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Original Article

Ibuprofen and diclofenac differentially affect cell viability, apoptosis and morphology changes of human cholangiocarcinoma cell lines

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الملخص

أهداف البحث: سرطان القنوات الصفراوية هو ورم خبيث يصيب القناة الصفراوية الظهارية عن طريق الالتهاب المزمن عن طريق عدوى الدودة المثقبة للكبد. يعتبر هذا السرطان مصدر قلق خطير للصحة العامة في المنطقة الفرعية لميكونغ الكبرى، ولا سيما في شمال شرق تايلاند. تم استخدام سلالات الخلايا البشريةلفحص تأثيرات الإيبوبروفين والديكلوفيناك، الأدوية المضادة للالتهابات غير الستيرويدية على نشاط تكاثر هذه الخلايا.

طرق البحث: تم تقييم صلاحية الخلية بواسطة اختبار "إم تي تي". تم استخدام مجهر ذو ضوء مقلوب متباين الطور ومجهر مسح إلكتروني ومجهر ناقل إلكتروني للتحقيق في التغيرات المورفولوجية للخلايا. وتم الكشف عن إنزيم كاسباس وبروتين أنيكسين بواسطة قارئ الصفيحة الدقيقة متعدد الأوضاع.

النتائج: أظهرت النتائج أن صلاحية كلا الخطين الخلويين قد انخفضت بسبب الإيبوبروفين و ديكلوفيناك. كما أظهرت النتائج أن الخلايا المعالجة بالإيبوبروفين أظهرت إصابة خلية قابلة للإصلاح. في كلا النوعين من الخلايا، كانت الخلايا المعالجة بالديكلوفيناك تحتوي على معظم الخلايا المصابة. الخلايا عرضت ميزات إصابة الخلية التي لا رجعة فيها. بالإضافة إلى ذلك، تم الكشف عن إنزيم كاسباس وبروتين أنيكسين في هذا التحقيق عن خصائص موت الخلايا المبرمج في وقت مبكر.

الاستنتاجات: تشير هذه النتائج إلى أن الأيبوبروفين والديكلوفيناك يمكن أن تثبط قابلية الخلايا السرطانية للحياة، حيث تشير هذه النتائج ضمنيا إلى أن هاذين الدوائين كلاهما يسببان تغيرا مورفولوجيا وموت الخلايا المبرمج.

الكلمات المفتاحية: موت الخلايا المبرمج؛ سرطان القنوات الصفراوية؛ ديكلوفيناك؛ ايبوبروفين؛ تغيرات شكلية

Abstract

Objectives: Cholangiocarcinoma is a malignant biliary epithelial duct neoplasm caused by chronic inflammation after liver fluke infection. It is a major public health concern in the Greater Mekong sub-region in northeast Thailand. Herein, the effects of the non-steroidal anti-inflammatory drugs (NSAIDs) ibuprofen and diclofenac on the cell proliferation activity of the human cholangiocarcinoma cell lines KKU-M139 and KKU-213B were studied.

Methods: Cell viability was assessed with MTT assays. Inverted phase-contrast light microscopy, scanning electron microscopy and transmission electron microscopy were used to investigate the cells' morphological alterations. Caspase 3/7 and Annexin V/PI were detected with a multimode microplate reader.

Results: Ibuprofen and diclofenac decreased viability in both cell lines, and ibuprofen-treated cells exhibited reversible cell injury. In both KKU-M139 and KKU-213B cell lines, the diclofenac-treated cells had the greatest injury. The cells exhibited features of irreversible cell injury. In addition, caspase 3/7 and Annexin V/PI detection revealed early cell apoptotic characteristics.

Conclusion: These findings suggest that NSAIDs may potentially suppress cell viability. Ibuprofen and diclofenac both induced morphological changes and apoptosis.

Keywords: Apoptosis; Cholangiocarcinoma; Diclofenac; Ibuprofen; Morphological changes

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Introduction

Cholangiocarcinoma (CCA) is a malignancy of the biliary epithelial cells with poor prognosis.[1–4] This cancer is one of the leading causes of mortality in Thailand. According to reports, this region has the highest rate of CCA cancer via chronic inflammation inducing by liver fluke infection in the world (*Opisthorchis viverrini*).[2,5] Aspirin has been demonstrated to have anti-proliferative and anti-inflammatory properties.[6] Both ibuprofen, a propionic acid derivative, and diclofenac, a phenylacetic acid derivative, are steroidal anti-inflammatory drugs (NSAIDs) with the same therapeutic uses as aspirin. These drugs inhibit the activity of cyclooxygenase enzymes (COX-1 and COX-2), which are involved in the synthesis of inflammatory stimulants, such as prostaglandins.[7] Although ibuprofen and diclofenac are in the same class as aspirin, they have lower adverse effects, whereas long-term use of aspirin irreversibly prevents the formation of thromboxane A2 in platelets.[8] The effects of ibuprofen and diclofenac have not been investigated in CCA. Therefore, in this study, we investigated the effects of ibuprofen and diclofenac on the proliferation activity of the human CCA cell lines KKU-M139 and KKU-213B through cell viability assays, and determined morphological changes through inverted phase-contrast light microscopy, scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Caspases and Annexin V/propidium iodide (PI) markers were assayed to evaluate apoptosis in cells with a multimode microplate reader.

Materials and Methods

Cell culture

The KKU-M139 and KKU-213B human intrahepatic CCA cell lines were used in this study. The cell lines were established from patients with CCA living in opisthorchiasis-endemic districts in northeastern Thailand. Faculty of Medicine, Khon Kaen University, KKU-M139 is from squamous carcinoma, whereas KKU-213B is from an adenocarcinoma that was moderately differentiated. The cell lines were cultured at 37 °C under 5% CO_2 in Ham's F12 medium containing 100 U/ml penicillin and 100 µg/ml streptomycin with 10% fetal bovine serum.

Chemicals

Ibuprofen (Sigma−Aldrich, product no. I4883) and diclofenac sodium salt (Sigma−Aldrich, product no. D6899) were used to treat the cells.

Cell viability assays

The cytotoxicity of CCA cell lines exposed to the drugs was determined with MTT assays. Briefly, suspensions of 2×10^4 cells were seeded in 96-well plates. The cells were treated with ibuprofen and diclofenac at concentrations of 0−2 mM for 48 h. Negative control cells were left untreated. The culture medium was subsequently removed, and 20 µl of MTT solution (0.25 mg/ml) was added. The treated cells were further incubated for 4 h. MTT dye converted to formazan crystals by living cells was dissolved in DMSO. The absorbance was measured at 540 nm with a microplate reader (EZ read 2000 microplate reader, Biochrom, Holliston, Germany). The concentrations of drugs required to inhibit cell proliferation by 50% (IC_{50}) were determined by plotting of the percentage of cell growth viability versus drug concentration.

SEM

For SEM, cells were cultured on glass coverslips and treated with ibuprofen and diclofenac (2 mM). The obtained cells were fixed with 2.5% glutaraldehyde overnight and rinsed twice with PBS, postfixed with 1% osmium tetroxide (OsO_4) for 1−2 h, and dehydrated with a concentration gradient of ethyl alcohol. The samples were dried in a K850 (Quorum Technology, UK) critical point dryer. The samples were mounted on stubs and sputter coated with 1−2 nm gold-palladium for approximately 40 s (SPI-MODULE, SPI supply, West Chester, PA).[9] The morphological changes were observed by SEM (Jeol, model JSM-6010LV, Japan).

TEM

Cells were cultured and treated with ibuprofen and diclofenac 2 mM. The obtained cells were harvested by trypsinization and processed for electron microscopy studies as described above for SEM, with a final step of propylene incubation for 10 min. For embedding, the cells were placed in a mixture of resin and propylene at a 1:3, 1:1 and 3:1 ratios for 1 h each, and in pure resin overnight at room temperature, then embedded in pure resin and polymerized in a 60 °C oven for 24−48 h. Ultra-thin sections were cut with an ultramicrotome (Powertome XL, RMC Products) and then stained with uranyl acetate and lead citrate.[9] Morphological changes were observed with TEM (Jeol, model JEM-1400, Japan).

Annexin V-FITC/PI assays

Cells were stained with a FITC Annexin V Apoptosis Kit (cat 556547, BD Pharmingen)[10] according to the manufacturer's instructions. The cells were cultured in 96-well plates (2×10^4 cells/well) and treated with 2 mM of ibuprofen and diclofenac for 24 h. The apoptosis control cells were induced with H_2O_2 (750 µM) for 24 h, and the primary necrosis control cells were heated to 55 °C for 90 min. A multimode microplate reader (BMG, CLARIO Star) was used for intensity analysis.

Caspase assays

Active caspases 3 and 7 were detected with Caspase-Glo 3/7 Assays (Promega, Madison, USA) as recommended by the manufacturer.[10,11] CCA cells (2×10^4 cells/well) were seeded in a 96-well plate and treated for 24 h with 2 mM ibuprofen and diclofenac. The primary necrotic cells (dead cell control) and the apoptotic control cells were produced as described above. The untreated control cells and the

drug-treated cells were incubated with 100 µl of Caspase-Glo 3/7 Reagent and examined with a luminescence microplate reader (BMG, CLARIO Star).

Statistical analysis

All data are expressed as the mean ± SEM or mean ± SD of three independent experiments. Two-way ANOVA was used to compare the scores of significant differences in GraphPad Prism statistical software, version 9. Statistical significance was defined as *p < 0.05, **p < 0.01 and ***p < 0.001.

Results

Cytotoxic effects of ibuprofen and diclofenac on KKU-M139 and KKU-M213B cells

The effects of ibuprofen and diclofenac on the viability of KKU-M139 and KKU-213B cells were assessed with MTT assays, as shown in Figure 1. A slight dose-dependent decrease in cell viability was observed, particularly with diclofenac treatment. The IC_{50} values of ibuprofen and diclofenac were 1.87 mM and 1.24 mM, respectively, in KKU-M139 cells. The IC_{50} values for ibuprofen and diclofenac were 1.63 mM and 1.12 mM, respectively, in KKU-213B cells.

Effects of ibuprofen and diclofenac on cells morphological changes, visualized by light microscopy

The morphology of KKU-M139 and KKU-213B cells was observed under an inverted phase contrast microscope. Untreated KKU-M139 cells showed mainly homogenous flattened morphologies, whereas KKU-213B cells were pleomorphic and larger than KKU-M139 cells. However, both cell lines generally displayed prominent large nuclei with multiple nucleoli.

Both cell lines treated with 500 µM of ibuprofen and diclofenac showed a marked decrease in cell numbers. KKU-M139 and KKU-213B cells showed decreases in size. Alterations in shape were observed, including shrinking and adoption of stellate morphology. The cell foot processes
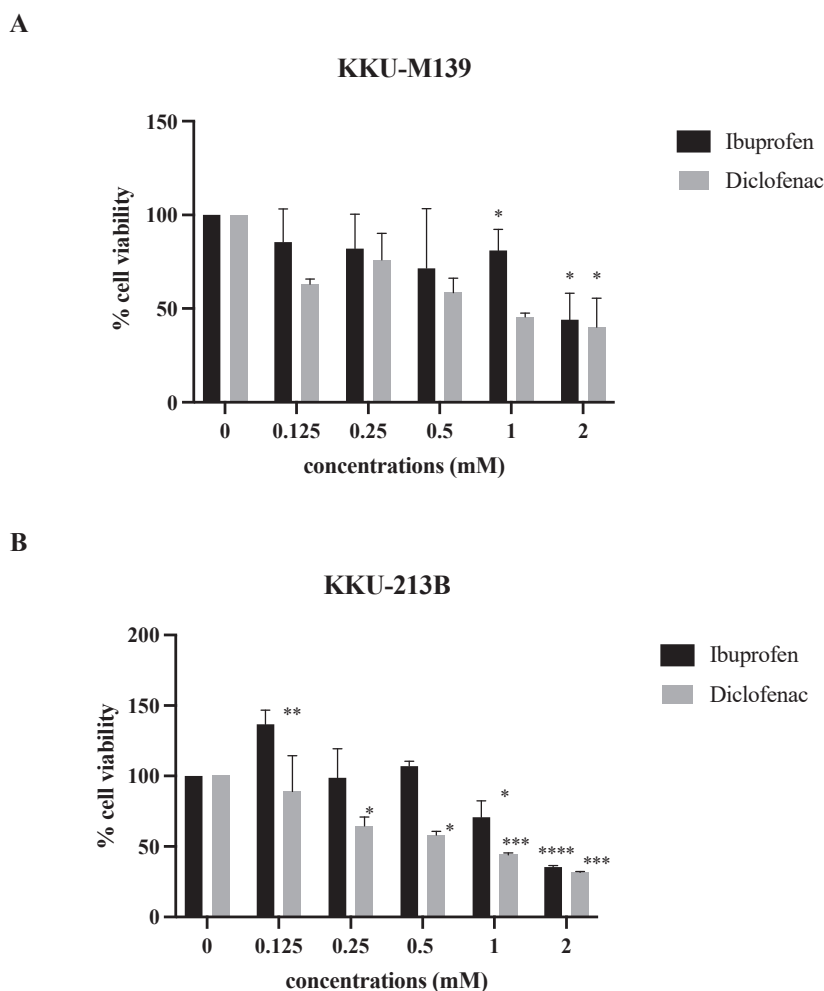
A

B

Figure 1: Effects of ibuprofen and diclofenac on KKU-M139 and KKU-213B cell viability. The data represent the mean ± SEM of three independent experiments. Statistically significant differences are indicated: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 compared with the control group.
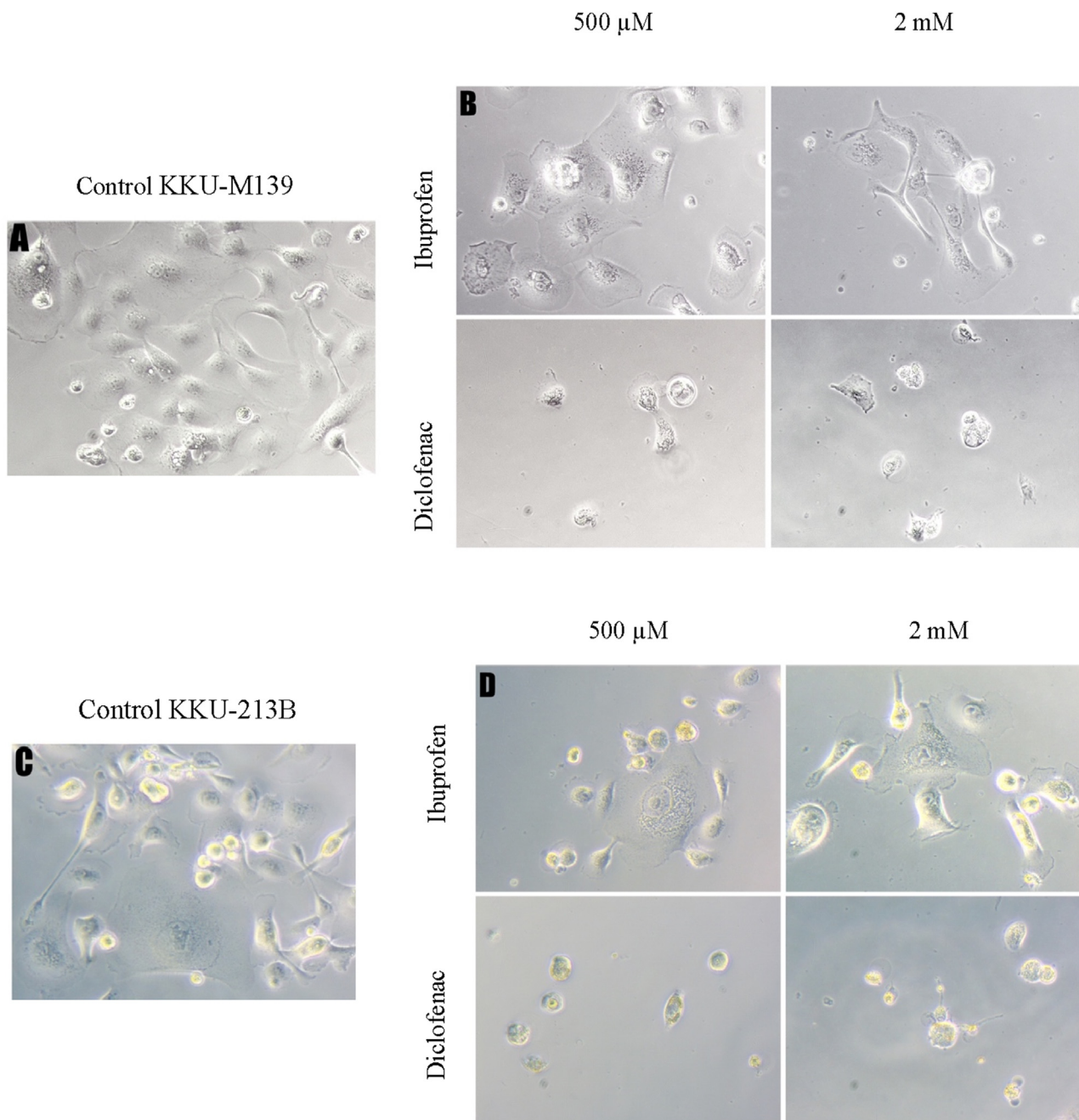
Figure 2: Morphological changes in KKU-M139 and KKU-213B cell lines. (A): Untreated control KKU-M139 cells. (B): KKU-M139 cells treated with ibuprofen and diclofenac concentrations of 500 μM and 2 mM. (C): Untreated control KKU-213B cells. (D): KKU-213B cells treated with ibuprofen and diclofenac with concentrations of 500 μM and 2 mM, respectively (×20 and ×40 magnification).

detached from the surfaces of the flasks, and the cells adopted a round morphology. Numerous cells were observed floating in the liquid medium. The cell number clearly declined at high treatment doses of 2 mM, particularly with diclofenac treatment (see Figure 2).

Effects of ibuprofen and diclofenac on cell morphological changes, visualized by SEM

The surface characteristics of the control KKU-M139 cells revealed a polygonal squamous cell shape. The cells attached to the bottoms of the culture flasks in a brick-like arrangement. Microvilli covered the cell surfaces, and the cells had uniform shape and size. The KKU-213B control cells showed similar features to those of the KKU-M139 cell line, but the cell surfaces had bulges and more microvilli.

Both cell lines treated with ibuprofen and diclofenac at 2 mM showed a significant decrease in the number of cells, particularly under diclofenac treatment. Both cell lines showed characteristics of cell injury under ibuprofen treatment and severe damage due to cell death under diclofenac

treatment. All ibuprofen treated cells showed cell retraction, shrinkage and loss of cell substrate interaction, thus resulting in round cell morphology. Under high dose treatment with diclofenac, numerous cells in both cell lines showed stellate morphology; diminished numbers of microvilli; shorten, unappealing, diffuse blebbing membranes; numerous pores; ruptured membranes and irregular spreading (Figures 3 and 4).

Effects of ibuprofen and diclofenac on cell changes, visualized by TEM

The ultrastructure of the control (untreated) groups of KKU-M139 and KKU-213B cell lines showed features of healthy cells with normal shape and size. Regular size and spreading of microvilli were observed around the cell membrane. The nuclei showed irregular shapes with normal heterochromatin, euchromatin and prominent nucleoli in both cell lines. Several cells had more than one nucleolus. The cell organelles displayed normal features, including numerous mitochondria, rough endoplasmic reticulum, smooth endoplasmic reticulum and vesicles.
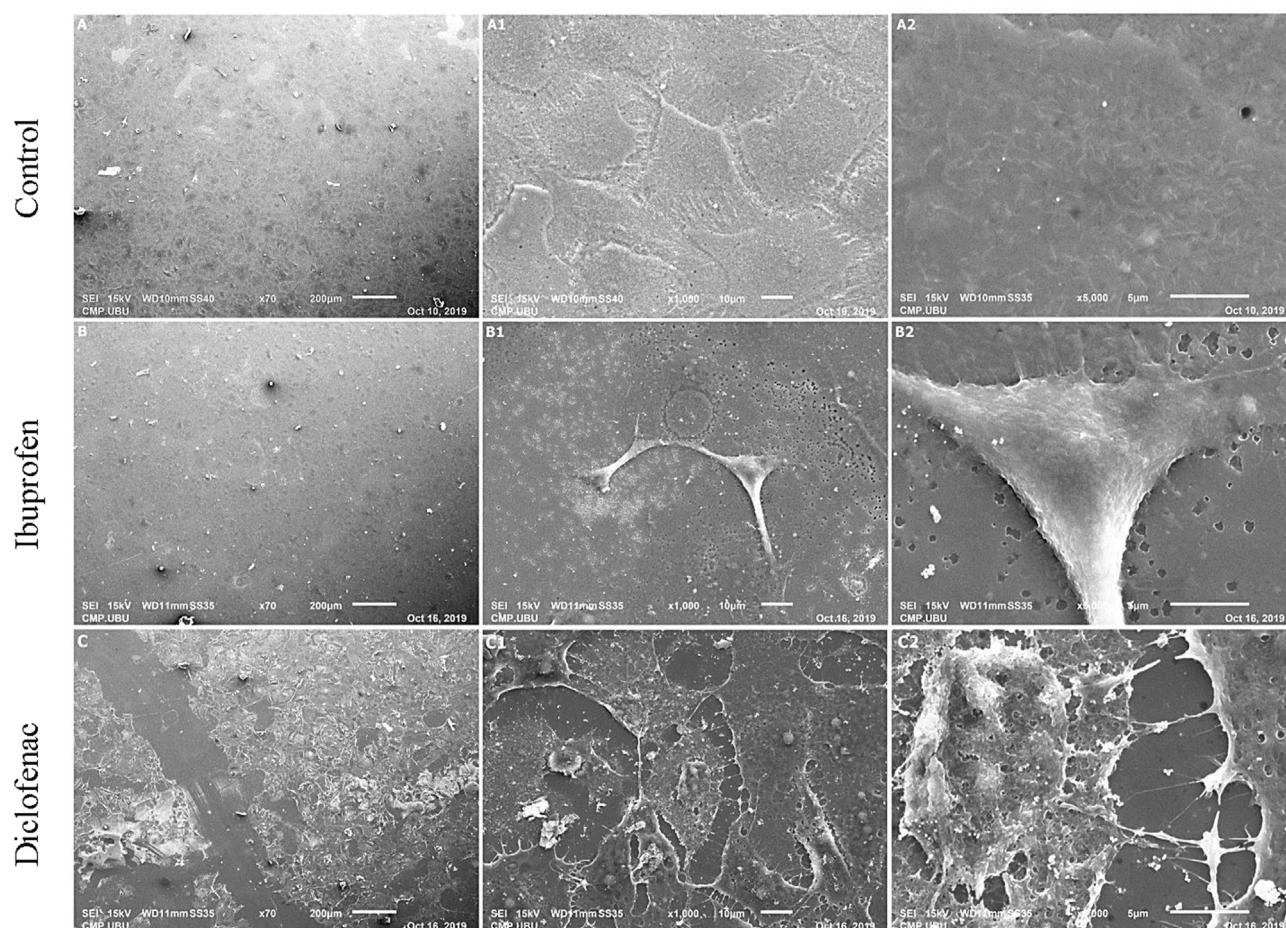
KKU-M139

Figure 3: SEM micrographs of the KKU-M139 cell line. (A, A1, A2): untreated control KKU-M139 cells. (B, B1, B2): KKU-M139 cells treated with 2 mM ibuprofen. (C, C1, C2): KKU-M139 cells treated with 2 mM diclofenac (×70, ×1000 and ×5000 magnification, respectively).
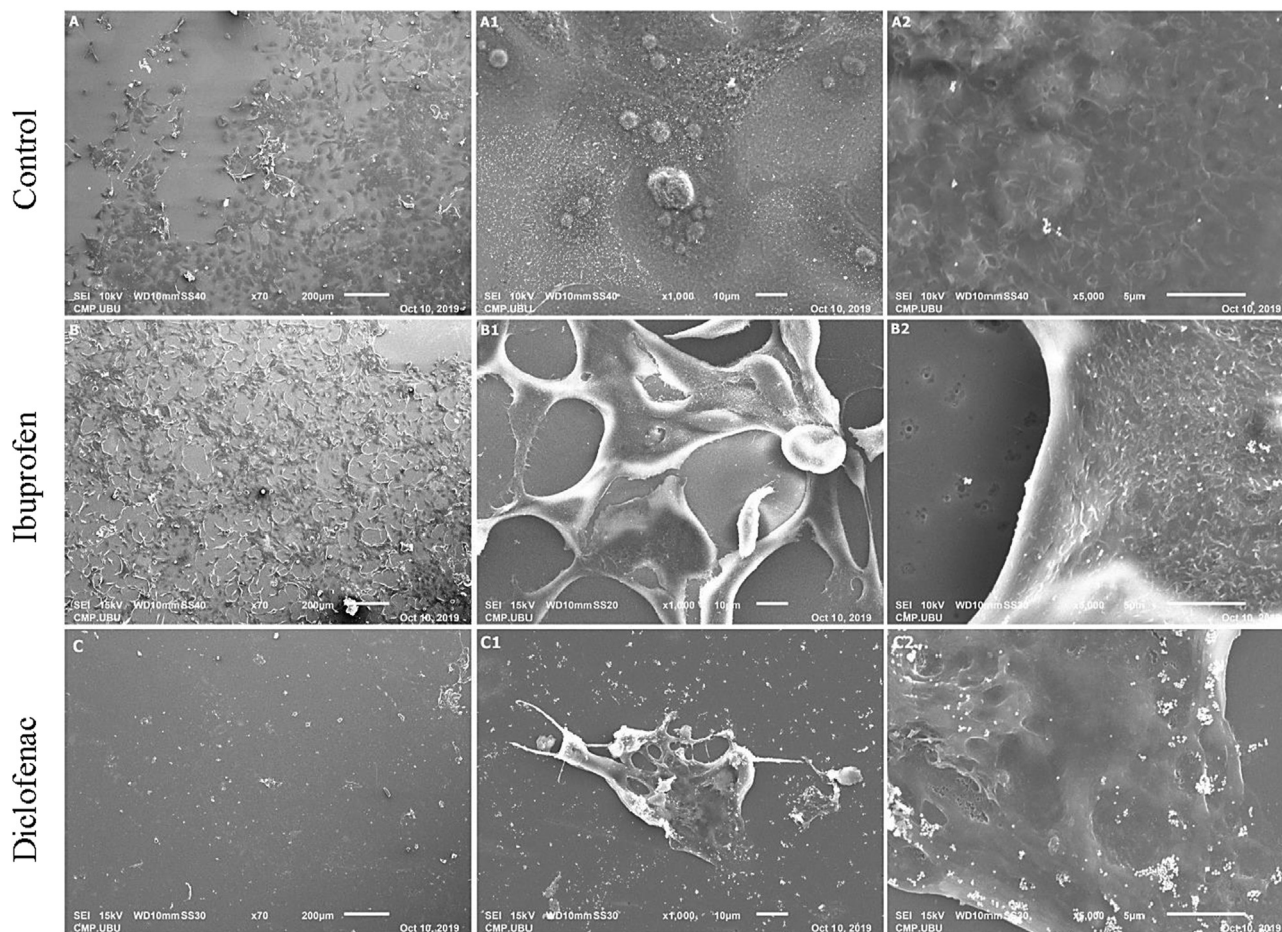
KKU-213B

Figure 4: SEM micrographs of the KKU-213B cell line. (A, A1, A2): untreated control KKU-213B cells. (B, B1, B2): KKU-213B cells treated with 2 mM ibuprofen. (C, C1, C2): KKU-213B cells treated with 2 mM diclofenac (×70, ×1000 and ×5000 magnification, respectively).

In both cell lines treated with a high dose of ibuprofen (2 mM), the morphological changes were similar to those of reversible cell injury. The microvilli appeared healthy but were sparser and shorter than those in the control group. The nuclear membrane and cell membrane remained intact; the nuclei showed slight pyknosis, chromatin condensation, clumping and marginalization; and the nucleoli remained normal. All cell organelles, particularly the rough endoplasmic reticulum, showed swelling, and myelin pattern is caused by increased ribosome dissociation and vacuoles. Notably, the nuclear membranes of KKU-213B cells showed more swelling, and appeared thinner and rounder, than those in KKU-M139 cells. Chromatin aggregation into small clusters, marginalization and lysis were also observed.

Both cell lines treated with diclofenac at 2 mM showed characteristics of irreversible cell injury and cell death. The microvilli were undetectable. The nucleoli were clearly reticular, and the nuclei showed pyknosis, chromatin clumping and marginalization. Degeneration of the nucleoli was observed, with the nuclear membrane still intact. Some cells showed chromatin degradation and reticular nucleoli. Several

cells showed lysis of all cell organelles and rupture of the cytoplasmic membrane. Numerous cells in both cell lines exhibited cytoplasm characteristics of complete lysis (Figures 5 and 6).

Annexin V-FITC and PI assays

The KKU-M139 cell ratios of Annexin V-FITC/PI were as follows: control cells, 85.37/14.63; ibuprofen treated cells, 87.64/12.36; diclofenac treated cells, 82.32/17.68; H_2O_2 treated cells, 88.3/11.7; and 55 °C-heated cells, 50.81/49.19.

The KKU-213B cell ratios of Annexin V-FITC/PI were as follows: control cells, 83.28/16.72; ibuprofen treated cells, 83.26/16.74; diclofenac treated cells, 75.77/24.23; H_2O_2 treated cells, 89.75/10.25; and 55 °C-heated cells, 47.88/52.12.

According to the findings, ibuprofen, diclofenac and H_2O_2 resulted in early apoptotic cell death in the KKU-M139 and KKU-213B cell lines. Both 55 °C-heated cell lines showed late apoptosis/necrotic cell death (Figure 7).
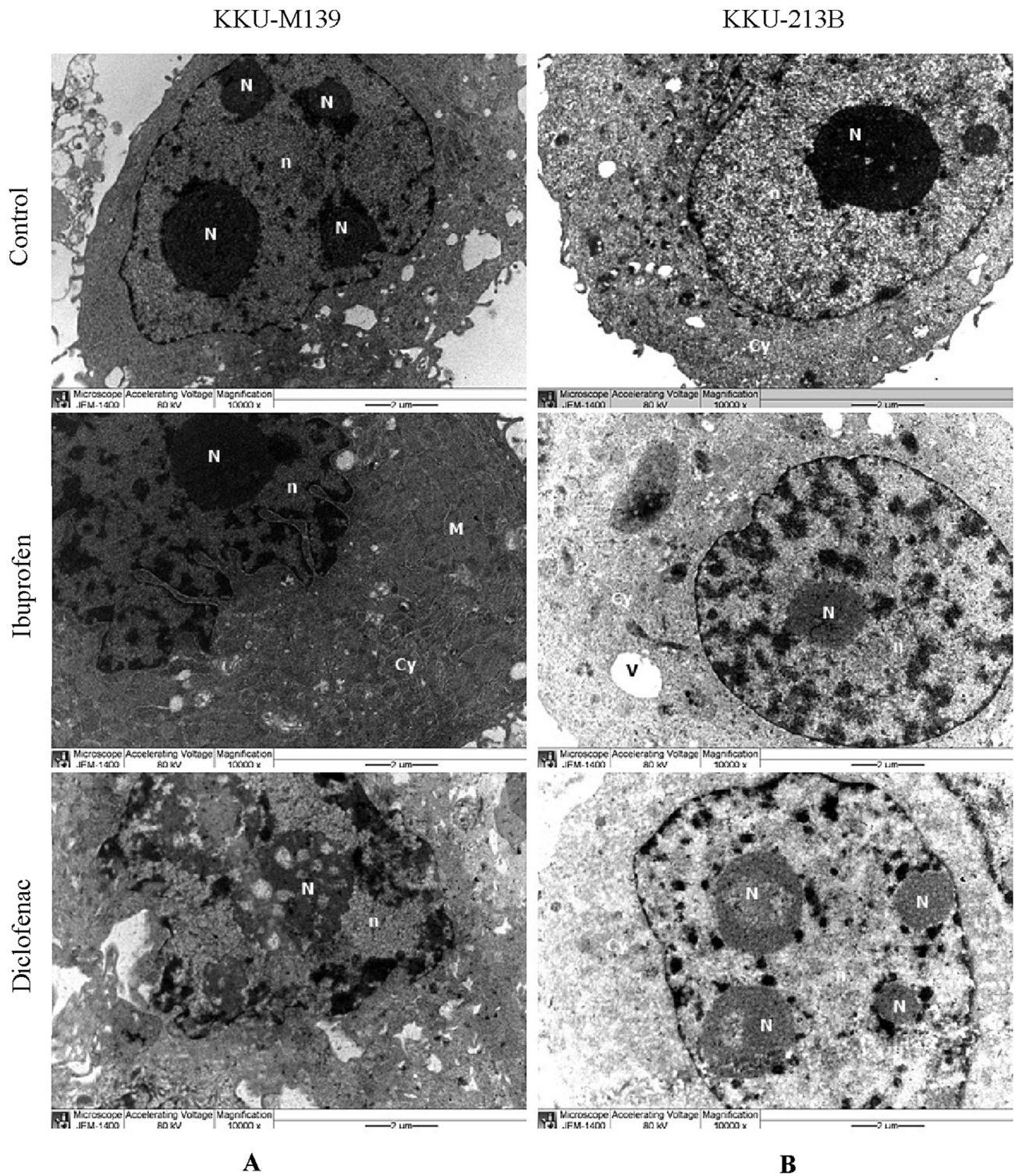
KKU-M139   KKU-213B

Figure 5: TEM micrographs of KKU-M139 and KKU-213B cell lines. (A): KKU-M139 treated with 2 mM ibuprofen and diclofenac. (B): KKU-213B treated with 2 mM ibuprofen and diclofenac. M, mitochondria; n, nucleus; N, nucleolus; Cy, cytoplasm; V, vacuole (×10,000 magnification).

Diclofenac

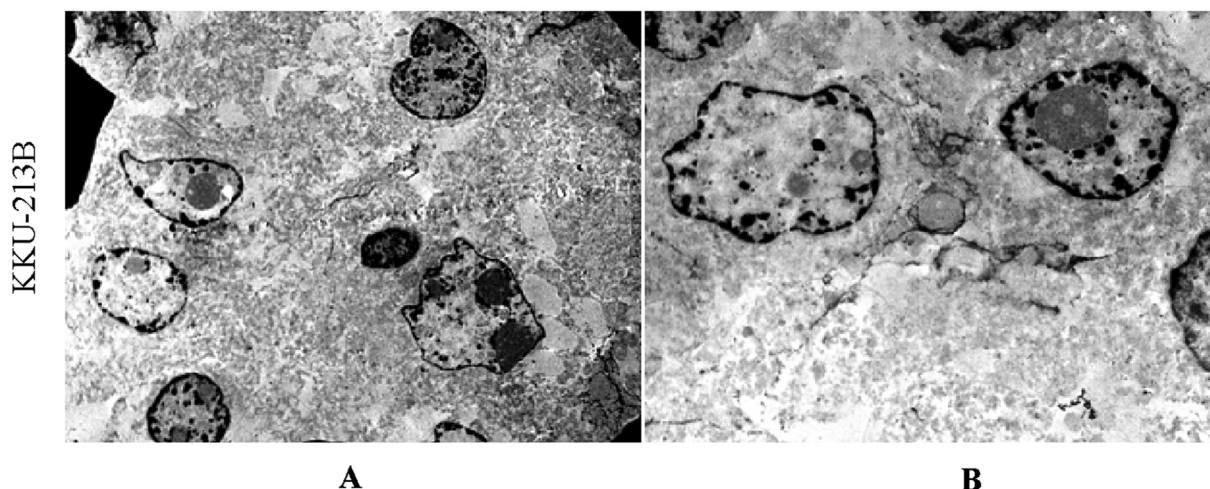**Figure 6:** TEM of KKU-213B cells treated with 2 mM diclofenac, showing nuclear swelling and slightly round shape. (A): ×3000 magnification. (B): ×5000 magnification.

Apoptosis detection by caspase activity assays

The caspase 3/7 activity was highest in both CCA cell lines heated to 55 °C. In KKU-M139 cells, diclofenac treatment and induction by H_2O_2 resulted in higher effective caspase activity than ibuprofen treatment. The caspase 3/7 activity after H_2O_2 treatment of KKU-213B cells was higher than that after NSAID treatment.

These findings suggested that ibuprofen, diclofenac and H_2O_2 treatment induced early apoptosis in both CCA cell lines. In addition, heating to 55 °C induced late apoptosis (Figure 8).

Discussion

Aspirin (acetylsalicylic acid), an antiplatelet and anti-inflammatory drug, has been widely used to reduce pain, fever and inflammation. In addition, aspirin inhibits the activity of cyclooxygenase enzymes (COX-1 and COX-2), which are involved in synthesis of inflammatory stimulants, such as prostaglandin.[7] According to a previous study, aspirin prevents the growth of many types of cancer cells, such as liver, colorectal, skin, breast and pancreatic cancer cells.[12,13] However, the use of aspirin is restricted by its adverse effects with long term use.
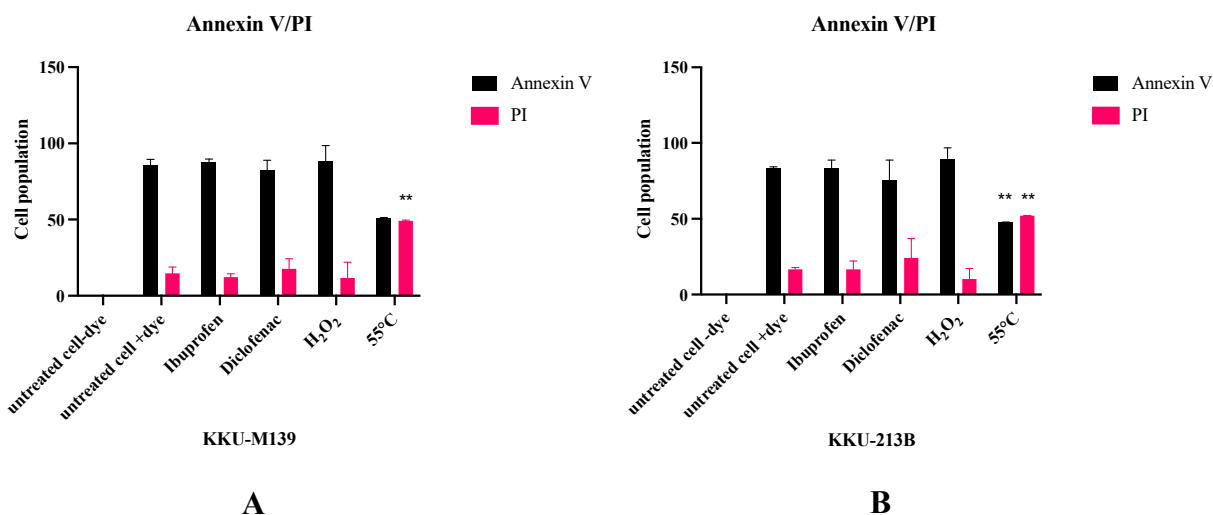

Figure 7: CCA cell lines treated with 2 mM ibuprofen and diclofenac. (A): KKU-M139. (B): KKU-213B. The data represent the mean ± SD of three independent experiments. Statistically significant differences are indicated: **$p < 0.01$ compared with the control group.
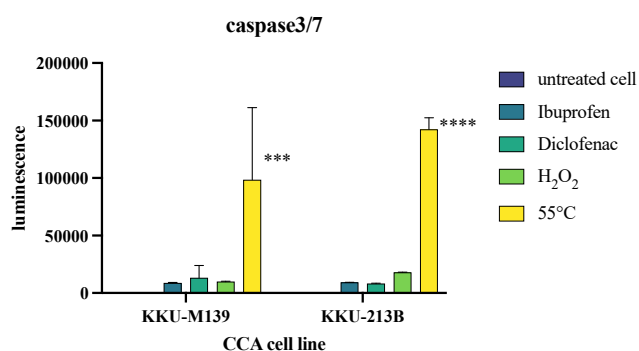
caspase3/7

Figure 8: CCA cell lines treated with 2 mM ibuprofen and diclofenac. The data represent the mean ± SD of three independent experiments. Statistically significant differences are indicated: ***$p < 0.001$, ****$p < 0.0001$ compared with the control group.

Aspirin irreversibly blocks the formation of thromboxane A2 in platelets. Aspirin can be replaced by a variety of drugs, for example, ibuprofen and diclofenac, which have fewer adverse effects.[8]

The present study focused on the effects of ibuprofen, a propionic acid derivative, and diclofenac, a benzene acetic acid derivative. Both drugs are NSAIDs in the same group as aspirin. Several studies have reported that ibuprofen and diclofenac show superior effectiveness to other NSAIDs in suppressing cell proliferation and inducing cell apoptosis in various cancers, such as those of the pancreas, breast and liver.[14,15] Our study indicated that both ibuprofen and diclofenac slightly inhibited the proliferation of KKU-M139 and KKU-213B cells in a dose-dependent manner (0−2 mM), particularly diclofenac. Our results are consistent with those from several previous studies indicating that ibuprofen and diclofenac inhibit the division of cells, such as pancreatic acinar, glioma and endothelial cells, through many mechanisms.[14−16] Our findings were consistent with those of a previous study showing that ibuprofen induces apoptosis in gastric cancer cells.[17,18]

According to light microscopy and inverted phase-contrast microscopy observations, the morphological changes differed with the drug concentration. Both cell lines clearly showed a significant decrease in the number of cells and a pattern of cell shrinkage and detachment from the flask surface. Although these characteristics indicated that the cells had been damaged, the data were insufficient to reveal how the cells were injured and how they changed.

Therefore, SEM and TEM were used to provide qualitative images of the cell surfaces and intracellular organelles at high magnification. These techniques enable morphological studies determining the decrease in proliferation due to treatment with drugs likely to cause cell injury, apoptosis and cell death. The results of SEM surface studies clearly indicated differences in the cell number and cell surface appearance between the control and treated groups. Under high dose ibuprofen, both cell lines showed reversible cell injury and early apoptosis features. Whereas under high dose diclofenac, both cell lines showed irreversible cell injury and cell death characteristics. The presence of a membrane pore

indicates that it may be used for drug transport. Convoluted membranes, diffuse blebbing of membranes, discontinuous membranes and the presence of numerous pores are considered to be typical apoptosis features. These alterations in the membrane, cell shape and organelle distribution patterns result from cytoskeletal rearrangement.[9,19] The TEM ultrastructure investigations revealed that high dose ibuprofen resulted in reversible cell injury, whereas high dose diclofenac resulted in irreversible cell injury and cell death. The current findings were consistent with previously reported results also showing the ultrastructural features[19] in cultured breast cancer cells treated with three commonly used platinum-based drugs. Breast cancer cells have been found to exhibit different responses to the various drugs.[9]

In addition, we observed the effects of high concentration diclofenac (2 mM) on both CCA cell lines. Clearly irreversible cell injury was observed in numerous cells, such as chromatin clumping and marginalization, reticular nucleoli and cell organelle degeneration. These features suggested karyolysis, particularly in KKU-M139 cells. Clear cytoplasm degradation and cell organelle degeneration were observed in both CCA cell lines. Our findings showed that high dose diclofenac decreased proliferation in both cell lines by inducing severe cell injury. This research supports the findings of Al-Bahlani indicating that breast cancer cells respond differently to various medications, as visualized by TEM.[9] In both cell lines, a high dose of ibuprofen induced reversible cell injury, whereas a high dose of diclofenac caused irreversible cell injury and cell death. Nevertheless, the typical apoptosis morphology of cell death is an inconspicuous appearance in this study. In this investigation, the ultrastructural characteristics of apoptotic cell death, nuclear fragmentation, apoptotic vacuoles and increased numbers of lysosomes were not found. Ibuprofen resulted in reversible cell injury, as observed through light microscopy, SEM and TEM.

Caspase 3/7 activity and Annexin V-FITC/PI assays were used to confirm cell apoptosis with a microplate reader and fluorescence detection of caspase activity, as previously described.[20,21] Annexin V-FITC/PI staining is used to detect apoptosis on the basis of staining of phosphatidylserine molecules that have translocated outside the cell membrane. Whereas viable cells with intact membranes exclude PI, the membranes of dead and damaged cells are permeable to PI. Therefore, viable cells (Annexin V-FITC negative, PI negative), early apoptotic cells (Annexin V-FITC positive, PI negative) and late apoptotic cells (Annexin V-FITC positive, PI positive) can be detected. The results of Annexin V-FITC/PI analyses indicated that these drugs had slight effects on the ratio of apoptotic cell death in both cell lines. The Caspase 3/7 assay is a luminescent assay to measure the activity of caspase 3/7, which are universally activated during apoptosis. Ibuprofen elicited lower caspase 3/7 activity than diclofenac in KKU-M139 cells. A previous study has indicated that aspirin induces cell cycle arrested in G0/G1 phase in cholangiocarcinoma (HuCCT1 cells).[22] Further research on other active compounds of these medications with the same anti-inflammatory activity as COX may reveal different findings.

Conclusion

The observed morphological changes suggested that in both KKU-M139 and KKU-213B cell lines, diclofenac is more effective than ibuprofen in decreasing cell proliferation and viability. In both cell lines, ibuprofen induced reversible cell injury, and high dose diclofenac induced irreversible cell injury and cell death. However, the characteristics of apoptosis determined by Annexin V/PI assays and the differences in caspase assay results were not statistically significant. Therefore, these drugs may serve as alternative treatments for preventing carcinogenesis and proliferation in CCA by targeting apoptosis.

Recommendations

None.

Source of funding

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Conflict of interest

The authors have no conflicts of interest to declare.

Ethical approval

In vitro experiments were performed on the cell lines KKU-M139 and KKU-213B, which have a long history in research and published literature. This experiment that was approved by a committee at Ubon Ratchathani University and did not require ethical approval.

Authors contributions

The study was created and designed by RTN. Under the supervision of RTN, KMR conducted research, provided research materials, and collected and organized data. RTN used Prism to evaluate and interpret data. All authors contributed to the first and final drafts of the paper, and provided appropriately resolved information. All authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

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