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Low-Dose Cyclophosphamide and Piroxicam Inhibit Growth, Migration, and Invasion of Canine Oral Malignant Melanoma Cell Line

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Abstract

The effect of low-dose chemotherapy on tumor motility of canine oral malignant melanoma has been investigated on an *in vitro* model using low-dose cyclophosphamide and piroxicam. Cyclophosphamide, at a concentration of 0.1 mg/mL in culture medium, significantly inhibited tumor cell migration/invasion through trans-well assays and suppressed both ERK and NF- κ B signaling, the latter of which was observed through I κ B- α and P-I κ B α expression. Furthermore, in combination with a COX-2 inhibitor, i.e. piroxicam (0.38 mg/mL in culture medium), cyclophosphamide revealed a potential anti-migration effect over the single piroxicam treatment on a wound assay. This result suggested that low-dose cyclophosphamide might have influence on tumor cell motility by inhibition of the NF- κ B/ERK cascade.

Keywords: canine, low-dose cyclophosphamide, motility, oral malignant melanoma, piroxicam

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Introduction

Canine oral malignant melanoma is one of the most common oral malignancies in dogs (Smith et al., 2002). Biological behavior of canine oral malignant melanoma is highly aggressive with high incidence of local invasion and distant metastasis (Bergman, 2007). Despite various treatments, the survival time of canine oral malignant melanoma patients was less than one year (Ramos-Vara et al., 2000). Dogs often died due to distant metastasis but not local recurrence of the tumor (Bergman, 2007).

Recently, low-dose cyclophosphamide has emerged as a promising chemotherapeutic protocol in human malignant melanoma patients with metastasis (Borne et al., 2010) and in a mouse model of metastatic melanoma (Cruz-Munoz et al., 2009). Although low-dose cyclophosphamide generally induces anti-angiogenic activity (Mutsaers, 2009), previous studies demonstrated that this regimen also influenced tumor metastasis and decreased both number and diameter of metastatic lung nodules, particularly when combined with a cyclooxygenase 2 (COX-2) inhibitor (Mainetti et al., 2011).

Accumulated evidences show that high expression of COX-2 correlates with malignancy in cancer patients (Pires et al., 2010). COX-2 precedes tumor proliferation and cell motility via the prostaglandin cascade, and non-steroidal anti-inflammatory drugs (NSAIDs) significantly retained tumor cell proliferation and migration (Cha and DuBois, 2007).

Although the aforementioned studies revealed the effect of low-dose cyclophosphamide on tumor metastasis (Matar et al., 1998), information about its effect on canine malignant melanoma is still unknown. In this study, we conducted *in vitro* experiments to investigate the effect of low-dose cyclophosphamide and/or piroxicam on tumor cell motility using a canine oral malignant melanoma cell line.

Materials and Methods

A canine oral malignant melanoma cell line, CMM1, which was established from a canine oral malignant melanoma clinical stage III patient (Ohashi et al., 2001) was maintained in RPMI-1640 (Wako, Osaka, Japan) supplemented with 10% heat-inactivated fetal bovine serum (Gibco BRL, NY, USA) and 5 mg/L gentamicin (Sigma-Aldrich, MO, USA) at 37°C with 5% CO₂.

Cyclophosphamide monohydrate (Sigma-Aldrich) was dissolved in sterile distilled water at a concentration of 20 mg/mL, and piroxicam (Sigma-Aldrich) was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich) at 50 mg/mL. Both stock solutions were kept at -20°C before use.

To determine the appropriate dosage, CMM1 was incubated with the treatment medium containing either cyclophosphamide or piroxicam at 10, 1, 0.1, 0.01, 0.001, or 0.0001 mg/mL for 48 h. Then, cell viability was assessed using the cell proliferation kit I MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylterazolium bromide)-based kit (Roche Diagnostics GmbH, NJ, USA). The highest nontoxic dose was

selected for cyclophosphamide (CyLD), as to imitate low-dose cyclophosphamide. The fifty percent inhibitory concentration (IC₅₀) of piroxicam (Px) was determined through the linear regression equation.

CMM1 was incubated for 0, 3, 6, 12, 24, or 48 h with treatment media which were control, CyLD, Px, or combination treatment (CyPx). Viable cells from each treatment at different time points were collected and counted by trypan blue staining. In addition, prostaglandin E2 (PGE2) concentrations in the treatment media from each treatment at 12 and 24 h were measured by using the Prostaglandin E2 EIA kit (Cayman, MI, USA). The PGE2 concentrations were normalized by the number of tumor cells at each time point and expressed as the concentration of PGE2/10⁴ cells.

Confluent monolayers of tumor cells in a 12-well plate were scraped to create a scratch using a sterile 200-μL pipette tip. After removing the floating cells by double washing with complete medium, the cells were incubated with each regimen at 37°C with 5% CO₂ and migrated cell images were obtained from the same field at 0, 3, 6, 12, and 24 h by using a phase-contrast microscope. Images at each time point were analyzed by Photoshop CS5 (Adobe System Inc., CA, USA) and distances of the scratch gap (μm) at different time points were expressed as the percentage of scratch width normalized to the gap width at 0 h.

Both the trans-well migration and invasion assays of CMM1 cells treated with each regimen were carried out after the cells were in culture for 24 h at 37°C with 5% CO₂. In brief, 2 × 10⁴ CMM1 cells were seeded in a 24-multi-well insert system containing a polyethylene terephthalate membrane with an 8 μm pore (BD Biosciences; CA, USA) for the migration assay or containing a polycarbonate membrane with an 8 μm pore coated with Matrigel™ (BD Biosciences) for the invasion assay. After 24 h in culture, nonmigrated cells or noninvading cells were removed by cotton swabs. Subsequently, the inserts were fixed and stained with Wright-Giemsa. Cells that attached to the surface were counted in 5 random non-overlapping views using a light microscope. Tumor cells that were able to move through the polyethylene terephthalate membrane were considered as migrated cells, while tumor cells that were able to degrade the Matrigel™ before migration were considered to be invading cells.

Several molecules in the signaling pathways that might inhibit tumor cell migration and invasion through the anti-inflammatory process were investigated by western blot analysis. In brief, equal amounts of lysates after each treatment were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto a polyvinylidene fluoride (PVDF) membrane (Bio-Rad Laboratories; CA, USA). After blocking for nonspecific proteins, the membranes were probed with the following primary antibodies at 4°C overnight: monoclonal mouse anti-actin antibody (1:10000) and monoclonal mouse anti-PKCα antibody (1:1000) (Millipore, MA, USA); monoclonal mouse anti-Ras antibody (1:500), monoclonal mouse anti-c-Raf antibody (1:2000) and monoclonal mouse anti-COX-2 antibody (1:1000) (BD Transduction Laboratories, KY); polyclonal rabbit anti Raf-B antibody (1:200) and polyclonal rabbit anti-IκB-α antibody (1:1000) (Santa

Cruz Biotechnology, CA, USA); monoclonal rabbit anti-human ERK antibody (1:1000), monoclonal rabbit anti-human phosphorylated-ERK (P-ERK) antibody (1:2000) and monoclonal mouse anti-phospho-IκBα (P-IκBα) antibody (1:1000) (Cell signaling Technology, MA, USA). Prior to visualization, the membranes were incubated with HRP-conjugated secondary antibodies against mouse or rabbit IgG (GE Healthcare, Buckinghamshire, US) and the antigen-antibody complex was visualized by using enhanced chemiluminescence (ECL) Prime detection (GE Healthcare).

All quantitative measurement was performed in triplicate and data were expressed as mean and standard error (SEM). Differences between groups were analyzed by ANOVA, followed by post-hoc analysis using Tukey's LSD methods (Graphpad Software, CA, USA). Differences were statistically significant when $p < 0.05$.

Results

After the preliminary determination, the dosage of 0.1 mg/mL cyclophosphamide (CyLD) was selected because it was the highest nontoxic concentration. The IC50 of Px was 0.38 mg/mL. These doses were used in the following experiments.

Tumor growth and PGE2 concentration after treatment with each regimen are shown in Figure 1. The tumor growth was comparable between 0-12 h. However, after 24 h, the control and CyLD groups showed rapid cell growth compared to the Px and CyPx groups. A significant difference in viable cell number was noted between the control and Px groups ($p < 0.05$), between the CyPx and CyLD groups ($p < 0.05$), and between the CyLD and Px groups ($p < 0.01$; Fig 1A). At 48 h, the viable cell numbers of the groups without Px were significantly higher ($70.75 \pm 4.20 \times 10^4$ cells for control and $65.72 \pm 2.96 \times 10^4$ cells for CyLD; p

< 0.001) than those with Px ($16.00 \pm 3.92 \times 10^4$ cells for Px and $14.16 \pm 1.15 \times 10^4$ cells for CyPx). According to these results, all of the following experiments were performed at 24 h to eliminate the factor of difference in cell concentration, particularly in the trans-well migration/invasion assay.

PGE2 concentrations in the treatment medium at 12 and 24 h are shown in Figure 1B. At 12 h, the mean PGE2 concentration was 4.82 ± 1.83 pg in the control group and was significantly higher than those of the Px and CyPx groups (1.90 ± 0.41 pg and 2.17 ± 0.89 pg, respectively) ($p < 0.01$). The PGE2 concentration of the CyLD group (3.77 ± 0.19 pg) was also significantly higher than that of the Px group ($p < 0.05$). At 24 h, the mean PGE2 concentration of the control (6.43 ± 0.89 pg) and the CyLD groups (4.41 ± 0.78 pg) was significantly higher than those of the Px (2.02 ± 0.23 pg) and the CyPx groups (1.77 ± 0.77 pg) ($p < 0.001$ and $p < 0.01$). In addition, the PGE2 concentration of the control group was significantly higher than that of the CyLD group ($p < 0.05$).

The effect of treatments on tumor cell migration observed through wound assay is shown in Figure 2A and 2B. Wound gaps of the CyLD and Px groups were comparable and significantly broader than that of the control group at 12 h ($26.22 \pm 10.05\%$, $33.62 \pm 10.19\%$ and $33.33 \pm 9.82\%$ in the control, CyLD, and Px groups, respectively; $p < 0.05$). In addition, the value for the CyPx group revealed the widest wound gap ($37.53 \pm 6.41\%$) compared to the other groups; however, a significant difference was detected only in comparison to the control group ($p < 0.001$). At 24 h, all the treatments significantly inhibited tumor cell migration. Means of wound gap were $2.36 \pm 4.24\%$ for the control, $9.14 \pm 6.54\%$ for the CyLD, $20.98 \pm 10.07\%$ for the Px and $31.38 \pm 6.19\%$ for the CyPx groups, respectively. Significant differences were noted between the groups as shown in Figure 2B.

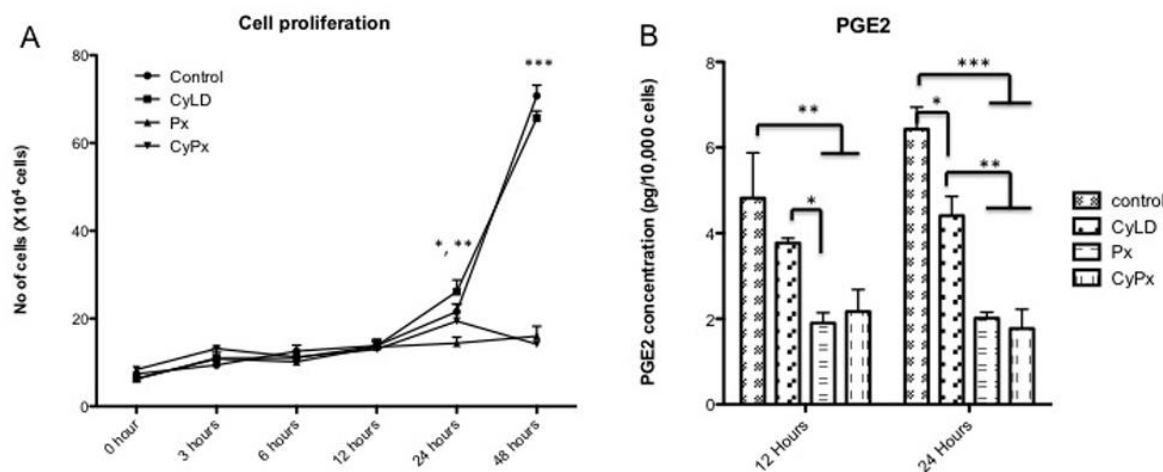


Figure 1 Effect of low-dose cyclophosphamide or piroxicam alone or in combination on canine oral malignant melanoma cell proliferation (A) and PGE₂ production (B). Tumor cells in each treatment group were comparable between 0-6 h. After 12 h of treatment, the number of tumor cells in the CyLD group was greater while those in the Px and CyPx group were lower than that of the control. A significant difference in viable cell number was noted between the control and Px groups ($p < 0.05$ [*]), between the CyPx and CyLD groups ($p < 0.05$ [*]), and between the CyLD and Px groups ($p < 0.01$ [**]). At 24 h, marked differences in tumor cell numbers were detected between groups that were treated with piroxicam (Px and CyPx) and groups that were not treated with piroxicam (control and CyLD) ($p < 0.001$ [***]). (B) Treatments with piroxicam (Px and CyPx) lowered PGE₂ concentration compared to those of the control and CyLD groups. Moreover, PGE₂ in the CyLD group was lower than that of the control group at both time points, especially at 24 h ($p < 0.05$ [*]).

In the trans-well assay, the Px and CyPx treatments significantly inhibited tumor cell migration (61.8 ± 13.6 cells for Px and 64.8 ± 12.3 cells for CyPx) compared to the control (168.5 ± 32.3 cells) or CyLD groups (137.5 ± 28.3 cells) ($p < 0.001$). CyLD also significantly inhibited tumor cell migration when compared to the control group ($p < 0.01$) (Fig 2C). The number of invading cells revealed a similar trend as the trans-well migration assay, which all treatments significantly inhibited tumor cell invasion compared to the control group (means; 20.8 ± 9.6 cells, 13.3 ± 5.3 cells, 5.3 ± 2.8 cells, and 4.9 ± 2.7 cells for the control, CyLD, Px and CyPx groups, respectively, with P levels of 0.01 and 0.001). Moreover, among the treatment groups, the Px and CyPx groups had significantly lower invading cell numbers compared to the CyLD group ($p < 0.01$) (Fig 3).

The western blot analysis of protein expression after 24 h of treatment is shown in Figure 4. The expression of PKC α did not change by any of the treatments while Ras appeared to have the lowest expression with the CyLD. Ras expression in the Px and CyPx treatments appeared lower than that in the control group. Raf-B and c-Raf appeared to have lower expression in the CyPx group compared to the other groups. Expression of ERK and P-ERK appeared lower in all of the treatment groups, especially the CyPx group. Furthermore, expression of I κ B- α appeared lower in the CyLD and CyPx groups, while P-I κ B α expression appeared lower in all the treatment groups compared to that in the control group. COX2 expression appeared lower in all the treatment groups, particularly the CyPx group.

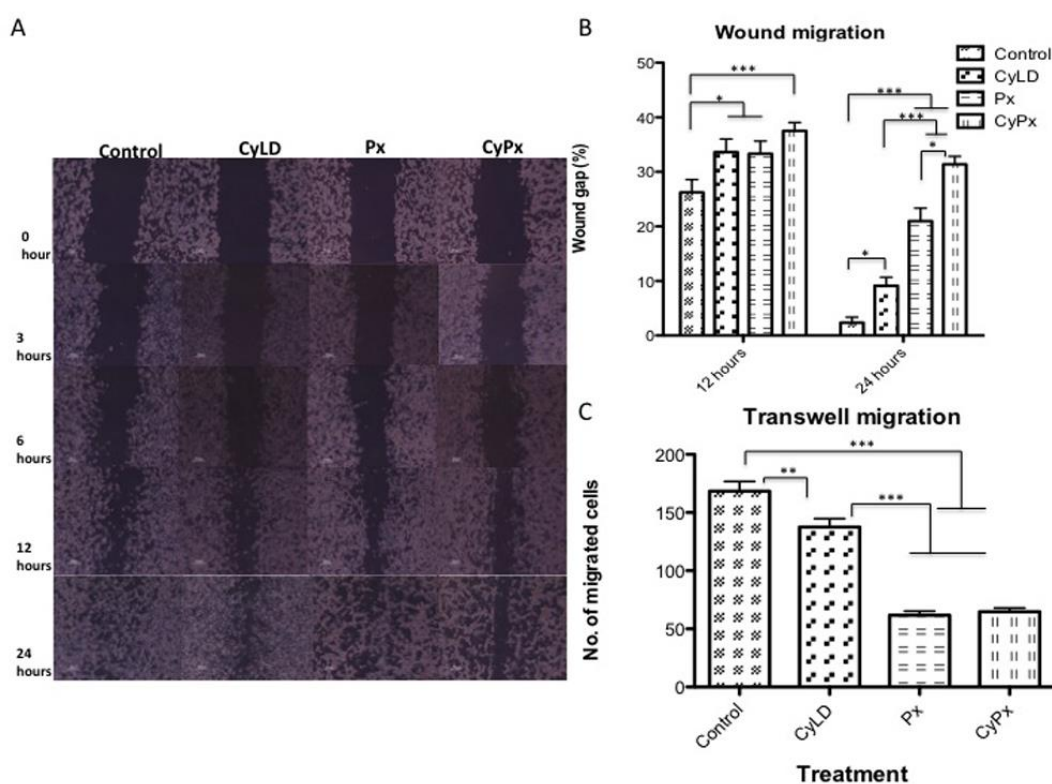


Figure 2 Anti-migration effect of low-dose cyclophosphamide or combination treatment on canine oral malignant melanoma cells. In a wound migration assay (A and B), all treatments inhibited tumor cell migration compared to the control group at 12 h ($p < 0.05$ [*] for CyLD and Px compared to control, and $p < 0.001$ [***] for CyPx compared to control). Moreover, at 24 h, Px, and CyPx significantly inhibited tumor cell migration compared to CyLD ($p < 0.001$ [***]), and CyPx revealed a superior anti-migration effect than Px ($p < 0.05$ [*]). Fig. 2C reveals an anti-migration effect of each treatment in the trans-well migration assay. Tumor cell migration was significantly inhibited in treatments that used piroxicam (Px and CyPx) compared to the control and CyLD groups ($p < 0.001$ [***]). Moreover, CyLD treatment resulted in significantly lower numbers of migrated cells compared to the control group ($p < 0.01$ [**]).

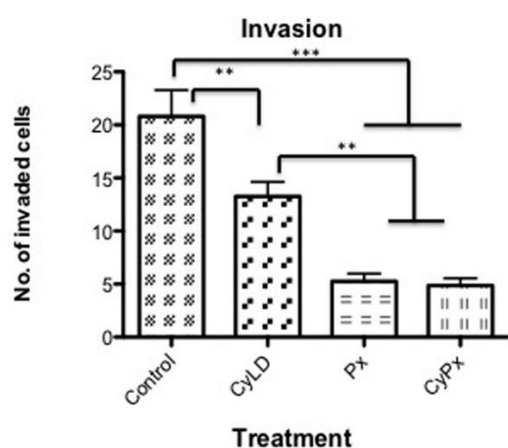


Figure 3 Effect of low-dose cyclophosphamide or combination treatment on tumor cell invasion. Numbers of invading cells in treatment groups were significantly lower than that of the control group ($p < 0.01$ [**] for CyLD compared to control, and $p < 0.001$ [***] for Px and CyPx compared to control). Furthermore, among the treatment groups, Px and CyPx had significantly lower numbers of invading cells compared to the CyLD group ($p < 0.01$ [**], $p < 0.001$ [***]). Moreover, CyLD treatment resulted in significantly lower numbers of migrated cells compared to the control group ($p < 0.01$ [**]).

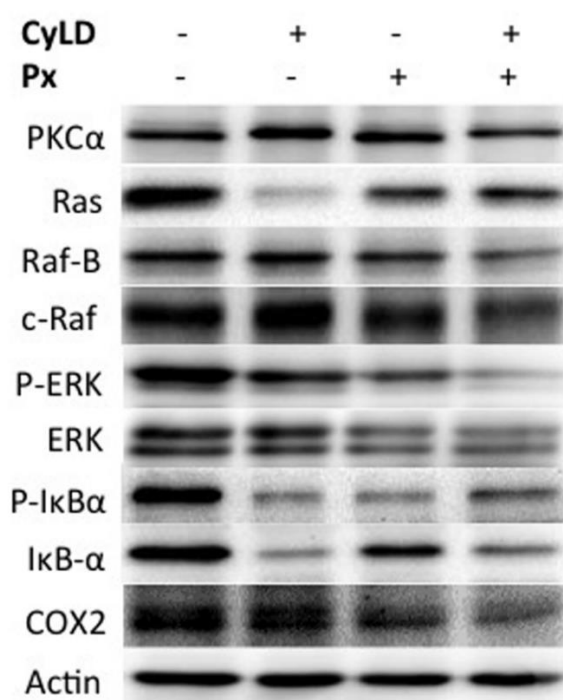


Figure 4 Protein expression in all treatment groups by western blot analysis. Several proteins such as Raf-B, c-Raf, ERK signaling pathway proteins, and COX-2 appeared to have lower expression in the CyPx group while Ras and the NF- κ B signaling pathway proteins IkB- α and phospho-IkB α had lower expression in the CyLD group. Moreover, PKC α expression in the CyPx group was comparable to that in the control group while those in the CyLD and CyPx groups appeared to have higher expression.

Discussion

It is well known that the COX-2/PGE2 cascade plays as a crucial role in tumor progression and motility (Wu, 2006). In this study, not only piroxicam, but also low-dose cyclophosphamide unexpectedly showed a significant effect on the PGE2 expression and cell motility. This concurs with previous studies which concluded that low-dose cyclophosphamide could affect PGE2 production by dose- and time-dependent mechanisms (Macedo et al., 2008) and this regimen could inhibit tumor cell motility and metastasis (Mainetti et al., 2011).

To ensure the effect of treatments on tumor cell motility, the wound and trans-well migration/invasion assays were conducted. In this study, all the treatments of low-dose cyclophosphamide, piroxicam, or the combination revealed anti-motility effects and these effects showed similar results to the PGE2 expression. This indicated that CMM1 cell motility might be controlled through several malignant cascades related to PGE2 signaling.

Among several signaling pathways

controlling tumor cell motility, a member of the serine/threonine kinase family such as PKC α did not show any changes by these treatments. The Ras/ERK pathways, in contrast, appeared lower expressed by all the treatments. Ras/ERK are molecules of the signaling pathway related to tumor cell motility (Song and Moon, 2006) and can be inhibited by COX-2 inhibitors (Cha and DuBois, 2007) or low-dose cyclophosphamide as observed in our study. Furthermore, we observed the upstream signaling cascade responsible for COX-2 expression through NF- κ B signaling (Wang et al., 2009). Surprisingly, the treatments, especially the low-dose cyclophosphamide treatment, markedly resulted in reduced IkB- α and P-IkB α expression, which may prevent nuclear localization of NF- κ B, which then inhibits transcription of several malignant genes such as COX-2. Previous studies revealed that NF- κ B activation was modulated by MAPK/ERK kinase kinase-1 (MEKK1), which influenced site-specific phosphorylation of IkB- α and activated the IkB kinase (IKK) complex (Lee et al., 1997). However, in this study, the starting point of the low-dose cyclophosphamide effect on MEKK1 was still

controversial. Further studies to investigate the beginning point in the cascade need to be conducted.

This result suggested that low-dose cyclophosphamide or a combination treatment with piroxicam might inhibit CMM1 motility through the NF- κ B/ERK pathway. Our study provides better insight into the effect of low-dose cyclophosphamide on tumor cell migration/invasion demonstrated by a canine oral malignant melanoma cell line.

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บทคัดย่อ

ผลของไซโคลฟอสฟาไมด์ขนาดต่ำร่วมกับไพร์อกซิแคมในการยับยั้งการเจริญเติบโต การเคลื่อนที่ และการรุกรานของเซลล์มะเร็งเมลาโนมาช่องปากของสุนัข

แนน ซ้อยสุนิรชอน^{1,2} โคตะ โยชิตะ¹ โคเฮ ซาเอกิ¹ ยูโยโกะ ทานะกะ¹ มานาบุ โมชิซึกิ¹ เรียวเฮ นิชิมูระ¹
ทาเกะยุกิ นาคากาวะ^{1*}

การศึกษาครั้งนี้เป็นการศึกษาประสิทธิผลของยาเคมีบำบัดขนาดต่ำโดยใช้ไซโคลฟอสฟาไมด์ขนาดต่ำร่วมกับไพร์อกซิแคมต่อการเคลื่อนที่ของเซลล์มะเร็งเมลาโนมาช่องปาก ผลการศึกษาพบว่า ไซโคลฟอสฟาไมด์ขนาด 1 มิลลิกรัมต่อมิลลิตรของอาหารเลี้ยงเซลล์สามารถยับยั้งการเคลื่อนที่และการรุกรานของเซลล์มะเร็งในการทดสอบข้ามหลุมเจาะ รวมทั้งการยับยั้งโปรตีนกลุ่ม ERK และ NF-KB โดยการตรวจสอบ NF-KB นั้นผ่านการตรวจหาโปรตีน IKB- α และ P-IKB α นอกจากนี้ ในการทำทดสอบการเคลื่อนที่แบบบาดแผล เมื่อผสมสารยับยั้งเอนไซม์ COX-2 ชนิดไพร์อกซิแคมลงในอาหารเลี้ยงเซลล์ร่วมกับไซโคลฟอสฟาไมด์ขนาดต่ำ (0.38 มิลลิกรัมต่อมิลลิตร) พบว่าอาหารเลี้ยงเซลล์ที่มีส่วนผสมของยาทั้งสองประเภทสามารถยับยั้งการเคลื่อนที่ของเซลล์มะเร็งได้ดีกว่าอาหารเลี้ยงเซลล์ที่มีส่วนผสมของไพร์อกซิแคมเพียงอย่างเดียว ผลการศึกษาครั้งนี้บ่งชี้ว่า ไซโคลฟอสฟาไมด์ขนาดต่ำ อาจจะมีผลยับยั้งการเคลื่อนที่ของเซลล์มะเร็งเมลาโนมาผ่านการยับยั้งกระบวนการ NF-KB และ ERK

คำสำคัญ: สุนัข ไซโคลฟอสฟาไมด์ขนาดต่ำ การเคลื่อนที่ มะเร็งเมลาโนมาช่องปาก ไพร์อกซิแคม

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