Improvement of antioxidant balance in diabetes mellitus type 1 mice by glutathione supplement

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Abstract: Diabetes mellitus (DM) type 1 is a chronic disease characterized by hyperglycemia and lacking of insulin. Oxidative stress participates in development and progression of DM, in which changes of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione (GSH) content were noted in DM mice. In this study, the effects of GSH supplement on anti-oxidation system in streptozotocin-induced DM type 1 Imprinting Control Region (ICR) mice were determined. The co-treatment of insulin and GSH significantly lowered the hepatic manganese superoxide dismutase (Mn-SOD), CAT, and GPx mRNA expression. Moreover, co-administration of insulin and GSH restored SOD and CAT activities to non-DM group except that of the CAT activity in the kidney. The GSH contents and GSH/GSSG ratio in the mouse livers were normalized to the normal levels by the GSH treatment and the co-administration of insulin and GSH. These observations reveal that GSH supplement potentially has the protective roles in delaying diabetic progression via the improvement of antioxidant balance.

Keywords: Glutathione, redox status, diabetes mellitus type 1, antioxidant system.

INTRODUCTION

Oxidative stress occurs according to the loss of balance between free radicals and antioxidant systems (Adly. 2010); an increase of cellular oxidant products, *i.e.,* superoxide, hydrogen peroxide, nitric oxide, and a decrease of antioxidants either non-enzyme glutathione (GSH) or anti-oxidation enzymes including catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) (Haskins *et al.*, 2003). Diabetes mellitus (DM) is a chronic metabolic disorder identified by abnormally high blood sugar level (Gil-del Valle *et al.*, 2005). The regular clinical signs and symptoms have been found with several major biochemical changes such as extended oxidative stress from non-enzymatic glycation and advance glycation end products production, glucose and advance glycation end products autoxidation and changes in activity of polyol pathway, subsequently influenced the cells and organs (Evans *et al.*, 2003). DM type 1 (DM1) is characterized by cellular mediated autoimmune character with progressive destruction of β-cells of pancreas (Varvarovská *et al.*, 2004), therefore stopped secreting normal insulin and lack of insulin, blocking off glucose influx to cell for change to energy (Jakus *et al.*, 2012). Prolonged high glucose level with fatty acids caused pathogenic effect (Brownlee, 1995, Koya and King. 1998) is activated to a great extent through elevating production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) and subsequent oxidative stress (Rosen *et al.*, 2001). The strong oxidizing species, ROS and RNS, directly damage DNA, proteins, and lipids components (Ceretta *et al.*, 2012). In addition to the direct action to devastate macromolecules, ROS and RNS indirectly induce tissue injury (Evans *et al.*, 2003).

Glutathione (GSH; fig. 1), a tri-peptide antioxidant present in millimolar-concentrations in a cell (Chatterjee, 2013), has been described for a long time as a defensive agent against the action of either endogenous or exogenous toxic xenobiotics including drugs, pollutants, and carcinogens (Coles and Kadlubar, 2003). GSH involved in cell protection from oxidative stress (Pompella *et al.*, 2003) according to capability to balance oxidative stress with adjustable changes in GSH metabolism-regulated enzymes (Seghrouchni *et al.*, 2002). The present study aims to assess an impact of GSH supplement on the oxidant-antioxidant balance in the DM1 mice with regulatory expression and activity of enzymatic antioxidants including the ratio of non-enzymatic antioxidant GSH to GSSG contents as metabolic parameters of the disease to describe systemic oxidative stress at the beginning period of diabetes. In the brains and the livers of the DM1 mice, levels of SOD and CAT activities were greatly augments while the ratio of GSH/GSSG in the livers was excessively decreased. This is the first time noted that glutathione supplement recovers the oxidant-antioxidant balance in the DM1 mice.

MATERIALS AND METHODS

Chemicals - Streptozotocin (STZ) was a product of Wako Pure Chemical Industries (Osaka, Japan). Reduced β-

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nicotinamide adenine dinucleotide phosphate (NADPH) was supplied by Oriental Yeast Co., Ltd. (Tokyo, Japan). 4-vinylpyridine (4-VP), 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), glutathione reductase, reduced form of glutathione (GSH), oxidized form of glutathione (GSSG), nitroblue tetrazolium (NBT), standard superoxide dismutase (SOD) from bovine erythrocytes, and xanthine oxidase were supplied by Sigma Aldrich (St.Louis, MO, USA). Mixtard30 HM Novo was a product of Novo Nordisk (Bagsværd, Denmark). Hydrogen peroxide (H_2O_2) was obtained from Fisher Scientific (Leicestershire, UK). 2-Thiobarbituric acid (TBA) was supplied by Fluka Chemika Co (Steinheim, Switzerland). Trizol® was a product of Invitrogen® (Carlsbad, CA). ReverTraAce® and HelixAmp™ Taq DNA polymerase were purchased from Toyobo Co., Ltd. (Osaka, Japan) and NanoHelix RM (Daejeon, South Korea), respectively. Random primers and RNase inhibitor were products of Takara Bio Inc. (Shiga, Japan). Forward and reverse primers of CAT, CuZn-SOD, Mn-SOD, GPx, and GAPDH were synthesized by Bio Basic, Inc. (Markham, Ontario, Canada). Table 1 showed the data of primers and product sizes of each gene. All other laboratory chemicals were of the highest available purity from commercial suppliers.

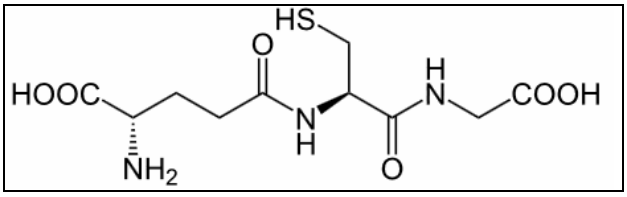

Fig. 1: Chemical structure of glutathione

Animals

Male ICR mice at 5 weeks of age were supplied by the National Laboratory Animal Center, Mahidol University (Nakhon Pathom, Thailand), and housed in the Northeast Laboratory Animal Center (NELAC), Khon Kaen University (Khon Kaen, Thailand). The research protocol was approved by the Animal Ethics Committee for Use and Care of Khon Kaen University (AEKKU 06/2553). The animal handling and treatment were performed under supervision of a certified veterinary doctor of the NELAC.

Induction of diabetes mellitus type 1 in mice

A single intraperitoneal injection of streptozotocin (STZ) at a dose of 100 mg/kg was employed to induce DM1 (Srinivasan and Ramarao, 2007; Hayashi *et al.*, 2006). STZ was freshly prepared in 0.05 M sodium citrate, pH 4.5. Two weeks after the STZ injection, the fasting blood glucose (FBG) level was determined from the tail vein blood. The mice with the FBG level higher than 250 mg/dL were included in the study. All mice were weekly monitored the FBG level before and after the treatments using a glucometer (Accu-Check® Advantage II Performa kits, Roche Diagnostics, Manheim, Germany). During fasting, mice were deprived of food for 18 hours but had free access to water

Experimental design

Mice were divided into two groups consisting of the normal (NT-NT; n=5) and the DM1 groups (n=20), in which the DM1 was randomly divided into four groups (n=5) including the control (DM1-NT) with daily subcutaneous injection of phosphate-buffer saline (PBS) 0.1 ml, once a day, for 2 weeks, the insulin treated group (DM-Ins) with daily subcutaneous injection of Mixtard30 HM Novo at a dose of 100 U/kg once a day, for 2 weeks, the glutathione treated group (DM-GSH) with weekly intravenous administration of glutathione at a dose of 30 mg/kg, for 2 weeks, and the co-treatment of insulin and glutathione (DM-Ins+GSH) group. The mice were sacrificed at 24 hours after the last treatment and the livers, brains, and kidneys were immediately excised and kept at -80ºC for further analysis.

Expression of hepatic CAT, SOD, GPx and GAPDH mRNA expression

Mouse CAT, CuZn-SOD, Mn-SOD, GPx, and glyceraldehydes 3-phosphate dehydrogenase (GAPDH) mRNAs were semi-quantified by RT-PCR. Total RNA was reverse-transcribed using ReverTraAce® (Toyobo Co., Ltd.), and the complementary DNA (cDNA) was amplified under the conditions recommended by the supplier of NanoHelix RM (Daejeon, South Korea). The conditions of PCR program were modified from the method of Mouatassim *et al.* (1999), Lao-ong *et al.* (2012) and Kondo *et al.* (2011). After separation of the PCR products by 1.5% agarose gel electrophoresis, the target cDNA was detected under ultraviolet (UV) light in the presence of Novel Juice of GeneDirex® (Bio-Helix Co., Ltd., Taiwan) and semi-quantified by Syngene® gel documentation (Ingenius L, Cambridge, UK) and the GeneTools match program (Syngene®). The cDNA levels of the target genes were normalized to that of GAPDH.

Measurement of glutathione contents and the ratio of GSH to GSSG in the livers

Glutathione contents including total glutathione, reduced glutathione (GSH), and oxidized glutathione (GSSG) contents were performed according to the method of Chatuphonprasert *et al.* (2013). The liver homogenate was deproteinized with sulfosalicylic acid (SSA), then kept at 2-8°C for 10 min before subjected to centrifuge at 10,000×*g* at 4°C for 10 min. To determine total glutathione content, the supernatant was mixed with the reaction mixture, which contained potassium phosphate buffer (pH 7.0), EDTA, NADPH, DTNB, and glutathione reductase. Absorbance of the thiol anions at a wavelength of 405 nm (A_{405}) was immediately measured every 60 sec for 5 min. The total glutathione content was determined by comparing A_{405}/min (or slope) of the sample to the series of the slope of the standard glutathione. The GSH content was calculated by subtraction of the total

Table 1: Forward and reverse primers of investigated genes

Genes	Reverse primers	Forward primers	Product size (bp)	References
CAT	5′-GTAGAATGTCCGCA-CCTGAG-3′	5′-GCAGATACCTGTG-AACTGTC-3′	229	Mouatassim et al., 1999
CuZn-SOD	5′-CAGGTCTCCAACAT-GCCTCT-3′	5′-AAGGCCGTGTGCG-TGCTGAA-3′	246	Lao-ong et al., 2012
Mn-SOD	5′-AGCCTCCAGCAACT-CTCCTT-3′	5′-GCACATTAACGC-GCAGATCA-3′	241	Lao-ong et al., 2012
GPx	5′-CAATGTCGTTGCG-GCACACC-3′	5′-CCTCAAGTACGTC-CGACCTG-3′	197	Lao-ong et al., 2012
GAPDH	5′-TAGACTCCACGAC-ATACTCAGC-3′	5′-TCCACTCACGGCA-AATTCAACG-3′	145	Kondo et al., 2011

glutathione content with the GSSG content (total GSH = GSH + GSSG).

For the GSSG content, 4-VP was incubated with an aliquot of the sample supernatant or the GSSG standard for 60 min at room temperature (4-VP: sample=2:50) before determination of the GSSG content as same as described for the determination of the GSH content.

Determination of GPx activity

Level of GPx activity was assessed according to the method of Chatuphonprasert *et al.* (2013) with some modifications. Briefly, the reaction mixture consisted of the sample homogenate, sodium phosphate buffer (pH 7.4), EDTA, sodium azide was incubated at 30°C for 10 min, and followed by adding GSH. The reaction was started by adding H_2O_2, and terminated by SSA. Then, the reaction mixture was subjected to centrifuge at 1,900 rpm for 15 min. The supernatant was employed for assessment of GPx activity as described for the determination of the GSSG content and the value was expressed as unit/mg protein, while a unit of GPx was defined as mmol(s) of the formed GSSG/min at 30°C and pH 7.4.

Determination of CAT activity

Level of CAT activity was determined according to the method of Chatuphonprasert *et al.* (2013). Briefly, the sample was incubated in a H_2O_2 substrate at 37°C for 1 min before terminated by ammonium molybdate. The yellow complex was measured at a wavelength of 405 nm. The percentage of inhibition of the complex was compared with the standard hepatic bovine catalase.

Determination of SOD activity

Level of SOD activity was determined by degree of inhibition of formazan formation (Chatuphonprasert *et al.*, 2013). In brief, an aliquot of homogenate was extracted by mixture of chloroform and ethanol, and the supernatant was employed for SOD assay. Either the supernatant or bovine Cu-Zn SOD standard was mixed with the reagent mixture of xanthine, EDTA, NBT, Na_2CO_3 and BSA, followed by xanthine oxidase. The reaction was incubated at 25°C for 20 min and terminated by $CuCl_2$. The absorbance of formazan was measured at a wavelength of 550 nm. The formazan inhibitory percentage was compared with the standard SOD.

STATISTICAL ANALYSIS

The results were analyzed by using one-way analysis of variance (ANOVA) followed by Tukey *post-hoc* test (version 19; SPSS Inc., Chicago, IL, USA). $p \leq 0.05$ was considered to be statistically significant.

RESULTS

Effect of glutathione supplement on the expression of hepatic CuZn-SOD, Mn-SOD, CAT and GPx mRNAs in the DM1 mice

To examine how DM1 status and glutathione influenced the oxidant-antioxidant balance, hepatic mRNA expression of anti-oxidation enzyme related genes, including CuZn-SOD, Mn-SOD, CAT and GPx, was determined in the DM1 mice. The expression of all investigated genes was significantly upregulated in the DM1 mouse livers (fig. 2). Glutathione significantly suppressed the expression of CuZn-SOD (fig. 2A) and GPx (fig. 2D) mRNA while insulin decreased only the expression of GPx mRNA. The co-administration of insulin and glutathione significantly lowered the expression levels of Mn-SOD, CAT and GPx mRNA, in which the level of Mn-SOD was comparable to that of the normals. These observations demonstrated antioxidant potential of glutathione supplement in the livers of DM1 mice.

Effect of glutathione supplement on activities of CAT and SOD enzymatic antioxidants

Since glutathione lowered the levels of CuZn-SOD and GPx mRNA expression in the DM1 mouse livers, the activities of related enzymatic antioxidants, ***i.e.,*** CAT, SOD, and GPx enzymes, were investigated in the livers, brains and kidneys of the DM1 mice. Corresponding to those of mRNA expression, the CAT and SOD activities

in all investigated organs (livers, brains, and kidneys) were increased in the DM1 mice (figs. 3-4). Both insulin and glutathione supplement significantly reduced the CAT and SOD activities in the livers (figs. 3A, 4A) and brains (figs. 3B, 4B) of the DM1 mice to the levels comparable to those of the normal, though these two enzymes were not extensively reduced in the DM1 mouse kidneys. The co-administration of insulin and glutathione drew both CAT and SOD activities in almost all investigated organs back to the normal levels. These findings underlined anti-oxidation activity of glutathione supplement in the DM1 mice.

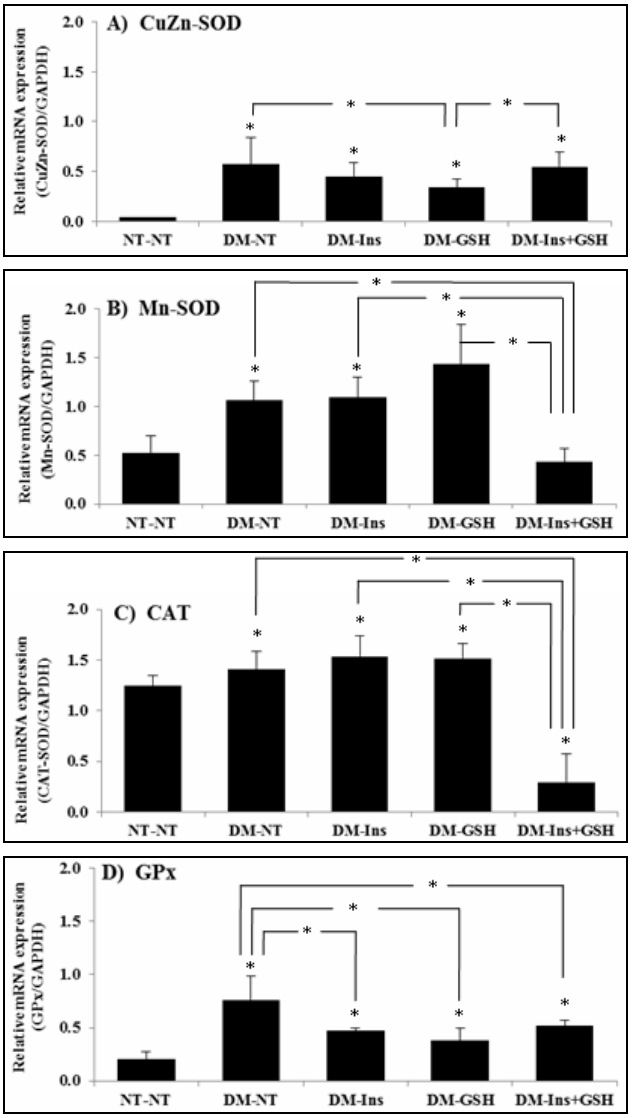

Fig. 2: Effect of glutathione supplement on the expression of hepatic (A) CuZn-SOD, (B) Mn-SOD, (C) CAT, and (D) GPx mRNAs in the DM1 mice. The male DM1 mice were daily subcutaneously injected with Mixtard30 HM Novo at a dose of 100 U/kg (DM-Ins) and/or weekly intravenously administered glutathione at a dose of 30 mg/kg (DM-GSH), for 2 weeks. The DM1 control mice (DM-NT) were daily subcutaneously given phosphate-buffer saline 0.1 ml/mouse, for 2 weeks. The normal control mice (NT-NT) were left untreated. The relative mRNA expression levels were normalized by that of GAPDH. The data are presented as the mean ±SD (n=5) from at least 3 independent experiments. A significant difference was examined by ANOVA followed by Tukey *post hoc* test. *$p<0.05$.

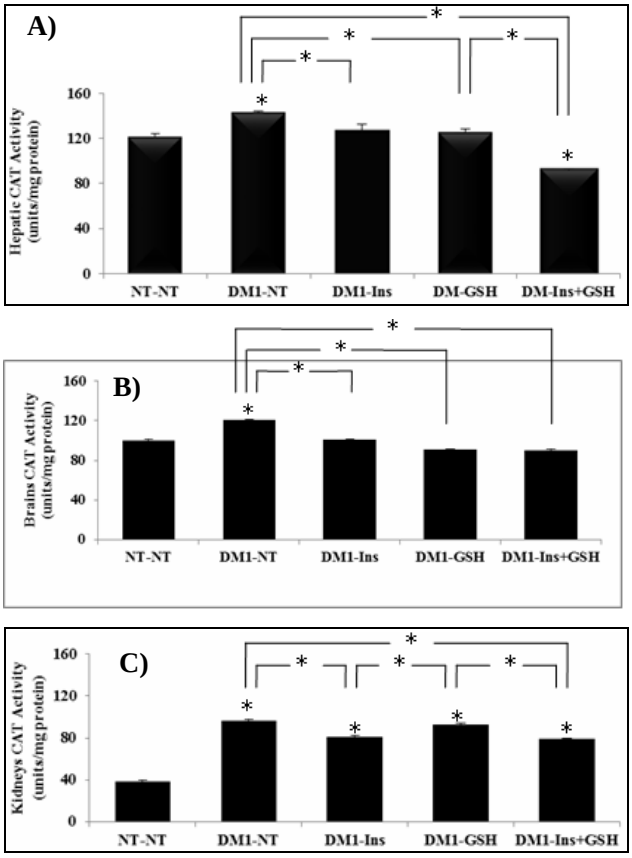

Fig. 3: Effect of glutathione supplement on activity of CAT enzymatic antioxidant in the DM1 mouse (A) livers, (B) brains, and (C) kidneys. The male DM1 mice were daily subcutaneously injected with Mixtard30 HM Novo at a dose of 100 U/kg (DM-Ins) and/or weekly intravenously administered glutathione at a dose of 30 mg/kg (DM-GSH), for 2 weeks. The DM1 control mice (DM-NT) were daily subcutaneously given phosphate-buffer saline 0.1 ml/mouse, for 2 weeks. The normal control mice (NT-NT) were left untreated. The data are presented as the mean ±SD (n=5). A significant difference was determined by one-way analysis of variance (ANOVA) followed by Tukey post hoc test. *$p<0.05$.

Effect of glutathione supplement on activity of GPx enzymatic antioxidants and non-enzymatic antioxidant glutathione content in the DM1 mouse livers

To examine whether GPx activity and non-enzymatic antioxidant glutathione contents in the DM1 mouse liver were positively affected as the hepatic CAT and SOD activities, the total glutathione, GSH, and GSSG contents, and the ratio of GSH/GSSG, including the GPx activity

were evaluated in the DM1 mouse livers. The hepatic GPx activity was suppressed in the DM1 mice (fig. 5A). Insulin improved the hepatic GPx activity in the DM1 mice, while the single glutathione treatment and the co-treatment did not. The ratio of hepatic GSH/GSSG was significantly decreased in the DM1 mice (1.78), compared to normal mice (2.99) (fig. 5B). Though all treatment patterns significantly raised the ratio of GSH/GSSG, only the single glutathione (2.16) and co-treatment (2.26) regained the GSH/GSSG ratio to the levels closed to the normal.

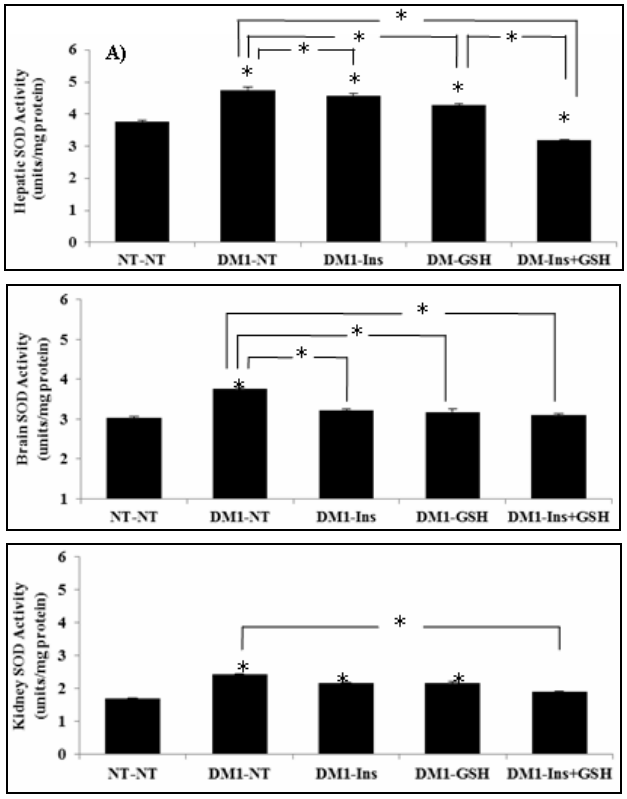

Fig. 4: Effect of glutathione supplement on activity of SOD enzymatic antioxidant in the DM1 mouse (A) livers, (B) brains, and (C) kidneys. The male DM1 mice were daily subcutaneously injected with Mixtard30 HM Novo at a dose of 100U/kg (DM-Ins) and/or weekly intravenously administered glutathione at a dose of 30 mg/kg (DM-GSH), for 2 weeks. The DM1 control mice (DM-NT) were daily subcutaneously given phosphate-buffer saline 0.1 ml/mouse, for 2 weeks. The normal control mice (NT-NT) were left untreated. The data are presented as the mean ±SD (n=5). A significant difference was determined by one-way analysis of variance (ANOVA) followed by Tukey post hoc test. *p< 0.05.

DISCUSSION

There were extensively revealed the relationship between hyperglycemia and increased production of ROS (Brownlee, 2001). The proton gradient produced the high electrochemical potential difference across the inner mitochondria membrane, subsequently extended the half-life of the intermediate from superoxide-generating electron transport such as the coenzyme Q (Brownlee, 2001). Du *et al.* (2001) proposed that hyperglycemia increased the proton gradient above this threshold value as a result of overproduction of electron donors by the tricarboxylic acid (TCA) pathway. This effect caused a marked increase in the production of superoxide by endothelial cells. The overexpression of mitochondrial superoxide dismutase, Mn-SOD, abolished the ROS-generated signal (Brownlee, 2001; Du *et al.*, 2001). In the present study, hepatic Mn-SOD mRNA (fig. 2B) and the SOD activities in the livers, brains, and kidney of the DM1 mice (fig. 4) were higher due to hyperglycemia-induced overproduction of ROS. Correspondingly, Cederberg *et al.* (2000) mentioned an increase of Mn-SOD and CAT mRNA expression in embryo of the diabetic rats. The GSH concentration and the GPx activity were decreased in both livers and kidney in the STZ-induced diabetic Balb/cA mice (Hsu *et al.*, 2004). In addition, the GPx activity in plasma of a DM type 1 patient was lowered (Ruiz *et al.*, 1999). Although we observed the decrease of hepatic GPx activity, the expression of hepatic GPx mRNA was elevated. The mRNA expression might not be a representative of protein expression or activity of enzyme, in some case. However, Lao-ong *et al.* (2012) noted the overexpression of hepatic GPx mRNA in the diabetic ddY mice, consistent with the present study.

Insulin, a typical biological drug for treatment of DM1, showed slightly anti-oxidative effect. The ROS generated in bovine aortic endothelial cells incubated with insulin was less than that produced in cells with absence of insulin (Yano *et al.*, 2004). The anti-oxidative property of insulin might be explained due to the relationship between hyperglycemia and ROS production. If blood glucose is decreased, ROS production must be lowered (Brownlee, 2001). In this study, the single insulin treatment showed the slight effect on lowering SOD and CAT activities.

Glutathione (GSH) is an endogenous antioxidant abundant in cells, especially in the liver cells, and has been described for a long time as a non-enzymatic antioxidant compound encountered the toxicants, *i.e.*, drugs, pollutants, carcinogens. As an antioxidant prototype, it has been involved in a process of cell protection from the noxious effect by the excess of oxidant stress (Pompella *et al.*, 2003). GSH supplement increased the myocardial GSH content and protected the heart from oxidative damage in rats (Ramires and Ji, 2001). Moreover, GSH showed a benefit to protect kidney cells against oxidative injury (Hagen *et al.*, 1988). Therefore, in the present study the advantage of GSH has been revealed in the DM1 condition. There were extensive researches showed GSH lost in the diabetic status while an impact of GSH on the DM status was still

less. GSH is a tri-peptide with a gamma peptide linkage between the amine group of cysteine and the carboxyl group of the glutamate side-chain (Pompella *et al.*, 2003). The study of Hsu *et al.* (2004) mentioned the effect of five cysteine containing agents in the diabetic mice. The benefit of cysteine containing agents to increase the CAT and GPx activities and the GSH content in both livers and kidneys were observed (Hsu *et al.*, 2004). Accordingly, GSH improved several anti-oxidation systems in the present study including restoration of the expression level of CuZn-SOD, Mn-SOD and GPx mRNAs, drawn back SOD, CAT and GPx activities to the normals, and an increase of the GSH contents and the ratio of GSH/GSSG to the normal levels.

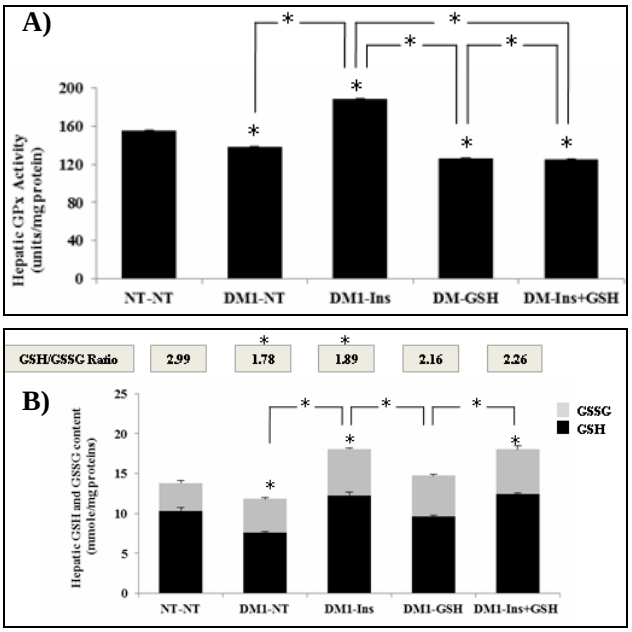

Fig. 5: Effect of glutathione supplement on (A) activity of GPx enzymatic antioxidant and (B) non-enzymatic antioxidant glutathione contents in the DM1 mouse livers. The male DM1 mice were daily subcutaneously injected with Mixtard30 HM Novo at a dose of 100 U/kg (DM-Ins) and/or weekly intravenously administered glutathione at a dose of 30 mg/kg (DM-GSH), for 2 weeks. The DM1 control mice (DM-NT) were daily subcutaneously given phosphate-buffer saline 0.1 ml/mouse, for 2 weeks. The normal control mice (NT-NT) were left untreated. The data are presented as the mean ±SD (n=5). A significant difference was determined by one-way analysis of variance (ANOVA) followed by Tukey post hoc test. *p<0.05.

In conclusion, DM1 status increased the level of oxidative stress in several models, leading to imbalance of antioxidant system, namely increasing of SOD and CAT, while lowering of GPx and GSH. Interestingly, the GSH supplement resulted in enhancing antioxidant protection in the DM1 mice. GSH reduced oxidative stress by its antioxidant property, which further relieved other diabetic complications from ROS such as the overproduction of inflammatory cytokines and coagulation predomination (Hsu *et al.*, 2004). These data suggested for the first time that the exogenous GSH potentially holds a number of protective roles for delaying diabetic degeneration.

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