The Effect of Grape Seed Extract and Cetuximab Drug on Mucositis of Experimental Rats

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Abstract

Objectives: Cetuximab is an anticancerous drug which causes oral mucositis as a major side effect. Oral mucositis can be reduced by the effect of grape seed extract which modulates the inflammatory pathways. Our aim is to evaluate the effect of Grape seed extract and Cetuximab drug on mucositis and bacterial infection in tongue mucosa of experimental rats. Design: 24 rats were housed in the animal house and divided into 4 groups receiving Cetuximab alone, Cetuximab and Grape seed extract together or Grape seed extract one week ahead of Cetuximab drug and compared with the control group which received no treatment. After sacrifice the tongue was dissected and paraffin blocks were prepared. Tissue sections were stained by routine hematoxylin and eosin stain for examination by the light microscope. Tissue blocks for scanning electron microscopic examination were also prepared. Results: The mean number of inflammatory cells decreased in rats receiving Grape seed extract and Cetuximab especially if Grape seed extract was given before Cetuximab. Scanning electron microscopic examination revealed atrophy of filliform papillae and many bacterial colonies in rats taking Cetuximab alone, which decreased after adding Grape seed extract to Cetuximab drug. Conclusion: Grape seed extract reduced oral mucositis and bacterial infection caused by Cetuximab drug.

Key Words: Grape seed extract, Cetuximab, Oral mucositis, Scanning electron microscopy, Bacterial colonization

Introduction

Cetuximab is an anticancerous drug that is directed against the [1]. Cetuximab binds with high affinity to the extracellular domain of epidermal growth factor receptor competing with its natural ligands, thus preventing the activation of epidermal growth factor receptor and inhibiting cancer [2].

Anticancerous agents play a role in reducing mortality and increasing the quality of life for cancer patients [3]. However, chemotherapy destroys rapidly dividing cells, thus the general side effects of it include bone marrow suppression,varying degrees of cytopenias and gastrointestinal tract problems [4]. Severe oral mucositis has also been reported from chemotherapy and leads to unplanned stoppage of the therapy [5]. Myelosuppression and disturbance in the oral flora contribute to Oral mucositis [6].

Grape is a natural source of polyphenols with exceptional biological activities which includes antioxidant, anti-inflammatory and antimicrobial properties [7]. It has been shown that Grape seeds extract is a mixture of polyphenolic components, but proanthocyanidins are considered to be a major fraction of Grape seeds extract [8].

Grape seeds extract decreases inflammation either by modulation of inflammatory pathways or by reducing Reactive oxygen species levels. As a natural compound, it can target multiple pathways to overcome chronic inflammation so it is more effective than mono-targeted anti-inflammatory drugs [9]. The mechanism of anti-inflammation of procyanidins is by inhibition of release of proinflammatory factors [10]. It has been demonstrated that the high anti-inflammatory action of proanthocyanidins is mediated by their ability to scavenge free radicals thus inhibiting formation of pro-inflammatory cytokines. [11]. Grape seeds extract exhibited some kind of antibacterial activity especially against the Gram-positive strain which is more sensitive than the Gram negative strain as the less complex structure of the cell wall in the Gram-positive bacteria makes it more permeable to the antimicrobial compounds [12].

Materials and Methods

The Cetuximab drug (Erbutix 5 mg/ml solution of infusion) was purchased from Merck Company (Kenil Worth, U.S.A) and was administrated by an intraperitoneal injection every 3 days for 5 total injections at a dose level of 0.25 mg/injection [13]. Grape seeds extract pure powder was purchased from Bulk Supplements Company (Henderson, U.S.A) and was given orally by a gastric tube at a daily dose of 20 mg/day till the sacrifice [14].

Twenty four adult male rats of weight ranging from 150-200 gms were divided into four groups (*Table 1*). Rats were housed under a controlled environment (temperature 25 ± 2°C and 12 hr dark/light cycles) in stainless-steel cages and were maintained on regular rat chow and distilled water. The rats were housed in the animal house of the Faculty of Medicine, Cairo University, Egypt according to the recommendations and approval of the Ethics Committee on Animals Experimentation.

Rats were divided into four groups: Group A represents the control group that received no treatment, Group B represents rats taking Cetuximab drug alone, Group C represents rats taking Cetuximab drug with Grape seed extract in parallel and Group D represents rats taking Grape seed extract one week ahead of Cetuximab (*Table 1*). The rats were sacrificed on day 16 from the start of Cetuximab administration (6 rats in each group) by an overdose of the anesthetic solution (1 ml/100 gm) according to the Research Animal Guidelines of Euthanasia.

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Table 1. Showing study design and animal grouping.

	Group A (Control Group)	Group B (CMAB Drug Group)	Group C (CMAB + GSE Group)	Group D (GSE one Week Ahead of CMAB Drug Group)
Number of rats	12	12	12	12
Cetuximab dose and route	No	0.25 mg/i.p Injection	0.25 mg/i.p Injection	0.25 mg/i.p Injection
Grape Seed Extract dose and route	No	No	20 mg/day orally	20 mg/day orally
Sacrifice date	Day 16	Day 16	Day 16	Day 16

Tissue preparation for hematoxylin and eosin (H&E) stained sections

Tongue specimens were fixed in 10% buffered formalin for 24 hours, dehydrated in ascending grades of ethyl alcohol (70%, 80%, 90% and 100%), cleared in xylene and embedded in paraffin. Sections of 5 microns thickness were cut from paraffin blocks and mounted on glass slides for H&E staining and subsequent examination under the ordinary light microscope.

The Computer image analyzer system, Leica Qwin 500 software (Germany) was used in counting the number of inflammatory cells in each field. Three fields from each slide were chosen in a standard measuring frame using a magnification of x400 by light microscopy transferred to the monitor's screen. The number of the inflammatory cells was described as mean values ± standard deviation (± SD). One way analysis of variance (ANOVA) test was used to compare between the studied groups. It was followed by Tukey Post Hoc multiple 2-group comparisons. The significance level was set at $p<0.05$.

Tissue preparation for scanning electron microscope examination

The dissected tongue was treated with a solution of glutraldehyde (2.5%), alcohol dehydrated and air dried at 37°C [15]. The specimen was the subjected to gold-palladium sputting for Scanning electron microscope analysis. Specimens were examined using QUANTA FEG 250 Scanning electron microscopy (U.S.A). Specimens were examined under magnification power (x800) for detecting fine surface details and changes in tongue papillae.

Results

Examination of H&E stained sections

In group A, examination revealed that normal tongue mucosa is covered by keratinized stratified squamous epithelium. Normal filliform papillae could be detected. The underlying connective tissue stroma was composed of collagen fibers, fibroblasts and few blood vessels.

In group B, examination showed atrophy of the filliform papillae in large areas with focal areas of complete loss. The connective tissue showed an intense inflammatory cell infiltrate and numerous dilated blood vessels (*Figure 1*).

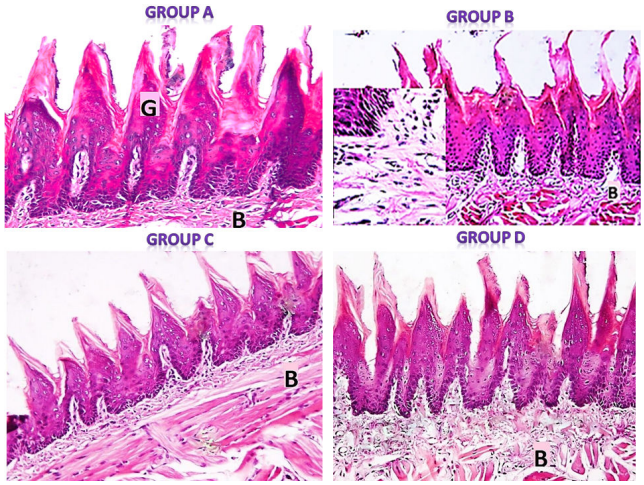

Figure 1. *Photomicrograph of H&E stained sections of: Group A showing normal tongue mucosa showing giant conical (G) filliform papillae and few blood vessels in the connective tissue (B) (H&E x200). Group B showing atrophy and loss of the tongue papillae and chronic inflammatory cells and numerous dilated blood vessels (B) in the connective tissue (H&E x200). A higher magnification of inflammatory cells is seen in the inset (H&E x400). Group C showing atrophy of few papillae and sporadic inflammatory cells and few dilated blood vessels (B) in the connective tissue (H&E x200). Group D showing slight atrophy of the filliform papillae, few inflammatory cells and few dilated blood vessels (B) (H&E x200).*

In group C, examination showed atrophy of few tongue papillae. Sporadic inflammatory cells and some dilated blood vessels were seen in the connective tissue. In group D, examination showed slight atrophy of filliform papillae and few chronic inflammatory cells infiltrating the connective tissue as well as few dilated blood vessels (*Figure 1*).

Scanning electron microscopy (SEM) examination

Group A revealed normal filliform papillae having uniform thickness and length. Group B showed severe destruction and loss of tongue papillae in many areas. Damage of the tissues in between the papillae was noticed. Colonization with large numbers of microorganisms was detected in many areas (*Figure 2*).

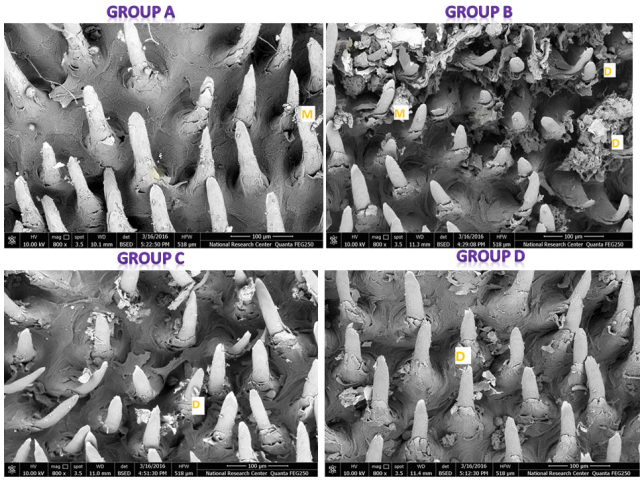

Figure 2. *Scanning electron micrograph of: Group A showing regular arranged filliform papillae and few microorganisms (M) (x800). Group B showing severe destruction of filliform papillae in many areas (D). Colonies of microorganisms (M) can be detected between the destructed tissue (x800). Group C showing irregularity of tongue surface with moderate destruction (D) of filliform papillae in some areas (x800). Group D showing slight destruction (D) of filliform papillae in many areas(x800).*

Group C showed moderated destruction of tongue papillae in some areas and maintaining normal tongue surface architecture in small areas. In comparison to group B, smaller and less frequent colonies of microorganisms were observed in the destructed tissue. Group D revealed slight destruction of tongue papillae in fewer areas in comparison to group B, with maintaining of normal tongue architecture in the other areas. Small sporadic colonies of microorganisms were also detected (*Figure 2*).

Table 2. **Significant at p<0.0001, all groups are statistically significant (ANOVA test). Means with same superscript letters are not significant different as groups (B&C), (C&D). Means with different superscript letters are significantly different. As groups (A&B&D) (Tukey's post hoc test).*

	Group A	Group B	Group C	Group D
Mean	3.33^{c}	14.83^{a}	$11.58^{a,b}$	9.5^{b}
SD	0.98	4.35	2.41	1.87
Min	2.5	9	9	7
Max	5	20	16	12
F Value	19.301			
P Value	<0.0001*			

Statistical analysis

The greatest mean number of inflammatory cells was recorded in group B (14.83 ± 4.35) followed by group C (11.58 ± 2.41), then group D (9.5 ± 1.87), with the least value recorded in the control group A (3.33 ± 0.98). ANOVA test revealed that the difference between the groups was statistically significant (P<0.0001). Tukey's post hoc test for pairwise comparison revealed a significant difference between groups B&D but no significant difference between groups (B&C) and (C&D) (*Table 2*) (*Figure 3*).

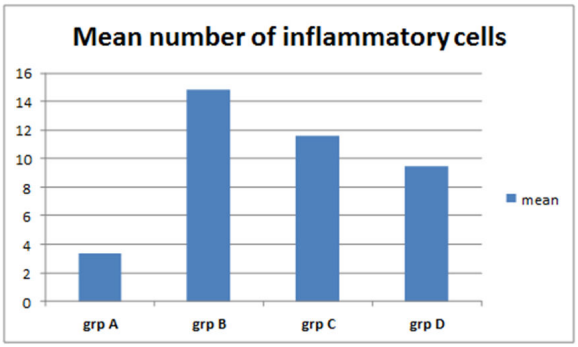

Figure 3. *Column chart showing mean number of inflammatory cells in the studied groups.*

Discussion

In this study, the Cetuximab drug was administrated by intraperitoneal injection by infusion of the drug directly into the peritoneal cavity, as this method is documented by Jaaback et al. [16] while Grape seed extract is stable in acid base environment so oral administration is preferable [17].

In this study, a significant decrease in the mean number of inflammatory cells was noticed in the rats receiving Grape seed extract a week ahead of Cetuximab compared to those taking Cetuximab alone. As mentioned by Cheah et al. [18], Grape seed extract has the ability to prevent (Nuclear factor kappa B) activation and subsequently reduce the activation of nitric oxide and pro-inflammatory cytokines. These results confirm the capability of Grape seed extract to reduce oral mucositis. This is in accordance with Cheah et al. [19] who noted reduction of experimentally induced inflammation in colon and small intestine of rats after taking of Grape seed extract.

Similar findings to our study were previously reported by Olaku et al. [20] who mentioned the effect of Grape seed extract in reducing mucositis caused by chemotherapy. On the other hand, Worthington et al. [21] concluded a weak benefit from using the cytoprotective agent (Amifostine) to prevent mucositis before and during the chemotherapy. These conflicting findings could be attributed to the differences in the dose, duration, chemical structures and mode of administration of the used drugs.

In this experiment, filliform papillae were chosen for examination since they are widely distributed on the dorsal surface of the tongue and undergo changes such as loss and atrophy faster and earlier than other papillae. This is in accordance with Abayomi et al. [22] who stated that the filliform papillae are of high metabolic activity, so any enzymatic disturbance or drug toxicity may result in their atrophy.

In this work, H&E stained sections of tongue mucosa showed filiform papillae atrophy in large areas and complete loss in focal areas in rats receiving Cetuximab alone. This was confirmed in a previous study by AL-Azri et al. [23] who explained the cytotoxic effect of chemotherapy on the rapidly dividing basal cells leading to reduced thickness of the epithelium (atrophy).

Atrophy of sporadic filiform papillae was detected in rats taking Cetuximab and Grape seeds extract in parallel. This could be attributed to the ability of Grape seed extract to decrease Cetuximab induced toxicity on the filiform papillae. This is in accordance with Cheah et al. [24] who reported a reduction in intestinal damage induced by 5-fluorouracil in animals receiving Grape seed extract. Minimal atrophy of papillae was observed in rats receiving Grape seeds extract one week ahead of Cetuximab and this suggests that the use of Grape seeds extract before chemotherapy had a better effect than if both drugs were given in parallel.

In this context, Scanning electron microscope was used to detect surface changes of the tongue. Rats which received Cetuximab only showed severe loss and destruction of filiform papillae in many areas, damage of the tissues in between the papillae, as well as colonies of microorganisms. These changes illustrated the adverse effects of Cetuximab administration. This is also in accordance with AL-Refai et al. [25] who reported mucotoxic effects of chemotherapy on the oral mucosa. Colonies of microorganisms detected in this group may be attributed to the effect of Cetuximab in inhibition of the immune system of the body thus enhancing bacterial infections [26].

Rats taking Grape seeds extract in parallel with Cetuximab showed a milder effect appearing as moderate destruction of tongue papillae in some areas with fewer colonies of microorganisms. However, taking Grape seeds extract one week ahead of Cetuximab showed the best results. This confirms the benefits of using Grape seeds extract before starting the anticancerous treatment [26].

Conclusion

Grape seeds extract is a potent neutraceutical agent capable of decreasing inflammation, bacterial infection and filliform papillae damage in tongue mucosa of rats. Therefore, daily administration of Grape seeds extract is advised especially with anti-cancer treatment. Administration of Grape seeds extract one week ahead of chemotherapy had a better effect.

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Conflict of Interest

None.

References

1. Kang SP, Saif MW. Infusion-related and hypersensitivity reactions of monoclonal antibodies used to treat colorectal cancer-identification, prevention, and management. *The Journal of Supportive Oncology.* 2007; **5**: 451-457.

2. Egloff AM, Grandis J. Epidermal growth factor receptor–targeted molecular therapeutics for head and neck squamous cell carcinoma. *Expert Opinion on Therapeutic Targets.* 2006; **10**: 639-647.

3. Suggitt M, Bibby MC. 50 years of preclinical anticancer drug screening: empirical to target-driven approaches. *Clinical Cancer Research.* 2005; **11**: 971-981.

4. Withrow SJ, Vail DM, Page R (2007) Cancer of the gastrointestinal tract: Small Animal Oncology. 2007; Saunders Elsevier, Philadelphia, London, Toranto.

5. Villa A, Sonis ST. Mucositis: pathobiology and management. *Current Opinion in Oncology.* 2015; **27**: 159-164.

6. Viet CT, Corby PM, Akinwande A, Schmidt BL. Review of preclinical studies on treatment of mucositis and associated pain. *Journal of Dental Research.* 2014; **93**: 868-875.

7. Ganapathy M, Bhunia S. Nutraceuticals: The new generation therapeutics. *Advanced Techniques in Biology & Medicine.* 2016; **4**: 1-5.

8. Nandakumar V, Singh T, Katiyar SK. Multi-targeted prevention and therapy of cancer by proanthocyanidins. *Cancer Letters.* 2008; **269**: 378-387.

9. Sung B, Prasad S, Gupta SC, Patchva S, Aggarwal BB (2012) Regulation of inflammation-mediated chronic diseases by botanicals. Academic Press: Oxford, UK.

10. Panico AM, Cardile V, Avondo S, Garufi F, Gentile B. The in vitro effect of a lyophilized extract of wine obtained from Jacquez grapes on human chondrocytes. *Phytomedicine.* 2006; **13**: 522-526.

11. Georgiev V, Ananga A, Tsolova V. Recent advances and uses of grape flavonoids as nutraceuticals. *Nutrients.* 2014; **6**: 391-415.

12. Papadopoulou C, Soulti K, Roussis IG. Potential antimicrobial activity of red and white wine phenolic extracts against strains of Staphylococcus aureus, Escherichia coli and Candida albicans. *Food Technology and Biotechnology.* 2005; **43**: 41-46.

13. Luo FR, Yang Z, Dong H, Camuso A, McGlinchey K, et al. Correlation of pharmacokinetics with the antitumor activity of Cetuximab in nude mice bearing the GEO human colon carcinoma xenograft. *Cancer Chemotherapy and Pharmacology.* 2005; **56**: 455-464.

14. Banerjee B, Bagchi D. Beneficial effects of a novel IH636 grape seed proanthocyanidin extract in the treatment of chronic pancreatitis. *Digestion.* 2001; **63**: 203-206.

15. Li H, Fan H, Wang Z, Zheng J, Cao W. Potentiation of scutellarin on human tongue carcinoma xenograft by low-intensity ultrasound. *PloS one.* 2013; **8**: e59473.

16. Jaaback K, Johnson N, Lawrie TA. Intraperitoneal chemotherapy for the initial management of primary epithelial ovarian cancer. *The Cochrane Database of Systematic Reviews.* 2011; **11**.

17. Record IR, Lane JM. Simulated intestinal digestion of green and black teas. *Food Chemistry.* 2001; **73**: 481-486.

18. Cheah KY, Howarth GS, Bastian SEP. Grape seed extract dose-responsively decreases disease severity in a rat model of mucositis; concomitantly enhancing chemotherapeutic effectiveness in colon cancer cells. *PloS one.* 2014; **9**: e85184.

19. Cheah KY, Bastian SE, Acott TM, Abimosleh SM, Lymn KA, et al. Grape seed extract reduces the severity of selected disease markers in the proximal colon of dextran sulphate sodium-induced colitis in rats. *Digestive Diseases and Sciences.* 2013; **58**: 970-977.

20. Olaku OO, Ojukwu MO, Zia FZ, White JD. The role of grape seed extract in the treatment of chemo/radiotherapy induced toxicity: a systematic review of preclinical studies. *Nutrition and Cancer.* 2015; **67**: 730-740.

21. Worthington HV, Clarkson JE, Bryan G, Furness S, Glenny AM, et al. Interventions for preventing oral mucositis for patients with cancer receiving treatment. *The Cochrane Database of Systematic Reviews.* 2010; **10**: 4.

22. Abayomi TA, Ofusori DA, Ayoka OA, Odukoya SA, Omotoso EO, et al. A comparative histological study of the tongue of rat (*Rattus norvegicus*), bat (*Eidolon helvum*) and pangolin (*Manis tricuspis*). *International Journal of Morphology.* 2009: **27**: 1111-1119.

23. Al-Azri AR, Gibson RJ, Keefe DMK, Logan RM. Matrix metalloproteinases: do they play a role in mucosal pathology of the oral cavity?. *Oral Diseases.* 2013; **19**: 347-359.

24. Cheah KY, Howarth GS, Yazbeck R, Wright TH, Whitford EJ, et al. Grape seed extract protects IEC-6 cells from chemotherapy-induced cytotoxicity and improves parameters of small intestinal mucositis in rats with experimentally-induced mucositis. *Cancer Biology & Therapy.* 2009; **8**: 382-390.

25. Al-Refai AS. Immunohistochemical study of the effect of chamomile extract on 5-fluorouracil induced intestinal mucositis in albino rats. *Journal of Clinical and Cellular Immunology.* 2014; **5**: 1-10.

26. Xia EQ, Deng GF, Guo YJ, Li HB. Biological activities of polyphenols from grapes. *International Journal of Molecular Science.* 2010; **11**: 622-646.