

Full Length Research Paper

Effect of allicin on THP-1, MT-2 and WISH cell apoptosis induced by vesicular stomatitis virus (VSV) and the molecular mechanism involved

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Vesicular stomatitis virus (VSV) has been reported to induce apoptosis and the onset of apoptosis may play an important role in virus-associated diseases. This study was conducted in order to investigate the protective effect of the herbal constituent allicin on VSV-induced apoptosis in the human monocyte line THP-1, human T lymphocytic leukemia cell line MT-2 and human amniotic cell line WISH and to determine the possible molecular mechanism involved. The THP-1, MT-2 and WISH cells were incubated with VSV in the absence or presence of different doses of allicin (10, 25 and 50 µg/ml). To study apoptosis, the cells were assessed by MTT and annexin V-propidium iodide double-staining flow cytometry. To investigate the molecular mechanism by which allicin regulates VSV-induced THP-1, MT-2 and WISH cell apoptosis, the expression of active cleavage products of caspases 3, 6, 7 and 9 and NF-κB was analyzed by western blotting. Our results indicated that allicin did not affect the adhesion and entry of VSV into THP-1, MT-2 or WISH cells. Using different concentrations of allicin, a dose-dependent protective effect on cell apoptosis was observed. In addition, the VSV-induced expression of active cleavage products of caspases 3, 6, 7 and 9 and NF-κB in THP-1, MT-2 and WISH cells was also significantly reduced by allicin at the protein level. We concluded that allicin protects THP-1, MT-2 and WISH cells from VSV-induced apoptosis by inhibiting the activation of caspases 3, 6, 7 and 9 and NF-κB, thereby suggesting a potential protective effect for allicin against virus-associated diseases.

Key words: Allicin, vesicular stomatitis virus (VSV), apoptosis, caspases, NF-κB.

INTRODUCTION

Allicin, the major bioactive molecule in garlic, is a volatile substance that is obtained by distillation (Li et al., 2002). Numerous researchers have studied various aspects of garlic, including its chemistry, pharmacology and clinical effects. Studies have indicated that allicin has a range of pharmacological functions, including antibacterial (Cutler and Wilson, 2004), antitumor (Oommen et al., 2004) and antiviral (Zhou et al., 1997) effects. In recent years, increasing attention has been paid to its antiviral activity.

There has been a recent increase in the number of virus-associated diseases, particularly AIDS and this has led to the search for suitable therapeutic methods for curing such diseases. Unfortunately, there is no perfect solution for the treatment of these diseases. Increasingly, researchers are turning to medicinal plants with potential antiviral activities to treat such diseases and there is a growing recognition that apoptosis may play a considerably more important role in the pathogenesis of virus-associated diseases than hitherto thought. It is believed that the pharmacological manipulation of apoptosis might offer new possibilities for the prevention and treatment of these diseases (Peng, 2000). In this study, using a vesicular stomatitis virus (VSV)-induced apoptosis model,

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we examined the effect of allicin on the human monocyte line THP-1, the human T lymphocytic leukemia cell line MT-2 and the human amniotic cell line WISH and attempted to determine the possible molecular mechanism involved. We aimed to identify the possible antiviral mechanism of allicin, thereby enabling us to suggest potential treatments for virus-associated diseases such as AIDS.

MATERIALS AND METHODS

Cell lines, antibodies and reagents

All cell culture medium components were purchased from Invitrogen Life Technologies (San Diego, CA, USA) unless otherwise noted. THP-1, MT-2 and WISH cell lines were all obtained from ATCC (Manassas, VA, USA). VSV was provided by the Immunology Research Institute of Zhejiang University. Allicin was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), Batch Number 100384-200501, with a purity of 88.4%. 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma (Hattiesburg, MS, USA) and an annexin V/FITC-PI assay kit was purchased from Bender MedSystems GmbH (Vienna, Austria). Antibodies used in the western blot analysis were rabbit anti-active caspase-3, caspase-6, caspase-7, caspase-9 (Chemicon, CA, USA), which recognizes only the cleaved large subunit (17 kDa of caspase-3, 15 kDa of caspase-6, 20 kDa of caspase-7, 37 kDa of caspase-9), rabbit anti-NF- κ B/p65 and rabbit anti- β -actin polyclonal primary antibodies (Chemicon, CA, USA). The secondary antibody was horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody.

Apoptosis model

In order to build the apoptosis model and choose the appropriate multiplicity of infection (MOI), WISH cells (5×10^3 cells per well) were seeded in 96-well microtiter plates. After adherence (approximately 4 to 6 h), the cells were incubated for 72 h with VSV diluted with the culture medium to 8 different serial concentrations (0 , 5^{-7} , 5^{-6} , 5^{-5} , 5^{-4} , 5^{-3} , 5^{-2} and 5^{-1}) and each concentration of VSV was placed into 6 repeat wells. Cell viability and apoptosis was measured using the MTT assay and Annexin V-FITC/PI assay.

Observation of the toxicity of allicin

THP-1, MT-2 and WISH cells were incubated at 5×10^3 cells/well in 96-well plates. The cells were treated with different concentrations of allicin (0 to 200 μ g/ml) for 72 h. Each concentration was placed into 6 repeat wells. Cell viability was measured using the MTT assay.

The effect of allicin on the adhesion of VSV

To test the effect of allicin on the adhesion of VSV, cells were incubated at 5×10^3 cells/well in 96-well plates. The cells were treated with allicin (50 μ g/ml) or medium control for 4 h and then infected with VSV (MOI 50:1) for an additional 2 h. After removing the supernatants, the cells were washed with PBS thrice. RNA was extracted and VSV adhering to the cells were detected by quantitative RT-PCR using SYBR Green I. Primer sequences were designed with Primer Premier 5.0 (www.PremierBiosoft.com) by using the sequence published in GenBank (accession number: NC 001560); the primer sequences were as follows: forward primer,

5'-AGT TCA ATG ATT TCA ATG-3' and reverse primer, 5'-TTA ACC AAC ATT CCC AT-3'. The PCR product size was 203 bp.

MTT assay

To investigate the effect of allicin on the growth of cells infected by VSV, THP-1, MT-2 and WISH, cells were incubated at 5×10^3 cells/well in 96-well plates. The cells were pre-incubated with allicin (50, 25 and 10 μ g/ml) for 4 h and then were treated with VSV (MOI 50:1) for an additional 72 h, followed by treatment with 2 mg/ml of MTT for 6 h. Dimethyl sulfoxide (DMSO) was added to each well and the absorbance at 570 nm was measured by ELISA-Reader.

Flow cytometry analysis

Recovery of the cells was monitored by examining the levels of apoptosis at 72 h after VSV and allicin treatment. Annexin V binding and propidium iodide (PI) staining were determined by flow cytometry. Flow cytometry was performed with a 488 nm laser coupled to a cell sorter (BD Biosciences, San Jose, CA, USA). Cells stained with both PI and annexin V (FITC+/PI+) were considered to be necrotic and late apoptotic, whereas the cells stained only with annexin V (FITC+/PI-) were considered to be early apoptotic.

SDS-PAGE and western blot analysis

Cells were cultured and treated with allicin and VSV as mentioned earlier in 100 mm dishes. Cell lysates were prepared for western blot analysis of caspases 3, 6, 7 and 9 and NF- κ B. The concentration of proteins in each cell lysate was determined using a BCA protein assay kit (Pierce, Rockford, IL, USA). Western blot analysis was performed using specific anti-active caspase 3, 6, 7 and 9 (1:500 in PBS) and NF- κ B p65 antibodies (1: 1000 in PBS) and was detected by ECL reaction. Results were recorded and analyzed using VersaDoc imaging system (Bio-Rad, Hercules, CA, USA). Each protein blot was a representative of at least three independent experiments.

Statistical analysis of the data

All the experiments were repeated at least three times. The data were expressed as the means \pm S.D. Statistical comparisons were made using a one-way analysis of variance (ANOVA). P-values of <0.05 and <0.01 were considered to be significant and highly significant, respectively.

RESULTS

In order to detect VSV-induced apoptosis of WISH cells, we used the MTT assay. As shown in Table 1, WISH cells treated with a 5^{-4} diluted concentration of VSV medium for 72 h exhibited an approximately 50% reduction in absorbance. The MOI we chose was 50:1.

The cytotoxic effect of allicin on THP-1, MT-2 and WISH cells was examined by exposing the cells to 6 different concentrations of allicin for 72 h. By using the MTT assay, we found that the highest dose of allicin (200 μ g/ml) had a statistically significant cytotoxic effect. Although, the cytotoxicity observed at 100 μ g/ml was not statistically significant ($P > 0.05$), the OD absorbance was smaller

Table 1. Cell viability assay of WISH cells induced by different concentrations of VSV (n = 6).

Concentration of VSV	OD absorbance
0	1.83±0.13*
5 ⁻⁷	1.80±0.21
5 ⁻⁶	1.78±0.14
5 ⁻⁵	1.62±0.17
5 ⁻⁴	0.94±0.11**
5 ⁻³	0.75±0.16**
5 ⁻²	0.68±0.11**
5 ⁻¹	0.54±0.10**

** , P < 0.01 compared with *.

than the others (Table 2). Consequently, we were able to select high, midrange, and low (50, 25 and 10 µg/ml, respectively) doses as our experimental concentrations.

As shown in Figure 1, allicin had no effect on the adhesion or penetration of VSV. Additionally, Table 3 shows that the 3 different concentrations of allicin increased the absorbance of the THP-1, MT-2 and WISH cells in the MTT assay. Allicin at concentrations of 10 and 25 µg/ml clearly increased the absorbance of the THP-1, MT-2 and WISH cells (P < 0.05) and 50 µg/ml of allicin significantly increased the absorbance of THP-1, MT-2 and WISH cells in the MTT assay (P < 0.01). Therefore, we concluded that allicin can increase the survival rate of THP-1, MT-2 and WISH cells induced by VSV.

Accordingly, we used flow cytometry to detect the percentage of early and late staged apoptotic cells (apoptosis rate). The apoptosis rates of VSV-induced THP-1, MT-2 and WISH cells were the highest (Figures 2 to 4). Allicin at concentrations of 10 and 25 µg/ml clearly decreased the apoptosis rates of the THP-1, MT-2 and WISH cells (P < 0.05), and 50 µg/ml of allicin significantly decreased the apoptosis rate of these cells (P < 0.01). As the concentration of allicin increased, the apoptosis rates of the THP-1, MT-2 and WISH cells were lowered (Figure 5). In particular, the apoptosis rates of the high-dose groups were only slightly higher than those of the control group.

As the caspase family members and NF-κB are important proteins and are highly expressed in apoptosis, we examined the expression levels of active cleavage products of caspases 3, 6, 7 and 9, and NF-κB in THP-1, MT-2 and WISH cells. Western blot analysis indicated that the active cleavage products of all the four caspases and NF-κB were highly expressed in THP-1, MT-2, and WISH cells that were induced by VSV for 72 h (Figure 6). Compared with the VSV groups, the groups treated with 10 and 25 µg/mL of allicin showed reduced expression of the active cleavage products of caspases 3, 6, 7 and 9 and NF-κB in THP-1, MT-2 and WISH cells to different degrees. Moreover, 50 µg/ml of allicin significantly reduced the expression of the active cleavage products of

caspases 3, 6, 7 and 9 and NF-κB in THP-1, MT-2 and WISH cells to a level almost equivalent to that of the control groups. Thus, these results suggest that allicin can inhibit the apoptosis of THP-1, MT-2 and WISH cells induced by VSV through downregulation of the expression of caspase family members and NF-κB.

DISCUSSION

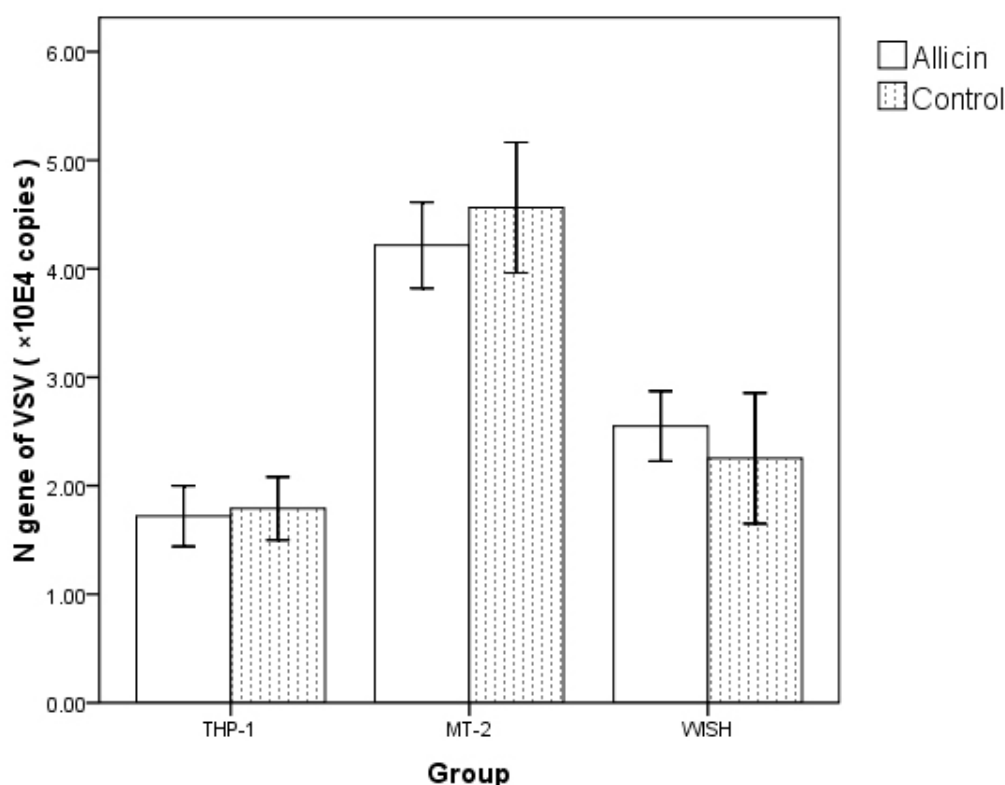
Vesicular stomatitis virus (VSV), a vesiculovirus that belongs to the family Rhabdoviridae, is a minus-chain enveloped RNA virus (Koyama et al., 2001). VSV can induce apoptosis and lead to typical changes in apoptotic morphology. Apoptosis is a mechanism responsible for cell death and appears to be intrinsically programmed. Typical morphological changes associated with apoptosis include cell shrinkage, chromatin condensation, formation of cytoplasmic blebs and apoptotic bodies and the phagocytosis of apoptotic cells or bodies by adjacent parenchymal cells or macrophages (Kopecky and Lyles, 2003; Tsai et al., 1985). Consequently, VSV is an appropriate virus with which to establish an apoptosis model.

Allicin, the major bioactive molecule of garlic, plays a complicated role in regulating the life and death of cells. The antitumor (Sun and Wang, 2003) and antifungal (Ogita et al., 2009) activities of allicin have previously been demonstrated and it has also been known to inhibit apoptosis of macrophages that are in a depleted nutritional state (Cho et al., 2006). More recently, however, studies have shown that allicin functions as an inhibitor that can suppress the replication of several viruses, including murine cytomegalovirus (MCMV) and herpes simplex virus-1 (HSV-1) *in vitro* (Tsai et al., 1985; Li and Dong, 1999). Nevertheless, the precise mechanism of action of allicin is yet to be clearly established. Allicin can also inhibit apoptosis induced by ischemia-reperfusion in animal models (Ren and Li, 2005; Shi and Gu, 2005; Zhang et al., 2008). Our prior experiments confirmed that VSV can induce apoptosis of the THP-1, MT-2 and WISH cells; however, allicin had little effect on the adhesion of VSV to the cell surface (Figure 1). In order to determine if allicin can suppress activation of viruses through an anti-apoptotic pathway in cells, we conducted the study reported here. As shown in Table 3 and Figures 2 to 5, allicin at all the concentrations investigated, exerted inhibitory effects against the apoptosis of THP-1, MT-2 and WISH cells, as measured by MTT and flow cytometry, and this inhibition was dose dependent. These results are consistent with our expectations. Moreover, our study suggested that the mechanism by which allicin regulates cell apoptosis differs in different cell lines. As previously demonstrated, apoptotic cell death can be induced through either the death receptor or mitochondria-mediated signaling pathways (Fumarola and Guidotti, 2004). Members of the caspase family of proteases play an important role in the initiation and execution of apoptosis. They include initiator caspases, such as caspases 2,

Table 2. Viability assay of THP-1, MT-2 and WISH cells treated with different concentrations of allicin (n = 6).

Concentration of allicin ($\mu\text{g/ml}$)	OD absorbance		
	THP-1 group	MT-2 group	WISH group
0	1.80 \pm 0.11 Δ	1.78 \pm 0.08 Δ	1.83 \pm 0.13 Δ
5	1.81 \pm 0.14	1.78 \pm 0.15	1.83 \pm 0.20
10	1.88 \pm 0.25	1.81 \pm 0.21	1.85 \pm 0.05
25	1.93 \pm 0.16	1.84 \pm 0.23	1.87 \pm 0.18
50	1.96 \pm 0.12	1.87 \pm 0.17	1.90 \pm 0.16
100	1.76 \pm 0.18	1.71 \pm 0.22	1.73 \pm 0.21
200	1.16 \pm 0.25*	1.24 \pm 0.19*	1.29 \pm 0.32*

*, P < 0.05 compared with Δ .

**Figure 1.** The number of gene transcript copies of VSV ($M \pm 2SD$). Quantitative RT-PCR analyses of VSV-*N* gene expression. Control: Medium control, allicin: 50 $\mu\text{g/ml}$.

8, 9 and 10, which activate downstream of the executioner caspases, resulting in an amplification of cascade activity. The executioner caspases, such as caspases 3, 6 and 7, directly cleave crucial cellular protein substrates, resulting in cell destruction (Hofmann et al., 1997). Previous studies have demonstrated that caspase 3 plays a key role in apoptosis (Patel and Gores, 1995; Petersen et al., 2005; Tamada et al., 2000; Yao et al., 2003), suggesting that a mitochondria-mediated signaling pathway is involved. In addition, *in vitro* studies have identified caspase 9, Apaf1

and cytochrome *c* as participants in a complex that is important for caspase 3 activation (Li et al., 1997). Caspase 3, in turn, mediates both DNA fragmentation and membrane PS exposure (Chong et al., 2002; Maiese and Vincent, 2000). In order to investigate the role of effector caspases 3, 6 and 7 and initiator caspase 9 in the VSV-induced apoptosis of THP-1, MT-2 and WISH cells and the molecular mechanism by which allicin protects these cells from apoptosis, we examined the expression of active cleaved caspases 3, 6, 7 and 9 by western blotting. As

Table 3. Viability assay of THP-1, MT-2 and WISH cells treated with different concentrations of allicin and VSV (n = 6).

MOI	Concentration of allicin ($\mu\text{g/ml}$)	OD absorbance		
		THP-1 group	MT-2 group	WISH group
0	0	1.81 \pm 0.09	1.78 \pm 0.06	1.82 \pm 0.07
50:1	0	0.95 \pm 0.12 Δ	0.88 \pm 0.22 Δ	0.94 \pm 0.18 Δ
50:1	10	1.37 \pm 0.14*	1.31 \pm 0.15*	1.42 \pm 0.17*
50:1	25	1.55 \pm 0.11**	1.52 \pm 0.16**	1.63 \pm 0.13**
50:1	50	1.76 \pm 0.21**	1.73 \pm 0.20**	1.78 \pm 0.14**

*, P < 0.01 compared with Δ ; **, P < 0.01 compared with *.

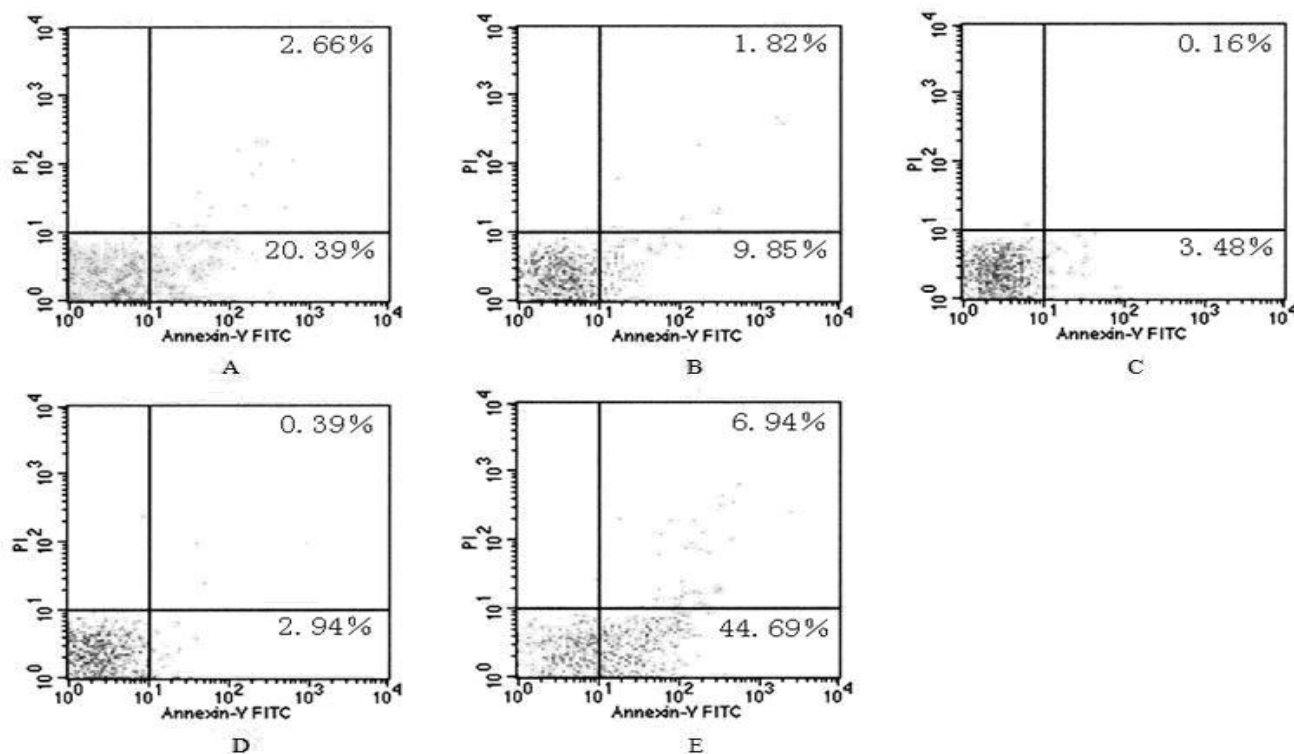


Figure 2. Cell apoptosis rate of THP-1 cells treated with different concentrations of allicin and VSV. Flow cytometry was performed to examine the apoptosis level of THP-1. A, 10 $\mu\text{g/ml}$ of allicin + VSV; B, 25 $\mu\text{g/ml}$ of allicin + VSV; C, 50 $\mu\text{g/ml}$ of allicin + VSV; D, medium control, E, VSV.

shown in Figure 6, the stimulation of THP-1, MT-2 and WISH cells by VSV resulted in a significant increase in the cleaved caspase 3, 6, 7 and 9 expression at the protein level. However, in the presence of different concentrations of allicin (10, 25 and 50 $\mu\text{g/ml}$), the VSV-induced expression of cleaved caspase 3, 6, 7 and 9 was significantly reduced in a dose-dependent manner.

NF- κ B is known for its general role in inflammatory and immune responses, as well as in the control of cell division and apoptosis. It is an important factor in regulating apoptosis (Baeuerle and Henkel, 1994). When cells are infected with viruses, NF- κ B is activated and the activated

NF- κ B can regulate the expression of an inhibitor of apoptosis proteins (IAPs). The latter in turn activates caspase 9, thereby stimulating downstream caspases 3, 6 and 7 (Roy et al., 1997). Accordingly, we hypothesized that a possible apoptotic pathway is as follows: VSV \rightarrow NF- κ B \rightarrow IAPs \rightarrow caspase 9 \rightarrow caspases 3, 6 and 7 \rightarrow cell apoptosis. Therefore, we also examined the expression of NF- κ B by western blotting in order to investigate the role of NF- κ B in VSV-induced cell apoptosis and allicin-mediated protection of the THP-1, MT-2 and WISH cells. Interestingly, the results obtained for NF- κ B were similar to the results observed for caspases 3, 6, 7 and 9 (Figure 6). Thus,

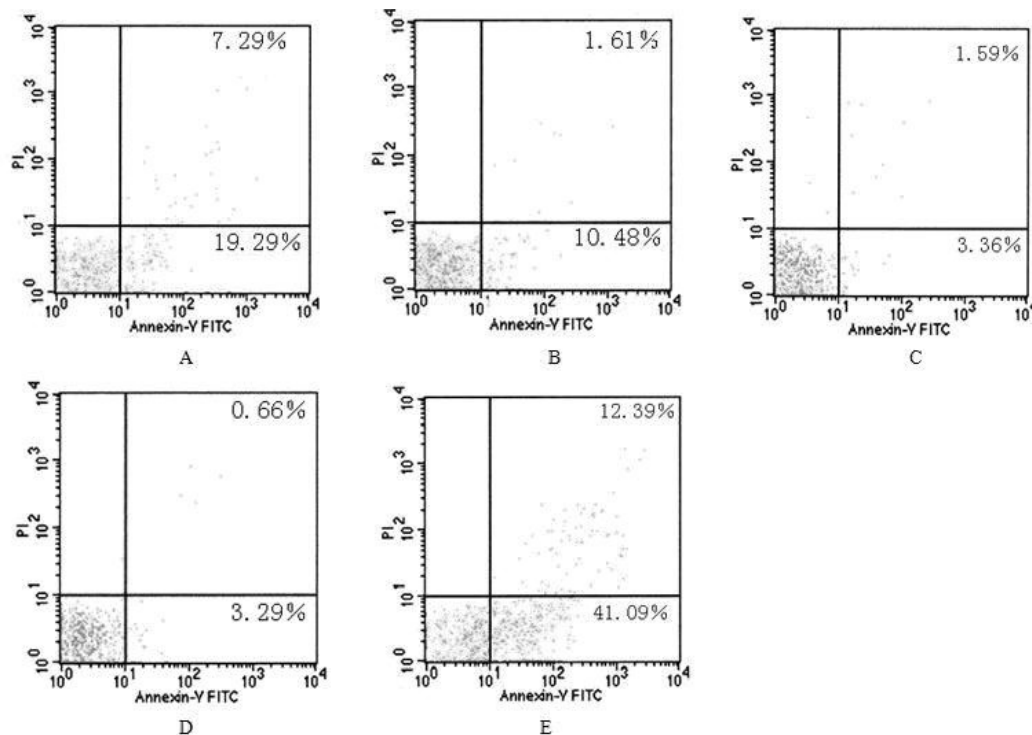


Figure 3. Cell apoptosis rate of MT-2 cells treated with different concentrations of alicin and VSV. Flow cytometry was performed to examine the apoptosis level of MT-2. A, 10 µg/ml of alicin + VSV; B, 25 µg/ml of alicin + VSV; C, 50 µg/ml of alicin + VSV; D, medium control, E, VSV.

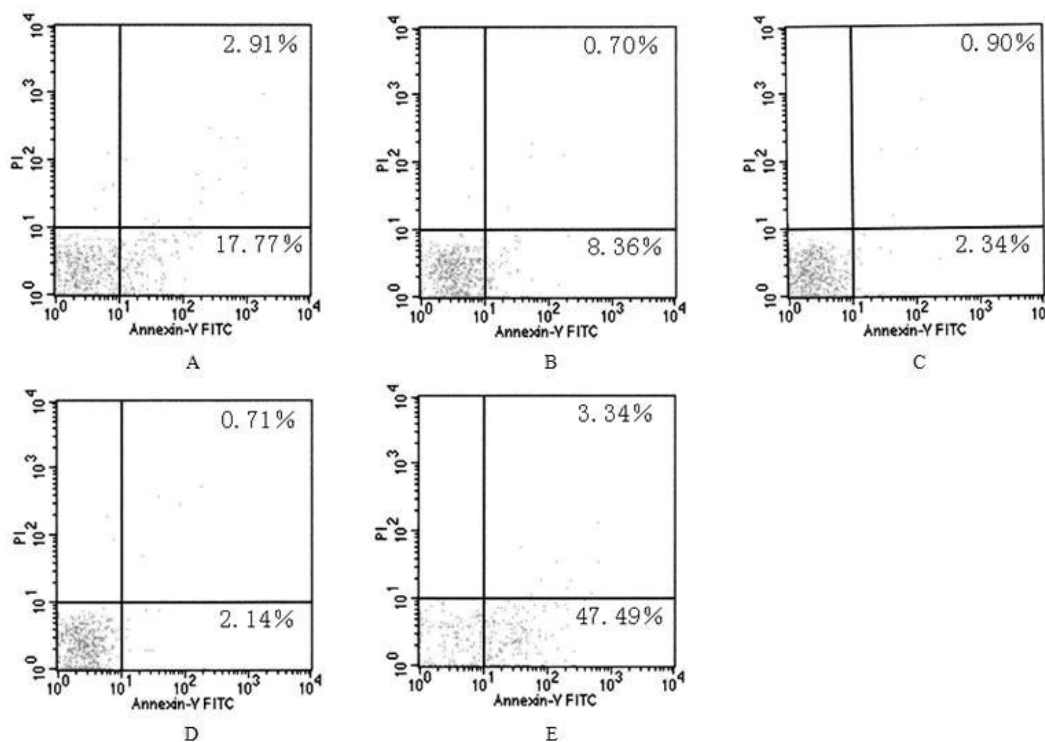


Figure 4. Cell apoptosis rate of WISH cells treated with different concentrations of alicin and VSV. Flow cytometry was performed to examine the apoptosis level of WISH cells. A, 10 µg/ml of alicin + VSV; B, 25 µg/ml of alicin + VSV; C, 50 µg/ml of alicin + VSV; D, medium control, E, VSV.

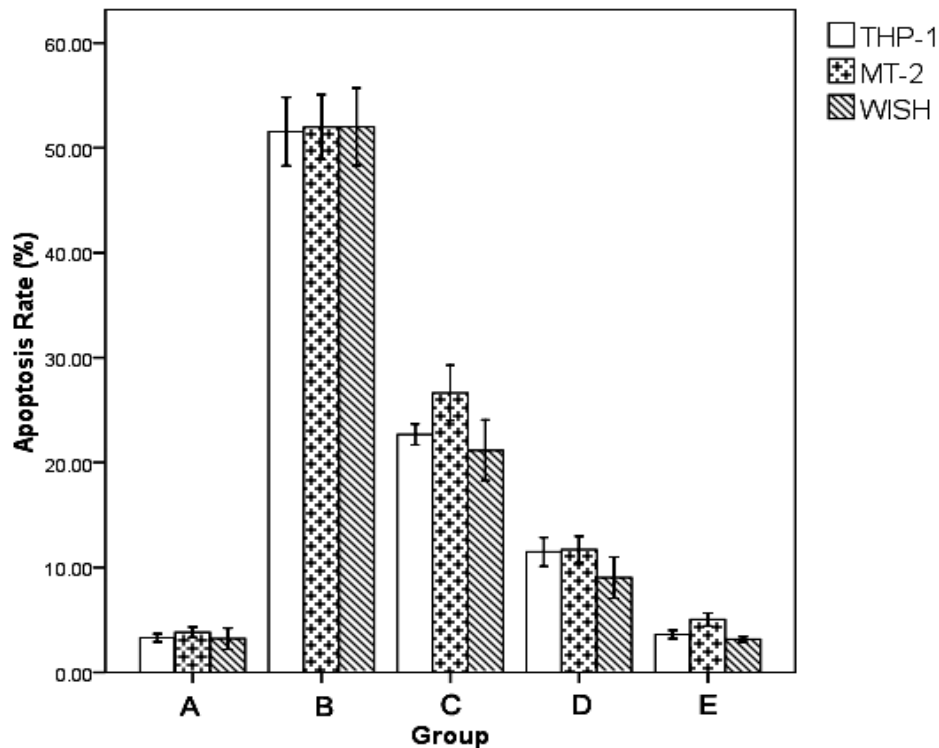


Figure 5. Cell apoptosis rate of THP-1, MT-2 and WISH cells treated with different concentrations of alliin and VSV ($M \pm 2SD$). The apoptosis level was examined by flow cytometry and each concentration was placed into 6 repeat wells. A, Medium control; B, VSV; C, 10 $\mu\text{g/ml}$ of alliin + VSV; D, 25 $\mu\text{g/ml}$ of alliin + VSV; E, 50 $\mu\text{g/ml}$ of alliin + VSV.

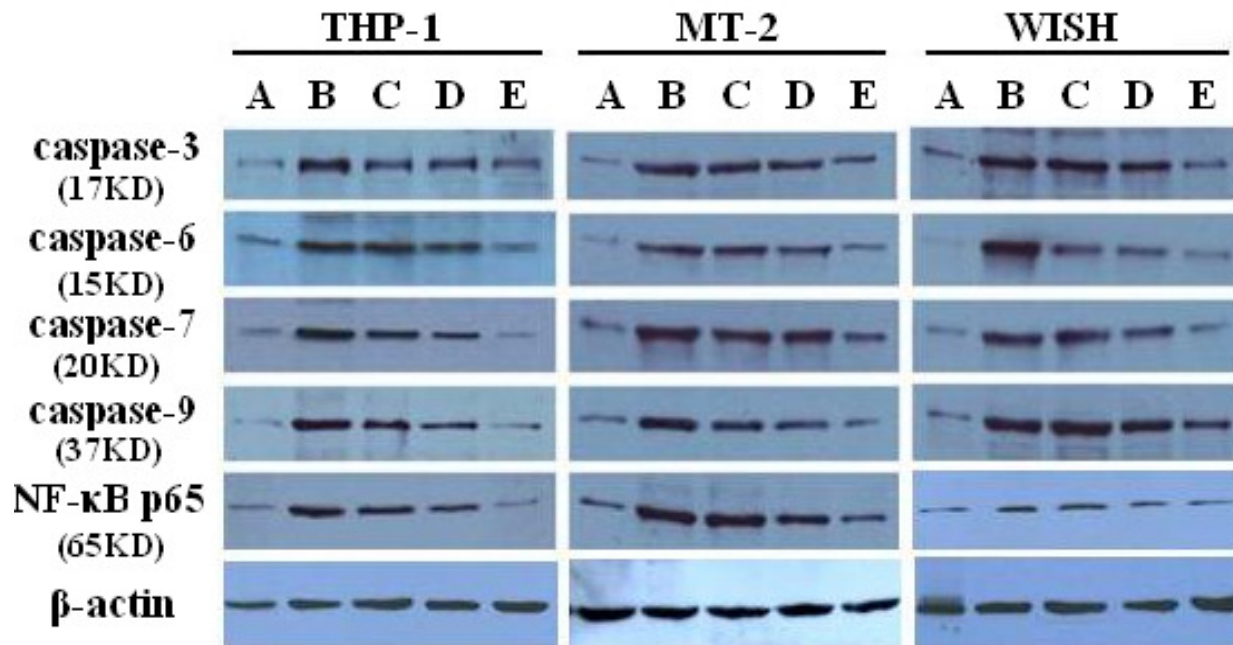


Figure 6. Caspases and NF- κ B expression of THP-1, MT-2 and WISH cells treated with different concentrations of alliin and VSV. Western blot analysis was performed using specific anti-active caspase 3, 6, 7 and 9 and NF- κ Bp65 antibodies and were detected by ECL reaction. Results were recorded and analyzed using VersaDoc imaging system. A, Medium control; B, VSV; C, 10 $\mu\text{g/ml}$ of alliin + VSV; D, 25 $\mu\text{g/ml}$ of alliin + VSV; E, 50 $\mu\text{g/ml}$ of alliin + VSV.

we could concluded from the cell culture experiments that there exists a signaling apoptotic pathway dependent on both NF- κ B and caspases 3, 6, 7 and 9 during VSV-induced apoptosis of THP-1, MT-2 and WISH cells. Furthermore, allicin can suppress the apoptosis of THP-1, MT-2 and WISH cells, at least in part, through the inhibition of the expression of NF- κ B and caspases 3, 6, 7 and 9.

In conclusion, our results demonstrate that allicin can significantly protect THP-1, MT-2 and WISH cells from apoptosis induced by VSV. This protection is mediated by inhibition of the expression of NF- κ B and caspases 3, 6, 7 and 9, which are considered to constitute a putative anti-apoptosis signaling pathway. Moreover, this may also constitute the potential antiviral mechanism of allicin.

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