Chemopreventive effect of celecoxib and expression of cyclooxygenase-2, Caspase-3 and AGNOR on chemically-induced rat submandibular salivary gland neoplasm.

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Abstract: BACKGROUND: Cyclooxygenase-2 (COX)-2 inhibitor (Celecoxib) is a non-steroidal anti-inflammatory drug (NSAIDs) and over-expression of COX-2 protein and mRNA has been reported in various cancer tissues. Therefore, it has been suggested that COX-2 is related to carcinogenesis. METHODS: Twenty five albino rats were used. They were divided into 3 groups; group I (normal control) and group II and III which was delivered 4-NQ in the drinking water. Meanwhile group III was given 1500 ppm celecoxib. Submandibular salivary glands were obtained after 32 weeks. Immuno-histochemical staining for COX-2 was performed to determine the COX-2 level and Caspase-3 immuno-expression was done for detection of apoptosis and silver nitrate staining of nucleolar organizer regions (AgNORs) was done for estimating the proliferating cells. The data were analyzed using Student's independent t-test and one-way analysis of variance (ANOVA). RESULTS: The group II and III showed pathological evidence of cancer. COX-2 immuno-staining was stronger in group II than in Group III. Caspase-3 immuno-reaction was statistically highly significant in group III (p<0.05). Meanwhile proliferation estimated by AgNOR nuclear count was statistically highly significant group II (p<0.05). CONCLUSION: The COX-2 expression was increased in group II (untreated group) than group III. Administration of celecoxib demonstrated the chemopreventive potential against the carcinogenesis through induction of apoptosis and suppression of tumor growth and proliferation.


Key words: Oral cancer, Cyclooxygenase-2, submandibular salivary gland

11. Introduction

Oral cancer is one of 10 most frequent cancers in the world. Squamous cell carcinoma (SCC) is the most common malignant tumor of the oral cavity, accounting for over 90% of the malignant neoplasms in this region. Furthermore; recent epidemiologic data have indicated that the incidence of oral cancer is increasing, Nishimura et al. (2004). Despite a better understanding of the disease and the advent of modern technology and rationally targeted drugs, the incidence and cure rate of cancer have not improved, Bharat and Shishir (2006). Therefore, new molecular targets are needed for the prevention and treatment of oral cancer.

Chemoprevention is to use of pharmacological or natural agents to prevent, suppress, interrupt, or reverse the process of carcinogenesis Garay & Engstrom (1999). Regular use of aspirin has been shown to lower the risk of colon cancer in man, Thun et al. (1991) and also Reddy et al. 1993; Rao et al. 1995 added that NSAIDs can suppress colon carcinogenesis induced by azoxymethane in rats ,Schreinemachers & Eversion (1994); Harris et al. (1996). They demonstrated that there are significant reductions of breast cancer risk with the use of NSAIDs. In this field Lin and Nelson (2003) added that the experimental and epidemiologic studies have demonstrated that the NSAIDs are effective in the prevention of human cancers.

It is known that NSAIDs decrease prostanoid synthesis through the inhibition of cyclo-oxygenase (COX) activity, Van (1971). There are two different isoforms of COX, COX-1 and COX-2. COX-1 is constitutively expressed in most normal tissues to maintain stable physiological conditions such as cytoprotection in the stomach, vasodilatation in the kidney, and production of the pro-aggregatory prostanoid thromboxane by platelets Vane, (1994). Where as COX-2 is transiently induced by pro-inflammatory cytokines and growth factors of epithelial cells involved in inflammation and mitogenesis, Herschman (1996) and its pathophysiological role has been primarily connected to PG production in response to inflammation ,O’Banion et al. (1992); Crofford et al. (1994).

COX-2 has been recently reported to be up regulated and over-expressed in various of human malignancies, such as colon, lung, stomach esophagus, pancreas, endocervix, urinary bladder, prostate and skin and breast cancers, and also in squamous cell carcinoma of the head and neck Leong et al. (1996); Wilson et al. (1998); Chan et
al. (1999); Tucker et al. (1999); Stolina et al. (2000); Yoshimura et al. (2000) and can be modulated by a variety of cytokines, hormones and tumor promoters Parrett et al. (1997;Wolff et al. (1998). In addition COX-1 and COX-2 have been detected in rat mammary gland tumors induced by various carcinogens, including DMBA,Robertson et al. (1998), N-nitrosomethyl urea,Hamid et al. (1999) and 2-amino-1-methyl-6-henylimidazo[4,5-b] pyridine ,Nakatsug et al. (2000). Moreover, COX-2 has been implicated in the development of colon cancer and may play a role in promoting invasion, metastasis, and angiogenesis in established tumors,Dubois et al. (1998) and Tsujii et al.,(1998).

Conventional NSAIDs such as aspirin, sulindac and indomethacin block both COX-1 and COX-2, resulting in unwanted side effects including gastritis and gastric ulceration. Therefore, when NSAIDs are used over long period as chemopreventive agent, a selective COX-2 inhibitor must be used .Jang et al. (2002). Celecoxib is new NSAIDs that inhibits COX-2 and has significant anti-inflammatory and analgesic properties Seibert et al. (1994). Harris et al. (2000) reported that the celecoxib shows inhibitory effects on the development of rodent mammary cancer.

In this study, we examined the COX-2 expression in rodent sub-mandibular salivary gland neoplasm induced by 4-NQ and the chemopreventive effect of celecoxib, a selective COX-2 inhibitor, for tumor development with correlation to its induction of apoptosis and proliferation.

2. MATERIALS AND METHODS

This study was performed in accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals, the National Institute of Health Guide for the Care and Use of Laboratory Animals, and the Animal Welfare Act (A 3775-01).

2.1. Materials:

2.1.1. Celecoxib and an animal model

Twenty five adult male albino rats weighing (150-200 gm) were selected and divided into three groups.

The rats were watched for 1 week before the study. Three rats were allocated to each cage and were fed standard laboratory chow and water and libitum. The animals were housed and caged separately in the animals' house of the Faculty of Medicine, Cairo University.

Celecoxib (Celebrex) was manufactured and supplied by Pfizer Egypt S.A.E under Authority of G. D. Searle &CO.USA.

The rats have been then randomly divided into 3 groups: (I) control – 5 rats; (II & III) 10 rats for each.

The weight of all hamsters was recorded prior to the medication and subsequently on the end of this study.

2.2. Methods:

2.2.1. Induction of cancer

Carcinogenesis was induced in the animals of both group II and group III in which the carcinogen 4-NQ was obtained as a powder (Sigma, St. Louis, MO, A, cat. # N8141) and dissolved in the drinking water for rats of both groups to a final concentration of 0.02 g/l (20ppm). The prepared drinking water was changed once a week and the rats were allowed access to the drinking water at all times during the experiment is given in the drinking water in addition to the standard laboratory chow diet.

2.2.2. Chemoprevention and follow-up

Celecoxib was given to group III only from the beginning of the experiment after addition of one capsule, 100 mg into fine powder which was added to the laboratory chow of the rats every day. Clinically, physicians usually use daily 200 mg or 400 mg Celecoxib for treatment of rheumatoid arthritis or other diseases, and both doses have been approved by FDA. There is no specific guidance and dose for its application in animal model. However, currently 1,500 p.p.m probably is the most common dose to be used in rodent models. Kawamori et al. (1998) reported their findings to establish a therapeutic blood level of Celecoxib for chemoprevention in such models. The study identified daily administration of 1,500 p.p.m as an ideal dose to significantly suppress the colonic aberrant crypt foci formation. These findings served as the basis for our dose selected for this study. Rats were allowed access to the drinking water at all times during the experiment. At the 20th week of the experiment, two rats from each group were sacrificed and analyzed for precancerous and cancerous lesions. The study period was extended for thirty two weeks. After that all the remaining rats were sacrificed and the submandibular salivary glands were dissected.

Salivary glands biopsy specimens were taken for histological examination. The specimens were fixed in 10% formalin, processed in a standard manner, and stained with hematoxylin and eosin and examined under light microscope.

2.2.3. Immuno-histochemical analysis:

For immuno-histochemical staining, paraffin embedded tissues, sectioned at 4 µm and collected at
serial sections on positive charged slides (SuperFrost Plus-Menzel GmbH) were deparaffinized and dehydrated. Antigen retrieval was performed by boiling the slides in 10Mm citrate buffer, pH 6.0 for 20 minutes in a domestic microwave. Slides were left to cool for 30 minutes at room temperature. Sections were incubated in 3% hydrogen peroxide for 20 minutes. Novocastra protein block (RE7102 Novocastra, UK) was applied for 10 minutes after which the slides were incubated with the primary monoclonal Mouse anti body COX-2 [(CX-294 Dako,Glostrup, Denmark] for COX-2 immunostaining and while the other slides with the primary rabbit monoclonal antibody: anti-caspase 3 for caspase-3 immunostaining [(CPP32) Ab-4 Thermo Fisher Scientific, USA] diluted 1:100; for 30 min. at room temperature in a humidified chamber. After rinsing twice with TBS (Tris Buffered Saline, Amresco-USA), sections were treated with biotinylated secondary antibody (RE7103 Novocastra, UK) then labeled with streptavidin-biotin kit (RE110-k Novocastra, UK). The sections were then incubated in 3,3'diaminobenzidine (RE7190-k Novocastra, UK) for 5 minutes and counterstained with Mayer's hematoxylin (RE7107 Novocastra, UK).

2.2.4. Silver nucleolar organizer regions (AgNOR) staining technique:

Equal proportions of 50% silver nitrate soln. and gelatin soln. were mixed immediately before use. Sections were dewaxed in xylene and hydrated through ethanol's to water then the slides were rinsed in distilled water. The Sections were then incubated in freshly prepared AgNOR working solution for 45 minutes at room temperature. After that the slides were washed in distilled water for one minute, then dehydrated, cleared and mounted in non aqueous mounting medium.

2.2.5. Cox-2, Caspase-3 and AgNOR staining assessment:

The histological sections were examined using light microscope to assess the prevalence of positive ones. For Cox-2 and caspase-3 positive cytoplasmic immuno-expression, the percentage of positive cells was measured in the form of an area and area percent inside a standard measuring frame of area 11434.9 micrometer$^2$ per 10 fields using a magnification (x200), using image analysis software (Leica-Qwin) system. In addition, the number and area percent of AgNOR positive dots were counted per 10 fields using the image analysis. The nuclei that were overlapped or those with indiscernible AgNORs were excluded.

2.2.6. Statistical analysis: Quantitative data of the image analyzer were statistically evaluated and presented as means and standard deviation (SD) values. Student's t-test was used to compare mean values of Cox-2 and caspase-3 immuno-expression related parameters in group II & III; however the ANOVA (analysis of variance) test was used to compare the mean values of AgNOR obtained data between the two experimental groups. The significance level was set at $P \leq 0.05$.

3. Results:

3.1. H&E examinations revealed the rodent submandibular salivary glands showed signs of dysplasia and malignancy after administration of 4-NQ which appeared sever in group II while in group III (treated by celecoxib) appeared to be less affected.

In Group II the serous acini showed dramatic changes ranged from shrinkage of the lobules to total atrophy. As the serous cells demonstrated disruption of its characteristic acinar arrangement, with appearance of isolated acinar cells and significant shrinking of the acinar cells and necrosis were termed as “abnormal acini” the acinar cell become disrupted, distorted and detached from adjacent acinar cells. (Fig. 1A). The nuclei were hyperchromatic and showed pleomorphism (Fig. 1A) the intralobular Ducts that demonstrated considerable cellular hyperplasia and atypia with extravasation of red blood cells (fig. 1A), while inter lobular duct showed hyperplasia where as in other areas revealed atrophy and necrosis (Fig. 1C). While mucous acini were more resistant to effect of carcinogenic agent (Fig. 1B).

In group III, by administration of celecoxib the evidence of cancer appeared to be reduced or cured. The serous cells were arranged in abnormal acinar arrangement (Fig. 2A). The acinar cells became smaller in size. In other area showed loss their acinar arrangement (Fig. 2B). The nuclei showed piknosis and hyperchromatism (Fig. 2B). The duct did not show significant changes but show extravasations of RBC. (Fig. 2A).

3.2. COX-2 immunostaining:

The group II showed strong positive immunoreactivity in serous acini more than group I &III (Fig. 3A). While the mucous acini show positive peri-nuclear immunoreactions (Fig. 3B). The duct revealed strong cytoplasmic immuno-expression (Fig. 3 B). While in group III showed occasionally positive immunoreactions in serous acini and intralobular duct (Fig. 3C).
3.3. Caspase-3 immunostaining:

Occasional immunoreactivity was observed for caspase-3 in group II (4-NQ treated group) less than group I [Fig.3A]. On the other hand, sections of the serous acini of group III revealed a higher immunostaining of caspase-3 (Fig.4B) in duct cells (original magnification, x 400).

Fig. 1: Photomicrographs of serous and mucous glands of group II (A) show disruption and loss of the characteristic acinar arrangement, appearance “Abnormal serous acini” (ABS) of isolated acinar cells, and significant shrinking of the acinar cells (H&E. original magnification, x 200). (B) Mucous acini retain their normal morphology (H&E original magnification, x 400). (C) The interlobular duct shows in some areas hyperplasia and in other areas shows disruption, loss of continuity and atrophy arrows (H&E. original magnification, x 200).

Fig. 2: Photomicrographs of group III demonstrate (A). smaller serous acini and become atrophied. The duct appeared less effected and show lesser degree of extravasation piknotic and hyper chromatic. (H&E. original magnification, x 400).

Fig. 3: Photomicrographs of (A) group II show strong cox-2 immuno-expression in serous acini (original magnification, x 200). (B) A mucous acini show Cox-2 positive peri-nuclear immunoreaction & strong cytoplasmic immunostaining in ducts. (Original magnification, x 200). (C) Group III showing few positive cox-2 immuno-expression in acinar cell with positive immunoreactions in ducts (original magnification, x 400). of RBC. (H&E. original magnification, x 200). (B) The nuclei become.

Fig. 4: Photomicrographs of caspase 3 immunostaining (A) group II show negative immunoreaction with few positive cells (original magnification, x 400). (B) While in group III demonstrate higher positive immunoreactions in acinar cells with positive

Fig.5: (A) Silver stained section of group II showing numerous silver stained nuclei in of serous cell (original magnification, x400). (B) Group III stained with silver showing less number of AgNOR dots in serous acini than group II (original magnification, x400)
3.4. AgNOR staining:
AgNOR were strictly located within the nucleus and were distinctly stained in black, being visible as dots. Silver stained dots were great in group II sections, almost all of the nuclei were stained (Fig.5a), meanwhile group III revealed lesser silver stained nuclei, restricted to acinar cell (Fig.5b).

3.5. Statistical analysis:
The results of the student's t-test revealed a non significant area percentage of the immunoreaction of COX-2 (p>0.01) between the group II & group III (table 1& fig. 6).

Table (1): Comparing the area percentage of COX-2 positive cells in group II and group III

<table>
<thead>
<tr>
<th>Statistical profile</th>
<th>Group II</th>
<th>Group III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean±SD</td>
<td>110.6±0.8</td>
<td>108±1.0</td>
</tr>
<tr>
<td>Student's t-test</td>
<td>1.9</td>
<td></td>
</tr>
<tr>
<td>P-value</td>
<td>0.084391</td>
<td></td>
</tr>
</tbody>
</table>

* Significant at $P \leq 0.05$

Fig.6: Bar chart illustrating the difference in area percentage of COX-2

And a highly significant area percentage of the immunoreaction of caspase-3 (p<0.01) between the two group II & group III (table 2& fig. 7).

Table (2): Comparing the area percentage of caspase-3 positive cells in group II and group III

<table>
<thead>
<tr>
<th>Statistical profile</th>
<th>Group II</th>
<th>Group III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean±SD</td>
<td>114.2±1.3</td>
<td>179.8±0.78</td>
</tr>
<tr>
<td>Student's t-test</td>
<td>46.5939</td>
<td></td>
</tr>
<tr>
<td>P-value</td>
<td>&lt;0.001*</td>
<td></td>
</tr>
</tbody>
</table>

* Significant at $P \leq 0.05$

Fig.7: Bar chart illustrating the difference in area percentage of caspase-3 immunoreactivity in group II & group III

In addition, the ANOVA test showed a highly significant area percentage of the positive AgNOR dots (p<0.01) between group II & group III (table 3& fig. 8).

Table (3): Comparing the area percentage of the AgNOR positive nuclei in group II and group III

<table>
<thead>
<tr>
<th>Statistical Profile</th>
<th>Group II</th>
<th>Group III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean±SD</td>
<td>8.5±0.66</td>
<td>16±1.6</td>
</tr>
<tr>
<td>F-value</td>
<td>4.23259</td>
<td></td>
</tr>
<tr>
<td>P-value</td>
<td>&lt;0.001*</td>
<td></td>
</tr>
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* Significant at $P \leq 0.05$

Fig.8: Bar chart illustrating the difference in the area percentage of the positive AgNOR dots in the groups II &III.

4. Discussion:

In the present study, we investigated the chemo-preventive effects of celecoxib in chemically induced 4-NQ cancer in submandibular rodent
salivary gland. We found that nearly all rats of (group II & III) that received 4-NQ suffered from developing salivary gland cancers 19/20 cases. This result is in accordance with Tang et al. (2004) as they demonstrated that murine 4-NQ induced oral and esophageal carcinogenesis model simulates many aspects of human oral cavity and esophageal carcinogenesis. They added that the availability of this mouse model should permit analysis of oral cavity and esophageal cancer development in various mutant and transgenic mouse strains. This model will also allow testing of cancer chemo-preventive drugs in various transgenic mouse strains COX-2 immuno-expression in group II was up regulated than in group III (treated by Celecoxib). These results are in accordance with Hwang et al. (1998); Mohammed et al. (1999); Kulkarni et al. (2001). They found that COX-2 was over expressed in various malignant tumor, such as cervical cancer, breast, skin also in squamous cell carcinoma of the head and neck. In this field, Nishimura et al. (2004) as they demonstrated that COX-2 was known to be over expressed in a variety of human pre-malignant and malignant lesions including oral ones. They also found that the administration of celecoxib produced inhibitory effects against oral SCC development both at the initiation phase ,during promotion and progression phases of carcinogenesis. The inhibition of COX-2 production led to marked lymphocytic infiltration of the tumor and reduced tumor growth, Stolina et al., (2000) 

In this regard, Sawaoka et al., (1998); Chan et al., (1999) cleared that the mechanism(s) by which NSAIDs inhibit tumor growth was not clearly understood, but it could involve blockage of COXs, which suppress PGs production and might affect cell proliferation, apoptosis and immune response. And also, Tsujii et al.,(1998) added that there were evidences in colon cancer cells suggested that excessive PG production due to COX-2 over-expression played a role in tumor growth and spread. While, Lin and Nelson (2003) explained in more detail that COX-2 may be involved in carcinogenesis via 2 distinct mechanisms: (1). DNA damage and (2). PG-mediated effects. Reactions mediated by COX-2 form reactive oxygen species that could directly induce the oxidation of DNA or instigate the bioactivation of carcinogens. Prostaglandin E2, a byproduct of COX-2-mediated arachidonic acid metabolism, exhibits several biologic actions that have been shown to promote tumorigenesis and tumor progression. These actions include increased cell proliferation, promotion of angiogenesis, and the elevated expression of the antiapoptotic protein Bel-2. In addition, PGE2 decreases natural killer cell activity and alters immune surveillance.

Caspase are fundamental component of the mammalian apoptotic machinery. Caspase 3 is a prototypical enzyme that becomes activated during apoptosis in a wide variety of tissues, Woo et al., (1998). The quantification of immuno-expression of caspase 3 might constitute a good method for measuring apoptotic activity in prostate cancer,Santamaria et al. (2005). In our study the immuno-expression of Caspase in group II was weak or negative while in group III the positive cell was abundant denoting the apoptosis process was markedly noticed. This result are in accordance with Pasricha et al., (1995) as they demonstrated that one of the most striking events in which PGs have been implicated in tumorigenesis is the inhibition of apoptosis in the colonic epithelium of familial adenomatous polyposis patients. More directly, Tsujii and DuBois (1995) showed that rat intestinal cells increasing COX-2 expression by gene transfer became resistant to butyrate induced apoptosis, which can be overcome by addition of the non-specific COX inhibitor sulindac sulfide. In this field, Gupta and Dubois (2001) reported that the studies in colonic cancer showed that induction of COX-2 was associated with inhibition of apoptosis increased in angiogenesis, and metastatic potential. Also, Hashitani et al. (2003) reported that celecoxib induced apoptosis in cultured head and neck cancer cell lines to a significantly greater degree than sulindac Nucleolar organizer regions (NORs) are the sites of ribosomal RNA which reflects protein synthesis. AgNOR dots are the visualized structures of NORs that can be selectively stained by a silver colloid technique and can be visualized as black dots under the transmission microscope,Uno et al. (1998). According to Derenzini and Trerè (1994), the higher the number of NORs, the lower is the duration of the cell cycle and the higher is the velocity of cell proliferation. Such relationship makes the quantitative analysis of NORs an excellent indicator of the proliferation activity of the cells and a valuable diagnostic tool, since it enables the differentiation of benign from malignant cells and even predicts the prognosis of different types of cancer ,Trerè et al. (1991). The results of this work showed that group II showed a higher number of AgNOR dots than group III. Chatterjee et al. (1997) demonstrated that the number of AgNORs rises with increasing the proliferative activity of cells, thus the number of AgNOR dots in malignant lesions is higher than normal or benign lesions. Moreover, Reddy et al. (1996) found that 1500 ppm celecoxib inhibits aberrant crypt foci multiplicity by 40–49% without...
gross changes in the intestines. In this field, Wang, (2005) added that the over-expression of Cox-2 enhances cell proliferation, inhibits apoptosis and increases metastatic potential, thereby contributing to carcinogenesis. Eslami et al. (2006) concluded that there is an increase in the number of AgNOR dots with the advancement of malignancy.

Consequently, the findings of the present work suggested that celecoxib might exert a chemopreventive effect on rodent salivary gland tumor through suppression PG production and inhibition of tumor growth and proliferation of cells and induction of apoptosis. In this regard, Ning et al. (2005) added Celecoxib is a highly selective inhibitor of COX-2, with less toxicity than traditional COX inhibitors. It may be used for reversing or stopping oral carcinogenesis at an early stage of disease.

In conclusion, our present results clearly indicated COX-2 protein to be increasingly expressed in the malignant salivary gland and provided the evidence that celecoxib, possessed the chemopreventive potential by delayed onset of tumor development, retarded tumor growth and inducing apoptosis. From these findings, it was indicated that celecoxib could serve as a potent chemo-preventive agent with low toxicity against oral carcinogenesis. In addition, further studies are recommended on determining the mechanism of anticancer activity of celecoxib and its active components against oral cancers.

5. References


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