ผลของภาวะระดับน้ำตาลสูงต่อการแสดงออกของกลูโคสทรานสปอร์ตเตอร์ 9 ในเซลล์เอ็มดีซีเค

นายจำรัส กาญจนไพบูลย์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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EFFECT OF HYPERGLYCEMIC CONDITION ON GLUCOSE TRANSPORTER 9 EXPRESSION IN MDCK CELL

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A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Biomedicinal Chemistry
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จำรัส กาญจนไพบูลย์ : ผลของภาวะระดับน้ำตาลสูงต่อการแสดงออกของกลูโคสทรานสปอร์ตเตอร์ 9 ในเซลล์เอ็มดีซีเค (EFFECT OF HYPERGLYCEMIC CONDITION ON GLUCOSE TRANSPORTER 9 EXPRESSION IN MDCK CELL) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ภญ. ดร.ธิติมา เพ็งสุภาพ, 91 หน้า.

กลูโคสทรานสปอร์ตเตอร์ 9 (GLUT9) มีความสำคัญต่อกระบวนการดูดซึมกลูโคสกลับเข้าสู่ระบบไหลเวียนเลือดที่ไต ซึ่งพบว่ามีความสัมพันธ์กับภาวะระดับน้ำตาลในเลือดสูง อย่างไรก็ตามกลไกการควบคุมการทำงานดังกล่าวยังไม่มีรายงานที่ชัดเจน การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อศึกษาปัจจัยที่เกี่ยวข้องกับภาวะน้ำตาลสูง ในการควบคุมการแสดงออกของ GLUT9 ในเซลล์เพาะเลี้ยง MDCK โดยการหาปริมาณ mRNA และ โปรตีนของ GLUT9 ใช้วิธี RT-PCR และ Western blot analysis เมื่อเซลล์ได้รับกลูโคส หรือซอร์บิทอล ที่ความเข้มข้น 25 มิลลิโมลาร์ เป็นระยะเวลา 72 ชั่วโมง พบว่าปริมาณของ GLUT9 mRNA ลดลงอย่างมีนัยสำคัญทางสถิติ โดยเลือกใช้ซอร์บิทอลเพื่อเป็นตัวแทนในการศึกษาผลของภาวะ hyper-osmotic stress ในขณะที่เมื่อเซลล์ได้รับกลูโคสในปริมาณสูงพบว่าปริมาณโปรตีนของ GLUT9 เพิ่มขึ้น นอกจากนี้ ATP และ adenosine ซึ่งมีรายงานว่าพบในปริมาณที่เพิ่มขึ้นในภาวะน้ำตาลสูง สามารถเพิ่มปริมาณโปรตีน GLUT9 ได้อย่างมีนัยสำคัญทางสถิติ โดยมีผลสูงสุดที่ระยะเวลา 6 ชั่วโมง Adenosine ทำให้มีการเพิ่มขึ้นของโปรตีน GLUT9 ผ่าน adenosine receptors ในขณะที่สารกระตุ้นการทำงานของ protein kinase A (PKA) สามารถยับยั้งผลของการตอบสนองดังกล่าวได้ การวิจัยในครั้งนี้แสดงให้เห็นว่า การลดลงของปริมาณ GLUT9 mRNA ในภาวะที่ได้รับกลูโคสที่ความเข้มข้นสูงเป็นเวลานานนั้น อาจเป็นผลมาจากภาวะ hyper-osmotic stress ในขณะที่ ATP และ adenosine ซึ่งพบสูงขึ้นในภาวะน้ำตาลในเลือดสูง อาจเป็นสารสำคัญที่ควบคุมการเพิ่มขึ้นของการแสดงออก GLUT9 ในระดับโปรตีน ผ่านทาง purinergic system โดยควบคุมผ่าน adenosine receptors และ PKA

ภาควิชา ชีวเคมีและจุลชีววิทยา ลายมือชื่อนิสิต .....

สาขาวิชา ชีวเวชเคมี ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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KEYWORDS: GLUCOSE TRANSPORTER / EXPRESSION / HYPERGLYCEMIC CONDITION / KIDNEY / PURINERGIC SYSTEM / ADENOSINE RECEPTOR

JAMRAS KANCHANAPIBOON: EFFECT OF HYPERGLYCEMIC CONDITION ON GLUCOSE TRANSPORTER 9 EXPRESSION IN MDCK CELL. ADVISOR: ASSOC. PROF. THITIMA PENGSUPARP, Ph.D., 91 pp.

A novel glucose transporter 9 (GLUT9), which is involved in glucose re-absorption processes in kidney, has been shown to be associated with hyperglycemia. However, the regulation mechanisms have not been thoroughly explained. This study aimed to investigate the contributing factors of hyperglycemic condition that could affect GLUT9 expression. The GLUT9 mRNA and protein were measured in semi-quantitative approach by using RT-PCR and Western blot analysis, respectively. The GLUT9 mRNA was significantly decreased by 25 mM glucose and 25 mM sorbitol, which was used to evaluate hyper-osmotic stress effect, whereas, the GLUT9 proteins were noticeably increased by glucose in the MDCK cells treated for 72 hours. Furthermore, extracellular ATP and adenosine, that have been reported to be increased in hyperglycemic condition, significantly increased the GLUT9 protein levels and reached maximum effect at 6 hours period. The GLUT9 protein enhancement effect of adenosine was likely occur through adenosine receptors. Stimulation of protein kinase A could inhibit the effect of adenosine treatment. Our results demonstrated that the decrease of GLUT9 mRNA expression in long-term treatment with glucose at high concentration might relate to hyper-osmotic stress effect. Moreover, the extracellular ATP and adenosine, found to be increased during hyperglycemia, may be the major components that enhance GLUT9 protein expression *via* purinergic system preferentially through adenosine receptor activation and protein kinase A inhibition.

Department: Biochemistry and Microbiology Student's Signature .....
Advisor's Signature .....
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LIST OF ABBREVIATIONS

%	Percentage
µg	Microgram (s)
µl	Microliter (s)
µM	Micromolar
µm	Micrometer (s)
^{0}C	Degree Celsius (centigrade)
cAMP	Adenosine 3',5'-cyclic monophosphate
cDNA	Complementary deoxyribonucleic acid
cm^{2}	Centimeter square
CO_2	Carbon dioxide
DNA	Deoxyribonucleic acid
g	Gram (s)
Ig	Immunoglobulin
MDCK	Madin-Darby Canine Kidney
mg	Milligram (s)
ml	Milliliter (s)
mM	Millimolar
mRNA	Messenger ribonucleic acid
nm	Nanometer (s)
PBS	Phosphate buffer saline
pH	The negative logarithm of hydrogen ion concentration
qs.	Make to volume
RNA	Ribonucleic acid
rpm	Round per minute
RT-PCR	Reverse transcription and polymerase chain reaction
V	Volt

CHAPTER I INTRODUCTION

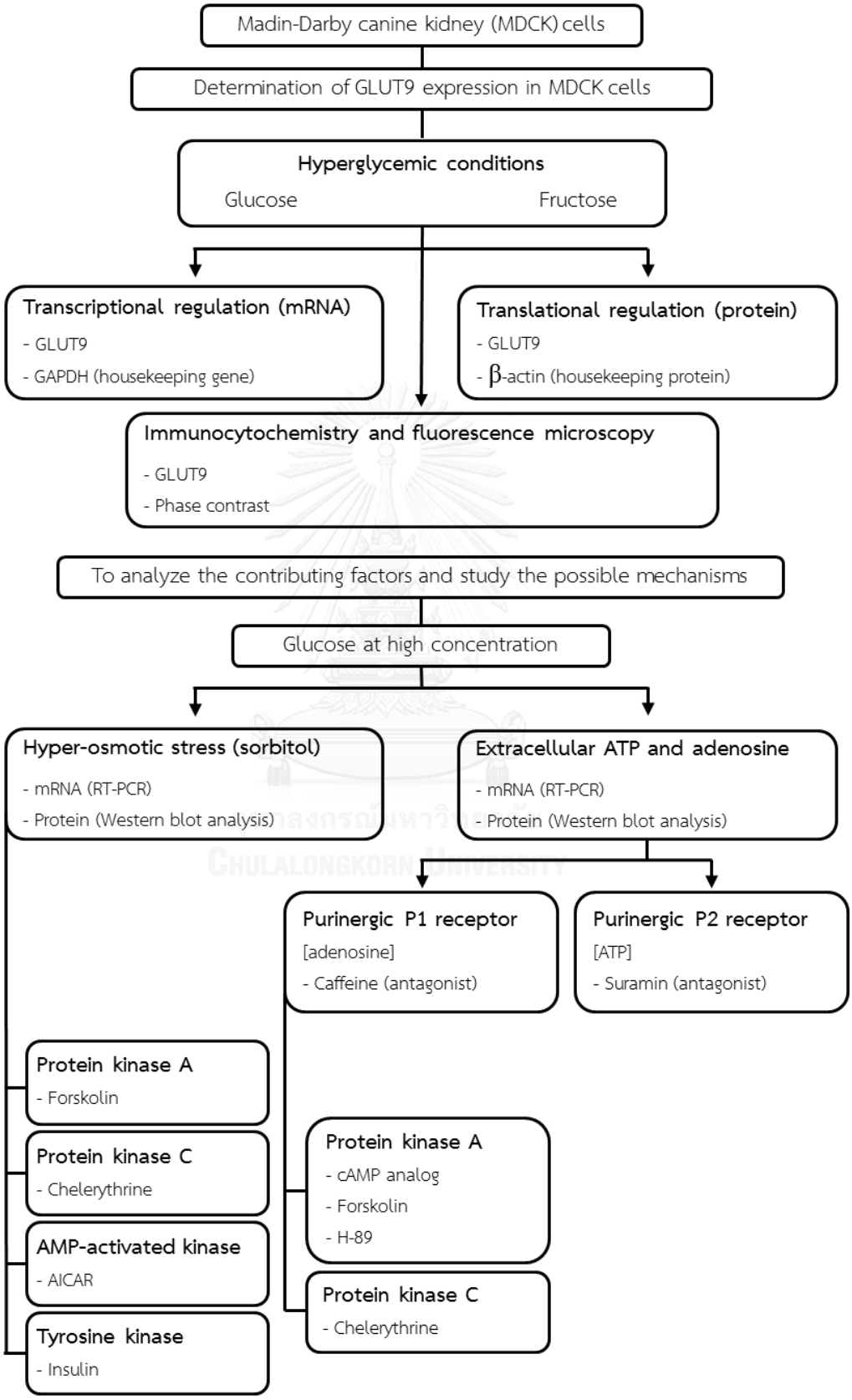
Hyperglycemia is defined as a condition characterized by a high plasma glucose level, and is a major cause leading to complications in diabetic patients. Diabetes is a chronic metabolic disease that occurs with insulin deficiency and/or insulin resistance (1). Insulin plays a role in maintaining glucose homeostasis. The glucose re-absorption in kidney contributes to maintain the balance by glucose transporters more than 25% (2). A facilitative diffusion glucose transporter 9 (GLUT9) encoded by the solute carrier family 2 member 9 gene (*SLC2A9*) plays a key role in uric acid and glucose homeostasis by re-absorption processes in kidney (3, 4). The GLUT9 also mediates transportation of fructose (4-6). In addition, GLUT9 is identified to be involved in insulin secretion in pancreatic beta cells and embryo implantation in placenta (7-10). Observations in animals and humans suggest that its expression is associated with hyperglycemia as well as hyperuricemia (5, 11, 12). The protein expression of GLUT9 was increased in streptozotocin-induced diabetic rats (13) and in gestational diabetes from human placenta (14). The GLUT9 protein also significantly increased in male *ob/ob* mice, a type 2 diabetic animal model, but the mRNA level did not alter when compared with control group (15). Therefore, hyperglycemic condition such as high concentration of glucose and fructose might be a contributing factor that influences the expression of GLUT9 in kidney.

Previous observations have reported that long-term glucose treatment at high concentrations noticeably enhanced content of extracellular adenosine triphosphate (ATP) in cell culture model. For example, extracellular ATP was increased two fold, whereas, intracellular ATP remained constant in rat mesangial cells cultured in 25 mM glucose for 72 hours (16). This result was similar to the study of Chen's group which observed an increase of ATP in culture medium when HK-2 cells were cultured in high glucose condition for 48 hours (17). Extracellular ATP exerts its activities to regulate pathological and physiological properties in autocrine and/or paracrine system of many tissues, such as the kidney, by binding to purinergic P2 receptors. Transportation of ATP through exocytosis, connexin hemichannels, or transporters

such as vesicular nucleotide transporter increases the extracellular ATP, which is immediately broken-down by ecto-nucleotidases to ADP, AMP, and adenosine (18-20). Extracellular adenosine, an ATP metabolite, can regulate the purinergic system through purinergic P1 or adenosine receptors. These receptors are divided to 4 subtypes: A1, A2a, A2b, and A3 receptors, which are detectable in kidney (21). All of them are coupled with G-proteins and associated with stimulation or inhibition of adenylate cyclase resulting to increase or decrease the cAMP concentration, respectively (20). Intracellular cAMP is able to trigger the protein kinase A (PKA) signaling pathway, but activation of adenosine receptors has also been attributed to protein kinase C (PKC) activity (22). The expression of adenosine receptors in terms of mRNA and protein are also increased in renal tubular cells of diabetic rats (23). Furthermore, the content of adenosine increased six-fold in glomeruli when compared with normal rats (24). These observations indicated the important and involvement of the extracellular ATP and adenosine in hyperglycemic condition.

In spite of the fact that GLUT9 expression was regulated under hyperglycemic condition, the mechanisms of regulation have not been thoroughly explained. Therefore, this study aimed to investigate the contributing factors such as glucose, fructose, ATP, and adenosine levels, including possible pathways that influence GLUT9 expression. These factors might have a potential role in the mechanisms of GLUT9 regulation under hyperglycemic conditions.

Conceptual framework



Objectives

1. To study the effect of hyperglycemic conditions on GLUT9 expression in terms of mRNA and protein levels.
2. To study signaling proteins involved in the regulation of GLUT9 expression under hyperglycemic conditions.

Scope of study

This study covered *in vitro* assays of the effect of hyperglycemic condition on GLUT9 expression in MDCK (NBL-2) cells derived from the kidney of a normal adult female cocker spaniel as a renal tubular cell model (25). First of all, the expression of GLUT9 in terms of mRNA and protein levels was determined using RT-PCR and Western blot analysis, respectively. Immunocytochemistry was performed to confirm GLUT9 protein expression in MDCK cell culture. Secondly, the effect of hyperglycemic conditions, *i.e.* increased glucose and fructose, on GLUT9 mRNA and protein expression was investigated. This study focused on chronic effects, so the experiments were performed for 24, 48, and 72 hours. Thirdly, hyperglycemic conditions have been reported to be related with hyper-osmotic stress and increased extracellular ATP, so these conditions were also used in this study. Sorbitol was used to mimic hyper-osmotic stress effect. Extracellular ATP and its metabolite, adenosine, were investigated for their effect on GLUT9 mRNA and protein within 24 hour period. Since ATP and adenosine could play a role in regulation of pathological and physiological properties through purinergic receptors, their involvement was evaluated using the non-selective antagonist of purinergic P2 and adenosine receptors, respectively. Finally, the involvement of signaling proteins was also investigated. The resulting hyper-osmotic stress has been demonstrated to be regulated through PKA, PKC, AMP-activated kinase, and tyrosine kinase. Furthermore, adenosine receptors are coupled with G-proteins which are associated with stimulation or inhibition of adenylate cyclase, resulting in activation of the PKA signaling pathway (20). However, PKC was reported to involve in activation of

adenosine receptors (22). Therefore, the involvement of PKA and PKC in the effect of adenosine on GLUT9 expression was also investigated.

Contributions of the study

The present study would provide a better understanding on the effect of hyperglycemic conditions, especially the level of glucose, fructose, osmolality, ATP, and adenosine, which can influence the regulation of GLUT9 expression in renal proximal tubular cells (MDCK cells). Furthermore, it would identify the possible intracellular signaling proteins that may explain how cells respond to hyperglycemic conditions. This knowledge may be useful for designing a novel target for controlling glucose homeostasis.

CHAPTER II LITERATURE REVIEWS

Hyperglycemia and diabetes

Hyperglycemia is a condition which is characterized by high glucose level in the blood plasma, and is a major cause of complications in diabetic patients (26). The normal range for fasting glucose level in plasma is 80-100 mg/dl. The American Diabetes Association guideline considers the subject with the fasting glucose concentration of more than 100 mg/dl to be hyperglycemic; whereas, one with glucose concentration above 126 mg/dl as diabetic. When the fasting glucose level is below 70 mg/dl, the subject is considered hypoglycemic (1, 27). Early signs of hyperglycemia are headaches, increased thirst, blurred vision, and frequent urination. The plasma glucose level is not only increased in hyperglycemia, but insulin and fructose levels as well (28, 29). Thorburn's group demonstrated a reduction of insulin sensitivity in rats that received high glucose and fructose diets (30). In 2002, diabetes patients from Teikyo University Hospital in Japan were monitored for their serum and urinary fructose concentrations. These fructose concentrations in the diabetic patients were higher than in healthy subjects. However, the concentration of fructose rapidly decreased when diabetic patients were treated by diet therapy, oral hypoglycemic therapy, and/or insulin therapy (29). These results indicated that hyperglycemia was associated with the increase in the fructose concentration in plasma and urine. Furthermore, hyperglycemia has been reported to be involved in changing physiological osmolality (31). In the hyperglycemic hyperosmolar state, the plasma glucose level can be higher than 600 mg/dl which causes osmolality to be greater than 320 mOsm/kg (32, 33).

Diabetes is a chronic metabolic disease that occurs with insulin deficiency and/or insulin resistance resulting in increased plasma glucose concentrations (34, 35). There are three types of diabetes. First, insulin-dependent diabetes mellitus (Type 1 Diabetes) is lack of insulin in blood circulation because of destruction of pancreatic beta cells, where synthesize and secrete insulin. A major cause of the pathology of type 1 diabetes is the attack of activated cytotoxic T-lymphocytes on

the pancreatic beta cells (36). Second, insulin-independent diabetes mellitus (Type 2 Diabetes) is due to the defective responsiveness to insulin which referred to as insulin resistance (37, 38). Third is gestational diabetes that involves with insufficient insulin secretion and dysfunctional insulin receptor during pregnancy (37). The gestational diabetes is a risk factor of macrosomic baby, congenital malformation, perinatal morality, and the development of maternal type 2 diabetes (39).

Since hyperglycemia causes complications such as ketoacidosis, cardiovascular disease, chronic renal failure, retinal damage, and hyperosmolar coma, the control of optimal plasma glucose level in patients is the main goal of therapy for hyperglycemia and diabetes (34, 35, 37). Treatment for type 1 diabetes is insulin replacement therapy, while type 2 diabetes is required combination drug therapy. A biguanide group, such as metformin, is one of the most common treatments. The efficacy of metformin is mediated by AMP-activated protein kinase (AMPK) stimulation to increase the insulin sensitivity in fat and muscle cells. The thiazolidinedione group, observed in drugs such as rosaglitazone and pioglitazone, is an insulin sensitizer similar to the biguanide group, but it activates the peroxisome proliferator-activated receptor, which act as a transcription factor regulating gene expression of proteins involved in metabolism. On the other hand, the sulfonylurea and meglitinide groups are insulin secretagogues. Sulfonylurea binds to the sulfonylurea receptor, a subunit of the ATP-dependent potassium channel, to activate the insulin secretion from pancreas (38). Furthermore, incretin belongs to a group of gastrointestinal hormones that can increase the amount of insulin secreted from pancreatic beta cells after eating. Incretin rapidly degraded by dipeptidyl peptidase 4, so that its levels can be increased by the inhibitor of dipeptidyl peptidase 4, which is used to treat diabetes type 2. Although anti-diabetic drugs are not widely available, treatment of diabetes depends on insulin secretion and/or function. Prolonged elevation of glucose can cause progressive decline in function and mass of beta pancreatic cells and can decrease insulin sensitivity. Therefore, a new agent that can reduce plasma glucose concentration in an insulin-independent manner is in great interest. Inhibitors of kidney glucose reabsorption may help to

achieve the goal of diabetic treatment because the kidney plays an important role in glucose homeostasis by reabsorbing all of the filtered glucose from the glomeruli. Some of the selective inhibitors of SGLT2 are dapagliflozin, canagliflozin, and remogliflozin. SGLT2 is high capacity for glucose transportation. The SGLT2 inhibitors could decrease the fasting plasma glucose level in diabetic patients, and some of them are effective in moderate renal impairment (40-42). In addition, GLUT9 as a novel glucose transporter has also been reported to involve in glucose reabsorption (3, 43). Not only glucose is allowed to transport *via* GLUT9 but fructose and uric acid transportation are also. GLUT9 has high affinity for glucose and fructose, but high capacity for uric acid (5). According to co-existing between hyperglycemia and hyperuricemia, high concentration of uric acid in plasma has been reported as a high risk of developing diabetes type 2 (44, 45). An increase of serum uric acid in pre-diabetes and insulin resistance may involve in renal re-absorption of uric acid (46, 47). Moreover, there was reported about the increase of GLUT9 protein in kidney of potassium oxonate-induced hyperuricemic rats (48). These observations suggest that inhibition of glucose transporters especially GLUT9 in the kidney has a potential role for further development as a treatment for diabetes.

Compounds/factors involve in hyperglycemic condition

I: Glucose

Carbohydrate products, which consist of glucose molecules, play an important role in maintaining good health. Cells uptake glucose from the blood and use it to synthesize metabolic energy (27, 49). Plasma glucose is maintained at a constant concentration of 5 mM, including after meal and overnight periods, by glucose homeostatic processes. In the kidney, approximately 162 g (900 mmole) of glucose is filtered per day by the glomeruli, but over 99% of filtered glucose is reabsorbed through the proximal tubular cells by glucose transporters. Generally, the glucose is not found in urine, but for hyperglycemic it is because the excess glucose levels beyond the ability of reabsorption processes in proximal tubule (2, 41).

Glucose reabsorption across the proximal tubule occurs *via* SGLT1- and SGLT2-mediated uptake at the brush border membrane and facilitated transport across the basolateral membrane mediated by GLUT1, GLUT2, and GLUT5 (2, 41). Recently, GLUT9 expressed at brush border-membrane and basolateral membrane of renal proximal tubular cells has been reported to involve in glucose re-absorption (3, 43).

In hyperglycemia, the high concentration of glucose has shown the physiological properties that contribute to its transportation in intestine and kidney. When glucose concentration in the intestinal lumen is higher than 25 mM, the intracellular GLUT2 is triggered to move to brush border membrane and basolateral membrane (50). Furthermore, this condition can also enhance the expression of GLUT5. In the kidney, previous studies have shown that glucose transporters such as SGLT2, GLUT2, and GLUT9 were increased in streptozotocin-induced diabetic rats (13, 51). Using porcine renal proximal tubular LLC-PK1 cells as a model, the mRNA level of SGLT1 and GLUT1 were down-regulated when the cells were cultured in high glucose condition (52).

II: Fructose

Fructose is a monosaccharide mostly found in honey and many plants. Sugar cane is a commercially product that contains fructose. Fructose is added to commercial foods and beverages for taste enhancement because its relative sweetness is 173 times that of sucrose (27, 53). Fructose is directly absorbed by specific fructose transporter, GLUT5, from intestinal lumen into enterocyte and GLUT2 at basolateral membrane (54, 55). The presence of fructose in the lumen increased mRNA and protein levels of GLUT5 leading to increase fructose transportation (56, 57). In hepatocytes, the GLUT2 plays role in operating transportation of fructose into the cell. The intracellular fructose is metabolized by fructokinase to fructose-1-phosphate before the metabolite is enrolled to the citric acid cycle for synthesized metabolic energy similar to glucose. Liver has been shown to be a major organ for fructose metabolism because the rate of metabolism is rapid,

whereas, fructose is poorly metabolized in extra-hepatic organs (53, 58). Fructose is also formed from glucose by aldose reductase and sorbitol dehydrogenase especially in kidney, lens, nerves, and heart tissues.

Although plasma fructose level is in the range of micromolar, their products can lead to the diabetic complication such as nephropathy, retinopathy, neuropathy, and atherosclerosis (58). Reducing sugars are spontaneously formed with proteins, lipids, or nucleic acids to form advanced glycation endproducts (AGEs) such as hemoglobin A_{1c}. This process is carried out using the non-enzymatic glycation mechanism. The AGEs are found to be increased in the extracellular and intracellular structures of diabetes patients, so that these structures and related biochemical processes are damaged (59, 60). Many observations have demonstrated that glucose and fructose can increase the rate of glycation, the cross-linking of glycated collagen, and the polymerization of proteins resulting to increase AGEs. Since fructose composes of highly reactive chain structures and reactive 3-deoxyglucosone, which is an intermediate of the dicarbonyl formation from fructose, fructose is considered as a critical reducing sugar (58). In addition, fructose is associated with the increased glucose in plasma and urine, which increases the chance of developing diabetes (29).

III: Osmolality imbalance

The Hyperglycemic hyperosmolar state and ketoacidosis are serious complications of diabetes. Although the hyperglycemic hyperosmolar state is found less in hospital admission of diabetes patients, this state causes the mortality rate in the range of 10-50% in older patients, whom have uncontrolled diabetes and/or diabetes type 2 patients (32, 33). However, recent case report has revealed that hyperglycemic hyperosmolar state is increased in children (61). The osmolality value in hyperglycemic patients, absent of ketoacidosis, is greater than 320 mOsm/kg while the glucose plasma level is greater than 600 mg/dl (33 mM). Furthermore, the infections of urinary tract and pneumonia promote the hyperglycemic hyperosmolar state (32, 33, 61).

A changing of serum osmolality can influence physiological and biochemical processes. The osmolality level of patients in the hyperglycemic hyperosmolar state can be increased to over 320 mOsm/kg, while the normal level is 300 mOsm/kg (33). Recently, hyper-osmotic stress has been reported to increase the risk of cellular insulin resistance. These observations may be involved in PKB dephosphorylation, IRS1 lysosomal degradation, and IRS1 phosphorylation (62). Hyper-osmotic stress has been shown to regulate glucose transporters such as GLUT2 and GLUT4. For example, the expression of GLUT2 on brush border membrane of renal proximal tubular cells was significantly increased when the rats were infused with mannitol to increase osmolality level in plasma. The enhancement of GLUT2 protein has been revealed that these processes involved in increasing of PKC-β expression (63). Furthermore, the translocation of GLUT4 from intracellular vesicles to plasma membrane in muscle and fat cells has been enhanced by hyper-osmotic stress to increase glucose uptake. This observation suggests that effect of hyper-osmotic stress on intracellular signaling proteins depends on cell types. In muscle cells, AMPK triggers phosphorylation of nitric oxide systhase and ERK and inhibits GLUT4 endocytosis using tyrosine kinase independent processes, whereas, the regulation in fat cell is tyrosine kinase dependence (62).

VI: Purine nucleotides and nucleosides

Adenosine 5'-triphosphate (ATP) is a purine nucleotide composed of adenine, ribose, and phosphate groups and is a precursor of cellular energy. ATP is mainly generated by mitochondrial oxidative phosphorylation to provide energy to many biological reactions, including intracellular signaling processes. Recently, extracellular ATP is demonstrated to regulate biologic and physiologic processes in numerous cell types such as epithelial cells, endothelial cells, fibroblasts, pancreatic beta cells, hepatocytes, neuron cells, muscle cells, and platelets (64). Although ATP plays a role in supporting intracellular energy, it can be released through exocytosis, connexin hemichannels, or transporters such as vesicular nucleotide transporter (65, 66). The secretion of ATP is an important step that is closely regulated in response to

changing physiologic conditions such as mechanical stress, osmotic stress, and cell injury including long-term glucose treatment at high concentration. For example, extracellular ATP was increased two fold but the intracellular ATP was not altered in rat mesangial cells, cultured in 25 mM glucose for 72 hours (16). This result was similar to the study of Chen's group which observed an increase of ATP in cultured medium when HK-2 cells were cultured in high glucose condition for 48 hours (17). However, the extracellular ATP is immediately broken-down by ecto-nucleotidases into ADP, AMP, and adenosine. The extracellular adenosine content has been also revealed to be significantly increased in cultured rat podocytes with 30 mM glucose (67). Furthermore, the content of adenosine increased in the glomeruli to a level six fold higher than in normal rats (24). Recently, the extracellular ATP and its metabolites have reported that exert potent autocrine and paracrine effects on cellular function by binding to purinergic P1 and P2 receptors.

Purinergic receptors

Purinergic system is an autocrine and/or paracrine in maintaining pathological and physiological properties of many tissues including kidney. Recently, many scientists have reported that purinergic system involves in diabetes, hypertension, inflammation, and platelet aggregation (68). The effect of purinergic activation depends on cell types and specific purinergic receptor population. The principle of purinergic signaling composes of the releasing of nucleotides and nucleosides, the selectivity between agonists and purinergic receptors on cell membrane, the transduction of intracellular signal, and the degradation processes of agonist and receptors (19). The purinergic receptors consist of two major classes based on the response of the receptor on various agonists. Purinergic P1 receptor or adenosine receptor recognizes adenosine, whereas, the purinergic P2 class is wildly bind to many nucleotides such as ATP, ADP, UTP, and UDP. As shown in Figure 1.

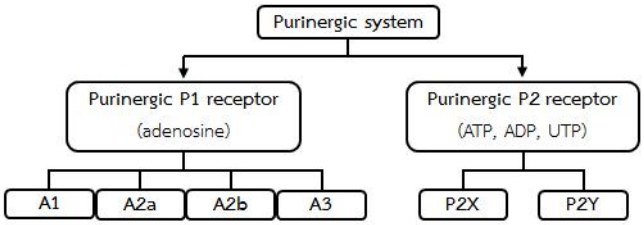


Figure 1 Diagram of purinergic receptors.

Adenosine receptors belong to a family of G protein-coupled receptors that are associated with stimulation or inhibition of adenylate cyclase resulting to increase or decrease the intracellular cAMP concentration, respectively. The cAMP plays a role to trigger PKA as a major intracellular signaling pathway. However, the stimulation of adenosine receptors has been reported to partly regulate through PKC. Adenosine receptors are divided to four subtypes including A1, A2a, A2b, and A3. The adenosine receptors A1 and A3 inhibit activity of adenylate cyclase preferentially through G_{α_i} protein, whereas, this enzyme is likely stimulated through G_{α_s} protein by A2a and A2b receptors (69, 70). Each subtype of adenosine receptors is detectable in different area in renal tubular cells. In diabetic rats, adenosine receptors also increased in renal tubular cells (23). However, all of them are inhibited by methylxanthines such as caffeine and theophylline (71).

Purinergic P2 receptors are divided to two subtypes including P2X and P2Y. The P2X is ligand-gate ion channel that maintaining the monovalent and divalent permeability. On the other hand, the P2Y belongs to G protein-coupled receptor that activates the inositol phospholipid signaling. The P2Y activation generates inositol-1,4,5-triphosphate (IP3) and diacylglycerol resulting to increase the calcium secretion from endoplasmic reticulum and to activate PKC, respectively (19, 20). Recently, the P2 receptors which respond to ATP have demonstrated in kidney including renal cell culture model such as MDCK cells (72, 73).

Glucose transporter

Glucose transportation is carried out by using specific transporters to allow the movement of glucose across cell membrane. Glucose cannot be transported by paracellular and transcellular transport without transporters because plasma membrane composes of hydrophobic compartment. Two families of glucose transporters have been described in mammalian tissues: active sodium-dependent glucose co-transporters (SGLTs) and facilitative diffusion glucose transporters (GLUTs) (43).

Sodium-dependent glucose cotransporters (SGLTs), a solute carrier family 5 (*SLC5A*), are exhibited in intestinal enterocytes and renal proximal tubular cells. The SGLTs are high affinity but low capacity transporter for glucose. The transportation processes by SGLTs needs intracellular ATP to energize Na^+/K^+-ATPase for maintaining the extracellular sodium ions. In extracellular compartment, glucose is coupled with sodium ions before they are transported across plasma membrane. Therefore, this process is called "secondary active transport" (55). Recently, the SGLT is found as three isoforms: SGLT1, SGLT2, and SGLT3. The localization of SGLT1 is expected in brush border membrane of the enterocytes and renal proximal tubular cells, while SGLT2 is exhibited only renal proximal tubular cells. The SGLT3 is found on plasma membrane of enterocytes and muscle cells (43). The expression of SGLT1 is increased depend on cellular differentiation state, but negatively regulated by glucose concentration (52).

Facilitative diffusion glucose transporters (GLUTs), a solute carrier family 2 (*SLC2A*), are exhibited in many organs such as intestine, liver, kidney, adipocytes, muscle, islets, testis, and brain. The mechanism of glucose transportation through the GLUTs depends on the concentration gradient. All member of GLUT proteins contain tweleve transmembrane domains, N- and C-terminal cytoplasmic domains, and a single glycosylation site on extracellular loops which are believed to be a glucose binding site (3). Moreover, they are classified into three classes based on sequence homology and substrate specificity. First, the GLUT class I or classical transporter consists of GLUT1 to 4 that recognize to glucose and galactose as major

substrates. Second is the GLUT class II which prefers fructose to glucose, because these transporters have NXV/NXI motif at extracellular domain, that contributes to binding between GLUTs and fructose (74). The GLUT5, GLUT7, GLUT9, and GLUT11 have been reported as the glucose transporter class II. Finally, the GLUT class III has structure similar to GLUT class II, but they carry N-glycosylation site at the fifth extracellular loop.

Glucose transporter 9 (GLUT9)

A novel glucose transporter 9 (GLUT9), which has been identified as a glucose transporter class II, is a high affinity glucose and fructose transporter and high capacity uric acid transporter (5, 74). In GLUT9-overexpressing *Xenopus laevis* oocyte, the K_m for glucose was showed to be 0.61 mM and the K_m for fructose was 0.42 mM (5). The apparent affinity and capacity for uptake of uric acid represented in K_m of 1 mM and V_{max} of 15 pmol.oocyte^{-1}.min^{-1}, respectively. The K_m for efflux direction is 1 mM, but the V_{max} value is limited to achieve because of solubility of uric acid. The uric acid transport across GLUT9 in GLUT9-overexpressing *Xenopus* oocytes is neither inhibited by D-glucose nor D-fructose, and the glucose transport is unaffected by uric acid at high concentration (6). The transportation of uric acid is indicated that depends on electrical gradient when membrane potential and extracellular chloride ion are changed (75). Decreasing the external chloride concentration enhance the uptake of uric acid, whereas, chloride ion is not required for uric acid transport by GLUT9 (76).

The gene of GLUT9 belongs to solute carrier 2A9 (SLC2A9) and is recently found to be encoded for two alternative RNAs, which referred to GLUT9a and GLUT9b. The two splice variants differ in their N-terminal sequences and are expressed in different area. The GLUT9a exhibits in various tissues, while expression of GLUT9b is specific in kidney, liver, and placenta (8, 13). Recently, these transporters are investigated for their functions to be involved in glucose reabsorption in kidney, insulin secretion in pancreatic beta cells, and embryo

implantation in placenta (7, 9, 10). Furthermore, the expression between both variants in renal proximal tubular cells and syncytiotrophoblast, which have polarize property of epithelial cells, exhibited in different sites. The GLUT9a is expressed at basolateral membrane, whereas, the GLUT9b is found at brush border membrane (8, 14).

In hyperglycemic condition, the protein expression of GLUT9 was increased 1.3-fold to 2-fold in liver and kidney when mice were induced to be diabetes by streptozotocin for 10 days. Although the mRNA level of GLUT9b was significantly increased, the mRNA level of GLUT9a had no effect (8). These observations may be due to differences of their promoters, indicated as an alternative splicing between both isoforms (77). In human term placenta from gestational diabetes as well as insulin-controlled gestational diabetes, the regulation of GLUT9a and GLUT9b was investigated by Western blot analysis and immunocytochemistry. The GLUT9a on basolateral membrane of placenta was increased under pregestational and gestational diabetes, while the GLUT9b on brush border membrane and basolateral membrane of placenta were increased under insulin-controlled gestational diabetes (14). These finding suggested that factors of hyperglycemic and hyperinsulinemic conditions might enhance the GLUT9 expression.

MDCK cell as a model for transport studies in kidney

The mechanisms, physiological roles, drug interactions, and regulation studies of transporters have been increasingly conducted by cell culture systems to avoid the complexity of organ and animal experiments (78). The parental cultured cells such as LLC-PK1, HK2, and MDCK cell lines were used as model that closely exhibit functional and physiological of the renal proximal tubular cells (79). The MDCK cell (NBL-2), which is a parental cell line, derived from kidney of a normal adult female cocker spaniel. This cell exhibits heterogeneity in features and presence of polarize epithelial structure including microvilli on the apical side of the cells. The formation of tight junction is carried out and shown the transepithelial resistance in range of

120-190 $\Omega.cm^2$ in confluent MDCK monolayer (78). Transport parameters such as apparent permeability coefficient appear to correlate well in both passive diffusion and active transport compounds (80). MDCK cell exhibits the transporter expression and function similarity to renal proximal tubular cell such as Na^+/H^+ exchanger (81). The cell is recently developed properties and isolated many strains. MDCK1 and MDCK2 are available by the American Type Culture Collection (ATCC), and MDCK I and MDCK II are supplied by the European Collection of Cell Cultures (ECACC) (25). However, all strains have been used to study epithelial cell polarity and permeability screening. As a consequence, MDCK cell is a useful tool for assessing the membrane permeability properties of early drug discovery compounds and epithelial development (80, 82).

CHAPTER III MATERIALS AND METHODS

Materials

MDCK (NBL-2) cell passage number 55 was obtained from American Type Culture Collection (ATCC; MD, USA). Minimum Essential Medium (MEM), fetal bovine serum, 0.25% trypsin-EDTA, TRIzol® reagent, and UltraPure™ distilled water were purchased from Invitrogen corperation (N.Y., USA). D-glucose, D-fructose, dimethyl sulfoxide (DMSO), ethanol (A.R. grade), hydrochloric acid (A.R. grade), potassium dihydrogen phosphate (KH_2PO_4), and sodium hydrogen phosphate dihydrate ($Na_2HPO_4.2H_2O$) were purchased from Merck (Darmstadt, Germany). Sorbitol and neutral red were purchased from BDH (Poole, England). Adenosine, AICAR, 8-(4-chlorophenyl-thio)adenosine 3',5'-cyclic monophosphate sodium salt (cAMP analog), forskolin, genistein, H-89 dihydrochloride hydrate, suramin sodium salt, chelerythrine chloride, 2-mercaptoethanol, paraformaldehyde, sodium deoxycholate, thiazolyl blue tetrazolium bromide (MTT), Tris base, bovine serum albumin, ethidium bromide, and Hoechst 33342 were purchased from Sigma Chemical (MO, USA). Adenosine 5'-triphosphate disodium salt (ATP), protease inhibitor cocktail set I, and agarose were purchased from Calbiochem (NJ, USA). Caffeine anhydrous and Triton X-100 were purchased from Fluka (Buchs, Switzerland). Boric acid, sodium chloride, and potassium chloride were purchased from Ajax Finechem (NSW, Australia). Omniscript® reverse transcription kit and *Taq* DNA polymerase are obtained from Qiagen (Hilden, Germany). Iso-propanol (A.R. grade) was purchased from QReC (New zealand). Chloroform (A.R. grade) was purchased from Lab Scan (BKK, Thailand). Primers are synthesized by 1st BASE Custom Oligos (FBCO; the Gemini Singapore Science Park II, Singapore). Ammonium persulfate, 30% acrylamide/bis (29:1), and sodium dodecyl sulfate were purchased from Bio-Rad laboratories (CA, USA). Tween 20 (enzyme grade) and brilliant blue G-250 were purchased from Fisher Chemical (NJ, USA). Glycerol (molecular biology grade) was purchased from Promega (WI, USA). Luminata™ Crescendo Western HRP Substrate was purchased from Millipore Corporation (MA, USA). BioTrace™ polyvinylidene fluoride transfer membrane (PVDF)

0.45 μm was purchased from Pall Corporation (FL, USA). Rabbit polyclonal anti-GLUT9 antibody (ab82910), horseradish peroxidase-coupled goat polyclonal secondary antibody against rabbit IgG (ab6721) and horseradish peroxidase-coupled goat monoclonal anti-actin antibody (ab20272) were purchased from abcam (MA, USA). Alexa Fluor® 488 antibody was purchased from Molecular Probes (OR, USA). The plastic ware for cell culture and thin wall PCR tube were obtained from Corning incorporation (NY, USA). Pipette tips and microcentrifuge tube were purchased from Axygen BioScience (CA, USA).

Cell culture (82)

MDCK cells were cultured in MEM supplemented with 10% (v/v) fetal bovine serum and maintained in humidified atmosphere of 5% CO_2 at 37°C. The cells were subcultured every 2-3 days with 0.25% (w/v) trypsin and 0.53 mM EDTA. The detached process was terminated by adding the MEM with fetal bovine serum. The cell suspension was centrifuged at 130 x g for 5 minutes. The supernatant was discarded and the cell pellet was resuspended with the medium. A subculture ratio was 1:10. For experiment, MDCK cells passage number in range of 65-82 were carried out. The cells were seeded on multi-well culture plate or cell culture dish at density of 20,000 cells per cm^2 and cultured for 4 days.

Cytotoxicity

This experiment was performed to consider concentrations of examined compounds that unchange the proportion of viable MDCK cell. The non-toxic concentrations were used in further study. The cell viability was measured by MTT reduction assay and neutral red dye uptake assay. MTT is a yellow water-soluble tetrazolium dye that is reduced by living cells to be purple formazan crystal, so the cell viability is evaluated from metabolic activity (83). For neutral red dye uptake assay, the dye is took up and retained in lysosome, but these processes are not maintained by nonviable cells (83, 84). Therefore, the properties of cell membrane

and lysosome are evaluated for survival cells in this assay. For experiment, MDCK cells were treated with various concentrations of glucose, fructose, and sorbitol for 72 hours. The ATP, adenosine, and other compounds which were conducted to investigate the involvement of signaling proteins were treated for 24 hours. After treatment, the cells were measured for viability by both assays. The statistically difference was considered under *p*-value less than 0.05 when compared with the control group. Moreover, the concentrations that change the percentage of viable cell proportion more than 10% of control was decision as toxic effect.

MTT reduction assay

MTT was dissolved in MEM without fetal bovine serum at a concentration of 0.2 mg/ml. The treated medium was changed to MTT solution and the cells were incubated for 1 hour in CO_2 incubator. Intracellular MTT was reduced by mitochrondrial reductase in viable cells to generate purple formazan crystal. The crystal was further dissolved with 200 µl DMSO and measured the absorbance at 570 nm.

Neutral red dye uptake assay

Briefly, neutral red was dissolved with PBS to a final concentration of 0.33% (w/v) before the experiment for 18-24 hours. The neutral red solution was centrifuged at 12,000 rpm for 10 minutes. The supernatant was diluted to 10% (v/v) with MEM without fetal bovine serum. The treated medium was changed to the diluted neutral red solution. The cells were incubated for 30 minutes in CO_2 incubator and then were washed three times with PBS. The intracellular neutral red was dissolved with 200 µl solubilizer solution containing 1% (v/v) acetic acid in 50% (v/v) ethanol and measured the absorbance at 560 nm.

Osmolality determination

Glucose, fructose, or sorbitol was dissolved in the MEM containing 10% (v/v) fetal bovine serum. The glucose and sorbitol were prepared at final concentrations of 5.5, 12.5, 25, 50, and 100 mM, while fructose was prepared at concentrations of 0.25, 0.5, 1, and 2 mM. The osmolality, calculated with comparative measurements from the freezing points of pure water and of samples, was measured by automatic cryoscopic osmometer. Each concentration was performed in triplicate. The osmolality values were compared with the medium, which contains glucose at 5.5 mM without fructose and sorbitol.

Treatment condition

The GLUT9 expression was determined by using RT-PCR analysis, Western blot analysis, and immunocytochemistry. Before MDCK cells were conducted either RNA isolation or protein harvest, the medium was replaced with medium containing tested substances. The concentrations of test substances chosen for treatment were relevant with pathophysiology of hyperglycemic condition and non-toxic to MDCK cells. The glucose was performed at concentrations of 12.5 and 25 mM. The fructose was performed at concentrations of 0.5 and 1 mM. The sorbitol at a concentration of 25 mM was performed to determine the effect of hyper-osmotic stress. Cells were treated with these substances for 24, 48, and 72 hours. Moreover, the signaling pathways that involved in long-term effect of hyper-osmotic stress were investigated by using 2 μM forskolin, 200 μM AICAR, 10 nM insulin, and 2 μM chelerythrine which are PKA activator, AMPK activator, tyrosine kinase activator, and PKC inhibitor, respectively. The cells were treated by these activators and inhibitor for 24 hours before determination of GLUT9 mRNA levels.

The exogenous ATP and adenosine, which can regulate purinergic system, were also investigated in this study. They were used at a concentration of 100 μM for 3, 6, 12, 18, and 24 hours. The involvement of purinergic P2 receptors and adenosine receptors were determined by using their antagonists, 100 μM suramin and 500 μM

caffeine, respectively. The antagonists were treated alone for 30 minutes before co-treated with 100 µM ATP or 100 µM adenosine for 6 hours. Moreover, cAMP analog at a concentration of 10 µM, forskolin at a concentration of 2 µM, H-89 at a concentration of 10 µM, and chelerythrine at a concentration of 2 µM were performed to confirm the possible signaling proteins that involve in regulating GLUT9 expression. Each compound was treated alone for 30 minutes before co-treated with 100 µM adenosine for 6 hours.

RT-PCR analysis for mRNA expression of *SLC2A9* gene (85)

The MDCK cells were washed twice by iced-cold PBS. The TRIzol® reagent was added and triturated by pipette. The homogenized sample was kept for 2-5 minutes before adding 150 µl of chloroform. The sample tube was vigorously shaken by hand and was centrifuged at 12,000 rpm, 4°C for 15 minutes. After centrifugation, the mixture was separated into an upper aqueous phase, an interphase, and a lower phenol-chloroform phase. The aqueous phase was collected for 150 µl. Total RNA was precipitated by adding iso-propanol 300 µl and keeping in refrigerator for 10 minutes. The RNA precipitate formed a gel-like pellet at the bottom of the tube after centrifugation at 12,000 rpm, 4°C for 10 minutes. The pellet was washed two times by 300 µl 75% (v/v) ethanol and centrifuged at 9,500 rpm, 4°C for 5 minutes. The pellet was dried by exposure to the air. After drying, the pellet was dissolved with 40 µl RNAse-free distilled water. The concentration of RNA was determined by measuring the absorbance at 260 nm using spectrophotometer. The purity of RNA product can be determined as the absorbance ratio of 260 nm / 280 nm higher than 1.6.

RT-PCR process, one microgram of total RNA was reverse-transcribed and amplified by Omniscript® reverse transcription kit and Taq DNA polymerase, respectively, following the manufacturer's instruction. Primers were designed from the NCBI primer design tool to recognize the mRNA of Canis lupus familiaris *SLC2A9* under NCBI reference sequence of NM_001130835.2. Furthermore, glyceraldehydes 3-

phosphate dehydrogenase (GAPDH) was used as a housekeeping gene for normalization. Their forward primers and reverse primers were shown in Table 1. The cycling protocol of cDNA amplification for 35 cycles consisted of denaturing temperature at 95°C for 45 seconds, annealing temperature at 55°C for 45 seconds, and extending temperature at 72°C for 45 seconds. The amplified products were fractionated with a constant 80 V for 1 hour on 2% (w/v) agarose gel. The PCR products on agarose gel were stained by ethidium bromide for 5 minutes, and then washed by distilled water for 30 minutes before detection of stained products using gel documentation. Intensity of the each band was analyzed by Image J software.

Oligo name	Sequence	Tm (°C)
GLUT9-forward	5'- TGA GAA GCA TGA CCA GGC AG -3'	66.1
GLUT9-reverse	5'- TCA GCC CAA AGC CAC CTA TG -3'	66.3
GAPDH-forward	5'- GTT TGT GAT GGG CGT GAA CC -3'	65.5
GAPDH-reverse	5'- AAG GTG GAA GAG TGG GTG TC -3'	65.6

Table 1 Primer sequences of GLUT9 and GAPDH genes.

Western blot analysis for GLUT9 protein expression (86)

The cells were washed and scraped in iced-cold PBS before centrifuged at 150 x g, 4°C for 10 minutes. The pellet was collected at -70°C until determination. The harvested cells were lyzed by RIPA buffer containing 50 mM Tris-hydrochloride (pH 8.0), 150 mM sodium chloride, 0.1% (w/v) sodium dodecyl sulfate, 0.5% (w/v) sodium deoxycholate, 1% (v/v) Triton X-100, and protease inhibitor cocktail and stood at 4°C for 1 hour. The sample was centrifuged at 12,000 rpm, 4°C for 15 minutes. Its supernatant was collected to measure the protein content using Bradford's method and determine the amount of GLUT9 protein (87).

The protein fractions were loaded at 10 µg total protein and separated on 12.5% (w/v) sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) with a constant 80 V for 3 hours. The separated proteins were transferred onto PVDF membrane with a constant 60 V for 2 hours. The membrane was blocked by 5% (w/v) bovine serum albumin in phosphate buffer saline with 0.1% (v/v) Tween20. Then, it was incubated with rabbit polyclonal anti-GLUT9 antibody (1:3,000) overnight at 4°C. This antibody recognizes the synthetic peptide VVTVIVTMAC YQLCGLNAIW FYTNSIFGKA GIPPAKIPYV TLSTGGIETL, which corresponding to internal amino acids sequence 295-344 of GLUT9 protein (NP_001124307.2) (Figure 2). Horseradish peroxidase-coupled goat polyclonal secondary antibody against rabbit IgG (1:5,000) and horseradish peroxidase-coupled goat monoclonal anti-actin antibody (1:50,000) were used to determine GLUT9 and actin proteins, respectively. The membrane was incubated with these antibodies for 2 hours at 4°C. The detection of GLUT9 and actin proteins on the membrane was performed using Luminata™ Crescendo kit and detected by gel documentation. Intensity of the each band was analyzed by Image J software.

solute carrier family 2, facilitated glucose transporter member 9 [Canis lupus familiaris]
Sequence ID: ref|NP_001124307.2| **Length:** 535 **Number of Matches:** 1

Range 1: 295 to 344 GenPept Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Method	Identities	Positives	Gaps
98.6 bits(244)	2e-25	Compositional matrix adjust.	46/50(92%)	48/50(96%)	0/50(0%)

```
Query  1    VVTVIVTMACYQLCGLNAIWFYTNSIFGKAGIPPAKIPYVTLSTGGIETL  50
           V+TVIVTMACYQLCGLNAIWFYTNSIFGKAGI P KIPY+TLSTGGIETL
Sbjct  295  VITVIVTMACYQLCGLNAIWFYTNSIFGKAGISPEKIPYITLSTGGIETL  344
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Figure 2 Blast searching between synthetic peptide that the anti-GLUT9 antibody recognizes and amino acid sequence of GLUT9 protein.

Immunocytochemistry for determined GLUT9 protein localization (13, 88)

Briefly, all process of this experiment was conducted at room temperature after the cells were cultured for 4 days on a cover slide in cultured dish. The cells were washed twice by PBS and fixation process was immediately performed by 4%

(w/v) paraformaldehyde in PBS for 15 minutes. The cell membrane was permeabilized by 0.1% (v/v) Triton-X100 for 5 minutes. A blocking reagent containing 5% (w/v) bovine serum albumin in PBS was carried out to incubate the cell monolayer for 30 minutes. The cells on cover slide were incubated overnight with the rabbit polyclonal anti-GLUT9 antibody (1:200) and then incubated with Alexa488-coupled goat polyclonal antibody against rabbit IgG for 1 hour. Each process was washed 3-5 times by PBS. The nucleus were stained with 1 μg/ml Hoechst 33342 for 10 minutes and washed by PBS. The cover slide was mounted with 70% (v/v) glycerol on glass slide before sealed by nail polish. The GLUT9 proteins and nuclei were determined using fluorescence microscope.

Statistical analysis

All values were expressed as mean ± SEM which was calculated from at least three independent experiments. Each experiment was conducted in duplicate. Statistical differences were analyzed by either one sample *t* test or independent-sample *t* test. In all cases, *p*-value less than 0.05 were considered to be statistically significant.

CHAPTER IV RESULTS

Determination of GLUT9 expression in MDCK cells

MDCK was mostly used as a model to assess the permeability of early drug discovery compounds as well as to examine the epithelial development of renal tubular cells. Since renal permeability is influenced by excretion and re-absorption processes, specific transporters involving in either processes play an important role in the determining the total transport of many compounds. GLUT9 is a novel glucose transporter that involves in glucose, fructose, and uric acid transportation in kidney. Therefore, we first investigated GLUT9 mRNA and protein expressions in MDCK cells by using RT-PCR and Western blot analysis, respectively. The MDCK cells were seeded at density of 20,000 cells per cm^2 and cultured for 4 days, which was the duration recommendation for permeability studies.

In RT-PCR experiment, the amplified product from GLUT9 primer was fractionated in lane 1 produced a single band at 389 bp, whereas, the PCR product of GAPDH was showed in lane 2 at 495 bp (Figure 3A). These sizes of mRNA products were similar to their predicted sizes. These observations indicated that GLUT9 mRNA can be detectable in MDCK cells. Moreover, duplex PCR technique which was amplification of the two PCR products in a single reaction tube was performed in this study. The result showed in Figure 3B demonstrated that the PCR products of GLUT9 and GAPDH displayed at same sizes when compared with each amplified product, which confirmed by two individual experiments. Therefore, this RT-PCR condition will be used further to study the effects hyperglycemic condition on GLUT9 mRNA expression.

In terms of GLUT9 protein, the total proteins extracted from MDCK cells were loaded at 10, 15, and 20 µg to find a proper condition for protein determination in this study. As seen in Figure 4A using Western blot analysis with anti-GLUT9 protein antibody, only one band of GLUT9 protein approximately appeared at 50 kDa. The results were detectable in every lane, and the intensity of each band depended on

the quantity of loaded proteins. Therefore, the total protein at 10 µg was chosen for GLUT9 protein investigation. An actin protein was used as a housekeeping protein for normalization of GLUT9 protein content. In Figure 4B, GLUT9 and actin proteins were showed at approximately 50 kDa and 42 kDa, respectively. Both molecular weights were similar to those observed in previous reports. Furthermore, the expression of GLUT9 protein in MDCK cells was confirmed by using immunocytochemistry technique. Figure 5 illustrated that GLUT9 proteins could be detected as fluorescence intensity of Alexa488-coupled antibody and represent GLUT9 proteins in MDCK cells. A negative control was conducted by cultured MDCK cells for 4 days and incubated with Alexa488-coupled antibody without pre-incubated with anti-GLUT9 antibody. The intensity of fluorescence in this condition was not detected.

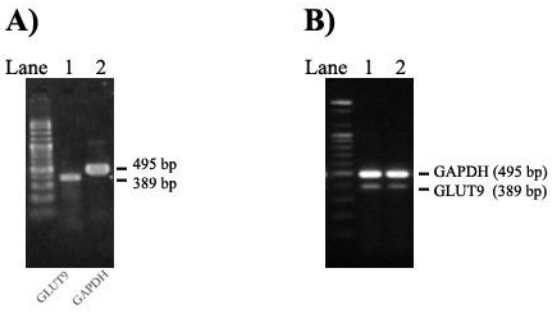


Figure 3 The expression of GLUT9 and GAPDH mRNA in MDCK cells.

The PCR products of GLUT9 (lane 1) and GAPDH (land 2) were fractionated in 2% (w/v) agarose gel and stained with ethidium bromide (A). The duplex PCR technique was conducted to amplify the PCR products of GLUT9 and GAPDH. The fractionated results in lane 1 and 2 come from individual experiment (B).

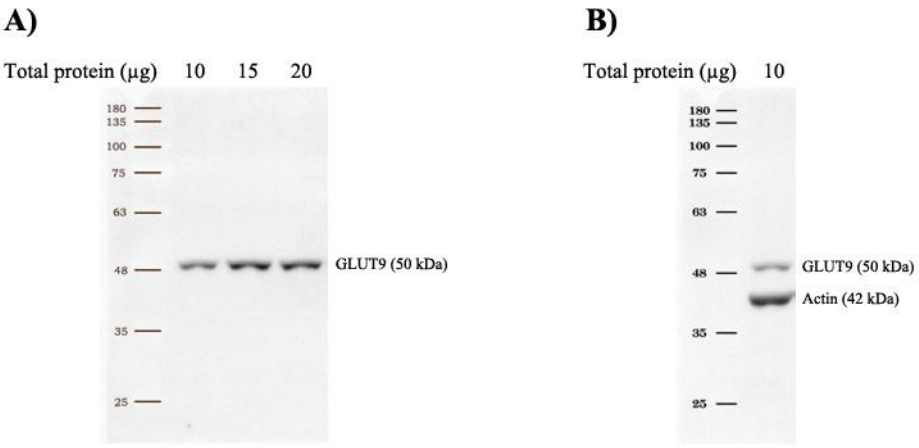


Figure 4 The expression of GLUT9 and actin proteins in MDCK cells.

The total protein isolated from MDCK cells was loaded at 10, 15, and 20 µg, and determined GLUT9 protein by using Western blot analysis (A). GLUT9 and actin proteins were determined in the same membrane (B).

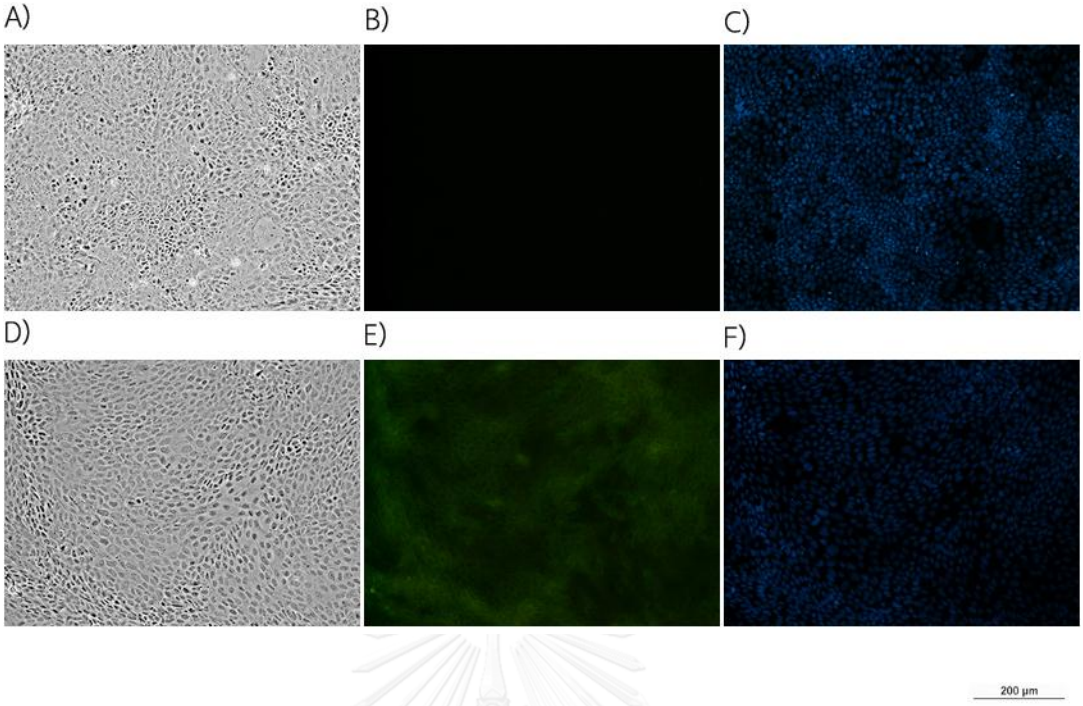


Figure 5 The fluorescence of GLUT9 protein in MDCK cells.

The GLUT9 protein was determined by using immunocytochemistry technique. MDCK cells were cultured for 4 days. The upper column showed negative control represented phase contrast (A), GLUT9 protein (B), and nuclei (C) under condition that was incubated with Alexa-488 antibody alone. The lower column showed control represented phase contrast (D), GLUT9 protein (E), and nuclei (F) under condition that was incubated with anti-GLUT9 antibody and Alexa-488 antibody, and Hoechst33342, respectively. Scale bar displayed 200 µm. This figure showed the result of one representative experiment from three individual experiments.

Cytotoxic determination

The cytotoxic effect of tested substances in MDCK cells were investigated for choosing their concentrations that did not affect cell survival for further experiments. The non-toxic concentrations were determined by using MTT reduction assay and neutral red dye uptake assay that measured based on metabolic activity and property of cell membrane, respectively. The glucose, fructose, and sorbitol were treated for 72 hours, which considered as the long-term effect, and their concentrations were performed in physiological range of these compounds found in hyperglycemic condition.

Glucose was investigated in concentration range of 12.5-100 mM represented hyperglycemic condition, whereas, glucose at a concentration of 5.5 mM was used as a control which related with concentration in plasma and MEM medium. In the MTT reduction assay, cell viability was enhanced by glucose but not significantly (Figure 6A). However, the results of neutral red dye uptake assay was not altered when compared with control (Figure 6B). This result indicated that intracellular metabolic activities were slightly increased, but properties of cell membrane were not changed. Therefore, the tested concentrations of glucose up to 100 mM could be used to investigate the effects hyperglycemic condition on GLUT9 expression. Moreover, the effect of fructose was also evaluated in this study. The concentration of fructose is very low in plasma, but it is increased to micromolar range in hyperglycemic condition. The concentration range of 0.25-2.00 mM of fructose was determined for the cytotoxic effect. The percentage of cell viability was unchanged by fructose in both cytotoxic assays, as shown in Figure 7.

In this study, cytotoxic effect of sorbitol was determined by using concentrations of 12.5-100 mM. The cell viability determined by MTT reduction assay was likely to increase when cells treated with sorbitol at concentration of 100 mM but not significantly (Figure 8A). However, the cell viability measured using neutral red dye uptake assay was significantly decreased to 87.1% and 82.7%, when treated with sorbitol at concentrations of 50 mM and 100 mM, respectively (Figure 8B).

Therefore, 25 mM sorbitol was chosen as the highest concentration that did not affect cell viability of MDCK cells.

Extracellular ATP and adenosine were reported to increase in cells cultured in high glucose concentration (16, 17). Therefore, they were evaluated for their effect on GLUT9 expression which their non-toxic concentrations were determined, first. However, these compounds were not stable, so the investigation of cytotoxic effect was conducted within 24 hours. The exogenous ATP was determined in concentration of 125-1,000 μM in this study. The results revealed that ATP did not affect the cell viability of MDCK cells determined by using MTT reduction assay and neutral red uptake assay (Figure 9). Adenosine concentration in range of micromolar has been reported to relevance with activation in physiological property, but its solubility was limited at maximum concentration of 200 μM in 0.5% (v/v) DMSO. Therefore, the exogenous adenosine was determined in concentration of 25-200 μM and 0.5% (v/v) DMSO was used as control. The results showed that the cell viability was not changed by adenosine at any concentrations. Therefore, the highest concentration of adenosine at 200 μM was used (Figure 10).

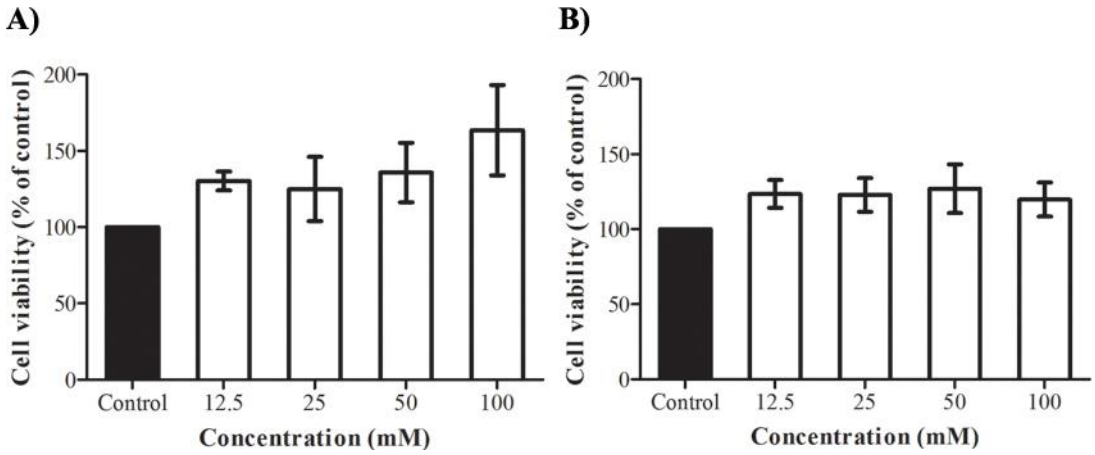


Figure 6 Cytotoxic effect of glucose at various concentrations.

The MDCK cells were treated with glucose for 72 hours. The cell viability compared with control group was measured by MTT assay (A) and neutral red uptake assay (B). Each value represented the mean ± SEM from 3 experiments.

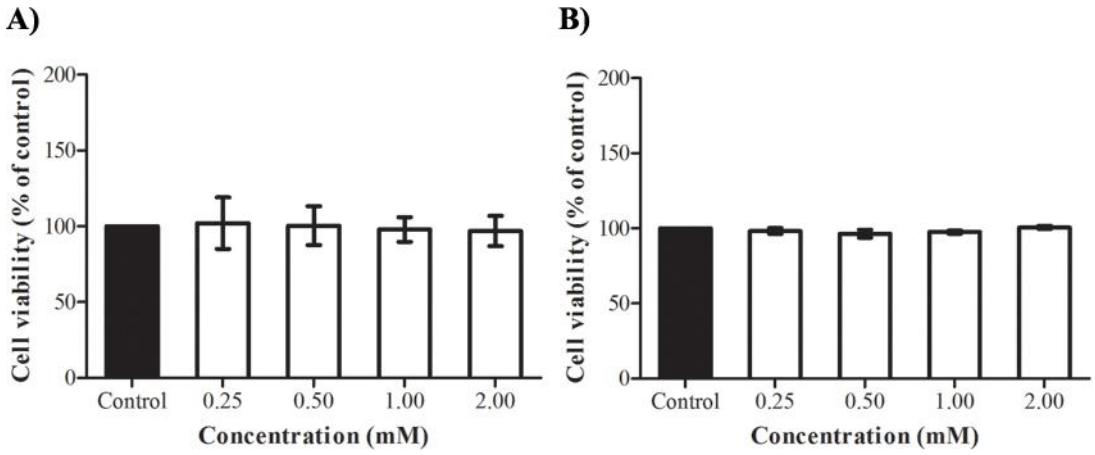


Figure 7 Cytotoxic effect of fructose at various concentrations.

The MDCK cells were treated with fructose for 72 hours. The cell viability compared with control group was measured by MTT assay (A) and neutral red uptake assay (B). Each value represented the mean ± SEM from 3 experiments.

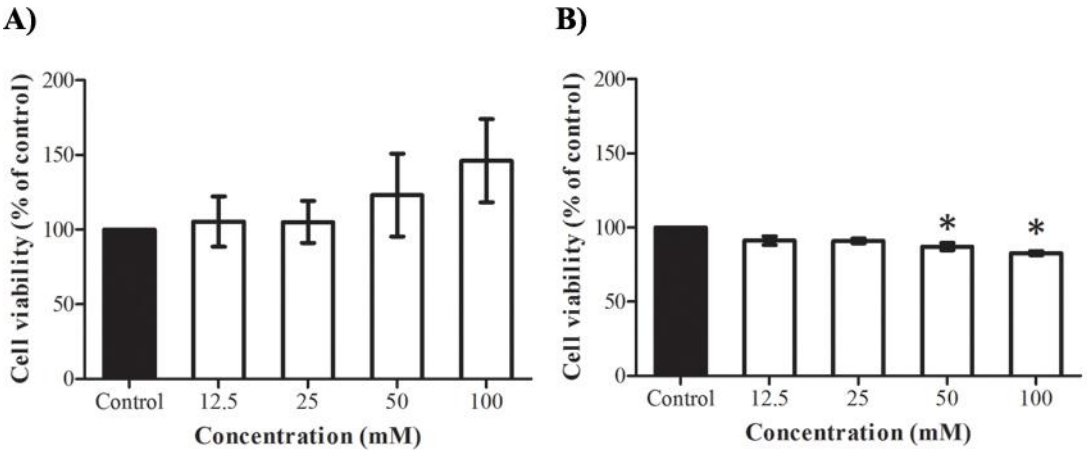


Figure 8 Cytotoxic effect of sorbitol at various concentrations.

The MDCK cells were treated with sorbitol for 72 hours. The cell viability compared with control group was measured by MTT assay (A) and neutral red uptake assay (B). Each value represented the mean ± SEM from 3-4 experiments. *, $p < 0.05$; significantly different versus control group.

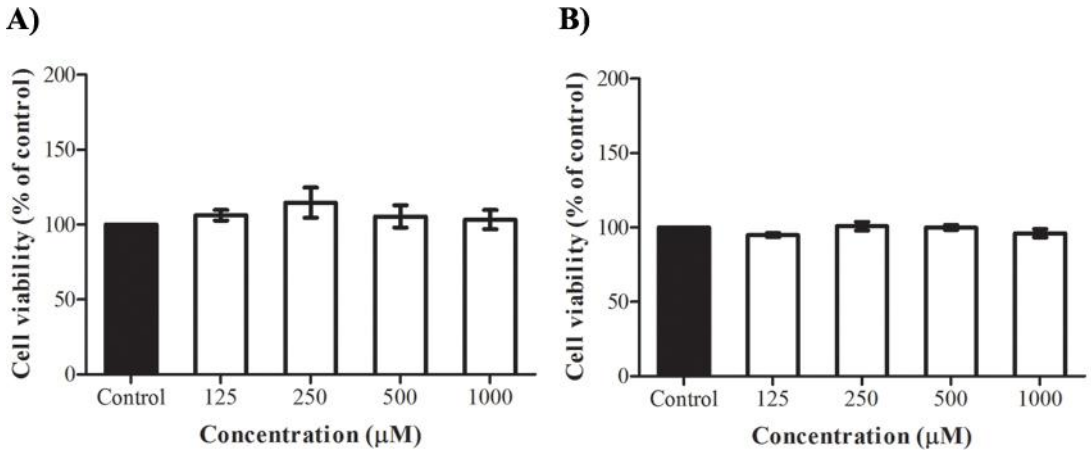


Figure 9 Cytotoxic effect of ATP at various concentrations.

The MDCK cells were treated with ATP for 24 hours. The cell viability compared with control group was measured by MTT assay (A) and neutral red uptake assay (B). Each value represented the mean ± SEM from 3 experiments.

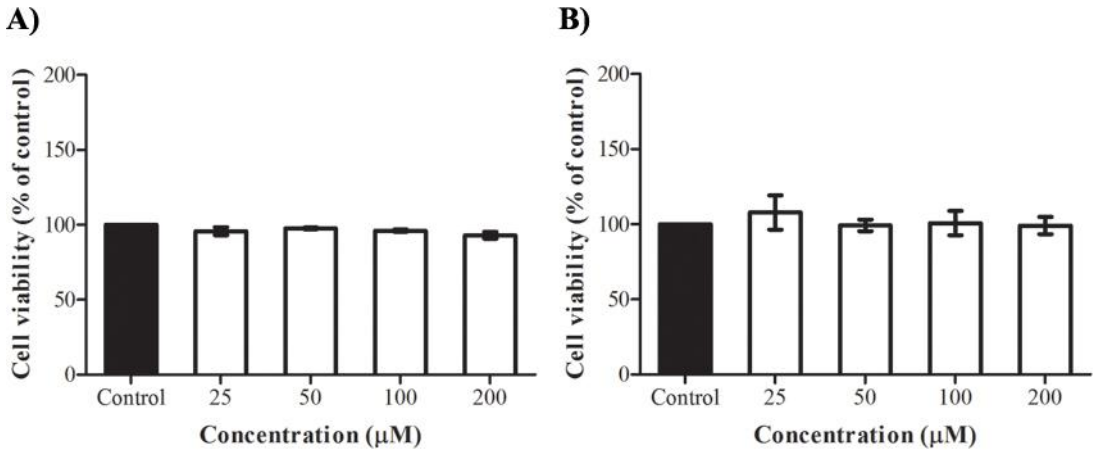


Figure 10 Cytotoxic effect of adenosine at various concentrations.

The MDCK cells were treated with adenosine for 24 hours. The cell viability compared with control group was measured by MTT assay (A) and neutral red uptake assay (B). Each value represented the mean ± SEM from 3 experiments.

Effect of compounds involved in hyperglycemic condition on GLUT9 expression

Glucose and fructose

Hyperglycemia is defined as a condition which has a high plasma glucose level and is a major cause of complication in diabetic patients (1). The long-term of hyperglycemic condition is a risk of diabetic development. Not only glucose that is involved in hyperglycemic condition, but fructose is also increased in plasma and urine (29). Although plasma fructose level is found in range of micromolar, it can lead to the diabetic complication such as nephropathy, retinopathy, neuropathy, and atherosclerosis (58). Moreover, both substances have been reported as substrates of GLUT9 for transportation across the renal tubular cells. Therefore, this study investigated the effect of glucose and fructose which involved in hyperglycemic condition on GLUT9 expression in long-term treatment.

The glucose at concentrations of 12.5 mM and 25 mM, represented as hyperglycemic condition, was used to determine their effect on GLUT9 expression in terms of mRNA and protein. As shown in Figure 11A, the level of GLUT9 mRNA was not altered by 12.5 mM glucose when the cells were treated for 24, 48, and 72 hours. However, glucose at concentration of 25 mM decreased the GLUT9 mRNA in time-dependent manner. The mRNA level was significantly decreased by 43.2% after cells were treated with 25 mM glucose for 72 hours. These observations indicated that GLUT9 mRNA was decreased only when cells were treated with 25 mM glucose for 72 hours. The effect of 12.5 mM and 25 mM glucose that treated for 24, 48, and 72 hours on GLUT9 protein levels were illustrated in Figure 11B. The protein levels seemed to increase when the cells were treated by 25 mM glucose for 48 and 72 hours but not significantly. The mean of GLUT9 protein levels were increased 1.3 ± 0.1-fold by 25 mM glucose for 72 hours. As shown in Figure 12, there were discrepancy between the effect of 25 mM glucose on GLUT9 mRNA and GLUT9 protein when treated cells for 72 hours.

From Figure 11, glucose seemed to have an effect on GLUT9 proteins, so the concentration-response of glucose was determined. The cells were treated with glucose at concentrations of 12.5 mM, 25 mM, and 50 mM for 72 hours before the GLUT9 proteins were investigated. As seen in Figure 13, the GLUT9 protein levels were enhanced by glucose in a concentration-dependent manner, and significantly increased to 1.9 ± 0.2-fold by glucose at a concentration of 50 mM when compared with control.

Fructose is another contributing factor of hyperglycemic condition being investigated for its effect on GLUT9 expression in MDCK cells. The cells were treated with 0.5 mM and 1.0 mM fructose, which were considered as a high concentration in hyperglycemic condition, for 24, 48, and 72 hours before GLUT9 mRNA levels were determined. Figure 14A illustrated that the GLUT9 mRNA levels were not changed when cells were treated with fructose in all conditions compared with that of control. Furthermore, 1 mM fructose was used to determine the effect on GLUT9 protein levels. The results were found that the GLUT9 protein levels were not altered by treated cells with 1 mM fructose (Figure 14B). Therefore, it can be concluded that fructose had no effect on GLUT9 expression both of mRNA and protein levels as shown in Figure 15.

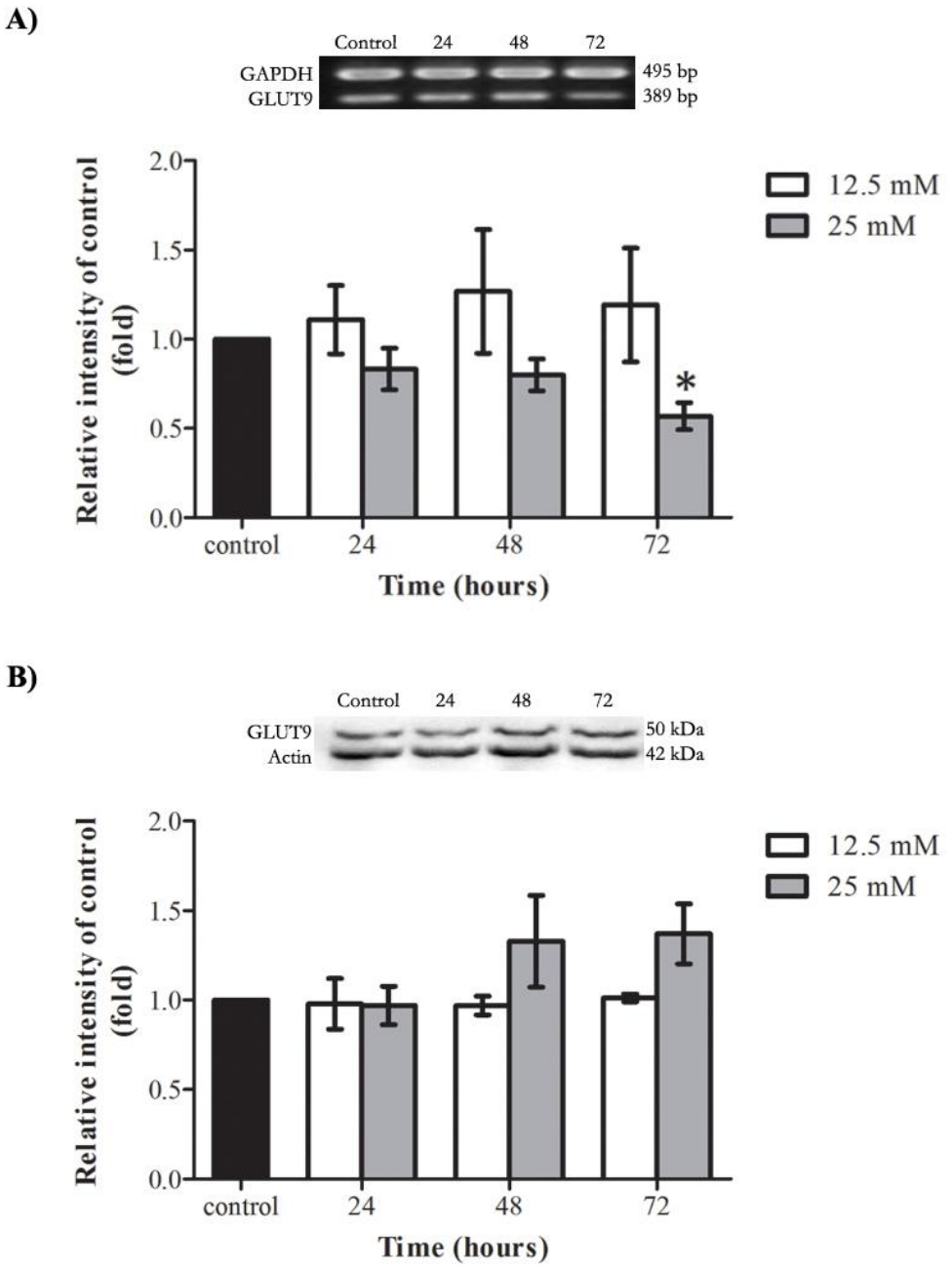


Figure 11 Effect of glucose on the GLUT9 expression.

Effect of glucose on the GLUT9 expression in terms of mRNA (A) and protein (B). The glucose was conducted at concentration of 12.5 mM (open bar) and 25 mM (close bar) for 24, 48, and 72 hours. The upper photograph showed the results of one representative experiment from treated with 25 mM glucose. Each value represented the mean ± SEM from 3-4 experiments. *, $p < 0.05$; significantly different versus control group.

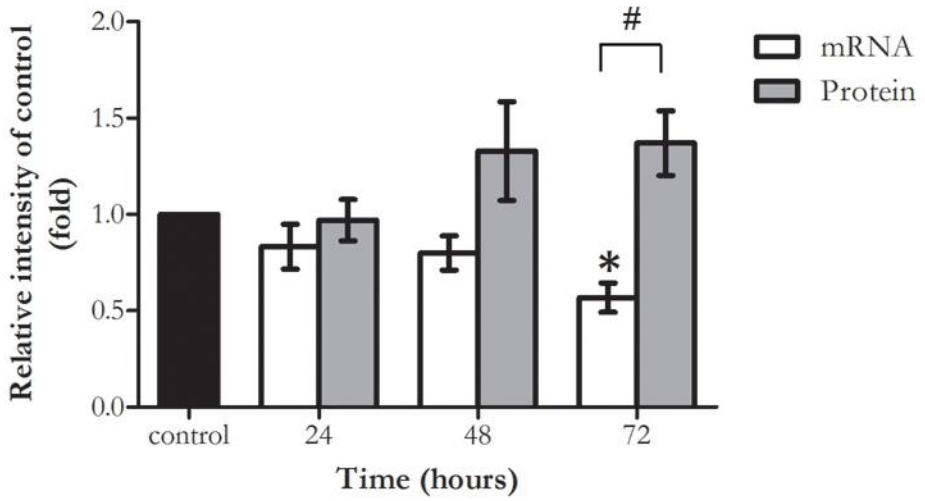


Figure 12 Effect of 25 mM glucose on the GLUT9 expression.

The cells were treated with 25 mM glucose for 24, 48, and 72 hours. The expression was showed in GLUT9 mRNA level (open bar) and in GLUT9 protein level (close bar). Each value represented the mean ± SEM from 3-4 experiments. *, $p < 0.05$; significantly different versus control group. #, $p < 0.05$; significantly different between mRNA level and protein level.

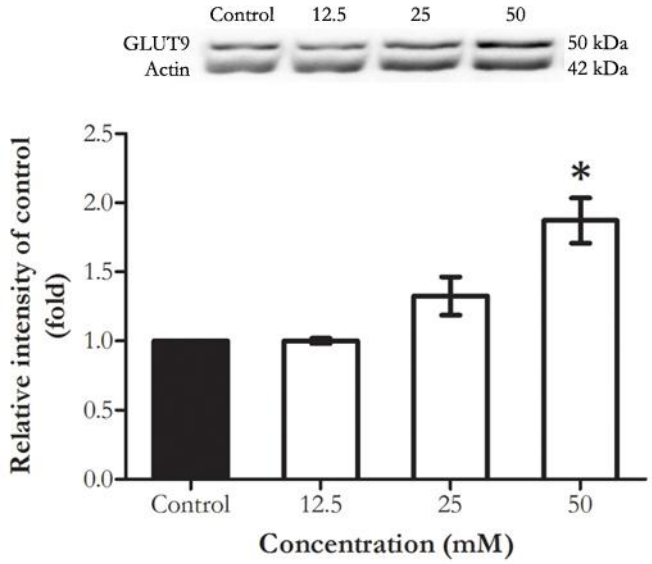


Figure 13 Concentration-response of glucose on GLUT9 protein expression.

The cells were treated with glucose at various concentrations for 72 hours. The upper photograph showed the results of one representative experiment. Each value represented the mean ± SEM from 4-5 experiments. *, $p < 0.05$; significantly different versus control group.

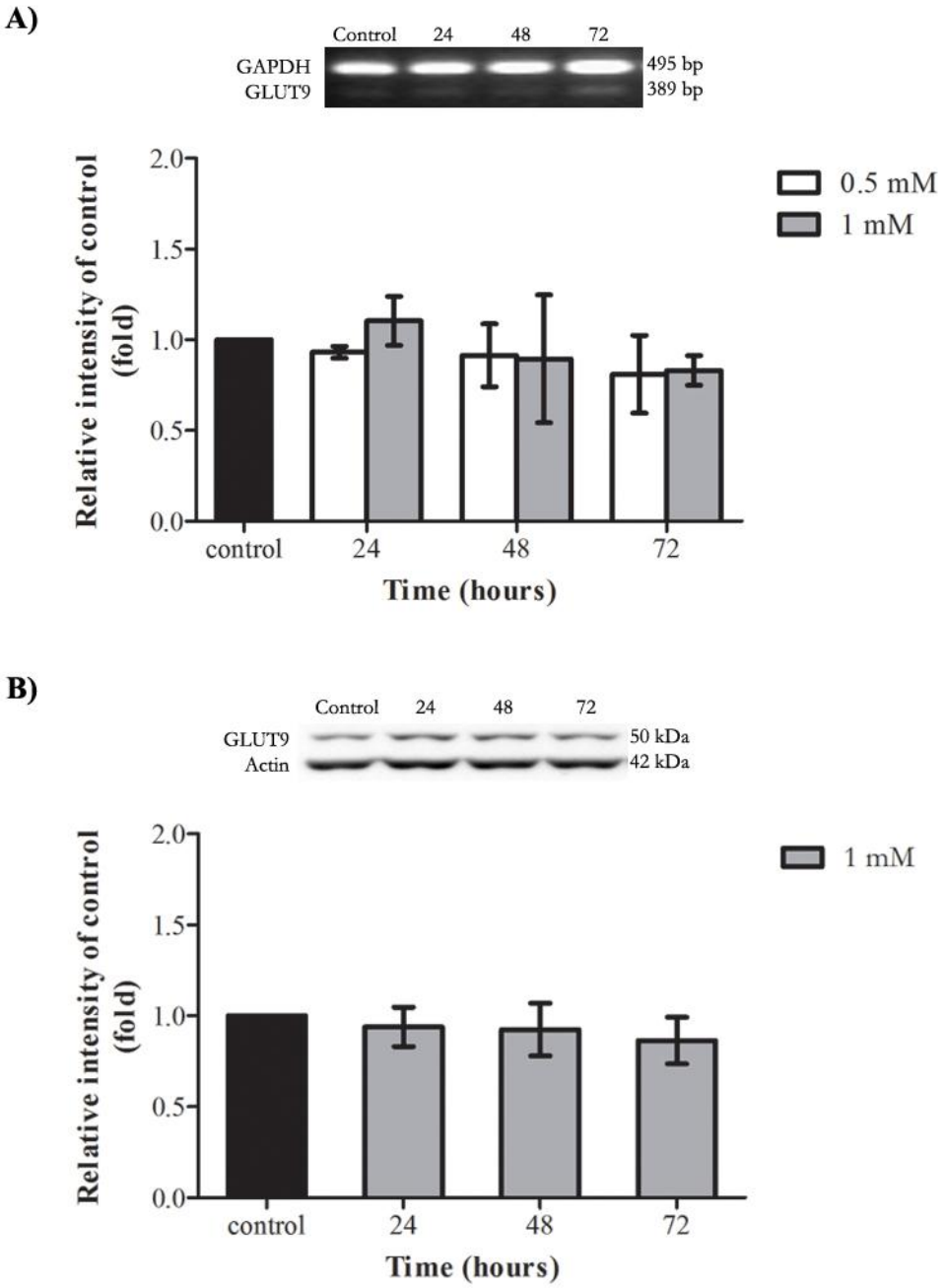


Figure 14 Effect of fructose on the GLUT9 expression.

Effect of fructose on the GLUT9 expression in terms of mRNA (A) and protein (B). The fructose was conducted at concentration of 0.5 mM (open bar) and 1 mM (close bar) for 24, 48, and 72 hours. The upper photograph showed the results of one representative experiment from treated with 1 mM fructose. Each value represented the mean ± SEM from 3-4 experiments.

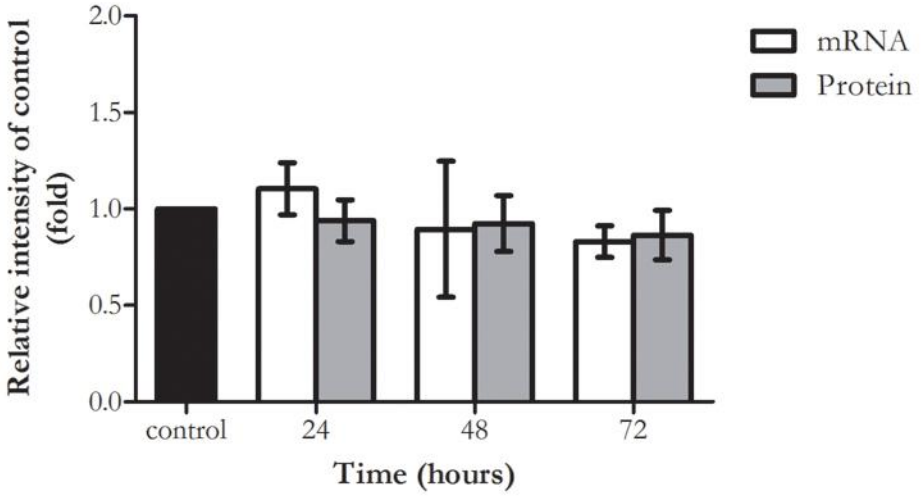


Figure 15 Effect of 1 mM fructose on GLUT9 expression.

The cells were treated with 1 mM fructose for 24, 48, and 72 hours. The expression was showed in GLUT9 mRNA level (open bar) and in GLUT9 protein level (close bar). Each value represented the mean ± SEM from 3-4 experiments.

Osmolality determination

Hyperosmolar state in diabetic patient is also involved in hyperglycemic condition. The osmolality value is depended on the level of plasma glucose. The changing of osmolality can influence cellular physiological and biochemical properties. Therefore, the osmolality values of sugar such as glucose, fructose, and sorbitol were evaluated.

The MEM medium containing 10% (v/v) fetal bovine serum was conducted as a control. The osmolality measurement of the medium was shown the value equal to 306.3 ± 2.7 mOsmol/Kg, which was similar to physiological osmolality in plasma. The osmolality of medium was significantly increased by glucose in concentration range of 12.5-100 mM when compared with the complete medium, containing 5.5 mM glucose. In contrast, fructose in concentration range of 0.25-2.00 mM had no effect on osmolality of the medium.

Sorbitol, a glucitol or slow-metabolizing sugar, can increase the osmolality value similar to glucose (89). Thus, present study determined the osmolality values of sorbitol in concentration range of 5.5-100 mM. The osmolality was increased by sorbitol in a concentration-dependent manner. The osmolality value of 5.5 mM sorbitol was similar with control group that containing 5.5 mM glucose. The osmolality values of sorbitol and glucose at the same concentration were compared in Figure 16. The results were found that each tested concentrations showed similar osmolality values. Therefore, sorbitol can be used to determine the hyper-osmotic stress effect of glucose in the following experiments. However, the sorbitol at concentration of 50 mM and 100 mM had some effect on MDCK cell viability (Figure 8). Therefore, sorbitol at a concentration of 25 mM was used as a non-toxic concentration, as a representator of 25 mM glucose, for determination of hyper-osmotic stress effect on GLUT9 expression in MDCK cells in next study. The osmolality value was increased to 326.5 ± 1.7 and 327.4 ± 3.3 mOsmol/Kg by glucose and sorbitol, respectively, at concentration of 25 mM.

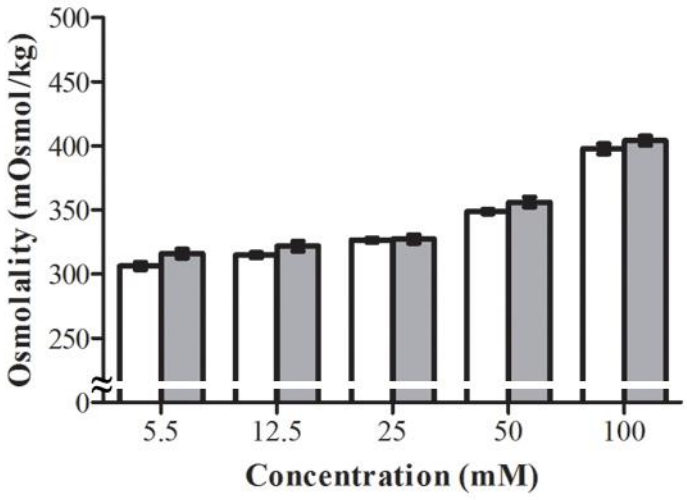


Figure 16 Comparative of osmolality values between glucose and sorbitol.

Comparative of osmolality values between glucose (open bar) and sorbitol (close bar) at the same concentration. Each value represented the mean ± SEM from 3-4 experiments.

Effect of hyper-osmotic stress on the GLUT9 expression

Elevation of blood glucose can cause hyperosmolar hyperglycemic state which is a life-threatening endocrine emergency (33). The high concentration of glucose can increase the osmolality in cell culture medium. Since previous observations have reported that hyper-osmotic stress involved in regulation of intracellular physiological and biochemical properties, the present study also determined the effect of hyper-osmotic stress on GLUT9 expression. Sorbitol at concentration of 25 mM was chosen to determine the hyper-osmotic stress effect of 25 mM glucose on GLUT9 expression in MDCK cells. The cells were treated with 25 mM sorbitol before determination of GLUT9 mRNA and protein. Figure 17 illustrated that the mRNA levels of GLUT9 were significantly decreased by treated cells with 25 mM sorbitol for 48 and 72 hours. The GLUT9 mRNA levels decreased by sorbitol were compared with the result of glucose that also significantly decreased the mRNA level after treated for 72 hours (Figure 18). Although the GLUT9 mRNA levels were decreased by hyper-osmotic stress, the GLUT9 proteins were not altered. As shown in Figure 19, the GLUT9 protein levels were not changed when cells treated with 25 mM sorbitol for 24, 48, and 72 hours, when compared with control.

The present study also investigated the effect of possible signaling proteins that involved in hyper-osmotic stress on GLUT9 mRNA, since 25 mM sorbitol showed an effect only on GLUT9 mRNA. Previous reports demonstrated that hyper-osmotic stress can trigger numerous types of signaling proteins such as PKA, AMPK, tyrosine kinase, and PKC. Therefore, the signaling pathways that involved in long-term effect of hyper-osmotic stress were investigated by using 2 μM forskolin, 200 μM AICAR, 10 nM insulin, and 2 μM chelerythrine which are PKA activator, AMPK activator, tyrosine kinase activator, and PKC inhibitor, respectively. The MDCK cells were treated with activators or inhibitors of signaling proteins at non-toxic concentrations for 24 hours. The GLUT9 mRNA was significantly decreased only by 2 μM forskolin, but was not changed by the other compounds (Figure 20).

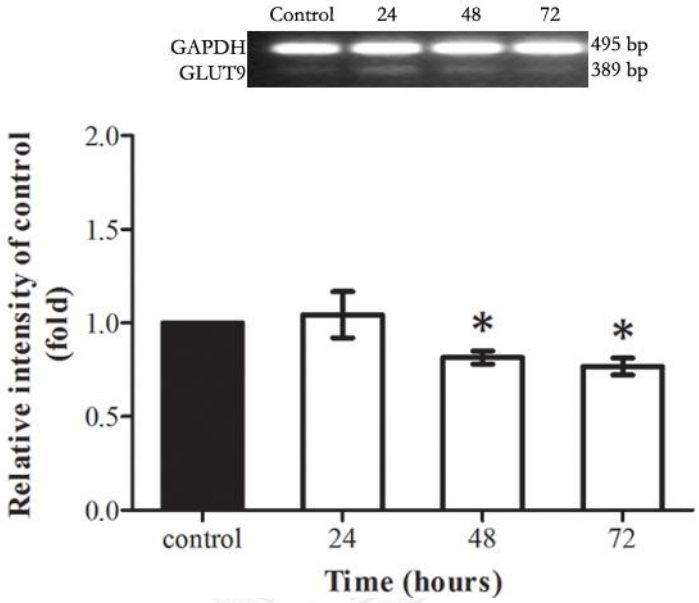


Figure 17 Effect of sorbitol on the GLUT9 mRNA expression.

The sorbitol at concentration of 25 mM was conducted for 24, 48, and 72 hours. The upper photograph showed the results of one representative experiment. Each value represented the mean ± SEM from 3-4 experiments. *, $p < 0.05$; significantly different versus control group.

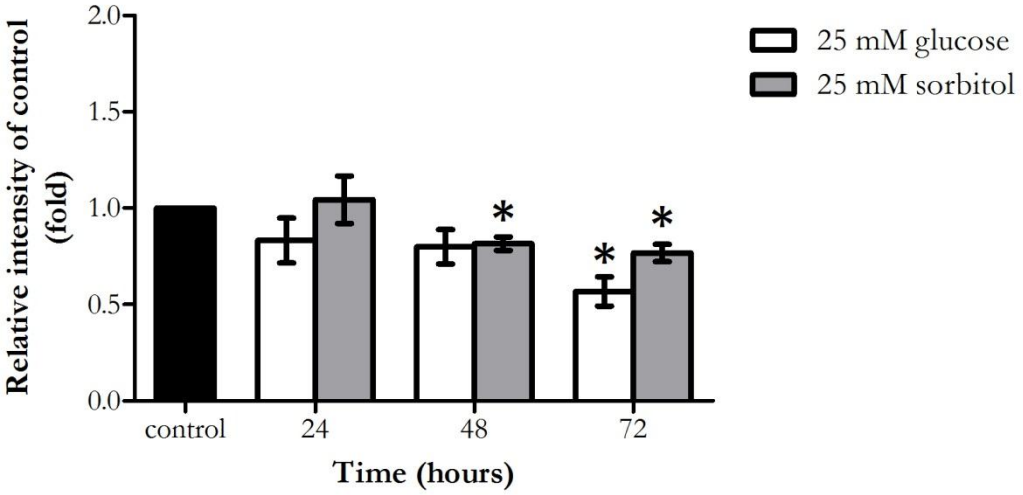


Figure 18 Comparative effects of glucose and sorbitol on the GLUT9 mRNA level.

The glucose and sorbitol at concentration of 25 mM were conducted for 24, 48, and 72 hours. Each value represented the mean ± SEM from 3-4 experiments. *, $p < 0.05$; significantly different versus control group.

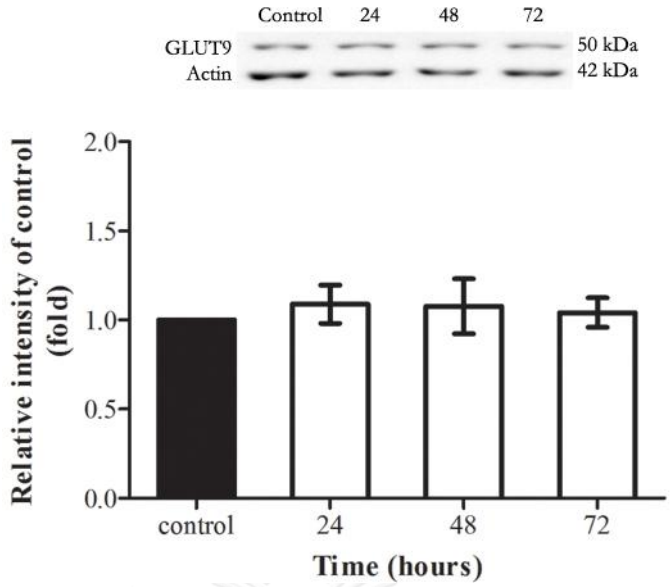


Figure 19 Effect of sorbitol on the GLUT9 protein expression.

The sorbitol at concentration of 25 mM was conducted for 24, 48, and 72 hours. The upper photograph showed the results of one representative experiment. Each value represented the mean ± SEM from 3-4 experiments.

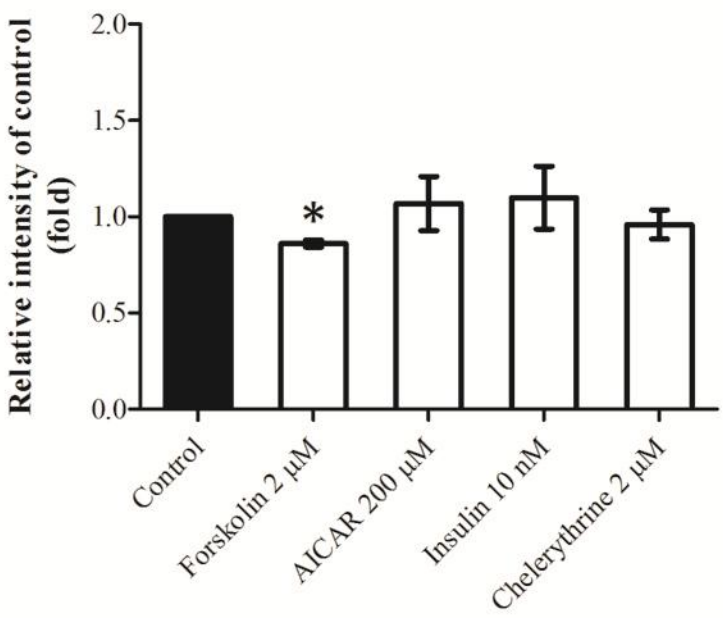


Figure 20 Effect of activators and inhibitor of signaling proteins on the GLUT9 mRNA.

The cells were treated with 2 μM forskolin (PKA activator), 200 μM AICAR (AMPK activator), 10 nM insulin (tyrosine kinase activator), or 2 μM chelerythrine (PKC inhibitor) for 24 hours. Each value represented the mean ± SEM from 3-4 experiments. *, $p < 0.05$; significantly different versus control group.

Effect of exogenous ATP on GLUT9 expression

Our observations revealed that high concentration of glucose in long-term treatment decreased the GLUT9 mRNA, but increased the GLUT9 protein significantly. The suppression of GLUT9 mRNA by glucose may due to its hyper-osmotic stress, as demonstrate in our previous experiments using 25 mM sorbitol. However, the GLUT9 protein levels were not changed by sorbitol, whereas, they were increased by glucose. Therefore, this study investigated the other mechanisms in term of hyperglycemic condition that influence GLUT9 protein levels.

Glucose as primary source of energy synthesis generates ATP and previous observations have demonstrated that long term-glucose treatment in cell culture model can enhance the extracellular ATP (16, 17). Therefore, the exogenous ATP was conducted to determine its effect on GLUT9 expression. The cells were treated with 100 μM ATP for evaluation of time-response on GLUT9 expression. As shown in Figure 21, the GLUT9 mRNA was not significantly altered by ATP. On the other hand, the ATP significantly increased GLUT9 protein levels during the course of 3-12 hours which reach maximum at 6 hours, whereas, this effect was decreased to the control level after 18-hour periods. Since the effect of ATP on GLUT9 protein expression was highest at 6 hours, this condition was conducted for concentration-response study in following experiments. The cells were treated with ATP in concentration range of 25-400 μM before the GLUT9 protein levels were determined. The level of GLUT9 proteins were significantly increased by ATP at concentrations of 50 μM and 100 μM, whereas, this effect was decreased to control level at concentrations of 200 μM and 400 μM (Figure 22).

The extracellular ATP regulates intracellular physiological and biochemical properties through purinergic P2 receptors. The effects of extracellular ATP on GLUT9 protein expression *via* purinergic P2 receptors were investigated using suramin as a non-selective P2 antagonist at a concentration of 100 μM. As seen in Figure 23, the level of GLUT9 protein was not altered by 100 μM suramin when compared with control. Moreover, the cells were performed in co-treatment condition of 100 μM ATP and 100 μM suramin, the mean of GLUT9 protein level was similar with control

and 100 μM suramin alone treatment. These observations indicated that 100 μM suramin had no effect on GLUT9 protein expression.

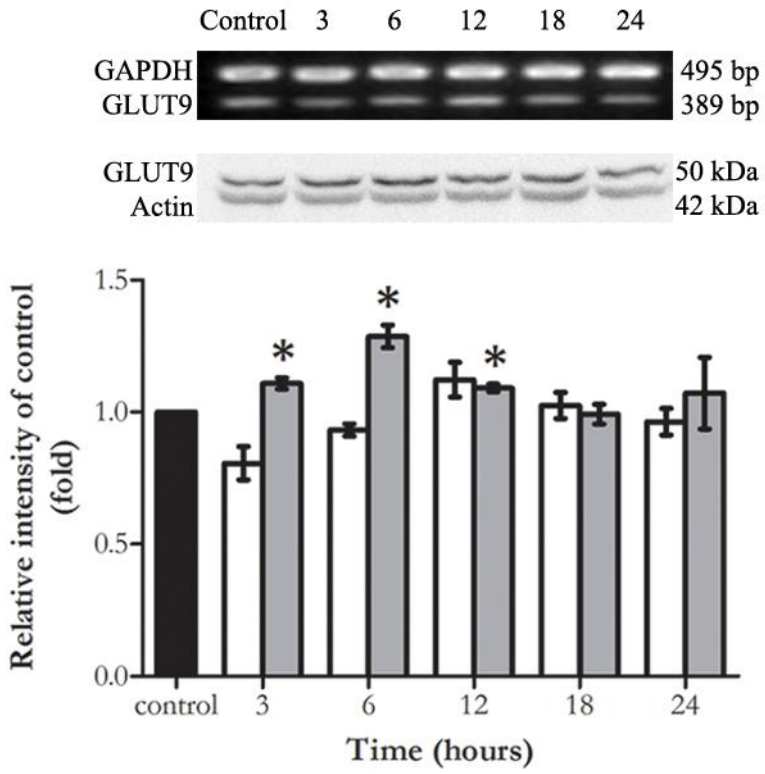


Figure 21 Effect of exogenous 100 μM ATP on the GLUT9 expression.

Data was shown the level of GLUT9 mRNA (open bar) and the level of GLUT9 proteins (close bar). The upper photograph showed the results of one representative experiment from RT-PCR and Western blot analysis. Each value represented the mean ± SEM from 3-6 experiments. *, $p < 0.05$; significantly different versus control group.

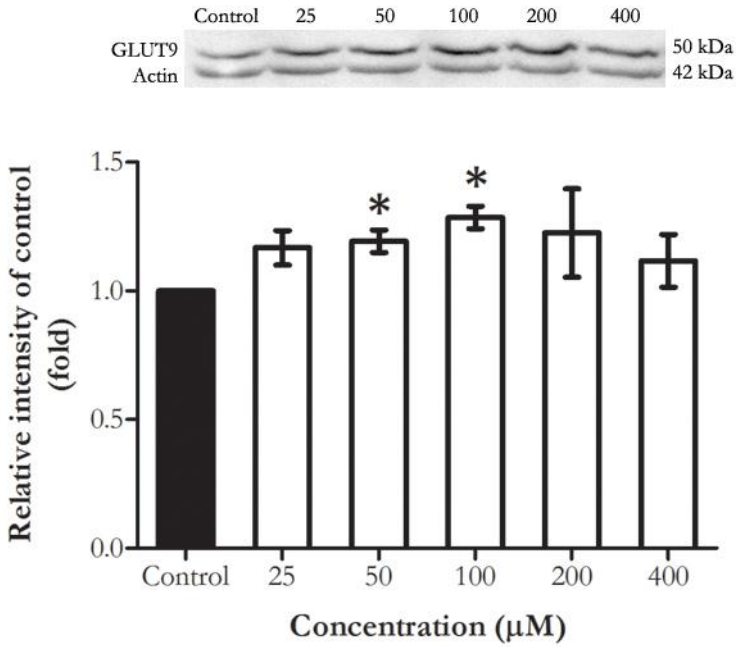


Figure 22 Concentration-response of exogenous ATP on the GLUT9 proteins.

The cells were treated with various concentrations of ATP for 6 hours. The upper photograph showed the results of one representative experiment from RT-PCR and Western blot analysis. Each value represented the mean ± SEM from 3-6 experiments. *, $p < 0.05$; significantly different versus control group.

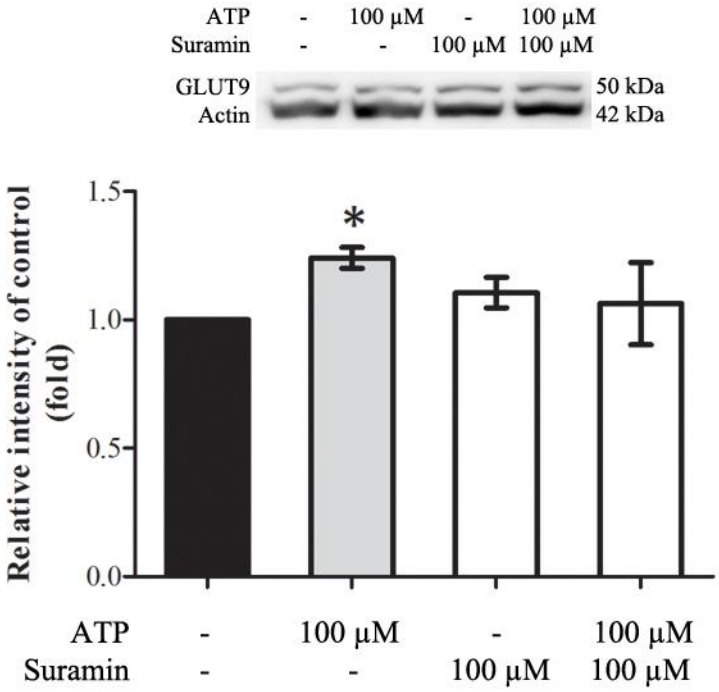


Figure 23 Effect of suramin on the GLUT9 protein expression.

Effect of purinergic P2 receptor antagonist, suramin, on the GLUT9 protein expression. The cells were treated by ATP and/or suramin for 6 hours. The upper photograph showed the results of one representative experiment. Each value represented the mean ± SEM from 3-5 experiments. *, $p < 0.05$; significantly different versus control group.

Effect of exogenous adenosine on GLUT9 expression

Since extracellular ATP was immediately broken-down by brush-border enzymes to be adenosine, previous reports showed that it regulated cell function through adenosine receptors (20). Therefore, the effects of exogenous ATP on GLUT9 expression through adenosine effect were evaluated. Present study, the MDCK cells were treated with 100 μM adenosine which related with concentration effect of ATP for evaluation of time-response on GLUT9 expression. Figure 24 illustrated the time-response of adenosine effect on GLUT9 expression in term of mRNA and protein levels. The 100 μM adenosine significantly decreased the level of GLUT9 mRNA when the cells were treated for 6 hours, while this effect was increased to the control level after 12-hours periods. In contrast, the GLUT9 protein levels were significantly increased by 100 μM adenosine for 6 hours. However, the GLUT9 protein levels were significantly decreased when treated cells for 18 hours and recovered to the control level after 24 hours. These observations indicated that the effects of adenosine on GLUT9 expression in term of mRNA and protein were highest at 6 hours, so this condition was used in following experiments.

The extracellular adenosine can also regulate the cell function through purinergic system by specific binding with adenosine receptors. Caffeine as a non-selective adenosine receptor antagonist was used to study the involvement of these receptors. GLUT9 mRNA was significantly decreased in cells treated with 100 μM adenosine for 6 hours. The mRNA levels were determined after treated cells with 100 μM adenosine alone or together with 500 μM caffeine. As seen in Figure 25, the GLUT9 mRNA levels were not changed by 500 μM caffeine, whereas, it was significantly decreased when co-treatment with 100 μM adenosine and 500 μM caffeine. The resulting of co-treatment cells with 100 μM adenosine and 500 μM caffeine suggested that the effect of adenosine on GLUT9 mRNA was slightly inhibited by 500 μM caffeine but not significantly. On the other hand, the effect of adenosine on GLUT9 protein was significantly inhibited by 100 μM and 500 μM caffeine, whereas, the GLUT9 protein levels were not changed when treated cells with 500 μM caffeine alone, as shown in Figure 26. These results indicated that

caffeine could suppress GLUT9 protein levels-induced by adenosine in a concentration-dependent manner. This finding suggested that adenosine can increase GLUT9 protein level *via* adenosine receptors.

The adenosine receptors which coupled with G-protein are associated with stimulation or inhibition of adenylate cyclase resulting to increase or decrease the intracellular cAMP concentration, respectively (20, 70). The cAMP then trigger PKA as a major signaling pathway, so a contributing pathway involved in inducing GLUT9 protein expression in MDCK cells was evaluated by using PKA activator and inhibitor. However, activation of adenosine receptors can also partly regulate through PKC. Since the effect of adenosine through these receptors resulted in increasing GLUT9 protein levels, the present study focused on investigation of possible signaling proteins such as PKA and PKC. As seen in Figure 27, the PKA activators, *i.e.* 10 μM cAMP analog and 2 μM forskolin, decreased the GLUT9 protein levels, whereas, the PKA inhibitor, 10 μM H-89, slightly increased the GLUT9 protein but not significantly. The co-treatment condition between 100 μM adenosine and either 10 μM cAMP analog or 2 μM forskolin resulted in significantly decrease the GLUT9 protein when compared with 100 μM adenosine treatment alone. Moreover, the resulting of co-treatment was also similar the level of control in the absence of adenosine. On the other hand, the effect of 100 μM adenosine on GLUT9 protein was not inhibited by 10 μM H-89.

In addition, a chelerythrine at concentration of 2 μM was chosen as a PKC inhibitor to evaluate the possible signaling protein that might involve in regulation of GLUT9 protein through adenosine receptors *via* PKC. The results were shown in Figure 28. The GLUT9 protein levels were not altered when compared with control in cells treated with 2 μM chelerythrine. Moreover, 2 μM chelerythrine could not alter the induction effect of 100 μM adenosine on GLUT9 protein.

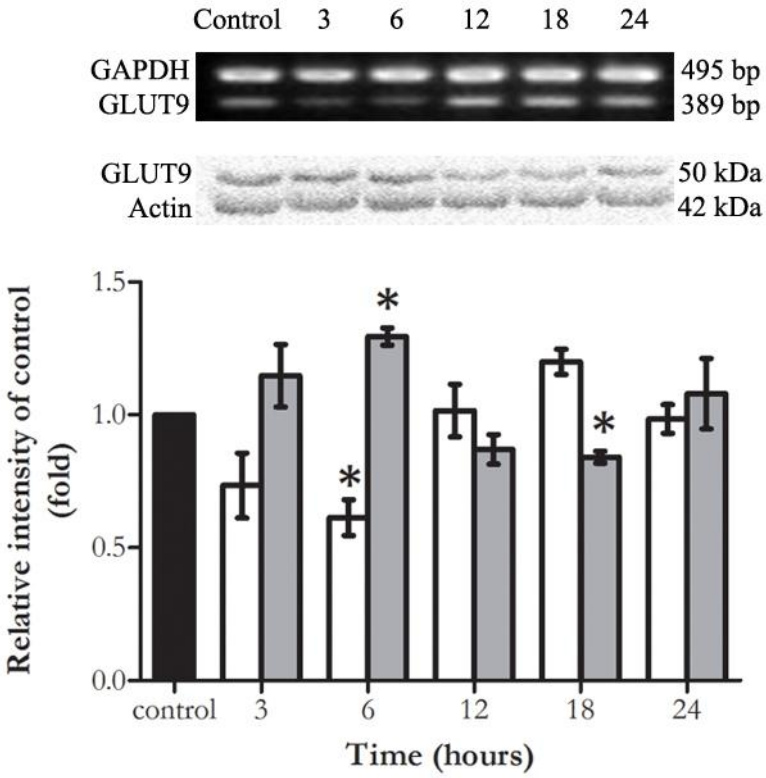


Figure 24 Effect of exogenous 100 μM adenosine on the GLUT9 expression.

Data was shown the level of GLUT9 mRNA (open bar) and the level of GLUT9 protein (close bar). The upper photograph showed the results of one representative experiment from RT-PCR and Western blot analysis. Each value represented the mean ± SEM from 3-6 experiments. *, $p < 0.05$; significantly different versus control group.

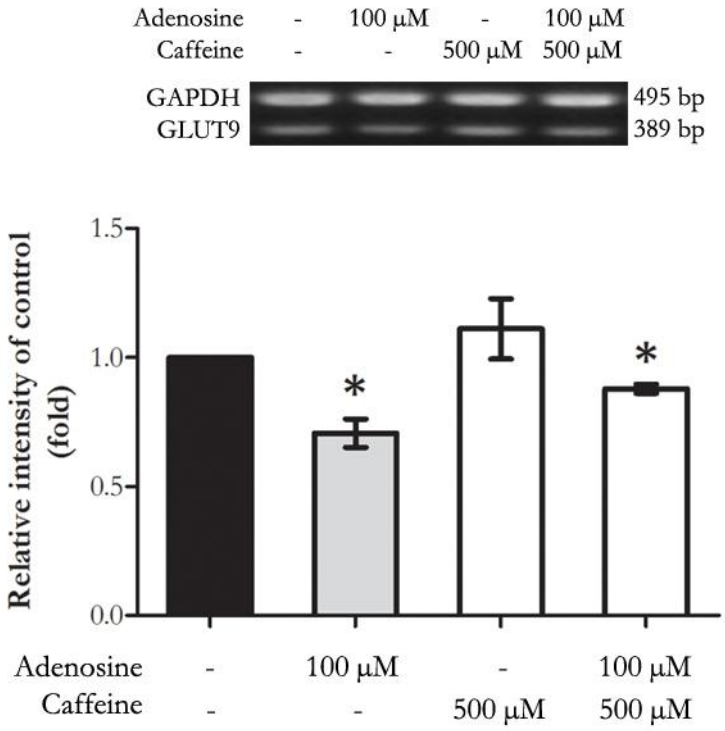


Figure 25 Effect of caffeine on the GLUT9 mRNA expression.

Effect of adenosine receptor (P1) antagonist, caffeine, on the GLUT9 mRNA expression. The cells were treated by adenosine and/or caffeine for 6 hours. The upper photograph showed the results of one representative experiment. Each value represented the mean ± SEM from 3-6 experiments. *, $p < 0.05$; significantly different versus control group.

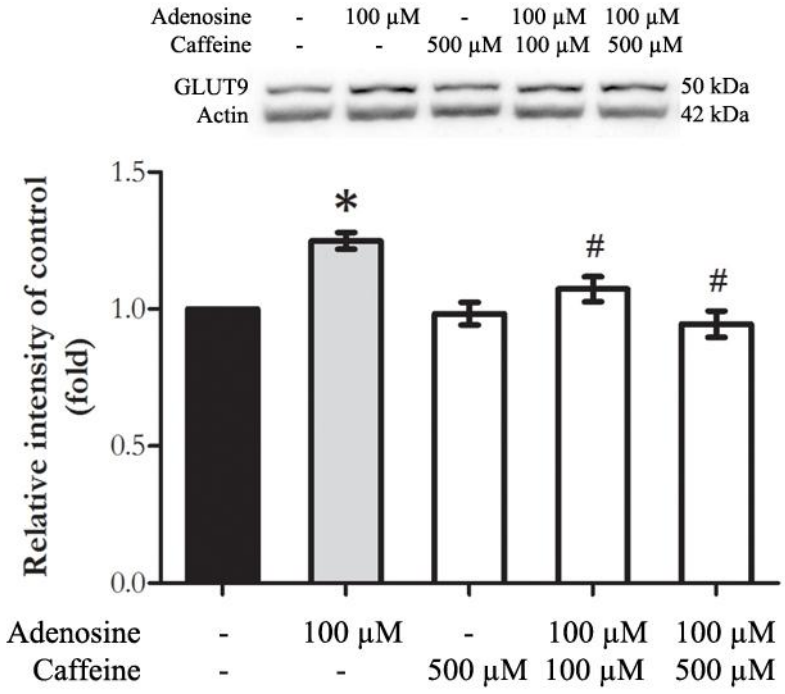


Figure 26 Effect of caffeine on the GLUT9 protein expression.

Effect of adenosine receptor (P1) antagonist, caffeine, on the GLUT9 protein expression. The cells were treated by adenosine and/or caffeine for 6 hours. The upper photograph showed the results of one representative experiment. Each value represented the mean ± SEM from 4-6 experiments. *, $p < 0.05$; significantly different versus control group. #, $p < 0.05$; significantly different versus 100 µM adenosine alone treatment.

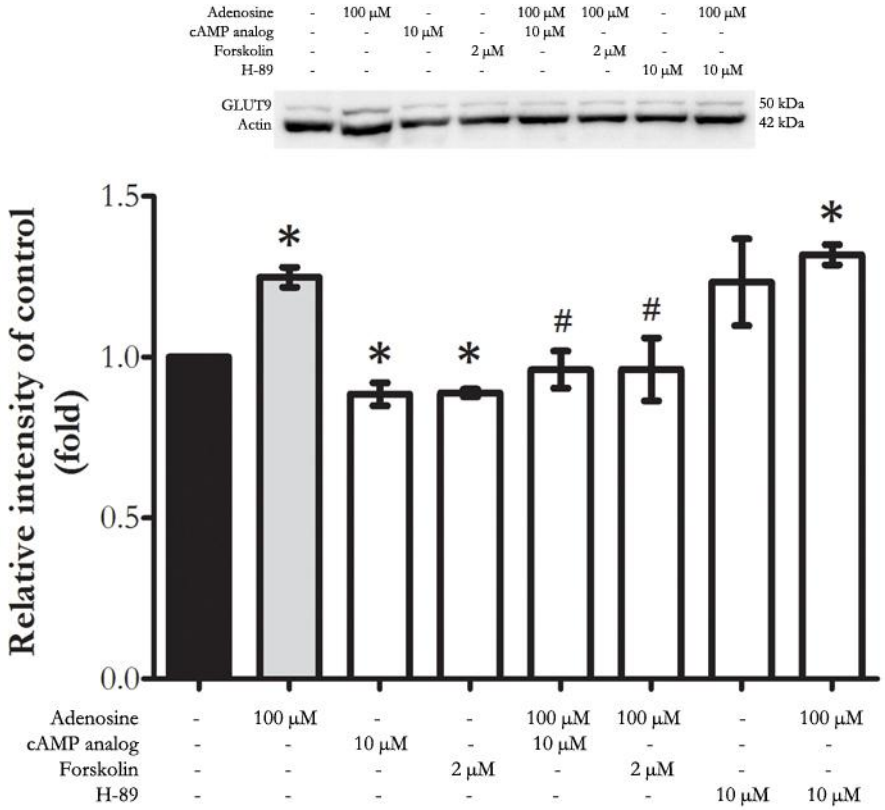


Figure 27 Effect of protein kinase A on the GLUT9 protein expression.

The activators and inhibitor were conducted for 6 hours. The upper photograph showed the results of one representative experiment. Each value represented the mean ± SEM from 3-6 experiments. *, $p < 0.05$; significantly different versus control group. #, $p < 0.05$; significantly different versus 100 μM adenosine alone treatment.

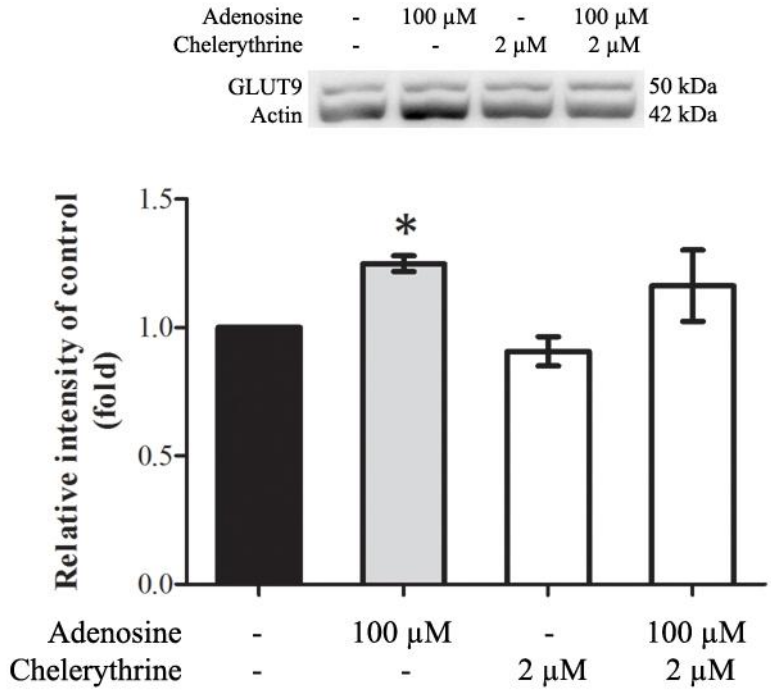


Figure 28 Effect of protein kinase C on the GLUT9 protein expression.

The cells were treated by chelerythrine as a PKC inhibitor for 6 hours. The upper photograph showed the results of one representative experiment. Each value represented the mean ± SEM from 3-6 experiments. *, $p < 0.05$; significantly different versus control group.

CHAPTER V DISCUSSION

The present study initially investigated the effect of substances that occur in hyperglycemic condition. In animals and humans, it has demonstrated that the expression of GLUT9 might involve in hyperglycemia. The GLUT9 protein was increased in streptozotocin-induced diabetic rats and in human term placenta from gestational diabetes (13-15). Since GLUT9 plays a key role in uric acid and glucose homeostasis by re-absorption processes in kidney, the GLUT9 expression might involve in pathophysiological properties (5, 11, 90). However, contributing factors in the regulation mechanisms have not been explained. Therefore, this study focuses on substances that involve in hyperglycemic condition such as glucose, fructose, ATP, and adenosine as well as possible mechanisms that influence GLUT9 expression in MDCK cells.

The MDCK cell, derived from kidney of normal adult cocker spaniel, is a useful tool for assessing the membrane permeability properties of early drug discovery compounds and epithelial development, so it was chosen as a renal tubular model (80, 82). The cells were cultured for 4 days, a condition that is recommended for permeability determination. Our experiment presented first evidence of GLUT9 expression in MDCK cells. The GLUT9 mRNA and proteins were detected using RT-PCR (Figure 3) and Western blot analysis (Figure 4), respectively. In addition, the expression of GLUT9 proteins in MDCK cells was also confirmed by using immunocytochemistry technique. The result was shown that fluorescence intensity of GLUT9 protein can be detectable after the cells were cultured for 4 days (Figure 5). Therefore, these results suggested that GLUT9 was spontaneously expressed in MDCK cells. These findings also support the idea that GLUT9 has been reported to express in renal tubular cells for glucose re-absorption processes (8, 91, 92). GLUT9 has been reported to encode in 2 variants, GLUT9a and GLUT9b (8, 13). Since the primers for GLUT9 gene and the anti-GLUT9 antibody in this study were recognized at conservative region, the results were represented as the whole amount of GLUT9 mRNA and proteins, respectively. Thus, our results suggested that MDCK

cells were suitable for investigating the effect of any substances on GLUT9 expression. However, in order to understand more, further studies determining the effects on each variant expression and localization are necessary.

The role of GLUT9, which is specifically exhibited in kidney and liver, has been identified to allow glucose, fructose, and uric acid transportation through the cell membrane (4, 6, 8, 93). The expression of GLUT9 in kidney has been associated with hyperglycemic conditions. In this study, MDCK cells were treated with glucose and fructose at high concentrations represented hyperglycemic conditions and then determined the GLUT9 expression in terms of mRNA and protein levels. Our results indicated that GLUT9 mRNA was decreased when treated cells with 25 mM glucose for 72 hours but it was not altered when treated cells with fructose (Figure 11A and Figure 14A). On the other hand, 25 mM glucose and 1 mM fructose had no effect on the level of GLUT9 protein when compared with control (Figure 11B and Figure 14B). Although GLUT9 protein levels were not significantly changed after cells cultured in 25 mM glucose for 72 hours, they were likely to increase in concentration-dependent and time-dependent manner. Glucose at a concentration of 50 mM can significantly induce GLUT9 protein levels in MDCK cells cultured for 72 hours (Figure 13). These observations suggested that long-term treatment of glucose at high concentrations may suppress GLUT9 mRNA but increase GLUT9 protein levels. The discrepancy between GLUT9 mRNA and protein expression was supported by previous study in diabetic rats. GLUT9 protein was significantly increased in the type 2 diabetic animal model, *ob/ob* mice, when compared with control mice but the GLUT9 mRNA was not altered (15). This absence of correlation between transcription and translation may due to protein half-lives and transcriptional mechanisms such as ribosomal occupancy (94, 95).

Hyperglycemia causes many complicated condition such as alteration of osmolality, hypoxia, and glucose consumption. In hyperglycemic hyperosmolar state, the plasma glucose level can be higher than 600 mg/dl (33 mM) which make osmolality greater than 320 mOsm/kg (32, 33). A changing of serum osmolality can influence the physiological and the biochemical processes. Our observations

demonstrated that the osmolality values were increased by glucose and sorbitol in a concentration-dependent manner. Moreover, the osmolality of each concentration of glucose and sorbitol showed similar values (Figure 16). Thus, 25 mM sorbitol, the highest non-toxic concentration, was chosen to determine the hyper-osmotic stress effect of 25 mM glucose that might involve in decreasing GLUT9 mRNA. The results were found that the GLUT9 mRNA level was significantly decreased after treated MDCK cells with sorbitol for 48 and 72 hours (Figure 17) but the GLUT9 protein was not changed (Figure 19). Our findings suggested that the hyper-osmotic stress might be a contributing factor to suppress the GLUT9 expression particularly at the transcriptional level. Since hyper-osmotic stress had no effect on GLUT9 protein levels, it might not contribute to the effect of high glucose on increasing GLUT9 protein levels. Furthermore, the hyper-osmotic stress has also been reported to involve the expression of other glucose transporters such as GLUT2 and GLUT4. The expression of GLUT2 on brush border membrane of renal proximal tubular cells was increased when osmolality in plasma was increased by infusing mannitol (63). The GLUT4 protein was regulated by hyper-osmotic stress through tyrosine kinase dependent and independent processes in fat and muscle, respectively (62). From previous observations suggested that osmotic stress could trigger many intracellular signaling proteins such as PKA, PKC, AMPK, and tyrosine kinase, so the effect on these proteins were determined in this study. MDCK cells were treated with forskolin (PKA activator), AICAR (AMPK activator), insulin (tyrosine kinase activator), or chelerythrine (PKC inhibitor). The GLUT9 mRNA was only significantly decreased when cells were treated with 2 μM forskolin (Figure 20). From these results suggested that the suppression of GLUT9 mRNA expression occur *via* activation of PKA. However, effect of hyper-osmotic stress on GLUT9 mRNA through PKA should be confirmed by co-treatment with PKA inhibitor and determination of intracellular cAMP content in further studies.

The result of extracellular ATP and adenosine was another factors which may regulate GLUT9 expression because their increasing have been showed by cultured cells in high concentrations of glucose (16, 17). The extracellular ATP was increased

two-fold but the intracellular ATP did not alter in mesangial cells cultured in 25 mM glucose for 72 hours (16). This result was similar to the study of Chen's group which demonstrated an increase of ATP in cultured medium when HK-2 cells were cultured in high glucose condition (17). The enhancement of ATP content may be another contributing factor that resulted from long-term treatment of glucose at high concentration. The intracellular ATP can be released through exocytosis, connexin hemichannels, or transporters such as vesicular nucleotide transporter and it binds the purinergic receptors on outer membrane. The extracellular ATP plays a role in maintaining pathological and physiological properties of many tissues including kidney through purinergic system (19, 96, 97). In addition, MDCK cells, as a renal tubular cell model, have been reported to contain purinergic system (22, 72, 98). The present study revealed that GLUT9 protein levels were significantly increased, but the GLUT9 mRNA was unchanged, when treated cells with 100 μM of ATP (Figure 21). The exogenous ATP increased GLUT9 protein levels during the course of 3-12 hours which reach maximum at 6 hours, whereas, this effect was decreased to the control level after 18-hour periods. Our results were supported in the same trend of exogenous ATP effect on glucose uptake that has been previously observed in human fibroblasts and mouse myoblasts. The uptake rate of 2-deoxyglucose and the expression of glucose transporters such as GLUT1 and GLUT4 were increased by exogenous ATP (99, 100). Moreover, the effect of ATP was also demonstrated by Lee's group that exogenous ATP at a concentration of 100 μM significantly increased the uptake rate of α-methyl-D-glucopyranoside by 1.5-fold when treated for 6 hours and this effect was decreased to the control level when treated for 24 hours (101). Although α-methyl-D-glucopyranoside is substrate of sodium-dependent glucose transporters, this may consider the possibility of a response of ATP to glucose transportation. The extracellular ATP exerted its effect *via* purinergic P2 receptors. The effect of ATP on GLUT9 protein *via* purinergic P2 receptors was confirmed by using non-specific P2 antagonist, suramin. Our results illustrated that suramin could not inhibit an increasing of GLUT9 protein from the exogenous ATP treatment (Figure 23). These finding suggested that P2 receptors might not involve in regulation of

GLUT9 protein expression. However, this observation could be explained by the fact that the ATP was immediately broken-down by ecto-nucleotidases on brush-border membrane to be ADP, AMP, and adenosine. Therefore, one of its metabolites might cause the increase of the GLUT9 protein.

Adenosine is an endogenous purine nucleoside that can regulate the cell function *via* purinergic system by specific binding with adenosine receptors. The extracellular adenosine is produced by ATP dephosphorylation and transportation (21, 96). Previous observations have reported that the content of adenosine in glomeruli in streptozotocin-induced diabetic rats was increased 6-fold higher than in normal rats (24) and adenosine receptors also increased in renal tubular cells (23). Likewise, an adenosine analog (CADO) can decrease the plasma glucose levels and the renal glucose excretion in diabetic rats (102). Our results demonstrated that GLUT9 mRNA was decreased but the protein was increased when the cells were treated with 100 μM adenosine for 6 hours (Figure 24). A decrease of the mRNA at 6 hours might responsible for the decrease of the level of GLUT9 protein at 18 hours. This discrepancy between the effect of adenosine on level of mRNA and protein was similar to that of glucose, so it might support the relevance of adenosine with long-term treatment of glucose at high concentration. Therefore, these observations suggested that GLUT9 protein half-lives and transcriptional processes might contribute to the effect of adenosine (94, 95).

In order to evaluate the involvement of adenosine in alteration of GLUT9 expression through adenosine receptors, this study used caffeine, a non-selective antagonist of P1 receptors. The effect of 100 μM adenosine on decreasing GLUT9 mRNA did not alter when it was co-treated with 500 μM caffeine for 6 hours (Figure 25). This observation could be explained by adenosine activation. Previous studies have been reported that adenosine can inhibit adenylate cyclase through purinergic receptors and directly inhibit intracellular adenylate cyclase, which is not suppressed the effect by methyxanthine (103). Therefore, our results suggested that the regulation of GLUT9 mRNA expression might not involve in purinergic system. On the other hand, the enhancement of GLUT9 protein expression by adenosine could be

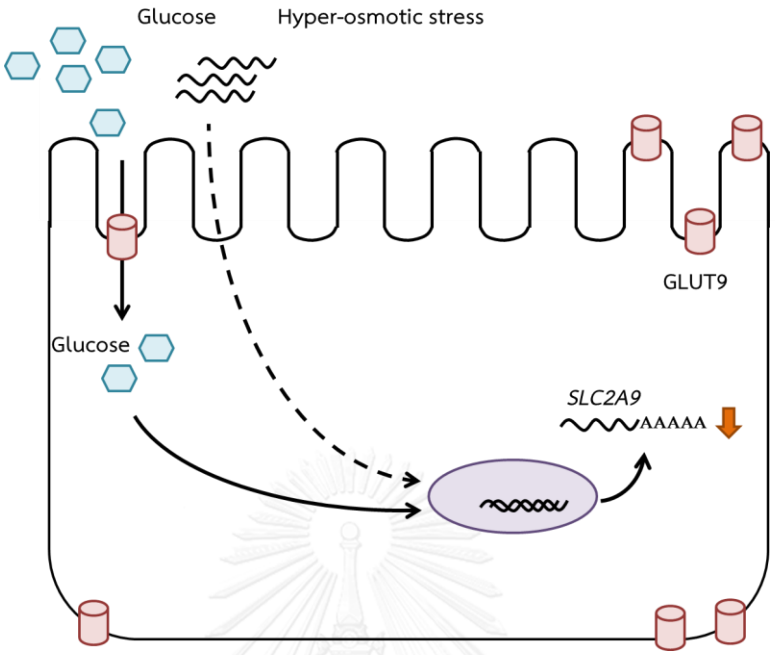
inhibited by 100 μM and 500 μM caffeine, suggested the possibility of the protein expression was likely a positive response on adenosine and its receptors (Figure 26).

The adenosine receptors which coupled with G-protein are associated with stimulation or inhibition of adenylate cyclase resulting to increase or decrease the intracellular cAMP concentration, respectively (20, 70). The cAMP plays a role to trigger PKA as a major signaling pathway, so a contributing pathway was evaluated using PKA activators and inhibitor. The activators of PKA, cAMP analog and forskolin, can increase the intracellular cAMP concentration, precursor of PKA stimulation, while H-89 is competitive inhibitor at cAMP binding site. From our investigation found that activation of adenosine *via* adenosine receptors could increase GLUT9 protein levels. The results demonstrated that PKA activators could significantly decrease the GLUT9 protein levels by themselves and also inhibit the effect of adenosine (Figure 27). On the other hand, H89 slightly increased the GLUT9 protein levels but not significantly. The co-treatment between H-89 and adenosine study showed that GLUT9 protein was not altered when compared with adenosine treatment alone (Figure 27). The activation of PKA could decrease the level of GLUT9 protein in cells cultured in the presence or absence of adenosine. This phenomenon was similar to the PKA stimulation in L6E9 myotubes that can increase level of GLUT1 protein but decrease level of GLUT4 protein (104). Our results presented first evidence that PKA might be involved in the regulation of GLUT9 protein expression *via* P1 receptors. Interestingly, the adenosine receptors A1 and A3 inhibit activity of adenylate cyclase preferentially through $G_{\alpha i}$ protein, whereas, this enzyme is likely stimulated through $G_{\alpha s}$ protein *via* A2a and A2b receptors (69, 70). Our results suggested that the $G_{\alpha i}$ subunit was more likely to mediate the action of adenosine in terms of regulating GLUT9 protein. Moreover, the possible signaling protein such as protein kinase C (PKC) which may involve in trigger adenosine receptors was also investigated by using chelerythrine, as a PKC inhibitor (22). Chelerythrine had no effect on adenosine-induced GLUT9 protein expression (Figure 28). Therefore, PKC might not involve in regulation of GLUT9 expression.

CHAPTER VI CONCLUSION

The present study suggested that long term-high glucose concentration treatment had an effect on GLUT9 expression. This condition could decrease GLUT9 mRNA but increase GLUT9 protein levels in MDCK cells. Since high glucose concentration can cause hyper-osmotic stress environment, 25 mM sorbitol was used to evaluate the hyper-osmotic stress effect of high glucose on GLUT9 expression. As shown in Figure 29, our results suggested that hyper-osmotic stress could decrease GLUT9 mRNA expression, but it had no effect on GLUT9 protein levels. The induction effect of high glucose concentration on GLUT9 protein expression was evaluated by using the action of exogenous ATP and adenosine, since both compounds were reported to increase in cells, long term treatment with high glucose level. Adenosine could increase GLUT9 protein through adenosine receptors and $G\alpha_i$ protein which in turn inhibit the activity of PKA. Therefore, the effect of hyperglycemic condition on GLUT9 expression can be explained in 2 ways, as shown in Figure 29. In term of hyper-osmotic stress, it decreased GLUT9 mRNA expression. Second, *via* the increasing of adenosine levels, it increased GLUT9 protein through adenosine receptors, $G\alpha_i$ protein, and PKA. This effect may not involve in transcriptional processes but may be translational processes.

A)



B)

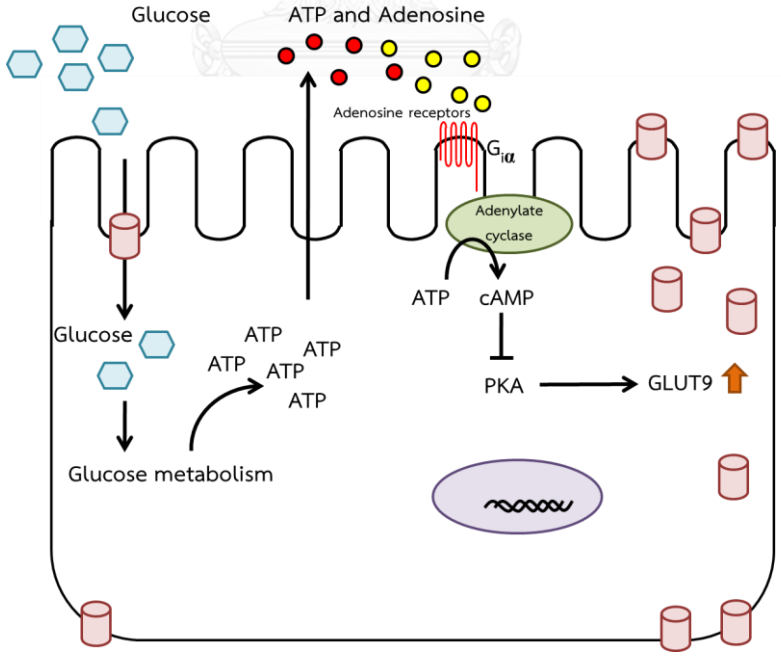


Figure 29 Possible mechanisms of hyperglycemic condition involved in GLUT9 expression in terms of mRNA (A) and protein (B).

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APPENDIX

APPENDIX A

PREPARATION OF REAGENTS

Phosphate buffer saline solution

KCl	0.2 g
KH_2PO_4	0.2 g
NaCl	8.0 g
$Na_2HPO_4.2H_2O$	1.4 g
Ultrapure water qs. to	1 L

Reverse transcription master mix

Component	1 Reaction	Final concentration
10 x RT buffer	2 µl	1.0 x
dNTPs mix (5 mM of each dNTP)	2 µl	0.5 mM
10 µM Oligo dT	2 µl	1.0 µM
RTO	1 µl	4.0 Unit
RNase-free water	3 µl	
Template RNA	10 µl	1.0 µg
Total	20 µl	

Polymerase chain reaction master mix

Component	1 Reaction	Final concentration
10 x PCR buffer	2.50 µl	1.0 x
25 mM $MgCl_2$	-	1.5 mM
dNTPs mix (10 mM of each dNTP)	0.50 µl	0.2 mM
10 µM GLUT9 forward primer	0.50 µl	0.2 µM
10 µM GLUT9 reverse primer	0.50 µl	0.2 µM
10 µM GAPDH forward primer	0.20 µl	0.08 µM
10 µM GAPDH reverse primer	0.20 µl	0.08 µM
Taq DNA polymerase	0.12 µl	2.5 Unit
RNase-free water	19.48 µl	
Template cDNA	1.00 µl	
Total	25 µl	

5 x TBE Buffer

Tris base	5.40	g
Boric acid	2.75	g
0.5 M EDTA (pH 8.0)	2.00	ml
Ultrapure water qs. to	100	ml

For experiment, the concentration for gel electrophoresis is prepared in 0.5 x TBE buffer.

Bradford reagent

Coomassie Brilliant Blue G250	10	mg
Absolute ethanol	5	ml
85% (v/v) phosphoric acid	10	ml
Ultrapure water qs. to	100	ml

2 x Sample buffer

1 M Tris-HCl (pH 6.7)	170	µl
10% SDS	210	µl
Glycerol	210	µl
2-mercaptoethanol	105	µl
0.1% (w/v) bromophenol blue	80	µl
Ultrapure water qs. to	1	ml

5 x Running buffer

Glycine	72.0	g
Tris base	14.5	g
SDS	2.5	g
Ultrapure water qs. to	500	ml

For experiment, the concentration for running buffer is prepared in 1 x running buffer.

5 x Transfer buffer

Glycine	36.0	g
Tris base	7.5	g
Ultrapure water qs. to	500	ml

For experiment, the concentration for transfer buffer is prepared in 1 x transfer buffer and containing 20% (v/v) methanol.

12.5% Separating gel

Component	8 ml for 2 gel	Final concentration
Ultrapure water	1.54 ml	
1 M Tris-HCl (pH 8.9)	3.00 ml	375 mM
10% (w/v) SDS	0.08 ml	0.1 % (w/v)
30% (w/v) acrylamide/bis (29:1)	3.33 ml	12.5 % (w/v)
10% (w/v) ammonium persulfate	0.04 ml	0.05 % (w/v)
TEMED	4 µl	0.05 % (v/v)

4.0% Stacking gel

Component	4 ml for 2 gel	Final concentration
Ultrapure water	2.90 ml	
1 M Tris-HCl (pH 6.7)	0.50 ml	125 mM
10% (w/v) SDS	0.04 ml	0.1 % (w/v)
30% (w/v) acrylamide/bis (29:1)	0.53 ml	4.0 % (w/v)
10% (w/v) ammonium persulfate	24 µl	0.05 % (w/v)
TEMED	4 µl	0.10 % (v/v)

VITA

Mr. Jamras Kanchanapiboon was born on September 7, 1978 in Bangkok, Thailand. Now he is working in the Institute of Medicinal Plant Research which belongs to the Department of Medical Sciences under the Ministry of Public Health. His position is Pharmacist in professional level. The job description focuses on evaluating pharmacokinetic profiles of traditional plants, area of interest is the involvement pathways of transportation in absorption and excretion processes including the metabolism in small intestine using cell culture model.

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