Do NSAIDs Inhibit Growth of Precancerous Cervical Cells *In Vitro*?

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Background: *Cervical cancer is one of the most common cancers worldwide. A promising, novel strategy for cancer treatment is chemoprevention. Non-steroidal anti-inflammatory drugs (NSAIDs) have chemopreventive effects on several cancers including those of cervix. There are few clinical trials of the effects of NSAIDs on precancerous cervical lesions but data from in vitro studies are lacking.*
Objective: *To study growth inhibitory effects of nonselective and selective NSAIDs on immortalized cervical cells in vitro.*
Material and Method: *Cytotoxicity of ibuprofen and celecoxib on immortalized cervical cells was analyzed by Cell Proliferation (MTT) Assay. Propidium Iodide (PI) Assay was used to analyze apoptotic cell death.*
Results: *Ibuprofen and celecoxib had significant growth inhibitory effects with IC_{50} of 3.00 $\pm$ 0.44 mM and 30.00 $\pm$ 11.00 μM, respectively. Both drugs significantly induced apoptosis.*
Conclusion: *Ibuprofen and celecoxib can inhibit growth and induce apoptotic cell death in immortalized cervical cells. Results from the present study highlight the need for further in vivo researches and clinical trials in search of novel strategies for cervical cancer prevention.*

Keywords: *Precancerous cervical cells, Cervical dysplasia, Immortalized cervical cells, Cervical intraepithelial neoplasia,, CIN, Squamous intraepithelial lesion, Cervical cancer, NSAIDs, Non-steroidal anti-inflammatory drugs, Ibuprofen, Celecoxib, Chemoprevention, Growth inhibition, Cancer prevention*

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Cervical cancer is one of the most common cancers worldwide. According to the latest survey by the International Agency for Research on Cancer (IARC), it is the second most common cancer among women in developing countries[1]. Human Papilloma Virus (HPV) is currently considered to be its most important causative agent[2]. Cervical infection by HPVs can initially result in low-grade lesions termed mild dysplasia or cervical intraepithelial neoplasia grade I (CIN I). These lesions exhibit only mildly altered patterns of differentiation and many of them regress to normal[3]. However, some of these lesions persist for several decades and may turn to be high-grade intraepithelial lesion (CIN II, III) or moderate to severe dysplasia, and eventually invasive cancer, if left untreated[4]. Standard treatment of CIN II, III include ablation and cervical conization[5]. However, these procedures may induce adverse reproductive consequences[6,7], thus alternative treatment would be beneficial. Although HPV vaccine is a new strategy to prevent cervical cancer, its effects will be known after decades of follow and it will be too expensive for developing countries[8-10]. Another promising, novel strategy for cancer treatment is chemoprevention.

Chemoprevention is the use of natural or synthetic chemicals to prevent, suppress, or reverse the process of carcinogenesis[11,12]. In the group of synthetic chemicals, non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used[13]. NSAIDs are a

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chemically heterogenous group of compounds, characterized by varying degree of anti-inflammatory and analgesic activity. Mechanism of their therapeutic effects is the inhibition of prostaglandin (PG) synthesis. PGs are lipid mediators derived from arachidonic acid by the action of cyclooxygenase (COX)-1 and COX-2[14]. COX-1 plays a role in cytoprotection of gastric mucosa, platelet aggregation and renal blood flow. COX-2 expression is inducible by cytokines, tumor promoters, and mitogens[15]. NSAIDs can be classified by their ability to inhibit COX isoforms. Nonselective NSAIDs, such as aspirin, ibuprofen, inhibit both COX-1 and COX-2. Selective COX-2 inhibitors, like celecoxib do not significantly inhibit COX-1[14]. Although NSAIDs have chemopreventive effects on several cancers[16-21] including cervix[22,32], there are very few clinical trials of NSAIDs on precancerous cervical lesions[33,34] and data from *in vitro* studies are lacking. Our objective is to study growth inhibitory effects of nonselective and selective NSAIDs on immortalized cervical cells, the *in vitro* model of precancerous cervical lesion[35].

Material and Method

Cell Culture

The immortalized ectocervical squamous epithelium cells CRL-2614 (American Type Culture Collection, USA) were grown in keratinocyte serum-free medium with penicillin (50 IU/ml) and streptomycin (50 g/ml). SIF-56; a normal human fibroblast cell line, were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) with penicillin (50 IU/ml) and streptomycin (50 g/ml). Both cells were maintained at 37°C in a humidified incubator with a 5% CO_2 atmosphere. Cells were seeded at a density of 3.3 x 10^4 cells/cm^2 in the appropriate culture vessels 24 hours before drug treatment. After 24 hours, fresh medium containing ibuprofen or celecoxib of various concentrations was added and cells were incubated for 24 or 48 hours.

Drug Treatment

Ibuprofen (Sigma Chemical, St. Louis, MO) and celecoxib (Pharmacia, USA) were separately dissolved in 100% dimethyl sulfoxide (DMSO) at various desired concentrations, filter-sterilized (0.2 µM Millipore) and were stored in dark-colored bottles at -20°C as stock solutions. The stock was diluted to the required concentration immediately before use with growth media. The cells were exposed to the drug at different concentrations for 24 or 48 hours. Cells grown in media containing 0.1% DMSO served as control.

Cell Proliferation (MTT) Assay

Cells were seeded in 96-well plates at a density of 3.3 x 10^4 cells/cm^2 (10^4 cells/well) and were allowed to grow for 24 hours then fresh medium containing drugs at various concentrations was added and the plates were incubated for 24 or 48 hours. In each plate, different concentrations of each drug were applied in triplicate. At the end of the drug exposure period, the medium was removed from all the wells containing cells and 200 µl of fresh medium was fed to each well then the cells were allowed to grow for additional 24 hours. The plates were fed with 200 µl/well of fresh medium at the end of the growth period and 50 µl of MTT (3,4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide) solution (Sigma Chemical Co, St. Louis, MO) was added to each well. The plates were then wrapped in aluminium foil and were incubated for 4 to 8 hours in a humidified atmosphere at 37°C. The medium containing MTT was removed from the wells and the remaining MTT-formazan crystals were dissolved by adding 200 µl of DMSO with 25 µl glycine buffer to each well. The absorbance at a wavelength of 570 nm was recorded immediately using a microplate reader. The wells which contained medium and MTT but no cells, were used to blank the plate reader.

All experiments were performed in triplicate. The percentage of relative cell viability was calculated by using the following formula:

$$\% \text{ relative cell viability} = \frac{\text{Optical density of treated sample}}{\text{Optical density of untreated sample}} \times 100$$

For each drug, a graph of the absorbance (y-axis) against the concentration of drug (x-axis) was plotted. The mean absorbance reading from the blanks was used as the control absorbance and the IC_{50} was determined as the drug concentration that was required to reduce the absorbance to half that of the control. The data were then converted to a percentage-inhibition curve.

Cellular Morphology

Cells were seeded in 6-well plates at a density of 3.3 x 10^4 cells/cm^2 (3.3 x 10^5 cells/well) and were allowed to grow for 24 hours then fresh medium containing drugs at the concentration of IC_{50} or two-fold of IC_{50} was added and the plates were incubated for 24 hours at 37°C in a humidified incubator with a 5% CO_2 atmosphere. At the end of drug exposure time, changes in morphology of treated cells compared to those of untreated cells were observed under phase-contrast light microscope.

In Vitro Apoptotic Assay (Propidium Iodide (PI) Assay)

Cells were seeded in 6-well plates at a density of 3.3 x 10^4 cells/cm^2 (3.3 x 10^5 cells/well) and were allowed to grow for 24 hours then fresh medium containing drugs at the concentration of IC_{50} or two-fold of IC_{50} was added and the plates were incubated for 24 hours at 37°C in a humidified incubator with a 5% CO_2 atmosphere. The cells fed with culture medium containing 0.1% DMSO were used as controls.

To detect DNA strand breaks, which represent apoptosis, at the end of the drug exposure time the treated and untreated cells were collected from culture plates by trypsinization. The trypsinized cells and cells floating in medium were pooled together and the cell suspension was centrifuged 6 min at 200 x g at room temperature. After the supernatant was removed, cells were suspended in 0.5 ml PBS then cells were fixed by adding suspension to 4.5 ml of 70% ethanol in a 12 x 75-mm tube on ice. Cells were centrifuged 5 min at 200 x g, ethanol was decanted, the cell pellet was suspended in 5 ml PBS and centrifuged 5 min at 300 x g, room temperature. Cell pellet was suspended in 1 ml of propidium iodide staining solution, containing propidium iodide (PI) 100 µg/ml; DNase-free RNase-A (Sigma Chemical, St. Louis, MO) 50 µg/ml and 0.1% (v/v) Triton X-100 in PBS. After incubated in the dark for 15 min at 37°C the percentage of apoptotic cells was analyzed using the FACSCalibur Flow Cytometer (Becton Dickinson, USA) and CellQuest 3.0.1 software (Becton Dickinson, USA). All experiments were performed in duplicate.

Statistical analysis

Unless otherwise specified, data were presented as mean ± standard error of mean (SEM). The analysis of variance (ANOVA) test was used to analyze the significance of differences between the observed data. P-value of less than 0.05 was considered to be statistically significant.

Results

Cell Proliferation (MTT) Assay

Both ibuprofen and celecoxib showed significant growth inhibitory effects on CRL-2614 cells. Percentage of cell viability of CRL-2614 after the treatment with ibuprofen at the concentration of 0.1, 0.5, 1 and 5 mM for 24 and 48 hours using the cells treated with 0.1% DMSO as the control was significantly decreased in dose-dependent manner (Fig. 1). The CRL-2614 cells treated with celecoxib at the concentration of 1, 5, 10 and 50 µM for 24 and 48 hours showed significant decrease in percentage of cell viability in dose-dependent manner compared to the control (Fig. 2). Cytotoxic effect of each drug on CRL-2614 cells and normal fibroblastic cells (SIF-56) were compared (Fig. 3, 4). Both ibuprofen and celecoxib showed less

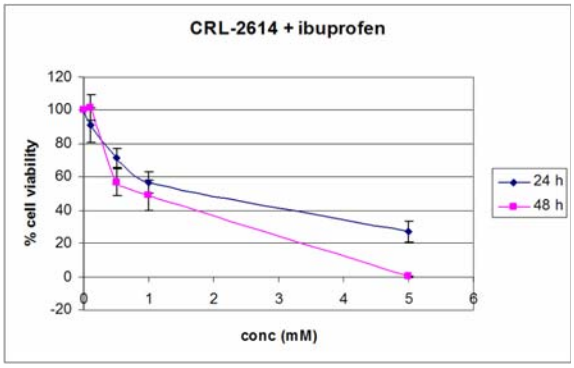

Fig. 1 Effect of ibuprofen on cellular proliferation of CRL-2614 compared to untreated cells. CRL-2614 were seeded in 96-well plate at the density of 1 x 10^4 cells/well with keratinocyte serum-free medium containing ibuprofen at different concentrations of 0.1, 0.5, 1 and 5 mM for 24 and 48 h. After drug exposure, the cells were allowed to grow for 24 h and cell viability was determined by MTT assay. Each point represents the mean ± SEM of data from three independent experiments

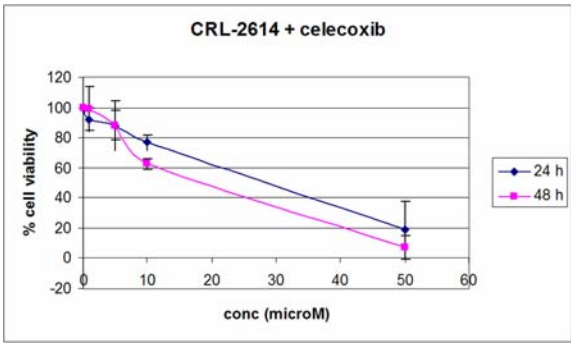

Fig. 2 Effect of celecoxib on cellular proliferation of CRL-2614 compared to untreated cells. CRL-2614 were seeded in 96-well plate at the density of 1 x 10^4 cells/well with keratinocyte serum-free medium containing celecoxib at different concentrations of 1, 5, 10 and 50 µM for 24 and 48 h. After drug exposure, the cells were allowed to grow for 24 h and cell viability was determined by MTT assay. Each point represents the mean ± SEM of data from three independent experiments

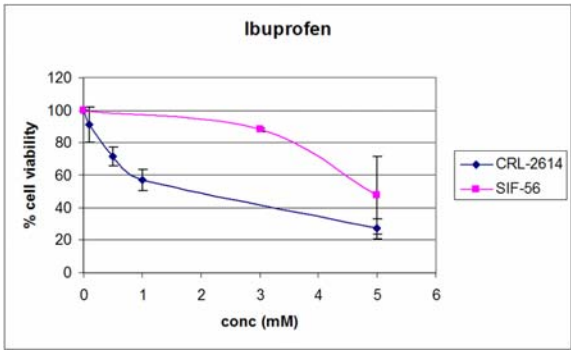

Fig. 3 Effect of ibuprofen on cellular proliferation of CRL-2614 compared to SIF-56; the normal human fibroblasts. Cells were seeded in 96-well plate at the density of 1 x 10^4 cells/well with keratinocyte serum-free medium (CRL-2614) or DMEM supplemented with 10% FBS (SIF-56) containing ibuprofen at different concentrations for 24 h. After drug exposure, the cells were allowed to grow for 24 h and cell viability was determined by MTT assay. Each point represents the mean ± SEM of data from three independent experiments

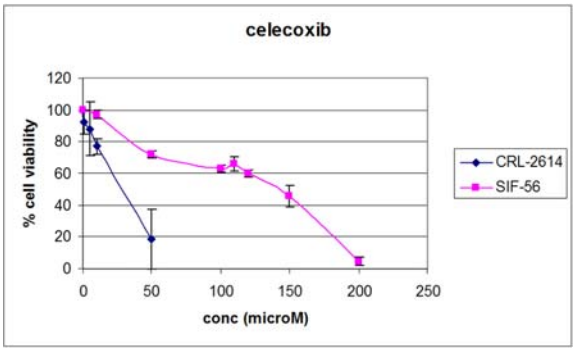

Fig. 4 Effect of celecoxib on cellular proliferation of CRL-2614 compared to SIF-56; the normal human fibroblasts. Cells were seeded in 96-well plate at the density of 1 x 10^4 cells/well with keratinocyte serum-free medium (CRL-2614) or DMEM supplemented with 10% FBS (SIF-56) containing celecoxib at different concentrations for 24 h. After drug exposure, the cells were allowed to grow for 24 h and cell viability was determined by MTT assay. Each point represents the mean ± SEM of data from three independent experiments

significant growth inhibitory effect on normal fibroblasts compared to the CRL-2614 cells.

To analyze the IC_{50} value of each drug, the 24 hour-data were used and for CRL-2614 cells, the IC_{50} value of ibuprofen and celecoxib was 3.00 ± 0.44 mM, and 30.00 ± 11.00 μM, respectively. In SIF-56 cells, the IC_{50} value of ibuprofen and celecoxib was 5.87 ± 0.62 mM and 145.94 ± 11.02 μM, respectively (Table 1).

Cellular morphology

After incubated with drugs at concentration of IC50 and two-fold of IC50 for 24 hours, the treated cells revealed morphologic changes of apoptosis such as cytoplasmic blebs, and apoptotic bodies in dose-dependent manner, compared to the control (Fig. 5A, 6A).

Propidium iodide assay

Apoptosis of CRL-2614 in response to ibuprofen and celecoxib at the concentration of IC_{50} and two-fold of IC_{50} was analyzed by PI assay and the results including the percentage of sub-G1 fraction of which represented the percentage of apoptotic cells were shown in Fig. 5B-C, and 6B -C, respectively.

Ibuprofen at the concentration of IC_{50} (3 mM) and two-fold of IC_{50} (6 mM) induced 0.69 ± 0.18 and 4.46 ± 0.98% of apoptosis, respectively. Celecoxib at the concentration of IC_{50} (30 μM) and two-fold of IC_{50} (60 μM) induced 0.68 ± 0.38 and 5.77 ± 0.49% of apoptosis, respectively. Compared to the control group, ibuprofen at the concentration of 6 mM significantly induced apoptosis of CRL-2614 cells ($p = 0.025$). In case of celecoxib, the dosage of two-fold of IC_{50} (60 μM) significantly induced the apoptotic cell death ($p = 0.003$).

Discussion

COX-2 overexpression has been demonstrated in various types of cancers and precancerous lesions including those of head and neck, skin, lung, liver, pancreas, urinary bladder, stomach and cervix[36-40]. Both classes of NSAIDs have been reported to inhibit several cancers *in vitro*, *in vivo* and at clinical level[16-21]. Most studies of chemopreventive effects of

Table 1. IC_{50} values of ibuprofen and celecoxib against immortalized ectocervical cells (CRL-2614) and fibroblasts (SIF-56)

Drug	IC_{50}	
	CRL-2614	SIF-56
Ibuprofen (mM)	3.00 ± 0.44	5.87 ± 0.62
Celecoxib (μM)	30.00 ± 11.00	145.94 ± 11.02

Results represent the mean ± SEM of data from three independent experiments

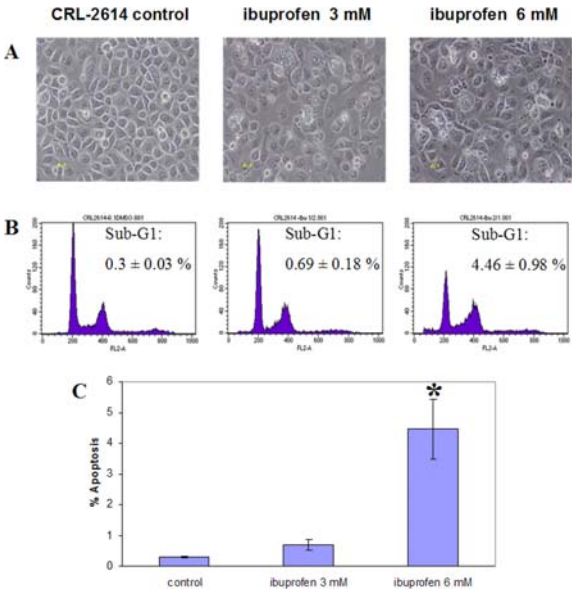

Fig. 5 Apoptosis of CRL-2614 in response to ibuprofen at the concentration of IC_{50} (3 mM) and two-fold of IC_{50} (6 mM). The cells were seeded in 6-well plate at the density of 3.3 x 10^5 cells/ well, fed with keratinocyte serum-free medium and were allowed to grow for 24 h then fresh medium containing ibuprofen at the concentration of 3 or 6 mM was added and plates were incubated for 24 h at 37°C in a humidified incubator with a 5% CO_2 atmosphere. After drug exposure, cells were trypsinized, pooled with nonadherent cells from the media, washed, stained with PI and percentage of apoptotic cells (sub-G1 fraction) was analyzed by flow cytometer. The cells fed with culture medium containing 0.1% DMSO were used as controls. (A) Cellular morphology of treated cells compared to the control, visualized by phase-contrast microscope. (B) Percentage of the cells in sub-G1 fraction. (C) Bar diagram comparing the percentage of apoptosis between the treated cells and control. The results are mean ± SEM of two individual experiments: * $p < 0.05$

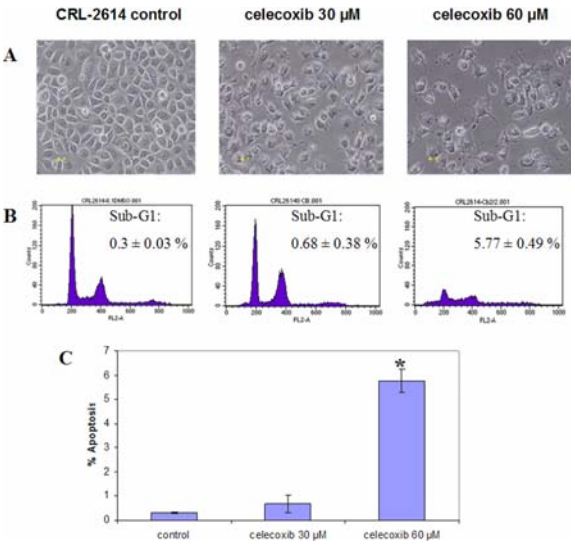

Fig. 6 Apoptosis of CRL-2614 in response to celecoxib at the concentration of IC_{50} (30 µM) and two-fold of IC_{50} (60 µM). The cells were seeded in 6-well plate at the density of 3.3 x 10^5 cells/well, fed with keratinocyte serum-free medium and were allowed to grow for 24 h then fresh medium containing celecoxib at the concentration of 30 or 60 µM was added and plates were incubated for 24 h at 37°C in a humidified incubator with a 5% CO_2 atmosphere. After drug exposure, cells were trypsinized, pooled with nonadherent cells from the media, washed, stained with PI and percentage of apoptotic cells (sub-G1 fraction) was analyzed by flow cytometer. The cells fed with culture medium containing 0.1% DMSO were used as controls. (A) Cellular morphology of treated cells compared to the control, visualized by phase-contrast microscope. (B) Percentage of the cells in sub-G1 fraction. (C) Bar diagram comparing the percentage of apoptosis between the treated cells and control. The results are mean ± SEM of two individual experiments: * $p < 0.05$

NSAIDs in cervical neoplasms have been focused on cancer cell lines or in invasive cancer patients. Studies in cervical cancer cell lines have shown growth inhibitory effect and apoptosis induction of celecoxib[22-24] and aspirin[25-28]. There are few clinical trials in cervical cancer patients. Most of them focused on the effects of celecoxib in combination with chemoradiotherapy[29-31]. Farrandina et al have demonstrated decreased expression of COX-2 and a cell proliferation index in tumor biopsies from the celecoxib-treated cervical cancer patients[32].

There are very few clinical studies of NSAIDs on precancerous cervical lesions. Farley et al[33] reported the efficacy of daily 200-mg celecoxib in the regression of CIN II, III in a randomized double-blind placebo-controlled trial with 2-month intervals follow-up of colposcopy, cervical cytology and cervical biopsy for 6-month period. The treated group (n = 12) revealed significantly higher clinical response than those of the placebo group (n = 13) (75% versus 31%; $p < 0.03$). Moreover, 33% of the patients receiving celecoxib had

complete pathologic response, compared to 15% of the control group. Another prospective, randomized, placebo-controlled, double-blind study of 25-mg daily rofecoxib, a selective COX-2 inhibitor which already withdrawn from the market, in patients with CIN II and III for 6 months revealed higher regression rate in the treated group (n = 8) than that of the placebo group (n = 8) (25% versus 12.5%), but no statistical significance was demonstrated[34]. However, studies of the effects of NSAIDs on the precancerous cervical epithelial cells *in vitro* are still lacking. Their results may support further application of these drugs for prevention of the malignant transformation of cervical precancerous lesions.

To study the effects of NSAIDs on the precancerous cervical cells, the authors used the immortalized ectocervical epithelium cell line CRL-2614 which has been established from human ectocervical epithelia immortalized by expression of human papillomavirus-16 E6/E7[35] as the *in vitro* model of the precancerous cervical lesion. Expression of HPV E6 and E7 viral oncogenes was shown to increase proliferation capacity of the epithelium but did not result in malignant transformation of the immortalized cell lines. In the literature, HPV-induced immortalized cervical epithelial cells have been used to study chemo-preventive effects of cisplatin[41], arsenic trioxide[42] and retinoic acid[43]. In the present study, both nonselective NSAIDs, ibuprofen; and selective COX-2 inhibitor, celecoxib, show significant growth inhibitory effect on the immortalized ectocervical epithelium cell line with induction of apoptotic cell death. Results from the present study may be applied for further *in vivo* researches and clinical trials in search of new way of cervical cancer prevention. And to avoid the cardio-vascular risks of the selective COX-2 inhibitors, more studies of non-selective NSAIDs should be considered. In addition, alternative drug administration methods such as topical application might be worth a try.

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Potential conflicts of interest

None.

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ยาต้านการอักเสบที่ไม่ใช่สเตียรอยด์สามารถยับยั้งการเจริญของเซลล์เยื่อบุปากมดลูกระยะก่อนเป็นมะเร็งได้หรือไม่

ดุษฎี สกลยา, ไพรัตน์ ฐาปนาเดโชพล, อรกัญญ์ ภูมิโคกรักษ์, แสงสูรย์ เจริญวิไลศิริ

ภูมิหลัง: *มะเร็งปากมดลูกพบมากเป็นอันดับต้นๆของโลก มีผลการวิจัยว่า ยาต้านการอักเสบที่ไม่ใช่สเตียรอยด์ (NSAIDs) มีฤทธิ์ยับยั้งมะเร็งในหลายอวัยวะรวมทั้งปากมดลูก และมีการศึกษาผลของNSAIDs ทางคลินิกในผู้ป่วยระยะก่อนเป็นมะเร็งปากมดลูก แต่ยังขาดข้อมูลการศึกษาระดับนอกกาย*

วัตถุประสงค์: *เพื่อศึกษาฤทธิ์ของ NSAIDs ต่อการเติบโตของเซลล์เยื่อบุปากมดลูกระยะก่อนเป็นมะเร็ง*

วัสดุและวิธีการ: *วิเคราะห์ฤทธิ์ความเป็นพิษของยาไอบูโปรเฟน(ibuprofen)และซีลีคอกซิบ(celecoxib) ต่อเซลล์เยื่อบุปากมดลูกระยะก่อนเป็นมะเร็งโดย Cell Proliferation (MTT) Assay และวิเคราะห์ปริมาณการตายแบบอะพอพโทซิส (apoptosis) โดย Propidium Iodide (PI) Assay.*

ผลการศึกษา: *ยาไอบูโปรเฟนและซีลีคอกซิบสามารถยับยั้งการเติบโตของเซลล์เยื่อบุปากมดลูกระยะก่อนเป็นมะเร็งได้ โดยมีค่า IC_{50} = 3.00 $\pm$ 0.44 mM และ 30.00 $\pm$11.00 μM ตามลำดับ และยาทั้งสองยังเหนี่ยวนำให้เซลล์ดังกล่าวมีการตายแบบอะพอพโทซิส อย่างมีนัยสำคัญ*

สรุป: *ยาไอบูโปรเฟนและซีลีคอกซิบสามารถยับยั้งการเจริญของเซลล์เยื่อบุปากมดลูกในระยะก่อนเป็นมะเร็งได้ และทำให้เซลล์ตายแบบอะพอพโทซิส*