ฤทธิ์ป้องกันของกรดไอโซเฟอรูลิกต่อปฏิกิริยาไกลเคชั่นที่ถูกเหนี่ยวนำด้วยน้ำตาลโมเลกุลเดี่ยว
และสารเมทิลไกลออกซอล

นางสาวอร่ามศรี มีพร้อม

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาชีวเคมีคลินิกและอณูทางการแพทย์ ภาควิชาเคมีคลินิก
คณะสหเวชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2558

THE PREVENTIVE EFFECT OF ISOFERULIC ACID ON MONOSACCHARIDES- AND METHYLGLYOXAL-MEDIATED GLYCATION

Miss Aramsri Meeprom

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Clinical Biochemistry and Molecular Medicine
Department of Clinical Chemistry
Faculty of Allied Health Sciences
Chulalongkorn University
Academic Year 2015

Thesis Title	THE PREVENTIVE EFFECT OF ISOFERULIC ACID ON MONOSACCHARIDES- AND METHYLGLYOXAL-MEDIATED GLYCATION
By	Miss Aramsri Meeprom
Field of Study	Clinical Biochemistry and Molecular Medicine
Thesis Advisor	Associate Professor Sirichai Adisakwattana, Ph.D.
Thesis Co-Advisor	Professor Catherine B Chan, Ph.D.

Accepted by the Faculty of Allied Health Sciences, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

.....Dean of the Faculty of Allied Health Sciences

(Associate Professor Prawit Janwantanakul, Ph.D.)

THESIS COMMITTEE

.....Chairman

(Associate Professor Rachana Santiyanont, Ph.D.)

.....Thesis Advisor

(Associate Professor Sirichai Adisakwattana, Ph.D.)

.....Thesis Co-Advisor

(Professor Catherine B Chan, Ph.D.)

.....Examiner

(Assistant Professor Tewin Tencomnao, Ph.D.)

.....Examiner

(Kittana Makynen, Ph.D.)

.....External Examiner

(M.L. Narudee Kashemsant, Ph.D.)

อร่ามศรี มีพร้อม : ฤทธิ์ป้องกันของกรดไอโซเฟอรูลิกต่อปฏิกิริยาไกลเคชั่นที่ถูกเหนี่ยวนำด้วยน้ำตาลโมเลกุลเดี่ยวและสารเมทิลไกลออกซอล (THE PREVENTIVE EFFECT OF ISOFERULIC ACID ON MONOSACCHARIDES- AND METHYLGLYOXAL-MEDIATED GLYCATION) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. สิริชัย อดิศักดิ์วัฒนา, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ. ดร. Catherine B Chan, 152 หน้า.

ไกลเคชั่นของโปรตีนเป็นปฏิกิริยาไร้เอนไซม์ที่ถูกเหนี่ยวนำด้วยน้ำตาลรีดิวซ์ อาทิ กลูโคสและฟรุกโตส หรือสารประกอบไดคาร์บอนิล อาทิ เมทิลไกลออกซอล ส่งผลต่อการเปลี่ยนแปลงโครงสร้างและหน้าที่ของโปรตีน เกิดเป็นสารประกอบเชิงซ้อนในขั้นตอนสุดท้ายที่เรียกว่า แอดว้านซ์ไกลเคชั่นเอนด์โปรดักซ์ ซึ่งสารประกอบชนิดนี้มีส่วนเกี่ยวข้องกับการเกิดพยาธิสภาพและภาวะแทรกซ้อนของโรคหลายชนิด รวมทั้งโรคชราและโรคเบาหวาน กรดไอโซเฟอรูลิกเป็นอนุพันธ์หนึ่งของกรดซินนามิกที่พบมากในส่วนรากของพืชตระกูล *Cimicifuga* ซึ่งพบว่ามีฤทธิ์ในการต้านอนุมูลอิสระและลดระดับน้ำตาลในเลือดได้ การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาฤทธิ์ของกรดไอโซเฟอรูลิกในการต้านปฏิกิริยาไกลเคชั่นที่เกิดกับโปรตีนอัลบูมิน ดีเอ็นเอ และในเซลล์เบต้าของตับอ่อน ผลการศึกษาพบว่า กรดไอโซเฟอรูลิกสามารถยับยั้งการสร้างสารประกอบเชิงซ้อนในขั้นตอนสุดท้ายของปฏิกิริยาไกลเคชั่นและการสร้างฟรุกโตซามีน ยับยั้งปฏิกิริยาออกซิเดชั่นโดยลดการเติมหมู่คาร์บอนิลและลดการสูญเสียหมู่ไทออลในโปรตีน รวมถึงสามารถลดการเกิดโครงสร้างอะไมลอยด์ครอสเบต้าในโปรตีนอัลบูมินที่ถูกเหนี่ยวนำด้วยน้ำตาลกลูโคส ฟรุกโตส และสารเมทิลไกลออกซอล นอกจากนี้ กรดไอโซเฟอรูลิกสามารถยับยั้งการทำลายดีเอ็นเอจากอนุมูลอิสระที่เกิดขึ้นในปฏิกิริยาไกลเคชั่นระหว่างสารเมทิลไกลออกซอลกับกรดอะมิโนชนิดไลซีน และลดการเกิดของอนุมูลอิสระชนิดซูเปอร์ออกไซด์และไฮดรอกซิล โดยไม่ผ่านการจับกันโดยตรงระหว่างกรดไอโซเฟอรูลิกกับสารเมทิลไกลออกซอล การทดลองในเซลล์เบต้าของตับอ่อนพบว่า กรดไอโซเฟอรูลิกช่วยป้องกันเซลล์จากการทำลายของสารเมทิลไกลออกซอลโดยสามารถลดปริมาณของอนุมูลอิสระภายในเซลล์ ซึ่งส่งผลให้มีการแสดงออกของโปรตีนอันคัปลิงชนิดที่ 2 ลดลง ช่วยให้เซลล์กลับมาหลั่งอินซูลินได้มากขึ้น แม้ว่ากรดไอโซเฟอรูลิกจะไม่สามารถเพิ่มปริมาณสารเอทีพีในเซลล์เบต้า แต่กรดดังกล่าวสามารถเพิ่มการอยู่รอดของเซลล์ เพิ่มการทำงานของเอนไซม์ไกลออกซาเลสในการกำจัดสารเมทิลไกลออกซอล และลดการทำงานของแคสเปส 3 ซึ่งเป็นโปรตีนสำคัญในกระบวนการตายของเซลล์เบต้าที่เกิดจากการเหนี่ยวนำของสารเมทิลไกลออกซอล จากผลการศึกษานี้ อาจนำไปสู่การประยุกต์ใช้กรดไอโซเฟอรูลิกในการป้องกันภาวะแทรกซ้อนของโรคเบาหวานอันเกิดจากสารประกอบเชิงซ้อนของปฏิกิริยาไกลเคชั่นได้

ภาควิชา	เคมีคลินิก	ลายมือชื่อนิสิต .....
สาขาวิชา	ชีวเคมีคลินิกและอณูทางการแพทย์	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
ปีการศึกษา	2558	ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5377407237 : MAJOR CLINICAL BIOCHEMISTRY AND MOLECULAR MEDICINE
KEYWORDS: PROTEIN GLYCATION / ADVANCED GLYCATIOIN END PRODUCTS / OXIDATIVE DNA DAMAGE / BETA CELL DYSFUNCTION / METHYLGLYOXAL / ISOFERULIC ACID

ARAMSRI MEEPROM: THE PREVENTIVE EFFECT OF ISOFERULIC ACID ON MONOSACCHARIDES- AND METHYLGLYOXAL-MEDIATED GLYCATION. ADVISOR: ASSOC. PROF. SIRICHAI ADISAKWATTANA, Ph.D., CO-ADVISOR: PROF. CATHERINE B CHAN, Ph.D., 152 pp.

Protein glycation is a non-enzymatic modification of protein by reducing sugars (such as glucose and fructose) or dicarbonyl molecules (such as methylglyoxal), resulting in the formation of advanced glycation end products (AGEs) which have been considered to be a significant contributor of age-related diseases and diabetic complications. Isoferulic acid (IFA), one of cinnamic acid derivatives in plants and rhizome of *Cimicifuga* species, has been shown various pharmacological activities including anti-oxidation and anti-hyperglycemia. The objectives of the present study were to investigate anti-glycation property of IFA in different models including bovine serum albumin (BSA), deoxyribonucleic acid (DNA) and pancreatic β cell. The results showed that IFA (1.25-5 mM) inhibited formation of fluorescent and non-fluorescent AGE, Amadori product (fructosamine), protein carbonyl content, loss of protein thiol groups, and amyloid cross β structure in BSA glycation induced by glucose, fructose and methylglyoxal. IFA (0.1-1 mM) prevented oxidative DNA strand breakage and suppressed superoxide anion and hydroxyl radical production induced by MG and lysine, however, the result from HPLC indicated that IFA did not directly trap MG. In pancreatic β cell, IFA (0.1 mM) prevented β-cell dysfunction by reducing intracellular reactive oxygen species (ROS) and uncoupling protein 2 (UCP2) expression, and improving insulin secretion. Although IFA could not elevate ATP level, it increased glyoxalase I activity in MG-detoxification system and β-cell viability, and also inhibited caspase-3 activity in β cell induced by MG. From these findings, IFA might be considered or applied to use in prevention of AGE-mediated diabetic complications.

Department:	Clinical Chemistry	Student's Signature .....
Field of Study:	Clinical Biochemistry and Molecular Medicine	Advisor's Signature .....
		Co-Advisor's Signature .....
Academic Year:	2015	

ACKNOWLEDGEMENTS

I am deeply thankful to my thesis advisor, Associate Professor Dr. Sirichai Adisakwattana for giving of chance, encouragement, advice and admonishment, and for offering help and care in everything since my master's degree until now. I really appreciate it.

I am greatly thankful to my co-advisor, Professor Dr. Catherine B Chan for her kindness on giving me an opportunity to be a part of Chan's lab. Thank you for suggesting on my work and taking really good care of me all over the time I have been at University of Alberta. It was such a wonderful experience.

I am also thankful to my thesis defense's committee, Associate Professor Dr. Rachana Santiyanont, Assistant Professor Dr. Tewin Tencomnao, Dr. M.L. Narudee Kashemsant and Dr. Kittana Mäkynen for devoting time in my thesis defense and giving suggestion and correction to the thesis.

I would like to give many thanks to all my labmates in both Chulalongkorn University and University of Alberta for their kind help. I really appreciate for all massive things they have done.

I always realize and appreciate my mother for always being there for me no matter what happened, supporting me in every way she can. Thank you so much for being patient and trust in me.

Finally, I am grateful to the RGJ-PhD program (PHD53K011) of the Thailand Research Fund (TRF) and Chulalongkorn University, and the 90th Anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund) for the financial support.

CONTENTS

Page

Page

LIST OF FIGURES

Page

CHAPTER I

INTRODUCTION

1.1 Background and significance of the present study

Protein glycation is the non-enzymatic reaction between carbonyl group of reducing sugar and free amino group of protein that has been found to be contributed from chronic hyperglycemia (Buongiorno et al., 1997; Sheetz and King, 2002). This reaction initiates a complex cascade of repeated condensations, rearrangements and oxidative modifications, resulting in the formation of poorly characterized heterogeneous byproducts termed advanced glycation end products or AGEs (Ahmed, 2005; Ardestani and Yazdanparast, 2007a; Negre-Salvayre et al., 2009). AGEs play an important role in further cross-linking or modification of other proteins and generating oxidizing intermediates, resulting in induction of oxidative stress in vascular cells as well as other tissues (Ulrich and Cerami, 2001). For this reason, the excessive formation and accumulation of AGEs have been considered to be a significant contributor of age-related diseases (Khazaei et al., 2010), atherosclerosis, and diabetic complications such as retinopathy, nephropathy and neuropathy (Kousar et al., 2009).

Current scientific evidence indicates a dicarbonyl molecule generated from glycolysis, lipid peroxidation and polyol pathway namely methylglyoxal as a reactive precursor to produce AGEs. It is supposed to be a causative factor mediated an oxidative modification and subsequent damage on cellular components including proteins (Seneviratne et al., 2011) and DNA (Kang, 2003; Suji and Sivakami, 2007). Recent studies demonstrate that methylglyoxal induces mitochondrial dysfunction

by suppressing respiratory chain and ATP synthesis, and increasing oxidative stress (Wang et al., 2009a; Remor et al., 2011). Additionally, methylglyoxal has also been found to impair glucose-stimulated insulin secretion in pancreatic β cell (Fiory et al., 2011). Thus, the possible way to reduce a risk of diabetic complications is to inhibit AGE formation and methylglyoxal accumulation (Wu et al., 2011). Anti-glycating agent such as aminoguanidine has received the most interest from a clinical trials perspective due to its inhibitory effect on AGEs formation (Ihm et al., 1999; Kousar et al., 2009). However, recent studies have indicated some deleterious properties of aminoguanidine in patient with diabetic nephropathy (Bolton et al., 2004). Therefore, much effort has been extended in search of effective phytochemical compounds from dietary fruits, plants and herbal medicines on inhibition of AGE formation (Vinson and Howard III, 1996; Ardestani and Yazdanparast, 2007a; Tupe and Agte, 2010) or that act as antioxidant, metal ion chelator, or methylglyoxal trapper (Peng et al., 2011).

Cinnamic acid and its derivatives are widely distributed among fruits and vegetables. They exert many biological activities such as anti-inflammation (Kim et al., 2012), anti-oxidation (Natella et al., 1999), and anti-hyperglycemia (Choi et al., 2011). Isoferulic acid (IFA), one of cinnamic acid derivatives, is a major active ingredient derived from the rhizome of *Cimicifuga* species which have been traditionally used as anti-inflammatory drug in oriental countries (Sakai et al., 2001). Recently, IFA has been reported to have anti-inflammatory activity in virus-infected mice (Hirabayashi et al., 1995; Sakai et al., 1999; Sakai et al., 2001), antioxidant activity as a free radical scavenger and metal ion chelator (Hirata et al., 2005; Cai et al., 2006; Wang et al., 2011), and anti-diabetic property in streptozotocin-induced

diabetic rats (Liu et al., 2000). Interestingly, IFA reduces plasma glucose concentration by activating α_1-adrenoceptors to enhance the secretion of β-endorphin, which could stimulate the opioid μ-receptors to increase glucose use and/or reduce hepatic gluconeogenesis in streptozotocin-induced diabetic rats (Liu et al., 2003). However, studies regarding the inhibitory effects of IFA on protein glycation have not been investigated. Therefore, it would be interesting to examine the proventive effect of IFA on reducing monosaccharides- or methylglyoxal-induced glycation in various models including bovine serum albumin (BSA), deoxyribonucleic acid (DNA) and pancreatic β cell. The expected benefits from the present study can be used to consider or apply IFA as an anti-glycating agent in order to prevent AGE-mediated protein glycation.

1.2 Objectives of the present study

1. To investigate the inhibitory effect of IFA on protein glycation induced by glucose, fructose and methylglyoxal.

2. To examine the preventive effect of IFA on methylglyoxal-induced oxidative DNA damage.

3. To elucidate the preventive effect of IFA on methylglyoxal-induced pancreatic β-cell dysfunction.

1.3 Hypotheses of the present study

1. IFA could inhibit glucose-, fructose- and methylglyoxal-mediated protein glycation by decreasing formation of AGEs, Amadori products, and amyloid cross β structure as well as oxidation-dependent protein damage.

2. IFA could prevent methyglyoxal-induced oxidative DNA damage by reducing ROS production and DNA fragments.

3. IFA could prevent methylglyoxal-induced pancreatic β-cell dysfunction by suppressing ROS production and UCP2 expression, improving ATP synthesis and insulin secretion, and preventing β-cell damage

CHAPTER II

LITERATURE REVIEW

2.1 Diabetes

The International Diabetes Federation (IDF) defines that “diabetes is a chronic condition that occurs when the body cannot produce enough insulin or cannot use insulin, and is diagnosed by observing raised levels of glucose in the blood. Insulin is a hormone produced in the pancreas; it is required to transport glucose from the bloodstream into the body’s cells where it is used as energy. The lack, or ineffectiveness, of insulin in a person with diabetes means that glucose remains circulating in the blood. Over time, the resulting high levels of glucose in the blood (known as hyperglycaemia) causes damage to many tissues in the body, leading to the development of disabling and life-threatening health complications.” (IDF, 2015).

A chronic hyperglycemia indicated by long-termed exposure to high concentration of glucose in blood circulation of diabetic patients is associated with development of diabetic complications in blood vessel (Nathan et al., 2005) leading to the pathogenesis in tissues and organs such as eyes (Hove et al., 2004), kidneys (Looker et al., 2003), brain (Seki et al., 2004) and heart (Nathan et al., 2005). Many risk factors were reported to involve in hyperglycemia including obesity, prolonged consumption of high fat or high carbohydrate diet, low exercise and activity, increasing age, as well as oxidative stress. An accumulation of ROS resulting in oxidative stress is a key factor in several degenerative diseases and involves in the pathological process of diabetes (Robertson et al., 2004; Hou et al., 2008). Also, an elevation of intermediate stage and late stage products of glycation, a non-enzymatic

reaction between glucose and biological macromolecules, is found to associate with the occurrence and pathology of diabetic complications especially diabetic nephropathy, neuropathy and retinopathy (Brownlee et al., 1988).

2.2 Glycation

2.2.1 Definition of glycation

Glycation is a process of spontaneous chemical transformation of amine-containing molecules by reducing sugars as called that Maillard reaction (Brownlee et al., 1984). Within the body, free amino groups of protein, lipid, and nucleotide in DNA can react non-enzymatically with carbonyl groups of reducing sugars and other reactive carbonyl-containing molecules to initiate a complex cascade of repeated condensations, rearrangements and oxidative modifications that leads to the formation of poorly characterized heterogeneous molecules termed advanced glycation end products (AGEs), which takes several days to weeks to complete the process (Negre-Salvayre et al., 2009). This reaction contributes the alteration of physiologic processes including enzyme activity, binding of regulatory molecules, crosslinking of proteins, susceptibility to proteolysis, macromolecular recognition and endocytosis, immunogenicity, and function of nucleic acids (Brownlee et al., 1984).

2.2.2 Mechanism of protein glycation

AGEs are cross-linked structures formed as irreversible byproducts from the cascade of glycation that affect an alteration of protein structure leading to modification of biological activities and the susceptibility of protein to proteolysis (Ulrich and Cerami, 2001). A complex process of protein glycation (Figure 1) is

initiated by non-enzymatic interaction between free amino groups of protein (mostly lysine, arginine, and cysteine residues) and carbonyl group in reducing sugars (such as glucose (Ardestani and Yazdanparast, 2007b) and fructose (Ardestani and Yazdanparast, 2007a) or reactive dicarbonyl molecules (such as glyoxal, methylglyoxal and 3-deoxyglucosone (Singh et al., 2001), leading to the formation of reversible structure called Schiff's bases, which occurs over a period of hours. This structure can further rearrange, through the formation of enaminol intermediate, into the more stable ketoamine termed Amadori products, such as fructosamine (Ardestani and Yazdanparast, 2007b) and HbA1c (Rahbar, 2005), which occurs over a period of days. These products undergo further autoxidation which leads to release of some free radicals, generation of dicarbonyl intermediates, and subsequent formation of AGEs (Brownlee et al., 1988; Negre-Salvayre et al., 2009). In the presence of transition metals, superoxide anion converted to hydroxyl radicals via the Fenton reaction (Ahmed, 2005). An accumulation of these reactive oxygen species (ROS) leads to oxidative stress which contributes to protein oxidation. The oxidation of protein is also found to diminish the free sulfhydryl or thiol groups (-S-H-) in protein (Aćimović et al., 2009). Thus, an increased content of protein carbonyls and loss of protein thiol groups reflect oxidative protein damage (Dalle-Donne et al., 2003; Balu et al., 2005). Furthermore, glycation is found to cause the conformational change of protein observed by increasing level of amyloid cross β structure (Khazaei et al., 2010), leading to an aggregation of protein which is mostly found in various neurodegenerative disorders such as Alzheimer's disease (Bouma et al., 2003).

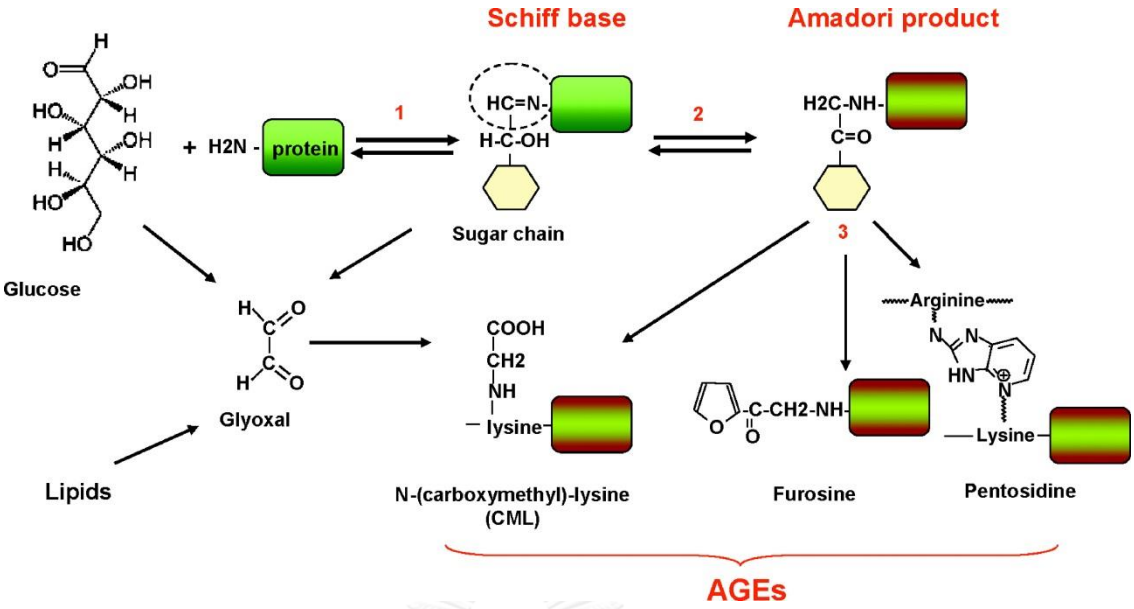

Figure 1 Protein glycation leading to AGEs formation via intermediates Schiff's bases and Amadori products.
(Negre-Salvayre et al., 2009)

2.2.3 Effect of protein glycation and AGEs

Several types of detectable AGEs in tissue can be categorized dependently upon fluorescent and cross-linking capabilities as shown in Figure 2. The most characterized fluorescent, cross-linked AGE that has been shown to increase in diabetes and Alzheimer's disease is pentosidine (Ardestani and Yazdanparast, 2007b), whereas a major non-crosslinked AGE derived from oxidative breakdown of Amadori product is N^{ε}-(carboxymethyl)-lysine (CML) (Bär et al., 2003). This lysine-modified AGE is widely used as an indicator for AGEs formation in human (Puddu et al., 2010; Wu et al., 2011). Formation and accumulation of CML have been demonstrated to associate with atherosclerosis (Wang et al., 2012), Alzheimer's disease, as well as diabetic complications (Gironès et al., 2004).

Deleterious effect of AGEs is the ability to either intramolecularly or intermolecularly cross-link to proteins, leading to protein modification and dysfunction, such as an impairment of enzyme activity, ligand binding, and immunogenicity, both in blood circulation and tissues (Ahmed, 2005). AGEs were shown to exert adverse effects on insulin-secreting cells in several studies. Luciano and colleagues indicated that 5-day incubation period of HIT-T15 cells, the pancreatic islet cell line, with glycated serum causes the reduction of cell proliferation demonstrating by an elevation of cell apoptosis and necrosis (Luciano Viviani et al., 2008). Glycated serum-treated HIT-I15 cells markedly increased oxidative stress, whereas decreased glucose-stimulating insulin secretion and intracellular insulin content (Luciano Viviani et al., 2008). Recent publication has revealed that AGEs also decreased insulin biosynthesis in pancreatic β-cell through suppressing transcription factor Pdx-1 protein expression (Shu et al., 2011).

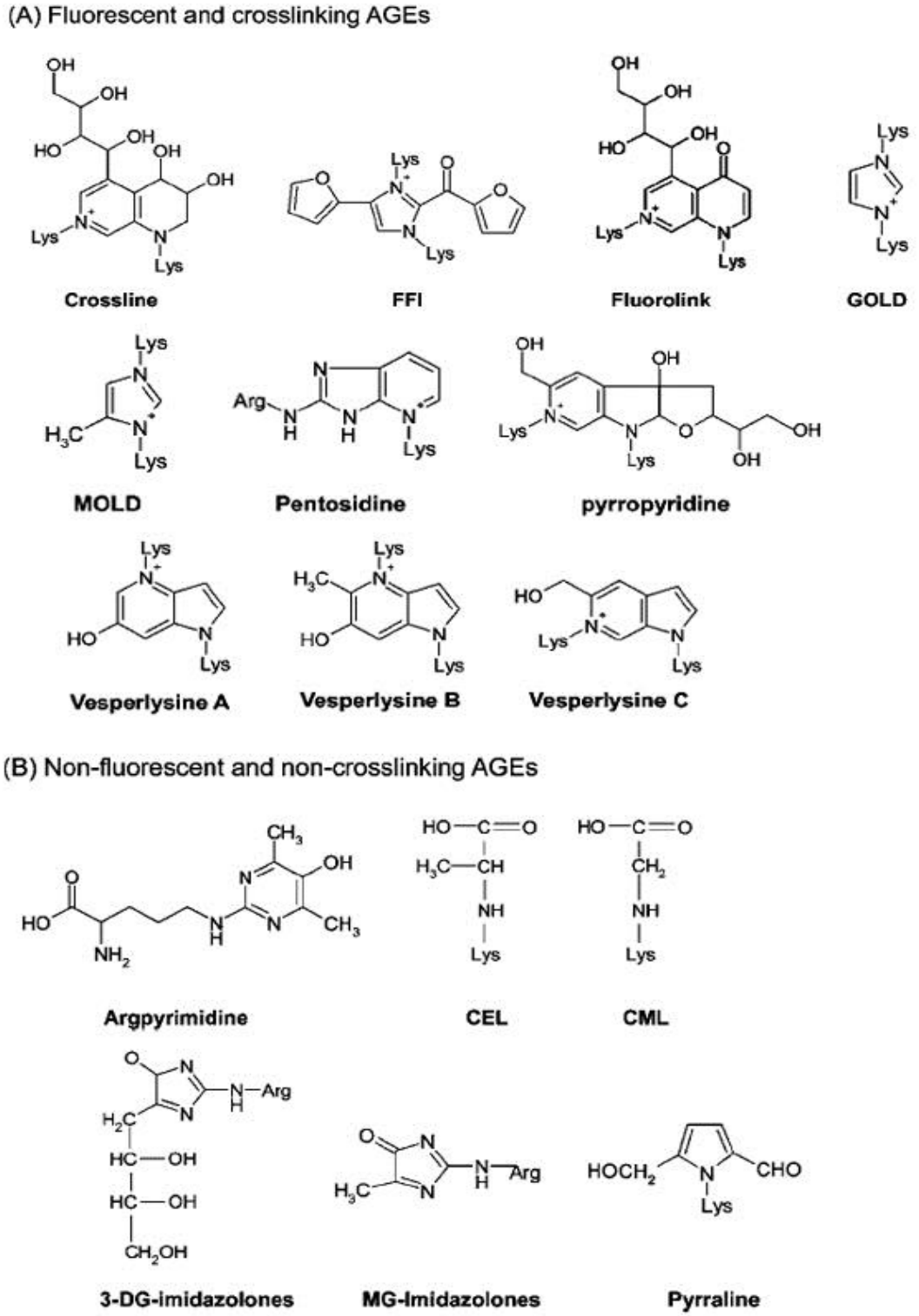

Figure 2 Chemical structures of different type of AGEs.

Moreover, AGEs themselves can also bind to the receptor of advanced glycation end products or RAGEs expressed on the cell surface of, in particular, structural components of the connective tissue matrix and basement membrane components such as type IV collagen (Goh and Cooper, 2008). Binding and stimulation of RAGEs by AGEs lead to intracellular signal transduction involved in aging, inflammation, vascular complications, and neurodegeneration through the key target transcription factor nuclear factor-κB (NF-κB) (Sparvero et al., 2009).

From these reasons, AGEs have been implicated in the development of aging as well as the pathogenesis of age-related disorders including Alzheimer's disease and diabetic complications (Booth et al., 1997).

2.2.4 Methylglyoxal

Methylglyoxal is a 72-dalton molecule which is a potent cross-linking agent significant in diabetic complications (Vander Jagt, 2008). Current scientific evidence indicates methylglyoxal, one of α-oxoaldehydes, as a reactive precursor to produce AGEs (Lo et al., 1994; Westwood and Thornalley, 1995). Methylglyoxal (see structure in Figure 3) is a reactive dicarbonyl molecule formed from dihydroxyacetone-phosphate (DHAP) and acetoacetate in glycolytic pathway and lipid peroxidation, respectively (Kalapos, 1999). Also, it is generated as an intermediate in polyol pathway by aldose reductase (Vander Jagt et al., 2001) and during early and intermediate stages of glycation pathway (Singh et al., 2001) as shown in Figure 4 and Figure 5.

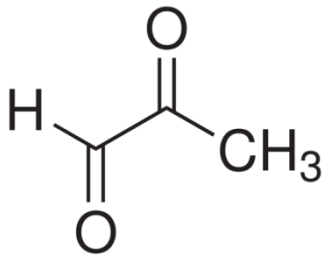

Figure 3 Chemical structure of methylglyoxal
(Wu et al., 2011)

Previous study has demonstrated that methylglyoxal involves the development of atherosclerosis induced on the glycated low-density lipoprotein (LDL), generated fluorescent AGEs and superoxide anion radicals (Schalkwijk et al., 1998). In type 2 diabetic patients with nephropathy, an increased methylglyoxal level has been found to positively correlate with increased fasting blood glucose, hemoglobin A1c (HbA1c), and urinary albumin/creatinine ratio (Lu et al., 2011) and also associate to an elevated cytokines in response to inflammation, which impairs β-cell function and causes microvascular damage (Sheader et al., 2001; Lu et al., 2011). Methylglyoxal was found to decrease insulin synthesis, impair insulin signaling (Fiory et al., 2011), suppress glucose-stimulated insulin secretion (Cook et al., 1998; Fiory et al., 2011), and also possess cytotoxic action by causing apoptosis in insulin secreting cells (Sheader et al., 2001). Furthermore, there are several intensive evidences demonstrated an oxidative effect of methylglyoxal on nucleic acid, particularly DNA structure. For example, human lens epithelial HLE-B3 cells exposed to methylglyoxal performed an elevation of 8-hydroxydeoxyguanosine (8-OHdG), which was used as an indicator of oxidative DNA damage (Kim et al., 2010). Moreover, methylglyoxal-induced lysine modification caused oxidation-dependent DNA strand breaks (Kang, 2003; Chan and Wu, 2006).

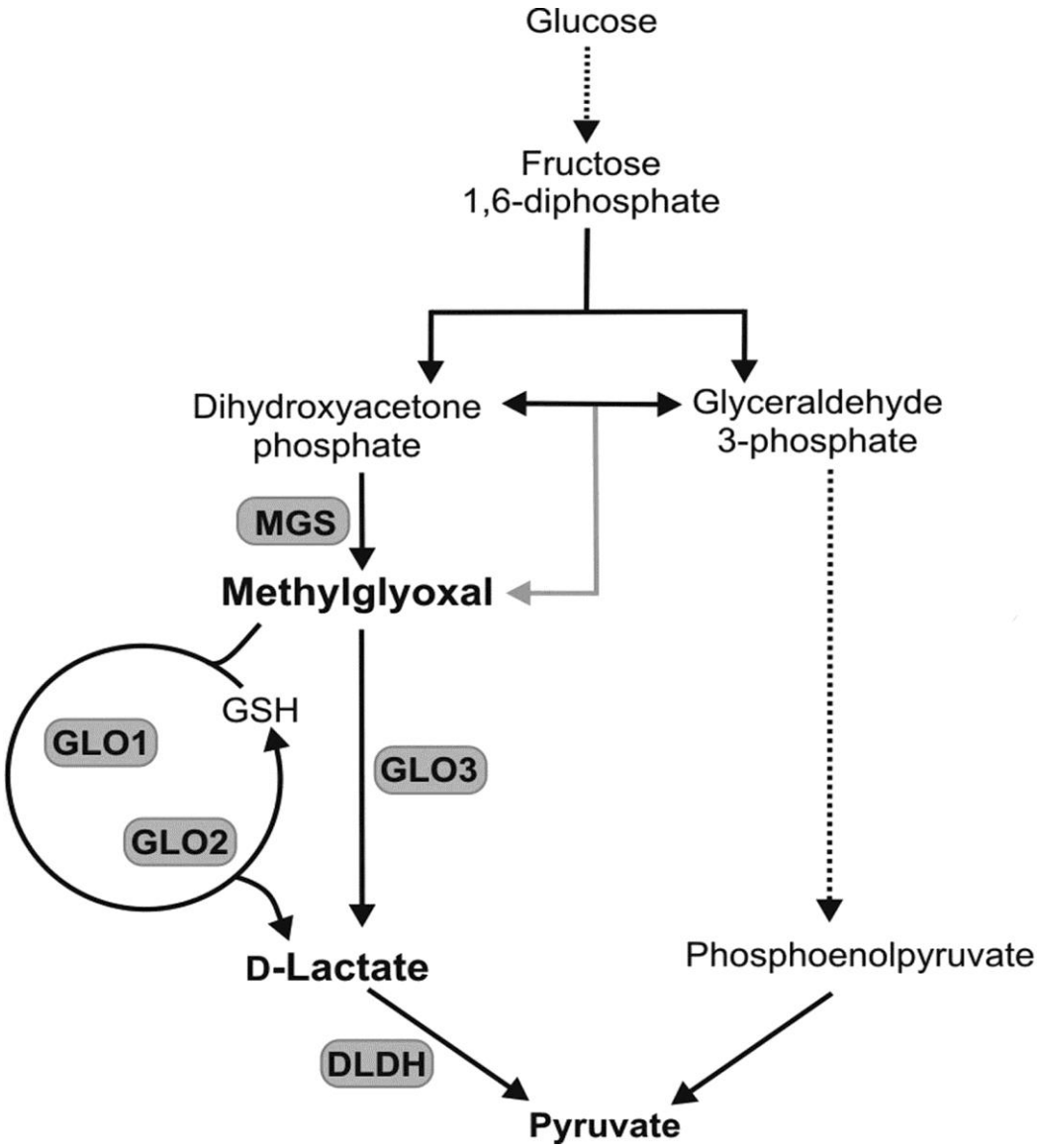

Figure 4 The generation of methylglyoxal from glycolysis
(Sousa Silva et al., 2013)

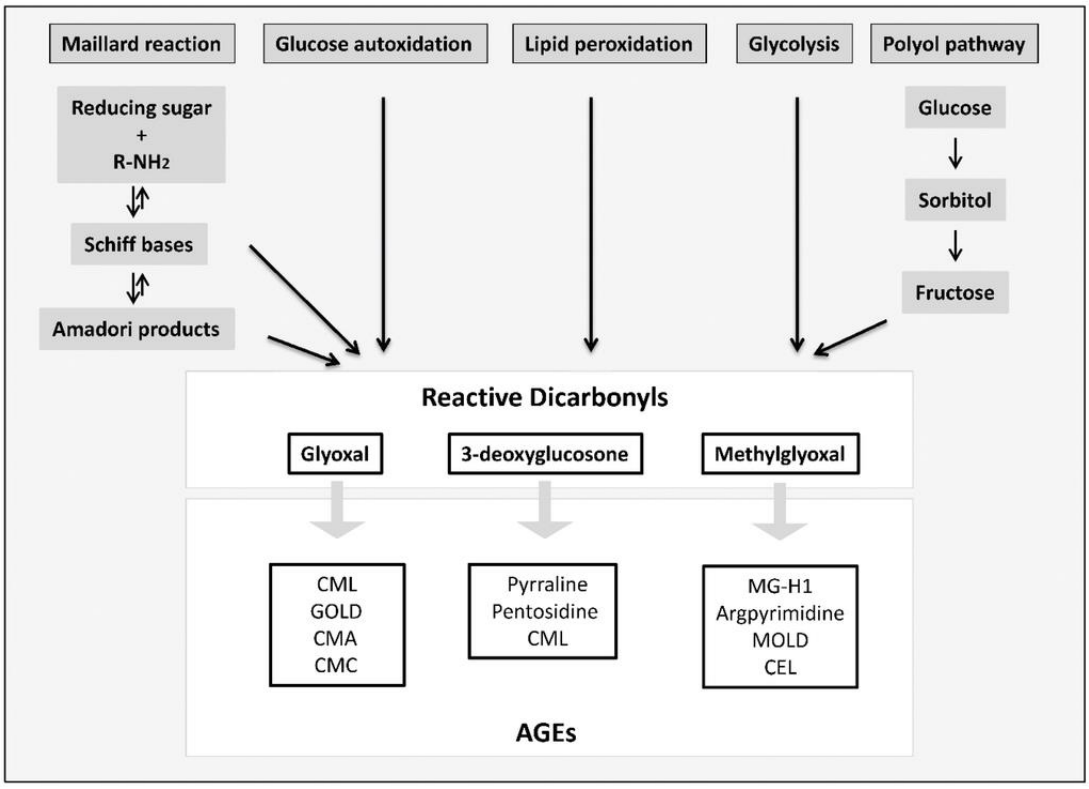

Figure 5 The generation of methylglyoxal from other mechanisms including polyol pathway, lipid peroxidation, glucose autoxidation and maillard reaction (glycation pathway).
(Nowotny et al., 2015)

2.2.5 Detoxification of methylglyoxal (Glyoxalase system)

Apart from alteration of methylglyoxal to pyruvate in glycolytic pathway, generated methylglyoxal can be eliminated by changing to a non-toxic molecule via detoxification process called glyoxalase system, which is a cytoplasmic reaction that converses methylglyoxal to lactate (Thornalley, 1994; Rabbani and Thornalley, 2012). This system comprises two major enzymes, glyoxalase I and glyoxalase II, working together with reduced glutathione (GSH). In the presence of GSH, methylglyoxal is non-enzymatically conversed to a reversible hemithioacetal and further catalyzed to an intermediate S-D-lactoylglutathione by glyoxalase I. After that, glyoxalase II

catalyses the hydrolysis of S-D-lactoylglutathione to D-lactate and regenerates reduced glutathione (Rabbani and Thornalley, 2014) (Figure 6).

This system plays a critical role in the enzymatic defense against glycation (Thornalley, 2003; Rabbani and Thornalley, 2014). An overexpression of glyoxalase I was found to reduce hyperglycemia-induced levels of AGEs and oxidative stress in diabetic rats (Brouwers et al., 2011), and also lowered the carbonyl stress (Inagi et al., 2002).

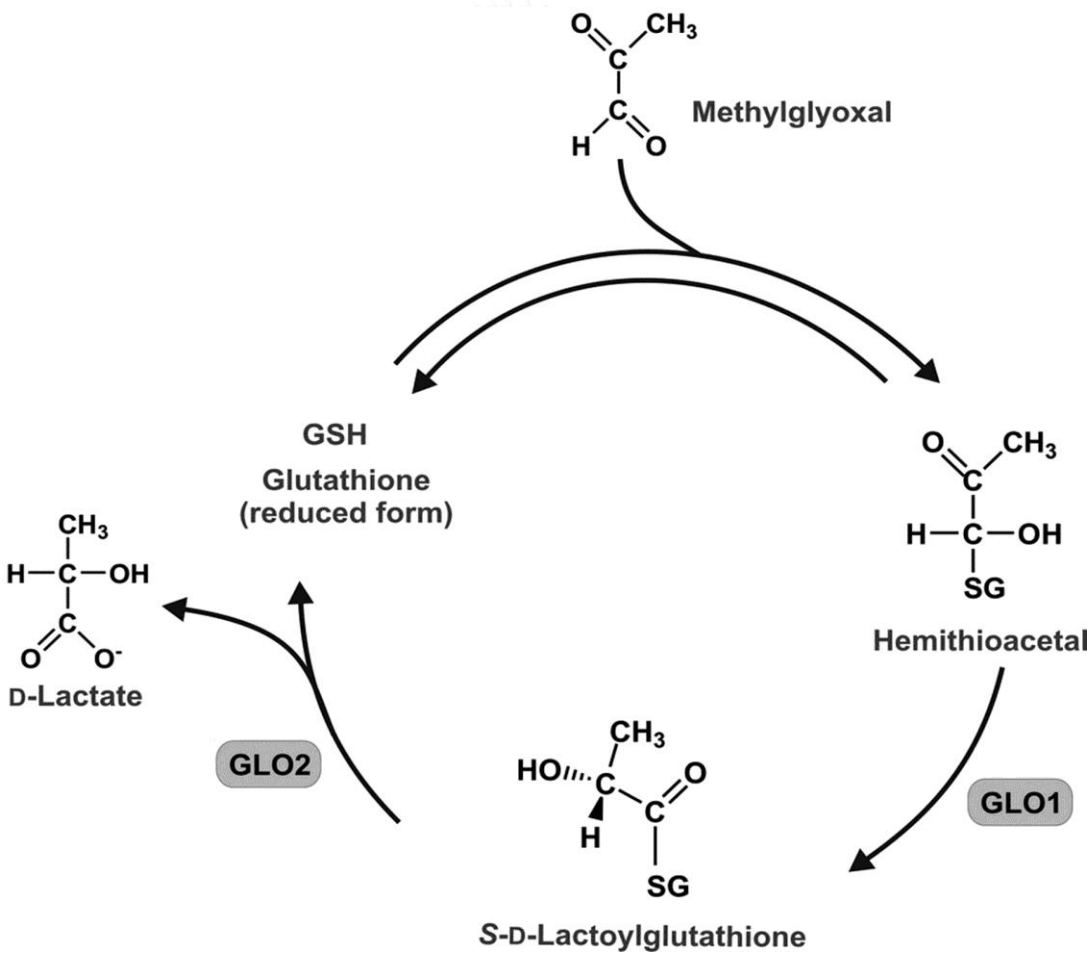

Figure 6 Glyoxalase (GLO) system
(Sousa Silva et al., 2013)

2.2.6 Anti-glycating agents

Anti-glycating agents are classified into 2 major groups according to the mechanism underlying their action; 1) AGE inhibitors which are both natural and synthetic compounds that inhibit each step of AGE formation, and 2) AGE breakers which are compounds that can destroy the generated AGEs by breaking the covalent cross-links of AGEs (Nagai et al., 2012).

The proposed mechanisms of AGE inhibitors include compounds that can act as the following:

1. Binding of amino groups that targeted to be the glycation sites of protein to prevent the reaction between protein and carbonyl-containing molecule in early stage of glycation. The sample of this mechanism is aspirin (Rendell et al., 1986).

2. Binding of carbonyl group in reducing sugars and/or reactive dicarbonyl compounds generating as intermediates in glycation such as methylglyoxal. Aminoguanidine (AG) is the sample that acts via this mechanism (Thornalley, 2003).

3. Scavenging of free radicals generating by oxidation during glycation process. Antioxidants and ROS scavengers, such as vitamin C and E (Kutlu et al., 2005) and glutathione (Thornalley, 1998), are proposed in this mechanism.

4. Degradation of Amadori products, such as the enzyme from *Aspergillus* called amadoriases (Takahashi et al., 1997).

2.3 Pancreatic β cell

Pancreatic β cell is well known as an insulin-producing cell and plays a significant role in glucose homeostasis due to its function on insulin secretion (Brand et al., 2004) (Figure 8). The impairment in function or damage of β cell leads to the failure in response to hyperglycemia and results in the the development of diabetes (Fiory et al., 2011).

2.3.1 Glycation and β cell dysfunction

ATP synthesis occurred by oxidative phosphorylation in inner mitochondrial membrane is an important step driven an exocytosis of insulin from pancreatic β cell (Brand et al., 2004). Therefore, mitochondrial dysfunction with a change of oxidative activity may associate with a defect of pancreatic insulin secretion (Souza et al., 2011).

Previous investigation in other tissues demonstrates an involvement of methylglyoxal-mediated AGEs and mitochondrial dysfunction. It found that methylglyoxal impairs mitochondrial respiratory chain and ATP synthesis by significantly inhibiting complex I-III activities in mitochondria from liver, skeletal muscle (Remor et al., 2011) and vascular smooth muscle (Wang et al., 2009a). In addition, an excessive production of ROS is reported to be a key causative factor contributes loss of mitochondrial membrane potential, leading to a defect of mitochondrial function in ATP synthesis (Rolo and Palmeira, 2006). Evidence from recent study reveals that methylglyoxal induces oxidative stress by increasing the production of mitochondrial peroxynitrite (ONOO), superoxide anion (O_2^-) and nitric oxide (NO) (Wang et al., 2009a).

In β cell, methylglyoxal is found to decrease insulin synthesis by suppressing mRNA expression of insulin-involving genes including insulin-1 (*Ins1*), glucokinase (*Gck*) and pancreatic duodenal homeobox-1 (*pdx1*), impair insulin signaling by inhibiting insulin receptor substrate (IRS) tyrosine phosphorylation and phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB) activation (Fiory et al., 2011), and also suppress glucose-stimulated insulin secretion (GSIS) (Cook et al., 1998; Fiory et al., 2011). The most recent evidence indicates that exposure of insulin secreting cell to high concentration of methylglyoxal in short period induces significantly high production of mitochondrial ROS which initiates an increasing expression of uncoupling protein 2 (UCP2) in response to regulate redox homeostasis in the cell, whereas ATP content is found to decrease. These events result in a decline of GSIS and reflect β cell dysfunction mediated by methylglyoxal (Bo et al., 2016).

2.3.2 Glycation and β cell damage

Methylglyoxal has been investigated the effect on cellular damage in different cell types such as neuronal cell (Heimfarth et al., 2013) and hepatic cell (Seo et al., 2014). Recent studies demonstrate the cytotoxicity of methylglyoxal in pancreatic β cell via activation of cell apoptosis which involves methylglyoxal-induced excessive ROS production and mitochondrial dysfunction (Bo et al., 2016; Gao et al., 2016). The study in pancreatic β-cell line including mouse insulinoma (MIN-6) and rat insulinoma (INS-1) cells indicated that methylglyoxal induced an increase of apoptotic rate and caspase-3 activity corresponding with an elevation of ROS (Bo et al., 2016). Moreover, INS-1 cell treated with 0.25-2 mM methylglyoxal for 24 h exhibited a decrease of ATP level correlating with an over production of intracellular of ROS, a release of

cytochrome *c* and apoptosis-inducing factor (AIF) from mitochondria, and also up-regulated the expression of activated caspase-3 and caspase-9 (Gao et al., 2016).

Activation of caspases in apoptotic cascade pathway initiates cell self-digestion and nuclear DNA fragmentation, resulting in cell damage and dysfunction. A proteolytic cleavage of caspase-3 is a downstream activation of caspase-8 from death receptor pathway and caspase-9 from mitochondrial pathway for cell apoptosis. Thus, once caspase-3 is activated, it reflects the response of cell in programing cell death (Gao et al., 2008).

Taken together, it seems to be that ROS overproduction, UCP2 upregulation, ATP depletion and cell apoptosis activation in β cell are the key linkage between the role of methylglyoxal and the pathology of diabetes and diabetic complications.

2.4 Uncoupling protein 2 (UCP2)

Uncoupling proteins (UCPs) are a group of proton transporter proteins locating in inner mitochondrial membrane. The term “uncoupling protein” was originally used for uncoupling protein 1 (UCP1) which is called a thermogenin due to its function in thermogenesis via influx of proton uncoupling to electron transport chain and ATP synthase (Jastroch et al., 2010) (Figure 7). Thus, UCP1 is uniquely present in mitochondria of brown adipocytes, the thermogenic cells that maintain body temperature (Rousset et al., 2004).

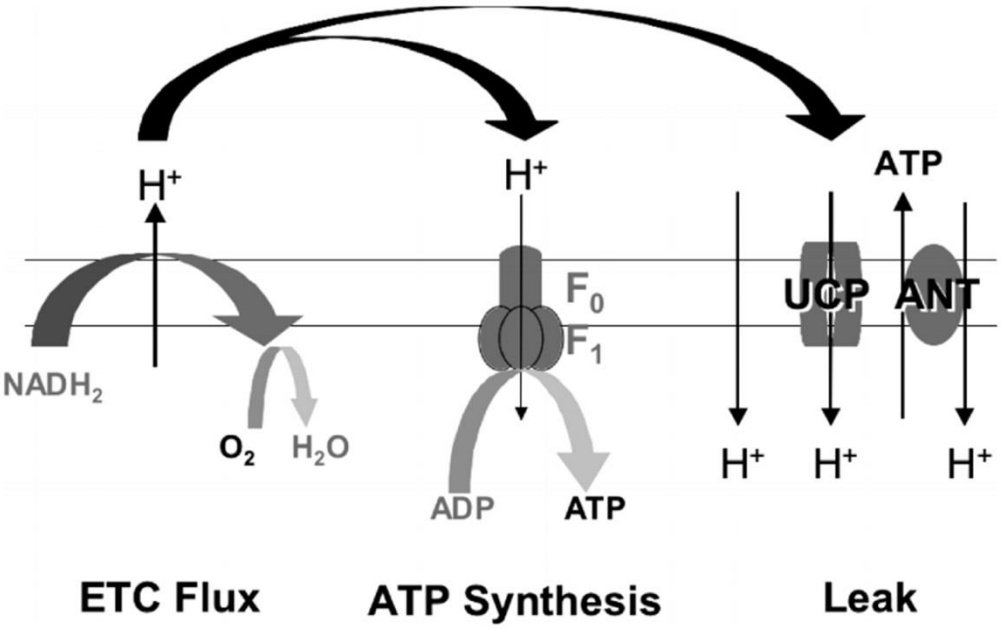

Figure 7 The location and function of uncoupling protein (UCP) in inner mitochondrial membrane (IMM).
ETC, electron transport chain; H^+, proton (Conley et al., 2007).

Uncoupling protein 2 (UCP2) is a novel member of uncoupling protein family and found to express in several cell types including pancreatic β cell (De Souza et al., 2007). Thus, the major function of UCP2 is not a thermogenesis as found in UCP1.

2.4.1 Role of UCP2 as a responsive regulator of reactive oxygen species (ROS)

Mitochondrial overproduction of ROS is proposed to be one of the main mechanisms linking hyperglycemia to diabetic vascular complications (Souza et al., 2011). Besides an increased action of antioxidant enzymes, increasing expression in UCP2 has been found to response in an overproduction of ROS (Pi and Collins, 2010). An expression of UCP2 is associated with the protection against oxidative stress evidenced by an elevation of ROS production in UCP2 knockout mice (Arsenijevic et al., 2000). In contrast, an overexpression of UCP2 prevents H_2O_2-induced β-cell death (Li et al., 2001).

2.4.2 Role of UCP2 as a negative regulator of glucose-stimulated insulin secretion (GSIS)

Evidence from study in rat islet demonstrates that an overexpression of UCP2 significantly inhibits GSIS (Chan et al., 2001), whereas an increase of GSIS is found in UCP2 gene deleted mice (Zhang et al., 2001). In addition, an increased expression of UCP2 has been reported to involve in down-regulation of ATP synthesis (Kashemsant and Chan, 2006). Therefore, possible functions of UCP2 may include control of ATP synthesis and ROS production, resulting in regulation of GSIS in pancreatic β cells.

Figure 8 describes how UCP2 is supposed to be as a negative modulator for insulin secretion. Insulin is normally secreted from β cell in response to the

concentration of blood glucose. The mechanism starts with glucose enters β cell through glucose transporter 2 (GLUT2) and passes glycolytic metabolism in cytoplasm, tricarboxylic-acid (TCA) cycle in mitochondrial matrix, and electron transport chain in inner mitochondrial membrane to synthesize ATP which subsequently triggers close of potassium channel, membrane depolarization, influx of calcium, and release of insulin. During the process of electron transport, superoxide anion is generated and then eliminated by antioxidant enzymes and UCP2. In normal condition, UCP2 is not highly expressed in β cell so its activity is relatively low (Figure 8a), whereas, in hyperglycemia, large amounts of superoxide are produced and subsequently stimulate the response of the cell to up-regulate the expression of UCP2 which is uncoupled to ATP synthesis, leading to low production of ATP and lower rate of insulin secretion (Figure 8b) (Krauss et al., 2005). From these evidences, UCP2 may play pivotal role in response to the induction of the pathogenesis of diabetic complications.

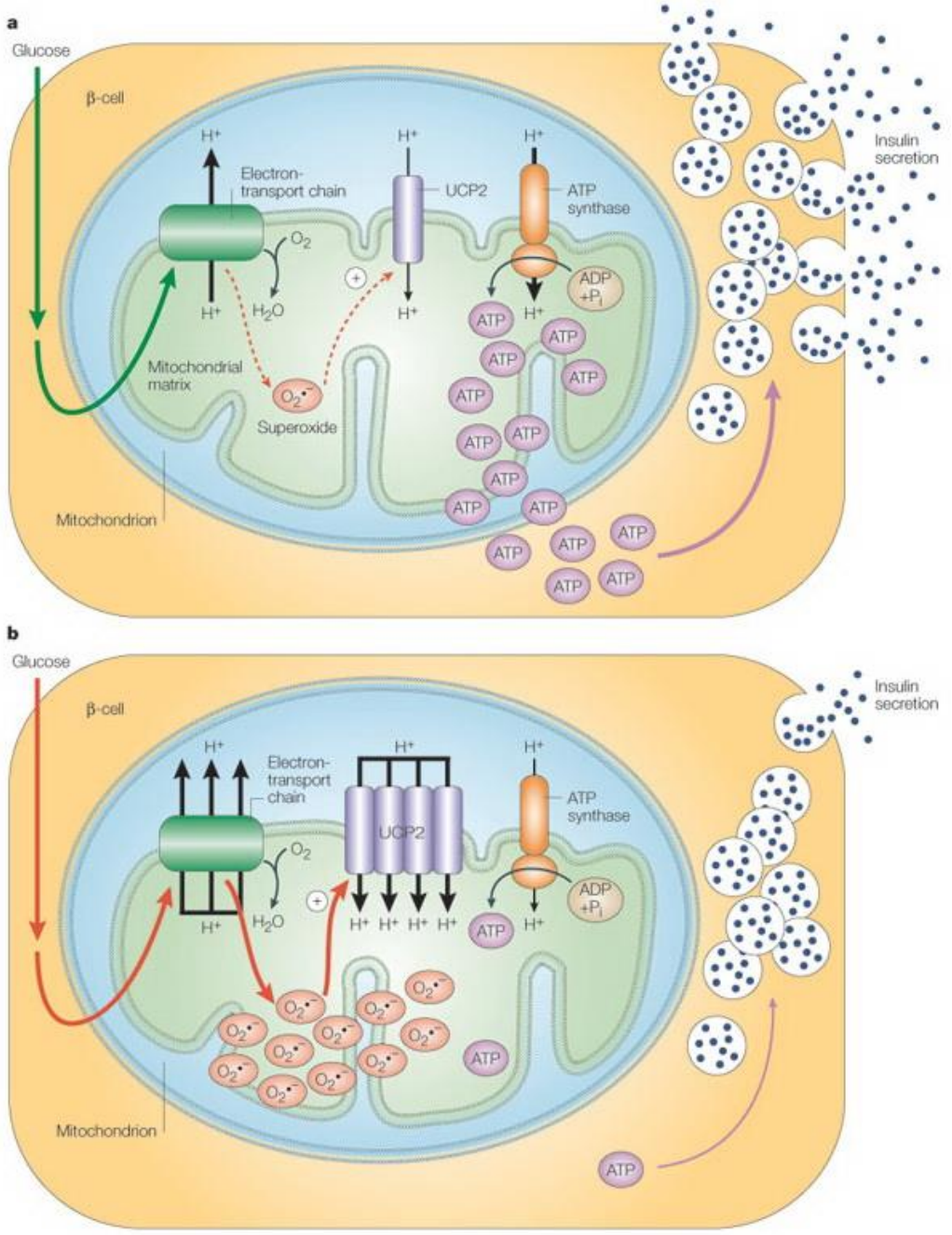

Figure 8 Role of uncoupling protein 2 (UCP2) on ROS production and glucose-stimulated insulin secretion (GSIS) in pancreatic β cells in normal (a) and hyperglycemic (b) conditions.
(Krauss et al., 2005)

2.5 Cinnamic acid and its derivatives

For the reason that a process of protein glycation involves in ROS generation and accumulation, antioxidants or free radical scavenging compounds are the alternative ways to prevent the harmful effects of glycation. Cinnamic acid and its derivatives are one of the major classes of phenolic compounds that possess many biological activities including anti-inflammation (Kim et al., 2012), anti-hyperglycemia (Choi et al., 2011) as well as anti-oxidation, illustrated by their ability to scavenge various free radicals (Natella et al., 1999).

2.6 Isoferulic acid (IFA)

2.6.1 General information of IFA

Isoferulic acid (IFA) or 3-Hydroxy-4-methoxy cinnamic acid is one of a hydroxycinnamic acid derivatives (see structure in Figure 9). It is abundant in rhizome of plants in genus *Cimicifuga* which are known as bugbane or cohosh, such as *C. heracleifolia*, *C. dahurica* and *C. racemosa* (black cohosh or Sheng ma) (Mukhopadhyay et al., 2006), and is also found in coffee (Monteiro et al., 2007) and artichoke leaf (Wittemera et al., 2005). Many kinds of *Cimicifuga* species have been used as an anti-inflammatory drug in traditional medicine in oriental countries such as Japan and China (Sakai et al., 1999).

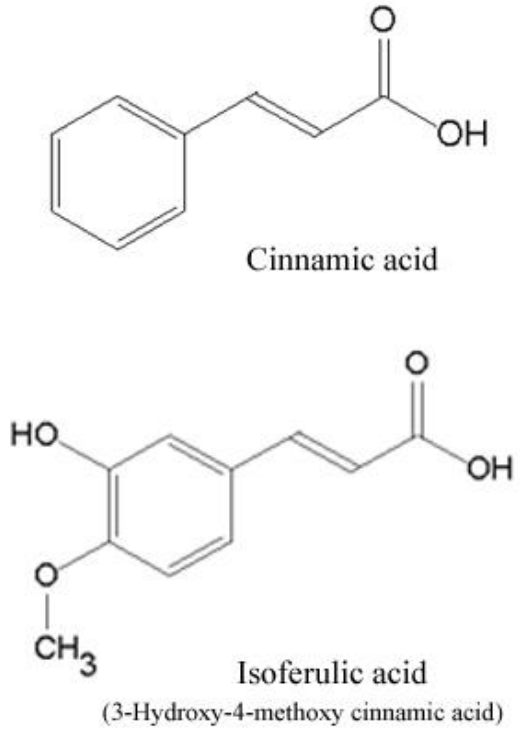

Figure 9 Chemical structures of cinnamic acid (upper) and isoferulic acid (IFA) (lower).

2.6.2 Pharmacokinetics of IFA

A pharmacokinetic study of IFA in rat demonstrated that plasma concentration of IFA reached the highest level (1.6 μg/mL and 2.9 μg/mL) within 10 min after administrating IFA and Rhizoma *Cimicifugae* extract, respectively, and could be eliminated with a half-life ($T_{1/2}$) of 1 – 1.5 h (Si et al., 2008). In human study, IFA could be determined in plasma from 14 volunteers within 15 min after single oral administration of Artichoke leaf extract and reached the maximum concentration (C_{max}, 7.87 – 10.51 ng/mL) within 0.83 – 1.1 h. The elimination half-life ($T_{1/2}$) was between 2.69 – 3.46 h (Wittemer et al., 2005). Another study was done in 6 participants by drinking a cup of coffee and monitoring the concentration of IFA in plasma and urine. Baseline concentration of IFA in normal subjects' urine was 0.05 –

2.07 μmol/mmol creatinine and was up to 4 times (0.2 – 9.6 μmol/mmol creatinine) in 4 h after having a cup of coffee (Monteiro et al., 2007).

2.6.3 Beneficial properties of IFA

2.6.3.1 Anti-inflammatory property

IFA has been previously reported to have anti-inflammatory activity by lowering interleukin-8 (IL-8) and macrophage inflammatory protein 2 (MIP-2) levels in virus-infected mice (Hirabayashi et al., 1995; Sakai et al., 1999; Sakai et al., 2001) and inhibiting cyclooxygenase-2 (COX-2) expresstion (Hirata et al., 2005).

2.6.3.2 Anti-oxidative property

Current studies have demonstrated the potent anti-oxidative effects of IFA. For instance, it has been found that IFA diminished oxidative stress *in vitro* by scavenging free radicals such as 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) ABTS (Hirata et al., 2005). Different antioxidant approaches, including anti-lipid peroxidation, DNA damage protection, superoxide anion ($\cdot O_2^-$) and hydroxyl radical (·OH) scavenging, DPPH·and ABTS· scavenging, and transition metal ion (such as copper ion (Cu^{2+}) and ferric ion (Fe^{3+})) chelating assays, were used to examine the antioxidant effect of the phenolic compounds derived from Rhizoma *Cimicifugae*. Three of all phenolic acids including caffeic acid, ferulic acid and isoferulic acid possessed the antioxidant activity by showing the positive correlation between chemical content and antioxidant level. The average R values were 0.51, 0.50, and 0.51, respectively (Li et al., 2012).

2.6.3.3 Anti-diabetic property

IFA possesses an anti-hyperglycemic action by inhibiting gluconeogenesis and/or increasing the glucose utilization in peripheral tissues, resulting in a decrease of plasma glucose in streptozotocin-induced diabetic rats (Liu et al., 2000). Interestingly, IFA reduced plasma glucose concentration in streptozotocin-induced diabetic rats by activating α_1-adrenoceptors to enhance the secretion of β-endorphin, which could stimulate the opioid μ-receptors to increase glucose use and/or reduce hepatic gluconeogenesis (Liu et al., 2003).

CHAPTER III

MATERIALS AND METHODS

3.1 Chemicals, reagents and assay kits

Chemicals and reagents	Companies, Countries
1-Deoxy-1-morpholino-D-fructose (1-DMF)	Sigma-Aldrich, USA
2,4-Dinitrophenylhydrazine (DNPH)	Ajax Finechem, Australia
2',7'-Dichlorofluorescein diacetate (DCF-DA)	Sigma-Aldrich, USA
2-Deoxy-D-ribose	Sigma-Aldrich, USA
2-Mercaptoethanol	Sigma-Aldrich, USA
2-methylquinoxaline (2-MQ)	Sigma-Aldrich, USA
3-hydroxy-4-methoxycinnamic acid (Isoferulic acid, IFA)	Sigma-Aldrich, USA
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	Amnesco, USA
5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB)	Sigma-Aldrich, USA
5-methylquinoxaline (5-MQ)	Sigma-Aldrich, USA
Agar A	Bio Basic, USA
Agarose	BioExpress, USA
Aminoguanidine hydrochloride (AG)	Sigma-Aldrich, USA
Ampicillin	AppliChem, USA
Anhydrous sodium carbonate	Fisher Scientific, USA
Antibiotic-antimycotic drug	Gibco, USA
Boric acid	Bio-Rad, USA
Bovine serum albumin (BSA)	Sigma-Aldrich, USA
Cacium chloride dihydrate	Fisher Scientific, USA

Cupric sulfate ($CuSO_4$)	Sigma-Aldrich, USA
Cytochrome *c*	Affymetrix, USA
D-fructose	Fisher Scientific, USA
D-glucose	Ajax Finechem, Australia
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, USA
DNA ladder	Thermo Scientific, USA
DNA loading dye	Thermo Scientific, USA
Ethanol	Merck, Germany
Ethedium bromide	Sigma-Aldrich, USA
Ethyl acetate	Merck, Germany
Ethylenediaminetetraacetic acid (EDTA)	Merck, Germany
Fetal bovine serum (FBS)	Gibco, USA
Folin-Ciocalteau reagent	Sigma-Aldrich, USA
Glutathione	Sigma-Aldrich, USA
Guanidine hydrochloride	Fluka, Germany
HEPES	Fisher Scientific, USA
Hydrochloric acid (HCl)	Merck, Germany
Hydrogen peroxide	Sigma-Aldrich, USA
Insulin (^{125}I) 10uCi	Perkin Elmer, USA
L-cysteine	Sigma-Aldrich, USA
L-lysine hydrochloride	Himedia, India
Luria-Bertani (LB) medium	Bio Basic, USA
Magnesium chloride	Ajax Finechem, Australia
Magnesium sulfate	Fisher Scientific, USA

Malondialdehyde tetrabutylammonium salt (MDA)	Sigma-Aldrich, USA
Methylglyoxal (MG), 40% in H_2O	Sigma-Aldrich, USA
Nitroblue tetrazolium (NBT)	Sigma-Aldrich, USA
o-Phenylenediamine (*o*-PDA)	Sigma-Aldrich, USA
Phosphate-buffered saline, 10X (PBS)	Gibco , USA
Plasmid pUC19	Invitrogen, USA
Potassium chloride	Fisher Scientific, USA
RPMI 1640 medium (#31800-022)	Gibco , USA
S-D-lactoylglutathione	Sigma-Aldrich, USA
Sodium azide (NaN_3)	Sigma-Aldrich, USA
Sodium bicarbonate ($NaHCO_3$)	EMD, USA
Sodium carbonate (Na_2CO_3)	Ajax Finechem, Australia
Sodium chloride	Fisher Scientific, USA
Sodium phosphate dibasic	Ajax Finechem, Australia
Sodium phosphate monobasic	Ajax Finechem, Australia
Sodium pyruvate	Gibco, USA
Taq DNA polymerase	Thermo Scientific, USA
Thiazolyl blue tetrazolium bromide (MTT)	Sigma-Aldrich, USA
Thiobarbituric acid (TBA)	Sigma-Aldrich, USA
Thioflavin T	Sigma-Aldrich, USA
Trichloroacetic acid (TCA)	Merck, Germany
Tris base	Bio-Rad, USA
Trizol	Invitrogen, USA
Trypsin/EDTA	Gibco, USA

Assay kits	Companies, Countries
OxiSelect™ N^{ε}-(carboxymethyl) lysine (CML) ELISA kit	Cell Biolabs, USA
QIAprep Spin Miniprep kit	Qiagen, Netherlands
ATPlite	Perkin Elmer, USA
EnzChek caspase-3 assay kit	Thermo Scientific, USA

3.2 Lab devices and instruments

Lab devices	Companies, Countries
Cell culture flask (25, 75, 150 cm^2)	Corning, USA
Cell culture plate (6, 24, 96 wells)	Corning, USA
Centrifuge tube (15, 50 mL)	Sterilin, UK
Cryotube	Thermo Scientific, USA
Filter paper (0.22 μM)	Milipore, USA
Filter paper (0.45 μM)	Whatman, UK
Glass bottle	Duran, Germany
Glassware	Pyrex, USA
Microcentrifuge tube (0.6, 1.5 mL)	Hycon, UK
Microcentrifuge tube (for cell culture)	Axygen, USA
Mini cell scraper	LEAP Biosciences, Canada
Pipette tip (20, 200, 1000 μL)	Axygen, USA
Plastic ware	Corning, USA
Plastic plates (6, 24, 96 wells)	Thermo Scientific, USA
Plastic serological pipette (5, 10, 25 mL)	Stripette, Costar
Reusable Bottle Top Filter Holder	Nalgene, USA

Lab instruments	Companies, Countries
Analytical balance	A&D, Japan
Autoclave	Hirayama, Japan
Automatic Gamma Counter	Perkin Elmer, USA
Autopipette (10, 20, 200, 1000 µL)	Labnet, USA
Centrifuge	Hettich, Germany
CO2 incubator	Slimcell,
Dispenser	Hamilton, Switzerland
Fluorescence Imager (Typhoon 9400)	GE Healthcare, UK
Freezer (-20°C)	Sanyo, Japan
Gel documentation (gel doc) system	Syngene, UK
Gel electrophoresis and power supply	Bio Rad, USA
Heating block	Labnet, USA
Hemacytometer	Sigma-Aldrich, USA
HPLC	(Shimadzu Corp., Japan)
Light microscope	Olympus, Japan
Luminar flow cabinet	Esco, USA
Magnetic stirrer	IKA, Malaysia
Microplate shaking incubator	Labnet, USA
Nano Drop 1000 spectrophotometer	Thermo Scientific, USA
pH meter	Thermo Electron, USA
Refrigerator	Sharp, Japan
Repeating pipette	HandyStep, USA
Spectrofluorometer	Perkin Elmer, USA

Spectrophotometer	Molecular Devices, USA
Thermal Cycler (PTC-200)	Bio-Rad, USA
Vortex mixer	Thermo Scientific, USA
Water bath	Memmert, Germany

3.3 Conceptual framework

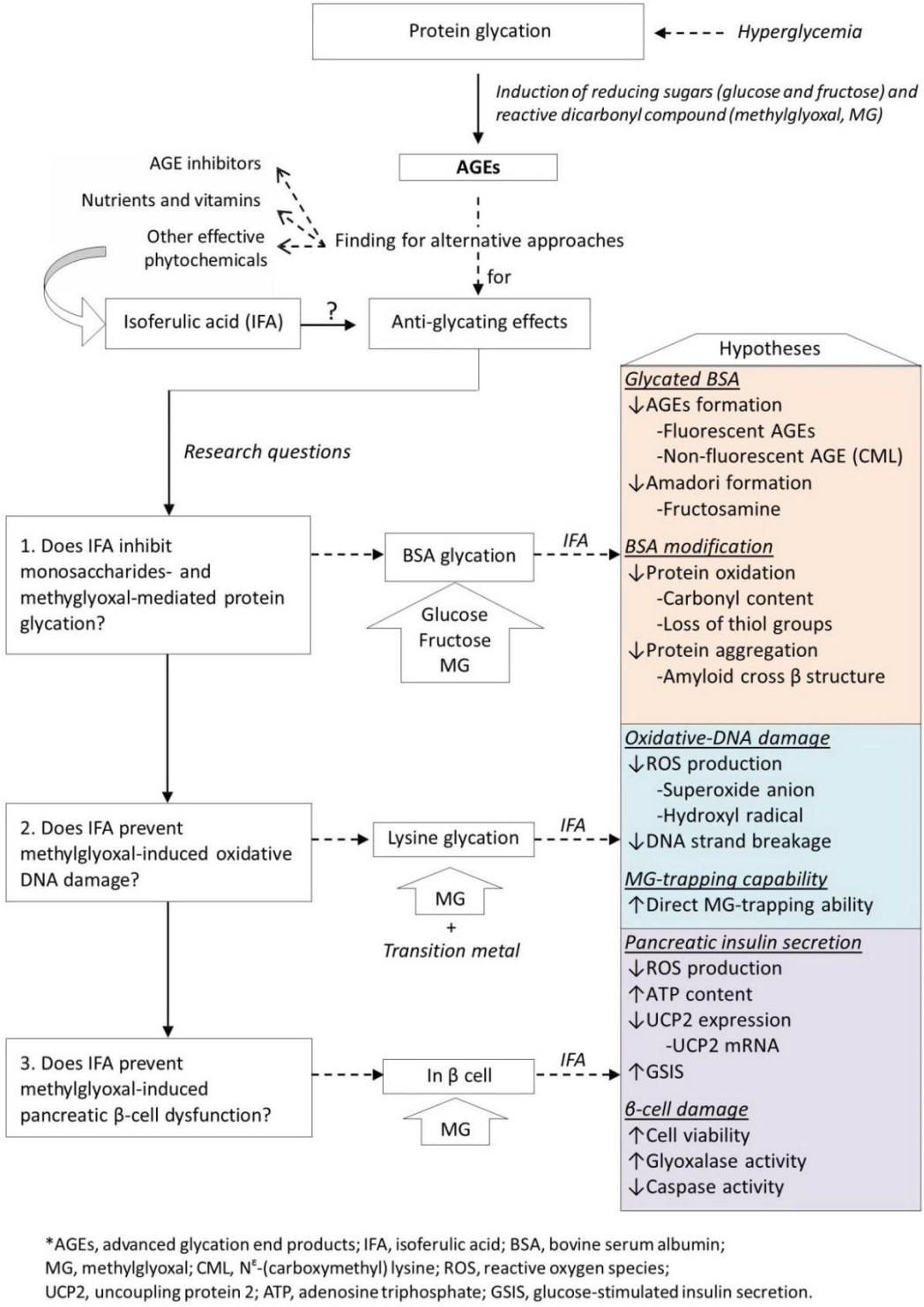

Figure 10 Conceptual framework of the study

3.4 Experiment 1: To investigate the effects of IFA on BSA glycation induced by glucose, fructose and methylglyoxal

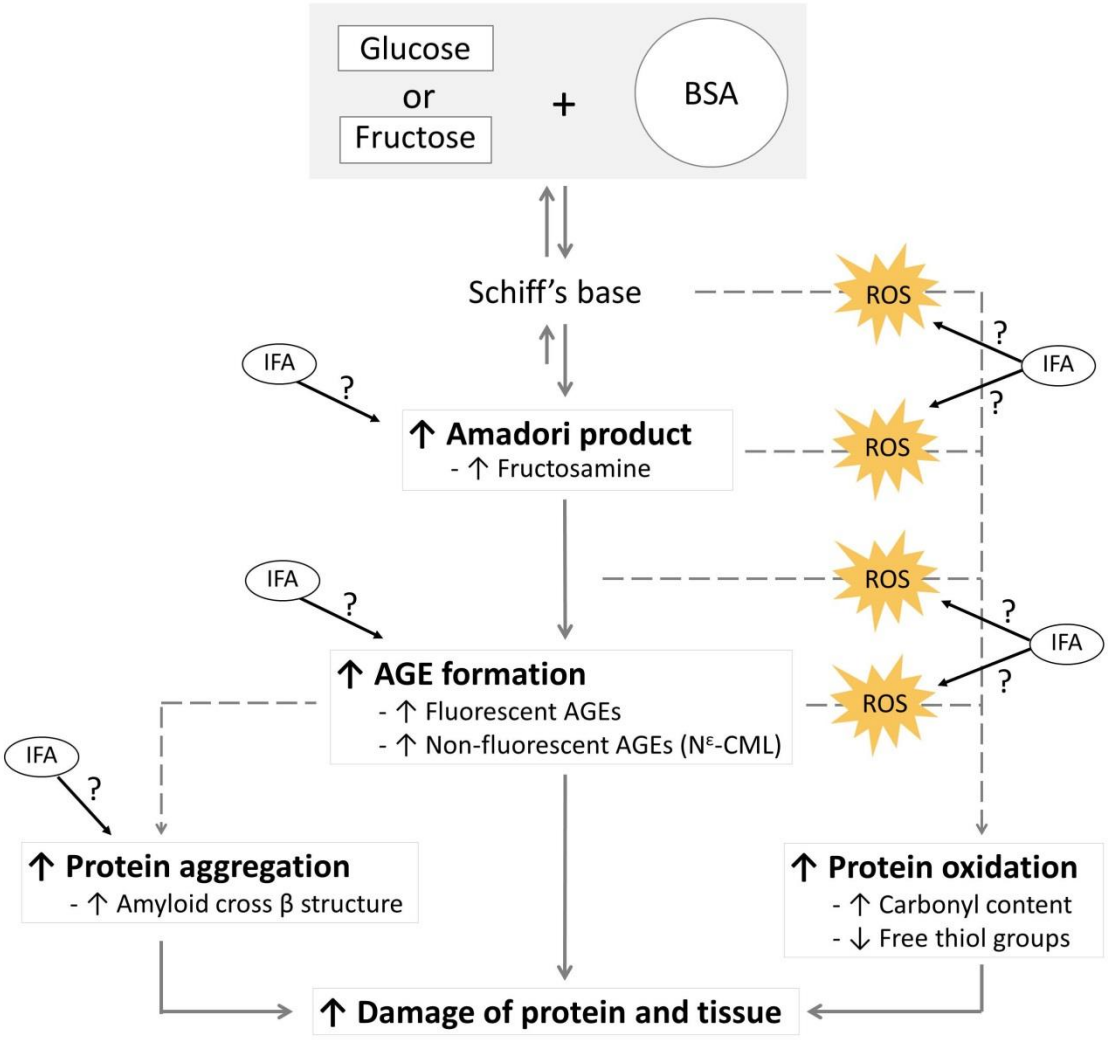

Figure 11 Framework of experiment 1: Investigation of the effects of IFA on BSA glycation induced by glucose and fructose.

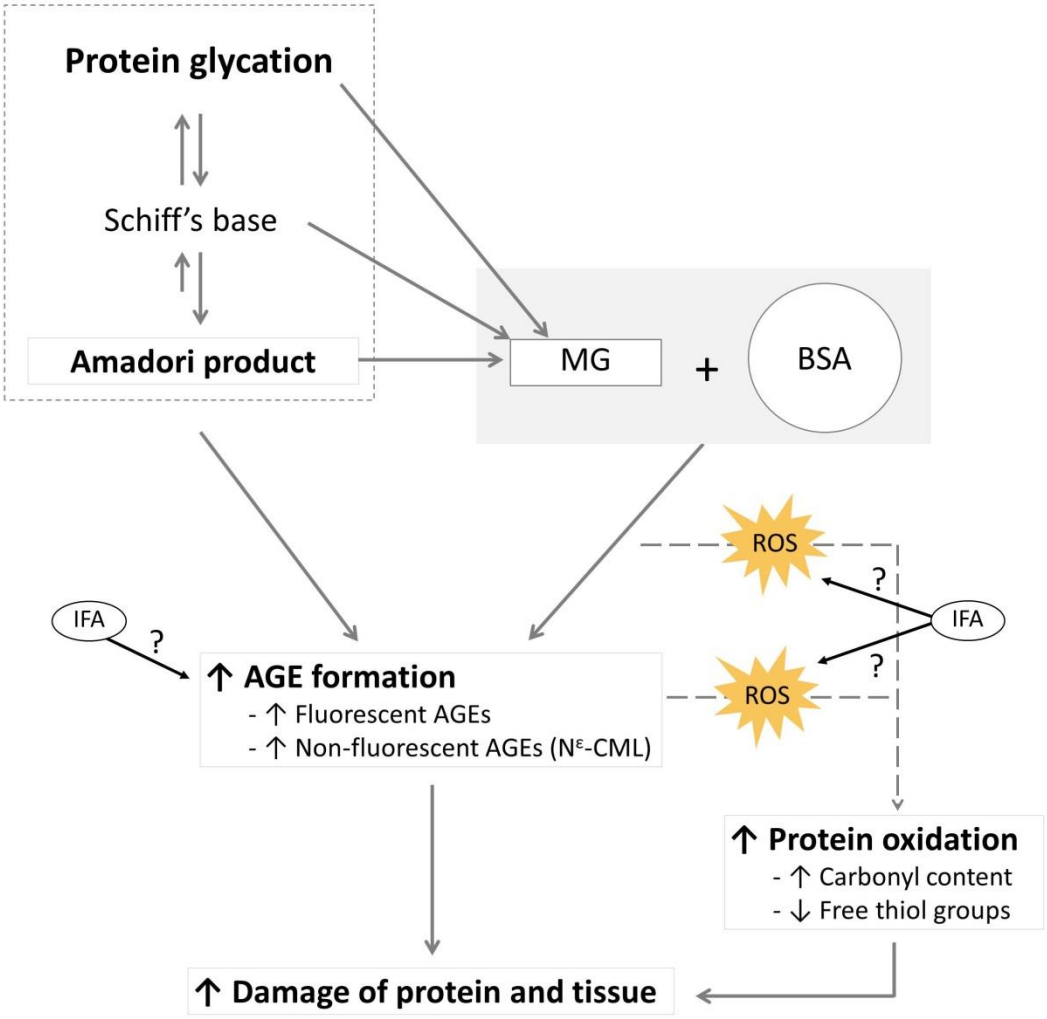

Figure 12 Framework of experiment 1: Investigation of the effects of IFA on BSA glycation induced by methylglyoxal (MG).

3.4.1 Preparation of in vitro monosaccharide-induced glycation of bovine serum albumin (BSA)

The formation of monosaccharide-induced glycated BSA in 1-mL reaction was done by incubating 500 µL of 20 mg/mL BSA (final concentration: 10 mg/mL) with 460 µL of 1.09 M glucose or fructose (final concentration: 0.5 M) in 0.1 M phosphate buffer saline (PBS), pH 7.4 containing 0.02% sodium azide (NaN_3) at 37 $^{\circ}$C for 4 weeks in the absence or presence of 40 µL of 25X isoferulic acid (IFA) or 25X aminoguanidine (AG) in 100% dimethylsulfoxide (DMSO), which were 31.25, 62.5 and 125 mM of IFA and 125 mM of AG, (final concentration of IFA: 1.25, 2.5, and 5 mM in 4% DMSO; final concentration of AG: 5 mM in 4% DMSO). The untreated BSA and reaction blanks were also prepared. Aliquots of the reaction mixtures were then assayed for AGE and CML formation, fructosamine, protein carbonyl content, thiol group and amyloid cross β structure.

3.4.2 Preparation of in vitro methylglyoxal (MG)-induced glycation of BSA

The preparation of MG-induced glycated BSA was done according to BSA/monosaccharide glycation method with slight modification. The 1-mL reaction containing 500 µL of 20 mg/mL BSA (final concentration: 10 mg/mL), 460 µL of 2.17 mM MG (final concentration: 1 mM) in 0.1 mM PBS (pH 7.4), and 40 µL of various 25X concentrations of IFA in 100% DMSO (final concentration: 1.25, 2.5, and 5 mM) or 25X of AG (final concentration: 1.25 mM) was incubated at 37 $^{\circ}$C for 2 weeks. Aliquots were then assayed for AGE and CML formation, and protein oxidation including protein carbonyl content and thiol group.

3.4.3 Determination of fluorescent AGE formation

The fluorescent intensity was weekly measured to assess total fluorescent AGE formation by using spectrofluorometer at excitation and emission wavelengths of 355 and 460 nm, respectively. The inhibitory effect of IFA and AG on the formation of AGEs was evaluated by the calculation of percentage inhibition compared with maximum glycation elicited by glucose, fructose or MG as shown in the formula below.

$$\%\ Inhibition\ = \frac{Fluorescent\ intensity\ of\ (glycated\ BSA\ -\ inhibitor)}{Fluorescent\ intensity\ of\ glycated\ BSA} \times 100$$

Where glycated BSA was BSA/glucose or BSA/fructose or BSA/MG, and inhibitor was BSA/glucose or BSA/fructose or BSA/MG with IFA or AG.

3.4.4 Determination of non-fluorescent AGE formation (N^{ε}-CML)

N^{ε}-(carboxymethyl) lysine (N^{ε}-CML), a major antigenic AGE structure, was detected at the end of incubation by enzyme linked immunosorbant assay (ELISA) kit. According to the manufacturer's protocol, the glycated samples were diluted to final concentration of 1 µg/mL (10000-fold dilution) before using in the assay. Each diluted sample (100 µL) was incubated in the 96-well protein binding plate at 37°C for at least 2 h. After washing with 0.1 M PBS, an assay diluent (200 µL) was added and further incubated for 2 h at room temperature on an orbital shaker. Three times washes with wash buffer were needed before incubating for 1 h with 100 µL of anti-CML antibody and with 100 µL of secondary antibody-HRP conjugate, respectively. The substrate solution (100 µL) was incubated in each well for 20 min before adding stop solution in an equal volume. The absorbance of samples was measured

immediately at 450 nm and compared with the absorbance of CML-BSA standard providing in the assay kit. The results were expressed as ng/mL.

3.4.5 Determination of Amadori product (fructosamine)

The concentration of the Amadori product fructosamine in BSA/monosaccharide glycation was weekly measured by NBT assay (Ardestani and Yazdanparast, 2007b). Glycated BSA (10 µL) was incubated with 100 µL of 0.5 mM NBT in 0.1 M carbonate buffer, pH 10.4 at 37 $^{\circ}$C. The absorbance was measured at 530 nm at 10 and 15 min time points. The concentration of fructosamine was calculated compared to 1-DMF as the standard.

3.4.6 Determination of protein carbonyl content

The carbonyl group in glycated BSA, a marker for protein oxidative damage, was determined following a method in previous publication with slight modifications (Lo et al., 1994). Briefly, 400 µL of 10 mM DNPH in 2.5 M HCl was added to 100 µL of each glycated sample and incubated in the dark for 1 h. After that, 500 µL of 20% (w/v) trichloroacetic acid (TCA) was added to precipitate protein on ice for 5 min and then centrifuged at 10,000*g* for 10 min at 4 $^{\circ}$C. The protein pellet was washed with 500 µL of ethanol/ethyl acetate (1:1) mixture three times and resuspended in 250 µL of 6 M guanidine hydrochloride. The absorbance was measured at 370 nm using the UV microplate. The carbonyl content of each sample was calculated based on the extinction coefficient for DNPH (22,000 $M^{-1}cm^{-1}$). The results were expressed as nmol carbonyl/mg protein.

3.4.7 Determination of thiol group

The free thiols in glycated samples were measured by Ellman's assay with minor modifications (Westwood and Thornalley, 1995). Briefly, 70 µL of each glycated sample was incubated with 5 mM DTNB in 0.1 M PBS, pH 7.4 at 25 $^{\circ}$C for 15 min. The absorbance of samples was measured at 410 nm. The concentration of free thiols was calculated from L-cysteine standard and expressed as nmol/mg protein.

3.4.8 Thioflavin T assay

Thioflavin T, a marker of amyloid cross-β structure, was measured according to a previous method with minor modifications (Kalapos, 1999). Briefly, 100 µL of 64 µM thioflavin T in 0.1 M PBS, pH 7.4 was added to 10 µL of each glycated sample and incubated at 25 $^{\circ}$C for 60 min. The fluorescence intensity was measured at excitation wavelength of 435 nm and emission wavelength of 485 nm.

3.5 Experiment 2: To investigate the effects of IFA on MG-induced oxidative DNA damage

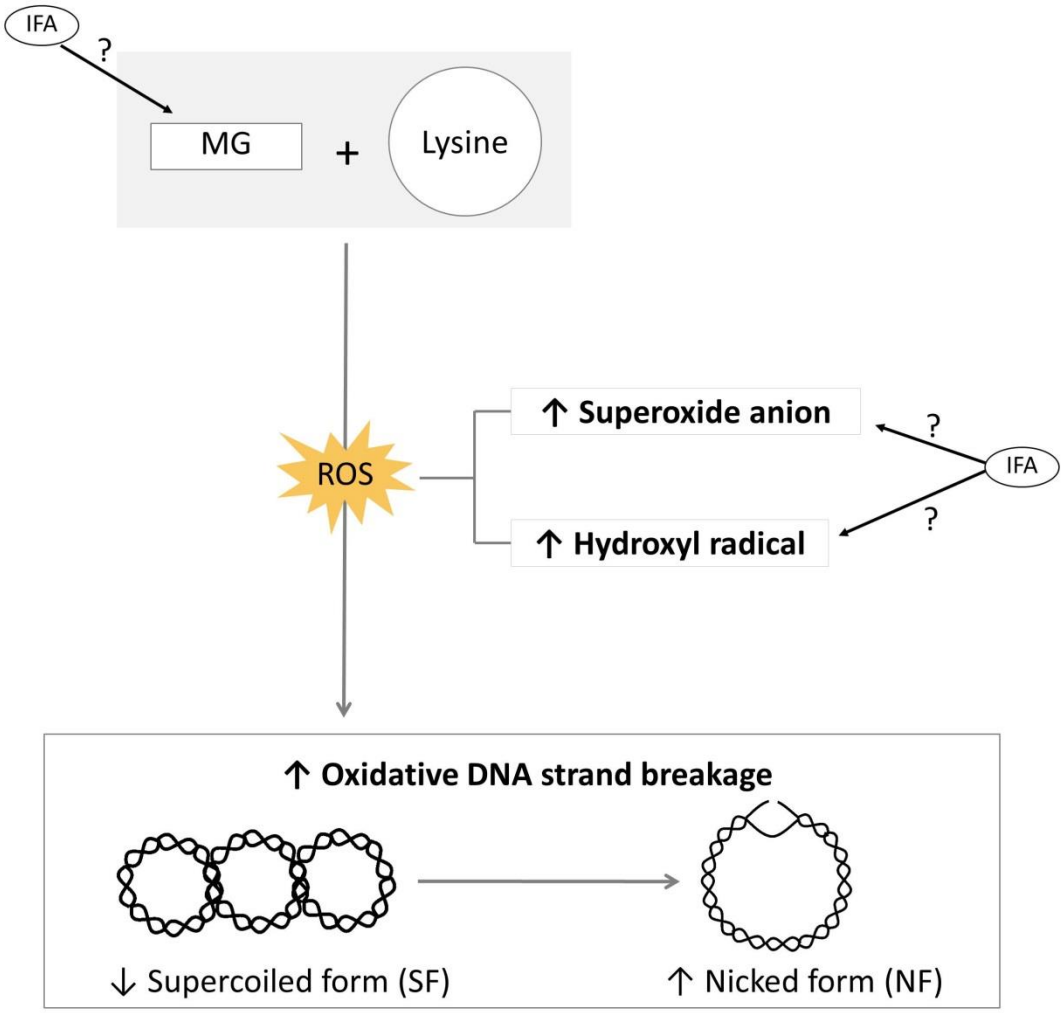

Figure 13 Framework of experiment 2: Investigation of the effects of IFA on MG-induced oxidative DNA damage.

3.5.1 Plasmid extraction

The pUC19 plasmid was purified from competent *Escherichia coli* by using plasmid DNA purification (QIAprep Spin Miniprep) kit according to the manufacturer's protocol. An overnight culture (12-16 h) of a single colony of pUC19 plasmid transformed *E. coli* in 5-ml Luria-Bertani (LB) broth containing 100 µg/mL ampicillin at 37 °C was centrifuged at 11,000g for 30 sec to remove non-cell medium before resuspending cell pallet in buffer A1 (250 µL). An equal volume of buffer A2 was added and gently mixed to initiate cell lysis before adding 350 µL of buffer A3. After gentle mix and centrifugation, supernatant was applied to the column and centrifuged to discard the flow-through portion. The column containing DNA was then washed and centrifuged by buffer, and placed in the new tube. After that, the Milii-Q water (50 µL) was added to the column, waited and centrifuged for 1 min to elute bound DNA. DNA concentration was measured using the NanoDrop-1000 spectrophotometer. The plasmid was kept at -20 °C until use.

3.5.2 Determination of DNA strand breakage

An extracted plasmid containing 0.25 µg of DNA was incubated with 2 µL of 250 mM lysine (final concentration: 50 mM), 2 µL of 250 mM MG (final concentration: 50 mM), and 2 µL of IFA at various 5X concentrations (final concentrations: 0.1, 0.25, 0.5 and 1 mM) with or without 1 µL of 3 mM $CuSO_4$ (final concentration: 300 µM) in the total volume of 10 µL (adjusted by PBS) at 37 °C for 3 h. The reaction was stopped at -20 °C for 90 min before applying to 6X DNA fluorescent loading dye and loading to 0.8% agarose gel in TBE buffer. The gel was electrophoresed under constant voltage (80 V) for 60 min and photographed by Gel Doc imager. DNA

fragments expressed on agarose gel consisted of supercoiled form (SF) and nicked form (NF). The intensity of each band was analyzed using GeneTools software (Syngene, UK) and calculated the percentage of nicked form (% NF) using the following formula before subtracting by those of control (DNA alone). The results were expressed as relative % NF.

$$\% NF = \frac{The\ band\ intensity\ of\ NF}{The\ band\ intensity\ of\ (NF + SF)} \times 100$$

3.5.3 Determination of superoxide anion

Superoxide anion was determined by cytochrome *c* reduction assay. Reaction mixtures with equal volumes (200 µL) of 50 mM lysine and 50 mM MG (final concentration: 10 mM) with or without 100 µL of IFAs (final concentrations: 0.1, 0.25, 0.5 and 1 mM) were adjusted the volume to 900 µL by PBS before adding 100 µL of 300 µM cytochrome *c* (final concentration: 30 µM) and monitoring the production of superoxide anion by measuring reduced cytochrome *c* at a wavelength of 550 nm every 10 min until 180 min. The concentration of reduced cytochrome *c* in each time point was calculated using its molar extinction coefficient (27,700 $M^{-1}cm^{-1}$) and then subtracted by baseline (at 0 min). The results were expressed as nmol/mL.

3.5.4 Determination of hydroxyl radical

Hydroxyl radical was determined by measuring thiobarbituric acid reactive 2-deoxy-D-ribose oxidation products (TBARS). The reaction containing equal volumes (20 µL) of 50 mM lysine, 50 mM MG (final concentration: 10 mM) and 100 mM 2-deoxy-D-ribose (final concentration: 20 mM) with or without 20 µL of IFAs (final concentrations: 0.1, 0.25, 0.5 and 1 mM) was adjusted the volume to 100 µL using 10

mM PBS before incubating at 37 °C. After 180 min of incubation, all mixtures were added equal volumes (100 µL) of 10 mM PBS, 2.8% (w/v) TCA and 1% (w/v) thiobarbituric acid (TBA), followed by heating at 100 °C for 10 min and cooling down. The degradation of 2-deoxy-D-ribose was measured using spectrophotometer at a wavelength of 532 nm. The concentration of TBARS was calculated from malondialdehyde (MDA) standard and the results were expressed as nmol/mL.

3.5.5 Determination of the MGO-Trapping Capacity

The capacity of IFA in direct trapping to MG was investigated by high performance liquid chromatography (HPLC). The mixture of 1 mM MG with IFA (1.25, 2.5 and 5 mM) or 1.25 mM AG in phosphate buffer solution (pH 7.4) was incubated at 37 °C for 1 - 24 h. After that, quantification of MG was based on the determination of its derivative compound, 2-methylquinoxaline (2-MQ) using HPLC with 5-methylquinoxaline (5-MQ) as the internal standard. A hundred µL of 20 mM *o*-phenylenediamine (*o*-PDA) containing 100 µL of 5 mM 5-MQ was added to the sample vials immediately after MG/compound incubation. MG derivatization took place at the room temperature for 30 min. Then, each sample was filtered and ready for analysis. The remaining MG in samples was quantified using HPLC equipped with a LC-10AD pump, SPD-10A UV-Vis detector and LC-Solution software. A C_{18} (Inertsil ODS 3V) column (250 × 4.6 mm i.d.; 5-µm particle size) was used for 2-MQ analysis. The column temperature was maintained at room temperature. The mobile phase for the HPLC system consisted of HPLC grade water (solvent A) and methanol (solvent B) with a constant flow rate set at 1.2 mL/min. In brief, aliquots of 10 µL were subjected to HPLC analysis. An isocratic program was performed with 70% solvent B

and 11-min running time per sample. The 2- and 5-MQ was monitored at 315 nm. The percentage of MG reduction was calculated using the equation below:

$$\%\ Reduction\ = \frac{Amount\ of\ (MG\ in\ control - MG\ in\ test\ compound)}{Amount\ of\ MG\ in\ control} \times 100$$

3.6 Experiment 3: To investigate the effects of IFA on MG-induced β-cell dysfunction

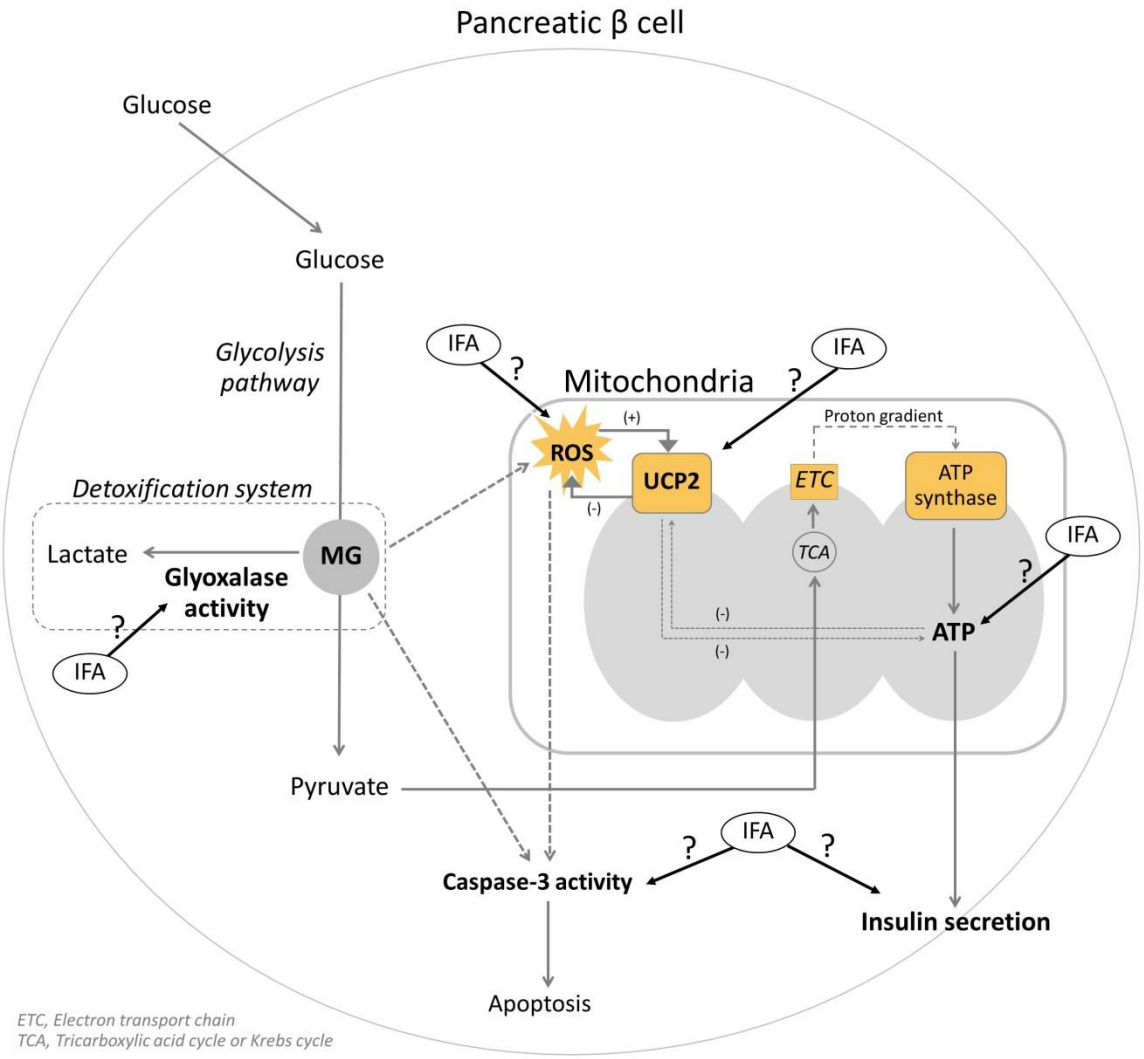

Figure 14 Framework of experiment 3: Investigation of the effects of IFA on MG-induced β-cell dysfunction.

3.6.1 Pancreatic β-cell culture

Rat insulin-secreting cell line (INS-1 832/13; passage 70-100) were obtained from Li Ka Shing Research Center and Health Innovation (University of Alberta, Edmonton, Alberta, Canada) and cultured in Roswell Park Memorial Institute (RPMI) 1640 medium containing 11 mM glucose and 2 mM L-glutamine. A liter of RPMI 1640 contained 100 mL of heat-deactivated fetal bovine serum (FBS) (final concentration: 10% (v/v)), 10 mL of 1 M HEPES (pH 7.4) (final concentration: 10 mM), 10 mL of 100 mM sodium pyruvate (final concentration: 1 mM), 2 g of sodium bicarbonate ($NaHCO_3$) (final concentration: 2 g/L), 1 mL of 50 mM β-mercaptoethanol (final concentration: 50 μM) and 10 mL of 100 mM antibiotic/antimycotic drug (final concentration: 100 IU/mL penicillin and 100 mg/mL streptomycin) at 37 °C in a humidified condition of 95% air and 5% CO_2. Cells were grown in 25-ml or 75-ml flask until achieving 70-80% confluency before trypsinization and starting a new passage or seeding for experiments.

3.6.2 Cell viability assay

The viability of the cells was evaluated by MTT assay according to general protocol. INS-1 cells were seeded into 96-well microplate (30,000 cells/well) and cultured for 24 h. Medium containing IFA (0.01-0.1 mM in 0.1% DMSO) was pretreated to the cells for 24 h or 48 h before incubating with different concentrations of MG (0-3 mM) for different times (0 - 6 h). The MTT solution (0.5 mg/mL MTT in media) was applied to each well for 3 h and was then replaced with DMSO to dissolve formazan. The absorbance was measured at the wavelength of 570 nm with the reference

wavelength of 630 nm. The results were expressed as percentage of cell viability related to MG-untreated control cells.

3.6.3 Glucose-stimulated insulin secretion (GSIS) assay

INS-1 cells were seeded into 24-well plate (250,000 cells/well) and cultured for 24 h before pretreating with medium containing various concentrations of IFA for 48 h. Cells were washed three times with Krebs-Ringer Bicarbonate (KRB) buffer containing 0.1% BSA and incubated with different concentrations of glucose (2.8, 5.5, 11 and 22 mM) in KRB buffer containing 0, 2 and 3 mM MG for 2 h. The glucose containing buffer was then collected from each well for measuring the releasing insulin, whereas the cells were added 3% acetic acid and kept for determining the total content of insulin using radioimmunoassay (RIA).

3.6.4 Radioimmunoassay (RIA)

Collected samples both releasing and total insulin portions from GSIS assay were diluted with RIA buffer (0.04 M phosphate buffer, pH 7.5 containing 0.1% BSA) in polypropylene tubes and incubated with anti-insulin antibody (1:20 dilution in RIA buffer) at 4 $^{\circ}$C overnight. The ^{125}I-labeled insulin solution (1:1000 dilution in RIA buffer) was then applied to each tube and further incubated at 4 $^{\circ}$C for a night. After adding charcoal, the sample tubes were then centrifuged 3000 rpm at 4 $^{\circ}$C for 15 min and discarded supernatant. All tubes were subjected to measure emitted radiation in automatic gamma counter and calculate the amount of insulin compared with standard. All processes were done in radioactive area under the guidelines and care of certificated person.

3.6.5 ROS production measurement

Intracellular ROS was determined by fluorometry using 2',7'-dichorofluorescein diacetate (DCF-DA). INS-1 cells were seeded into 96-well microplate (30,000 cells/well) and cultured for 24 h. After pretreatment of IFA for 48 h, cells were then treated with 0 and 2 mM MG for 0.5 - 2 h. Cells were washed with KRB buffer once prior to adding 10 μM DCF-DA and incubating for 1 h. After removing DCF-DA and washing, KRB buffer was added to each well. The fluorescence intensity was then detected at the excitation and emission wavelengths of 488 and 520 nm.

3.6.6 Uncoupling protein 2 (UCP2) expression

3.6.6.1 Cell lysis and RNA extraction

INS-1 cells were seeded into 6-well plate (200,000 cells/well), cultured for 24 h and pretreated with or without 0.1 mM IFA in 0.1% DMSO for 48 h. After removing IFA, cells were treated with 0 and 2 mM MG for various incubation periods (0.5, 1, 2, 3, 4, 5 and 6 h). Then, cell lysis and RNA extraction was processed using cooled Trizol reagent (1 mL/well). After incubating on ice for 10-15 min, cell lysates were transferred to new 1.5-mL microtubes. The cooled chloroform (0.2 mL) was added to each tube before mixing thoroughly until the color of solution became milky pink. Further incubated for a few minutes and centrifuged at 12,000 g for 15 min. The solution would be separated to 3 phases of protein, DNA and RNA. Collected the top clear phase of solution which contained RNA into new tubes (approximately 400-450 μL) and incubated with cooled isopropanol (0.5 mL/tube) for 5-10 min before centrifuging at 12,000 g for 10 min to precipitate RNA. After discarding supernatant,

the RNA pallets were washed with 75% ethanol (1 mL/tube) and centrifuged at 7,500 g for 5 min. After removing isopropanol, RNA pallets were dried and resuspended in 40 µL of milli-Q water, and warmed at 55 °C for 10 min before measuring RNA concentration using NanoDrop-1000 spectrophotometer or keeping in the -20 °C freezer until use.

3.6.6.2 DNase treatment

DNase treatment was done in the master mix of 2 µL of DNase, 2 µL of DNase buffer, 6 µL of water, and 10 µL of RNA sample (20 µL/reaction), and then incubated at 37°C for 30 min. The reaction was stopped by adding 2 µL of DNase stop solution and warmed at 65°C for 10 min.

3.6.6.3 Reverse transcription

After DNase treatment, RNA samples (13 µL) were pre-warmed at 65°C for 5 min before mixing with 7 µL of the prepared master mix containing 4 µL of 5X RT buffer, 2 µL of 10 mM dNTPs, and 1 µL of reverse transcriptase (RT) enzyme. The reverse transcription was done by incubating at 42°C for 1 h and then deactivated by heating to 72 °C for 10 min.

3.6.6.4 Polymerase chain reaction (PCR)

A PCR master mix per 1 reaction (23 µL) was prepared by mixing 2.5 µL of 10X PCR beffer, 0.5 µL of 10 mM dNTPs, 2.5 µL of 10 µM forward and reverse promers, 2.5 µL of 25 mM $MgCl_2$, 1.25 µL of DMSO for PCR, 11 µL of water, and 0.25 µL of 5 U/µL *Taq* DNA polymerase. The whole volume of master mix was calculated for the

whole number of reaction (plus one extra). Then, the aliquotes of master mix (23 µL) was added to the PCR tube containing 2 µL of template DNA/reaction.

PCRs (25 µL/reaction) were carried out in Peltier Thermal Cycler (PTC-200). Reaction conditions included 35 cycles of denaturating at 95 $^{\circ}$C for 15 s, annealing at 56 $^{\circ}$C for 45 s, and extension at 72 $^{\circ}$C for 20 s.

The sequences of rat *Ucp2* forward primer was 5' TGGGACAGCAGCCTGTATTG 3' and rat *Ucp2* reverse primer was 5' CCCGAAGGCAGAAGTGAAGT 3' (product size = 610 bp). The sequences of 18s rRNA forward primer was 5' CTTTGGTCGCTCGCTCCTC 3' and 18s rRNA reverse primer was 5' CTGACCGGGTTGGTTTTGAT 3' (product size = 130 bp).

3.6.6.5 Gel electrophoresis

Each PCR product and DNA ladder was mixed with fluorescent DNA Loading dye before loading onto 2 % agarose gel in Tris-borate-EDTA (TBE) buffer. The gel was electrophoresed at 80 V for 60 min, photographed by Gel Doc imager, and quantified the band intensity by GeneTool software (Syngene, UK).

3.6.7 ATP content

INS-1 cells were seeded into 96-well microplate (10,000 cells/well) and cultured for 24 h before pretreating with or without 0.1 mM IFA in 0.1% DMSO for 48 h. After removing IFA, cells were treated with 0 and 2 mM MG for various incubation times (0.5, 1, 2, 3, 4, 5 and 6 h). After that, cells were lysed and total ATP content was determined using ATPlite luminescence assay kit (Perkin Elmer) according to

manufacturer's protocol. ATP concentration was calculated using provided ATP standard.

3.6.8 Glyoxalase activity assay

INS-1 cells were seeded into 12-well plate (100,000 cells/well) and cultured for 24 h before pretreating with or without 0.1 mM IFA in 0.1% DMSO for 48 h. After removing IFA, cells were treated with 0 and 2 mM MG for various incubation times (0.5, 1, 2, 3, 4, 5 and 6 h). After that, cells were washed with 1X PBS and lysed using cooled lysis buffer (400 µL/well). Centrifugation of homogenate was done at 2,100 g for 10 min (4 $^{\circ}$C) to collect supernatant and then keep in the -20 $^{\circ}$C freezer until glyoxalase activity assay was performed.

3.6.8.1 Total protein determination

Protein concentration was determined using Bradford assay. Supernatant (5 µL) was incubated at room temperature with Bradford reagent (250 µL) for 10 min before measuring the absorbance at the wavelength of 595 nm. The concentration of protein in each sample was calculated using BSA standard curve and adjusted equal to others before measuring glyoxalase activity.

3.6.8.2 Glyoxalase I assay

This assay was used to determine the activity of glyoxalase I enzyme which catalyzes hemithioacetal adduct (from spontaneous reaction between reduced glutathione and MG) in the formation of S-D-lactoylglutathione which is the physiological intermediate of the glyoxalase system.

Firstly, the reaction of 2 mM reduced glutathione and 2 mM MG was prepared in Tris buffer, pH 6.6 at 37 °C for 10 min to generate hemithioacetal adduct. After that, 90 µL of this reaction was added to 10 µL of each sample in 96-well UV plate and immediately read the absorbance at 240 nm for 30 min. The absorbance in each time point was subtracted by baseline (the first reading).

3.6.9 Caspase activity assay

The activity of caspase-3 was evaluated using EnzChek caspase-3 assay kit according to a manufactorer's protocol. INS-1 cells were seeded and cultured for 24 h before pretreating with medium containing 0.1 mM IFA for 48 h followed by incubation of 2 mM MG for up to 3 h. Then, cells were washed one time with PBS (pH 7.4) and lysed with 500 µL of lysis buffer. Cell lysates were determined caspase-3 activity by incubation of 50 µM Z-DEVD-R110 substrate in the reaction buffer containing 10 µM DTT at room temperature for 30 min. The fluorescence intensity was measured at the excitation wavelength of 495 nm and the emission wavelength of 520 nm.

3.7 Statistical analysis

All data are presented as means ± standard error of mean (SEM). The statistical significance of results from *in vitro* experiments was evaluated by one-way ANOVA using a Duncan post-hoc comparison to analyze the difference between mean. The statistical significance of results from cell culture experiments was evaluated by one-way ANOVA or two-way ANOVA using a Tukey post-hoc comparison. A p-value < 0.05 was considered statistical significance.

CHAPTER IV

RESULTS

4.1 The effects of IFA on BSA glycation induced by glucose and fructose

4.1.1 Fluorescent AGE formation

The formation of fluorescent AGEs in BSA/sugar solutions is presented in Figure 15. A significant elevation of fluorescent intensity was observed when the duration of incubation increased in both BSA/glucose and BSA/fructose systems (17.6-fold increase in BSA/fructose and 8.0-fold increase in BSA/ glucose at week 4 when compared to baseline). An addition of IFA (1.25 - 5 mM) to the BSA/sugar reactions was found to significantly reduce the formation of fluorescent AGEs in a concentration-dependent manner throughout the study period. At week 4 of incubation, the percentage inhibition of AGE formation by IFA at the concentrations of 1.25, 2.5, and 5 mM was 40.2%, 54.3%, and 71.4%, respectively, for BSA/fructose system, and 42.9%, 53.6%, and 73.0%, respectively, for BSA/glucose system. Moreover, an anti-glycation drug aminoguanidine (AG) inhibited the formation of AGEs in BSA/fructose and BSA/glucose by 87.3% and 73.1% , respectively.

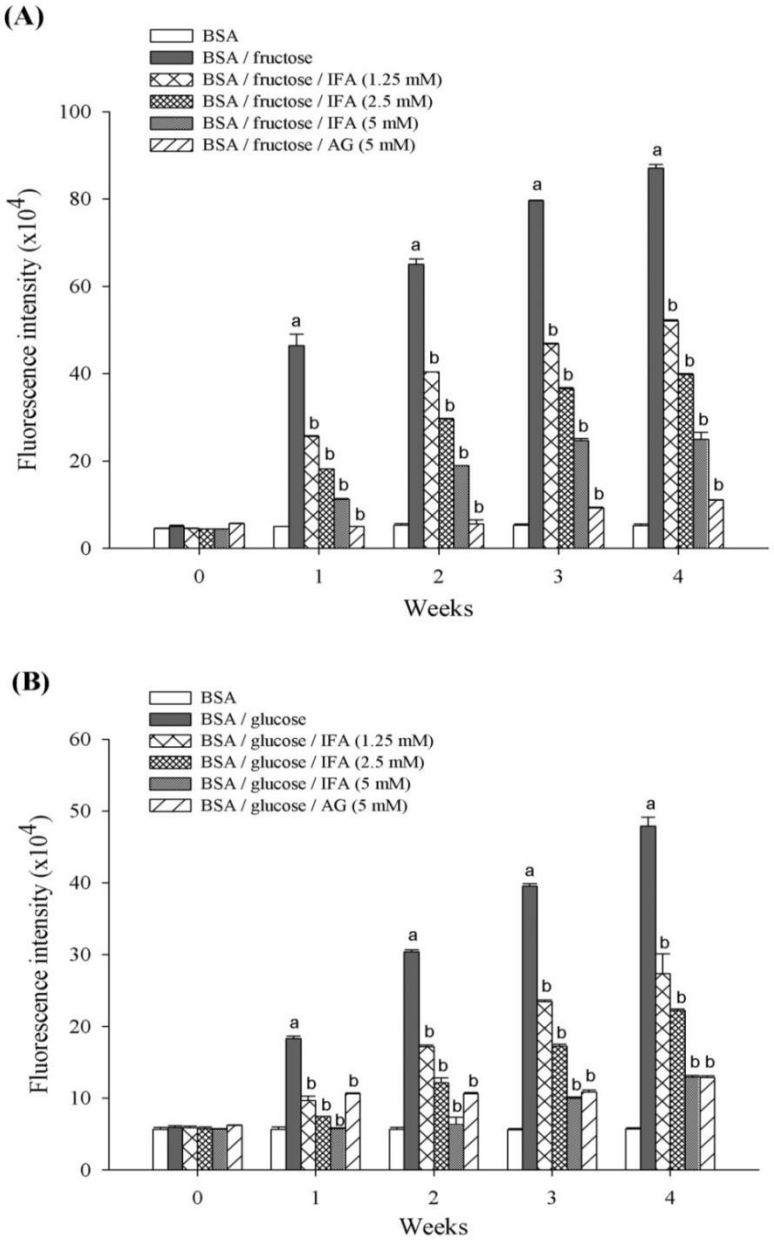

Figure 15 Effect of IFA on fluorescent AGE formation in BSA/fructose (A) and BSA/glucose (B) systems.

Results are presented as mean ± SEM (n=3). One-way ANOVA (Duncan post hoc) was used to analyze statistical significance of results at the same time of incubation. [a]$p<0.05$ when compared to BSA, [b]$p<0.05$ when compared to BSA/fructose or BSA/glucose.

4.1.2 Non-fluorescent AGE formation

The formation of N^{ε}-CML, a non-fluorescent AGE, is shown in Figure 16. After 4 weeks of incubation, glycated BSA induced by fructose presented a 9.2-fold elevation in CML formation, whereas there was 1.6-fold increase in glucose-induced glycated BSA when compared to non-glycated BSA. IFA at a concentration of 5 mM significantly inhibited CML formation by 47.0% in BSA/fructose system and 21.9% in BSA/glucose system. Similarly, AG significantly reduced the formation of CML by 65.8% and 20.2% for BSA/fructose and BSA/glucose systems, respectively.

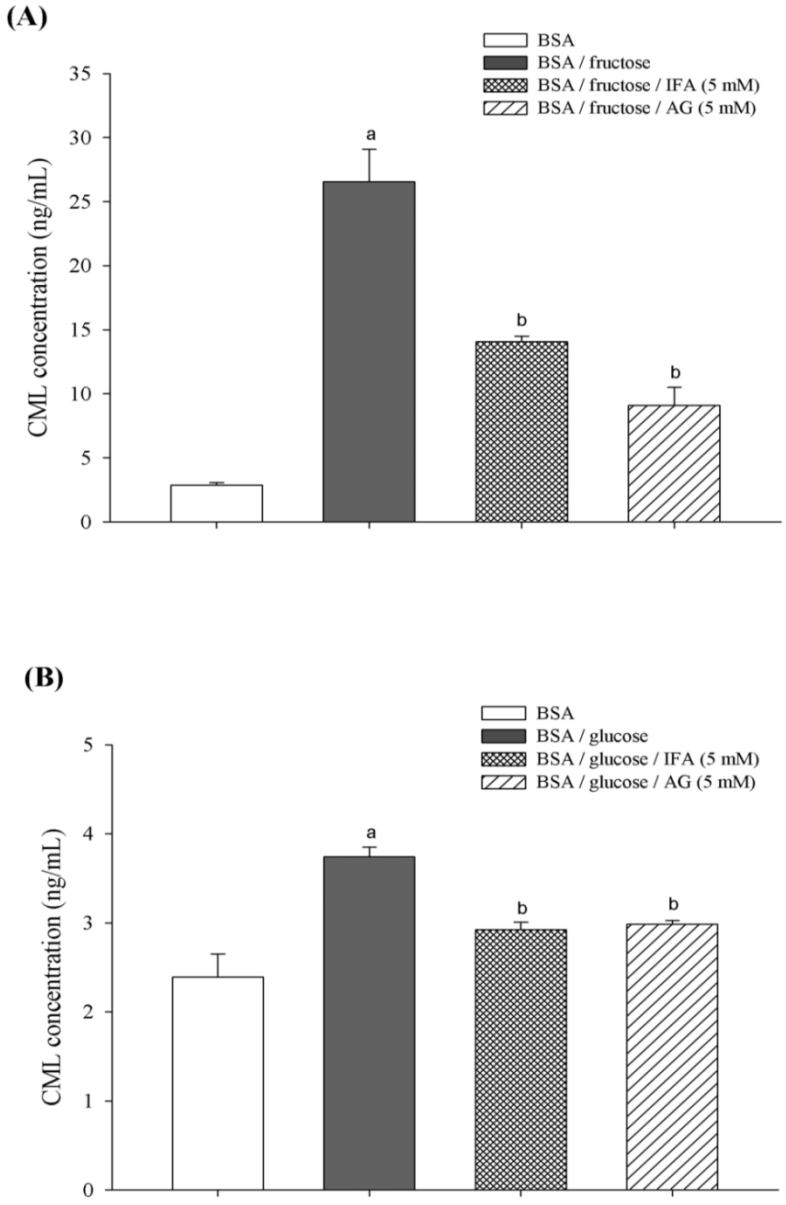

Figure 16 Effect of IFA on the formation of N^{ε}-(carboxymethyl) lysine (CML) in BSA/fructose (A) and BSA/glucose (B) systems at week 4.

Results are presented as mean ± SEM (n=3). One-way ANOVA (Duncan post hoc) was used to analyze statistical significance. $^{a}p<0.05$ when compared to BSA, $^{b}p<0.05$ when compared to BSA/fructose or BSA/glucose.

4.1.3 Amadori product formation

The level of fructosamine, an Amadori product derived from BSA/sugar glycation, is shown in Figure 17. Low detectable concentrations of fructosamine were observed at baseline in each group and over 4 weeks of incubation in non-glycated BSA. BSA incubated with both monosaccharides exhibited an increasing level of fructosamine throughout the study period, however, less formation of fructosamine was observed in BSA/fructose than BSA/glucose system. The addition of IFA and AG significantly suppressed the production of fructosamine. At the end of the study period, the concentrations of IFA at 1.25, 2.5 and 5 mM inhibited the formation of fructosamine by 20.6%, 30.0%, and 33.4%, respectively, in BSA/fructose system and by 7.3%, 15.0%, and 20.1%, respectively, in BSA/glucose system, whereas the inhibitory effect of AG at 5 mM in BSA/fructose and BSA/glucose systems was 34.3% and 10.7%, respectively. The results also found that IFA was more effective to reduce fructosamine formation in BSA/fructose than BSA/glucose system.

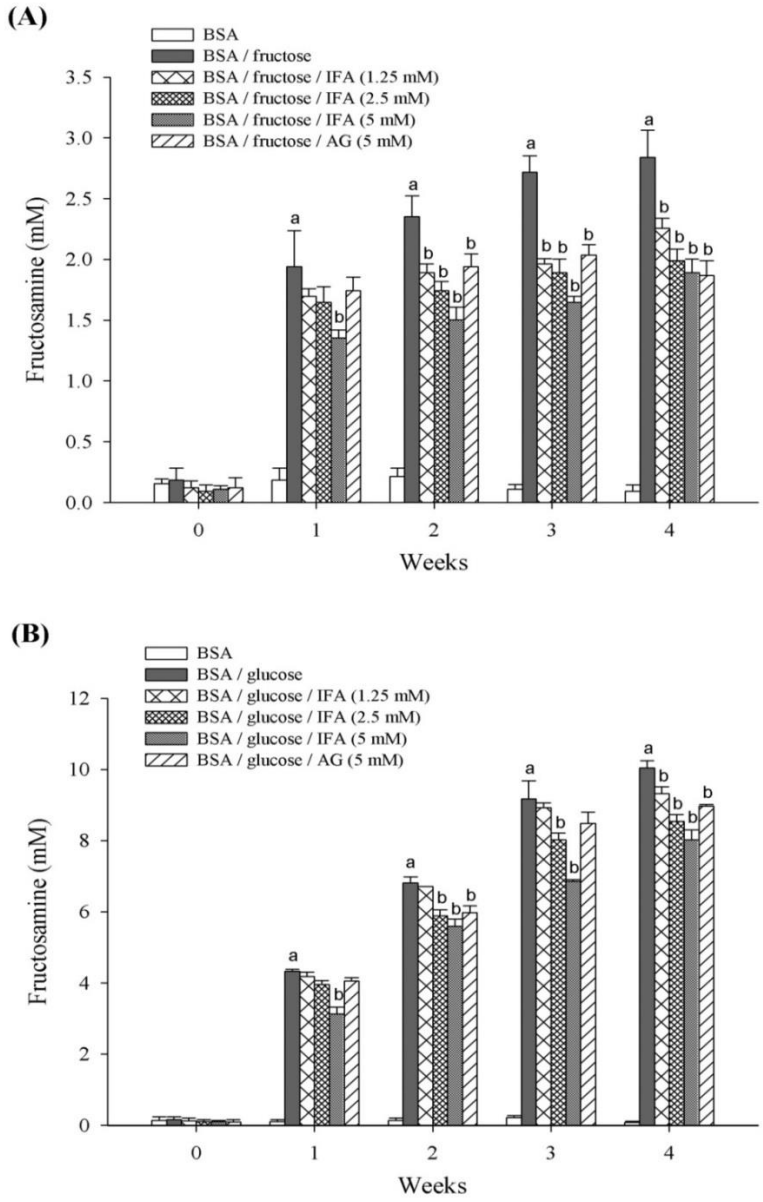

Figure 17 Effect of IFA on the Amadori production in BSA/fructose (A) and BSA/glucose (B) systems.

Results are presented as mean ± SEM (n=3). One-way ANOVA (Duncan post hoc) was used to analyze statistical significance of results at the same time of incubation. [a] $p<0.05$ when compared to BSA, [b] $p<0.05$ when compared to BSA/fructose or BSA/glucose.

4.1.4 Protein oxidation

The protein oxidation occurred during the process of glycation was assessed by measurement of carbonyl content and free thiol groups in BSA as shown in Figure 18 and 19, respectively. The carbonyl content of glycated BSA at week 4 of study period was significantly higher than non-glycated BSA approximately 12.9 times in BSA/fructose system, and 9.5 times in BSA/glucose system. Incubation of IFA (1.25, 2.5 and 5 mM) for 4 weeks exhibited a concentration-dependent reduction in protein carbonyl content by 36.5%, 46.1%, and 60.5% in BSA/fructose system, and 48.1%, 58.7%, and 70.6% in BSA/glucose system, respectively, whereas addition of AG decreased the protein carbonyl content about 40.2% and 58.1% in BSA/fructose and BSA/glucose systems, respectively.

In contrast to an increase of carbonyl content, oxidation of protein led to the loss of free thiol groups in its structure. Fructose- and glucose-mediated glycated BSA had lower level in free thiol groups than non-glycated BSA over the period of study. At week 4, the results showed that fructose caused a decrease in thiol groups in BSA by 22.4%, whereas the loss of thiol groups in BSA induced by glucose was 20.2%. There was a significant improvement in the level of thiol after adding IFA at various concentrations (1.25-5 mM) as well as AG. At week 4 of incubation, the percentage prevention of decreased thiol groups by IFA was 10.6%, 11.3%, and 16.0% for BSA/fructose system, and 4.2%, 11.7%, and 18.9% for BSA/glucose system, respectively, whereas AG prevented loss of thiol by 17.5% in BSA/fructose and 15.6% in BSA/glucose.

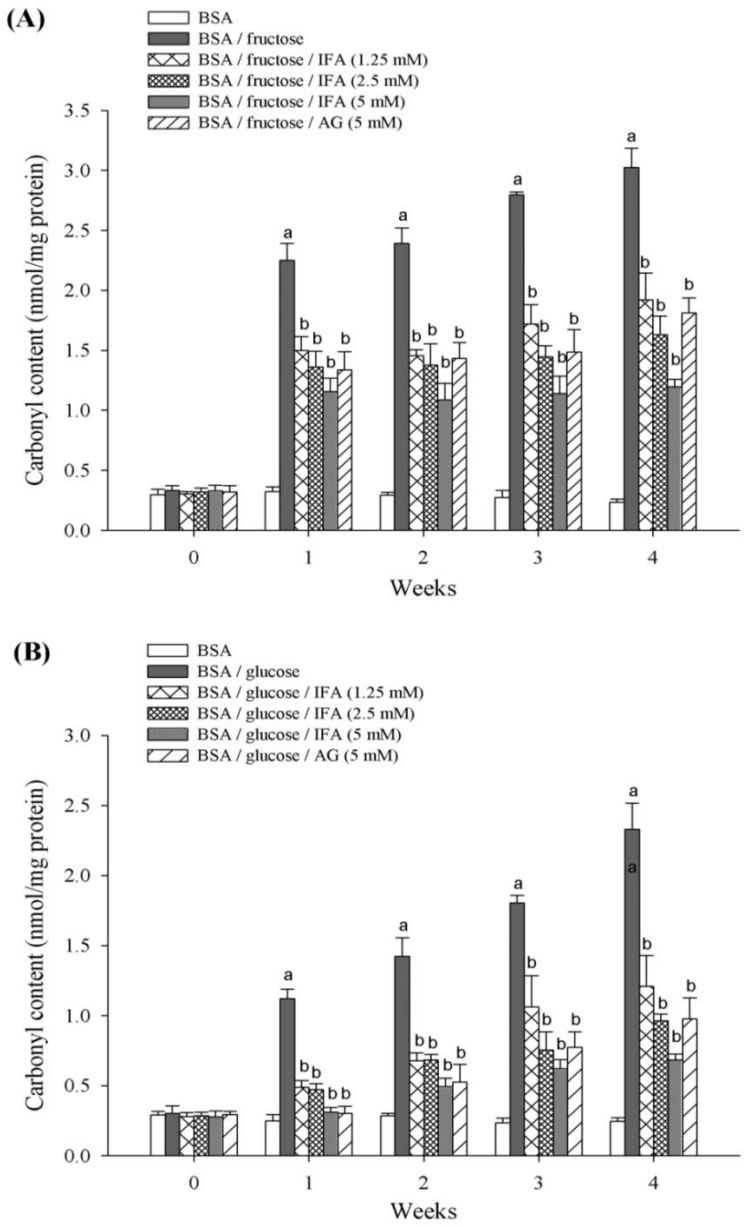

Figure 18 Effect of IFA on protein carbonyl content in BSA/fructose (A) and BSA/glucose (B) systems.

Results are presented as mean ± SEM (n=3). One-way ANOVA (Duncan post hoc) was used to analyze statistical significance of results at the same time of incubation. [a] $p<0.05$ when compared to BSA, [b] $p<0.05$ when compared to BSA/fructose or BSA/glucose.

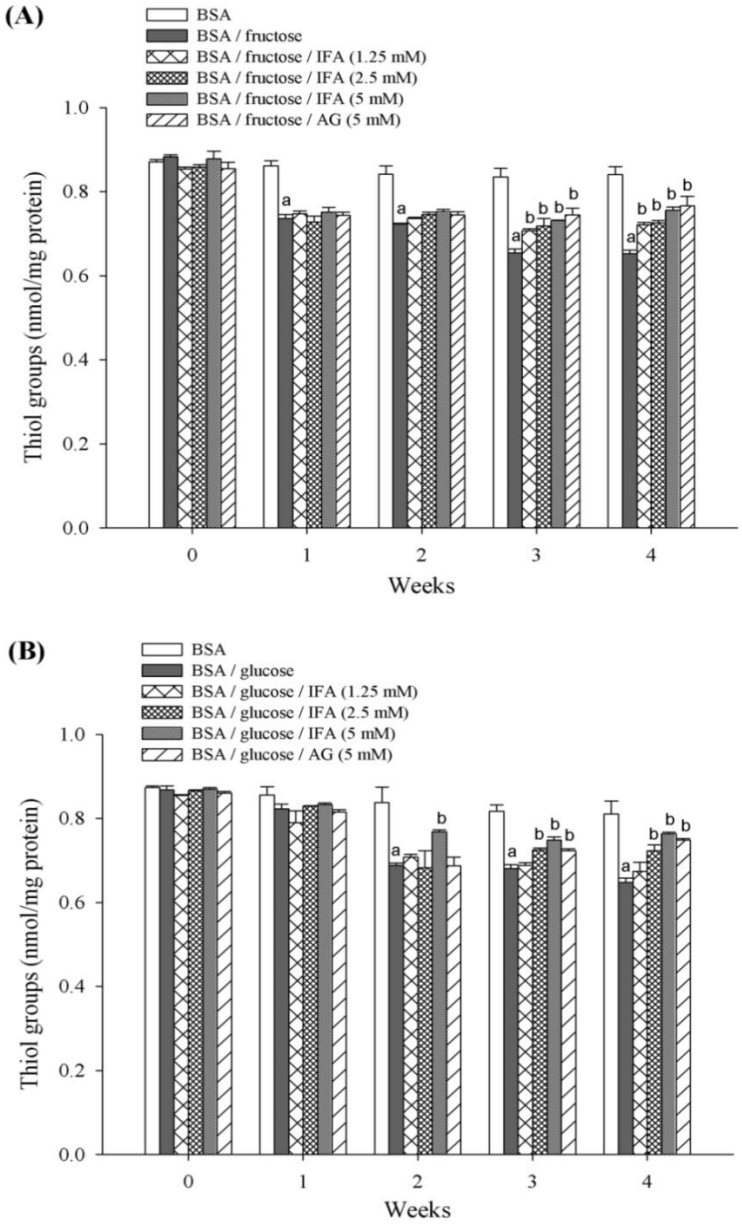

Figure 19 Effect of IFA on free thiol groups in BSA/fructose (A) and BSA/glucose (B) systems.

Results are presented as mean ± SEM (n=3). One-way ANOVA (Duncan post hoc) was used to analyze statistical significance of results at the same time of incubation. [a] $p<0.05$ when compared to BSA, [b] $p<0.05$ when compared to BSA/fructose or BSA/glucose.

4.1.5 Protein aggregation

The thioflavin T is a common assay used to quantify the amount of amyloid cross β structure which indicated protein aggregation in glycated BSA. As shown in Figure 20, the glycated BSA induced by fructose and glucose exhibited an elevation of amyloid cross β conformation up to 1.6-fold and 1.2-fold, respectively, over non-glycated BSA at week 4 of incubation. IFA at concentrations of 1.25 - 5 mM suppressed the level of amyloid cross β structure in a concentration-dependent manner for both BSA/fructose (12.0%, 17.7%, and 21.5%) and BSA/glucose (0.2%, 3.1%, and 7.3%) systems. Likewise, a significant decrease in the level of amyloid cross β structure (7.1% in BSA/fructose and 12.4% in BSA/glucose) was observed in the presence of AG at the end of study.

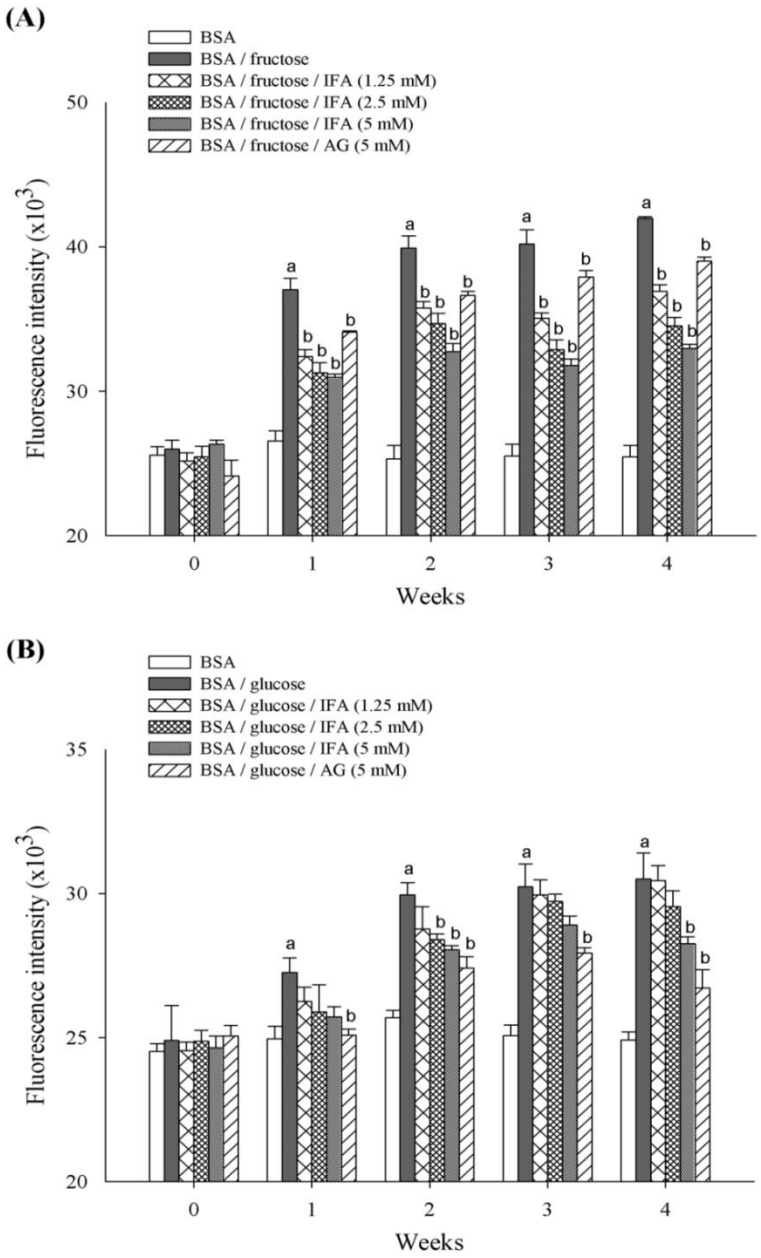

Figure 20 Effect of IFA on the amyloid cross **β**-structure in BSA/fructose (A) and BSA/glucose (B) systems.

Results are presented as mean ± SEM (n=3). One-way ANOVA (Duncan post hoc) was used to analyze statistical significance of results at the same time of incubation. [a]$p<0.05$ when compared to BSA, [b]$p<0.05$ when compared to BSA/fructose or BSA/glucose.

4.2 The effects of IFA on BSA glycation induced by methylglyoxal

4.2.1 Fluorescent AGE formation

Figure 21 illustrates the fluorescence intensity of BSA incubated with MG and IFA for up to 2 weeks. A 16- and 20-fold increase of fluorescence intensity was observed for BSA incubated with MG after 1 and 2 weeks, respectively. When IFA (1.25-5 mM) was incubated with MG, the fluorescence intensity significantly decreased in a concentration-dependent manner. The percentage inhibition in BSA/MG system by IFA (1.25-5 mM) was 25.4%, 37.4%, and 51.6% at week 1 and was 27.5%, 40.5%, and 54.9% at week 2. In addition, AG (1.25 mM) completely inhibited MG-derived fluorescence of glycation.

4.2.2 Non-fluorescent AGE formation

The presence of non-fluorescent AGEs represented by N^{ε}-CML was determined at the end of 2-week incubation. The results in Figure 22 demonstrate that MG caused a 2-fold increase of N^{ε}-CML formation when compared to BSA control. Incubation with IFA (1.25-5 mM) significantly reduced the formation of N^{ε}-CML by 29.9%, 31.7%, and 41.3%, respectively. A similar effect was observed with 1.25 mM AG (37.0% inhibition).

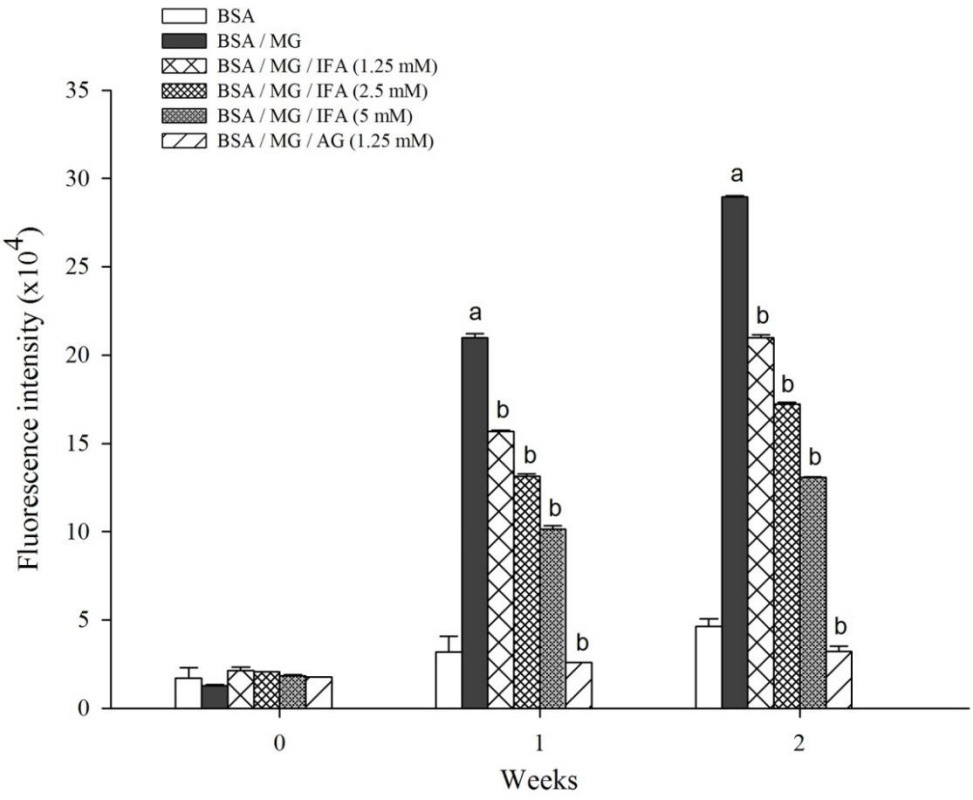

Figure 21 Effect of IFA on fluorescent AGE formation in BSA/MG system.
Results are presented as mean ± SEM (n=3). One-way ANOVA (Duncan post hoc) was used to analyze statistical significance of results at the same time of incubation. [a]$p<0.05$ when compared to BSA, [b]$p<0.05$ when compared to BSA/MG.

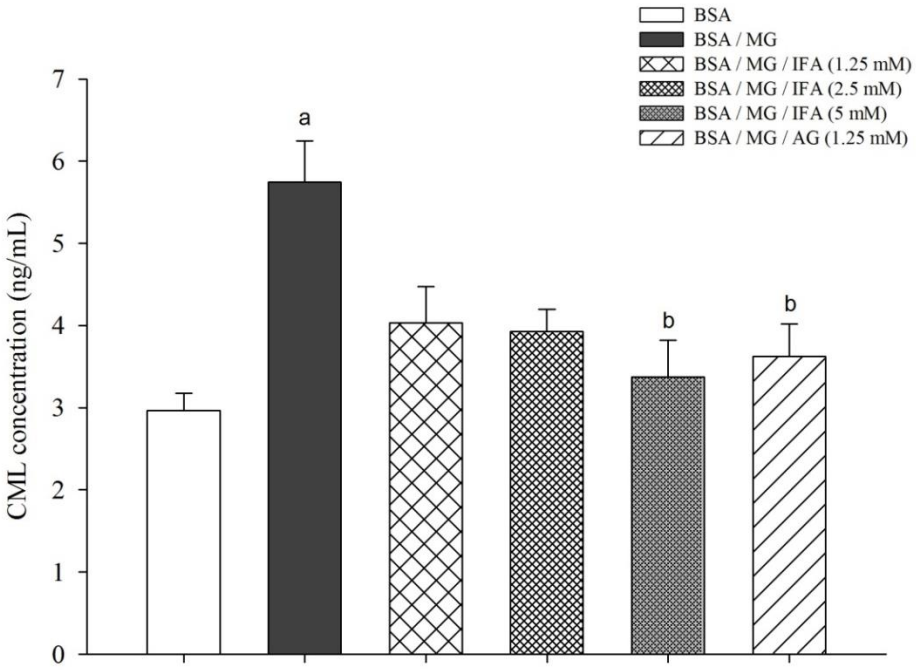

Figure 22 Effect of IFA on the formation of N^{ε}-(carboxymethyl) lysine (CML) in BSA/MG system at week 2.

Results are presented as mean ± SEM (n=3). One-way ANOVA (Duncan post hoc) was used to analyze statistical significance. [a]$p<0.05$ when compared to BSA, [b]$p<0.05$ when compared to BSA/MG.

4.2.3 Protein oxidation

The protein oxidation represented by protein carbonyl content and free thiol groups was determined after 1 and 2 weeks of incubation period. The protein carbonyl concentration (Figure 23) in BSA incubated with MG was 13.5-fold higher than that of BSA alone. AG suppressed MG-induced carbonylation in BSA by 82.1% at week 1 and 78.3% at week 2, whereas IFA at concentration of 2.5 and 5 mM significantly reduced the elevated carbonyl content by 48.6% and 52.3%, respectively, at week 2.

The level of free thiol groups (Figure 24) in BSA incubated with MG at week 1 and week 2 of incubation was approximately 0.9-fold lower than BSA alone. Addition of AG significantly reduced loss of free thiol group by 6.0% at week 1 and 5.0% at week 2, whereas IFA (1.25 – 5 mM) significantly improved the level of thiol groups in BSA/MG system ranging from 4.0% to 4.8% at week 2.

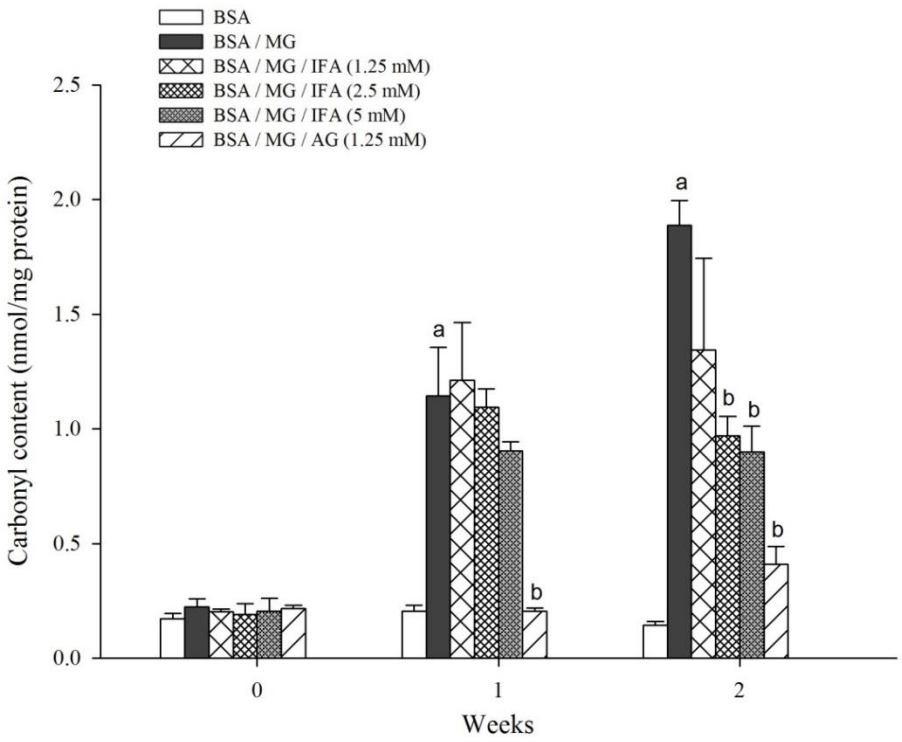

Figure 23 Effect of IFA on protein carbonyl content in BSA/MG system.
Results are presented as mean ± SEM (n=3). One-way ANOVA (Duncan post hoc) was used to analyze statistical significance of results at the same time of incubation. [a]$p<0.05$ when compared to BSA, [b]$p<0.05$ when compared to BSA/MG.

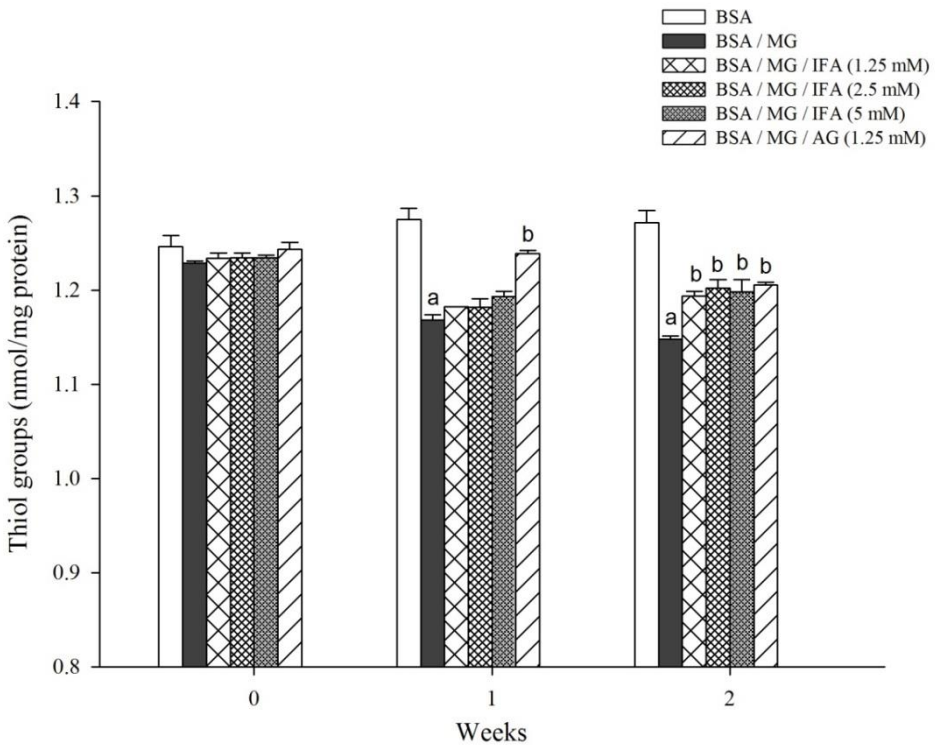

Figure 24 Effect of IFA on free thiol groups in BSA/MG system.

Results are presented as mean ± SEM (n=3). One-way ANOVA (Duncan post hoc) was used to analyze statistical significance of results at the same time of incubation. [a]$p<0.05$ when compared to BSA, [b]$p<0.05$ when compared to BSA/MG.

4.3 The effects of IFA on MG-induced oxidative DNA damage

4.3.1 DNA strand breakage

The band intensity of DNA fragments on agarose gel indicating the strand breakage of plasmid DNA after incubating with or without lysine or MG or $CuSO_4$ or IFA (0.1 and 1 mM) is presented in Figure 25. Untreated and damaged DNA were detected as major bands of supercoiled form (SF) and nicked form (NF), respectively. The results from control experiments showed that there was a slight increase of NF band when DNA was incubated with lysine or MG or $CuSO_4$ or IFA, however, no difference in the band intensity of both SF and NF was observed between those treatments.

The effect of IFA on preventing DNA strand breakage in the absence of Cu^{2+} is demonstrated in Figure 26. The incubation of DNA together with lysine and MG markedly induced DNA stand breakage with 2-fold increase in the intensity of NF band when compared to untreated DNA. IFA significantly reduced DNA damage at 0.5 mM (23.0%) and 1 mM (24.9%) whereas IFA at 0.25 mM and 0.1 mM did not have a significant effect.

The cleavage of lysine/MG-treated DNA was enhanced by addition of Cu^{2+} as shown by an increase in the intensity of NF band and a decrease in the intensity of SF band (Figure 27). In the presence of Cu^{2+}, DNA strand breakage was inhibited by IFA (0.1-1 mM) with the percentage inhibition of 24.0%, 39.1, 47.2, and 57.0%, respectively.

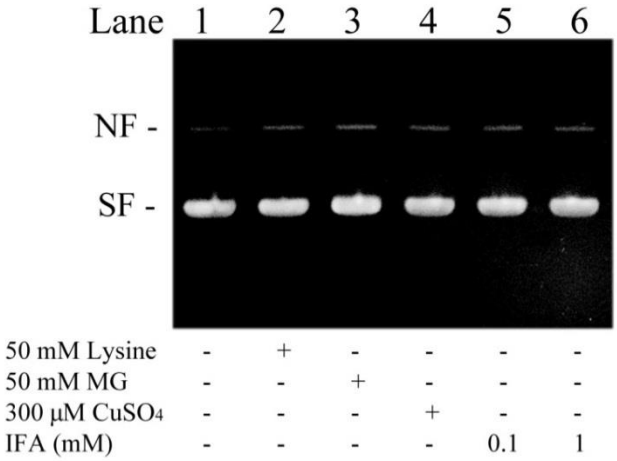

Figure 25 The band intensity of nicked form (NF) and supercoiled form (SF) of plasmid DNA after incubating pUC19 (0.25 µg) with different treatments.
Lane 1-6: no treatment, 50 mM lysine, 50 mM MG, 300 µM $CuSO_4$, 0.1 mM IFA and 1 mM IFA, respectively.

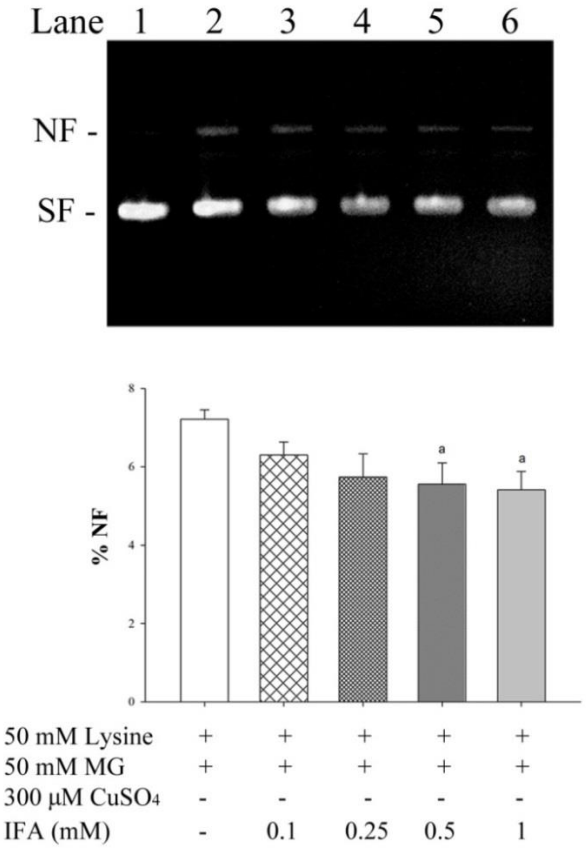

Figure 26 Effect of IFA on lysine/MG-induced DNA strand breakage in the absence of Cu^{2+}.

Gel image expresses the intensity of nicked form (NF) and supercoiled form (SF) of plasmid DNA after pUC19 (0.25 µg) was incubated with Lysine and MG in the absence or presence of various IFA concentrations (0.1-1 mM) without addition of $CuSO_4$. The percentage of NF (%NF, see calculation method in Chapter III) in each treatment was subtracted by %NF of untreated DNA (lane 1 on gel) before expressing in bar graph. Results are presented as mean ± SEM (n=3). One-way ANOVA (Duncan post hoc) was used to analyze statistical significance. $^{a}p<0.05$ when compared to lysine/MG.

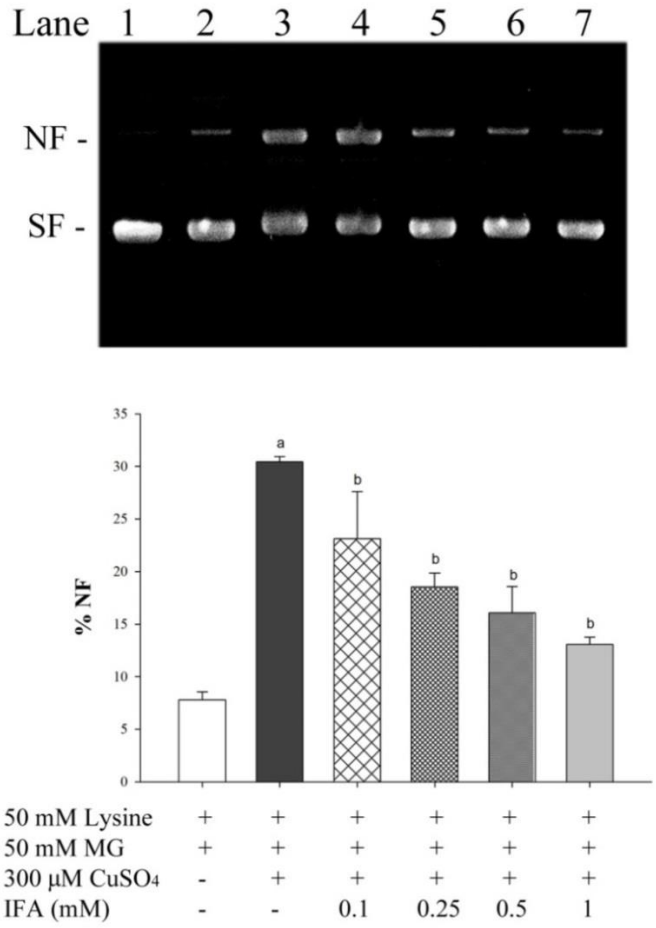

Figure 27 Effect of IFA on lysine/MG-induced DNA strand breakage in the presence of Cu^{2+}.

Gel image expresses the intensity of nicked form (NF) and supercoiled form (SF) of plasmid DNA after pUC19 (0.25 µg) was incubated with Lysine and MG in the absence or presence of various IFA concentrations (0.1-1 mM) co-incubating with $CuSO_4$. The percentage of NF (%NF, see calculation method in Chapter 3) in each treatment was subtracted by %NF of untreated DNA (lane 1 on gel) before expressing in bar graph. Results are presented as mean ± SEM (n=3). One-way ANOVA (Duncan post hoc) was used to analyze statistical significance. $^{a}p<0.05$ when compared to lysine/MG, $^{b}p<0.05$ when compared to lysine/MG/Cu^{2+}.

4.3.2 Superoxide anion production

To monitor the generation of superoxide anion induced by the reaction between lysine and MG and also the effect of IFA in prevention, the reduction of cytochrome *c* was used as an indicator. Figure 28 represents a time-dependent increase of the reduced form of cytochrome *c,* corresponding to increased superoxide anion production during 180 min of incubation. Superoxide anion produced by the interaction of lysine with MG created 13.8 nmol/mL reduced cytochrome *c* at 180 min. The generation of superoxide anion was suppressed by IFA after 10 min of incubation. At the end of incubation, IFA (0.1-1 mM) inhibited lysine/MG-induced superoxide anion production by 5.0%, 8.0%, 13.9%, and 25.0%, respectively, as shown in Figure 29.

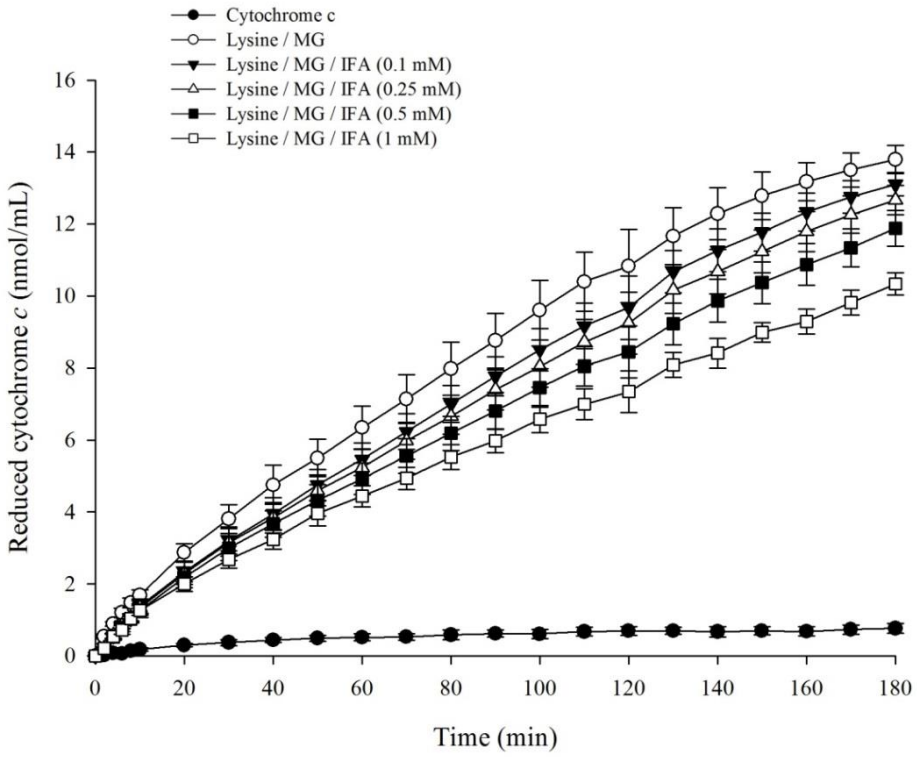

Figure 28 Effect of IFA on the production of superoxide anion in lysine/MG-induced glycation as measured by cytochrome *c* reduction within 180 min.
Results are presented as mean ± SEM (n=3).

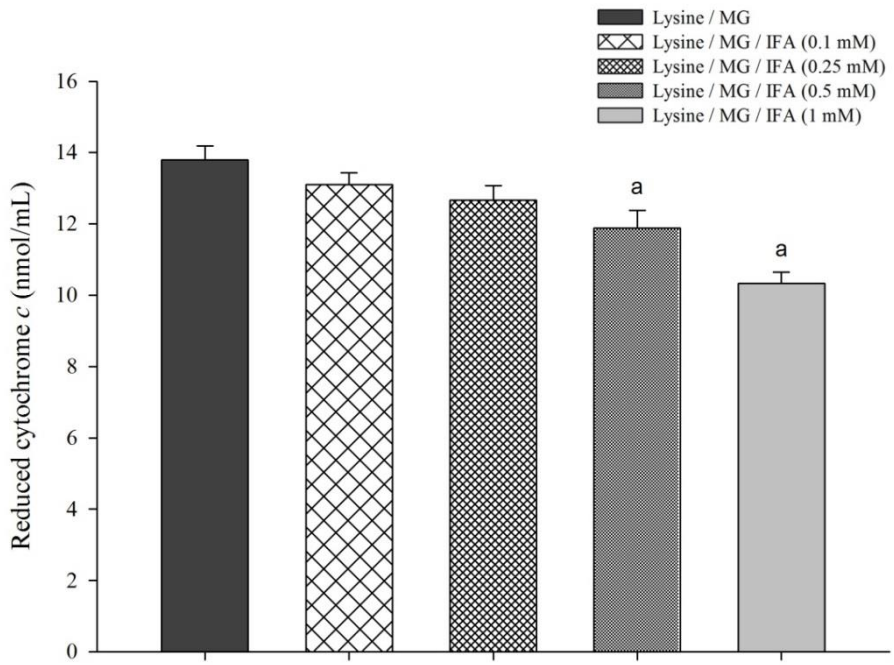

Figure 29 Effect of IFA on the production of superoxide anion in lysine/MG glycation at the incubation time of 180 min.

Results are presented as mean ± SEM (n=3). One-way ANOVA (Duncan post hoc) was used to analyze statistical significance. [a]$p<0.05$ when compared to lysine/MG.

4.3.3 Hydroxyl radical production

Figure 30 shows the TBARS concentration in the lysine/MG system indicating the generation of hydroxyl radicals. Similar to the effect on superoxide anion formation, IFA had the ability to reduce the generation of hydroxyl radical. The percentage inhibition of hydroxyl radical generation by IFA (0.1-1 mM) was 26.1%, 39.5%, 41.6%, and 44.6%.

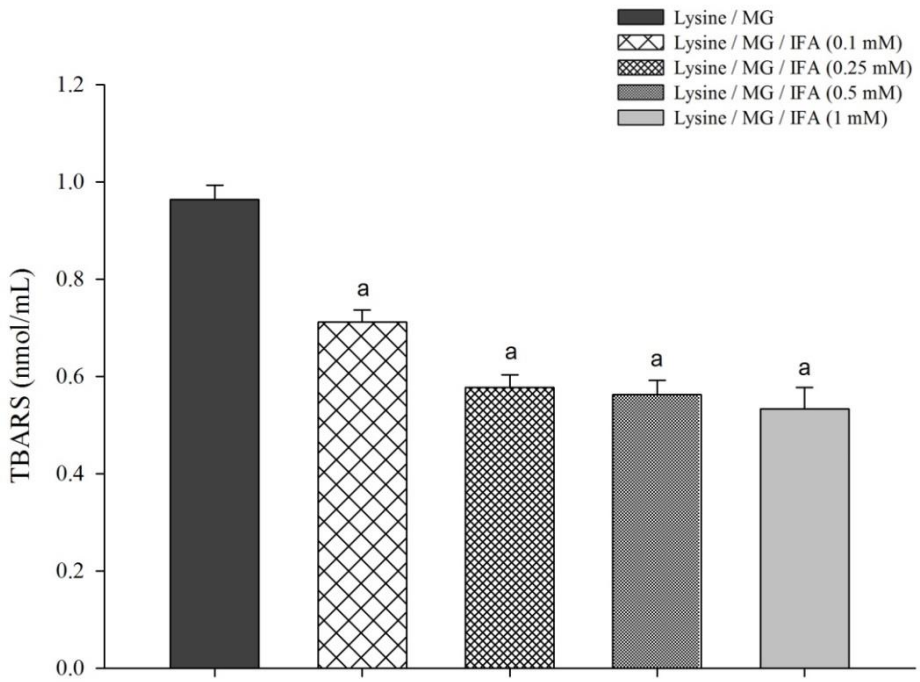

Figure 30 Effect of IFA on the production of hydroxyl radical in lysine/MG glycation at 180 min.

Results are presented as mean ± SEM (n=3). One-way ANOVA (Duncan post hoc) was used to analyze statistical significance. [a]$p<0.05$ when compared to lysine/MG.

4.3.4 MG-trapping capability

An evaluation of direct MG-trapping capacity was carried out in order to investigate whether IFA could directly scavenge MG. Figure 31 demonstrates the direct MG-trapping ability of IFA and AG after 1 h and 24 h of incubation. The level of 2-MQ, a product from the reaction of MG and *o*-PDA, represented free MG remaining from the trapping reaction. The chromatogram results showed that AG exhibited MG-trapping ability with the values of 82.9% at 1 h and 93.5% at 24 h. However, IFA (1.25-5 mM) had no MG-trapping ability after 1 h and 24 h of incubation.

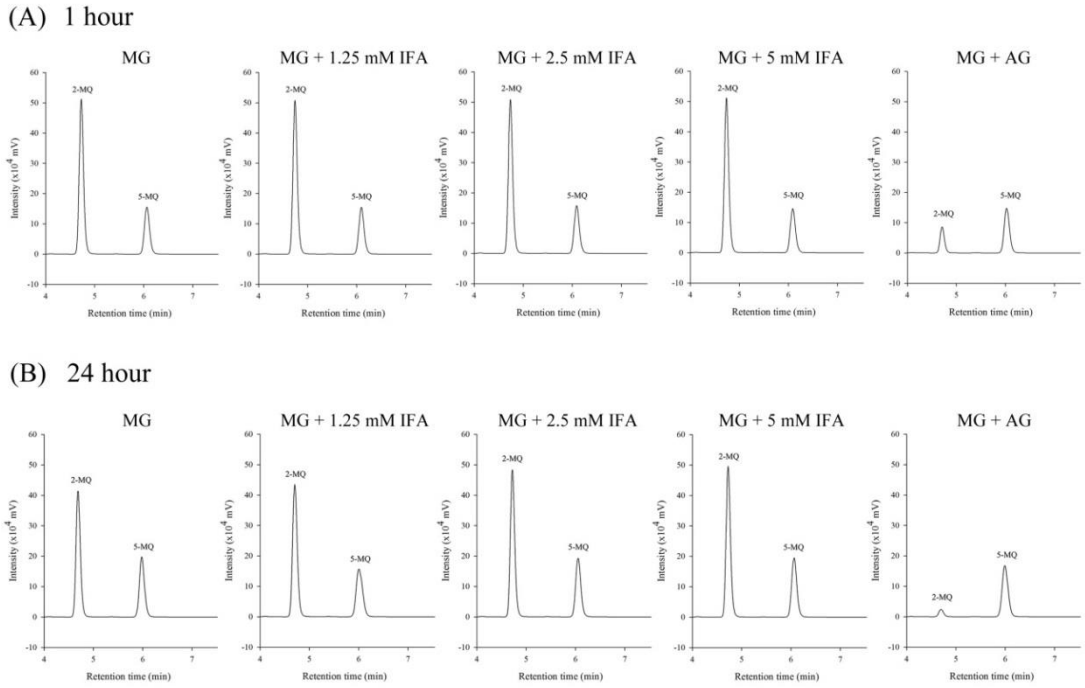

Figure 31 The HPLC chromatogram of 1 mM MG after reacting with IFA (1.25-5 mM) or AG (1.25 mM) at the incubation time of 1 h (A) and 24 h (B).

MG was detected as 2-methylquinoxaline (2-MQ) after derivatization using *o*-phenylenediamine (*o*-PDA) at 315 nm. 5-methylquinoxaline (5-MQ) was used as the internal standard.

4.4 The effects of IFA on MG-induced β-cell dysfunction

4.4.1 Cell viability

To monitor the concentration-dependent and time-dependent effect of MG on cell viability, INS-1 cells were treated with various concentrations of MG (0.5 - 4 mM) at different incubation period (1 – 6 h) and assessed using MTT assay. As shown in Figure 32, cells treated with 0.5 and 1 mM MG exhibited a slight increase but no significance in cell viability at every period of incubation, whereas cells treated with 2 mM MG for 6 h exhibited a significant decrease in cell viability approximately 75% when compared to untreated cells. In addition, the highest concentration of MG (4 mM) significantly suppressed the viability of INS-1 cells in a time-dependent manner. At 6 h of incubation, 4 mM MG caused a decrease in cell viability more than 50%.

The incubation period of 6 h was chosen and MG at multiple concentrations was treated to the INS-1 cells pretreated with 0.1% DMSO (which was a solvent for IFA) for 24 h. The results from Figure 33 demonstrate that 2 mM and 3 mM MG induced a decrease of cell viability in the range of 25% to 50% when compared to control, whereas 4 mM exhibited high toxicity to the cells.

IFA at various concentrations (0.01-0.1 mM) was also treated to the cells to evaluate the cytotoxicity. The results from Figure 34 show that all concentrations of IFA did not cause a decrease in cell viability in both 24 h and 48 h of incubation.

The effect of IFA pretreatment (24 h and 48 h) on MG-treating cells for 6 h is presented in Figure 35 and Figure 36, respectively. In cells treating with 2 mM MG, preincubating with IFA for 24 h improved cell viability up to approximately 95% of

viability, whereas it failed to increase the viability of cells treated with 3 mM MG. Similar results to 24-h preincubation, pretreatment of IFA for 48 h improved the viability of INS-1 cells. Additionally, IFA (0.1 mM) significantly improved the viability of cells treated with 3 mM MG when it was preincubated to the cells for up to 48 h.

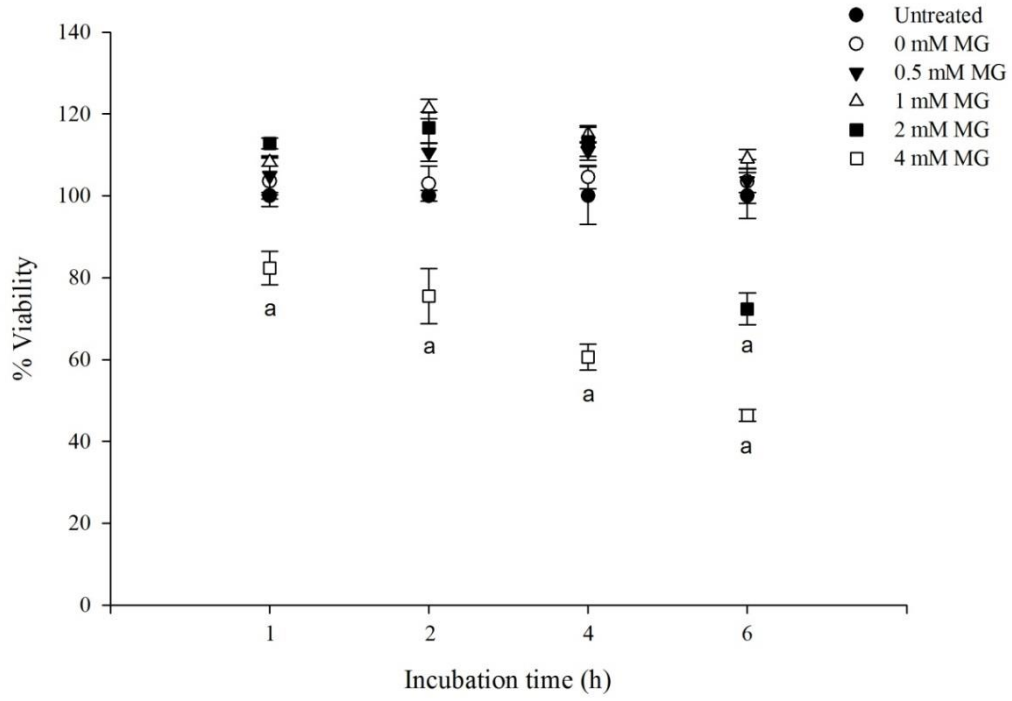

Figure 32 The viability of INS-1 cells treated with various concentrations of MG (0.5–4 mM) at different times of incubation (1 – 6 h).

Results are presented as mean ± SEM (n=3). One-way ANOVA (Tukey post hoc) was used to analyze statistical significance of results at the same time of incubation. [a] $p<0.05$ when compared to untreated cells.

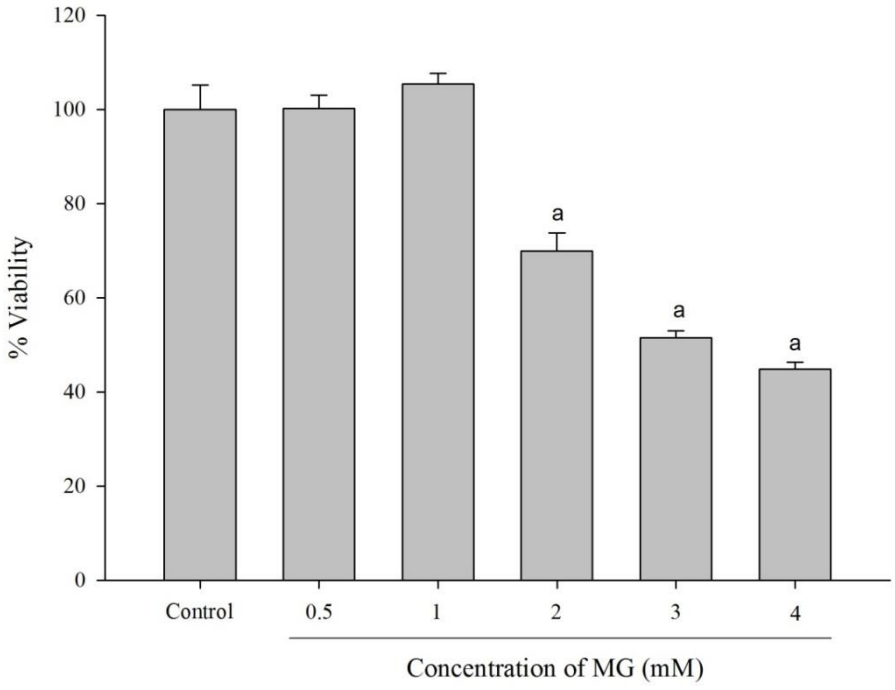

Figure 33 The viability of INS-1 cells treated with various concentrations of MG (0.5–4 mM) at 6 h of incubation.

Results are presented as mean ± SEM (n=3). One-way ANOVA (Tukey post hoc) was used to analyze statistical significance. $^{a}p<0.05$ when compared to control (INS-1 cells pretreating with 0.1% DMSO for 24 h).

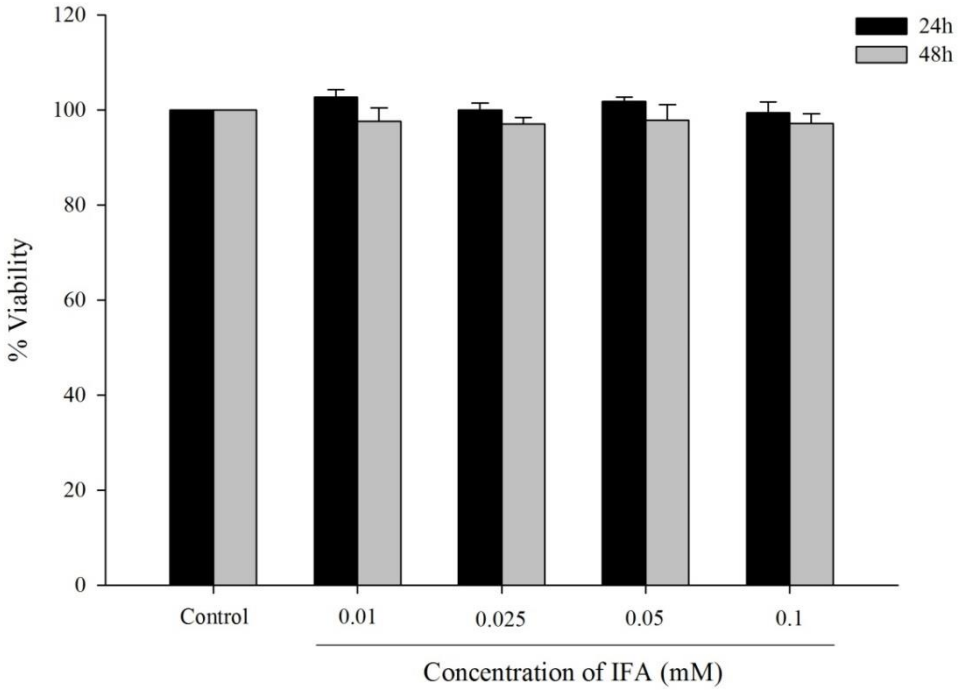

Figure 34 The viability of INS-1 cells after treating with various concentrations of IFA (0.01–0.1 mM) for 24 h and 48 h.

Results are presented as mean ± SEM (n=3). One-way ANOVA (Tukey post hoc) was used to analyze statistical significance of results at the same time of incubation. Control was INS-1 cells treated with 0.1% DMSO.

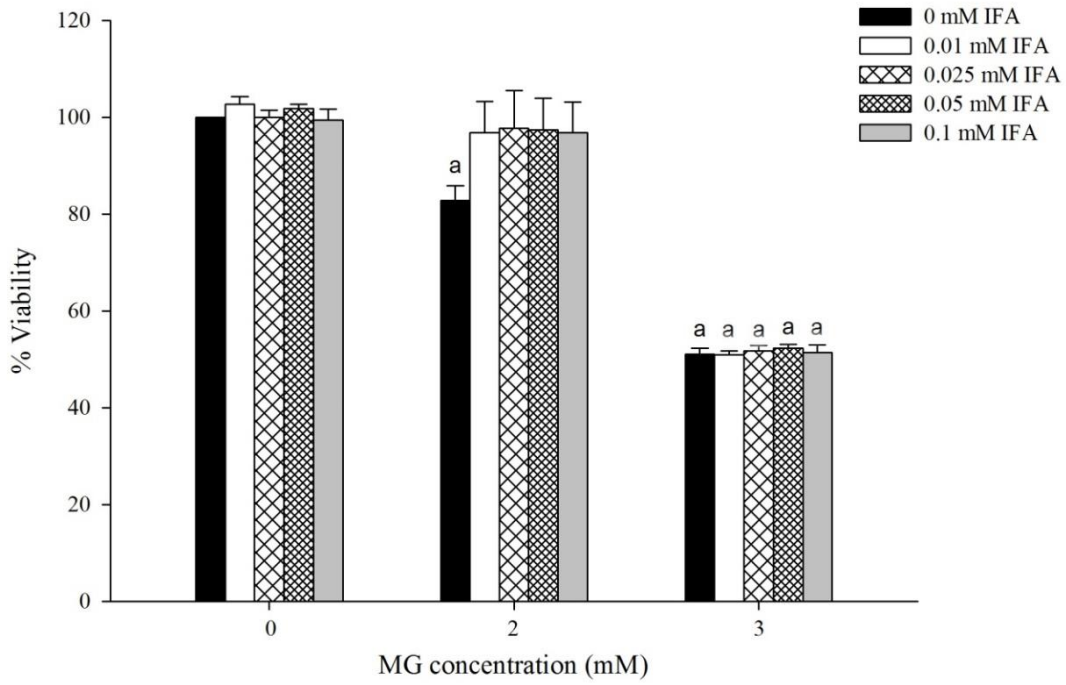

Figure 35 Effect of IFA pretreatment for 24 h on the viability of INS-1 cells incubated with MG (2 mM and 3 mM) for 6 h.

Results are presented as mean ± SEM (n=3). Two-way ANOVA (Tukey post hoc) was used to analyze statistical significance. $^{a}p<0.05$ when compared to 0 mM MG (MG-untreated cells).

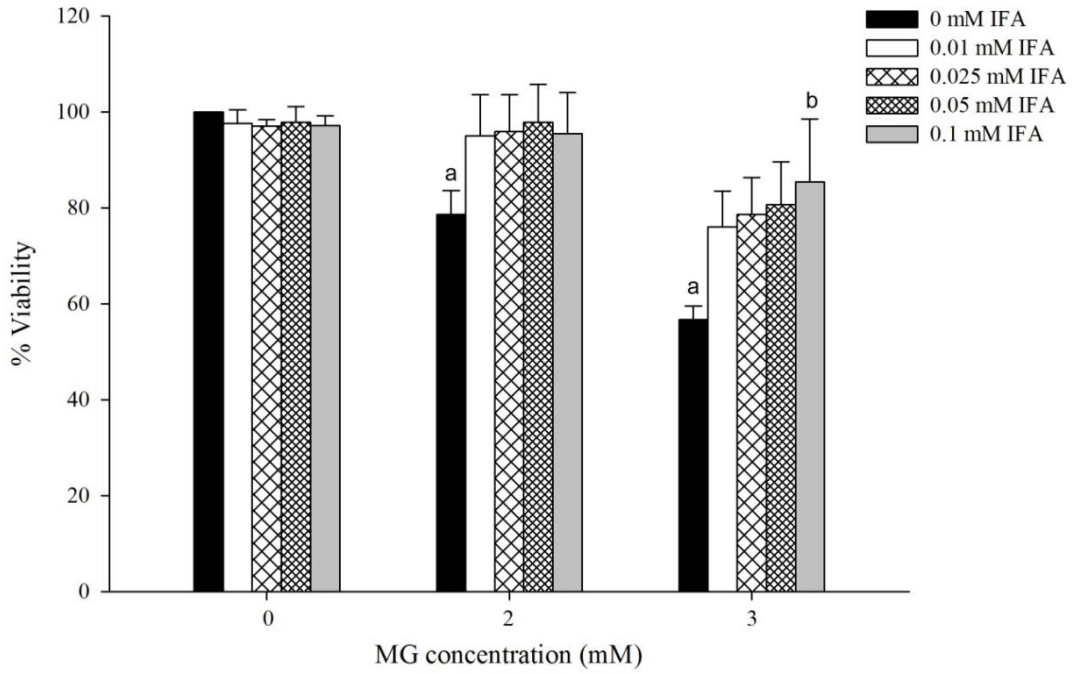

Figure 36 Effect of IFA pretreatment for 48 h on the viability of INS-1 cells incubated with MG (2 mM and 3 mM) for 6 h.

Results are presented as mean ± SEM (n=3). Two-way ANOVA (Tukey post hoc) was used to analyze statistical significance. [a]$p<0.05$ when compared to 0 mM MG (MG-untreated cells), [b]$p<0.05$ when compared to 0 mM IFA (IFA-untreated cells) at the same concentration of MG.

4.4.2 Glucose-stimulated insulin secretion (GSIS)

INS-1 cells were determined the insulin secretion in response to glucose at low, normal and high concentrations after incubating with MG at the concentrations of 2 and 3 mM as presented in Figure 37. Without MG, cells increased the secretion of insulin in response to increasing concentration of glucose. The two highest concentrations of glucose (11 and 22 mM) showed 2.7-fold and 4.6-fold of secreted insulin over the lowest concentration (2.8 mM). In the presence of MG, GSIS was significantly suppressed particularly at the high concentration of glucose. The percentage inhibition by 2 mM and 3 mM MG was 32.8% and 43.3%, respectively, at 11 mM glucose, and was 55.2% and 60.9%, respectively, at 22 mM glucose.

Figure 38 demonstrates the effect of IFA on GSIS. After pretreating to the cells for 48 h, IFA (0.01-0.1 mM) did not change the level of insulin secretion at all concentrations of glucose. In contrast, there was an increasing level of high glucose-induced insulin secretion observed in cells pretreated with IFA before incubation of 2 mM MG (Figure 39). However, this effect of IFA did not occur in cells incubated with 3 mM MG (Figure 40).

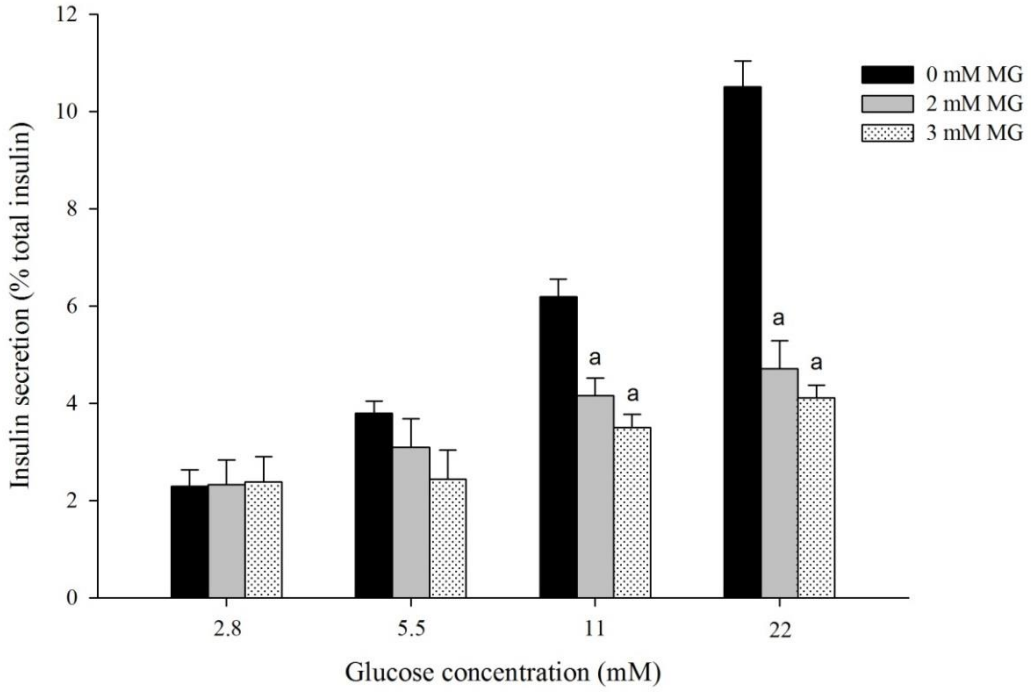

Figure 37 Glucose-stimulated insulin secretion (GSIS) of INS-1 cells treated with 0 mM, 2 mM and 3 mM MG.

Results are presented as mean ± SEM (n=3). One-way ANOVA (Tukey post hoc) was used to analyze statistical significance of results at the same concentration of glucose. [a] $p<0.05$ when compared to 0 mM MG (MG-untreated cells) at the same concentration of glucose.

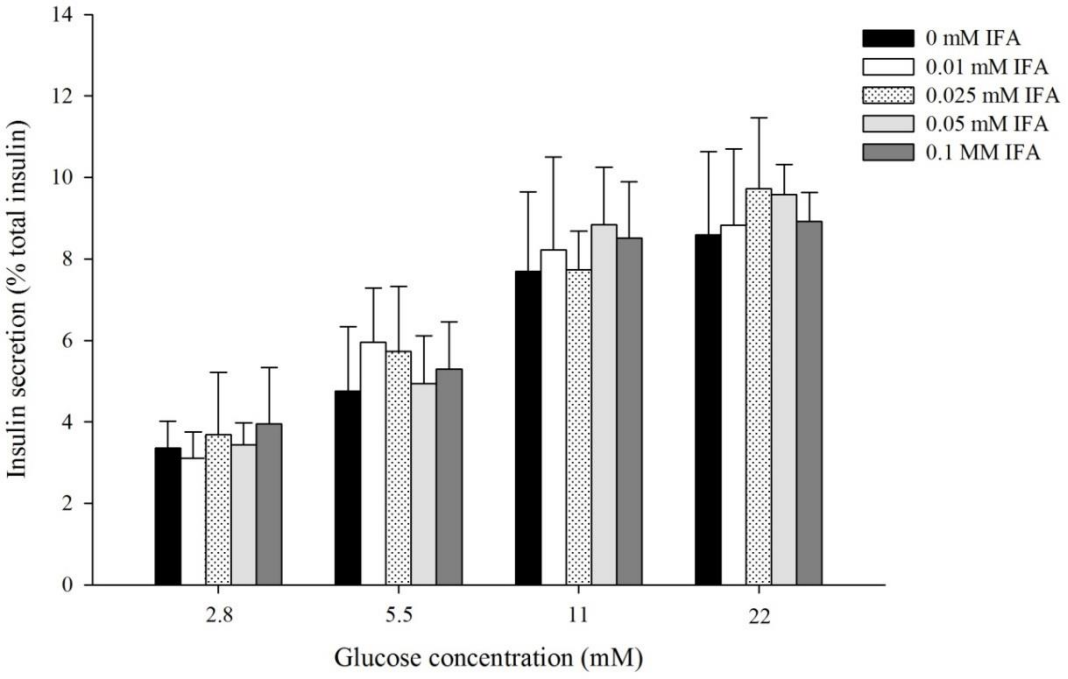

Figure 38 Glucose-stimulated insulin secretion (GSIS) of INS-1 cells treated with various concentrations of IFA (0.01 – 0.1 mM) for 48 h.

Results are presented as mean ± SEM (n=3). One-way ANOVA (Tukey post hoc) was used to analyze statistical significance of results at the same concentration of glucose.

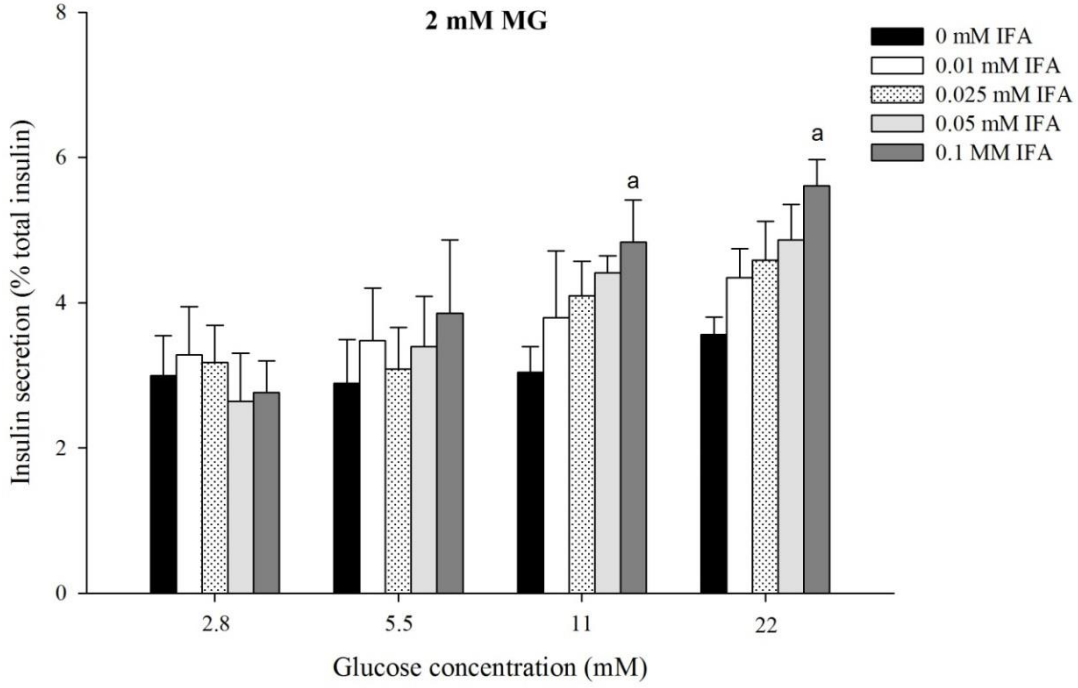

Figure 39 Effect of IFA pretreatment for 48 h on glucose-stimulated insulin secretion (GSIS) of INS-1 cells incubated with 2 mM MG.

Results are presented as mean ± SEM (n=3). One-way ANOVA (Tukey post hoc) was used to analyze statistical significance of results at the same concentration of glucose. $^{a}p<0.05$ when compared to 0 mM IFA (IFA-untreated cells) at the same concentration of glucose.

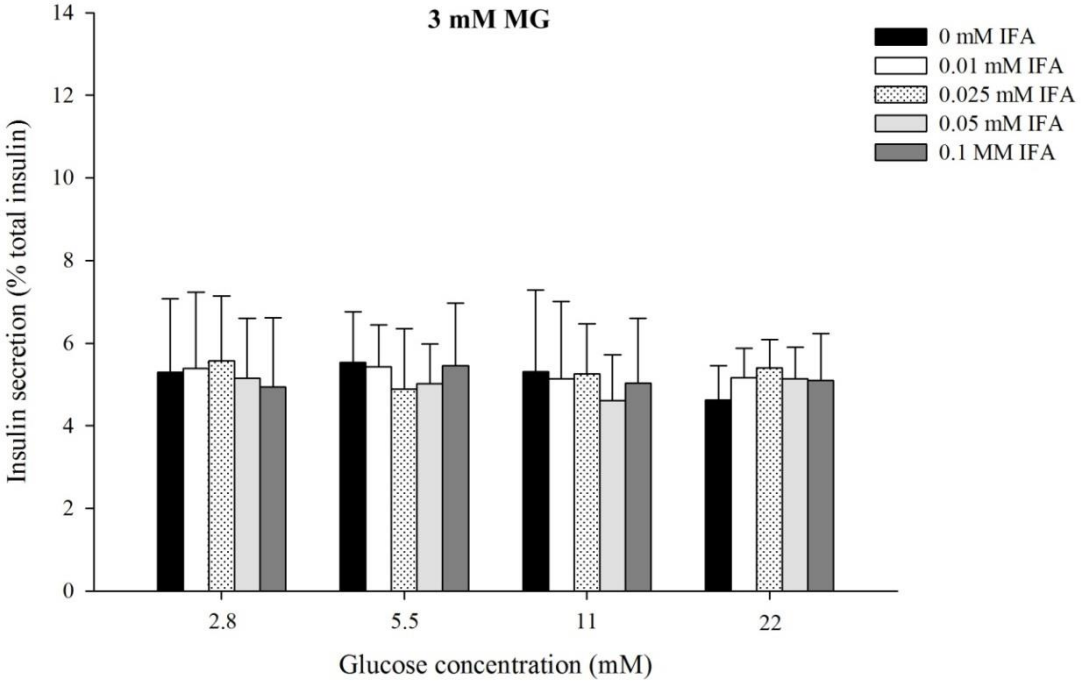

Figure 40 Effect of IFA pretreatment for 48 h on glucose-stimulated insulin secretion (GSIS) of INS-1 cells incubated with 3 mM MG.

Results are presented as mean ± SEM (n=3). One-way ANOVA (Tukey post hoc) was used to analyze statistical significance of results at the same concentration of glucose.

4.4.3 ROS production

To determine the production of ROS inside INS-1 cells after incubating with MG in the absence or presence of IFA, DCF-DA method was used. MG at the concentration of 2 mM was incubated to INS-1 cells for 30 min, 1 h and 2 h after 48-h pretreatment of various concentrations of IFA (0.01 – 0.1 mM). Figure 41 shows that the cells incubating with MG for 30 min had an elevated production of ROS. Although the percentage of increasing level was not significant, IFA at had a trend to reduce the production in a concentration-dependent manner and showed significance at the highest concentration (0.1 mM). Figure 42 demonstrates a significant increase in ROS production after incubating the cells with MG for 1 h. At this incubation period, 0.05 and 0.1 mM IFA significantly decreased ROS level by 25.3%, and 29.8%, respectively. Similarly, IFA at 0.05 and 0.1 mM significantly suppressed increasing level of ROS in cells incubating with MG for 2 h as presented in Figure 43. From this result, IFA at 0.1 mM was chosen to use in the next experiment.

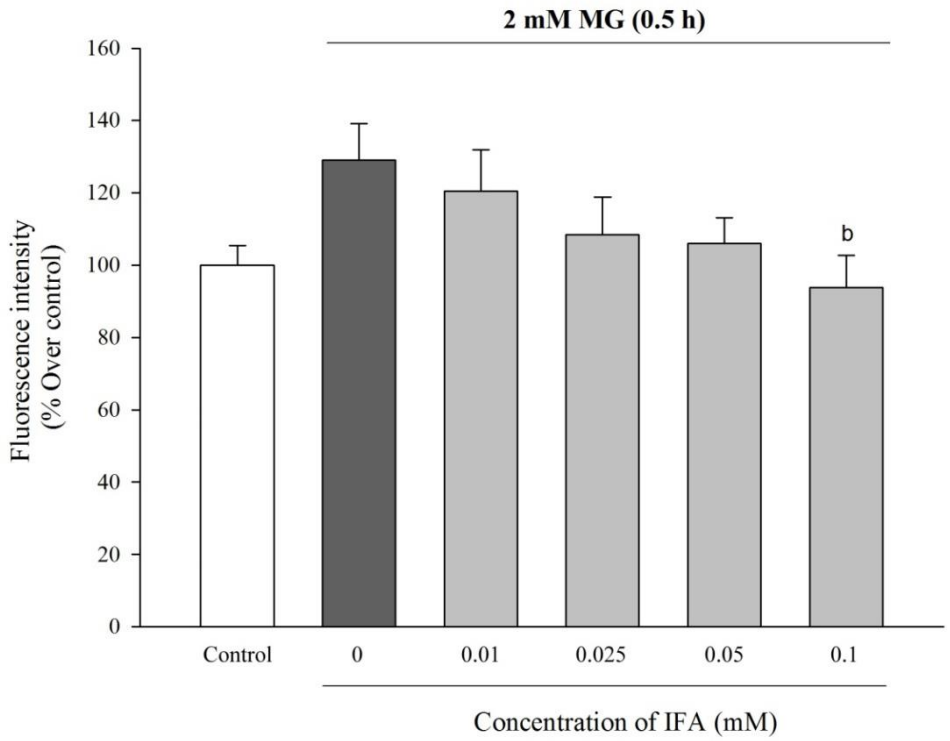

Figure 41 Effect of IFA (0.01 - 0.1 mM) pretreatment for 48 h on the production of reactive oxygen species (ROS) in INS-1 cells incubated with 2 mM MG for 30 min. Results are presented as mean ± SEM (n=3). One-way ANOVA (Tukey post hoc) was used to analyze statistical significance. [a] $p<0.05$ when compared to control (INS-1 cells pretreating with 0.1% DMSO), [b] $p<0.05$ when compared to 2 mM MG.

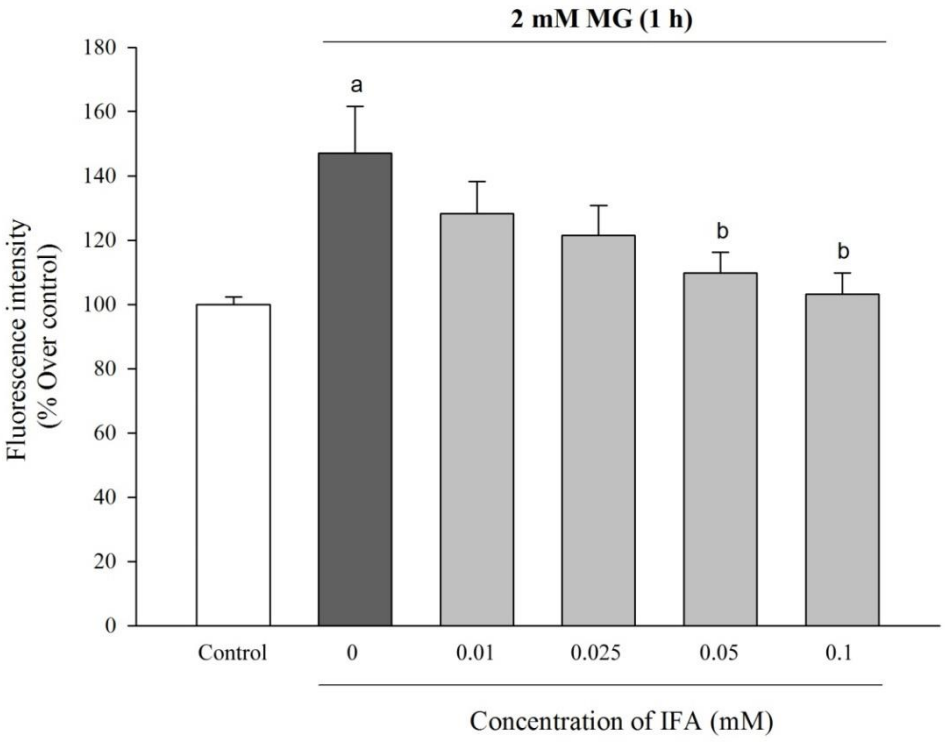

Figure 42 Effect of IFA (0.01 - 0.1 mM) pretreatment for 48 h on the production of reactive oxygen species (ROS) in INS-1 cells incubated with 2 mM MG for 1 h.
Results are presented as mean ± SEM (n=3). One-way ANOVA (Tukey post hoc) was used to analyze statistical significance. [a] $p<0.05$ when compared to control (INS-1 cells pretreating with 0.1% DMSO), [b] $p<0.05$ when compared to 2 mM MG.

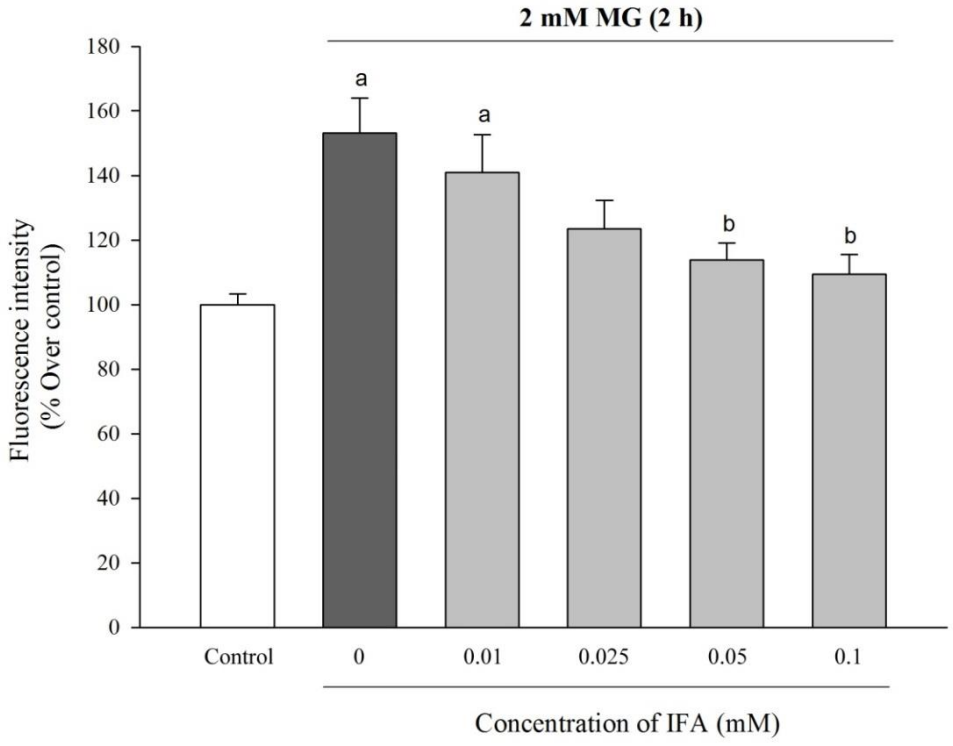

Figure 43 Effect of IFA (0.01 - 0.1 mM) pretreatment for 48 h on the production of reactive oxygen species (ROS) in INS-1 cells incubated with 2 mM MG for 2 h.
Results are presented as mean ± SEM (n=3). One-way ANOVA (Tukey post hoc) was used to analyze statistical significance. [a]$p<0.05$ when compared to control (INS-1 cells pretreating with 0.1% DMSO), [b]$p<0.05$ when compared to 2 mM MG.

4.4.4 Uncoupling protein 2 expression

To investigate the expression of UCP2 which is known as a regulator responding to high accumulation of ROS, the RT-PCR was performed. *Ucp2* mRNA level in untreated, DMSO-treated and IFA-treated INS-1 cells was expressed in agarose gel. After normalizing with 18s rRNA, 0.1 mM IFA did not cause any change in the expression of *Ucp2* mRNA as shown in Figure 44.

To examine the effect of MG on UCP2 expression, 2 mM MG was incubated to INS-1 cells at different period (0.5-6 h). There was an increasing level of UCP2 expression corresponding to increasing time of incubation particularly at the period of 1 to 3 h, and then the expression level started decreasing at the period of 4 h (Figure 45 and Figure 46). Based on the percentage of UCP2 expresssion by MG in Figure 46, IFA at the concentraion of 0.1 mM was pretreated to the cells for 48 h before incubating with 2 mM MG in order to invastigate its effect on MG-induced increase of UCP2. The results in Figure 47 showed that IFA did not change the expression level of UCP2 when the cells were treated with MG for 30 min. However, the presence of IFA in MG treated cells significantly lowered the increased level of UCP2 throughout the incubation preiod of 1 h to 4 h. The inhibitory effect of IFA was 14.2%, 17.0%, 20.9%, and 17.5% for 1 h to 4 h of MG incubation period, respectively.

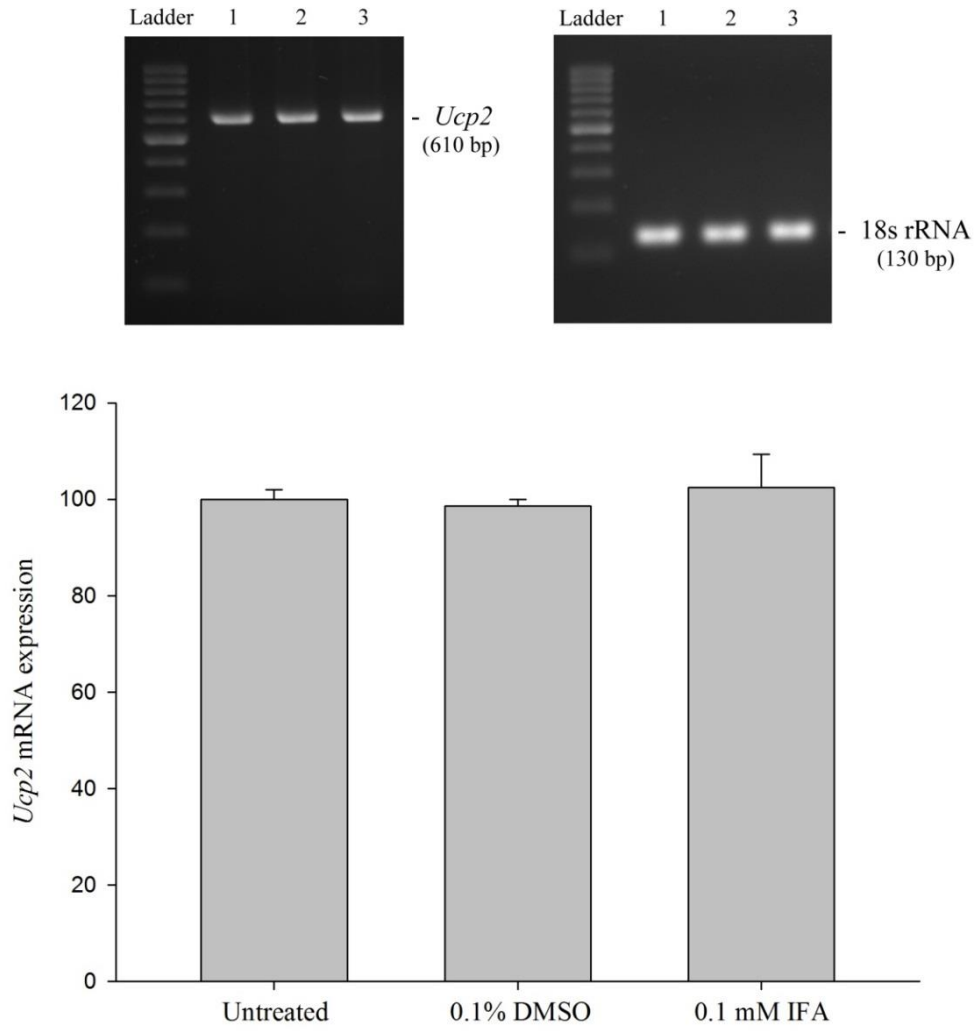

Figure 44 *Ucp2* mRNA expression in untreated (1), DMSO-treated (2) and IFA-treated (3) INS-1 cells.

The percentage of *Ucp2* expression was calculated and normalized by 18s rRNA. Results are presented as mean ± SEM (n=3). One-way ANOVA (Tukey post hoc) was used to analyze statistical significance.

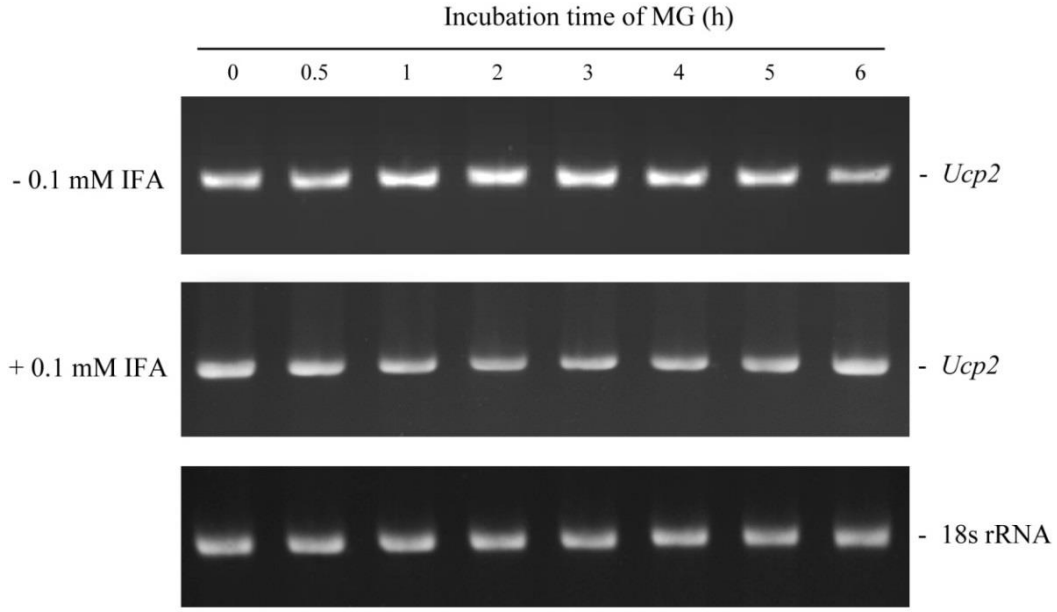

Figure 45 *Ucp2* mRNA expression in INS-1 cells treated with 2 mM MG (-0.1 mM IFA), and in INS-1 cells pretreated with IFA (+0.1 mM IFA) before treatment of 2 mM MG at various times of incubation (0.5 – 6 h).
Results are presented as mean ± SEM (n=3).

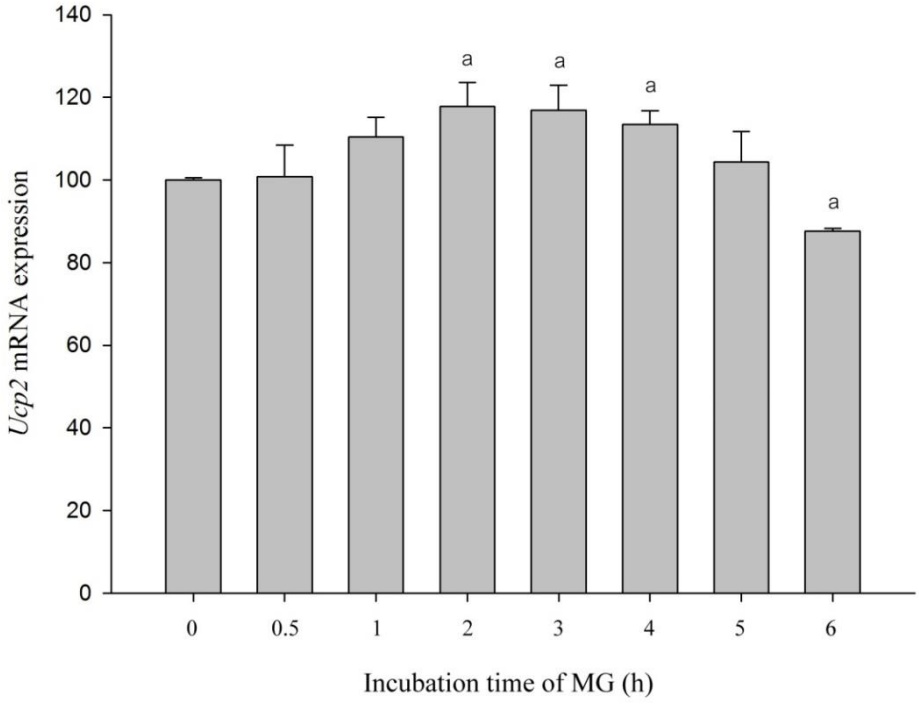

Figure 46 *Ucp2* mRNA expression in INS-1 cells treated with 2 mM MG at various times of incubation (0.5 – 6 h).

Results are presented as mean ± SEM (n=3). One-way ANOVA (Tukey post hoc) was used to analyze statistical significance. [a]$p<0.05$ when compared to 0 h.

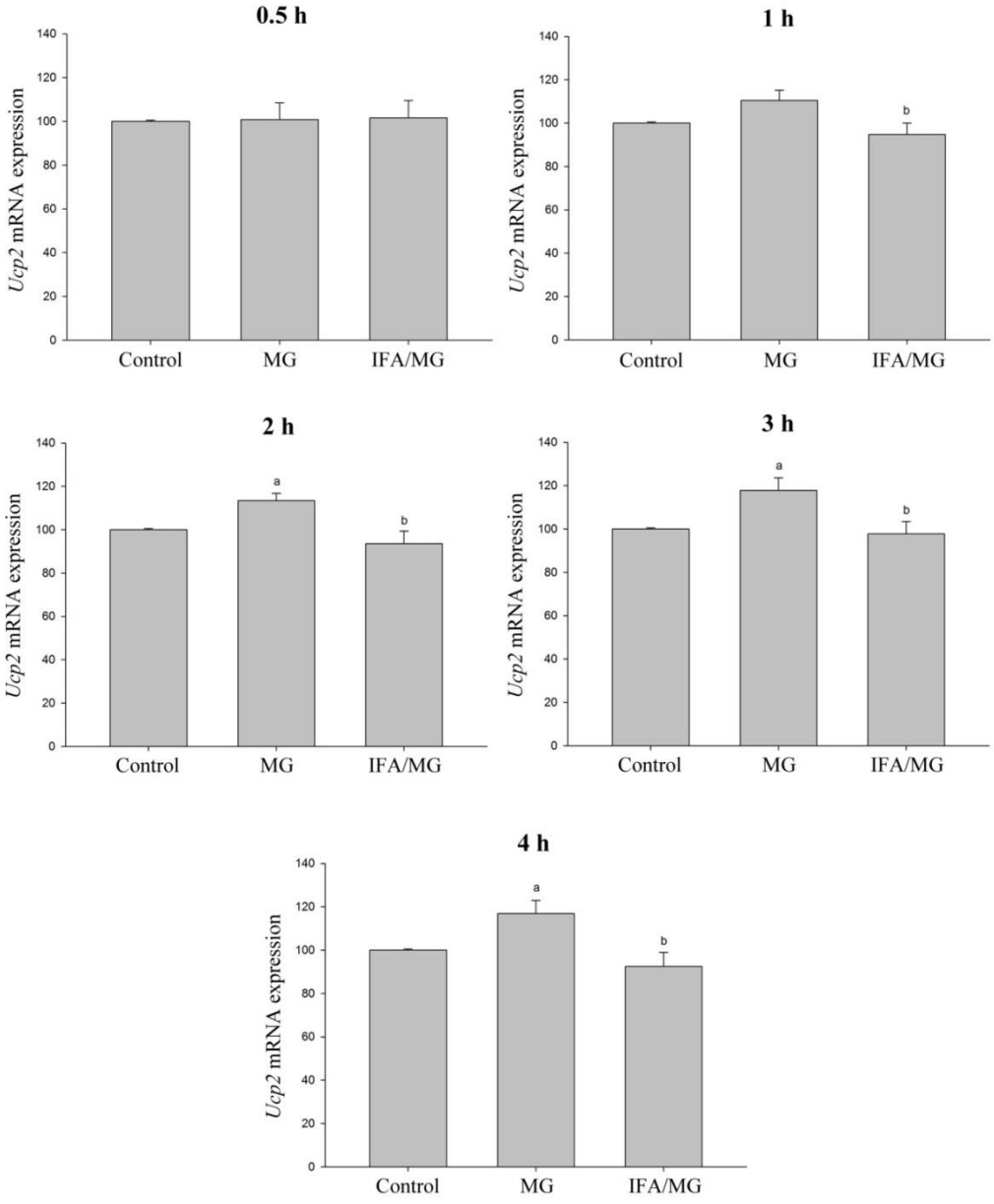

Figure 47 Effect of IFA (0.1 mM) on *Ucp2* mRNA expression in INS-1 cells treated with 2 mM MG for 0.5 - 4 h.

Results are presented as mean ± SEM (n=3). One-way ANOVA (Tukey post hoc) was used to analyze statistical significance. [a]$p<0.05$ when compared to control, [b]$p<0.05$ when compared to MG.

4.4.5 ATP content

The concentration of ATP was measured using luminescence assay. Firstly, 0.1 mM IFA was treated to INS-1 cells for 48 h and found that there was no difference in ATP content observed when compared to untreated or 0.1% DMSO treated cells (Figure 48).

To investigate the effect of MG on ATP content, INS-1 cells was incubated with 2 mM MG and monitored the ATP level for different incubation time up to 6 h. The results showed that MG-treated cells had a reduction of ATP concentration in a time-dependent manner. A significant decrease was found at longer incubation period than 1 h (2h – 6h) with the percentage reduction ranging from 27.7% to 35.7% as presented in Figure 49.

Figure 50 depicts the effect of IFA pretreatment on MG-treating cells. Surprisingly, the presence of 0.1 mM IFA could not reverse the decrease of ATP content at every incubation period of MG.

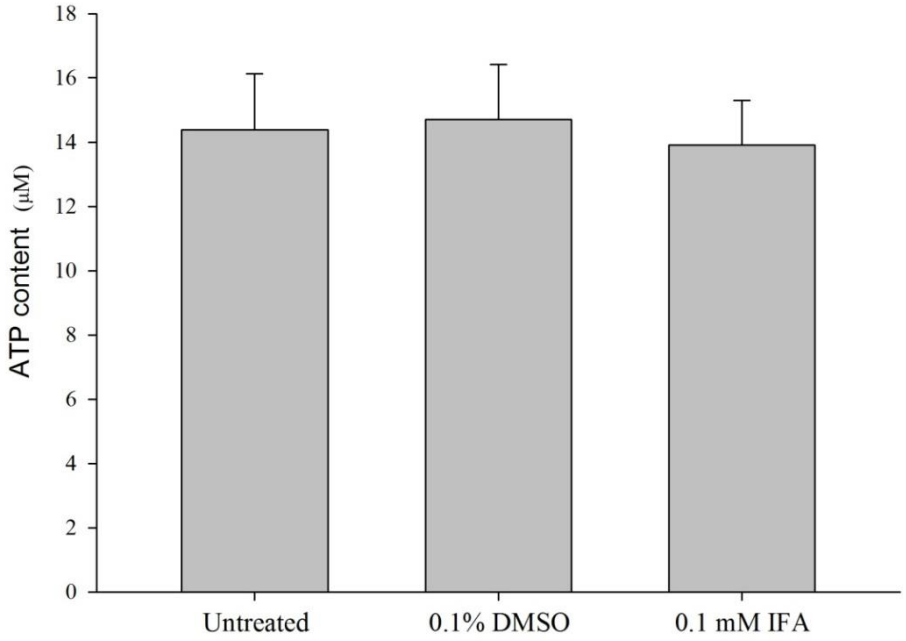

Figure 48 ATP content in untreated, DMSO-treated and IFA-treated INS-1 cells. Results are presented as mean ± SEM (n=3). One-way ANOVA (Tukey post hoc) was used to analyze statistical significance.

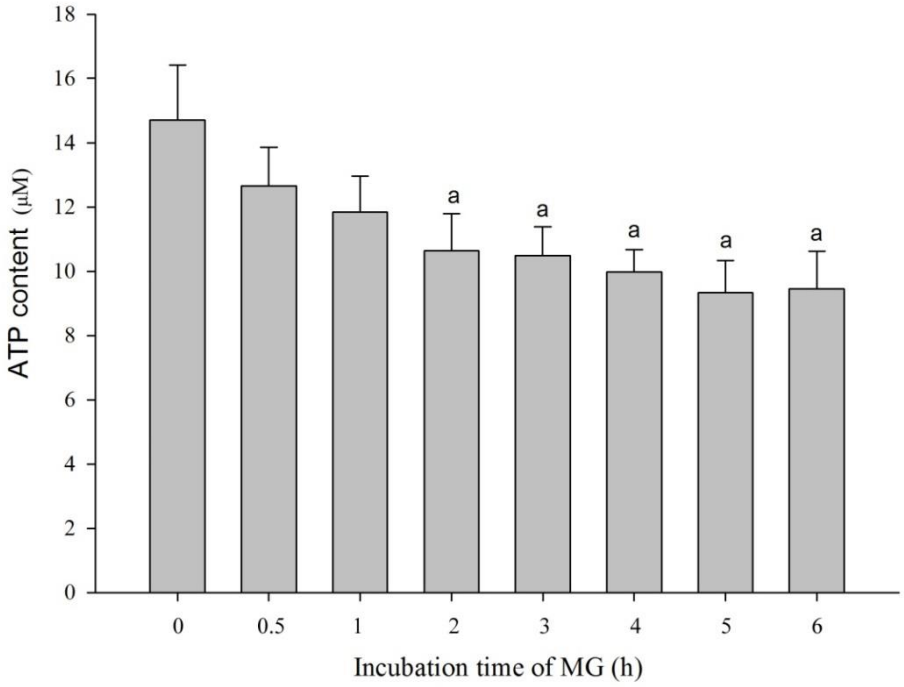

Figure 49 ATP content in INS-1 cells treated with 2 mM MG at various times of incubation (0.5 – 6 h).

Results are presented as mean ± SEM (n=3). One-way ANOVA (Tukey post hoc) was used to analyze statistical significance. [a]$p<0.05$ when compared to 0 h.

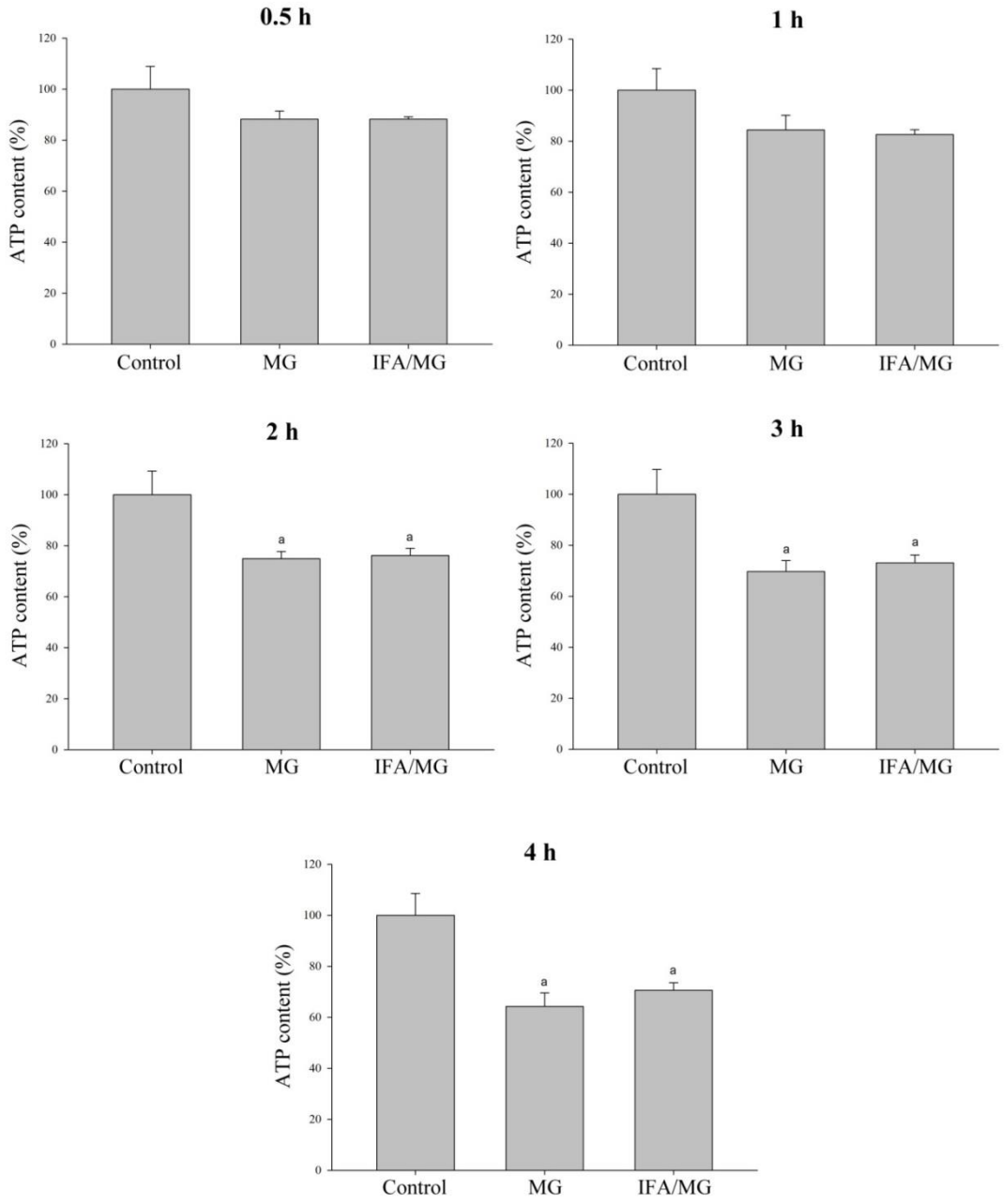

Figure 50 Effect of IFA (0.1 mM) on ATP content in INS-1 cells treated with 2 mM MG for 0.5 - 4 h.

Results are presented as mean ± SEM (n=3). One-way ANOVA (Tukey post hoc) was used to analyze statistical significance. [a]$p<0.05$ when compared to control.

4.4.6 Glyoxalase I activity

To investigate the underlying mechanism of IFA in protecting INS-1 cells, the activity of MG's detoxification enzyme glyoxalase I (GLO I) was also determined. IFA at various concentrations (0.01 – 0.1 mM) was treated to INS-1 cells for 48 h. The results from Figure 51 expresses the increasing level of GLO I activity, which was indicated by the formation of S-D-lactoylglutathione, in response to the increasing concentration of IFA. IFA at the concentration of 0.05 and 0.1 mM significantly induced the activity of GLO I by 8.0% and 8.6%, respectively.

Figure 52 exhibits the GLO I activity when incubating INS-1 cells with 2 mM MG at different period of incubation (0.5 – 6 h). Treatment of 2 mM MG for 0.5 or 1 h did not affect the activity of GLO I in the cells, whereas the longer period (2 – 6 h) of incubation showed lower activity of the enzyme.

The incubation time of MG during 0.5 h to 4 h was selected to examine the effect of IFA on GLO I activity in MG-treated cells as demonstrated in Figure 53. The results shown that preincubation of 0.1 mM IFA for 48 h had a trend to prevent loss of enzyme activity in cells treating with 2 mM MG at all selected incubation period. The percentage improvement was 4.1%, 6.0%, 12.3%, 10.1%, and 6.4%, respectively. However, a significant increase in GLO I activity by 0.1 mM IFA was shown only at 1 and 2 h of MG incubation.

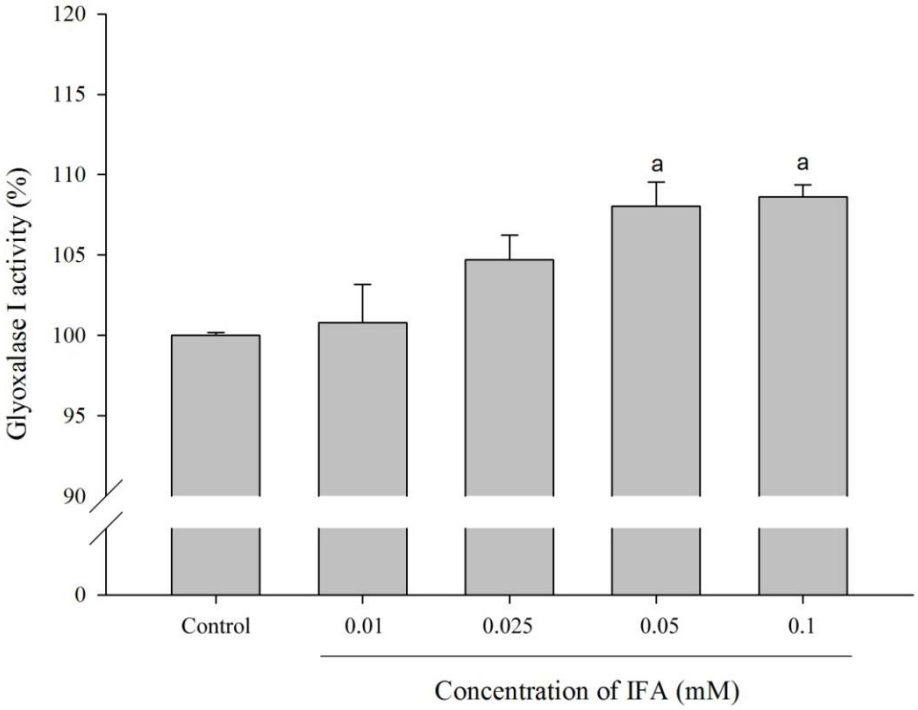

Figure 51 Glyoxalase I (GLO I) activity in INS-1 cells treated with various concentrations of IFA (0.01 – 0.1 mM) for 48 h.

Results are presented as mean ± SEM (n=3). One-way ANOVA (Tukey post hoc) was used to analyze statistical significance. [a]$p<0.05$ when compared to control.

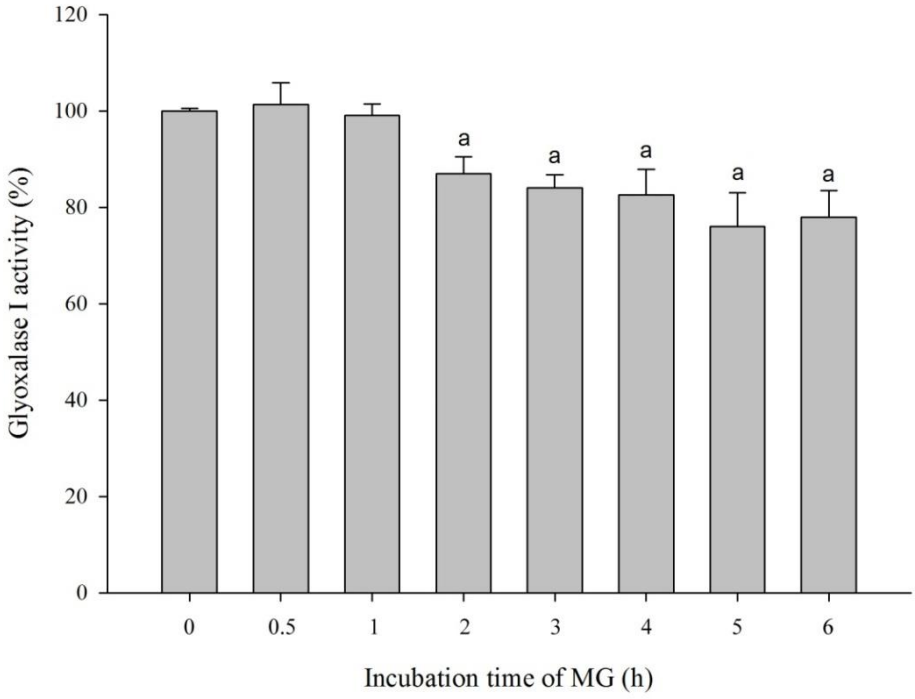

Figure 52 Glyoxalase I (GLO I) activity in INS-1 cells treated with 2 mM MG at various times of incubation (0.5 – 6 h).

Results are presented as mean ± SEM (n=3). One-way ANOVA (Tukey post hoc) was used to analyze statistical significance. [a]$p<0.05$ when compared to 0 h.

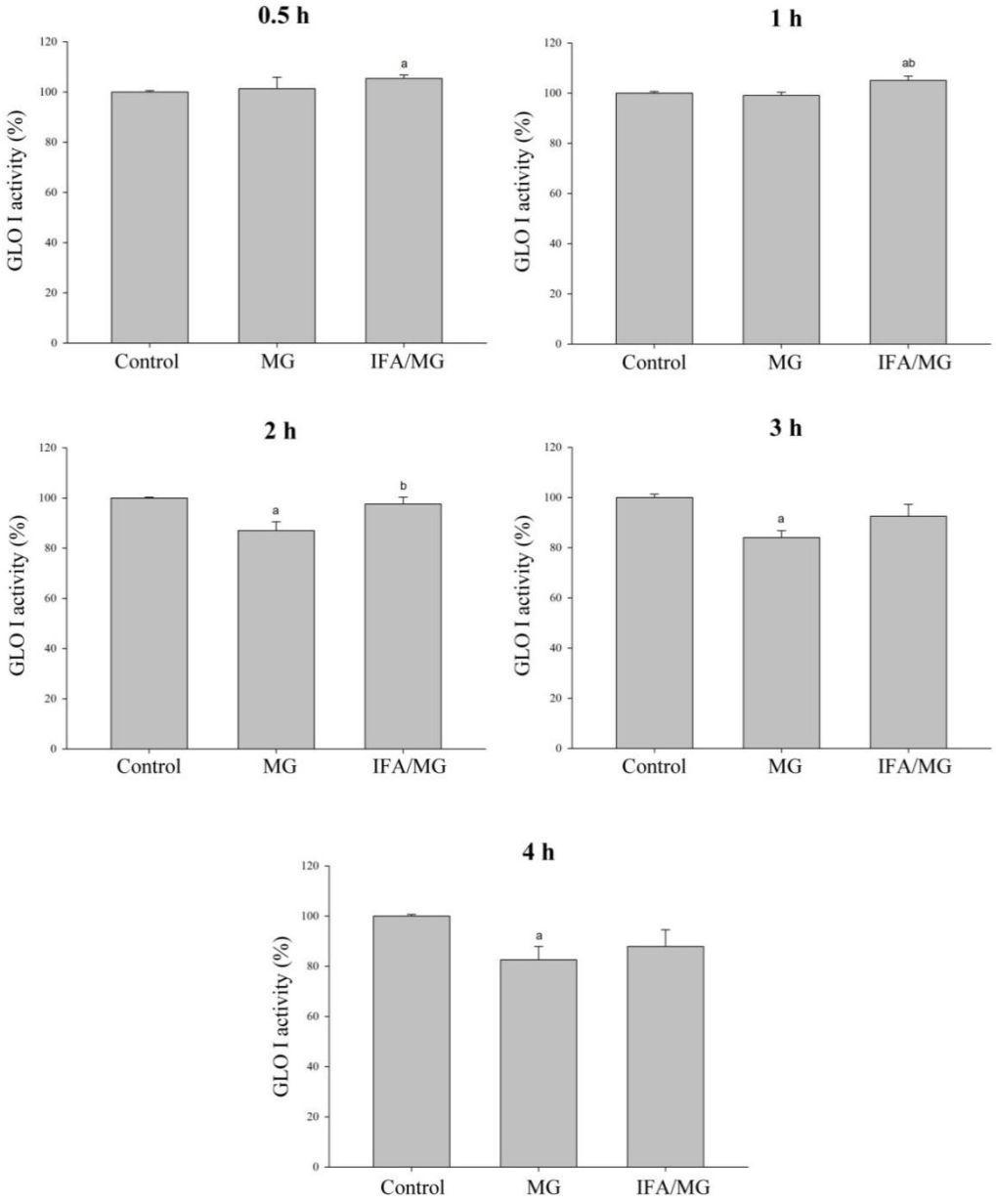

Figure 53 Effect of IFA (0.1 mM) on glyoxalase I (GLO I) activity in INS-1 cells treated with 2 mM MG for 0.5 - 4 h.

Results are presented as mean ± SEM (n=3). One-way ANOVA (Tukey post hoc) was used to analyze statistical significance. [a]$p<0.05$ when compared to control, [b]$p<0.05$ when compared to MG.

4.4.7 Caspase activity

The activity of caspase-3 was used to examined the preventive effect of IFA on the initiation of cell apoptosis in INS-1 cells. After incubating the cells with 2 mM MG for 1 h, 2 h and 3 h, MG significantly induced 1.8-fold, 2.6-fold, and 3.4-fold increase in caspase-3 activity in a time-dependent manner as presented in Figure 54.

At 3 h of MG incubation, cells pretreating with 0.1 mM IFA for 48 h expressed the inhibitory effect on caspase-3 activity with the percentage reduction of 22.4% (Figure 55).

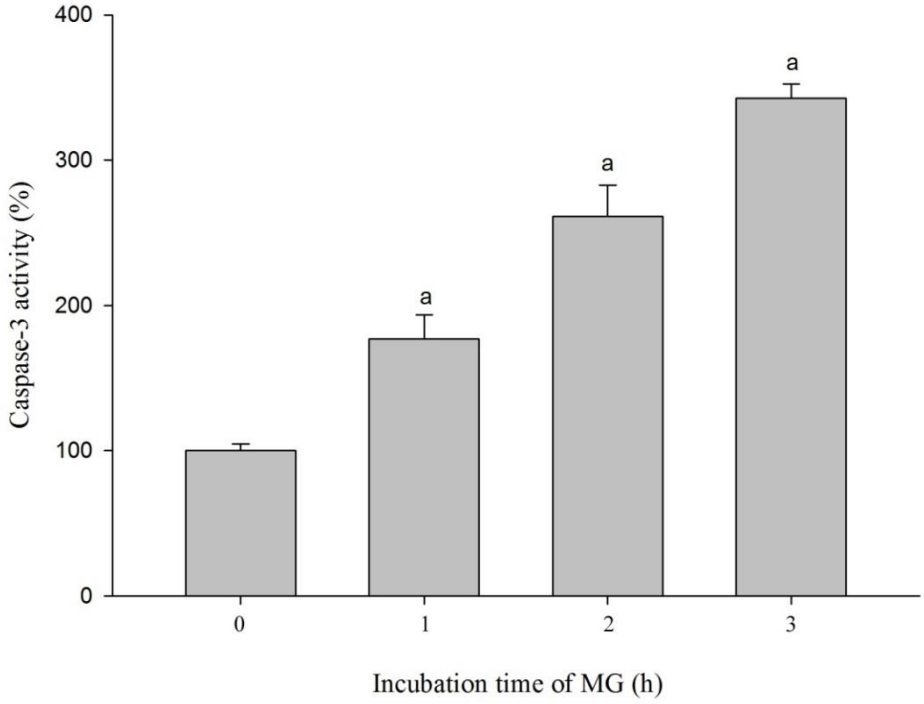

Figure 54 The activity of caspase-3 in INS-1 cells treated with 2 mM MG at various times of incubation (1 – 3 h).

Results are presented as mean ± SEM (n=3). One-way ANOVA (Tukey post hoc) was used to analyze statistical significance. [a]$p<0.05$ when compared to 0 h.

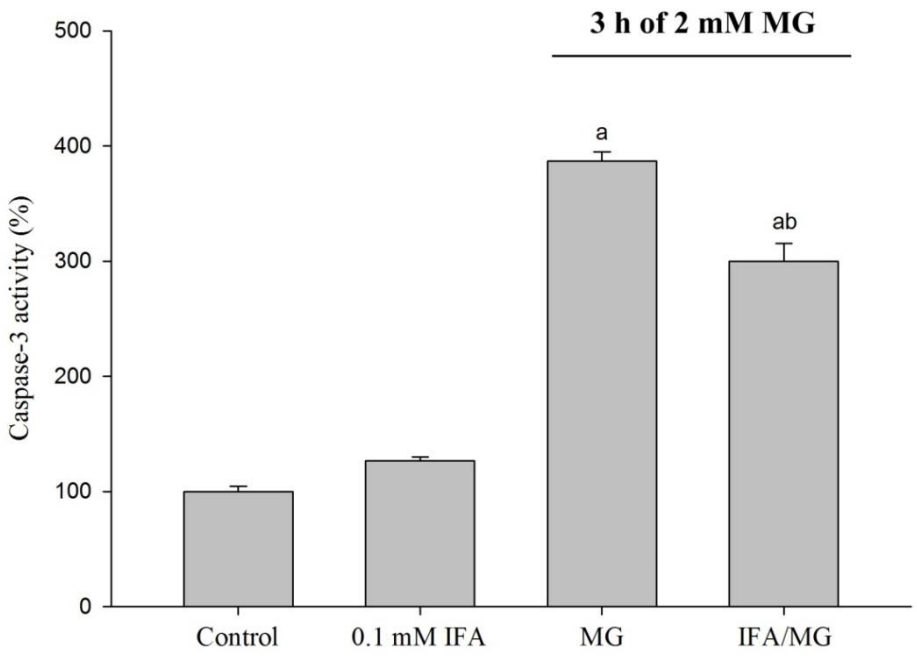

Figure 55 Effect of IFA on caspase-3 activity in INS-1 cells treated with 2 mM MG for 3 h.

Results are presented as mean ± SEM (n=3). One-way ANOVA (Tukey post hoc) was used to analyze statistical significance. [a]$p<0.05$ when compared to control. [b]$p<0.05$ when compared to MG.

CHAPTER V

DISCUSSION

5.1 Effects of Isoferulic acid (IFA) on protein glycation in BSA induced by glucose and fructose

Protein glycation is a spontaneous chemical modification of proteins or amino acids by reducing monosaccharides, such as glucose and fructose (Ardestani and Yazdanparast, 2007a; Adisakwattana et al., 2012), and reactive dicarbonyl molecules. This reaction is a cascade of rearrangement and oxidative-dependent modification through the formation of reversible Schiff's bases and more stable Amadori products to generate irreversible heterogeneous byproducts termed advanced glycation end products (AGEs) which are implicated in the development of aging as well as the pathogenesis of age-related disorders including Alzheimer's disease and diabetic complications (Booth et al., 1997; Goh and Cooper, 2008). Amino acids that contain the positively charged side chain (such as lysine and arginine) or the polar side chain consisting sulfhydryl group (such as cysteine) have been reported to be the target sites of sugars or dicarbonyl compounds in generating protein glycation (Johansen et al., 2006; Ansari et al., 2011), whereas the rate of protein glycation depends on type, concentration and exposure duration of reducing sugars (Ledesma-Osuna et al., 2008; Wei et al., 2009).

Glycation has been studied in both short- and long-lived proteins such as hemoglobin (Turk et al., 1998) and collagen (Aronson, 2003), respectively. However, many studies have been done on bovine serum albumin (BSA) due to the high

content of lysine in its structure (Hori et al., 2012) and high sequence homology to human serum albumin (HSA) (Sadowska-Bartosz et al., 2014).

In this study, BSA was used to investigate the effect of reducing sugars including glucose and fructose on protein glycation. The formation of AGEs and other intermediate products was monitored for up to 4 weeks. Both glucose and fructose induced the formation of fluorescent AGEs in a time-dependent manner, however, fructose exhibited the higher rate on generating fluorescent AGEs than glucose. Similar effect was observed in the formation of non-fluorescent N^{ε}-(carboxymethyl)lysine (N^{ε}-CML), which is an indicator of AGEs formation generated either from oxidative breakdown of Amodori product (Wu et al., 2011) or polyol pathway mediated by α-oxoaldehydes (Singh et al., 2001). This can be described by the lower reducing property of glucose when compared to fructose (Takagi et al., 1995). Other supporting evidence demonstrates that the rate and extent of protein-bound fluorescence generation upon nonenzymatic glycation by fructose is about 10 times that by glucose (Suárez et al., 1989).

Isoferulic acid (IFA) has been found to reduce the elevation of blood glucose in rats (Liu et al., 2000), however, this is the first study demonstrated the effect of IFA on glucose- and fructose-mediated protein glycation. In the presence of IFA, the formation of fluorescent and non-fluorescent AGEs (N^{ε}-CML) in BSA mediated by glucose and fructose was inhibited in a concentration-dependent manner.

In the early stage of glycation, unstable Schiff's bases are formed and turned to Amadori products. Apart from hemoglobin A1c (HbA1c) which is the Amadori product from glycation of hemoglobin in blood circulation (Turk et al., 1998),

fructosamine is one of the Amadori products generated from glycated albumin which mostly being concerned and monitored as a clinical indicator for short term control of blood sugar in diabetic patients (Ardestani and Yazdanparast, 2007a). Reduction of fructosamine, therefore, is a therapeutic way to delay the incident of vascular complications (Shield et al., 1994). In the present study, fructosamine was found to increase in both glucose- and fructose-induced BSA glycation. The presence of IFA markedly suppressed the formation of fructosamine as well as AGE formation.

Glycation is not only a major cause of AGE-mediated protein modification, but it also induces oxidation-dependent tissue damage, leading to the development of complications of diseases including diabetes (Hunt and Wolff, 1991; Kalousová et al., 2002). An increase in carbonyl content and the loss of free thiol groups by modification of cysteine residues directly reflect the oxidation of protein (Dalle-Donne et al., 2003; Aćimović et al., 2009). Oxygen-containing free radicals, such as superoxide anion and hydroxyl radical, generated during glycation and glycoxidationare are able to oxidize side chains of amino acid residues in protein to form carbonyl derivatives and also diminish an oxidative defense of protein by decreasing thiol groups, leading to damage of cellular proteins (Balu et al., 2005). Apart from a significant role in directly resisting against these free radicals, most antioxidants also possess the ability to inhibit oxidative damage-mediated glycation and glycoxidation by modulating abnormal levels of protein carbonyls and thiol groups (Balu et al., 2005; Ardestani and Yazdanparast, 2007a). A marked increase in protein carbonyl formation and oxidation of thiols was observed when BSA reacted with fructose and glucose. In the present study, IFA significantly suppressed the protein carbonyl formation and oxidation of thiols in BSA. Several previous studies

demonstrated that IFA potently reduced oxidative stress and free radicals *in vitro*, including scavenging of DPPH and ABTS radicals (Hirata et al., 2005; Cai et al., 2006), superoxide anion and hydroxyl radicals, as well as reduction of lipid peroxidation (Wang et al., 2011). Thus, the ability of IFA to modulate glycation-mediated BSA oxidation might be from its anti-oxidant activity.

Furthermore, scientific data reveals that protein glycation is an important factor affecting the conformational changes and aggregation of polypeptides, especially the amyloid cross β structure (Bouma et al., 2003). Amyloid cross β is an aggregated structure of protein commonly found and accumulated as the deposited fibrils in the brains of patients with glycation-related diseases such an Alzheimer's (Hardy and Selkoe, 2002; Khazaei et al., 2010). Moreover, β-amyloid deposits in pancreas have also been found to be pathologic lesion in pancreatic β cells of type 2 diabetic patients (Marzban et al., 2005). Notably, accumulation of protein aggregation induces pancreatic islet amyloidosis, which directly damages β cell and impairs insulin secretion (Tokuyama et al., 1997; Marzban and Verchere, 2004). The present findings show that the formation of amyloid cross β structure can be reduced by addition of IFA to fructose- and glucose-induced glycation in BSA. This beneficial effect of IFA might help to reduce the risk of developing debilitating degenerative diseases in diabetic patients.

Previous investigation has demonstrated the inhibitory effects on protein glycation induced by fructose of cinnamic acid and its derivatives (Adisakwattana et al., 2012). Also, ferulic acid which is IFA's isomer has recently found to inhibit AGE formation, fructosamine production, protein oxidation, and amyloid cross β structure

in BSA glycation induced by glucose, fructose and ribose (Sompong et al., 2013). This may support the anti-glycation effect of IFA due to their similar structure. Moreover, some kinds of natural compounds or antioxidants, such as vitamin C and E (Desai and Wu, 2007), polyphenol extracts of alligator pepper, ginger and nutmeg (Kazeem et al., 2012), and feruloyl oligosaccharides from wheat bran (Wang et al., 2009b), have been found to inhibit AGE formation through metal ion chelating and free radical scavenging activities. So, it may suggest that anti-oxidation activity of IFA is one of beneficial effects resulting in the prevention of protein glycation or possible mechanism explaining anti-glycating property of IFA.

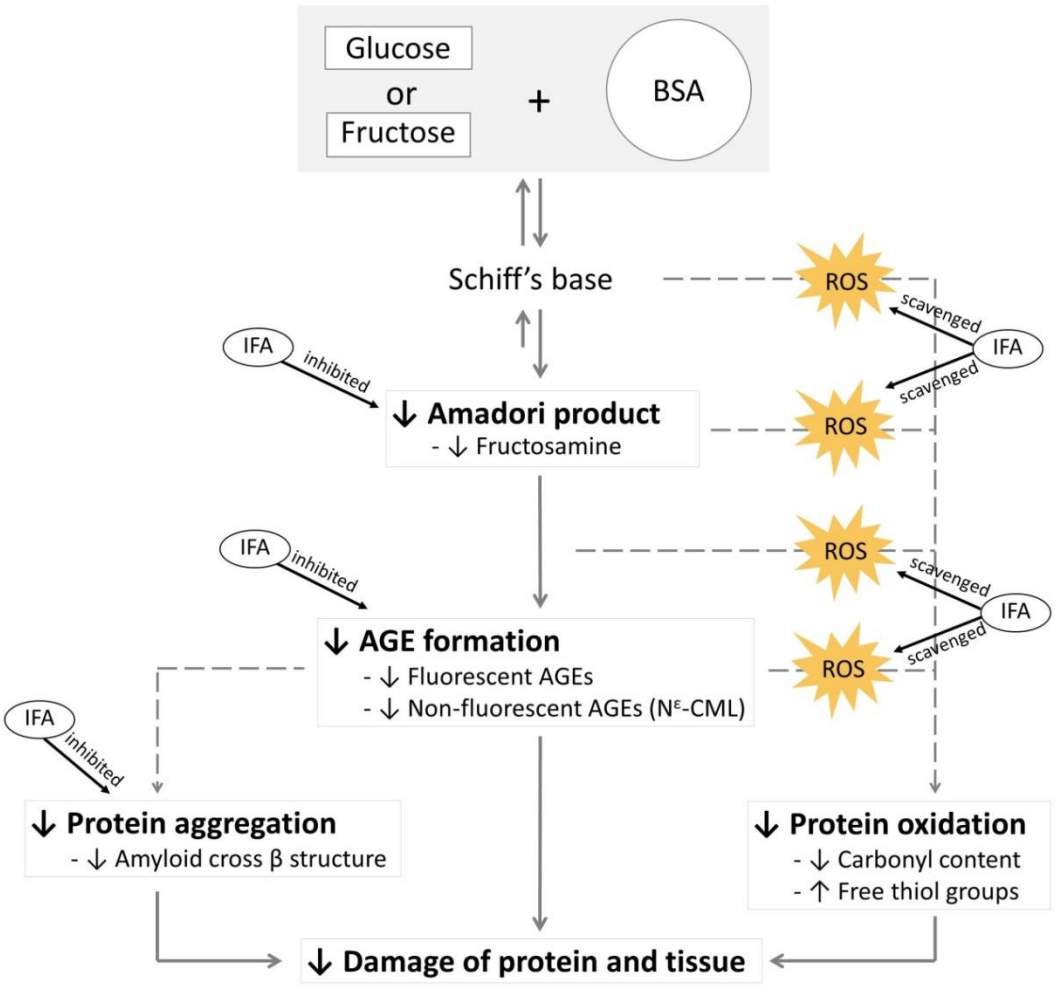

Figure 56 Proposed effects of IFA on BSA glycation induced by glucose and fructose.

5.2 Effects of IFA on BSA glycation induced by methylglyoxal (MG)

Methylglyoxal (MG) is commonly recognized as the most reactive glycating agent and irreversibly reacts with lysine residues in proteins to form fluorescent crosslinking and non-fluorescent crosslinking AGEs in the late stage of glycation (Li et al., 2007; Li et al., 2008). In the present study, BSA glycation inducing by MG was monitored for up to 2 weeks and found that MG markedly increased the formation of fluorescent AGEs at the late stage of glycation with more rapid rate than fructose and glucose in the previous experiment. This result confirms the high reactivity of MG as a reactive precursor of glycation in protein (Luers et al., 2012). Moreover, the formation of non-fluorescent AGE (N^{ε}-CML) was also found to increase by the incubation of MG with BSA. The glycation-derived oxidation indicating by the carbonyl content and free thiol groups was examined and found that MG induced protein oxidation at the intermediate stage of glycation by exhibiting higher level of carbonyl content and loss of free thiol groups as observed in BSA/sugar glycation.

From the inhibitory property of IFA on BSA glycation mediated by glucose and fructose in the previous experiment, IFA was also investigated the effect on MG-mediated BSA glycation. The present study showed that MG-induced formation of AGEs was attenuated by IFA at both intermediate and late stage of glycation. These findings, taken together, suggest that IFA can protect BSA from the intermediate stage of glycation, leading to inhibition of AGE formation in the late stage.

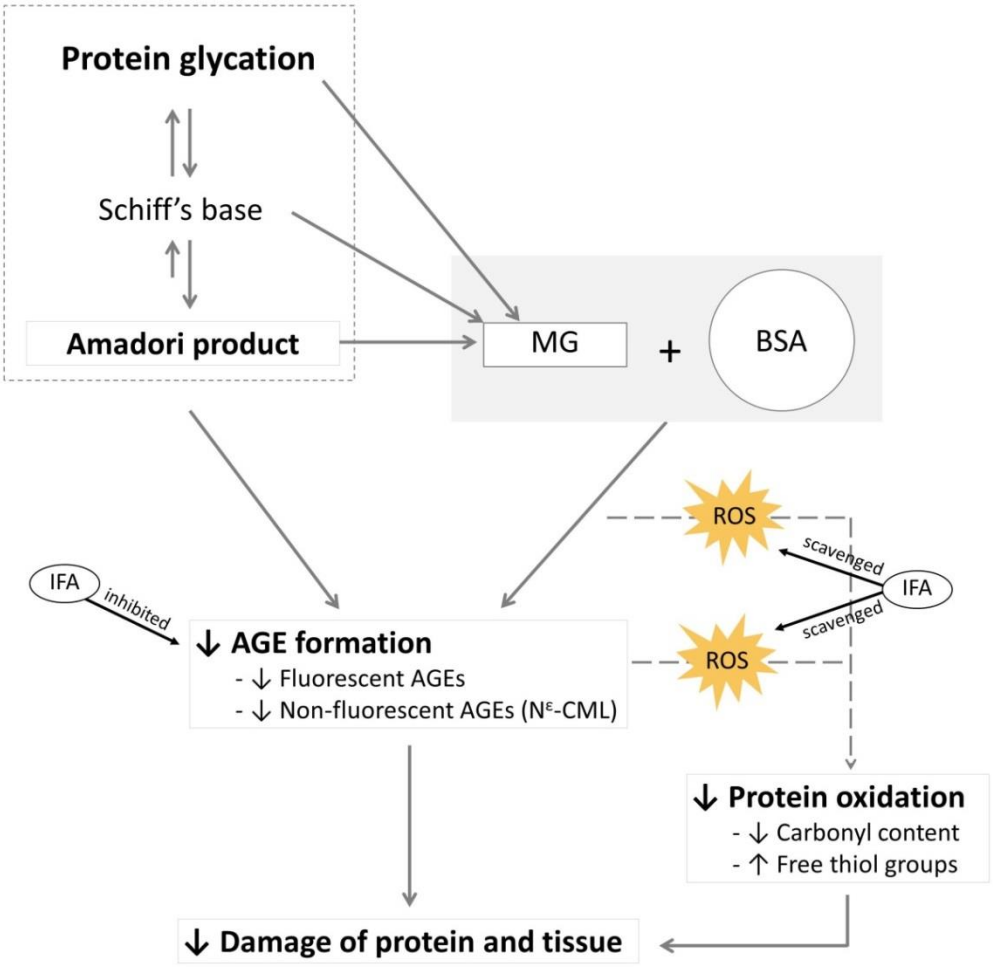

Figure 57 Proposed effects of IFA on BSA glycation induced by MG.

5.3 Effects of IFA on MG-induced oxidative DNA damage

Besides direct glycation damage to protein, MG has been reported to contribute an oxidative DNA damage and DNA strand breakage (Wu and Chan, 2007; Tatone et al., 2011). Reactive oxygen species (ROS) generating during the process of glycation was supposed to be a key factor that mediates this effect (Yim et al., 1995). The oxidation-dependent DNA damage has been found to involve in several diverse biological processes such as mutagenesis (Tamae et al., 2011), carcinogenesis and age-related diseases (Ames, 1983). The previous study demonstrated that MG reacting with lysine induced the cleavage of DNA strand associating with the increase production of ROS (Kang, 2003).

Several lines of evidence show that superoxide and hydroxyl radicals can be generated from the reaction between lysine and MG (Kang, 2003). It has been reported that MG-induced albumin modification generates the cross-linked methylglyoxal dialkylimine radical cation and the enediol radical anion of methylglyoxal during the glycation process (Yim et al., 1995; Suji and Sivakami, 2007). The formation of these intermediates leads to protein cross-linking and formation of radical cation sites on the cross-linked proteins. The presence of trace metal ions (copper and iron ions) enhances hydroxyl radical generation by reacting with hydrogen peroxide (H_2O_2) through the Fenton reaction (Kang, 2003). ROS generated from this reaction contributes oxidative modification of protein and DNA (Kang, 2003).

In the present study, co-incubation of lysine and MG persuaded oxidative damage of DNA indicating by the elevation of nicked or open circular form of DNA (Kang, 2003; Suji and Sivakami, 2007; Wu and Chan, 2007). MG was used at the higher

concentration than physiological condition in order to demonstrate clear cleavage of DNA in a relatively short period of time as observed in the previous investigation (Kang, 2003). The presence of DNA nicked form was higher when Cu^{2+} was added to the reaction implying that oxidative DNA damage was enhanced by Cu^{2+}. However, IFA could prevent this oxidative damage to DNA indicated by a reduction of nicked DNA. This protective effect became clearer when Cu^{2+} was presented in the system. This result strongly supported the metal-chelating property of IFA as demonstrated in the previous work (Li et al., 2012).

In addition, the formation of superoxide anion and hydroxyl radicals confirmed by the observed increase in reduced cytochrome *c* and TBARS, respectively, was found to be generated from lysine/MG reaction which was consistent with previous studies (Kang, 2003; Suji and Sivakami, 2007). However, when the MG and lysine was incubated with IFA, the increase in superoxide anion and hydroxyl radicals was attenuated suggesting that IFA scavenges lysine/MG-derived ROS. Considerable interest has been devoted to phytochemical compounds due to their ability to prevent lysine/MG-induced protein glycation and DNA damage by acting as free radical scavengers (Wu and Yen, 2005), and the present findings indicate that IFA also acts in this manner.

A small synthetic hydrazine-like compound Aminoguanidine (AG) is wildly used as an AGE inhibitor due to its effective property in blocking the carbonyl groups in the reducing sugars and reactive dicarbonyl precursors of glycation (Nagai et al., 2012). In the present study, this carbonyl blocker showed the potential inhibition of glycation-mediated AGE formation in both BSA/sugars and BSA/MG systems. One of

the mechanisms possibly underlying this effect was MG-trapping capability (Shao et al., 2008; Lv et al., 2011). Trapping MG by binding to its carbonyl group is a direct way to block MG-induced glycation and AGE formation, and is a therapeutic target of some AGE inhibitors such as AG (Thornalley, 2003). As presented in this study, AG clearly exhibited a direct trap to MG performing by HPLC. In contrast, IFA, which possessed inhibitory effect on sugars- and MG-induced protein glycation, did not show the MG-trapping ability as AG did.

Previously, there were some natural substances possessed anti-glycation and AGE formation through direct trapping of MG such as curcumin (Hu et al., 2012) and ginger (Zhu et al., 2015). Although cinnamic acid and its derivatives have also been found to inhibit glycation, less evidence examined their MG-trapping capability. However, a study on MG-trapping capacity of ferulic acid, IFA's isomer, clearly showed the failure to trap MG (Hu et al., 2012). Currently, there was an interesting study demonstrated that phenolic compounds composed of a benzene structure with a mono-hydroxyl substitute cannot react with MG, and also suggested that the carbon electron charges on benzene ring are the influential factors in reactivity (Lo et al., 2011).

From these findings, it may imply that carbonyl scavenging activity is not the anti-glycation mechanism of IFA and this may support the indirect way of IFA's action such as anti-oxidation.

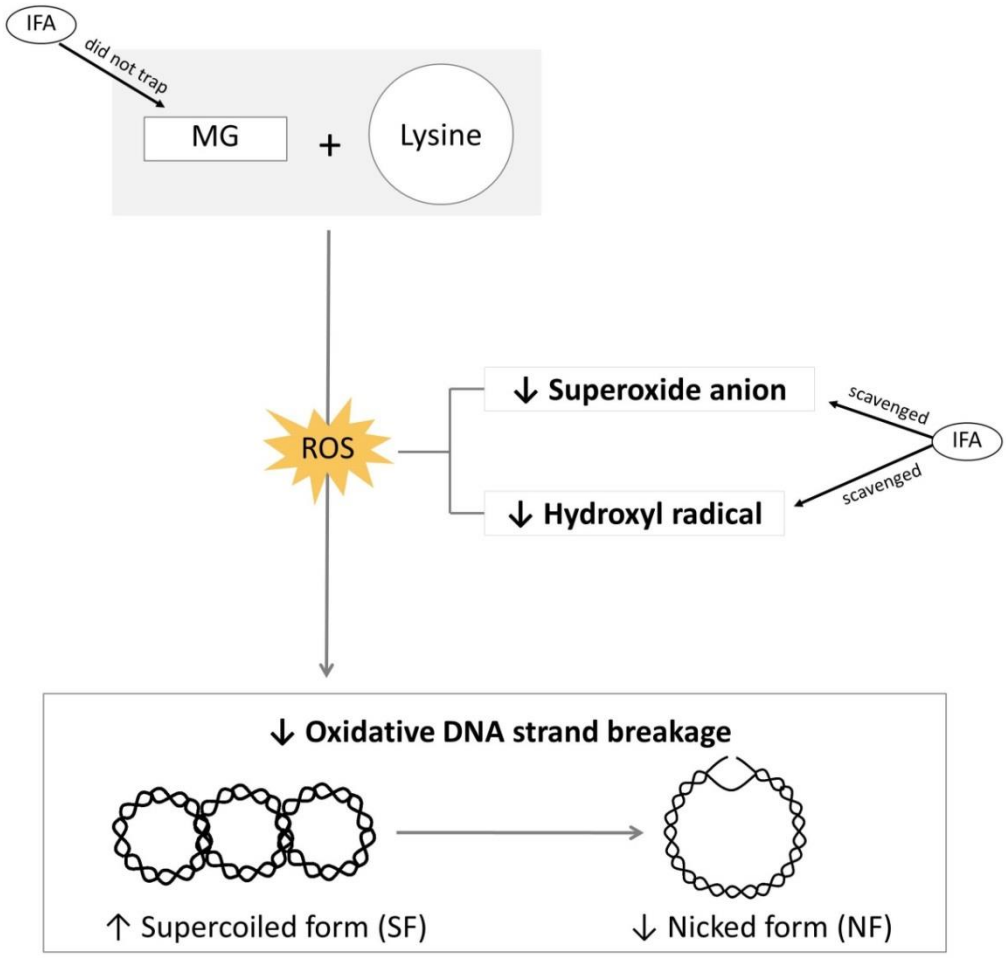

Figure 58 Proposed effects of IFA on MG-induced oxidative DNA damage.

5.4 Effects of IFA on MG-induced β-cell dysfunction

Pancreatic β cell is well known as the insulin-producing cell and plays a significant role in glucose homeostasis due to its function on insulin secretion. The impairment or damage of β cell leads to the failure in response to hyperglycemia and results in the the development of diabetes (Fiory et al., 2011).

Pancreatic insulinoma INS-1 (832/13) cells were used as a model in GSIS and other functions in the present study. The viability assay was used to assess enzyme activity in mitochondria which related to cell proliferation and survival. The presence of 1 mM MG and lower concentration within 6 h in this study did not cause a decrease in cell viability consistent with the previous investigation that defined 1 mM MG as a non-cytotoxic concentration in INS-1E cells (Fiory et al., 2011), however, the higher concentrations exhibited more toxicity to the cells when time of incubation was increased. To investigate the preventive effect of IFA, MG at 2 and 3 mM which possessed loss of cell viability ranging from 25-50% in 6 h was selected to incubate to the cells after IFA pretreatment. The findings indicated that IFA was non-toxic and safe to be treated to INS-1 cells for 24 and 48 h. The longer period of IFA pretreatment had an effect on preventing cytotoxicity demonstrating by the improvement of cell viability observed in cells treating with 2 and 3 mM MG after pretreatment of IFA for 48 h.

MG has been reported to induce damage in β cells resulting in the defect of β-cell function particularly glucose-stimulated insulin secretion (GSIS). Oxidative stress was supposed to be one of causative factors mediating cellular dysfunction including β cell (Kajimoto and Kaneto, 2004). Intracellular ROS is generated from three main

sources including endoplasmic reticulum (ER), peroxisomes and mitochondria (Holmström and Finkel, 2014). Apart from antioxidant enzymes, mitochondria regulates overproduction of ROS by upregulating the expression of uncoupling protein 2 (UCP2) in order to increase proton influx to the matrix, leading to the decrease of mitochondrial membrane potential and resulting in the decrease of ROS production (Yang et al., 2016). In addition, an over-expression of UCP2 had a result in suppression of ATP production through ATP synthase (Kashemsant and Chan, 2006). These explain how oxidative stress affects insulin secretion in β cells.

In the present study, MG induced ROS production and UCP2 expression in INS-1 cells, whereas ATP content and insulin secretion were suppressed. MG has been previously found to induce oxidative stress by increasing the production of mitochondrial oxigen- and nitrogen-containing radicals (Wang et al., 2009a). Recently, there was a study revealed that MG induced ROS production and over-expression of UCP2 in pancreatic β cell (Bo et al., 2016). These might be the reason of the decrease in ATP content observed in this study. However, evidences in other tissues demonstrate that MG impairs mitochondrial respiratory chain by inhibiting complex I-III activities resulting in the reduction of ATP synthesis (Wang et al., 2009a; Remor et al., 2011). Taken togather, these might be the proposed mechanisms of MG in reducing insulin secretion from β cells.

The presence of IFA could inhibit the overproduction of ROS, downregulate UCP2 expression, and improve insulin secretion, however, it failed to increase ATP content in MG-treated INS-1 cells. UCP2 has supposed to be a regulator for ROS production in mitochondria. An expression of UCP2 appears to be higher in response

to excessive production of ROS and lower when ROS level decreases (Li et al., 2001; Pi and Collins, 2010). It would be possible that IFA directly scavanges to ROS due to its antioxidant activity against free radicals (Hirata et al., 2005; Li et al., 2012), leading to a lower accumulation of ROS and subsequently resulting in a decrease expression of UCP2. Although IFA could not elevate ATP content, it improved a decrease of insulin secretion in MG-treating cells. There might be other ATP-independent actions of IFA underlying the mechanism involved insulin secretion that have not been revealed. More studies are required for this point.

Glyoxalase sytem is a MG-detoxification process during glycolytic pathway (Thornalley, 2003). An over-expression or increase in the activity of enzymes in this system leads to the prevention of cells from glycation-derived AGEs (Brouwers et al., 2011). In contrast, inhibition of enzymes or co-enzyme (GSH) in this process results in the increasing formation of AGEs (Hanssen et al., 2014). In the present study, pretreatment of IFA increased the glyoxalase I (GLO I) activity in INS-1 cells, whereas prolonged incubation time of MG exhibited the lowering activity of GLO I. However, IFA could reverse the decrease in GLO I activity at least in the shorter incubation period of MG than 3 h. This finding suggests the beneficial effect of IFA in prevention of MG-treated INS-1 cells via induction of MG-detoxifying system.

In order to more understanding in the preventive effect of IFA on β-cell damage, cell progression that leads to apoptosis was also considered by investigation of caspase-3 activity in INS-1 cells. Caspase-3, which is a member of cysteine-aspartic acid protease family, plays an important role in downstream of caspase-dependent apoptosis of both intrinsic (mitochondrial mediated) and extrinsic (death receptor

mediated) pathways (Li and Yuan, 2008). Activation of caspases initiates cascade signal involving inflammation and cell death (McIlwain et al., 2013). ROS accumulation has been counted to be one of important factors contributing cell apoptosis through caspase-3 activation (Chen et al., 1998).

In the present study, the activity of caspase-3 was observed to be increased by the existence of MG in INS-1 cells consistent with previous reports demonstrating that MG initiated apoptotic pathway in RINm5F cells by inducing caspase-3 and caspase-9 activities (Bao et al., 2013; Cheng et al., 2013), and in bovine retinal pericytes by mediating ROS induction (Kim et al., 2004). However, MG-mediated increase in activity of caspase-3 was reduced in INS-1 cells pretreating with IFA. This suggests that the preventive effect of IFA on cell survival relates to suppression of caspase-3 activation which may be subsequent from its ROS scavenging activity.

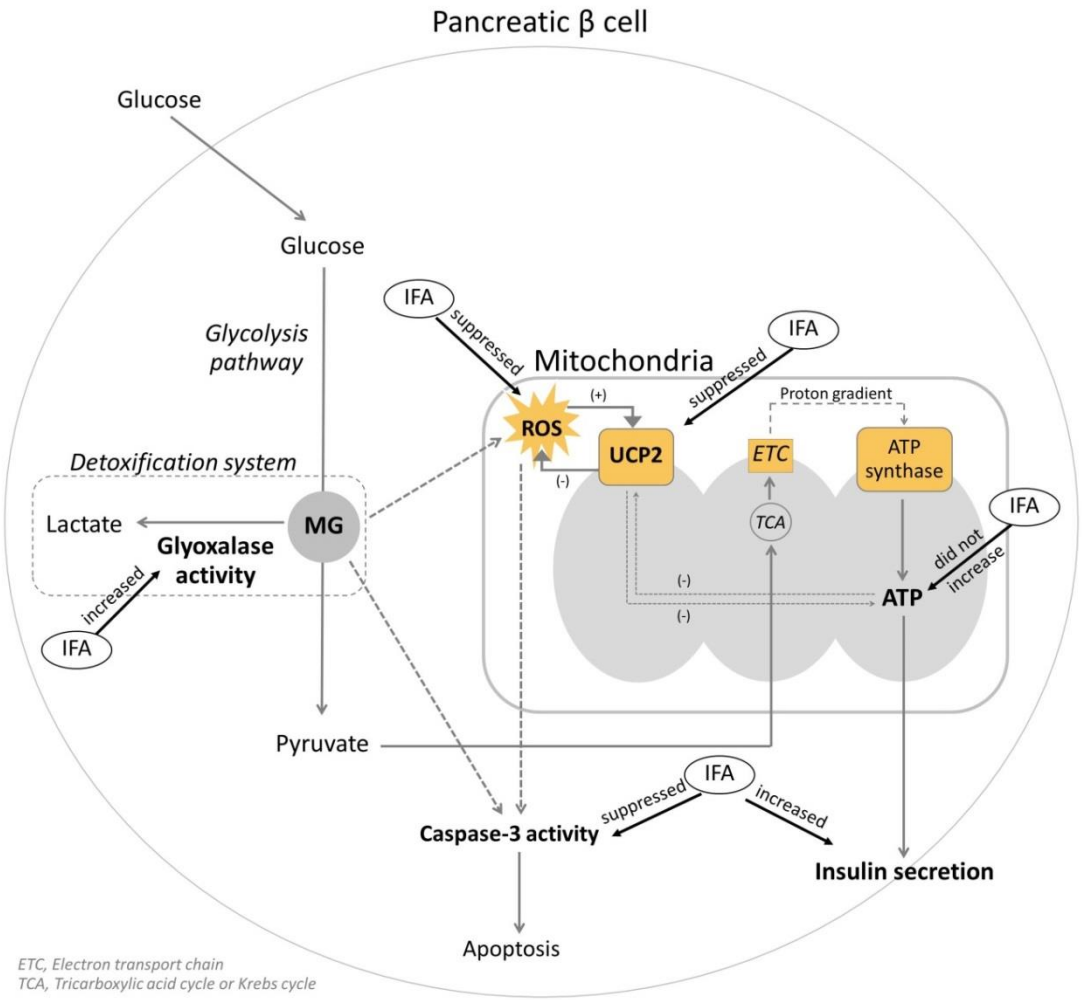

Figure 59 Proposed effects of IFA on MG-induced β-cell dysfunction.

CHAPTER VI

CONCLUSION

Isoferulic acid (IFA) markedly inhibited glucose-, fructose- and methylglyoxal-mediated protein glycation in BSA by decreasing formation of fluorescent and non-fluorescent AGEs as well as Amadori products, inhibited protein oxidation by decreasing formation of protein carbonyl content and loss of protein thiol groups, and inhibited protein aggregation by decreasing formation of amyloid cross β structure.

IFA also prevented oxidative DNA damage induced by glycation of lysine and methyglyoxal by reducing DNA fragmentation, relating to reduction of superoxide anion and hydroxyl radical production.

Moreover, IFA prevented methylglyoxal-induced pancreatic β-cell dysfunction via suppression of ROS production which may trigger downregulation of UCP2, resulting in improvement of insulin secretion, and also prevented damage of β cells via activation of glyoxalase I in MG-detoxifying system and inhibition of caspase-3 activity.

These findings reveal IFA as an anti-glycating agent and effective antioxidant of DNA and pancreatic β cell. Based on these beneficial effects, IFA might be considered or applied to use as a compound that prevents AGE-mediated diabetic complications.

REFERENCES

Aćimović JM, Stanimirović BD, Mandić LM (2009) The role of the thiol group in protein modification with methylglyoxal. J Serb Chem Soc 74:867-883.

Adisakwattana S, Sompong W, Meeprom A, Ngamukote S, Yibchok-anun S (2012) Cinnamic acid and its derivatives inhibit fructose-mediated protein glycation. Int J Mol Sci 13:1778-1789.

Ahmed N (2005) Advanced glycation endproducts--role in pathology of diabetic complications. Diabetes Res Clin Pract 67:3-21.

Ames BN (1983) Dietary carcinogens and anticarcinogens. Oxygen radicals and degenerative diseases. Science 221:1256-1264.

Ansari NA, Ali M, Ali R (2011) Glycated lysine residues: A marker for non-enzymatic protein glycation in age-related diseases. Dis Markers 30 317-324.

Ardestani A, Yazdanparast R (2007a) Inhibitory effects of ethyl acetate extract of *Teucrium polium* on *in vitro* protein glycoxidation. Food Chem Toxicol 45:2402-2411.

Ardestani A, Yazdanparast R (2007b) Cyperus rotundus suppresses AGE formation and protein oxidation in a model of fructose-mediated protein glycoxidation. Int J Biol Macromol 41:572-578.

Aronson D (2003) Cross-linking of glycated collagen in the pathogenesis of arterial and myocardial stiffening of aging and diabetes. J Hypertens 21:3-12.

Arsenijevic D, Onuma H, Pecqueur C, Raimbault S, Manning BS, Miroux B, Couplan E, Alves-Guerra MC, Goubern M, Surwit R, Bouillaud F, Richard D, Collins S, Ricquier D (2000) Disruption of the uncoupling protein-2 gene in mice reveals

a role in immunity and reactive oxygen species production. Nat Genet 26:435-439.

Balu M, Sangeetha P, Murali G, Panneerselvam C (2005) Age-related oxidative protein damages in central nervous system of rats: modulatory role of grape seed extract. Int J Dev Neurosci 23:501-507.

Bao HL, Wei HH, Hsu YW, Pan TM (2013) Dimerumic acid protects pancreas damage and elevates insulin production in methylglyoxal-treated pancreatic RINm5F cells. J Funct Foods 5:642–650.

Bär KJ, Franke S, Wenda B, Müller S, Kientsch-Engel R, Stein G, Sauer H (2003) Pentosidine and N(epsilon)-(carboxymethyl)-lysine in Alzheimer's disease and vascular dementia. Neurobiol Aging 24:333-338.

Bo J, Xie S, Guo Y, Zhang C, Guan Y, Li C, Lu J, Meng QH (2016) Methylglyoxal impairs insulin secretion of pancreatic β-cells through increased production of ROS and mitochondrial dysfunction mediated by upregulation of UCP2 and MAPKs. J Diabetes Res 2016:1-14.

Bolton WK, Cattran DC, Williams ME, Adler SG, Appel GB, Cartwright K, Foiles PG, Freedman BI, Raskin P, Ratner RE, Spinowitz BS, Whittier FC, Wuerth J-P (2004) Randomized trial of an inhibitor of formation of advanced glycation end products in diabetic nephropathy. Am J Nephrol 24:32-40.

Booth AA, Khalifah RG, Todd P, Hudson BG (1997) In vitro kinetic studies of formation of antigenic advanced glycation end products (AGEs). Novel inhibition of post-Amadori glycation pathways. J Biol Chem 272:5430-5437.

Bouma B, Kroon-Batenburg LM, Wu YP, Brünjes B, Posthuma G, Kranenburg O, de Groot PG, Voest EE, Gebbink MF (2003) Glycation induces formation of amyloid cross-beta structure in albumin. J Biol Chem 278:41810-41819.

Brand MD, Affourtit C, Esteves TC, Green K, Lambert AJ, Miwa S, Pakay JL, Parker N (2004) Mitochondrial superoxide: production, biological effects, and activation of uncoupling proteins. Free Radic Biol Med 37:755-767.

Brouwers O, Niessen PM, Ferreira I, Miyata T, Scheffer PG, Teerlink T, Schrauwen P, Brownlee M, Stehouwer CD, Schalkwijk CG (2011) Overexpression of glyoxalase-I reduces hyperglycemia-induced levels of advanced glycation end products and oxidative stress in diabetic rats. J Biol Chem 286:1374–1380.

Brownlee M, Vlassara H, Cerami A (1984) Nonenzymatic glycosylation and the pathogenesis of diabetic complications. Ann Intern Med 101:527-537.

Brownlee M, Cerami A, Vlassara H (1988) Advanced glycosylation end products in tissue and the biochemical basis of diabetic complications. N Engl J Med 318:1315-1321.

Buongiorno AM, Morelli S, Sagratella E, Castaldo P, Di Virgilio A, Maroccia E, Ricciardi G, Sciullo E, Cardellini G, Fallucca F, Sensi M (1997) Levels of advanced glycosylation end-products (AGE) in sera of pregnant diabetic women: comparison between type 1, type 2 and gestational diabetes mellitus. Ann Ist Super Sanita 33:375-378.

Cai Y-Z, Sun M, Xing J, Luo Q, Corke H (2006) Structure-radical scavenging activity relationships of phenolic compounds from traditional Chinese medicinal plants. Life Sci 78:2872-2888.

Chan CB, De Leo D, Joseph JW, McQuaid TS, Ha XF, Xu F, Tsushima RG, Pennefather PS, Salapatek AM, Wheeler MB (2001) Increased uncoupling protein-2 levels in beta-cells are associated with impaired glucose-stimulated insulin secretion: mechanism of action. Diabetes 50:1302-1310.

Chan WH, Wu HJ (2006) Protective effects of curcumin on methylglyoxal-induced oxidative DNA damage and cell injury in human mononuclear cells. Acta Pharmacol Sin 27:1192-1198.

Chen YC, Lin-Shiau SY, Lin JK (1998) Involvement of reactive oxygen species and caspase 3 activation in arsenite-induced apoptosis. J Cell Physiol 177:324-333.

Cheng A-S, Cheng Y-H, Chang T-L (2013) Resveratrol protects RINm5F pancreatic cells from methylglyoxal-induced apoptosis. J Funct Foods 5:1774–1783.

Choi R, Kim BH, Naowaboot J, Lee MY, Hyun MR, Cho EJ, al. e (2011) Effects of ferulic acid on diabetic nephropathy in a rat model of type 2 diabetes. Exp Mol Med 43:676–683.

Conley KE, Amara CE, Jubrias SA, Marcinek DJ (2007) Mitochondrial function, fibre types and ageing: new insights from human muscle in vivo. Exp Physiol 92:333-339.

Cook LJ, Davies J, Yates AP, Elliott AC, Lovell J, Joule JA, Pemberton P, Thornalley PJ, Best L (1998) Effects of methylglyoxal on rat pancreatic beta-cells. Biochem Pharmacol 55:1361-1367.

Dalle-Donne I, Rossi R, Giustarini D, Milzani A, Colombo R (2003) Protein carbonyl groups as biomarkers of oxidative stress. Clin Chim Acta 329:23-38.

De Souza CT, Araújo EP, Stoppiglia LF, Pauli JR, Ropelle E, Rocco SA, Marin RMF, K.G., Carvalheira JB, Saad MJ, Boschero AC, Carneiro EM, Velloso LA (2007)

Inhibition of UCP2 expression reverses diet-induced diabetes mellitus by effects on both insulin secretion and action. FASEB J 21:1153-1163.

Desai K, Wu L (2007) Methylglyoxal and advanced glycation endproducts: new therapeutic horizons? Recent Pat Cardiovasc Drug Discov 2:89-99.

Fiory F, Lombardi A, Miele C, Giudicelli J, Beguinot F, Van Obberghen E (2011) Methylglyoxal impairs insulin signalling and insulin action on glucose-induced insulin secretion in the pancreatic beta cell line INS-1E. Diabetologia 54:2941-2952.

Gao L, Laude K, Cai H (2008) Mitochondrial pathophysiology, reactive oxygen species, and cardiovascular diseases. Vet Clin North Am Small Anim Pract 38:137-155.

Gao Y, Liu C, Wan G, Wang X, Cheng X, Ou Y (2016) Phycocyanin prevents methylglyoxal-induced mitochondrial-dependent apoptosis in INS-1 cells by Nrf2. Food Funct 7:1129-1137.

Gironès X, Guimerà A, Cruz-Sánchez CZ, Ortega A, Sasaki N, Makita Z, Lafuente JV, Kalaria R, Cruz-Sánchez FF (2004) N epsilon-carboxymethyllysine in brain aging, diabetes mellitus, and Alzheimer's disease. Free Radic Biol Med 36:1241-1247.

Goh SY, Cooper ME (2008) Clinical review: The role of advanced glycation end products in progression and complications of diabetes. J Clin Endocrinol Metab 93:1143-1152.

Hanssen NMJ, Brouwers O, Gijbels MJ, Wouters K, Wijnands E, Cleutjens JPM, al e (2014) Glyoxalase 1 overexpression does not affect atherosclerotic lesion size and severity in ApoE−/− mice with or without diabetes. Cardiovasc Res 104:160-170.

Hardy J, Selkoe DJ (2002) The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. Science 297:353-356.

Heimfarth L, Loureiro SO, Pierozan P, de Lima BO, Reis KP, Torres EB, Pessoa-Pureur R (2013) Methylglyoxal-induced cytotoxicity in neonatal rat brain: a role for oxidative stress and MAP kinases. Metab Brain Dis 28:429-438.

Hirabayashi T, Ochiai H, Sakai S, Nakajima K, Terasawa K (1995) Inhibitory effect of ferulic acid and isoferulic acid on murine interleukin-8 production in response to influenza virus infections *in vitro* and *in vivo*. Planta Med 61:221-226.

Hirata A, Murakami Y, Atsumi T, Shoji M, Ogiwara T, Shibuya K, Ito S, Yokoe I, Fujisawa S (2005) Ferulic acid dimer inhibits lipopolysaccharide-stimulated cyclooxygenase-2 expression in macrophages. In Vivo 19:849-853.

Holmström KM, Finkel T (2014) Cellular mechanisms and physiological consequences of redox-dependent signalling. Nat Rev Mol Cell Biol 15:411-421.

Hori M, Yagi M, Nomoto K, Ichijo R, Shimoda A, Kitano T, Yonei Y (2012) Experimental models for advanced glycation end product formation using albumin, collagen, elastin, keratin and proteoglycan. J Anti Aging Med 9:125-134.

Hou N, Torii S, Saito N, Hosaka M, Takeuchi T (2008) Reactive oxygen species-mediated pancreatic beta-cell death is regulated by interactions between stress-activated protein kinases, p38 and c-jun N- terminal kinase, and mitogen-activated protein kinase phosphatases. Endocrinology 149:1654-1665.

Hove MN, Kristensen JK, Lauritzen T, Bek T (2004) The prevalence of retinopathy in an unselected population of type 2 diabetes patients from Arhus County, Denmark. Acta Ophthalmol Scand 82:443-448.

Hu TY, Liu CL, Chyau CC, Hu ML (2012) Trapping of methylglyoxal by curcumin in cell-free systems and in human umbilical vein endothelial cells. J Agric Food Chem 60:8190-8196.

Hunt JV, Wolff SP (1991) Oxidative glycation and free radical production- a causal mechanism of diabetic complications. Free Radic Res Commun 12-13:115-123.

IDF (2015) International Diabetes Federation (IDF) Diabetes Atlas - Seventh Edirion. Belgium.

Ihm SH, Yoo HJ, Park SW, Ihm J (1999) Effect of aminoguanidine on lipid peroxidation in streptozotocin-induced diabetic rats. Metabolism 48:1141-1145.

Inagi R, Miyata T, Ueda Y, Yoshino A, Nangaku M, Strihou CVYD, Kurokawa K (2002) Efficient in vitro lowering of carbonyl stress by the glyoxalase system in conventional glucose peritoneal dialysis fluid. Kidney Int 62:679-687.

Jastroch M, Divakaruni AS, Mookerjee S, Treberg JR, D.Brand M (2010) Mitochondrial proton and electron leaks. Essays Biochem 47:53-67.

Johansen MB, Kiemer L, Brunak S (2006) Analysis and prediction of mammalian protein glycation. Glycobiology 16:844-853.

Kajimoto Y, Kaneto H (2004) Role of oxidative stress in pancreatic beta-cell dysfunction. Ann N Y Acad Sci 1011:168-176.

Kalapos MP (1999) Methylglyoxal in living organisms: chemistry, biochemistry, toxicology and biological implications. Toxicol Lett 110:145-175.

Kalousová M, Krha J, Zima T (2002) Advanced glycation end products and advanced oxidation protein products in hemodialyzed patients. Physiol Res 51:597-604.

Kang JH (2003) Oxidative damage of DNA induced by methylglyoxal *in vitro*. Toxicol Lett 145:181-187.

Kashemsant N, Chan CB (2006) Impact of uncoupling protein-2 overexpression on proinsulin processing. J Mol Endocrinol 37:517-526.

Kazeem MI, Akanji MA, Hafizur RM, Choudhary MI (2012) Antiglycation, antioxidant and toxicological potential of polyphenol extracts of alligator pepper, ginger and nutmeg from Nigeria. Asian Pac J Trop Biomed 2:727-732.

Khazaei MR, Bakhti M, Habibi-Rezaei M (2010) Nicotine reduces the cytotoxic effect of glycated proteins on microglial cells. Neurochem Res 35:548-558.

Kim EO, Min KJ, Kwon TK, Um BH, Moreau RA, Choi SW (2012) Anti-inflammatory activity of hydroxycinnamic acid derivatives isolated from corn bran in lipopolysaccharide-stimulated Raw 264.7 macrophages. Food Chem Toxicol 50:1309-1316.

Kim J, Son JW, Lee JA, Oh YS, Shinn SH (2004) Methylglyoxal induces apoptosis mediated by reactive oxygen species in bovine retinal pericytes. J Korean Med Sci 19:95-100.

Kim J, Kim NH, Sohn E, Kim CS, Kim JS (2010) Methylglyoxal induces cellular damage by increasing argpyrimidine accumulation and oxidative DNA damage in human lens epithelial cells. Biochem Biophys Res Commun 391:346-351.

Kousar S, Sheikh MA, Asghar M, Sarwar M (2009) Effect of aminoguanidine on advanced glycation end products (AGEs) using normal and diabetic plasma. J Chem Soc Pak 31:109-114.

Krauss S, Zhang CY, Lowell BB (2005) The mitochondrial uncoupling-protein homologues. Nat Rev Mol Cell Biol 6:248-261.

Kutlu M, Naziroğlu M, Simşek H, Yilmaz T, Sahap Kükner A (2005) Moderate exercise combined with dietary vitamins C and E counteracts oxidative stress in the

kidney and lens of streptozotocin-induced diabetic-rat. Int J Vitam Nutr Res 75:71-80.

Ledesma-Osuna AI, Ramos-Clamont G, Vázquez-Moreno L (2008) Characterization of bovine serum albumin glycated with glucose, galactose and lactose. Acta Biochim Pol 55:491-497.

Li J, Yuan J (2008) Caspases in apoptosis and beyond. Oncogene 27:6194-6206.

Li LX, Skorpen F, Egeberg K, Jørgensen IH, Grill V (2001) Uncoupling protein-2 participates in cellular defense against oxidative stress in clonal beta-cells. Biochem Biophys Res Commun 282:273-277.

Li X, Lin J, Gao Y, Han W, Chen D (2012) Antioxidant activity and mechanism of Rhizoma *Cimicifugae*. Chemistry Central Journal 6:1-10.

Li Y, Dutta U, Cohenford MA, Dain JA (2007) Nonenzymatic glycation of guanosine 5'-triphosphate by glyceraldehyde: an in vitro study of AGE formation. Bioorg Chem 35:417-429.

Li Y, Cohenford MA, Dutta U, Dain JA (2008) The structural modification of DNA nucleosides by nonenzymatic glycation: an in vitro study based on the reactions of glyoxal and methylglyoxal with 2'-deoxyguanosine. Anal Bioanal Chem 390:679-688.

Liu IM, Chen WC, Cheng JT (2003) Mediation of beta-endorphin by isoferulic acid to lower plasma glucose in streptozotocin-induced diabetic rats. J Pharmacol Exp Ther 307:1196-1204.

Liu IM, Hsu FL, Chen CF, Cheng JT (2000) Antihyperglycemic action of isoferulic acid in streptozotocin-induced diabetic rats. Br J Pharmacol 129:631-636.

Lo CY, Hsiao WT, Chen XY (2011) Efficiency of trapping methylglyoxal by phenols and phenolic acids. J Food Sci 76:H90-96.

Lo TW, Westwood ME, McLellan AC, Selwood T, Thornalley PJ (1994) Binding and modification of proteins by methylglyoxal under physiological conditions. A kinetic and mechanistic study with N alpha-acetylarginine, N alpha-acetylcysteine, and N alpha-acetyllysine, and bovine serum albumin. J Biol Chem 269:32299-32305.

Looker HC, Fagot-Campagna A, Gunter EW, Pfeiffer CM, Narayan KM, Knowler WC, Hanson RL (2003) Homocysteine as a risk factor for nephropathy and retinopathy in Type 2 diabetes. Diabetologia 46:766-772.

Lu J, Randell E, Han Y, Adeli K, Krahn J, Meng QH (2011) Increased plasma methylglyoxal level, inflammation, and vascular endothelial dysfunction in diabetic nephropathy. Clin Biochem 44:307-311.

Luciano Viviani G, Puddu A, Sacchi G, Garuti A, Storace D, Durante A, Monacelli F, Odetti P (2008) Glycated fetal calf serum affects the viability of an insulin-secreting cell line *in vitro*. Metab Clin Exp 57:163-169.

Luers L, Rysiewski K, Dumpitak C, Birkmann E (2012) Kinetics of advanced glycation end products formation on bovine serum albumin with various reducing sugars and dicarbonyl compounds in equimolar ratios. Rejuvenation Res 15:201-205.

Lv L, Shao X, Chen H, Ho CT, Sang S (2011) Genistein inhibits advanced glycation end product formation by trapping methylglyoxal. Chem Res Toxicol 24:579-586.

Marzban L, Verchere CB (2004) The role of islet amyloid polypeptide in type 2 diabetes. Can J Diabetes 28:39-47.

Marzban L, Soukhatcheva G, Verchere CB (2005) Role of carboxypeptidase E in processing of pro-islet amyloid polypeptide in β-cells. Endocrinology 146:1808-1817.

McIlwain DR, Berger T, Mak TW (2013) Caspase functions in cell death and disease. Cold Spring Harb Perspect Biol 5:1-29.

Monteiro M, Farah A, Perrone D, Trugo LC, Donangelo C (2007) Chlorogenic acid compounds from coffee are differentially absorbed and metabolized in humans. J Nutr 137:2196-2201.

Mukhopadhyay S, Luthria DL, Robbins RJ (2006) Optimization of extraction process for phenolic acids from black cohosh (*Cimicifuga racemosa*) by pressurized liquid extraction. J Sci Food Agric 86:156-162.

Nagai R, Murray DB, Metz TO, Baynes JW (2012) Chelation: a fundamental mechanism of action of age inhibitors, age breakers, and other inhibitors of diabetes complications. Diabetes 61:549-559.

Natella F, Nardini M, Di Felice M, Scaccini C (1999) Benzoic and cinnamic acid derivatives as antioxidants: structure-activity relation. J Agric Food Chem 47:1453-1459.

Nathan DM, Cleary PA, Backlund JY, Genuth SM, Lachin JM, Orchard TJ, Raskin P, Zinman B (2005) Intensive diabetes treatment and cardiovascular disease in patients with type 1 diabetes. N Engl J Med 353:2643-2653.

Negre-Salvayre A, Salvayre R, Augé N, Pamplona R, Portero-Otín M (2009) Hyperglycemia and glycation in diabetic complications. Antioxid Redox Signal 11:3071-3109.

Nowotny K, Jung T, Höhn A, Weber D, Grune T (2015) Advanced glycation end products and oxidative stress in type 2 diabetes mellitus. Biomolecules 5:194-222.

Peng X, Ma J, Chen F, Wang M (2011) Naturally occurring inhibitors against the formation of advanced glycation end-products. Food Funct 2:289-301.

Pi J, Collins S (2010) Reactive oxygen species and uncoupling protein 2 in pancreatic β-cell function. Diabetes Obes Metab 12:141-148.

Puddu A, Storace D, Durante A, Odetti P, Viviani GL (2010) Glucagon-like peptide-1 counteracts the detrimental effects of advanced glycation end-products in the pancreatic beta cell line HIT-T 15. Biochem Biophys Res Commun 398:462-466.

Rabbani N, Thornalley PJ (2012) Methylglyoxal, glyoxalase 1 and the dicarbonyl proteome. Amino Acids 42:1133-1142.

Rabbani N, Thornalley PJ (2014) The critical role of methylglyoxal and glyoxalase 1 in diabetic nephropathy. Diabetes 63:50-52.

Rahbar S (2005) The discovery of glycated hemoglobin: a major event in the study of nonenzymatic chemistry in biological systems. Ann N Y Acad Sci 1043:9-19.

Remor AP, de Matos FJ, Ghisoni K, da Silva TL, Eidt G, Búrigo M, de Bem AF, Silveira PC, de León A, Sanchez MC, Hohl A, Glaser V, Gonçalves CA, Quincozes-Santos A, Borba Rosa R, Latini A (2011) Differential effects of insulin on peripheral diabetes-related changes in mitochondrial bioenergetics: involvement of advanced glycosylated end products. Biochim Biophys Acta 1812:1460-1471.

Rendell M, Nierenberg J, Brannan C, Valentine JL, Stephen PM, Dodds S, Mercer P, Smith PK, Walder J (1986) Inhibition of glycation of albumin and hemoglobin by acetylation in vitro and in vivo. J Lab Clin Med 108:286-293.

Robertson RP, Harmon J, Tran POT, Poitout V (2004) Beta-cell glucose toxicity, lipotoxicity, and chronic oxidative stress in type 2 diabetes. Diabetes 53:S119-S124.

Rolo AP, Palmeira CM (2006) Diabetes and mitochondrial function: role of hyperglycemia and oxidative stress. Toxicol Appl Pharmacol 212:167-178.

Rousset S, Alves-Guerra M-C, Mozo J, Miroux B, Cassard-Doulcier A-M, Bouillaud Fdr, Ricquier D (2004) The biology of mitochondrial uncoupling proteins. Diabetes 53:S130-S135.

Sadowska-Bartosz I, Galiniak S, Bartosz G (2014) Kinetics of glycoxidation of bovine serum albumin by methylglyoxal and glyoxal and its prevention by various compounds. Molecules 19:4880-4896.

Sakai S, Ochiai H, Mantani N, Kogure T, Shibahara N, Terasawa K (2001) Administration of isoferulic acid improved the survival rate of lethal influenza virus pneumonia in mice. Mediators Inflamm 10:93-96.

Sakai S, Kawamata H, Kogure T, Mantani N, Terasawa K, Umatake M, Ochiai H (1999) Inhibitory effect of ferulic acid and isoferulic acid on the production of macrophage inflammatory protein-2 in response to respiratory syncytial virus infection in RAW264.7 cells. Mediators Inflamm 8:173-175.

Schalkwijk CG, Vermeer MA, Stehouwer CD, te Koppele J, Princen HM, van Hinsbergh VW (1998) Effect of methylglyoxal on the physico-chemical and biological properties of low-density lipoprotein. Biochim Biophys Acta 1394:187-198.

Seki M, Tanaka T, Nawa H, Usui T, Fukuchi T, Ikeda K, Abe H, Takei N (2004) Involvement of brain-derived neurotrophic factor in early retinal neuropathy of streptozotocin-induced diabetes in rats: therapeutic potential of brain-derived neurotrophic factor for dopaminergic amacrine cells. Diabetes 53:2412-2419.

Seneviratne C, Dombi GW, Liu W, J.A. D (2011) The *in vitro* glycation of human serum albumin in the presence of Zn(II). J Inorg Biochem 105:1548-1554.

Seo K, Ki SH, Shin SM (2014) Methylglyoxal induces mitochondrial dysfunction and cell death in liver. Toxicol Res 30:193-198.

Shao X, Bai N, He K, Ho CT, Yang CS, Sang S (2008) Apple polyphenols, phloretin and phloridzin: new trapping agents of reactive dicarbonyl species. Chem Res Toxicol 21:2042-2050.

Sheader EA, Benson RS, Best L (2001) Cytotoxic action of methylglyoxal on insulin-secreting cells. Biochem Pharmacol 61:1381-1386.

Sheetz MJ, King GL (2002) Molecular understanding of hyperglycemia's adverse effects for diabetic complications. JAMA 288:2579-2588.

Shield JP, Poyser K, Hunt L, Pennock CA (1994) Fructosamine and glycated haemoglobin in the assessment of long term glycaemic control in diabetes. Arch Dis Child 71:443-445.

Shu T, Zhu Y, Wang H, Lin Y, Ma Z, Han X (2011) AGEs decrease insulin synthesis in pancreatic β-cell by repressing Pdx-1 protein expression at the post-translational level. PLoS One 6:e18782.

Si D, Sun X, Qi D, Chen X, Bi K (2008) Determination and pharmacokinetics of isoferulic acid in rat plasma by high-performance liquid chromatography after

oral administration of isoferulic acid and Rhizoma Cimicifugae extract. J Pharmacol Biomed Anal 47:140-145.

Singh R, Barden A, Mori T, Beilin L (2001) Advanced glycation end-products: a review. Diabetologia 44:129-146.

Sompong W, Meeprom A, Cheng H, Adisakwattana S (2013) A comparative study of ferulic acid on different monosaccharide-mediated protein glycation and oxidative damage in bovine serum albumin. Molecules 18:13886-13903.

Sousa Silva M, Gomes RA, Ferreira AE, Ponces Freire A, Cordeiro C (2013) The glyoxalase pathway: the first hundred years... and beyond. Biochem J 453:1-15.

Souza BM, Assmann TS, Kliemann LM, Gross JL, Canani LH, Crispim D (2011) The role of uncoupling protein 2 (UCP2) on the development of type 2 diabetes mellitus and its chronic complications. Arq Bras Endocrinol Metabol 55:239-248.

Sparvero LJ, Asafu-Adjei D, Kang R, Tang D, Amin N, Im J, Rutledge R, Lin B, Amoscato AA, Zeh HJ, Lotze MT (2009) RAGE (Receptor for Advanced Glycation Endproducts), RAGE ligands, and their role in cancer and inflammation. J Transl Med 7:1-21.

Suárez G, Rajaram R, Oronsky AL, Gawinowicz MA (1989) Nonenzymatic glycation of bovine serum albumin by fructose (fructation). Comparison with the maillard reaction initiated by glucose. J Biol Chem 264:3674-3679.

Suji G, Sivakami S (2007) DNA damage during glycation of lysine by methylglyoxal: assessment of vitamins in preventing damage. Amino Acids 33:615-621.

Takagi Y, Kashiwagi A, Tanaka Y, Asahina T, Kikkawa R, Shigeta Y (1995) Significance of fructose-induced protein oxidation and formation of advanced glycation end product. J Diabetes Complications 9:87-91.

Takahashi M, Pischetsrieder M, Monnier VM (1997) Isolation, Purification, and Characterization of Amadoriase Isoenzymes (Fructosyl Amine-oxygen Oxidoreductase EC 1.5.3) from Aspergillus sp. J Biol Chem 272:3437-3443.

Tamae D, Lim P, Wuenschell GE, Termini J (2011) Mutagenesis and repair induced by the DNA advanced glycation end product N2-1-(carboxyethyl)-2'-deoxyguanosine in human cells. Biochemistry 50:2321-2329.

Tatone C, Heizenrieder T, Di Emidio G, Treffon P, Amicarelli F, Seidel T, al. e (2011) Evidence that carbonyl stress by methylglyoxal exposure induces DNA damage and spindle aberrations, affects mitochondrial integrity in mammalian oocytes and contributes to oocyte ageing. Hum Reprod 26:1843-1859.

Thornalley PJ (1994) Methylglyoxal, glyoxalases and the development of diabetic complications. Amino Acids 6:15-23.

Thornalley PJ (1998) Glutathione-dependent detoxification of alpha-oxoaldehydes by the glyoxalase system: involvement in disease mechanisms and antiproliferative activity of glyoxalase I inhibitors. Chem Biol Interact 24:137-151.

Thornalley PJ (2003) Use of aminoguanidine (Pimagedine) to prevent the formation of advanced glycation endproducts. Arch Biochem Biophys 419:31-40.

Tokuyama T, Yagui K, Yamaguchi T, Huang CI, Kuramoto N, Shimada F, Miyazaki J, Horie H, Saito Y, Makino H, Kanatsuka A (1997) Expression of human islet

amyloid polypeptide/amylin impairs insulin secretion in mouse pancreatic beta cells. Metabolism 46:1044-1051.

Tupe R, Agte V (2010) Interaction of zinc, ascorbic acid, and folic acid in glycation with albumin as protein model. Biol Trace Elem Res 138:346-357.

Turk Z, Mesić R, Benko B (1998) Comparison of advanced glycation endproducts on haemoglobin (Hb-AGE) and haemoglobin A1c for the assessment of diabetic control. Clin Chim Acta 277:159-170.

Ulrich P, Cerami A (2001) Protein glycation, diabetes, and aging. Recent Prog Horm Res 56:1-21.

Vander Jagt DL (2008) Methylglyoxal, diabetes mellitus and diabetic complications. Drug Metabol Drug Interact 23:93-124.

Vander Jagt DL, Hassebrook RK, Hunsaker LA, Brown WM, Royer RE (2001) Metabolism of the 2-oxoaldehyde methylglyoxal by aldose reductase and by glyoxalase-I: roles for glutathione in both enzymes and implications for diabetic complications. Chem Biol Interact 130-132:549-562.

Vinson JA, Howard III TB (1996) Inhibition of protein glycation and advanced glycation end products by ascorbic acid and other vitamins and nutrients. J Nutr Biochem 7:659-663.

Wang H, Liu J, Wu L (2009a) Methylglyoxal-induced mitochondrial dysfunction in vascular smooth muscle cells. Biochem Pharmacol 77:1709-1716.

Wang J, Sun B, Cao Y, Tian Y (2009b) Protein glycation inhibitory activity of wheat bran feruloyl oligosaccharides. Food Chem 112:350-353.

Wang X, Li X, Chen D (2011) Evaluation of antioxidant activity of isoferulic acid *in vitro*. Nat Prod Commun 6:1285-1288.

Wang Z, Jiang Y, Liu N, Ren L, Zhu Y, An Y, Chen D (2012) Advanced glycation end-product N**ε**-carboxymethyl-Lysine accelerates progression of atherosclerotic calcification in diabetes. Atherosclerosis 221:387-396.

Wei Y, Chen L, Chen J, Ge L, He RQ (2009) Rapid glycation with D-ribose induces globular amyloid-like aggregations of BSA with high cytotoxicity to SH-SY5Y cells. BMC Cell Biol 10:1-15.

Westwood ME, Thornalley PJ (1995) Molecular characteristics of methylglyoxal-modified bovine and human serum albumins. Comparison with glucose-derived advanced glycation endproduct-modified serum albumins. J Protein Chem 14:359-372.

Wittemera SM, Plocha M, Windecka T, llerb SCM, Drewelowb B, Derendorfc H, Veit M (2005) Bioavailability and pharmacokinetics of caffeoylquinic acids and flavonoids after oral administration of Artichoke leaf extracts in humans. Phytomedicine 12:28-38.

Wu CH, Yen GC (2005) Inhibitory effect of naturally occurring flavonoids on the formation of advanced glycation endproducts. J Agric Food Chem 53:3167-3173.

Wu CH, Huang SM, Lin JA, Yen GC (2011) Inhibition of advanced glycation endproduct formation by foodstuffs. Food Funct 2:224-234.

Wu HJ, Chan WH (2007) Genistein protects methylglyoxal-induced oxidative DNA damage and cell injury in human mononuclear cells. . Toxicol In Vitro 21:335-342.

Yang Y, Yang Y, Hou J, Ding Y, Zhang T, Zhang Y, Wang J, Shi C, Fu W, Cai Z (2016) The hydroxyl at position C1 of genipin Is the active inhibitory group that affects mitochondrial uncoupling protein 2 in Panc-1 cells. PLoS One 11:e0147026.

Yim HS, Kang SO, Hah YC, Chock PB, Yim MB (1995) Free radicals generated during the glycation reaction of amino acids by methylglyoxal. A model study of protein-cross-linked free radicals. J Biol Chem 270:28228–28233.

Zhang CY, Baffy G, Perret P, Krauss S, Peroni O, Grujic D, Hagen T, Vidal-Puig AJ, Boss O, Kim YB, Zheng XX, Wheeler MB, Shulman GI, Chan CB, Lowell BB (2001) Uncoupling protein-2 negatively regulates insulin secretion and is a major link between obesity, beta cell dysfunction, and type 2 diabetes. Cell 105:745-755.

Zhu Y, Zhao Y, Wang P, Ahmedna M, Sang S (2015) Bioactive ginger constituents alleviate protein glycation by trapping methylglyoxal. Chem Res Toxicol 28:1842-1849.

APPENDIX

VITA

My given name is Aramsri Meeprom. I was born on August 28th, 1984 in Surin, Thailand. I finished primary school and high school from Muang Surin School and Sirindhorn School, respectively, and graduated Bachelor's Degree of Sciences in Medical Technology and Master's degree of Sciences in Clinical Biochemistry and Molecular Medicine from Faculty of Allied Health Sciences, Chulalongkorn University, Bangkok, Thailand.