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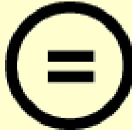

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碩士學位 請求論文

Antioxidant Effects of Diallyl sulfide, Capsaicin, Gingerol and Sulforaphane in H_2O_2-Stressed HepG2 cells

2011

誠信女子大學校 大學院

食品營養學科

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이 論文을 碩士學位 論文으로 提出함

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認　准　書

李 昭 兗 의 碩士學位 論文을 認准함.

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誠信女子大學校 大學院

Abstract

Oxygen is necessary to sustain life, yet the very process of oxygen metabolism in the cells creates destructive elements called free radicals. Reactive oxygen species (ROS) are generated during oxidative stress, including hydroxyl radical superoxide, peroxyl radical, hydrogen peroxide and singlet oxygen. Free radicals, or oxidants, are chemically unbalanced, carrying free electrons that can damage molecules in our cells while trying to achieve balance - potentially damaging the cell itself. Fortunately, the body has its own free radical defense system. Virtually every cell produces antioxidant enzymes called Superoxide Dismutase (SOD), catalase and glutathione peroxidase. These enzymes protect the cells during oxygen metabolism, safely breaking down harmful free radicals to balanced elements like H_2O.

However, our antioxidant defense system can become overwhelmed. Many studies indicate the levels of SOD, catalase and GPx decrease with age. Also, certain conditions are related to the increased production of unstable oxygen derivatives, including physical stress, health challenges and exposure to environmental toxins such as smoking and pollution [1].

For this reason, many antioxidant products, including supplement and functional foods, are being developed. However, the bioactivity of dietary antioxidants is often quite low, and the amounts needed to be consumed daily to remove free radicals and inhibit oxidative stress are expensive and unsafe. In addition, this approach is designed to correct a possible antioxidant deficiency, and does not specifically promote the body' s own endogenous antioxidant defenses. Natural products are rich source of pharmacologically active compounds in which plant materials deserves an important position. Naturally occurring organosulphur compounds from garlic and onion mediate significant chemopreventive activities against the initiation stage of carcinogenesis induced by various chemical carcinogens [2,3].

Therefore, this study examined the effect of Antioxidant capacity in Korea traditional seasoning ingredients that usually eat a lot. Garlic organosulfur compounds are recognized as a group of potential chemopreventive compounds. among these, diallyl sulfide(DAS) is known that can modulate drug metabolism systems, especially various phase II detoxifying enzymes, though the mechanism underlying their inductive effect on these enzymes remains largely unknown. antioxidant enzymes.

Capsaicin (CAP), N-vanillyl-8- methyl-1-nonenamide, is a pungent ingredient in varieties of red pepper of the genus Capsicum. Gingerol (GGR), a major component of ginger, has antioxidant, anti-apoptotic, and anti-inflammatory activities. also Outstanding antioxidant gingerol inhibited the tumor promotion stage in skin carcinogenesis mice has been reported [4]. Sulforaphane (SFN), is already revealed as biologically active compound extracted from cruciferous vegetables, and possessing potent anti-cancer and anti-inflammatory activities.

In the present study, investigated the antioxidnt enzyme levels of superoxide dismutase(SOD), catalase(CAP), glutathione(GSH) contents and glutathione peroxidase and production of Lipid peroxidation (LPO) and the protein level of transcription factor, Nuclear factor-kB(NFkB), nuclear factor E2-related factor 2 (Nrf2), after administration of four natural compound DAS, CAP, GGR and SFN in human hepatoma HepG2 cells.

So the aim of this study was to investigate the antioxidant effect of DAS, CAP, GGR, SFN and response of the antioxidant defense system to oxidative stress, hydrogen peroxide(H_2O_2) in HepG2 cells. The results of this study were as following ;

1. CCK data

First, appropriate concentration and incubation time were decided 200μM H_2O_2 to induce 30% cell death and each antioxidant as following ; DAS(100, 200μM), CAP(25, 50μM), GGR(50, 100μM) and SFN(12.5, 25μM) by safe range of the cytotoxicity. And then each antioxidants were treated after H_2O_2. As a result each groups elevated significantly ($p<0.05$) the restoration compared to only H_2O_2-treated cells. But there was no drastic induction of viability.

2. ROS data

ROS is important mediators of liver damage. The result shown that exposure to H_2O_2 elevated directly amount of ROS by uo to 220% and all of the treatment significantly reduced intracellular ROS level compared to the negative control by up to almost similar with control group [Fig 12A]. Interestingly, ROS could be more efficiently removed by concentration of each chemicals such as DAS : 200μM, CAP : 25μM, GGR : 50 μM and SFN : 12.5μM than another concentration

3. Enzyme data

In our experimental conditions, treatment of HepG2 cells with H_2O_2-induced cells significantly decrease in the activity of antioxidant enzymes such as SOD, CAT and GPx. whereas GSH in cells treated with 200 μM DAS, 25 μM CAP, 50μM GGR and 12.5μM SFN for 12, 24hr showed a significant recovery. this result suggest that H_2O_2-induced oxidative stress in HepG2 cells depleted endogenous antioxidant defense system.

4. LPO

LPO concentration was increased significantly in cells treated with H_2O_2 compared to control cells after 12h and 24hr. However, the LPO level with DAS, CAP, GGR, SFN treatment in the presence of H_2O_2 caused significantly decreased the level of LPO in 12 than 24hr.

5. Western blotting

In this study confirm that DAS, CAP, GGR and SFN increased transcription factor Nrf2 related with antioxidant system and Nrf2 was regulated by activation of the MAPK-JNK. Futhermore, each antioxidants inhibited activation of TNFα

and NF-kB by downregulation of phospholylation of IkBα. The inhibition of TNFα and NF-kB mean that DAS, CAP, GGR and SFN decreased inflammation factor induced by ROS.

In conclussion, these results indicate protective effects of DAS CAP, GGR and SFN against H_2O_2-induced oxidative stress. By increasing the SOD, CAT and GPx activity which removes peroxides and superoxides, also might prevent the accumulation of ROS by removing them. These modulations in the antioxidant enzyme system, which upregulate the host detoxification process, might be associated with reduced risk of pathologies which are related to the ROS-mediated cellular damage. therefore, Garlic, Red pepper, Ginger and Brocoli intake might contribute to the dietary prevention of those.

Contents

Abstract

Ⅰ. Introduction ··1

1. Reactive Oxygen Species and Antioxidant Mechanism ·······1
2. Cell signaling : Stress and Antioxidation ·······························9
3. Natural Antioxidant Materials ···13

Ⅱ. Materials and Methods ···20

1. Materials ···21
2. Cell Culture ···22
3. Cell Treatment ··22
4. Cell proliferation -CCK8 ···23
5. Measurement Of Intracellular ROS ·····································24
6. Antoxidant Enzyme Assay ···25

1) Superoxide Dismutase (SOD) Activity 25
2) Catalase (CAT) Activity 27
3) Glutathione (GSH) Levels 28
4) Glutathione Peroxidase (GPx) Activity 29
7. Lipid Peroxidaion(LPO) production 30
8. Western Blotting 31
9. Statistical Analysis 32

Ⅲ. Results 33

1. Cell Proliferation 33
2. Intracellular ROS Formation 37
3. Antoxidant Enzyme Activity 41
1) Superoxide Dismutase (SOD) Activity 41
2) Catalase (CAT) Activity 44
3) Glutathione (GSH) Content 47
4) Glutathione Peroxidase (GPx) Activity 50
4. Lipid Peroxidaion(LPO) production 53
5. Protein Expression in HepG2 cells 56

Ⅳ. Discussion ······ 65

Ⅴ. Conclusion ······ 76

References

국문개요

Appendix

List of Tables

Table 1. Effect of DAS, CAP, GGR and SFN on the activity of SOD in H_2O_2 induced Human Hepatoma HepG2 Cells ……42

Table 2. Effect of DAS, CAP, GGR and SFN on the activity of CAP in H_2O_2 induced Human Hepatoma HepG2 Cells ……45

Table 3. Effect of DAS, CAP, GGR and SFN on the level of GSH in H_2O_2 induced Human Hepatoma HepG2 Cells ……48

Table 4. Effect of DAS, CAP, GGR and SFN on the activity of GPx in H_2O_2 induced Human Hepatoma HepG2 Cells ……51

Table 5. Effect of DAS, CAP, GGR and SFN on MDA reduction in H_2O_2 induced Human Hepatoma HepG2 Cells ……54

List of Figure

Figure 1. Relevant sites of production of ROS and antioxidant systems in a generic cell type ······ 2
Figure 2. Factors of ROS generation and Reductoin process ····· 4
Figure 3. Three important intracellular enzymes constitute antioxidant defense; superoxide dismutase (SOD), catalase, and the GSH peroxidase/GSSG reductase system ······ 6
Figure 4. Oxidative stress regulation the activation of the transcription factor Nrf2 by MAPK ······ 10
Figure 5. Oxidative stress regulation the activation of the transcription factor NF-kB by phospholylation of IkB-α ······ 12
Figure 6. Structure of Diallyl sulfide (DAS) ······ 15
Figure 7. Structure of Capsaicin (CAP) ······ 16
Figure 8. Structure of Gingerol (GGR) ······ 17
Figure 9. Structure of Sulforaphane (SFN) ······ 18
Figure 10. The experimental design of this study ······ 20
Figure 11. Cell was treated with each antioxidant after treatment 200μM H2O2 for 10min and 12 or 24hr later, cell was

detected with CCK-8 ······36
Figure 12. Inhibitory effect of DAS, CAP, GGR and SFN on the H2O2-induced intracellular accumulation of ROS ······40
Figure 13. Effect of DAS, CAP, GGR and SFN on the activity of SOD in H2O2 induced Human Hepatoma HepG2 Cells ······43
Figure 14. Effect of DAS, CAP, GGR and SFN on the activity of CAP in H2O2 induced Human Hepatoma HepG2 Cell ······46
Figure 15. Effect of DAS, CAP, GGR and SFN on the level of GSH in H2O2 induced Human Hepatoma HepG2 Cells ······49
Figure 16. Effect of DAS, CAP, GGR and SFN on the activity of GPx in H2O2 induced Human Hepatoma HepG2 Cells ······52
Figure 17. Effect of DAS, CAP, GGR and SFN on LPO reduction in H2O2 induced Human Hepatoma HepG2 Cells ······55
Figure 18. Effect of DAS, CAP, GGR and SFN on the expression of oxidative stress markers ······61
Figure 19. Effect of DAS, CAP, GGR and SFN on the expression of antioxidative signals ······64

Figure 20. The cooperative regulation of Nrf2 and NF-κB has been shown to play a crucial role in translation cellular stress signal into an anti-inflammatory response ……75
Figure 21. Diagram for effect of DAS, CAP, GGR and SFN on the anioxidant system and cell signaling ……78

<Appendix>

Figure 22. Cell proliferation of HepG2 cells treatmented with H_2O_2
Figure 23. Cell proliferation of HepG2 cells treatmented with DAS, CAP, GGR and SFN

Ⅰ. Introduction

1. Reactive Oxygen Species and Antioxidant Mechanism

1) Reactive Oxygen Species (ROS)

Reactive oxygen species (ROS) include the superoxide anion radical ($O_2\cdot^-$), hydroxyl radical ($OH\cdot$), singlet oxygen (O_2) and hydrogen peroxide (H2O2) are generated residual product of normal cellular metabolism or results of exogenous factors. [Fig 1] Generation of ROS is inevitable for aerobic organisms, and, in healthy cells, occurs at a controlled rate. Under conditions of oxidative tress, ROS production is dramatically increased, resulting in subsequent alteration of membrane lipids, proteins, and nucleic acids. Oxidative damage of these biomolecules is associated with a variety of pathological events including atherosclerosis, carcinogenesis, ischemic reperfusion injury, neurodegenerative disorders[5,6] and with aging [7,8].

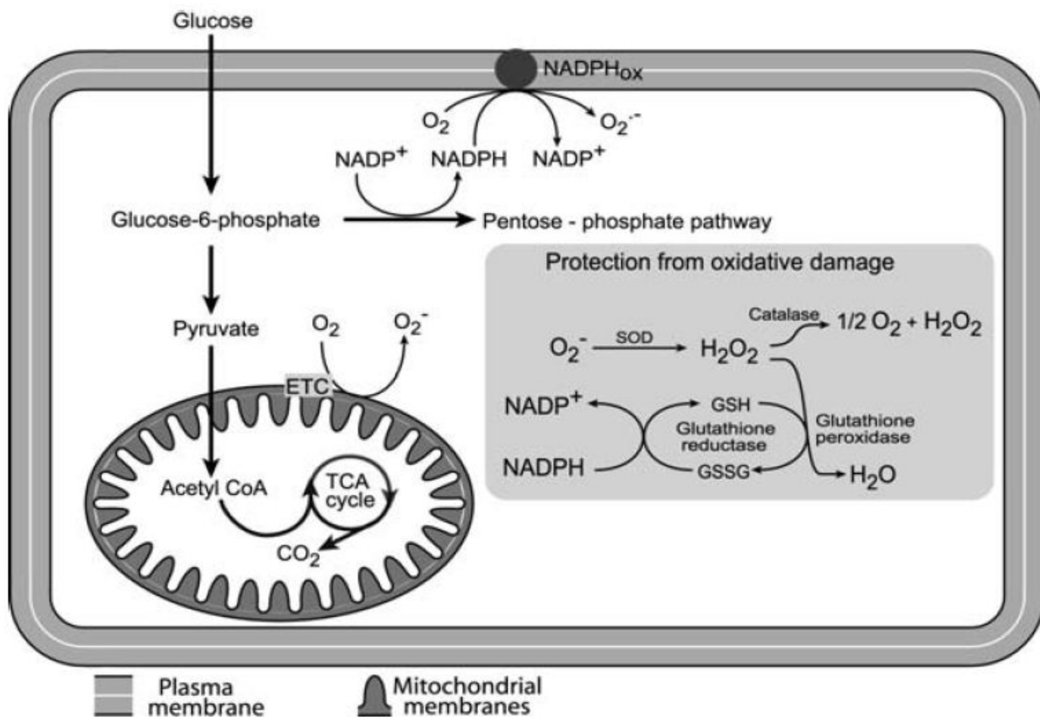


Fig 1. Relevant sites of production of ROS and antioxidant systems in a generic cell type.

Although the mitochondrial oxidation-reduction system and cytochrome P450 monooxygenase are well known generators of superoxide anion radicals, there are several other potential candidates for ROS generation, including xanthine oxidase, lipoxygenase, mitochondrial oxidase, NO synthase (NOS), and NADH/ NADPH oxidase. Superoxide anion radicals are converted to H2O2. [Fig 2] [9]

Under conditions of elevated metabolism or enhanced mitochondrial activity many tissue specific cells are continuously subject to insult from reactive oxygen species (ROS). The damage inflicted by ROS has been implicated in conditions of inflammation, diabetes mellitus, age-related degeneration and tumour formation [10]. Futhermore overproduction of ROS or a failure in intracellular defences against ROS will result in pathogenesis of disease [11].

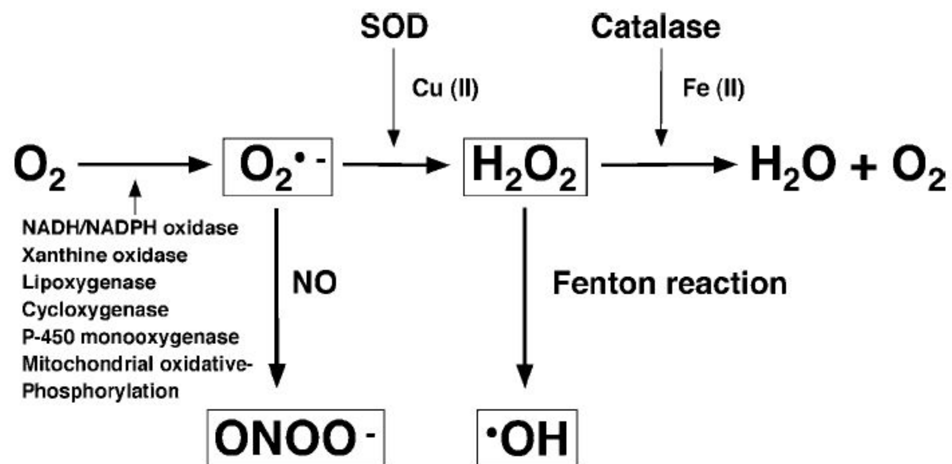


Fig 2. Factors of ROS generation and Reductoin process

2) Antioxidant Mechanism

Cells require antioxidant systems to neutralize ROS. For example, superoxide anions are enzymatically converted to hydrogen peroxide by a manganese superoxide dismutase (MnSOD) within mitochondria. Hydrogen peroxide can then be rapidly removed by the mitochondrial enzyme glutathione (GSH) peroxidase. A further antioxidant enzyme, catalase, is the major hydrogen peroxide detoxifying enzyme found exclusively in peroxisomes [9].

The normal function of superoxide dismutase (SOD) is to convert toxic superoxide radicals into H2O2 that are subsequently inactivated by catalase. In amyotrophic lateral sclerosis this antioxidant protein is converted into a pro-oxidant protein [12]. Superoxide dismutase (SOD) is an enzyme that removes the superoxide (O2-) radical. Simply stated, SOD out competes damaging reactions of superoxide, thus protecting the cell from superoxide toxicity. The reaction of superoxide with non-radicals is spin forbidden.

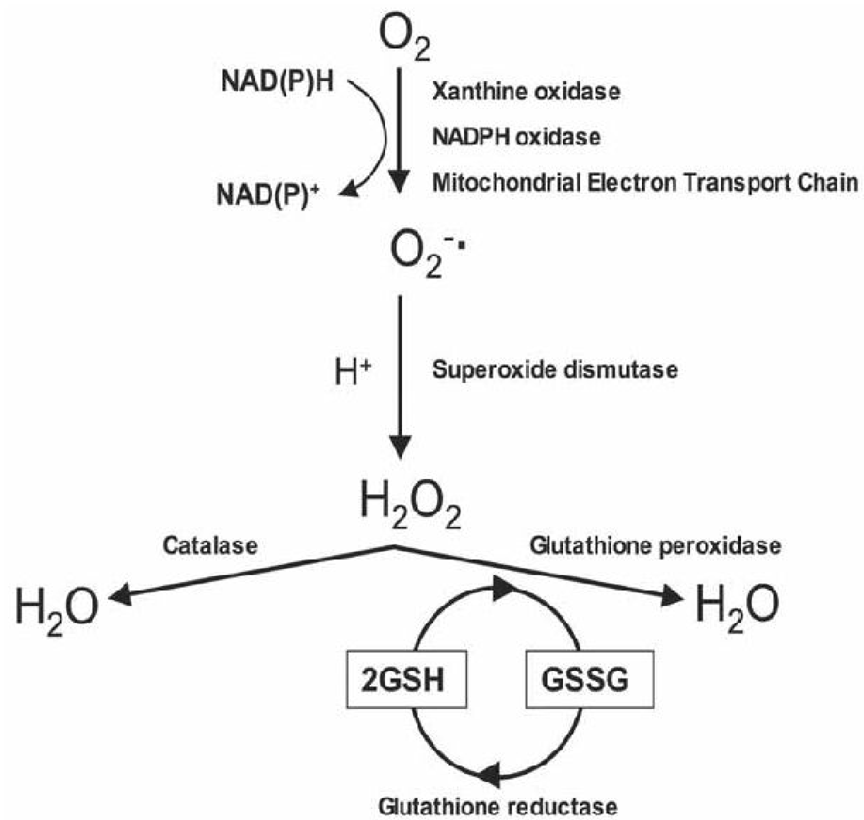
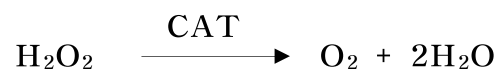


Fig 3. Three important intracellular enzymes constitute antioxidant defense; superoxide dismutase (SOD), catalase, and the GSH peroxidase/GSSG reductase system

In biological systems, this means its main reactions are with itself (dismutation) or with another biological radical such as nitric oxide (NO) or a metal. The superoxide anion radical (O2−) spontaneously dismutes to O2 and hydrogen peroxide (H2O2) quite rapidly (at pH 7). SOD is necessary because superoxide reacts with sensitive and critical cellular targets.

Catalase is usually located in a cellular organelle called the peroxisome. Catalase is a common enzyme found in nearly all living organisms that are exposed to oxygen, where it functions to catalyze the decomposition of hydrogen peroxide to water and oxygen [13]. Catalase has one of the highest turnover numbers of all enzymes; one molecule of catalase can convert millions of molecules of hydrogen peroxide to water and oxygen per second [14]. catalase is frequently used by cells to rapidly catalyze the decomposition of hydrogen peroxide into less reactive gaseous oxygen and water molecules[15].



Glutathione (L-γ-glutamyl-L-cysteinylglycine; GSH) is a tripeptide widely distributed in both plants and animals, and usually located in a cellular cytosol and mitocondria. GSH plays a key role in many biological processes, including the synthesis of proteins and DNA, the transport of amino acids, and the protection of cells against oxidation. Harmful hydrogen peroxide cellular levels are minimized by the enzyme glutathione peroxidase (GPx) using GSH as a reductant. The GSSG, oxidized GSH dimer, is produced during the reduction of hydroperoxide by GPx reaction (see below). however GSSG is reduced to GSH by glutathione reductase (GR) and it is the reduced form that exists mainly in biological system.

2. Cell signaling : Oxidative Stress and Antioxidation

Even if cells have a number of antioxidant mechanisms available, it is still possible for ROS to avoid antioxidant defence mechanisms, resulting in a slow accumulation of chronic damage. So the mitochondrion and nucleus contain a variety of DNA repair enzymes to correct oxidant-induced modifications [16]. Tanscription factor, nuclear factor -E2-related factor-2 (Nrf2) serves as a cellular stress sensor and is a key regulator for induction of hepatic detoxification and antioxidative stress systems [17]. Several stimuli, including oxidative stress, lead to the activation of Nrf2 signaling involves cytosolic stabilization and subsequent nuclear translocation of Nrf2 via oxidation or covalent modification of distinct Keap1 cysteine residues, enhanced degradation of Keap1, and phosphorylation of Nrf2. After translocation to nucleus, Nrf2 forms a heterodimer with small Maf protein and binds to antioxidant response element (ARE). The ARE is a cis-acting regulatory element in promoter regions of several genes encoding phase II detoxification enzymes and antioxidant proteins. These include glutathione peroxidase (GPx), g-glutamylcysteine ligase (GCL), glutathione S-tranferase (GST), superoxide dismutase (SOD), and NAD(P)H: quione oxidoreductase 1 (NQO1) [18,19]. [Fig 4]

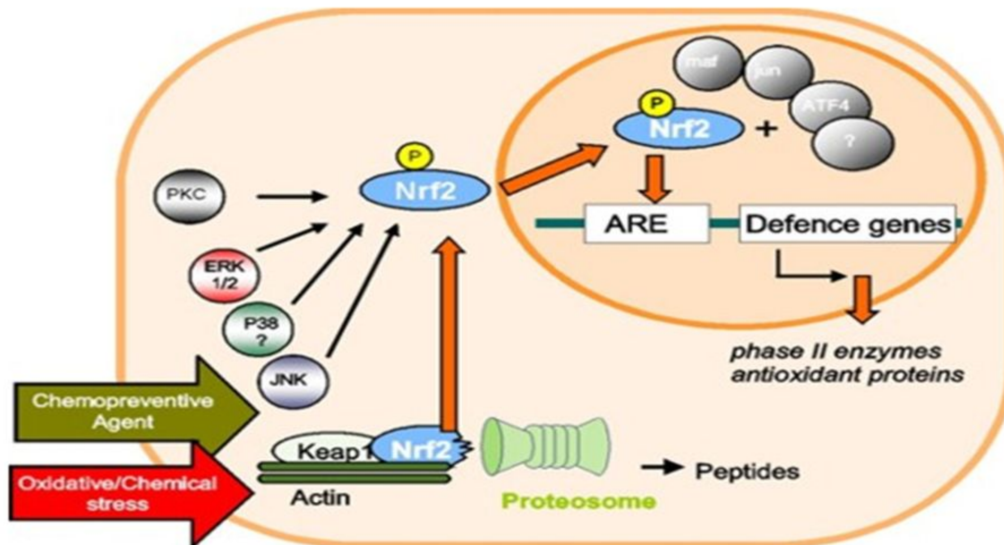


Fig 4. Oxidative stress regulation the activation of the transcription factor Nrf2 by MAPK

While NF-kB can be also activated by a wide variety of stimuli such as tumor necrosis factor (TNF)-a, interleukin-1 (IL-1), UV light, and reactive oxygen species (ROS) [20]. Of these factors, ROS have been reported to play a key role in carcinogenesis by inducing oxidative DNA damage [21]. In addition, ROS are thought to contribute to carcinogenesis through interference with signal cascade systems, including nuclear factor kB (NF-kB). Also, ROS has been deeply implicated in TNF-α-mediated NF-kB activation through phosphorylation of IkB-α and p65 [22].

In the most cells, NF-kB is usually sequestered by IkB-α complexes in the cytoplasm [6.8]. Rapid phosphorylation and degradation of IkBa allows for the translocation of the NF-kB complex into the nucleus [23]. [Fig 5]

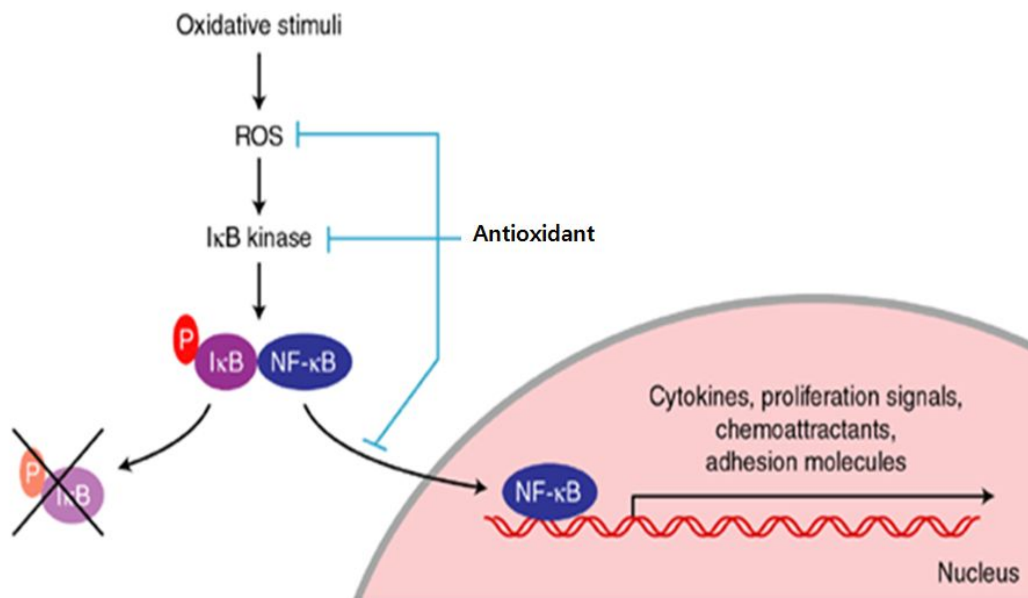


Fig 5. Oxidative stress regulation the activation of the transcription factor NF-kB by phospholylation IkB-α

3. Natural Antioxidant Materials

Consumption of fruit and vegetables containing large amounts of antioxidative nutraceuticals has been associated with the balance of the free radicals/antioxidants status, which helps to minimize the oxidative stress in the body and to reduce the risk of cancers [24].

A naturally occuring plant-derived flavonoid, has been at the centre of several genetic toxicity and carcinogenicity investigations. Especially garlic and ginger and chili, as the traditional foods that can be easily consumed a lot, possesses a wide range of both potentially detrimental and protective biological characteristics. First, garlic has been recognized since ancient times not only as a flavoring agent for food but also for its medicinal properties, including bactericidal, antineoplastic, hypolipidaemic and hypocholesterolaemic effects [25]. This chemopreventive effect has been further confirmed by many animal studies, in which garlic extract and compound administration inhibited tumorigenesis in tissues including liver [26], colon [27], and breast [28].

Hot chili peppers that belong to the plant genus capsicum are among the most frequently consumed spices throughout the world. The principal pungent ingredient present in hot red pepper and chili pepper is the phenolic substance named

capsaicin. Capsaicin inhibited constitutive and induced activation of NF-KB in buman malignant-melanoma cells, leading to inhibition of melanoma-cell proiitcration. Capsaicin also induced apoptosis in cultured Jurkat cells through generation of reactive oxygen species(ROS) [29,30].

Ginger (Zingiber officinale Roscoe, Zingiberaceae) is among the most frequently and heavily consumed dietary condiments throughout the world. In our country, Ginger are widely used as material of kimchi, snacks, drinks, teas and other things. Besides its extensive use as a spice, the rhizome of ginger has also been used in traditional oriental herbal medicine for the management of such symptoms as common cold, digestive disorders, rheumatism, neurologia, colic and motion- sickness [31-35].

Sulforaphane (SFN) is one of the most abundant isothiocyanates in some cruciferous vegetables, especially broccoli [36]. Already many studies present SFN have focused on the chemopreventive effects that lead to increased expression of multiple antioxidant proteins. The cytoprotective effect exerted by this compound is mediated through the transcription factor Nrf2 [37].

Garlic (Allium sativum) has been extensively used as a medicine for many centuries, and its positive effects on human health have been demonstrated throughout this long-term usage [38]. When garlic is cut, chopped or crushed, the clove's membrane is disrupted and S-allylcysteine sulfoxide is transformed enzymatically into allicin by allinase. The main bioactive components of garlic are sulfur compounds, which can be classified as either water-soluble, such as allicin, Sallyl cysteine, or oil-soluble, such as diallyl sulfide (DAS), diallyl disulfide(DADS) and diallyl trisulfide (DATS). Especially DAS has been shown to inhibit several chemically induced forms of cancer, such as benzo(a)pyrene-induced forestomach tumors and pulmonary adenomas [39], diethylstilbestrol- induced breast cancer in rats [40] and cyclophosphamide -induced chromosomal aberrations in Swiss albino mice [41].

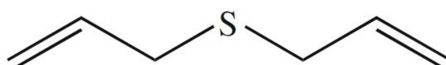


Fig 6. Structure of Diallyl sulfide (DAS)

For centuries, humans have utilized capsicum for the preparation of spicy foods and traditional medications.
capsaicinoids are the principal pungent substances found in "hot" peppers. Six naturally occurring compounds have been characterized as follows: capsaicin, dihydrocapsaicin, nordihydrocapsaicin, homocapsaicin, homodihydrocapsaicin, and nonivamide. capsaicin is the most abundant of the capsaicinoids, constituting approximately 40-60% of the total capsaicinoid contentin hot pepper products; dihydrocapsaicin constitutes 20-40%, with the remainder being the other capsaicinoids. Many studies has also shown The antioxidant activity of capsaicin. Additionally capsaicin rapidly have reduced the mitochondrial O_2 consumption in cultured skin [42], lung [43], and pancreatic carcinoma cells. So, capsaicin is thought to function as antioxidants in the intracellular hepatoma HepG2 cells.

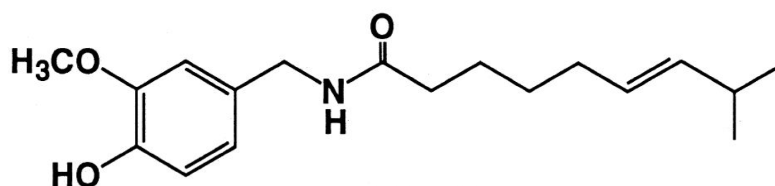


Fig 7. Structure of Capsaicin (CAP)

The oleoresin from rhizome of ginger contains pungent ingredients including Gingerol, Shoagol, and Zingerone. The major pungent principle of ginger, [6]-gingerol, chemical structure of that is shown in [Fig 8].

[6]-gingerol have been found to possess many interesting pharmacological and physiological activities, such as anti-oxidant, anti-tumor promoting activities [44], anti-inflammatory, analgesic and cardiotonic effects [45,46]. The antioxidative properties and other constituents of ginger already have been confirmed in various in vitro and in vivo test systems [47,48]. Gingerol also exerts an inhibitory effect on xanthine oxidase [49] responsible for generation of superoxide anion[50].

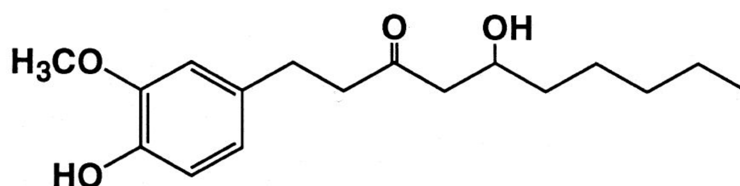


Fig 8. Structure of Gingerol (GGR)

Sulforaphane (1-isothiocyanato-4-(methylsulfinyl)-butane) is a naturally occurring isothiocyanate isolated from cruciferous vegetables such as broccoli. That is already well known, Sulforaphane is a potent cancer chemo -preventive agent that functions by inducing phase II detoxification enzymes and antioxidant proteins through the activation of antioxidant response element (ARE)-mediated transcriptional activity. Recent studies also showed that SFN inhibited inflammation by inhibiting direct NF-kB binding to DNA or interaction with redox regulators [51,52]

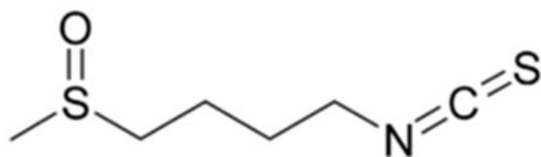


Fig 9. Structure of Sulforaphane (SFN)

It is known that many antioxidants and dietary components can protect humans and animals against insults from environmental toxins and carcinogens through the enhancement of cytoprotective machinery. The chemopreventive properties of these agents have been partially ascribed to their ability to induce detoxifying enzymes, which in turn decrease the chemical reactivity of carcinogens and their metabolites through conjugation, reduction, and hydrolysis to facilitate their elimination.

Huamn hepatoma HepG2 cells represent a sell-characterized, reliable model that have been widely used to study the biochemical variation in antioxidant defence systems. So we will specifically discuss oxidative stress in HepG2 cells [53].

In the present study, we aimed to investigate the effects of Korean traditional natural flavoring substances, DAS, CAP, GGR and SFN on the antioxidant enzyme activity such as SOD, CAT, GSH, GPx and regulating cell signal protein levels, and examined the roles of Nrf2, stress signals such as NFkB, TNF-α and upstream protein kinases in DAS, CAP, GGR and SFN-induced cytoprotective event using human hapatoma HepG2 cells.

Ⅱ. Materials and Methods

The experimental design for this study is following [Fig 10].

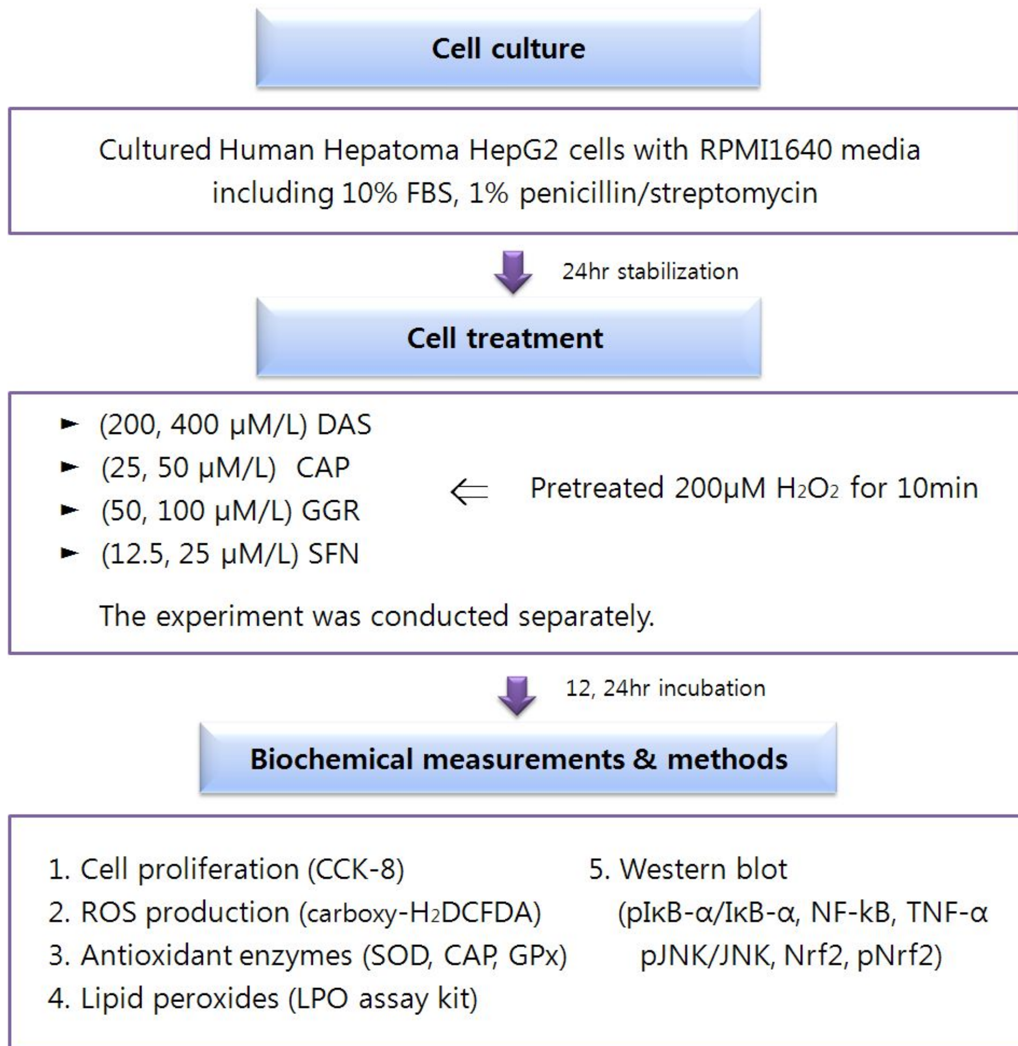


Fig 10. The experimental design of this study

1. Materials

The human hepatomablastoma HepG2 cell line was purchased from Korean Cell Line Bank (KCLB) (Seoul, Korea). RPMI1640 medium and other materials for cell culturing including penicillin-treptomycin, trypsin-EDTA, fetal bovine serum (FBS) and PBS buffer (pH 7.2) were purchased from Gibco BRL Carlsbad,CA).

Dimethylsulfoxide (DMSO), H2O2, Diallyl Sulfide(DAS), Capsaicin(CAP), Gingerol(GGR), trypan-blue, 5-(and-6) -carboxy-2’ ,7’ -dichlorofluoresceindiacetate (carboxy-H_2 DCFH-DA) were purchased form Sigma -Aldrich, Co. (St. Louis, MO, USA). Sulforaphane was obtained from LKT Laboratories (St. Paul, MN, USA)

A Cell-Counting Kit-8 (CCK-8) for cell proliferation was purchased from Dojindo Molecular Technologies (Baltimore, MD), and antioxidant enzymes kits for SOD (No. 706002), CAT (No. 707002) and GPx (No.703102) were purchased from Cayman Chemical Company (Ann Arbor, MI).

GSH/GSSG detection kit was product of ArborAssays compny (Michigan, USA). lipid peroxidation (LPO) assay kit for The cellular malondialdehyde (MDA) level was purchased from Calibiochem, La Jolla (California, USA)

Antibodies for stress-activated proteins such as IkB-α, pIkB-α, nuclear factor-kB(NF-kB), tumour necrosis factor-α(TNF-α) and conjugated secondary antibodies were purchased from Cell Signaling Technology (Danvers, MA). c-Jun-Nterminal kinase (JNK), pJNK and nuclear factor E2-related factor 2 (Nrf2) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). pNrf2 was product of Abcam (Cambridge, UK).

2. Cell culture

The hepatomablastoma HepG2 cells were cultured in RPMI-1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin mixture in a humidified atmosphere of 95% air with 5% CO_2 at 37 ° C and when the cells reached at 70% confluency, subculture was conducted at a cell density of 1 × 10^6 cells/dish.

3. Treatment of the experimental materials

For the treatment of HepG2 cells, appropriate concentrations of each antioxidant chemicals(DAS, CAP, GGR, SFN) were dissolved in DMSO and diluted with nutrient free media to

final concentrations of DAS (100, 200 μmol/L), CAP (25, 50 and μmol/L), GGR (50, 100μmol/L), SFN (12.5, 25 μmol/L) which were treated respectively. To estimate the roles of DAS, CAP, GGR, SFN on the anti-oxidant effect in HepG2 cell, the cells were preincubated with H_2O_2 for 10 min, and then washed twice by PBS (pH 7.4) before cultivation with DAS, CAP, GGR and SFN for 12h and 24hr. The protein concentration of the lysed cells was determined using a bovine serum albumin (BSA) protein assay kit (Biorad, Illinois, USA), and the data were expressed as a mean values per milligram of protein basis.

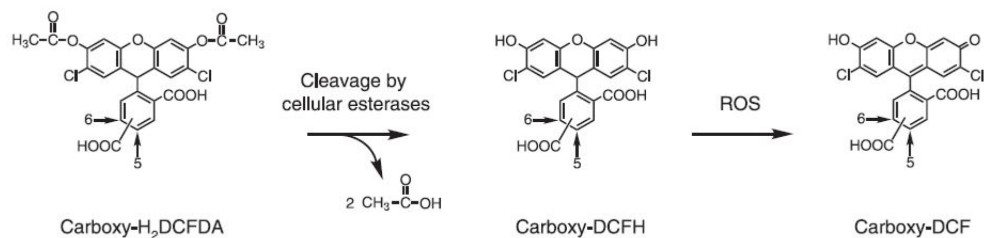
4. Cell proliferation -CCK8

Cells (1×10^4/well) were seeded in a 96-well plate. And the plate was need to stabilize for 24 hour. Pretreated with 200μM H_2O_2 in nutrient free media after 10min, the plates were washed with PBS and antioxidants ; 100, 200μM DAS, 25 or 50μM capsaicin, 50,100μM GGR and 12.5, 25μM SFN, were added with RPMI1640. And then added 10 ul CCK-8 solution using WST-8 [2-(2-methoxy -4-nitrophenyl) -5- (2,4-disulfophenyl) -2H-tetrazolim, monosodium salt] to produce a water-soluble formazan dye in the presence of an electron mediator

released from mitochondrial NADH. Concentration of NADH was measured in the absorbance at 450 nm using a Multiskan spectrum microplate spectropotometer (Thermo).

5. Measurement of Intracellular ROS

The level of ROS in HepG2 cells was examined by fluorescence microscope (Nicon Eclips TS100), using 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA ; Sigma). The oxidation product of carboxy-H_2DCFDA can be observed using fluorescein filter and appeared green color. Reaction process is shown as following this :

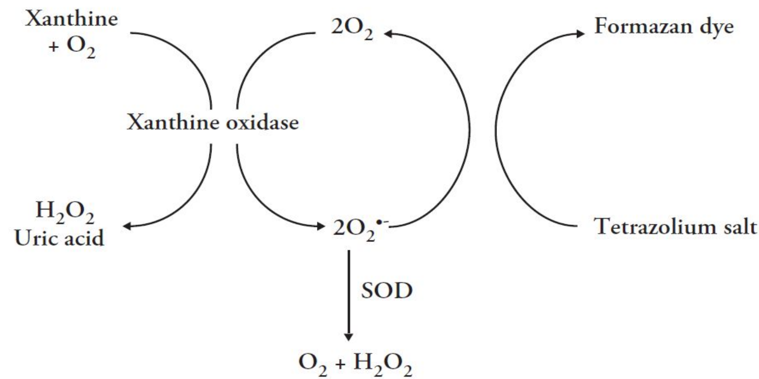


To detect the ROS changes, approximately 5×10^4 cells were seeded on each well of 6-well plates and incubated with each anioxidants for 12 h after pretreated 200μM H_2O_2 for 10min. Cells were washed out once with warm HBSS/Ca/Mg. carboxy-H_2DCFDA was added to the culture plates at a final concentration of 25uM in HBSS/Ca/Mg and plates were incubated for 30 min at 37℃ protected from light. Gently wash the plate three times again with warm HBSS/Ca/Mg, the cells were examined immediately by fluorescence microscope with an excitation wavelength of 495 nm and emission wavelength of 529 nm.

6. Antioxidant Enzymes

1) Superoxide Dismutase (SOD) Activity

SOD activity was detected by using SOD Assay Kit (Cayman) according to manufacturer's instruction. This study was determined by using the xanthine and xanthine oxidase system. Xanthine is converted to uric acid by the xanthine oxidase. A tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. Reaction process is shown as following this :



To conduct this assay, cell lysate was prepared by homogenizing with cold 20mM HEPES buffer (1mM EGTA, 210mM mannitol, and 70mM sucrose) on ice. The detection was performed at 25℃ and started by adding the radical detector (tetrazolium solution) and reaction was started by adding 20 μL of xanthine oxidase. After incubation at room temp for 20 min, plate was measured at 340 nm using a plate reader and then, the enzyme activity was obtained from linear regression of the standard curve. One unit was defined as the amount if enzyme needed to exhibit 50% dismutation of the superoxide radical and expressed as nmol/min/mg protein.

2) Catalase activity

Catalase activity was measured using Catalase Assay Kit (Cayman, MI, USA) according to manufacturer' s instruction. This method is based on the reaction of the enzyme with methanol in the presence of optimal concentration of H_2O_2. The formaldehyde produced is measured colormetrically with 4-amino- 3-hydrazino- 5-mercapto-1,2,4-triazole (Purpald) as the chromogen . Purpald specifically forms abicyclic geterocycle with aldehydes, which upon oxidation changes from colorless to a purple color. The rate of decompostion of H_2O_2 was measured at 540 nm and the enzyme activity was obtained from linear regression of the standard curve. One unit of catalase was defined as the amount of enzyme required to decompose 1 mM of H_2O_2 per 1min. The enzyme activity was expressed as nmol/min/mg protein.

CAT activity (nmol/min/mL)

$$= \frac{\text{Fomaldehyde } \mu\text{M}}{\text{20min}} \times \text{dilution factor}$$

3) Glutathione (GSH) contents

The concentration of GSH and oxidized glutathione was evaluated by Glutathione detection kit (Arborassay, MI, USA). This method measures the total GSH and reduced GSH (GSSG) products, while GSH values were calculated as the following equation.

$$GSH = Total\ GSH\ conc - Oxidized\ GSH\ conc$$

4×10^{6} cells were deproteinized in 5% sulfosalicylic acid. The acid-precipitated protein was pelleted by centrifugation at 4° C for 10 min at 15,000 g. The total protein content of each sample was determined using bradford. By using 2-Vinylpyridine (2VP) to block any free GSH in the sample, Oxidized Glutathione (GSSG) can be determined. Any samples that have not been treated with 2-VP will yield Total GSH levels. The Free GSH concentration in the sample is calculated from the difference between the Total GSH determined and the GSH generated from Oxidized Glutathione for the 2-VP treated samples. The concentration of GSH can be determined by measuring the rate of color development at 405 nm.

4) Glutathione Peroxidase (GPx) Activity

GPx activities were determined by GPx assay kit (Cayman, MI, USA). This assay was based on the oxidation of GSH to GSSG catalyzed by GPx, which is then coupled to the recycling of GSSG to GSH using GR and NADPH. The decreasing of NADPH absorbance during the oxidation of NADPH to NADP+ is indicative of GPx activity, since GPx is the rate limiting factor of the coupled reactions. The reation was perfomed at 25℃ and started by adding th the reaction co-substrate mixtures consisting of NADPH, glutathione, glutathiome peroxidase and assay buffer(5mM EDTA, 50mM Tris-HCl, pH 7.6). The reaction was initiated by the addition of GPx cumen hydroperoxide. GPx activity was measured as the rate of NADPH oxidation at 340 nm using a plate reader every minute to obtain at least 5 times points. One unit of GPx was defined as the amount required to oxidize 1 nM of NADPH to NADP+ per minute at 25℃. The activity of GPx was expressed as anmol/min/mg protein in each group.

GPx activity (nmol/min/mL)

$$= \frac{\triangle A_{340}/\text{min}}{0.00373\ \mu M^{-1}} \times \frac{0.19\ \text{ml}}{0.02\ \text{ml}} \times \text{dilution factor}$$

7. Lipid Peroxides(LPO) production

The cellular Lipid peroxides level was determined with a LPO assay kit (Calibiochem, California, USA) according to the manufacturer' s protocol. Lipid hydroperoxides was measured directly utilizing redox reactions with ferrous ions. Hydroperoxides are highly unstable and react readily with ferrous ions to produce ferric ions. The resulting ferric ions are detected using thiocyanate ion as the chromogen. To perform this assay, approximately 4×10^6 cells were plated onto 100mm dish and at the end of treatment the cells were scraped into 1ml of double distilled H_2O and lysed by sonicator on ice. Lipid hydroperoxides must be extracted from the lysate with chloroform and then added the required reagents. Each samples were transfered into 96well and measured at 500nm in comparison to the standard curves. The data for LPO production in hepatocyte were expressed as nmol/mg protein basis.

8. Western Blotting

Whole cell extracts from treated cells with DAS, CAP, GGR and SFN or without that (4×10^6 cells) were prepared in a lysis buffer (50 mM Tris, pH 8, 1% Triton x-100, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 0.5% Sodume deoxycholate, Protein inhibitor cocktail contains inhibitors against the major classes of proteases and phosphatases). After the extracts were centrifuged (14,000×g for 20min), the resulting supernatant was subjected to immunoblot analyses. The total proteins (20 μg) were electrophoresed on 8% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and transferred to nitrocellulose membrane. The immunoblot was incubated for 2 h with blocking solution (Tris-buffered saline/Tween 20, TBS) containing 5% skim milk at room temperature, followed by incubation overnight at 4 ℃ with a primary antibodies against each target protein such as 1:1000 dilution of IkB-α, pIkB-α, NFkB, 1:500 dilution of JNK, pJNK, Nrf2, 1:5000 dilution of pNfr2. The β-actin was used as a standard protein. The membrane was washed four times with TBS, and then incubated with goat anti-rabbit or mouse IgG secondary antibody for 1 h at room temperature. The membrane was washed three times and then Each immunoreactive antigen was visualized by using the ECL kit (Biorad) and exposed to X-ray film (Kodak XAR)

to identify the target proteins. And according to the manufacturer's instructions, quantified using a Bio ImageJ Analyzer (NIH, Bethesda, USA).

9. Statistical analysis

The results are expressed as mean ± SD and differences were determined by one-way analysis of variance (ANOVA). Statistical significance was accepted within $P < 0.05$ as determined by Duncan's multiple range test . and significant differences between two groups were expressed by T-test to verify the efficacy of each chemical compared to negative control.

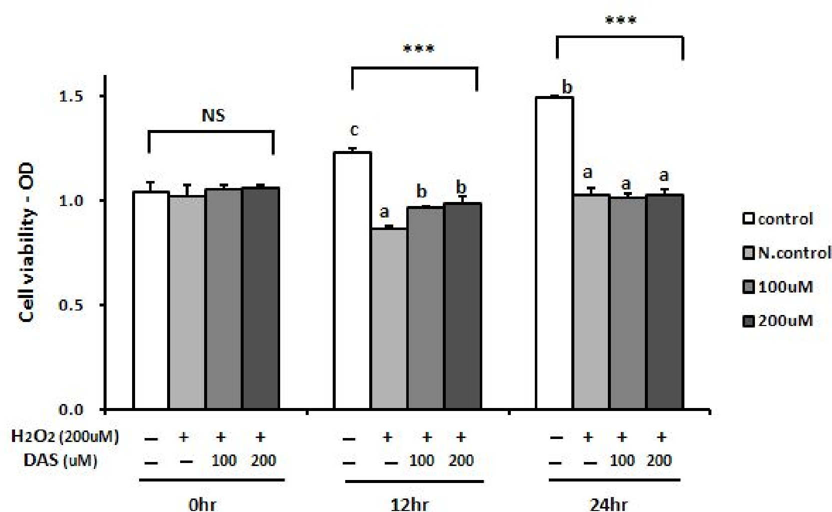
Ⅲ. Result

1. Cell Proliferation

To determine prevent effect of the various antioxidants (DAS, CAP, GGR and SFN) on increased the cell death by oxidative stress, viability of HepG2 cells was determined by a CCK-8 kit. First, H_2O_2 increased a cell death in HepG2 cells, but, the antioxidants decreased the H_2O_2-induced cell death in a each concentration. Viability of HepG2 cells was rapidly decreased from early time for 10min by 200μM H_2O_2 and arrived to approximately 70%. All of the antioxidant was efficient in improving HepG2 cell viability in spite of oxidative stress. The increased cell viability effect of DAS were shown in [Fig 11A]. The treated levels of 100 and 200μM of DAS increased HepG2 cell survival at 74 and 76% of total cells at 12hr respectively but not 24hr. Effects of CAP on cell survival [Fig 11B] shows the induction of viability by different levels of 25, 50μM. CAP increased HepG2 cell survival by 83.7 and 79.3% of total cells at 12hr, 71.3 and 72.4% at 24hr respectively. Effects of GGR on survival [Fig 11C] shows the induction of viability. The concentrations of 50, 100μM GGR increased

HepG2 cell survival by 77.5 and 75.2% of total cells at 12hr, 79.2 and 74.9% at 24hr respectively. The increased cell viability effect of SFN were shown in [Fig 11D]. The concentration of 12.5, 25 μM SFN increased HepG2 cell survival by 84.1 and 81.6% of total cells at 12hr, 84.6 and 67.8% at 24hr respectively. In this result, The treated levels of each antioidants were not diferent on cell survival except for CAP during the 12hr incubation. However, the cell viability were more affected by lower levels of antioxidants than by higher level except for DAS. In conclusoin, 25μM CAP and 12.5μM SFN were the most effective at the each time.

A



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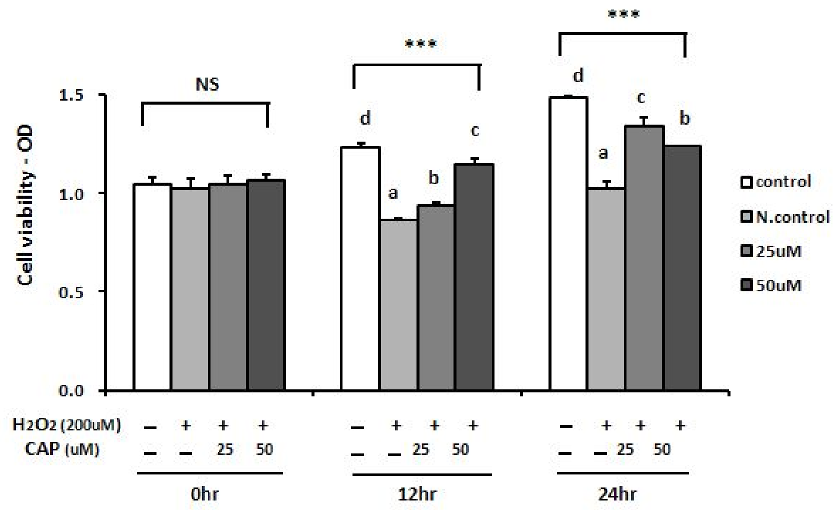

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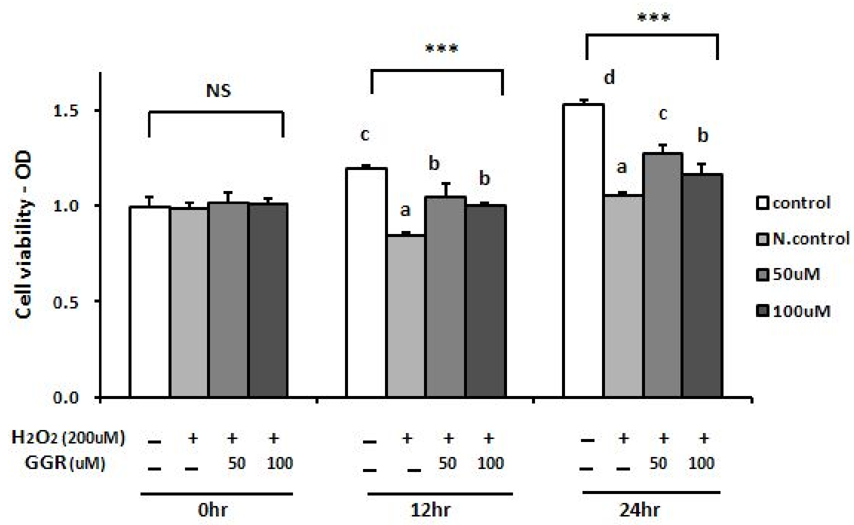

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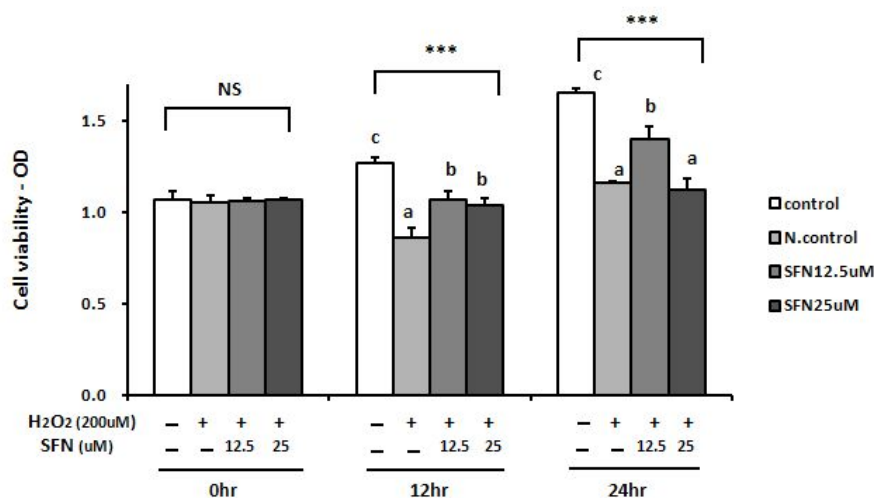


Fig 11. cell was treated with each antioxidant after treatment 200 μM H_2O_2 for 10min and 12 or 24hr later, cell was detected with CCK-8.

A. Effects of DAS on viability in H_2O_2-treated Hepg2 cells.

B. Effects of CAP on viability in H_2O_2-treated Hepg2 cells.

C. Effects of GGR on viability in H_2O_2-treated Hepg2 cell.

D. Effects of SFN on viability in H_2O_2-treated Hepg2 cell.

Data are presented as mean ± SD.

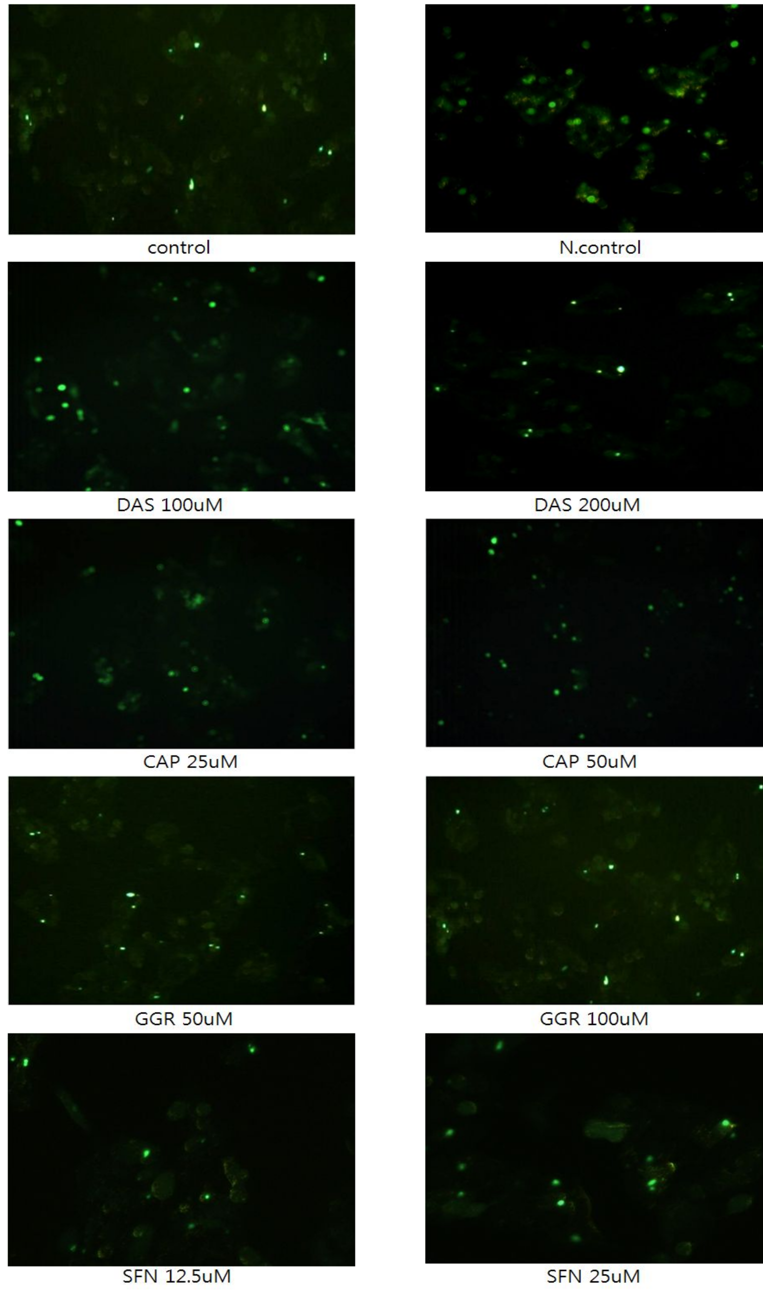
a, b, c : Significant difference between the groups were tested by ANOVA. (*p < 0.05, **p < 0.01, **p < 0.001)

2. Intracellular ROS Formation

Direct evaluation of ROS yields is a very good indication of the oxidative damage to living cells. The intracellular ROS level was detected using a fluorescence microscope with fluorescence probe 5-(and-6)-carboxy -2′, 7′ -dichloro -dihydrofluorescein diacetate (carboxy-H2DCFDA). The ROS formation was evaluated with cells were treated with each antioxidant 200 μM H_2O_2 exposure. Treatment of H_2O_2 increased significantly the intracellular ROS levels up to 220% compared to the control [Fig 11]. However, 200 μM level of DAS, intracellular ROS level was noticeably reduced by 45.5% compared to the negative control. Treatment of DAS at lower concentration (100 μM) also significantly reduced intracellular ROS level by up to 61.4% compared to the negative control. In case of CAP, at the low concentration (25 μM), intracellular ROS level was reduced by up to 43.2% compared to the negative control. Treatment of CAP at higher concentration (50 μM) also significantly reduced intracellular ROS level by up to 47.7% compared to the negative control. Also GGR treatment low concentration (50 μM) was more effective to reduce intracellular ROS level by up to 56.8% compared with higher concentration (100 μM)

reduced by up to 61.4%. Finally SFN was the most effective to decreased the formation ROS. As CAP and GGR, at the low concentration of SFN(12.5μM) reduced intracellular ROS by up to 59.1% more than the higher concentration (25 μM/L) by up to 86.4%. [Fig 12] In this result, all of concentration DAS and 25μM CAP were the most effective.

A



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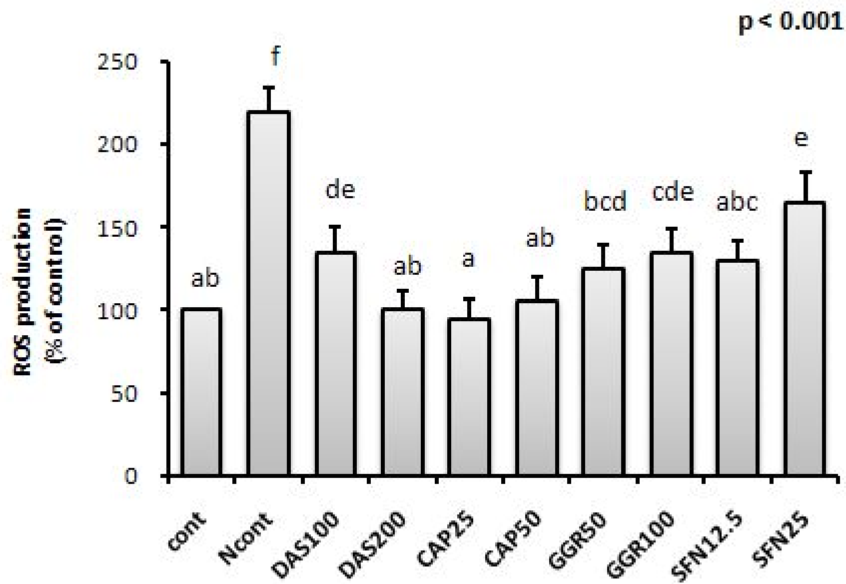


Fig 12. Inhibitory effect of DAS, CAP, GGR and SFN on the H_2O_2-induced intracellular ROS production.

(5×10^4)HepG2 cells were treated with 200μM H_2O_2 for 10 min and further incubated with antioxidant (each concentration) for 12 hr and detected immediately by Fluorescence Microscopy (A). The change in the fluorescence of the oxidized probe was determind and each value was expressed as means ± SD (B). a, b, c : Significant difference between the groups were tested by ANOVA. (*$p < 0.05$, **$p < 0.01$, **$p < 0.001$)

3. Antoxidant Enzyme Assay

1) Superoxide Dismutase (SOD) Activity

Superoxide Dismutase (SOD) protect the cell from superoxide toxicity by converting superoxide anions to hydrogen peroxide. so we examined the effect of each antioxidant about induction SOD level in HepG2 cells. Fist, SOD levels were significantly decreased by up to 75.2 % in only H_2O_2 treated cells compared to the control. On the other hand, DAS treatment at the concentration of 200 μM inhibited the reduction of enzyme activity by up to 120.5 % compared to negative control. And 25 μM CAP increased by up to 107.8 %, 50 μM GGR increased by up to 105.9 % and 12.5 μM SFN increased by up to 129.9 %, SFN treatment was best efficient in 12hr. However, in incubation time 24hr, DAS was no efficiency to increasing the SOD levels. Otherwise, 25 μM CAP increased SOD levels by up to 108.2 %, 50 μM GGR increased by up to 114.5 % and 12.5 μM SFN increased by up to 104.7 % compared with negative control, GGR treatment was best efficient in longer incubation time. [Fig 13]

Table 1. Effect of DAS, CAP, GGR and SFN on the SOD activity in H_2O_2 induced Human Hepatoma HepG2 Cells.

	12hr	24hr	p-value[5)]
control	1.704±0.02[f]***[4)]	1.426±0.02[d]***	0.000
H_2O_2	1.167±0.03[a] [2)]	1.114±0.03[a]	NS
H_2O_2 + DAS[5)]	1.299±0.01[c]**	1.117±0.03[a]	0.000
H_2O_2 + CAP[6)]	1.219±0.03[b]	1.205±0.02[b]*	NS
H_2O_2 + GGR[7)]	1.491±0.03[d]***	1.276±0.06[c]**	0.005
H_2O_2 + SFN[8)]	1.647±0.02[e]***	1.166±0.01[ab]*	0.000
p-value[3)]	0.000	0.000	

1) Values are expressed mean±SD

2) a, b, c : Difference in continous variables between each group were tested by ANOVA

3) P-values for the significance with the various antioxidants treatment by ANOVA

4) Significantly difference each groups compared to negative control(only H_2O_2). (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

5) Significantly difference between of 12 and 24hr

6) 10min pretreatmet of 200μM H_2O_2 and 200μM DAS

7) 10min pretreatmet of 200μM H_2O_2 and 25μM CAP

8) 10min pretreatmet of 200μM H_2O_2 and 50μM GGR

9) 10min pretreatmet of 200μM H_2O_2 and 12.5μM SFN

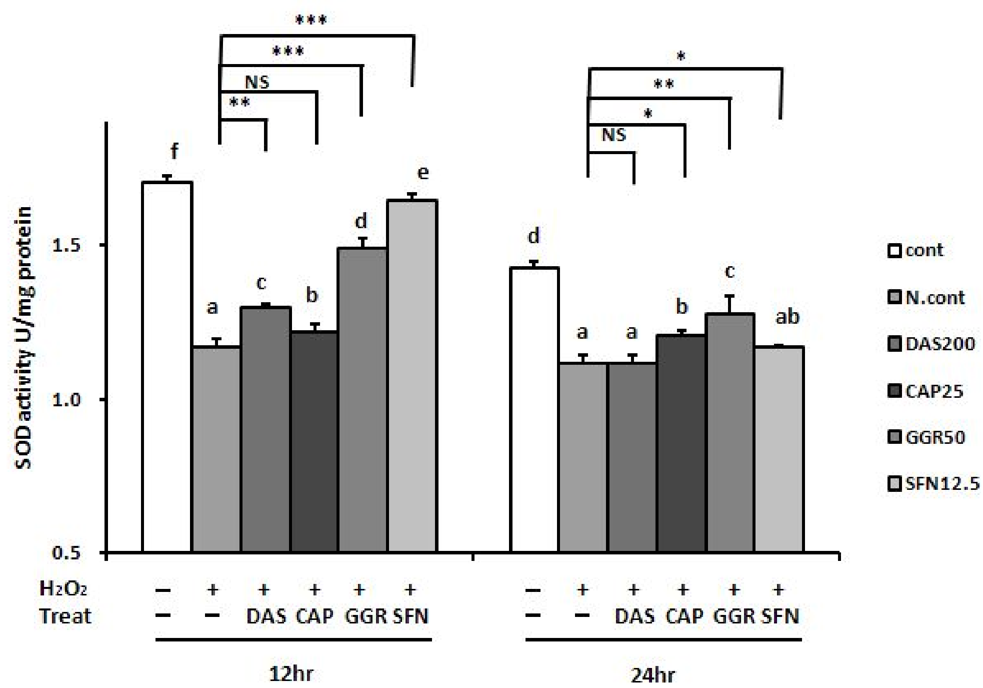


Fig 13. Effect of DAS, CAP, GGR and SFN on the SOD activity in H_2O_2 induced Human Hepatoma HepG2 Cells.

Data are presented as mean ± SD.

a, b, c : Significant difference between the groups were tested by ANOVA.

*p < 0.05, **p < 0.01, **p < 0.001 : Significantly different each groups compared to negative control(only H_2O_2).

2) Catalase (CAT) Activity

Catalase is a common enzyme found in nearly all living organisms which are exposed to oxygen, where it functions to catalyst catalyze the decomposition of hydrogen peroxide to water and oxygen. This study shown the changes of CAT level in HepG2 Cells as presented in [Fig 14]. CAT levels were significantly decreased by up to 76.8% in only H_2O_2 treated cells compared to the control. whereas a significant induction in CAT level was observed at the concentration of 50㎛ GGR by up to 111.6%, 12.5㎛ SFN treatment by up to 123.3% after 12hr compared to negative control. Otherwise, there was no significant difference the level of CAT in HepG2 treated with 200㎛ DAS, 25㎛ CAP. Likewise, each antioxidants did not affect CAT activity at 24 hr, except for GGR treatment, so only GGR induced CAT level by up to 118.6 %. So in this result, SFN treatment was also best efficient in 12hr GGR treatment was best efficient in longer incubation time as results of SOD level. [Fig 13]

Table 2. Effect of DAS, CAP, GGR and SFN on the activity of CAT in H_2O_2 induced Human Hepatoma HepG2 Cells.

	12hr	24hr	p-value[5)]
control	66.31±3.50[c]**[4)]	57.77±4.81[d]*	NS
H_2O_2	50.95±2.02[a2)]	43.90±2.96[ab]	0.027
H_2O_2 + DAS[5)]	48.96±2.52[a]	41.74±1.04[a]	0.010
H_2O_2 + CAP[6)]	51.23±2.59[a]	48.24±2.25[bc]	NS
H_2O_2 + GGR[7)]	56.87±0.89[b]**	52.04±1.21[c]*	0.005
H_2O_2 + SFN[8)]	62.84±2.04[c]**	42.53±3.92[a]	0.001
p-value[3)]	0.000	0.000	

1) Values are expressed mean±SD
2) a, b, c : Difference in continous variables between each group were tested by ANOVA
3) P-values for the significance with the various antioxidants treatment by ANOVA
4) Significantly difference each groups compared to negative control(only H_2O_2).
(* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)
5) Significantly difference between of 12 and 24hr.
6) 10min pretreatmet of 200 μM H_2O_2 and 200 μM DAS
7) 10min pretreatmet of 200 μM H_2O_2 and 25 μM CAP
8) 10min pretreatmet of 200 μM H_2O_2 and 50 μM GGR
9) 10min pretreatmet of 200 μM H_2O_2 and 12.5 μM SFN

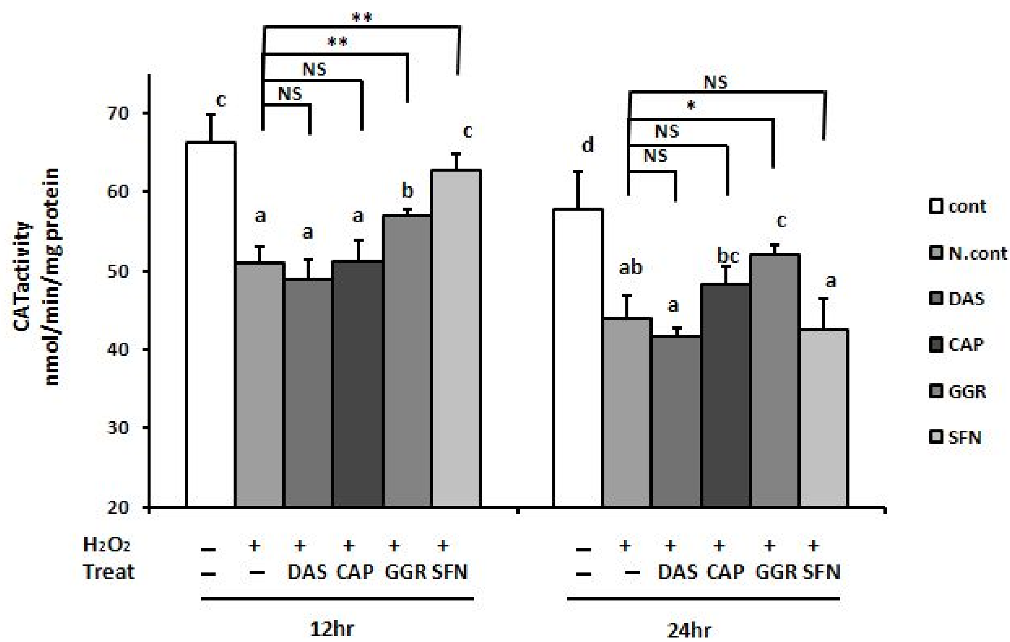


Fig 14. Effect of DAS, CAP, GGR and SFN on the activity of CAT in H_2O_2 induced Human Hepatoma HepG2 Cells.

Data are presented as mean ± SD.

a, b, c : Significant difference between the groups were tested by ANOVA.

*p 〈 0.05, **p 〈 0.01, **p 〈 0.001 : Significantly different each groups compared to negative control(only H_2O_2).

3) Glutathione (GSH) levels

GSH is the main non-enzymatic antioxidant defense within the cell and plays an important role in protection against oxidative stress, and GSH depletion reflects intracellular oxidation. [Fig 15] represents the change of GSH level in HepG2 cells. In this study the concentration of hepatic GSH was significantly decreased in 200μM H_2O_2-pretreated cells compared with control by up to 90.2%. in the other hand, the post-treatment with 200μM DAS to the cells resulted in a statistically increase in GSH levels up to 110.4% compared with the negative control. And 25μM CAP increased by up to 124 %, 50μM GGR increased by up to 112.4 % and 12.5μM SFN increased by up to 130.9 %, SFN treatment also was best efficient in 12hr as other result. Futhermore, in incubation time 24hr, only DAS increased the GSH levels by up to 112.4 % and other antioxidants did not affect the increment.

Table 3. Effect of DAS, CAP, GGR and SFN on the level of GSH in H_2O_2 induced Human Hepatoma HepG2 Cells.

	12hr	24hr	p-value[5)]
control	12.64±0.29[b]*[4)]	12.52±0.95	NS
H_2O_2	11.40±0.57[a2)]	12.22±0.56	NS
H_2O_2 + DAS[5)]	12.59±0.21[b]*	13.54±0.18*	0.004
H_2O_2 + CAP[6)]	14.13±1.20[c]*	12.56±0.76	0.030
H_2O_2 + GGR[7)]	12.81±0.62[b]*	12.44±1.50	NS
H_2O_2 + SFN[8)]	14.93±0.28[c]**	13.32±1.08	NS
p-value[3)]	0.000	0.471	

1) Values are expressed mean±SD
2) a, b, c : Difference in continous variables between each group were tested by ANOVA
3) P-values for the significance with the various antioxidants treatment by ANOVA
4) Significantly difference each groups compared to negative control(only H_2O_2). (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)
5) Significantly difference between of 12 and 24hr.
6) 10min pretreatmet of 200μM H_2O_2 and 200μM DAS
7) 10min pretreatmet of 200μM H_2O_2 and 25μM CAP
8) 10min pretreatmet of 200μM H_2O_2 and 50μM GGR
9) 10min pretreatmet of 200μM H_2O_2 and 12.5μM SFN

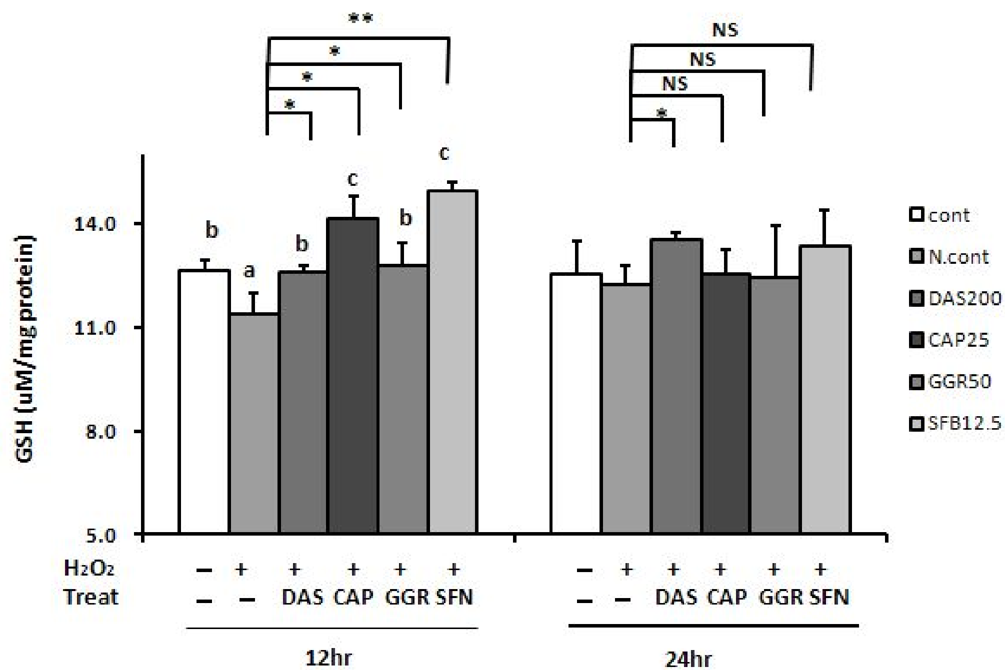


Fig 15. Effect of DAS, CAP, GGR and SFN on the level of GSH in H_2O_2 induced Human Hepatoma HepG2 Cells.

Data are presented as mean ± SD.
a, b, c : Significant difference between the groups were tested by ANOVA.
*p < 0.05, **p < 0.01, **p < 0.001 : Significantly different each groups compared to negative control (only H_2O_2).

4) Glutathione Peroxidase (GPx) Activity

GPx catalyses GSH oxidation to oxidized glutathione at the expense of H_2O_2 or other peroxides and GR recycles oxidized glutathione back to GSH. therefore, their activities are essential for the cell-damaging peroxide species and the effective recovery of the steady-state concentration of GSH. In this study, the level of GPx was significantly reduced in 200μM H_2O_2-pretreated cells compared to control by up to 86.3%. Administration of 200μM DAS could significant recover the level of GPx up to 118.2% compared to negative control in liver cell. And 25μM CAP increased by up to 130 %, 50μM GGR increased by up to 111.9 % and 12.5μM SFN increased by up to 146 %, SFN treatment also was best efficient at 12hr. Futhermore, in incubation time 24hr, DAS increased the SOD levels by up to 108.7 %. And 25μM CAP increased by up to 115.3 %, 50μM GGR increased by up to 112.6 % and 12.5μM SFN increased by up to 112.4 %, therefore CAP treatment was best efficient to increase the GPx activity at 24hr. But, there was no apparent difference between each groups as result of 12hr. [Fig 16]

Table 4. Effect of DAS, CAP, GGR and SFN on the activity of GPx in H_2O_2 induced Human Hepatoma HepG2 Cells.

	12hr	24hr	p-value[5)]
control	105.97 ± 4.76^{b}*[4)]	93.65 ± 3^{b}	0.019
H_2O_2	91.43 ± 3.12^{a}[2)]	87.58 ± 3.27^{a}	NS
H_2O_2 + DAS[5)]	108.05 ± 4.5^{b}**	95.22 ± 2.36^{bc}*	0.012
H_2O_2 + CAP[6)]	118.96 ± 6.79^{c}**	100.97 ± 3.06^{c}**	0.014
H_2O_2 + GGR[7)]	102.34 ± 2.38^{b}**	98.63 ± 3.03^{bc}*	NS
H_2O_2 + SFN[8)]	133.5 ± 3.24^{d}***	98.42 ± 3.86^{bc}*	0.000
p-value[3)]	0.000	0.003	

1) Values are expressed mean±SD
2) a, b, c : Difference in continous variables between each group were tested by ANOVA
3) P-values for the significance with the various antioxidants treatment by ANOVA
4) Significantly difference each groups compared to negative control(only H_2O_2).
(* p <0.05 , ** p <0.01, *** p<0.001)
5) Significantly difference between of 12 and 24hr.
6) 10min pretreatmet of 200 μM H_2O_2 and 200 μM DAS
7) 10min pretreatmet of 200 μM H_2O_2 and 25 μM CAP
8) 10min pretreatmet of 200 μM H_2O_2 and 50 μM GGR
9) 10min pretreatmet of 200 μM H_2O_2 and 12.5 μM SFN

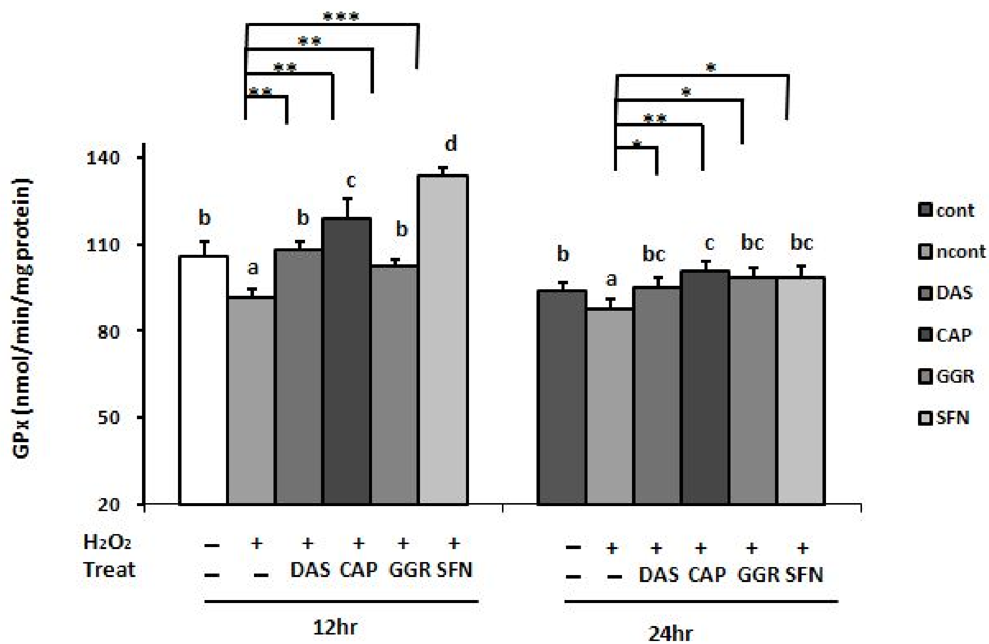


Fig 16. Effect of DAS, CAP, GGR and SFN on the activity of GPx in H_2O_2 induced Human Hepatoma HepG2 Cells.

Data are presented as mean ± SD.

a, b, c : Significant difference between the groups were tested by ANOVA.

*p < 0.05, **p < 0.01, **p < 0.001 : Significantly different each groups compared to negative control(only H_2O_2).

4. Lipid Peroxidaion(LPO) production

Lipid Peroxidation (LPO) is used commonly as an indicator of oxidative stress in tissues or cells. We studied the effect of DAS, CAP, GGR and SFN inhibitor, on the LPO production as well as enzyme activities. As a result, we have found that a 10min treatment of HepG2 with 200 μM H_2O_2 caused a significant increase of about 14% in the cellular concentration of LPO after 12h. futhermore after 24hr, H_2O_2 caused a significant increase of about 26.2% so this result indicated the LPO product was induced as time goes by. But LPO was also strongly inhibited by DAS up to 85.7% compared to negative control, GGR inhibited up to 90.1% and SFN inhibited up to 81.9% at 12hr of incubation [Fig 17]. Otherwise, at the longer exposure all of each antioxidants was effective to decrease level of LPO. The case of 200μM DAS, the level was decreased by 94%, 25μM CAP decreased by 96.5%, 50μM GGR decreased by 78.8% and 12.5μM SFN decreased by 89.6%, so it indicate that SFN treatment also was best efficient at 12hr likewise all of the antioxidant enzyme results. One the other side GGR was best role about reduction of LPO product in 24hr as SOD and CAT results.

Table 5. Effect of DAS, CAP, GGR and SFN on LPO reduction in H_2O_2 induced Human Hepatoma HepG2 Cells.

	12hr	24hr	p-value[5)]
control	2.178±0.06[b]**[4)]	2.340±0.06[a)]***	0.031
H_2O_2	2.483±0.5[c2)]	2.952±0.03[d]	0.000
H_2O_2 + DAS[5)]	2.127±0.02[ab]	2.774±0.08[bc]*	0.000
H_2O_2 + CAP[6)]	2.450±0.05[c] *	2.848±0.11[cd]	0.004
H_2O_2 + GGR[7)]	2.236±0.09[b]	2.328±0.10[a]**	NS
H_2O_2 + SFN[8)]	2.033±0.07[a] **	2.645±0.05[b]**	0.000
p-value[3)]	0.000	0.000	

1) Values are expressed mean±SD

2) a, b, c : Difference in continous variables between each group were tested by ANOVA

3) P-values for the significance with the various antioxidants treatment by ANOVA

4) Significantly difference each groups compared to negative control(only H_2O_2).
(* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

5) Significantly difference between of 12 and 24hr.

6) 10min pretreatmet of 200μM H_2O_2 and 200μM DAS

7) 10min pretreatmet of 200μM H_2O_2 and 25μM CAP

8) 10min pretreatmet of 200μM H_2O_2 and 50μM GGR

9) 10min pretreatmet of 200μM H_2O_2 and 12.5μM SFN

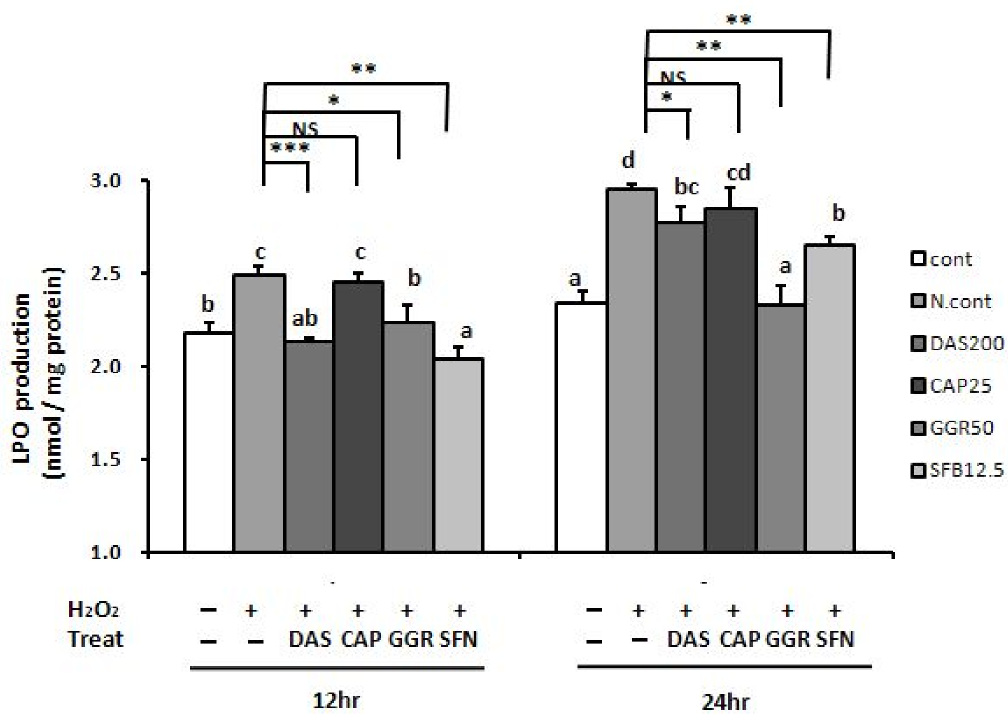


Fig 17. Effect of DAS, CAP, GGR and SFN on LPO reduction in H2O2 induced Human Hepatoma HepG2 Cells.

Data are presented as mean ± SD.
a, b, c : Significant difference between the groups were tested by ANOVA.
*p < 0.05, **p < 0.01, **p < 0.001 : Significantly different each groups compared to negative control(only H_2O_2).

5. Protein Expression in HepG2 cells

To examine the antioxidant mechanisms of DAS, CAP, GGR and SFN against H^2O^2-induced oxidative damage, HepG2 cells were treated with each antioxidants (DAS : 200μM, CAP : 25μM, GGR 50μM and SFN 12.5μM) for 30min and determined by western blot analysis. There was no significant difference of protein expression on 12 and 24 hr incubation (Figure was not shown in this data). We have examined the cellular Protein Expression of NF-E2-related factor 2 (Nrf2). It is known that Nrf2 protein can function as a direct oxidative stress sensor, and that phase II detoxifying enzymes are major coordinator of the cellular defense system against oxidative stress [42]. Treatment of HepG2 cells with DAS, CAP, GGR and SFN in the expression of pNrf2 show significantly ($P < 0.05$) increased in all groups including negative control, compared to control[Fig 19B]. We also, verified activation of pNrf2. As a result, nuclear accumulation of pNrf2 was increased similar appearance with Nrf2 result [Fig 19C]. Among the MAPKs, the activation of JNK is one of the pathways for phospholylation of Nrf2. All of the them completely increased phospholylation of JNK so they was partially considered to increase significantly ($p<0.05$) the expression of pNrf2 on H_2O_2-induced HepG2 cells [Figs 19B,

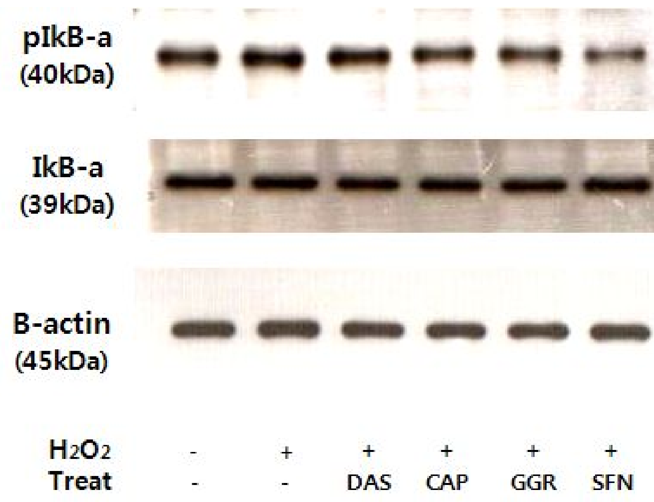
C]. This result shown that similar appearance with pJNK and pNrf2 expression except for GGR, so this indicated GGR induced directly activation of phospholylation of Nrf2. But there was no detection of onther MAPK which are related with activation of pNrf2, therefore we need to prove this signal by more research.

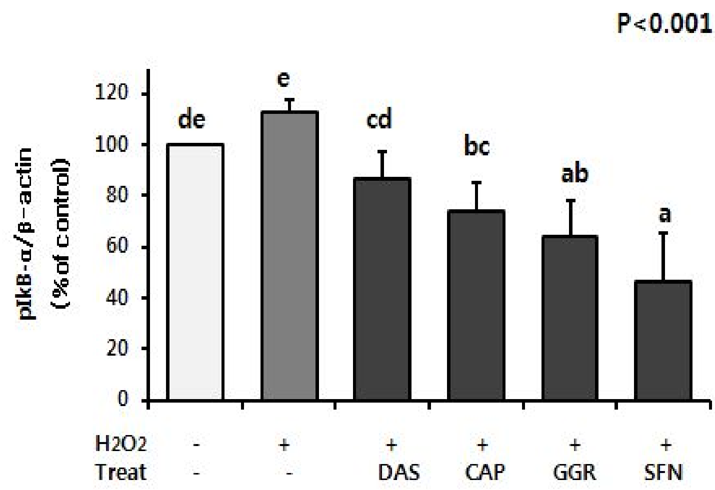
Futhermore we have investigated the inhibitory effect of the this materials in relation to potential oxidant stress on nuclear factor kB (NF-kB) binding activity in HepG2 cells. Oxidative stress might be involved in the upregulation of NF-kB level and NF-KB regulates transcription of genes encoding for cytokines such as IL-1, IL-6, IL-8, TNF-a. Therefore, we tested whether DAS, CAP, GGR and SFN blocked H_2O_2-induced NF-kB activity. First, H_2O_2 treatment induced significantly ($P < 0.05$) the expression of NFkB protein compared to control. But administration of DAS, CAP, GGR and SFN was able to suppress significantly ($P < 0.05$) the expression of NF-kB protein especially in SFN(12.5μM) compared to negative control [Fig 18B]. And we confirmed expression of pIkBα/IkBα ratio to prove the signal. Phospholylation of IkBα is a crucial mediator of NF-kB activation. As a result, the level of phospholylation of IkBα was decreased by each antioxidants with simiar appearance of NF-kB result except for DAS treatment [Fig 18A]. Additionally we examined the level of

TNF-a derived from NF-kB, and also that was decreased compared to negative control with simiar appearance of NF-kB result [Fig 18C].

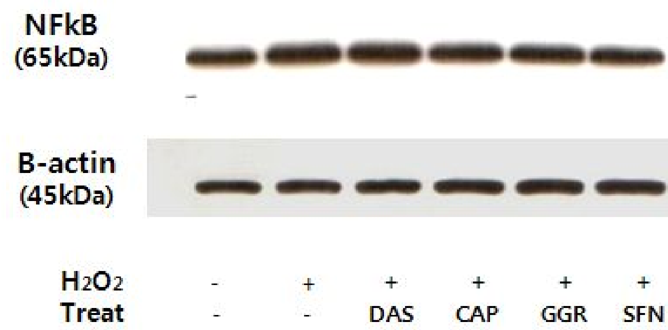
In conclusion, SFN decreased all of the stress-induced signaling proteins such as IkBα, NF-kB, TNF-α and was most effective to increase the activation of Nrf2. Following, there was more effective as GGR, CAP and DAS in that order even though there was no significant difference.

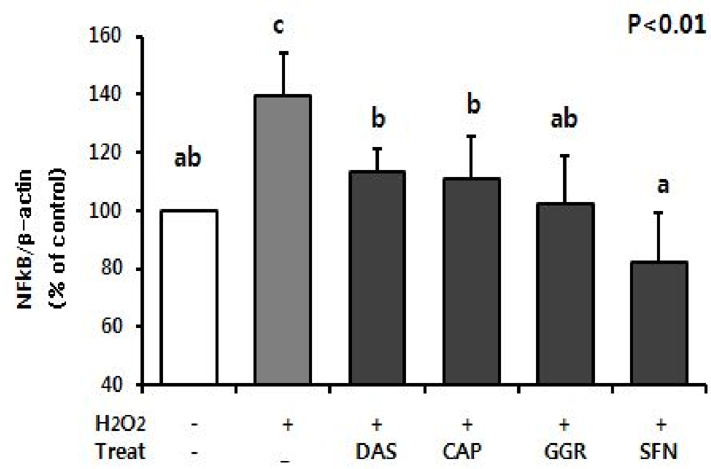
A





B





C

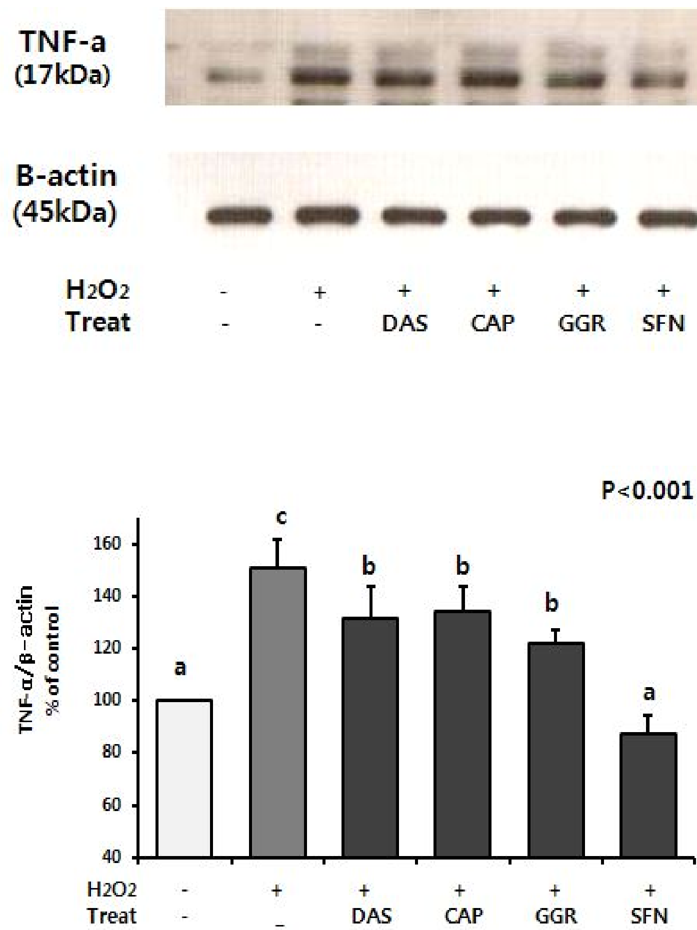
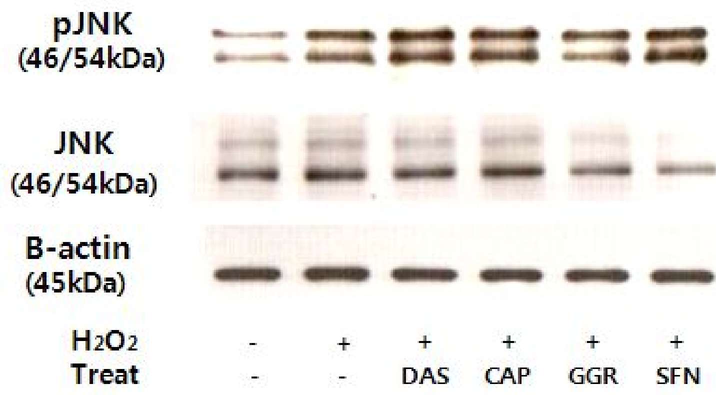
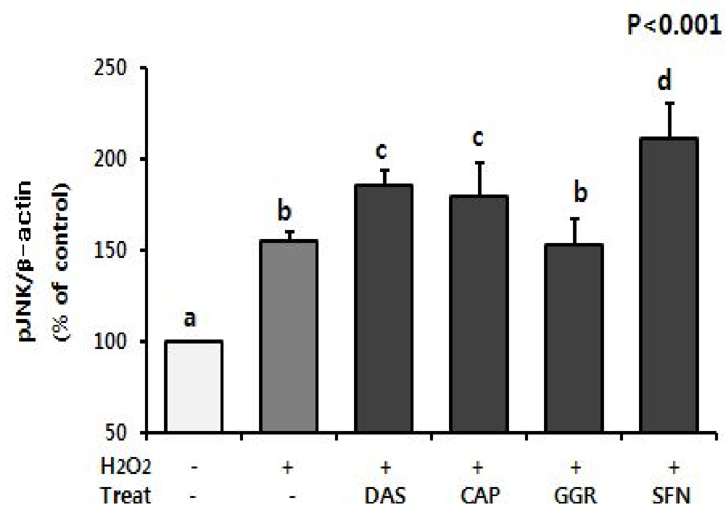


Fig 18. Effect of DAS, CAP, GGR and SFN on the expression of oxidative stress markers, pIkBα (A), NFkB (B), TNFα (C) against H_2O_2-pretreated HepG2 cells. Actin levels were compared to ensure equal amount of protein loading.

A





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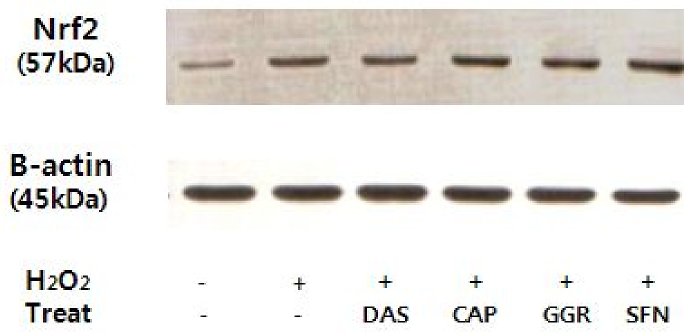
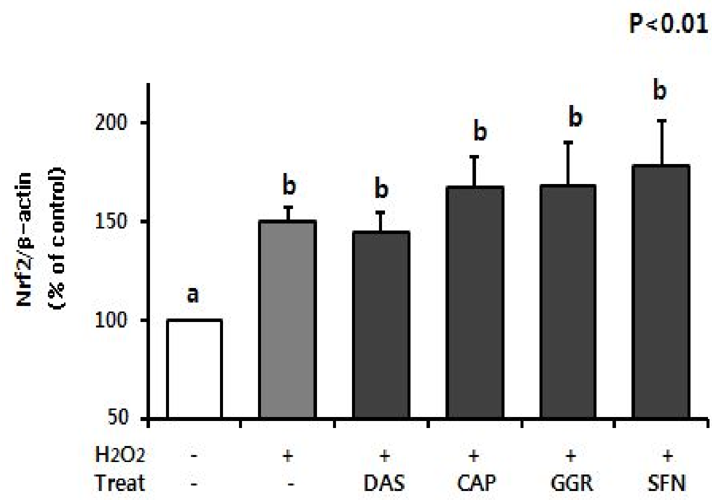




C

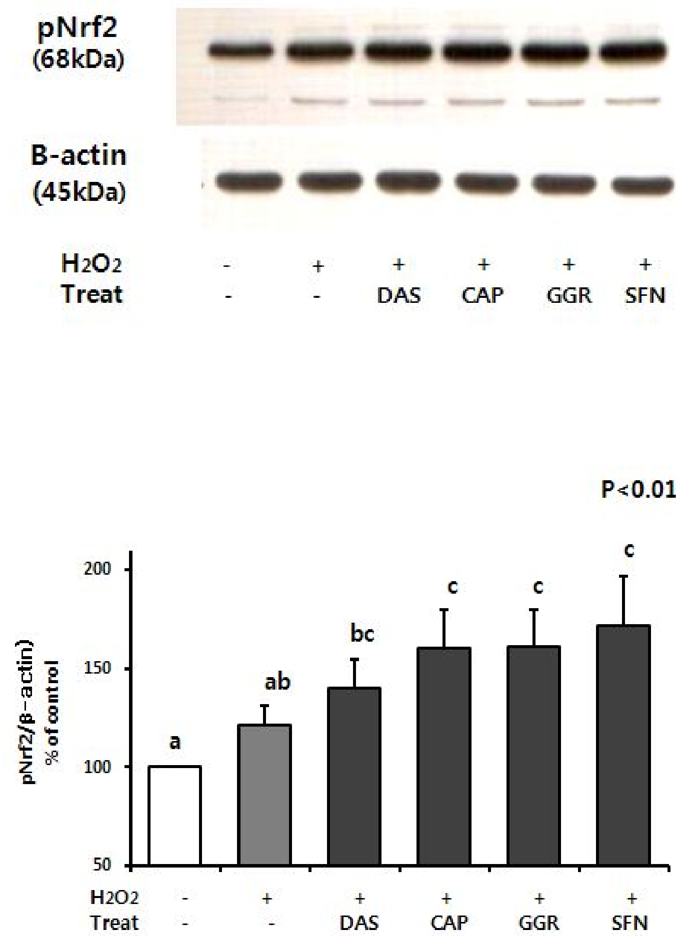


Fig 19. Effect of DAS, CAP, GGR and SFN on the expression of antioxidative signals, JNK (A), Nrf2 (B), pNrf2 (C) against H_2O_2-pretreated HepG2 cells. Actin levels were compared to ensure equal amount of protein loading.

Ⅳ. Disscusion

The present study was performed to investigate the hepato-protective effects of DAS, CAP, GGR and SFN against oxidative stress and liver injury induced by H_2O_2 in vitro system. We decide HepG2 cells(Human liver carcinoma cell line as experimental model to examine the antioxidant effect of DAS, CAP, GGR and SFN. The reason why, liver is the major detoxification organ in the body with a high metabolic rate as well as HepG2 cells have been used a lot as liver experimental model.

Fisrt, to determine the concentration of H_2O_2 and each antioxidants, HepG2 cells were exposed to different concentrations of exposed to different concentrations of H_2O_2 from 10 to 60min and DAS, CAP, GGR and SFN from 6 to 48 hr. As a result we decided the incubation time 10min and concentration of 200μM H_2O_2 to induce 30% cell death As shown in [Fig 20] and concentrarion of 100, 200μM DAS, 25, 50μM CAP, 50, 100μM GGR and 12.5, 25μM SFN by safe range of the cytotoxicity As shown in [Figs 21A-D]. In case of CAP and SFN, shown that high concentration was harmful for cell survival. And then we Determined concentration was treated with H_2O_2, cell viability of four treatment groups was

significantly ($p<0.05$) elevated compared to only H_2O_2-treated cells. but there was no drastic induction of viability.

So, to study the effects of each antioxidants on ROS elimination, HepG2 cells were exposed to different concentrations of DAS, CAP, GGR and SFN for 12hr after treatmen of H_2O_2 for 10min. ROS is important mediators of liver damage. The result shown that exposure to H_2O_2 elevated directly amount of ROS by uo to 220% and all of the treatment significantly reduced intracellular ROS levels compared to the negative control by up to almost similar with control group [Fig 12A]. Interestingly, ROS could be more efficiently removed by concentration of each chemicals such as DAS : 200 μM, CAP : 25μM, GGR : 50μM and SFN : 12.5μM than another concentration [Fig 12B]. Recovery of the intracellular ROS level by treatment with antioxidants indicate that its protective effect against H_2O_2-induced liver damage was mainly due to the inhibition of ROS production. So this result suggest that the integrity of the each antioxidants treated cells was fully Scavenged against the ROS production. Interestingly, protective effect of decrease ROS has been previously reported. Capsaicin pretreatment also protects against the ROS -induced pulmonary damage in rats exposed to such gaseous chemical irritants as sulfur dioxide and nitrogen dioxide [54]. additionally SH-SY5Y neuroblastoma cells were treated

[6]-gingerol (3 or 10 mM) with Aβ_{25-35}(20 mM) and intracellular accumulation of ROS) intracellular levels were significantly decreased by [6]-gingerol pretreatment to the control(Aβ_{25-35})[55].

The cellular antioxidant enzyme system plays a crucial role and changes in the activity of antioxidant enzymes can be considered as biomarkers of the antioxidant response[56]. So maintaining appropriate antioxidant enzyme activity of above a while increasing a stressful situation represents an advantage for cell survival. For these reason, we detected the results that each antioxidants defense against oxidative stress by increasing antioxidant enzymes activity. The concentration of hepatic antioxidant enzymes (SOD, CAP, GPx) was decreased in negative control. The results suggest the following : depletion of antioxidant enzymes from H_2O_2 exposure may be affected by quick using for detoxification over the ROS. But each result of induction of antioxidant enzymes level was different depend on each chemcals and incubation time. This result indicate intracellular antioxidant enzyme system would be more reaction in 12hr than 24hr but CAP only worked in 24 hr with SOD level. And in case of SOD, CAT levels, tendency of result was similar to each other in 12hr, 12.5 SFNμM was highest, on the other hand, GGR was reach up to SFN by 81.5%, whereas in 24hr, GGR shown highest effect on SOD,

CAT levels, this results suggest that GGR could be extended to maintain of activation of antioxidant enzymes. Interestingly, a protective effect of 200μM CAPand 0.63~5μM SFN on Human cells has been previously reported. (57,58), in case of GGR, have been confirmed in many in vitro and in vivo test systems [59]. And also DAS was effectively reduced the frequency of occurrence of lipid peroxidation and enhanced the level of antioxidant enzymes CAT, SOD, GR and GST in prostate and liver in a male mice[60].

It is usually assumed that GSH level reflects intracellular oxidation, whereas a balanced GSH concentration could be expected to prepare the cell against a potential oxidative stress[61]. whereas the oxidation-reduction status of GSH is central to many investigations involving oxidative stress and free radical pathologies and low reduced GSH, high GSSG, and a lower GSH/GSSG ratio have been found in blood from patients with various pathologies[62]. Futhermore reduction of GSH concentrations is associated with aging and the pathogenesis of many diseases, including liver disease, Alzheimer disease, rheumatoid arthritis, muscular dystrophy etc[63]. In fact these, suggest that is important to maintain appropriate level of reduced GSH intracellular. So this study was performed both of reduced GSH contents and GPx activity to verify each ressult. In our experimental conditions,

treatment of HepG2 cells with H_2O_2-induced cells significantly decrease in the concentration of GSH. whereas GSH in cells treated with 200 μM DAS, 25 μM CAP, 50μM GGR and 12.5μM SFN for 12 hr showed a significant recovery. Maintaining GSH concentration above a while increasing a stressful situation represents an advantage for cell survival. Interestingly, reduced GPx was significantly increased by each antioxidant treatment in the same manner as GSH was shown [Fig 15, 16]. This result is considered that all of chemicals were performed role of increment of activity of Glutathione reductse(GR) as well as GPx. Same tendency of result has been proved these fact. In the previous other study, treatment with DAS effectively reduced the frequency of occurrence of testosterone-induced lipid peroxidation and enhanced the level of antioxidant enzymes CAT, SOD and GR in prostate and liver in vivo) in a dose dependent manner[64] and suggested that the protective effect of DAS might be caused by increasing activity of GST, which catalyzes conjugation of electrophillic compounds with GSH. The intracellular content of GSH increases within the cells following treatment with DAS. This phenomenon is beneficial to the detoxification capabilities of hepatocytes [65]. In this regard, the different expression levels of antioxidant enzymes in this study implies that the relationship between intrcellular

ROS levels and antioxidant defense system cannot be interpreted only by the role of individual antioxidant enzymes.

Lipid peroxidation(LPO) refers to the oxidative degradation of lipids. It is the process whereby free radicals electrons from the lipids in cell membranes, finally LPO cause damage to cellular macromolecules by generation of ROS [66], which is considered to enhance cell membrane damage, carcinogenesis and other disease. Traditionally, lipid peroxidation is quantified by measuring malondialdehyde(MDA) and 4-hydroxynonenal (4-HNE), the degradation products of polyunsaturated fatty acid (PUFA) hydroperoxides. However, these assays are non-specific and often lead to an over-estimation of lipid peroxidation. There are important additional problems in using these byproducts as indicators of lipid peroxidation. For example, 4-HNE is formed only from ω-6 PUFA hydroperoxides and is catalyzed by transition metal ions like ferrous ions. Decomposition of hydroperoxides derived from abundant cellular lipids such as cholesterol and oleic acid does not produce MDA or 4-HNE. These factors can lead to an under-estimation of lipid peroxidation. As the reason, we used the LPO assay kit. In experiment, a slight statistically significant increase of LPO was observed in cells treated with the 200μM H_2O_2 at 12 and 24hr. This indicated oxidative damage to cell membrane lipids. Whereas, treatment of DAS,

CAP, GGR and SFN decreased LPO level after treatment with 200μM H_2O_2 in our study, especially effect of GGR is almost same with SOD, CAT and LPO data and SFN treatment also was best efficient at 12hr likewise all of the antioxidant enzyme results. Thease result indicated that SFN was best effective on short time, GGR could maintain the activity of antioxidant enzymes and raised the efficiency to maximum on 24hr. as a result, LPO levels were decreased dramatically.

Therefore LPO level corresponded with that of result of antioxidant enzymes as in other researches. Actually prior study shown that reduction of GSH levels increased lipid peroxidation in a human hepatic cell line [67]. These results supported a role of ROS increasing LPO production and suggested that LPO might be involved in H_2O_2-mediated cell death and antioxidant enzymes system.

In western blot result, shown that expression of Nrf2 and pNrf2 protein level was increased including negative control by DAS, CAP, GGR and SFN [Figs 19B, C]. it is possible that oxidative stress might be involved in the upregulation of phase II detoxifying enzymes, ARE-mediated gene expression [68]. Actually, earlier study shown that ROS play an important role in inducing of Nrf2 expression [69]. Recent studies have presented the effect on antioxidant of Nrf2. Nrf2-null mice have decreased expression of antioxidant genes, cellular

antioxidant capacity and increased oxidative stress[70-74]. In addition, decreased Nrf2 trancriptional activity in Nrf2-null mice fibroblasts was also reported to cause lossing of mRNA of GSH synthesis by up to 15%[75]. whereas the other study, suggest that transcription of ARE was activated by over expression of Nrf2 in HepG2 cells. and this activatoin was more increased by ROS generator, tert- butylhydroquinone [76]. Therefore we know that our data such as pNrf2 increasig is similar with previous studies. Recently, many studies have shown that MAP kinases such as p38, ERK, JNK have sensitivity to oxidative stress [77]. So this study confirm that H_2O_2 rapidly and significantly stimulated MAP kinase family member JNK compared to control [Fig 19A]. Additionally, MAP kinase activation regulates phosphorylation of downstream target proteins and activation of transcription factors such as Nrf2 [78]. So we examined that interestingly, activation of phospholylaton JNK and Nrf2 is similar trend except for GGR [Fig 19C]. In prior study, ERK is activated by DAS and ERK pathways appear to mediate the ho-1 gene activation by DAS. in other word, compared with the untreated HepG2 cell, DAS stimulates a transient increased ROS and ERK activation and subsequently stimulated Nrf2 protein synthesis [79].

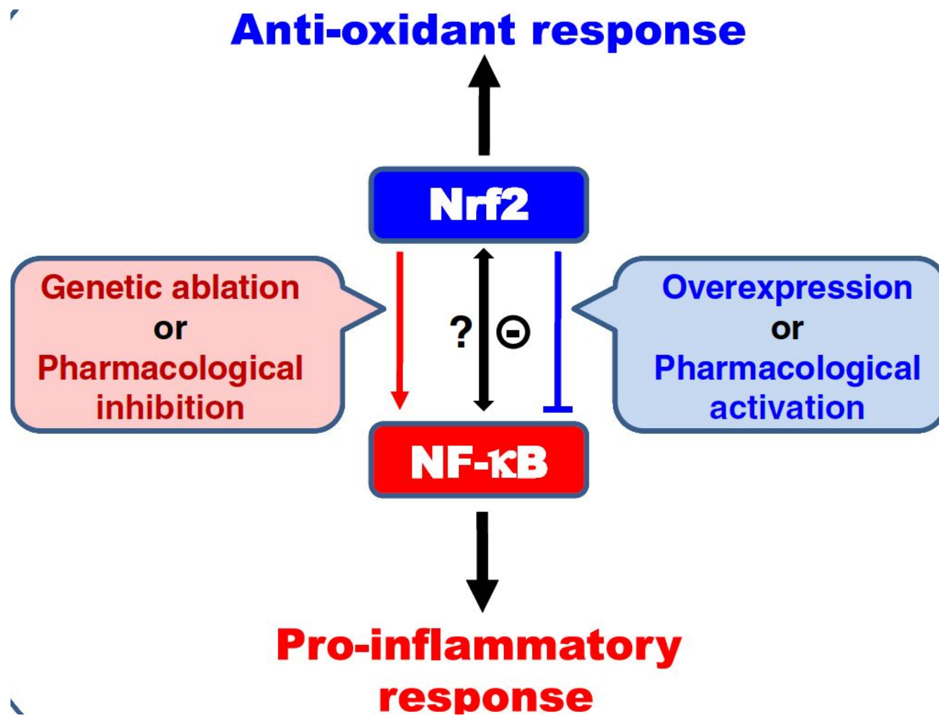
whereas in case of GGR, tendency is especially different beetwen pJNK and pNrf2. in other word, pNrf2 was increased

compared to negative control, DAS even though pJNK was not. so this observation indicate results obtained above that Nrf2 may not be the only factor of control the gene expression by GGR treatments. Futhermore this observation demand additional research directions about wether GGR did activation pNrf2 directly or not.

Already, Hydrogen peroxide (H_2O_2) has been reported to both enhance NF-kB transcription factor activity in HeLa cells and to induce DNA strand breaks after conversion to the hydroxyl radical via the Fenton reaction [80-82]. For these reason, In the current study we have used human-derived HepG2 cells to assess the effects of DAS, CAP, GGR and SFN on H_2O_2-mediated activation of inflammation marker, NF-kB transcription factor activity and TNF-α by confirming the activation of IkBα. As a result, all of chemicals was effective on reduction of expression that. In other study, DAS significantly reduced the production of proinflammatory cytokines such as IL-1β, IL-6, TNF-α which are known proangiogenic factors[83]. But our study, SFN was best effective on inhibition of inflammation marker such as NF-kb and TNF-α protein by blocking phospholylation of IkBα. On the other hand, GGR was also Markedly decreased expression of NF-kB and TNF-α protein. NF-KB regulates transcription of genes encoding for cytokines (such as IL-1β, IL-6, TNF-α)

and growth factors. So expression of inflammatory cytokines is dependent on activated NF-kB. Thus inflammatory cytokines use NF-kB to increment their own signals[84,85]. In additoin, other study shown that 6-gingerol exhibited an anti-inflammatory effect. 6-Gingerol could decrease inducible TNF-a expression through suppression of IkBα phosphorylation, NF-kB nuclear activation and they mentioned that may be developed as a useful agent for the chemoprevention of cancer or inflammatory diseases [86]. In summary our western blot data, activation of Nrf2 and NF-kB was apposite each other likewise other study, treatment with an Nrf2 inducer, sulforaphane, or transient over expression of Nrf2 inhibited NF-κB activation through suppression of H_2O_2 production [87]. Likewise, genetic elimination of Nrf2 aggravated lung injury as revealed by increased serum levels and tissue expression of cytokines by blocking the activation of NF-κB signaling in acute lung injury after traumatic brain injury [88]. Intestinal levels of NF-κB, pro-inflammatory cytokines and ICAM-1 in Nrf2-null mice were significantly higher than those in Nrf2 wild-type mice after challenge with brain injury [89].

These studies demonstrate, Nrf2 is a role as modulator of inflammatory responsesas as shown in [Fig 20]. There are negative correlation between Nrf2 and NF-κB. Nrf2 signal transduction may inhibit NF-κB signaling in contrary.



[Fig 20] The cooperative regulation of Nrf2 and NF-κB has been shown to play a crucial role in translation cellular stress signal into an anti-inflammatory response.

Ⅴ. Conclusion

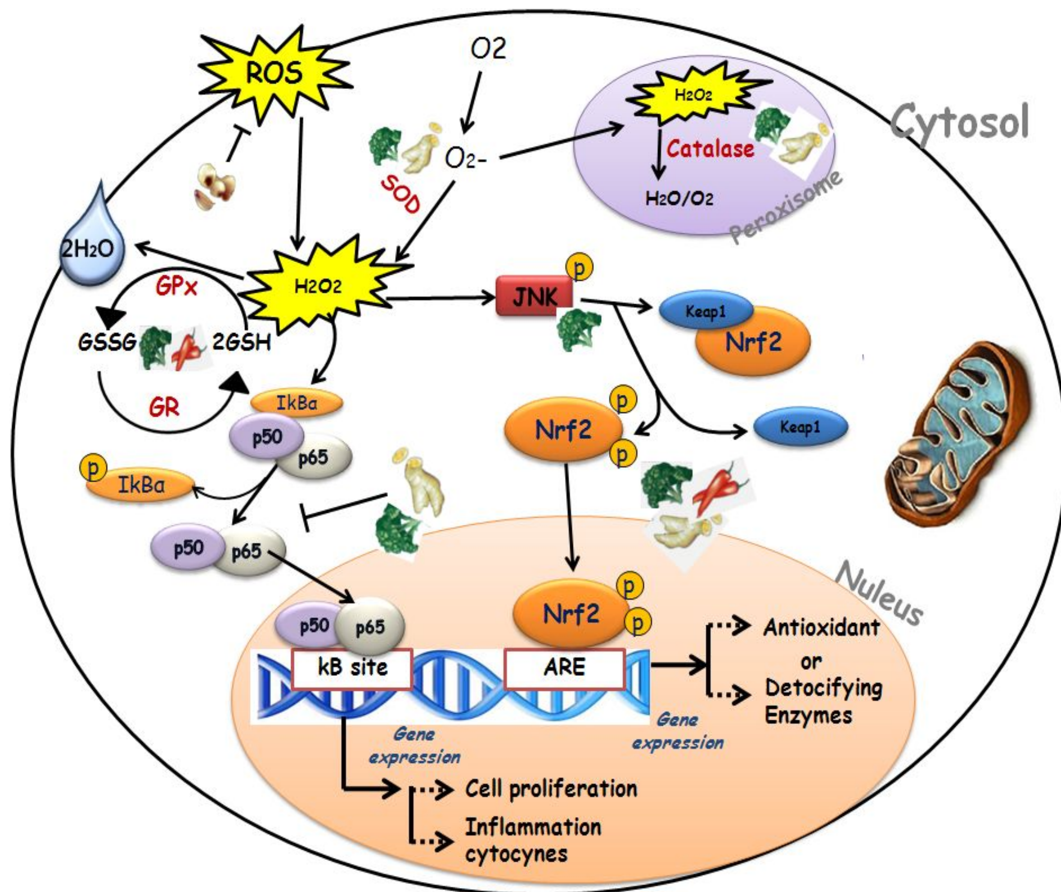
Present study show, for the first time, that a short and long term treatment of human HepG2 cells with DAS, CAP, GGR and SFN prevents the increase of cell death induced by oxidative stress [Fig 11]. And shown a significant decrease of accumulation of ROS and increase the activity all of antioxidant enzymes, SOD, CAT and GPx is observed in HepG2 cells treated with each antioxidant (Figs 13-16) Even though the different time and different levels of each other. Thus, the decreased ROS production reduces the necessity of activity of SOD, CAT and peroxide detoxification through GPx and reduced GSH recovery from GSSG through GR.

Western blot result shown that DAS, CAP, GGR and SFN increased transcription factor Nrf2 related with antioxidant system and Nrf2 was regulated by activation of the MAPK-JNK. Additionally, each antioxidants inhibited activation of TNFα and NF-kB by downregulation of phospholylation of IkBα. The inhibition of TNFα and NF-kb mean that DAS, CAP, GGR and SFN decreased inflammation factor induced by ROS. This result indicated that treatment with an Nrf2 inducer, DAS, CAP, GGR and SFN, increased the antioxidant activity and inhibited NF-kB activation through suppression of H2O2 production. Although a potential antioxidant enzymes

gene expression throughout the Nrf2 can not be found out, the protective mechanism of each antioxidants or its metabolites could be shown in terms of regulation of the antioxidant defense system activity.

Therefore, this study supports that in very common Korean foodstuffs such as Garlic, Red pepper, Ginger and Brocoli intake might contribute to the dietary prevention against many chronic disease are related to the ROS-mediated cellular damage.

In fact there is lack of signal evidence, so more study of concrete signaling and multiple observations is necessary to verify the relationship of these mechanism and the relationship of expression of Nrf2 and NF-kB in the HepG2 cells antioxidant systems with DAS, CAP, GGR and SFN.



[Fig 21] Diagram for effect of DAS, CAP, GGR and SFN on the anioxidant system and cell signaling

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국 문 개 요

산화 스트레스가 유도된 인체 간암세포(HepG2 cell)에서 Diallyl sulfide, Capsaicin, Gingerol 과 Sulforaphane의 항산화 효과

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생체는 외부의 에너지를 섭취하여 산화과정을 통하여 체내 유용한 에너지인 고인산 에너지(ATP)를 생성한다. 이러한 생체 산화과정은 무수한 형태의 활성산소(reactive oxygen substrate: ROS)가 생성을 동반하며 이들은 높은 반응성으로 인해 생체 내 거대분자인 단백질, 지질, 유전자에 산화적 손상을 유발하게 된다. 이러한 이유로 산화스트레스에 의한 여러 가지 질병을 예방하기 위해 우리가 자주 섭취하는 천연식품에서 항산화 물질을 찾으려는

노력이 끊임없이 이어지고 있다. 연구에 의하면 천연식품성분 중의 항산화물질들은 생체 내에서 산화적 활성화과정에 관여하는 free radical을 소거하여 지질의 과산화를 억제하며, 결과적으로 DNA손상 즉 돌연변이 발현을 저해하고 여러 가지 퇴행성 질병 등을 예방하고 건강한 삶을 누릴 수 있게 해 준다.

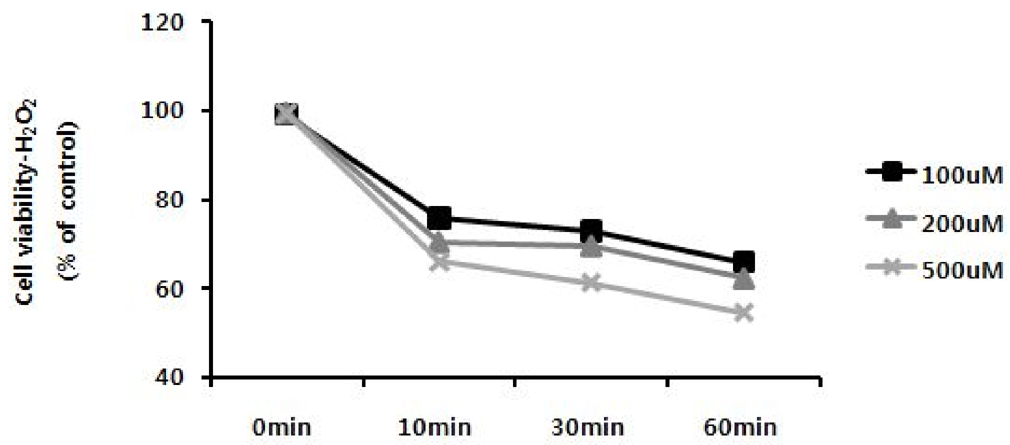
우리나라 대표 향신료 식물인 마늘, 고추, 생강은 김치의 필수 재료이며 주성분은 Diallyl sulfide, Capsaicin과 Gingerol으로 우리나라 식생활에서 중요하게 여겨지고 있으며 이미 오래전부터 음식의 감칠맛을 살리는 향신료, 조미료, 식재료로 사용되어져왔다. 브로콜리의 경우 주성분은 Silforaphane이며, 항산화능이 알려진 이래로 우리나라에서도 비교적 쉽게 섭취할 수 있는 식품이 되었다. 생체 내에서 간은 외부로부터 유입된 물질 뿐 아니라 대사 생성물에 의한 독성으로부터 보호와 해독 기능에 깊이 관여하는 중요한 기관으로 산화스트레스에 의한 간 손상은 여러 가지 질병의 원인이 된다고 알려져 있다. 본 연구에서 선정한 HepG2 cell은 human hepatic carcinoma 로부터 분리된 간암세포로서 인체 간 기능의 실험적 모델로 널리 이용되고 있으며 정상 간과 생화학적 대사가 유사한 범위 안에 있다고 알려져 있다. 따라서 본 연구에서는 HepG2 cell 에 가장 독성이 강한 산화스트레스 인자인 H_2O_2를 처치하여 산화스트레스를 유발하고 DAS, CAP, GGR과 SFN을 농도별로 각각 또는 함께 처치하여 세포의 보호효과, 항산화효소 활성, 지질과산화 생성억제와 항산화와 관련된 세포내 신호전달과정을 분자적 수준에서 확인하고자 한다.

본 실험에서 H_2O_2처리는 HepG2 세포의 손상을 직접적으로 유발시켰고, DAS, CAP, GGR and SFN의 첨가는 손상된 세포의 보호효과를 가지는 것이 확인 되었다 (DAS: 100, 200㎛M, CAP : 25, 50㎛M, GGR : 50,

100μM, SFN : 12.5, 25μM). 또한 세포 내 ROS의 생성을 강하게 억제시키는 것으로 확인 되었으며, 세포내 항산화 효소활성 증가와 과산물 생성억제 효과를 나타냈다. 또한, Western blot으로 확인한 결과 30분동안 처리하였을 때 DAS(200μM), CAP(25μM), GGR(50μM) and SFN(12.5μM)은 항산화와 관련된 전사인자(Nrf2)의 신호 전달 체계를 활성화시켰고 산화스트레스에 의해 활성화되는 단백질(NFkB)의 발현을 억제시킴으로써 연차적으로 염증성 사이토카인 단백질(TNF-a)의 활성을 저해하여 산화스트레스에 의한 세포손상을 막는 것으로 나타났다.

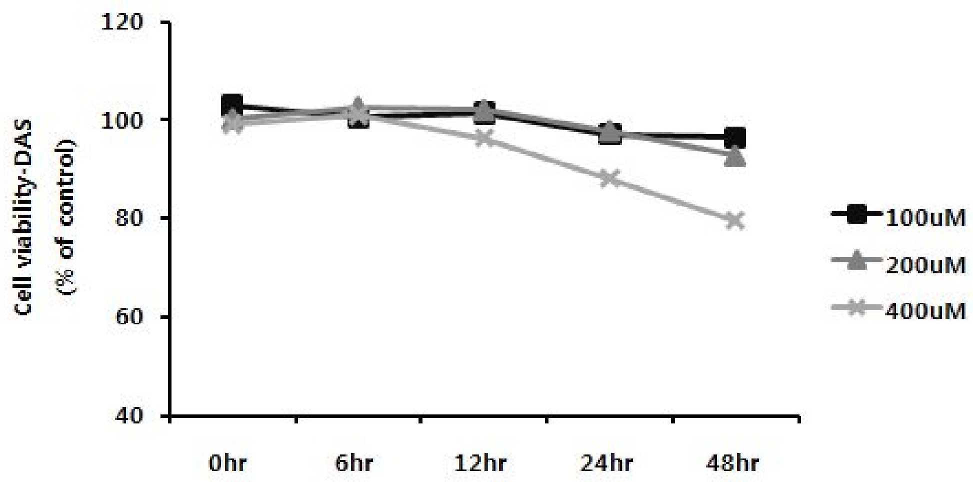
최종적으로, 우리전통 고유의 음식으로부터 유래된 DAS, CAP, GGR과 브로콜리의 SFN은 항산화효소 활성을 증진시키고 이와 관련된 세포내 단백질발현을 조절함으로써 ROS로부터 유도된 산화적 스트레스에서 간세포를 보호하는 것으로 사료된다.

Appendix

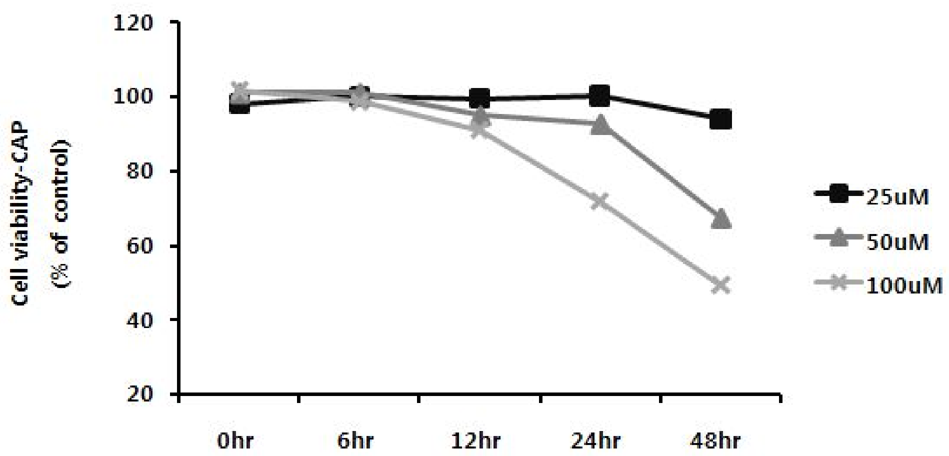


[Fig 22] Cell proliferation of HepG2 cells treatmented with H_2O_2

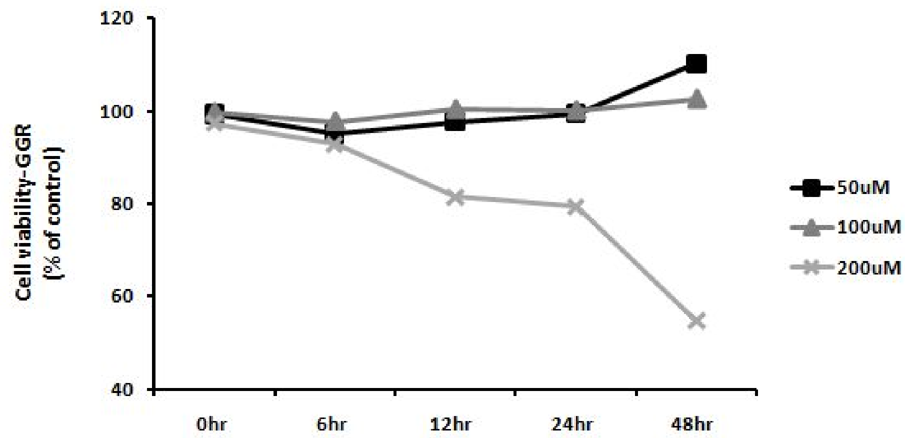
A



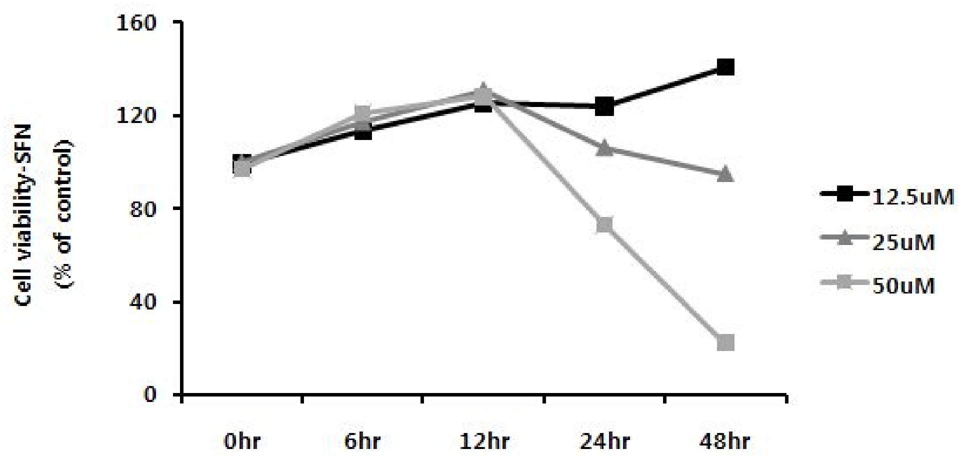
B



C



D



[Fig 23] Cell proliferation of HepG2 cells treatmented with DAS (A), CAP (B), GGR (C) and SFN (D)