行政院國家科學委員會專題研究計畫　成果報告

DAS、DADS、DATS 在體外和體內影響大腸癌細胞入侵及轉移之機轉

計畫類別：個別型計畫
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中國醫藥大學醫學系鍾景光國科會計畫編號：**NSC93-2320-B-039-033-**
計畫題目：**DAS**、DADS、DATS 在體內和體外影響大腸癌細胞入侵及轉移之機轉

結案報告

Summary

Background: Diallysulfid (DAS), Diallyl disulfide (DADS), Dially trisulfide (DATS), an oil-soluble organosulfur component of garlic, is well known to induce growth inhibition and apoptosis in human cancer cell types. DAS, DADS, DATS have anti-carcinogenic and anti-tumorigenic activity in chemical carcinogenesis in vivo. In this study, we investigated the effects of DAS, DADS, DATS on human colo 205 colon cancer cells to better understand its effect on cell cycle arrest, apoptosis and apoptosis-related factors in vitro.

Methods: Cell viability, cell cycle arrest, reactive oxygen species , caspases-3 activity, Mitochondria membrane potential, Calcium release were measured to determine their variations by flow cytometric assay,

Results: After 24-hour treatment with DADS, a dose- and time-dependent decrease in the viability of colo 205 cells was observed and the approximate IC50 was 39.4 μM. The decreased percentages of viable cells are associated with the production of reactive oxygen species which is induced by DADS, DAS, DATS. Treatment of colo 205 cells with DADS resulted in arrest of cell cycle in G2/M phase and led to apoptosis occurrence. Treatment of colo 205 cells with DAS, DATS resulted in arrest of cell cycle in G0/G1 phase and led to apoptosis occurrence. DADS, DAS, DATS induced apoptosis was further examined and confirmed by morphological assessment . A significant increase was found in apoptosis induction when cells were treated with DADS, DAS, DATS compared to without DADS, DAS, DATS treated groups. DADS, DAS, DATS also promoted caspase-3 activity after exposure for 6, 24, and 48 hr, which led to induce apoptosis.

Conclusions:

1. DADS induced apoptosis were accompanied by increasing the levels of Fas, phosph-Ask1 and –JNK, p53 and decreasing the mitochondrial membrane potential which then led to release the cytochrome c, cleavage of pro-caspase-9 and -3. Inhibition of caspase-3 activation completely blocked DADS-induced apoptosis on colo 205 cells.
2. DATS, DAS induced apoptosis was further examined and confirmed by morphological assessment A significant increase was found in apoptosis induction

when cells were treated with DATS, DAS compared to without DATS, DAS treated groups. DATS, DAS also promoted caspase-3 activity, calcium and reactive oxygen species release, decrease the mitochondris membrane potential after exposure for different time, which led to induce apoptosis.

3. Cell viability：cytotoxicity DADS＞DATS＞DAS
4. Cell cycle analysis：DADS arrest at G2/M phase. DAS, DATS arrest at G0/G1 phase.
5. Reactive oxygen species：DAS 50 μM at 8 hr ROS product. DADS 50 μM at 24 hr ROS product. DATS various concentrations at 2 hr all produce ROS.
6. Calcium release：after 24hr exposure of DAS, DADS, DATS, calcium release % of cells are DAS ＞DADS＞DATS.
7. Mitochondria membrane potential：after 24 hr treatment of DAS, DADS, DATS, and they all presented dose- dependent.
   At time course (12, 24, 48 hr), decreased the mitochondria membrane potential DATS＞ DAS＞ DADS.
8. Caspase-3 activity：after 6, 24, 48 hr exposure , DAS, DADS, DATS all could see the Caspase-3 activity increase..

Introduction

Apoptosis, or programmed cell death, is a normal component of the development and health of multicellular organisms. Many studies have focused on selectively killing tumor cells through the induction of apoptosis (1). Apoptosis is a kind of cell death, that upon receiving specific signals instructing the cells to undergo apoptosis, causes specific morphological and biochemical modification such as plasma and nuclear membrane blebbings, chromatin condensation, proteases activation, and DNA fragmentation that are considered as landmarks of the apoptotic process (2, 3). Thus, apoptosis occurs under normal physiological conditions in eliminating abundant and unwanted cells during embryonic development, growth and differentiation. Therefore, apoptosis plays an essential role as a protective mechanism against neoplastic cells which are damaged or excess cells that have been improperly produced. It is well known that the inappropriate regulation of apoptosis is associated with a variety of diseases (4-6). Apoptosis represents an active, gene-directed mechanism, and it is possible that controlling the apoptosis process may lead to therapeutic methods. Therefore, how to induce apoptosis of cancer cells is a major strategy for cancer chemotherapy. In Taiwan about 15.03 persons per 100 thousand people die per year in colon cancer from the reports of the "People Health Bureau of Taiwan". Surgery, radiotherapy and chemotherapy are used for clinical therapy in human colon cancer. Currently the strategies for treatment of human colon cancer are not yet satisfactory.

Garlic is a plant commonly used as a food throughout the world population, and its medicinal properties have been described long ago. It was reported that enhanced garlic consumption is closely related with reduced cancer incidence (7, 8). Diallysulfid (DAS), Diallyl disulfide (DADS), Dially trisulfide (DATS), an important oil-soluble organosulfur component of garlic (*Allium sativum*), has been reported to inhibit the growth of human cancer cells such as colon, lung, skin, and breast (9-12). It was also reported that DADS induced apoptosis of HL-60 cells by the generation of H_2O_2, induction of p53 and activation of caspase-3 (10, 11). DADS can induce apoptosis in HCT-15 cells through the sequential mechanism of Ca^{+2} homeostasis disruption, accumulation of H_2O_2, and resulting caspase-3 activation (13). It was reported that DADS induced cell death through reactive oxygen species-dependent c-jun NH2-terminal kinase/c-jun signaling cascade mediates neuroblastoma (14). Recently, DADS and the specific inhibitors of MAPKs induced apoptosis in HepG2 hepatoma cells and the MAPKs inhibitors further enhanced the apoptotic effect in DADS-treated HepG2 hepatoma cells (15). Although DADS as an antitumor agent has been established, the exact mechanism of cytotoxic effect is not completely clear, and the potential mechanism of apoptosis through which the signal is transduced within the cell remains to be evaluated. Therefore, the aim of present study was to dissect the mechanisms underlying DADS, DATS, and DAS cytotoxicity and apoptosis in human colon colo 205 cancer cells.

Materials & Methods

Chemicals and Reagents. Diallysulfid (DAS), Diallyl disulfide (DADS), Dially trisulfide (DATS), was purchased from Fluka Chemika Co. (Bucha, Switzerland). Catalase, ribonuclease-A, trypan blue, Tris-HCl, triton X-100, propidium iodide (PI) and epigallocatechin-3-gallate (EGCG) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). SP600125 was obtained from Calbiochem (San Diego, CA, USA). Potassium phosphates, dimethyl sulfoxide (DMSO), and TE buffer were purchased from Merck Co. (Darmstadt, Germany). RPMI-1640, penicillin-strptomycin, trypsin-EDTA, fetal bovine serum (FBS), and glutamine were obtained from Gibco BRL (Grand Island, NY, USA). Caspase-3 activity assay kit was bought from Boehringer Mannheim (Mannhein, Germany).

Human colon cancer colo 205 cell line. Human colon cancer colo 205 cell line was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). The cells were immediately placed into 75 cm^3 tissue culture flasks and grown at 37°C under a humidified 5% CO_2 and 95% air at one atmosphere in RPMI 1640 medium supplemented with 10% FBS, 1% penicillin-streptomycin (10 ng/ml penicillin and 10 ng/ml streptomycin) and 1% glutamine.

Morphological changes and cell viability of colo 205 cells treated with or without DADS were determined by using phase light microscopy and trypan blue exclusion and flow cytometry. The colo 205 cells were plated in 12 well plates at a density of $2x10^5$ cells/well and grown for 24 hours. They were then added to different concentrations of DADS, DATS, DAS for final concentration 0, 10, 25, 50, 75 and 100μM, while only adding DMSO (solvent) for the control regimen and grown at 37°C, 5% CO_2 and 95% air for a different period of time. For examinations of morphological changes, the cells in the plates were photographed by phase light microscopy. For determining cell viability, the trypan blue exclusion protocol was used. Briefly, about 10 μl of cell suspensions in PBS were mixed with 40 μl of trypan blue, and the numbers of stained (dead cells) and unstained cells (live cells) were counted using a hemocytometer (17) or for flow cytometric assay as described previously (18,19).

Flow cytometry analysis of DNA content for cell cycle and apoptosis from colo 205 cells treated with different concentrations of DADS, DATS, DAS. About $2x10^5$ cells/well of colo 205 cells in 12-well plate with concentrations (0, 10, 25, 50, 75 and 100μM) of DADS, DATS, DAS were incubated in an incubator for different time periods, and then the cells were harvested by centrifugation. The cells were fixed gently (drop by drop) by putting 70% ethanol (in PBS) in ice overnight and were then resuspended in PBS containing 40 μg/mL PI and 0.1 mg/mL RNase (Sigma, St. Louis, MO, USA) and 5% triton x-100 in dark room. After 30 minutes at 37°C, the cells were analyzed with a flow-cytometry (Becton-Dickinson, San Jose, CA, USA) equipped with an argonion laser at 488 nm wave-length. Then the cell cycle was determined and analyzed (19).

Detection of reactive oxygen species (ROS) in human colon cancer colo 205 cells by flow cytometry. The level of ROS of the colo 205 cells was determined by flow cytometry (Becton Dickinson FACS Calibur), using the 2,7-Dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma). H_2DCF-DA is a fluorogenic freely permeable tracer specific for ROS assessment. The colo 205 cells were treated with or without various concentrations (10, 25, 50, 75 and 100 μM) of DADS, DATS, DAS for 24 hours to detect the changes of ROS. The cells were harvested and washed twice, re-suspended in 500 μl of 2,7-Dichlorodihydrofluorescein diacetate (10 μM) and incubated at 37°C for 30 min and analyzed by flow cytometry (19).

Caspase activity determination of colo 205 cells treated with or without DADS, DATS, DAS. About $2x10^5$ cells/well of colo 205 cells in 12-well plate with concentrations 0, 25, 50, 75 and 100 μM of DADS, DATS, DAS were incubated in an

incubator for different time periods. Cells were harvested by centrifugation and the medium were removed. We then add 50 μL of 10 μM substrate solution (PhiPhilux is a unique class of substrates for caspase-3) to cell pellet ($1x10^5$ cells per sample). We did not vortex the cells. Cells then were incubated at 37℃ for 60 minutes. Then the cells were washed once by adding 1 mL of ice cold PBS and re-suspend in fresh 1 mL. Cells were analyzed with a flow-cytometry (Becton-Dickinson, San Jose, CA, USA) equipped with an argonion laser at 488 nm wave-length. Then the caspase-3 activity was determined and analyzed (20).

Detection of Ca^{+2} concentrations in human colon cancer colo 205 cells after treated with DADS, DATS, DAS by flow cytometry. The level of Ca^{+2} of the colo 205 cells was determined by flow cytometry (Becton Dickinson FACS Calibur), using the Indo 1/AM (Calbiochem; La Jolla, CA). Cells were treated with or without 50 μM concentrations of DADS, DATS, DAS for 6, 12, 24, 48 hours to detect the changes of Ca^{+2} concentration. The cells were harvested and washed twice, re-suspended in Indo 1/AM (3 μg/ml) and incubated at 37°C for 30 min and analyzed by flow cytometry (13).

Detection of mitochondrial membrane potential ($\Delta\Psi_m$) in human colon cancer colo 205 cells by flow cytometry. The level of mitochondrial membrane potential of the colo 205 cells was determined by flow cytometry (Becton Dickinson FACS Calibur), using the $DiOC_6$ (4 mol/L). Cells were treated with or without various concentrations (10, 25, 50, 75 and 100 μM) of DADS, DATS, DAS for 24 hours to detect the changes of mitochondrial membrane potential. The cells were harvested and washed twice, re-suspended in 500 μl of $DiOC_6$ (4 mol/L) and incubated at 37°C for 30 min and analyzed by flow cytometry (19, 22).

Results

1. Effects of various concentrations of DADS, DATS, DAS on cell viability of human colon cancer colo 205 cells. For control group, the data indicated that <3% of colo 205 cells were stained by trypan blue or propidium iodide when they were incubated in medium containing 10% FBS only. The morphological examinations showed cells are significantly different between DADS, DATS, DAS treated group and control (Figure 1 A, B and C). In the presence of DADS, DATS, DAS (10, 25, 50, 75 and 100 μM), the cell culture was increased by staining after the time and concentration increased, suggesting that DADS, DATS, DAS exerted a dose- and time-dependent cytotoxic effect on colo 205 cells (Figure 1 A, B, C and D).

2. DADS, DATS, DAS induced cell cycle arrest and apoptosis in human colon cancer colo 205 cells. The data indicated that during the 48 hours time period, DADS increased the percentage of G2/M phases, and the percentage of G0/G1 phase

was decreased. Control cells showed a typical pattern of DNA content that reflected G0/G1-, S- and G2/M- phase of the cell cycle. The DADS treated cells showed a typical pattern of DNA content together with a sub-G0/G1- phase (corresponding to apoptotic cells) as shown in (Figure 2.) DADS induced a distinct sub-G1 peak representing an apoptotic cell population.

DATS, DAS increased the percentage of G0/G1 phases, and the percentage of G2/M phase was decreased. The percentages of apoptosis in various concentrations of DADS, DATS, DAS treatments are shown in (Figure 2.)

3. Effects of DADS, DATS, DAS on reactive oxygen species expresion on colo 205 cells. The changes of ROS levels in response to the effect with various concentrations of DADS, DATS, DAS were studied by staining with 2,7-Dichlorodihydrofluorescein diacetate and analyzed by flow cytometry. The results showed that ROS levels significantly increased after cells were treated with DADS, DATS, DAS (Figure 3).

4. Flow cytometric assays for caspase-3 activity of colo 205 cells treated with or without DADS, DATS, DAS.

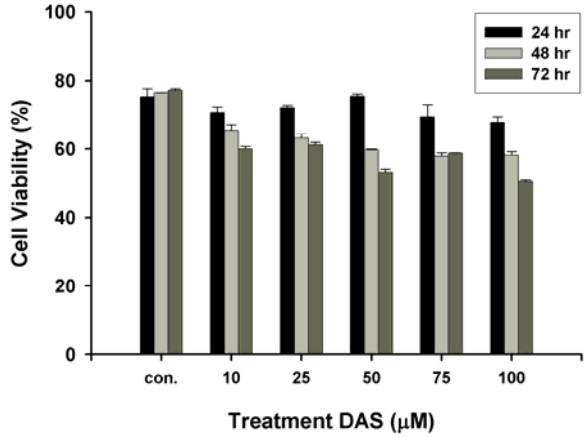
About 2x105 cells/well of colo 205 cells in 12-well plate with concentrations 0, 10, 25, 50, 75 and 100 μM of DADS, DATS, DAS were incubated in an incubator for different time periods. The results showed that caspase-3 activity significantly increased after cells were treated with DADS, DATS, DAS (Figure 4).

5. Effects of DADS, DATS, DAS on the levels of Ca^{+2} on colo 205 cells after treated with various concentrations of DADS, DATS, DAS. The changes of Ca^{+2} levels in response to the effect with 50 μM concentration of DADS, DATS, DAS were studied by staining with Indo 1/AM and then analyzed by flow cytometry. The data is showed in (Figure. 5.) Fig. 4 shows that Ca^{+2} levels increased in treatment with DADS, DATS, DAS..

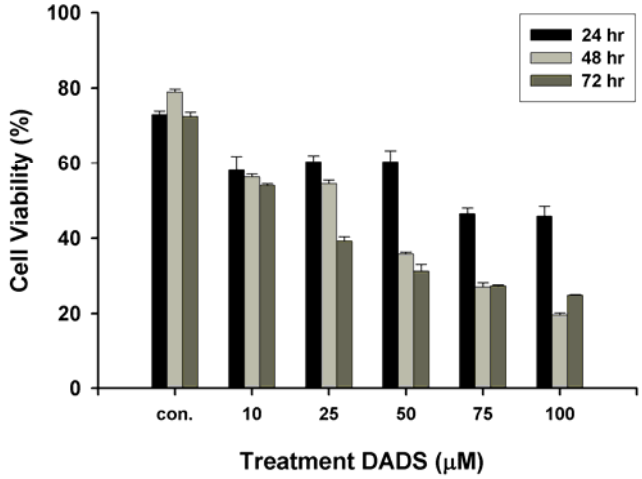
6. Effects of DADS, DATS, DAS on mitochondrial membrane potential expresion on colo 205 cells. The changes of mitochondrial membrane potential levels in response to the effect with various concentrations of DADS, DATS, DAS were studied by staining with DiOL6 and then analyzed by flow cytometry. The representative data is showed in (Figure 6.) Figure 6 showed that mitochondrial membrane potential levels decreased in treatment with DADS, DATS, DAS.

Fig.1. DAS.DADS.DATS decreased the percentage of viable Colon 205 cells.

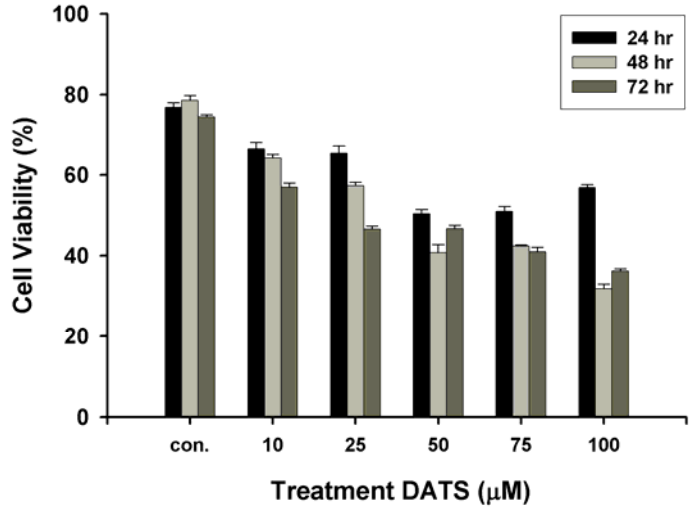
(A).


24 hr
48 hr
72 hr
Cell Viability (%)
100
80
60
40
20
0
con.
10
25
50
75
100
Treatment DAS (μM)

(B).


24 hr
48 hr
72 hr
Cell Viability (%)
100
80
60
40
20
0
con.
10
25
50
75
100
Treatment DADS (μM)

(C).


24 hr
48 hr
72 hr
Cell Viability (%)
100
80
60
40
20
0
con.
10
25
50
75
100
Treatment DATS (μM)

(D). 綜合比較圖

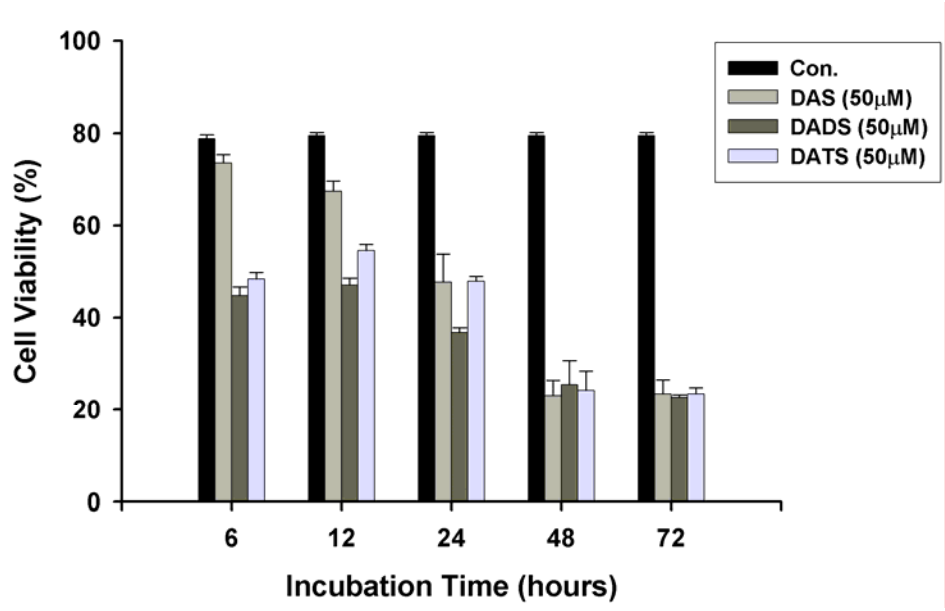
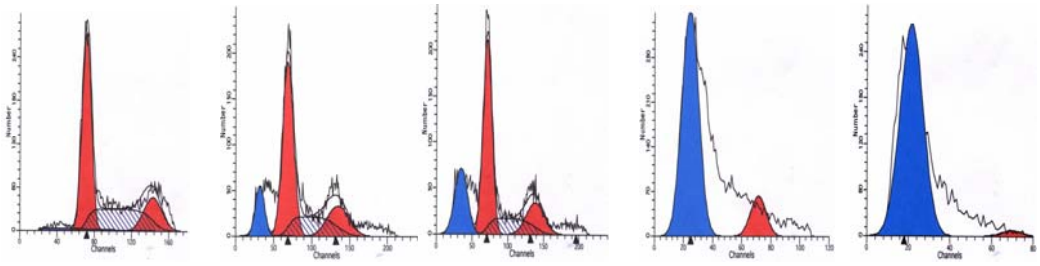
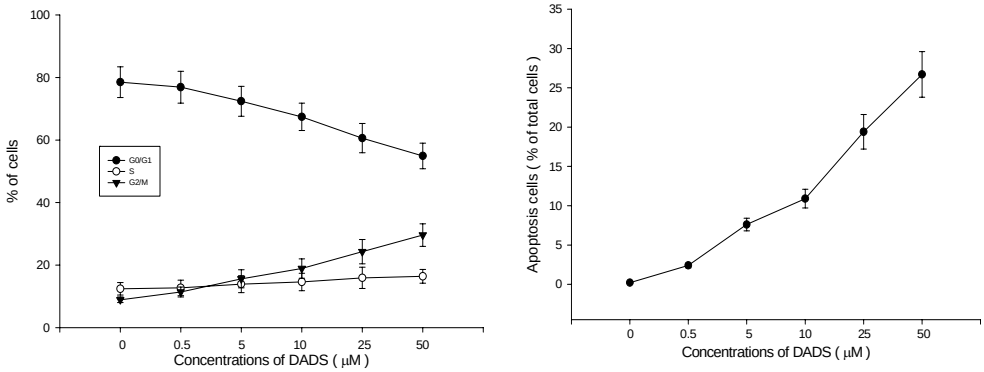


Fig.2 DADS induced G2/M arrest and apoptosis in Colon 205 cells.
DAS, DATS induced G0/G1 arrest and apoptosis in Colon 205 cells.

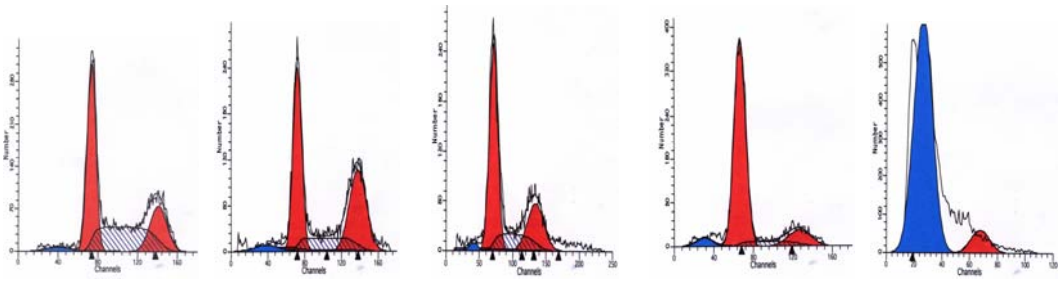
(A).DADS (50 μM)



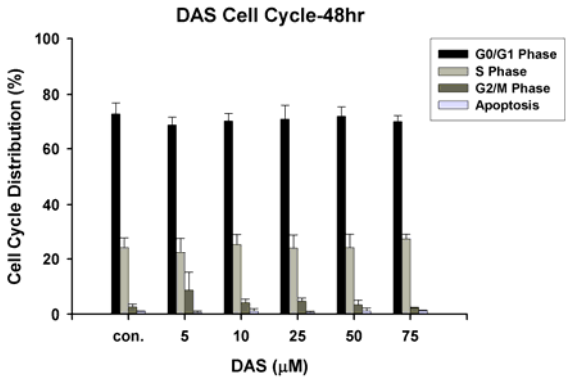
Con. 12hr 24hr 48hr 72hr

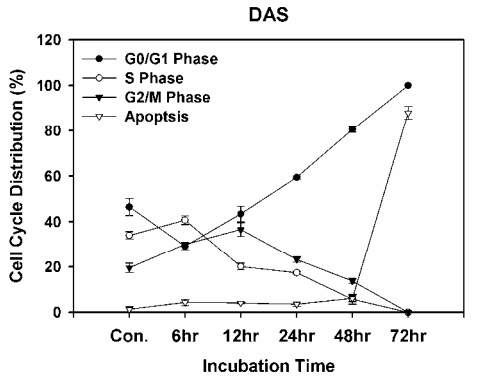


(B). DAS (50 μM)

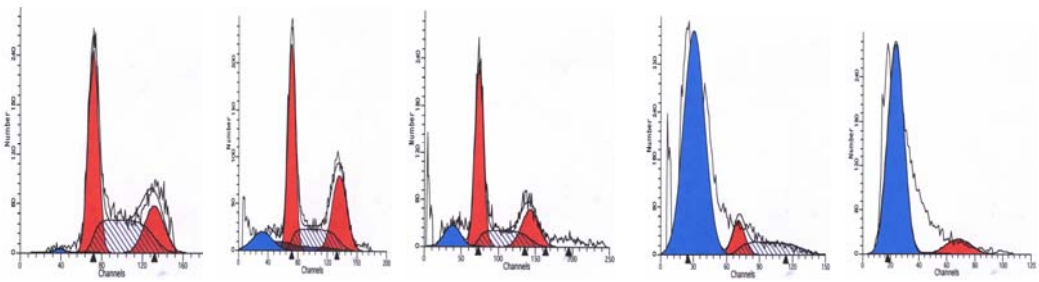


Con. 12hr 24hr 48hr 72hr

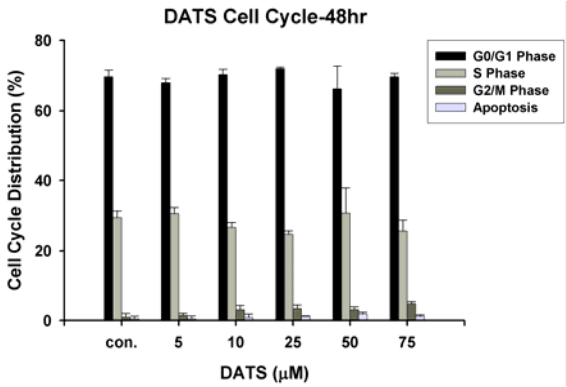




(C).DATS (50 μM)



Con. 12hr 24hr 48hr 72hr



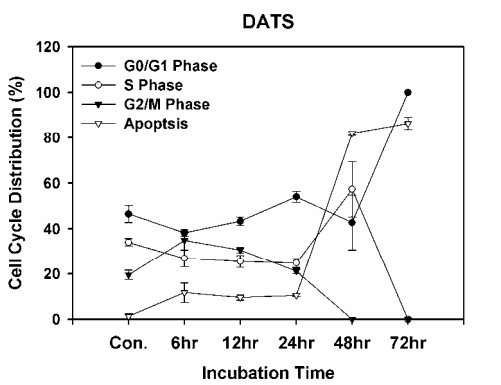
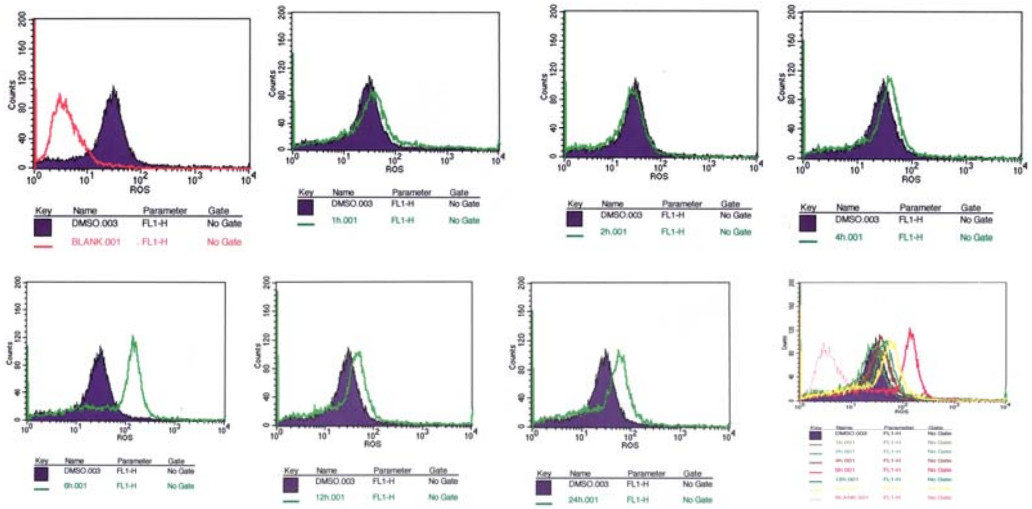
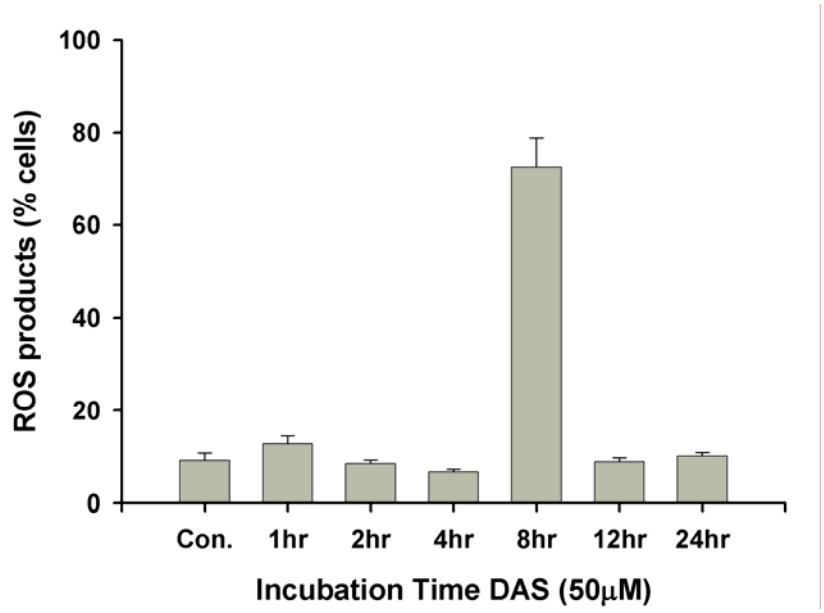


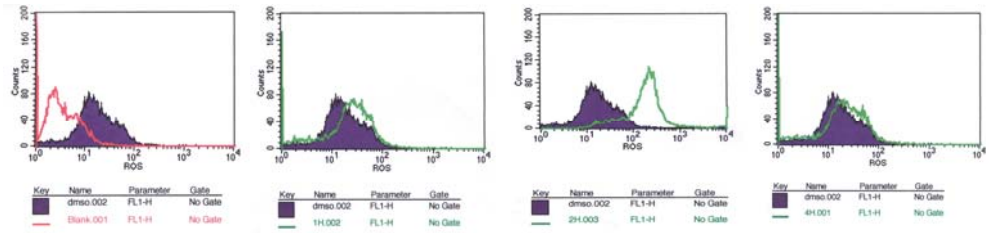
Fig. 3. DAS.DADS.DATS induced the production of reactive oxygen species (ROS) in Colon 205 cells.

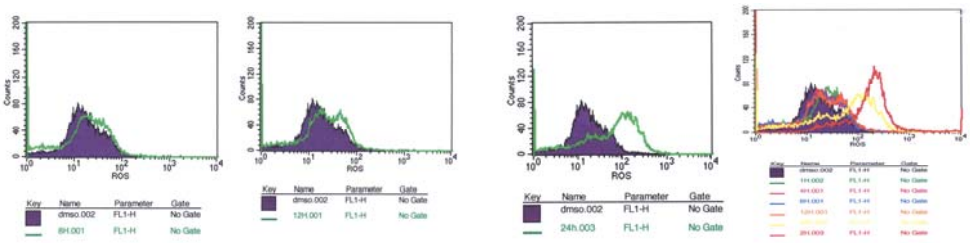
(A).

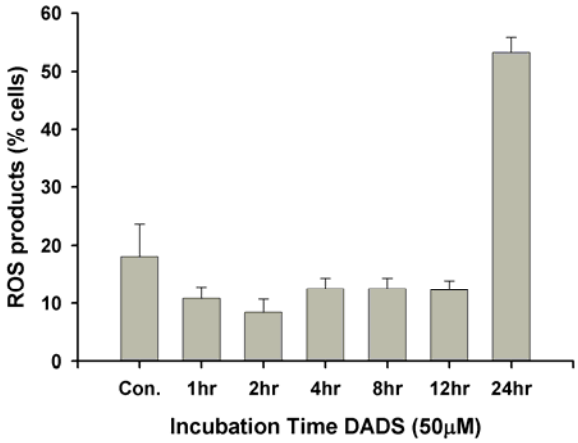




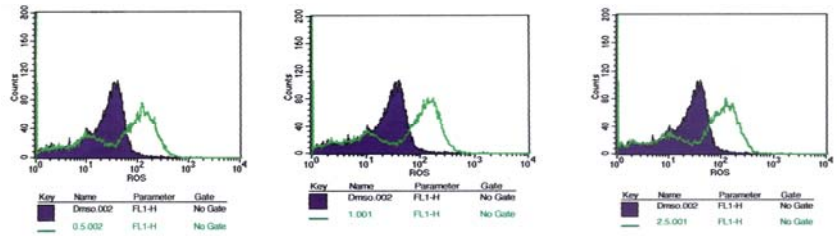
(B).

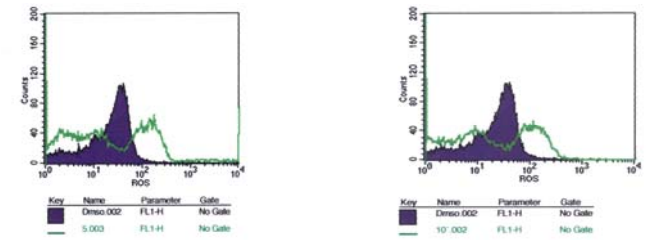






(C).DATS-2hr ROS production





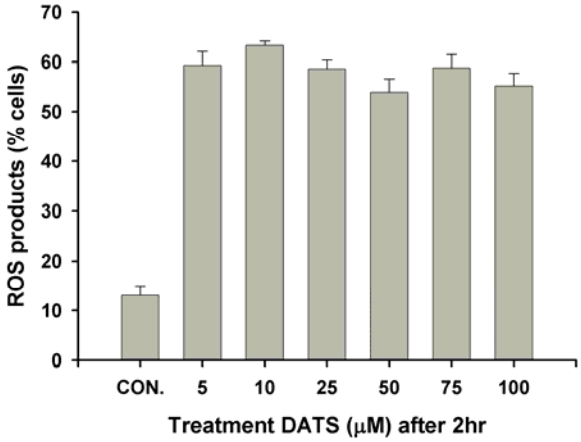
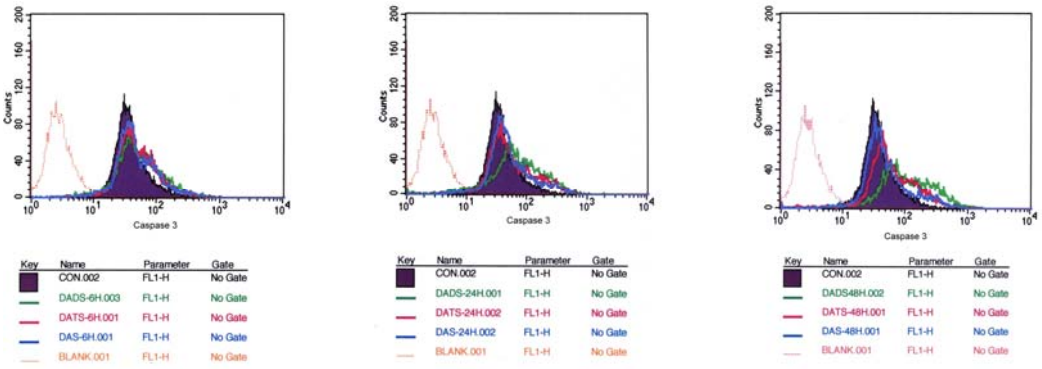


Fig.4 DAS, DADS, DATS induced caspase-3 activity in Colo 205 cells.

(A).



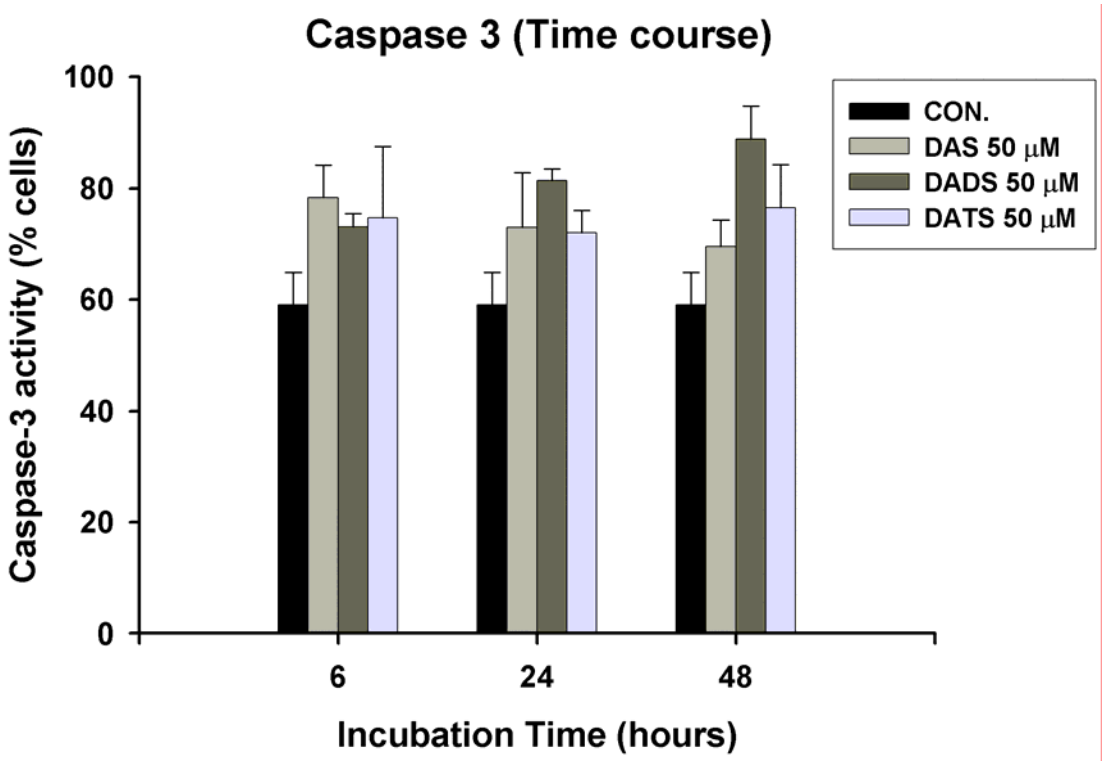
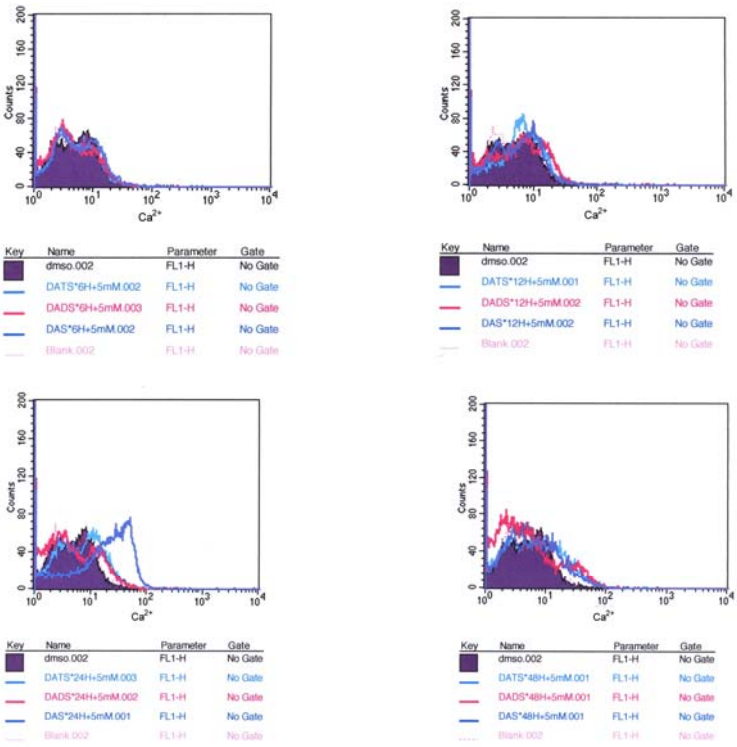


Fig.5 DAS, DADS, DATS induced the Ca^{2+} production in Colo 205 cells.

(A).



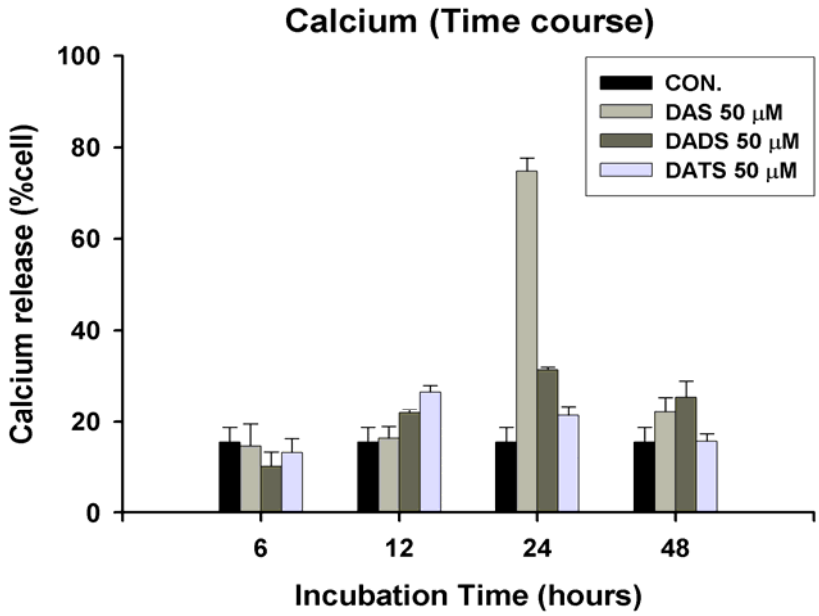
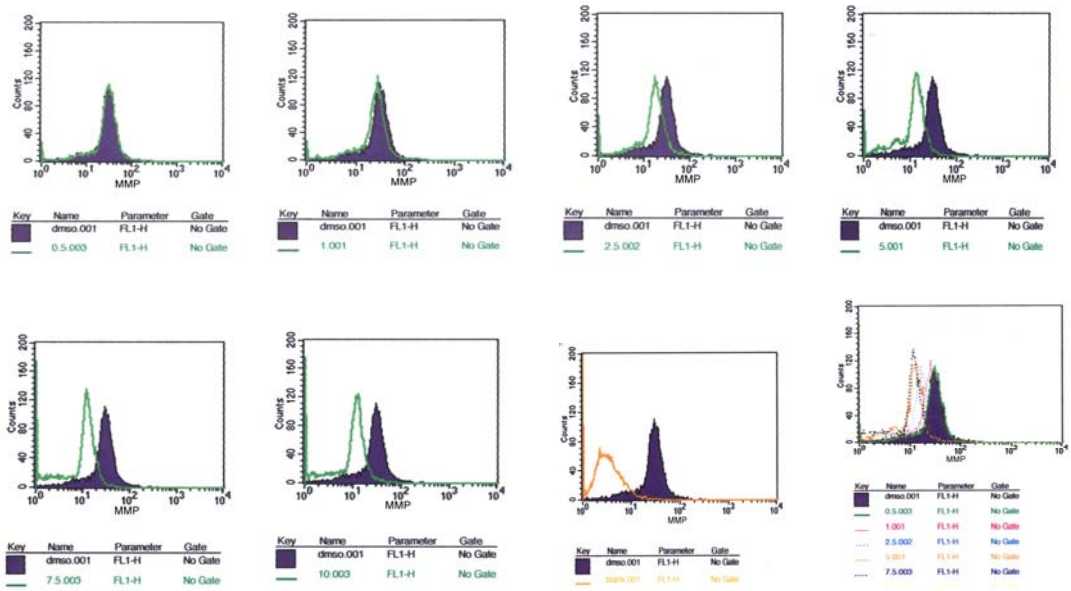
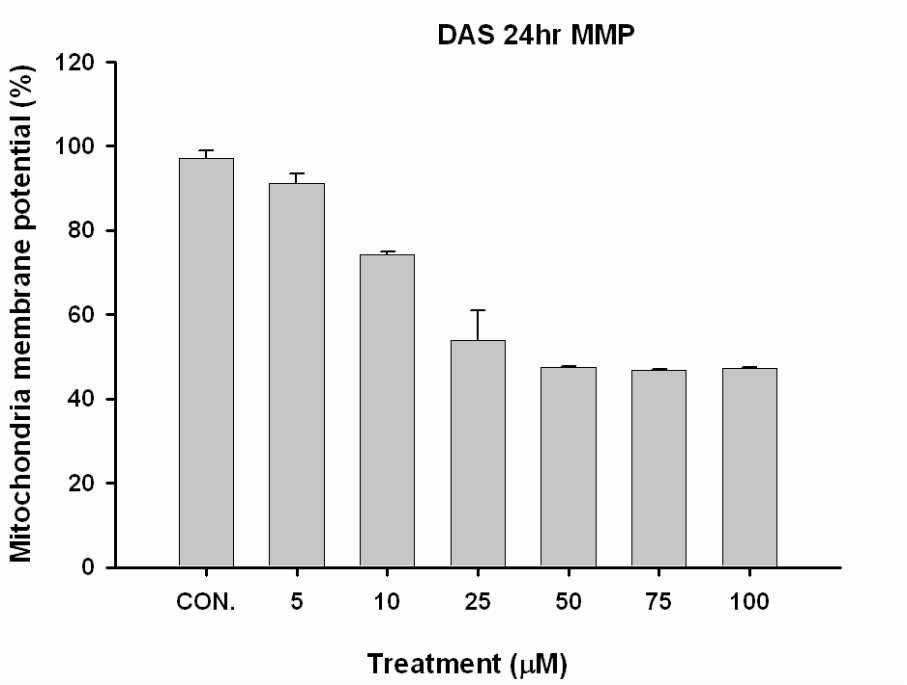


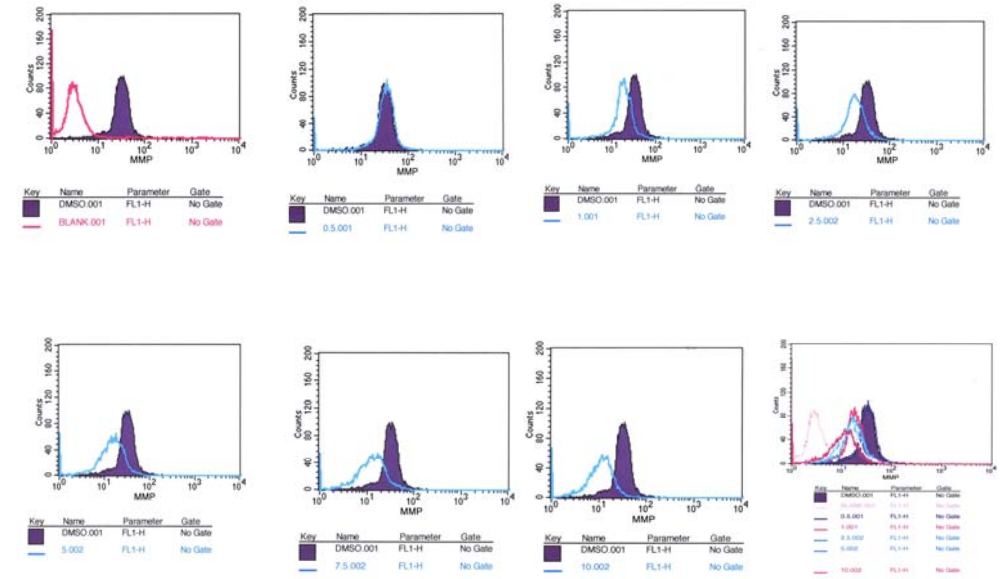
Fig.6 DAS, DADS, DATS decreased the levels of mitochondria membrane potential ($\Delta\Psi_m$) in Colo 205 cells.

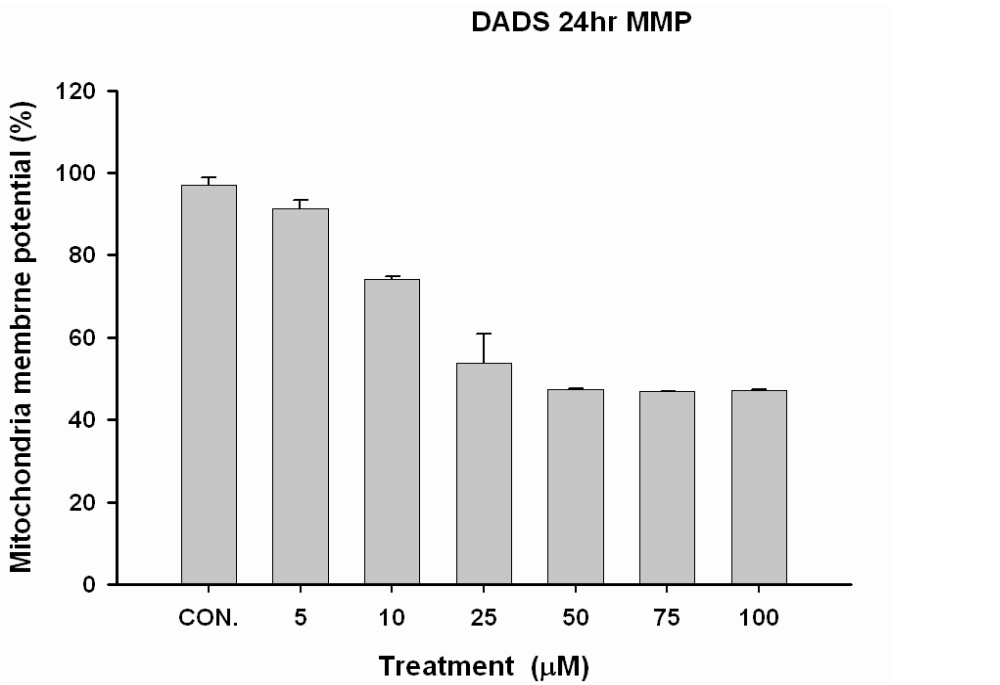
(A). DAS-24hr MMP



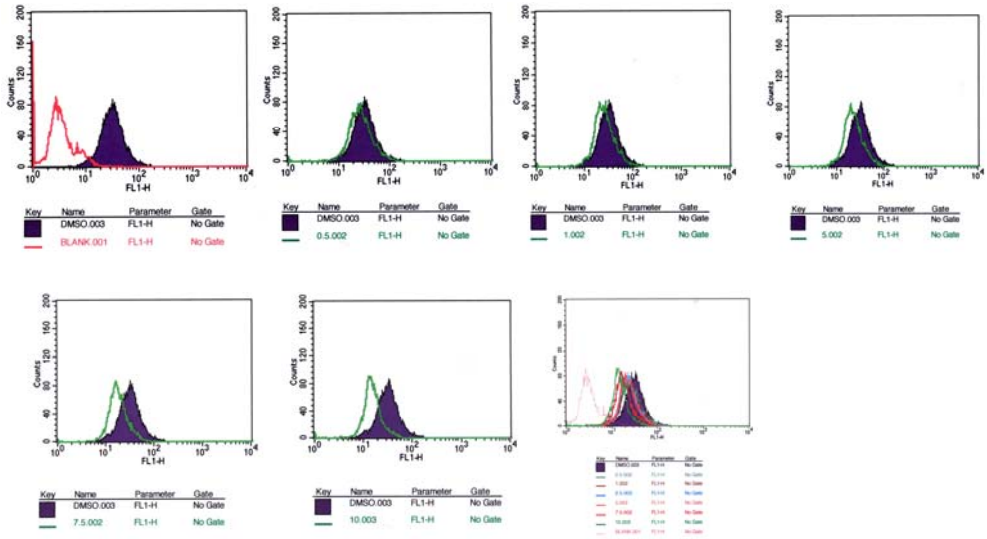


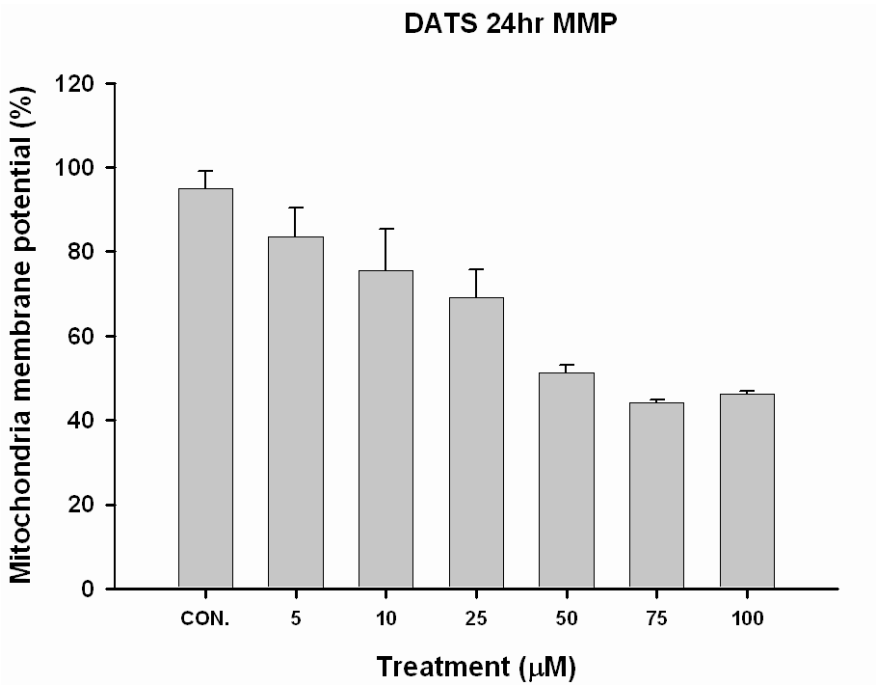
(B). DADS-24hr MMP





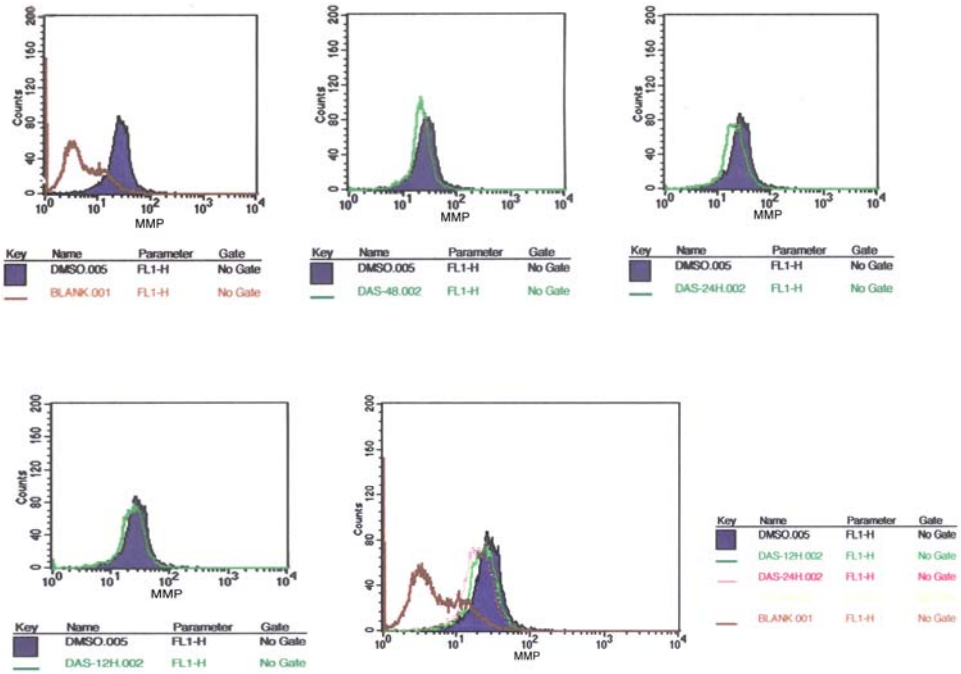
(C). DATS-24hr MMP



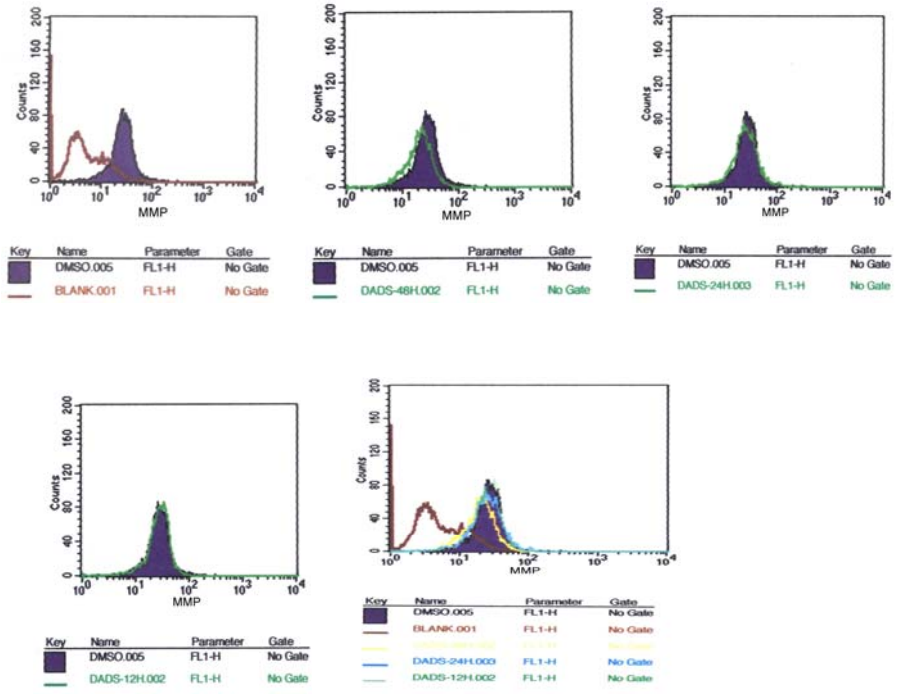


Time course

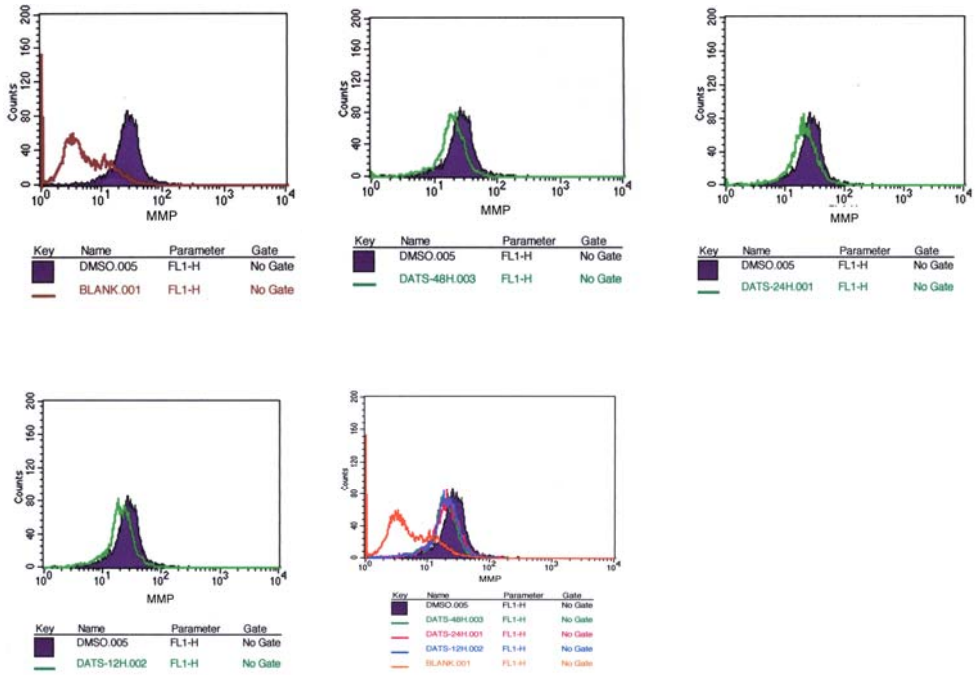
(D). DAS-5mM 時間差



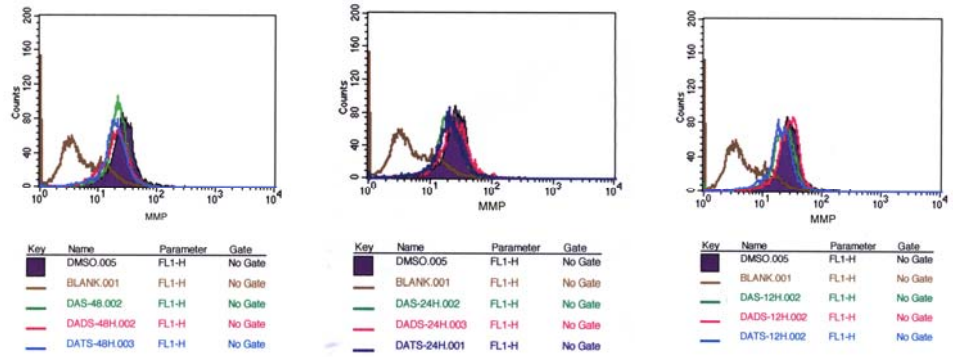
(E). DADS-5mM 時間差



(F).DATS-5mM 時間差



(G).綜合比較圖



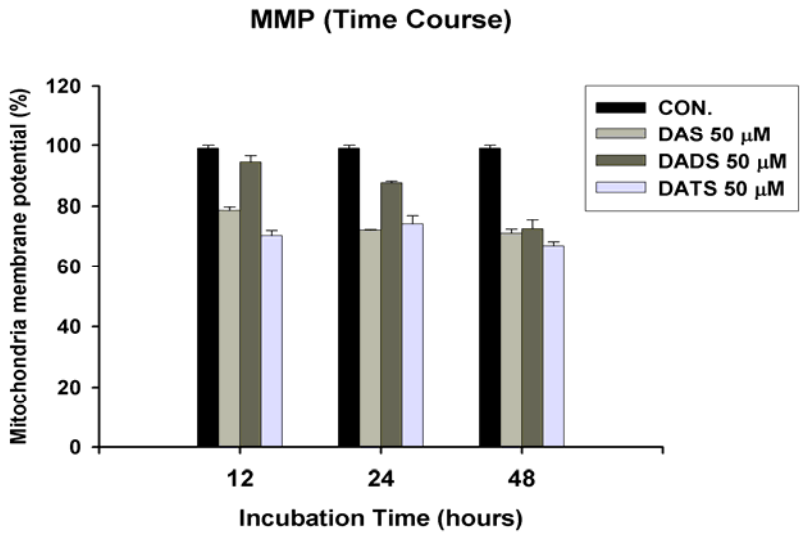
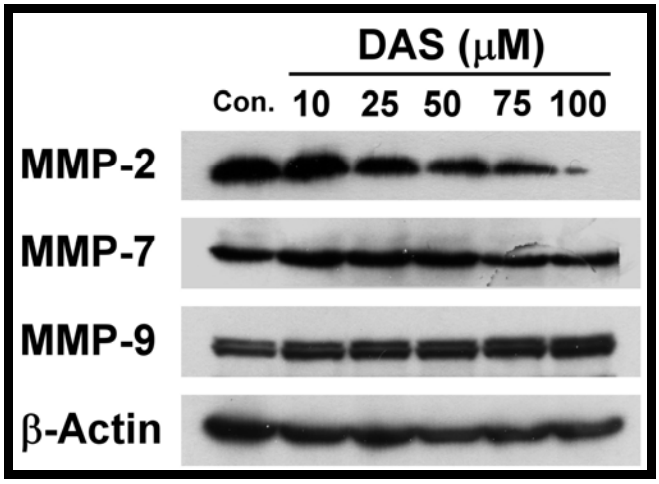
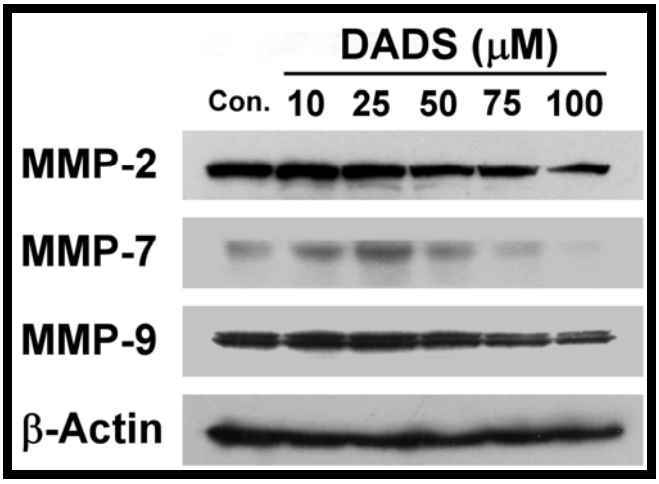


Figure 7. The expression of MMP-2, 7, and 9 on Colo 205 cells after treated with Diallysulfid (DAS), Diallyl disulfide (DADS), and Dially trisulfide (DATS) for 48 hours at various concentrations (10, 25, 50, 75, and 100 μM).

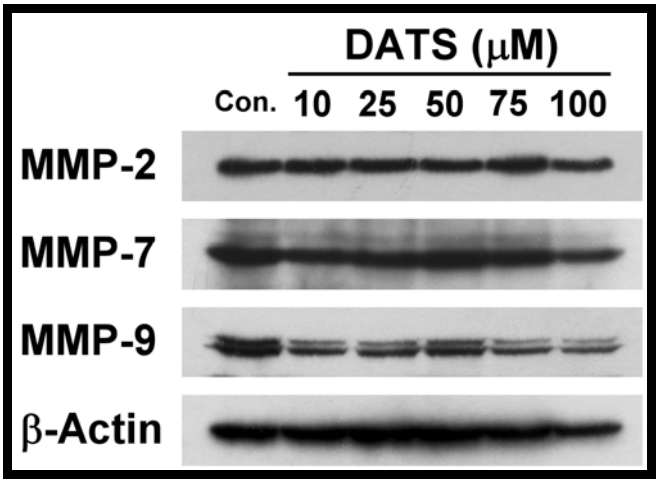
(A)



(B)



(C)



Discussion

The present study is to focus on the role of Ca^{2+} in DAS, DADS, DATS-induced colo 205 cell apoptosis and also examined the cell cycle arrest. DAS, DADS, DATS caused an increase in the productions of ROS and Ca^{2+}, accompanying down-regulation of mitochondria membrane potential ($\Delta\Psi_m$) in mitochondria, and in turn caused apoptosis in colo 205 cells. These findings suggest that Ca^{2+} plays a potential role in capsaicin-induced apoptosis of colo 205 cells. So far, the anticancer activity of capsaicin is still controversial, our results clearly demonstrated that DADS induces G2/M phase arrest and apoptosis, DAS, DATS induces G0/G1 phase arrest and apoptosis in examined human colon cancer colo 205 cells. This is in agreement with other reports who demonstrated that DADS induces G2/M phase arrest and apoptosis,DAS, DATS induces G0/G1 phase arrest ,and apoptosis in examined human colon cancer colo 205 cells. However, they did not show the role of Ca^{2+} on the DAS, DADS, DATS induced apoptosis in colo 205 cells. These findings provide important new insights into the possible molecular mechanisms of the anticancer activity of DAS, DADS, DATS. Apparently, in the future, We expected that we can studies focusing on cell signaling and biological significance of DAS, DADS, DATS -induced apoptosis and cell cycle arrest in vivo could lead to explore the mechanisms of chemotherapeutic potency of DAS, DADS, DATS in human cancer.