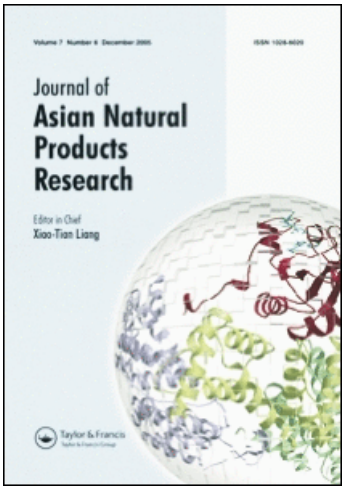

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Tai-Ping He[a]; Wei-Hong Yan[b]; Li-Er Mo[c]; Nian-Ci Liang[a]
[a] Guangdong Medical College, Institute of Biochemistry and Molecular Biology, Zhanjiang, China [b] Toronto Western Research Institute, Toronto Western Hospital, Toronto, Canada [c] Guangdong Key Laboratory for Research and Development of Natural Drugs, Guangdong Medical College, Zhanjiang, China

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Tai-Ping He[a], Wei-Hong Yan[b], Li-Er Mo[c] and Nian-Ci Liang[a]*

[a]*Guangdong Medical College, Institute of Biochemistry and Molecular Biology, Zhanjiang 524023, China;* [b]*Toronto Western Research Institute, Toronto Western Hospital, Toronto, Canada M5T 2S8;* [c]*Guangdong Key Laboratory for Research and Development of Natural Drugs, Guangdong Medical College, Zhanjiang 524023, China*

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Aloe-emodin (AE) has been demonstrated to have antitumor activity in several tumor cells. However, no information is available on the effect of AE on metastasis in human carcinoma cells. This study was designed to investigate the inhibitory effect of AE on the metastasis potential of HO-8910PM cell line *in vitro*, and the role of AE in focal adhesion kinase (FAK) expression. Transwell chamber assay was performed to determine the effect of AE on the invasion and migration capacities of the cells. The effect of AE on the adhesion potential of HO-8910PM cells was determined by cell–Matrigel adhesion assay. We found that AE significantly inhibited invasion, migration, and adhesion capacities of HO-8910PM cells, and, furthermore, reduced the protein and mRNA expression of FAK. These findings suggest that the possible mechanistic explanation for the inhibitory effect of AE on metastasis potential *in vitro* is involved in FAK expression.

Keywords: aloe-emodin; invasion; migration; adhesion; focal adhesion kinase

1. Introduction

Aloe-emodin (AE), an anthraquinone derivative isolated from the root and rhizome of *Rheum palmatum*, has laxative, antifungal, antibacterial, antiviral, and hepatoprotective effects. AE has also been reported to have antitumor activity by inducing the arrest of cell cycle in HL-60 cells[1] and apoptosis in T24 human bladder cancer cells,[2] t-HSC/Cl-6 cells,[3] and human lung non-small cell carcinoma cells.[4] However, to our knowledge, there is no available information about the effect of AE on metastasis in human carcinoma cells *in vitro*. Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase which provides signaling and scaffolding functions at sites of integrin adhesion. It is involved in the regulation of turnover of these adhesion sites, a process that is crucial in the control of cell migration. Evidence has also showed that the overexpression of FAK is associated with the invasive potential of tumors.[5,6]

Therefore, the major purpose of our research was to investigate the inhibitory effect of AE on the metastasis potential of HO-8910PM cells *in vitro*, and to study its possible role of AE in FAK expression.

2. Results and discussion

2.1 Results

2.1.1 Effect of AE on the growth of HO-8910PM cells

Our study showed that AE had low cytotoxicity for HO-8910PM cell line at high

*Corresponding author. Email: ncliang@gdmc.edu.cn

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Table 1. Effect of AE on the migration ability of HO-8910PM cells to matrigel.

Group	Cell count per field	Inhibitory rate (%)
Control	51.2 ± 5.9	0
0.1% (DMSO)	51.2 ± 6.8	0.0 ± 13.3
AE (20 μmol L^{-1})	31.0 ± 4.0*	39.5 ± 7.8*
AE (40 μmol L^{-1})	30.8 ± 3.3*	39.8 ± 6.4*
AE (80 μmol L%$^{-1}$)	24.4 ± 3.0*	52.3 ± 5.8*

Data are means ± SD ($n = 3$). *$P < 0.05$ *vs.* control group.

concentrations. Its IC_{50} value was 138.90 ± 2.34 μmol L^{-1} after 24 h treatment. Compared with the control group, 10–320 μmol L^{-1} AE treatment for 6 h showed no significant effect on cell cytotoxicity as determined by MTT assay. Its IC_{50} value was 203.72 ± 2.02 μmol L^{-1}. To rule out the possibility that the inhibition of tumor cell migration, invasion, and adhesion was due to AE-induced cytotoxicity, we chose 20, 40 and 80 μmol L^{-1} AE, which were much lower than IC_{50} values, as the experimental concentrations in our study.

2.1.2 AE inhibits the migrative ability of HO-8910PM cells

As shown in Table 1 and Figure 1(A),(B), treatment with 20, 40 and 80 μmol L^{-1} AE resulted in a significant reduction in the migrated cell number. Inhibitory rates were 39.5 ± 7.8, 39.8 ± 6.4 and 52.3 ± 5.8%, respectively.

2.1.3 AE inhibits the invasive ability of HO-8910PM cells

As shown in Table 2 and Figure 1(C),(D), the invasion ability of HO-8910PM cells was significantly different from that of the control group. After the treatment of the cells with 20, 40 and 80 μmol L^{-1} AE for 6 h, inhibitory

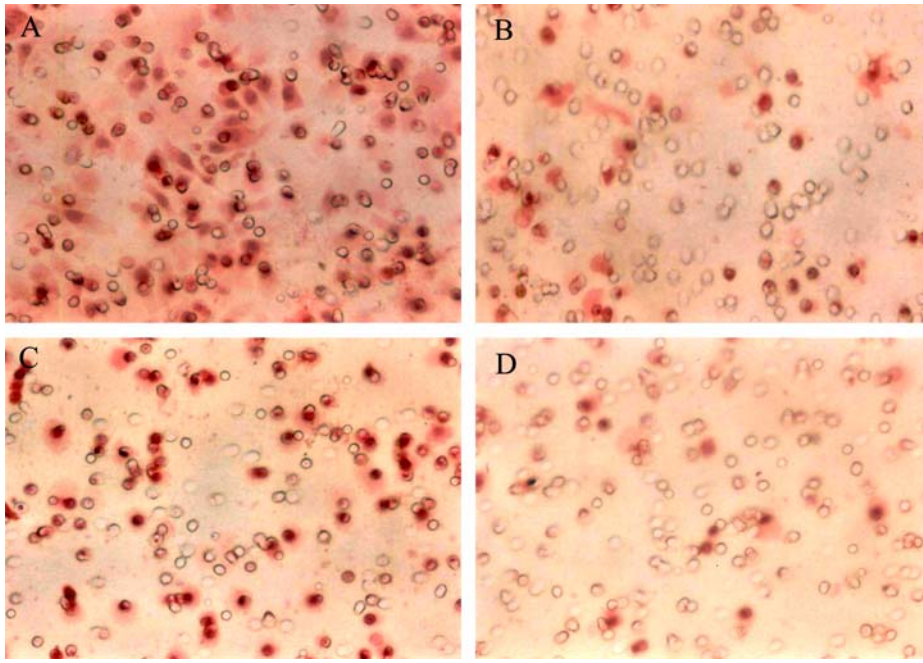

Figure 1. HO-8910PM cells passed through the PVPF membrane after treatment with AE for 6 h. (A) Control cells in the migration test (PBS). (B) AE-treated cells in the migration test (80 μmol L^{-1}). (C) Control cells in the invasion test (PBS). (D) AE-treated cells in the invasion test (80 μmol L^{-1}). Results were representative of three independent experiments.

Table 2. Effect of AE on the invasion ability of HO-8910PM cells.

Group	Cell count per field	Inhibitory rate (%)
Control	37.4 ± 9.5	0
0.1% (DMSO)	31.0 ± 8.5	17.1 ± 22.7
AE (20 μmol L^{-1})	33.6 ± 1.3	10.2 ± 3.6
AE (40 μmol L^{-1})	23.0 ± 3.9*	38.5 ± 10.4*
AE (80 μmol L^{-1})	20.0 ± 2.7*	46.5 ± 7.1*

Data are means ± SD ($n = 3$). *$P < 0.05$ *vs.* control group.

rates were 10.2 ± 3.6, 38.5 ± 10.4 and 46.5 ± 7.1%, respectively.

2.1.4 AE inhibits the adhesive ability of HO-8910PM cells to reconstituted matrigel membrane

After incubation for 4 h, 20, 40 and 80 μmol L^{-1} AE showed a significant decrease in adhesion to Martigel compared with the control group. Inhibitory rates were 28.8 ± 1.7, 36.8 ± 0.3 and 45.3 ± 1.0%, respectively, whereas 0.1% DMSO showed no effect on the adhesion of HO-8910PM cells (Table 3).

2.1.5 AE reduces the expression of FAK

Western blot analysis showed that AE could decrease the expression of FAK (Figure 2). After the treatment of the cells with 20, 40 and 80 μmol L^{-1} AE, the relative levels of FAK protein were 0.83 ± 0.06-, 0.47 ± 0.04-, and 0.41 ± 0.04-fold of the control group, respectively. The difference between the AE-treated group and the control group was significant ($P < 0.05$).

Table 3. Effect of AE on the adhesion ability of HO-8910PM cells to Matrigel.

Group	A570 nm	Inhibitory rate (%)
Control	0.843 ± 0.125	0
0.1% (DMSO)	0.753 ± 0.180	8.2 ± 5.7
AE (20 μmol L^{-1})	0.628 ± 0.114*	28.8 ± 1.7*
AE (40 μmol L^{-1})	0.517 ± 0.055*	36.8 ± 0.3*
AE (80 μmol L^{-1})	0.468 ± 0.075*	45.3 ± 1.0*

Data are means ± SD ($n = 3$). *$P < 0.05$ *vs.* control group.

2.1.6 AE decreases FAK mRNA expression

RT-PCR result showed that the expression of FAK mRNA was downregulated. After the treatment of the cells with 20, 40 and 80 μmol L^{-1} AE, the relative mRNA levels of FAK, expressed as the FAK/GAPDH ratio, were 0.23 ± 0.04, 0.18 ± 0.05 and 0.13 ± 0.05-fold of the control group, respectively. The relative mRNA level of FAK was also significantly different between the AE-treated group and the control group ($P < 0.05$, Figure 3).

2.2 Discussion

Ovarian carcinoma is one of the most aggressive female cancers in the world, which is commonly first diagnosed in an advanced stage with distal metastasis. The major cause of treatment failure and recurrence is the invasion of tumor cells into the surrounding normal tissue or distal area. Prophylactic chemotherapy is a promising way to prevent the further development of ovarian carcinoma. Large numbers of active components of Chinese medicine, such as herbals, have been reported to exhibit certain potential in inhibiting different stages of carcinogenesis.[8,9] To aim at developing novel anticancer drugs selective to tumor cells and with low toxicity for normal host cells, we focus on AE which has been proved promising in the treatment of a variety of diseases. In terms of tumor therapy, AE has been demonstrated to have tumor-suppressing activity by inducing apoptosis in several human tumor cells.[2–4] However, no study about AE on metastasis of human carcinoma cells *in vitro* has been reported so far. In our study, we have demonstrated a possible role that AE may play to directly inhibit the migration, invasion, and adhesion of HO-8910PM cells *in vitro*. Our data also showed that AE did induce a dose-dependent

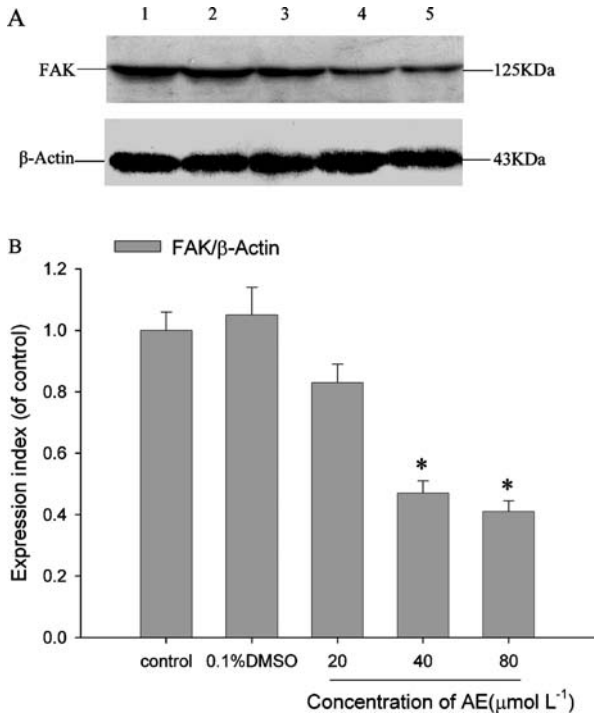

Figure 2. Effect of AE on the expression of FAK in HO-8910PM cells. (A) The effect of AE on the expression of FAK and β-actin in HO-8910PM cells. Cell lysates were analyzed by 10% SDS-PAGE and then probed with FAK and β-actin antibody as described in the Western blot assay. Lane 1, PBS; lane 2, 0.1% DMSO; lane 3, 20 μmol L^{-1} AE; lane 4, 40 μmol L^{-1} AE and lane 5, 80 μmol L^{-1} AE. Results were representative of three independent experiments. (B) Image analysis of the expression of FAK by the Scion Image software. The expression of FAK and β-actin of HO-8910PM cells were quantified by the densitometric analysis of X-ray film. The relative level of FAK was expressed as the FAK/β-actin ratio. Data are means ± SD ($n = 3$). $^{*}P < 0.05$ *vs.* control group.

inhibitory effect on the adhesion of HO-8910PM cells to the reconstituted Matrigel membrane.

Tumor metastasis is a multistep process that involves cell detachment from the primary tumor, entry into the vascular or lymphatic system, dispersal through the circulation, and proliferation after extravasation in target organs.[10] Tumor cell invasion through matrix and tissue barriers requires the combined effects of increased cell motility and regulated proteolytic degradation of the matrix. Cell migration is an important component of the metastatic process and requires repeated adhesion to and detachment from the extracellular matrix microenvironment.[11] Therefore, tumor cell migration, invasion, and adhesion are crucial steps in the metastatic cascade of cancer cells. Interruption of these steps is considered to be a potential strategy in the prevention and treatment of tumor metastasis. Our study demonstrated that AE inhibited the malignant tumor cell metastasis-associated behavior *in vitro* most likely by blocking these essential steps of tumor metastasis including the invasion, migration, and adhesion of HO-8910PM cell line.

Nevertheless, the underlying mechanism of the action of AE has remained largely unclear. FAK is a non-receptor tyrosine kinase which is overexpressed in various kinds of cancer tissues and cancer cell lines, and induces survival, proliferation, and motility of cells in culture. Evidence has proved levels of FAK expression correlating

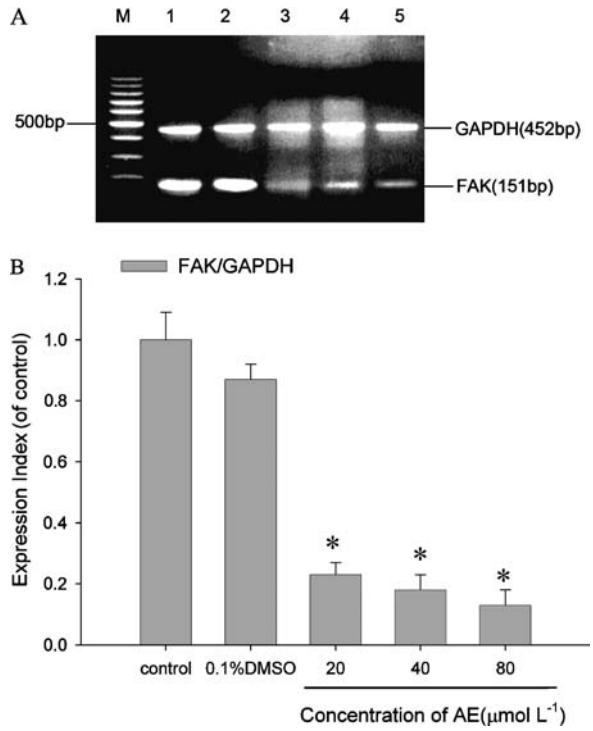

Figure 3. Effect of AE on the expression of FAK mRNA in HO-8910PM cells. (A) The expression of FAK mRNA was detected by RT-PCR. HO-8910PM cells were incubated with or without various concentrations of AE for 24 h. RNA samples were prepared from control or AE-treated cells. PCR products were run on a 2% agarose gel. Lane M, marker; lane 1, PBS; lane 2, 0.1% DMSO; lane 3, 20 μmol L^{-1} AE; lane 4, 40 μmol L^{-1} AE and lane 5, 80 μmol L^{-1} AE. Results were representative of three independent experiments. (B) Image analysis of the FAK mRNA expression by the Scion Image software. The mRNA levels of FAK and GAPDH on gel electrophoresis were quantified by densitometric analysis of gel photographs and expressed as the FAK/GAPDH ratio. Data are means ± SD ($n = 3$). $^{*}P < 0.05$ *vs.* control group.

with the invasive potential of tumors. The inhibition of FAK function might provide an attractive anticancer target.[12] Van de Water and colleagues have demonstrated that FAK is required for metastasis in a syngeneic rat model. Specifically, inducible expression of an inhibitory FAK protein, FAK-related non-kinase, in MTLn3 mammary adenocarcinoma cells, suppressed the growth of primary tumors and blocked metastasis formation in the lungs.[13] Huang reported that FAK activity can be suppressed by specific FAK siRNA, luteolin and quercetin, and that the siRNA targeting FAK almost completely inhibited FAK protein expression in MiaPaCa-2 cells and potently blocked the cell migration mediated by FAK.[14] In our study, we examined FAK protein and mRNA expression after the treatment of HO-8910PM cells with AE. A significant decrease in FAK mRNA expression and suppression in FAK protein expression were observed in AE-treated cells. This is consistent with Huang's study, which demonstrates that decreased FAK expression in tumor cells inhibits migration, invasion, and adhesion. Emodin, the analog of AE, has recently been demonstrated to inhibit cell adhesion of various human cancer cells through suppressing the recruitment of FAK to integrin beta,[6] so our result also suggests that AE and emodin have a very similar mechanism in this aspect.

In summary, AE can inhibit the migration, invasion, and adhesion of HO-8910PM cells, and its possible mechanism may be associated with a decrease in FAK expression. Given this clear evidence of the inhibitory effect of AE, further study on anti-metastasis of the compound *in vivo* may be warranted.

3. Experimental

3.1 Materials

AE was purchased from Sigma Co. (St. Louis, MO, USA) dissolved in DMSO as a stock solution (20 mmol L^{-1}), and then stored at 4°C until use. The compound was diluted in RPMI-1640 medium immediately before use. Calf serum and RPMI-1640 were purchased from Invitrogen, Co., CA, USA. Transwell chamber (6.5 mm in diameter, 8 μm pore size polyvinyl pyrrolidone-free (PVPF) membrane) was obtained from Corning, Inc. (Corning, NY, USA) Matrigel was obtained from BD Biosciences. Fibronectin was purchased from Sigma, Co. FAK mouse monoclone antibody was purchased from Neomarkers Co. (Fremont, CA, USA). Rabbit polyclonal β-actin antibody and HRP-labeled goat antimouse IgG were purchased from Santa Cruz Biotechnology. One-step RT-PCR assay kit was obtained from Qiagen, Co., Germany. Super ECL Plus Detection Reagent kit was purchased from Applygen, Co., China.

3.2 Cells and cell culture

Human highly metastatic ovarian carcinoma HO-8910PM cell line[7] was grown in RPMI-1640 supplemented with 10% heat-inactivated calf serum, at 37°C in a 5% CO_2 humidified atmosphere.

3.3 Cell cytotoxicity assay

HO-8910PM cells were subcultured into a 96-well plate at 1×10^4 cells per well. Various concentrations of AE diluted in culture medium were added. Triplicate wells were used for each determination. The plates were incubated at 37°C in 5% CO_2 for 6 or 24 h when the control cells reached 90% confluence, and MTT solution was then added to each well and the plates were incubated for another 4 h. The plates were read on enzyme-linked immunosorbent assay reader at a wavelength of 570 nm. The IC_{50} was defined as the concentration of the AE required to reduce the optical density by 50% in treated cells compared with the control cells.

3.4 Cell migration assay

Cell migration assay was performed using Transwell chamber system. In brief, the under surface of the PVPF membrane was coated with 5 μg fibronectin. Cells (1×10^5) in 0.5 ml serum-free medium containing 0.1% BSA were placed in the upper chamber, and the lower chamber was filled with 0.6 ml of RPMI-1640 culture medium with 0.1% BSA. After treatment with 20, 40 and 80 μmol L^{-1} AE for 6 h, the cells on the upper surface of the membrane were removed using cotton tips. The migrant cells attached to the under surface were fixed in methanol for 1 min and stained for 3 min with hematoxylin, followed by washing with water, and stained for 3 s with eosin. The number of migrated cells on the lower surface of the membrane was counted under a microscope in five fields of × 200 magnification. Triplicate samples were conducted. Data were expressed as the average cell number of 15 fields. The inhibitory rates were calculated according to the following formula:

$$\text{Inhibitory rate}\,(\%) = \left(1 - \frac{\text{the mean of treated group}}{\text{the mean of control group}}\right) \times 100\%.$$

3.5 Cell invasion assay

Matrigel invasion ability of cells was assayed using a Transwell chamber. The upper surface of the membrane was coated with 5 μg Matrigel, and the under surface of the membrane was coated with 5 μg fibronectin,

and then allowed to air-dry overnight. The next steps were performed according to the cell migration assay.

3.6 Cell adhesion assay

In brief, 96-well, flat-bottom culture plates were coated with 10 μg Matrigel, blocked with 0.2% BSA for 2 h, and then washed with PBS. Cells (5×10^4 per well) were added to each well in triplicate and treated by 20, 40 and 80 μmol L^{-1} AE for 4 h. Plates were washed with serum-free RPMI-1640 medium to remove unbounded cells. The remaining cells attached to the plates were quantified with MTT assay.

3.7 Western blot assay

Cells were seeded at 2×10^5 cells per well in a six-well plate. After treatment with 20, 40 and 80 μmol L^{-1} AE for 24 h, confluent cells were washed with cold PBS and lysed with cold lysis buffer. Total protein concentration was determined according to the method of Bradford protein assay. Protein samples were loaded onto a 10% SDS-polyacrylamide gel and run at 150 V using running buffer. The protein was transferred onto a PVDF (polyvinylidene difluoride) membrane and then blocked for 1 h with 5% non-fat milk in TBS. Primary antibody was incubated for 1 h. After being washed, the membrane was incubated with horseradish peroxidase-labeled second antibody for 1 h, and then Western blot was visualized for the enhanced chemiluminescence according to the kit instructions. The results were documented on an X-ray film. Image analysis result was performed by the Scion Image software, with β-actin as an inner control.

3.8 RT-PCR analysis

Total RNAs were isolated using an RNeasy Mini kit, and RT-PCR analysis was performed with a one-step RT-PCR assay kit. Primers for RT-PCR amplification were as follows: FAK mRNA forward, 5′-ATGTTCTGGTGTCCTCAAATG-3′; FAK mRNA reverse, 5′-GAGGTAAAACGTCGAAAATTG-3′; GAPDH mRNA forward, 5′-ACCACAGTCCA TGCCATCAC-3′; and GAPDH mRNA reverse, 5′-TCCACCACCCTGTTGCTGTA-3′. After an initial incubation at 48°C for 45 min and denaturation at 94°C for 2 min, the following cycling parameters (36 cycles) were used: denaturation at 94°C for 30 s, annealing at 53°C for 30 s, and elongation at 68°C for 2 min. RT-PCR products underwent electrophoresis in a 2% agarose gel. The mRNA levels of FAK and GAPDH on gel electrophoresis were quantified by the densitometric analysis of gel photographs and expressed as the FAK/-GAPDH ratio.

3.9 Statistical analysis

Data were expressed as means ± SD. Student's *t*-test was used to evaluate the difference between the two groups. All statistical tests were two-sided and carried out with the SPSS statistical software package (version 10.0). *P* values less than 0.05 were considered statistically significant.

Acknowledgement

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