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# Hangambujeongsan or Kangai Fuzheng Powder shows the anti-cancer effect by enhancing macrophage activation

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## ABSTRACT

**Objectives :** Many of currently used anti-cancer drugs were developed to target cell death mechanisms and had serious side effects by causing damage to normal cells. Hangambujeongsan or Kangai Fuzheng Powder was a mixture based on the traditional Chinese medicine. It had been used in the local Chinese hospitals to treat cancer patients for decades and had shown a certain level of beneficial effects without major toxic effects. But its mechanism of action had not been elucidated yet. Thus this study aimed to investigate the effects of Kangai Fuzheng Powder in an *in vitro* experiment.

**Methods :** Cancer lines or RAW264.7 mouse macrophage cells were treated with Kangai Fuzheng Powder. Cell viability was measured by MTT assay, and morphological observation was also performed. Gene expression of cytokines in macrophages was determined by real-time polymerase chain reaction. Phagocytic function assay was also performed in macrophage cells.

**Results :** Kangai Fuzheng Powder had no direct detrimental effect on cancer cells. When macrophages were co-cultured with cancer cells, Kangai Fuzheng Powder had toxic effect on cancer cells. After exposing macrophages to Kangai Fuzheng Powder, macrophages transformed into activated form and the mRNA level of tumor necrosis factor-alpha, interleukin-1beta, interleukin-6, interleukin-10 and monocyte chemotactic protein-1 was significantly enhanced. Phagocytic activity of macrophages was dramatically potentiated.

**Conclusions :** We demonstrated that anti-cancer effect of Kangai Fuzheng Powder was related to activation of macrophages including enhanced cytokine production and phagocytic function.

**Key words :** Hangambujeongsan or Kangai Fuzheng powder, Macrophage, cytokine, phagocytosis, traditional Chinese medicine

## Introduction

Cancers have been one of the most detrimental diseases to human, and a leading cause of death in many countries. For the last decade, a lot of new anti-cancer drugs has been developed and contributed to the successful treatment of cancer. Some of recently developed drugs have shown specific targets, reducing unnecessary side effects by chemotherapy[1-2]. However, most of the currently used anti-cancer drugs still have serious side effects such as toxicity to rapidly proliferating normal cells including bone marrow, hair, and gastrointestinal mucosa cells[3-6]. Especially, bone marrow suppression could cause immune deficiency and lead to serious infection of the patient[7-10]. Therefore there have been trials to develop a new type of anti-cancer drugs which have no serious side effects. Some of the traditional Chinese medicines are known to treat cancer by killing cancer cells directly while others are known to enhance the immunity, thereby terminate cancer cells indirectly[11-13].

Hangambujeongsan or Kangai Fuzheng Powder is a mixture of many ingredients based on the traditional Chinese medicine remedy used in the clinical treatment of malignant tumors without major toxic or

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side effects[14]. However, its mechanisms for therapeutic effects has not been investigated yet. Therefore this study aims to investigate the effects of Kangai Fuzheng Powder on cancer cells and macrophages using *in vitro* culture system.

# Materials and Methods

## 1. Reagents and materials

Kangai Fuzheng Powder is a powder form of medicine made of mixture of many traditional Chinese traditional medicinal ingredients such as Zhi Ke (*Citrus aurantium* L.), Zang Hong Hua (Crocus sativus), Yún Nán Zhòng Lóu (*Rhizoma Paridis Yunnanensis*), Quán Xiē (Scorpion), She Tui (*Elaphe carinata*), Jia Yu (*Trionychidae*), Ganqi (*Resina toxicodendri*). Kangai Fuzheng Powder is developed by Mr. Yang WanQuan and his private pharmaceutical facility at Heqing County (Yunnan Province, China) is in charge of the production and supply of this drug. 10g of powder was mixed with 100ml of culture media used for the cell culture study in this experiment and sonicated for 10 minutes on ice. The suspension was filtered with 0.22μm membrane filter from Millipore (Darmstadt, Germany) to remove debris and endotoxin. Filtered solution was isolated and kept in −70℃ as a stock solution (100mg/ml). The stock solution was thawed and diluted with culture media before use. The final concentrations of Kangai Fuzheng Powder in the culture media were 0.01, 0.1, 1, 10, 100, 1,000 and 10,000 μg/ml. Other chemical reagents were obtained from Sigma Chemical Co. (St Louis, MO, USA) and culture media and supplements were from Invitrogen (Carlsbad, CA, USA) and HyClone (South Logan, UT, USA).

## 2. Cell Culture and treatment

RAW264.7 (mouse macrophage), BV2 (mouse microglia), A549 (human lung cancer cell), Dul45 (human prostatic cancer cell), Hep3B (human hepatic cancer cell), SK−BR−3 (human breast cancer cell), MCF7 (human breast cancer cell) and SNU354 (human hepatic cancer cell) cell lines were used in this experiment. Cells were cultured in culture media as suggested by provider's manual. The cells were incubated at 37℃ under a humidified atmosphere of 95% air and 5% $CO_2$. To observe the indirect effect of Kangai Fuzheng Powder we co−cultured macrophages on the membrane of Transwell insert (Corning, USA) and cancer cells on the bottom of the same plate well while direct effect was measured by applying Kangai Fuzheng Powder to the cells in the regular culture plate. Lipopolysaccharide (LPS, 1 μg/ml) was used as the activator of macrophages and LPS treated macrophage sample was used as a positive control. Morphological changes were observed under inverted microscope (Olympus IX51, Japan).

## 3. Cell viability assay

Cells were seeded in 24−well culture plates ($4 \times 10^4$ cells/well) and incubated at 37℃ in complete media for 16 h before the drug treatment. Kangai Fuzheng Powder was treated into the culture media for 48 hours and cellular injury was observed morphologically using microscope (Olympus IX51, Japan) and biochemically using 3−(4, 5−dimethylthiazol−2−yl)−2, 5−diphenyltetrazolium bromide (MTT) assay. MTT assay was done as previously described[15]. Briefly, MTT was added to each well, and the cells were incubated for 4 h at 37℃. After discarding the culture media, dimethyl sulfoxide (DMSO) was added to dissolve the formazan dye. Absorbance at 570 nm was measured with the GENius Plus microplate reader (Tecan, Männedorf, Switzerland).

## 4. Real−time polymerase chain reaction (PCR) analysis

Total RNA was isolated from cultured cells using RNeasy Mini Kit (Qiagen, USA). Aliquots of total ribonucleic acid (RNA) were reverse transcribed into single−stranded complementary deoxyribonucleic acid (cDNA) by High−Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA). Sequence−specific primers for the SYBR Green assay were designed with Primer Express 1.0 software (Applied Biosystems, Foster City, CA). Real−time PCR was done in triplicate on a 7500 Real time PCR system (Applied Biosystems, Foster City, CA). Relative gene expression was analysed with the comparative Ct method with glyceraldehyde 3−phosphate dehydrogenase (GAPDH) as the endogenous control after confirming that the efficiencies of the target and the endogenous control amplifications were approximately equal. Results are presented as target gene expression normalized to GAPDH. The kit contains HotStarTaq polymerase which is included to avoid false positives in the quantitative PCR. Nucleotide sequences of the primers were displayed in Table 1.

Table 1 : Primers for Real-time PCR

| Gene name | Sequence (5' -> 3' ) |
|---|---|
| TNF-alpha F | GAT CTC AAA GAC AAC CAA CAT GTG |
| TNF-alpha R | CTC CAG CTG GAA GAC TCC TCC CAG |
| IL-1beta F | CCA GGA TGA GGA CAT GAG CAC C |
| IL-1beta R | TTC TCT GCA GAC TCA AAC TCC AC |
| IL-6 F | ACT TCC ATC CAG TTG CCT TC |
| IL-6 R | TTC TCA TTT CCA CGA TTT CC |
| IL-10 F | AGT GGA GCA GGT GAA GAG TG |
| IL-10 R | TTC GGA GAG AGG TAC AAA CG |
| MCP-1 F | CCC ACT CAC CTG CTG CTA CT |
| MCP-1 R | TCT GGA CCC ATT CCT TCT TG |
| GAPDH F | GCC AAG GTC ATC CAT GAC AAC |
| GAPDH R | AGT GTA GCC CAA GAT GCC CTT |

### 5. Phagocytosis assay

Phagocytosis assay kit (Molecular probes, V6694; FITC-*E.Coli*, USA) was purchased and all the experiments were done according to the vender' s manual. Briefly, 2 h after adding fluorescein isothiocyanate (FITC) labeled bacterial cell debris into the cultured macrophage, observation of the phagocytosis of bacterial cell debris by macrophage was done with inverted microscope (Olympus IX51, Japan) and measurement of FITC fluorescence signal using the GENius Plus microplate reader (Tecan, Männedorf, Switzerland) was performed.

### 6. Statistical analysis

Data were represented as the mean ± SE. Comparisons between groups were performed using ProStat software (Poly Software International, Inc, USA). Significant difference was considered at $p<0.05$ using one-way analysis of variance (ANOVA) followed by Tukey' s multiple comparison procedure.

## Results

### 1. Cellular toxicity

Kangai Fuzheng Powder showed no direct toxicity to cancer cells at the dosages lower than 1 mg/ml and 10 mg/ml was the only concentration demonstrating direct toxicity (Fig. 1). At the lower concentration (1 and 10 μg/ml), some cancer cells even showed proliferating tendency. 10 mg/ml is a very high therapeutic dosage compared to the usual range of other anti-cancer drugs and should be over the clinically used dosage range of Kangai Fuzheng Powder since patients are prescribed to take 2-3 grams three times per day orally. Immune cells such as macrophages and microglia showed a little different pattern. Kangai Fuzheng Powder caused direct toxicity to these cells even at lower concentrations. Macrophages were more susceptible to Kangai Fuzheng Powder than microglia (Fig. 2).

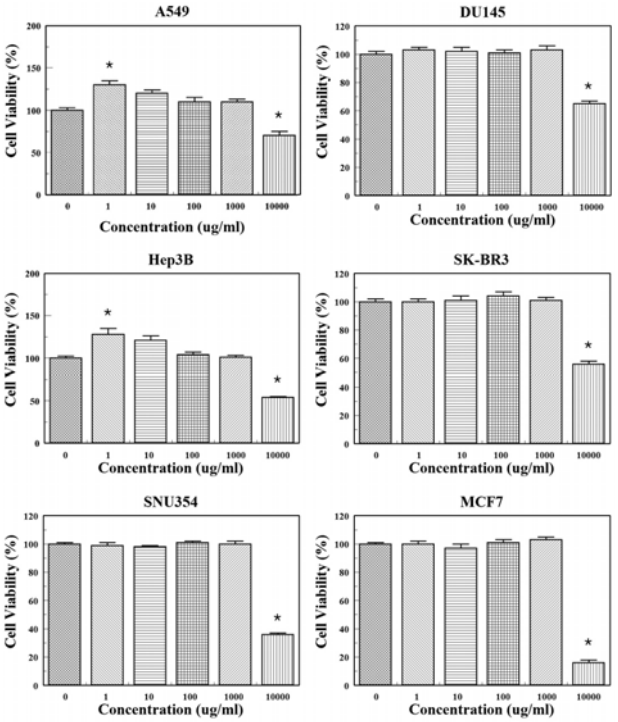


Fig. 1. Cytotoxicity assay in cancer cells after Kangai Fuzheng Powder treatment. Cytotoxic effect of Kangai Fuzheng Powder was measured with MTT assay. All the cancer cells tested in this experiment including A549, Dul45, Hep3B, SK-BR-3, MCF7 and SNU354, showed no damage at concentrations up to 1 mg/ml. Cellular toxicity was obvious only at very high concentration (10 mg/ml) after 48 h of drug treatment. Statistically significant at $P < 0.05$ of values * compared with the concentration at 0.

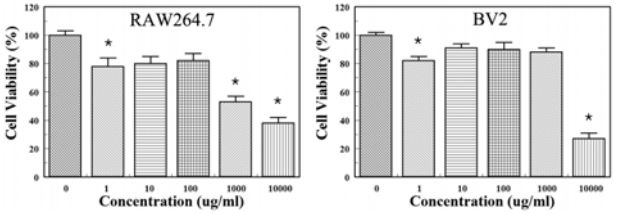


Fig. 2. Cytotoxicity assay in immune cells after Kangai Fuzheng Powder treatment. Cytotoxic effect of Kangai Fuzheng Powder was measured with MTT assay. Immune cells, RAW264.7 macrophages and BV2 microglia, showed direct damage by drug treatment at lower doses. Statistically significant at $P < 0.05$ of values * compared with the concentration at 0.

We expect that this direct injury should be mediated by macrophages themselves and macrophage mediated toxicity might be related with anti-cancer effect shown in clinical cases. To investigate the macrophage mediated toxicity, we observed indirect effect of Kangai Fuzheng Powder in the cancer cell and macrophage co-culture system. By plating MCF7 cancer cells in the bottom of culture plate and seeding the macrophages on the membrane of Transwell insert, two cells are placed in the same culture media with some distance. MCF7 cells showed no direct toxicity to Kangai Fuzheng Powder at the dosages below 1 mg/ml but demonstrated indirect toxicity even at 1 μg/ml. Indirect toxicity was increased dose dependently

(Fig. 3). This data indicate that some biologically active materials secreted from the macrophages caused toxicity to cancer cells. To further investigate the precise reason of indirect toxicity we performed more experiments.

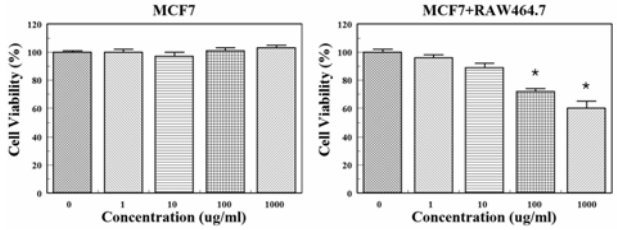


Fig. 3. Cytotoxicity assay in co-culture system after Kangai Fuzheng Powder treatment. Cytotoxic effect of Kangai Fuzheng Powder was measured with MTT assay. MCF7 cancer cells co-cultured with RAW264.7 macrophages showed damage after drug treatment while there was no toxicity in MCF7 cells without macrophages. Statistically significant at P 〈 0.05 of values * compared with the concentration at 0.

## 2. Morphological changes of macrophage

When macrophages were treated with Kangai Fuzheng Powder, there were dramatic morphological changes in macrophages. Normal resting macrophages were small and round shaped cells. When cells were exposed to lipopolysaccharide (LPS, 1 μg/ml), they transformed into larger activated cells as it is well demonstrated by other previous studies. At lower doses (1 and 10 μg/ml), Kangai Fuzheng Powder activated cells are minimal and macrophages became bigger cells with multiple podia on the surface at higher doses (100 and 1000 μg/ml). When macrophages were activated, the number of cells observed under the microscope was less than non-activated cells (Fig. 4). This morphological transformation implies Kangai Fuzheng Powder can stimulate macrophages. Activation of macrophages was further confirmed by measuring cytokine induction and phagocytosis activity in the next step.

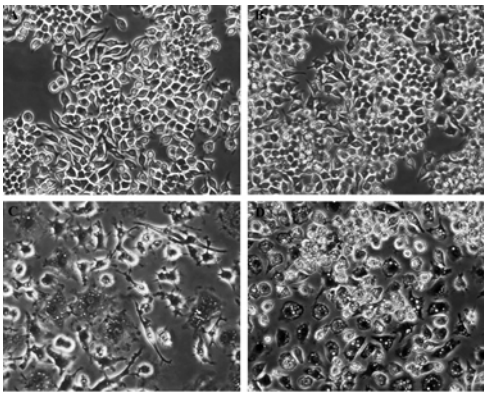

Fig. 4. Morphological changes under microscope. Morphological features of Raw264.7 macrophage cells under normal culture condition (A), after activation by LPS (1 μg/ml) (B), and 10 μg/ml (C) or 1000 μg/ml (D) of Kangai Fuzheng Powder treatment.

## 3. Cytokine gene expression of macrophage

Kangai Fuzheng Powder was administered to macrophages and RNA sampling was done at 1, 24 and 48 h after drug treatment and induction of cytokines was observed. From Kangai Fuzheng Powder treated macrophages total RNA was isolated and reverse transcription was performed to obtain cDNA. Using these cDNAs we performed real-time PCR analysis of some representative cytokines. Induction of tumor necrosis factor (TNF)-alpha and interleukin (IL)-1beta was marked at 1 h while IL-6, IL-10 and monocyte chemotactic protein (MCP)-1 were prominently induced at 24 and 48 h. This induction of cytokines was dose-dependently increased with highest level at 1000 μg/ml (Fig. 5). The time profiles of cytokines induction by Kangai Fuzheng Powder was similar to LPS mediated induction.

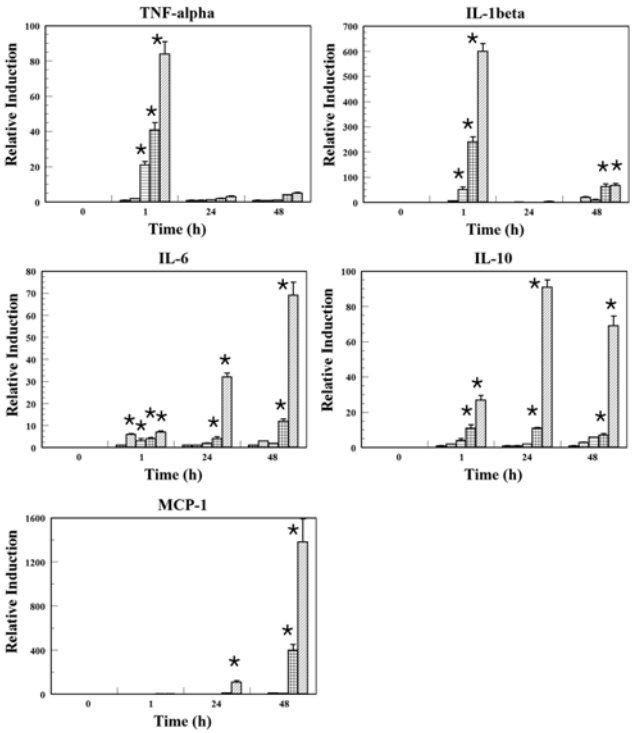


Fig. 5. Cytokine expression using realtime PCR. Effect of Kangai Fuzheng Powder (0, 1, 10, 100 & 1000 μg/ml) on induction of cytokines in Raw264.7 macrophage cells was evaluated using real-time PCR analysis at 1, 24, & 48 h after drug treatment. Gene expression of TNF-alpha, IL-1beta, IL-6, IL-10 & MCP-1 was markedly enhanced by drug treatment in a dose dependent manner. Statistically significant at P 〈 0.05 of values * compared with the time point at 0.

## 4. Phagocytic activity of macrophage

In addition to cytokine induction, phagocytotic activity of foreign substances was measured to evaluate activation of macrophages. Kangai Fuzheng Powder treatment enhanced the phagocytic activity of macrophages when FITC labeled bacterial debris was incubated for 2 h

with macrophages. Normal resting macrophages had a lower level of phagocytic activity but it was potentiated by Kangai Fuzheng Powder treatment in a dose dependent manner (Fig. 6).

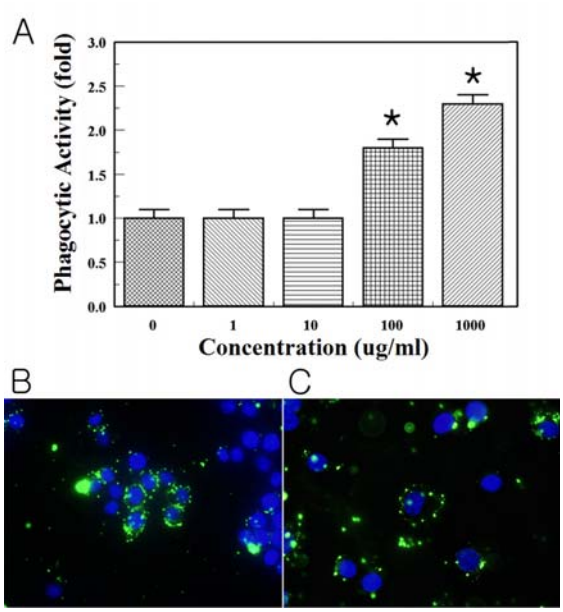


Fig. 6. Phagocytosis assay. Effect of Kangai Fuzheng Powder (0, 1, 10, 100 & 1000 μg/ml) on phagocytic function of Raw264.7 macrophage cells was observed. Phagocytosis was increase in a dose dependent manner. Statistically significant at $P < 0.05$ of values * compared with the concentration at 0 (A). Representative images of phagocytosis were obtained in LPS (1 μg/ml) treated macrophages as a positive control (B) and macrophages treated with Kangai Fuzheng Powder (1000 μg/ml) for 48 h (C).

## Discussions

Treatment of Kangai Fuzheng Powder was not toxic to cancer cells when applied directly but was toxic when macrophages were co-cultured with cancer cells. This result suggests that anti-cancer effect of Kangai Fuzheng Powder might be mediated by some indirect mechanisms. Macrophages and microglia showed direct toxicity by Kangai Fuzheng Powder while other cells showed no toxic response. We speculate the susceptibility of immune cells to Kangai Fuzheng Powder is related to immune reaction caused by cell themselves. Once immune cells are exposed to Kangai Fuzheng Powder, the cells undergo activation process and then cause apoptosis to terminate its action spontaneously as it is shown in most inflammation or immune cases. Indirect toxicity observed in cancer cells co-cultured with macrophages supported our speculation that Kangai Fuzheng Powder activates macrophages. We hypothesize that some biologically active materials, such as cytokines, are secreted from the macrophages and cause injury to cancer cells. After treatment of Kangai Fuzheng Powder, there was a dramatic morphological change of macrophages from small round resting to bigger activated macrophages. When macrophages were activated, the number of live cells observed under the same magnigication of microscope was less than resting cells. There were some dead cells observed as well. This change provides direct evidence that Kangai Fuzheng Powder can trigger macrophage activation. Activation of macrophages was confirmed by measuring the cytokine induction and phagocytic activity. During the acute period, induction of TNF-alpha and IL-1beta was marked and IL-6, IL-10 and MCP-1 were prominently at delayed period. Phagocytosis of bacterial debris was also enhanced by Kangai Fuzheng Powder treatment. Both cytokine induction and phagocytosis was dose dependently increased.

## Conclusions

In order to investigate the effects of Kangai Fuzheng Powder on cancer cells and macrophages using *in vitro* culture system, we had conclusion as follows.

1. Kangai Fuzheng Powder had no toxic effects on cells, with a wide safety margin, since it only has toxic effects on cells at dosages over 100 times more concentrated than anti-cancer dosages.

2. Kangai Fuzheng Powder's mechanism of anti-cancer action is through the activation of the phagocytic activity of macrophages

3. Kangai Fuzheng Powder also induced cytokines.

We conclude that Kangai Fuzheng Powder can be used as a new approach to develop anti-cancer with lesser side effects and can be used as immune promoting drug as well.

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