group and the other three control groups regarding the epithelial thickness at day four (p=0.076) and eight (p=0.268), so Saline/Water treated group was considered the reference one for the control groups at day four and eight.

At day four, the mean epithelial thicknesses in the CPA/Water and CPA/Oil treated groups were 20.8 ± 1.30 and 22.3 ± 1.92µm respectively. At day eight, the mean epithelial thicknesses in the CPA/Water and CPA/Oil treated groups were 29.4±2.50 and 31.3±3.03 µm respectively. Statistical analysis showed that there were no significant differences (p>0.05) present between them at day four (0.468) and eight (p=0.718), so CPA/Water treated group was considered the reference one for these two study groups at day four and eight.

The results showed that there were significant differences in epithelial thickness (P<0.05) present between the Saline/Water and CPA/Water, CPA/Vitamin C or CPA/Vitamin E treated groups at day four and eight respectively (table 1). At day four and eight, the results showed that there were no significant differences
(P>0.05) present between CPA/Water and CPA/Vitamin C treated groups, but it the relation was significant (p<0.05) with CPA/Vitamin E treated group at these days (p<0.05) as seen in (table 2). At day four and eight, comparison of mean epithelial thickness between CPA/Vitamin C and CPA/Vitamin E treated groups also showed significant differences (p<0.05) as seen in (table 3).

Table 1: Comparison of mean epithelial thickness (µm) between the Saline/Water treated group and the CPA/Water, CPA/Vitamin C, or CPA/Vitamin E treated groups in rat tongue at different experimental duration.

| Duration | Saline/Water | CPA/Water | CPA/Vitamin C | CPA/Vitamin E | P-value |
|----------|--------------|-----------|---------------|---------------|---------|
| Day 4    | 48.2±2.63    | 20.8 ± 1.30| 23.4±2.58     | 36.2±2.94     | *0.0120 <0.05 S |
|          |              |           |               |               | **0.0120 <0.05 S |
|          |              |           |               |               | ***0.0120 <0.05 S |
| Day 8    | 48.8±1.30    | 29.4±2.50 | 32.002±0.67   | 42.2±1.16     | *0.0120 <0.05 S |
|          |              |           |               |               | **0.0120 <0.05 S |
|          |              |           |               |               | ***0.0366 <0.05 S |

X: Mean. SD: Standard deviation. S: Significant.
* Comparison between Saline/Water and CPA/Water.
** Comparison between Saline/Water and CPA/Vitamin C.
*** Comparison between Saline/Water and CPA/Vitamin E.

Table 2: Comparison of mean epithelial thickness (µm) between CPA/Water treated group and the CPA/Vitamin C or CPA/Vitamin E treated groups in rat tongue at different experimental duration.

| Duration | CPA/Water | CPA/Vitamin C | CPA/Vitamin E | P-value |
|----------|-----------|---------------|---------------|---------|
| Day 4    | 20.8 ± 1.30| 23.4±2.58     | 36.2±2.94     | *0.1443 >0.05 NS |
|          |           |               |               | **0.0120 <0.05 S |
| Day 8    | 29.4±2.50 | 32.002±0.67   | 42.2±1.16     | *0.116 >0.05NS |
|          |           |               |               | **0.0120 <0.05 S |

X: Mean. SD: Standard deviation. NS: Non-significant. S: Significant.
* Comparison between CPA/Water and CPA/Vitamin C.
** Comparison between CPA/Water and CPA/Vitamin E.

Table 3: Comparison of mean epithelial thickness (µm) between CPA/Vitamin C and the CPA/Vitamin E treated groups in rat tongue at different experimental duration.

| Duration | CPA/Vitamin C | CPA/Vitamin E | P-value |
|----------|---------------|---------------|---------|
| Day 4    | 23.4±2.58     | 36.2±2.94     | 0.0120 *<0.05 S |
| Day 8    | 32.002±0.67   | 42.2±1.16     | 0.0120 *<0.05 S |

X: Mean. SD: Standard deviation. S: Significant.
* Comparison between CPA/Vitamin C and CPA/Vitamin E.

3.3 Damage score:
At day four, the mean damage scores in the Saline/Water, Saline/Oil, Saline/Vitamin C, and Saline/Vitamin E treated groups were 0.23±0.14, 0.21±0.08, 0.4±0.19, and 0.28±0.88 respectively. At day eight, the mean damage scores in the Saline/Water, Saline/Oil, Saline/Vitamin C, and Saline/Vitamin E treated groups were 0.21±0.01, 0.19 ±0.77, 0.49±0.71, and 0.32±0.56 respectively. Statistical analysis showed that there were no significant differences (p>0.05) present between Saline/Water treated group and the other three control groups regarding the epithelial thickness at day four (p=0.927) and eight (p=0.847), so Saline/Water treated group was considered the reference one for the control groups at day four and eight.

At day four, the mean damage scores in the CPA/Water and CPA/Oil treated groups were 3.68 ± 0.31 and 3.12 ± 0.92 respectively. Statistical analysis showed that there were no significant differences present between them at day four (p=0.278) and eight (p=0.278). At day eight, the mean damage score in the CPA/Water and CPA/Oil were 2.44±0.380 and 2.34 ±0.21 respectively. Statistical analysis showed that there were no significant
differences (p>0.05) present between them (p=0.277), so CPA/Water treated group was considered the reference one for these two study groups at day four and eight.

The results also showed that there were significant differences in damage scores (P<0.05) present between the Saline/Water and the CPA/Water, CPA/ Vitamin C or CPA/ Vitamin E treated groups at day four and eight (table 4).

At day four and eight, there were no significant difference in damage scores (P>0.05) present between CPA/Water and CPA/Vitamin C, but it was significant with CPA/Vitamin E treated groups (p<0.05) at these days as seen in (table 5). At day four and eight, comparison of mean damage score between CPA/Vitamin C and CPA/Vitamin E treated groups showed significant differences(p<0.05) as seen in (Table -6).

Table 4: Comparison of mean damage scores between Saline/Water treated group and the CPA/Water, CPA/Vitamin C, or CPA/Vitamin E treated groups in rat tongue at different experimental duration.

| Duration | Saline/Water X±SD | CPA/Water X±SD | CPA/Vitamin C X±SD | CPA/Vitamin E X±SD | P- value |
|----------|-------------------|----------------|-------------------|-------------------|---------|
| Day 4    | 0.23±0.14         | 3.68 ± 0.31    | 3.2±0.26          | 2.52±0.81         | *0.0120 <0.05 S |
|          |                   | **0.0120 <0.05 S | ***0.0120 <0.05 S |                   |         |
| Day 8    | 0.21±0.01         | 2.44±0.380     | 2.093±0.203       | 1.52±0.24         | *0.0120 <0.05 S |
|          |                   | **0.0120 <0.05 S | ***0.0120 <0.05 S |                   |         |

Table 5: Comparison of mean damage scores between CPA/Water treated group and the CPA/Vitamin C or CPA/Vitamin E treated groups in rat tongue at different experimental duration.

| Duration | CPA/Water X±SD | CPA/Vitamin C X±SD | CPA/Vitamin E X±SD | P- value |
|----------|----------------|-------------------|-------------------|---------|
| Day 4    | 3.68 ± 0.31    | 3.2±0.26          | 2.52±0.81         | *0.0522 >0.05 NS  |
|          | **0.0120 <0.05 S |                   |                   |         |
| Day 8    | 2.44±0.380     | 2.093±0.203       | 1.52±0.24         | *0.094 >0.05 NS   |
|          | **0.0120 <0.05 S |                   |                   |         |

Table 6: Comparison of mean damage score between CPA/Vitamin C with CPA/Vitamin E treated groups in rat tongue at different experimental duration.

| Duration | CPA/Vitamin C X±SD | CPA/Vitamin E X±SD | P- value |
|----------|-------------------|-------------------|---------|
| Day 4    | 3.2±0.26          | 2.52±0.81         | *<0.05 S |
|          |                   | 0.0164            |         |
| Day 8    | 2.093±0.203       | 1.52±0.24         | 0.0120 *<0.05 S |
|          |                   |                   |         |

3.4 Immunohistochemical result:
Caspase-3 immune expression: All sections in the control groups appear negatively stained for caspase-3 at day four and eight and were almost absent, especially in Saline/Water group (figure 6). The CPA/Water,

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CPA/Oil and CPA/Vitamin C groups appear mildly stained at day four, but the CPA/Vitamin E treated group appeared negatively stained at the same day (figure 7). All the study groups appeared negative at day eight.

The mean caspase-3 immune expressions in the CPA/Water, CPA/Oil and CPA/Vitamin C at day four were 9.12±0.30, 8.79±0.59, and 8.56±0.38 respectively. Statistical analysis of these three groups, at day four, showed that there were no significant differences (p>0.05) present between them (p=0.0621). So CPA/Water treated group was considered the reference one for these groups at day four. Statistical analysis showed significant difference present between CPA/Water and CPA/Vitamin E treated group, at day four (p=0.0120) and eight (p=0.0120) respectively (table 7).

Figure 6: Immunohistochemical result for caspase -3 in the epithelium of the control groups: A1 and A2: Saline/Water treated group at day four and eight respectively showing negative immune expression of caspase-3 (A: Immunohistochemistry x100: B: Immunohistochemistry x400).

Figure 7: Immunohistochemical result for caspase -3 in the epithelium of the study groups: CPA/Water treated group showing mild positive (A1) and negative (A2) immune expression of caspase-3 at day four and eight respectively (Immunohistochemistry x400). CPA/Oil treated group showing mild positive (B1) and negative (B2) immune expression of caspase-3 at day four and eight respectively (Immunohistochemistry x400). CPA/Vitamin C treated group showing mild positive (C1) and negative (C2) immune expression of caspase-3 at
day four and eight respectively (Immunohistochemistry x400). CPA/Vitamin E treated group showing negative immune expression of caspase-3 at day four (D1) and eight (D2) respectively (Immunohistochemistry x400).

Table 7: Comparison in caspase-3 immune expression between CPA/Water treated group and CPA/Vitamin E treated groups in rat tongue at different experimental duration.

| Duration | CPA/Water X±SD | CPA/Vitamin E X±SD | P-value |
|----------|----------------|--------------------|---------|
| Day 4    | 9.12±0.30      | 0.92±0.173         | *0.0120 <0.05 S |
| Day 8    | 0.97±0.035     | 0.29±0.064         | *0.0120 <0.05 S |

X: Mean. SD: Standard deviation. S: Significant. NS: Non significant
* Comparison between CPA/Water and CPA/Vitamin E.

3.5 PCNA immune expression
The effect of vitamin C as well as vitamin E treatment on cell proliferation was evaluated using PCNA. figure 8 shows that all sections in the control groups for Saline/Water, Saline/Oil, Saline/Vitamin C, and Saline/ Vitamin E appear moderately stained for PCNA at day four and eight. The positive cells appear in the basal and supra basal cells layers. The mean PCNA immune expressions of these groups at day four were (20.10±0.31, 19.57±0.12, 18.99±0.81, 19.14±0.58 respectively), but at day eight, it was (19.65± 0.40, 20.05±0.65, 18.92±0.62, 19.48±0.37 respectively). Statistical analysis showed non-significant differences (P>0.05) present between them regarding the immune expression of PCNA at day four (p=0.018) and eight (p=0.027). So Saline/Water treated group was considered the reference one for these groups at day four and eight.

In the study groups (figure 9), the mean PCNA immune expressions in the CPA/Water and CPA/Oil at day four were 15.17±0.40 and 15.29±0.522 respectively. At day eight the mean PCNA immune expressions in the CPA/Water and CPA/Oil were 17.29±1.15 and 17.83±0.096 respectively. Statistical analysis showed non-significant differences (P>0.05) present between them regarding the immune expression of PCNA at day four (p=0.0601) and eight (p=0.144). So CPA/Water treated group was considered the reference one for these groups at day four and eight.

The results showed that there were significant differences in mean PCNA immune expression (P<0.05) present between the Saline/Water and CPA/Water, CPA/ Vitamin C or CPA/ Vitamin E treated groups at day four. The results also showed that there were significant differences (P<0.05) present between the Saline/Water and CPA/Water or CPA/ Vitamin C treated group, but the relation was non-significant with the CPA/ Vitamin E treated groups at day eight (Table -8).

At day four and eight, there were significant difference in mean PCNA immune expression (P<0.05) present between the CPA/Water and CPA/Vitamin C, CPA/Water and CPA/Vitamin E treated groups as seen in (table 9).

At day four and eight, comparison of mean PCNA immune expression between CPA/Vitamin C and CPA/Vitamin E treated groups showed significant difference (p<0.05) as seen in (table 10).
Figure 8: Immunohistochemical result for PCNA in the epithelium of the control groups: A1 and A2: Saline/Water treated group at day four and eight respectively showing moderate immune expression of PCNA (A: Immunohistochemistry x100; B: Immunohistochemistry x400). B1 and B2: Saline/Oil treated group at day four and eight respectively showing moderate immune expression of PCNA (Immunohistochemistry x400). C1 and C2: Saline/Vitamin C treated group at day four and eight respectively showing moderate immune expression of PCNA (Immunohistochemistry x400). D1 and D2: Saline/Vitamin E treated group at day four and eight respectively showing moderate immune expression of PCNA (Immunohistochemistry x400). In all sections the positive cells appear in basal and supra basal cells layer.
Figure 9: Immunohistochemical result for PCNA in the epithelium of the study groups: CPA/Water treated group at day four and eight respectively showing moderate immune expression of PCNA (A1: Immunohistochemistry x400; A2: Immunohistochemistry x100). CPA/Oil treated group at day four and eight respectively showing moderate immune expression of PCNA (B1, Immunohistochemistry x400; B2 Immunohistochemistry x100). In A1 and B1, arrows show some negative cells. CPA/Vitamin C treated group at day four and eight respectively showing moderate immune expression of PCNA (C1, Immunohistochemistry x40; C2, Immunohistochemistry x100). CPA/Vitamin E treated group at day four and eight respectively showing moderate immune expression of PCNA (Immunohistochemistry x100). In C2, D1, and D2, the positive cells appear in basal and suprabasal cells layer.

Table 8: Comparison of mean PCNA immune expression between CPA/Water treated group and the CPA/Vitamin C or CPA/Vitamin E treated groups in rat tongue at different experimental duration.

| Duration | CPA/Water X±SD | CPA/Vitamin C X±SD | CPA/Vitamin E X±SD | P-value |
|----------|----------------|--------------------|--------------------|---------|
| Day 4    | 15.17± 0.40    | 17.35± 0.31        | 18.76±0.26         | *0.0120 <0.05 S |
|          |                |                    |                    | **0.0120 <0.05 S |
| Day 8    | 17.64±0.21     | 18.59±0.23         | 19.37±0.18         | *0.0120 <0.05 S |
|          |                |                    |                    | **0.008 <0.05 S |

X: Mean. SD: Standard deviation. S: Significant. NS: Non significant
* Comparison between CPA/Water and CPA/Vitamin C.
** Comparison between CPA/Water and CPA/Vitamin E.

Table 9: Comparison of mean PCNA immune expression between CPA/Vitamin C with CPA/Vitamin E treated groups in rat tongue at different experimental duration.

| Duration | CPA/Vitamin C X±SD | CPA/Vitamin E X±SD | P-value |
|----------|--------------------|--------------------|---------|
| Day 4    | 17.35±0.31         | 18.76±0.26         | 0.0120 *<0.05 S |
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4 Discussion

In the present study, statistical analysis of the data collected at day four and eight, showed that there were no significant differences (p>0.05) present between the Saline/Water treated group and the other three control groups regarding the epithelial thickness, damage scores, and rate of proliferation. In addition, all these groups appear negative for caspase -3 at day four and eight. Other studies found that vitamin C affect the tumor cells but not the normal cell [17], and vitamin E toxicity has rarely been documented in humans [18].

The present study showed that the dorsal surface of the tongue in the CPA/Water treated group showed significant decrease in epithelial thickness, significant increase in damage scores. Zhao et al [19] found the same changes in the tongue of mice, but following radiation. The significantly higher histopathological score is due to the increases in the release of proinflammatory cytokines which cause tissue damage and inflammatory response resulting in increased subepithelial vascularity [20]. Cawley and Benson [21] found that chemotherapy generates ROS which are deleterious to the DNA of epithelial cells. The present study also showed significant increase in caspase-3 immune expression and significant decrease in PCNA immune expression in comparison with the Saline/Water treated group. Cytokines released from keratinocytes, endothelial cells and the cells of the lamina propria enhance cell damage; concomitantly, chemotherapeutic drugs activate enzymes that increase apoptosis [22]. Aboushady et al [23] found obvious reduction in PCNA expression in tongue epithelia in irradiated rats. The histopathological changes in the present study seen decreased at day eight. Jezernik et al [24] found that the toxicity of CPA regarding the increased apoptosis and decreased proliferation decreased gradually after 24 hour from intraperitoneal injection, and these actions were decreased with CPA-treated days.

Even the statistical difference was not significant; the result showed that vitamin C can decrease the severity of oral mucositis. Vitamin C can repair the normal connective tissue, and accelerate the healing of wounds, can promote resistance to infections, and has anti-inflammatory effect [24]. Vitamin C has been shown to impede the release of C-reactive proteins, IL-6, TNF-α, and ROS from several types of cells involved in inflammation [25].

The simultaneous treatment with vitamin E in the group receiving vitamin E and CPA, greatly compensated the harmful effects of CPA on tongue tissue. Previous studies showed that vitamin E as an antioxidant can reduce ROS levels [27]. Singh et al [28] also found that vitamin E, has been shown to decrease the release of proinflammatory cytokines, the chemokine IL-8 and plasminogen activator inhibitor-1 levels. Haendeler et al [29] found that vitamin E can increase Bcl-2 immune expression. The vitamin E anti-apoptotic effect could be related to its antioxidant effect since oxidative stress is one of the main triggers of apoptosis [30]. Furthermore, vitamin E was reported to have the ability to repair DNA damage [31]. Vitamin E was also shown to inhibit Protein Kinase B and activate protein tyrosine phosphatase, both altering cell proliferation and survival [32].

In conclusion, the result showed that vitamin E can significantly ameliorate the cytotoxic effect of CPA.

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| Duration | CPA/Vitamin C | CPA/Vitamin E | P-value |
|----------|--------------|--------------|---------|
|          | X±SD         | X±SD         |         |
| Day 4    | 17.35±0.31   | 18.76±0.26   | 0.0120  | *<0.05 S |
| Day 8    | 18.59±0.23   | 19.37±0.18   | 0.008   | *<0.05 S |

X: Mean. SD: Standard deviation. S: Significant.
* Comparison between CPA/Vitamin C and CPA/Vitamin E.
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