Anti-Herpes Simplex Virus Mechanism of Trichosanthin

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Declaration of Originality

The work contained in this thesis is original research carried out by the author in the Department of Physiology, Faculty of Medicine, the Chinese University of Hong Kong, starting from August 2007 to August 2011. No part of the work described in this thesis has already been or is being submitted to any other degree, diploma or other qualification at this or any other institutions.

Abstract of the thesis entitled:

Anti-Herpes simplex virus mechanism of Trichosanthin

Submitted by HE Dongxu

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Trichosanthin (TCS) is a type I ribosome inactivating protein (RIP), it was found to inhibit human simplex virus type 1 (HSV-1) but the anti-HSV-1 mechanism is unclear. HSV-1 is a widely distributed DNA virus, it causes large range of human diseases. During the lytic life cycle of HSV-1, highly regulated cascade of genes are expressed to interfere with host cell metabolism and immune response. In this context the anti-HSV-1 mechanism of TCS in human epithelial carcinoma HEp-2 cells was studied.

Firstly, we demonstrated that TCS reduced HSV-1 antigen and DNA content, The IC_{50} (half maximal inhibitory concentration) of TCS on HSV-1 replication was 2.5 ± 0.23 μg/ml. The anti-HSV-1 effect of TCS was related to interfering with viral replication during 3 to 15 hours after infection which coincide with early to late viral replication period. TCS had no effect on HSV-1 attachment, penetration or immediate early gene expression. However, the expression of early gene, late gene and virion release were diminished.

TCS is toxic to cell because its RIP activity, killing of the viral host cells certainly inhibits virus expansion, only when it kills more infected cells, the material could be considered as an anti-viral agent. It was found TCS induced losing of cell viability and enhancing in apoptosis in HEp-2 cells and HSV-1 infected HEp-2 cells. The decrease of cell viability and increase of apoptosis ratio were enhanced when HEp-2 cells were infected with HSV-1 compared with uninfected ones. The 50% of effect concentration (EC_{50}) in cytotoxicity and apoptosis were decreased from 24.64 $\pm$ 1.17 μg/ml and 37.57 $\pm$ 1.47 μg/ml in uninfected HEp-2 cells to 3.01 $\pm$ 1.30 μg/ml and 3.89 $\pm$ 1.31 μg/ml in HSV-1 infected HEp-2 cells respectively.

Thirdly, TCS activated different apoptotic pathways, namely type I and type II apoptotic pathways, between uninfected and infected cells. The type I apoptotic pathway bypasses the dependence on the mitochondrial but quickly activates a large amount of caspase-8 at the CD95 (Fas/Apo-1) formed death inducing signaling complex (DISC), which amplifies the signal. By contrast, the formation of the DISC in the type II apoptotic pathway is strongly reduced. It depends on loss of the mitochondrial transmembrane potential ($\Delta \Psi_m$) and release of cytochrome c and capase-9 activation to mediate apoptosis signal transduction. We found in HSV-1 infected an uninfected HEp-2 cells, TCS induced the loss of $\Delta \Psi_m$, this $\Delta \Psi_m$ losing was increased when HEp-2 cells were infected with HSV-1. Furthermore, when there were no HSV-1 infection, TCS induced caspase-dependent type I apoptosis pathway that quickly activated large amount of caspase-8 after TCS treatment. However, when infected with HSV-1, this pathway turned into mitochondrial dependent type II pathway involving caspase-9 response, whose apoptosis ratio was diminished by over expressed Bcl-2, which is a hallmark defining

type I or type II apoptosis.

The reason of type I to type II apoptosis pathway transition might due to the activity change of death receptor on HEp-2 cells. The type I apoptotic pathway induced by TCS was related to CD95 (Fas/Apo-1) system activation and signaling pathway. When HEp-2 cells were infected with HSV-1, the CD95 (Fas/Apo-1) was suppressed by HSV-1 infection. As a result, TCS triggered a less CD95 (Fas/Apo-1) dependent type II apoptotic pathway in the infected cells.

Fourthly, the role of the nuclear factor-κB (NF-κB) in the anti-HSV-1 effect of TCS was explored. NF-κB initiates cell survival pathways. It is widely involved in viral infection and replication to make sure virus overcomes the host cells immune response. We found HSV-1 enhanced the activity of NF-κB in HEp-2 cells by triggering its translocation from cytoplasm to nuclear. However, during the anti-HSV-1 effect of TCS, TCS suppressed HSV-1-aroused NF-κB translocation in HEp-2 cells, the inhibition of NF-κB activity in HSV-1-infected cells by TCS treatment tend to abolish the anti-apoptosis effect developed by HSV-1, so that the host cells suffered more extracellular stress and showed more apoptosis ratio than uninfected ones.

Finally, NF-κB and another transcriptional regulator p53 are usually tightly related in their control of cell survival. Opposite to NF-κB, p53 mediates cell death signals, usually activated under DNA damage and subsequently involved in cell growth control, DNA damage repair or apoptosis. It was found in this study, DNA damage and cell cycle arrest responses tended to participate with the anti-HSV-1 activity. Although in HEp-2 cells, TCS induced more DNA damage ratio and S and G_2/ M phase arrest proportion than HSV-1 infected cells, but more p53 was expressed and activated by phosphorylating at

Ser 15 by TCS in HSV-1 infected HEp-2 cells than uninfected ones. In the same time the activation of BAX, which promotes the apoptotic function of p53, increased during TCS treatment when infected with HSV-1, the p53 therefore regulates apoptosis in HSV-1 infected cell during TCS treatment.

Taken together, this study demonstrated TCS interfered with HSV-1 early to late infection period. TCS selectively induced more HSV-1 infected HEp-2 cells to apoptosis than uninfected ones, the selectivity of TCS was due to apoptotic signaling pathway switching from CD95 (Fas/Apo-1)-mediated type I to type II apoptotic pathway. Furthermore, during TCS induced-apoptosis in HSV-1 infected cells, TCS suppressed NF-κB activation that triggered by HSV-1 infection. At the meanwhile, p53 participated in the TCS-induced apoptosis regulation in infected cell.

論文摘要

天花粉蛋白（TCS）屬於一型核糖體滅活蛋白。研究表明 TCS 具有抗單純皰疹病毒一型（HSV-1）活性，但其抗 HSV-1 機制仍未被完全闡明。HSV-1 是一種廣泛存在的 DNA 病毒，在其急性感染的裂解複製週期中，複製過程可分爲即刻早期（IE）、早期(E)和晚期(L)三個階段，有序地表達了一系列病毒蛋白，以確保病毒順利複製並干擾宿主細胞的免疫反應系統。本研究旨在從病毒複製、宿主細胞凋亡信號轉導方面，探討 TCS 抗 HSV-1 的機制。

研究首先發現，TCS 有效抑制了 HSV-1 病毒複製，其 IC50 (半有效抑制濃度) 爲 2.5 ± 0.23 μg/ml。TCS 對 HSV-1 的抑制作用，與其干擾病毒複製的 E 至 L 階段相關，但對 IE 階段，包括病毒與 HEp-2 細胞膜結合、融合及 IE 蛋白表達無明顯效果。TCS 可抑制 E 至 L 階段的蛋白表達，繼而影響成熟病毒粒子的包裝和釋放。

本研究進一步探究 TCS 誘導的 HSV-1 感染的 HEp-2 細胞的凋亡機制。實驗表明，TCS 選擇性地誘導了更多的 HSV-1 感染的 HEp-2 凋亡。在 HSV-1 未感染細胞中，TCS 的 CC_{50}（半有效細胞毒性濃度） 及 EC_{50}（半有效致凋亡濃度）分別爲 24.64 ± 1.17 μg/ml 及 37.57 ± 1.47 μg/m，但是在感染細胞中，CC_{50} 和 EC_{50} 降爲 3.01 ± 1.30 μg/ml 和 3.89 ± 1.31 μg/ml。在凋亡過程中，HSV-1 感染細胞比未感染細胞顯示了更多線粒體膜電位、Bcl-2 蛋白損失，過量表達的 Bcl-2 蛋白抑制了 TCS 在感染細胞中誘導的凋亡。此外，caspase-8 在感染細胞中的活化比未感染細胞顯著

減弱和推遲，但 caspase-9 活化比未感染細胞顯著增加，感染細胞的凋亡過程中對 CD95 (Fas/Apo-1)死亡受體的信號轉導依賴性較未感染細胞顯著減小。這些證據表明 TCS 誘導未感染細胞致一型凋亡信號途徑，感染 HSV-1 後，凋亡轉換爲二型凋亡信號途徑。

在 TCS 所誘導的 HSV-1 感染或未感染的 HEp-2 細胞的凋亡基礎上，我們分別從核因數- κB（NF-κB）及 p53 蛋白介導細胞生存或凋亡的信號通路角度進一步探討了 TCS 抗 HSV-1 機制。以往研究表明，HSV-1 活化 NF-κB 信號通路以抵抗宿主細胞免疫反應和凋亡信號。我們發現，當 TCS 作用於 HSV-1 感染細胞之後，HSV-1 在 HEp-2 中活化的 NF-κB 通路被顯著抑制。另一方面，TCS 導致了 HEp-2 細胞的 DNA 損傷和細胞週期阻滯，並活化了少量的 p53 蛋白。然而，當 HSV-1 感染細胞後，DNA 損傷和細胞週期阻滯率顯著減少，卻有顯著增加的 p53 和 p53 凋亡介導蛋白 BAX 蛋白被活化，從而影響了 TCS 誘導 HSV-1 感染 HEp-2 細胞的凋亡途徑。

總而言之，我們證明了 TCS 抗 HSV-1 病毒機制主要作用於病毒感染的早、晚期，TCS 選擇性誘導 HSV-1 感染細胞至二型凋亡途徑，並阻滯了 HSV-1 感染引發的 NF-κB 信號通路活化，同時啓動了 p53 凋亡信號通路活化。

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After 1416 days in the Chinese university of Hong Kong, finally the thesis is fulfilled. I still remember the first day when I arrived at CU, I was shocked by its scenic beauty and believed I must be the luckiest person in the world to pursue a Ph.D degree here.

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Standing at the end of this final semester, I realized this probably be the last few months of being a "student" in a school or college. However, I would always be the student of all my mentors: not only my supervisor, but also Professor Zheng YT, Shaw PC, Zhang CK, HE XH and so on. I will always keep my respect and gratitude in mind to them, for their profound specialized knowledge, rigorous doing scholarly research attitudes and indefatigable noble virtue of my profound influence.

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This thesis is a gift to myself, to people whom I love, people who love me.

Table of content

Abbreviations

AIDS	Acquired immunodeficient diseases syndromes
ACV	Acyclovir
APAF-1	Apoptotic protease activating factor 1
BSA	Bovine serum albumin
CHX	Cycloheximide
CytoC	Cytochrome C
DISC	Death inducing signaling complex
E gene	HSV-1 early gene
EC_{50}	50% of effect concentration
ELISA	Enzyme linked immunosorbent assay
ER	Endoplasmic reticulum
FasL	Fas ligand
FADD	Fas-Associated protein with death domain
HIV	Human immunodeficiency virus
HSV-1	Human simplex virus type 1
IC_{50}	Half maximal inhibitory concentration
ICP	Infect cell protein

IE gene	HSV-1 immediate early gene
LBP	LPS-binding protein
IL	Interleukin
IL-1R	Interleukin 1 receptor
L gene	HSV-1 late gene
IκB	Inhibitor-κB
IKK	IκB kinase
MAPK	Mitogen activated protein kinase
MDM2	Murine double minute
MMP	Mitochondrial transmembrane potential
MOI	Multiplicities of infection
NF-κB	Nuclear factor-κB
TCS	Trichosanthin
$TCID_{50}$	50% tissue culture infective dose
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
$\Delta \Psi_m$	Mitochondrial transmembrane potential

Chapter 1

General Introduction

1.1 Trichosanthin

Trichosanthin (TCS) is a 27 kDa protein isolated from the root tuber of the Chinese medicinal herb *Trichosanthes kirilowii Maximowicz* (Tian Hua Fen). TCS is a type 1 ribosome inactivating protein (RIP), it possesses a broad spectrum of biological and pharmaceutical activities including inducing abortion, antitumor, antiviral effects.

1.1.1. General properties

The crystal of TCS protein is purified from the root tubers by employing CM-Sephadex chromatography and everse-phase HPLC. In a 0.1 M barbiturate buffer at pH 8.5, it represents 0.014% of total weight of root and exhibits an isoelectric point of 9.4.

TCS consists of one polypeptide with 289 amino acids with high content of serine (7.7 %) but without cysteine and carbohydrate attachment in the protein, it includes a 23-residue signal peptide at the N-terminus and a 19-residue leader peptide at the C-terminus.

The complete gene encoding the 289 amino acids of recombinant TCS was sequenced, cloned and synthesized in Escherichia coli [1;2]. There are no introns inside the recombinant DNA of TCS, it includes an 850 bp open reading frame encoding full length

of the protein. Among the recombinant DNA of TCS, the first 23 residues encoded contain a putative secretory signal peptide, the last 19 residues comprise a carboxyl extension, and this extension is thought to be cleaved after the encoded protein reaches reticulum or Golgi apparatus of cells to produce an active TCS. These two residues are not natural products of TCS DNA from its root tuber.

A native TCS shares a highly similar secondary structure with ricin A-chain, a type II RIP (Contains a toxic A chain and lectin-like guiding B chain) from castor. At the pH of 4.7, 7 and 9, the structure showed by CD spectra of TCS is nearly identical to that of ricin A-chain [3]. The single chain of TCS is composed of about 29% alpha-helix and 42% beta-sheet but no beta-turn.

The potential active site of TCS is located from 110 to 174 of its amino acids [4], especially, the amino acids 120-123 are essentially involved in the ribosome inactivating activity of TCS, any deletion of these part of amino acids was found to induce dramatic decrease in the activity. By sequence alignment of TCS with other RIP, six residues among the amino acids of TCS, including Tyr-70, Tyr-lll, Glu-160, Ala-161, Arg-163 and Trp-192 were found to play an important role in substrate binding and catalysis reaction of TCS, they are on the similar active-site cleft as other RIP. The Glu-160 and 189 were found to be involved in the catalytic reactions [5], they are responsible to maintain the negative charge on the face of secondary structure of the protein and stabilize the transition-state complex. The Trp-192 was found to affect the RIP activity of TCS [6], deletion or mutation on this site induces losing of ribosome inactivating ability. The Arg-163 forms an active pocket inside the TCS crystal, it has N-glycosidase activity and

provides proton for the enzyme activity of TCS [7]. The Leu-240 stablizes relationship between N- and C-terminal by hydrogen bonds between the two domains. Furthermore, the Tyr-l4 and Arg-22 are closed to the active-site cleft [8], they are responsible for the folding process of TCS, a mutation on Arg-22 may induce formation of hydrogen bond instead of intermolecular bond, results in a less stable protein structure and decrease in activity.

1.1.2. Biological activities

1.1.2.1 Ribosome inactivating activity

TCS is a type I ribosome inactivating protein (RIP). The RIPs are a group of protein synthesis inhibitors. Members of the family include type I (e.g. TCS and luffin), type II (e.g. Ricin agglutinin and abrin) and type III (e.g. active mutant of maize RIP) [9;10] RIPs. The type I RIP contains one peptide chain, this single chain is responsible for both protein entry and the toxicity. On the other hand, type II RIP contains two chains, including a toxic A chain and lectin-like guiding B chain, the two-chain structure therefore potentiates the toxicity of type II RIP. The A-chain of type II RIP is structurally related with type I RIP, The secondary structure of native TCS is nearly identical to ricin A-chain. Furthermore, the type III RIP, a newly founded RIP, contains one chain structure composed of a precursor with a 25-residue internal inactivation region, during activation, the internal inactivation region will be removed by proteinase.

The RNA-N-glycosidase activity of TCS is composed of two subsites in the active

pocket of TCS [11]. One is responsible to recognize catalytic site, the other inactivates the 60s subunit of eukaryotic ribosomes, then the conformation of the rRNA is altered, binding of elongation factors is decreased and protein synthesis is inhibited [12].

1.1.2.2 Abortifacient activity

TCS was used in ancient China to induce abortion in mid-gestation and to treat ectopic pregnancy, hydatidiform mole, invasive mole and choriocarcinoma derived from fetal trophoblast cell in the uterus because it is highly toxic to trophoblast. The therapeutic effect in the inhibition of early pregnancy of TCS was found in man, mouse, rat and rabbit [13;14], placental unit may be affected by TCS, result in dislodging of the placenta premature delivery.

1.1.2.3 Antitumor activity

TCS has been proved very effective in treating malignant moles and choriocarcinoma clinically and is more toxic to specific tumor cell lines than normal cells lines. TCS is cytotoxic on choriocarcinoma cell line JAR[15], leukemia cell line k562 and HL60 [16;17] , mouse macrophage-like tumor cell line IC21[18], lymphoma, cervical adenocarcinoma Hela [19] and breast adenocarcinoma [20].

1.1.2.4 Immunomodulatory activity

TCS at non-toxic concentration is capable of interfering humoral immunity and immune-related processes [21]. TCS was showed to trigger a cytotoxic T-lymphocyte

response and suppress activated lymphoproliferative response. Similarly, the interleukin IL-2 production in activated splenocytes, delayed type hypersensitivity (DTH) response in activation of natural killer (NK) cells and humoral antibody formation to SRBC could be inhibited by TCS. TCS was found to suppress inflammation by increasing cell death of ovalbumin-specific T cell via nitro oxide-mediated apoptosis pathway [22]. TCS could induce expression of IL-4 in mesenteric lymph node cells and ovalbumin cells, the IL-4 was believed involved in the TCS inducement of ovalbumin-specific immunoglobulin E (IgE) production [23]. In peritoneal macrophage, TCS could enhance antigens, such as IL-10 and monocyte chemoattractant protein 1 (MCP-1) secretion, and reduce antigens, such as IL-12 and tumor necrosis factor (TNF)-α expression, then in the aftermath facilitate the major T helper 2 (Th2) response. Moreover, TCS was found to interact with chemokine receptors and then activate chemokines for stimulating chemotaxis and G protein activation [24].

1.1.2.5 Antiviral activity

TCS is the first RIP found to inhibit human immunodeficiency virus type 1 (HIV-1) replication in both acutely infected T-lymphoblastoid cells and chronically infected macrophages in vitro in 1980s [25], TCS inhibits the serum HIV-1 p24 antigen level and increases CD4+ T cell counts in HIV-1 infected patient during phase I / II clinical trials [26], and its anti-HIV activity of TCS in vitro partly relates to its ribosome inactivating (RI) activity [27;28] . Zhao and colleagues further showed TCS inhibited HIV-1 fusion by enhancing interaction between chemokines and chemokine receptors [24].

Because herpes simplex virus (HSV) infections usually can be lethal to HIV-1 infected patients, researches on HSV inhibition aroused widely concerns during recent years. TCS was found to show anti-HSV type 1 (HSV-1) activity [12;29] and this activity is related to Mitogen-Activated Protein Kinase (MAPK) signal transduction pathways [30], moreover, anti-HSV-1 activity of ACV was found to be enhanced by combining with TCS treatment [29]. However by now, the exact anti-HSV-1 mechanism of TCS is still unsure.

Moreover, the anti-viral activity of TCS was found in transgenic tobacco [31]. Transgenic tobacco plants expressing recombinant TCS exhibited inhibition effect on cucumber mosaic virus and Tobacco mosaic virus (TMV) infection.

1.2 Cell death

Cell death takes two distinct forms, namely, apoptosis and necrosis. These two types of cell death could be identified from their morphological differences. The apoptosis or programmed cell death is an energy dependent process; it usually shows nuclei and cytoplasmic condensation, nuclear DNA cleavage and apoptosis bodies formation. The features of necrosis, on the other hand, including swelling of cytoplasm and mitochondrial, breaking of plasma membrane, leaking of lysosomal enzymes and losing of organelles such as nuclei, it takes place independently of energy.

1.2.1 Apoptosis

1.2.1.1 The network of apoptosis (Figure 1.1)

Apoptosis is widely studied because its important role in development, tissue maturation, immune regulation and tumor suppression, it is usually found when cells are confronting physiological and pharmaceutical stimuli from both extracellular and intracellular sources.

Apoptosis is an energy dependent process controlled by a diverse group of proteins for its signal transmission. Those cytotoxic signal proteins interact with receptors on plasma membrane, their cognate ligands, other cellular constituents such as mitochondrial and other signal proteins for the death execution. Within the apoptosis network, one of the major players is death receptors on cell surface. The death receptors belong to a large

super family of tumor necrosis factor receptors (TNFR), including TNFR1, CD95, TNF-related apoptosis-inducing ligand receptor-1 (TRAILR1) and TRAILR2. The death receptors provide a death domain (DD) in the intracellularly which facilitates recruitment of caspase.

Another group of major apoptotic players is caspase families, including initiator caspases (caspase-8,10) and executioner caspases (caspase 3,6,7,9) [32]. Caspases are a family of cysteine proteases that are well known by their essential role during execution of apoptosis. An apoptotic program requires different caspases during different phases of apoptosis. In response to apoptotic signals, the initiator caspases undergo proteolytic activation by DD of TNFRs, then they activate executioner caspases, and subsequently cleave other downstream substrates.

The role of caspases is not limited to apoptotic functions, it can regulate cell proliferation, cell cycle, or interestingly sometimes promote cell survival [33]. Caspase-8, known as an initiator caspase, was shown to regulate cell proliferation, patient with defect in capase-8 showed defect in T cell proliferation and IL-2 production. Caspase-3, was reported to regulate cell cycle when they were presented in the nuclei of cells, it is also be able to regulate cell cycle proteins [34]. Several studies showed caspase-8 and 10 may activate nuclear factor-κB (NF- κB) pathway in favor of cell survival [35].

The TNFRs and caspases related apoptosis pathway may be defined as extrinsic pathways. Oppositely, mitochondrial, may lead to an intrinsic pathway, mediated by mitochondrial and sometimes the endoplasmic reticulum (ER) [36]. The mitochondrial transmembrane potential ($\Delta\Psi_m$) between inner and outer mitochondrial membrane is

crucial for the function of mitochondrial and electron transport. In apoptosis, there is a rapid reduction of $\Delta\Psi_m$, the pro-apoptosis proteins BAX and BID may be recruited to enhance the losing of $\Delta\Psi_m$. The cytochrome C then releases from inter membrane space and interact with other downstream substrates, which ultimately enhance the apoptotic signal. The calcium store inside ER, on the hand, could be released during apoptosis, the released Ca^{2+} can then lead to rupture of $\Delta\Psi_m$.

Another group of participators in apoptosis includes MAPK families. The MAPK super family is one of the major members involved in cell proliferation, differentiation and embryogenesis. The MAPK consists of protein serine/ threonine kinases mediate signal transduction in response to variety of stimuli. Generally, three sequential protein kinase activation steps are activated through the MAPK cascade: MAPK kinase kinases (MAPKKKs)-MAPK kinases (MAPKKs)-MAPKs. There are six types of MAPK cascades in mammalian cells, including ERK 1/2, JNK 1/2/3, p38 α /β /γ /δ , ERK 5, NLK and ERK3 α/β pathways [37;38]. The extracellular signal regulated kinase (ERK) is the most widely studied MAPK pathway. Its signal transduction pathway is related to cell proliferation, differentiation regulation, it is usually activated in response to growth factors. The signal cascade follows the three MAPK pathway activation steps with different kinases: Raf- MAPK/ ERK kinase (MEK)- ERK. Opposite to ERK pathway activated by growth factors, the (c-Jun kinase/ stress activated protein kinase) JNK/ SAPK and p38 are cascades response to stress stimuli including UV irradiation, growth factor deprivation, heat shock or inflammatory cytokines stimulation, they share the pathways parallel to the Raf-MEK-ERK pathway. Therefore, both of JNK/ SAPK may be

activated in response to apoptosis, their activation results in transcriptional activity which in turn stimulates apoptosis-related gene expression.

Some transcriptional regulators were showed related to apoptosis. Most commonly the p53 and NF- κB are involved. The pro-apoptotic p53 and anti-apoptotic NF- κB mediate gene transcriptions in response to control cell apoptosis or survival.

1.2.1.2 Cell death receptors mediated apoptosis

After a cell receives stimulus, two models separated by different plasma membrane receptors were suggested to induce apoptosis [39;40], the TNFR and CD95 (APO-1/ Fas) systems. The proteins involved in the two models belong to TNFR family.

In the TNFR-mediated apoptosis, TNF-α plays an important role to trigger apoptosis by binding to the TNFR, which is a protein crosses plasma membrane. After interacting with TNF-α, the intracellular death domain of TNFR, TNFR associated death domain (TRADD), activates Fas-associated protein with death domain (FADD). Then downstream caspase families are recruited to induce apoptosis. Alternatively, the TNF--α sometimes mediates survival signaling, the TRADD may recruit MAPK or NF-κB pathways to promote survival.

In the CD95 (also known as APO-1 and Fas) mediated apoptosis, the transmembrane protein Fas, extracellular Fas ligand (FasL), intracellular FADD and intracellular pro-caspase-8 form a death-inducing signal complex (DISC). The caspase families (including caspase-3, 6, 7, 8, 9 and 10), Bcl-2 families (including Bcl-2, Bcl-xL, Bax and Bid) and mitochondrial are all related to the downstream signaling.

1.2.1.3 CD95 (APO-1/ Fas) mediated two types of apoptosis

The CD95 (APO-1/ Fas) is the one of the most studied death receptors that initiates a cascade of pro-apoptosis signals. When exposed to stress signals, usually with a process of FasL trimerization [41], the adaptor protein FADD and two isoforms of pro-caspase-8 are recruited at the DD of Fas and the DISC is formed. Upon DISC formation, the pro-caspase-8 is quickly processed and the following apoptotic cascades was triggered. The pathways are divided into extrinsic or intrinsic categories, also known as CD95-mediated type I or type II apoptotic pathways respectively, based on the level of mitochondrial dependence.

The type I apoptotic pathway bypasses the dependence on the mitochondrial. This pathway quickly activates a large amount of caspase-8 at the DISC, the substrate caspase-3 is then recruited to amplify the signal.

By contrast, the formation of the DISC in the type II apoptotic pathway is strongly reduced but the dependence on mitochondrial is increased. In this pathway, the Bcl-2 family plays an important role in signal transmission with their major site of action on the mitochondrial. The Bcl-2 family is composed of two opposite groups of molecules. These proteins share a Bcl-2 homology (BH) domain. The anti-apoptotic molecules, such as Bcl-2, Bcl-xl, Mcl-1, A1 and ORF16 related cell survival signal transmissions, may benefit cell survival and protect cell from apoptosis. And the pro-apoptotic families, such as BAX, Bak, Bok and Bid, promote cell death signal. When exposed to stress, the

formation of DISC in cells using type II apoptotic pathway only requires a limited and delayed caspase-8 process, the small quantity of caspase-8 is enough to cleave Bcl-2 family member Bid, a BH3-domain-only protein, at the mitochondrial membrane, but the amount of caspase-8 is insufficient to activate caspase-3. The cleaved Bid then interact with BAX, induces the oligomerization of BAX [42;43], BAX with conformation changes inserts tightly within the outer mitochondrial membrane and induces a loss of the $\Delta\Psi_m$. When the $\Delta\Psi_m$ is broken, cytochrome c may easily release from inter membrane space and mediate apoptosis signal transduction. Cytochrome c, along with apoptotic protease activating factor 1 (APAF-1) oligomerization, mediates capase-9 activation during signal transmission [40;44;45], which can in turn activates caspase-3 leading to apoptosis.

Although these two apoptotic pathways can work through different pathways, they are usually connected to each others. Because the $\Delta\Psi_m$ depolarization itself is a symbol of cell apoptosis, so the depolarization can be observed in both type I and II pathways. But only the type II apoptotic cells depend on mitochondrial for cell survival. Therefore, protecting of the mitochondrial from depolarization by over-expressing Bcl-2, a homolog to the anti-apoptotic C.elegans protein CED-9 [46], the caspase-8 activated in type II apoptotic pathways is not sufficient to initiate downstream caspase, and subsequently diminishes apoptosis in type II apoptotic cells but not in type I apoptotic cells [47].

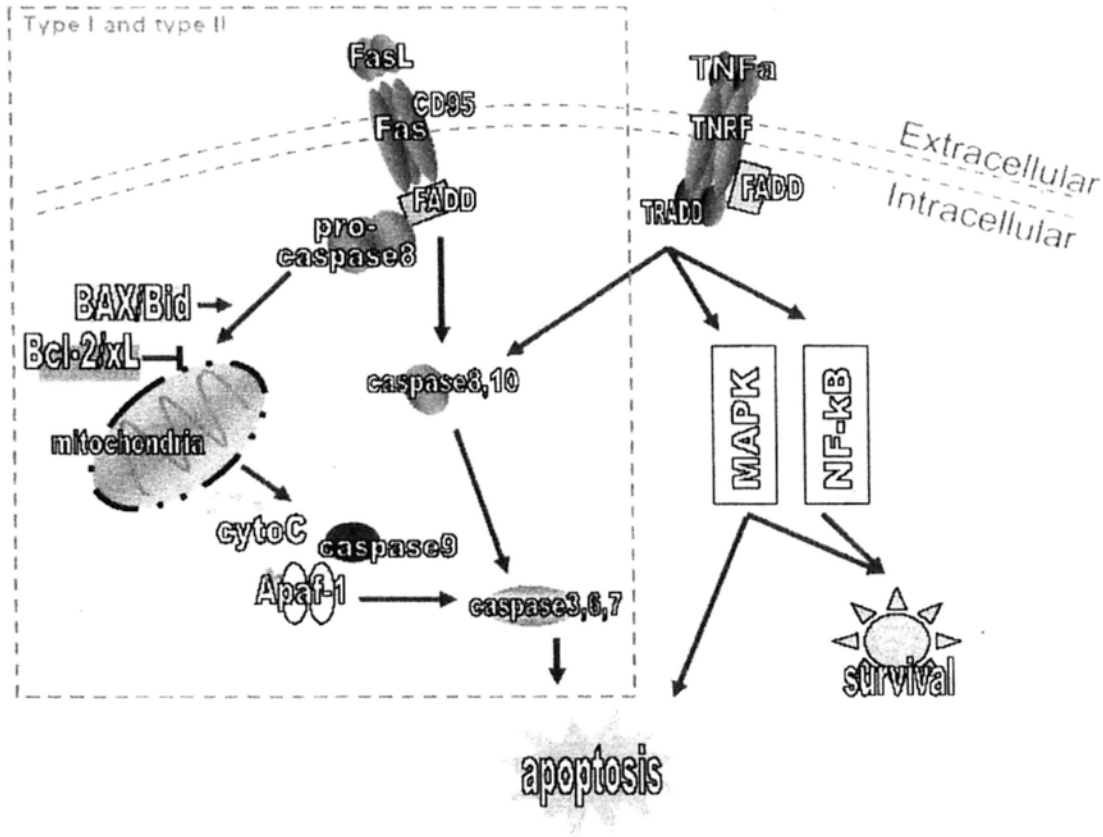


Figure 1.1 The TNRF and Fas mediated apoptotic pathway and the CD95 mediated type I and type II apoptotic pathway.

1.2.2 Necrosis

Necrosis is a cell death phenomenon that usually caused by injury, infection, cancer, infarction, poisons, and inflammation, it begins with swelling of cytoplasm and mitochondrial, breaking of plasma membrane and digestion of chromatin. In the late necrosis, the lysosomal enzyme is leaked and organelles are broken down, the cells finally suffer lysis. While the apoptosis is a natural process of cellular death benefit to development, immune response and so on, necrosis is a fatal process and detrimental. By contrast to apoptosis, which requires energy and a large, complex network of molecules

and cells, necrosis does not depend on energy, and usually does not send signals to nearby cells and does not involve any chemical reaction between molecules.

The apoptosis sometimes is reversible. There are numerous of chemicals to date to be used as an inhibitor of apoptosis, such as the most commonly used caspase inhibitor Z-VAD-FMK and the death receptors inhibitor CD95 Fc Chimera. Also there are plenty of activators that activates anti-apoptosis signals to antagonize apoptosis pathway, such as PKC activator PMA and NF-kB activator IL-1a. However, necrosis is irreversible after the initial cause of the necrosis is introduced.

1.3 Herpes Simplex Virus Type 1

The HSV-1 is a member of the herpes viruses termed as *Herpesviridiae*, which includes herpes simplex virus type 2 (HSV-2), cytomegalovirus, varicella zoster virus, Epstein-Barr virus, human herpesviruses 6, 7 and 8, and Kaposi's associated herpesvirus. A virion of herpesvirus is like a sphere with a diameter of 120 to 130 nm, the core of a virion is about 75 nm.

HSV-1 and HSV-2 are the most widely studied important human pathogens that cause extensive range of human diseases such as mucocutaneous lesions of mouth, eyes or genitalia [48], they are structural related but HSV-1 commonly infects oral mucosa, whereas HSV-2 infects genital mucosa. Because the virus is covered by a glycoprotein formed envelop, the virus is sensitive to organic solvent, such as ether, acetone and chloroform. HSV-1 is stable at room temperature and -70 ℃ but can be inactivated at 56 ℃.

1.3.1 HSV-1 structure

The HSV-1 is a DNA virus. The virion is composed of an icosahedral protein capsid coated with a lipid envelop with a diameter of about 125 nm, the capsid is constructed with 162 caposomeres encasing a 153-kilobase linear double-stranded DNA. The capsid and envelop together join with each other by means of a tegument. Several glycoproteins are included on the surface of HSV-1, the proteins not only compose an enclosed structure covering the viral DNA, but also be able to attach to and then fuse with the

plasma membrane of host cells with glycosaminoglycan chains on cell surface [48] in order to inject viral DNA into cells.

The 153-kb DNA contains 75 open reading frames, encodes at least 84 proteins involving proteins control viral replication, infection and package into integrate virion [49]. The HSV-1 genome consists of two unique regions, U_L (unique long) and U_S (unique short). U_L comprises 82% of the viral genome and encodes 56 viral proteins, the U_S comprise18% of the genome with 11 genes.

1.3.2 HSV-1 life cycle

1.3.2.1 HSV-1 replication steps

HSV-1 replication is able to go trough a lytic cycle to produce infectious progeny or stay silence during a latent status. During the lytic life cycle of HSV-1, the virus mainly infects epidermis and peripheral nervous near mouth, face and eyes, this kind of acute infection is prevalent. But in newborn infant or individuals with immunodeficiency, the infection could be disseminated and lethal. One lytic life cycle includes cell attachment, penetration, gene expression and progeny releasing.

The attachment of HSV-1 to the host cells relies on a group of envelop glycoproteins involving gB, gC, gD and heterodimers of gH-L. Generally, gC bind to the heparin sulfate proteoglycans on the host cell surface. Subsequently, gD will further bind to the herpesvirus entry mediators (HVEM), including TNF receptor family [48], nectin-1 and 2 and isoforms of 3-*O*-sulfotransferases [50;51]. When bound to specific receptors on the

cell surface, a pore will be formed as a bridge between the envelop and cell surface membrane by means of fusing, the gH-gL were reported to be important during the fusion [52] because the heptad repeat structure of gH exhibits structural-functional features typical in viral fusion glycoproteins, it can physical interacts with heptad repeat on the cell membrane. The binding of glycoprotein to the cell surface is not essential for viral entry, it is a reversible process and the detached virus is still infectious. Scine testis and Chinese hamster ovary were found to restrict to bound-virus entry [53;54].

After the pore between the virus and the host cell have been made, it is followed with a penetration process. The viral genome is inoculated quickly into the cell nucleus through capsid portal when the capsid is transported to the nucleus. In the meanwhile, viral tegument protein VP16, a transcriptional activator of immediate-early (IE) gene product is delivered to nuclear pore, which may be facilitated by focal adhesion kinase (FAK) on the cell surface [55]. Then the gene expression from IE genes, early (E) genes to late (L) genes is serially triggered.

Then viral genes are expressed and the proteins are synthesized, the viral particles, including viral genome and capsid, are packaged in the nucleus. The viral envelop is acquired by a budding process. The virus first buds into the inner nuclear membrane and the capsid is covered by a primary envelop, the primary envelop then fuses with outer nuclear membrane when the virus moves through the membrane. The final viral envelop is obtained when virus buds into cytoplasmic vesicles and finally packages into a complete virion.

1.3.2.2 HSV-1 gene expression

The HSV-1 gene expression is a highly regulated process. As soon as the tegument protein VP16 reaches nucleus, it will start the gene expression together with cellular octamer DNA-binding protein Oct-1 [49]. The origin of HSV-1 DNA replication is formed by three *cis*-acting elements: one copy of ori_L and two copies of ori_S.

The first group of genes expressed is IE genes, such as infect cell protein 0 (ICP0) ICP4, ICP22, ICP27 and α4 [56], are expressed under VP16 and Oct-1 facilitation by binding to the TAATGARAT located on the promoter during 2-4 h post infection that later will regulate the expression of other HSV-1 genes. The IE gene expression or replication is independent on the E gene products that are essential for viral DNA synthesis, but they serve as transactivators of E gene expression. For example, the ICP0 was reported to be a positive regulator of viral replication through transcription, interacts with the translation elongation factor 1δ and interferes with transcription efficiency [57]. ICP27 was found to regulate E and L DNA replication, transcription and gene expression and control host protein synthesis [58]. ICP4 plays a dual role in E and L gene expression as activator or repressor [57].

Then the second group of gene expressed is E gene during the 5-7 h after infection, such as U_L30 and U_L52, are involved in viral-DNA synthesis. The products of this group of genes are thought to be essential for origin-specific DNA replication, these products include origin-binding protein (U_L9), replicative helicase (U_L52), primase (U_L53), DNA polymerase (U_L30/ 42), single strand DNA binding protein (U_L29), DNA polymerase clamp (U_L30), RNase H (U_L30) and 5'-3'-Exonuclease (U_L12), viral DNA synthesis

begins after the E gene appearance.

Later L genes are expressed up to 15 h post infection, such as glycoprotein D and B (gD and gB), primarily encode structural proteins [49].

1.3.2.3 HSV-1 latent cycle

The life cycle of all the herpesviruses, including HSV-1, is able to enter latent infection in neurons of peripheral ganglia. During the latent state of HSV-1, there is no infectious progeny production. Although the entire HSV genome is presented as either a circle or a long linear cocatemer in latently infected tissues [59], only a limited number of viral genes are expressed during latency, such as a group of latency-associated transcript (LAT) genes , which were showed to be implicated during latency of HSV-1 to interfere with host cell immunoresponse, gene expression and cell death. The LATs are not required for the establishment or maintenance of latency, but essential for the effectiveness of reactivation. Some IE genes, such as ICP0 and ICP34.5 [60], are detected to cover the range of LAT gene. Three LATs with 2.0, 1.5 and 1.45 kb in size, are sharing the ICP0 RNA in an opposite direction, the ICP0 was also shown to be needed for the establishment of latency and reactivation [61]. Another IE gene ICP4, is important in establishment of latency and gene expression silencing during latency. One element, the repressor element-1/neuronal restrictive silencer element (RE-1/NRSE), was found to locate around ICP4 during latency, suppresses the expression of ICP4 as a transactivator [62].

With appropriate stimulus, which results in systemic changes in immune modulators

and neurotransmitters, the latently infected cells will be reactivated into the lytic cycle and produce of infectious viral particles, transporting from neurons to the dorsal root ganglis.

1.3.3 HSV-1 pathology and epidemiology

HSV-1 causes a variety of diseases in human. The HSV-1 infection is usually acquired during childhood; it also could be transmitted sexually or transmitted by close personal contact with infectious area of the skin during symptomatic reactivations of the virus. HSV-1 infection is not life threatening and could be kept latency, but in certain cases, such as in infants, transplant recipients and immunocompromised patients, HSV-1 infection may be fatal. In infants or embryos, the symptom of first HSV-1 infection usually could be so serious to threat life, because there are little antibodies produced inside the body, the mortality of infected infants without treatment is as high as 60%. But the infectious chances are minimal if the mother does not show symptom of infection or reactivation during pregnancy or delivery.

The typical symptom of HSV-1 infection or reactivation would be visible skin scores around the lips, keratitis and conjunctivitis, the HSV-1 induced keratitis is one of the mainly course of viral-induced blindness. Herpes encephalitis is another important disease induced by HSV-1 infection, it happens at a high ratio (around 10%) among encephalitis and develop rapidly with a 70% mortality. The placental infection of HSV-1 affects the mitosis of embryo, which may then induce abortion, congenital malformation, premature birth and brain damage.

1.3.4 Clinic treatment of HSV-1

Currently, there is no effective way to eradicate HSV infection once get infected. Avoid close personal contact with patient with infectious symptoms could reduce possibility of infection. However, the asymptomatic reactivation virus could be contagious through body secretory products and saliva.

The anti-HSV-1 drug targets at HSV-1 replication from attachment to package of virion. The vaccine for HSV-1 is in clinic trials but the effectiveness has not been demonstrated. In the 1960s, three antiviral agents, namely 5-iododexoyuridine and trifluoromethyldeoxyuridine and adenine arabinoside, were found to have good anti-HSV-1 activity, but they showed toxicity to be given parenterally or at a limited therapeutic index [63].

To date, the most potent and well-studied anti-HSV agent is a nucleoside analogues Acyclovir (ACV). An antiviral agents are very often to be toxic, but its toxicity on HSV-1 is much more than normal host cells. The chemical structure ACV is similar to one of the DNA component guanine deoxyriboside, only lacks the 2'- and 3' carbons and 3'-hydroxyl group of the deoxyribose ring [63]. Inside the cell, the thymidine kinase of HSV-1 phosphorylates ACV into acyclo-guanosine monophosphate. After the HSV-1 specified phosphorylation, a cellular guanylate kinase converts the monophosphate into the active biphosphate form, acyclo-guanosine biphosphate. Other cellular enzymes, such as phosphoglycerated kinase and phosphoenol pyruvate kinase, convert biphosphate to triphosphate form, acyclo-guanosine triphosphate. The acyclo-guanosine triphosphate is a

very potent viral DNA polymerase inhibitor, it exhibits 100 times greater affinity for viral than cellular polymerase [64].

However, the anti-HSV ability of ACV is attenuated when initial treatment is delayed [63;65]. Furthermore, in immunocompromised individuals, ACV-resistant HSV-1 strains often arise [66].

1.3.5 HSV-1 and apoptosis

Apoptosis is essential in cellular response to viral infection to reduce the producing of progeny virus, it is an important way of host cells to protect themselves from viral infections. Viruses have therefore developed mechanisms to modulate host cell apoptosis, to prolong the cell survival and maximize viral replication. The first evidence for such viral modulation came from study of adenoviruses [67]. One adenoviral protein E1B is responsible to interfere with host cell apoptotic response.

The anti-apoptosis ability of wild-type HSV-1 has been widely discussed. The HSV-1 was showing to trigger apoptosis immediately after infection and does not require *de novo* viral protein synthesis [68]. However, HSV-1 successfully evolved mechanism to block the apoptosis induced by its infection. Furthermore, HSV-1 is also able to inhibit apoptosis induced by TNFa, antibody to Fas , C2-ceramide, sorbitol, thermal shock [68] and cycloheximide (CHX) [69] etc. This apoptotic inhibition seems to be a network between different apoptosis pathway, it was thought to be related to caspase pathways, death receptor pathways [68], mitochondrial apoptotic pathways [69;70] and MAPK pathways [71].

By studying on several HSV-1 mutant lacking certain viral genes, several viral proteins were demonstrated to be involved in the host cell apoptosis prevention. The most important one is ICP27 [58]. ICP27 protein is a 63 kDa essential IE gene product interferes with host cell pre-mRNA splicing and shuts off host cell protein synthesis. It recruits cellular RNA polymerase II and facilitates viral RNA transporting away from nucleus to cytoplasm in favor of E and L gene expression. The N- terminal of ICP27 protein is necessary for nuclear localization during its shuttling between nucleus and cytoplasm, the C-terminal is involved in the transactivation and repression functions. Studies have found the ICP27-deficient virus vBS△27, lacks the ability to express ICP27 protein, fails to protect host cells from apoptosis [68] induced by CHX, the vBS△27 itself becomes a apoptotic virus, its infection could induce host cell apoptosis, which involves caspase pathway. These results indicate the ICP27 play an important role in the apoptosis interfering system of HSV-1.

Other HSV-1 genes were also found to be implicated in the apoptotic repression of host cells. A HSV-1 mutant d120 [68] with an IE gene, α4ICP4 deletion, failed to protect cells from hyperthermia induced apoptosis and the mutant could induce apoptosis [72]. This apoptosis inducing ability was thought to be related to a failure in produce ICP4 protein, which plays a crucial role for optimal expression of viral DNA. Another mutant tsB7 [68] within the U_S3 gene gene deleted, is also able to induce apoptosis. The U_S3 protein is a protein kinase, it was thought to activate protein kinase A by phosphorylating protein kinase A substrate to block apoptosis, and it was found to inhibit the apoptosis induced by d120 [73].

1.4. Objective of the present study

HSV-1 is one of the most widely distributed virus for a broad spectrum of human infectious diseases, it establishes lifelong infection once the subject gets infected. There are only limited numbers of therapies effective to control the symptom of HSV-1 infection or reactivation, but these therapies may be invalidated under certain circumstances, including viral mutant development and treatment delay. Therefore, it is necessary to explore more anti-HSV-1 agencies to more effectively control the HSV-1 infection.

TCS is the first RIP found to repress HIV, this anti-HIV activity evokes the interest to study HSV inhibitory effect of TCS. The anti-HSV-1 activity of TCS is thought to be related to its toxicity, but the mechanism is uncertain by now. TCS is an abortifacient agency, it some times causes reaction of anaphylaxis and other side effects, so the clinical application of TCS on HSV-1 treatment requires more clinic trials or development of derivatives with less antigenicity, but understanding of anti-HSV-1 mechanism of TCS is necessary and meaningful to provide more information to understand the replication and nature of HSV-1 and help to develop anti-HSV-1 agencies.

In order to explore the anti-HSV-1 mechanism of TCS, this thesis aims to achieve the following:

1. To investigate if TCS inhibits HSV-1 replication in our system;
2. To understand which viral replication step, TCS exhibits its antiviral ability at;
3. To understand whether the cytotoxicity of TCS is related to its anti-HSV-1

activity;

4. To find out which signaling pathways are involved during the antiviral activity of TCS.

Chapter 2

TCS inhibits HSV-1 proliferation

2.1 Introduction

TCS is the first RIP found to inhibit human immunodeficiency virus type 1 (HIV-1) replication in both acutely infected T-lymphoblastoid cells and chronically infected macrophages *in vitro* [25]. It has been shown to inhibit serum HIV-1 p24 antigen levels and increases $CD4^+$ T cell counts in HIV-1 infected patient during phase I / II clinical trials [26]. Also, the anti-HIV activity of TCS *in vitro* is partly related to its ribosome-inactivating (RI) activity [27;28]. Additionally, Zhao and colleagues have shown that TCS inhibits HIV-1 fusion by enhancing the interaction between chemokines and their receptors [24] so that chemokines could compete with HIV to attach to the chemokine receptors.

Because herpes simplex virus (HSV) infections are usually lethal to HIV-1 infected patients, research into HSV inhibition has been a focus in recent years. Herpes simplex virus type 1 (HSV-1) is a widely prevalent DNA virus that causes large range of human diseases. During HSV-1's lytic life cycle, a highly regulated cascade of genes is expressed, IE, E and L genes. The IE genes, such as ICP27 and α4, are expressed to regulate the expression of other HSV-1 genes. The E genes, such as U_L30 and U_L52, are

involved in viral-DNA synthesis. The L genes, such as glycoprotein D and B (gD and gB) primarily encode structural proteins [49].

Currently, the most potent and well-studied anti-HSV agent is Acyclovir (ACV). However, ACV's anti-HSV ability is attenuated when initial treatment is delayed [64;65]. Furthermore, in immunocompromised individuals, ACV-resistant HSV-1 strains often occur and result in reduced effectiveness of ACV [66]. Therefore, the development of new anti-HSV agents is important. Because of the unique anti-HIV-1 mechanism of TCS, the anti-HSV-1 effect of TCS was studied and is reported here. TCS is known to have anti-HSV-1 activity [12;29], which is related to Mitogen-Activated Protein Kinase (MAPK) signal transduction pathways [30]. Moreover, the anti-HSV-1 activity of ACV was enhanced when combined with TCS treatment [29]. However, the exact anti-HSV-1 mechanism of TCS is still unknown. In this study, the mechanism of inhibition of TCS on different replication steps of HSV-1 was investigated.

2.2 Materials and Methods

2.2.1 Virus and cell culture

Human epithelial carcinoma HEp-2 cells and HSV-1 (F) were obtained from the American Type Culture Collection (Rockville, MD, USA). HEp-2 cells were grown in DMEM with 10% fetal bovine serum (FBS) at 37℃ in a humidified, 5% CO_2 atmosphere. HEp-2 cells were infected with HSV-1 and the viral tilters were determined by multiplicities of infection (MOIs) calculated by the $TCID_{50}$ (50% tissue culture infectious doses) assay. For cultures with indicated treatments, DMEM with 5% FBS was used as a maintenance medium.

2.2.2 In situ sandwich ELISA assay

The quantity of HSV-1 antigen was determined by in situ ELISA. 100 μl HEp-2 cells (1×10^5) were seeded on a 96-well microtiter plate and cultured overnight. The cells were then infected with HSV-1 and treated with TCS (Shanghai Jinshan Pharmaceutical Limited Company, Shanghai, PRC) for the required tilter-concentration and period. After fixing the confluent monolayers of HSV-1-infected HEp-2 cells with 0.05% glutaraldehyde for 15 min at 4 ℃, primary antibodies of human anti-HSV serum (1:2000) were added for 1 h. Then, secondary Goat-anti-human IgG-HRP antibodies (1: 3000, Southern Biotech, USA) were added for 1 h. Tetramethylbenzidine (TMB) was used as substrate for color development (OD_{450}).

2.2.3 Assessment of anti-HSV-1 activity of TCS

HEp-2 cells were infected with HSV-1 from 5 to 5×10^{-8} MOI (10 fold serially diluted)

for 2 h and then washed two times with PBS. Infected cells were then incubated with 5 μg/ml of TCS for 5,15 and 24 h at 37 ℃. In experiments studying the IC_{50} of TCS, HEp-2 cells were infected with 0.5 MOI HSV-1 for 2 h and then washed two times with PBS. Then cells were treated with 100 - 0.39 μg/ml (two fold serially diluted) of TCS for 24 h at 37 ℃. HSV-1 antigen was assessed by ELISA as described above.

2.2.4 Assessment of TCS effect on virus particles

HSV-1 at a MOI of 5 was pretreated with 100 - 0.39 μg/ml (two fold serially diluted) of TCS for 2 h at 37 ℃. The mixture of each TCS concentration with HSV-1 was then 50 fold diluted and co-incubated with HEp-2 cells for 24 h at 37 ℃. HSV-1 antigen was assessed by ELISA as described.

2.2.5 HSV-1 attachment and penetration assay

The attachment and penetration assay followed procedures previously described, with the following modifications [65;74]. For the attachment assay, 100 - 0.39 μg/ml (two fold serially diluted) TCS or 50 - 0.20 μg/ml (two fold serially diluted) ACV (EMD Chemicals Inc., USA) were combined with 0.5 MOI HSV-1 and incubated with HEp-2 cells for 2 h at 4 ℃. TCS and HSV-1 solutions were then washed away with PBS, and the cells were incubated for 24 h, followed by analysis by ELISA assay.

For the penetration assay, 0.5 MOI HSV-1 was co-incubated with HEp-2 cells at 4 ℃ for 2 h and washed twice with PBS. Then, 100 - 0.39 μg/ml (two fold serially diluted) TCS or 50 - 0.20 μg/ml (two fold serially diluted) ACV were added and incubated at 37 ℃ for 10 min to allow for viral penetration. Citrate acid buffer (0.1 M, pH 3) was then

used to inactivate virion that had not penetrated. Finally, the culture was incubated for 24 h at 37 ℃ and subsequently assayed via ELISA.

2.2.6 Time-Of-Addition and Time-Of-Removal assay

Time-Of-Addition and Time-Of-Removal assays were employed as previously described, with some modifications [75]. In the Time-Of-Addition assay, HEp-2 cells were incubated with 0.5 MOI HSV-1. At 0, 3, 5 or 15 h after HSV-1 infection, 5 μg/ml TCS or 0.5 μg/ml ACV was added. ELISA was then performed 24 h after HSV-1 infection.

In the Time-Of-Removal assay, HEp-2 cells were incubated with 0.5 MOI HSV-1 and immediately treated with TCS (5 μg/ml) or ACV (0.5 μg/ml) for 0, 3, 5 or 15 h. The TCS solution was then washed out, and the culture was incubated up to 24 h after infection. ELISA was then performed as described.

2.2.7 PCR and real-time PCR

HEp-2 cells were infected with 0.5 MOI HSV-1 and then incubated with or without 5 μg/ml TCS or 0.5 μg/ml ACV for 3 to 24 h. Genomic DNA and total RNA were extracted using the DNeasy tissue kit and Trizol protocols (both from Qiagen, USA).

PCR for general DNA was performed using the PCR Master Mix (Promega, USA) for 30 cycles of 94 ℃ for 60 s; 62 ℃ for 45 s and 72 ℃ for 90 s. Products were resolved on 1% agarose gels.

RNAs were reverse transcribed to yield single-stranded cDNA via oligo $(dT)_{12-18}$ primers. The mixture containing RNA, oligo $(dT)_{12-18}$ and dNTPs was first heated at 65 ℃ for 5 min and then quickly chilled on ice. Subsequently, Superscript™ II reverse

transcriptase (Invitrogen, USA) was added to the mixture and incubated at 50 ℃ for 60 min, at 70 ℃ for 15 min and then chilled on ice. Real-time PCRs were performed using IQ™ SYBR Green Supermix (Bio-Rad, USA) for 40 cycles of 95 ℃ for 15 s and 62 ℃ for 40 s. The primer pairs for ICP27 were 5'-AGCCTCTATCGCACTTTTGC-3' (forward) and 5'-ATGTCCTTAATGTCCGCCAG-3' (reverse). For UL-30, the primers were 5'-GGTCGACAGGCACCTACAAT-3' (forward) and 5'-GTTGTACCCGGTCACGAACT-3' (reverse). For gD, the primers were 5'-AGCAGGGGTTAGGGAGTTGT-3' (forward) and 5'-CCATCTTGAGAGAGGCATCC-3' (reverse).

2.2.8 Virus release assay

HEp-2 cells were infected with 0.5 or 1 MOI HSV-1 for 2 h and then washed two times with PBS. Medium containing 50, 30 or 5 μg/ml TCS was then added to the cells and incubated for 24 h at 37 ℃. The supernatant was collected and virus titer was determined by ELISA.

2.2.9 Statistical analyses

All data are expressed as mean ± SEM (n=3). Two-way ANOVA with Bonferroni's post hoc test was used for statistical analysis ($p<0.05$ was considered statistically significant). Non-liner regression was employed to analyse the dose response to different treatments. EC_{50} and IC_{50} were calculated by GraphPad Prism Software. The inhibition ratio of TCS was calculated as decrease in HSV antigen (% of control).

2.3 Results

2.3.1 Effect of TCS on HSV-1 yield

To evaluate the time and dose effect of TCS on viral replication, HEp-2 cells were first infected with 10-fold serially diluted HSV-1 solution for 5, 15 and 24 h and treated with 5 μg/ml of TCS. Alternatively, 100 - 0.39 μg/ml (two fold serially diluted) TCS was added to 0.5 MOI infected HEp-2 cells for 24 h. Data in figures 2.1A and B show that TCS significantly decreased HSV-1 antigen, and the effect of TCS inhibition was time and dose dependent ($p<0.05$). The EC_{50}s were 0.001 ± 0.01, 0.05 ± 0.01 and 0.48 ± 0.03 MOI at 5, 15 and 24 h respectively when the effect of different TCS treatment times was examined. The 24 h IC_{50} of TCS on HSV-1 was 2.5 ± 0.23 μg/ml (Figure 2.1B).

To study whether TCS could inhibit HSV DNA synthesis, PCR was performed to amplify HSV-1 gD to measure the amount of viral DNA. As shown in Figure 2.1C, TCS significantly decreased the amount of HSV-1 genomic DNA detected ($p<0.05$), suggesting that TCS could inhibit HSV-1 DNA synthesis in HEp-2 cells. Additionally, ACV significantly decreased the amount of HSV-1 genomic DNA detected after treatment ($p<0.05$ at 15 h; $p<0.01$ at 24 h).

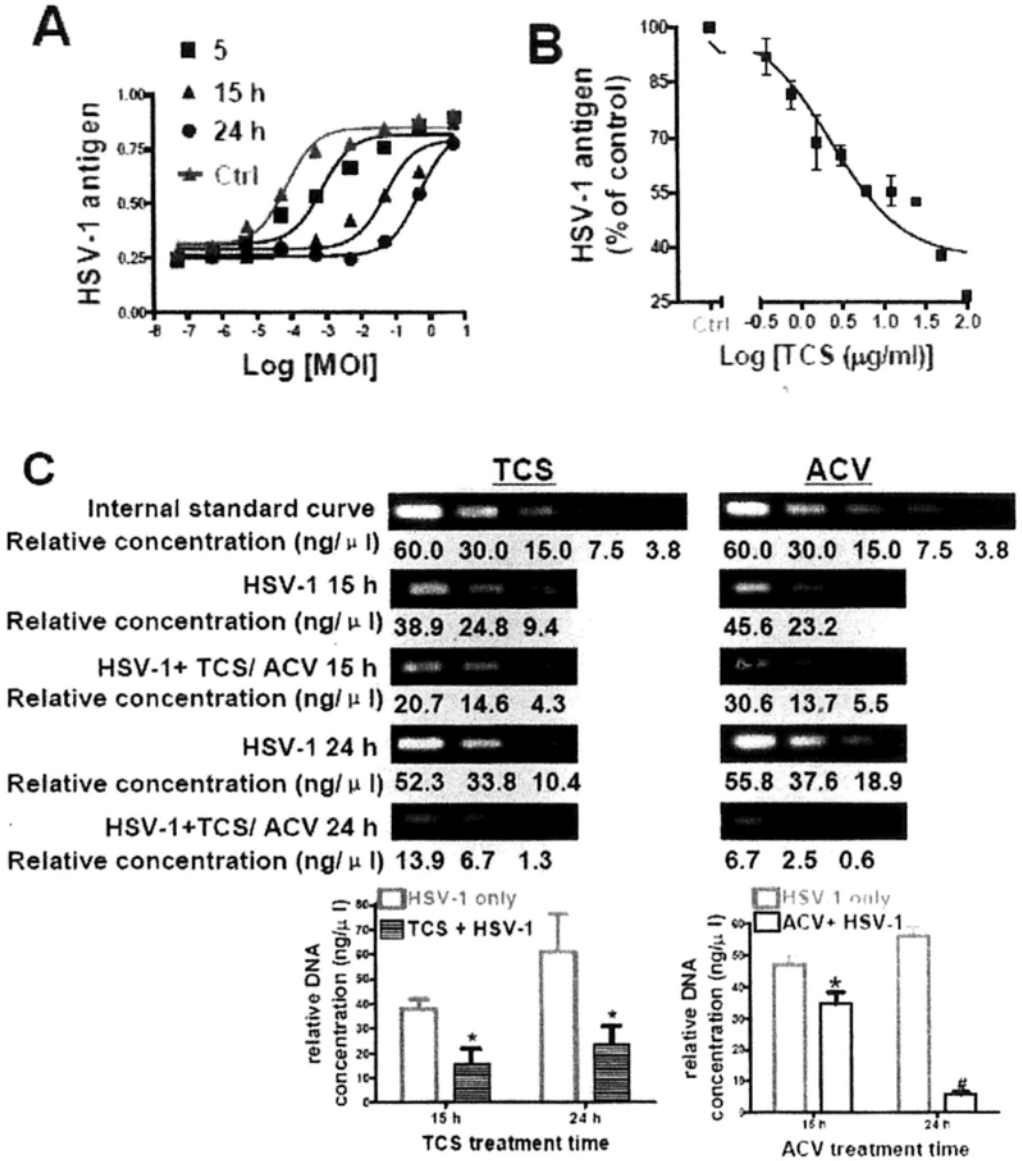


Figure 2.1. Effect of TCS on HSV-1 yield. (A) HEp-2 cells were infected with HSV-1 from 5 to 5×10^{-8} MOI (ten fold serially diluted) for 2 h and then washed two times with PBS. 5 μg/ml of TCS was then added to the cells and incubated for 5 (square), 15 (triangle) and 24 h (circle) at 37 ℃. HSV-1 antigen was assessed by ELISA. (B) HEp-2 cells were infected with 0.5 MOI HSV-1 for 2 h and then washed two times with PBS. Cells were treated with 100 - 0.39 μg/ml (two fold serially diluted) of TCS for 2 h at 37

℃. HSV-1 antigen was assessed by ELISA. (C) HEp-2 monolayer cells in a 6-well plate were infected with 0.5 MOI HSV-1 and treated with (filled bar) or without (unfilled bar) 5 μg/ml of TCS or 0.5 μg/ml of ACV for either 15 or 24 h. DNA was extracted and amplified by PCR using primers for the gD gene. Products were two fold serially diluted and resolved on a 1% agarose gel. The signal intensity of the DNA band on an agarose gel was semi-quantitated by Genesnap software. Values displayed below the image represent the relative concentration (ng/μl) compared with an internal standard sample. The internal standard is a pool consisting of equal amounts of each of the experimental samples, which were two fold serially diluted from 60 ng/μl for PCR. *$p<0.05$, #$p<0.01$ vs. HSV-1 only. Data are shown as mean ± SEM (n=3).

2.3.2 Inactivation of viral infectivity by TCS

Because TCS solutions were 50 fold diluted after co-incubation with HSV-1, the cytotoxicity of TCS could be ignored when the mixture was added to HEp-2 cells. As the results show in Figure 2.2 A, TCS at concentrations from 100 - 0.39 μg/ml had no effect on HSV-1 particle integrity. There were no significant differences in the level of virus antigen at various TCS-treated groups compared with control.

To determine whether the anti-HSV-1 activity of TCS can be attributed to blocking the entry of the virus into host cells, TCS's effects on HSV-1 attachment and penetration were studied. As shown in Figure 2.2 B, TCS did not significantly inhibit HSV-1 attachment or penetration. Therefore, the anti-HSV-1 activity of TCS was not attributable to interference with viral entry. In this assay ACV was employed as a positive control. As shown in Figure 2.2 C, ACV did not significantly inhibit HSV-1 attachment or penetration.

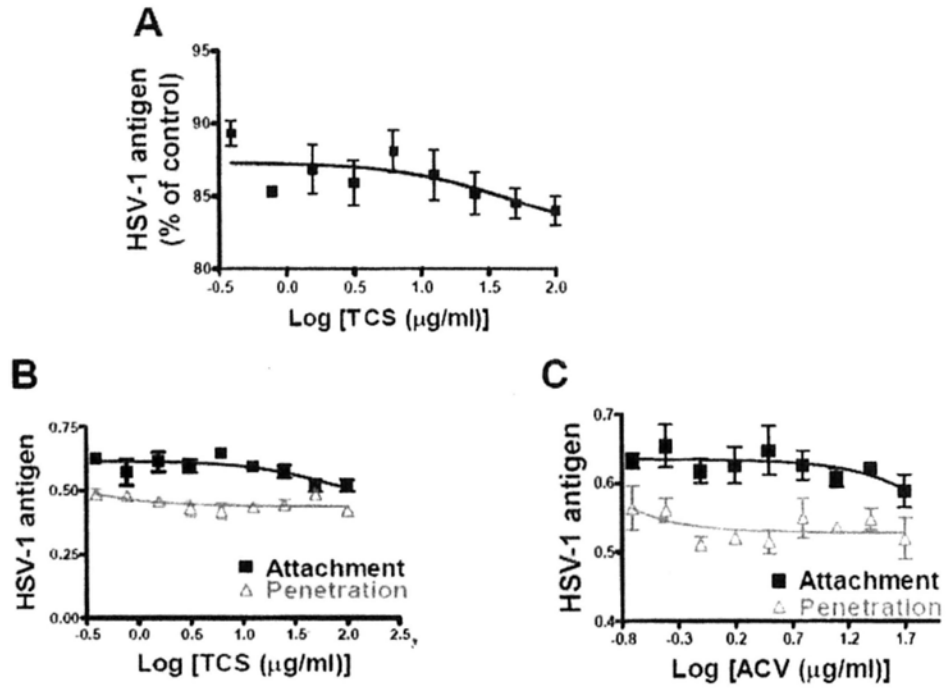


Figure 2.2. Effect of TCS on HSV-1 infectivity. (A) Effect of TCS on HSV-1 particle integrity. 5 MOI HSV-1 was pretreated with 100 - 0.39 μg/ml (two fold serially diluted) of TCS for 2 h at 37 ℃. The mixtures of HSV-1 with each TCS concentration were then 50 fold diluted and co-incubated with HEp-2 cells for 24 h at 37 ℃. HSV-1 antigen was assessed by ELISA. (B) Effect of TCS on HSV-1 attachment (filled square) and penetration (unfilled-triangle). In attachment assay, 100 - 0.39 μg/ml (two fold serially diluted) of TCS or 50 - 0.20 μg/ml (two fold serially diluted) of ACV were combined with 0.5 MOI HSV-1 then added to monolayer HEp-2 cells in a 96-well plate for 2 h at 4 ℃. TCS and virus were then washed out and the cells were cultured for another 24 h at 37℃. The HSV-1 antigen levels were examined by in situ ELISA. During penetration assay, HEp-2 cells in a 96-well plate were infected with 0.5 MOI HSV-1 at 4℃ for 2 h and washed out. 100 - 0.39 μg/ml (two fold serially diluted) of TCS or 50 - 0.20 μg/ml (two fold serially diluted) of ACV were added at 37℃ for 10 min and washed out. Citrate acid buffer (0.1 M, pH 3) was used to inactivate extracellular virus. After a 24 h

incubation at 37℃, the HSV-1 antigen was examined by in situ ELISA. Data are shown as mean ± SEM (n=3).

2.3.3 Effect of TCS on different periods of HSV-1 replication and on HSV-1 gene expression

To determine the effect of TCS on different HSV-1 replication stages, Time-Of-Addition and Time-Of-Removal assays were performed. As shown in Figure 2.3 A, in the Time-Of-Addition assay the inhibitory effect of TCS was significantly decreased when TCS was added 5 or 15 h after HSV-1 infection compared with groups in which TCS was added simultaneously or 3 h after infection with HSV-1 ($p<0.05$ or $p<0.01$ respectively). Furthermore, in the Time-Of-Removal assay (Figure 2.3 A), the inhibitory effect of TCS was significantly increased in groups for which TCS was removed 5 to 15 h post infection compared with removal 0 to 3 h post infection ($p<0.01$). These data indicate that TCS inhibition is most effective during 3 to 15 h post-infection period.

Effect of TCS on the expression of different HSV-1 genes was studied by real-time PCR. The HSV-1 genes ICP27 (IE), U_L30 (E) and gD (L) were chosen as representative of the different HSV-1 replication periods. As the data in Figures 2.3 C-E show, TCS significantly decreased the expression of both U_L30 and gD after 5 h treatment ($p<0.05$) but had less effect on ICP27 expression.

When ACV was compared to TCS, data in the Time-Of-Addition assays showed that the inhibitory effect of ACV did not change during 0-15 h post infection, and in the Time-Of-Removal assays ACV required at least 15 h incubation with HSV-1 infected cells to exhibit its anti-viral effect ($p<0.01$). These results suggest ACV works most

effectively only after the 15 h post infection period (Figure 2.3 B). Only E-L gene expression was significantly ($p<0.01$) inhibited by ACV after 15 h treatment (Figure 2.3 C-E).

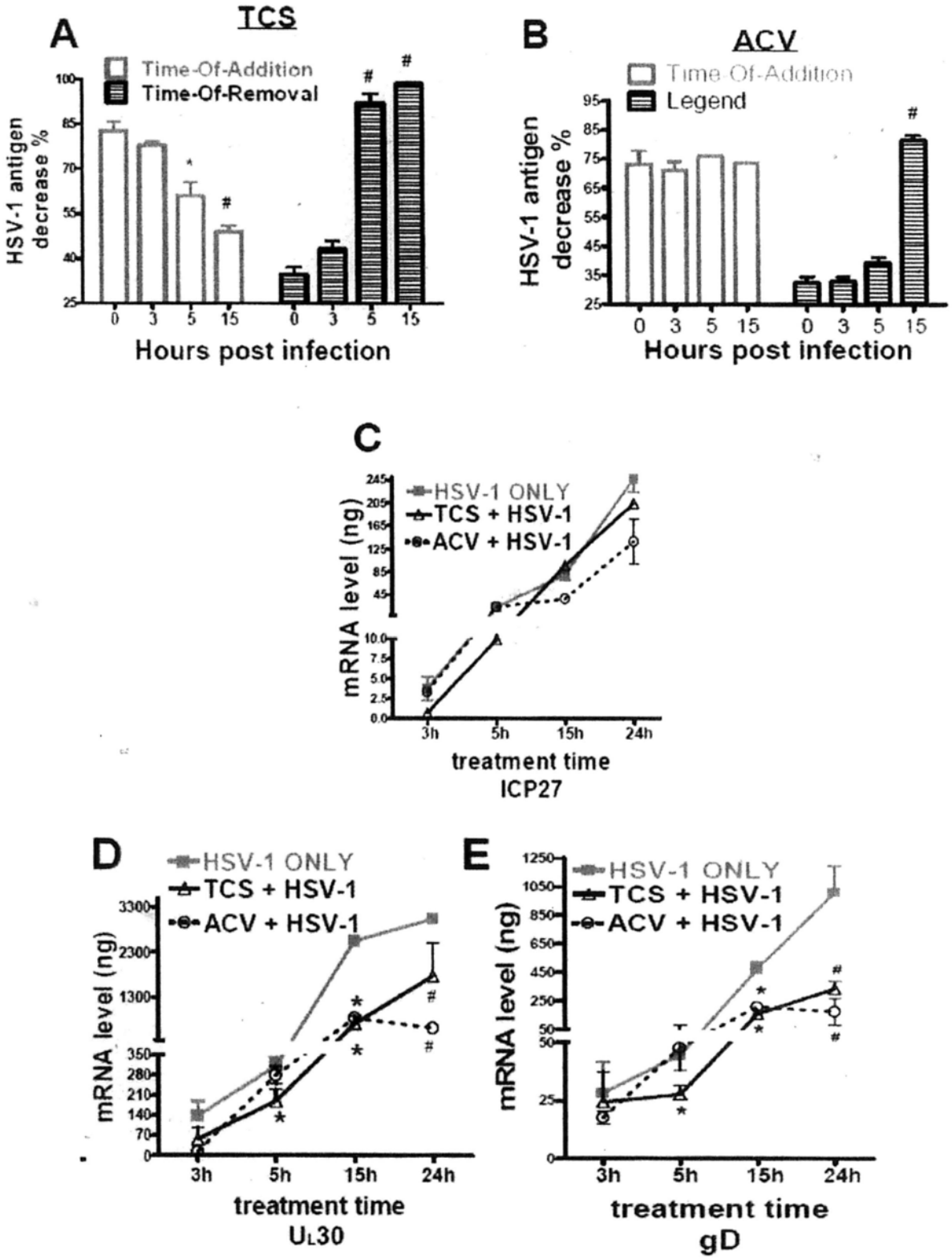

A
TCS
Time-Of-Addition
Time-Of-Removal
HSV-1 antigen decrease %
Hours post infection
B
ACV
Time-Of-Addition
Legend
HSV-1 antigen decrease %
Hours post infection
C
HSV-1 ONLY
TCS + HSV-1
ACV + HSV-1
mRNA level (ng)
treatment time
ICP27
D
HSV-1 ONLY
TCS + HSV-1
ACV + HSV-1
mRNA level (ng)
treatment time
UL30
E
HSV-1 ONLY
TCS + HSV-1
ACV + HSV-1
mRNA level (ng)
treatment time
gD

Figure 2.3. TCS effect on different periods of HSV-1 replication and gene expression. (A, B) During Time-Of-Addition assay (unfilled column), HEp-2 cells in a 96-well plate were incubated with 0.5 MOI HSV-1. 5 μg/ml of TCS (A) or 0.5 μg/ml of ACV (B) was added at 0, 3, 5 and 15 h post infection. ELISA was then performed 24 h post infection. During Time-Of-Removal assay (filled column), HEp-2 cells were incubated with 0.5 MOI HSV-1 and immediately treated with TCS (5 μg/ml) or ACV (0.5 μg/ml) for 3, 5 and 15 h. TCS or ACV was washed from the cells, which were then incubated up to 24 h post-treatment. ELISA was then used to analyze viral antigen. Data are shown as mean ± SEM (n=3). * $p<0.05$, # $p<0.01$ vs. 0 and 3 h post infection. (C-E) Effect of TCS or ACV on HSV-1 gene expression. Monolayer HEp-2 cells on a 6-well plate were infected with 0.5 MOI HSV-1 in either absence (filled-square line) or presence (unfilled-triangle line) of 5 μg/ml of TCS or 0.5 μg/ml ACV (unfilled circle dashed line). The cells were cultured for 3, 5, 15 and 24 h, after which RNA was extracted for real-time PCR. (C) Effect of TCS on HSV-1 ICP27 gene expression. (D) Effect of TCS on HSV-1 U_L30 gene expression. (E) Effect of TCS on HSV-1 gD gene expression. Data were analysed by iCycler iQ™ Real-Time Detection System Software and are shown in relative nanograms compared with a standard curve as mean ± SEM (n=3). * $p<0.05$, # $p<0.01$ vs. HSV-1 only. Standard curves were prepared by a dilution series constructed from blended cDNA samples of the same quantity (ng).

2.3.4 Effect of TCS on HSV-1 release

To determine whether inhibition of HSV-1 release contributes to TCS's anti-HSV-1 activity, the extracellular virus titer under TCS treatment was studied. The data demonstrated that HSV-1 release was significantly decreased in the presence of TCS compared with normal HSV-1-infected HEp-2 cells ($p<0.05$) (Fig. 2.4).

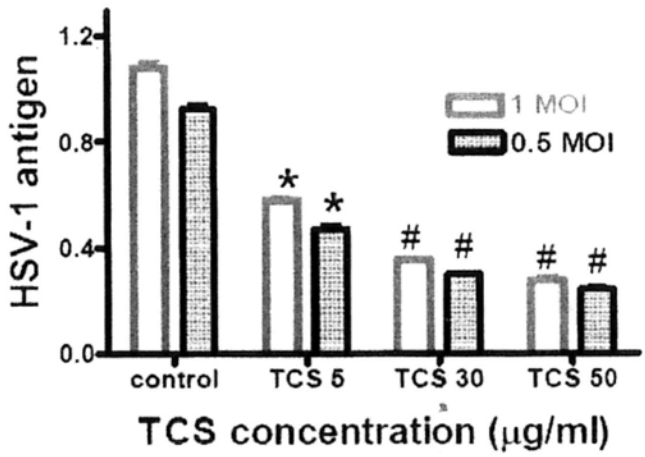


Figure 2.4. Effect of TCS on HSV-1 release. HEp-2 cells were infected with HSV-1 (0.5 and 1 MOI) for 2 h, and then virus solution was washed out. 50, 30 or 5 μg/ml TCS was added and incubated for 24 h at 37℃, and then supernatant was collected and virus titer was determined by in situ ELISA. Data are shown as mean ± SEM (n=3). *$p<0.05$, # $p<0.01$ vs. control.

2.4 Discussion and Conclusion

In this study, TCS treatment was shown to decrease HSV-1 antigen in host cells (Figs. 2.1 A, B). The anti-HSV-1 activity of TCS is believed to be derived from its ability to induce apoptosis. However, the exact mechanism of TCS's anti-HSV-1 effect remains uncertain.

The anti-HSV-1 activity of TCS is described in this work, with a focus on different viral replication steps. According to the ELISA for HSV-1 antigen and PCR of HSV-1 DNA (Fig. 2.1), TCS treatment decreased HSV-1 total antigen on the surface of host HEp-2 cells and DNA abundance in HEp-2 cells, providing evidence of TCS's HSV-1 inhibition. The inhibition was further evaluated in Time-Of-Addition and -Removal assays. These data revealed that the most effective period of TCS inhibition is between 3 and 15 h post infection (Fig. 2.3 A). This 3 to 15 h post-infection period coincides with the E-L infection period [49]. These results were supported by real-time PCR tests (Figs. 2.3 B-D) in which the genes ICP27, U_L30 and gD were picked as representative markers [49;58;76] of IE, E and L gene expression levels, respectively. These data demonstrated that U_L30 and gD expression levels were diminished by TCS treatment, but there was less of an effect on ICP27. These data further indicated that TCS interferes with HSV-1's E-L replication period. In our previous studies, it was found that TCS takes at least 3 h to enter cells [18] but HSV-1 IE gene expression occurs within the first 3 h of infection. Therefore, in HEp-2 cells, it is reasonable to suspect that TCS acts intracellularly only after 3 h. TCS is an RIP that can affect protein translation. Interference with the

translation of proteins required for viral E-L life cycle events, including synthesis of enzymes related to transcription, might result in inhibition of gene expression. Interestingly, we found that transcription of the IE gene ICP27 increased even after long period of TCS treatment, possibly because IE genes play a very important role in HSV-1 life cycle. This might require HSV-1 to developed mechanism to effectively express IE genes, such as the addition of enhancer motifs to IE genes [77;78], with the result that the ribosome-inactivating activity of TCS may not be as strong as it is for to E-L genes.

We compared the anti-HSV-1 effect of TCS with that of ACV, a widely used and well-studied anti-HSV-1 agent. We found that ACV had no effect either on entry (Fig. 2.2) or the IE gene expression stage (Fig. 2.3) of HSV-1, but strongly inhibited the L stage (about 15 h post infection) during HSV-1 replication and diminished U_L30 and gD gene expression after 15 h incubation. These results are consistent with the known activity of ACV, a viral DNA polymerase inhibitor, whose activation depends on the expression of HSV thymidine kinase (HSV-Tk) gene expression [63]. Because HSV-Tk is an early gene, it is expected that ACV only works during the late E to L stages. Therefore, the differences between TCS and ACV suggest that TCS could be a substitute for ACV when a more rapid drug response is required.

Because the U_L30 protein is necessary for HSV-1 DNA replication [79], and the gD protein mediates HSV-1 attachment and packaging of the virion [74;80], it is reasonable that TCS's inhibition of these two proteins would affect the maturation of HSV-1. This hypothesis was supported by the observation that TCS inhibited HSV-1 release from the host cell (Fig. 2.4), indicating that TCS suppresses DNA replication and expression of

proteins related to virion maturation [49]. Thus TCS interrupts HSV-1's replication life cycle and disrupts the production of new viral progeny.

Some anti-HSV-1 chemicals have been shown to interfere with viral entry [75;81;82]. Other studies have suggested that TCS interferes with the interactions between chemokines and their receptors [83], a mechanism implicated in HSV-1 infection [84;85]. Therefore it is possible that TCS may interfere with the infectivity of HSV-1. However, in this study, TCS did not significantly inhibit either attachment, penetration of HSV-1 during infection or the integrity of virus particles (Fig. 2). This suggests that TCS does not directly impact HSV-1 entry into host cells, although the cytokines that TCS recruits to inhibit HSV-1 infection may be absent in these in vitro experiments. Therefore, the effect of TCS on HSV-1 entry into host cells requires further examination.

In summary, the present study demonstrates that the anti-HSV-1 effect of TCS is related to the inhibition of E to L replication of HSV-1 and DNA expression as well as the blockage of viral release.

Chapter 3

The anti-HSV-1 mechanism of TCS is related to an apoptotic signaling pathway transition from type I to type II apoptotic pathway

3.1 Introduction

It has been suggested that the anti-HIV activity of TCS is related to its selective cytotoxicity [86] on infected cells. HSV-1 expresses a highly regulated cascade of genes to hijack host cell metabolism pathways. Although the anti-HSV-1 mechanism of TCS is still unclear, it is possible the selectivity of TCS in apoptosis induction is still involved because TCS has been shown to interfere with anti-apoptotic signals triggered by HSV-1 [30].

Apoptosis is regulated by a complex network of positive and negative effectors. The CD95 (also known as Fas or Apo-1) system is a key member of the cell death receptor family on the cell surface [87-89]. Fas initiates apoptosis by forming a complex with Fas ligand (FasL) on ligand binding during the trimerization of FasL. The FasL/ Fas complex plays a critical role in mediating the transduction of apoptotic signals [90]. Upon FasL and Fas binding, a death domain (DD) aggregated. Caspase families and the

mitochondrial are thought to be involved in CD95 mediated apoptosis [32;47;91;92]. The pathways are divided into two categories, CD95-mediated type I or type II, based on the level of mitochondrial dependence. The type I apoptotic pathway bypasses the dependence on the mitochondrial. This pathway quickly activates a large amount of caspase-8 at the Fas formed death inducing signaling complex (DISC) with FADD, FasL and caspase-8 itself. Then the large amount of caspase-8 is executed, the activated caspase-8 subsequently triggers caspase-3 process, which in turn induces apoptosis. Certain cell types, such as H9, CH1, SKW6.4 and SW480, prefer the type I apoptotic pathway, they are defined as type I cells. However, all of these cells do not strictly chose one apoptotic pathway during apoptosis, what we always bear in mind is that apoptosis pathways may shift under certain circumstance or there are interactions between different pathways.

By contrast, the formation of the DISC in the type II apoptotic pathway is strongly reduced. The formation requires a limited and delayed caspase-8 process to activate Bcl-2 family member BAX at the mitochondrial membrane. In this pathway, a loss of the mitochondrial transmembrane potential ($\Delta \Psi_m$) by BAX insertion into mitochondrial transmembrane and the release of cytochrome c are triggered to mediate apoptosis signal transduction. Cytochrome c, along with apoptotic protease activating factor 1 (APAF-1) oligomerization, mediates capase-9 activation during signal transmission [44;45]. Thus, protecting of the $\Delta \Psi_m$ by over-expressed Bcl-2, a homolog to the anti-apoptotic C.elegans protein CED-9 [46], diminishes apoptosis in type II apoptotic cells but not in type I apoptotic cells [47]. There are only limited cells, including Jurkat, HL-60 and CEM,

chose type II apoptotic pathway during their apoptosis, they are thereby named as type II cells. Still, there is possibility of apoptotic pathways transition between each other when cell are exposed to certain stimuli [93].

In the present investigation, the anti-HSV-1 mechanism of TCS in human epithelial carcinoma HEp-2 cells was explored by studying TCS-induced apoptosis. Our results show that TCS was more toxic to HSV-1 infected HEp-2 cells than uninfected ones. TCS-induced type I apoptosis in uninfected HEp-2 cells and type II apoptosis in HEp-2 cells infected with HSV-1. We found that the conversion from the type I to the type II apoptotic pathway was related to FasL regulation during TCS treatment.

3.2 Materials and Methods

3.2.1 Cell, virus and reagents

Human epithelial carcinoma HEp-2 cells and HSV-1(F) were obtained and maintained as previously described. The cells were infected at a multiplicity of infection (MOI) of 0.5.

TCS was obtained from Shanghai Jinshan Pharmaceutical Limited Company (Shanghai, PRC), and cycloheximide (CHX) was purchased from Sigma Life Science (USA).

Anti-caspase-8, anti-caspase-9, anti-Bcl-2 and anti-FasL antibodies were all purchased from Cell Signaling Technology (USA). Goat anti-rabbit IgG – horseradish peroxidase (HRP) was purchased from Southern Biotech (USA). Alexa Fluor 488 conjugated goat anti-rabbit IgG was purchased from Invitrogen (USA).

3.2.2 Cell viability detection by MTT assay

MTT was employed to assess the viability of cells following the MTT Kit protocol (Cell Proliferation Kit I, Roche Applied Science). Briefly, 10^4 per 100 μL HEp-2 cells were seeded into 96-well plates overnight. For experiments studying TCS cytotoxicity, cells were infected or left uninfected (cultured by virus free medium) with HSV-1 for 2 h and then treated for 24 h with different concentration of TCS solutions (200-0.78 μg/mL; two fold serially diluted). For experiments comparing the effects of TCS and CHX, TCS (200-0.78 μg/mL; two fold serially diluted) or CHX (50-0.20 μg/mL; two fold serially diluted) were added 0, 3 and 6 h post-infection (hpi) with HSV-1, and the treated cells

were compared to uninfected cells. During experiments studying HSV-1 cytotoxicity, HEp-2 cells were infected with 0.5 MOI HSV-1 for 1-10, 12, 18 and 24 h for the MTT test. In experiments studying FasL inhibition, 10^4 HEp-2 cells per 100 µL were seeded into 96-well plates overnight in the absence (control) or presence of 0.5 µg/mL biotin mouse anti-human CD178 (NOK-1) (BD Pharmingen™, USA). The cells were either infected with 0.5 MOI HSV-1 for 2 h or left uninfected and then treated with TCS (200-0.78 µg/mL; two times serially diluted) for 24 h in the absence (control) or presence of 0.5 µg/mL NOK-1. After the treatments, to measure the number of viable cells, 10 µL MTT was added to each well and incubated for 4 h. The formazan crystal was dissolved in Solubilization solution over night. Absorbance was read at 570 nm.

3.2.3 Apoptosis by ELISA detection

The first group of cells were either infected with HSV-1 or left uninfected for 2 h before incubation with TCS (200-0.78 µg/mL; two times serially diluted) for 24 h. The second group was either infected with HSV-1 or left uninfected for 2 h before incubation with 10 µg/mL TCS for 3, 6, 18 or 24 h. Apoptosis was determined with a Cell Death Detection ELISA (Roche Biochemical, Germany) assay based on the detection of mono- and oligonucleosomes in the cytoplasm, which indicate DNA degradation in apoptotic cells. Briefly, cells were lysed and the lysate was coated on streptavidin coated microplates and incubated with anti-Histone Biotin and anti-DNA OPD, The immune reaction was detected with ABTS and ABTS stop solution at 490 nm.

3.2.4 Flow cytometry analysis apoptosis and $\Delta \Psi_m$

Apoptosis was analyzed with the Annexin-V-FLUOS Staining Kit (Propidium Iodide

(PI) and Annexin V-FITC double staining, Roche Applied Science, Germany) and the Tunel Apoptosis Kit (Beyotime institute of Biotechnology, PRC). The $\Delta\Psi_m$ depolarization ratio (MD, ratio of the median fluoresecense change for treated cells to control cells: (Fluoresent treated cells-fluoresent control cells)/ fluorescent control cells) was analyzed with the MitoCapture™ mitochondrial apoptosis detection kit (BioVision, USA). Briefly, HEp-2 cells were either infected with HSV-1 or left uninfected 2 h before treatment with either TCS (10 or 30 μg/mL) for 24 h or with 10 μg/mL TCS for 3, 6, 18 or 24 h. The cells were then stained with Annexin V-PI, Tunel or MitoCapture™ dye. Fluorescence was detected under a FACSCalibur flow cytometer at FITC or PE-Texas Red channels.

3.2.5 DNA transfection

Bcl-2 pCDNA3 was acquired from Prof. W.Y. Lau Prince of Wales hospital, Hong Kong). HEp-2 cells (2.5×10^5 cells /mL) were pre-seeded onto 96-well plates overnight prior to transfection. The cells were transfected with Lipofectamine™ 2000 (Invitrogen, US) according to the manufacture's protocol. Briefly, 0.2 μg/ 50 μL Bcl-2 over-expression plasmid or empty pCDNA3 (control) was mixed with Lipofectamine™ 2000 and incubated with the cells for 4 h. The transfected cells were then infected with 0.5 MOI HSV-1 for 2 h and treated with 200-0.78 μg /mL (two times diluted) TCS for 24 h. Cell viability was assayed with MTT. To quantify the Bcl-2 expression level, 10^6 cell/ well HEp-2 cells were seeded onto a 6-well plate and transfected with the Bcl-2 over-expression plasmid or pCDNA3 at 4 μg /well. The cells were then lysed, and western blot analysis was performed.

3.2.6 Immunoblotting analysis

Proteins were separated by 12% SDS-PAGE and transferred to a PVDF membrane (Immobilon P, Millipore, Bedford, MA, USA). The membrane was incubated with a generic protein (5% skimmed milk proteins in TBST) for 1 h at room temperature. Primary antibodies were added to the solution and incubated overnight. Secondary antibodies were added to the reaction for 1 h. The antibody binding was detected with an Odyssey imaging system (LI-COR biosciences, USA). Anti-β-actin antibody was used as the loading control.

3.2.7 Preparation of cytoplasmic protein extractions

In our study, 2×10^6 cells were either infected with 0.5 MOI HSV-1, or they were left uninfected and treated with 10 μg/mL TCS or 5 μg/mL of CHX for 3, 6, 18 or 24 h for protein extraction. Cytoplasmic extracts were prepared according to the protocol from the Abcam website (www.abcam.com) with some modifications. Briefly, cells were rinsed twice with cold PBS, harvested by scraping and lysed with 50 μL general lysis buffer containing 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 150 mM NaCl, 0.5% NP-40, and freshly prepared 20 μg/mL aprotinin, 5 μg/mL leupeptin and 1 mM PMSF. Samples were incubated on ice for 10 min and spun at 4 °C at 500 x g for 5 min. The supernatant was collected as the cytoplasmic extract.

3.2.8 Real-time PCR

Cells were infected or left uninfected with 0.5 MOI HSV-1 and incubated in the absence or presence of 10 μg/mL TCS for 3, 6, 18 or 24 h. Total RNA was extracted using Trizol (Qiagen, USA). RNAs were reverse transcribed to yield single-stranded cDNA according to the iScript™ cDNA Sythesis Kit (Bio-rad, CA) protocol. Briefly,

RNA samples were mixed with the 5× iScript reaction mix and the iScript reverse transcriptase and reacted at 25°C for 5 min, 42°C for 30 min and 85°C for 5 min. cDNA products were stored at -20°C.

Real-time PCR was performed using IQ™ SYBR Green Supermix (Bio-Rad, CA) for 40 cycles of 95°C for 15 s and 50°C for 40 s. Data were analyzed with the iCycler Multicolor Real-time PCR Detection System (Bio-rad Labratories, USA) Software. The primer pairs for FasL were as follows: forward: 5'-CAA GTC CAA CTC AAG GTC CAT GCC-3', reverse 5'-CAG AGA GAG CTC AGA TAC GTT TGA C-3'[41]; for FLICE inhibitory protein (FLIP) were: forward: 5'-TGT TGC TAT AGA TGT GG-3', reverse 5' CAG GTC TAT TCT GTG GA-3'[94].

3.2.9 In situ sandwich Enzyme-linked immunosorbent assay (ELISA) assay

The quantity of FasL on the plasma membrane was determined by *in situ* ELISA. 100 μL HEp-2 cells (1×10^5) were seeded onto a 96-well plate and cultured overnight. The cells were either infected with 0.5 MOI HSV-1 or left uninfected and treated with TCS 10 μg/mL for 3, 6, 18 or 24 h. After fixing the confluent monolayers of the HSV-1-infected HEp-2 cells with 0.05% glutaraldehyde for 15 min at 4°C, primary antibodies of rabbit anti-FasL Ab (1:100) were added for 1 h. Then, the secondary antibody, Goat-anti-rabbit IgG-HRP (1: 3000), was added for 1 h. Tetramethylbenzidine (TMB) was used as the substrate for color development (OD_{450}).

3.2.10 Immunostaining on plasma membrane FasL

1×10^6 HEp-2 cell samples were seeded on circle cover glass (25 mm) in 6-well plates overnight. The cells were then treated with 10 μg/mL TCS, with or without 0.5

MOI HSV-1 for 24 h. Cells were fixed with 3.5% formalin and incubated with primary antibody, rabbit anti-FasL Ab (1:100), for 1 h and secondary antibody, Alexa Fluor 488 conjugated goat anti-rabbit IgG (1:1000), for 1 h. Fluorescence signals were detected with a FV1000 laser scanning confocal system (Olympus, Japan)

3.2.11 Statistical analyses

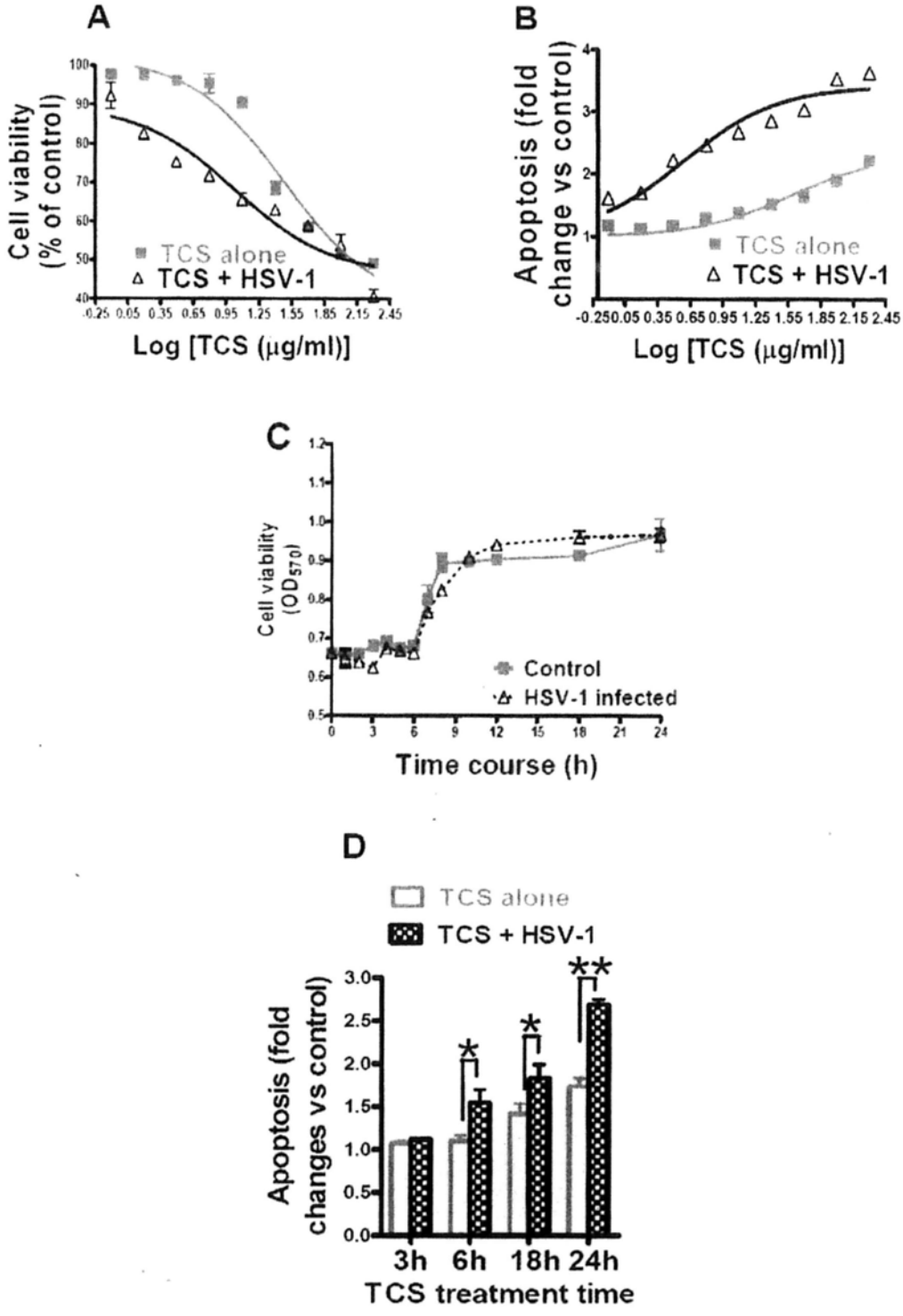
Data were analyzed using a two-way ANOVA. A p-value <0.05 was considered as statistically significant. Dose response and EC_{50} (the dose that gives half of the maximal response) were calculated by non-liner regression using the Graphpad Prism software. All data are expressed as mean ± SEM.

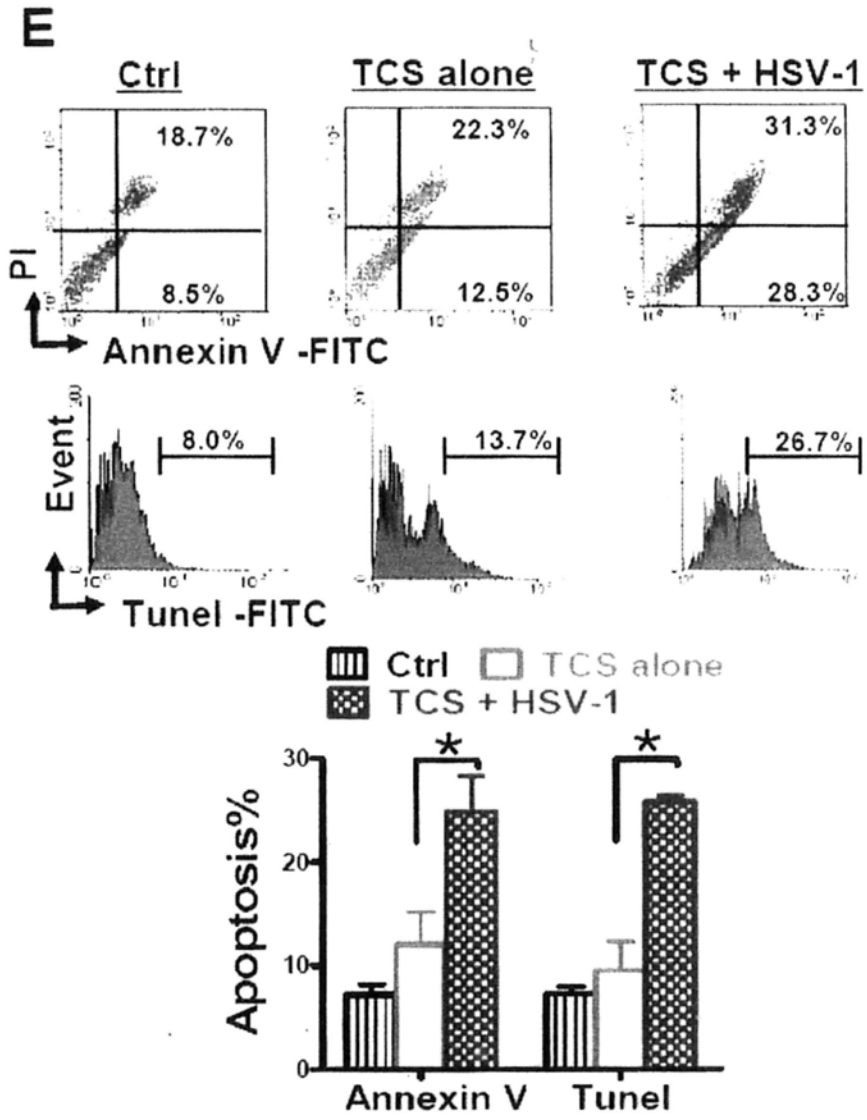
3.3 Results

3.3.1 The anti-viral effect of TCS is related to apoptosis

To compare the effects of TCS on healthy and HSV-1-infected HEp-2 cells, the cells were either infected with HSV-1 or left uninfected for 2 h prior to the TCS treatment. MTT and a cell death ELISA were employed to assess cell viability and apoptosis. As illustrated in figure 3.1 A and B, TCS decreased the viability of HEp-2 cells (EC_{50} 24.64 $\pm$ 1.17 μg/mL) and increased the apoptosis ratio (EC_{50} 37.57 $\pm$ 1.47 μg/mL). In addition, when the cells were infected with HSV-1, the viability of infected cells was further decreased (EC_{50} 3.01 $\pm$ 1.30 μg/mL) by TCS and the apoptosis ratio increased (EC_{50} 3.89 $\pm$ 1.31 μg/mL) compared to uninfected cells ($p<0.01$). The decrease in cell viability and the increase in apoptosis ratio were time dependent from 3 h of TCS treatment to 24 h (Fig. 3.1 D). HSV-1 alone did not bring cytotoxicity to HEp-2 cells (Fig. 3.1.C).

To further demonstrate the anti-viral capability of TCS, the effect of TCS was compared with CHX in HSV-1 infected HEp-2 cells with a MTT assay. TCS or CHX was added at 0 hpi, 3 hpi or 6 hpi to HSV-1-infected HEp-2 cells. Consistent with previous reports [68], CHX decreased the viability of HEp-2 cells at 0 hpi (EC_{50} 0.30 $\pm$ 2.72 μg/mL) and 3 hpi (EC_{50} 0.21 $\pm$ 2.67 μg/mL), but the cell viability increased ($p<0.05$) when CHX was added 6 hpi (EC_{50} 4.48 $\pm$ 1.53 μg/mL; Fig. 3.1 E and F). By contrast, TCS decreased the viability of all HSV-1-infected HEp-2 cells treatment groups (EC_{50} 5.62 $\pm$ 1.15 μg/mL at 0 hpi, 3.92 $\pm$ 1.11 μg/mL at 3 hpi and 3.99 $\pm$ 1.14 μg/mL at 6 hpi, respectively).


A
Cell viability (% of control)
TCS alone
TCS + HSV-1
Log [TCS (μg/ml)]
B
Apoptosis (fold change vs control)
TCS alone
TCS + HSV-1
Log [TCS (μg/ml)]
C
Cell viability (OD_{570})
Control
HSV-1 infected
Time course (h)
D
TCS alone
TCS + HSV-1
Apoptosis (fold changes vs control)
3h 6h 18h 24h
TCS treatment time


E
Ctrl
TCS alone
TCS + HSV-1
18.7%
8.5%
22.3%
12.5%
31.3%
28.3%
PI
Annexin V -FITC
Event
8.0%
13.7%
26.7%
Tunel -FITC
Ctrl
TCS alone
TCS + HSV-1
Apoptosis%
30
20
10
0
*
*
Annexin V
Tunel

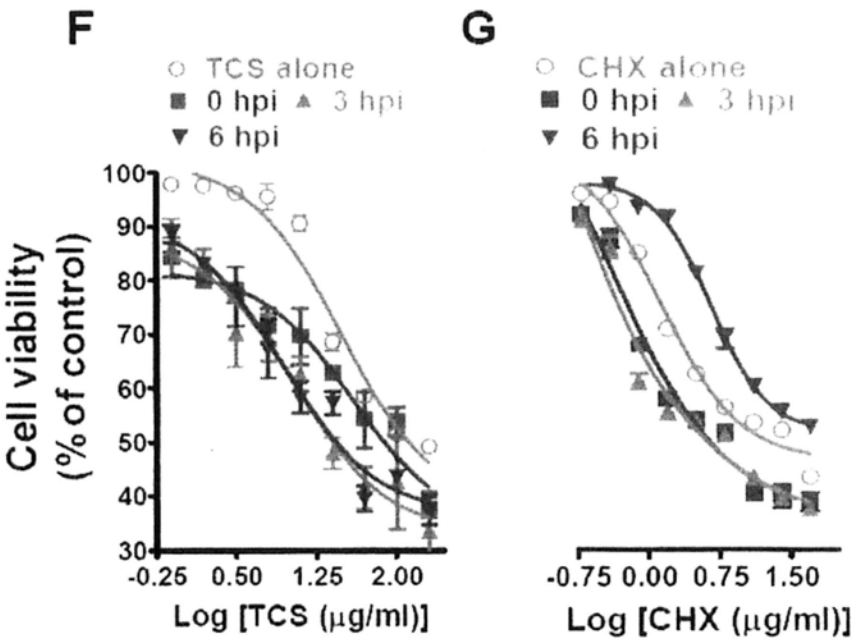


Figure 3.1. Cytotoxicity of TCS. HSV-1-infected or uninfected HEp-2 cells were treated with TCS. Cell viability and apoptosis was determined with MTT and cell death ELISA respectively at TCS concentrations of (A, B) 200-0.78 μg/mL two folds serially diluted for 24 h, or (D) 10 μg/mL for 3, 6, 18 or 24 h (ELISA only). The filled square line and the unfilled column represent TCS treatment on HEp-2 cells without HSV-1 infection. The unfilled triangle line and the dot filled column represent HSV-1-infected HEp-2 cells treated with TCS. (C) MTT analysis of cell viability changes in HSV-1-infected HEp-2 cells. Cells were infected with 0.5 MOI HSV-1 or left uninfected and cultured for the indicated number of hours for MTT tests. Filled square line represents cells without infection, unfilled triangle dot line represents HSV-1 infected ones. (E, F) CHX was used as a positive control for TCS, and the effects were assessed by using MTT to determine cell viability. HSV-1 infected or uninfected HEp-2 cells were treated with (E) TCS (200-0.78 μg/mL, two folds serially diluted) or (F) CHX (50-0.20 μg/mL, two folds series diluted) at 0, 3 and 6 hpi and cultured for 24 h. The unfilled circle line represents TCS/ CHX treatment on HEp-2 cells without HSV-1 infection. The filled square, up triangle and down triangle lines represent HSV-1 infected HEp-2 cells treated with TCS/ CHX 0, 3, 6 hpi, respectively. Data represent the mean ± SEM (n=3). * $p < 0.05$ vs. TCS

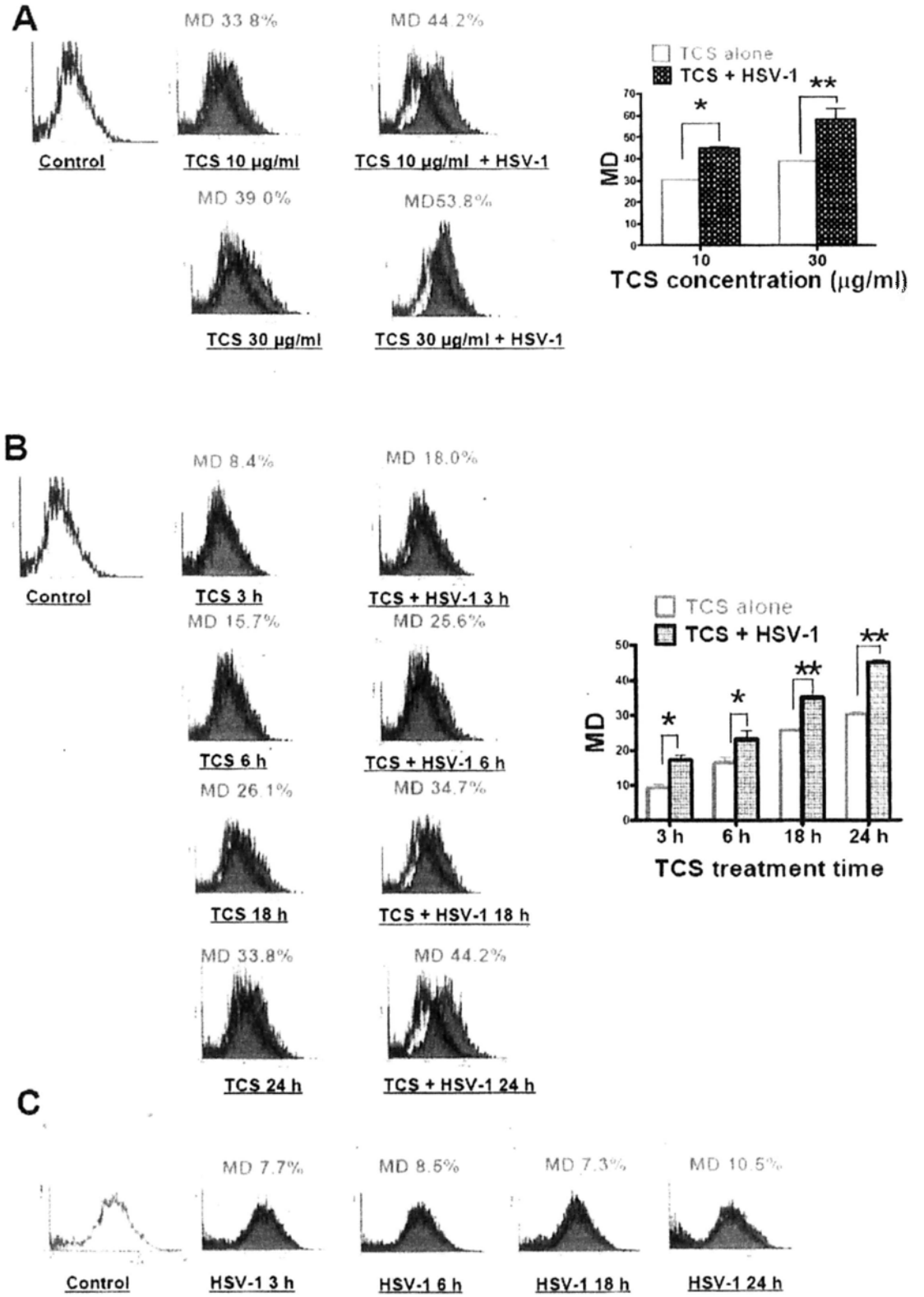
only, **p< 0.01 vs. TCS only.

3.3.2 The type I to type II apoptotic signaling transition during the anti-HSV-1 process of TCS

3.3.2.1 TCS induced different ratios of mitochondrial transmembrane depolarization in control and HSV-1-infected HEp-2 cells

To identify the involvement of the mitochondrial during apoptosis in HEp-2 cells induced by HSV-1 infection and TCS treatment, control or HSV-1-infected HEp-2 cells were treated with various concentrations of TCS along different time courses. Percentages of mitochondrial transmembrane depolarization (MD) were determined by flow cytometry at the FITC channel. As illustrated in figure 3.2 A, after treatment with 10 μg/mL TCS, the FITC fluoresence ratio increased compared to control cells. In HSV-1-infected cells treated with TCS, the FITC ratio was further increased. When the TCS concentration increased from 10 to 30 μg/mL, the depolarization increased both in the TCS and HSV-1 plus TCS groups, but the differences between the uninfected and HSV-1-infected groups were still significant. There was also an effect of time course (Fig. 3.2 B). Longer TCS treatments resulted in larger $\Delta \Psi_m$ depolarizations ($p<0.05$). HSV-1 infection alone did not induce changes in $\Delta \Psi_m$ (Fig. 3.2 C).

The effect of CHX on $\Delta \Psi_m$ depolarization was also studied. As illustrated in figure 3.2 D, CHX induced a dose-dependent loss of $\Delta \Psi_m$ in both the uninfected and HSV-1-infected HEp-2 cells. There was no significant difference between the loss of $\Delta \Psi_m$ in infected and uninfected cells.


A
MD 33.8%
MD 44.2%
Control
TCS 10 μg/ml
TCS 10 μg/ml + HSV-1
MD 39.0%
MD53.8%
TCS 30 μg/ml
TCS 30 μg/ml + HSV-1
TCS alone
TCS + HSV-1
MD
TCS concentration (μg/ml)
B
MD 8.4%
MD 18.0%
Control
TCS 3 h
TCS + HSV-1 3 h
MD 15.7%
MD 25.6%
TCS 6 h
TCS + HSV-1 6 h
MD 26.1%
MD 34.7%
TCS 18 h
TCS + HSV-1 18 h
MD 33.8%
MD 44.2%
TCS 24 h
TCS + HSV-1 24 h
TCS alone
TCS + HSV-1
MD
3 h
6 h
18 h
24 h
TCS treatment time
C
MD 7.7%
MD 8.5%
MD 7.3%
MD 10.5%
Control
HSV-1 3 h
HSV-1 6 h
HSV-1 18 h
HSV-1 24 h

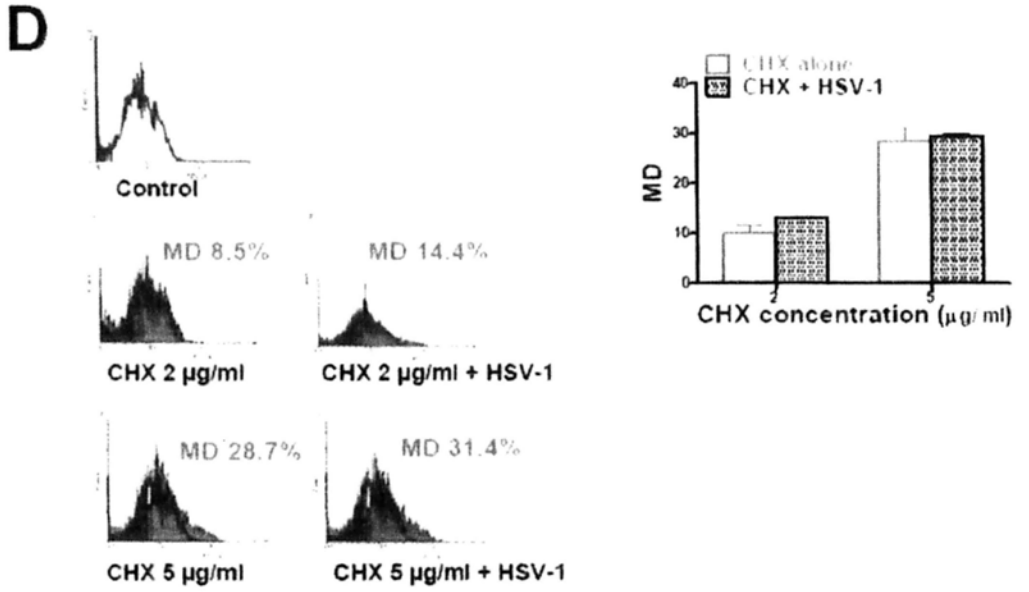
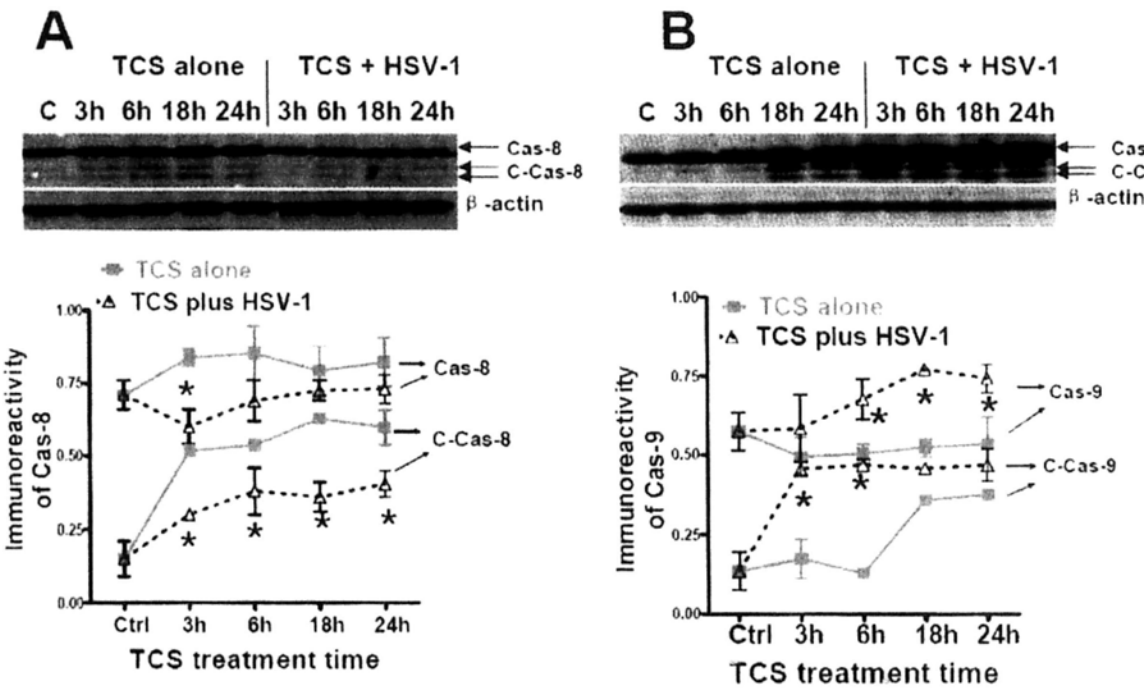


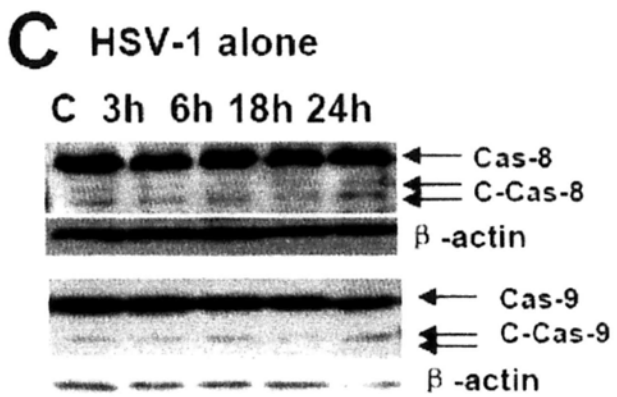
Figure 3.2. Flow cytometry analyzes changes in the mitochondrial transmembrane potential. Cells were preloaded with MitoCapture™ dye. The mitochondrial depolarization was induced by TCS in uninfected HEp-2 cells and HSV-1-infected HEp-2 cells and detected at FITC channel. (A, B, D) Representative figures for flow cytometry data in uninfected and HSV-1-infected HEp-2 cells treated with (A) 10 or 30 µg/mL TCS for 24 h, (B) 10 µg/mL TCS for 3, 6, 18 or 24 h and (D) 2 or 5 µg/mL CHX for 24 h. MD represents the percentage of cells with $\Delta \Psi_m$ depolarization (MD was determined as the ratio of the median FITC change for treated cells to control cells: (Fluoresent treated cells-fluoresent control cells)/ fluorescent control cells). Unfilled columns represent HEp-2 cells treated with TCS, and filled columns represent HSV-1-infected cells treated with TCS. (C) Representative figures for the flow cytometry data analyzing mitochondrial transmembrane potential changes in HSV-1-infected HEp-2 cells at 3, 6, 18 and 24 hpi. Data represent the mean ± SEM (n=3). * $p<0.05$ vs. TCS only. ** $p<0.01$ vs. TCS only.

3.3.2.2 Involvement of caspase in TCS-induced apoptosis

Western blot was employed to identify the effect of TCS on the activation of caspase-8 and -9 in HSV-1-infected and uninfected HEp-2 cells (Fig. 3.3 A and B). Cells were treated with 10 μg/mL TCS for 3, 6, 18, or 24 h. TCS recruited and processed more caspase-8 at the beginning of the TCS treatment in uninfected HEp-2 cells than in HSV-1-infected cells. In the HSV-1-infected cells, the activation of caspase-8 was delayed and attenuated. However, the downstream substrate of mitochondrial apoptosis, caspase-9, was strongly recruited after 3 h of TCS treatment and activated during the 24 h treatment in HSV-1-infected HEp-2 cells. In the uninfected cells, a gradual response of both un-cleaved and cleaved caspase-9 was detectable, but diminished. HSV-1 alone did not induce caspase-8 and 9 activation until 18 h infection (Fig. 3.3 C).

The effect of CHX on caspase activation was also studied. As illustrated in figure 3.3 D and E, caspase-8 was activated to a detectable level in both the uninfected and HSV-1-infected cells, whereas caspase-9 was not activated during the 24 h CHX treatment.


A
TCS alone
TCS + HSV-1
C 3h 6h 18h 24h 3h 6h 18h 24h
Cas-8
C-Cas-8
β -actin
TCS alone
TCS plus HSV-1
Immunoreactivity of Cas-8
Cas-8
C-Cas-8
Ctrl 3h 6h 18h 24h
TCS treatment time
B
TCS alone
TCS + HSV-1
C 3h 6h 18h 24h 3h 6h 18h 24h
Cas-9
C-Cas-9
β -actin
TCS alone
TCS plus HSV-1
Immunoreactivity of Cas-9
Cas-9
C-Cas-9
Ctrl 3h 6h 18h 24h
TCS treatment time


C HSV-1 alone
C 3h 6h 18h 24h
Cas-8
C-Cas-8
β -actin
Cas-9
C-Cas-9
β -actin

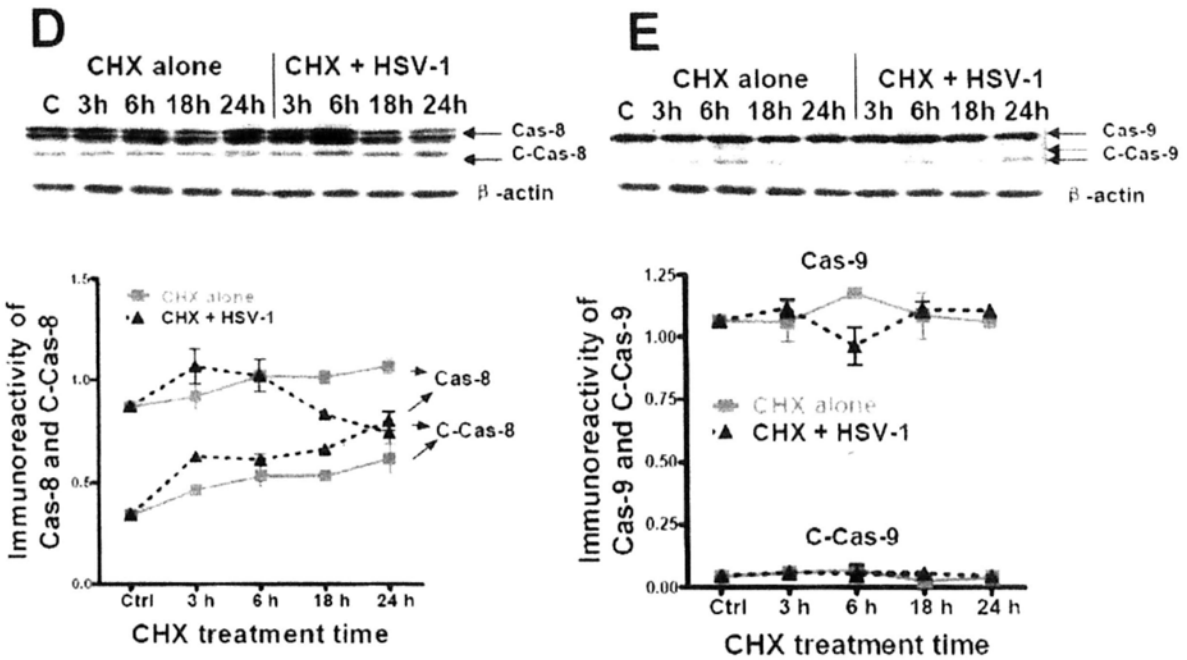


Figure 3.3 The immunoreactivity of full-length and cleaved caspase-8 and -9. HEp-2 cells were infected with 0.5 MOI HSV-1 or left uninfected for 2 h before treatment with 10 μg/mL of TCS or 5 μg/mL CHX. After TCS or CHX treatment, cells was further cultured for 3, 6, 18 or 24 h. Data are represented as the relative level of immunoreactivity determined by densitometric analysis and a representative western blot gel. (A, C and D) Immunoreactivity of caspase-8 (Cas-8, ~ 57 kDa) and cleaved caspase-8 (C-Cas-8, ~43/41 kDa); (B, C and E) Immunoreactivity of caspase-9 (Cas-9, ~47 kDa) and cleaved caspase-9 (C-Cas-9, ~37/35 kDa). The square lines represent HEp-2 cells treated with TCS or CHX, and the triangle lines represent HSV-1-infected cells treated with TCS or CHX. Data represent the mean ± SEM (n=3). *$p<0.05$ vs. TCS only.

3.3.2.3 Involvement of Bcl-2 in the TCS-induced apoptosis in HSV-1-infected HEp-2 cells

HSV-1-infected and uninfected HEp-2 cells were transfected with the Bcl-2 over-expression plasmid (Fig. 3.4 C) to study whether the over-expression of Bcl-2 could affect the apoptosis induced by TCS. In uninfected cells, transfection with the Bcl-2 over-expression plasmid slightly increased cell viability (EC_{50} 19.7 $\pm$ 6.24 μg/mL) compared to untransfected cells (EC_{50} 16.84 $\pm$ 5.31 μg/mL), but the effect was not significant (Fig. 3.4 A). On the other hand, in HSV-1-infected cells, the TCS-induced decrease in cell viability was significantly increased from EC_{50} 3.67 $\pm$ 0.51 μg/mL (without Bcl-2 over-expression plasmid transfection) to EC_{50} 18.56 $\pm$ 3.37 μg/mL (with Bcl-2 over-expression plasmid transfection) after transfection with Bcl-2 ($p < 0.05$) (Fig. 3.4 B).

Western blot was used to identify the effect of TCS on the activation of Bcl-2 in uninfected and HSV-1-infected HEp-2 cells (Fig. 3.4 D). Cells were treated with 10 μg/mL TCS for 3, 6, 18, or 24 h. TCS treatment decreased the total Bcl-2 level in HSV-1-infected cells, but had less effect on the uninfected cells.

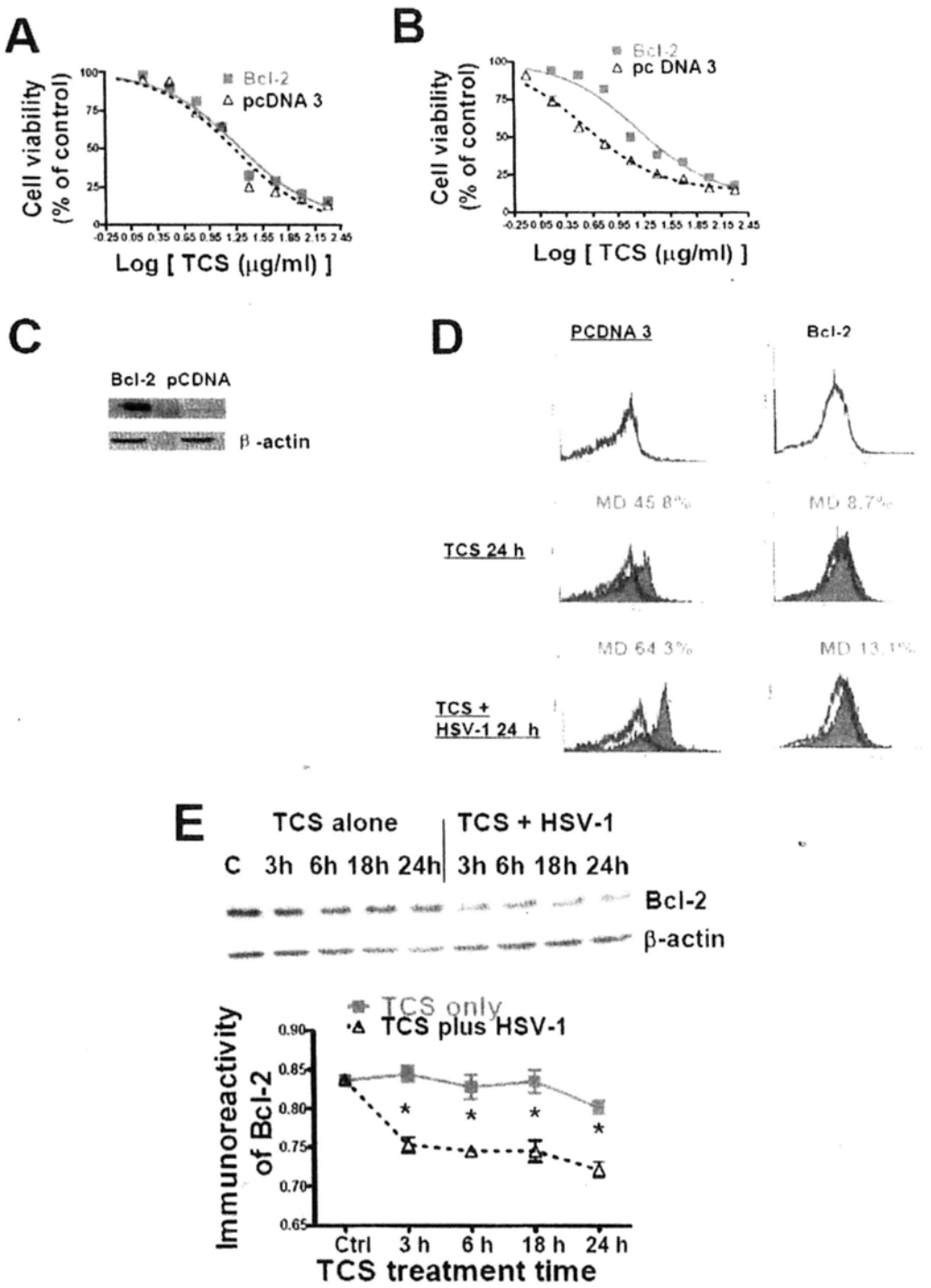

A
Cell viability (% of control)
Bcl-2
pcDNA 3
Log [TCS (μg/ml)]
B
Cell viability (% of control)
Bcl-2
pc DNA 3
Log [TCS (μg/ml)]
C
Bcl-2 pCDNA
β -actin
D
PCDNA 3
Bcl-2
TCS 24 h
TCS + HSV-1 24 h
E
TCS alone
TCS + HSV-1
C 3h 6h 18h 24h 3h 6h 18h 24h
Bcl-2
β-actin
TCS only
TCS plus HSV-1
Immunoreactivity of Bcl-2
Ctrl 3 h 6 h 18 h 24 h
TCS treatment time

Figure 3.4. Bcl-2 is implicated in the anti-HSV-1 effect of TCS. (A, B) Bcl-2 over-expression inhibits the effect on TCS cytotoxicity. HEp-2 cells were sequentially transfected with the Bcl-2 over-expression plasmid or pCDNA3 (control) infected (B) or not (A) with 0.5 MOI HSV-1 and treated with TCS (200-0.78 μg/mL, two folds serially diluted) for 24 h. The filled square line represents HEp-2 cells transfected with Bcl-2, and the unfilled triangle line represents cells transfected with the pcDNA3 vector. Cell viability was assessed with MTT. (C) Immunoreactivity of Bcl-2 (~28 kDa) in over-expression plasmid. (D) MD was detected by MitoCapture™ dye in the FITC channels by flow cytometry. (E) Immunoreactivity of Bcl-2 (~28 kDa) in HEp-2 cells with TCS or HSV-1 treatments. Cells were infected with 0.5 MOI HSV-1 or left uninfected 2 h before treatment with 10 μg/mL TCS. After TCS treatment, cells were cultured for 3, 6, 18 or 24 h. The filled square lines represent HEp-2 cells treated with TCS, and the unfilled triangle lines represent HSV-1-infected cells treated with TCS. Data represent the mean ± SEM (n=3). *$p<0.05$ vs. TCS only.

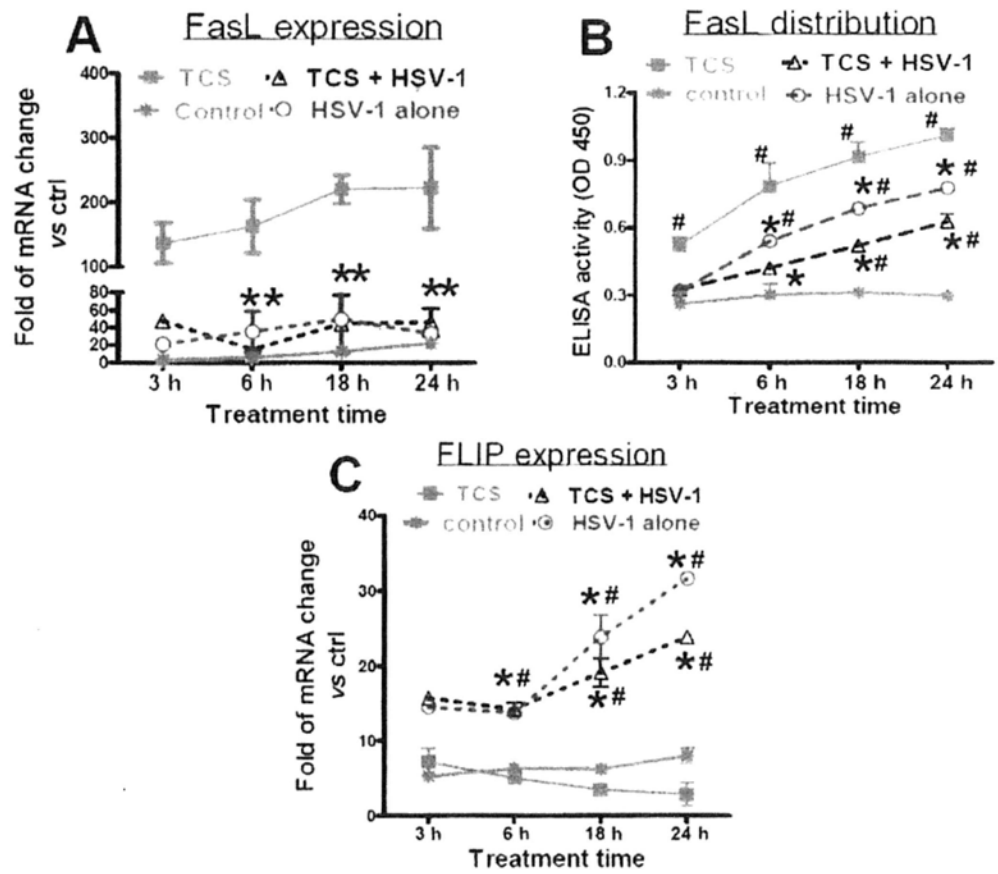
3.3.2.4 Involvement of FasL in TCS-induced apoptosis

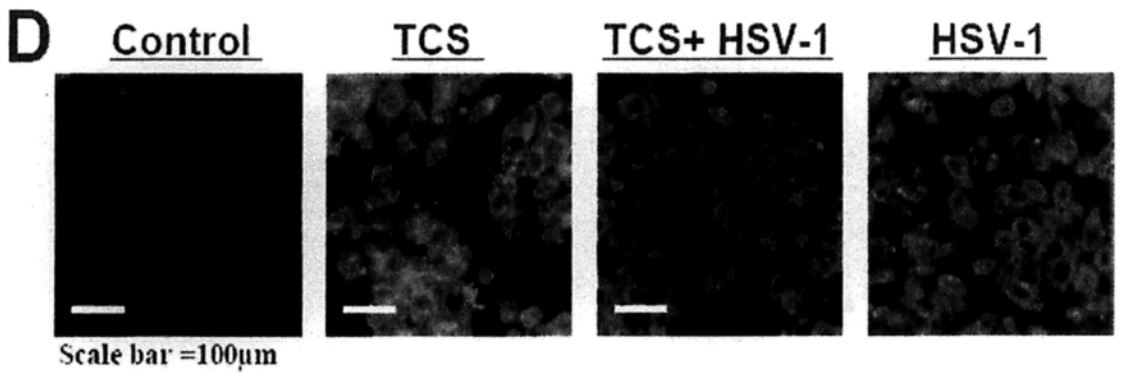
Real-time PCR, ELISA and immunostaining on FasL were used to study the apoptosis regulating effect of the CD95 (Fas/Apo-1) system. TCS significantly increased the expression of FasL (Fig. 3.5 A), but this effect was decreased in HSV-1-infected cells with TCS treatment. ELISA and immunostaining showed that the distribution of FasL in the plasma membrane induced by TCS only was increased compared to control cells (Fig. 3.5 C and D). Cells infected with HSV-1 and treated with TCS significantly decreased expression and distribution of FasL compared to TCS-treated, uninfected cells. However, the expression was still significantly increased compared to control cells. In untreated cells infected with HSV-1, the FasL expression and cell-membrane distribution level showed significant ($p< 0.05$) increase versus control.

At the meanwhile, the expression level of CD95 signaling pathway inhibitor FLIP [94;95] was studied (Fig. 3.5 B). It was found in TCS-treated cells, without HSV-1 infection, the FLIP expression level were significantly decreased after 3 h TCS treatment ($p<0.05$ at 3, 6 h, $p<0.01$ after 18 h). When infected with HSV-1, there were significantly decrease of FLIP induced by TCS only till 18 h treatment. In HSV-1 infected cell, the expression FLIP maintained the same level as control and was significantly increased versus control ($p<0.05$) after 18 h infection.

The FasL inhibitor NOK-1 was employed to demonstrate the effect of FasL during TCS-induced apoptosis. We found that (Fig. 3.5E and F) NOK-1 decreased the TCS-induced cytotoxicity in uninfected cells (From 26.77 ± 3.35 to 40.64 ± 8.75 μg/mL; $p<0.05$). In cells infected with HSV-1, the cytotoxicity of TCS was not significantly

affected by NOK-1 (4.10 ± 0.86 to 6.93 ± 1.09 μg/mL).


A
FasL expression
TCS
TCS + HSV-1
Control
HSV-1 alone
Fold of mRNA change vs ctrl
Treatment time
3 h
6 h
18 h
24 h
B
FasL distribution
ELISA activity (OD 450)
C
FLIP expression


D
Control
TCS
TCS+ HSV-1
HSV-1
Scale bar =100μm

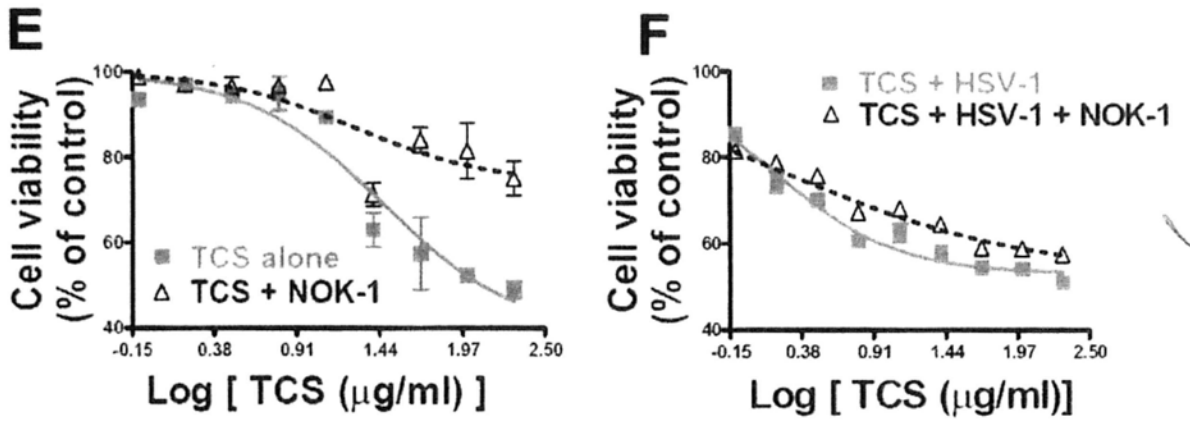


Figure 3.5. FasL are involved in TCS-induced apoptosis. (A, B) Real-time PCR analyzing FasL and FLIP expression. Monolayer HEp-2 cells on a 6-well plate were infected with 0.5 MOI HSV-1 or left uninfected in either the absence or presence of 10 μg/mL TCS. The cells were cultured for 3, 6, 18 or 24 h, after which RNA was extracted for real-time PCR. The filled square line, the unfilled triangle and the circle line represent TCS only, TCS + HSV-1 and HSV-1 only, respectively. (C, D) In situ ELISA and immunostaining were used to analyze the FasL distribution on the cell membrane. Treated cells were fixed and conjugated with primary antibodies, rabbit anti-FasL Ab, and secondary antibodies, Goat-anti-rabbit IgG-HRP (ELISA) or Alexa Fluor 488 conjugated goat anti-rabbit IgG (Immunostaining). Signals were detected at 450 nm (ELISA) by a plate reader or by a FV1000 laser scanning confocal system. Filled and unfilled square lines represent treatment with 10 μg/mL TCS, respectively, The filled and unfilled triangle lines represent treatment with 10 μg/mL TCS with HSV-1 infection, respectively, The filled circle and star dashed lines represent treatment with HSV-1 only and control, respectively. (E, F) HEp-2 cells were pretreated with 0.5 μg/mL NOK-1 overnight and treated with TCS or HSV-1 for 24 h. Cell viability was assessed by MTT. * $p<0.05$ vs.

control, **p<0.01 vs. control, #p<0.05 vs. TCS only.

3.4 Discussion and Conclusion

Killing the cells certainly inhibits viral replication, only when agents show selectivity on infected cell in their ability to induce cell death, they could be anti-viral agents. At the beginning of this study, the selectively apoptosis-inducing ability of TCS between HSV-1 infected and uninfected HEp-2 cells was described. TCS induced a greater loss of cell viability and progression to apoptosis in HSV-1-infected than in uninfected ones. This selectivity of TCS is interesting because HSV-1 interferes with host cell immune response or antagonizes the stress response in the host cell to favor viral proliferation [56;69;96]. The ability of HSV-1 to block CHX-induced apoptosis has been widely discussed [56;69], and we compared the effect of CHX on HSV-1 infected HEp-2 cells with the effect of TCS. As previously described [68], HSV-1 antagonized the cell death induced by CHX when CHX was administered 6 hpi. However, from 0-6 hpi, CHX was able to inhibit the anti-apoptotic protein synthesis of HSV-1[49] , inducing apoptosis in HSV-1 infected cells. By contrast, TCS induced apoptosis in HSV-1 infected HEp-2 cells even when TCS treatment was applied at 6 hpi. The effect of HSV-1 on cell viability in these tests could be excluded because no decrease in cell viability was detected during the 24 h of HSV-1 infection. Although previous studies have suggested that ribosome inactivity or cytotoxicity usually went hand in hand with antiviral activities [97-99], the difference between TCS and CHX showed that the RIP activity of TCS may not completely account for its anti-HSV-1 activity. Wang *et al.* [28] also found exceptions in the connection between ribosome inactivity and virus inhibition. Thus, in addition to interfering with cell

ribosome activity, there may be other mechanisms that contribute to the anti-HSV-1 ability of TCS. The selectivity of TCS in HSV-1-infected cells offers insight into the anti-HSV-1 mechanisms of TCS.

The function of the mitochondrial in apoptosis has become a highly discussed topic [100;101]. The $\Delta \Psi_m$ depolarization and related proteins activation, such as the Bcl-2 family and cytochrome c, are widely thought to be involved in apoptotic control. In fact, the loss of $\Delta \Psi_m$ is considered to be a hallmark of apoptosis. Both type I and type II apoptotic signaling pathways involve $\Delta \Psi_m$ depolarization, but they are distinguished by the level of dependence on mitochondrial [32;92;102]. In this study, it was found both HSV-1-infected and uninfected HEp-2 cells showed TCS-induced time- and dose-dependent mitochondrial transmembrane depolarization, suggesting that the mitochondrial is involved in TCS-induced apoptosis. However, amount of mitochondrial requirement for apoptosis was different. HSV-1-infected HEp-2 cells showed a higher percentage of $\Delta \Psi_m$ loss than uninfected cells ($p<0.05$). This difference between HSV-1 infected and uninfected cells was not due to infection with HSV-1, as HSV-1 infection alone had no effect on $\Delta \Psi_m$. The two types of apoptosis activate caspase in different ways. Type I is dependent on the rapid recruitment of a large amount of caspase-8 to send the apoptotic signal, whereas in type II, only a very small amount of caspase-8 was needed to activate BAX to expand the apoptotic signal, at which point caspase-9, downstream of the mitochondrial, was activated [103]. We found TCS induced a rapid response of caspase-8 expression and consumption in uninfected HEp-2 cells. By contrast, when the HEp-2 cells were infected with HSV-1, TCS did not induce the rapid activation

of caspase-8, but triggered the activation of caspase-9 instead. Additionally, former studies have suggested that the over-expression of Bcl-2 has a protective effect that could be a defining feature of type I or type II apoptosis. Large amounts of Bcl-2 could protect cells from losing $\Delta \Psi_m$ and therefore reduce type II apoptosis whereas having less effect on type I apoptosis [92;102-104]. In this study, when HSV-1-infected HEp-2 cells were transfected with Bcl-2, the cytotoxicity of TCS was significantly diminished ($p< 0.05$), however, this Bcl-2 protection was decreased in uninfected cells. In untransfected HEp-2 cells, TCS induced more endogenous Bcl-2 breakdown in infected cells than uninfected ones ($p< 0.01$). Suggesting Bcl-2 play a more important role during TCS induced apoptosis when infected with HSV-1. Taken together, these data indicated that TCS induces type I apoptosis in uninfected HEp-2 cells and induces type II apoptosis in HSV-1-infected cells. As described elsewhere [32;92;104;105], the type I apoptosis pathway depends solely on caspase activation. By contrast, the type II apoptosis pathway consists of a wider set of signaling molecules and is sensitive to a number of apoptosis inhibitors or activators. Therefore, HSV-1 infection in HEp-2 cells may enable TCS to utilize the type II apoptosis pathway, during which a larger amount of apoptotic signaling molecules are engaged.

The effect of CHX on mitochondrial function was compared with TCS. Consistent with the results of other studies showing that CHX typically utilizes type I apoptosis [93;106], we found that there were no significant differences in the $\Delta \Psi_m$ depolarization between HSV-1 uninfected and infected cells. We saw an activation of capsase-8, but not caspase-9. Usually, this contrast between TCS and CHX strongly indicates that the

anti-viral ability of TCS may be not completely dependent on killing the host cell like CHX does, TCS may chose a more sensitive type II apoptosis to kill more infected cells but protect uninfected ones.

Additionally, the reasons of apoptotic type change were further discussed. The formation of DISC is a critically early step in both type I and type II apoptosis. The amount of DISC activation is directly related to the amount of caspase-8 activation, the step in which mitochondrial dependence is decided. Viral infection interferes with CD95 (Fas/APO-1) activity in different ways between various cell types. Some virus, (e.g., HIV) trigger CD95 (Fas/APO-1) activation in human T cells [107;108], whereas HSV-2 sequesters the apoptotic function of CD95 in favor of viral survival [109]. The Us5 and Us3 proteins of HSV-1 may interfere with Fas-mediated apoptosis in Jurkat cells [110]. In this study, we found that TCS treatment triggered FasL expression and activation as early as 3 h after treatment, but this TCS-induced increase in FasL activity was significantly decreased when there were HSV-1-infected cells. The decrease in FasL activity may be due the HSV-1 interfering effect, because HSV-1 infection alone tends to suppressed FasL activation by expressing inhibitor of CD95 induced apoptosis FLIP. Therefore, when the CD95 (Fas/APO-1) system is detained by HSV-1 infection, TCS is likely to bypass the CD95 (Fas/APO-1) complex and exert its cytotoxicity directly through the mitochondrial signaling pathway, i.e., when infected with HSV-1, TCS utilizes the type II apoptotic signaling pathway instead of the type I pathway.

In conclusion, this study provides evidence for the anti-HSV-1 mechanism of TCS. TCS selectively induces apoptosis in HSV-1 infected HEp-2 cells compared to uninfected

cells. This selectivity is related to the replacement of the type I apoptosis pathway with the type II apoptosis pathway in HSV-1 infected HEp-2 cells. CD95 (Fas/APO-1) is involved in the activation of TCS-induced type I apoptosis, but when the CD95 (Fas/APO-1) apoptotic response is altered by HSV-1, TCS bypasses the CD95 (Fas/APO-1) requirement and induces type II apoptosis.

In figure 3.6, we provide a sketch graph demonstrating possible anti-HSV-1 mechanism of TCS by using type I to type II transition. The key issue is the ability to induce a more potent apoptosis in infected cells over non-infected cells. It works by modulating the CD95 (Fas)-mediated type I and II apoptotic pathways. In normal cells, TCS activates CD95 (Fas), which in turn stimulates caspase-8 and eventually leading to CD95 (Fas)-mediated type I apoptosis. In HSV-1 infected cells, the infection process blocked CD95 (Fas) activation. The action of TCS is changed to a more potent CD95 (Fas) type II mitochondrial depolarization dependent apoptotic pathway.

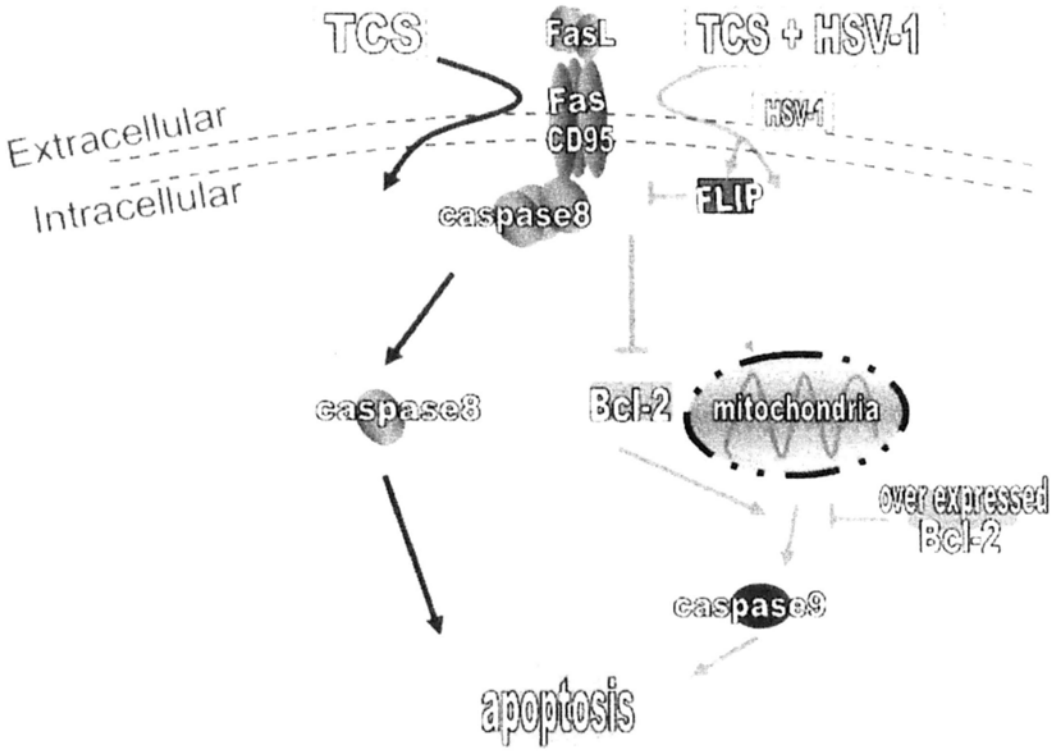


Figure 3.6. Proposed mechanism of anti-HSV-1 mechanism of TCS by using type I to type II apoptotic pathway transition. TCS induces a more potent apoptosis in infected cells over non-infected cells. It works by modulating the CD95-mediated type I and II apoptotic pathways. In normal cells, TCS activates CD95, which in turn stimulates caspase-8, eventually leading to CD95-mediated type I apoptosis. In HSV-1 infected cells, the infection process blocks CD95 activation. The action of TCS is changed to the more potent CD95 type II apoptotic pathway.

Chapter 4

Nuclear factor-κB signaling pathway participates the anti-HSV-1 mechanism of TCS

4.1 Introduction

The transcriptional factor NF-κB is a key regulator implicated in immune response, inflammation operating, oncogenesis modulation and apoptosis regulation. It belongs to the Rel family containing five members: c-Rel, NF-κB1 (p50/p105), NF-κB2 (p52/p100), RelA (p65) and RelB[111]. All proteins of the NF-κB family share a Rel homology domain in their N-terminus. The c-Rel, RelA and RelB contain a transactivation site at their C- terminal. But the NF-κB1 and NF-κB2 were produced as large precursors, p105, and p100. The p105 then was processed into subunit p50 while p100 processed into p52, to grow activated and participate following pathways of NF-κB. However, there is no transactivation site on the C-terminal in p50 and p52, they modulate NF-κB by forming a NF-κB hetero-dimmer with c-Rel, p65 or RelB.

Activation of NF-κB is naturally inhibited by cytoplasmic inhibitory protein inhibitor-κB (IκB) which inhibits the translocation of NF-κB dimmer (p50/p65, most abundant) into nucleus and binding to DNA. In response to certain stimuli, IκB Kinase

(IKK) phosphorylates IκB, following degradation of IκB by ubiquitin, the NF-κB is then released and translocates into the nucleus [111-113].

The transcriptional activity of NF-κB does not depend on activation of other factors. Upon stimulation, it responses quickly and acts as a "first responder". The NF-κB could be activated by toll-like receptors (TLRs) and interleukin 1 receptor (IL-1R), which is an important mechanism in innate and adaptive immunity and inflammatory response [114]. Upon interaction between IL-1 and IL-1R or TLR and LPS-binding protein (LBP), the IL-1R and TLR will trigger signaling pathways, involving phosphorylates IκB, in turn initiates NF-κB related gene transcription.

The role of NF-κB during apoptosis is widely discussed. Activation of apoptosis could lead to suppression in NF-κB, therefore, NF-κB dysfunction may result in tumor genesis. NF-κB mediated apoptosis was showed to induced by TNFR [114], because mice with knocking out of the TNFR failed activated NF-κB pathway and underwent apoptotsis [115].

The role of NF-κB in inmmunoresponse and apoptosis regulation is demonstrated during the HSV-1 infection. The NF-κB was showed to be involved in HSV-1 infection [113;116-119], HSV-1 immediate gene expression induced translocation of NF-κB to nucleus in order to regulate several target gene, including genes for HSV-1 escaping from host cell immune response and suppressing host cell apoptosis [118]. The HSV-1 ICP27 protein, a important IE gene product regulates E and L DNA, controls host protein synthesis and inhibits host cell apoptosis triggered by TNFa, antibody to Fas, C2-ceramide sorbitol and CHX [58;96], was shown to trigger the NF-κB activation. After

ICP27 protein activation during HSV-1 infection, the protein triggers phosphorylation of IκB, which depends on integrity of ICP27 protein amino acid 21 to 63, and then ICP27 protein induces a robust and persistent translocation of NF-κB and binding to the DNA [117]. This translocation of NF-κB usually initiates at 3 hour post infection, coincides with the time point of ICP27 protein activation.

In this study, NF-κB activation is studied during HSV-1 infection and TCS treatment to demonstrate the possible anti-HSV-1 mechanism of TCS in human epithelial carcinoma HEp-2 cells.

4.2 Materials and Methods

4.2.1 Cell, virus and reagents

Human epithelial carcinoma HEp-2 cells and HSV-1 (F) were obtained and maintained as previously described. The cells were infected at a multiplicity of infection (MOI) of 0.5 .

TCS was obtained from Shanghai Jinshan Pharmaceutical Limited Company (Shanghai, PRC), NF-κB activator recombinant human IL-1α and inhibitor BAY 11-7082 were purchased from PeproTech INC. (USA) and Sigma Life Science (USA) respectively.

4.2.2 Real-time PCR

Cells were infected with 0.5 MOI HSV-1 and simultaneously incubated in the absence and presence of 10 μg/ml TCS for 3, 6, 18 and 24 h. Total RNAs were extracted using Trizol (Qiagen, USA). RNAs were reverse transcribed to yield single-stranded cDNA according to iScript™ cDNA Sythesis Kit (Bio-rad, CA) protocal. Briefly, RNA samples were mixed with 5× iScript reaction mix, iScript reverse transcriptase and reacted at 25℃ for 5 min, 42 ℃ for 30 min, 85 ℃ for 5 min. cDNA products were stored at -20 ℃.

Real-time PCR were performed using IQ™ SYBR Green Supermix (Bio-Rad, CA) for 40 cycles of 95 ℃ 15 s and 62 ℃ 40 s. Data were analyzed by iCycler Multicolor Real-time PCR Detection System (Bio-rad Labratories, USA) Software. The primer pairs for NF-κB were: forward: 5'-ACAAATGGGCTACACCGAAG-3', reverse: TAGGGCTTTGGTTTACACGG-3'; for IKK were: forward: 5'-CCTATGGAAGACGTCAGGGA-3'; reverse: 5'-TCATGTTCTGCTGAAGTCGG-3'.

4.2.3 Preparation of cytoplasmic and nuclear extracts

2×10^6 cells were mock or infected with 0.5 MOI HSV-1 and treated with 10 μg/ml TCS for 3, 6 and 18 h. during the experiments with NF-κB activator and inhibitor, the NF-κB activator IL-1α (100 ng/ml) and inhibitor BAY 11-7082 (30 μM) were pretreated for 1h before TCS or HSV-1 addition. Cytoplasmic and nuclear extracts were prepared according to protocol from Abcam website (www.abcam.com) with some modifications. Briefly, cells with certain treatment or infection were rinsed twice with cold PBS, harvested by scraping from 60 mm culture dish and lysed with 50 μl general lysis buffer containing: 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 150 mM NaCl, 0.5% NP-40, and freshly prepared 20 μg/ml aprotinin, 5 μg/ml leupeptin, 1 mM PMSF. Samples were incubated on ice for 10 min and spinned at 4℃, 500 g for 5 min. Supernatant were saved as cytoplasmic extract.

The nuclear pellets were then lysed with 40μl nuclear lysis buffer containing: 20 mM Hepes (pH 7.2), 400 mM NaCl, 1 mM EDTA (pH 8.0) with freshly prepared 1 mM DTT, 20 μg/ml aprotinin, 5 μg/ml leupeptin and 1 mM PMSF. The resuspended nuclear pellet was vortexed at 4℃ for 60 min and spinned at 12000 rpm for 5 min at 4 ℃, supernatant were saved as nuclear extract.

4.2.4 Immunoblotting analysis

Proteins (100 μg) were separated with 12% SDS –PAGE then transferred to PVDF membrane (Immobilon P, Millipore, Bedford, MA, USA), Rabbit polyclonal antibodies against NF-κB p65 (1:1000) or β-actin (1:1000) (Cell Signaling Technology Inc., Beverly, MA.) were probed as primary antibody, followed by secondary antibody goat- anti-rabbit

(1:7000) (LI-COR biosciences, USA) binding. The antibody-binding was detected with odyssey imaging system (LI-COR biosciences, USA).

4.2.5 NF-κB EMSA assay

The electrophoretic mobility shift assay (EMSA) was performed according to the protocol of NF-κB EMSA Probe Set (Panomic Inc. USA). Briefly, the nuclear extracts at 24 h TCS or infection treatment were probed with NF-κB specific probe labeled with biotin and separated by 6% non- denaturing PAGE. The separated proteins were then transferred to probe with Streptavidin-HRP. The HRP signal was exposed to Hyperfilm ECL (Amersham, United Kingdom).

4.2.6 ELISA assay assess the effect of TCS on HSV-1 antigen yeild

100 μl of HEp-2 cells (1×10^5) were seeded on 96-well microtiter plate and cultured overnight, cells were pretreated with or without (control) NF-κB activator IL-1α (100 ng/ml) and inhibitor BAY 11-7082 (30 μM) for 4h and then infected with 0.5 MOI HSV-1, treated with 200-0.78 μg/mL, two folds serially diluted of TCS followed by 24 h incubation. Then HSV-1 infected HEp-2 cells were fixed with 0.05% glutaraldehyde for 15 min at 4℃, primary antibody human anti-HSV serum (1:2000) were added for 1 h, then secondary Goat anti-human IgG- HRP (1:3000, Southern Biotech Assoc) were incubated for 1 h. TMB was used as substrate for color development (OD_{450}).

4.2.7 Statistical analysis

All result in the texts and figures are expressed as means ± SEM (n=3). $P< 0.05$ values were considered statistically significant. Two-way ANOVA with Bonferroni's *post hoc* test was used for statistical analysis.

4.3 Results

4.3.1 TCS affected NF-κB and IKK gene expression during HSV-1 infection in HEp-2 cells

To exam whether the NF-κB signaling pathway is implicated in anti-HSV-1 effect of TCS, the expression of NF-κB and IKK gene were studied by real-time PCR. As shown in figure 4.1 A and B, HSV infection tended to gradually enhance both IKK and NF-κB gene expression during 24 h infection period. When the HSV-1-infected cells were treated with TCS at the same time with infection and additionally cultured for 3, 6, 18 and 24h, the NF-κB and IKK gene expression level were firstly elevated and then gradually decreased.

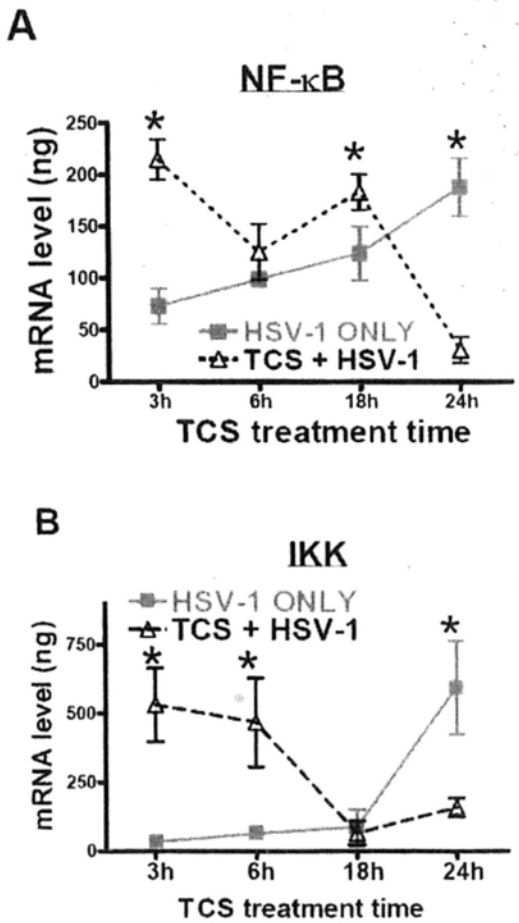


Figure 4.1. TCS interferes with HSV-1 infected HEp-2 cell NF-κB and IKK gene expression. HEp-2 cells were infected with 0.5 MOI HSV-1 in the absence (The filled square line) or presence (The unfilled triangle line) of 10 μg/ml TCS, cells were cultured for 3, 6, 18 and 24 h and RNA were extracted for real-time PCR. (A) TCS effect on NF-κ B gene expression; (B) TCS effect on IKK gene expression. Data were shown in relative nanogram compared with standard curve as mean ± SEM (n=3). Standard curve were formed with a dilution series consisted of blended cDNA samples with same qualities (ng).* $p< 0.05$ vs HSV-1 only.

4.3.2 Modification of NF-κB translocation by TCS in HSV-1 infected HEp-2 cells

To study the activation of NF-κB, the translocation of NF-κB were investigated. As shown in figure 4.2 A, HSV-1 triggered translocation of cytoplasmic NF-κB p65 subunit to the nucleus as previously reported [118-123]. However, HSV-1-induced translocation of NF-κB into nucleus were suppressed by TCS, the cellular level of NF-κB p65 subunit were gradually dropped during 3-24 h treatment period ($p< 0.01$ after 18 h), the nuclear level of NF-κB largely decreased ($p< 0.01$ in 3 and 6 h, $p< 0.001$ in 18 and 24 h) by TCS versus HSV-1 infected ones. At the same time, when the DNA binding activity of NF-κB was studied by EMSA at 24 h treatment (Figure 4.2 B), it was found TCS significantly ($p<0.001$) decreased the DNA binding in HSV-1 infected cells. TCS alone did not show effect on NF-κB activation versus control.

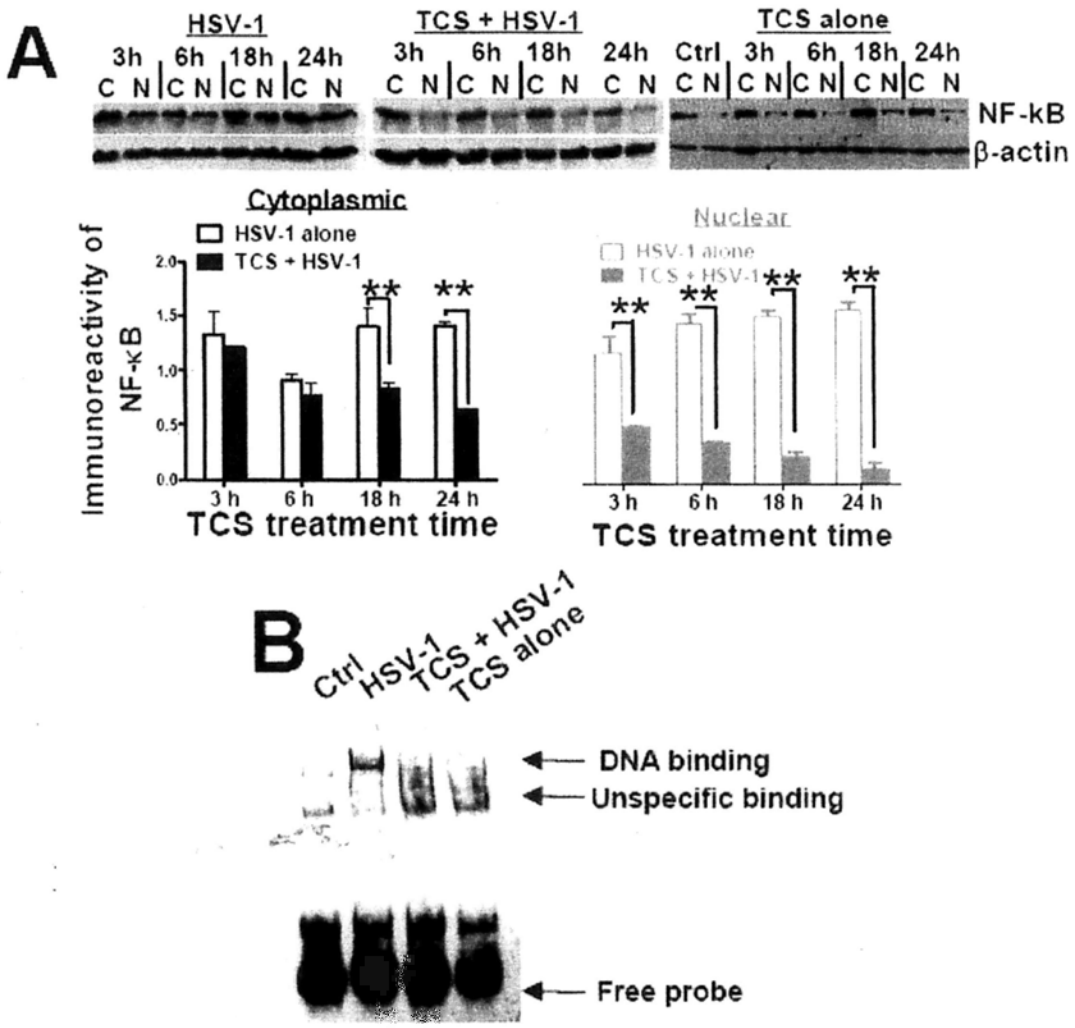


Figure 4.2. TCS interferes NF-kB activation of HSV-1 infected HEp-2 cell. HEp-2 cells were infected with 0.5 MOI HSV-1 in the absence (unfilled) or presence (filled) of 10 μg/ml or2 00-0.78 μg/ml (two folds serially diluted) TCS, cells were cultured for 3, 6, 18 and 24 h. (A) TCS suppresses NF-kB subunit (~65 kDa) translocation from cytoplasm (C) to nucleus (N) induced by HSV-1 infection. (B) EMSA analyzes TCS inhibition on NF-kB bind to DNA. Data were showed as mean ± SEM (n=3). **p< 0.01 vs HSV-1

alone.

4.3.3 Inhibition on HSV-1 replication of TCS was enhanced by NF-κB inhibitor but decreased by NF-κB activator

Our pervious studies have showed TCS is able to decrease the HSV-1 antigen during viral infection. To further elucidate the role of NF-κB in the anti-HSV-1 activity of TCS, the NF-κB activator IL-1α (100 ng/ml) and inhibitor BAY 11-7082 (30 μM) were employed to observe their effects on HSV-1 inhibition of TCS. (Both IL-1α and BAY 11-7082 alone did not show cytotoxicity at the indicated concentrations) According to the data in figure 4.3 A a, when treated with IL-1α, the antigen of HSV-1 infected HEp-2 cells increased significantly ($p < 0.05$) after treatment with TCS, giving an EC_{50} of 11.49 $\pm$ 4.13 μg/ml versus the IL-1α-untreated cells that showed an EC_{50} of 3.14 $\pm$ 1.16 μg/ml. On the other hand, when HSV-1 infected HEp-2 cells were treated with BAY 11-7082 in the presence of TCS (Figure 4.3 A b), the HSV-1 antigen decreased significantly ($p < 0.05$) (EC_{50} 1.19 $\pm$ 0.57 μg/ml) versus BAY 11-7082-untreated cells (3.14 $\pm$ 1.16 μg/ml). The absence of anti-HSV-1 effect of TCS after the administration of IL-1α was related to the ability of IL-1α to enhance NF-κB translocation, whereas BAY 11-7082 inhibits NF-κB translocation (Figure 4.3 C c).

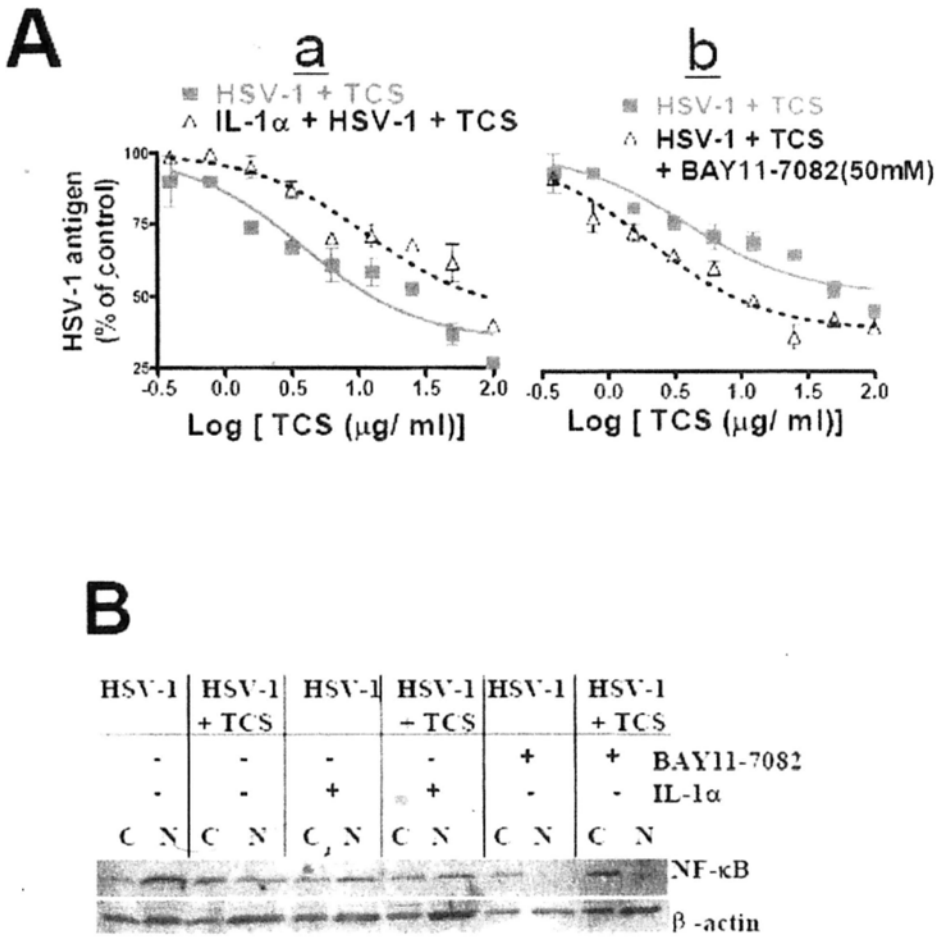


Figure 4.3. Inhibition on HSV-1 replication of TCS is enhanced by NF-κB inhibitor and reduced by NF-κB activator. HEp-2 cells were pretreated with or without 100 ng/ml IL-1α or 30 μM BAY11-7082 for 1h and then infected with 0.5 MOI HSV-1, followed by TCS treatment and additional 24h incubation. (A) ELISA was employed to exam HSV-1 antigen. The filled square line represents group with TCS treatment in HSV-1 infected HEp-2 cells. The unfilled triangle line represent groups with IL-1α or BAY11-7082 treatment respectively. (B) Western blot was employed to analyze the translocation of NF-κB under IL-1α and BAY11-7082 treatment. Data were shown as mean ± SEM (n=3).

4.4 Discussion and Conclusion

The anti-HSV-1 activity of TCS is usually thought to be related to its selective cytotoxicity. In our former studies (Figure 3), we found TCS are capable of selectively inhibiting HSV-1 infected cells growth (EC_{50} 3.01 + 1.30 μg/mL at 24 h treatment) but killing less normal cells (EC_{50} 24.64 + 1.17 μg/mL) than infected ones. However, it was already known wild type HSV-1 was able to express some anti-apoptosis genes and ignite host cell survival signaling pathway in order to antagonize apoptotic signal to its host cell and favor the viral proliferation [56]. Especially, the ability of HSV-1 to block CHX-induced apoptosis was widely discussed [56]. The CHX is a eukaryotic protein synthesis inhibitor works by interfering with the translocation step in protein synthesis. The anti-HSV-1 activity of CHX only effect when it can inhibit HSV-1immediate early gene expression, but the anti-HSV-1 activity is abolished when CHX treatment is delayed after immediate early infection period (6 h post infection) [68]. Whereas in our former experiments, we found even after early period of HSV-1 infection, treatment of TCS still showed HSV-1 inhibitory effect. Therefore, the difference between this two protein synthesis inhibitor, TCS and CHX, as well as the selectively apoptosis inducing activity of TCS between HSV-1 infected and uninfected cells, suggest the RIP activity of TCS may not tightly related to its anti-HSV-1 ability [28]. The evidences arouse our interesting to find out by what mechanism TCS uses to surmount the anti-apoptosis effect of HSV-1.

In this study, we demonstrated the role of NF-κB during the anti-HSV-1 effect of TCS. NF-κB is a key regulator implicated in immune response and apoptosis regulation. NF-κB was showed to be involved in HSV-1 infection [118]. HSV-1 stimulates NF-κB

activation and in the aftermath promotes host cell and itself proliferation. In this study, we found HSV-1 infection induced NF-κB translocated from cytoplasm to nucleus and bonded to DNA in HEp-2 cells as described. However, when HEp-2 cells were treated with TCS during HSV-1 infection, translocation of NF-κB from cytoplasm to nucleus and binding to DNA were markedly and persistently inhibited. At the same time, we found the expression of NF-κB and IκB inhibitor IKK increased during HSV-1 infection, further indicating the activation of NF-κB activation..These works on NF-κB suggest the possibility that anti-HSV-1 of TCS is related to NF-κB inactivation. They also show one aspect to explain the selective cytotoxicity of TCS, TCS suppresses the NF-κB pathway raised by HSV-1 infection, weakens the power by which virus escaping from host cell immune response and resisting apoptosis-inducing stimuli, therefore more HSV-1 infected HEp-2 cells are killed by TCS.

Interestingly, it is worth to notice expression of NF-κB in HSV-1 infected HEp-2 cells was elevated by TCS compared with HSV-1 only groups after 3 h treatment, and then gradually decreased by TCS. This increase in NF-κB perhaps due to initial anti-apoptosis function of de novo synthesized (2-6 h post infection) immediate early (IE) protein of HSV-1 [73]. These proteins, including ICP27 [117], ICP4 [124] and ICP0 [125], were reported to arouse noticeable signaling pathways, including NF-κB signaling pathway, to antagonize the surrounding stress. Indeed, in another part of our experiment (Fig. 2.3) focusing on TCS's effect on HSV-1 IE protein ICP27 synthesis, although the overall 24 h treatment of TCS did not show significance in suppression of ICP27 level, fluctuation in ICP27 expression level was observed during 3-6 h post infection, ICP27 were firstly

elevated by TCS at 3 h post infection and then drop back to base level at 6 h post infection.

In other experiments (Figure 2.1), one of the characteristics in anti-HSV-1 ability of TCS was showed to decrease HSV-1 antigen during HSV-1 infection. In this study, both NF-κB activator and inhibitor were applied in HSV-1–antigen-detecting ELISA to further elucidate the role of NF-κB in anti-HSV-1 activity of TCS. Data showed BAY11-7082 significantly enhanced TCS ability to suppress HSV-1 proliferation, suggesting the anti-viral efficiency of TCS could be potentiated by NF-κB inhibition. On the other hand, NF-κB activator IL-1α significantly increased HSV-1 antigens, therefore attenuated TCS's inhibition effect on HSV-1 proliferation. These data further support our former conclusion that the benefit of NF-κB on HSV-1 replication could be interfered by TCS.

By now, the most widely used anti-HSV-1 medicine is acyclovir (ACV), ACV is phosphorylated to triphosphate by viral thymidine kinase and cellular enzyme during treatment of HSV-1 infected subjects and cells, this triphosphate inhibits viral DNA polymerases [63;64]. Because mutated HSV-1 may show resistance to ACV when there are alternation in viral thymidine kinase activity, and at the same time a unique anti-HSV-1 mechanism of TCS was gradually demonstrated [29;30], suggesting the possibility of applying TCS as new anti-HSV agent. In summery, we now furthermore propose the NF-κB signaling pathway is implicated in anti-viral effect of TCS, TCS down regulates NF-κB activity in HSV-1 infected HEp-2 cells. The anti-HSV-1 activity of TCS can be amplified by NF-κB inhibitor.

Chapter 5

P53 signaling pathway participates the anti-HSV-1 mechanism of TCS

5.1 Introduction

In contrast to the role of NF-κB benefits cell survival, wild-type tumor suppressor protein p53, an important transcriptional activator, oppositely suppresses growth signals [126;127]. The transactivation domain of p53 is located on its amino terminus, it interacts with DNA sequence with 5'-PuPuPuC(A/T)(T/A)G Py-PyPy-3' [128], and recruits transcriptional machineries including TATA box binding proteins and its related factors, such as multisubunit transcription factor IID (TFIID) which includes TAFII40 and TAFII60 [129].

P53 is a tumor suppressor whose functions have been widely discussed. The gene mutation of p53 is often involved in human cancers. Cell growth suppression of p53 induced by genotoxic and non genotoxic stress is related to its tumor suppression function by promoting tumor cell death [127;130]. The polyproline region is essential for p53 to induce apoptosis following the transcriptional domain of p53, but not related to cell cycle arrest [131]. The expressions of some apoptotic proteins were showed to be related to p53 apoptotic transcriptional activation, such as p21/WAF1, growth arrest and

DNA damage (GADD45) [132;133], but the most important one is BAX [128]. The BAX gene promoter contains at least four p53-binding sites, one is CACGTG motifs at 5'UTR of the BAX gene. At positions -486 bp to -448 bp, including one perfect located on 5' of the TATAA box that is about 70 bp and two imperfect 10 bp site for p53 binding.

P53 also plays a major role in cellular response to DNA damage or other genotoxic stress [134-137], it in turn regulates cell cycle [135;138], and DNA damage repair [136;137;139]. In response to DNA damage, p53 is phosphorylated by ATM at Ser 15, leading to the decrease in the interaction between p53 and its negative regulator murine double minutes 2 (MDM2) [140] which is a ubiquitin ligase targets p53, the p53 is then accumulated and activated. Whether the cells go to apoptosis or cell cycle arrest may dependent on the level of p53 activation. Low level of p53 activation usually induces cell cycle arrest, but high level of p53 activation, usually accompanied with BAX insertion into mitochondrial transmembrane and cytochrome C release [141;142].

The activation of p53 is strictly regulated. There are at least three proteins, MDM-2, Cop-1 and Pirh-2, could auto-inhibit p53 activation [143]. The most important regulation of p53 is by MDM2. The MDM2 is a 97 kDa protein, it can bind to the p53 in the cytoplasma or transport p53 out of the nucleus. Although it is commonly believed the export of p53 from nucleus is associated with MDM2 binding to and transport of p53 [144], there are other evidence indicates a COOH-terminal-located nuclear export signal (NES) on the p53 may directly mediate its own nuclear export [145]. The binding domain of MDM2 is in the N-terminal region of p53 consensus a 15-residue transactivation domain peptide. The MDM2 deep hydrophobic cleft formed as an amphipathic α helix

with p53 [146]. MDM2 also plays as an ubiquitin ligase to ubiquitinate p53 protein [147]. The ubiqutination activity of MDM2 dependent on activity is dependent on its RING finger. Dependent on a feedback loop, p53 could affect the amount of MDM2 by positive transcriptional activation. The $p19^{ARF}$ protein was showed to be involved during the feed back signal [143], it can either binds to and inhibits MDM2 or binds to and activates p53.

The activation of p53 is also managed by another group of enzymes. The ataxia telangiectasia mutated (ATM) kinase [148;149], ataxia telangiectasia and Rad3 related kinase (ATR) [150], DNA-dependent protein kinase (DNA-PK) and the CHK-2 kinase [151] can phosphorylate the p53 protein and then activate p53. These kinase that phosphorylate p53 belong to the phosphoinositede-3 kinase-related kinase (PIK) superfamily, the ATM, ATR and CHK-2 kinases primarily regulate cell cycle checkpoints in response to DNA damage [152], and the DNA-PK participates DNA damage repair process [150]. There are at least 7 phosphorylation site of p53, including Ser 6,9,15, 20, 37, 46 and 392, they are phosphorylated in response to different stimuli. Another protein CREB-binding protein (CBP) was reported to facilitate p53 activation. The CBP interacts with the N terminus of p53 after it was phosphorylated, usually at the Ser 15, and at the same time acetylates its C terminus in order to increase the sequence-specific DNA-binding activity of p53 [153].

Phosphorylation of the Ser 15 within the p53 N-amino-terminal region is one of the important responses to stabilizing p53 when cells are exposed to genotoxic stress. The Ser 15 phosphorylation that is conducted by either ATM, ATR or DNA-PK can directly impair the connection between p53 and MDM2 and lead to subsequent event, including

accumulation of p53, functional activation of p53, expression of the p21 cyclin-dependent kinase inhibitor, prevention of the progression from G1 to S phase and DNA repair. At the same time, phosphorylation of p53 on Ser 20 and 37 at N-terminal also play a role after DNA damage to stabilize p53 [154;155].

In the present investigation, the anti-HSV-1 mechanism of TCS on human epithelial carcinoma HEp-2 cells with wild type p53 was studied from p53 signaling pathways. It was found, TCS induced DNA damage and S to G_2/ M phase arresting in HEp-2 cells, this DNA damage and cell cycle arrest ratio decreased when cells were infected with HSV-1, but in HSV-1 infected HEp-2 cells, the level of p53 and BAX activation, MDM2 degradation were enhanced by TCS than uninfected cells.

5.2 Materials and Methods

5.2.1 Cell, virus and reagents

Human epithelial carcinoma HEp-2 cells and HSV-1(F) were obtained and maintained as previously described. The cells were infected at a multiplicity of infection (MOI) of 0.5.

TCS was obtained from Shanghai Jinshan Pharmaceutical Limited Company (Shanghai, PRC). Anti-phospho-p53 (Ser 15) and anti-BAX antibodies were purchased from Cell Signaling Technology (USA). Anti-Bcl-2 and anti-MDM2 antibodies were purchased from Santa Cruz Biotechnology Inc. (USA). Anti-p53 antibody was obtained from Calbiochem (USA).

5.2.2 Preparation of cytoplasmic extractions

2×10^6 cells were infected or left uninfected with 0.5 MOI HSV-1 and treated with 10 μg/ml TCS for 3, 6, 18 and 24 h for protein extraction. Cytoplasmic and extracts was prepared according to protocol from Abcam website (www.abcam.com) with some modifications. Briefly, cells with indicated treatment or infection were rinsed twice with cold PBS, harvested by scraping and lysed with 50 μl general lysis buffer containing: 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 150 mM NaCl, 0.5% NP-40, and freshly prepared 20 μg/ml aprotinin, 5 μg/ml leupeptin, 1 mM PMSF. Samples were incubated on ice for 10 min and spun at 4℃, 500 g for 5 min. Supernatant were saved as cytoplasmic extract.

5.2.3 Immunoblotting analysis

Proteins were separated by 12% SDS –PAGE then transferred to PVDF membrane

(Immobilon P, Millipore, Bedford, MA, USA). The membrane was incubated with a generic protein (5% skimmed milk proteins in TBST) for 1h at room temperature. Primary antibodies were added to the solution and incubated over night. Secondary antibodies were added to the reaction for 1 h. The antibody binding was detected with an Odyssey imaging system (LI-COR biosciences, USA). Anti-β-actin antibody was used as the loading control.

Real-time PCR

Cells were infected with 0.5 MOI HSV-1 and incubated in the absence and presence of 10 μg/ml TCS for 3, 6, 18 and 24 h. Total RNAs were extracted using Trizol (Qiagen, USA). RNAs were reverse transcribed to yield single-stranded cDNA according to iScriptTM cDNA Sythesis Kit (Bio-rad, CA) protocal. Briefly, RNA samples were mixed with 5× iScript reaction mix, iScript reverse transcriptase and reacted at 25℃ for 5 min, 42 ℃ for 30 min, 85 ℃ for 5 min. cDNA products were stored at -20 ℃.

Real-time PCR were performed using IQTM SYBR Green Supermix (Bio-Rad, CA) for 40 cycles of 95 ℃ 15 s and 62 ℃ 40 s. Data were analyzed by iCycler Multicolor Real-time PCR Detection System (Bio-rad Labratories, USA) Software. The primer pairs for p53 were: forward: 5'-GTGGAAGGAAATTTGCGTGT-3', reverse TCTGAGTCAGGCCCTTCTGT-3'.

5.2.4 Flow cytometry analysis cell cycle.

HEp-2 cells (10^6) were infected or left uninfected with 0.5 MOI HSV-1 2 h before treatment of TCS with a concentration of 10 μg/ml for 3, 6, 18 and 24 h, Cells (10^6) were then freshly harvested fixed with 70% ethanol in 4℃ overnight, then stained with PI

(Sigma Life Science, USA) solution (100μg/ml) with RNase. The PI fluorescence was detected under FACSCalibur flow cytometry with PE-Texas Red channel.

5.2.5 Cytokinese block micronucleus (CBMN) assay

HEp-2 cells were infected or left uninfected with 0.5 MOI HSV-1 and incubated with 10 or 30 μg/ml TCS for 24 h. The treated cells were incubated with 4.5 μg/ml Cytochalasin B (Sigma Life Science, USA) for 28 h to generate binuclear (BN) cells, cells were then detached by trypsin and prepared for slide by cytocentrifuge slide maker (Xiangyi centrifuge LTD. of Changsha, PRC). The slides were fixed with methanol: glacial acetic acid (3:1, v/v) at 4℃ for 10 min, stained with Giemsa dye (Number three reagent factory of shanghai, China) two times for 5 min. The frequency of Micronucleusd (MNs), nucleoplasmic bridges (NPBs) and nuclear buds (NBuds) were determined by scoring 1000 BN cells.

5.2.6 Statistical analyses

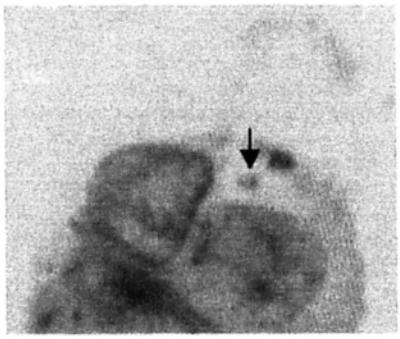
Data were analyzed using two-way ANOVA. A p-value <0.05 was considered as statistically significant. Dose response and EC_{50} (the dose that gives half of the maximal response) were calculated by non-liner regression using the Graphpad Prism software. All data were expressed as mean ± SEM (n=3).

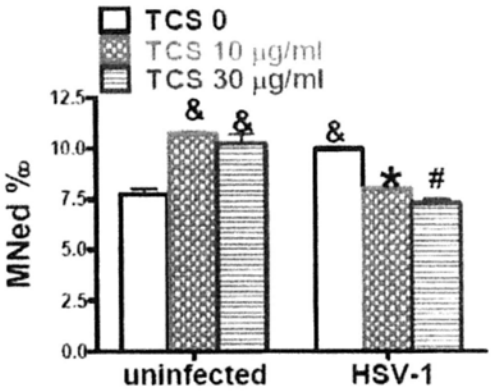
5.3 Results

5.3.1 TCS-induced DNA damage decreased in HSV-1 infected HEp-2 cells

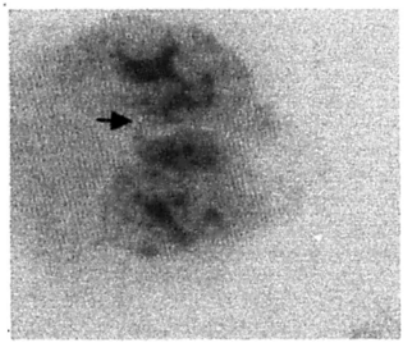
To assess TCS induced DNA damage, CBMN assay was employed and the frequencies of MN, NPB and NBud were observed [156-158]. As data showed (Figure 5.1), TCS induced significantly increase in percentage of MN in BN cells versus control. However, percentage of MN significantly decreased when infected with HSV-1 and treated with TCS versus uninfected ones and did not show significant change compared to control (uninfected, TCS 0). When infected with HSV-1 alone, the percentage of MN and NBud significantly increased compared to control ($p< 0.05$).

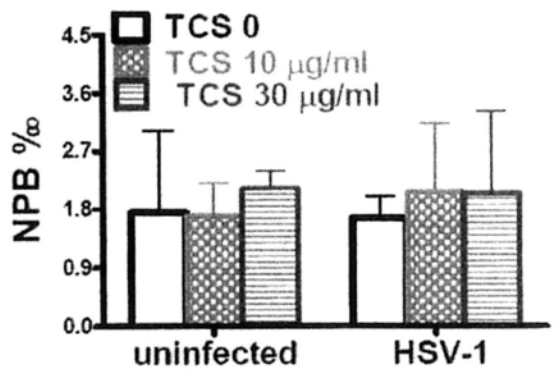
MNed BN cell



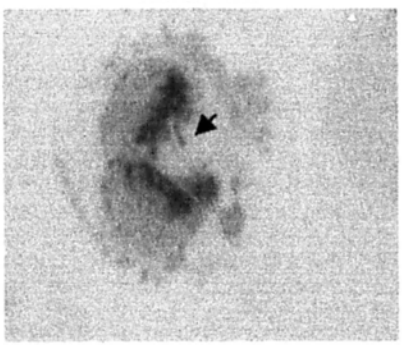


NPB





NBud



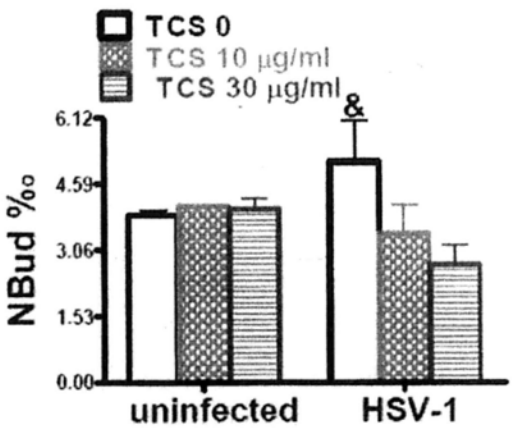
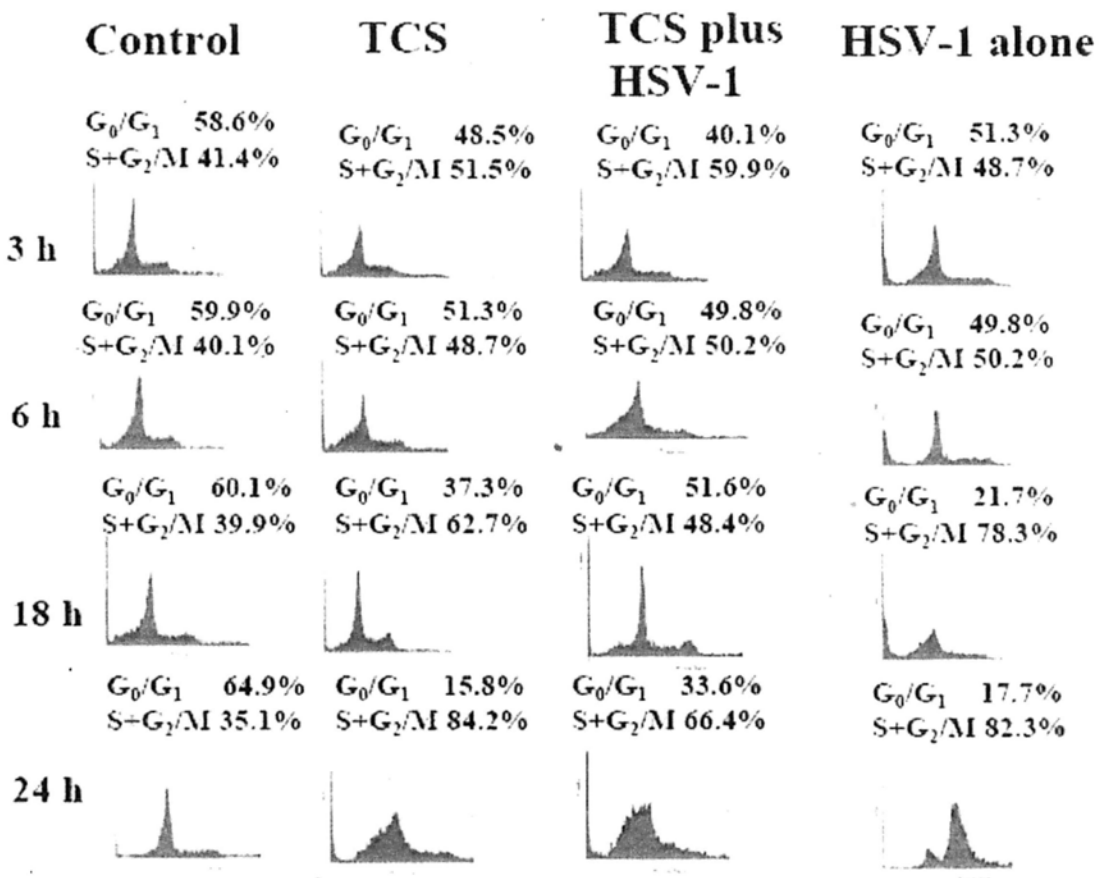


Figure 5.1. Differences in percentage of DNA damage cells under TCS treatment and HSV-1treatment in HEp-2 cells. HEp-2 cells were infected with 0.5 MOI HSV-1 and incubated with 10 and 30 μg/ml TCS for 24 h. The treated cells were then incubated with 4.5 μg/ml Cytochalasin B for 28 h and stained with Giemsa dye. MNs, nucleoplasmic bridges (NPBs) and nuclear buds (NBuds) were determined by scoring 1000 BN cells. Data were shown as mean ± SEM (n=3). Unfilled columns represent infected or uninfected HEp-2 cells without TCS treatment, dot filled column represent infected or mock HEp-2 cells treated by 10 μg/ml TCS, and line filled column represent infected or mock HEp-2 cells treated by 30 μg/ml TCS. & $p<0.05$ v.s. control (uninfected, TCS 0) ; * $p<0.05$ v.s. uninfected with TCS 10 μg/ml; # $p<0.05$ v.s. uninfected with TCS 30 μg/ml.

5.3.2 Cells cycle arrestment participated anti-HSV-1 activity of TCS

To test whether the DNA damage induced by TCS causes cell cycle arrest, the cell cycle distribution of TCS in HSV-1 infected or uninfected HEp-2 cells was studied and compared with normal HEp-2 cells. As the data showed (Figure 5.2), most of the TCS-treated cells were arrested in G_2/ M, as an increase in cells with a 4n DNA content was clearly detected after 18 h of exposure to TCS; these TCS-treated HEp-2 cells showed significant decreases in the G_1 peak at 18 and 24 h, respectively. However, this TCS-induced cell cycle blockage significantly decreased when HEp-2 cells were infected with HSV-1, but was still at a significantly greater level versus the control after 18 h of treatment. Meanwhile the cell cycle of HSV-1 infected HEp-2 cells were explored. The infected cells showed significant decreases in the G_1 peak at 6, 18 and 24 h respectively.


Control
TCS
TCS plus HSV-1
HSV-1 alone
3 h
G_0/G_1 58.6%
$S+G_2/M$ 41.4%
G_0/G_1 48.5%
$S+G_2/M$ 51.5%
G_0/G_1 40.1%
$S+G_2/M$ 59.9%
G_0/G_1 51.3%
$S+G_2/M$ 48.7%
6 h
G_0/G_1 59.9%
$S+G_2/M$ 40.1%
G_0/G_1 51.3%
$S+G_2/M$ 48.7%
G_0/G_1 49.8%
$S+G_2/M$ 50.2%
G_0/G_1 49.8%
$S+G_2/M$ 50.2%
18 h
G_0/G_1 60.1%
$S+G_2/M$ 39.9%
G_0/G_1 37.3%
$S+G_2/M$ 62.7%
G_0/G_1 51.6%
$S+G_2/M$ 48.4%
G_0/G_1 21.7%
$S+G_2/M$ 78.3%
24 h
G_0/G_1 64.9%
$S+G_2/M$ 35.1%
G_0/G_1 15.8%
$S+G_2/M$ 84.2%
G_0/G_1 33.6%
$S+G_2/M$ 66.4%
G_0/G_1 17.7%
$S+G_2/M$ 82.3%

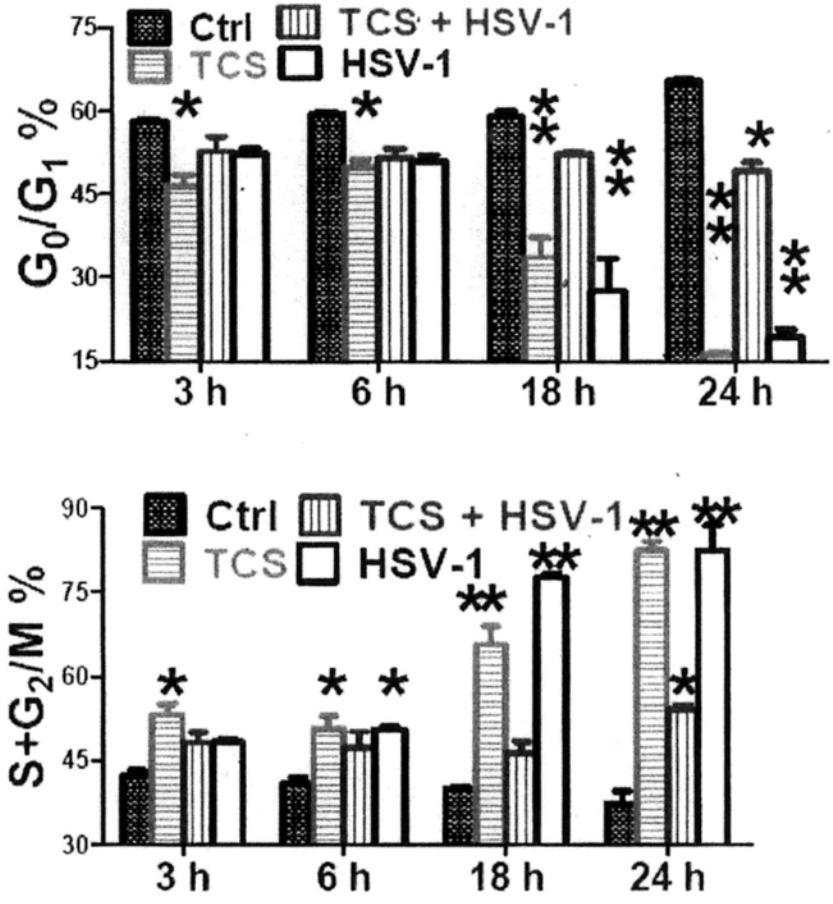


Figure 5.2. TCS interferes with cell cycle of HEp-2 cells. HEp-2 cells were infected or left uninfected with HSV-1 2h before treatment of TCS with a concentration of 10 μg/ml for 3, 6, 18 and 24 h, Cells then stained with PI solution (100 μg/ml) with RNase. The PI fluorescence was detected under FACSCalibur Flow cytometry with PE-Texas Red channel. * and ** $p < 0.05$ and 0.01 vs. control (uninfected, TCS 0).

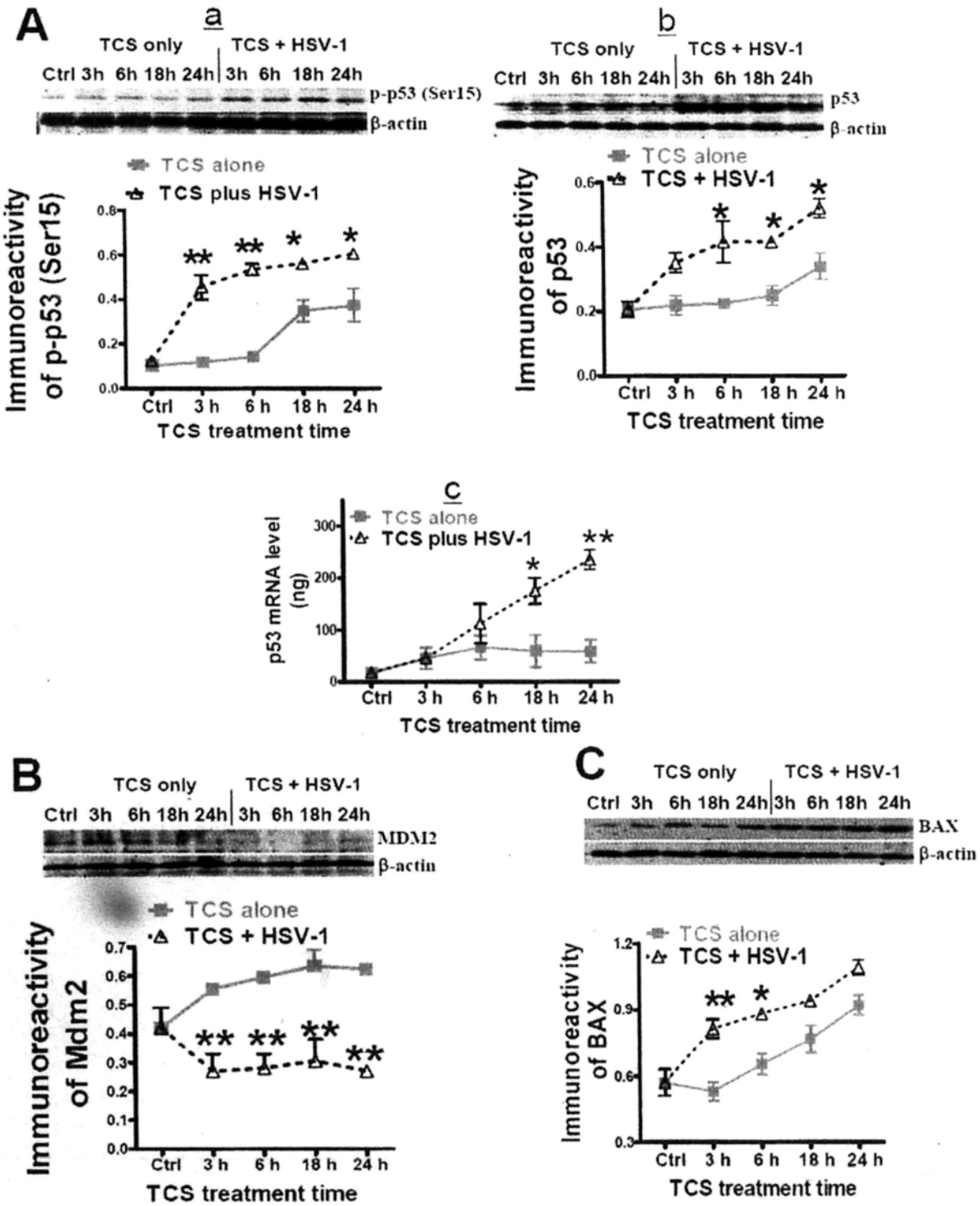
5.3.3 Activation of p53 and BAX during anti-HSV-1 activity of TCS

To further study the role of TCS induced DNA damage in its anti-HSV-1 activity, activation of p53, MDM2 and BAX were studied. As western blot in figure 5.3 A a and b showed, in HEp-2 cells, TCS triggered p53 phosphorylation at Ser 15 at 18 and 24 h treatment, the phosphorylation of p53 and total intracellular p53 increased when infected with HSV-1. Additionally, the expression of p53 was studied by real-time PCR and as data showed (figure 5.3 A c), under the treatment of TCS, the expression level of intracellular p53 increased when Hep-2 cells infected with HSV-1, but had less effect without infection.

At the meanwhile, intracellular level of p53 inhibitor MDM2 decreased ($p<0.05$) by TCS when cells infected with HSV-1 (Figure 5.3 B).

Furthermore, TCS stimulated BAX expression till HEp-2 cells were treated for 6 h (Figure 5.3 C). In contrast, when infected with HSV-1, TCS treatment triggered increase in total amount of BAX in HEp-2 cells.

However, it was also found, the p53, MDM2 and BAX did not showe significant changes in when HEp-2 cells were infected with HSV-1 alone (Figure 5.3 D).


A
a
TCS only
TCS + HSV-1
Ctrl 3h 6h 18h 24h 3h 6h 18h 24h
p-p53 (Ser15)
β-actin
TCS alone
TCS plus HSV-1
Immunoreactivity of p-p53 (Ser15)
TCS treatment time
b
p53
Immunoreactivity of p53
C
p53 mRNA level (ng)
B
MDM2
Immunoreactivity of Mdm2
C
BAX
Immunoreactivity of BAX

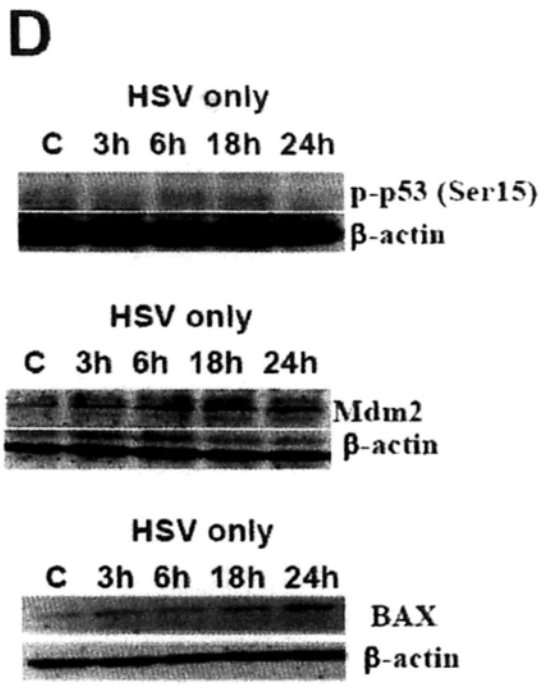


Figure 5.3. TCS activated p53 and BAX. HEp-2 cells were infected or left uninfected with 0.5 MOI HSV-1 2h before 10 μg/ml TCS treatment for 3, 6, 18 and 24 h. Cytoplasmic extract were collected and immune activity of p53, MDM2 and BAX were studied. The filled square line represents TCS alone treatment, the unfilled triangle dash line represents cells infected with HSV-1 and then treated with TCS. (A a-b, B and C) Representative western blotting and immunoreactivity demonstrate TCS effect on p53 phosphorylation (p-p53 Ser15, ~53 kDa), p53 expression (~53 kDa), MDM2 degradation (~90 kDa) and BAX activation (~20 kDa) respectively. (A c) The p53 RNA level during TCS treatment was analyzed by real-time PCR. Standard curve were formed with a dilution series consisted of blended cDNA samples with same qualities (ng). (D) Western blotting demonstrates HSV-1 infection effect on p53, MDM2 and BAX activation in HEp-2 cells respectively *P<0.05, **p<0.01 v.s. TCS only, data were shown as mean ± SEM (n=3).

5.4 Discussion and Conclusion

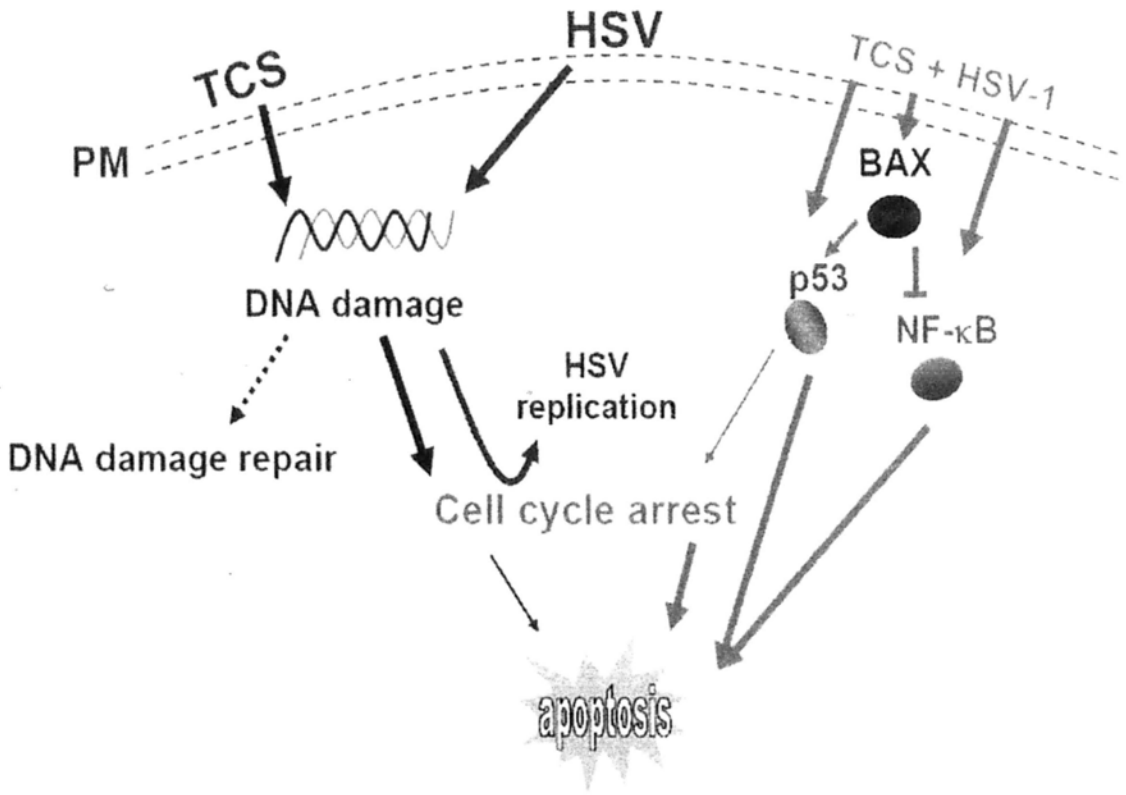


Figure 5.4. Proposed mechanism of anti-HSV-1 mechanism of TCS by using NF-κB and p53 pathways. TCS suppresses the anti-apoptotic function of HSV-1 by suppressing the NF-κB to enhance apoptosis in HSV-1 infected cell. The apoptotic function of p53 is also activated by TCS to transmit cytotoxic signal of TCS.

In chapter 4, the role of NF-κB in ant-HSV-1 effect of TCS was discussed. The p53 gene may provide a binding site for NF-κB within the p53 gene promoter to trigger p53 activation and initiated a cell repair process [159]. Some times the p53 activation directly suppressed NF-κB [160]. Therefore, the NF-κB shows close relationship with p53 in regulating cell growth, but has opposite effect to p53 [126;127;130]. In this study the role

of p53 was explored to demonstrated anti-viral effect of TCS.

Many studies have confirmed p53 activation is a critical response to DNA damage [161;162] induced by ionising radiation, radio-mimetic drugs, ultraviolet light [150;163;164] and chemicals [165]. The p53 activation involves quick expression of p53 and accumulation of phosphorylated p53, then the phosphorylated p53 translocate into nucleus with an enhanced ability to bind DNA and mediate transcriptional activation, and then either cell cycle regulation or apoptosis are initiated. Governing of p53 activation is dependent on phosphorylation and dephosporylation of p53 within its N-terminal and C-terminal regions. Triggered by DNA damage, p53-specific transcription and cell-cycle arrest are aroused to deal with cellular stress. However by now, studies on the role of p53 during cell cycle arrest were controversial, some argued p53 work closely on cell cycle arrest [166], others suggested cell cycle arrest was irrespective of p53 activation [141;167]. Still, all of these studies showed evidences supporting p53 can regulate cell cycle processing and determine proliferation of DNA damaged cells [163;168]. Because the MN, NPB and NBud in cells originates from broken DNA strand or chromosome separated from spindle apparatus [156-158], DNA breakage effects of drugs is usually accompanied with MN, NPB and NBud formation, so in this context the DNA damage was studied by observing ratio of MN, NPB and NBud in BN cells. As the data showed, when treated with TCS, uninfected HEp-2 cells showed significantly increase in MN, NPB and NBud than control cells. Subsequently, a high ratio of G_1 to S and G_2/ M cell cycle phase arrest was triggered, together with a slightly but detectable phosphorylation of p53 at serine 15 that is significantly lower than those in TCS-treated infected cell. The

p53 activation and cell cycle arrest are typical response to DNA damage [161;162]. In these series of responses, p53 is an important factor that determines the fate of DNA damaged cells [168], with less p53 activation, cells may not respond acutely to the DNA damage, or this small amount of p53 activation may stimulate cell cycle checkpoints that subsequently triggers the self-protection system to repair the DNA. Because only a small amount of uninfected HEp-2 cells were killed by TCS, we can conclude p53 is more likely to be activated to allow cells to repair damaged DNA and recover from stress of TCS during the arrested cell cycle [169]. The 10% cell death may originate from cells with severe DNA damage induced by TCS, when the severe DNA damage cannot be repaired, apoptosis of cells is triggered. But the percentage of cells suffering severe DNA damage is much lower than percentage of cells with cell cycle arrest (About 50 %, defined as the percentage differences between TCS treated and control cells in S and G_2/ M phase), the finding further supports our conclusion that the majority of the cells are undergoing a process of repair.

Activation of p53 is not completely related to the response to DNA damage, it can also induce apoptosis. We found in HSV-1 infected cells, although there was no detectable DNA damage and less G_1 to S and G_2/ M phase arrest when treated with TCS versus those in uninfected cells, p53 activation was largely increased by TCS treatment compared to uninfected ones, suggesting that p53 responds in different ways as a result of TCS treatment during HSV-1 infection. Consist with previous studies [170;171], p53 is tightly connected with BAX in its role of induction of cell death, we found that BAX was strongly activated in HSV-1 infected cells by TCS and the 30% cell death was eventually

stimulated. This activation of BAX is related to acute p53 activation triggered by TCS because p53 is a direct transcriptional activator of *bax* gene when the apoptotic signal is introduced [128], then the activated BAX will stimulates a apoptotic pathway. Therefore, based on findings that anti-HSV-1 effect is related to the cytotoxicity of TCS, this part of findings suggests that the activation of p53 by TCS plays an important role in inducing cell death in HSV-1-infected cells to inhibit viral expansion.

Meanwhile, it was found that HSV-1 infection alone induced a noticeable enhancement in MN and NBud ratio but did not show p53 and BAX activation versus the control. According to former studies [172], the DNA damage response of host cells, triggered by dsDNA virus, may benefit the viral replication by prolonging S phase. Indeed, we found that HSV-1 induces an increase in G_1 to S and G_2/ M phase arrest as early as 6 h after infection but the infection alone did not trigger host cell death (Supplemental figure B). These data further support our former conclusion that p53 play an important role in determining the fate of DNA damaged HEp-2 cells. With little or without p53 activation, although there are DNA damage and cell cycle arrest in both TCS treated HEp-2 cells and HSV-1 infected but TCS untreated cells, the activation of apoptosis is limited by the status of p53.

In conclusion, this study suggests evidence that how the cytotoxicity relates to the anti-HSV-1 mechanism of TCS. The NF-κB pathway, activated by HSV-1 infection to benefit viral replication, is inhibited by TCS. Meanwhile, TCS induces p53 related cell death signaling that killed HSV-1 infected cells. In Figure 5, we provide a sketch graph demonstrating a possible anti-HSV-1 mechanism of TCS by modulating the NF-κB and

p53 pathways.

Chapter 6

General discussion and conclusion

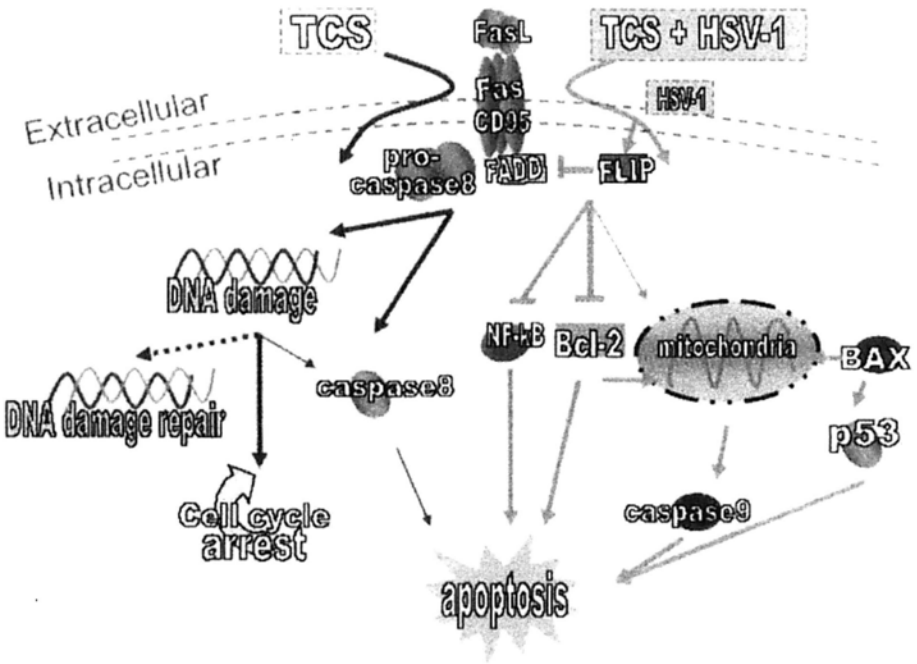


Figure 6.1. Proposed mechanism of anti-HSV-1 mechanism of TCS. In HSV-1 infected cells, TCS induces mitochondrial-dependent type II apoptotic pathway. At the same time, the apoptotic function of p53 is activated by TCS, playing as another apoptotic pathway induced by TCS in infected cells. When the CD95 is suppressed by HSV-1 infection, the p53 activation may also transmit the apoptotic signal of TCS onto mitochondrial through BAX activation. On the other hand, NF-κB pathway was suppressed by TCS to weaken the anti-apoptotic ability of HSV-1, therefore, the TCS-induced apoptosis pathway is potentiated.

The pharmacological activity of TCS is ranging from abortificiency and antitumor to apoptosis-inducing and anti-virus. The mechanism of both TCS-generated apoptosis and antiviral effect has been discussed when studies have found TCS is able to inhibit HIV in vitro through host cell apoptosis [86], which is participated by RIP and MAPK pathway [27;30] . In this study, we focused on another virus, HSV-1, to demonstrate the antiviral mechanism of TCS and its relationship with apoptosis pathways. This study aims to provide more information to understand the action principle of TCS, the replication principle of HSV-1 and potential ways to inhibit and interfere with HSV-1 replication.

At the very beginning, it was found TCS exhibited an anti-HSV-1 activity by interfering with the viral early to late gene expression. This HSV-1 inhibition ability is related to an enhanced cytotoxicity of HSV-1 infected cells than uninfected cells. However, the HSV-1 is a fine evolved DNA virus stay in organism either in latent or infectious status, it can not only utilize critical factors in the host cell in favor of its replication, but also established complicate mechanism to suppress host cell immunoresponse. The most typical examples of HSV-1 interference were demonstrated by interactions between the virus and a widely applied protein synthesis inhibitor, CHX. When CHX is applied to induce cell apoptosis without suppressing HSV-1 de novo anti-apoptosis protein synthesis, HSV-1 inhibits the apoptosis induced by CHX [69]; when CHX is used to inhibit HSV-1 anti-apoptosis protein synthesis, apoptosis inhibitory function of HSV-1 was abolished [68]. These findings have drawn our interests because TCS is also a protein synthesis inhibitor effects by its ribosome inactivating activity and HSV-1 was not able to inhibit TCS induced apoptosis even when TCS did not suppress

HSV-1 de novo anti-apoptosis protein synthesis. According to the data, the IE gene especially ICP27, which is responsible for host cell apoptosis inhibition, was still expressed during effectively anti-viral TCS treatment. Additionally, when the TCS treatment time point was delayed as late as 3 h after infection when the de novo anti-apoptosis protein already synthesized, TCS also killed infected cell. These evidences consist with former studies that TCS's anti-HSV-1 activity is related to its cytotoxicity [29] and suggest TCS may not only rely on ribosome inactivating activity to induce HSV-1 infected cells to apoptosis.

Therefore, we subsequently worked on several different apoptosis or apoptosis related pathways to demonstrate reasons of why TCS kills more HSV-1 infected cell regardless anti-apoptosis function of the virus.

Firstly, the TCS-induced CD95 (APO-1/ Fas) mediated apoptotic pathways were studied. The study showed TCS-induced apoptosis involves CD95 (APO-1/ Fas) mediated pathways, however, HSV-1 infection exhibited impact on the CD95 (APO-1/ Fas) signaling by stimulation a DISC inhibitor, FLIP, expression that attempts to suppresses the infection-induced immunoresponse, so when TCS was applied, the CD95 (APO-1/ Fas) dependent pathway (type I apoptotic pathway) turned into CD95 (APO-1/ Fas) less dependent but mitochondrial dependent type II apoptotic pathway. The HSV-1 tried to ensure host cell alive by expressing FLIP, it at the meanwhile render the cell to the type II apoptotic pathway under TCS treatment, but the type II apoptosis pathway consists of a wider set of signaling molecules and is more sensitive to a number of apoptosis inhibitors or activators than type I pathway [32;92;104;105], therefore more

HSV-1 infected cells are killed by TCS.

At the meanwhile, by combining the result of BAX and Bcl-2 in chapter 3 and 5, the activity ratio of Bcl-2/ BAX is higher in TCS treated cell than infected cell treated with TCS. Some studies suggested the lower ratio of Bcl-2/ BAX is indicative of activation of mitochondrial related pathway [141;173]. Therefore the data additionally supported our conclusion that TCS triggers HSV-1 infected cell undergo type II apoptotic pathway.

By this step, we still can not explain why TCS could surmount anti-apoptotic function of HSV-1. Then we processed to study the NF-κB, an important nuclear factor widely implicated during viral infections. NF-κB activated as early as 3 h after HSV-1 infection triggered by ICP27 synthesis [117], is required for apoptosis prevention during viral infection [118]. This nuclear factor may participate or initiate the anti-apoptosis viral function, inhibition of NF-κB activity induces cell death of HSV-1 infected host cells. We found in our system, the HSV-1 infection induced robust and persistent translocation and DNA binding of NF-κB, suggesting the natural anti-apoptosis role of HSV-1. Interestingly, TCS strongly inhibited such activation of NF-κB, both translocation and DNA binding of NF-κB were diminished. Therefore on one hand, the NF-κB suppression by TCS explains the reason why TCS killed more HSV-1 infected cell, because HSV-1 is unable to utilize NF-κB to facilitates its interfering on host cell immunoresponse, so the HSV-1 itself is a stress to the host cell with additional stress from TCS, the cell suffers more cytotoxicity. On the other hand, because TCS inhibited NF-κB activation, expression of many proteins that benefit cell survival, such as expression of Bcl-2 showed in our formal experiment, is abolished, so it is suggesting the reason of why TCS

could surmount the anti-apoptotic effect of HSV-1, it is possible that the synthesis of some host cell proteins required for HSV-1 signaling interfering and apoptosis inhibiting decreased when the NF-κB is no longer unavailable.

P53, a transcriptional activator, plays opposite role to NF-κB. We thereby continue to investigate the p53 modulation during anti-viral effect of TCS. P53 usually activated in response to genotoxic agencies inducing DNA damage. The activation of p53 associates with cell cycle regulation and apoptosis. Here, we demonstrated although TCS induced more DNA damage and cell cycle arrest in normal cells, but more p53 and BAX were activated by TCS in infected cells. The p53 majorly plays an apoptotic role when there are BAX participates its activation [142], Additionally and importantly, we found HSV-1 alone induced noticeable DNA damage and cell cycle arrest without p53 and BAX activation in order to maximize HSV-1 replication [172]. This data between TCS treated and untreated infected cells support the idea that TCS induces infected cell directly into apoptosis with p53 and BAX activation, perhaps because the less deadly damage caused by HSV-1 is intensified by TCS when TCS itself also induces DNA damage, at this stage, with NF-κB related anti-apoptosis effect abolished, the double stress is too serious to go through a mild repairing process, so the cells undergo apoptosis. At the same time, the BAX participation was demonstrated in infected cells treated with TCS, because $\Delta \Psi_m$ is usually broke down by BAX insertion into mitochondrial transmembrane to lead the type II apoptotic pathway, so this part of data supports the former conclusion that TCS induce HSV-1 infected cell into type II apoptotic pathway.

In conclusion, this study produced evidence on anti-HSV-1 mechanism of TCS. TCS

shows its anti-HSV-1 activity by enhancing cell death in infected cells, the enhanced cell death is related to a type I to type II apoptotic pathway when there is HSV-1 infection. At the meanwhile, during TCS induced apoptosis in HSV-1 infected Hep-2 cells, the HSV-1 induced NF-κB activation is suppressed by TCS, and apoptotic function of p53 is triggered, further contributes to the enhanced cell death in HSV-1 infected cells triggered by TCS. In figure 6.1, a sketch map on the anti-HSV-1 mechanism of TCS was provided.

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