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Oxidative and Anti-oxidative Status in Blood of Streptozotocin-induced Diabetic Piglets*

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ABSTRACT : Eight LW×D crossbred, castrated weanling piglets were used to examine the effect of hyperglycemia by streptozotocin (STZ)-injection on oxidative and anti-oxidative status in circulating fluid. Every two of the eight piglets were intravenously administrated STZ at a dose of 0 (control), 100, 125 or 150 mg/kg BW, respectively, and on 15th day after the STZ-injection, some markers of the oxidative stress in circulating fluid were measured to evaluate oxidative and anti-oxidative status in the piglets. First, piglets with hyperglycemia were selected from the STZ-injected piglets as measured by the levels of fasting plasma glucose (FPG) during 2 weeks after the STZ-injection. Additionally, data obtained from the intravenous glucose tolerance test (IVGTT) on 14th day were analyzed. Secondly, the data obtained in this experiment were divided into the control group and the hyperglycemic (STZ) group, and compared. The FPG level or area under curve (AUC) for plasma glucose during the IVGTT in the STZ-induced diabetic piglets was slightly significantly (FPG, $p = 0.070$; AUC, $p = 0.072$) higher compared with the control. On the other hand, the plasma level of lipid peroxidation in the STZ-induced diabetic piglets was significantly ($p<0.05$) higher compared with the control. These results raise the possibility that STZ-induced diabetic piglets produced in this study can be used as a diabetic animal model to research the pathogenic mechanisms or therapy of complications in diabetic mellitus. (**Key Words :** Diabetes, Oxidative Stress Marker, Piglet, Streptozotocin)

INTRODUCTION

Many available animal models based on rodents have been well-characterized and widely used in the field of research for the diabetes mellitus in humans. Animal models for type 1 diabetes have been established mainly by chemical procedures, such as injection of streptozotocin (STZ) or alloxan. The mechanism of action of these chemicals in pancreatic β cells which are responsible for producing insulin is different, but both result in the production of active oxygen species (Szkudelski, 2001). STZ (a N-nitroso derivative of glucosamine) is synthesized by Streptomycetes achromogenes and contains D-glucopynarose as well as D-glucose. Since STZ is transported by the β cells via a glucose transporter 2 (GLUT2) and the formation of superoxide radicals is enhanced in the cells, the β cells undergo specifically the destruction by necrosis.

Recently, swine have attracted a great deal of attention as one of hopeful candidates for an animal model, because pigs, compared with rodents, may be more comparable to human on the basis of biochemical and physiological parameters and a lot of their organ systems resemble those of human (Larsen and Rolin, 2004). Although it is said that pigs are more resistant to the diabetogenic effect of STZ than rats and it may be difficult and unreliable to induce type 1 diabetes in pigs compared with rats by STZ, we succeeded induction of hyperglycemia (fasting plasma glucose level = 302-491 mg/dL) for 7 weeks and development of type 1 diabetes in piglets (Murakami et al., 2007) according to the procedure proposed by Korompai et al. (2000) with some modifications.

In recent years, evidence has accumulated suggesting that diabetic patients are under oxidative stress as a result of increased free radicals production or reduced activity of antioxidant system by hyperglycemia (Oberley, 1988) and that the development of complications in diabetes mellitus seem to be partially mediated by oxidative stress (Brownlee,

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2005). Bonnefont-Rousselot et al. (2000) reviewed the effect of diabetic status on the oxidant and anti-oxidant balance, pointing out that oxygenated free radical are able to alter vascular function and to disturb cellular homeostasis and that tight interrelations have been demonstrated between oxygenated free radicals and advanced protein glycation, since advanced glycation endproducts (AGEs) are themselves able to produce free radicals and to be involved in diabetes complications. However there is very little available information on the oxidative status in swine with hyperglycemia.

In this study, we measured some markers of oxidative stress to evaluate the oxidative and anti-oxidative status in the STZ-induced diabetic piglets and discussed whether these piglets could be used as a diabetic animal model to research the pathogenic mechanisms or therapy of complications in diabetic mellitus.

MATERIALS AND METHODS

Diabetes experiment and animal management

Eight castrated male crossbred (progeny of Landrace× Large White dam and Duroc sire) weanling piglets from one litter, weighing 12.1-21.8 kg, were fitted with indwelling jugular catheters according to the method described in our previous report (Murakami et al., 2007). Every two of the eight piglets were intravenously administrated STZ (549-00286, Wako pure chemical industries, Osaka) at a dose of 0 (diluting buffer; placebo), 100, 125 or 150 mg/kg BW, according to the method of our previous report (Murakami et al., 2007) with some modifications. All animal were observed closely until fully recovered. A commercial post-weanling diet (Hyper-A-EX, JA Kitakyushu Kumiai Shiryo, Fukuoka, Japan) supplemented 0.02% ampiciline (Meitime, Meiji seika kaisya LTD, Tokyo, Japan) was fed at three times (09.00, 13.00 and 17.00 h) into which the substantial amount of 4% of their body weights was divided. They had *ad libitum* access to water. The piglets were housed in an individual pen in an environmentally regulated building (room temperature 23°C, illumination 14 L:10 D). Blood sampling procedures to evaluate diabetes were conducted similar to those described in our previous report (Murakami et al., 2007). All procedures involving the care and handling of experimental animals were performed according to the guidelines established by the National Agricultural Research Center for Kyushu Okinawa Region. Animal use protocols were approved by the Animal Experiments Committee at the research center.

Sample collection and preparation

On 15 days from the administration of STZ, fresh blood sample was collected via the fitted catheter before the morning meal in all piglets, transferred in test tubes with heparin and cooled on ice as soon as possible. Plasma and erythrocytes from an aliquot of fresh heparinized blood were obtained by centrifuging at 1,800×*g* (4°C for 10 min). Heparinized blood, plasma and erythrocytes were submitted to the measurement of oxidative stress markers immediately after the preparation.

Plasma glucose and insulin analysis

Plasma glucose levels were determined using a commercially available kit (Glucose CII-Test, Wako pure chemical industries, Osaka). Plasma Insulin levels were measured in duplicate by a double-antibody RIA as previously described (Inoue et al., 2005). The sensitivity of the assay was 0.06 ng/ml. The intra-assay coefficient of variation was 7.4%.

Intravenous glucose tolerance test (IVGTT)

On 14 days from the administration of STZ, the intravenous glucose tolerance test (IVGTT) was carried out. All piglets, after deprivation of feed for 16 h, were given a bolus injection of 50% D-glucose solution at a rate of 0.5 g/kg BW, via the fitted with indwelling jugular catheter. Baseline blood samples were taken at -20, -10 and 0 min of the glucose load and blood samples were taken at 1, 3, 5, 10, 20, 30, 60, 90 and 120 min after the glucose load. Plasma glucose levels were determined using a commercially available kit (Glucose CII-Test, Wako pure chemical industries, Osaka).

Oxidative stress marker analysis

The activities of glutathione peroxidase (GPx) in blood and superoxide dismutase (SOD) in hemocytes were measured by using commercial assay kits, RANSEL (RS505, Randox Laboratories Ltd., Crumlin, UK) and RANSOD (SD125, Randox Laboratories Ltd., Crumlin, UK), respectively. Reduce glutathione (GSH) in plasma was estimated by the method of Ellman (1959). The concentrations of plasma thiobarbituric acid reactive substances (TBARS) and hydroperoxides, products derived from the oxidant of lipids, were measured by the methods of Yagi (1976) and Jiang et al. (1992), respectively. Malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) were used as lipid peroxide and hydroperoxide standards, respectively. Plasma ceruloplasmin level, which involves the anti-oxidant status through chelating Cu ion, was estimated by the method of Ravin (1961). Total antioxidant status (TAS) in plasma was estimated by using a commercial kit (NX2332, Randox Laboratories Ltd., Crumlin, UK) based on the assay of 2,2'-azino-di[3-ethylbenzthiazoline sulfonate] radical cation ($ABTS^{\bullet+}$) scavenging activity. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as an antioxidant standard.

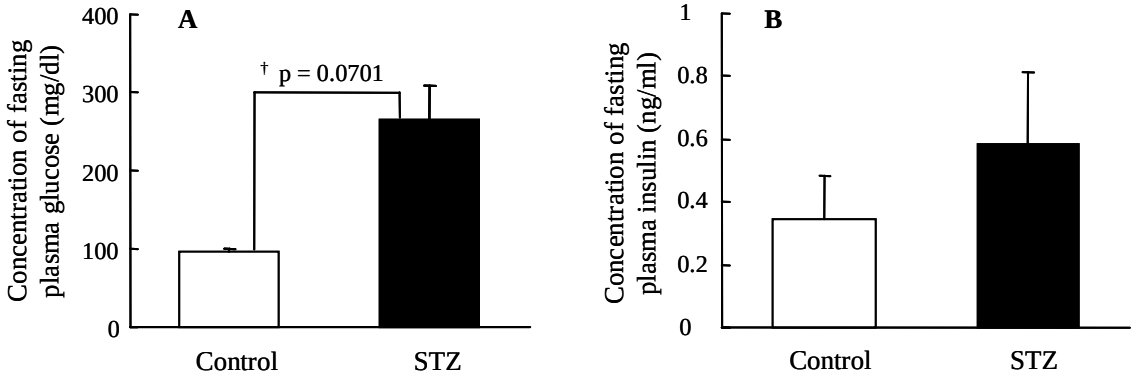

Figure 1. The concentration of fasting plasma glucose (A) and insulin (B) in streptozotocin (STZ)-induced diabetic piglets. The white or black bar represents the concentration in the control or STZ group, respectively. The vertical bar represents the standard error of the mean. † There is a marginally significant difference between the control and STZ group, p = 0.07.

Statistical analysis

Statistical analysis was performed by analysis of variance using the General Linear Model procedure of SAS (1988). Differences between means by treatment were tested using Student's t-test. p Values of <0.05 were considered significant. By a nonlinear regression analysis, data obtained from the IVGTT were fitted to a simple mono-exponential glucose decay curve predicted by the following equation:

$$G(t) = A \cdot \exp(-B \cdot t) + C$$

where G(t) represents the concentration at time t of plasma glucose after a sugar load at time 0 min. B means the fractional decay rate and C, basal glucose concentration, means the levels of fasting plasma glucose (FPG).

The nonlinear regression was performed by the Gauss-Newton method using NLIN procedure of SAS (1988). Thereafter, in the fractional decay rate, differences between means by treatment were tested using Student's t-test. p Values of <0.05 were considered significant.

RESULTS

Fasting plasma glucose and insulin level

Average of the fasting plasma glucose in every two piglets administrated STZ at a dose of 0, 100, 125 and 150 mg/kg BW, was 95.5, 142.5, 240.0 and 336.2 mg/dl, respectively. One the other hand, average of the fasting plasma insulin was 0.34, 0.87, 0.42 and 0.30 ng/ml, respectively.

During 2 weeks after the administration of STZ, the levels of FPG in all piglets administrated STZ except one, who was administrate at a dose of 100 mg/kg BW, exceeded 126 mg/dl, which was defined as one of criteria for the diagnosis of diabetes mellitus (Sacks et al., 2002). Therefore, the data obtained from the STZ-administrated piglet, which was not diagnosed with diabetes mellitus, was omitted.

On 15 days from the administration of STZ or placebo, the concentration of FPG in the STZ-induced diabetic piglets tended to be higher (p = 0.07) compared with one in the non-diabetic piglets (control) as shown in Figure 1A (STZ vs. Control: 265.0 vs. 95.5 mg/dl). On the other hand, there was no significant difference in the concentration of fasting plasma insulin between the diabetic and non-diabetic piglets (Figure 1B, STZ vs. Control: 0.584 vs. 0.345 ng/ml).

Plasma glucose response to intravenous glucose road

Plasma glucose concentrations during the IVGTT are shown in Figure 2. Plasma glucose concentration peaked within only 1 min following bolus intravenous injection. For both groups, plasma levels of glucose reduced exponentially and returned to the baseline at 20 min for control piglets or at 90 min for STZ-induced diabetic piglets, respectively. There were no significant differences in plasma glucose levels between diabetic and non-diabetic groups at any time point expect for 30 min. Parameters about characteristics of plasma glucose response during the IVGTT are shown in Table 1. There was no significant difference (p = 0.172) between diabetic and non-diabetic groups in the fractional decay rate which was produced by the nonlinear regression. On the other hand, the area under curve (AUC) of plasma glucose during the IVGTT (0-120 min following bolus intravenous glucose injection) tended to be larger in diabetic piglets compared with control piglets (p = 0.072).

Blood anti-oxidative activities and plasma oxidative damage

Average of some of oxidative stress markers measured

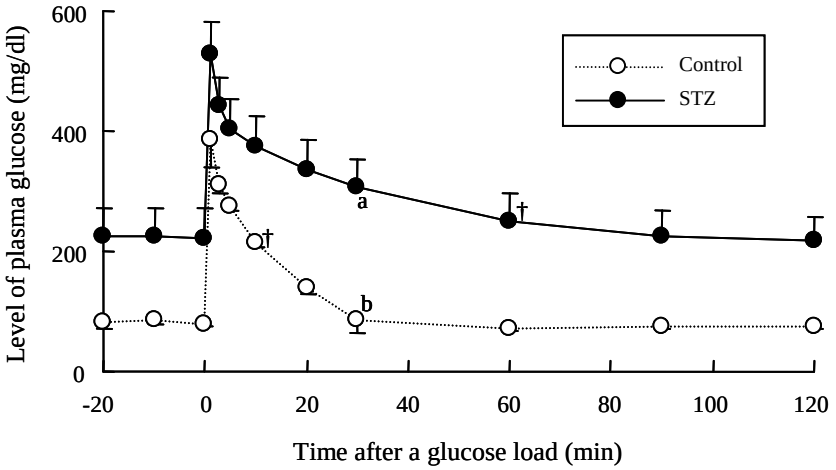

Figure 2. The change of blood glucose level during the intravenous glucose tolerance tests (IVGTT) in streptozotocin (STZ)-induced diabetic piglets. A solid circle with a vertical bar, joined by a solid line, or a null circle with a vertical bar, joined by a broken line, represents an average±SE of plasma glucose concentration in STZ-induced diabetic or control piglets, respectively. * $p<0.05$ and † $p<0.10$ compared with value at 0 min (the baseline). [a,b] Different alphabet shows significant difference between STZ-induced diabetic and control groups ($p<0.05$).

using blood sample from every two piglets administrated STZ at a dose of 0, 100, 125 and 150 mg/kg BW was as follows: plasma reduced glutathione level (μM), 88.5, 79.0, 106.0 and 122.2; whole blood glutathione peroxidase activity (U/ml), 50.2, 36.4, 41.7 and 41.6; erythrocyte superoxide dismutase activity (U/ml), 108.4, 81.8, 113.3 and 110.8; plasma ceruloplasmin (mg/dl), 95.1, 88.2, 89.9 and 76.4; plasma total antioxidant status (mmol Trorox/L), 1.02, 1.01, 1.01 and 1.06, respectively.

Comparison of the oxidative stress makers between the diabetic and non-diabetic piglets were summarized in Table 2. There was no significant difference between diabetic and non-diabetic groups in the level of reduced glutathione in plasma, the activities of GPx in whole-blood, SOD in erythrocyte and ceruloplasmin in plasma, and the radical scavenging activity (TAS) in plasma, measured using the blood on 15 days from the administration of STZ or placebo.

Average of the concentration of plasma lipid peroxide (TBARS) in every two piglets administrated STZ at a dose of 0, 100, 125 and 150 mg/kg BW, was 2.68, 3.61, 3.67 and 3.51 nmol MDA/ml, respectively. On the other hand, average of the level of plasma hydroperoxide, classified one of lipid peroxide, was 1.07, 1.17, 1.08 and 1.28 nmol H_2O_2/ml, respectively.

The concentration of lipid peroxide (TBARS) in plasma of the STZ-induced diabetic piglets was significantly higher compared with one of the non-diabetic piglets (control) on 15 days from the administration, as shown in Figure 3A (STZ vs. Control: 3.57 vs. 2.68 nmol MDA/ml). However, in the level of plasma hydroperoxide, there was no significant difference between diabetic and non-diabetic groups (Figure 3B).

Table 1. Characteristics of plasma glucose response to intravenous glucose road in STZ-induced diabetic piglets

Parameter	Control (n = 2)	STZ (n = 5)	SE	p-value
Fractional decay rate (B)[1]	0.0894	0.0568	0.0092	0.172
AUC[2] glucose 0-120 min(mg/dl/min)	12,495	33,056	4,080	0.072

[1] B value produced from a nonlinear regression analysis means the fractional decay rate of glucose decay curve (See text).
[2] AUC = Area under curve during intravenous glucose tolerance test.

Table 2. The concentrations of the oxidative stress makers in STZ-induced diabetic piglets

Oxidative stress marker	Control (n = 2)	STZ (n = 5)	SE	p-value
Plasma reduced glutathione (μM)	88.5	102.2	9.6	0.546
Whole blood GPx activity (U/ml)[1]	50.2	38.7	2.9	0.129
Erythrocyte SOD activity (U/ml)[1]	108.4	102.6	8.3	0.769
Plasma ceruloplasmin (mg/dl)	95.1	89.9	6.0	0.708
Plasma TAS (mmol Trorox/L)[1]	1.02	1.02	0.03	0.933

[1] GPx = Glutathione peroxidase, SOD = Superoxide dismutase, TAS = Total antioxidant status.

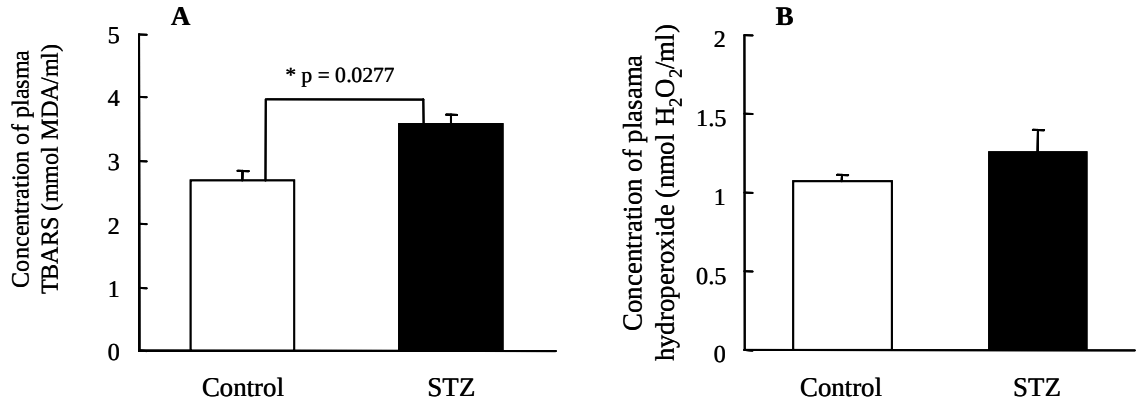

Figure 3. The concentration of plasma thiobarbituric acid reactive substance (TBARS) (A) and hydroperoxide (B) in streptozotocin (STZ)-induced diabetic piglets. The white or black bar represents the concentration in the control or STZ group, respectively. The vertical bar represents the standard error of the mean. * There is a significant difference between the control and STZ group, p = 0.028.

DISCUSSION

In the present study, the elevation in FPG was successful in five of the six piglets administrated intravenously STZ at a dose of 100-150 mg/kg BW. Therefore, a dose of 100 mg/kg BW was judged to be unreliable for inducing entirely diabetic mellitus in all subject piglets. On the other hand, a statistically significant difference was not observed in the concentration of fasting plasma insulin between diabetic and non-diabetic groups. The reason may be that the endogenous insulin secretion could decline and reach to nearly basal level just before breakfast in control non-diabetic piglets under a restricted feeding schedule of this study.

Since central venous catheters for human could be applied for piglets and the indwelling jugular catheters fitted with piglets were available in this study, the IVGTT with sampling at interval of a few minutes could be accurately carried out. According to a published report (Dyson et al., 2006), fitting of jugular catheters under anesthesia was performed on a day of the IVGTT and pigs were allowed to recover from anesthesia for at least 2 h before the IVGTT. Data obtained from experiments under such schedule may contain errors from anesthesia or catheterization. On the other hand, data of the IVGTT in this study were well fit to a simple compartment model suggesting glucose decay curve. The results of parameter about the characteristics of plasma glucose response to intravenous glucose injection show that the return to baseline in plasma glucose level tended to be slow in hyperglycemic piglets compared with non-diabetic piglets.

In patients with diabetes mellitus, free radical-induced damage to lipid or DNA in an organism arises from an imbalance between pro-oxidant and antioxidant systems (Bonnefont-Rousselot et al., 2000). In the present study, any oxidative stress markers as indices to evaluate the formation of free radicals could not be measured, because free radical measurement is very difficult given their high reactivity, their very short half-life and their low concentration. Recently a few sensitive methods have been developed to determine the generation of free radicals in blood, tissues or organelle of animals, based on the electron spin resonance (ESR) method (Hall et al., 1994; Hall et al., 2000; Mujahid et al., 2007; Lin et al., 2008). For these methods, the most important thing is how to take samples and mix with spin trap agent for a very short time period without changes of level in free radicals. Unfortunately, there was no available report showing the effect of hyperglycemia on the formation of free radicals in swine. Thus, in this study, some markers as indices to evaluate the enzymatic or non-enzymatic antioxidant status, and the damage of lipid in plasma were measured. Although the activity of SOD in erythrocyte was not significantly declined by hyperglycemia, a weak tendency toward reduced activity of GPx in whole blood by hyperglycemia was observed ($p = 0.129$). As SOD catalyzes the dismutation of superoxide (O_2^-) to H_2O_2 and O_2, and GPx reduce and detoxify H_2O_2 and various hydroperoxide in the presence of GSH and NADPH (Weiss, 1986), the lowered activity of GPx in whole blood may indicate the diminished capacity in extracellular enzymatic antioxidant system. Non-enzymatic antioxidant system involves free radical scavengers, such as vitamin E, vitamin C, GSH, uric acid, bilirubin, etc., and metal chelators, such as transferrin, ceruloplasmin, etc. In the present study, significant influence of hyperglycemia on the plasma concentration of GSH or ceruloplasmin was not observed and it would be predicted that non-enzymatic antioxidant system is not or slightly affected by hyperglycemia. A new method has been developed to evaluate the TAS based on the assay of a radical cation

trapping antioxidant parameter in plasma (Childs and Bardsley, 1975). According to some reports, it was obvious that plasma TAS in type 1 or type 2 diabetic patients significantly decreased (Tsai et al., 1994; Ceriello et al., 1997; Opara et al., 1999). But, in this study, no significant effect by hyperglycemia on plasma TAS was observed. The enzymatic or non-enzymatic antioxidant status, such as the activities of GPx and SOD, the level of GSH and TAS, in animals is strongly influenced by their nutritional status. Since piglets used in this study were reared under restricted feeding, hyperglycemia may not significantly affect the enzymatic or non-enzymatic antioxidant status.

Since measurement of oxidative injury as indirect markers is relatively easy, it is performed commonly to evaluate secondary products of lipid peroxidation such as TBARS and hydroperoxides or DNA oxidation such as 8-hydroxydeoxyguanosine (8-OHdG). Based on the accumulated evidence from type 1 and 2 diabetes patient or experimental diabetic animal studies, it was suggested that lipid and DNA damages in circulating fluid were elevated with or without reduction of anti-oxidative system (Gallou et al., 1994; Nourooz-Zadeh et al., 1995; Young et al., 1995; Park et al., 2001; Desco et al., 2002; Anwar and Meki, 2003). In this study, the development of oxidative injury, as indicated by the concentration of TBARS in plasma, was shown, although no significant effect of hyperglycemia on the concentration of hydroperoxides was observed. Lipids containing polyunsaturated fatty acids (PUFA) initially are oxidized to produce hydroperoxides by enzymatic and non-enzymatic oxidations and then the hydroperoxides produced are degraded further into secondary oxidation products such as a variety of aldehyde products. The TBARS assay is a method to measure typically aldehyde products derived from lipid peroxidation. Therefore, both data obtained from measurements of hydroperoxides and TBARS as a marker of oxidative stress lead frequently a similar conclusion. It is not clear why expected elevation of plasma hydroperoxides was not observed in STZ-induced diabetic piglets of this study.

In our other experiment, we confirmed the significant elevation of plasma TBARS in STZ-induced diabetic piglets up to 3 weeks after the STZ-injection at a dose of 150 mg/kg BW (unpublished data). Thus, from a result of development of plasma lipid damage, it is evident that experimental induction of hyperglycemia in piglets resulted in elevated oxidative stress. In addition, since no significant reduction of antioxidant system is observed except that GPx activity in whole blood tended to reduce by hyperglycemia, it can be easily estimated that free radical production in the piglets increase. These results raise the possibility that STZ-induced diabetic piglet produced in this study can be used as a diabetic animal model to research the pathogenic mechanisms or therapy of complications in diabetic mellitus.

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