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Evaluation of Chemoprevention Effect of Systemic Celecoxib on Induction of Tongue Neoplasms in Rat

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Abstract

***Background*:** Cyclo-oxygenase-2 (COX-2) specific inhibitors were examined for predication or treatment of different tumors and it is indicated that COX-2 specific inhibitors play an important regulatory role in apoptosis of tumoral tissues. Therefore, the present study was designed in order to examine the preventive effects of a COX-2 specific inhibitor called celecoxib on 4-nitroquinoline 1-oxide (4NQO)-induced squamous cell carcinoma on rat.

***Materials and Methods*:** In this experimental study, 30 Sprague Dawley rats (with the age of 3- 3.5 months) were selected and divided into three groups. In order to induce lingual carcinoma, 4NQO powder was prepared 3 times a week for each cage. In this study, celecoxib power was mixed with a basic food (basal diet) in order to examine the systematic effect. Tongue samples were sent to laboratory for immunohistochemical (IHC) staining and histological examination.

***Results*:** Based on morphological criteria and the ratio of apoptosis to cell proliferation, the prevalence of tongue precancerous lesions was reduced significantly by celecoxib.

***Conclusion*:** Celecoxib systematic has inhibitory effects on the 4-nitroquinoline 1-oxide (4NQO)-induced squamous cell carcinoma of tongue. The effect of celecoxib is probably via suppression of cell proliferation and induction of apoptosis.

Introduction

Cancers are the second leading cause of death in the United States; in the meanwhile, oral cancer and oropharyngeal cancer comprise 3% of all the cancers [1]. Despite advances in recent decades in the field of surgical methods, radiotherapy and chemotherapy, the 5-year survival rate for oral cancer has not improved and is 50-55% and according to the WHO (World Health Organization) report, it is one of the cancers which have the highest rate of mortality among malignancies [2]. Since the 5-year survival rate has a direct relation with the stage of disease at diagnosis, preventive measures and early detection will reduce the incidence of oral cancer [2]. COX-2 is an enzyme which is secreted from epithelial cells upon stimulation of growth factors, cytokines and mitogens and leads to the production of prostaglandin in response to inflammation [3, 4].

Nowadays, COX-2 specific inhibitors have been studied for prevention or treatment of different tumors and it is indicated that COX-2 specific inhibitors play an important regulatory role in apoptosis of tumoral tissues and epidemiologic studies have found an inverse an inverse relationship between their use and colorectal cancer [5, 6]. The combination of COX-2 specific inhibitors treatment and chemotherapy or radiotherapy will result in the increase of antitumor effects without destructive effects on normal tissues [7]. The efficacy of COX-2 specific inhibitors on oral squamous cell carcinoma has been examined restrictively and it is indicated that using 1500 ppm of celecoxib orally prevents rats from induced cancer [8], however, the current studies in this area are limited and inadequate. Therefore, the present study was designed in order to examine the efficacy of celecoxib systematic on the inductive cancer in the tongue of rat.

Materials and Methods

In this experimental study, carrying out in the drug applied research center of Tabriz during eight weeks, 30 Sprague Dawley rats aged 3-3.5 months purchasing from the animal breeding house of Tabriz university of medical sciences were used. Animals have been kept in the animal house of the pharmaceutical sciences research center,

Tabriz university of medical sciences and other matters related to study up to killing the animals have been also done in the animal surgical laboratory of this center.

All animals were put in special cages (five animals in each cage) bedded with sawdust and had free access to food and potable (drinking) water (except 2 to 3 hours after gel application) and they were under identical conditions, 6±5% humidity, 12-hour cycles light/dark cycle and temperature 30±2°C. All ethical considerations in working with animals were taken into account according to the instructions of drug research center of Tabriz and Zahedan universities of medical sciences. For lingual carcinoma induction, 4NQO powder was purchased from Sigma company, Germany. Three times a week regularly (on alternate days), 500 ml of NQO solution 30 ppm was prepared for each cage. For preparing the solution, 15 mg of 4NQO powder was measured with a sensitive digital scale, dissolved in 498.5 ml of water and then was kept in foil covered bottles in order to be protected against light.

Celecoxib power was purchased from Sigma company, Germany. To examine the systematic effect, the drug was combined with basal diet in this study. The combination plate was prepared three times a week regularly (on alternate days) 400 gr with dose of 2000 ppm for each cage. Therefore, 2 gr of celecoxib powder was measured with a sensitive digital scale and after combining with 998 gr wet powdered basal diet, it was changed into plate by a meat grinder. First, the plates were made dry in the oven at temperature of 50°C and then, they were brought into laboratory for consumption.

The animals were kept for 2 weeks in order to be adapted and adjusted to new conditions and then upon the beginning of the study, they were randomly divided into 3 groups. In each group, the animals received 4NQO and celecoxib as follows:

Group A: basal plate + 4NQO; Group B: combination plate + 4NQO; Group C: combination plate + normal water. Weight of rats in different groups was measured regularly 3 times a week during eight weeks (29 times) by a sensitive scale. The consumable food and water of animals was also measured in these times. At the end of the study 28 animals survived. During the study of animals in group B, 2 animals died.

Biopsy was conducted on the tongue of these animals. Thus, after inhalational anesthesia with an ether-stained cotton and separating the whole, anterior 2.3 (the mobile part or the oral tongue) was first cut into 2 halves longitudinally and then each piece was divided into 2 parts latitudinally and they were sent to laboratory for hematoxylin eosinophil staining and immunohisto-chemistry (IHC) staining.

TUNEL staining is a method for indicating apoptosis. In order to perform TUNEL on the tongue sections of animals in situ cell death detection kit, POD (cat No. 11684817910. DAB Substrate), and Proteinase K were purchased from the Roche company, Germany and used. This kit can be applied for paraffin blocks. For examination of proliferation, the samples were also stained with Ki67 immunohistochemistry methods.

Results

Statistical analysis of data by Kruskal- Wallis test and ANOVA indicated that the average weight of animals before intervention in different groups had no significant difference and was 170±10 g but after intervention, significant difference (p=0.001) was seen in the average weight of the animals in the experimental group vs. control group. So that, the average of final weight in group A, group B, and group C was 245.55±26.82, 212.33±26.54 and 169.14±31.36 g, respectively and the maximum weight loss was related to group C (receiver of oral CCB).

A significant decrease (p=0.001) was seen in the average food consumption of the animals since the 9th day of the study in all the control groups vs. the control group. So that, the average daily food intake of animals before intervention had not significant difference with each other and was 83±10.25 g on average. The average daily food intake of animals after intervention in group A, group B and group C was 97±10.27, 50.13±5.11 and 66.50±6.27 g, respectively.

The results of the data by measuring frequency indicated that on the 17th day of study, 4.2% (2 rats) of group B died. Quantitative analysis of histopathological changes in stained slide by hematoxylin-eosin and chi-squared test indicated that there was a significant difference between experimental groups in comparison with the control group. Hyperkeratosis i.e. hyperplasia of the keratic layer often accompanies with Keratin qualitative disorders. Dyskeratosis is abnormal keratinization occurring prematurely within individual cells or groups of cells below the stratum corneum (serrated layer).

Parakeratosis is a mode of keratinization characterized by the retention of nucleus in the stratum corneum; on mucous membranes, parakeratosis is normal. Table 1 shows the percentage of histological changes of different groups.

Staining the samples with TUNEL kit indicated that these cells can be observed in brown color and in group C (with prescription of oral celecoxib) as separate masses between other epithelial cells. TUNEL-positive cells were counted and apoptotic index was calculated which was 24.5±15 in group C. Figures 1, 2 and 3 indicate the cellular changes of the groups.

Discussion

The results of the study indicated that the systematic use of celecoxib with carcinogen decreases the occurrence of dysplasia and cancer in rat tongue. These results can be compared with the results of similar studies. Shiatani et al. used 4NQO in order to induce cancer in the rat tongue and they applied Nimesulide as COX-2 specific inhibitor. His study indicated that Nimesulide with a behavior related to dose will decrease the occurrence of dysplasia and cancer in rat and 300ppm dose of this drug has had the most decrease in creating tumor but he has not studied the rate of proliferation and apoptosis [9].

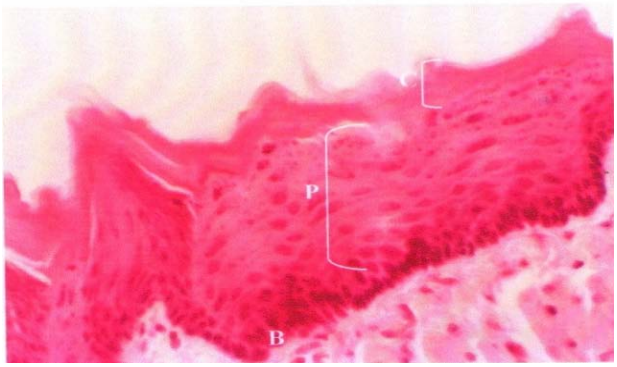

Figure 1. A microscopic view: part of the normal epithelium tissue of rat basal layer (B), prickle cell layer (P=Stratum spinosum), cornified layer (C= Stratum corneum)

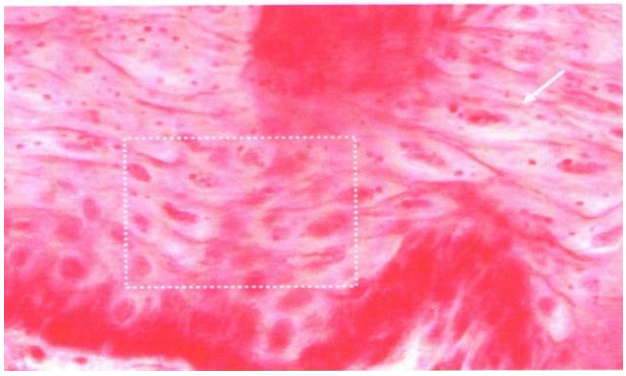

Figure 2. A perspective view of part of the tongue epithelium tissue in rats receiving only carcinogen. In some parts cell polarity is disrupted and signs of dysplastic changes can be observed

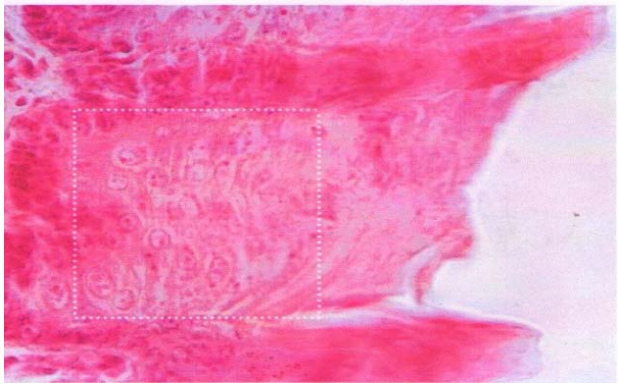

Figure 3. A perspective view: part of the tongue epithelium in rats receiving carcinogen plus celecoxib systematic; dysplasia is not observed

Table 1. The percentage of histological changes in different groups

Appoptptiv index / groups	Survival mice	TUNEL+	Ki67+
basal plate + 4NQO	10	11.68±1.9	27±5.6
combination plate + 4NQO	8	40.8±14.6	13.9±11.61
combination plate + normal water.	10	24.5±15	24.5±15

Table 2. The average of Ki67 and TUNEL-positive cells in different groups

Histological Changes / groups	Group A (%)	Group B (%)	Group C (%)
Mild Hyperkeratosis	0	0	62.5
Moderate Hyperkeratosis	0	44.4	0
Severe Hyperkeratosis	100	55.6	0
Dyskeratosis	0	100	0
Mild Dysplasia	10	88.9	0
Moderate Dysplasia	40	0	0
Severe Dysplasia	50	0	0
Tumoral cells	10	0	0

In another study conducted by Yoshida, Nimesulide was used for treatment after the induction of cancer by 4NQO and the amount of apoptotic cells was evaluated. His study indicated that using COX-2 specific inhibitor will increase the number of apoptotic cells significantly compared with the group who receive carcinogen alone [10].

In 2004, Nishimura induced cancer in hamster by DMBA and simultaneously with the beginning of animals' confrontation with carcinogen, different doses of celecoxib were given to animals. He used TUNEL staining in order to examine the amount of apoptosis and observed that in groups who receive celecoxib, the amount of apoptosis is more than the control group. He observed that the increase of apoptotic cells has a process related to dose so that in the dose of 1500 ppm of celecoxib the most number of celecoxib cells was observed which is similar to the results of the present study, but he has not evaluated the amount of cellular proliferation in different groups [8].

Irregularity of molecular events which manages the control of cellular cycle will be considered as the basis of oral carcinogenesis and cell proliferation programs, differentiation, senescence, and apoptosis will be in close relationship with cellular cycle regulation. Activated oncogenes or inactivated tumoral suppressor genes will increase the genomic inconsistency via multiple control points. Congenitally, carcinogenesis process is selected instead of apoptosis and has lead to the initiation, progress, and progression of malignant phenotype. Therefore, apoptosis is one of the most powerful defensive factors against cancer [11].

Therefore, animal studies will provide a method for studying multi-stage growth of carcinoma and intervention in the target paths of apoptosis in pre-malignant cells which are still relatively untouched (intact) may be an effective way in preventing cancer. 4NQO is a water-soluble quinoline derivative which can form some adhesions (stickiness) to DNA and it can also create oxygen free radicals in a cycle which itself will lead to mutations and some fractures in the DNA chain [9].

Our results have indicated that 4NQO brings about a series of typical precancerous symptoms and also morphological changes in tongue epithelium. The changes include weight loss, macroscopic lesions, changes in cellular size and pathologic changes. In step with our results, it has been indicated that 4NQO results in the creation of SCC in rat. Oral leukoplakia is the most common premalignant lesion of oral cancer and it was observed in the study that 20% of the patients having leukoplakia progress into invasive carcinoma. Oral cancer usually begins with hyperplasia and then changes into dysplasia and with exposure to carcinogen due to the existence of genetic cancer background; it grows into carcinoma [12].

Dysplasia will be specified with an increase in the number of basic stratum cells, an increase in mitotic forms, and increase in the ratio of cell to cytoplasm as well as the loss of cell polarity. Due to the concept of genetic background of cancer, it is assumed that molecular events and biochemistry will appear before cell morphological changes. Epithelial dysplasia is related to histopathological changes and increase in the risk of SCC malignancy growth. In our study this finding existed in animals receiving 4NQO. High COX-2 is related to several of carcinogenic mechanisms. mRNA and COX-2 protein expression in most of tissues can be induced by external stimulations such as: tumoral protumors, growth factors and cytokines. COX-2 seldom can be observed in normal epithelium but will be expressed highly in hyperplasia and SCC and it is specified that COX-2 plays an important role in initiation and post-initiation phases of carcinogenesis. Carcinogenic COX-2 mechanisms include abnormal expression of epithelial growth factors, epithelial and micro-vascular proliferation, resistance to apoptosis and angiogenesis increase and suppression of anti-tumoral security (immunity). In oral cancer of human, high expression of COX-2 has been observed.

Studies indicate that inhibition of COX-2 has a potential role in chemoprevention of head and neck SSC and direct evidences has been observed of COX-2 expression in tongue carcinogenesis of rat and dysplasia inhibition by prescription of NSAIDs but another study has indicated that the incidence of SSC with prescription of NSAID has decreased 31-32% in animals compared to the control group who didn't receive this treatment (71%) [13].

Celecoxib is the first NSAID selective inhibitor of COX-2 which is recorded and is effective in adults' arthritis treatment. Celecoxib has a strong chemopreventive effect in induced carcinogens of colon, bladder and breast via carcinogen and also carcinogen-induced skin via UV ray; it has also been able to effectively inhibit the growth of colon and breast cancers in rat [14]. A study conducted in 2004 with inducing cancer in hamster type via DMBA carcinogen indicated that celecoxib dependent to dose, prevented oral carcinogenesis [8]. It is also specified that celecoxib had anti-tumoral activity in cells or tissues which lacked COX-2 enzyme. Therefore, recently chemopreventive or therapeutic activity of celecoxib against different cancers is via clinical methods because it has been observed that celecoxib can induce apoptosis in several cells [15].

Although the mechanisms by which apoptosis would be induced are unknown to some extent, anti-apoptosis activity of celecoxib may be considered as its chemopreventive and therapeutic activity. Apoptosis is a genetic regulated active process, in which some changes have occurred in the cellular structure and will result in the cell self-destruction. On the one hand, apoptosis is proliferation supplement in regulating the cell populations in both physiologic and pathologic conditions. Therefore, cell proliferation is considered as one of the most important biological mechanisms in oncogenesis.

Cell proliferation in animals' tissues is the basis of toxicological and carcinogenesis studies and is also useful for gaining access to the efficiency of cytotoxic and chemopreventive drugs. Our results in methods done by TUNEL and Ki67 indicated that during tumorogenesis in group A, apoptosis has been in a low level and on the contrary proliferation has been high and also the ratio of apoptosis to proliferation in the group who received 4NQO was minimum and on the contrary apoptosis induction was high and low expression of Ki67 existed in groups B and C that were treated by celecoxib. If we have observed a significant decrease in animals' weight in treatment with oral CCB (2000 ppm), it may be a reflection of the existing deviations in the statistical studies and uncertainty because of the low number of samples or the interference of 4NQO carcinogen with celecoxib. It is notable that the ratio between apoptotic and proliferation indexes in dysplasia is higher than carcinomas and probably the reason is that apoptosis in invasive lesions has been hidden because of high proliferation.

Other findings of the study indicated that Ki67-positive cells in group A have been abundant and decreased with prescription of celecoxib in the experimental group. The results indicated that although 4NQO has increased proliferation as a carcinogen and has suppressed apoptosis, but these changes made reverse with prescription of celecoxib. This means that prescription of oral celecoxib is an effective chemopreventive substance in preventing carcinogen. Other findings indicated that TUNEL-positive cells increased with prescription of celecoxib showing that prescription of oral celecoxib can induce apoptosis.

The obtained results suggest that when prescribed in tumorogenesis initiation stages, celecoxib has the chemopreventive ability against induced cancer by 4NQO via an increase in apoptosis and proliferation suppression. The created lesions suggest that the balance between proliferation and apoptosis can be more important that the absolute role of each of them.

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Authors' Contributions

All authors had equal role in design, work, statistical analysis and manuscript writing.

Conflict of Interest

The authors declare no conflict of interest.

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