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学 位 論 文

Studies on the changes of oxidative stress and autophagy in the hearts of streptozotocin-induced early diabetes rats, and the improvement effects by azuki bean (Vina angularis) extract

（ストレプトゾトシン誘導初期糖尿病ラットの心臓における酸化ストレスおよびオートファジーの変化と小豆抽出物による改善効果に関する研究）

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Abstract

I examined the changes in oxidative stress and autophagy function of the heart of rats at the early stages of streptozotocin (STZ)-induced diabetes, and whether azuki bean extract (ABE) could influence these changes.

The experimental diabetic rats received 0 or 40 mg/kg of ABE orally for 4 weeks, whereas the control group rats received distilled water. 8-Hydroxy-2'-deoxyguanosine (8-OHdG) and expression of proteins associated with peroxisomal FA β-oxidation, oxidative stress markers as well as autophagy-related proteins were examined.

The levels of peroxisomal ACOX1 and catalase of the diabetic groups were significantly higher than those in the control group. ABE did not effect on the increased level of these enzymes. The levels of p62, phosphorylated-p62 (p-p62) and HO-1 in the STZ group were significantly higher than those in the control group, and the levels of p-p62, HO-1, and 8-OHdG were significantly lower by ABE administration as compared with those in the STZ group. The increase in peroxisomal ACOX1 and catalase in the heart of STZ-induced early-diabetic rats suggested that peroxisomal FA-oxidation is enhanced in the early-diabetic heart. Furthermore, the increase in the levels of oxidative stress markers p62, p-p62 and HO-1 suggested that the heart of early-stage diabetes is under strong oxidative stress. ABE has antioxidant effects and protects from oxidative damage of the heart of STZ-induced early-diabetic rats.

In addition to stress markers, p62 and p-p62 are known to be responsible for transporting substances such as damaged organelles and proteins that will be degraded by autophagy, to autophagosome. Marked accumulation of p62 and p-p62 was observed in STZ-induced early-diabetic rats. Thus, I examined next the changes in autophagy function of the heart of rats at the early stages of STZ-induced diabetes. The levels of mTOR and Beclin1 proteins that are rerated with the autophagy induction did not change in any of the groups. The levels of O-GlcNAc modification and LAMP2 protein related to the fusion of autophagosome and lysosome were significantly increased in the STZ group compared to the control group. ABE has the effect of suppressing the increase in the levels of LAMP2 and O-GlcNAc modification.

The present study showed that the STZ-induced early diabetes increases the levels of proteins related with peroxisomal FA β-oxidation, oxidative stress markers, and the fusion of autophagosomes and lysosomes in hearts. ABE protects diabetic hearts from oxidative damage and has the effect of maintaining autophagy function from the early stage of diabetes.

KEYWORDS: polyphenols, diabetes, heart, oxidative damage, autophagy

Chapter 1

Changes of oxidative stress in the hearts of streptozotocin-induced early diabetes rats, and the improvement effects by azuki bean (*Vigna angularis*) extract

Introduction

Diabetes is a metabolic disorder characterised by hyperglycaemia and causes dysfunction and complications of various organs and tissues. Diabetic cardiomyopathy, one of the chronic complications of diabetes, is a major cause of heart failure. Many studies have demonstrated that hyperglycaemia is central to the development of diabetic cardiomyopathy leading to excessive production of reactive oxygen species (ROS) and causing a series of events leading to oxidative stress, myocardial damage, and cardiomyopathy (Frustaci et al. 2000, Karbasforooshan et al. 2017, Li et al. 2006). Oxidative injury in the heart can cause myocardial cell death and fibrosis, hypertrophy, ventricular dilation, cardiac function deterioration, and ultimately heart failure (Senqupta et al. 2011).

The heart needs a constant supply of ATP to meet the high-energy requirements of contractile muscles which is produced primarily by the metabolism of fatty acids (FA) and carbohydrates. FA β-oxidation usually provides 60 to 70% of the ATP production by the heart but provides 90 to 100% in uncontrolled diabetes. Increased FA uptake and oxidation in the myocardium contributes to cardiac dysfunction (Lopashuk 2002, Huang et al. 2005).

Recent studies have shown that plant-derived polyphenolic compounds such as epigallocatechin gallate, quercetin and resveratrol have beneficial effects similar to exercise and food restriction for metabolic diseases, and are useful for the prevention of obesity, cardiovascular disease, and diabetes (Zhang et al. 2009, Chen et al. 2012, Tomas-Barberan et al. 2012, Cardona et al. 2013, Sato, Mukai et al. 2016). In previous studies, cardiac hypertrophy and fibrosis were reported in streptozotocin (STZ)-induced diabetic model mice at 16 weeks after the onset of diabetes. Long-term resveratrol treatment improved reduced cardiac function and oxidative injury in diabetes (Thirunavukkarasu et al. 2007). After the 3 weeks in diabetic animals, pro-inflammatory cytokine expression was

up-regulated in myocardia, and initial abnormalities in cardiomyocyte contractility occurred, and left ventricular mass was lowered by 23%, and a definite ventricular remodeling was already present (Savi et al. 2016, Stilli et al. 2007, Delucchi et al. 2012). The early resveratrol administration inhibited the pro-inflammatory cytokine production, leading to a recovery of cardiomyocyte contractile efficiency and reduced inflammatory cell recruitment, and improved ventricular function by reducing inflammatory state and decreasing ventricular remodeling (Savi et al. 2016, Delucchi et al. 2012).

The azuki bean (*Vigna angularis*) is rich in plant-derived polyphenols such as proanthocyanidins, catechins, and quercetin (Sato, Mukai et al. 2016). Proanthocyanidins are natural antioxidants that exert antioxidant activity in pathological conditions such as cardiovascular disease, inflammation, and diabetes (Mukai et al. 2013). Several studies have shown that azuki beans have beneficial effects on oxidative stress, hypertension, obesity, and diabetes (Liu et al. 2017, Yao et al.2012, Kitano-Okada et al. 2012, Mukai et al. 2011). Proanthocyanidins are natural antioxidants that exert antioxidant activity in pathological conditions such as cardiovascular disease, inflammation, and diabetes (Mukai et al. 2013). It is also known that azuki bean extract (ABE) reduces oxidative stress and stimulates autophagy in the kidneys of STZ-induced early diabetic rats (Sato, Kataoka et al. 2016).

However, little is known about whether ABE has beneficial effects on the cardiac function in early diabetes. Therefore, this study aimed to examine and to clarify the changes in FA β-oxidation and oxidative stress in the hearts of rats at the early stages of STZ-induced early diabetes, and whether ABE, which is enriched with polyphenols, could influence the changes.

Materials and methods

ABE

By using the Folin-Ciocalteu method, the total polyphenol content of ABE (provided by Endo Seian Co. Ltd.) was analysed. To analyse polyphenolic compounds, ABE was incubated with β-glycosidase at 43℃ for 3 h for hydrolysis, and the compounds were separated by

high-performance liquid chromatography. The total content of the polyphenol compound was 212 mg of (+)-catechin hydrate/100 g ABE and the total proanthocyanidin content was 74 mg procyanidin B1/100 g ABE. ABE was concentrated under reduced pressure at a temperature of 50-55℃, the final ABE concentration was adjusted to 4.0 mg/mL.

Animal treatments and sample collections

All procedures were carried out according to the Guidelines for Animal Experimentation by the Aomori University of Health and Welfare (Permission number: 12005). We used 6-week-old Male Wistar rats (CLEA Japan, Inc., Tokyo, Japan) weighing 179-202 g. We kept the animals under a 12-h light/dark cycle at 23 ± 1℃. To induce diabetes, the animals received a single intravenous tail vein injection of 55 mg/kg body weight of STZ (Sigma Chemical Co., St Louis, MO, USA) in 0.5 mL of a 0.05 mol/L citrate buffer (pH 4.6). Blood was collected from the tail vein of the rat 48 h after the STZ injection and the blood glucose level was determined. All the animals treated with STZ confirmed to be over 300 mg/dL. We started ABE administration the next day after confirmation. STZ+ABE group rats (n=6) received 40 mg/kg ABE solution via oral gavage every day for 4 weeks, and control group rats (n=6) and STZ group rats (n=6) received distilled water only, orally.

Previously, we found that the administration of 10 mg/kg azuki bean seed coat-containing diets (equal to approximately 54 mg/kg daily ABE) to Wistar Kyoto rats for 8 weeks did not show noticeable toxic signs (Mukai et al. 2011). Based on the previous finding, we assumed 40 mg/kg ABE to be non-toxic dose and used in this study. We checked the body weight of the animals every day and administered ABE solution that adjusted to be ABE 40 mg/kg body weight. All groups of rats were allowed unrestricted access to a standard commercial laboratory diet (MF diet; supplied by Oriental Yeast Co., Ltd., Tokyo, Japan) and distilled water. After four weeks of ABE administration, they fasted overnight, and the weight of the animals was measured, blood samples were collected under ether anaesthesia from the subclavian vein. The next day, they were killed. The hearts were immediately removed and, weighed. The separated tissues were immediately frozen in liquid nitrogen and stored at −80℃ until used in experiments.

Blood chemistry

Plasma samples were obtained by centrifugation (800×g, 15 min at 4℃), and then the plasma glucose level was measured using a commercially available kit (Wako Pure Chemical Industries, Ltd.).

Western blot analysis

The heart sample was added to 1 mL of homogenate buffer, containing 55mM Tris (pH 7.4), 2.2% sodium dodecyl sulphate (SDS), 5.5% β-mercaptoethanol, 11% glycerol, and 55 mM phenylmethane sulfonyl fluoride. We homogenised the sample four times for 15 sec with a Polytron (PCU Drehzahlregler, Kinematica, Switzerland), then centrifuged at 150×100 rpm, for 20 min at 4℃, and the supernatant was transferred to a fresh tube. The tubes were heated for 5 min at 100℃, and 0.1% BPB-glycerol was added. The protein concentration was measured by Bradford assay (Protein Assay, BIO-RAD, USA). Adjusted proteins were separated by SDS-PAGE (12.5% e-PAGEL, ATTO, Japan). Biotinylated protein molecular weight markers (M&S TechnoSystems, Japan) were used as protein standards. Proteins were electrophoretically transferred onto a nitrocellulose membrane (Bio-rad, USA) by using the iBlot transfer system (Invitrogen, USA). The membrane was incubated overnight at 4℃, in a blocking solution, containing a 40 mM Tris-HCl buffer (pH 7.4), 0.9% NaCl, 0.15% Tween 20, and 3% blocking reagent. The membrane was washed twice for 3 min with a 40 mM Tris-HCl buffer (pH 7.4), 0.9% NaCl, and 0.3% Tween 20, and then exposed to the diluted primary antibody. Anti-ACOX1 antibody, anti-catalase antibody, anti-FOXO-1a, anti-phosphorylated FOXO-1a (p-FOXO-1a, Ser256) antibodies, anti-Glutathione Peroxidase 1 antibody, anti-NDUFS3-C-terminal antibody, anti-Superoxide Dismutase 1 (Cu, Zn-SOD) antibody, anti-SOD2/Mn-SOD antibody, anti-UQCRC2 antibody, anti-βactin antibody [Abcam, Japan], COX IV (3E11) Rabbit mAb [Cell Signaling Technology, Danvers, MA], p62 (SQSTM1) antibody [MBL, Japan], Ser403-phosphorylated p62 antibody [Gene Tex, Unites States] and anti-HO-1 antibody [Enzo Life Sciences AG, Lausen, Switzerland] were incubated with the blot in a 1% blocking solution. Again, the membrane was washed 3 times for 3 min, and then exposed to the secondary

antibody: Anti-Rabbit IgG IRDye 680 or Anti-Mouse IgG IRDye 800 (M&S Techno Systems, Japan). Finally, the membrane was washed 5 times for 3 min and the protein bands were quantitated with an Odyssey Infrared Imaging System (M&S Techno Systems). All experiments were performed in triplicate. In the analysis, protein levels were quantified as fold values of control levels after adjusting for endogenous β-actin.

8-OHdG levels in the heart

DNA was extracted based on the high pure PCR template preparation kit and hydrolysed using an 8-OHdG assay preparation reagent set (Wako Pure Chemical Industries, Ltd., Japan). 8-OHdG was measured by ELISA (enzyme-linked immunosorbent assay) using a monoclonal antibody specific to 8-OHdG (Highly Sensitive 8-OHdG Check ELISA, JaICA, Nikken Seil Co., Ltd., Japan). All experiments were performed in triplicate.

Statistical analysis

Each value is expressed as mean ± SEM. Statistical analyses were performed by one-way analysis of variance, followed by a multiple comparison test (Fisher's LSD test) using BellCurve for Excel (Social Survey Research Information Co., Ltd.). In all cases, $p<0.05$ was considered statistically significant.

Results

Body weight, heart mass, and plasma glucose level

The body weights of diabetic groups (STZ group and STZ+ABE group) were significantly lower than those of the control group. The relative weights of the hearts in the diabetic groups were higher than those in the control group (Table 1). The plasma glucose levels of the diabetic groups were significantly higher than those of the control group (Fig. 1). Pathological changes such as collagen accumulation or fibrosis were not confirmed in early diabetic rat hearts (data not shown).

Table 1. Morphological characteristics

	Control	STZ	STZ+ABE
Body weight‡ (g)	328.7±10.9	258.5±2.6[a]	254.1±4.2[a]
Heart (g)	0.900±0.027	0.804±0.020[a]	0.775±0.015[a]
Heart/Body weight (g/kg)	2.752±0.029	3.173±0.061[a]	3.067±0.039[a]

‡ At sacrifice. Values are means ± SEM (n = 6). [a]p< 0.05 compared with the control group.

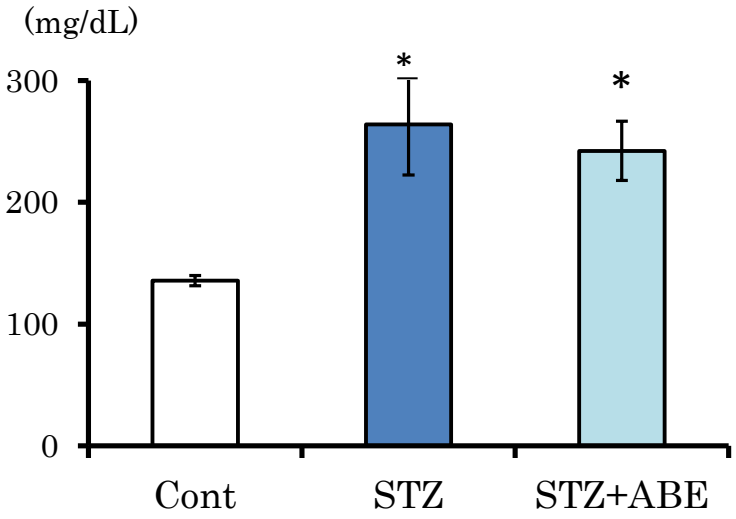

Fig 1. Plasma glucose level

Values are shown as mean ± SEM (n = 6). *: p < 0.05 compared with the control group.

The protein levels of peroxisomal ACOX1 and catalase in diabetic rat hearts.

Acyl-CoA oxidase 1 (ACOX1) is the first rate-limiting enzyme of the peroxisomal straight-chain FA oxidation system. The level of ACOX1 was significantly higher in the diabetic groups than in the control group (Fig. 2(A)). The levels of peroxisomal catalase in the diabetic groups were significantly higher than those in the control group (Fig. 2(B)).

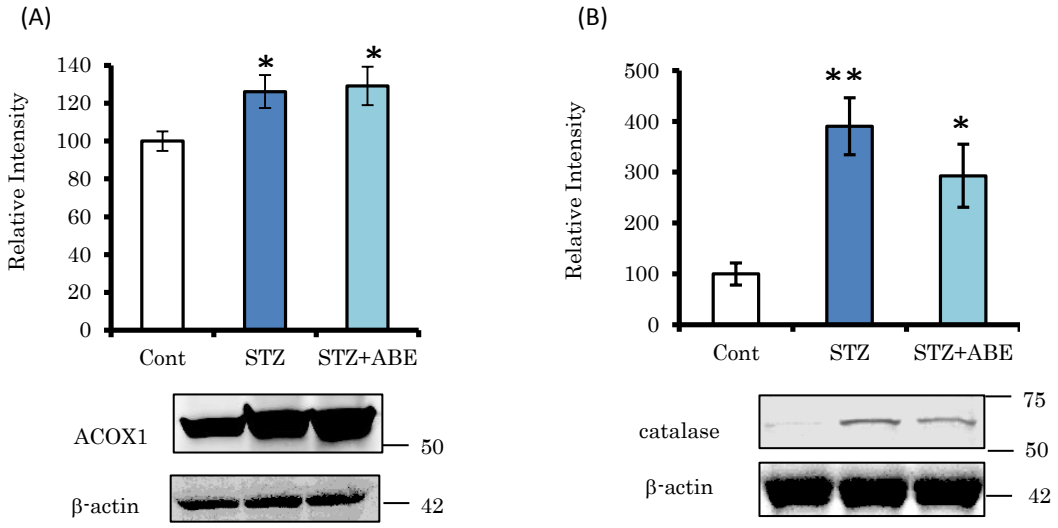

Fig 2. The protein levels of peroxisomal ACOX1 (A) and catalase (B) in diabetic rat hearts. ACOX1 (A) is the first rate-limiting enzyme of the peroxisomal straight-chain FA oxidation system and catalase (B) converts of H_2O_2 to oxygen and water, at peroxisome.

Values are shown as mean ± SEM (n = 6). **: $p < 0.01$, *: $p < 0.05$ compared with control group. Each band in figure is representative of the average behavior of each group.

The protein levels of COX IV, UQCRC2 and NDUFS3 in diabetic rat hearts.

The level of cytochrome oxidase subunit IV (COX IV), which is inferred to reflect the number of mitochondria (Lomax et al. 1992), did not change significantly in any of the groups (Fig. 3(A)). The cytochrome b-c1 complex subunit 2 (UQCRC2) and NADH dehydrogenase iron-sulphur protein 3 (NDUFS3) are present in the mitochondrial matrix and produce O_2^- during the electron transfer process. The levels of UQCRC2 and NDUFS3 did not change significantly in any of the groups (Fig. 3(B) (C)).

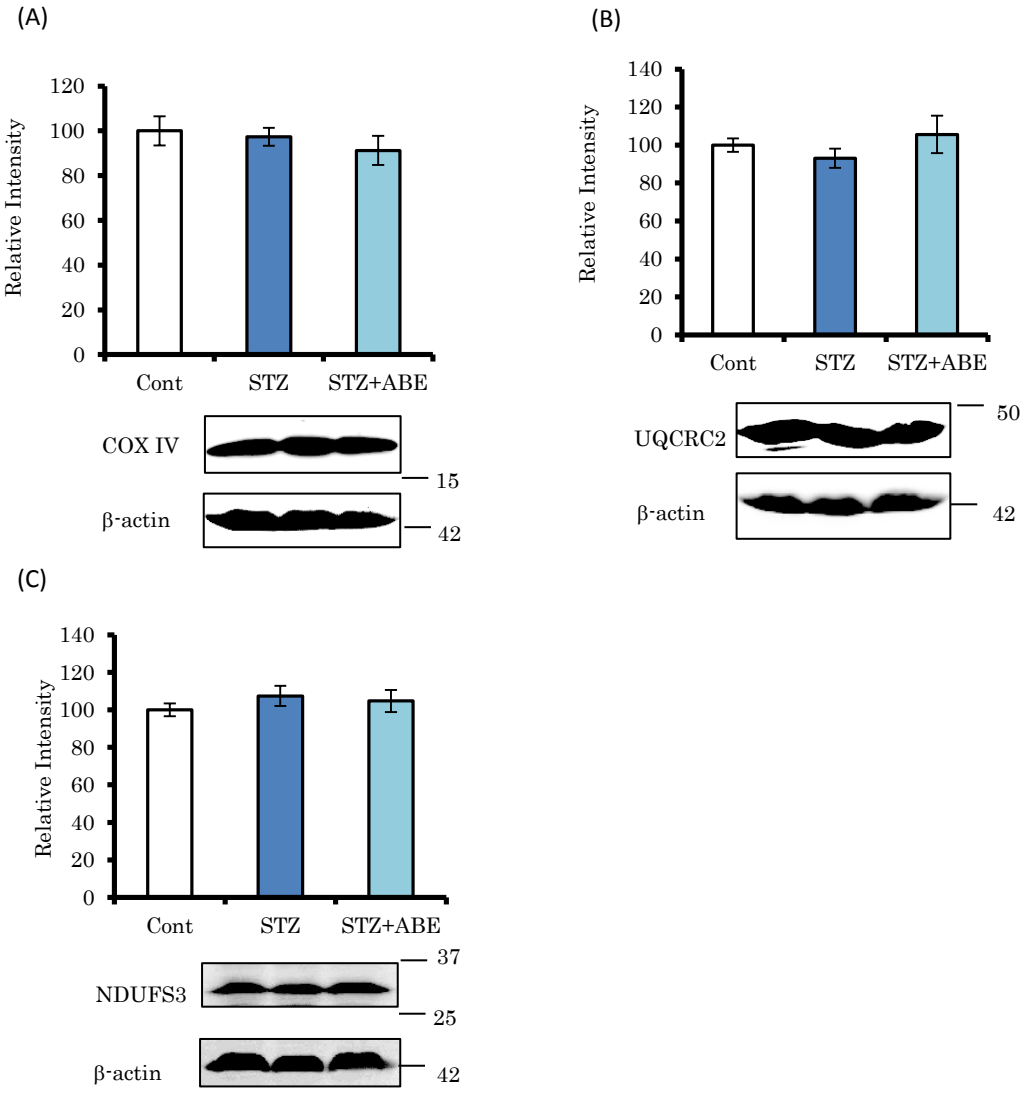

Fig 3. The protein levels of COX IV (A), UQCRC2 (B) and NDUFS3 (C) in diabetic rat hearts. COX IV(A) is inferred to reflect the amounts of mitochondria. UQCRC2 (B) is a core protein of complex III and NDUFS3 (C)is a protein present in complex.

Values are shown as mean ± SEM (n = 6).

Each band in figure is representative of the average behavior of each group

The protein levels of p62/SQSTM1, p-p62, and HO-1 in diabetic rat hearts.

The levels of p62 in the STZ group were significantly higher than those in the control group, no significant difference was observed between the STZ+ABE group and the control group (Fig. 4(A)). The levels of p-p62 in the STZ group were significantly higher than those in the control group, and the levels in the STZ+ABE group were significantly lower than those in the STZ group (Fig. 4(B)). The levels of HO-1 in the STZ group were significantly higher than those in the control group, and the HO-1 levels in the STZ+ABE group were significantly lower than those in the STZ group (Fig. 4(C)).

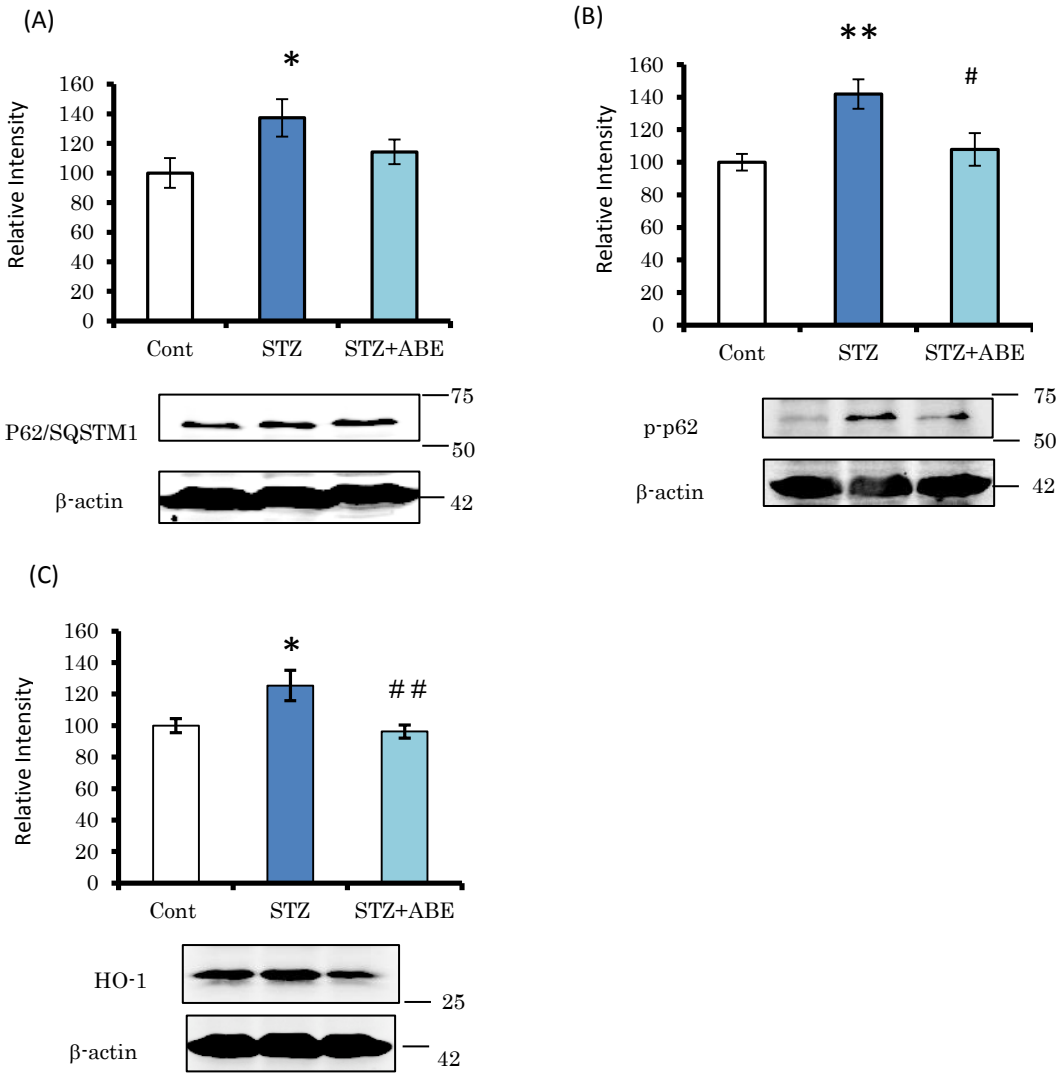

Fig 4. The protein levels of p62/SQSTM1 (A), p-p62 (B), and HO-1 (C) in diabetic rat hearts. They are stress maker proteins. Values are shown as mean ± SEM (n = 6).

**: $p < 0.01$, *: $p < 0.05$ compared with control group. $^{\#\#}p$: < 0.01, $^{\#}$: $p < 0.05$ compared with STZ group. Each band in figure is representative of the average behavior of each group.

The levels of index of oxidative DNA damage 8-OHdG in diabetic rat hearts.

The levels of 8-OHdG in the STZ + ABE group were significantly lower than those in the STZ group (Fig. 5).

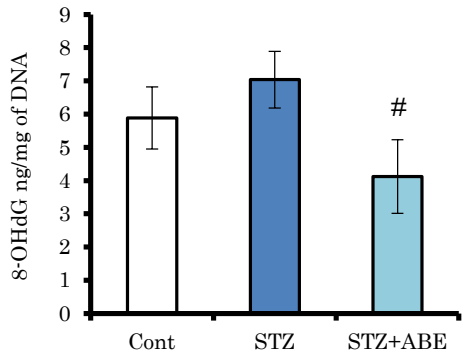

Fig 5. The levels of 8-OHdG in diabetic rat hearts.

8-OHdG is index of oxidative DNA damage.Values are shown as mean ± SEM (n = 6).

[#]: $p < 0.05$ compared with the STZ group.

The protein levels of Cu, Zn-SOD, Mn-SOD and GPx in diabetic rat hearts.

We focused on the copper-zinc superoxide dismutase (Cu, Zn-SOD), manganese superoxide dismutase (Mn-SOD) and glutathione peroxidase (GPx) to investigate key antioxidant enzymes. The levels of these enzymes did not change significantly in any of the groups (Fig. 6).

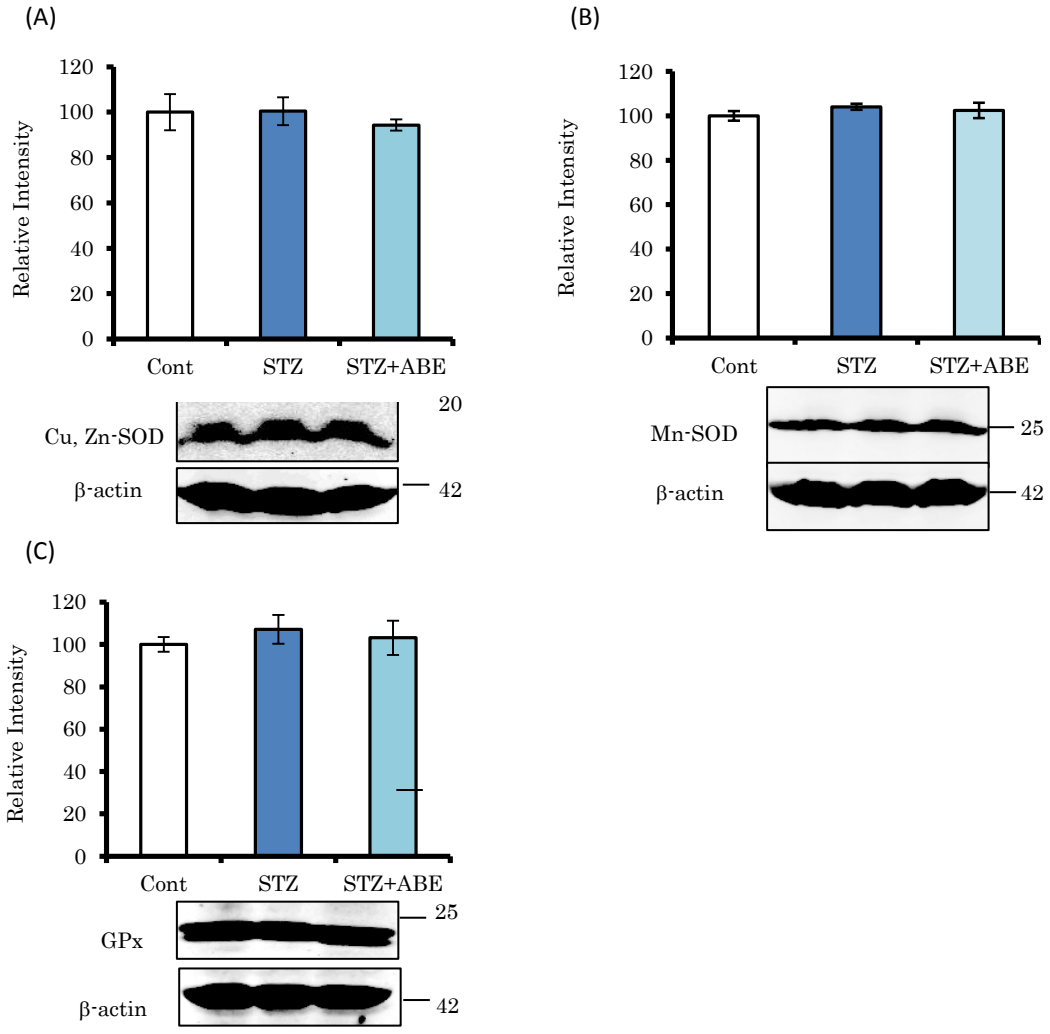

Fig 6. The protein levels of Cu, Zn-SOD (A), Mn-SOD (B) and GPx (C) in diabetic rat hearts. They are antioxidant enzymes. Values are shown as mean ± SEM (n = 6).

Each band in figure is representative of the average behavior of each group.

The protein levels of the transcription factors proteins of antioxidant enzymes.

FOXO transcription factors are known to regulate the expression of antioxidant enzymes such as Mn-SOD, GPx, and catalase. The total level of FOXO-1a did not change significantly in any of the groups (Fig. 7(A)). However, the levels of p-FOXO-1a in the diabetic groups were significantly higher than those in the control group (Fig. 7(B)).

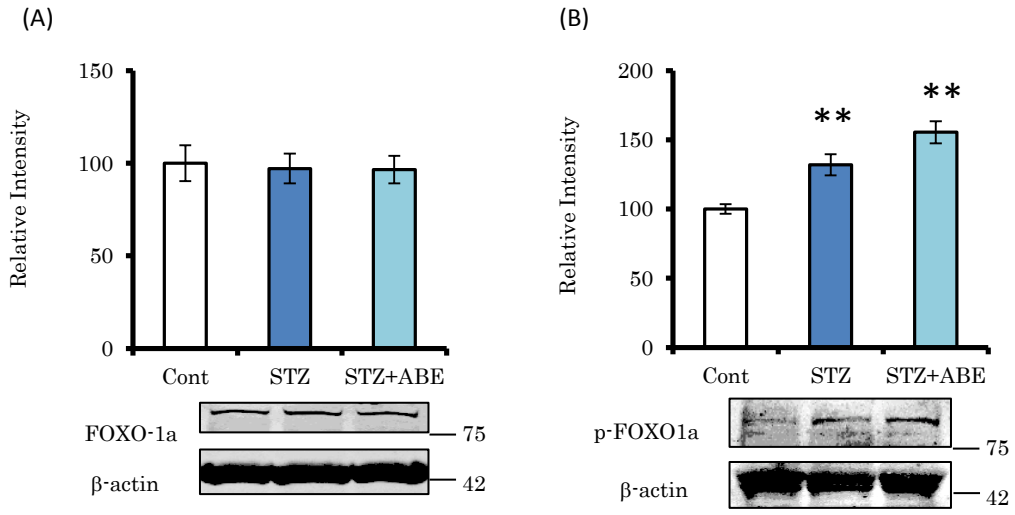

Fig 7. The protein levels of the transcription factors proteins of antioxidant enzymes.

Total FOXO-1a (A) and p-FOXO1a (B) in diabetic rat hearts.

Values are shown as mean ± SEM (n = 6). **: $p < 0.01$ compared with control group.

Each band in figure is representative of the average behavior of each group.

Discussion

In previous studies, cardiac hypertrophy and fibrosis were reported in the STZ-induced diabetic model at 16 weeks after the onset of diabetes (Wang et al. 2014). In this study, pathological changes such as collagen accumulation or fibrosis were not confirmed. Previous reports showed that after the 3 weeks in diabetic animals, pro-inflammatory cytokine expression was up-regulated in myocardia, and initial abnormalities in cardiomyocyte contractility occurred, and left ventricular mass was

lowered by 23%, and a definite ventricular remodeling was already present (Savi et al. 2016, Stilli et al. 2007, Delucchi et al. 2012). In diabetic rats 5 weeks after STZ injection, there were significant increase in cross-section area and myocardial collagen deposition of LV cardiomyocyte, and the levels of LV end-diastolic volume and stroke volume were decreased (Gao et al.2012). After 26 days of diabetes there were significant decreases in resting LV systolic pressure, developed pressure, and maximal +dP/dt, whereas LV end-diastolic pressure increased and the time constant of LV relaxation was prolonged. LV cavity/wall volume and end-diastolic volume were increased (Litwin et al.1990). It is also widely known that oxidative stress and moderate inflammation of cells existing in the early stages of diabetes are closely related to the pathogenesis of diabetic cardiomyopathy. ABE did not reduce glucose level, however, we confirmed variations in the levels of proteins related to oxidative stress in the diabetic heart.

Because glucose metabolism cannot be carried out normally in the diabetic heart. To meet the high-energy demands of the contracting muscle, the heart produces a constant and available supply of ATP. In uncontrolled diabetes, FA β-oxidation provides 90 to 100% of the ATP production in the heart. When FA β-oxidation is carried out, ROS such as H_2O_2 are produced in the process. Therefore, we focused on proteins related to FA β-oxidation. ACOX1 is the first rate-limiting enzyme of the peroxisomal straight-chain FA oxidation system. In the diabetic groups, the level of peroxisomal ACOX1 was enhanced suggesting that peroxisomal FA β-oxidation and H_2O_2 production increased. In the process of peroxisomal FA β-oxidation, ACOX1 generates H_2O_2, a target substrate of catalase (Varanasi et al. 1994). Catalase localized in peroxisomes catalyses the conversion of H_2O_2 to oxygen and water (Chelikani et al. 2004). We observed up-regulation of the ACOX1 level in the diabetic groups, leading to that H_2O_2 production increased in the process of FA β-oxidation at peroxisomes. Catalase is localized in peroxisome and is one of the antioxidative enzyme involved in elimination of H_2O_2. In response to the increased H_2O_2, the peroxisomal catalase, which acts as a defense system, is also induced to protect the cell from oxidative stress. ABE had no effect on increased level of the peroxisomal ACOX1 induced by STZ injection.

In our study, the levels of catalase were significantly higher in the diabetic groups than those in the

control group. Increased level of catalase was reported in several studies of STZ-diabetic rats (Mayyas et al. 2017, Ivanović-Matić S et al. 2010), while decreased in some studies (Vuppalapati et al. 2016, Roslan et al. 2017). At the end of the 2nd week STZ administration, catalase activity exhibited a maximal increase of 150% and remained 80% above the control level throughout 16 weeks (Ivanović-Matić S et al. 2010). From these reports, the activity of catalase is also related to the time course from the onset of diabetes. These discrepancies between catalase levels findings might be related to the differences in the used rat strain, gender and age, as well as the dose, duration, and administration route of STZ that affect DM severity (Deeds et al. 2014).

Next, we observed proteins related to ATP synthesis in mitochondria. A large amount of ROS is produced as a product of incomplete reduction of molecular oxygen in the electron transfer system in mitochondria during respiration. In particular, a large amount of ROS is produced during the transfer of electrons from complex I to III in mitochondria (Brunyanszki et al. 2016, Cadenas et al. 2000). COX IV is inferred to reflect the amounts of mitochondria (Lomax et al. 1992). UQCRC2 is a core protein of complex III (Fernandez-Vizarra et al. 2015) and NDUFS3 is a protein present in complex I (Lim et al. 2013). They constitute part of the mitochondrial respiratory chain. The levels of COX IV, UQCRC2, and NDUFS3 did not change significantly in diabetic groups.

However, previous studies reported the abnormalities in energy metabolism and dysfunctions in cardiac mitochondria in diabetes (Thirunavukkarasu et al. 2007), and impairment in mitochondrial respiratory capacity, alterations in mitochondrial structure and change in expression of respiratory chain complexes, in hearts of STZ-induced diabetic animals (Bugger et al. 2010). These results suggest that mitochondrial dysfunction might occur in early diabetes, possibly increasing ROS production, although we did not find changes in levels of some mitochondrial proteins in the heart of 4 weeks from the onset of diabetes.

Next, we examined stress marker proteins, since it has been reported that hyperglycaemia increases ROS that cause oxidative damage. p62 protein is upregulated with an increase in peroxidised oxygen concentration and the accumulation of autophagosomes in STZ-induced diabetes model heart increases with the increase of p62 (Wang et al. 2014). Autophagy dysfunction occurs in the diabetic

hearts which leads to the accumulation of mitochondrial ROS (He et al. 2013, Tal et al. 2009). Excessive ROS also bring about the dysfunction of autophagy (Oh et al. 2012). p-p62 (Ser403) increases the affinity of its ubiquitin-associated domain and enhances the recruitment of polyubiquitinated proteins into the autophagosome. Heme oxygenase-1 (HO-1) is an enzyme induced by oxidative stress which protects the heart from oxidant-mediated damage in diabetic animals (Chang et al. 2011, Rodriguez et al. 2011).

The levels of p62 were significantly higher in the STZ group than those in the control group. The levels of p-p62 and HO-1 were significantly higher in the STZ group than those in the control group, and they were significantly decreased in the STZ+ABE group compared to the STZ group. It was revealed that the increases in the levels of p62, p-p62 and HO-1 were caused by oxidative stress. These results suggest that the hearts of the diabetic groups were exposed to oxidative stress compared with the control group. We also observed a reduction in the expression levels of p-p62 and HO-1 by ABE administration.

8-OHdG is an index of oxidative DNA damage. The levels of 8-OHdG in the STZ+ABE group were markedly lower than those in the STZ group. Hyperglycaemia increases various ROS that cause oxidative damage. p62, p-p62 and HO-1 are oxidative stress marker proteins, and they increased with the oxidative damage induced by STZ injection. ABE decreased the protein levels of oxidative stress marker proteins (p-p62 and HO-1), and diminished DNA oxidative damage in the STZ-induced early diabetic rat. These results suggested that ABE has antioxidant effects and protects from oxidative damage of the heart in the STZ-induced early diabetic rats. In previous report, a significant increasing in 8-OHdG was observed in the diabetic rat compared with healthy control group in the 4 weeks experiment from the onset of diabetes (Farhangkhoee et al. 2006). However, in this study, there was no significant difference between control and STZ (diabetic) group. Differences in the used rat strain, age, sample size and dose of STZ, and used measurement sample (urine and heart tissue) and measurement method (immunohistochemistry and ELISA) might have influences on the results.

We then focused on antioxidant enzymes. Cu, Zn-SOD is located in the cytoplasm and the

mitochondria crista, and Mn-SOD is located in the mitochondria matrix. They convert O_2^-, a highly reactive type of ROS, to benign H_2O_2. GPx-1 is the most abundant isozyme of GPx, and is located in the cytoplasm and mitochondria and removes the H_2O_2 (Schriner et al. 2005, Beckman et al. 1998, Valko et al. 2006, Yakunin et al. 2014). The levels of Cu, Zn-SOD, Mn-SOD, and GPx did not change significantly in early diabetic groups.

FOXO transcriptional regulators are known to regulate the expression of antioxidant enzymes such as Mn-SOD, GPx, and catalase. Under strong oxidative stress conditions, FOXO-1a is not phosphorylated and remains in the nucleus to promote transcription of catalase (Bartholome et al. 2010). FOXOs have protective roles in resistance to oxidative stress through regulation of antioxidant activity (Greer et al. 2005, Paik et al. 2007). The total level of the FOXO-1a protein did not change in any of the groups. However, the levels of p-FOXO-1a in the diabetic groups were significantly higher than those in the control group, indicating a control mechanism suppressing the transcription of antioxidants. The reason why the level of phosphorylated FOXO1 is significantly higher in the diabetic group is not clear. Further research is needed to clarify the role of p-FOXO1 in the early diabetes.

Chapter 2

Changes of autophagy function in the hearts of streptozotocin-induced early diabetes rats, and the improvement effects by azuki bean (*Vigna angularis*) extract

Introduction

Diabetes is a metabolic disorder characterized by hyperglycaemia and causes dysfunction and complications of various organs and tissues. Diabetic cardiomyopathy, one of the chronic complications of diabetes, develops in patients with diabetes without coronary artery disease and hypertension, and is a major cause of heart failure in these patients. Many studies have demonstrated that there is hyperglycaemia at the center of the development of diabetic cardiomyopathy. Hyperglycaemia induces excessive production of reactive oxygen species (ROS) and causing a series of events leading to oxidative stress, cardiomyocyte apoptosis, myocardial damage, and cardiomyopathy, and cardiac dysfunction (Frustaci et al. 2000, Karbasforooshan et al. 2017, Li et al. 2006). Oxidative injury in the heart can cause myocardial cell death and fibrosis, hypertrophy, ventricular dilation, cardiac function deterioration, and ultimately heart failure (Senqupta et al. 2011). Increased ROS and dysfunction of ROS clearance contribute to the onset and progression of diabetic cardiomyopathy (Giacco F et al. 2010).

Autophagy occurs at the basal rate of most cells to maintain homeostasis, eliminating damaged organelles such as protein aggregates and mitochondria. Recent studies have demonstrated autophagy dysfunction in the heart of diabetes, and it has been revealed that accumulation of mitochondrial ROS increases due to autophagy dysfunction (He C et al 2013, Tal MC et al. 2009, Oh JM et al. 2012). Studies have demonstrated that STZ‐induced diabetic mice exhibit autophagosome accumulation in the heart (Xie Z et al. 2011, Wang B et al. 2014). The overactivation or inhibition of any step of the process of autophagy can cause myocardial injury (Huang L et al. 2018). Significant increases in the O‐GlcNAc modification and accumulation of the autophagy markers microtubule‐associated protein 1 light chain 3α II/I and P62, which suggest that autophagic flux is inhibited, were observed in rats 8 weeks following STZ induction. Therefore, there is crosstalk between autophagy

and oxidative stress.

Recent studies have shown that plant-derived polyphenolic compounds such as epigallocatechin gallate, quercetin and resveratrol have beneficial effects similar to exercise and food restriction for metabolic diseases, and are useful for the prevention of obesity, cardiovascular disease, and diabetes (Zhang et al. 2009, Chen et al. 2012, Tomas-Barberan et al. 2012, Cardona et al. 2013, Sato, Mukai et al. 2016).There have reported that resveratrol has a beneficial effects on diabetic cardiomyopathy. Cardiac hypertrophy, fibrosis and increased autophagosomes observed at 16 weeks after onset of diabetesin STZ-induced, and the number of increased autophagosomes in the heart of diabetic model mice decreased with resveratrol (Wang B et al. 2014). Long-term resveratrol treatment improved reduced cardiac function and oxidative injury in diabetes (Thirunavukkarasu et al. 2007). After the 3 weeks in diabetic animals, pro-inflammatory cytokine expression was up-regulated in myocardia, and initial abnormalities in cardiomyocyte contractility occurred, and left ventricular mass was lowered by 23%, and a definite ventricular remodeling was already present (Savi et al. 2016, Stilli et al. 2007, Delucchi et al. 2012). The early resveratrol administration inhibited the pro-inflammatory cytokine production, leading to a recovery of cardiomyocyte contractile efficiency and reduced inflammatory cell recruitment, and improved ventricular function by reducing inflammatory state and decreasing ventricular remodeling (Savi et al. 2016, Delucchi et al. 2012).

The azuki bean (*Vigna angularis*) is rich in plant-derived polyphenols such as proanthocyanidins, catechins, and quercetin (Sato, Mukai et al. 2016). Proanthocyanidins are natural antioxidants that exert antioxidant activity in pathological conditions such as cardiovascular disease, inflammation, and diabetes (Mukai et al. 2013). Several studies have shown that azuki beans have beneficial effects on oxidative stress, hypertension, obesity, and diabetes (Liu et al. 2017, Yao et al.2012, Kitano-Okada et al. 2012, Mukai et al. 2011). It is also known that azuki bean extract (ABE) reduces oxidative stress and stimulates autophagy in the kidneys of STZ-induced early diabetic rats (Sato, Kataoka et al. 2016).

Two major pathways accomplish regulated protein catabolism in eukaryotic cells: the ubiquitin-proteasome system (UPS) and the autophagy-lysosomal system (Monika L et al. 2014).

The UPS serves as the primary route of degradation for thousands of short-lived proteins and many regulatory proteins and contributes to the degradation of defective proteins (Wolf DH et al. 2000). Autophagy, by contrast, is a major protein degradation system that targets primarily long-lived cytoplasmic proteins, and is known to include 3 main forms: chaperone-mediated autophagy, microautophagy, and macroautophagy (Kanamori H et al. 2015). Macroautophagy (hereafter referred to as autophagy) is a physiological self-degradation and recycling process that proceeds within double-membrane organelles via the lysosomal digestive pathway and functions to maintain the intracellular environment (Levine B et al. 2004). Autophagy is an evolutionarily conserved catabolic process used for degradation surplus and damaged proteins and organelles from the cytoplasm (Hubert V et al. 2016). Autophagy has most likely evolved both as a pathway for restoring intracellular nutrient supply during starvation and as a quality control mechanism to protect the cell against damage caused by toxic macromolecules and damaged organelles (Terje J et al. 2011). Following lysosomal degradation, recycling occurs to replenish the cell with nutrients and building blocks for anabolic processes (Mizushima N. 2007). Autophagy normally occurs at a basal level, but it is accelerated by a variety of stresses such as starvation, accumulation of abnormal proteins, organelle damage and pathogen infection (Nakamura S et al. 2017). Autophagy has a critical role in many physiological processes, such as cellular quality control, stress responses, and development (Deretic and Levine, 2009; Levine and Kroemer, 2008; Levine et al., 2011; Mizushima and Komatsu, 2011; White et al., 2010). During autophagy, a small cisterna, called the isolation membrane (phagophore), elongates and surrounds a part of the cytoplasm to form a double-membraned structure, called the autophagosome. Autophagosomes either fuse with late endosomes to form amphisomes, which then fuse with lysosomes, or they fuse directly with lysosomes (Berg et al., 1998; Fader et al., 2008). After fusion with the lysosome, they are called autolysosomes and the sequestered contents are digested (Nakamura S et al. 2017).

In the normal myocardium, autophagy is maintained at a low level (Gustafsson AB et al.

2008). The abnormal energy metabolism in diabetes is caused by high glucose, and autophagy can be overactivated or inhibited (Yorimitsu T and Klionsky DJ. 2005.). Furthermore, insufficient energy

due to low levels of ATP causes a cellular starvation state, and accumulated adenosine monophosphate (AMP) induces an increase in autophagy and myocardial injury by activating the AMP activated protein kinase pathway (Liu S and Li Y. 2017).

Autophagy is initiated by formation of the phagophore, a crescentshaped double membrane that expands and fuses to form a double-membrane vesicle, the autophagosome (Terje J et al. 2011, Mizushima et al. 2011, Tooze and Yoshimori. 2010). Formation of the autophagosome is regulated by the hierarchical function of a number of autophagy-related (Atg) proteins. All these Atg proteins except LC3 detach from completed autophagosomes (Fujita et al., 2008; Itakura and Mizushima, 2010). The kinase mTOR (mammalian target of rapamycin) is a critical regulator of autophagy induction, with activated mTOR (Akt and MAPK signaling) suppressing autophagy, and negative regulation of mTOR (AMPK and p53 signaling) promoting it. mTOR activity reflects cellular nutritional status (Wullschleger S et al. 2006). Following amino-acid starvation or mTOR inhibition, the activated ULK1 phosphorylates Beclin1 (the mammalian homologue of yeast Atg6) on Ser14, thereby enhancing the activity of the the class III phosphatidylinositol 3-kinase complex, which comprises PI3K (Vps34 in yeast), Beclin 1, VPS15 (PIK3R4) and ATG14L (ATG14) (Nakamura S et al. 2017). Beclin1 is part of the class III PI3K complex that is required for the formation of the autophagic vesicle, and interference with Beclin1 prevents autophagy induction (Levine B et al. 2005). The Beclin1 Ser14 phosphorylation by ULK is required for full autophagic induction in mammals and this requirement is conserved in Caenorhabditis elegans (Ryan CR et al. 2013). The subsequent elongation and closure of the isolation membrane are mediated by two ubiquitinlike ATG conjugation pathways, ATG5-ATG12 and LC3/Atg8. In mammals, there are seven Atg8 orthologues; MAP1LC3A/B/C, GABARAP and GABARAPL1/2/3 (all of which are hereafter referred to as LC3). LC3 is widely used as a marker for the microscopic detection of isolation membranes and autophagosomes. After synthesis, LC3 is processed at its C terminus by Atg4 and becomes LC3-I, which has a glycine residue at the C-terminal end. LC3-I is subsequently conjugated with phosphatidylethanolamine (PE) to become LC3-II by a ubiquitination-like enzymatic reaction that requires Atg7 and Atg3 (E1 and E2-like enzymes, respectively) (Nakamura S et al. 2017). In contrast

to the cytoplasmic localization of LC3-I, LC3-II associates with both the outer and inner membranes of the autophagosome. PE-conjugated LC3 (LC3-II) and unconjugated LC3 (LC3-I) can be detected separately by immunoblot analysis, and the amount of LC3-II is also widely used for the quantification of autophagic activity (Kabeya et al., 2000).

The protein p62, which binds ubiquitin and LC3 and is a selective substrate for autophagy, regulates the formation of protein aggregates (Komatsu M et al. 2010). Genetic ablation of p62 suppressed the appearance of ubiquitin-positive protein aggregates in autophagy-deficient mice (Komatsu M et al. 2007), indicating that p62 is important in the formation of inclusion bodies. Importantly, excess accumulation of p62 and inclusion bodies containing both ubiquitinated proteins (Ub proteins) and p62 have been identified in several human disorders, especially in neurodegenerative diseases (Kuusisto E et al. 2001), liver injuries (Stumptner C et al. 2002) and hepatocellular carcinoma (Zatloukal K et al. 2002). p62-mediated selective autophagy has been implicated as a compensatory pathway for proteasomal protein degradation (Komatsu M et al. 2007, Korolchuk V et al. 2009), as autophagy can remove protein aggregates (Filimonenko M et al. 2010, Martinez VM et al. 2010). There is growing evidence that p62 is an important mediator for targeting Ub proteins to the autophagy system and has a crucial role in the removal of aggregated proteins (Deretic V et al. 2010, Komatsu M et al. 2010). As p62 is itself degraded during selective autophagy, p62 mediating signal transduction should be distinct from the p62 managing selective autophagy. Phosphorylation of p62 at serine 403 increases the affinity of its ubiquitin-associated domain for polyubiquitinated chains. This enhances the recruitment of polyubiquitinated protein into the sequestosome that is a targeting unit for the autophagosome. Casein kinase 2 (CK2) phosphorylates Serine 403 of p62, and overexpression of CK2 reduces the large inclusion formation of the mutant huntingtin exon 1 fragment in a p62-dependent manner. The Ser403-phosphorylated p62 binding with the polyubiquitin chain is resistant to dephosphorylation. Thus, Ser403-phosphorylated p62 enhances autophagic degradation of Ub proteins, when the proteasome is malfunctioning or ubiquitinated proteins are accumulated (Matsumoto G et al. 2011).

The unwanted material is incorporated into autophagosomes that eventually fuse with lysosomes,

leading to the degradation of their cargo. Qa-SNARE syntaxin-17 (STX17) localizes to the ER under feeding conditions but, upon starvation, it relocalizes to ER mitochondria contact sites (Hamasaki et al., 2013). Here, STX17 binds to, and so recruits, ATG14L to ER-mitochondria contacts to initiate formation of the isolation membrane. By contrast, STX17, which is involved in autophagosome-lysosome fusion, is presumably recruited from the cytosol to closed autophagosomes (Itakura et al., 2012). Upon starvation in mammals, STX17 is recruited, presumably from the cytosol to closed autophagosomes, and mediates autophagosome-lysosome fusion by binding to its partners, the Qbc-SNARE Synaptosomal associated protein 29 (SNAP29) and the lysosomal R-SNARE vesicle‐associated membrane protein 8 (VAMP8) (Itakura et al., 2012, Hubert V et al. 2016). Lysosome membrane-associated protein-2 (LAMP-2) is essential for STX17 expression by the autophagosomes and this absence is sufficient to explain their failure to fuse with lysosomes and increased numbers of autophagosomes (Hubert V et al. 2016).

The lysosome is an organelle that is specialized for degradation. When the lysosome fuses with other cellular compartments, lysosomal hydrolases degrade materials inside the target compartments. STX17 localizes to the outer membrane of completed autophagosomes but not to the isolation membrane (unclosed intermediate structures); for this reason, the lysosome does not fuse with the isolation membrane. Depletion of STX17 causes accumulation of autophagosomes without degradation. STX17 has a unique C-terminal hairpin structure mediated by two tandem transmembrane domains containing glycine zipper-like motifs, which is essential for its association with the autophagosomal membrane (Itakura E et al. 2012). STX17, which plays several potentially diverse roles (Itakura et al. 2012, Hamasaki et al. 2013, Arasaki et al. 2015, Mc- Lelland et al., 2016), once recruited to autophagosomes forms a trans-SNARE complex by pairing with the R-SNAREs (e.g., VAMP8; Furuta et al., 2010; Itakura et al., 2012; Wang et al., 2016) located within the late endosomal/lysosomal membranes (Jahn and Scheller, 2006). It has been shown that STX17 inserts into the autophagosomal membrane as a hairpin-type tail-anchored protein coming from the cytosol or other locales (Itakura et al., 2012). Recruitment of STX17 to emerging autophagosomes is strongly influenced by the LC3 lipidation machinery (Tsuboyama et al., 2016). STX17 directly

interacts with the immunity-related GTPase M (IRGM), and efficient STX17 recruitment to autophagosomes requires IRGM. Both IRGM and Stx17 directly interact with mammalian Atg8 (LC3) proteins, thus being guided to autophagosomes.

Furthermore, the O-linked N-acetylglucosamine (O-GlcNAc) modification of SNAP29 negatively regulates SNARE-dependent fusion between autophagosomes and lysosomes (Guo et al., 2014). SNAP29 is an O‑GlcNAc substrate and mainly involved in the localization and fusion of organelle membranes in the cytoplasm (Hohenstein AC and Roche PA. 2001). The increase in O‑GlcNAc‑modified SNAP29 inhibits SNAP29‑STX17‑VAMP8 complex formation, thereby inhibiting the degradation of autophagy and exacerbating myocardial injury in type I diabetic rats. The posttranslational modification of O‑GlcNAc regulates proliferation, differentiation, apoptosis, autophagy and other physiological and pathological processes in a variety of cells, including cardiomyocytes (Yi W et al. 2012, Wang P and Hanover JA. 2013, Pekkurnaz G et al. 2014, Ruan HB et al. 2014, Peng C et al.2017, Ngoh GA et al. 2010).

However, little is known about whether ABE has beneficial effects on the cardiac function in early diabetes. Therefore, this study aimed to examine and to clarify the changes in autophagy in the hearts of rats at the early stages of STZ-induced diabetes, and whether ABE, which is enriched with polyphenols, could influence the changes.

Materials and methods

Western blot analysis

The heart sample was added to 1 mL of homogenate buffer, containing 55mM Tris (pH 7.4), 2.2% sodium dodecyl sulphate (SDS), 5.5% β-mercaptoethanol, 11% glycerol, and 55 mM phenylmethane sulfonyl fluoride. We homogenised the sample four times for 15 sec with a Polytron (PCU Drehzahlregler, Kinematica, Switzerland), then centrifuged at 150×100 rpm, for 20 min at 4℃, and the supernatant was transferred to a fresh tube. The tubes were heated for 5 min at 100℃, and 0.1% BPB-glycerol was added. The protein concentration was measured by Bradford assay (Protein Assay, BIO-RAD, USA). Adjusted proteins were separated by SDS-PAGE (12.5% e-PAGEL, ATTO, Japan).

Biotinylated protein molecular weight markers (M&S TechnoSystems, Japan) were used as protein standards. Proteins were electrophoretically transferred onto a nitrocellulose membrane (Bio-rad, USA) by using the iBlot transfer system (Invitrogen, USA). The membrane was incubated overnight at 4℃, in a blocking solution, containing a 40 mM Tris-HCl buffer (pH 7.4), 0.9% NaCl, 0.15% Tween 20, and 3% blocking reagent. The membrane was washed twice for 3 min with a 40 mM Tris-HCl buffer (pH 7.4), 0.9% NaCl, and 0.3% Tween 20, and then exposed to the diluted primary antibody. Anti-ATG7 antibody [EPR6251], anti-LAMP2 antibody, anti-LC3B antibody, anti-O-Linked N-Acetylglucosamine antibody [RL2], anti-SNAP29 antibody [EPR9199], anti-STX17 antibody, anti-VAMP8 / EDB antibody [EP2629Y] [Abcam, Japan], mTOR Antibody [Cell Signaling Technology, Danvers, MA], Beclin1 antibody [MBL, Japan] and mTOR antibody [Gene Tex, Unites States] were incubated with the blot in a 1% blocking solution. Again, the membrane was washed 3 times for 3 min, and then exposed to the secondary antibody: Anti-Rabbit IgG IRDye 680 or Anti-Mouse IgG IRDye 800 (M&S Techno Systems, Japan). Finally, the membrane was washed 5 times for 3 min and the protein bands were quantitated with an Odyssey Infrared Imaging System (M&S Techno Systems). All experiments were performed in triplicate. In the analysis, protein levels were quantified as fold values of control levels after adjusting for endogenous β-actin.

Statistical analysis

Each value is expressed as mean ± SEM. Statistical analyses were performed by one-way analysis of variance, followed by a multiple comparison test (Fisher's LSD test) using BellCurve for Excel (Social Survey Research Information Co., Ltd.). In all cases, $p<0.05$ was considered statistically significant.

Results

The protein levels of the autophagy induction related proteins mTOR and Beclin1 in diabetic rat hearts.

The levels of mTOR and Beclin1 proteins that are related with the autophagy induction did not change in any of the groups. The levels of mTOR and Beclin1 proteins that are critical regulators of autophagy induction and necessary for the initiation of autophagy and formation-maturation of autophagosome did not change in any of the groups (Fig.8 (A)(B)).

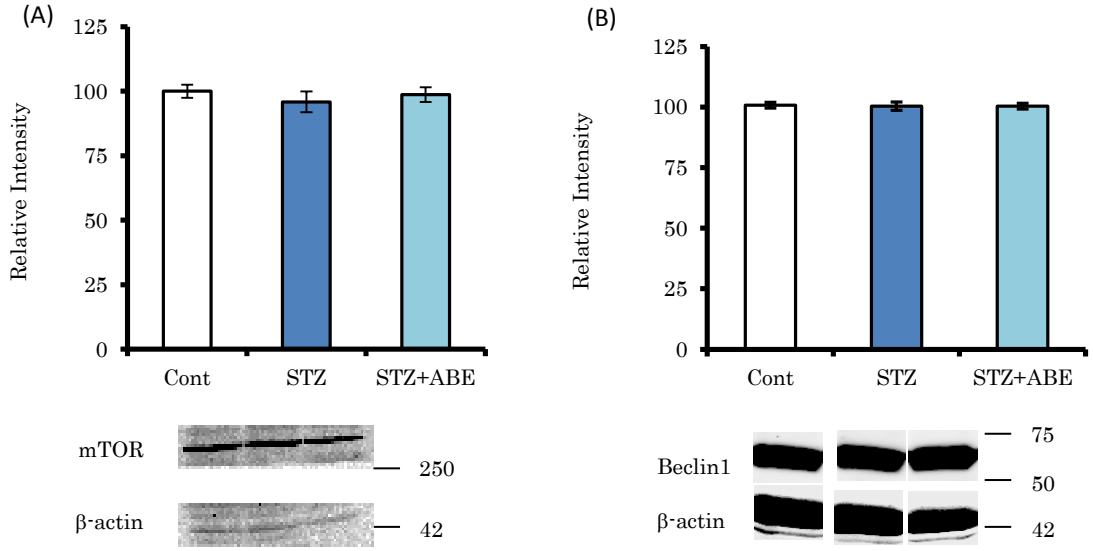

Fig 8. The protein levels of the autophagy induction related proteins in diabetic rat hearts.

mTOR (A) is a critical regulator of autophagy induction, Beclin1(B) is part of the class III PI3K complex that is required for the formation of the autophagic vesicle, and p-Beclin1(C) is required for full autophagic induction in mammals.

Values are shown as mean ± SEM (n = 6).

Each band in figure is representative of the average behavior of each group.

The protein levels of autophagosomes formation related proteins LC3 (LC3-Ⅱ/ LC3-Ⅰ), and Atg7 in diabetic rat hearts.

The levels of LC3 (LC3-Ⅱ/ LC3-Ⅰ) and Atg7 proteins that are necessary for the initiation of autophagy and formation-maturation of autophagosome did not change in any of the groups (Fig.9 (A)(B)).

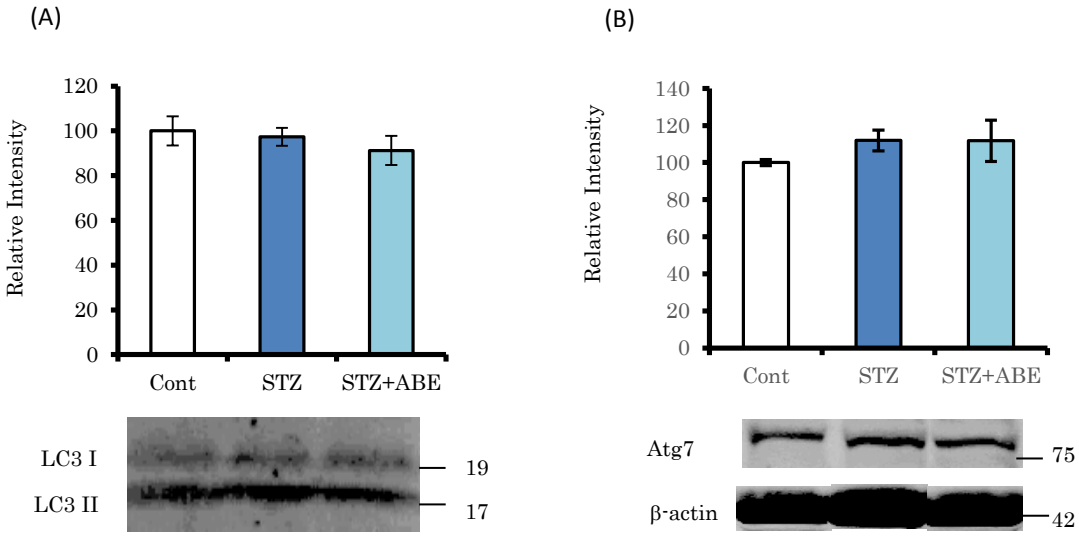

Fig 9. The protein levels of autophagosomes formation related proteins LC3 (LC3-Ⅱ/ LC3-Ⅰ)(A), and Atg7(B) in diabetic rat hearts. Values are shown as mean ± SEM (n = 6). Each band in figure is representative of the average behavior of each group.

The protein levels of p62/SQSTM1 and p-p62 in diabetic rat hearts.

p62 is an important mediator for targeting Ub proteins to the autophagy system and has a crucial role in the removal of aggregated proteins. Phosphorylation of p62 at serine 403 increases the affinity of its ubiquitin-associated domain for polyubiquitinated chains. This enhances the recruitment of polyubiquitinated protein into the sequestosome that is a targeting unit for the autophagosome.

The levels of p62 in the STZ group were significantly higher than those in the control group, no significant difference was observed between the STZ+ABE group and the control group (Fig.10 (A)).

The levels of p-p62 in the STZ group were significantly higher than those in the control group, and

the levels in the STZ+ABE group were significantly lower than those in the STZ group (Fig.10 (B)).

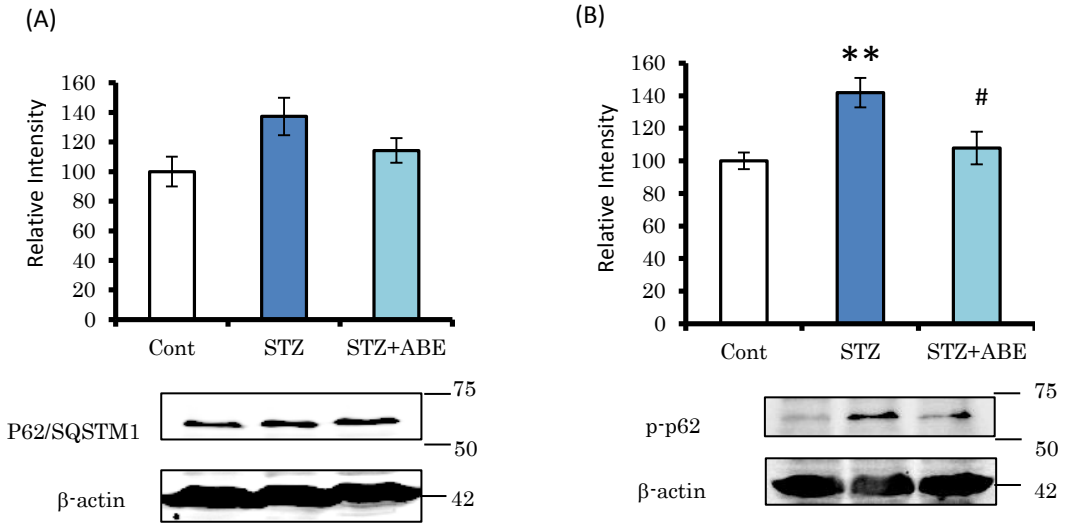

Fig 10. The protein levels of p62/SQSTM1 (A) and p-p62 (B) in diabetic rat hearts.

They are important mediators for targeting Ub proteins to the autophagy system and has a crucial role in the removal of aggregated proteins. Values are shown as mean ± SEM (n = 6). **: $p < 0.01$, *: $p < 0.05$ compared with control group. $^{\#}$:$p < 0.05$ compared with STZ group. Each band in figure is representative of the average behavior of each group.

The protein levels of autophagosome-lysosome fusion related proteins SNAP29, STX17, VAMP8 and LAMP2 in diabetic rat hearts.

STX17 is recruited from the cytosol to closed autophagosomes, and mediates autophagosome–lysosome fusion by binding to its partners, the SNAP29 and the lysosomal R-SNARE VAMP8. LAMP-2 is essential for STX17 expression by the autophagosomes and this absence is sufficient to explain their failure to fuse with lysosomes and increased numbers of autophagosomes. The levels of SNAP29, STX17 and VAMP8 proteins did not change in any of the groups (Fig.11 (A)(B)(C)). The levels of LAMP2 in the STZ group were significantly higher than those in the control group (Fig.11 (D)).

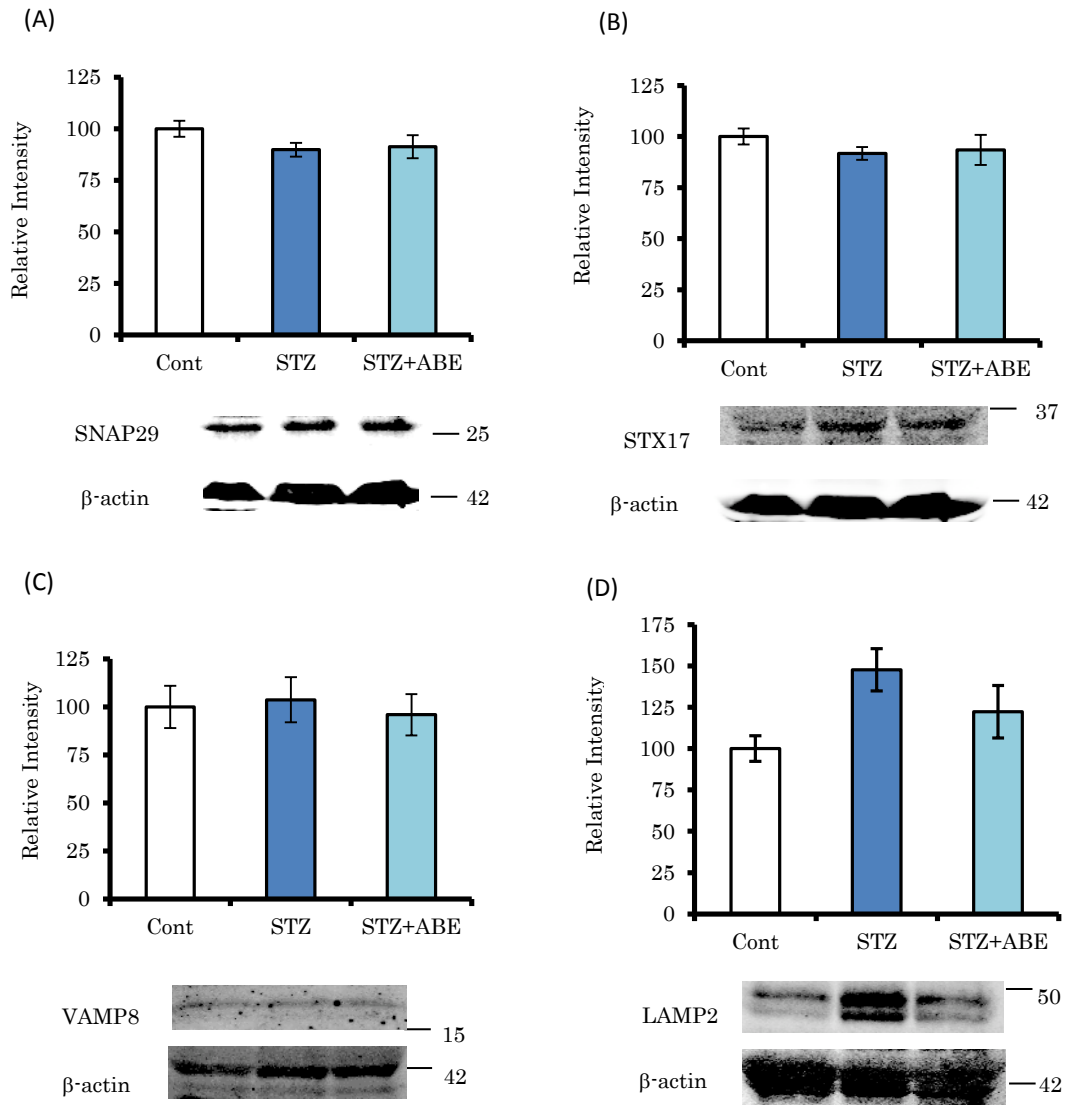

Fig 11. The protein levels of autophagosome-lysosome fusion related proteins SNAP29(A), STX17(B), VAMP8(C)and LAMP2(D) in diabetic rat hearts.

STX17 (A) is recruited from the cytosol to closed autophagosomes, and mediates autophagosome–lysosome fusion by binding to its partners, the SNAP29(B) and the lysosomal VAMP8(C). LAMP-2(D) is essential for STX17 expression by the autophagosomes. Values are shown as mean ± SEM (n = 6).).

*: $p < 0.05$ compared with control group.

Each band in figure is representative of the average behavior of each group.

The protein level of O-GlcNAc modification in diabetic rat hearts.

O-GlcNAc modification of SNAP29 negatively regulates SNARE-dependent fusion between autophagosomes and lysosomes. The levels of O-GlcNAc modification in the STZ group were significantly higher than those in the control group (Fig.12).

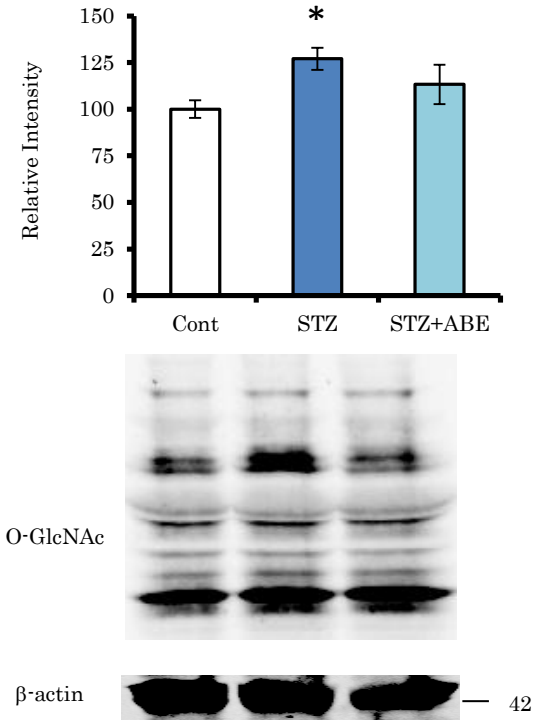

Fig 12. The protein levels of O-GlcNAc in diabetic rat hearts.

O-GlcNAc modification of SNAP29 negatively regulates SNARE-dependent fusion between autophagosomes and lysosomes.

Values are shown as mean ± SEM (n = 6). *: $p < 0.05$ compared with control group.

Each band in figure is representative of the average behavior of each gro

Discussion

There have been reported autophagy dysfunction in the heart of diabetes (He C et al. 2013, Tal MC et al. 2009, (Xie Z et al.2011, Wang B et al. 2014), and the accumulation of autophagosomes in STZ-induced diabetes model heart increases with the increase of p62 (Wang et al. 2014, Huang L et al. 2018). Autophagy dysfunction occurs in the diabetic hearts which leads to the accumulation of mitochondrial ROS (He C et al. 2013, Tal et al. 2009). Excessive ROS also bring about the dysfunction of autophagy (Oh et al. 2012). p-p62 (Ser403) increases the affinity of its ubiquitin-associated domain and enhances the recruitment of polyubiquitinated proteins into the autophagosome. So, we examined autophagosome key proteins p62 and p-p62.

In our study, the levels of p62 and p-p62 were significantly higher in the STZ group than those in the control group, and p-p62 were significantly decreased in the STZ+ABE group compared to the STZ group. It was revealed that the increases in the levels of p62 and p-p62 were caused by STZ-induced diabetes. These results suggest that the hearts of the diabetic groups were exposed to oxidative stress compared with the control group, and that autophagy functional abnormality may have occured in STZ group.

Since the levels of p62 and p-p62 were significantly increased in the STZ group, we presumed that in the heart of STZ-induced early-diabetic rats, some abnormality of autophagy function occurred compare with control group. To clarify this mechanism, we next focused on autophagy induction related proteins mTOR and Beclin1. The kinase mTOR is a critical regulator of autophagy induction. Beclin1 is part of the class III PI3K complex that is required for the formation of the autophagic vesicle, and interference with Beclin1 prevents autophagy induction (Levine B et al. 2005). The levels of mTOR and Beclin1 proteins that are related with the autophagy induction did not change in any of the groups.

Next, we observed autophagosomes formation related proteins LC3 (LC3-II / LC3-I), and Atg7. LC3 is widely used as a marker for the microscopic detection of isolation membranes and autophagosomes. After synthesis, LC3 is processed at its C terminus by Atg4 and becomes LC3-I. LC3-I is subsequently conjugated with phosphatidylethanolamine (PE) to become LC3-II by a

ubiquitination-like enzymatic reaction that requires Atg7 and Atg3. In contrast to the cytoplasmic localization of LC3-I, LC3-II associates with both the outer and inner membranes of the autophagosome. LC3-II and unconjugated LC3-I can be detected separately by immunoblot analysis, and the amount of LC3-II is also widely used for the quantification of autophagic activity (Kabeya et al., 2000). Previous genetic studies on the autophagy-essential protein Atg7 in the mouse showed that loss of autophagy caused a marked accumulation of p62 (Komatsu M et al. 2010). Accumulation of the autophagy markers microtubule‐associated LC3 II/I and P62, which suggest that autophagic flux is inhibited, were observed in rats 8 weeks following STZ induction(Huang L et al. 2018). In our study, the levels of p62 and p-p62 were siginificant increased in the STZ group, but the levels of LC3 (LC3-Ⅱ/ LC3-Ⅰ) , Atg7 proteins did not change in any of the groups in the early diabetic rats hearts.

Since the levels of proteins related induction and formation of autophagy did not a significant change, we considered that the fusion or degradation of autophagosomes and lysosomes may have some troubles. We then focused on autophagosome-lysosome fusion related proteins SNAP29, STX17, VAMP8, LAMP2, and O-GlcNAc modification. STX17 is recruited, presumably from the cytosol to closed autophagosomes, and mediates autophagosome-lysosome fusion by binding to its partners, SNAP29 and VAMP8 (Itakura et al., 2012). LAMP-2 is essential for STX17 expression by the autophagosomes and this absence is sufficient to explain their failure to fuse with lysosomes and increased numbers of autophagosomes. O-GlcNAc modification of SNAP29 negatively regulates SNARE-dependent fusion between autophagosomes and lysosomes (Guo et al., 2014).

The levels of SNAP29, STX17 and VAMP8 proteins did not change in any of the groups. But, the levels of LAMP2 in the STZ group were significantly higher than those in the control group, and the levels of O-GlcNAc modification in the STZ group were significantly higher than those in the control group.

LAMP-2 is essential for STX17 expression by the autophagosomes (Hubert V et al. 2016).

Continuous high‐glucose stimulation increased O‐GlcNAc modification of SNAP29 effectively, and inhibited the formation of the SANP29‐STX17‐VAMP8 complex, which acts as the mediator of

autophagosome and lysosome fusion , resulting in myocardial injury in the diabetic heart. Previous studies demonstrated that O‑GlcNAc modification was gradually enhanced over time in type I diabetic rat hearts.

The significant increaseing of LAMP2 and O-GlcNAc modification in the STZ group indicate that some abnormality has occurred anywhere up to the SANP29-STX17-VAMP8 complex binding. And these results suggest the fusion of autophagosomes and lysosomes is inhibited, the abnormality of autophagy function may occur.

In the STZ + ABE group, there was no significant change in the protein level of LAMP2, O-GlcNAc compared with the control group, which suggested that ABE has an effect on these proteins changes by diabetes.

These results indicate that there is no change in autophagy induction/initiation related proteins in the early stage of diabetes, but changes occur in the amount of p62 · p-p62 and fusion related proteins of autophagosome and lysosome. And it has been suggested that ABE has the effect of suppressing these changes.

Conclusion

I examined the changes in oxidative stress and autophagy function of the heart of rats at the early stages of streptozotocin (STZ)-induced diabetes, and whether azuki bean extract (ABE) could influence these changes.

STZ-induced early diabetes increased the levels of peroxisomal ACOX1 and catalase. ABE did not effect on the increased level of these enzymes. STZ-induced early diabetes increased the levels of p62, p-p62 and HO-1. ABE decreased the protein levels of oxidative stress marker proteins (p-p62 and HO-1). The levels of 8-OHdG in the STZ+ABE group were markedly lower than those in the STZ group. These results suggest that ABE has antioxidant effects and protects from oxidative damage of the heart in the STZ-induced early diabetic rats.

The p62 and p-p62 known to be responsible for transporting substances such as damaged organelles and proteins to autophagosome increased significantly in STZ group as compared with control group. The levels of and O‐GlcNAc modification and LAMP2 proteins related to the fusion of autophagosome and lysosome in the STZ group was significantly increased compared to the control group. Form these results, in STZ-induced early-diabetic rat hearts, it was suggested some possible that autophagy dysfunction was occurring. In addition, it has been suggested that autophagy dysfunction in the heart of diabetic animals may start from the inhibition of fusion of autophagosomes and lysosomes. ABE has the effect of suppressing the increase in the levels of LAMP2 and O-GlcNAc modification. It was suggested that ABE administration from the early stage of diabetes may have the effect of maintaining autophagy function.

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