Dietary capsaicin exhibits anti-steatosis activity in obese mice. High-fat diet (HFD)-induced mice is a highly studied approach to develop non-alcoholic fatty liver disease (NAFLD). In this study, we determined whether the topical application of capsaicin can improve lesions of NAFLD. The HFD-induced mice were treated with daily topical application of capsaicin for 8 weeks. Topical application of capsaicin reduced liver fat in HFD-fed mice. Capsaicin stimulated carnitine palmitoyl transferase (CPT)-1 and CD36 expression, which are associated with β-oxidation and fatty acids in flux of liver while it decreased the expression of key enzymes involved in the synthesis of fatty acids, such as acetyl Co-A carboxylase (ACC) and fatty acid synthase (FAS). Immunohistochemical analysis revealed the elevated level of adiponectin in liver tissue of the capsaicin-treated mice. These results suggest that the topical application of capsaicin suppresses liver fat accumulation through the upregulation of β-oxidation and de novo lipogenesis in HFD-induced NAFLD mice.

Capsaicin is the major pungent ingredient in chili peppers and is a highly selective agonist for the transient receptor potential vanilloid 1 (TRPV1) channels. Several in vitro and pre-clinical studies proved the efficacy of capsaicin in attenuating metabolic disorders through activation of TRPV1 (Panchal et al. 2018). A study with dietary capsaicin in fat-fed mice did indeed confirm that adipose expression of peroxisome proliferator-activated receptor (PPAR)-γ was increased in the treated mice, whereas gain in weight and visceral fat mass were blunted (Chen et al. 2015). Capsaicin-rich diets represent a beneficial intervention in populations at high risk for non-alcoholic fatty liver disease (NAFLD) (Hu et al. 2017). This beneficial effect of chronic dietary capsaicin intake on NAFLD is mediated by preventing fatty liver in vivo through TRPV1 activation (Li et al. 2013). TRPV1 activation by dietary capsaicin prevents NAFLD through PPAR-δ-dependent autophagy enhancement in mice. In addition, TRPV1 is highly expressed in liver tissues (Miao et al. 2008). Response of TRPV1 by capsaicin modulated AMP-activated protein kinase (AMPK), PPARα, uncoupling protein 1 (UCP1), and glucagon-like 1 (GLP-1) (Panchul et al. 2018).

In previous studies, we found that the topical application of capsaicin on the high-fat diet (HFD)-induced obese mice enhanced adiponectin level in visceral
adipose tissues (Lee et al. 2013). Visceral obesity is the main risk factor for NAFLD, and inappropriate storage of triglycerides in adipocytes and higher concentrations of free fatty acids may add to increased hepatic lipid storage and progressive liver damage (Buechler et al. 2011). Capsaicin attenuated inflammation in liver tissue, which might be induced by phosphorylation of AMPK signaling through the TRPV1 receptor (Kang et al. 2011). This hepatic AMPK activation is mediated by systemic increases in the concentration of adiponectin as the mechanisms of action of capsaicin. Treatment of topical capsaicin with exercise ameliorated the symptoms of metabolic syndrome induced by hypoestrogenism by activating AMPK (Medina-Contreras et al. 2017). In the current study, we studied whether the topical application of capsaicin improves fatty liver through the inhibition of lipogenesis and the promotion of fatty acid oxidation by AMPK activation. We used the HFD-induced NAFLD animal model for demonstrating the therapeutic effect of capsaicin from the mesenteric adipose tissue.

Materials and methods

Animal and capsaicin treatment

Male C57/BL6 mice (8 weeks of age) were purchased from Orient Bio Inc. (Gyeonggi, Korea). The mice (n = 7 per each group) were fed either 60% kcal HFD (Research Diets Inc., New Brunswick, NJ, USA) or methionine choline-deficient (MCD) (ICNBiomedicals, Costa Mesa, CA, USA) diet for 8 weeks. For topical applications, 0.075% capsaicin was mixed with hydrophilic cream base (Sigma, St, Louis, MO, USA). HFD-induced mice (HFD mice) were treated daily with 100 mg capsaicin cream onto shaved abdominal skin for 8 weeks. Hepatic fat accumulation was evaluated by measuring liver weight and Oil Red O staining. The expressions of lipid metabolism factors were analyzed by reverse transcription-polymerase chain reaction (RT-PCR) and western blot analysis.

All the experiments were performed in compliance with the guidelines of the University of Ulsan Animal Care and Use Committee and conformed to the guidelines of the National Institutes of Health.

Histological analysis

Liver specimens were fixed overnight in buffered formaldehyde (10%) and embedded in paraffin. To evaluate the accumulation of liver fat, hematoxyline and eosin stain were performed. For oil-red staining, the liver tissues were immediately frozen in liquid nitrogen and fixed in Tissue-TeK OCT compound (Sakura Finetek USA, Torrence, CA, USA). Sections were stained with 0.5% Oil Red O solution (Sigma) for 10 min at room temperature. The sections were stained with hematoxylin to visualize the nucleus and then washed with distilled water and mounted using a water-based mounting medium.

Plasma biochemical assay

Blood samples were collected from the heart in heparinized tubes, and the plasma was isolated immediately by centrifugation (3000 rpm, 4°C, 15 min). Plasma transaminase (ALT and AST) and triglyceride (TC and TG) were determined by EnzyChrom™ Aspartate Transaminase assay kit, EnzyChrom™ Alanine Transaminase Assay Kit, EnzyChrom™ Triglyceride Assay Kit, and EnzyChrom™ Cholesterol Assay Kit (BioAssay Systems, Hayward, CA, USA).

RNA extraction and RT-PCR

The collected liver tissues were homogenized with TRI reagent (Invitrogen, Carlsbad, CA, USA). Total RNA was reverse-transcribed using an oligo dT (Invitrogen) and M-MLV reverse transcription kit (Promega, Madison, WI, USA). PCR was performed with Go Taq DNA polymerase (Promega) under the following conditions: initial denaturation at 94°C for 2 min, cycling at 94°C for 30 s at appropriate annealing temperature for 30 s and 72°C for 30 s, with a final extension at 72°C for 5 min. Specific primer sequences were used in this study shown in Table 1. PCR product was electrophoresed on 1.5% agarose gel and stained with SYBR safe DNA Gel stain dye (Thermo Fisher Scientific, Waltham, MA, USA). Band intensity was determined by Image J software 1.42v (NIH, USA) and normalized to GAPDH.

Western blot analysis

Tissues were homogenized with 1x passive lysis buffer (Promega) supplemented with protease inhibitor

Table 1. Gene-specific primer sequences used in PCR amplification.

| Gene  | primer sequence (5′→3′)                                                                 |
|-------|----------------------------------------------------------------------------------------|
| ACC   | AGGGAGCCGCGATTTATCGAC                                                                  |
|       | TGACGGTGGCGCAAAAGTT                                                                   |
| CPT1a | GATTTAGGTTGAGCTTGA                                                                    |
| CD36  | GGACTAAGGTGTGACTAA                                                                   |
| FAS   | AATTAAACTCTAGGGACC                                                                      |
|       | TAGGCTGATTGGTCTGATCA                                                                  |
| F6Pase| CTGCCGAAACCTCCCGGGAATG                                                                 |
| GAPDH | AGGTCGGTGAGGCTGACC                                                                     |

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capsaicin was found to be increased in HFD between capsaicin treatment (Figure 1(c) and (d)). Cross-sections of liver tissue and oil-red staining also showed a marked decrease of lipid droplets in the capsaicin-treated HFD mice (Figure 1(e) and (f)). The standard HFD provokes decrease of lipid droplets in the capsaicin-treated HFD mice without capsaicin treatment (Figure 4(a)). Binding of plasma adiponectin to adiponectin receptor 2 (AdipoR2) on liver membrane activates PPAR-α pathways. We observed that the capsaicin treatment increased phosphorylation of AMPK and level of PPAR-α protein in HFD-induced obese mice (Figure 4(b) and (c)). These data suggest that lower fatty liver symptoms by topical application of capsaicin are induced by AMPK-mediated adiponectin signal.

Discussion
HFDIs a highly studied approach to develop NAFLD. Interestingly, we showed that HFD increased hepatic fat, but capsaicin treatment decreased it to the level of lean diet-fed mice. HFD-induced genes were involved in de novo lipogenesis and fatty acid combustion in
liver (Yamauchi et al. 2003; Machado and Cortez-Pinto 2014). These findings suggest that the topical application of capsaicin may promote lipogenesis and inhibit β-oxidation in HFD-induced mice. On the basis of our data, it is likely that the effect of capsaicin is associated with adipose tissues and leads to less fat accumulation in the liver of the HFD-induced obese mice.

HFD induces obesity and impaired metabolic syndrome. Most of the adipose tissue-derived proteins are elevated in obesity and may contribute to systemic inflammation and liver damage. Altered adipokines production of the adipose tissues in obesity has been implicated in the pathophysiology of diverse diseases including NAFLD (Radin et al. 2009). In our previous

Figure 1. Topical application of capsaicin decrease hepatic fat accumulation in the HFD-induced obese mice. Mice fed an HFD continuously for 14 weeks. After 7 weeks the mice were divided into two groups, one of which received treatment with 0.075% capsaicin cream for the remaining 7 weeks, the other control cream. Control group of mice fed a general diet continuously for 14 weeks. (a) Liver/body weight ratio, (b) Representative anatomical photographs of liver, (c) AST, (d) ALT, (e) Representative histological images of liver tissues (H&E staining), (f) Representative histological images of liver tissues (Oil-red O staining). Body and tissue weight were measured with an analytical balance. Data are mean ± s.e.m. *P < .01.

Figure 2. Capsaicin activates phosphorylation of AMPK in liver of the HFD-induced obese mice. (a) Western blot analysis of AMPK protein in the liver, (b) Immunolabeling of phosphor-AMPK in liver of the HFD-fed mice.

Figure 3. Capsaicin regulates the fatty acid metabolism-related genes in liver of HFD-induced obese mice. (a) Western blot analysis, (b) RT-PCR analysis of ACC, FAS, CPT1a, PEPCK-C, G6Pase, and CD36 in liver of the mice fed either with HFD or MCD diet.
study, we found that the topical application of capsaicin on HFD-induced obese mice increased adiponectin level in mesenchymal adipose tissues. Although adiponectin is highly abundant in human serum, its levels are reduced in obese patients and patients with hepatic steatosis of NASH. It is suggested that adiponectin could minimize fat accumulation in the liver (Awazawa et al. 2009). In the present study, we found that capsaicin treatment increased adiponectin level in the liver of HFD-fed obese mice. Adiponectin antagonizes excess lipid storage in the liver and prevents inflammation and fibrosis (Kadowaki et al. 2006). Since adiponectin secreted from mesenteric adipose tissues can act directly on hepatic tissues and produce beneficial effects on lipid metabolism, adiponectin seems to be involved in the capsaicin-induced inhibition of hepatic lipid accumulation. We sought to investigate whether adiponectin increase due to the topical application of capsaicin can reduce liver fat in the HFD-induced NAFLD animal models. Phosphorylation and activation of the AMPK stimulate adiponectin in the liver (Yamauchi et al. 2002). Capsaicin reduced inflammation in liver without changes in body weight and adiposity (Kang et al. 2011). The effect of capsaicin on this reduction was suggested by hepatic AMPK activation, systemic increases in the concentration of adiponectin and increased AdipoR2 in liver. Our study shows that the topical application of capsaicin resulted in AMPK activation, which might be stimulated by adiponectin in the liver. In addition, capsaicin treatment also stimulated CPT-1 and CD36 expression, which are associated with β-oxidation and fatty acids influx. The increased hepatic synthesis of fatty acid, and possibly their increased uptake, eventually reaches equilibration by an increased rate of mitochondrial β-oxidation and an increased rate of hepatic triacylglycerol secretion, allowing stabilization of the amount of fat in the liver. In contrast, capsaicin reduced molecules such as ACC, FAS, PEPCK-C, and G6Pase, which are involved in de novo lipogenesis and gluconeogenesis in the liver. AMPK activation results in the inhibition of ACC, the rate-limiting enzyme controlling fatty acid synthesis. The reduced expression of gluconeogenic enzymes such as PEPCK-C and G6Pase was associated with elevated phosphorylation of AMPK in adiponectin transgenic mice (Combs et al. 2004). Our data indicate that the topical application of capsaicin accounts for the inhibition of hepatic lipogenesis and endogenous glucose production.

The common causes of mild increase in AST and ALT levels include NAFLD, hepatitis C, and alcoholic fatty liver disease. AST and ALT are reasonably sensitive indicators of liver damage or injury from different types of diseases or conditions, although higher-than-normal levels of these liver enzymes should not be equated with liver disease (O’Shea et al. 2010). We showed that AST and ALT are recovered by capsaicin treatment in HFD mice. Although there is no proven pharmacotherapy for the treatment of NAFLD, therapeutic strategies to upregulate adiponectin levels, such as TZD administration are expected to be effective for the treatment of the metabolic syndrome including NAFLD (Iwaki et al. 2003; Sharabi et al. 2007). Recent study showed that the elevation of adiponectin level by natural product supplementation might benefit patients with metabolic syndrome and its animal model (Kessoku et al. 2016). The result of this study suggests that topical application of capsaicin could potentially be beneficial in preventing the complications of NAFLD.

Acknowledgements
This research was supported by Basic Science Research Program (2017R1D1A1A04030339 and 2013R1A1A4A01009559) through the National Research Foundation of Korea (NRF) funded by the Ministry of Education.

Disclosure statement
No potential conflict of interest was reported by the author(s).

Funding
This research was supported by Basic Science Research Program [2017R1D1A1A04030339 and 2013R1A1A4A01009559] through the National Research Foundation of Korea (NRF) funded by the Ministry of Education.
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