# Meloxicam Inhibit the Growth of Oral Squamous Cell Carcinoma Induced by Benzopyrenes

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ORIGINAL ARTICLE

# Meloxicam Inhibit the Growth of Oral Squamous Cell Carcinoma Induced by Benzopyrenes

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## ABSTRACT

**Introduction:** Meloxicam is a NSAID which able to inhibit the cyclooxygenase 2 (COX-2). The purpose of this research is to explain the effect of Meloxicam in inhibit the growth of oral squamous cell carcinoma (OSCC) induced by benzopyrenes. The apoptosis and proliferation of OSCC, the p53, Ras and COX-2 expression used as indicator. **Methods:** male mice were induced by benzopyrene 10 mg/kg body weight was given topically on buccal mucosa 2 times a week for 4 weeks for induced the OSCC. Meloxicam was given orally with 3 different doses was 50mg/kg, 100mg/kg, and 200mg/kg.b.w given once a day for 60 days. The control groups were given with CMC-1% 0.1ml/10g body weight. The buccal mucosa then biopsy and staining with immunohistochemistry to analyzed the p53, Ras, COX-2 expression, apoptosis and proliferation of OSCC. **Results:** The increase of Meloxicam dose is proportional to the increase in wild p53 expression and apoptosis, and inversely proportional to the expression of mutant race, cox-2 and the proliferation of OSCC. **Conclusion:** Meloxicam able to inhibit the growth of OSCC induced by benzopyrenes.

**Keywords:** Meloxicam, benzopyrene, oral squamous cells carcinoma

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## INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the epithelial malignancy with the incident around 9.2%. This number is quite high and will increase every year (1). The etiology of OSCC is complex and need attention. One of the OSCC etiology is chemicals exposure (2). Benzopyrenes is an aromatic polycyclic hydrocarbon compound that is carcinogenic and most often causes cancer in the oral cavity (3). Benzopyrenes is mostly found in cigarette smoke, vehicle smoke, smoke from the combustion process of organic materials and smoked food or baked. Benzopyrenes produces reactive metabolites that can bind covalently to DNA so that genetic mutations occur (4).

Oncogenesis is the complex process as result of mutations from apoptotic gene inhibitors, protooncogene, tumor suppressor gene and DNA repair. The p53 and p21 genes are associated with cell apoptosis and proliferation. The p53 gene has mechanism and role to stop the cell cycle if DNA damage occurs (5). Whereas one of the genes most often mutated in cancer cells is Ras oncogene. In the condition of pre-cancerous and neoplasia, the cyclooxygenase-2 (COX-2) expression is found. The cancer process of epithelial is closely related with COX-2 activity. The higher COX-2 activity and expression in the epithelial can inhibit the process of apoptosis and increase cancer cell formation (6).

The Non-Steroid Anti-inflammatory Drugs (NSAIDs) is cyclooxygenase-2 inhibitors are used for analgesics in cancer cases because they can provide analgesic and anti-inflammatory effects, do not cause severe side effects and are economically affordable. Meloxicam is a partial selective NSAID that can inhibit the COX-2, although there are side effects on the heart that are reversible so that if drug use is stopped the side effects will be controlled. Several epidemiological studies with case control design show that NSAID have the potential of preventing the development and growth of cancer cells (7). The problem of research to date is the mechanism of meloxicam in inhibiting the growth of OSCC due to benzopyrenes is not yet clearly known. The purpose of this research is to explain the possible mechanism of meloxicam in inhibiting the growth of OSCC through differences in p53, Ras, COX-2 expression, apoptosis

and proliferation of cell.

## MATERIALS AND METHODS

### Animals

This study was obtained ethical approval from the Ethical Clearance of Ethic Committee for Research in Faculty of Dental Medicine, Universitas Airlangga with registered number 106/HRECC.FODM/X/2012.

Fourty male mice (Mus musculus), 3 months, with body weight 30-40g was used as an animal model. This research was conducted in Animal Center, Faculty of Veterinary, Universitas Airlangga. The animal was conditioned in separated cages under room temperature (27°C) and lighted rooms on 12 hours light/12 hours dark cycle with free access to water and standard diet.

### Oral squamous cell carcinoma model

OSCC model was obtained with induced by benzopyrene (benzo[a]pyrene, Sigma Aldrich, USA). Benzopyrene 10 mg/kg body weight of mice was given by topically on buccal oral mucosa 2 times a week for 4 weeks. The control groups were given with Oleum olivarum 0.05 ml by topically on buccal oral mucosa 2 times a week for 4 weeks (Table I).

Meloxicam treatment for oral squamous cell carcinoma Meloxicam (Meloxicam, Kimia Farma, Indonesia) was given orally with 3 different doses was 50mg/kg.b.w, 100mg/kg.b.w and 200mg/kg.b.w (body weight). Each of dose was given once a day for 60 days. The control groups were given with CMC-1% (Carboxymethylcellulose sodium, Sigma Aldrich, USA) with dose 0.1ml/10g body weight of mice. Al groups distributed as described in Table I.

12 weeks after treatment with Meloxicam, all mice were sacrificed and the buccal of oral mucosa that had undergone changes was biopsy then were fixed using formalin buffer then dehydrated and paraffin block using standard methods.

**Table I: Animal groups of benzopyrenes induced OSCC and treatment with Meloxicam**

| Groups | Number of mice | OSCC model | Treatment |
|---|---|---|---|
| A (control -) | 8 | *Oleum olivarum* 0.05 ml | CMC 1% 0.1ml/10gr body weight |
| B (control +) | 8 | Benzopyrene 10 mg/kg body weight | CMC 1% 0.1ml/10gr body weight |
| C | 8 | Benzopyrene 10 mg/kg body weight | Meloxicam with dose 50mg/kg body weight |
| D | 8 | | Meloxicam with dose 100mg/kg body weight |
| E | 8 | | Meloxicam with dose 200mg/kg body weight. |

### Apoptosis and proliferation of OSCC

The apoptosis of OSCC was analyzed using TUNEL assay and proliferation of OSCC using proliferating cell nuclear antigen (PCNA).

### p53, Ras, COX-2 expression

Immunohistochemistry staining was used to determine the p53 (anti-p53 antibody ab131442, polyclonal, Abcam), Ras (anti-Ras antibody ab16907, monoclonal, Abcam) and COX-2 (Anti-COX2 ab15191, monoclonal, Abcam). All antibody was diluted with ratio 1:50.

The p53, Ras and COX-2 expression were counted from epithelial cells that expressed the p53, Ras and COX-2. The measurement using a light microscope (Nikon H600L microscope; Nikon, Japan) at a magnification of 400x (DS Fi2 300MP digital camera; Nikon, Japan, digital software imaging by Nikon Image System, Nikon, Japan).

Counting the number of OSCC cells undergoing apoptosis and proliferation and expressing p53, Ras and COX-2 per 100 cells. OSCC cells that are positive p53, Ras and COX-2 has given a brown color. Every one field of view was observed and counted in two places according to the direction of the hands of 6 and 12 using grateculae (counting rooms) and counters. Each preparation was observed in 4 visual fields (6)

### Statistical analysis

The data of p53, Ras and COX-2 expression, apoptosis and proliferation were presented as mean+standard deviation. The One-Way ANOVA then used for analysis the differences each indicator. The high significant differences (HSD) as post-hoc test was used with significance of difference $p<0.05$. The analysis was performed with SPSS software (7).

## RESULTS

The increase in Meloxicam dose is proportional to the increase in wild p53 expression and apoptosis of OSCC (Figure 1). The highest of p53 expression is found in the group E (Meloxicam 200 mg/kg.b.w) ($p<0.05$). The apoptosis of OSCC in the group E (Meloxicam 200 mg/kg.b.w) is higher than group D (Meloxicam 100 mg/kg.b.w) ($p=0.000$) and group C (Meloxicam 50 mg/kg.b.w) ($p=0.000$) (Figure 2).

The increase in Meloxicam dose is inversely proportional to the Ras, COX-2 expression and the proliferation of OSCC (Figure 2). The Ras expression in the group E (Meloxicam 200 mg/kg.b.w) is lower than group D (Meloxicam 100 mg/kg.b.w) ($p=0.020$) and group C (Meloxicam 50 mg/kg.b.w) ($p=0.001$).

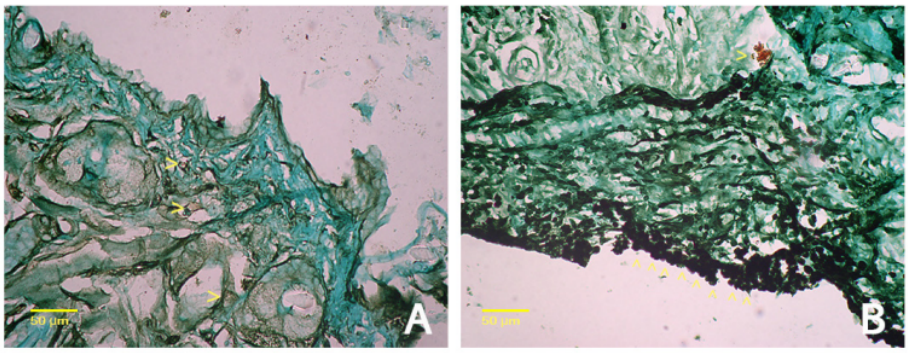


**Figure 1: The p53 expression was analyzed in the OSCC cells with immunohistochemistry.** (A) control groups (B) Meloxicam 200 mg/kg.b.w. the yellow arrow showed a positive cell. Magnification at 400x.

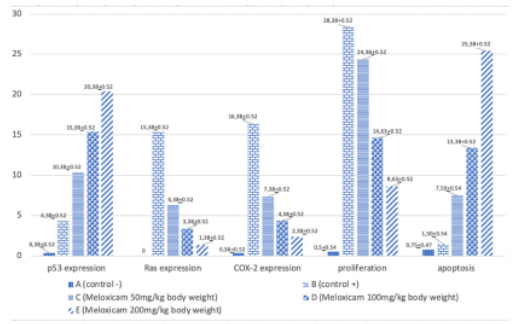


**Figure 2: The p53, Ras, COX-2, proliferation and apoptosis of OSCC.** The expression was analyzed in the OSCC cells with immunohistochemistry

The COX-2 expression in the group E (Meloxicam 200 mg/kg body weight) is lower than group D (Meloxicam 100 mg/kg.b.w) (p=0.01) and group C (Meloxicam 50 mg/kg.b.w) (p=0.001).

The proliferation of OSCC in the group E (Meloxicam 200 mg/kg.b.w) is lower than group D (Meloxicam 100 mg/kg.b.w) (p=0.02) and group C (Meloxicam 50 mg/kg.b.w) (p=0.000).

## DISCUSSION

This study explains the potential of meloxicam on inhibiting the OSCC induced by benzopyrenes by analyzed the difference of p53, Ras, COX-2 expression, apoptosis and proliferation of OSCC. The chemical stimulus in the oral epithelial such as benzopyrenes, which found in cigarettes, for a long time can causes changes in the gene coding for the molecule in G1 phase in the cell cycle. Especially in the phase of the restriction point that is the G1 phase to the S phase, where the DNA damage occurs and if not repaired there will be mutations in various negative molecular regulatory genes in the p53 and p21 pathway (8). The protein product of the p53 tumor suppressor gene is a WAF1 / CIP1 gene transcription factor to produce p21. The p21 gene has a mechanism of action that can directly inhibit excessive cell proliferation activity in the cell cycle. The p21 protein product can bind to CDK, so that the CDK-cyclin complex loses kinase activity, consequently RB phosphorylation does not occur and the cell cycle stops temporarily in the G1 phase. During the cell cycle stops, DNA repair works to repair the damage that occurs in DNA. If the repair is successful the cell cycle is continued into the S phase but if a failure occurs, the cell will program cell death with the apoptotic mechanism (9).

In this study it was found that the increase of Meloxicam dose was proportional to the increase in wild p53 expression and apoptosis, inversely proportional to the Ras, COX-2 and proliferation of OSCC. In this stage, we can inhibit the growth of OSCC induced by benzopyrenes. The dose of meloxicam in this study was 50 mg/kg.b.w until 200 mg/kg.b.w. The rational of this dose because the acute oral toxicity for meloxicam has been investigated in rats was greater than 200 mg/kg body weight and 98.4 mg/kg body weight for males and females, respectively (10).

Meloxicam can inhibit the growth of OSCC through the inhibition of COX-2 and also COX-2 independent through activating PPARγ activity. Meloxicam as partial selective COX-2 inhibitor that catalyzes the oxidation of B[a]P-7,8-dihydrodiol to B[a]P-diolepoxide (11). Meloxicam works by penetrating the hydrophobic channel blocking the arachidonate entrance in the active site COX-2 (12). Meloxicam is a selective COX-2 inhibitor, so that it can suppress PGE2 and activate PPARγ. Besides that, Meloxicam is suspected to be able to directly activate PPARγ without going through the COX-2 inhibition mechanism. The Over-expression of PPARγ also as indicator of reduce the cancer growth. COX-2 inhibition causes PPARγ activation (13). Other

mechanism explained that, Meloxicam able to inhibit the mechanism of Ras-Raf-Mitogen Activated Protein Kinase (MAPK)/ Erk kinase (MEK) - Mitogen Activated Protein Kinase (MAPK) - Murine Double Minute (MDM2) which can trigger activation of p53 (14). P53 expression causes transcription of the Bax gene to increase and transcription of the Bcl-2 gene to decrease. Bcl-2 is a Bax inhibitor, preventing Bax from forming homodimers by binding and heterodimers. Bax triggers the release of cytochrome-c from mitochondria with ATP binding to apoptotic protease activating factor 1 (Apaf 1) activating cysteine proteases with aspartate specificity caspase 9 and caspase 3. Caspase activity will cut DNA into fragments with 3OH ends and various apoptotic morphological changes resulting in an increase in apoptosis (15). In addition, activation of p53 induces p21 transcription factor so that it inhibits CDK-4 and CDK-6 in G1-phase, CDK-2 in S-phase and CDK-1 in M-phase. This causes no complex bonding between the CDK and cyclin so that the cell division cycle stops and cancer cell proliferation is inhibited (16).

## CONCLUSION

It can be concluded that there are differences in the number of cell proliferation, apoptosis, p53, Ras and COX-2 expression with meloxicam treatment in OSCC induced by benzopyrene. The increase in Meloxicam dose is proportional to the increase in wild p53 expression and apoptosis, and inversely proportional to the Ras, COX-2 expression and the proliferation of OSCC. Further research needs to be done on the mechanism of action of various gene expression that affects the mechanism of apoptosis and OSCC proliferation due to benzopyrenes exposure.

## ACKNOWLEDGEMENT

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