**Concomitant using of topical carrageenan-kappa and oral vitamin D against 7, 12-dimethylbenz[a]anthracene induced-oral cancer in rats: A synergism or an antagonism effects**

**ABSTRACT**

**Objective:** κ-carrageenan is a food stabilizer agent which has an antiproliferative effect, while vitamin D is a prohormone acts on the nuclear receptor and has a cytotoxic against cancer. This study aimed to show the synergistic effect of using topical κ-carrageenan and oral administration of the vitamin D on the 7, 12-dimethylbenz[a] anthracene (DMBA)-induced oral cancer.

**Material and Methods:** fifty four male albino rats were randomly divided into seven groups: Acetone-treated served as control (Group I), vitamin D (5000UI)-treated (Group II), κ-carrageenan (1%)- treated (Group III), DMBA (0.5%)-treated (Group IV), Acetone, κ-carrageenan and DMBA were administered topically on both cheeks and palate, five times weekly for 12 weeks, while the vitamin D was administered orally twice weekly for 12 weeks. Groups V, VI, and VII were animals treated with vitamin D, κ-carrageenan, and both vitamin D and κ-carrageenan for 8 weeks after induction of oral cancer. At the end of the study, blood samples were obtained by cardiac puncture for determination of TNF-α and EGFR.

**Results:** In the groups III and IV, serum EGFR showed significant low levels compared with Group I. In the Group VII, serum EGFR showed a significantly (p=0.014) low level compared with Group IV (614.3±69.7 pg/ml versus 882.4±45.6 pg/ml, respectively). Higher percentages of high levels of TNF-α were observed in the Groups VI and VII, while a lower percentage of EGFR was observed in the Group VI.

**Conclusion:** both κ-carrageenan and vitamin D have antiproliferative effect against DMBA-inducing oral cancer by increasing the levels of TNF-α and suppressing the signaling pathway of EGFR. Concomitant using κ-carrageenan and vitamin D reduces the antiproliferative effect of each other.

**KEYWORDS:** Oral cancer; 7, 12-dimethylbenz[a] anthracene, Vitamin D, κ-carrageenan; Epidermal growth factor receptor, Tumor necrosis factor-α

**INTRODUCTION**

7,12-dimethylbenz[a]anthracene (DMBA) is a carcinogenic chemical substance that used experimentally to induce a different variety of tumors in an attempt to study the pathogenesis of tumor and to assess the chemotherapeutic agents [1-3]. Experimentally, several studies showed the effectiveness of many substances in reducing or attenuating the effects of DMBA-induced tumorogenesis by improving the antioxidant status, augmenting the apoptosis, inducing antiproliferative, and activation of cytotoxic T cell-dependent tumor regression [4-7]. κ-carrageenan is a food stabilizer agent which used in different pharmaceutical formulation to prepare several compounds categorized as antioxidant, anti-inflammatory, antiinfective and antiproliferative [8-11]. Low molecular weight and sulfated cytotoxic substances derived from carrageenan showed anticancer property via inducing apoptosis and inhibiting the interactions between cell-to cell and cell-to matrix ground substances. Also, they can be used with anticancer agents to suppress the growth and the metastasis of the tumor [12]. Vitamin D is a prohormone acts on the nuclear receptor and has pleiotropic effects beyond its nutriceutical actions. Experimental studies carried on the cancer cell lines showed that vitamin D is cytotoxic against cancer cells by inducing apoptosis [13-14]. In human, the vitamin D reduces the risk of cancers and maintains high serum levels of vitamin D in cancer-patients which improve the survival rate [15]. The rational of the study is both κ-carrageenan and vitamin D have pleiotropic effects including anticancer through different mechanisms, and application of these substances via different routes can attenuate the oral cancer induced by DMBA. The aim of this study was to show the synergistic effect of using topical κ-carrageenan and oral administration of the vitamin D on the DMBA-induced oral cancer using epidermal growth factor receptor (EGFR) and tumor necrosis factor-α (TNF-α) as biomarkers.

**MATERIAL AND METHODS**

***Ethical Approval***

The Scientific Committee in the College of Dentistry at Baghdad University approved this experimental study.

***Animals***

The National Center for Drug Control and Research (NCDCR), Ministry of Health in Baghdadsupplied the animals and the Animal Care Department at the Biotechnology Research Center, The University of Al-Nahrin in Baghdad considered the animal housing and care. Male Wistar albino rats (weighing 150-175 g) were housed four per cage, under a standard laboratory control condition at room temperature of 25±2°C and humidity (50±10%), 12-hour light/dark cycle, fed with astandard rat chow pellet diet, and free access to tap water. After 1 week of acclimatization, the animals were divided into seven groups:

Group I (n=7) treated with an equal volume of acetone ( a solvent of DMBA) and served as the negative control.

Group II (n=9) treated with oral dose (5000UI) vitamin D twice weekly

Group III (n=7): treated with topical application of κ-carrageenan (1%).

Group IV (n=31): treated with topical application of DMBA (0.5% w/v, dissolved in acetone).

The animals of Group I, II, and IV were treated topically (the chemicals applied to both check and palate using a brush size #4 under light anesthesia), five times per week for 12 weeks. Then 21 rats of Group IV were subgrouped into:

Group V (n=7): treated with an oral dosage of vitamin D (5000UI) twice weekly via stainless feeding tube for 8 weeks.

Group VI (n=7): treated with topical application of κ-carrageenan (1%) for 8 weeks.

Group VII (n=7): treated with simultaneous administration of an oral dosage of vitamin D (5000UI) twice weekly and topical application of κ-carrageenan (1%) five times weekly for 8 weeks.

Water and pellets access was not allowed for at least two hours to avoid the washing out of the chemicals. During the study, the animals were checked for the appearance of mass or ulcerative lesion, and the number, size, and the characteristic features of the lesion were recorded. Also, access to water and pellets by the animals and the number of dead animals during the study were recorded. At the end of the study, blood samples were obtained by cardiac puncture under light anesthesia, and the check bearing tumor lesion was excised for further histopathological study. Blood samples were centrifuged at 3,000 R.P.M. for 15 minutes, the sera separated and kept in the deep freeze at -20ºC for further determination of TNF-α, and EGFR by Enzyme-Linked ImmunoSorbent Technology (ELISA) according to the instructions of the manufacturers.

***Statistical analysis***

The results are expressed as number, percentages, and mean ±SEM of the number of animals. The difference between means was analysed using one way analysis of variances (ANOVA) with *post hoc Boneferroni* two-tailed independent two sample test. P-value at a level of ≤0.05 considered significant. Statistical analyses and figure were performed by using Statistical Package for Social Sciences (SPSS-IBM, version 21) software for Windows.

**RESULTS**

Table 1 shows significant differences between treated groups (Group I, II, III, IV) in the means of the serum levels of EGFR (F=5.530, p=0.004). Animals treated with κ-carrageenan (Group III) showed a significantly lower serum level of EGFR compared with the corresponding level of Group I, which account a 22.2% decrease. Group IV (n=10) animals showed significantly lower serum EGFR levels compared with the corresponding value of Group I, which accounted for 21.1% (Table 1). There is a non- significant difference between the groups in the means of the serum TNF-α levels (Table 1). The mean serum levels of TNF- α decreased to 95.3% (Group II), 73.8% (Group III) and 86% (Group IV) of the serum level of Group I.

Table 2 shows significant differences between treated groups (Group IV, V, VI, VII) in the means of the serum levels of EGFR (F=3.844, p=0.021). Animals treated with simultaneous administration of an oral dosage of vitamin D and topical application of κ-carrageenan (Group VII) showed a significantly lower serum level of EGFR compared with the corresponding level of Group I (614.3±69.7pg/ml versus 614.3±69.7pg/ml, p=0.014), which accounts 30.4% decrease). Oral administration of vitamin D (Group V) and topical application of κ-carrageenan (Group VI) produced a non-significant decrease of the EGFR serum levels in rats treated with topical application of DMBA by 8.9% and 13.6% of the serum level of EGFR of rats treated with DMBA alone (Group IV). A non-significant increase of serum levels of TNF-α were observed in animals treated with oral doses of vitamin D (Group V), topical application of κ-carrageenan (Group VI), and both treatments (Group VII) by 26.8%, 25.6%, and 4.5%, respectively compared with animals treated with DMBA alone (Group IV).

Figures 1 and 2 show the effects of different pharmacological interventions on the serum levels of EGFR and TNF-α in animals bearing tumors which induced by a topical application of DMBA in rats.

**Table 1**- Baseline data of the effect of vitamin D, Carrageenan, combined vitamin D and carrageenan, and DMBA on the serum levels epidermal growth factor receptor (EGFR) and tumor necrosis factor (TNF)-α in rats

|  |  |  |
| --- | --- | --- |
| Treated groups | EGFR | TNF- α |
| Group I (n=7) | 1118.5±91.8  (633.4-1349.4) | 39.03±4.44  (21.18-51.55) |
| Group II (n=9) | 1072.3± 21.3  (1001.4-1199.4) | 37.16±5.68  (14.27-66.09) |
| Group III (n=7) | 869.7± 51.2  (705.4-1059.4 | 28.82±6.79  (2.46-51.54) |
| Group IV (n=10)  F-value | 882.4±45.6 (653.4-929.4)  5.530 | 33.55±6.09  (2.46-57.9)  0.506 |
| p-value | 0.004 | 0.681 |
| Comparison between Groups  Group I versus Group II  Group I versus Group III  Group I versus Group IV  Group II versus Group III  Group II versus Group IV  Group III versus Group IV | 0.932  0.025  0.020  0.063  0.054  0.998 | 1.000  1.000  1.000  1.000  1.000  1.000 |

The results are expressed as mean ±SEM (range). F-value was calculated by using ANOVA with *posthoc Boneferroni test* to calculate the p-value of the differences between groups. Group I: acetone-treatment, Group II: Vitamin D-treatment, Group III: carrageenan-treatment, and Group IV: DMBA-treatment

**Table 2**- Effect of vitamin D, Carrageenan, combined vitamin D and carrageenan, on the serum levels epidermal growth factor receptor (EGFR) and tumor necrosis factor (TNF)-α in rats treated with DMBA

|  |  |  |
| --- | --- | --- |
| Treated groups | EGFR | TNF-a |
| Group IV (n=10) | 882.4±45.6 (653.4-929.4) | 33.55±6.09  (2.46-57.9) |
| Group V (n=7) | 804.8±49.4  641.4-929.4) | 42.53±7.47  (1.182-59.73) |
| Group VI (n=7) | 762.8±71.7  (523.4-1135) | 42.14±8.20  (1.18-65.18) |
| Group VII (n=7) | 614.3±69.7  (363.4-489.4) | 35.05±10.48  (1.55-74.27) |
| F-value | 3.844 | 0.359 |
| p-value | 0.021 | 0.783 |
| Comparison between Groups  Group IV versus Group V  Group IV versus Group VI  Group IV versus Group VII  Group V versus Group VI  Group V versus Group VII  Group VI versus Group VII | 1.000  0.877  0.014  1.000  0.220  0.588 | 1.000  1.000  1.000  1.000  1.000  1.000 |

The results are expressed as mean ±SEM (range). F-value was calculated by using ANOVA with *posthoc Boneferroni test* to calculate the p-value of the differences between groups. Group IV: DMBA-treatment, Group V: Vitamin D followed DMBA-treatment, Group VI: carrageenan followed DMBA-treatment, and Group VII: Both vitamin D and carrageenan followed DMBA-treatment

**DISCUSSION**

The results of this study showed that DMBA as a carcinogenic substance induced significant changes in the serum EGFR and the therapeutic modalities in form of topical application of κ-carrageenan and systemic oral administration of vitamin D produce significant changes in the EGFR in rats bearing tumors but not in control rats. A non-significant difference in the serum level of the TNF-α in rats bearing tumors and control is contradictory to the previous studies. A significantly higher salivary TNF-α was observed in patients with oral squamous cell carcinoma compared with healthy subjects [16]. Other studies found that exogenous TNF-α induces the transition of inflammation in the tumor stroma [17]. On the other side, Schiegnitz et al [18] demonstrated that serum TNF-α level is not a useful associated or prognostic marker of oral squamous cell carcinoma patients as IL-6, IL-8, and soluble IL-2R. Conflicting results about the level of TNF-α relates to the methodology of each study. Topical application κ-carrageenan or systemic oral administration of vitamin D produced an increment of TNF-α levels in rats bearing tumor while concomitant administration of these agents does not produce considerable incremental. Carrageenan *per se* can induce inflammation and thereby increased the serum level TNF-α which explained our findings [19]. The explanation of systemic oral vitamin D produced an increment of TNF-α level is a little bit complicated. A significant weak inverse correlation between the serum level of TNF-α with the serum vitamin D was observed in patients with chronic periodontitis [20]. In breast cancer, the antiproliferative effect of vitamin D is enhanced by using TNF-α, in addition calcitriol enhances the synthesis of TNF-α [21]. Therefore, the positive effect of vitamin D on the serum level of TNF-α in rats bearing tumors confirmed the other studies that showed vitamin D has an antiproliferative effect. Concomitant using of oral administration of vitamin D and a topical application of κ-carrageenan resulted in a non-considerable increment of TNF-α levels indicating that each substance acts in different signalling pathway resulting in a term of antagonism in the synthesis or generation of TNF-α. Serum level of EGFR is significantly decreased in rats bearing tumor. Current studies demonstrated that the expression of EGFR in oral squamous cell carcinoma is less than other markers [22].

κ-carrageenan significantly decreases the serum level EGFR in rat bearing tumor while vitamin D does not have this effect. As early as 1997, He et al [23] reported that the selenocarrageenan suppressed the proliferation of breast cancer cell by a mechanism related to the regulation of EGFR. Again, the level of EGFR is higher in rats treated with combined therapy of vitamin D and κ-carrageenan indicating an antagonism between these substances. It is important to emphasize *herein* that EGFR level tended to be decreased in oral cell carcinoma, and a further decrease by PEG-8000 indicates a good prognosis.

**Conclusion**

We conclude that both κ-carrageenan and vitamin D have antiproliferative effect against DMBA-inducing oral cancer by increasing the levels of TNF-α and suppressing the signaling pathway of EGFR. Concomitant using κ-carrageenan and vitamin D reduces the antiproliferative effect of each other.

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**Conflicts of interest:** nil

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**Author contribution**: equally shared with this study

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**Figures legends**

Figure 1: Percentage changes in the serum levels of epidermal growth factor recptors in rats bearing tumors, treated with oral dosage of vitamin D (Group V), topical application of κ-carrageenan (Group VI), and simultaneous administration of oral vitamin D and topical κ-carrageenan (Group VII) compared wih rats bearing tumors induced by DMBA (Group IV).

Figure 2: Percentage changes in the serum levels of tumor necrosis factor-α in rats bearing tumors, treated with oral dosage of vitamin D (Group V), topical application of κ-carrageenan (Group VI), and simultaneous administration of oral vitamin D and topical κ-carrageenan (Group VII) compared wih rats bearing tumors induced by DMBA (Group IV)