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Effect of baicalein on migration and invasion of cutaneous squamous cell carcinoma cells and the underlying mechanism

[Efecto de la baicaleina en la migración e invasión de células de carcinoma cutáneo escamoso y el mecanismo subyacente]

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Abstract: This study aimed to investigate the effect of baicalein on migration and invasion of human cutaneous squamous cell carcinoma A431 cells. A431 cells were incubated with baicalein with concentrations of 0 (control), 2, 4, 8, 16 and 32 μmol/L, respectively. The cell proliferation, apoptosis, migration, invasion and expressions of matrix metalloproteinse-2 (MMP-2), matrix metalloproteinse-9 (MMP-9) and vascular endothelial growth factor (VEGF) protein were detected. Results showed that, with 32 μmol/L baicalein and treatment for 72 h, the cell proliferation inhibition and apoptosis rates were the highest. With 32 μmol/L baicalein and treatment for 48 h, the cell migration and invasion inhibition rates were the highest, and the MMP-2, MMP-9 and VEGF protein expression levels were the lowest. In conclusion, baicalein can inhibit the proliferation of A431 cells, promote the apoptosis, and inhibit the invasion and metastasis. The mechanism may be related to its down-regulation of MMP-2, MMP-9 and VEGF expressions.

Keywords: Baicalein; Squamous cell carcinoma; A431; Migration; Invasion

Resumen: Este estudio se propuso investigar el efecto del baicaleína en la migración e invasión de células de carcinoma cutáneo escamoso humano A431. Las células A431 se incubaron con baicaleína a concentraciones de 0 (control), 2, 4, 8, 16 y 32 μmol/L, respectivamente. Se detectaron la proliferación celular, apoptosis, migración, invasión y expresiones de proteínas de metaloproteinasa de matriz-2 (MMP-2), metaloproteinasa de matriz-9 (MMP-9) y factor de crecimiento endotelial vascular (VEGF). Los resultados mostraron que, con 32 μmol/L de baicaleína y tratamiento durante 72 h, las tasas de inhibición de proliferación celular y apoptosis fueron las más altas. Con 32 μmol/L de baicaleína y tratamiento durante 48 h, las tasas de inhibición de migración y invasión fueron las más altas, y los niveles de expresión de MMP-2, MMP-9 y VEGF fueron los más bajos. En conclusión, la baicaleína puede inhibir la proliferación de células A431, promover la apoptosis y inhibir la invasión y metástasis. El mecanismo puede estar relacionado con su regulación descendente de las expresiones de MMP-2, MMP-9 y VEGF.

Palabras clave: Baicaleína; Carcinoma cutáneo escamoso; A431; Migración; Invasión

INTRODUCTION

Cutaneous squamous cell carcinoma (CSCC) is a kind of malignant epithelial tumor forming from the keratinocytes of the epidermis or skin appendages. It is one of the most common skin malignant tumors (Cerra-Franco *et al.*, 2017). CSCC develops rapidly and has strong destruction to the tissues. It has a strong ability to invade and migrate, and the metastasis rate is very high (Glynne-Jones *et al.*, 2016). CSCC often spreads to regional and distant sites, which can markedly affect the clinical course of the disease and patient prognosis (Iancu *et al.*, 2024). The individualized program for comprehensive treatment is often advocated for CSCC. According to the patient's age, sex, location, and local and distant metastasis of CSCC, conventional surgical resection, chemotherapy, radiotherapy, laser microsurgery, photodynamic therapy and other methods are used alone or in combination (Soura *et al.*, 2015; Perez *et al.*, 2017; Winkelmann *et al.*, 2017). However, the recurrence rate and 5-year metastasis rate of CSCC are still high, and the prognosis is poor (Asgari, 2016). At present, the surgery combined with chemotherapy drugs often used for the treatment of metastatic lesions of CSCC. However, the effect of chemotherapy drugs cannot meet the expectations, and the side effects of chemotherapy drugs on normal cells are large (Kirby & Miller, 2010). Therefore, it is an urgent task to find new drugs to treat the CSCC in clinical and basic medical research.

Baicalein is a flavonoid extracted from the root of *Scutellaria baicalensis* Georgi, a common medical plant (Lee *et al.*, 2011). As a selective inhibitor of platelet-type 12-lipoxygenase, baicalein is widely used in disease prevention and treatment (Leung *et al.*, 2007). Studies have shown that, baicalein has antiallergic, anti-inflammatory, cerebral blood circulation improvement and anti-tumor effects, with the advantages of high efficiency and low toxicity (Shen *et al.*, 2003; Chao *et al.*, 2007; Bae *et al.*, 2016; Liang *et al.*, 2017). It is found that, baicalein can promote KDM4E to induce BICD1 and inhibit triple-negative breast cancer progression by blocking PAR1 signaling (Dong *et al.*, 2024), and induce gastric cancer cell pyroptosis through the NF-κB-NLRP3 signaling axis (Liu *et al.*, 2024). As a safe potential drug, baicalein does not cause the chromosomal abnormalities and mutations, and has no serious side effect in chemotherapy (Mu *et al.*, 2016).

The purpose of this study was to investigate the effect of baicalein on the migration and invasion of human CSCC A431 cells, and to provide an experimental basis for the further developing the anti-tumor value of baicalein.

MATERIALS AND METHODS

Cell culture

A431 cells (Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China) were cultured in RPMI 1640 medium containing 10% fetal bovine serum at 37°C and under 5% CO_2. The cells were passaged once every three days. The cells in the logarithmic growth phase were taken for the next experiments (Hao *et al.*, 2013).

Detection of cell proliferation

Inhibition of baicalein on proliferation of A431 cells was detected using the CCK-8 method. The cells with a concentration of 1×10^5 cells/mL were inoculated into 96-well plates, 100 μL in each well. Baicalein was diluted with dimethyl sulfoxide (DMSO) into 1 mg/mL mother liquor. Different amount of baicalein mother liquor was added to the wells. The final volume of each well was adjusted to 100 μL. In different treatment groups, the baicalein concentration was 2, 4, 8, 16 and 32 μmol/L. At the same time, the control group (the same as the treatment group except for adding baicalein) and the blank group (the same with treatment group except for adding DMSO, baicalein and cells) were set. Six repetitive wells were set for each group. The cells were cultured for 24, 48 and 72 h, respectively. After culture, 10 μL CCK-8 reagent

was added to each well. After 2.5 h of incubation, the optical density (OD) at 450 nm wavelength of the well was detected in the microplate reader. The proliferation inhibition rate of A431 cells was calculated as follows: proliferation inhibition rate (%) = ($OD_{control}$ - $OD_{treatment}$) / ($OD_{control}$ - OD_{blank}) × 100% (Xia *et al.*, 2016).

Detection of cell apoptosis

Cells with a concentration of 1×10^5 cells/mL were inoculated into the 96-well plate. After 12 h of culture, baicalein was added to the wells. In different groups, the baicalein concentration was 0 (control), 2, 4, 8, 16 and 32 μmol/L. Six repetitive wells were set for each group. The culture was performed for 24, 48 and 72 h, respectively. After washing the cells with phosphate-buffered saline (PBS), 500 μL of binding buffer was added to suspend the cells. Then, 5 μL fluorescein isothiocyanate-labeled annexin-V and 5 μL PI were added, followed by incubation at room temperature for 15 min. Finally, the apoptosis of A431cells was detected in a flow cytometer (Pal *et al.*, 2013).

Determination of cell migration and invasion

A431 cells were treated with 0 (control), 2, 4, 8, 16, 32 μmol/L baicalein for 48 h, respectively. The cells were collected, and the concentration was adjusted to 8×10^5 cell/mL. The configured Matrigel glue was spread on the inner membrane of Transwell chamber. A 100 μL cell solution was added to the upper layer of the chamber, while 600 μL medium containing 10% serum was added to the lower layer of the chamber. Three chambers were set for each baicalein concentration. The chamber was incubated for 24 h. Then, the chamber was taken out, followed by fixing with methanol and dyeing with crystal violet. After washing with water, the cells on the surface of membranes were wiped off using cotton swab dipped in normal saline. Three different fields of visual were randomly selected and observed under the microscope. The number of cells passing through the membranes was counted. The invasion inhibition rate was calculated as follows: invasion inhibition rate (%) = (1 - number of invading cells in treatment group / number of invading cells in control group) × 100%. The procedure of cell migration experiment is basically the same with the invasion experiment, but the Matrigel glue was not spread on the inner membrane of the Transwell chamber. The migration inhibition rate was calculated as follows: migration inhibition rate (%) = (1 - number of migrating cells in treatment group / number of migrating cells in control group) × 100% (Fan *et al.*, 2014).

Western blot assays

A431 cells were treated with 0 (control), 2, 4, 8, 16, 32 μmol/L baicalein for 48 h, respectively. Three repetitions were set for each group. The cells were collected and washed with PBS twice. The cells were lysated with RIPA. The total proteins were extracted by ultrasound, and the protein concentration was determined using the bicinchoninic acid method. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed to separate the proteins. The separated proteins were transferred to the polyvinylidene fluoride membranes, followed by blocking with 5% skimmed milk powder at room temperature for 1 h. The membranes were incubated with the primary antibody of matrix metalloproteinse-2 (MMP-2), matrix metalloproteinse-9 (MMP-9) and vascular endothelial growth factor (VEGF), respectively, overnight at 4°C, followed by incubating with the secondary antibody at 37°C for 1 h. After washing with PBS for three times, the membranes were developed using enhanced chemiluminescence substrates. Finally, the bands on membranes were visualized and analyzed using Image J imaging system. β-actin was used as the internal reference. The relative expression level of target protein was calculated (Rao *et al.*, 2024).

Statistical analysis

Data were presented as mean ± standard deviation. The single factor analysis of variance

was performed using the SPSS 18.0 statistical software. LSD-t test was used for pairwise comparison. The difference of comparison was statistically significant at $p<0.05$.

RESULTS

Effect of baicalein on proliferation of A431 cells

As shown in Table No. 1, after treatment for 24, 48 and 72 h, baicalein with different concentrations could inhibit the proliferation of A431 cells. The proliferation inhibition rate of A431 cells increased with the increase of baicalein concentration and the prolonging of treatment time, respectively. When the baicalein concentration was 32 μmol/L and the treatment time was 72 h, the proliferation inhibition rate was the highest, which was (62.55 ± 7.51) %.

Table No. 1
Effect of baicalein on proliferation of A431 cells (n = 6)

Baicalein concentration	Proliferation inhibition rate (%)		
(μmol/L)	24 h	48 h	72 h
0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
2	4.82 ± 0.56^{a}	12.78 ± 1.65$^{a\#}$	23.48 ± 3.28$^{a\#\&}$
4	12.62 ± 2.34^{ab}	25.23 ± 3.15$^{ab\#}$	32.37 ± 4.41$^{ab\#\&}$
8	22.40 ± 3.66^{abc}	31.52 ± 4.42$^{abc\#}$	42.16 ± 6.67$^{abc\#\&}$
16	34.69 ± 5.21^{abcd}	41.10 ± 6.46$^{abcd\#}$	47.62 ± 6.82$^{abc\#\&}$
32	43.18 ± 4.10^{abcde}	52.22 ± 7.55$^{abcde\#}$	62.55 ± 7.51$^{abcde\#\&}$

$^{a}p<0.05$ compared with 0 μmol/L baicalein; $^{b}p<0.05$ compared with 2 μmol/L baicalein; $^{c}p<0.05$ compared with 4 μmol/L baicalein; $^{d}p<0.05$ compared with 8 μmol/L baicalein; $^{e}p<0.05$ compared with 16 μmol/L baicalein. $^{\#}p<0.05$ compared with 24 h; $^{\&}p<0.05$ compared with 48 h

Effect of baicalein on apoptosis of A431 cells

Table No. 2 showed that, the apoptosis rate of A431 cells increased with the increase of baicalein concentration and prolonging of treatment time, respectively. When the baicalein concentration was 32 μmol/L and the treatment time was 72 h, the apoptosis rate was (46.26 ± 5.42) %, the highest among different groups.

Table No. 2
Effect of baicalein on apoptosis of A431 cells (n = 6)

Baicalein concentration	Apoptosis rate (%)		
(μmol/L)	24 h	48 h	72 h
0	0.88 ± 0.12	2.13 ± 0.43$^{\#}$	1.67 ± 0.23$^{\#\&}$
2	4.03 ± 0.60^{a}	7.18 ± 1.20$^{a\#}$	12.04 ± 1.29$^{a\#\&}$
4	7.16 ± 1.12^{ab}	11.32 ± 2.90$^{ab\#}$	14.30 ± 2.18$^{a\#\&}$
8	15.37 ± 2.75^{abc}	19.84 ± 3.22$^{abc\#}$	27.21 ± 3.05$^{abc\#\&}$
16	21.55 ± 4.28^{abcd}	26.62 ± 4.51$^{abcd\#}$	36.52 ± 6.31$^{abcd\#\&}$
32	24.42 ± 3.15^{abcd}	32.33 ± 4.33$^{abcde\#}$	46.26 ± 5.42$^{abcde\#\&}$

$^{a}p<0.05$ compared with 0 μmol/L baicalein; $^{b}p<0.05$ compared with 2 μmol/L baicalein; $^{c}p<0.05$ compared with 4 μmol/L baicalein; $^{d}p<0.05$ compared with 8 μmol/L baicalein; $^{e}p<0.05$ compared with 16 μmol/L baicalein. $^{\#}p<0.05$ compared with 24 h; $^{\&}p<0.05$ compared with 48 h

Effect of baicalein on migration and invasion of A431 cells

After treatment for 48 h, baicalein with different concentrations could inhibit the migration and invasion of A431 cells. The migration and invasion inhibition rates of A431 cells increased with the increase of baicalein concentration, respectively. When the baicalein concentration was 32 μmol/L, the migration and invasion inhibition rates were (46.63 ± 5.77) % and (40.56 ± 6.67) %, respectively, which were the highest among different groups (Table No. 3).

Table No. 3
Effect of baicalein on migration and invasion of A431 cells (n = 3)

Baicalein concentration (μmol/L)	Migration inhibition rate (%)	Invasion inhibition rate (%)
0	0.00 ± 0.00	0.00 ± 0.00
2	5.28 ± 1.05[a]	4.44 ± 0.78[a]
4	14.54 ± 2.38[ab]	12.68 ± 1.93[ab]
8	22.22 ± 4.05[abc]	18.31 ± 3.55[ab]
16	27.10 ± 3.39[abc]	24.29 ± 4.23[abc]
32	46.63 ± 5.77[abcde]	40.56 ± 6.67[abcde]

[a]*p*<0.05 compared with 0 μmol/L baicalein; [b]*p*<0.05 compared with 2 μmol/L baicalein; [c]*p*<0.05 compared with 4 μmol/L baicalein; [d]*p*<0.05 compared with 8 μmol/L baicalein; [e]*p*<0.05 compared with 16 μmol/L baicalein

Effect of baicalein on MMP-2, MMP-9 and VEGF protein expressions in A431 cells

The western blot assays showed that, after treatment for 48 h, the baicalein could down-regulate the MMP-2, MMP-9 and VEGF protein expressions in A431 cells. The relative expression level of each protein decreased with the increase of baicalein concentration. When the baicalein concentration was 32 μmol/L, the relative expression level of each protein was the lowest (Table No. 4).

Table No. 4
Effect of baicalein on MMP-2, MMP-9 and VEGF protein expressions in A431 cells (n = 3)

Baicalein concentration (μmol/L)	MMP-2/β-actin	MMP-9/β-actin	VEGF/β-actin
0	1.01 ± 0.09	0.88 ± 0.12	1.12 ± 0.10
2	1.07 ± 0.11	0.77 ± 0.07	1.04 ± 0.08
4	0.85 ± 0.10[ab]	0.73 ± 0.06[a]	0.92 ± 0.06[ab]
8	0.52 ± 0.03[abc]	0.46 ± 0.04[abc]	0.56 ± 0.05[abc]
16	0.49 ± 0.04[abc]	0.38 ± 0.05[abc]	0.47 ± 0.05[abc]
32	0.37 ± 0.03[abcde]	0.29 ± 0.02[abcd]	0.41 ± 0.03[abcd]

[a]*p*<0.05 compared with 0 μmol/L baicalein; [b]*p*<0.05 compared with 2 μmol/L baicalein; [c]*p*<0.05 compared with 4 μmol/L baicalein; [d]*p*<0.05 compared with 8 μmol/L baicalein; [e]*p*<0.05 compared with 16 μmol/L baicalein. MMP-2, matrix metalloproteinse-2; MMP-9, matrix metalloproteinse-9; VEGF, vascular endothelial growth factor

DISCUSSION

This study investigated the effect of baicalein on the proliferation, apoptosis, migration, and invasion of CSCC A431 cells. Results showed that, when A431 cells were treated with 32 μmol/L baicalein for 72 h, the proliferation inhibition rate and apoptosis rate of cells were (62.55 ± 7.51) % and (46.26 ± 5.42) %, respectively, which were the highest among different groups. When A431 cells were treated with 32 μmol/L baicalein for 48 h, the migration rate and invasion inhibition rate of cells were (46.63 ± 5.77) % and (40.56 ± 6.67) %, respectively, which were the highest among different groups. This indicates that, baicalein can inhibit the proliferation of A431 cells, promote the apoptosis, and inhibit the invasion and metastasis.

The tumor metastasis is a complex dynamic process, which includes the invasion, metastasis and other steps. The tumor cells must overcome the histological barrier before invasion and metastasis. The barrier consists of basement membrane and extracellular matrix (Mierke, 2008). In tumor cells, MMP-2 and MMP-9, members of MMPs family, belong to type IV collagenase. The main components of basement membrane and extracellular matrix are type IV collagen. When MMP-2 and MMP-9 are activated and over-expressed, they can decompose the type IV collagen, thus destroying the basement membrane and extracellular matrix. So, the histological barrier was destroyed, which promotes the invasion and metastasis of cancer cells (Lee *et al.*, 2008). MMP-2 and MMP-9 are highly expressed in CSCC and play an important role in the invasion and metastasis of CSCC cells (Chebassier *et al.*, 2002). Previous study (Yu *et al.*, 2014) has shown that, baicalein can significantly down-regulate MMP-2 and MMP-9 expressions in cervical cancer cells. In our study, after treatment for 48 h, the baicalein could down-regulate the MMP-2 and MMP-9 protein expressions in A431 cells. This suggests that baicalein may inhibit the invasion and metastasis of A431 cells by down-regulating the MMP-2 and MMP-9 expressions.

There is a large angiogenesis during the invasion and metastasis of tumor cells. VEGF, as one of the strongest factors to promote the angiogenesis, plays an important role in tumor invasion and metastasis. VEGF can increase the permeability of blood vessels, which is beneficial for tumor cells entering blood vessels and flowing to all parts of the body (Nagy *et al.*, 2006). In addition, VEGF can bind to the specific receptors, and stimulate the endothelial cell proliferation, thus promoting the angiogenesis (Scharpfenecker *et al.*, 2007). VEGF is highly expressed in CSCC cells, which is closely related to the tumor invasion and lymph metastasis, suggesting that VEGF plays an important role in the invasion and metastasis of CSCC (Bock *et al.*, 2008). It is confirmed that baicalein can regulate tumor angiogenesis by inactivating VEGF (Ling *et al.*, 2011). In addition, baicalein can down-regulate the expression of VEGF protein, thus exerting the anti-cancer effect on non-small cell lung cancer (Cathcart *et al.*, 2016). Results of this study showed that, the baicalein could down-regulate the VEGF protein expression in A431 cells. This indicates that, the inhibition of A431 cell migration and invasion by baicalein may be related to its down-regulating the VEGF expression.

In conclusion, baicalein can not only inhibit the proliferation of human CSCC A431 cells and promote their apoptosis, but also inhibit their migration and invasion. The mechanism may be related to its down-regulation of MMP-2, MMP-9 and VEGF expression. This study has enriched the theoretical basis of baicalein in the treatment of CSCC. However, the more detailed action mechanism of baicalein needs to be further verified.

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