

# Effects of Moderate Alcohol Intake in the Bladder of the Otsuka Long Evans Tokushima Fatty Diabetic Rats

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Received: 10 March 2015  
Accepted: 14 May 2015

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Funding: This study was supported by a grant of the Korea Health technology R&D Project, Ministry of Health & Welfare, Republic of Korea (A111378).

## INTRODUCTION

Diabetic bladder dysfunction is the most common lower urinary tract complication of diabetes mellitus (DM). The prevalence ranges from 25% and 83% (1). Diabetes is related with a number of cystopathic complications, including impaired bladder sensation, overactive bladder, urge incontinence and increased residual urine volume (2). Modifications of contraction have been reported, and interactions between receptor activity have been proposed in several types of smooth muscle tissues. Detrusor muscle expresses muscarinic receptors in various animals, and M3 receptors are reported to be more important for bladder contractions (3, 4). Muscarinic responses are also greater in the bladders of diabetic animals (5) and this has been linked with up-regulation of M3 receptors (6). Nobe et al. (7) demonstrated that increased glucose-dependent contraction is associated with the activation of the Rho kinase and calcium-independent protein kinase C pathways in diabetic bladder. This abnormal contraction may contribute to diabetic complications such as detrusor overactivity and lower capacity in urinary bladder.

Alcohol-drinking is known to be one of the risk factors for acute urinary retention. In addition to the diuretic and/or sympathomimetic effects, high blood levels of ethanol decrease detrusor contractility, increase bladder capacity with a decrease

Diabetes is related with a number of cystopathic complications. However, there have been no studies about the influence of alcohol consumption in the bladder of type 2 diabetes. Thus, we investigated the effect of moderate alcohol intake in the bladder of the Otsuka Long Evans Tokushima Fatty (OLETF) diabetic rat. The non-diabetic Long-Evans Tokushima Otsuka (LETO, n = 14) and the OLETF control group (n = 14) were fed an isocaloric diet; the LETO (n = 14) and the OLETF ethanol group (n = 14) were fed 36% ethanol 7 g/kg/day. After ten weeks, muscarinic receptors, RhoGEFs, myogenic change, and the level of oxidative stress were evaluated. Moderate alcohol intake significantly decreased excessive muscarinic receptor and Rho kinase expressions in the OLETF rats compared with the LETO rats. In addition, iNOS and collagen expression were not changed in the OLETF rats in spite of alcohol consumption. Superoxide dismutase levels, which is involved in antioxidant defense, in the LETO rats were significantly decreased after alcohol consumption, however those in the OLETF rats were similar. Moderate alcohol consumption reduces the oxidative stress, and may prevent molecular and pathologic changes of the bladder of rats with type 2 diabetes.

**Keywords:** Alcohols; Diabetes Mellitus; Urinary Bladder Disease

in voided volume, increase residual volume, and subsequently impair voiding efficacy (8). However, several studies reported that moderate alcohol consumption may have some favorable effects, especially regarding insulin resistance and type 2 DM (9, 10). More than 90% of DM patients have type 2 DM, which is described with hyperglycemia resulting from defects in insulin secretion, insulin action, and rages from predominantly insulin resistance (11). Cohort studies investigating the effect of alcohol intake on DM also demonstrated that moderate drinkers showed a decreased risk of DM and coronary heart disease (12).

However, there have been no studies about the influence of alcohol consumption on bladder function in type 2 DM. Thus, we investigated the effect of moderate alcohol intake on diabetic cystopathy in Otsuka Long Evans Tokushima Fatty (OLETF) rats, as an animal model of human type 2 DM.

## MATERIALS AND METHODS

### Animals

Tokushima Institute, Otsuka Pharmaceutical (Tokushima, Japan) kindly provided 5-week-old male OLETF rats and Long-Evans Tokushima Otsuka (LETO) rats of similar weight. The animals were fed standard laboratory chow (25 g/day) until 10 weeks of age. They showed no sign of pain or distress during the feed-

ing and experimental procedures. The rats were randomized into four treatment groups: 1) LETO-Control (LC,  $n = 14$ ), 2) LETO-Ethanol (LE,  $n = 14$ ), 3) OLETF-Control (OC,  $n = 14$ ), and 4) OLETF-Ethanol (OE,  $n = 14$ ). The rats in the alcohol-fed group were fed with Lieber-DeCarli Regular EtOH (Cat. No. 710260, Dyets Inc., USA) for 10 weeks. The liquid diet contained 34% fat, 11% carbohydrate, 18% protein, and 36% ethanol. We defined the 36% ethanol intake for 10 weeks as moderate ethanol intake based on a previous study (13). One previous study also showed that ethanol ingestion over a 4 week period in rats is equivalent to chronic moderate drinking in humans (14). The control rats consumed Lieber-DeCarli Control diet (Cat. No. 710027, Dyets Inc., USA) with the calories from ethanol replaced by maltose-dextran. The liquid diet contained 35% fat, 47% carbohydrate, and 18% protein. Pair-feeding controls were used with a synchronized pellet pairfeeding apparatus (Cat. No. 900006, Dyets Inc., USA) in order to regulate the different caloric ingestion; the animals were fed the same amount as the average of the OE group members. The body weight and food intake in each group of rats were recorded every week. All rats were bled weekly from a tail stab for glucose level measurements using an ACCU-CHEK®Go (Roche, Mannheim, Germany). At the end of the study, the rats of all groups were killed, urethane anesthetized by intraperitoneal injection and then the bladder tissues were excised.

#### IPGTT measurements

Intraperitoneal glucose tolerance test (IPGTT) was performed before sacrificing the rats. Blood glucose levels for IPGTT were measured at 0, 30, 60, 90, 120 min after an IP injection of a 50% dextrose solution (Sigma, St. Louis, USA) at 1.5 g/kg body weight. Glucose clearance was calculated after intraperitoneal bolus by calculating the area under the curve (AUC) of the glycemic profile.

#### RT-PCR analysis of M2, 3-muscarinic receptor and RhoGEF mRNA expression

Total RNA (2  $\mu$ g per reaction) extracted from rat bladder tissues using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and cDNA synthesis was performed using the SuperScript III First-Strand kit (Invitrogen) according to the manufacturer's instructions. Synthesized cDNA fragments were amplified by a PCR process in thermal cycler (S1000 Thermal Cycler; BIO-RAD Laboratories, CA, USA).

M2, 3-muscarinic receptors in our study were used published primers (6, 15). PDZ-RhoGEF, LARG, and p115RhoGEF were used published primers (16). And  $\beta$ -actin primer was designed bound on exon-exon base. The  $\beta$ -actin in the diabetic disease research has been used as a house-keeping gene because the expression of  $\beta$ -actin is not affected by the glucose. Each PCR reaction was carried out with the Maxime PCR PreMix I-StarTaq (iNtRON Biotechnology, Korea) using cDNA equal to 100 ng to-

tal RNA, under the following conditions: 94°C for 2 min and 35 cycles at 94°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec, followed for 7 min. The reaction products were analysed by electrophoresis on agarose gels. Densitometric analysis of band intensity was detected by Gel Documentation System (Gel Doc XR; BIO-RAD Laboratories, CA, USA) and measured using Quantity One 4.6.3 software (BIO-RAD, CA, USA).

#### Western blot analysis of RhoA, ROCK-I, and ROCK-II expression

The frozen bladder tissues were ground to a fine powder in a liquid nitrogen-cooled mortar and pestle. The analysis was performed as previously described (17). The bladder total protein was extracted using a cell lysis buffer. Protein extracts were quantified with the BCA Protein Assay Reagent (Thermo Scientific, Rockford, IL, USA). Quantitative proteins (40  $\mu$ g) were boiled in loading buffer (62.6 mM Tris-HCl pH 6.8, 2% sodium dodecyl sulfate [SDS], 0.01% bromophenol blue, 10% glycerol, and 100 mM DTT). Proteins were loaded per lane and resolved by 6 or 12% (for Rho, ROCK-I and ROCK-II) SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred onto Hybond-ECL nitrocellulose membrane (Amersham Biosciences, Germany) and equal protein loading was verified by Ponceau-S staining (Sigma-Aldrich). The membranes were blocked by treatment 5% non-fat milk in Tris-buffered saline containing 0.1% Tween 20 and membranes were probed with anti-Rho antibody (1:250; BD Biosciences), anti-ROCK-I/ROK $\beta$  antibody (1:250; BD Biosciences), anti-ROCK-II/ROK $\alpha$  antibody (1:1,000; BD Biosciences), and anti- $\beta$ -Actin antibody (1:10,000; SIGMA) for internal control. Immunoreactivation were emitted by using horseradish peroxidase conjugated secondary antibody (Santa Cruz Biotechnology, CA, USA). Densitometric analysis of band intensity was detected by Luminescent Image Analysis System (LAS-3000; FUJIFILM, Japan) and measured using Multi Gauge 3.0 software (FUJI Photo Film, Japan).

#### Measurement of oxidative stress

Superoxide dismutase (SOD) activity (CuZnSOD and Mn SOD) in bladder tissue was measured using a SOD Assay Kit-WST (Dojindo), monitoring the decrease in the rate of superoxide-mediated reduction of nitroblue tetrazolium at 450 nm using a spectrophotometer.

#### Histologic analysis

The bladder tissues were fixed in 4% paraformaldehyde for 1 day at 4°C and then paraffin-embedded. Seven  $\mu$ m thin slice sections were prepared for Masson's trichrome staining to observe the bladder muscle. After staining, the color distribution of the muscle tissue was assessed by using Optimas 6.5 software (Media cybernetics. L.P. Bethesda, USA). After the entire color distribution of the image was calculated, we selected the muscle

tissue distribution, expressed as the color red.

### Immunohistochemical analysis

The bladder tissues were fixed in 4% paraformaldehyde for 1 day at 4°C and processed in a routine manner for paraffin section. Sections (7 µm thick slices) of bladder tissues were then deparaffinized, rehydrated, treated with 3% hydrogen peroxidase to block endogenous peroxidase, rinsed and kept in 0.01 M phosphate-buffered saline (PBS, pH 7.4), microwaved to retrieve the antigen and then exposed to 10% normal serum to block nonspecific reaction. Sections were subsequently probed with anti-iNOS (1:100; Abcam, Cambridge, UK) and with HRP-conjugated secondary antibody (1:500; Abcam, Cambridge, UK). Control sections were incubated only with the secondary antibody. Colour was developed by the addition of diaminobenzidine (Invitrogen, Camarillo, CA, USA) for microscopic examination. Immunostain was visualized and obtained with an Olympus microscope (Olympus, Tokyo, Japan). For the quantitative analysis, the sections were examined by 1 urologist and 2 pathologists, respectively. The measurement was performed on 5 fields which were selected randomly in each sample. The mean iNOS-positive cells were determined using Optimas 6.5 software (Media cybernetics. L.P. Bethesda, USA).

### Statistical analysis

Statistical analyses and graphs were performed using SigmaPlot 10.0 (SPSS Inc., Chicago, USA), SPSS 15.0 (SPSS Inc., Chicago, USA) and Microsoft Excel 2007 (Microsoft Corp.). The data was expressed as mean ± standard deviation. Results of body weight, IPGTT, AUC, the mRNA levels and protein expressions were analyzed using one-way ANOVA of variance followed by Bonferroni post hoc test. The significance was set at  $P < 0.05$ .

### Ethics statement

All animal procedures were performed in accordance with the guidelines of the animal care and use committee of the College of Medicine of the Catholic University, Korea. All experimental protocols were reviewed and approved by the animal care and use committee of the College of Medicine, the Catholic University of Korea, Korea (CUMC-2012-0027-01). Animal care and experimental procedures conformed to the National Institute of Health Guidelines for the Care and Use of Laboratory Animals

(NIH Publications No. 80-23) revised in 1996.

## RESULTS

### Changes in body weight and IPGTT

Table 1 shows that there was no significant difference in body weights among the groups. Prior to glucose injection, the mean glucose levels in the OE, OC, LE, and LC groups were  $105.38 \pm 9.57$ ,  $116.54 \pm 29.58$ ,  $107.18 \pm 6.43$ , and  $117.36 \pm 4.43$  mg/dL, respectively (Table 1). Thirty minutes after intraperitoneal injection, the mean glucose levels in the OE, OC, LE, and LC groups were  $258.42 \pm 6.77$ ,  $208.25 \pm 31.30$ ,  $179.54 \pm 3.53$ , and  $200.45 \pm 32.46$  mg/dL, respectively; all groups showed increases in the mean glucose levels compared to baseline. The increased mean glucose level in the OE group was significantly higher than that in the OC group ( $P = 0.022$ ). The increased mean glucose level in the LE group was also higher than in the LC group, but the difference was not significant. The mean glucose levels in the OE, OC, LE, and LC groups were  $167.48 \pm 4.57$ ,  $121.50 \pm 11.52$ ,  $120.43 \pm 6.24$ , and  $113.65 \pm 9.55$  mg/dL, respectively, 120 min after intraperitoneal injection. All groups showed increases in the mean glucose levels compared to baseline. AUC-IPGTT was significantly different between the LETO and OLETF groups ( $P = 0.041$ ). However, the difference between the OLETF groups was not significant.

### RT-PCR analysis of M2, 3-muscarinic receptors mRNA expression

The expression of M2, 3-muscarinic receptor in the OC group was significantly higher than that in the LC group (M2,  $P = 0.025$ ; M3,  $P = 0.021$ , Fig. 1). The expression in the LE group was higher than that in the LC groups but not significant. However, the expression in the OE group was significantly decreased compared to that in the OC group.

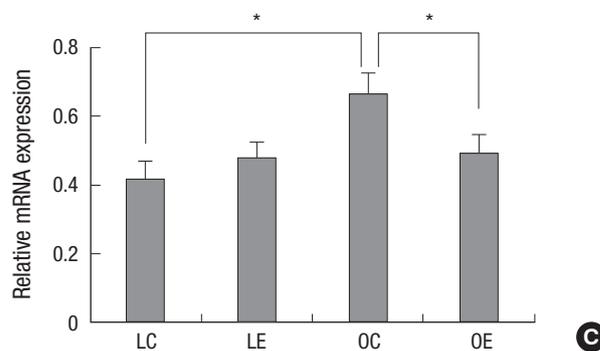
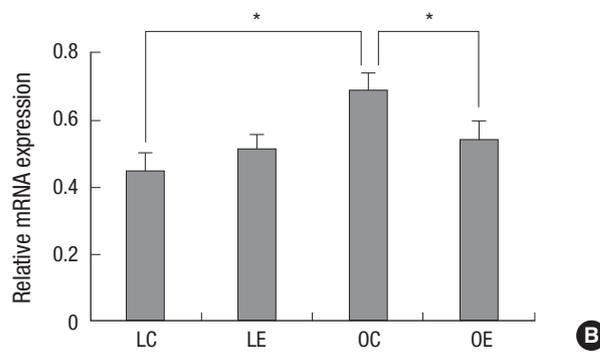
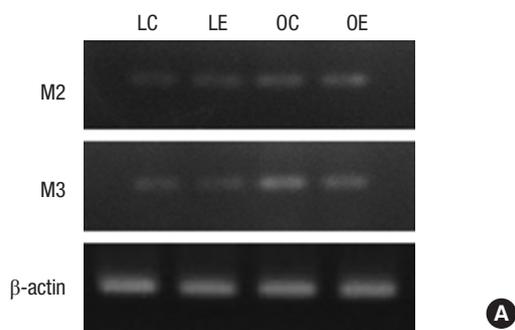
### RT-PCR analysis of RhoGEF mRNA expression and Western blot analysis of RhoA, ROCK-I, and ROCK-II expression

The expression of the three RhoGEFs in the OC group was significantly higher than in the LC group (Fig. 2A). In addition, the expression in the OE group was significantly decreased compared to that in the OC group.

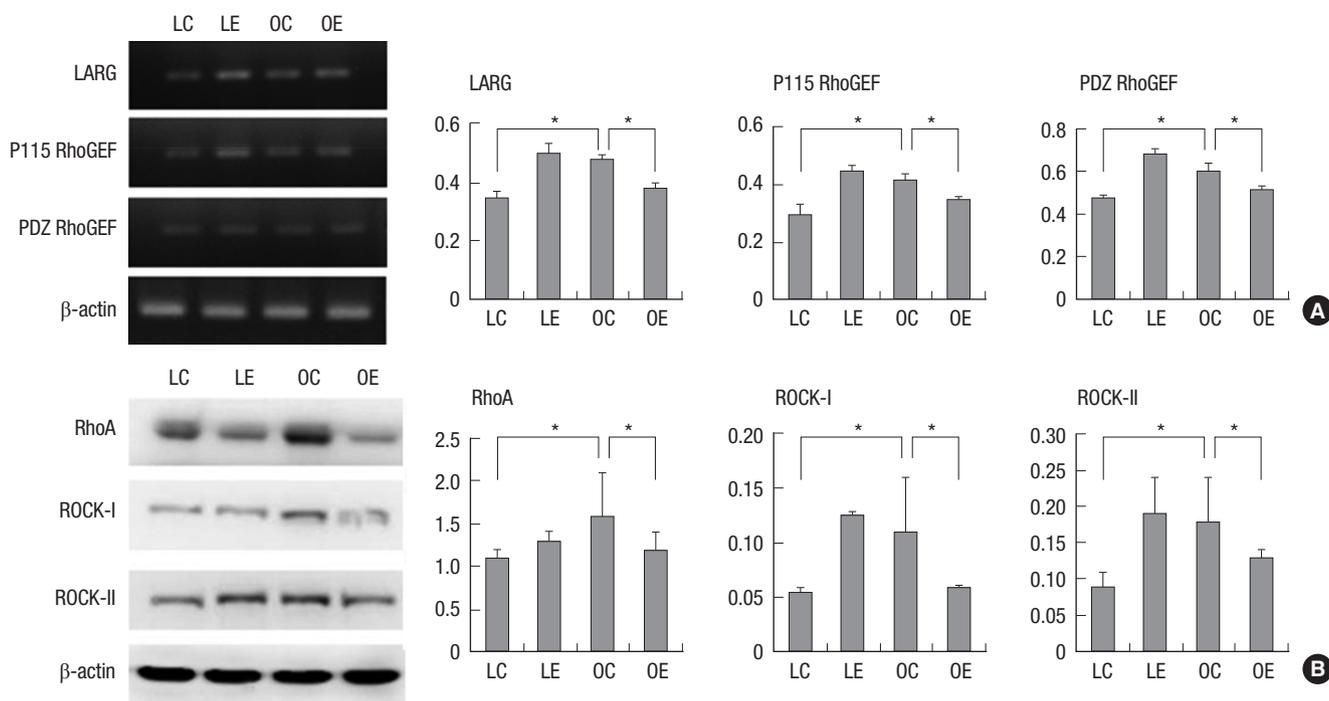
**Table 1.** Changes in body weight and IPGTT

Group	N	Weight (g)		Plasma glucose level (mg/dL)		
		Baseline	After 10 weeks	Initial	30 min	120 min
OE	14	382.52 ± 14.88	397.43 ± 20.45	105.38 ± 9.57	258.42 ± 6.77	167.48 ± 4.57
OC	14	388.14 ± 28.25	398.98 ± 28.94	116.54 ± 29.58*	208.25 ± 31.30*	121.50 ± 11.52*
LE	14	307.22 ± 24.73	350.51 ± 22.36	107.18 ± 6.43	179.54 ± 3.53	120.43 ± 6.24
LC	14	310.85 ± 18.61	356.28 ± 19.10	117.36 ± 4.43	200.45 ± 32.46	113.65 ± 9.55

Data are expressed as mean ± S.D. and were analyzed by one-way ANOVA. \* $P < 0.05$  compared with OE group. OE, OLETF-Ethanol; OC, OLETF-Control; LE, LETO-Ethanol; LC, LETO-Control.



**Fig. 1.** Comparison for bladder M2-mAChR, M3-mAChR mRNA expressions among 4 groups. (A) RT-PCR analysis (B) Densitometric analysis relative to beta-actin of M2-mAChR (C) Densitometric analysis relative to beta-actin of M3-mAChR. \* $P < 0.05$ . LC, LETO-Control; LE, LETO-Ethanol; OC, OLETF-Control; OE, OLETF-Ethanol.



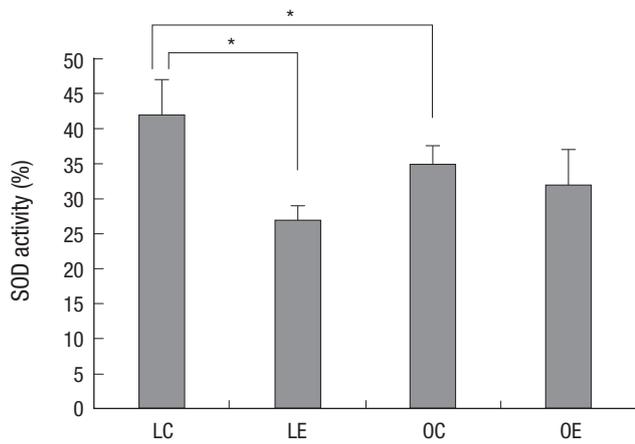
**Fig. 2.** Comparison for bladder RhoGEFs mRNA expressions (A) and RhoA, ROCK-I, ROCK-II protein expressions (B) among 4 groups. \* $P < 0.05$ . LC, LETO-Control; LE, LETO-Ethanol; OC, OLETF-Control; OE, OLETF-Ethanol.

The expression of RhoA, ROCK-I, and ROCK-II in the OC group was significantly higher than that in the LC group (RhoA,  $P = 0.027$ ; ROCK-II,  $P = 0.015$ ; ROCK-I,  $P = 0.018$ , Fig. 2B). The expression in the LE group was significantly higher than that in the LC groups. However, the expression in the OE group was significantly decreased compared to that in the OC group (RhoA,

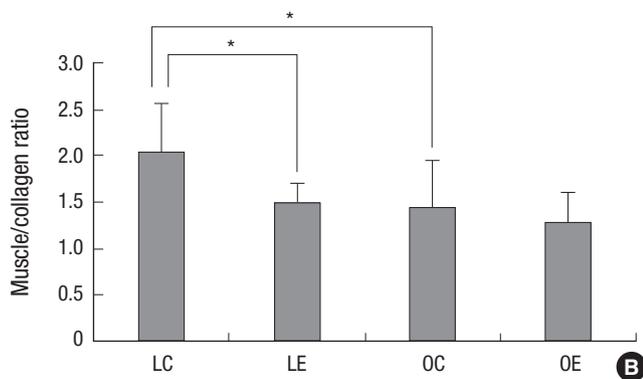
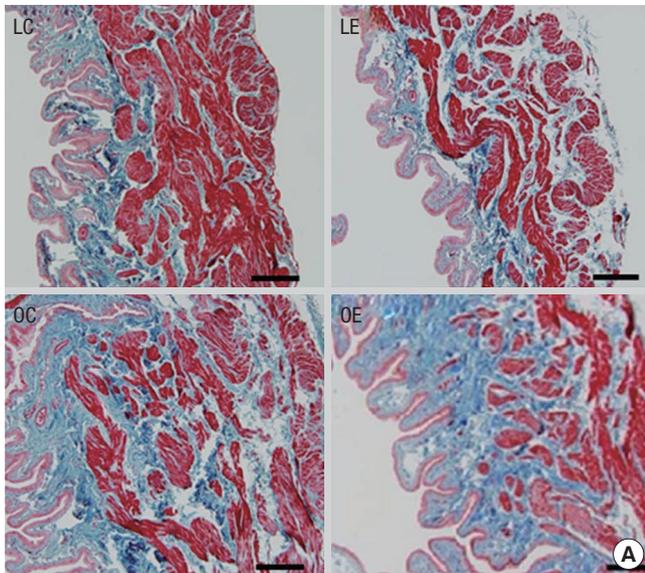
$P = 0.031$ ; ROCK-II,  $P = 0.035$ ; ROCK-I,  $P = 0.018$ ).

**Comparison of oxidative stress in bladder tissues**

There was a significant decrease in SOD levels in the OC group compared to the LC group ( $P = 0.032$ ); however, SOD levels in the OE group were slightly decreased compared with OC group

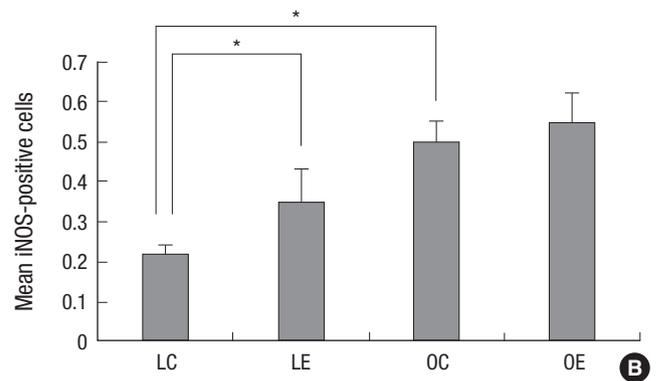
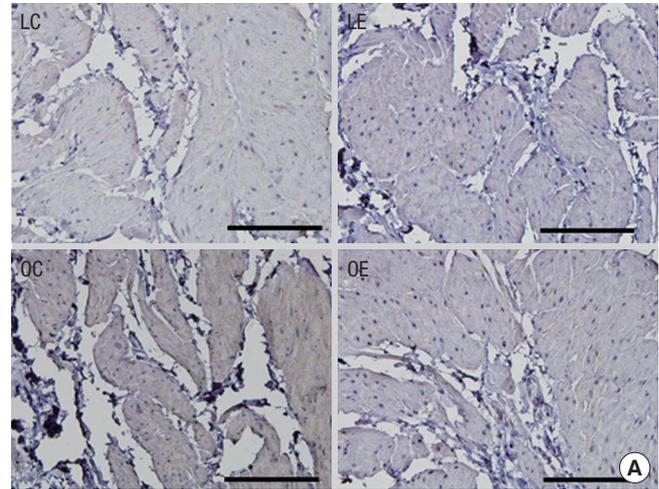


**Fig. 3.** Comparison of total superoxide dismutase expression. \* $P < 0.05$ . LC, LETO-Control; LE, LETO-Ethanol; OC, OLETF-Control; OE, OLETF-Ethanol.



**Fig. 4.** Comparison for histology among 4 groups (OE, OC, LE, and LC). (A) Masson's trichrome staining. (B) Bladder smooth muscle/collagen ratio. Scale bars shown in each figure represent 100  $\mu\text{m}$ . \* $P < 0.05$ . LC, LETO-Control; LE, LETO-Ethanol; OC, OLETF-Control; OE, OLETF-Ethanol.

but not significant. On the other hand, the expression in LE group was significantly decreased compared to that in the LC



**Fig. 5.** Comparison for immunohistochemistry among 4 groups (OE, OC, LE, and LC). (A) Immunohistochemical staining of iNOS. (B) Mean number of iNOS-positive cells. Scale bars shown in each figure represent 100  $\mu\text{m}$ . \* $P < 0.05$ . LC, LETO-Control; LE, LETO-Ethanol; OC, OLETF-Control; OE, OLETF-Ethanol.

group (Fig. 3).

**Histologic and immunohistochemical analysis**

In comparison with the LETO group, smooth muscle contents were decreased and collagen deposition was increased in the OLETF group (Fig. 4A). Collagen infiltration and disruption of the muscular structure were more prominent in the LE group than in the LC group. However, the mean muscle/collagen ratio was similar between the OC and OE groups (Fig. 4B).

The immunohistochemical studies of iNOS showed that there were more iNOS-positive cells in the OLETF groups than in the LETO groups (Fig. 5A). The mean number of iNOS-positive cells was significantly higher in the LE group *P* compared with the LC groups; it was similar between the OC and OE groups (Fig. 5B).

**DISCUSSION**

In order to understand the various contributions of neurogenic and myogenic causes to *in vivo* alterations in bladder function in diabetes, many investigators have evaluated the *in vitro* charac-

teristics of bladder contractility with conflicting results (18, 19). Tong et al. (20) reported significant increases in bladder acetylcholine (Ach) and catecholamine in streptozotocin induced diabetic rats, which can up-regulate muscarinic receptors in the diabetic detrusor muscle. Latifpour et al. (21) also reported that the quantity of muscarinic receptors in the bladder was significantly increased in eight-week streptozotocin induced diabetic rats. The possible mechanisms of this up-regulation of muscarinic receptors are explained by a decrease in cholinergic nerve density (22). Diabetic neuropathy may inhibit the release of Ach, in turn inducing the over-expression of muscarinic receptors in the diabetic detrusor muscle (23). In our study, the expression of M2, 3-muscarinic receptor was significantly elevated in diabetic rats compared with the control group. The expression of Rho and the Rho kinase pathway, which is associated with smooth muscle contractions, were examined in order to confirm the assistance of the calcium-independent signaling pathway. Nobe et al. (7) demonstrated that glucose-dependent enhancement of contraction in the diabetic bladder is involved in activation of the Rho kinase and calcium-independent protein kinase C pathways. The results of the present study also confirm previous findings; expression of M2, 3-muscarinic receptor and Rho kinase was increased in the OLETF rats compared with the LETO rats.

Oxidative stress is a relative overload of oxidants caused by increased free radical production that reduces cellular function and contributes to the pathophysiology of many diseases. Several reports suggested that diabetic patients are under oxidative stress and that various complications are related with oxidative stress (24, 25). Kanika et al. (26) demonstrated that oxidative damage of the smooth muscle cells may cause diabetic cystopathy. The effects of diabetes on iNOS induction in the bladder are not well understood. However, hyperglycemia has been shown to increase free radical production and raise the concentration of thiobarbituric acid reactive substance in the cells through various mechanisms (27). Advanced glycation end product (AGE), protein-bound oxidation products of sugars, can also increase free radicals (28). AGEs have been reported to induce iNOS expression in some cell cultures (29, 30). AGEs may induce iNOS in diabetic bladder smooth muscle cells by similar mechanisms.

The prevalence of type 2 DM is increasing and is highly influenced by lifestyle factors, such as consumption of high-calorie diets and inactive behavior (31). Alcohol use is also another major factor of lifestyle, and the association between alcohol use and mortality or coronary artery disease in the general population has best been described by a U-shaped curve, favoring moderate drinking (32). The mechanisms by which moderate alcohol drinking could afford these favorable effects are still hypothetical but could affect an enhancement of adiponectin levels (33). One important mechanism that moderate alcohol consumption ameliorates insulin sensitivity could be through increased adiponectin secretion and plasma levels. Adiponectin

is an adipocyte-secreted hormone and is present in the circulation of healthy humans at high concentrations (34). Because adiponectin plays a role in increases in glucose incorporation and insulin sensitization, it is thought that adiponectin is an endogenous antidiabetic factor (35). It has also been reported that adiponectin affects vascular smooth muscle contraction (36). In addition, Nobe et al. (37) demonstrated that adiponectin could control the bladder contraction. Although the adiponectin levels were not evaluated in this study, it is thought that moderate alcohol intake can affect a diabetic bladder.

Alcohol intake significantly decreased muscarinic receptor and Rho kinase expression in the OLETF rats compared with the LETO rats. In addition, iNOS and collagen expression were not changed in the OLETF rats after ethanol consumption. SOD levels in the LETO rats were significantly decreased, however those in the OLETF rats were similar after ethanol consumption. These findings indicate that moderate alcohol consumption ameliorates receptor and myogenic changes of the bladder, and is also tolerable to oxidative stress compared with the non-diabetic rats.

We note that our study has some limitations. First, we did not gather functional data for the bladder (cystometry) according to alcohol consumption. Daneshgari et al. (38) reported that diabetic bladders undergo a conversion from a compensated to a decompensated state, and the transition in the type 1 DM rat model may begin 9 to 12 weeks after induction. However, it takes much more time for transition in the type 2 DM because the progress of type 2 DM is noticeably slower than type 1 DM. Previous study showed that compliance of the diabetic bladder was increased and contractile response was decreased in the OLETF groups compared with the control groups at 40 and 60 weeks (39). Therefore, we need long-term studies for observing the functional change of the bladder from the type 2 DM. Second, there we did not evaluate the hepatic effects of a moderate consumption of ethanol. However, this study is the first to suggest that moderate alcohol intake may have an ameliorating effect on bladder dysfunction in type 2 DM. Thus, further studies are needed to determine whether moderate alcohol consumption cannot cause the liver damage despite an improvement of functional changes of the bladder.

Our results suggest that expression of the M2,3-muscarinic receptor and Rho kinase are increased in type 2 DM rats, but their activity is decreased after moderate alcohol intake concomitant with the alteration of oxidative stress. Moderate alcohol consumption reduces the oxidative stress, and may prevent molecular changes of the bladder of rats with type 2 diabetes.

## DISCLOSURE

The authors have no potential conflicts of interest to disclose.

## AUTHOR CONTRIBUTION

Conception and design of experiments: Lee JY. Performing experiments: Lee JY, Bae WJ, Choi YS, Kim SJ. Data analysis and interpretation: Lee JY, Bae WJ, Kim SW, Hwang T, Kim DJ. Contribution for reagents/materials/analysis tools: Lee JY, Bae WJ, Choi YS, Kim SJ, Cho HJ, Hong SH. Writing manuscript: Lee JY, Bae WJ.

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